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**INSTITUT DE RECHERCHE SUR LA BIOLOGIE DE L'INSECTE**

# THÈSE

présentée par :

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# MANIPULATION DES VÉGÉTAUX PAR LES ORGANISMES ENDOPHYTES :

## Dialogue chimique et moléculaire entre les insectes manipulateurs de plantes et leurs plante hôtes

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Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled.

WILLIAM RAMSAY

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# Résumé détaillé

Les insectes ont développés diverses stratégies pour compenser les faibles apports nutritionnels fournis par les végétaux et pour contourner leurs mécanismes de défense. En raison de leur rôle central dans la physiologie et le développement des plantes, les phythormones ont depuis longtemps été considérées comme des médiateurs déterminants dans la capacité des insectes à manipuler leur environnement végétal. Les insectes phytophages peuvent ainsi manipuler la balance et la communication phytohormonale de façon à réguler la croissance, les défenses ou le statut nutritionnel des plantes et ils sont désormais connus pour leur capacité à produire des phytohormones. Les mécanismes permettant aux insectes manipulateurs de contrôler la balance phytohormonale demeurent néanmoins pour la plupart inconnus en raison d'un manque de données sur la modulation phythormonale au niveau moléculaire et biochimique dans diverses conditions environnementales. De plus, malgré des décennies de recherche sur les interactions plantes-insectes, les mécanismes sous-jacents à la manipulation des plantes par les insectes galligènes et mineurs de feuilles sont largement méconnus, la plupart des études ayant été conduites sur les insectes broyeurs ectophages.

L'objectif de ma thèse était de fournir une caractérisation précise des capacités de modulation phytohormonale par les insectes manipulateurs de plantes avec un accent particulier sur le lépidoptère mineur de feuille *Phyllonorycter blancardella*. Nous avons tout d'abord identifié la réponse des plantes aux attaques des larves mineuses au niveau moléculaire ciblant les voies métaboliques impliquées dans la production d'hormones liées aux défenses et aux flux de nutriments. Cette première étude visait également à caractériser le profil phytohormonal des feuilles par LC-MS/MS. Nous avons ensuite établi une caractérisation dynamique des cytokinines (CKs) dans les larves et les feuilles attaquées et nous avons comparés les résultats obtenus chez *P. blancardella* avec ceux collectés chez une espèce proche (*P. mespilella*) partageant la même niche écologique mais étant dépourvue de bactéries symbiotiques de type *Wolbachia*. Ces étdues visaient à identifier l'origine des CKs impliquées dans la manipulation de la plante et savoir si elles étaient produites par la plante, l'insecte ou la bactérie symbiotique. Enfin, nous avons évalués la capacité des insectes mineurs à moduler la composition en auxines (AUXs) des plantes comme le font les insectes galligènes sachant que mineurs et galligènes présentent de nombreuses similitudes dans les mécanismes mis en jeu pour manipuler leur plante hôte.

En tant que régulateurs importants de la croissance, de la défense et du métabolisme des plantes, il n'est pas étonnant que les phytohormones aient été la cible privilégiée des insectes phytophages au cours de l'évolution. Notre étude démontre que *P. blancardella* induit une profonde modulation de la balance phytohormonale des plantes associée à une augmentation de l'approvisionnement en nutriments, une inhibition de la senescence et une atténuation des défenses. Une caractérisation exhaustive des CKs suggère fortement que les insectes produisent des CKs et les délivrent à la plante, particulièrement sur les feuilles jaunes, leur permettant ainsi de contourner la senescence programmée des feuilles. Ces altérations permettent à l'insecte de contrôler les apports nutritionels dans des conditions environnemenates fluctuantes. Cette modulation se fait en ciblant des voies métaboliques particulières soulignant des similitudes dans les stratégies adoptées par les arthropodes et les microorganismes associés aux plantes. Les analyses révèlent aussi de nombreuses similitudes entre les stratégies mises en place par des insectes aux mode de vie variés (ex : Galligènes *vs.* Mineurs de feuilles). Enfin, notre étude suggère que les bactéries symbiotiques d'insectes contribuent à la production de CKs par la synthèse de types spécifiques de CKs. Notre étude fournit ainsi un faisceau de données expérimentales convergent vers l'influence des bactéries symbiotiques dans la capacité des insectes mineurs à contrôler la physiologie de leur plante hôte avec des conséquences sur l'écologie et la diversification écologique des insectes mineurs.

Notre étude fournit ainsi des avancées significatives dans la compréhension des mécanismes impliqués dans une interaction étroite plante-insecte-microorganisme. Ceci est d'un intérêt majeur pour comprendre le rôle joué par les phytohomrones dans les manipulations des plantes par les insectes, leur origine et leur rôle adptatif pour les insectes. Notre étude suggère par ailleurs que l'étude des similarités dans la réponse phytohormonale induite par les insectes manipulateurs de plantes dans différents systèmes biologiques pourrait permettre d'identifier les fonctions végétales préférentiellement ciblées. Parce que les insectes manipulateurs attaquent souvent des plantes à haute valeur économique, les données obtenues sont par ailleurs susceptibles d'aider au développement de stratégies innovantes de lutte contre les insectes ravageurs.

**Mots clés**: Interactions plantes-insectes, manipulation, phytohormones, cytokinines, mineurs de feuilles, insectes, bactéries symbiotiques d'insectes, *Phyllonorycter blancardella, Phyllonorycter mespilella, Malus domestica, Wolbachia.*

# Résumé détaillé en anglais

Insects have developed a series of mechanisms and strategies to address the nutritional mismatch between what plants provide and what insects require but also to avoid physical and chemical plant defensive systems. Because 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. Herbivorous insects can manipulate the plant hormonal biosynthesis and the hormone-dependent signaling pathways to regulate plant growth, defense and/or nutritional status and they are now known to be able to produce phytohormones. Mechanistic understanding of how phytohormones operate in plant reconfigurations by plant-manipulating insects is lacking due to limited information on the molecular and biochemical phytohormonal modulation under various environmental conditions. Moreover, despite decades of research on plant-insect interactions, most studies have been conducted on ectophagous chewing insects and mechanisms underlying plant manipulation by gall-inducing and leaf-mining insects are still largely unresolved.

The objective of my Ph.D. was to provide an extensive characterization of how plant-manipulating insects modulate the plant global hormonal balance with a specific focus on the leaf-mining moth *Phyllonorycter blancardella*. We first investigated the plant response following attack by the leaf-miner by analyzing how the leaf-miner reprograms the host-leaf transcriptome to modulate phytohormones associated with nutrient mobilization and plant defense. This also included the analysis by LC-MS/MS of phytohormone profiles in attacked and un-attacked apple trees. We then conducted a time course characterization of cytokinins (CKs) in larvae and in attacked apple leaves and compared results obtained in *P. blancardella* with data collected on a closely related leaf-miner species (*P. mespilella*) sharing the same ecological niche but that differ in its *Wolbachia* infection status. This aimed to gain a deeper understanding into the possible origin of CKs involved in the plant manipulation to figure out if the plant, the insect or the symbionts, produces them. Finally, we investigated whether or not leaf-mining insects modulate the auxin (AUX) profile of their host-plant like their gall-inducer counterparts, gall-inducers and leaf-miners sharing numerous similarities in mechanisms used to manipulate their host-plant.

As important regulators of plant growth, defense and metabolism, it is not surprising that phytohormones have been the target of phytophagous insects over the course of evolution. Our

study demonstrates that leaf-mining by *P. blancardella* leads to a strong reprogramming of the plant phytohormonal balance associated with increased nutrient mobilization, inhibition of leaf senescence and mitigation of plant defense. The extensive identification and quantification of CKs 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. These alterations allow insects to control their nutritional supply under fluctuating environmental conditions. Alterations occur mainly through a modulation of specific pathways of CK biosynthesis with a common strategy shared by arthropods and plant-associated microorganisms. Analyses also suggest that strategies underlying the plant manipulation may be shared between herbivorous insects with distinct life histories (e.g. gallers vs leaf-miners). Our study further suggests that insect bacterial symbionts contribute to the production of CKs through the synthesis of specific CK forms. Our study thus provides converging experimental evidences pointing towards 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.

Ultimately, our study provides key findings towards the understanding of molecular mechanisms underlying an intricate plant-insect-microbe interaction. This is of great importance to gain a deeper understanding of the role played by phytohormones in insect-induced plant manipulation, their origin, and their adaptive significance for insects. Our study also suggests that looking at similarities in the phytohormonal responses elicited by plant reprogrammers in a diversity of biological systems may help to identify key plant functions that are targeted during plant manipulation. Because a great number of these plant-manipulators attack plants of high economic importance, information collected can also potentially help to develop innovative strategies to fight against these insect pests.

**Key words**: Plant-insect interactions, plant manipulation, phytohormones, cytokinins, leaf-miner, insects, insect bacterial symbionts, *Phyllonorycter blancardella, Phyllonorycter mespilella, Malus domestica, Wolbachia*.

# La structure de la thèse

**Objectifs de la thèse:**

L'objectif de ma thèse consiste à réaliser une caractérisation exhaustive des capacités de manipulation de la balance phytohormonale de la plante hôte par des insectes manipulateurs avec un focus spécifique sur le lépidoptère mineur de feuille *Phyllonorycter blancardella*. Les résultats acquis seront de première importance pour enrichir notre connaissance sur le rôle des phytohormones dans la régulation des interactions plante-insecte et plus spécifiquement dans les manipulations induites par les insectes. Ceci permettra également de mettre en évidence les mécanismes impliqués et d'identifier l'origine de ces médiateurs chimiques. Plus spécifiquement, ma thèse vise secondairement à identifier si les hormones impliquées sont produites par la plante, par l'insecte ou indirectement *via* la présence de symbiotes bactériens. Ces éléments seront déterminants pour comprendre le fonctionnement d'interactions plante-insecte-microorganisme complexes et pour identifier le rôle adaptatif de ces manipulations végétales pour les insectes. Les informations obtenues pourraient également permettre de développer des stratégies innovantes de lutte contre les insectes ravageurs, les insectes manipulateurs attaquant fréquemment des plantes à haute valeur économique.

**Organisation de la thèse :**

Ma thèse aborde donc les manipulations des végétaux par les organismes endophytes en étudiant le dialogue chimique et moléculaire mis en jeu entre les insectes manipulateurs de plantes et leurs plantes hôtes. Elle vise plus spécifiquement à étudier le lépidoptère mineur de feuille *Phyllonorycter blancardella* (Lépidoptère : Gracillaridae) se développant dans les feuilles du pommier *Malus domestica*. Au cours de ma thèse je me suis attaché à caractériser la réponse de la plante hôte aux attaques de l'insecte mineur en analysant au niveau moléculaire et biochimique les modifications phytohormonales induites en lien avec les flux de nutriments et les défenses des plantes (chapitre 2) (Fgure 1.3a). Ceci inclus l'analyse du profil phytohormonal dans les feuilles attaquées et non attaquées et plus spécifiquement les altérations au niveau des cytokinines (CKs) et des auxines (AUXs) (Chapitres 2 et 3) (Figures 1.3a et b) et leurs implications sur la blance phytohormonale globale (Chapitre 2) (Figure 1.3a). Les approches d'écologie moléculaire et chimique que j'ai développée visaient à fournir des

informations clés sur l'origine des phytohormones (et plus spécifiquement les CKs en tant que médiateurs chimiques de l'interaction) de façon à identifier si elles étaient produites par la plante, l'insecte et/ou les bactéries symbiotiques d'insectes (Chapitres 2 et 3) (Figures 1.3a et b). Les données obtenues sur *P. blancardella* ont été ensuite comparée à celles générées sur une espèce phylogénétiquement proche appartenant au complexe *blancardella*, utilisant la même niche écologique mais qui n'héberge pas *Wolbachia* (*P. mespilella*). Cette comparaison visait à évaluer le rôle des bactéries symbiotiques dans la modulation des CKs et des AUXs (Chapitre 4) (Figure 1.3c). Des informations sur la dynamique des altérations phytohormonales ont également été collectées (Chapitre 3) (Figure 1.3b). Ma discussion générale aborde les conséquences écologiques de ces manipulations au niveau des capacités de défense directe et indirecte de la plante, en particulier concernant la production de composés organiques volatils, et de la nutrition de l'insecte. De façon à renforcer notre compréhension du rôle des phytohormones dans les manipulations végétales induites par les insectes, les mécanismes sous-jacents et leur rôle adaptatif pour les insectes, j'aborde également des perspectives évolutives en comparant les données obtenues sur les systèmes mineurs avec celles issues de la littérature ou obtenues expériemntalement sur la modulation des CKs et des AUXs par les larves de l'insecte galligène *Mayetiola destructor* (Mouche de Hesse)

Ma thèse s'organise de la façon suivante :

**Chapitre 1. Introduction générale**

**Chapitre 2. Leaf-mining by *Phyllonorycter blancardella* reprograms the host-leaf transcriptome to modulate phytohormones associated with nutrient mobilization and plant defense.**

*Objectifs.*

Le premier objectif du chapitre 2 visait à produire une caractérisation exhaustive de la modulation des CKs par le lépidoptère mineur de feuille *P. blancardella*. Les résultats précédemment obtenus sur ce système montrent une capacité des larves à induire une « île-verte » sur feuilles sénescentes par l'accumulation de CKs dans la zone d'alimentation (Giron et al., 2007 ; Kaiser et al., 2010 ; Body et al., 2013). Néanmoins ces résultats avaient été obtenus par test immuno-enzymatique ELISA permettant la caractérisation d'un nombre extrêmement

limité de CKs (iP isopentenyladenine; iPR *N6*-isopentenyladenosine; Z zeatin; ZR zeatin riboside).

→ *Hypothèse de travail :* Nous prédisions une altération phytohormonale des feuilles impliquant une diversité bien plus importante de CKs.

Le second objectif visait à fournir des informations sur la possible origine des CKs accumulées dans la zone d'alimentation de façon à savoir si elles étaient produites par la plante ou par l'insecte.

→ *Hypothèse de travail :* Nous prédisions une activation des gènes impliqués dans la synthèse de CKs par la plante contribuant ainsi à l'augmentation des concentrations en CKS.

Le troisième objectif visait à caractériser l'influence des altérations en CKs sur la balance phytohormonale globale.

→ *Hypothèse de travail :* Nous prédisions une altération de la balance phytohormonale globale en lien avec l'apport en nutriments, l'inhibition de la sénescence et l'inhibition des défenses directes et indirectes.

*Méthodes.*

Pour atteindre ces objectifs, les phytohormones majeurs ainsi que l'activité transrciptionnelle des cellules en contact direct avec *P. blancardella*, ont été analysées par puce ADN et LC-MS/MS.

*Résultats.*

Nos résultats mettent en évidence une reprogrammation importante des zones minées au niveau moléculaire et biochimique soulignant la relation étroite entre l'insecte et sa plante hôte. Nous montrons que les CKs sont très fortement accumulées dans la zone d'alimentation de l'insecte malgré une très faible expression des gènes de biosynthèse des CKs chez la plante. L'activité de l'insecte est également associée à une augmentation de la biosynthèse des précurseurs de l'acide jasmonique (JA) mais pas de sa forme active, une faible altération de la synthèse d'acide

salicylique (SA) et une très nette inhibition de la voie de biosynthèse de l'acide abscissique (ABA).

*Conclusions.*

Notre étude démontre que la plante n'est pas à l'origine des CKs accumulées dans la zone d'alimentation. Elle confirme des résultats préliminaires suggérant que l'insecte produit des CKs qu'il délivre à la plante de façon à créer un environnement nutritif favorable.

Nous démontrons également que *P. blancardella* induit une profonde reconfiguration phytohormonale associée à l'approvisionnement en nutriments, l'inhibition de la sénescence et une atténuation (mais pas une inhibition totale) des défenses de la plante. Les implications de ces altérations sur l'expression physiologiques des défenses directes et indirectes restent à déterminer (voir discussion principale).

En raison du rôle des CKs dans l'induction des galles par les insectes et les bactéries, et de leur rôle plus général dans la modulation des interactions entre les plantes et les organismes biotiques, nos résultats sont susceptibles d'avoir des implications importantes pour d'autres systèmes biologiques. Des études supplémentaires sont désormais nécessaires pour évaluer les similitudes entre les stratégies mis en jeu par les insectes galligènes et les insectes mineurs de feuilles en particulier en étudiant le rôle joué par les auxines qui sont également des médiateurs chimiques importants pour les organismes galligènes (voir chapitre 5 et discussion générale).

Enfin, des études supplémentaires au niveau moléculaire et biochimique sont désormais nécessaires pour comprendre pleinement comment les modulations de phytohormones affectent la réponse des plantes aux endophytophages car l'essentiel de nos connaissances est issu d'études sur les insectes ectophages. Ceci nous permettra également une meilleure compréhension du rôle des phytohormones dans les phénotypes étendus induits par les insectes, les mécanismes de manipulation sous-jacents, l'origine des médiateurs chimiques impliqués et leur signification adaptative pour l'insecte. De manière ultime ceci nous fournira des informations clés sur le rôle des phythormones dans l'évolution des interactions plantes-insecte-microorganismes.

Ces résultats sont publiés dans *Journal of Insect Physiology*.

## Chapitre 3. Dynamics and origin of cytokinins involved in plant manipulation by a leaf-mining insect.

*Objectifs.*

Le premier objectif du chapitre 3 visait à approfondir nos connaissances sur l'origine possible des CKs impliquées dans la manipulation des plantes par l'insecte mineur *P. blancardella*. Plus particulièrement il s'agissait d'évaluer si ces CKs pouvaient être produites par la plante, l'insecte ou les bactéries symbiotiques de l'insecte. Les résultats acquis précédemment suggéraient que les CKs étaient vraisemblablement produits par l'insecte (Chapitre 2) et que les bactéries symbiotiques jouaient un rôle déterminant dans ces altérations phytohormonales (Giron et al., 2007 ; Kaiser et al., 2010 ; Body et al., 2013 ; Gutzwiller et al., 2015). Une corrélation forte entre la quantité de bactéries symbiotiques (*Wolbachia*), la quantité de CKs accumulées dans la mine et l'intensité du phénotype « île verte » avaient en effet été mis en évidence (Kaiser et al., 2010). Un traitement antibiotique des insectes avaient également permis de montrer que les insectes dépourvues de bactéries étaient dans l'incapacité d'induire une île verte et présentaient une concentration en CKs très faible (Body et al., 2013).

→ *Hypothèse de travail :* Nous prédisions la présence de CKs dans les larves, y compris des CKs spécifiques des bactéries, signature chimique du rôle des symbiotes dans la reconfiguration phytohormonale.

Le deuxième objectif visait à caractériser la dynamique spatio-temporelle des altérations en CKs.

→ *Hypothèse de travail :* Nous prédisions une altération en CKs identique dans des conditions environnementales distinctes mais avec des modifications strictement restreintes à la zone d'alimentation de l'insecte.

*Méthodes.*

Pour atteindre ces objectifs, nous avons réalisés une identification et une quantification des CKs par HPLC-(ESI+)-MS/MS sur feuilles de pommiers vertes et jaunes attaquées par *P. blancardella*. Une quantification des CKs dans les larves a également été réalisée de façon à évaluer leur contribution potentielle à la production de CKs. Notre profilage métabolique intègre des Méthyl-thioCKs dont certaines formes sont spécifiques des bactéries permettant ainsi de clarifier si les bactéries symbiotiques produisent directement des CKs.

*Résultats.*

Notre caractérisation dynamique des CKs dans les larves et les feuilles infestées montre un enrichissement en CKs aussi bien dans les feuilles jaunes que dans les feuilles vertes. La distribution spatiale des CKs montre que les altérations se limitent strictement à la zone minée. Les concentrations les plus importantes en CKs sont observées dans les larves avec la présence de CKs caractéristiques des bactéries.

*Conclusions.*

La caractérisation exhaustive des CKs sur feuilles vertes et sur feuille jaunede *M. domestica* attaquées par *P. blancardella* confortent l'hypothèse selon laquelle les insectes produisent des CKs administrées à la plante et ce plus particulièrement sur feuilles jaunes permettant ainsi à l'insecte de contourner le programme de sénescence programmée. Ces altérations permettent à l'insecte de contrôler ses apports en nutriments dans des conditions environnementales fluctuantes.

La manipulation se limite à la zone d'alimentation de l'insecte suggérant une absence de translocation de CKs depuis les autres zones de la feuille vers la mine. Les altérations des concentrations en CKs se font par une modulation de voies métaboliques spécifiques soulignant des similitudes dans la stratégie utilisée par les arthropodes et celle des micro-organismes de plantes. Nos analyses révèlent que les types de CKs prioritairement accumulés dans la zone minée sont identiques à ceux accumulés par les insectes galligènes suggérant des convergences dans les stratégies mis en jeu par des insectes phytophages ayant des styles de vie distincts (voir chapitres 4 et 5).

Notre étude suggère par ailleurs que les bactéries symbiotiques d'insectes pourraient contribuer à la production de CKs par la synthèse de 2-MeS-CKs spécifiques. Ceci est en accord avec des résultats obtenus précédemment suggérant que les bactéries contribuent aux phénotypes observés. L'origine évolutive des CKs reste à démontrer formellement et des tests fonctionnels sont désormais nécessaires pour valider la fonction spécifique des différents types de CKs et le rôle de chaque partenaire dans cette interaction plante-insecte-microorganisme (voir chapitre 4). De façon ultime note étude fourni des éléments clés dans la compréhension des mécanismes moléculaires impliqués dans cette interaction plante-insecte-microorganisme.

Ces résultats sont soumis à *Insect Science*.

**Chapitre 4. The *Wolbachia*-free leafmining species *Phyllonorycter mespilella* fails to modulate plant auxin and cytokinin levels under variable environmental conditions.**

*Objectifs.*

Le premier objectif du chapitre 4 visait à évaluer si les insectes mineurs modulent les auxines (AUXs) de leur plante hôte comme le font les insectes galligènes. Les AUXs sont connus pour moduler la croissance et la division cellulaire et jouent donc un rôle dans l'induction du tissu nutritionnel des galles dont les larves se nourrissent (Tooker & Helms, 2014). L'accumulation d'AUXs au niveau du site d'attaque a été mis en évidence dans de nombreux systèmes et les insectes galligènes peuvent produire et délivrer ces effecteurs à la plante (Yamaguchi et al., 2012). Par ailleurs, les galligènes et les mineurs présentent de nombreuses similitudes dans les mécanismes utilisés pour manipuler le plante hôte et dans les effets induits (Giron et al., 2016).

→ *Hypothèse de travail :* Nous prédisions une augmentation des concentrations en AUXs dans les mines.

Le deuxième objectif visait à évaluer les similitudes dans les stratégies de manipulation (et plus particulièrement concernant l'altération des CKs) entre deux espèces de mineuses

phylogénétiquement proches partageant la même niche écologique mais qui diffèrent par le statut d'infection à *Wolbachia*.

→ *Hypothèse de travail :* Nous prédisions que l'espèce dépourvue de *Wolbachia, P. mespilella*, allait induire une altération en CKs distincte de *P. blancardella*.

*Méthodes.*

Pour atteindre ces objectifs, nous avons réalisés une identification et une quantification exhaustive des AUXs et des CKs par HPLC-(ESI+)-MS/MS sur feuilles de pommiers vertes et jaunes attaquées par *P. mespilella.*

*Résultats.*

Notre caractérisation dynamique des phytohormones dans les feuilles infestées montre que les mines ne sont pas enrichies en CKs et en AUXs. Les concentrations totales en CKs et en AUXs sont plus faibles dans les mines aussi bien sur feuilles vertes que sur feuilles jaunes. Des augmentations des niveaux en CKs sont néanmoins observées dans les mines sur feuilles jaunes pour trois formes de CKs actives (iPR, *t*ZR et *t*Z).

*Conclusions.*

La caractérisation des AUXs et des CKs sur feuilles vertes et jaunes de pommier attaquées par *P. mespilella* montre que des espèces de mineuses qui diffèrent par le statut d'infection à *Wolbachia* induisent des altérations phytohormonales distinctes. Ceci démonte que des mineuses dépourvues de *Wolbachia* ne parviennent pas à moduler les niveaux de CKs et d'AUXs dans des environnements variables.

Une caractérisation de l'ensemble des CKs est nécessaire afin d'identifier les profils physiologiques susceptibles d'être importants pour l'interaction plante-insecte. L'accumulation de CKs n'est en effet observée que pour certaines formes de CKs actives et uniquement dans les mines sur feuilles jaunes. Cette accumulation résulte vraisemblablement d'une diminution

des CKs dans les zones non infestées en raison de la sénescence des feuilles plutôt que d'une accumulation *sensu stricto* dans la mine.

Notre étude suggère que les mécanismes mis en jeu par *P. mespilella* diffèrent de ceux de *P. blancerdella*, et que *P. mespilella* ne produit pas de CKs. Elle produit par ailleurs un faisceau de résultats convergents vers l'influence des bactéries symbiotiques dans la capacité des insectes mineurs à contrôler la physiologie de leur plante hôte avec des conséquences sur l'écologie et la diversification évolutive des insectes.

Ces résultats sont mis en forme pour une soumission à *Entomologia Experimentalis et Applicata.*

**Chapitre 5. Discussion générale et conclusions**

Notre étude démontre que les larves mineuses de *P. mespilella* qui n'hébergeant pas *Wolbachia* ne modulent pas les niveaux de CKs et Aux dans des conditions environnementales variables et que l'accumulation de CKs est strictement restreinte aux mines sur feuilles jaunes. Une caractérisation exhaustive et dynamique des phythormones et indispensable pour mettre en évidence des altérations physiologiques d'importance pour l'écologie des insectes mineurs, les concentrations totales en CKs étant plus faible sur feuilles vertes et sur feuilles jaunes. Ces résultats s'opposent à ceux obtenus sur une espèce de mineuse phylogénétiquement proche et sur d'autres mineuses et insectes galligènes qui présentent des modifications phytohormonales convergentes. Ces résultats suggèrent que les mécanismes sous-jacents aux interactions plantes-insectes sont différents entre les deux espèces de mineuses et que *P.mespilella* ne produit vraisemblablement pas de Cks. Les interactions entre AUXs et CKs sont complexes, de même que leurs effets physiologiques sur la croissance et l'immunité des plantes selon leur localisation spéciale dans la plante (Costacurta and Vanderleyden, 1995; Pernisová et al., 2011; Schaller et al., 2015) et le système biologique considéré (Kazan and Manners, 2009; Naseem et al., 2015). Notre compréhension de la régulation des interactions biotiques des plantes par les AUXs et les CKs et comment ces médiateurs interagissent avec les autres phytohormones proviennent essentiellement de travaux sur des plantes modèles comme *Arabidopsis thaliana* et *Oryza sativa* (Kazan and Manners, 2009; Naseem et al., 2015). Une trop grande généralisation ne permet pas de prendre en compte la complexité des interactions biotiques et

plus spécifiquement lorsqu'elle implique une interaction tripartite entre une plante, un insecte et des bactéries symbiotiques. Par conséquent, élucider mes mécanismes moléculaires sous-jacents aux diminutions d'AUXs et de CKs dans les tissus attaqués par *P. mespilella*, ainsi que leurs conséquences en termes de fitness pour l'insecte nécessite des études complémentaires. Néanmoins, des résultats expérimentaux convergents soulignent l'influence des bactéries symbiotiques d'insectes dans l'écologie et la diversification évolutive des insectes mineurs. Ils démontrent par ailleurs l'excitation qui entoure ces analyses et les espoirs qu'ils génèrent pour une compréhension globale des interactions entre les plantes et leur communauté biotique.

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# Chapter 1. General introduction

# 1.1. Plant-insect interactions

There are numerous species of plants and insects on earth and long-term evolutionary processes have led to very intimate and dynamic relationships between insects and their host-plants (Schoonhoven et al., 2005; Matthews and Matthews, 2009; Sauvion, et al., 2013; Willis and McElwain, 2014). Some interactions can be beneficial for the plant, as in the case of insect-mediated pollination or seed dispersion (Stebbins, 1972; Klein et al., 2007), and others are deleterious, as in the case of attack by herbivorous insects (Schoonhoven et al., 2005; **Zhang et al., 2013***). Plants are under selection pressure to maximize interactions with beneficial insects and minimize interactions with detrimental ones. To fight against antagonists, plants have developed an intricate defense network that includes specialized morphological structures or the production of secondary metabolites and proteins that have negative impacts on pathogens and herbivorous insects (Rani and Jyothsna, 2010; War et al., 2011a, 2011b). Plants defend themselves by influencing insects' preference, feeding efficiency, survival and/or reproduction (direct defense) but also by attracting other species such as natural enemies of the insects (indirect defense).

## 1.1.1. Plant manipulation as a strategy used by insects to improve plant nutritional quality

In the meantime, insects have developed a series of mechanisms and strategies to address the nutritional mismatch between what plants provide and what insects require (Schoonhoven et al., 2005; Behmer, 2009; Raubenheimer et al., 2009) but also to avoid physical and chemical plant defensive systems. A specific strategy involves insect reprogramming of host-plant development, resulting in new structures benefiting the parasitic herbivore at the expense of the plant (Price et al., 1987; Stone and Schönrogge, 2003; Giron et al., 2013). Insect-induced galls are key examples of such manipulations. Plants galls are tumor-like plant tissues that are induced by oviposition of the gall-inducers and/or the larval feeding activity of their offspring (Suzuki et al., 2015). Gall development often results in a combination of cell division

(hyperplasia) and growth (hypertrophy) (Oliveira and Isaias, 2010; Dias et al., 2013; Carneiro et al., 2014; Carneiro and Isaias, 2015; Suzuki et al., 2015). The gall induction often causes plant cell dedifferentiation and changes of cell walls and cell content/ultrastructure of the plant tissue (Carneiro et al., 2014). For example in the hessian fly-wheat system, one of the best-characterized examples, the larvae strongly modify the cells to induce a nutritive tissue that provides the developing larva with a diet rich in nutrients (Harris et al., 2006; Saltzmann et al., 2008).

Gall-inducers manipulation of host-plant development results in complex tissue reorganization, sometimes resulting in new plant organs (Mani, 1964; Rohfritsch, 1992; Harper et al., 2004; Shorthouse et al., 2005). But the ability of insects to manipulate their host-plants also refers to their capacity to strongly affect the plant primary and secondary metabolism. Nutrition is at the core of most interactions between organisms. In spite of different feeding methods, both sap-feeders and chewing insects can influence the plant quality by creating nutrient sinks and diverting nutrients away from the plant (Awmack and Leather, 2002; Giron et al., 2007; Schwachtje and Baldwin, 2008). Iconic examples of how insects manipulate their host-plants to increase their nutrition are galling and leaf-mining insects. Many studies have demonstrated that induced galls and mined tissues contain higher concentrations of nutritive compounds than control plant tissues. Gall nutritive tissues are usually rich in lipids, minerals, amino acids, sugars and/or starch to feed the developing larvae (Bronner, 1992; Awmack and Leather, 2002; Koyama and Akimoto, 2004; Zhu et al., 2008). Leaf-miners also reprogram their host plant to buffer seasonal variations of leaf nutritional quality and to avoid plant defenses (Body et al, 2013). This allows them to create an enhanced nutritional microenvironment especially in degenerating contexts when leaves enter senescence. Insects also developed various strategies to manipulate plant defenses. By altering the production of secondary metabolites such as phenolics, flavonoids and tannins (War et al., 2012), insects can overcome plant chemical defenses. Along with other strategies including contact and ingestion avoidance, excretion, sequestration, degradation of toxins and target-site mutations (Despres et al., 2007), insects can thus counteract host plant resistance.

## 1.1.2. Plant manipulation involves alteration of phytohormone levels

Because 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 (Tooker and Helms, 2014). Herbivorous insects can manipulate the plant hormonal biosynthesis and the hormone-dependent signaling pathways to regulate plant growth, defense and/or nutritional status and many organisms are now known to be able to produce phytohormones (Giron et al., 2013; Guiguet et al., 2016; see section 1.3). This includes gall-inducing bacteria (e.g. Stes et al., 2011, 2013), nodulating bacteria (e.g. Frugier et al., 2008), plant-associated fungi and viruses (e.g. Walters et al., 2008; Baliji et al., 2010), plant-parasitic nematodes (e.g. Siddique et al., 2015; Favery et al., 2016) and molluscan herbivores (Kästner et al., 2014). High levels of phytohormones have also been detected in the body, saliva or accessory glands of galling and leaf-mining insects suggesting their ability to produce and deliver these effectors to the plant (Giron et al., 2013; Bartlett and Connor, 2014; Tooker and Helms, 2014). As important regulators of plant growth, defense and metabolism, it is not surprising that phytohormones have been the target of phytophagous insects over the course of evolution (Schultz and Happel, 2004; Erb et al. 2012, Giron et al, 2013).

## 1.1.3 Main objectives of the thesis

Despite decades of research on plant-insect interactions, most studies have been conducted on ectophagous chewing insects and mechanisms underlying plant manipulation by gall-inducing and leaf-mining insects are still largely unresolved. More specifically, mechanistic understanding of how phytohormones operate in these plant reconfigurations is lacking due to limited information on the molecular and biochemical phytohormonal modulation following attack by plant-manipulating insects. **The objective of my Ph.D. is to provide an extensive characterization of how plant-manipulating insects modulate the plant global hormonal balance with a specific focus on the leaf-mining moth *Phyllonorycter blancardella*.** This is of great importance to gain a deeper understanding of the role played by phytohormones in

insect-induced plant manipulation, the underlying mechanisms, their origin, and their adaptive significance for insects. Because a great number of these plant-manipulators attack plants of high economic importance, information collected can also potentially help to develop innovative strategies to fight against these insect pests.

## 1.2. Phytohormones in plant-insect interactions

Plant hormones are a collection of structurally unrelated small molecules derived from various essential metabolic pathways (Santner et al., 2009). These molecules work at low concentrations (pmol range) (Venkatesan, 2015). Phytohormones are involved in essentially all plant physiological processes related to growth and development, including the regulation of quiescence and seed germination, root formation, flowering, branching and tillering, and fruit ripening (Tsavkelova et al., 2006). They also play a particularly important role in mediating plant responses to the environmental biotic and abiotic stress and induce or suppress the expression of genes involved in the synthesis of enzymes, pigments, and metabolites (Tsavkelova et al., 2006; Wasternack and Kombrink, 2009). They lie at the very core of key evolutionary trade-offs between growth, defense and reproduction (Herms and Mattson, 1992; Fujii and Zhu, 2009; Avanci et al., 2010; Giron et al., 2013).

The “classical” plant hormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) have rapidly emerged as key regulators of plants defense, plant physiology and plant ecology (Kessler and Baldwin 2002; Pieterse and Dicke 2007; Erb et al. 2008; Schwachtje and Baldwin 2008; Pieterse et al. 2012). There is a thorough understanding of the role of these three main phytohormones and they have been well studied at the molecular and ecological levels (Yang et al., 1980; Raskin, 1992; Santner et al., 2009, Dicke and Baldwin 2010, Erb et al. 2012). They are known to play major roles in regulating plant-insect interactions (Howe and Jander, 2008; Erb e al., 2012), albeit many other players are also implicated in this intimate interactions, such as abscisic acid (ABA), gibberellins (GBs), auxins (AUX) and cytokinins (CKs), which more recently have (re)emerged as important regulators as well (Robert-Seilaniantz et al., 2011, Giron et al. 2013).

A large number of plant-associated microorganisms such as bacteria, plant associated fungi and virus, plant-parasitic nematodes and molluscan herbivores are also able to synthesize phytohormones (Costacurta and Vanderleyden, 1995; Frugier et al., 2008; Walters et al., 2008; Stes et al., 2011; Kästner et al., 2014; Favery et al., 2016). High levels of phytohormones have also been found in eggs, neonates, body, saliva or accessory glands in insects strongly suggesting their ability to produce and deliver these phytohormones to the plant (Tooker and De Moraes, 2005; Giron et al., 2013; Bartlett and Connor, 2014; Tooker and Helms, 2014). Several studies have shown that gall-inducing insects have the biosynthetic ability to produce indole-3-acetic acid (IAA – the main form of AUX) from tryptophan (Mapes and Davies, 2001a; Yamaguchi et al., 2012; Tanaka et al., 2013). Recently IAA has also been found in non-galling silkworm (*Bombyx mori*) larvae (Suzuki et al., 2014) and in secretions and frass produced by the European corn borer *Ostrinia nubilalis* (Dafoe et al., 2013). Several studies have also identified ABA and CKs in several galling insects (Ohkawa, 1974; Van Staden and Davey, 1978; Dorchin et al., 2009; Straka et al., 2010; Tooker and De Moraes, 2011a, 2011b; Giron et al., 2013). High levels of CKs were also found in larval frass, gastrointestinal tracts and labial glands of leaf-mining insects *Ectoedemia argentipedella* and *E. Argyropeza* (Engelbrecht et al., 1969) and leaf-mining larvae of *Stigmella* spp. and *Phyllonorycter blancardella* (Engelbrecht, 1971; Body et al., 2013). By potentially producing these plant hormones, insects could suppress plant defenses and ensure an active nutrient supply for their own benefit but this could also potentially activate the plants to better defend themselves (Van Staden and Davey, 1978). Besides synthesizing phytohormones, insects could also directly act on the plant and influence the plant hormonal balance through 1) the mobilization of phytohormone precursors or storage forms, 2) the stimulation of phytohormone biosynthesis, and/or 3) the stimulation of phytohormone perception and/or signaling (**Figure 1**).

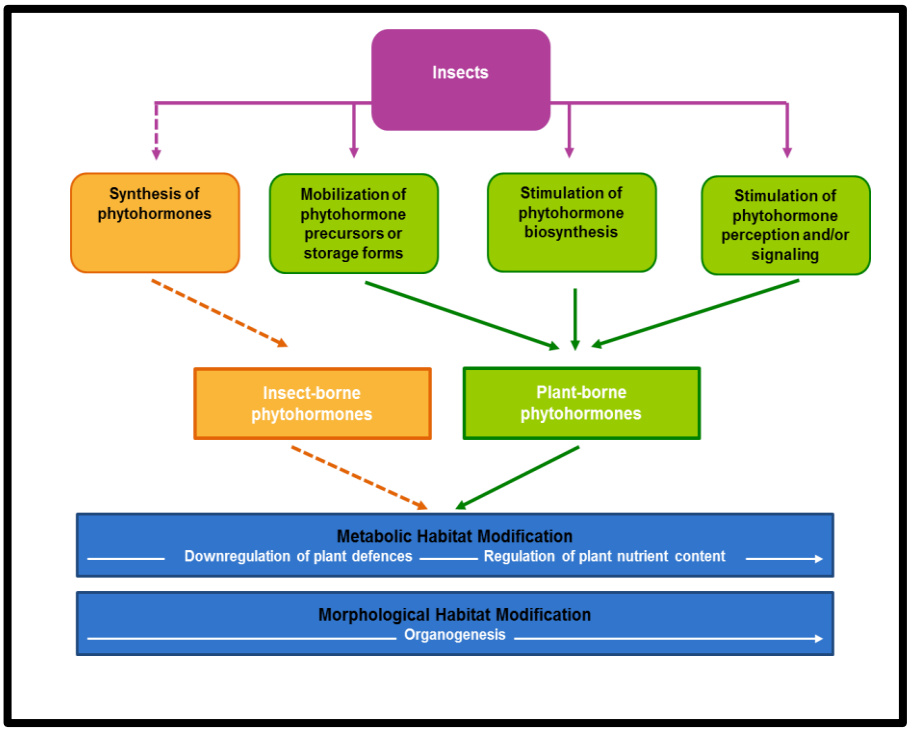


**Figure 1.** Modulation of phytohormones by insects and phytohormone-mediated plant modifications (Adapted from Giron and Glevarec, 2014).

There is also growing evidences that insect-associated microbes are active players in plant manipulation to the benefit of the insect host (Kaiser et al., 2010; Frago et al., 2012; Biere and Bennett, 2013; Body et al., 2013; Sugio et al., 2014; Su et al., 2015). They can affect, among other traits, insect host plant range (Hosokawa et al., 2007; Chu et al., 2013), feeding efficiency of the insect (Brune and Dietrich, 2015), insect metabolism (Douglas, 2013), ability of the insect to manipulate the plant physiology for their own benefit (Kaiser et al., 2010; Giron et al., 2016) and more generally insect diversification and speciation (Vavre and Kremer, 2014). Insect symbionts can indeed directly or indirectly affect the plant by interfering with plant signal transduction pathways, repressing or counteracting the expression of plant defense-related genes or altering plant primary and secondary metabolisms (Body et al., 2013; Giron et al., 2013; Sugio et al., 2014; Zhu et al., 2014). For example, the Colorado potato beetle (*Leptinotarsa decemlineata*) larvae release bacteria in their oral secretions inducing a SA-mediated plant response. Due to a negative crosstalk between SA and JA, this leads to the suppression of anti-herbivore defenses in tomato by decreasing production of JA and JA-

mediated anti-herbivore defenses (Chung et al., 2013). Insect symbionts can also affect plant-insect interactions through their direct or indirect effects on their insect host by providing new metabolic pathways (Moran et al., 2008; Douglas, 2013) and/or by altering insect reproduction (Engelstädter and Hurst, 2009; Ferrari and Vavre, 2011) or insect immunity with consequences on plant exploitation (Dubreuil et al., 2014). Finally, they can also modulate insect interactions with natural enemies or plant-associated organisms such as other herbivores, plant symbionts or plant pathogens (Frago et al., 2012; Biere and Bennett, 2013; Sugio et al., 2014; Chuche et al., 2016). Recent literature on manipulation of phytohormones by insect-associated bacteria is rapidly growing (Frago et al., 2012; Casteel and Hansen, 2014; Kazan and Lyons, 2014) and it is now much clearer that many phenotypes that are associated with insects can be attributed to their symbionts (Giron and Glevarec, 2014; Giron et al., 2016; Add ref Giron et al. 2017).

**Whether insects only influence plant-borne phytohormones or whether they produce phytohormones directly or indirectly through their symbiotic partners are still opened questions in most plant-insect interactions especially for plant manipulators.** A mechanistic understanding of how phytohormones operate in these plant reconfigurations requires focusing on the plant hormone biology, including their biosynthesis, transport, perception and how attacks by herbivorous insects and phytopathogenic microorganisms influence these processes.

## 1.2.1. Jasmonates

Jasmonic acid (JA) is a naturally occurring phytohormone found in higher plants (see chapter 2 for the biosynthetic pathway). Jasmonates are produced from the α-linolenic acid via the octadecanoid pathway (Wasternack et al., 2006). A combination of genetic, molecular, and biochemical analyses in *Arabidopsis* indicate that the main signal transduction chain linking JA synthesis to hormone-induced changes in gene expression involve five interacting players: the JA signal CORONATINE-INSENSITIVE1 (COI1), the putative receptor for jasmonates; the Skp/Cullin/F-box (SCF)-type E3 ubiquitin ligase SCFCOI1 destroying negative regulators of JA signaling; the JASMONATE ZIM-domain (JAZ) repressor proteins that are targeted by

SCFCOI1 for degradation by the ubiquitin/26S proteasome pathway; and transcription factors (TFs) that positively regulate the expression of JA-responsive genes (Katsir et al., 2008; Vasyukova and Ozeretskovskaya, 2009).

JA as well as its derivatives (jasmonates) play important roles in many aspects of plants growth and development, including sexual reproduction, growth control, and secondary metabolism (Parthier, 1991; Devoto et al., 2005; Avanci et al., 2010). For example, JA and methyl jasmonate treatment on soybean (*Glycine max*) can potently induce vegetative storage proteins gene expression in cell cultures, developing axes, leaves, and roots (Mason and Mullet, 1990). *Arabidopsis* mutant that is defective in the JA biosynthetic gene *CYP74A* (*allene oxide synthase*, *AOS*) shows severe male sterility due to defects in anther and pollen development and the sterile phenotype was completely rescued by exogenous application of methyl jasomonate and by complementation with constitutive expression of the *AOS* gene (Park et al., 2002). Additionally, they are also very important plant signaling molecules that orchestrate immune response to a wide range of inductive signals, including herbivory, pathogenesis, mechanical wounding and various other abiotic stresses, (Gundlach et al., 1992; Creelman and Mullet, 1997; Glazebrook, 2005; Vasyukova and Ozeretskovskaya, 2009; Santner et al., 2009). For example, research on JA signaling defective sterile mutant of tomato (*jasmonic acid–insensitive1* [*jai1*]) shows an inability to express JA-responsive genes and a severely compromised resistance to two-spotted spider mites (Li et al., 2004). *Arabidopsis* mutant (fad3–2 fad7–2 fad8) that is deficient in the jasmonate precursor linolenic acid contains negligible levels of jasmonate and shows extremely high mortality from attack by larvae of a common saprophagous fungal gnat, *Bradysia impatiens* (McConn et al., 1997). Jasmonate-insensitive mutant *coi1* of *Arabidopsis* shows enhanced susceptibility to infection by the fungal pathogens *Alternaria brassicicola* and *Botrytis cinereal* and treatment with MeJA elevates resistance to *A. brassicicola* (Thomma et al., 1998). JA is involved in plant protection by inducing the expression of genes leading to the production of proteinase inhibitors (Farmer and Ryan, 1990), flavonoids (Balbi and Devoto, 2008), sesquiterpenoids (Choi et al., 1994), or various antifungal proteins such as thionin and osmotin (Xu et al., 1994). However, microbes and insects can also counteract the JA-mediated plant immune response. For example, coronatine (COR) is a phytotoxin produced by several pathovars of *Pseudomonas syringae* (Bender et al., 1999), which is structurally similar to Jasmonoyl-isoleucine (JA-Ile). By activating the JA signaling pathway in a COI1-dependent way, the production of COR can thus reduce plants susceptibility to *P. syringae* (Farmer and Ryan, 1990; Zhao et al., 2003; Laurie-Berry et al., 2006; Katsir et al., 2008). Feeding by

whitefly (*Bemisia tabaci* type B) on *Arabidopsis* is suggested to repress JA-dependent RNAs thus suppressing JA-regulated plant defense (Zarate et al., 2007).

JA has also been reported to play important role in galler and leaf-miner/plant interactions. Gall-inducing insect *Eurosta solidaginis* likely avoid inducing JA synthesis, which could inhibit plant growth and hypertrophy, thereby inhibiting gall formation (Tooker and De Moraes, 2008). Feeding by leaf-miner fly (*Liriomyza sativae*) larvae activates SA-inducible genes (*PRb-1b* and *GluB*) which is likely to suppress JA-mediated plant defense (Kawazu et al., 2012). On the contrary, JA is also an effective defensive response to gall insects and leaf-miners. Tall goldenrod (*Solidago altissima*) plants exposed to male *E. solidaginis* volatile emissions exhibit more vigorous defense responses by significantly increasing JA production (Helms et al., 2013). Treatment of sweet pepper (*Capsicum annuum*) with JA causes a strong oviposition deterrence against the leaf-miner *Liriomyza trifolii* (Tebayashi et al., 2007).

## 1.2.2. Salicylic acid

Salicylic acid (SA) is synthesized through two different pathways that employ different precursors depending on the plant species, developmental stage and growth conditions (Tounekti et al., 2013; Wildermuth et al., 2001) (see chapter 2 for the biosynthetic pathway). The phenylpropanoid route starting in the cytoplasm from phenylalanine (phenylalanine ammonia-lyase pathway), and the isochorismate (IC) pathway operating in the chloroplasts of the cells (Tounekti et al., 2013). Interestingly, the IC pathway was first studied in bacteria (Shah, 2003; Tounekti et al., 2013). The Arabidopsis NPR1 protein (NONEXPRESSER OF PATHOGENESIS RELATED PROTEINS1, also called NON-INDUCIBLE IMMUNITY1, NIM1) is known as a link between the SA signaling molecule and defense-gene activation and acts as a receptor for the plant defense hormone SA (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996; Shah, 2003; Wu et al., 2012;). Overexpression of NPR1 could enhance the resistance in *Arabidopsis* and rice (Cao and Dong, 1998; Chern et al., 2001; Friedrich et al., 2001).

SA has been in focus of intensive research due to its crucial role in regulating plant physiological and biochemical processes and playing key roles in the regulation of their growth

and productivity (Arberg, 1981; Vlot et al., 2009). For example, after spraying with SA, a significant increase in growth characteristics, pigment contents and photosynthetic rate in maize is observed (Khodary, 2004). By inducing oxidative stress, applying SA can negatively regulate seed germination in *Arabidopsis thaliana* ((Rajou et al., 2006; Hayat et al., 2007; Xie et al., 2007). Additionally, SA is also highly involved in plant-microbe-insect interactions (Dempsey et al., 1999; DebRoy et al., 2004; Bostock 2005; Zarate et al. 2007; Giordanengo et al. 2010). SA has a key role in inducing systemic acquired resistance in plants (Durrant and Dong, 2004; Vlot et al., 2008), demonstrating the importance of SA in anti-herbivore/pathogen defense (White, 1979; Van Loon and Antoniw, 1982; Zarate et al., 2007). Transgenic tobacco and *A. thaliana* expressing the bacterial enzyme salicylate hydroxylase cannot accumulate SA, which makes the plants disable to induce systemic acquired resistance and leads to increased susceptibility to viral, fungal and bacterial pathogens (Delaney et al., 1994). Phloem feeding by the green peach aphid (*Myzus persicae*) on *Arabidopsis* induced expression of genes involved in the SA-mediated response pathway (Moran and Thompson, 2001). After the attack, SA levels could increase both at the site of infection and at distant sites (Santner et al., 2009). The response appears to trigger the synthesis of the volatile compound methyl salicylate (MeSA), which could attract insect predators in combination with other herbivore-induced volatile compounds (HIPVs Herbivore-Induced Plant Volatiles) (Seskar et al., 1998; James, 2003; De Boer and Dicke, 2004).

Recent researches have shown that pathogens and insects can manipulate SA-mediated plant defenses. For example, tomato treated with SA is more susceptible to *Alternaria solani* compared with wild-type plants, which suggests that SA is used by *A. solani* to promote its disease development in tomato (Rahman et al., 2012). Corn earworm uses salicylate from its host plant to activate four of its cytochrome P450 genes, which function in detoxifying potentially toxic secondary metabolites, thus making the induction of detoxifying enzymes rapid and specific (Li et al., 2002).

SA has also been reported to play a role in galler and leaf-miner/plant interactions. The leaf-miner fly *Liriomyza sativae* larvae feeding on leaf tissues optimize their survival by neutralizing JA-regulated defenses of the plant through induction of the SA pathway (Kawazu et al., 2012). The Asian rice gall midge (*Orseolia oryzae*) induces higher levels of SA and activates a resistance (*R*) gene of the plant when feeding on rice (Harris et al., 2003; Rawat et al., 2013).

Likewise, feeding by the gall midge *Dasineura marginemtorquens also* induce higher levels of SA in resistant genotypes of the willow *Salix viminalis* (Ollerstam et al., 2002).

## 1.2.3. Abscisic acid

Abscisic acid (ABA) is a cleavage product of carotenoids. It belongs to isoprenoid compounds of the sesquiterpene series (Tsavkelova et al., 2006) (see chapter 2 for the biosynthetic pathway). It is synthesized via a mevalonic-acid-independent (MEP-plastidal 2-C-methyl-D-erythritol-4-phosphate) pathway (Mehrotra et al., 2014). ABA synthesis starts with the $C_{40}$ carotenoid zeaxanthin precursor in the plastid, and ends with the synthesis of ABA aldehyde in the cytosol. It is then converted into ABA through oxidization (Seo and Koshiba, 2002). The oxidative cleavage reaction that leads to the formation of xanthoxin is considered to be the rate-limiting step and the enzyme 9-*cis*-epoxycarotenoid dioxygenases (NCED) is the key enzyme in ABA biosynthesis (Tan et al., 1997). The biosynthetic pathway of ABA was clarified through genetic researches primarily in maize and *Arabidopsis* (Nambara and Marion-Poll, 2005). A new family of soluble ABA receptors, named PYR/PYL/RCAR, has emerged as ABA sensors able to inhibit the activity of specific protein phosphatases type-2C (PP2Cs) in an ABA-dependent manner (Santiago et al., 2012).

ABA sometimes called the “stress hormone”, has been recognized to be associated with many important aspects of plant development, including synthesizing seed storage proteins and lipids, promoting seed desiccation tolerance and dormancy, and inhibiting the phase transitions from embryonic to germinative growth and from vegetative to reproductive growth (Addicott, 1983; Ruth et al., 2002; Santner et al., 2009; Tooker and Helms, 2014). Research on *Nicotiana plumbaginifolia* Viv. shows that dry dormant seeds contained more ABA than dry after ripened seeds which reveals an important role for ABA synthesis in dormancy maintenance in seeds (Grappin et al., 2000). ABA is also involved in the production of antioxidants (Jiang and Zhang, 2002).

ABA is also known to be involved in plant defense against insects and pathogens. Infestation by tobacco mosaic virus (TMV) can increases ABA concentrations in tobacco and treatment of

a local-lesion-forming cultivar of tobacco with exogenous ABA at low concentration significantly decreases susceptibility to infection with TMV (Fraser 1982; Whenham et al., 1986). ABA-deficient tomatoes support higher growth rates of caterpillars *Spodoptera exigua* and *Trichoplusia nicaterpillars* in comparison to wild-type plants, which demonstrates that ABA interact with resistance to insects (Thaler and Bostock, 2004). ABA can also activate JA-regulated plant defense responses to herbivore attack (Vos et al., 2013). For instance, *Pieris rapae* larvae feeding on *A. thaliana* or exogenous application of ABA lead to increased expression levels of MYC2, the transcription factor which regulate distinct branches of the JA signaling pathway.

ABA is also involved in galling and leaf-mining insect-plant interactions. ABA play a key role in senescence and abscission-promoting effects used by plants to fight against sessile insects (Lim et al., 2007). Leaf abscission is indeed an efficient way to fight against galling and leaf-mining herbivorous insects that are trapped in the plants (Lim et al., 2007). However, several results suggest that ABA concentrations may influence gall formation processes (Tooker and Helms, 2014). During larval feeding of the *Dryocosmus kuriphilus* and *Lipara lucens*, high concentrations of ABA have been found in gall tissues. (De Bruyn et al., 1998; Wood and Payne, 1998). After attack by gall-inducing leafhopper *Cicadulina bipunctata*, ABA levels were ten to thirty times higher in susceptible maize plants than control ones (Tokuda et al., 2013). In other systems ABA levels are decreased such as in gall induced by *Gnorimoschema gallaesolidaginis* on *S. altissima* stems (Tooker and De Moraes, 2011a). Similarly, attacks by hessian fly larvae *Mayetiola destructor* cause a decrease of ABA in both wheat and rice plants relative to unattacked controls (Zhu et al., 2011). Because ABA can trigger plant defense responses, occasionally acting independently of JA (Bostock, 1999; Erb et al., 2009), the gall-inducers may be actively manipulating ABA levels thus to avoid inducing plant defensive responses (Tooker and De Moraes, 2011a, Zhu et al., 2011; Tooker and Helms, 2014). But the information of ABA for leaf-miner are scarce.

## 1.2.4. Auxin

Auxin (AUX) is a class of plant hormones with some morphogen-like characteristics (Zhang et al., 2014) (see annex for the biosynthetic pathway). The most important naturally occurring active AUX in plants is IAA (Kobayashi et al., 1993) that could also be produced by bacteria (Patten and Glick, 1996). IAA biosynthesis can occur via two major routes: tryptophan (Trp) dependent and Trp independent pathways (Wright et al., 1991; Normanly et al., 1993; Woodward and Bartel, 2005). Feeding plants with labeled Trp results in the production of labeled IAA demonstrating that Trp is a precursor for auxin biosynthesis (Wright et al., 1991; Normanly et al., 1993). IAA can also be synthesized from Trp-independent pathways, including the indole-3-acetaldoxime (IAOx) pathway, which has been identified only in crucifers (Sugawara et al., 2009), the indole-3-acetamide (IAM) pathway that could come from both a IAOx dependent or independent pathway, and the indole-3-pyruvic acid (IPyA) pathway, the main contributor to the production of free IAA (Zhao, 2012). The molecular components of the Trp-independent pathway have not been identified so far (Zhao, 2014). There are two proteins which may function as auxin receptors: AUXIN BINDING PROTEIN 1 (ABP1), which binds two auxin molecules as a dimer (Napier et al., 2002), and TRANSPORT-INHIBITOR-RESISTANT1 (TIR1) that binds auxin in a sandwich complex consisting of TIR1, auxin and an IAA protein (Tan et al., 2007). It has been proposed that auxin signal transduction is mediated by a conserved signaling cascade consisting of three protein kinases: the mitogen-activated protein kinase (MAPK), MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK) (Kovtun et al., 1998). There are three major classes of AUX-responsive genes that involved in molecular mechanism of AUX action: *Aux/IAA* family, *GH3* family and small auxin-up RNA (*SAUR*) family (Guilfoyle, 1999; Woodward and Bartel, 2005).

AUX has been known for regulating all aspects of plant growth and development (Zhao, 2010) by determining the rate of cell division and expansion, or a cell's developmental fate (Weijers and Friml, 2009). It is also involved in modulating plant defense responses against various diseases (Kazan and Manners, 2009) and may negatively influence plant defense against insects (Tooker and De Moraes, 2007; Zhu et al., 2011). It has been shown that AUX responsive gene *GH3* play roles in the plant defense in *Arabidopsis* and rice (Bari and Jones, 2008). For example, *GH3.5* modulates AUX signaling during pathogen infection (Zhang et al., 2007). Over

expression of *GH3-8* results in enhanced resistance to the rice pathogen *Xanthomonas oryzae pv. oryzae* (Xoo) (Ding et al., 2008). However, exogenous application of AUX promotes disease caused by *Agrobacterium tumefaciens* (Yamada, 1993) and co-inoculation of *P. syringae* pv. *Maculicola* (Psm) 4326 and AUX promote both disease symptom and pathogen growth in *Arabidopsis* (Wang et al., 2007).

AUX appears to play a key role in plant abnormal growth such as gall initiation and formation, providing good examples on how insects manipulate their host plants for their own benefits. Larvae of *Eurosta solidaginis* (Diptera) and *Gnorimoschema gallaesolidaginis* (Lepidoptera) harbor high concentrations of IAA and induce higher levels of IAA in young galls (Mapes and Davies, 2001a, Tooker and De Moraes, 2001a). Interestingly, in the *E. solidaginis* system, galls continue to grow even without apical buds and leaves, the sources of IAA (Mapes and Davies, 2001a). IAA was also found in larvae of the gall-inducing pteromalid wasp *Trichilogaster acaciaelongifoliae*, and the concentrations were thousands of times more abundant than in gall or controlled tissues (Dorchin et al., 2009). Gall-inducing sawfly larvae (*Pontania* sp.) also contain high concentrations of IAA and the endosymbiotic bacteria may contribute to IAA production (Yamaguchi et al., 2012). These examples suggest that gallers are the source of IAA involved in gall formation. Evidence for how AUX works in leaf-miners are scarce, but is has been demonstrated that application of AUX and herteroauxin on poplar leaves could induce intumescences which are similar to those found in the tunnels of leaf-miners (La Rue, 1936,1937).

## 1.2.5. Cytokinin

Cytokinins (CKs) are essential plant hormones that derives from adenine (Silver et al., 1996) (see chapter 2 for the biosnthetic pathway). Naturally occurring CKs are a complex group of $N^6$ substituted adenine derivatives that include isoprenoid CKs or aromatic CKs, but the isoprenoid ones are more common and abundant in higher plants (Mok and Mok, 2001; Klanicová et al., 2006; Kamada-Nobusada and Sakakibara, 2009). Common naturally occurring isoprenoid CKs are *trans*-zeatin (*t*Z), isopentenyl adenine (iP), *cis*-zeatin (*c*Z) and dihydrozeatin (DZ) (Sakakibara, 2006).

In general, the rate-limiting step in CKs biosynthesis is catalyzed by the enzyme adenosine phosphate-isopentenyltransferase (IPT) that catalyzes the *N*-prenylation of adenosine 5'-phosphate (from adenosine monophosphate (AMP), adenosine diphosphate (ADP) or adenosine triphosphate (ATP)) at the $N_6$-terminus with dimethylallyl diphosphate (DMAPP) or 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBDP) (Kamada-Nobusada and Sakakibara, 2009). CK signaling involves a multistep two-component system through histidine (His) and aspartate (Asp) phosphorelay, which is similar to bacterial and yeast signal transduction pathways (Müller and Sheen, 2007b; To and Kieber, 2008). Studies in *A.thaliana* have shown that the CK receptors are predicted to signal through His phosphotransfer proteins to ultimately alter the phosphorylation state of the *Arabidopsis* response regulators (ARRs) in a multistep phosphorelay (Hutchison and Kieber, 2002). ARRs can be broadly classified into type A and type B (To et al., 2004). Genetic analysis has shown that Type A ARRs are generally thought to act as inhibitors of CK signaling while type B ARRs are able to activate the transcription of CK responsive genes (Sakai et al., 1998; Sakai et al., 2000; Sakai et al., 2001; To et al., 2004).

CKs control numerous processes in plants' development and response to external stimuli (Müller and Sheen, 2007a). These include for instance their role in the delay of plant senescence, the regulation of source-sink relationships and the response to external stress such as wounding (Gan and Amasino, 1995; Dervinis et al., 2010; Giron et al., 2013).

Recently there has been an increased interest for CKs, highlighting their important role in the regulation of plant defense against pathogens and insects (Siemens et al., 2006; Dervinis et al., 2010; Giron et al., 2013). Transgenic *Arabidopsis* overexpressing CK oxidase/dehydrogenase genes shows resistance against *Plasmodiophora brassicae* infection that suggest that CKs act as key regulators in the development of clubroot disease (Siemens et al., 2006). Exogenous applications of zeatin to the transgenic *plumbaginifolia Nicotiana* plants enhances the level of resistance to the tobacco hornworm and almost completely inhibits normal development of the green peach aphid nymphs (Smigocki et al., 1993).

Pathogens and insects to successfully colonize their host-plant can also use CKs. Bacteria commonly carry genes for CK synthesis in plasmids to form symbiotic or pathogenic relationships with plants, such as *Rhodococcus fascians* (syn: *Corynebacterium fascians*) (Scarbrough et al, 1973; Armstrong et al, 1976; Murai et al., 1980), *Agrobacterium tumefaciens* (Barry et al. 1984), *Pseudomonas savastanoi* (Powell and Morris 1986) and *Sinorhizobium meliloti* (Kisiala et al., 2013). Infection with *R. fascians* modifies CK biosynthesis by sending

a key enzyme into plastids of the host plant *Arabidopsis* to promote tumorigenesis (Sakakibara et al., 2005). Interestingly, the first identification of a gene encoding a CK *de novo* biosynthetic enzyme was carried out in bacteria (Akiyoshi et al., 1984; Barry et al., 1984). Additionally, several data strongly suggest that insect endosymbiotic bacteria could also produce CKs allowing insects to manipulate the host plant for their own benefits (Yamaguchi et al., 2012; Giron et al., 2013; Bartlett and Connor, 2014; Giron and Glevarec 2014).

Similar with AUX, CKs also play a role in plant growth and cell division (Davies, 2004), and recent data highly suggest that CKs may be part of the strategy used by gall-inducers and leaf-miners to colonize and manipulate their host-plant (Giron et al., 2013; Tooker and Helms, 2014; Giron et al., 2016). In two of the gall-inducing species *E. solidaginis* and *T. acaciaelongifoliae,* larvae not only contain high levels of AUX but also much higher levels of CKs than surrounding gall tissues, indicating that the insect could have been the source of CKs, in addition to AUX (Dorchin et al., 2009; Mapes and Davies, 2001b). Very large amounts of CKs have also been found in many leaf-mining insects. As early as 1969, Engelbrecht et al. already found that in lepidopteran leaf-miners *Stigmella argyropeza* Z. and *St. argentipedella Z.* infesting leaves of *Populus tremula* and *Betula pendula,* CKs were highly accumulated. Large quantities of CKs were also found in the larval labial glands, which highly suggest that the labial glands of the larvae were probably an active site of CK biosynthesis (Engelbrecht et al., 1969).

# 1.3. Crosstalks among different phytohormones

Interactions of plants with members of their ecological community, once perceived by the plant, can lead to a profound metabolic reconfiguration of the plant physiology, which favors beneficial organisms and deters antagonists like pathogens or herbivores (e.g. Kessler and Baldwin 2002; Pieterse and Dicke 2007; Schwachtje and Baldwin 2008). Because plants suffer from a vast array of herbivorous insects and microbial pathogens with diverse modes of attack, their ability to perceive and respond specifically to these attackers is the basis of their survival. Plant defense against insects and microbes involves multiple signal transduction pathways that are mediated by a network of plant hormones (Adie et al. 2007; RobertSeilaniantz et al. 2007).

A key step in this defense process is the recognition of the biotic partner and the activation of a signaling network that will regulate both locally and systemically, the biochemical reconfiguration of the plant (e.g. Pieterse et al. 2009). Plant defenses are usually

activated following detection of Pathogen, Herbivore or Damage-Associated Molecular Patterns (PAMPs, HAMPs and DAMPs respectively) by plant Pattern Recognition Receptors

(PRR) (Kazan and Lyons, 2014; Zhu et al., 2014; Zipfel, 2014). Such detection leads to the activation of signaling pathways that induce transcription factors and ultimately leads to production of defense secondary metabolites and Pathogenesis-Related (PR) proteins (Tsuda and Somssich, 2015). However, continuing activation of plant defense also relies on hormone-dependent signaling pathways. Crosstalks between phytohormones are a powerful regulatory mechanism that strongly influences the outcome of plant-insect-microbe interactions and allows plants to mount an appropriate defense response (Koornneef and Pieterse, 2008; Ponzio et al., 2013).

As mentioned in previous sections, JA, and SA have been well documented as the major players in regulating plant defense responses against biotic stress (Dong, 1998; Glazebrook, 2005). JA is considered as the most important phytohormone associated with plant defense against chewing herbivores and activates the expression of both direct and indirect defenses (Rani and Jyothsna, 2010; Shivaji et al., 2010; War et al., 2011a), while SA is usually linked with activating defense responses against pathogens and sap-feeding insects (Grant and Lamb, 2006). JA and SA usually act antagonistically. For example research with tobacco plants has shown that SA inhibits JA-mediated activation of basic pathogenesis-related (PR) gene expression while JA inhibits SA-mediated activation of acidic PR gene expression (Niki et al., 1998). Antagonistic interactions between SA and JA also affect the expression of PR genes in tomato (Thaler et al., 1999). *Arabidopsis* NPR1 (receptor of SA) (Conrath et al., 2001; Kohler et al., 2002) has emerged as a crucial modulator of the SA-JA crosstalk and is thought to play an important role in regulating the plant defense responses to the encountered attackers (Dong, 2004). However, many evidences have also shown that JA and SA can have, sometimes, synergistic interactions (Xu et al., 1994; Beckers and Spoel, 2006). For example, induction of JA synthesis by adding SA was observed in sorghum plants (Salzman et al., 2005).

AUX acts mostly in synergy with JA while there is an antagonistic interaction between AUX and SA (Kazan and Manners, 2009). For example treatment with JA could induce AUX

biosynthetic enzymes in cabbage while SA application has a counterproductive effect on AUX (Grsic et al., 1999). Additionally, activation of AUX signaling in *Arabidopsis* induces the suppression of SA biosynthesis and SA signaling thus inducing susceptibility towards an avirulent strain of *P. syringae* pathovar (pv.) tomato (Mohr and Cahill, 2007) while SA-treated *Arabidopsis* repress AUX signaling pathway thus to defend the plants against the pathogens *Hyaloperonospora parasitica* Noco2 (Wang et al., 2007). Interestingly, AUX and SA sometime also work synergistically. It has been reported that a member of AUX responsive GH3 family genes is required for elevated accumulation of SA and increased expression of PR gene in response to *P. syringae* (Zhang et al., 2007).

From the beginning of discovery as inducers of plant cell division in culture, CKs have been linked to AUX (Miller et al., 1955, 1956). Indeed, CKs and AUX have many similar functions, such as regulation of apical meristems, senescence, stress response, and gall formation (Allan, 1979; Kim et al., 2006; Tran et al., 2007; Toklikishvili et al., 2010; Murray et al., 2012). Recent studies also shed light on the molecular mechanisms how CKs and AUX strongly interact with each other to promote and maintain plant growth and development suggesting that both CKs and AUX may play a pivotal role in plant-insect-microbe interactions (Costacurta and Vanderleyden, 1995; Hirsch et al., 1997; Pernisová et al., 2011; Tooker and Helms, 2014; Schaller et al., 2015). CKs can also crosstalk with other phytohormones such as SA and JA. CKs application to wild type *Arabidopsis* can promote plant resistance against pathogens by enhancing the SA response through the activation of NPR1 (Choi et al., 2010). JA and methyl jasmonate strongly inhibit soybean callus growth induced by either kinetin, a purine cytokinin, or PCPU (an urea cytokinin) (Ueda and Kato, 1982). Biochemical experiments suggest that ABA has a synergistic effect with JA induced responses to insects (Wasternack and Parthier, 1997). In tomato, ABA also interferes with SA-mediated resistance to *Botrytis cinerea* (Audenaert et al., 2002). Research on *Arabdopsis* also shows that ABA suppresses plant immune responses to the bacterial phytopathogen *P. syringae* by down-regulating SA biosynthesis and SA-mediated defenses (de Torres Zabala et al., 2009).

# 1.4. Summary

Plant hormone signaling pathways are differentially regulated in different biological systems and over the time of the plant-herbivore/pathogen interactions. They can act individually, synergistically or antagonistically (Zhao et al., 2007) and the outcome of phytohormonal crosstalk can vary between different biological systems. The complex regulatory and interaction network occurring between plant hormone signaling pathways has been the target of plant attackers over the course of evolution to modify the physiology of their host for their own benefits (Schultz and Happel, 2004; Robert-Seilaniantz et al., 2011). Many insect herbivores and microbes involved in symbiotic or pathogenic interactions can produce plant hormones that potentially interfere with plant immunity, development and metabolism (Costacurta and Vanderleyden, 1995; Schultz, 2002; Felton and Tumlinson, 2008). For example CKs, AUX, ABA, JA and SA are all reported to be involved in interactions between gall-inducing insects and their host-plants (Wood and Payne, 1988; Hori, 1992; Straka et al., 2010; Tooker and De Moraes, 2011b; Zhu et al., 2011; Tooker and Helms, 2014). In contrast, data on other plant-manipulating insects such as leaf-miners still remain scarce. **The exact role of phytohormones in these systems and how they interact with each other is unclear and more specialized research is now needed to get a deeper understanding of how plant hormones operate in these plant-insect interactions. Because insect symbionts are key players in many plant-insect interactions, I suggest that they should be taken into account to elucidate the possible origin of the chemical stimuli involved in insect-induced plant manipulations. I also suggest that looking at similarities in the phytohormonal responses elicited by plant reprogrammers in a diversity of biological systems may help to identify key plant functions that are targeted during plant manipulation and their adaptive significance for insects.**

# 1.5. Thesis outline

My PhD study is focused on the "Chemical and Molecular interplays between plant-manipulating insects and their host-plants". It specifically focuses on *Phyllonorycter*

*blancardella* (Lepidoptera: Gracillariidae) developing on *Malus* domestica. This leaf-mining moth is a polyvoltine microlepidopteran of apple trees that performs two to five generations during the growing season (Pottinger and Leroux, 1971). *P. blancardella* is composed of a group of cryptic species that can co-occur on the same host-plant. The larva establishes and maintains a permanent 'feeding area' for its development (Mozuraitis et al., 1999; Body et al., 2013). The first three instars that feed on interstitial fluids are fluid-feeders and the last two instars that consume the lower and upper parenchyma are tissue-feeders (Body et al., 2013) (**Figure 2**). In autumn, insects manipulate the leaf physiology to induce 'green islands' which are characterized by photosynthetically active green patches in otherwise senescing leaves (Kaiser et al. 2010). They can also suppress the plant defense system, resist the senescence program and maintain the food supply (Giron et al., 2007). In this system, endosymbiotic bacteria *(Wolbachia)* associated with insects play a key role in the plant insect interaction allowing the insect to take the control of the plant machinery (Kaiser et al., 2010; Gutzwiller et al. 2015). This attack strategy can have a serious economic consequence by reducing the quantity and quality of apples (Pottinger and Leroux, 1971; Reissig et al., 1982). Manipulation of the plant is strongly regulated by phytohormones, some of them being presumably directly produced by the insect and/or its symbiotic bacteria. Previous works have already shown that CKs are involved in the plant manipulation by *P. blancardella* but only few CKs were quantified. Nothing was known about 1) the complete set of CKs involved in this plant-insect interaction, 2) the specific origin of these CKs and their dynamics, and 3) the potential modulation of other phytohormones and especially AUX.

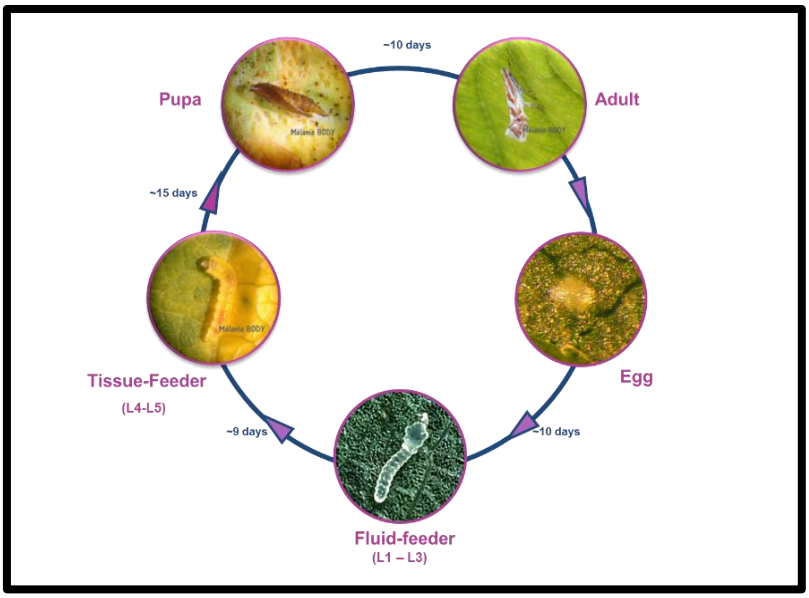


**Figure 2A.** Life circle of *Phyllonorycter blancardella.* (Pictures from Mélanie Body)

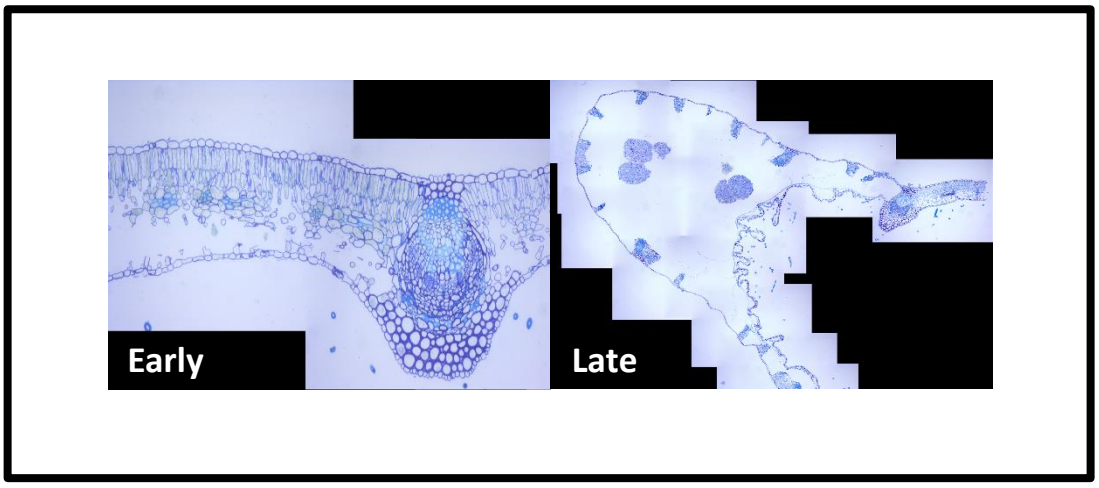


**Figure 2B.** Apple tree mined leaf tissues at the early and late larval stages. (Pictures from Antoine Guiguet). Larva starts by delimitating the total surface of their mine (L1) and by cutting through the spongy parenchyma (L2–L3). Some spongy parenchyma cells remain tied to the lower epidermal cells while others remain attached to the palisade parenchyma above. At this stage, palisade parenchyma tissues remain intact and linked to the upper epidermal cells. Area exploited by the insect is bordered by the major vein on one side and usually by a secondary vein on the other side. Mechanical stress on plant cells remains very limited at this stage with no major cell consumption, as just a few cells from the spongy parenchyma are damaged by the larva (Body et al., 2015). Tissue-feeder instars (L4–L5) cannot expand the total surface of the mine but progressively feed from internal tissues. The larvae consume mesophyll tissues, but leave vascular tissues and the epidermis intact. The mine is formed by the consumption of the mesophyll tissues, starting by the spongy parenchyma and followed by the palisade parenchyma. Areas where all but the epidermis has been consumed result in the formation of feeding windows appearing as white translucent patches. These feeding windows are separated by green, starch-containing uneaten tissues called interwindows. Larvae usually leave an intact area in the middle of the mine. Silk threads secreted by the larvae lead to the shrinkage of the lower epidermis giving the mine a tentiform shape. After the hypermetamorphosis of larval mouthparts, the feeding pattern of larvae evolves leading to more drastic consequences on the leaf anatomy with major cell consumption (Body et al., 2015).

My PhD work aims at elucidating the plant response following attack by the leaf-miner by analyzing how the leaf-miner reprograms the host-leaf transcriptome to modulate phytohormones associated with nutrient mobilization and plant defense (Chapter 2) (**Figure 3A**). This also includes the analysis of phytohormone profiles in attacked and un-attacked apple trees, especially CKs (Chapters 2/3) (**Figure 3A, B**) and AUX (Chapter 4) (**Figure 3C**) profiles, and how this might interfere with the global hormonal balance (Chapter 2) (**Figure 3A**). Molecular and chemical ecology approaches that I developed also aimed to provide key information towards the identification of the origin of phytohormones (and more specifically CKs as key chemical mediators of the interaction) altered by the insects thus to figure out if they are produced by the plant, the insect and/or the endosymbiotic bacteria (Chapters 2/3) (**Figure 3A, B**). Data collected on a species of the *P. blancardella* complex that use the same ecological niche but that does not host *Wolbachia* (*P. mespillela*) was used as a comparison with *P. blancardella* to investigate the role of the bacterial symbiont in the modulation of AUX and CKs (Chapter 4) (**Figure 3C**). Information about the dynamics of plant manipulation is also addressed (Chapter 3) (**Figure 3B**). My general discussion addresses the evaluation of the ecological consequences of the plant manipulation in terms of plant direct and indirect defenses, including volatiles, and insect nutrition. In order to get a deeper understanding of the role played by phytohormones in insect-induced plant manipulation, the underlying mechanisms and their adaptive significance for insects, I also address evolutionary perspectives by comparing results obtained on the leaf-miner system with data generated on CK and AUX profiles in the gall induced by the Hessian fly on wheat (along with data available in the literature).

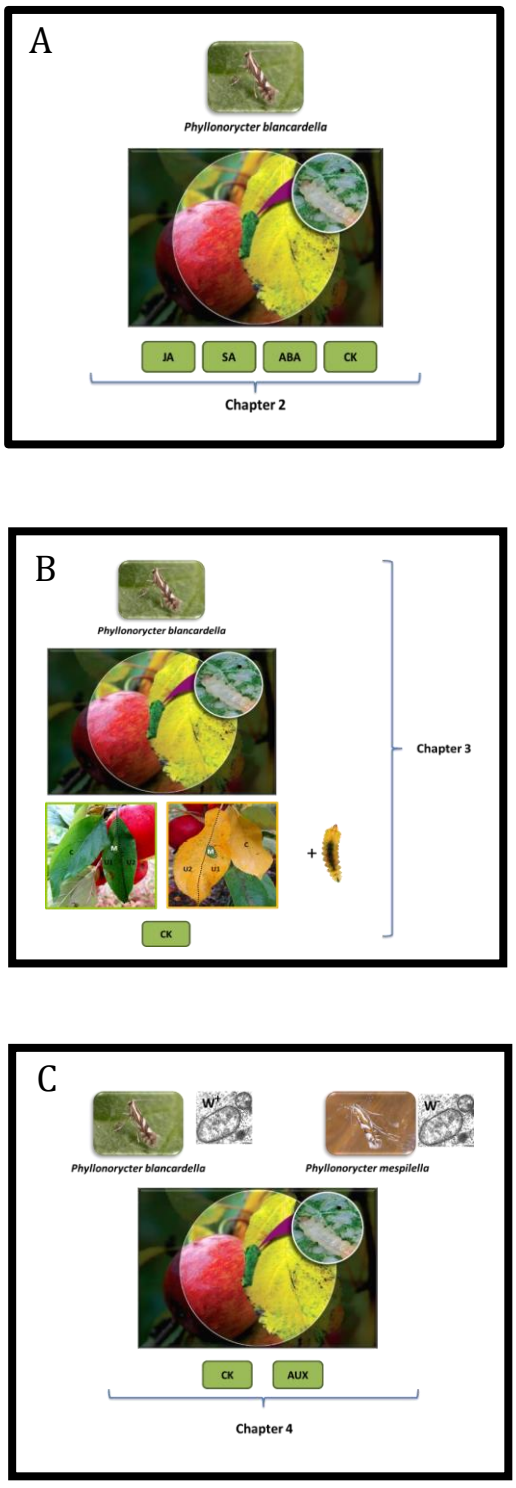


**Figure 3. Thesis outline. A.** Analysis of plant response at the gene expression level and at the biochemical level in leaves attacked by *P. blancardella*. **B.** Analysis of CK dynamics (green *vs.* yellow leaves) and CK origin in leaves attacked by *P. blancardella*. **C.** Analysis of AUX alterations and comparison of CK modulation between the *Wolbachia*-free leafminer *P. Mespilella* and *P. blancardella*.

# Chapter 2. Leaf-mining by *Phyllonorycter blancardella* reprograms the host-leaf transcriptome to modulate phytohormones associated with nutrient mobilization and plant defense

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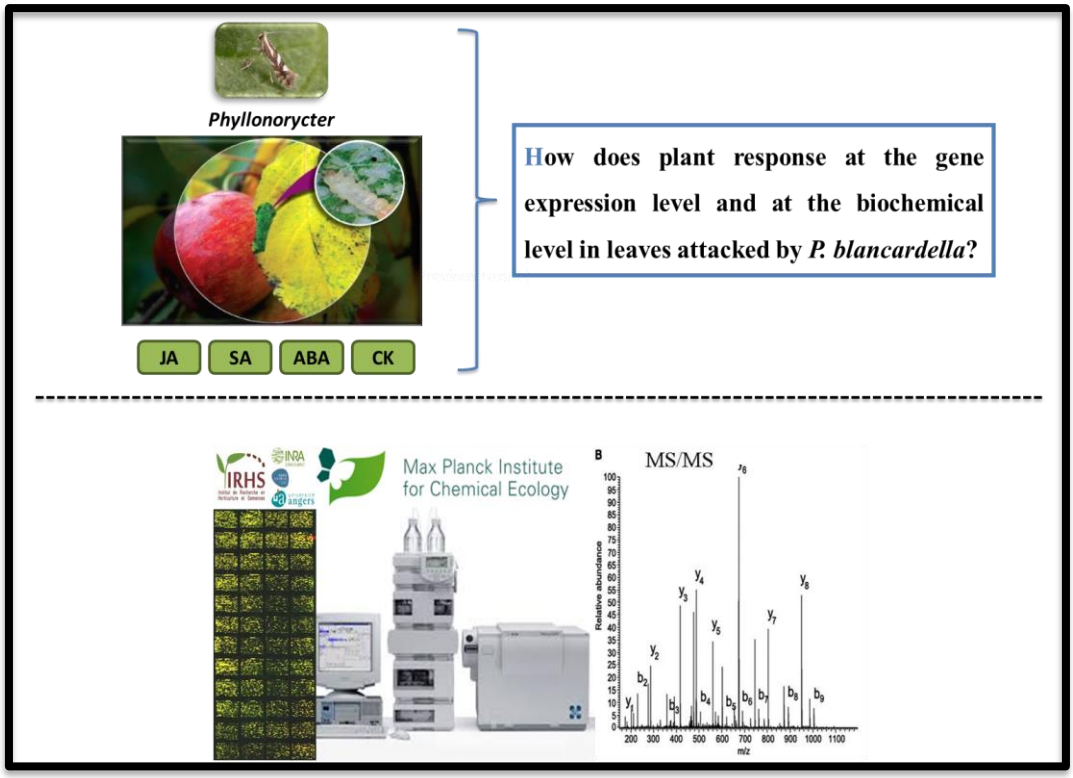
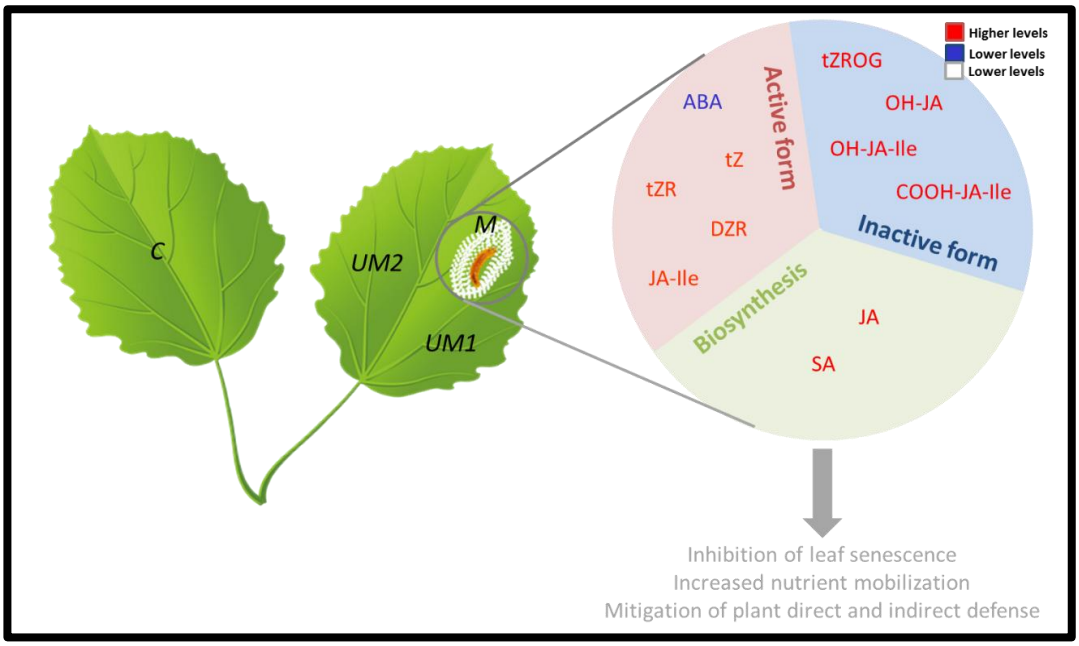
# 2.1. Graphical abstract



# 2.2. Introduction

## 2.2.1. Objectives.

The first objective of this chapter 2 was to provide an **extensive characterization** of how the leaf-miner *Phyllonorycter blancardella* modulates the CK profile in leaves of *Malus domestica*. Previous results on this system established that larvae are able to induce "green-islands" on senescing leaves and that the feeding area is characterized by similar or even greater levels of CKs than non-senescing green leaves (Giron et al., 2007; Kaiser et al., 2010; Body et al., 2013). However, these results were obtained by using a targeted enzyme-linked immunosorbent assay (ELISA), allowing the characterization of only a limited number of CKs (iP isopentenyladenine; iPR *N6*-isopentenyladenosine; Z zeatin; ZR zeatin riboside).

*Working hypothesis:* We expected that the phytohormonal alteration of leaves would involve a much larger panel of CKs.

The second objective was to provide key information towards the **identification of the origin of CKs** altered by the insects thus to figure out if they are produced by the plant or the insect. *Working hypothesis:* We expected that plant CK-biosynthetic genes would be overexpressed in the mined areas resulting in a global increase of CK levels (besides already characterized CK compounds).

The third objective was to study how the modulation of the plant CK profile might interfere with the **plant global hormonal balance**.

*Working hypothesis:* We expected that leaf-mining by *P. blancardella* woud lead to a strong reconfiguration of the plant phytohormonal balance associated with increased nutrient mobilization, inhibition of leaf senescence and inhibition of plant direct and indirect defense.

## 2.2.2 Methods.

To reach these objectives, major phytohormones and transcriptional activity of plant cells in contact with *P. blancardella* were monitored by microarray and LC-MS/MS and compared to that of control unmined leaf tissues.

## 2.2.3. Results.

Here, we report an important transcriptional and biochemical reprogramming in the mined area reflecting the intimate relationship between the insect and its host-plant. We show, that CKs strongly accumulate in mined tissues despite a weak expression of plant CK-related genes. Leaf-mining is also associated with enhanced biosynthesis of JA precursors but not the active form, a weak alteration of the SA pathway and a clear inhibition of the ABA pathway.

## 2.2.4. Conclusions.

Our study demonstrates that the plant is not responsible for the increased levels of CKs in mined areas. It rather consolidates previous results suggesting that insects may produce and deliver CKs to the plant as a strategy to manipulate the physiology of the leaf to create a favorable nutritional environment. Due to the specific association between *P. blancardella* and *Wolbachia* whether these phythormones originate from microbial symbionts remains to be answered (see chapters 4 and 5).

We also demonstrate that leaf-mining by *P. blancardella* leads to a strong reprogramming of the plant phytohormonal balance associated with increased nutrient mobilization, inhibition of leaf senescence and mitigation (but not total inhibition) of plant defense. How these phytohormonal alterations translate into effective plant physiological direct and indirect defense remains to be answered (see main discussion).

Because of CKs involvement in gall induction by bacteria and insects, and more generally in plant interactions with various biotic invaders, our results are likely to be relevant for other plant- associated interactions. Further research is needed to investigate similarities between strategies adopted by gall-inducers and leaf-miners, especially the possible role played by AUX that are key signaling molecules modulated by gall-inducers (see chapter 5 and main discussion).

Finally, more research at the biochemical and molecular scales are also necessary to understand fully how phytohormones affect a plant's response to endophytic herbivores, as most of our current knowledge comes from ectophytic leaf-feeding or sap-feeding insects. This will also allow us to gain a deeper understanding of the role played by phytohormones in insect-generated extended phenotypes, the mechanisms underlying plant manipulation, their origin, and their adaptive significance for insects. Ultimately, this will lead to a more comprehensive knowledge on the role of phytohormones in the evolution of plant-insect-microbe interactions.

These results are published in *Journal of Insect Physiology*.

# 2.3. Abstract

Phytohormones have long been hypothesized to play a key role in the interactions between plant-manipulating organisms and their host-plants such as insect-plant interactions that lead to gall or ‘green-islands induction. However, mechanistic understanding of how phytohormones operate in these plant reconfigurations is lacking due to limited information on the molecular and biochemical phytohormonal modulation following attack by plant-manipulating insects. In an attempt to fill this gap, the present study provides an extensive characterization of how the leaf-miner *Phyllonorycter blancardella* modulates the major phytohormones and the transcriptional activity of plant cells in leaves of *Malus domestica*. We show here, that cytokinins strongly accumulate in mined tissues despite a weak expression of plant cytokinin-related genes. Leaf-mining is also associated with enhanced biosynthesis of jasmonic acid precursors but not the active form, a weak alteration of the salicylic acid pathway and a clear inhibition of the abscisic acid pathway. Our study consolidates previous results suggesting that insects may produce and deliver cytokinins to the plant as a strategy to manipulate the physiology of the leaf to create a favorable nutritional environment. We also demonstrate that leaf-mining by *P. blancardella* leads to a strong reprogramming of the plant phytohormonal balance associated with increased nutrient mobilization, inhibition of leaf senescence and mitigation of plant direct and indirect defense.

**Keywords.** Cytokinins; Phytohormones; Insects; Leaf-mining

# 2.4. Introduction

Successful development of herbivorous insects and phytopathogenic microorganisms on a host plant relies on their ability to counteract the plant immune response and to divert host-plant resources to sustain their metabolic needs. Plant defenses are usually activated following detection of Pathogen, Herbivore or Damage-Associated Molecular Patterns (PAMPs, HAMPs and DAMPs respectively) by plant Pattern Recognition Receptors (PRR) (Kazan and Lyons, 2014; Zhu et al., 2014; Zipfel, 2014). Perception of such patterns leads to the activation of signaling pathways that induce transcription factors and ultimately lead to production of defense secondary metabolites and Pathogenesis-Related (PR) proteins (Tsuda and Somssich, 2015). However, sustained activation of plant defense also relies on hormone-dependent signaling pathways. Jasmonic acid (JA) and salicylic acid (SA) have long been known as major plant hormones involved in fine-tuning of plant defenses, although many complex interactions also involve other hormones, such as abscisic acid (ABA) and cytokinins (CKs) (Robert-Seilaniantz et al., 2011; Erb et al., 2012). To circumvent this plant defense activation process, pests and phytopathogenic microorganisms have evolved strategies to interfere with plant signaling pathways either by using proteinaceous effectors or by secreting phytohormones or phytohormone analogs (Schultz, 2002; Schultz and Appel, 2004; Kazan and Lyons, 2014; Harris et al., 2015; Guiguet et al., this issue; Nabity et al., this issue).

Phytohormones play important roles in regulating plant developmental processes and signaling networks involved in plant responses to a wide range of biotic and abiotic interactions (Bari and Jones, 2009). As important regulators of the balance between plant defense activation and growth, it is not surprising that phytohormones have often been the target of phytophagous insects over the course of evolution (Schultz and Appel, 2004; Erb et al., 2012; Giron et al., 2013). Targeting phytohormonal balances may help insects to invade a plant successfully by redirecting plant development and/or by reprogramming the plant primary and secondary metabolism for their own benefit. In particular, CKs are key players in the regulation of plant growth and development, but also mediate plant responses to various environmental stresses (Choi et al., 2011). Recent years have shown an increased interest for CKs, highlighting their pivotal role in plant defense against pathogens and insects and in the regulation of important

processes for the fate of pathogens or pests. This includes for example their role in the delay of plant senescence and in the regulation of source-sink relationships, in addition to their involvement in mediating responses to external stress such as wounding ( Body et al., 2013; Giron et al., 2013; Schäfer et al., 2015).

A large number of organisms such as insects, mites, nematodes, molluscs, protists, bacteria, fungi and viruses are known to use phytohormone-mediated mechanisms to take control of their host-plants making phytohormones key orchestrators of plant-biotic interactions (Chung et al., 2013; Giron et al., 2013; Kästner et al., 2014). More specifically, phytohormones play a key role in gall-induction and may even have facilitated the evolution of insect galls (Bartlett and Connor, 2014; Tooker and Helms, 2014). They are also key players in the interaction between leaf-miners and their host-plant with an intricate involvement of insect bacterial symbionts sometimes implicated (Connor and Taverner, 1997; Kaiser et al., 2010; Body et al., 2013; Giron and Glevarec, 2014). Leaf-mining and gall-inducing insects likely took advantage of their intimate relationship with their host-plant to manipulate their microenvironment, thus creating insect-generated shelters that avoid plant defenses, buffer against seasonal variations of leaf nutritional quality, and/or allow the insect to consume high nutritious plant tissues leading to a higher feeding efficiency (Price et al., 1987; Hespeinde, 1991; Connor and Taverner, 1997; Stone and Schönrogge, 2003; Sinclair and Hughes, 2010; Body et al., 2013; Giron et al., this issue). A mechanistic understanding of how phytohormones operate in these plant reconfigurations is lacking and requires, as a first step, the molecular and biochemical characterization of the plant phytohormonal reconfiguration following attack by plant-manipulating insects.

*Phyllonorycter blancardella* (Lepidoptera: Gracillariidae) is a polyvoltine leaf-mining microlepidopteran of apple trees. This insect interacts with its host-plant in a remarkable manner and has evolved strategies for infesting apple trees probably by manipulating fundamental elements of plant cell development. The larva establishes and maintains a permanent ‘feeding area’ (the mine) that constitutes the exclusive source of nutrients for its development. The first three larval instars (L1-L2-L3) 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 (L4-L5) are tissue-feeders and consume the lower and upper parenchyma (Body et al., 2015), resulting in the formation of feeding windows on a characteristic tentiform-shaped mine (Pottinger and LeRoux, 1971; Djemai et al., 2000). As in other leaf-miner systems, *P. blancardella* creates 'green-islands' around mining caterpillars on yellow (but also green) leaves that provide sugar-rich green tissues as well as creating an enhanced nutritional microenvironment in an otherwise senescent context (Giron et al., 2007; Body et al., 2013). Previous results established that 'green-islands' are characterized by similar or even greater levels of CKs than non-senescing green leaves and that these phythormones are likely to originate from microbial symbionts (Giron et al., 2007; Kaiser et al., 2010; Body et al., 2013). A strong correlation was found between the level of an endosymbiotic bacteria, *Wolbachia*, the amount of CKs in the mine and the intensity of the 'green-islands' phenotype (Kaiser et al., 2010). Insects treated with antibiotics created mines that contained significantly lower concentrations of CKs and failed to induce green-islands. Analysis of larvae showed a high concentration of CKs in their salivary glands but only when *Wolbachia* was present (Body et al., 2013). These results were obtained by using a targeted enzyme-linked immunosorbent assay (ELISA), allowing the characterization of a limited number of CKs (iP isopentenyladenine; iPR *N6*-isopentenyladenosine; Z zeatin; ZR zeatin riboside).

The objective of the present study is to provide an extensive characterization of how the leaf-miner *P. blancardella* modulates the plant CK profile in leaves of *Malus domestica* and how this might interfere with the plant global hormonal balance. Major phytohormones and transcriptional activity of plant cells in contact with *P. blancardella* were monitored and compared to that of control unmined leaf tissues. We expected that CK-biosynthetic genes would be overexpressed in the mined areas resulting in a global increase of CK levels (besides already characterized CK compounds). Here, we report an important transcriptional and biochemical reprogramming in the mined area reflecting the intimate relationship between the insect and its host-plant. Because of CKs involvement in gall induction by bacteria and insects, and more generally in plant interactions with various biotic invaders, our results are likely to be relevant for other plant- associated interactions.

# 2.5. Materials and methods

## 2.5.1. Biological material

The experiments were conducted on *Malus domestica* (Borkh. 1803) (Rosaceae) apple-tree leaves ('Elstar' cultivar) naturally infected by the spotted tentiform leaf-miner, *Phyllonorycter blancardella* (Fabricius, 1781) (Lepidoptera: Gracillariidae). This leaf-miner species is a polyvoltine microlepidopteran widely distributed in Europe (Pottinger and LeRoux, 1971).

L4-mined (only one mine per leaf) and unmined (an adjacent neighboring leaf) green leaves were simultaneously collected in the field between 08:00 a.m. and 09:00 a.m. in autumn (November) on *Malus domestica* apple-trees, in a biologically managed orchard in Thilouze, France (47°14′35″ North, 0°34′43″ East). Leaf tissues were dissected on site and immediately frozen in liquid nitrogen. Leaf-miner insects and frass were removed from the mine. Back in the lab, samples were stored at -80 °C until further analysis. Prior to chemical and gene expression analyses, insect species and infection status (presence of *Wolbachia* symbionts) of larvae removed from field leaf samples were checked according to the protocol developed for this species by Kaiser et al. (2010).

In order to study the phytohormone concentrations and the gene expression pattern of leaf tissues, mined areas (M) were dissected on ice following the exact outline of 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).

## 2.5.2. Phytohormones analysis

Phytohormones were analyzed following the protocol described in Schäfer et al. (2015): http://www.bio-protocol.org/e1167, and in Nakamura et.al. (2013). In brief, for each sample (n=14 for each area: M, U1, U2 and C), 30 mg of plant tissues were extracted in acidified aqueous methanol extraction buffer (750 ml MeOH, 200 ml $ddH_2O$, 50 ml HCOOH (Schäfer et.al. 2015). Isotopically labeled standards were added to each sample for quantification (1ng [$^2H_5$]-*trans*-zeatin ; 0.1ng [$^2H_5$]-*trans*-zeatin riboside ; 4ng [$^2H_5$]-*trrans*-zeatin-*O*-glucoside riboside ; 0.2ng [$^2H_6$]-$N^6$-isopentenyladenosine (all Olchemin); 40ng 9,10-[$^2H_2$]-9,10-dihydrojasmonic acid ; 40ng [$^2H_4$]-salicylic acid (Sigma-Aldrich); 40ng [$^2H_6$]-abscisic acid (Santa Cruz Biotechnology) ; and 8ng jasmonoyl-[$^{13}C_6$]-isoleucine).This extraction was followed by two solid-phase extraction (SPE) steps, which resulted in two fractions from the second SPE (HR-XC) step: (a) methanol eluate; and (b) 0.35 N $NH_4OH$ in 60% MeOH eluate. Fraction (a) was analysed for salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA), 11/12-hydroxy-jasmonic acid (OH-JA), jasmonoyl-isoleucine (JA-Ile), 12-hydroxy-jasmonoyl-isoleucine (OH-JA-Ile), and 12-carboxyjasmonoyl-isoleucin (COOH-JA-Ile) by LC-MS/MS (API 5000 mass spectrometer, Applied Biosystems, Darmstadt, Germany) in negative ionization mode and multiple reaction mode (MRM) as described in Nakamura et.al. (2013). The following MRMs were added to the method: *m/z* 225 →59 [collision energy (CE)-24 V; declustering potential (DP) -35 V] for OH-JA; *m/z* 338 →130 (CE -30 V; DP -50 V) for OH-JA-Ile; and *m/z* 352 →130 (CE -30 V; DP -50 V) for COOH-JA-Ile. Fraction (b) was analysed for cytokinins by LC-MS/MS (API 5000 mass spectrometer) in positive ionization mode and MRM mode according to Schäfer et.al. (2015). Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of OH-JA, 9,10-[$^2H_2$]-9,10-dihydrojasmonic acid was used as the internal standard applying a response factor of 1.0; for quantification of OH-JA-Ile and COOH-JA-Ile, jasmonoyl-[$^{13}C_6$]-isoleucine was used as the internal standard applying a response factor of 1.0. *Cis* isomers of cytokinins were quantified using the signal of the respective *trans* isomer standard and applying a response factor of 1.0

## 2.5.3 Microarray analysis

For gene expression, five different leaves were pooled for each biological replicate and two biological replicates per treatment were used and analyzed together. Mined tissues were obtained similarly to the procedure described above and compared to unmined ipsilateral tissues. Total RNAs were extracted from 50 mg of frozen plant tissues ground in liquid nitrogen using Nucleospin® RNA Mini Kit (Macherey-Nagel, Düren, Germany). mRNAs were amplified, labelled and cohybridized according to the protocol of Celton, Gaillard et al. (2014). In brief, aRNAs were produced with Message AmpII aRNA amplification kit (Ambion) from 200 ng of total RNA. Then, 5 μg of each aRNA was retrotranscribed and labelled with either Cyanine-3 or Cyanine-5 fluorescent dye (Interchim, Montluçon, France). Labelled samples were combined as 30 pmol for each dye and cohybridized to the NimbleGen microarray AryANE v1.0 containing 126,022 *M. domestica* sense-antisense gene pairs, as described in Celton, Gaillard et al. (2014). Deva software (NimbleGen) was used to extract pair-data files from the scanned images, obtained using the MS200 microarray scanner (Roche NimbleGen). Expression for each gene was worked out as the mean and standard error of two biological replicates hybridized on two independent arrays with fluorochrome reversal (dye switch). Intensity data were extracted using R (v.2.13.0). Data were normalized with the Lowess method, and differential expression analyses were carried out using the lmFit function and the Bayes moderated t test using the R package LIMMA (Smyth, 2005) from the Bioconductor project. To estimate gene expression levels, the normalized expression values were corrected from background. Genes were considered differentially expressed if the t-test P-values of the paired sample were below 1%

## 2.5.4. Data analysis

Annotation of *M. domestica* transcriptome was performed by blasting all sequences from the mRNA consensus version 1.0 available in the Genome Database for Rosaceae (http://www.rosaceae.org/) against the Uniprot database (BLASTx). Functional annotation was performed with HmmerScan against Pfam A and B (Finn et al., 2014) after predicting putative

protein sequences with Transdecoder (http://transdecoder.sourceforge.net/). Programs were run in the multithreaded or Message Passing Interface (MPI) mode when possible using the high-performance computing centre of the French Centre Val de Loire region (CCSC) of Orléans, France. Results were next integrated in a MySQL database with Trinotate (http://trinotate.sourceforge.net/). Gene Ontology terms were then browsed inside R to get genes associated with hormonal pathways. Hypergeometric testing was performed using the 'phyper' function with custom scripts.

### 2.5.5 Statistical analyses

Statistical analyses were performed using R version 2.13.0. Phytohormone concentrations were analysed using *Kruskal-Wallis tests* as data were not normally distributed. Where significant effects were observed, post-hoc comparisons were performed using *Mann-Whitney post-hoc tests* with Bonferroni correction.

## 2.6. Results

### 2.6.1. Cytokinins accumulate in mined-tissues

Only 8 compounds were present in significant amounts in our leaf samples (**Figure 4A**). We found an increase (*Kruskal-Wallis test, $P < 0.05$*) in *t*Z (*trans*-zeatin), *t*ZR (*trans*-zeatin riboside), *t*ZROG (*trans*-zeatin-*O*-glucoside riboside) and DZR (dihydrozeatin riboside is a combination of "*t*DZR" and "*c*DZR") in mined-leaf material when compared to unmined or control leaf tissues (**Figure 4A**), but no difference (*Kruskal-Wallis test, $P > 0.05$*) in the amount of iPR (*N6*-isopentenyladenosine-5'-diphosphate), *c*ZR (*cis*-zeatin riboside) and *c*ZROG (*cis*-zeatin-*O*-glucoside riboside) between the different leaves. *t*ZR and DZR were aproximately 3 times more concentrated in the mine, *t*Z was found to be 6 times more concentrated in mined tissues and *t*ZROG 12 times more concentrated. *t*ZROG is not an active form of a CK, but is

considered a storage form that can be activated by β-glucosidases (Mok et al., 2000). We also detected unknown forms of CKs that seem to be specific to the mined tissues; these were 400 times more abundant in mined areas than in unmined tissue (43 ng/g DW *vs* <1 ng/g DW in unmined and control zones).

## 2.6.2 Plant transcriptome reveals a general reprogramming in the mined zone

A total of 63,011 putative genes were analyzed by microarray to compare their expression levels in mined and unmined areas. We found 2,684 and 1,396 genes, respectively, that were significantly ($P < 0.05$) more or less expressed in the mined zone (**Figure 5**).

Gene set enrichment was tested (hypergeometric distribution) on Gene Ontology (GO) terms to determine biological processes that were significantly affected during infection by *P. blancardella*. The term 'response to chitin' (GO:0010200) was enriched with genes with higher expression levels in the mined area (**Table 1**). The up-regulated gene list was also enriched with terms related to 'lignin biosynthetic process' (GO:0009809) and 'isoprenoid biosynthetic process' (GO:0008299), as well as terms related to hormonal regulation (JA, GO:0009611; brassinosteroid, GO:0010268; ethylene GO:0009873), that may suggest an increased activation of the plant defense system in the presence of the leaf-mining insect. Seven genes having homologies with a quinone oxidoreductase-like protein of *Arabidopsis* (At1g23740) were found in the top 50 most expressed genes in the mined area, together with 4 genes homologous to a probable aldo-keto reductase from soybean. The significance of this apparent enrichment in reductase expression remains to be determined. Of the genes with lower expression levels in the mined zone, 28 were markedly related to 'photosynthesis' (GO:0015979) and 6 to sucrose 'biosynthetic processes' (GO:0005986). Similar to the list of genes more highly expressed in the mined zone, terms related to hormonal regulation were also found to be enriched in the unmined area (CKs, GO:0009691; ABA, GO:0009688). This global overview highlights important alterations of the plant transcriptional program when *P. blancardella* is present.

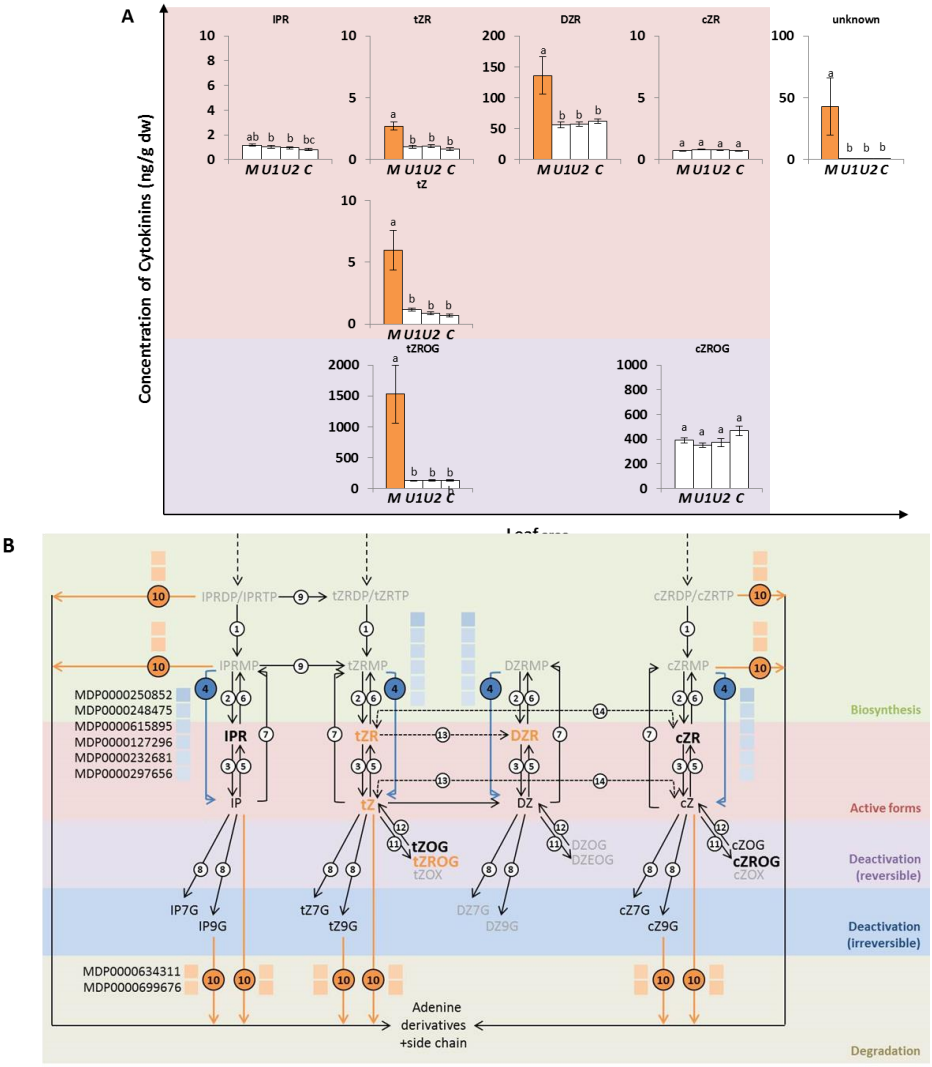



**Figure 4.** Changes in cytokinin (CK) levels and metabolism in apple tree mined tissues *vs* unmined tissues. (A) Levels of iospentenyladenosine (iPR), *trans*-zeatin (*t*Z), *trans*-zeatin riboside (*t*ZR), dihydrozeatin riboside (DZR), *trans*-zeatin riboside O-glucoside (*t*ZROG), *cis*-zeatin (*c*Z), *cis*-zeatin riboside (*c*ZR) and an unknown cytokinin form in mined (M) and unmined plant tissues (Unmined ipsilateral tissues U1, Unmined contralateral tissues U2 and Control tissues C). Statistical differences among means are shown by different letters (a, b, c). (B) Overview of the differentially expressed CK metabolism-related genes (adapted from Spíchal, 2012) in response to *P. blancardella*. Only genes with significantly different expression levels ($P < 0.05$) are shown. Ratios of expression levels of target transcript in mined and unmined areas are represented by a blue to orange palette, blue colors reflecting higher expression in the unmined area and orange colors higher expression in the mined area. (1) Phosphatase; (2) 5'-ribonucleotide phosphohydrolase; (3) adenosine nucleosidase; (4) CK phosphoribohydrolase 'Lonely guy'; (5) purine nucleoside phosphorylase; (6) adenosine kinase; (7) adenine phosphoribosyltransferase; (8) *N*-glucosyl transferase; (9) cytokinin hydroxylase; (10) cytokinin dehydrogenase; (11) zeatin-O-glucosyltransferase either *trans*-zeatin specific or *cis*-zeatin specific; (12) β-glucosidase; (13) zeatin reductase; (14) zeatin isomerase. IPRDP, *N*6-isopentenyladenosine-5'-diphosphate; IPRTP, *N*6-isopentenyladenosine-5'-triphosphate; iPRMP, *N*6-isopentenyladenosine-5'-monophosphate; iPR, *N*6-isopentenyladenosine; iP7G, *N6*-isopentenyladenosine-7-glucoside; iP9G, *N*6-isopentenyladenosine-9-glucoside, and the equivalents for *t*Z (*trans*-zeatin), DZ (dihydrozeatin) and *c*Z (*cis*-zeatin); *t*ZOG, *trans*-zeatin-*O*-glucoside; *t*ZROG, *trans*-zeatin-*O*-glucoside riboside and the equivalents for DZ and *c*Z; *t*ZOX, *trans*-zeatin-*O*-xyloside; cZOX, *cis*-zeatin-*O*-xyloside; Color code: light gray, compounds not measured; black, compounds measured but not detected; black bold, compounds detected with no significant change between tissues; orange bold, compounds accumulated in the mined zone/genes overexpressed in the mine; blue, compounds with lower level in the mined zone/genes underexpressed in the mine.

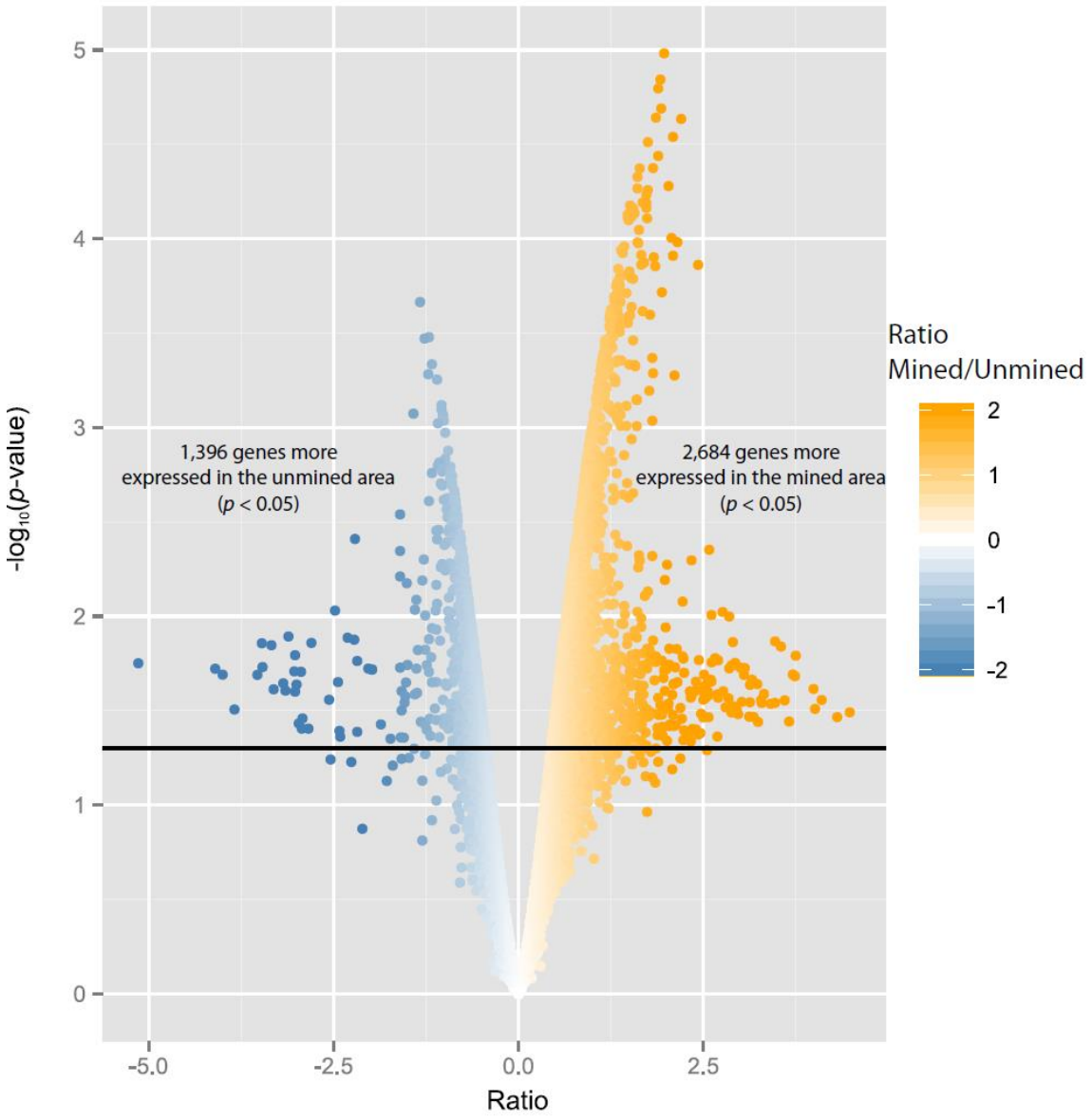



**Figure 5. Volcano Plot.** Overview of differentially expressed genes in apple tree leaves with mined and unmined areas. For each gene in apple tree genome, expression ratios (mined vs unmined) are plotted against -log10 of *P*-values of the significance test. Blue and orange dots, respectively, indicate genes more expressed in the unmined area (ratio < 0) and genes more expressed in the mined area (ratio > 0), with color intensities related to the amplitude of fold change. The horizontal line corresponds to the 0.05 significance threshold.

## 2.6.3. Expression of CK-related genes contrasts with CK accumulation patterns

A list of 219 genes was constructed by querying GO annotations with the term 'cytokinin'. Expression levels that differed significantly between mined and unmined leaf areas were observed for only 22 genes. We found an overall weaker expression of genes related to activation of CKs. Indeed, 6 CK phosphoribohydrolases 'lonely guy' genes (enzyme ❹ in **Figure 4B**), which encode enzymes converting iPRMP (*N*6-isopentenyladenosine-5'-monophosphate), *t*ZRMP (*trans*-zeatin riboside -5'-monophosphate), DZRMP (dihydrozeatin riboside-5'-monophosphate) and *c*ZRMP (*cis*-zeatin riboside-5'-monophosphate) into their active forms iP, *t*Z, DZ (dihydrozeatin) and *c*Z (*cis*-zeatin), respectively, were less expressed in mined tissues. One gene encoding a CK hydroxylase (enzyme ❾ in **Figure 4B**), which catalyzes an early step in the biosynthesis of *t*Z, was strongly expressed in the mined zone, but genes encoding enzymes involved in subsequent steps were less expressed in mined tissues, as mentioned above. In contrast, we found a higher expression of genes related to CK interconversion/degradation in the mined area. For example, 2 genes with homologies to a gene encoding adenine phosphoribosyltransferase (enzyme ❼ in **Figure 4B**) and 1 gene encoding adenosine kinase (enzyme ❻ in **Figure 4B**) were more expressed in the mined area (**Figure 4B**). Finally, 2 genes encoding cytokinin dehydrogenases (enzyme ❿ in **Figure 4B**) involved in CK degradation were also up-regulated in the mined area. While we detected a very high amount of *t*ZROG in the mine, genes related to CK inactivation processes were not differentially expressed. Overall, the analysis of CK-related gene expression in *M. domestica* did not reveal a clear pattern of regulation in mined tissus, contrasting with the strong accumulation of CK forms in the mined area.

**Table 1.** Hypergeometric testing of enrichment of GO terms related to biological processes.

| GO term | Search term | Eff genome[a] | Up-regulated[b] | *P*-value |
|---|---|---|---|---|
| GO:0010200 | Response to chitin | 263 | 48 | 3.06E−18 |
| GO:0009809 | Lignin biosynthetic process | 182 | 34 | 6.97E−14 |
| GO:0006032 | Chitin catabolic process | 80 | 21 | 1.61E−12 |
| GO:0009813 | Flavonoid biosynthetic process | 169 | 26 | 3.54E−09 |
| GO:0046148 | Pigment biosynthetic process | 27 | 10 | 5.68E−09 |
| GO:0009611 | Response to wounding | 405 | 44 | 6.15E−09 |
| GO:0010268 | Brassinosteroid homeostasis | 55 | 14 | 6.28E−09 |
| GO:0016131 | Brassinosteroid metabolic process | 45 | 12 | 3.00E−08 |
| GO:0009873 | Ethylene mediated signaling pathway | 418 | 42 | 1.21E−07 |
| GO:0008299 | Isoprenoid biosynthetic process | 54 | 12 | 3.18E−07 |
| GO:0000103 | Sulfate assimilation | 33 | 9 | 7.27E−07 |
| GO:0000272 | Polysaccharide catabolic process | 108 | 16 | 3.27E−06 |
| GO:0009825 | Multidimensional cell growth | 57 | 11 | 4.13E−06 |
| GO:0009741 | Response to brassinosteroid stimulus | 77 | 13 | 4.20E−06 |
| GO:0015936 | Coenzyme A metabolic process | 24 | 7 | 4.27E−06 |
| GO:0043255 | Regulation of carbohydrate biosynthetic process | 18 | 6 | 5.30E−06 |
| GO:0006730 | One-carbon metabolic process | 59 | 11 | 6.04E−06 |
| GO:0016998 | Cell wall macromolecule catabolic process | 62 | 11 | 1.03E−05 |
| GO:0009699 | Phenylpropanoid biosynthetic process | 35 | 8 | 1.17E−05 |
| GO term | Search term | Eff genome[a] | Down-regulated[c] | *P*-value |
| GO:0015979 | Photosynthesis | 219 | 28 | 7.13E−08 |
| GO:0005986 | Sucrose biosynthetic process | 27 | 6 | 1.06E−04 |
| GO:0035865 | Cellular response to potassium ion | 15 | 4 | 2.93E−04 |
| GO:0009691 | Cytokinin biosynthetic process | 34 | 6 | 4.93E−04 |
| GO:0009688 | Abscisic acid biosynthetic process | 25 | 5 | 5.23E−04 |
| GO:0009143 | Nucleoside triphosphate catabolic process | 19 | 4 | 9.84E−04 |
| GO:0022603 | Regulation of anatomical structure morphogenesis | 12 | 3 | 1.23E−03 |
| GO:0030001 | Metal ion transport | 44 | 6 | 2.42E−03 |
| GO:0010027 | Thylakoid membrane organization | 26 | 4 | 4.35E−03 |
| GO:0040019 | Positive regulation of embryonic development | 10 | 2 | 7.39E−03 |
| GO:0009851 | Auxin biosynthetic process | 19 | 3 | 7.62E−03 |
| GO:0010189 | Vitamin E biosynthetic process | 30 | 4 | 8.18E−03 |
| GO:0006561 | Proline biosynthetic process | 11 | 2 | 9.85E−03 |
| GO:0007166 | Cell surface receptor signaling pathway | 11 | 2 | 9.85E−03 |
| GO:0016123 | Xanthophyll biosynthetic process | 11 | 2 | 9.85E−03 |
| GO:0007568 | Aging | 21 | 3 | 1.10E−02 |
| GO:0009231 | Riboflavin biosynthetic process | 23 | 3 | 1.52E−02 |
| GO:0055114 | Oxidation–reduction process | 23 | 3 | 1.52E−02 |
| GO:0010089 | Xylem development | 35 | 4 | 1.56E−02 |
| GO:0006546 | Glycine catabolic process | 13 | 2 | 1.60E−02 |
| GO:0009773 | Photosynthetic electron transport in photosystem I | 13 | 2 | 1.60E−02 |

[a] Number of genes having this function in the genome.
[b, c] Number of genes having this function in the list of genes more highly expressed in the mined area and less expressed in the mined area respectively.

## 2.6.4. Leaf-mining is associated with biosynthesis of JA precursors but not the active JA-Ile form

A list of 396 genes was constructed by querying GO annotations with ‘jasmonic acid mediated signaling’ and ‘oxylipin biosynthetic process’ terms. Seventy-eight genes out of 396 displayed

expression levels that differed significantly between the two areas. According to our transcriptomic data, most of the steps leading to the formation of JA are likely to be activated in the mined area (**Figure 6**).

In fact, the GO term 'Response to wounding' (GO:0009611) was significantly enriched with genes having higher expression level in the mined area (hypergeometric, $P$ = 6.15e-09) (**Table 1**). This started with higher ($P < 0.05$) expression of several apparent lipid-related genes in this tissue, such as those encoding a triacylglycerol lipase (MDP0000157538) and various phospholipases (enzymes ❶ in **Figure 6**: A1-Iβ2, MDP0000286937; A1-Iγ3, MDP0000674122; C4, MDP0000126791; A1-lγ1, MDP0000674141; D-β1, MDP0000216575) that may act together to allow the release of α-linolenic acid, the first intermediate in JA biosynthesis, from chloroplastic phospholipids. In addition, a palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase (MDP0000779630) had one of the highest expression ratios. This enzyme is involved in the release of dinor-12-oxo-Phytodienoic acid (dinor-OPDA, the $C_{16}$ derivative of OPDA) during wounding in *Arabidopsis* (Buseman et al., 2006). The conversion of α-linolenic acid to JA requires 6 steps (**Figure 6**), of which we found genes for four that were more expressed in the mined area compared to control leaves, including allene oxide synthase (AOS, enzyme ❹ in **Figure 6**), allene oxide cyclase (AOC, enzyme ❺ in **Figure 6**); 12-oxo-phytodienoic acid reductase (OPR, enzyme ❻ in **Figure 6**), and ketoacyl coA thiolase (enzyme ❽ in **Figure 6**). The two remaining steps are catalyzed by a lipoxygenase chloroplastic 13-LOX (enzyme ❸ in **Figure 6**) and a 4-coumarate CoA like ligase (enzyme ❼ in **Figure 6**). We found that homologous genes putatively encoding these enzymes displayed contrasted expressions. However, the apple gene displaying the strongest homology with a 13-LOX [enzyme ❸ in **Figure 6**: MDP0000874800, 75 % identity (*e*-value=0) with O24371 from *Solanum tuberosum*] was more expressed in the mined area, suggesting that synthesis of 13-hydroperoxylinolenic acid is possible. It has been shown in potato that different 13-LOX genes had specific expression patterns (Vancanneyt, 1996), which could explain the different behavior observed here for apple. A putative gene encoding an enzyme for the step requiring 4-coumarate CoA ligase-like activity (enzyme ❼ in **Figure 6**) also showed this contrasting behavior. A 4-coumarate CoA ligase-like 7 was encoded by a probably duplicated gene in apple located on chromosome 11, MDP0000267064 and MDP0000277093, both of them being more expressed in the unmined area. By contrast, 4-coumarate CoA ligase like 5 and 9, respectively encoded by MDP0000716496 and MDP0000289629 (enzyme ❼ in **Figure 6**), were both more expressed

in the mined area. These 4 genes had high identity with *Arabidopsis* genes (> 60% identity, *e*-value=0) and could display specificities regarding their regulation profiles.

The apparent overall activation of JA biosynthesis was supported by the accumulation of JA derivatives in mined and unmined leaf areas (**Figure 6**). In particular, JA was present in high amounts in the mined zone (20 ng/g DW) but undetectable in unmined and control zones. Four JA derivatives (hydroxyjasmonic acid OH-JA, jasmonoyl isoleucine JA-Ile, hydoxyjasmonoyl isoleucine OH-JA-Ile, carboxylated jasmonic acid COOH-JA-Ile) also accumulated in relatively large amounts in the mined area. The most active form of JA is its conjugate to isoleucine, JA-Ile. Although no Jasmonic acid amido transferase (JAR) gene was found to be differentially expressed between the two zones, the large accumulation of JA-Ile (48 ng/g DW in mined zone *vs* 7 ng/g DW in unmined and control zones; JA-Ile-1 and JA-Ile-2 showed the exact same patterns/levels and were combined as 'JA-Ile') demonstrates the activation of the JA signaling pathway in the mined area. Oxidized derivatives of JA-Ile (OH-JA-Ile and COOH-JA-Ile) are inactive but also accumulated in the mined area, albeit to a lesser extent (1 and 13 ng/g DW respectively) than JA-Ile. Hydroxylated JA (OH-JA) (35 ng/g DW) was also found in a relatively higher amount in the mined areas. This form was previously shown to be inactive (Miersch et al., 2008). Recent results suggest that amidohydrolases are involved in the generation of OH-JA, as a degradation product of JA-Ile (Widemann et al., 2013). Our transcriptomic analysis revealed that 3 *Malus* genes (MDP0000454027, MDP0000249564 and MDP0000491020) having more than 60% identity with *Arabidopsis* IAA-amino acid hydrolase ILR1-like 6 (Q8VYX0) were significantly more expressed in the mined area than in the unmined zone. In addition, we observed a putative sulfotransferase (MDP0000857608: enzyme 12 in **Figure 6**) that was strongly expressed in the mined area. This gene may be required to convert OH-JA into the sulfated form ($HSO_4$-JA), which is also inactive (Miersch et al., 2008). We were unable to obtain $HSO_4$-JA concentrations in mined leaves.

## 2.6.5. Leaf-mining induces weak modifications of the SA signaling pathway

SA biosynthesis may either use phenylalanine or shikimate as precursors (Chen et al., 2014). To determine a possible involvement of this pathway in *P. blancardella* mined leaf areas, we analyzed the differential expression of 301 SA-related (with GO annotations 'salicylic acid biosynthetic process' or 'systemic acquired resistance') and 356 phenylpropanoid-related (with GO annotations 'cinnamic acid biosynthetic process' or 'phenylpropanoid biosynthetic process') genes. According to our transcriptomic data, expression level of genes involved in the biosynthesis of the two intermediates *t*-cinnamic acid and 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) may be increased in the mined zone (**Figure 7**).

In particular, 4 genes encoding DAHP synthases (enzyme ❶ in **Figure 7**) had log2 expression ratios > 1.6. The expression pattern for a putative chorismate synthase (MDP0000244469: enzyme ❸ in **Figure 7**), which catalyzes one of the subsequent steps, had a similar expression pattern, suggesting an overall activation of this biosynthetic branch. However, this contrasted with the expression of a gene encoding for a putative 3-dehydroquinate dehydratase (MDP0000193646: enzyme ❷ in **Figure 7**), which was more expressed in the unmined area. There was no difference in expression between mined and unmined leaf areas of a gene encoding for a putative chorismate mutase, an enzyme important for biosynthesis of isochorismate. While phenylalanine ammonia lyase (PAL: enzyme ❺ in **Figure 7**) encoding genes were strongly expressed in the mined area, the genes putatively encoding enzymes for the subsequent steps leading to SA were not differentially expressed. However, it should be noted that some of those steps are not clearly elucidated (Widhalm and Dudareva, 2015). In addition, many genes encoding enzymes located in the downstream biosynthesis of plant-defensive flavonoids are also more expressed in the mined area (see above, **Table 1**). The up-regulation of PAL might therefore reflect more an increased biosynthesis of phenylpro-

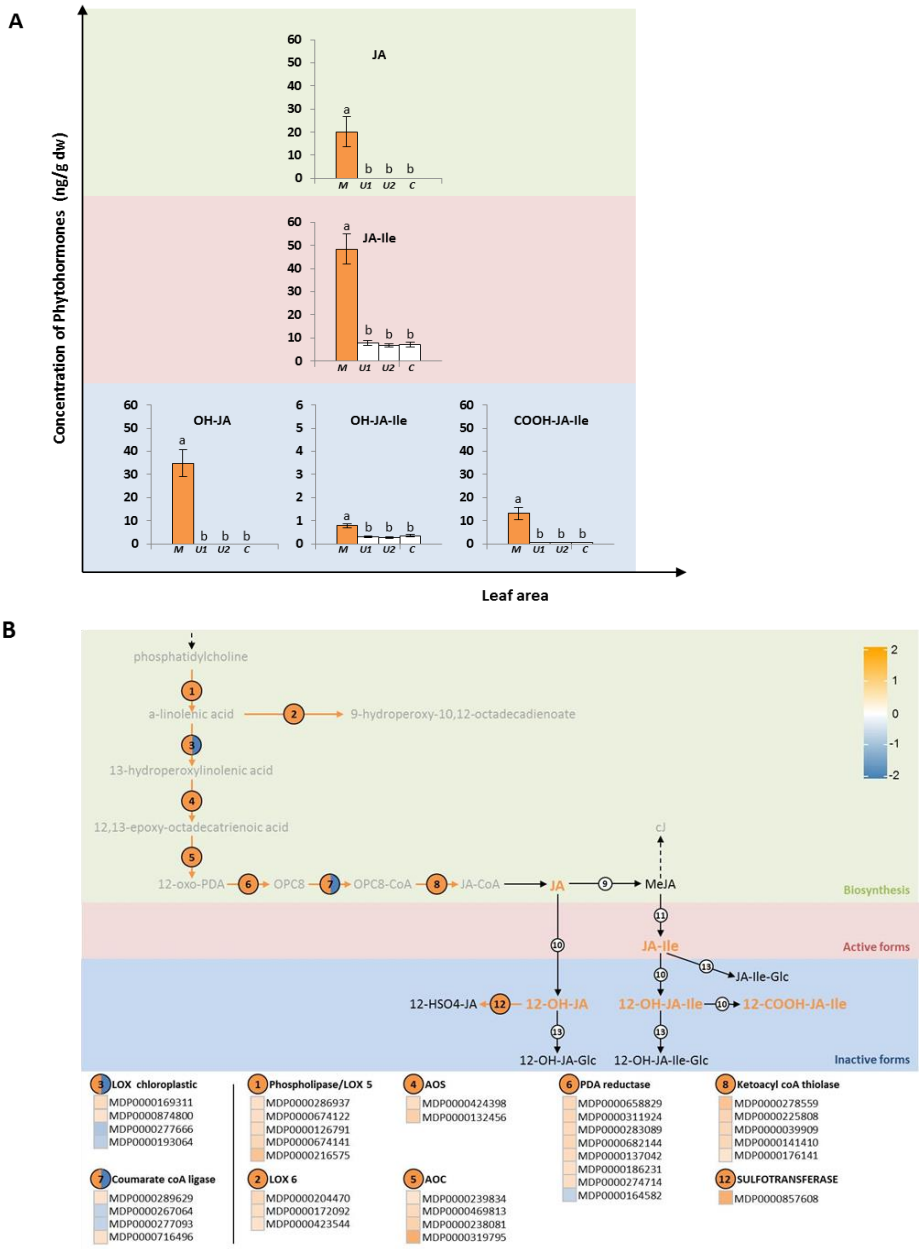



**Figure 6**. Changes in jasmonate levels and metabolism in apple tree mined tissues vs unmined tissues.
(A) Levels of jasmonic acid (JA), jasmonoyl-isoleucine (JA-Ile), hydroxy-JA (OH-JA), hydroxy-JA-isoleucine (OH-JA-Ile) and dicarboxy-JA-isoleucine (COOH-JA-Ile) in mined (M) and unmined plant tissues (Unmined ipsilateral tissues U1, Unmined contralateral tissues U2 and Control tissues C). Statistical differences among means are shown by different letters (a, b, c). (B) Overview of the differentially expressed JA metabolism-related genes in response to P. blancardella. The same color criteria is used for ratios of gene expression levels (orange: overexpressed in mined tissues, blue: downregulation in mined tissues; white: no significant change.) (1)Phospholipase; (2) Lipoxygenase 6 (LOX6); (3) Lipoxygenase chloroplastic (13-LOX); (4) Allene oxide synthase (AOS); (5) Allene oxide cyclase (AOC); (6) 12-oxophytodienoic acid reductase (OPDA reductase); (7) 3-oxo-2-(2-(Z)-pentenyl)-cyclopentane-1-octanoic coA ligase (OPC8 CoA ligase); (8) 3-ketoacyl-CoA thiolase (KAT); (9) JA methyl transferase (JMT); (10) cytochrome P450 hydroxylase (CYP94B3); (11) JA amido synthetase (JAR1); (12) Sulfotransferase; (13) Glucosylation; (14) amino acid hydrolase. 12-oxo-PDA , Oxo-phytodienoic acid; OPC8, 3-oxo-2-(2-(Z)-pentenyl)-cyclopentane-1-octanoic; JA, jasmonic acid; MeJA, methyljasmonate; JA-Ile, jasmonoyl-isoleucine; 12-OH-JA, 12-hydroxy-JA; 12-OH-JA-Ile, 12-hydroxy-JA-isoleucine; 12-COOH-JA-Ile, 12-dicarboxy-JA-isoleucine; JA-Ile-Glc; O-glucosyl-JA-isoleucine; 12-HSO4-JA, 12-hydroxyjasmonic acid sulfate; 12-OH-JA-Glc, 12-hydroxy-O-glucosyl-JA; 12-OH-JA-Ile-Glc, 12-hydroxy-O-glucosyl-JA-isoleucine; Color code: see **Fig. 4**.

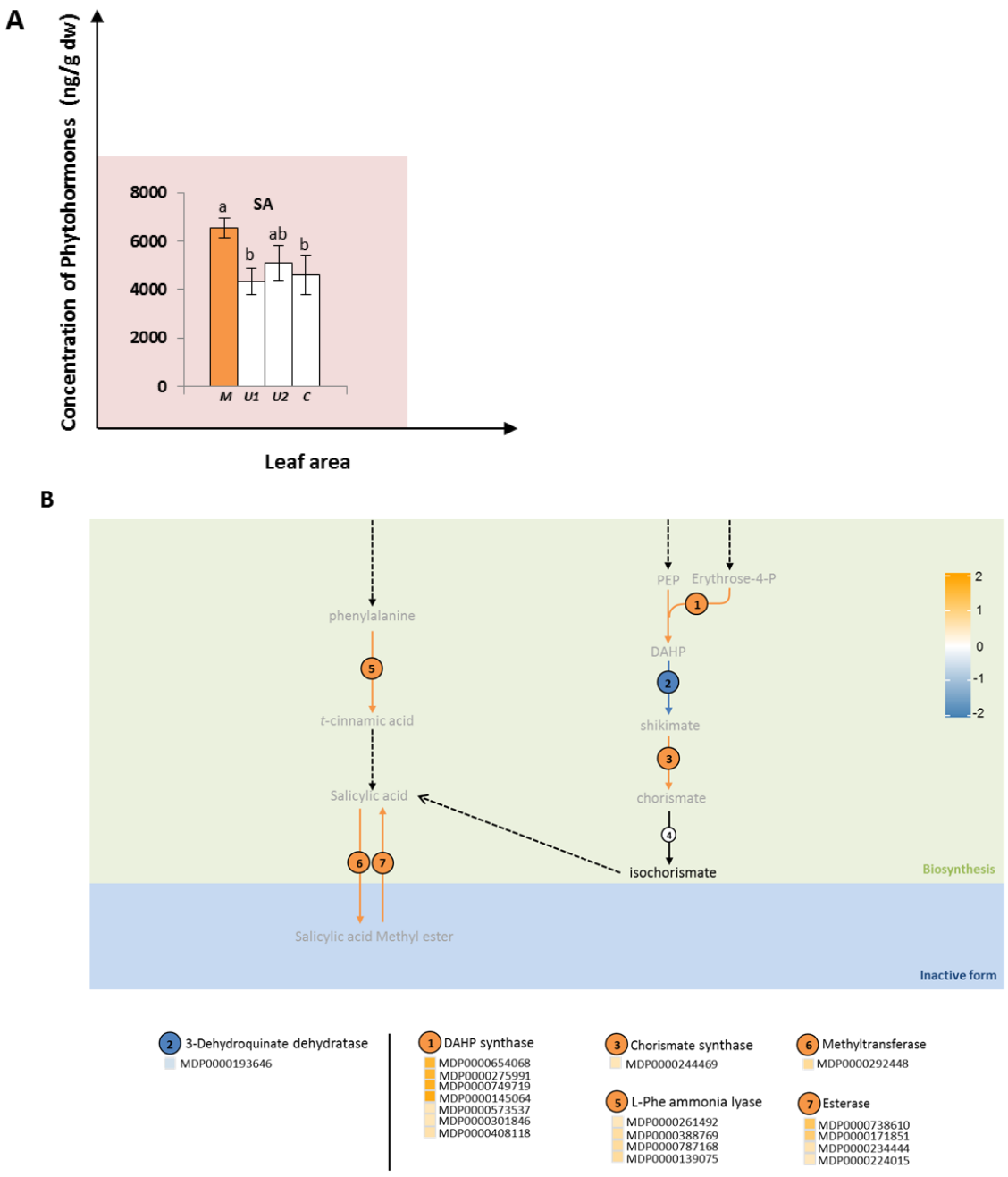



**Figure 7.** Changes in salicylic acid levels and metabolism in apple tree mined tissues vs unmined tissues. (A) Levels of salicylic acid (SA) in mined (M) and unmined plant tissues (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Statistical differences between means are shown by different letters (a, b, c). (B) Overview of the differentially expressed SA metabolism-related genes in response to P. blancardella. The same color criteria is used for ratios of gene expression levels (orange: overexpressed in mined tissues, blue: downregulation in mined tissues; white: no significant change). (1) 3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHP synthase); (2) 3-dehydroquinate dehydratase; (3) chorismate synthase; (4) Isochorismate synthase (ICS); (5) phenylalanine ammonia lyase (PAL); (6) SA methyltransferase; (7) SA esterase; color code: see **Fig. 4**.

The methyl ester derivative of SA, MeSA, corresponds to an inactive form and its role as a mobile signal remains questionable (Fu and Dong, 2013). According to expression patterns of

SA methyltransferase (enzyme ❻) and esterase (enzyme ❼), it seems that the equilibrium panoids and flavonoids (the corresponding GO terms are significantly enriched, see above) rather than that of SA.

between MeSA and SA is in favor of SA in the mined area, as 4 genes encoding SA esterases (enzyme ❼) were significantly more expressed in this zone (**Figure 7**). Taken together, these data are in favor of a stronger accumulation of SA in the mined area. This was confirmed by monitoring accumulation of SA in mined and unmined leaf area (**Figure 7**). SA was indeed found to accumulate in higher amounts in the mined zone (6550 ng/g DW *vs* ~4700 ng/g DW in unmined and control zones). However, only 24 genes out of 306 related to SA mediated signaling were differentially expressed, suggesting that modulation of this pathway is likely to be limited. Among those genes, 3 genes thought to encode glutaredoxins (MDP0000713715, MDP0000179654 and MDP0000135807) may be involved in the regulation of redox homeostasis and crosstalk with the jasmonate signaling pathway (Ndamukong et al., 2007).

## 2.6.6. Mined zones are associated with lower ABA levels

A list of 411 genes was constructed by querying GO annotations with 'abscissic acid metabolism' terms among which 34 evidenced different expression levels between the two leaf tissues. The biosynthesis of ABA appeared to be markedly decreased in mined tissues as several biosynthetic genes (zeaxanthin epoxidase (MDP0000142434 - MDP0000189613: enzyme ❶ in **Figure 8**) and xanthoxin dehydrogenase (MDP0000399796: enzyme ❺ in **Figure 8**) were less expressed than in the unmined area (**Figure 8)**. Moreover 3 apple genes (enzyme ❼: MDP0000276663, MDP0000192034, MDP0000326412) with strong homologies to ABA 8-hydroxylases of Arabidopsis (70 % identity) involved in the oxidative degradation of ABA were significantly more expressed in the mined area. Those results were constistent with our measures of ABA concentration in mined and unmined leaf area (**Figure 8**). Indeed, ABA accumulates in lower amounts in the mined zone (57 ng/g DW *vs* ~91 ng/g DW in unmined and control zones).

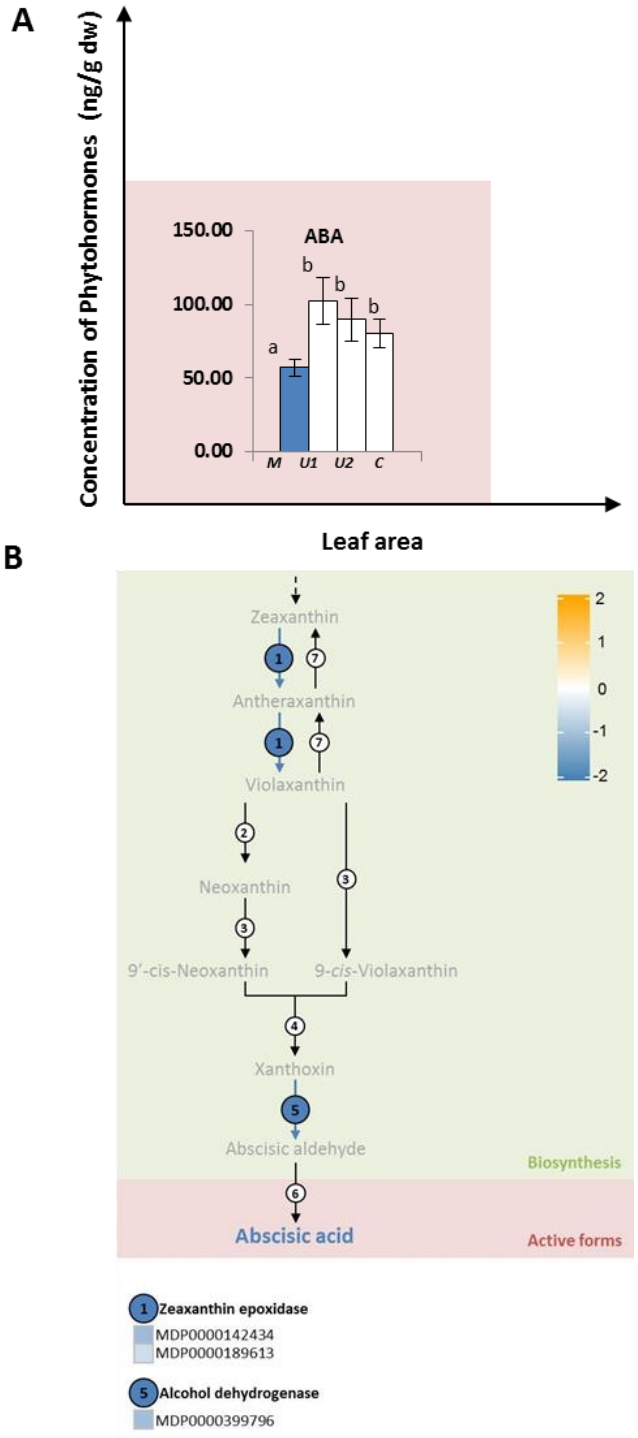



**Figure 8.** Changes in abscisic acid levels and metabolism in apple tree mined tissues *vs* unmined tissues. (A) Levels of abscisic acid (ABA) in mined (M) and unmined plant tissues (Unmined ipsilateral tissues U1, Unmined contralateral tissues U2 and Control tissues C). Statistical differences between means are shown by different letters (a, b, c). (B) Overview of the differentially expressed ABA metabolism-related genes in response to *P. blancardella*. The same color criteria are used for ratios of gene expression levels (orange: overexpressed in mined tissues, blue: downregulation in mined tissues; white:no significant change.) (1) Zeaxanthin epoxidase; (2) Neoxanthin synthase; (3) isomerase; (4) 9-*cis*-epoxycarotenoid dioxygenase; (5) xanthoxin dehydrogenase; (6) abscisic aldehyde oxidase (AAO). (7) Abscisic acid 8-hydroxylase Color code: see **Fig. 4.**

# 2.7. Discussion

Biochemical and transcriptional changes in plant cells in contact with *P. blancardella* larvae were monitored to better understand how leaf-mining modulates the plant CK profile and how

this might interfere with the plant global hormonal balance. Modulation of phytohormone levels is known to influence, either positively or negatively, insect development and is therefore a good indicator of the plant tissue physiological state (Erb et al., 2012). Indeed, plant hormones are known to regulate key processes related to plant development and responses to environmental stresses (Santner and Estelle, 2009). They are also the specific target of several phytophagous and phytopathogenic organisms as a strategy to successfully invade the host-plant, to modify or create new habitat structures and to obtain the nutrients

required to sustain their metabolic needs (Schultz and Appel, 2004; Body et al., 2013; Barlett and Connor, 2014; Tooker and Helms, 2014; Giron et al., this issue).

Previous studies revealed that *P. blancardella* is able to induce CK-enriched tissues in the mine on both green and yellow leaves contributing to the creation of an enhanced nutritional microenvironment (Giron et al., 2007; Kaiser et al., 2010; Body et al., 2013). Plant-associated bacteria are known to use fascinating molecular mechanisms that lead to the production of bacterial CKs and the modulation of plant-borne CKs which ultimately result in profound

metabolic and morphological plant modifications (Frugier et al., 2008: Stes et al., 2011; Giron and Glevarec, 2014). Several lines of evidence suggest that insect bacterial symbionts could be involved in the delivery of CKs to the plant in the *P. blancardella*/apple tree interaction (Kaiser et al., 2010; Body et al., 2013; Giron and Glevarec, 2014). It is noteworthy that control of hormonal balance by insects has been reported to be mediated by insect-associated bacteria or viruses (Frago et al., 2012; Chung et al., 2013; Kazan and Lyons, 2014).

Cytokinin content in the mined area of apple tree leaves was significantly increased in comparison to unmined zones despite an overall weaker expression of genes related to CK activation and a higher expression level of genes involved in CK interconversion and degradation. These results suggest that CKs accumulated in the mined area mostly originate from the insect itself rather than being produced by the plant. Alternatively, they could

potentially be actively transported to the mined area from other parts of the leaf/plant. Our transcriptomic data also suggest that the plant molecular activity is acting to reduce the CK active pool in a possible attempt to regulate the flow of CKs provided by the bacteria-insect interaction. Interestingly, a large amount of CK *O*-glucoside reversible storage forms (*t*ZROG) are accumulated in the mine. It is interesting to note that a similar pattern has recently been observed in another system where active CKs (iP, iPR and *c*ZR) as well as CK inactive forms (*t*ZROG, *c*ZROG, *t*Z7G *trans*-zeatin-7-glucoside) accumulate rapidly and to high levels upon wounding and application of *Manduca sexta* oral secretions in *Nicotiana attenuata* (Schäfer et al., 2015). These *O*-glucoside forms are considered to be resistant to degradation by CK oxidases (CKX) enzymes and are regarded as a storage form of CK able to be re-activated by β-glucosidases (Mok et al., 2000). Therefore the strong level of *t*ZROG in mined areas may contribute to maintain insect-induced effects over the entire lifecycle of the insect (40-50 days). Time course experiments will be required to characterize the phytohormone dynamics over the course of the plant-insect interaction. Additionally, the identification of the large fraction of unknown CKs accumulated in the mine may help us determine whether they are insect- or endosymbiotic bacteria-specific types of CKs (Giron and Glevarec, 2014).

Increased CK concentrations in the mine are expected to sustain the activation of the corresponding signaling pathway in the plant. However, increasing the content of CKs in mined tissues is also likely to impact other signaling pathways. Indeed, much crosstalk between phytohormones has been shown in plants, resulting in the activation of appropriate plant defense mechanisms or in the disruption of the plant anatomy and physiology leading to a favourable niche for the insect (Jaillais and Chory, 2010; Erb et al., 2012). Other phytohormonal signaling pathways have been shown to be impacted by the presence of *P. blancardella*. Leaf tissues infected by *P. blancardella* were marked by a increased expression of genes associated with the JA pathway, and, to a lesser extent, the SA pathway. Leaf-mining was also associated with a repressed expression of genes of the ABA pathway. Antagonism between SA and JA pathways, as well as SA and ABA are well established (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). However, crosstalk between JA and ABA pathways are less well understood (Pieterse et al., 2012). Inhibition of the JA pathway by ABA after infection by a fungal pathogen was reported (Anderson et al., 2004) whereas JA was more recently found to positively regulate expression of PYL4, the ABA receptor (Lackman et al., 2011). The interplay between CK and ABA or JA is currently not fully understood (O'Brien and Benková, 2013; Schäfer et al., 2015). A recent study showed that CKs accumulate rapidly upon wounding with application of oral

secretions from the grasshopper *Schistocerca gregata* indicating a possible crosstalk between CK and JA (Schäfer et al., 2015). Recently, methyl jasmonate (MeJA) treatment was shown to affect herbivory-elicited accumulation of CKs, by tending to increase *t*ZROG levels (Schäfer et al., 2015). Hence, the specific pattern of phytohormone accumulation in apple leaves infected by *P. blancardella* might reveal new crosstalk or indirect cascading effects between the corresponding pathways. Other signaling pathways appear to be induced by *P. blancardella*. for example two apple orthologs of *Arabidopsis* genes (ARR3: MDP0000328874 and ARR18: MDP0000202657) are overexpressed in mined tissues. Expression of AtARR3 was shown to be induced by the *Pseudomonas* bacterial effector HopQ, which contributes to shutting down the basal immunity that is activated following detection of microbial conserved patterns (Hann et al., 2013). Whether the expression of these genes is dependent on CK-signaling awaits investigation and a deeper understanding of insect CK-mediated signaling, an area that has received very few attentions so far (but see Dervinis et al., 2010; Schäfer et al., 2015).

Our data show that the JA-Ile active form is produced in high amounts in the mine, possibly as a result of the activation of the JA pathway. This likely reflects the plant defense against the leaf-miner, as physiological responses mediated by JA include seed and flower development, and also defense activation after wounding or during attacks by necrotrophic microorganisms and chewing insects (Erb et al., 2012; Wasternack and Hause, 2013; Farmer et al., 2014). In accordance with the presence of JA-Ile, we found that the expression of many genes involved in the phenylpropanoid/flavonoid pathway were similarly increased in the mined zone. Positive regulation of this pathway by jasmonates has been established several times (De Geyter et al., 2012). Increased production of jasmonates and phenylpropanoids was also reported in leaves of a resistant coffee genotype during infestation by the leaf-miner *Leucoptera coffella* (Cardoso et al., 2014). Whether such an activation of defense responses is required to limit the spread of the mine remains to be determined. In another leaf-mining insect, *Liriomyza huidrobrensis*, the JA pathway was activated locally in the host plant *Arabidopsis*, while the production of secondary metabolites was down-regulated (Zhang et al., 2012). Similar results were found in *P. blancardella*, in which the insect is able to mitigate phenylpropanoid biosynthesis (Glevarec, Giron et al., unpublished). Interestingly, another *Liriomyza* species, *L. sativae* suppresses JA-dependent responses as observed by the decrease in JA-related transcript expression, in contrast to overexpression of SA-related markers, in tomato (Kawazu et al., 2012). In addition, MeJA treatments of tomato leaves reduced performance of *L. sativae*. For *P. blancardella*, inactive forms of JA also accumulate in the mined zone, which may reveal an attempt of the leaf-miner

to control the jasmonic flux through deactivation processes. This would allow the leaf-miner to limit activation of the downstream components that act as a negative feedback against the insect. Taken together, our data reveal (*i*) a sustained activation of the JA pathway probably as a response to the presence of *Phyllonorycter* larva and (*ii*) an attempt to limit JA-Ile production by oxidizing JA and JA-Ile towards inactive compounds.

Our results show a minor activation of the SA pathway (weak accumulation of SA and few differentially expressed SA-related genes). This weak activation might result from a combination of (*i*) the possible perception of chitin oligomers known to trigger the SA pathways and (*ii*) a higher activation of the JA pathway which is able to negatively modulate the SA pathway (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). The phytohormone SA mediates resistance against biotrophic bioagressors, but the trigerring of the SA pathway by 'stealthy' insects such as sap- and cell content-feeders is still debated (Robert-Seilaniantz et al., 2011; Fu and Dong, 2013). For example, this pathway was reported to be involved in Mi-1 mediated resistance of tomato to the aphid *Macrosiphum euphorbiae* (Li et al., 2006). However, other studies reported SA/JA-independent transcriptome changes in response to other aphids, such as *Myzus nicotianae* on *Nicotiana attenuata* and *Myzus persicae* on *Arabidopsis* (Voelckel et al., 2004; De Vos et al., 2005). In fact, plant responses to sap- and cell content feeders are likely to be very different, with aphids triggering specific and strong reprogramming of the host metabolism (De Vos et al., 2005). The apple tree transcriptomic responses to *P. blancardella* resemble that of *Arabidopsis* responses to *Frankliniella occidentalis*, a cell content feeder, which were characterized by the accumulation of JA-related defenses and no activation of the SA pathway (De Vos et al., 2005; Kawazu et al., 2012). However, our transcriptomic data was obtained using older tissue-feeding instars. It is very likely that younger fluid-feeding instars of *P. blancardella* induce different plant responses, necessitating that the phytohormone dynamics be characterized over the entire course of the plant-insect interaction.

ABA has a pivotal role in protection against water loss in plants under desiccation stress (Wasilewska et al., 2008; Erb et al., 2011). ABA is also involved in plant responses to biotic stresses caused by a wide range of plant pathogens with various effects depending not only on pathogen lifestyles and overall infection biology (e.g. necrotrophic *vs* biotrophic pathogens) but also on specialized features of each interaction (Fan et al., 2009; Erb et al., 2012). More recently, ABA has been shown to play a role in plant resistance against insect herbivores through a modulation of JA-driven defense responses (Thaler and Bostock, 2004; Bodenhausen and

Reymond, 2007; Mondego et al., 2011; Erb et al., 2012 ). ABA also plays a key role in senescence and abscission-promoting effects, two processes of high importance for sessile organisms such as leaf-mining and gall-inducing insects (Lim et al., 2007). *P. blancardella* induces a marked decrease of ABA levels in the mined area due to the inhibition of the expression of several genes involved in ABA synthesis. Several leaf-miners and gall-inducers induce leaf abscission with, typically, a positive correlation between the density of insects and the probability of leaf abscission (Faeth et al., 1981; Williams and Whitham, 1986; Kahn and Cornell, 1989; Stiling and Simberloff, 1989; Hespeinde, 1991; Waddell et al., 2001; Mazía et al., 2012). Abscission can be more frequent when mines are clustered on one side of the midvein of a leaf rather than when leaf-miners are on both sides of a leaf (Stiling et al., 1987). Interestingly, several other *Phyllonorycter* species, such as *P. maestingella* on beech and *P. messaniella* on holm oak (*Quecus ilex*), show a lower abscission rate of mined-leaves compared to unmined-leaves with leaf abscission accounting for less than 3% of mortality (Pritchard and James, 1984a, b). However, other studies show induction of leaf abscission by a *Phyllonorcyter* sp. leaf-mining moth on *Salix lasiopis* resulting in increased insect mortality (Preszler and Price 1993) and an induced early leaf abscission by the apple blotch leaf-miner *Phyllonorycter crataegella* (Maier 1983). Our results show that *P. blancardella* reduces ABA levels that could contribute to non-senescing effects ('green-islands' being a striking example) in synergy with insect-induced accumulation of CKs. This could also favor the suppression of abscission during the critical larval feeding stage. Future studies will require to investigate whether leaf-mining by *P. blancardella* positively or negatively impacts leaf abscission.

Our study demonstrates that leaf-mining by *Phyllonorycter blancardella* leads to a strong reconfiguration of the plant phytohormonal balance associated with increased nutrient mobilization, inhibition of leaf senescence and mitigation of plant direct and indirect defense. Further research at the biochemical and molecular scales are necessary to understand fully how phytohormones affect a plant's defensive response to endophytic herbivores, as most of our current knowledge comes from ectophytic leaf-feeding or sap-feeding insects. This will also allow us to gain a deeper understanding of the role played by phytohormones in insect-generated extended phenotypes, the mechanisms underlying plant manipulation, their origin, and their adaptive significance for insects. Ultimately, this will lead to a more comprehensive knowledge on the role of phytohormones in the evolution of plant-insect-microbe interactions.

# 2.8. Acknowledgements

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# Chapter 3. Dynamics and origin of cytokinins involved in plant manipulation by a leaf-mining insect

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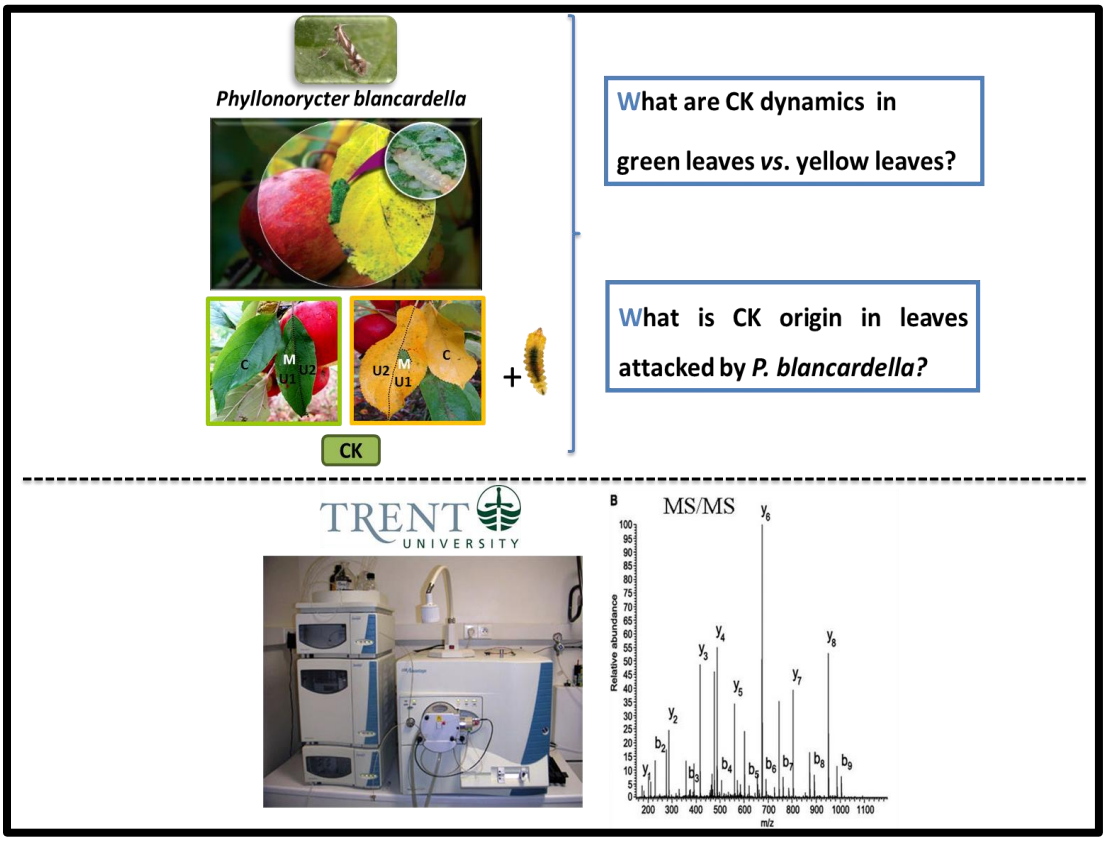
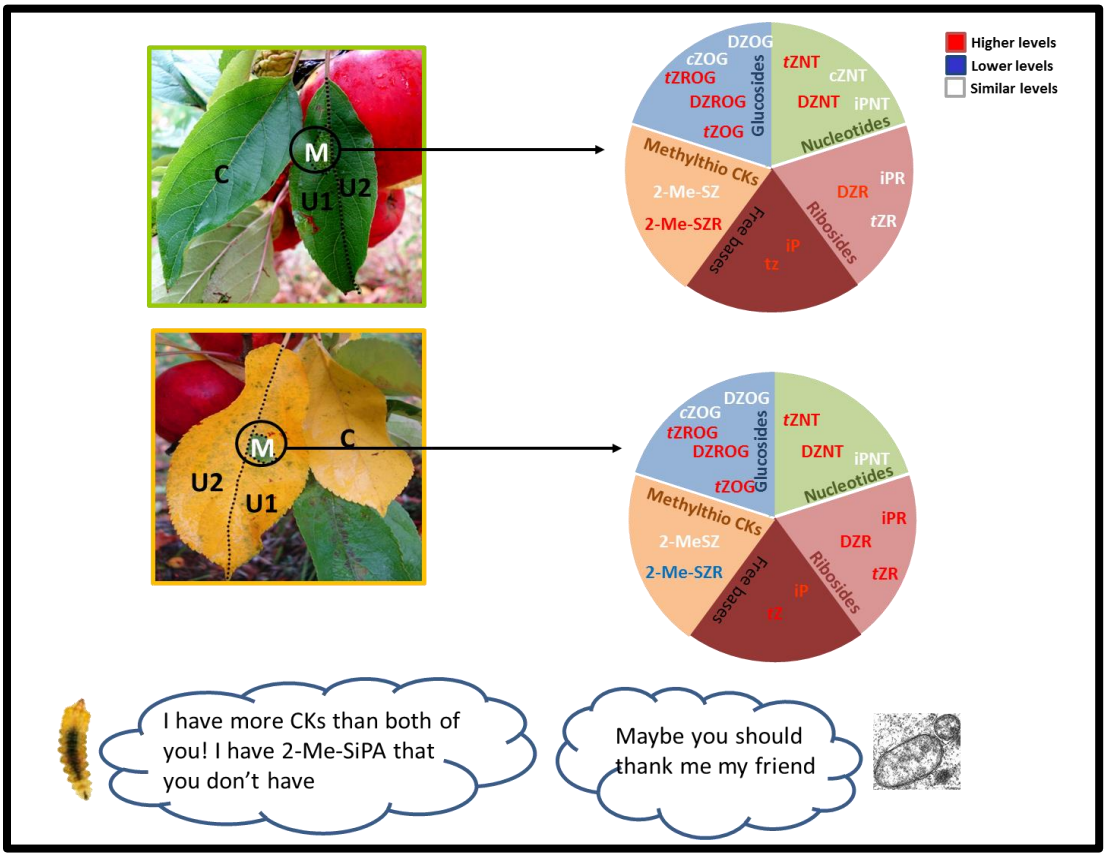
# 3.1. Graphical abstract



# 3.2. Introduction

## 3.2.1. Objectives.

The first objective of this chapter 3 was to gain a deeper understanding into the possible **origin of CKs** involved in the plant manipulation by the leaf-miner *P. blancardella* to figure out if they are produced by the plant the insect or the symbionts. Previous results established that CKs are likely to originate from the insect (Chapter 2) and that microbial symbionts play a key role in this phytohormonal alteration (Giron et al., 2007; Kaiser et al., 2010; Body et al., 2013; Gutzwiller *et al.* 2015). A strong correlation was found between the level of an endosymbiotic bacteria, *Wolbachia*, the amount of CKs in the mine and the intensity of the 'green-islands' phenotype (Kaiser et al., 2010). Insects treated with antibiotics created mines that contained significantly lower concentrations of CKs and failed to induce green-islands (Body et al., 2013).
*Working hypothesis:* We expected to find CKs in larvae, including bacteria-specific CKs as a chemical signature of the role played by endosymbionts in the plant phytohormonal remediation.
The second objective was to investigate the **dynamics of CK alterations** taking into account temporal and spatial aspects of the interaction.
*Working hypothesis:* We expected that CK alterations would operate in a similar fashion under various environmental conditions but that alterations would be restricted to the mined area.

## 3.2.2. Methods.

To reach these objectives, we conducted an extensive identification and quantification of CKs by HPLC-(ESI+)-MS/MS in both green and yellow leaves of *M. domestica* infested by *P. blancardella*. CKs were also characterized in larvae to investigate their possible contribution to the production of CKs. Our extended CK profiling included 2-Methylthio (2-MeS)-derivatives. Some 2-MeS-CKs seem to be strictly bacteria-specific CKs and their characterization could thus help clarify whether insect bacterial symbionts directly contribute to the production of CKs

involved in this plant-insect interaction.

## 3.2.3 Results.

Our time course characterization of CKs in larvae and in attacked apple leaves results show that mines are enriched in CKs both on green and yellow leaves. The spatial distribution of CKs within attacked leaves show that plant manipulation is strictly limited to the mine. Analyses reveal that CKs are detected in the highest levels in larvae and that bacteria-specific methylthio-CKs can be identified.

## 3.2.4 Conclusions.

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. These alterations allow insects to control their nutritional supply under fluctuating environmental conditions.

Manipulation of plant is limited to the feeding area suggesting the absence of CK translocation from distant leaf areas towards the insect feeding site. Alterations occur mainly through a modulation of specific pathways of CK biosynthesis with a common strategy shared by arthropods and plant-associated microorganisms. Analyses also 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 (see chapters 4 and 5).

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. This is consistent with

previous results suggesting that insect bacterial symbionts contribute to the observed phenotype. The evolutionary origin of CKs involved remains to be fully addressed but functional tests are now needed to validate the possible role of the various CKs and the specific role of each partner in this intricate plant-insect-microbe interaction (see chapter 4). Ultimately, our study provides key findings towards the understanding of molecular mechanisms underlying this intricate plant-insect-microbe interaction.

The results are submitted to *Insect Science*.

# 3.3. Abstract

Several herbivorous insects and plant-associated microorganisms control the phytohormonal balance, thus enabling them to successfully exploit the plant by inhibiting plant defences 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 time course characterization of CKs in larvae and in attacked apple leaves. Our results show that mines are enriched in CKs both on green and yellow leaves, allowing insects to control their nutritional supply under fluctuating environmental conditions. The spatial distribution of CKs within attacked leaves show that plant manipulation is strictly limited to the mine suggesting the absence of CK translocation from distant leaf areas towards the insect feeding site. Analyses revealed 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. They 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 towards the understanding of molecular mechanisms underlying this intricate plant-insect-microbe interaction.

**Keywords:** Cytokinins, Insect bacterial symbionts, Leaf-miners, Phytohormones, Plant-insect-microbe interactions, Plant manipulation

# 3.4. 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 and 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 recently re-emerged as important players regulating evolutionary trade-offs between plant growth and defence (Herms and Mattson, 1992; Giron et al., 2013) or plant defence and reproduction (van der Krieken et al., 1990; D'Aloia et al., 2011). Because CK-mediated effects on plant physiology include modulation of plant defence, 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 and 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 and Glevarec, 2014; Tooker and Helms, 2014; **Zhang et al., 2016***). This suggests that insects could be the sources of phythormones – rather than simply manipulating the plant phytohormonal balance/signalling – 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) and plant-associated fungi and viruses (e.g. Walters et al., 2008; Baliji et al., 2010). 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 and Connor, 2014; Tooker and Helms, 2014). They have been found in several gall-inducing insect species (Ohkawa, 1974; Mapes and Davies, 2001b; Dorchin et al., 2009; Straka et al., 2010; Tooker and 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 larvae were infected with *Wolbachia* and 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 the present 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 extended CK profiling includes *cis*-type CKs and 2-Methylthio (2-MeS)-derivatives. *Cis*-type CKs are a group of CKs that have been often ignored compared to *trans*-type CKs mainly due to the lack of appropriate analytical methods (Schäffer et al., 2015). In addition to being detected in plant species, the ability to produce *cis*-CKs has been identified 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 and 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 oxidase/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 defences (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 and 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 towards 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 and Armstrong, 1970; Ajitkumar and Cherayil, 1988; Mok and Mok, 1994). Characterization of 2-MeS-CKs could thus help clarify whether insect bacterial symbionts directly contribute to the production of CKs involved in this plant-insect interaction.

# 3.5. Materials and methods

## 3.5.1. 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 three instars (L1 - L2 - L3) 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 (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 and LeRoux, 1971; Djemaï et al., 2000). The insect suppresses the plant defence 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 one mine per leaf at the L4-L5 tissue feeders instar) and unmined (an adjacent neighbouring 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), U2 (unmined), and C (control) respectively. Larvae were also frozen immediately in liquid nitrogen and stored at -80°C for further CKs 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).

## 3.5.2. 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 re-suspended in extraction buffer Bieleski #2 ($CH_3OH$:H20: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 2**), and homogenized (ball mill, RetschMM300; 5min, 25Hz) at 4$^{o}$C with zirconium oxide grinding beads (Comeau Technique Ltd., Vaudreuil-Dorion, Canada). The samples were allowed to extract passively overnight (approximately 12 hours) at -20$^{o}$C. Pellets were removed by centrifugation (Thermo Scientific; Model Sorvall ST16, Ottawa, Canada; 10 min at 10000 RPM), the obtained samples were re-extracted with 1 mL extraction buffer at -20$^{o}$C for 30 min. The pooled supernatants were dried in a speed vacuum concentrator at 35$^{o}$C.

Extraction residues were reconstituted in 1 mL of 1M 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 6cc; 150mg, Mississauga, ON, Canada). Cartridges were activated with 5 mL of HPLC grade methanol and equilibrated using 5 mL of 1M formic acid (pH 1.4). After equilibration, each sample was loaded and washed with 5 mL of 1M formic acid (pH 1.4). CKs were eluted based on their chemical properties. The nucleotide fraction (CKNTs) was eluted using 5 mL of 0.35M 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 0.35M ammonium hydroxide in 60% methanol. All samples were evaporated to dryness in a speed vacuum concentrator at 35$^{o}$C, and stored at -20°C.

CKNTs were dephosphorylated using 3 units of bacterial alkaline phosphatase (12 ul) in 1 mL of 0.1M ethanolamine-HCL (pH 10.4) for 12 hours 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 re-constituted in 1.5 mL double distilled water for further purification on a reversed-phase C18 column (Canadian Life Sciences; C18/14, 3cc, 500mg; 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 re-constituted in 1.5 mL of starting conditions buffer ($CH_3COOH$:$CH_3CN$:dd$H_2O$ [0.08:5.0:94.92, vol/vol/vol].

## 3.5.3. 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). 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 $min^{-1}$. The initial conditions were 5% A and 95% B, changing linearly in 12 minutes to 95% A and 5% B. Conditions remained constant for 5 minutes, and then immediately returned back to initial conditions for 12 minutes. 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 product ion.

All data were analysed 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 $^2H$-labelled internal standards.

### 3.5.4. Statistical analyses

Statistical analyses were performed using Statistica 8.0. Phytohormone concentrations were analysed using Fisher LSD tests.

## 3.6. Results

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

In total, 16 CKs that belong to four isoprenoid types of CKs (iP, *t*Z, DZ and *c*Z-type CKs – see **Table 2** for abbreviations) are identified in both green and yellow leaves (**Figures 9 and 10**). Identified CKs include precursors (phosphorylated 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 (**Figure 13**). Mined areas are enriched in CKs both on green and yellow leaves with higher levels of CKs observed on yellow leaves (**Figure 9A**). The specific CK composition also differs between mines on green and on yellow leaves (**Figure 9B**) 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 is 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 (**Figure 9A**) and that CK profiles of control leaf tissues (C) are similar to those of adjacent (U1) and distant (U2) areas.

**Table 2.** Cytokinins (CKs), scanned for 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 (DZNT) | $^2H_3$[9RMP]DZ |
| 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 (DZR) | $^2H_3$[9R]DZ |
| 8. | $N^6$-isopentyladenosine (iPR) | $^2H_6$[9R]iP |
| | **Free bases (CKFBs)** | |
| 9. | *Trans*-zeatin (*t*Z) | |
| 10. | *Cis*-zeatin (*c*Z) | $^2H_3$DZ |
| 11. | Dihydrozeatin (DZ) | |
| 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 (DZOG) | $^2H_7$DZOG |
| 16. | *Trans*-zeatin-O-glucoside riboside (*t*ZROG) | $^2H_5$ZROG |
| 17. | *Cis*-zeatin-O-glucoside riboside *c*ZROG | |
| 18. | Dihydrozeatin-O-glucoside riboside (DZROG) | $^2H_7$DZROG |
| 19. | *Trans*-zeatin-9-glucoside (*t*Z9G) | $^2H_5$Z9G |
| 20. | *Cis*-zeatin-9-glucoside (*c*Z9G) | |
| 21. | Dihydrozeatin-9-glucoside (DZ9G) | $^2H_3$DZ9G |
| | **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 | Labeled CK standard |
| 26 | Benzyloaminopurine (BA) | $^2H_7$BA |
| 27 | Benzyloaminopurine riboside (BAR) | $^2H_7$BAR |

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 CKs that is present in all four CK fractions (CKNTs, CKRBs, CKFBs and CKGCs) (**Figure 10**). In green leaves, the concentration of CK precursor *t*ZNT is characterized by a two-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 re-activated 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 is 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.

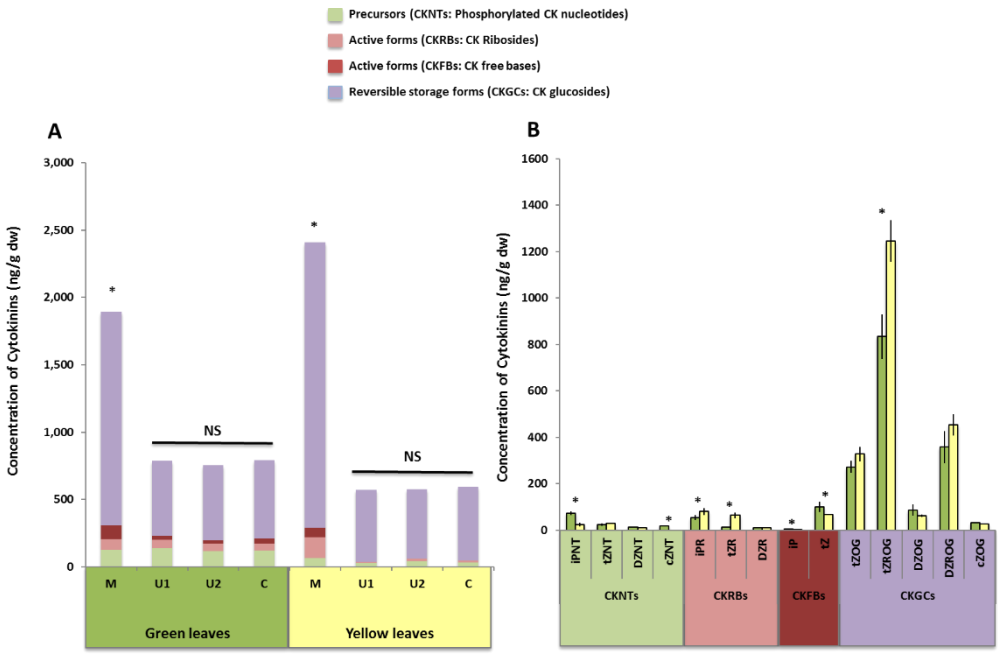



**Figure 9. A)** CK concentrations in green and yellow leaves in mined (M) and unmined plant tissues (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Light green = phosphorylated CK nucleotides (CKNTs: iPNT, *t*ZNT, DZNT and *c*ZNT); light red = CK active forms (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 shown by asterisks. See table 1 for abbreviations.

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 (**Figure 10**). 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 larger concentrations of iPR.

DZ-type of CKs show very similar patterns both on green and yellow leaves, with mined areas accumulating DZR and DZROG with similar amounts for mines on green and mines on yellow leaves (**Figure 10**). 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 affected on mines in yellow leaves where it cannot be detected in unmined and control tissues. DZ was not detected in any leaf tissues.

### 3.6.2. 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 amounts in yellow leaves (**Figure 11**). All types of CKs can be found in larvae from precursors to active and storage forms (**Figure 11**) but larvae mainly contain CKRBs and CKFBs (**Figure 12**) contrasting with leaf tissues that mainly contain CKGCs (**Figure 9**). 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 show greater amounts 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 increase in larvae from mines on yellow leaves compared to larvae from mines on green leaves (Figure **11**) while *t*Z concentrations are shown to be lower in mined tissues of yellow leaves compared to mined tissues on green leaves (**Figure 10**). Interestingly, other *t*Z-types of CKs (*t*ZNT, *t*ZR, *t*ZROG) also occur in higher concentrations in larvae from mines on yellow leaves compared to larvae from mines on green leaves. Total CK and total *t*Z-type CK levels are higher in mines than in larvae on green leaves due to high levels of CKGCs (**Figure 12**). The opposite trend is observed on yellow leaves with higher concentrations in larvae compared to mined tissues (**Figure 12**).

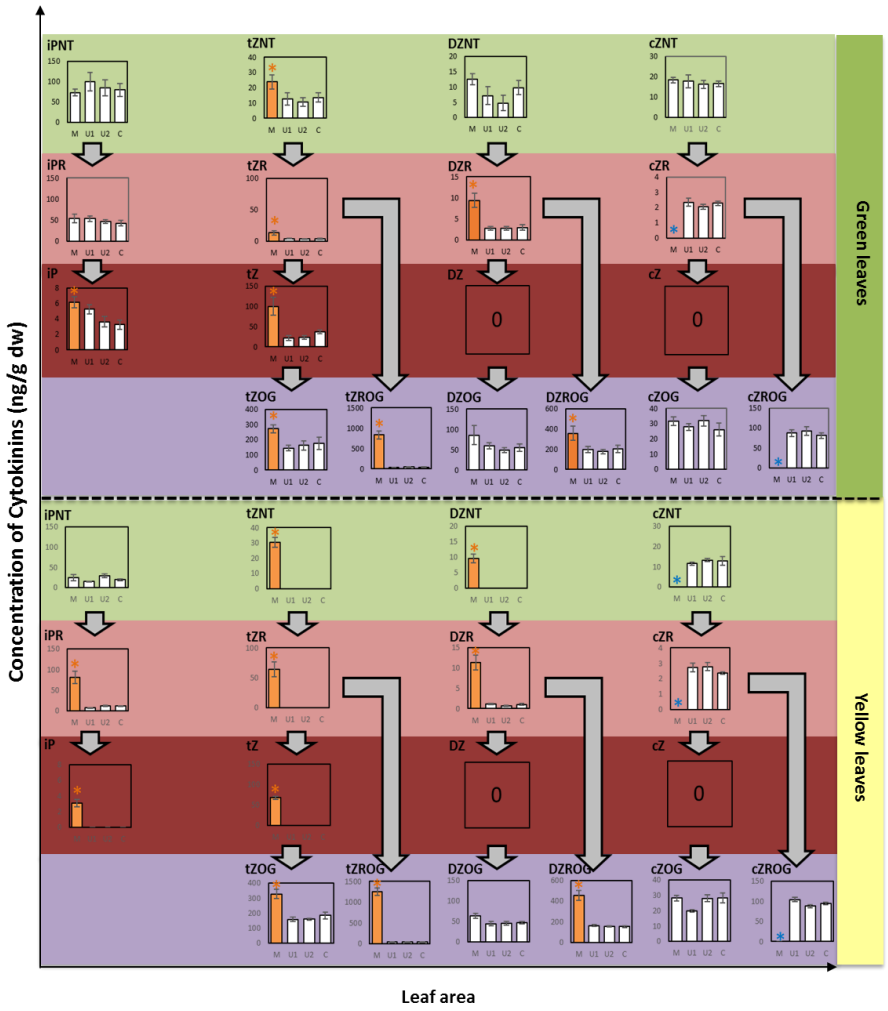



**Figure 10.** Changes in CK levels in green and yellow leaf tissues of mined (M) and unmined areas (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Data are presented as mean ± SEM. Statistical differences are shown by asterisk (orange asterisks indicate a significant increase while blue ones indicate a significant decrease). Orange bars shown significant increases of CK concentrations. See figure 9 for background colours and table 1 for abbreviations.

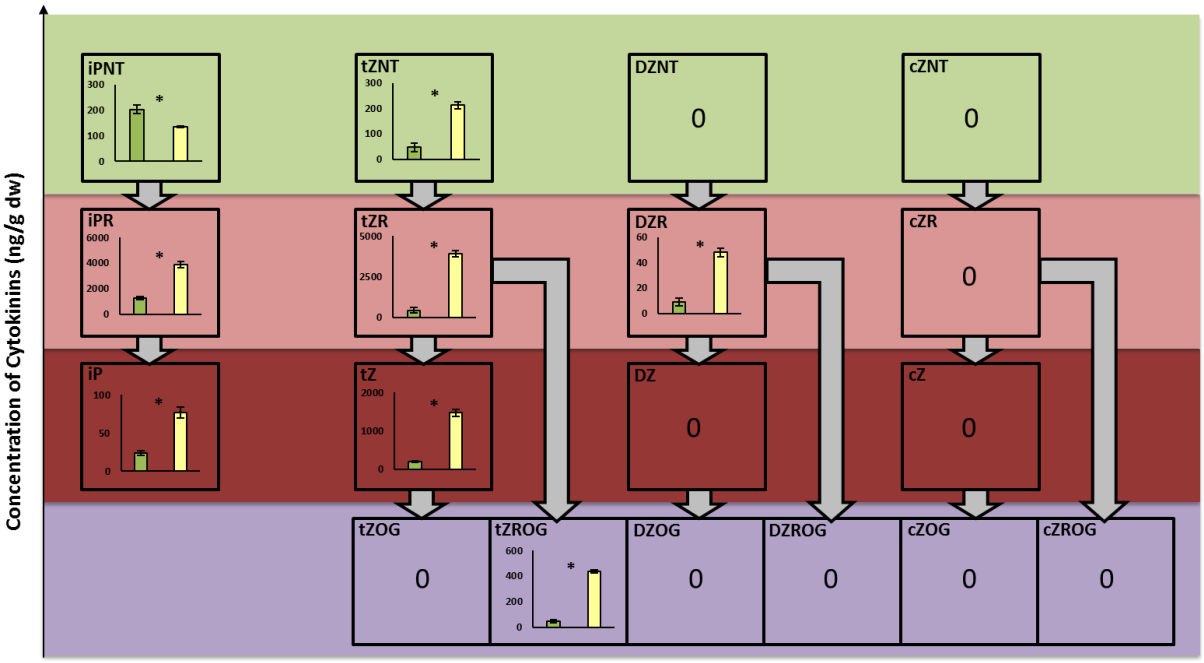



**Figure 11.** Concentrations of CKs in larvae from green (green bars) and yellow leaves (yellow bars). Data are presented as mean ± SEM. Statistical differences are shown by asterisk. See figure 9 for background colours and table 1 for abbreviations.

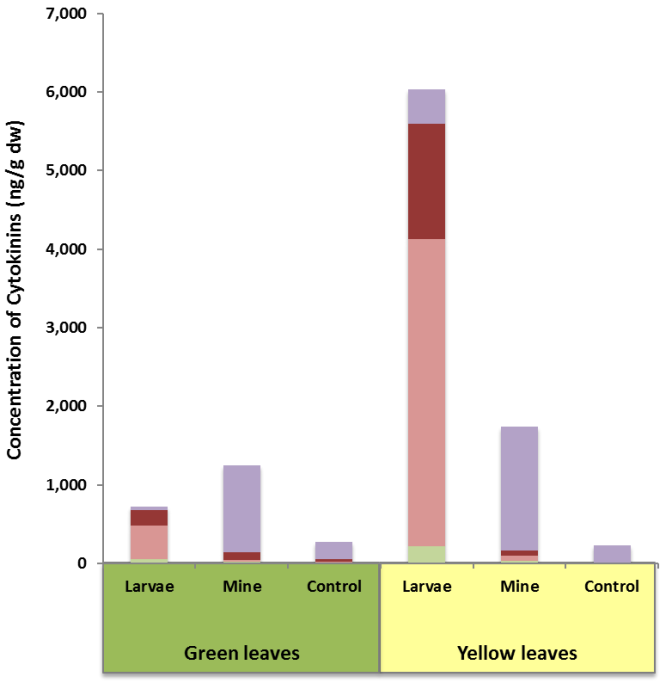



**Figure 12.** Levels of tZ-type CKs in larvae, and in mined and control leaf tissues both on green and yellow leaves. Light green = tZNT; light red = tZR; dark red = tZ; purple = tZOG, and tZROG).

## 3.6.3. High concentration of 2-MeS-CKs can be found both in larvae and leaf tissues but with differences between larvae and leaf tissues

High concentrations of 2-MeS-CKs are found both in larvae (**Figure 13A**) and leaf tissues (**Figure 13B**). Very high amounts of 2-MeS-CKs are detected in larvae, especially those 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 on green and yellow leaves. Overall 2-Me-SZR concentrations are higher in yellow leaves but with lower amounts in mined areas compared to control while the opposite is observed on green leaves.

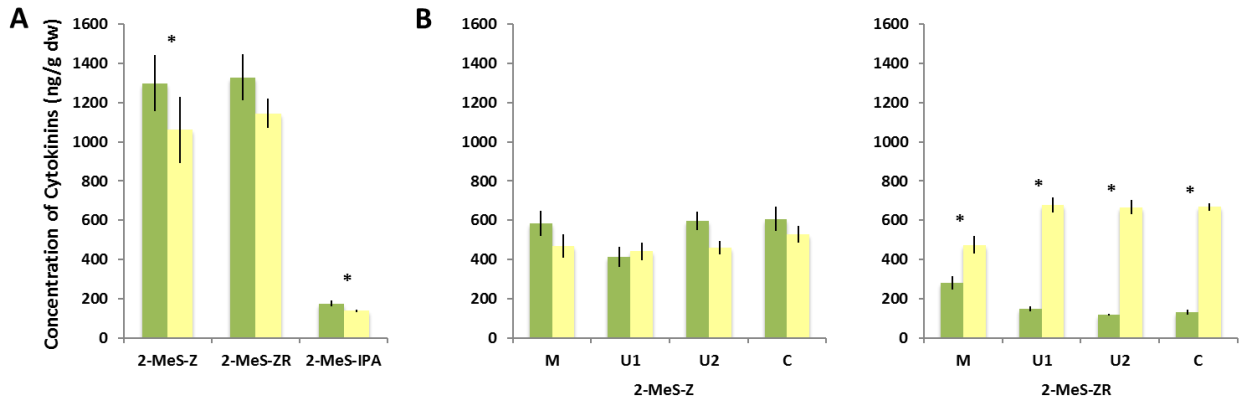



**Figure 13.** Concentration of 2-methylthio-CKs in larvae and leaf tissues. **A)** Concentrations of 2-MeS-CKs in larvae from green and yellow leaves. **B)** Concentrations of 2-MeS-CKs in green and yellow leaf tissues in mined (M) and unmined areas (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Data are presented as mean ± SEM. Statistical differences are shown 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).

# 3.7. 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 defence 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 and Connor, 2014; Tooker and Helms, 2014; Giron et al., 2016).

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 and 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 and 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. 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 and 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 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 and 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 areasof 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 suggested 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 favourable nutritional environment. Overall, CKs found in larvae correspond 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 oxidases enzymes and they can be re-activated by β-glucosidases (Mok et al., 2000). Therefore, they can help maintaining 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's physiology, with the conversion of a fraction of these CKs into CKGCs happening later in the

mine. High levels of isoprenoid CKs (iP, iPR, *t*Z, *t*ZR, *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 the very high metabolic demand required on yellow leaves to maintain a green-island phenotype on senescing leaves. Higher levels of all *t*Z-type CKs in larvae from yellow leaves compared to larvae from green leaves suggests that insects benefit from plant- and insect-borne CKs on green leaves but shift on yellow leaves to (almost) exclusively insect-borne CKs produced through the MEP pathway. This is further supported by the great amount of active CKs (both CKRBs and CKFBs) in larvae and higher amounts of CKs in larvae compared to mined tissues on yellow leaves while the opposite trend was observed on green leaves.

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 and Glevarec, 2014) as well as on gall-inducing insects that reported high concentrations of *t*Z (Mapes and Davies, 2001a, 2011b; Dorchin et al., 2009; Straka et al., 2010; Tokuda et al., 2013; Bartlett and Connor, 2014; Tooker and 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 CKs concentration and the high biological activity of *t*Z contribute to the alteration of the plant's 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; Schaller et al., 2015; Yamaguchi et al., 2012). Increased *t*Z-type CK levels can also favour nutrient translocation toward the insect's feeding site, inhibition of leaf senescence and mitigation of direct and indirect plant defences (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 signalling pathways (Frago et al., 2012; Biere and 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 in the absence of detectable CKs in larvae compared to the non-treated 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 and 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 and 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 and 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 degraded 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-Me-SiPA is a predominant and specific form of CKs found in prokaryotes (Skoog and 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 thus potentially contributing to allow persistent effects, while avoiding CK degradation by the plant, deleterious effects on plant development and CK-mediated plant defences (Ajitkumar and 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 and Glevarec, 2014) and suggests that CKs of bacterial origins could be involved in the interaction between *P. blancardella* and its host-plant.

# 3.8. 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 plant 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 now needed to validate the possible role of the various CKs and the specific role of each partner in this intricate plant-insect-microbe interaction.

# 3.9. Acknowledgments

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# Chapter 4. The *Wolbachia*-free leafmining species *Phyllonorycter mespilella* fails to modulate plant auxin and cytokinin levels under variable environmental conditions

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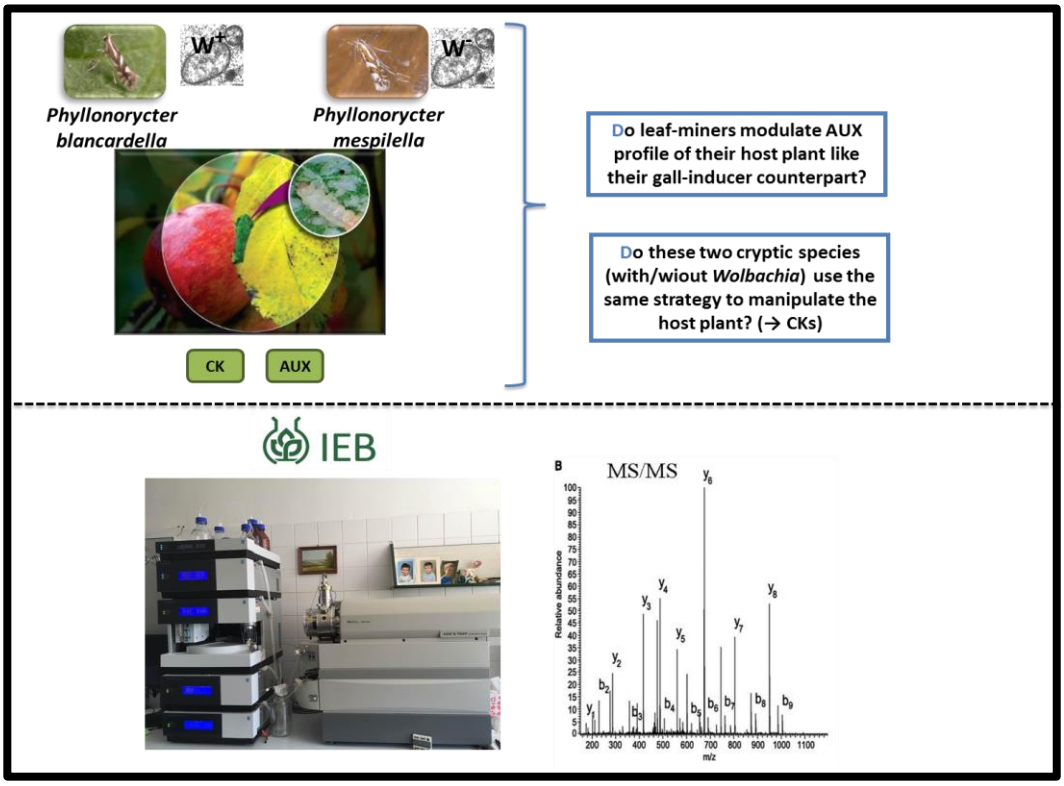
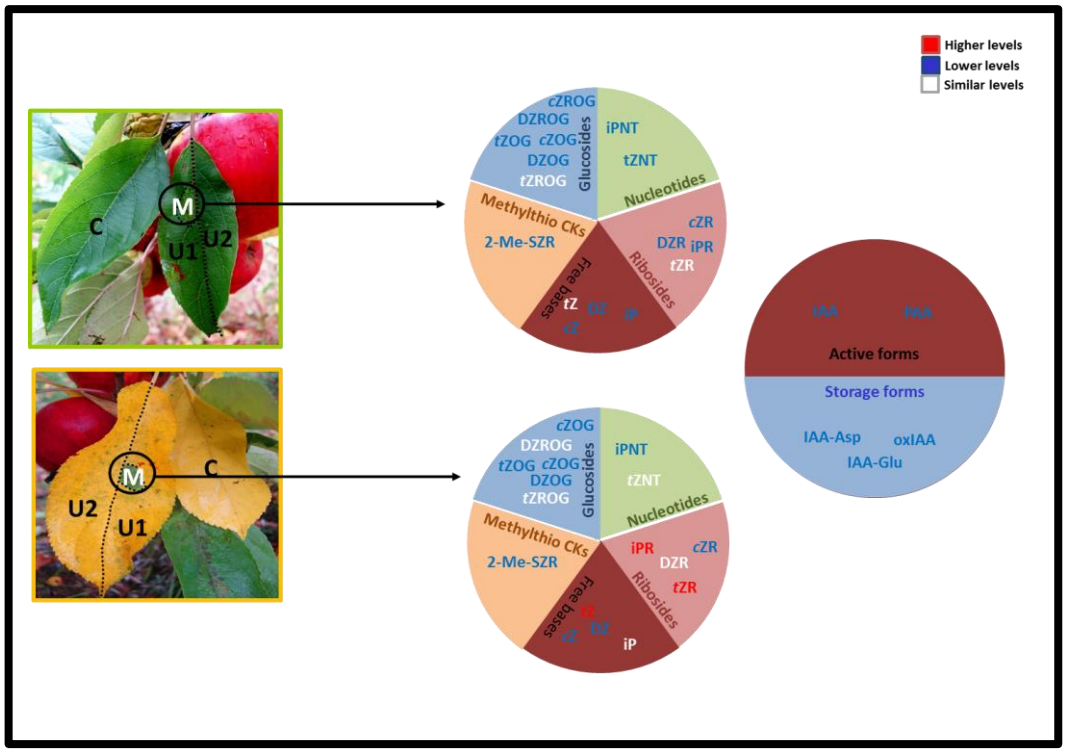
# 4.1. Graphical abstract



# 4.2. Introduction

## 4.2.1. Objectives.

The first objective of this chapter 4 was to investigate whether or not leaf-mining insects modulate the auxin (AUX) profile of their host-plant like their gall-inducer counterpart. AUX is well known to cause plant cells to grow and divide, therefore potentially playing a role in generating the nutritive tissue found in galls at larval feeding sites (Tooker and Helms, 2014). AUX accumulation at infection sites has been reported in several systems and gall-inducing larvae can produce and deliver these effectors to the plant (Yamaguchi et al., 2012). Additionally, gall-inducers and leaf-miners share numerous similarities in mechanisms involved in plant manipulation and insect-induced effects on plants (Giron et al., 2016).

*Working hypothesis:* We expected to find increased levels of AUX in mined areas.

The second objective was to see if closely related leaf-miner species sharing the same ecological niche but that differ in their *Wolbachia* infection status developed similar strategies to manipulate their host-plant, especially regarding the alteration of Cytokinins (CKs).

*Working hypothesis:* We expected that *Wolbachia*-free *Phyllonorycter mespilella* larvae would induce distinct CK alterations compared to *P. blancardella*.

## 4.2.2. Methods.

To reach these objectives, we conducted an extensive identification and quantification of AUX and CKs by HPLC-(ESI+)-MS/MS in both green and yellow leaves of *Malus domestica* infested by *P. mespilella*.

## 4.2.3. Results.

Our time course characterization of phytohormones in attacked apple leaves show that mines are not enriched in CKs and AUX. Total CKs and total AUX levels are lower in mines both on green and yellow leaves. Increased levels of CKs can only be observed in mines on yellow leaves for 3 CK active forms (iPR, *t*ZR, *t*Z).

## 4.2.4. Conclusions.

The characterization of AUX and CKs in both green and yellow leaves of *M. domestica* infested by *P. mespilella* show that leaf-mining moth species that differ in their *Wolbachia* infection status induce distinct phytohormonal alterations of their host-plant. This shows that *Wolbachia*-free leaf-miners fail to modulate plant AUX and CK levels under variable environmental conditions.

An extensive identification and quantification of CKs is necessary to identify physiological patterns relevant for the plant-insect interactions, accumulation of CKs being observed only for specific active forms and strictly restricted to mines on yellow leaves. This pattern is most likely due to the strong decrease of CKs in non-infested leaf areas resulting from the senescing programme rather than a CK accumulation in the mine *per se*.

Our study suggests that mechanisms underlying the plant-insect interaction are different between the two leaf-miner species and that *P. mespilella* larvae most likely do not produce CKs. It further provides converging experimental evidences pointing towards 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.

The results are prepared for a submission in *Entomologia Experimentalis et Applicata*

# 4.3. Abstract

Because 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) and auxins (AUX) are phytohormones widely recognized to be involved in plant manipulation by insects. Our results show that the *Wolbachia*-free leaf-mining moth *Phyllonorycter mespilella* fails to modulate plant AUX and CK levels under variable environmental conditions contrasting with results previously observed in the closely related moth species *P. blancardella* that produce and deliver CK to the plant through and intricate interaction with *Wolbachia*. While AUX have been demonstrated to play a key role in plant manipulation by gall-inducing insects and that larvae can supply the hormones themselves, our results show that leaf tissues infected by *P. mespilella* have lower levels of AUX. Our results show that the leaf-mining moth *P. mespilella* does not modulate AUX despite the fact that gallers and leaf-miners share numerous similarities in mechanisms involved in plant manipulation and insect-induced effects on plants. They further suggest that closely related leaf-miner species sharing the same ecological conditions but that differ in their *Wolbachia* status most likely rely on distinct mechanisms to colonize their host-plant and adapt to fluctuating environmental conditions. Converging experimental evidences point towards the influence of bacterial symbionts in the ability of leaf-miners to control the plant phytohormonal balance with important consequences for the ecology and the evolutionary diversification of leaf-mining moths.

**Keywords:** Plant-insect-microbe interactions, Phytohormones, Cytokinins, Auxins, Leaf-miners, Symbionts

# 4.4. 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; Raubenheimer et al., 2009; Body, 2013). It also requires them to avoid the 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 and Tumlinson, 2008; Bruessow, et al., 2010) and/or improved nutritional benefits for the parasitic herbivore at the expense of the plant (Awmack and Leather, 2002). Some of the most spectacular insect-induced plant manipulations are the strong reprogramming of the host-plant development leading to new plant structures such as galls (Price et al., 1987; Stone and Schönrogge, 2003). These insect-generated shelters provide insects with protection against natural enemies and abiotic factors but also an enhanced nutritional environment (Stone and Schönrogge, 2003). Other phenotypes such as 'green-islands' induced by several leaf-mining insects are also clear examples of plant manipulation with numerous similarities with gall-inducers in possible mechanisms involved and insect-induced effects on plants (Giron et al., 2016; **Chapter 3**).

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*/chapter 3**). These compounds are key regulators of plant growth and developmental processes and modulate plant responses to their biotic and abiotic environment (Pieterse and 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 the evolution allowing them to successfully colonize and exploit various host-plants (Schultz and 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 antiherbivore defenses due to an accumulation of salicylic acid (SA) and a negative crosstalk between SA and JA (Chung et al., 2013). This manipulation decoy the host plant to induce an antibacterial rather than an anti-insect response. Several studies on Hessian fly and other gall-inducers such as *Eurosta solidaginis* and *Gnorismoshema gallaesolidaginis* 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 and Davies, 2001; Tooker and De Moraes, 2011a, 2011b; Tooker and Helms, 2014).

The ability to control the plant phytohormonal balance is a well-characterized strategy used by several plant-associated microorganisms to colonize and exploit the plant and molecular mechanisms involved have been intensively studied (Jameson 2000; Robert-Seilaniantz et al. 2007; Pertry et al., 2009; Giron and Glevarec, 2014). Many plant-associated microbes potentially influence the levels of phytohormones by inducing plant genes involved in phytohormone biosynthesis, metabolism, degradation or response, but they can also produce and secrete plant hormones themselves (e.g. Costacurta and Vanderleyden, 1995; Spaepen and Vanderleyden, 2011 Giron et al., 2013). One of the best examples is the actinomycete *Rhodococcus fascians*, a grampositive bacterium that induces differentiated galls (known as leafy galls), on a wide variety of plants (Stes et al., 2011). *R. fascians* employs virulence genes located on a linear plasmid allowing the production of a mixture of synergistically acting cytokinins (CKs) that increase nutrient release, suppress defenses and promote disease establishment (Choi et al., 2011; Depuydt et al., 2009; Pertry et al., 2009, 2010; Stes et al., 2011, 2013; Giron and Glevarec, 2014). In *Agrobacterium tumefaciens*, a gramnegative bacterium causing crown galls on a wide variety of host-plants, CKs are produced by a CK biosynthesis enzyme which is encoded on the T-DNA region of a tumor-inducing plasmid that is transferred and integrated into the plant genome after infection (Choi et al. 2011; Jameson 2000). Several herbivores have also developed the ability to manipulate the plant signaling but underlying mechanisms still remain to be discovered for the great majority of them. However, significant amounts of phytohormones have been found in some insect saliva, body and accessory glands suggesting that insects may also directly produce relevant levels of phytohormones or effectors with mimicking functions. (Bartlett and Connor, 2014; Giron et al., 2013; Tooker and Helms, 2014; **Zhang et al. 2017a***/**Chapter 3**).

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 and Bennett, 2013; Giron et al., 2013; Sugio et al., 2014; Giron 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 the insect host (Body et al., 2013; Kaiser et al., 2010; Sugio et al., 2014; **Zhang et al., 2016***/**Chapter 2**; Giron et al., 2017; **Zhang et al. 2017a*/Chapter 3**). Metabolic profiling looking for bacterial chemical

signatures (Zhang et al., 2017a/Chapter 3), and comparisons between insects deprived of their endosymbiotic bacteria through antibiotic treatments (Kaiser et al. 2010) or between distinct populations that harbor or not endosymbionts (Barr et al., 2010) have provided key indirect evidences of the role of symbionts in the modulation of plant phytohormones. However, without labeling experiments, artificial rearing media or knockout mutants it remains often 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).

CKs and AUX are phytohormones widely recognized to be involved in plant manipulation by gall-inducers and leaf-miners (Giron et al., 2013; Tooker and Helms, 2014; Giron et al., 2016). These phytohormones regulate many plant growth and developmental processes and interact in a complex manner that varies according to their precise spatial distributions within plant tissues (Costacurta and Vanderleyden, 1995; Pernisová et al., 2011; Schaller et al., 2015). Because most galls grow via hypertrophy and/or hyperplasia, attention has rapidly focused on AUX and CKs due to their influence on cell growth and division (Bartlett and Connor, 2014; Davies, 2004; Straka et al., 2010; Tooker and Helms, 2014; Tooker and De Moraes, 2011a; Yamaguchi et al., 2012). IAA (indole-3-acetic acid) is the main AUX in plant tissues and is well known to cause plant cells to grow and divide, therefore potentially playing a role in generating the nutritive tissue found in galls at larval feeding sites. Increased CK levels can also favor cell division but also nutrient translocation toward the insect's feeding site and delay senescence thus contributing to gall induction and a green-island phenotype (Lara et al., 2004; Walters and McRoberts, 2006; Walters et al., 2008; Mok and Mok, 2001; **Chapter 2, 3**).

AUX and/or CKs accumulation at infection sites have been reported in several systems providing indirect evidences that insects control the AUX:CK ratio playing a role in the development and maintenance of galls (Schaller et al., 2015) or green-islands (Giron et al., 2013). Studies on *Mayetiola destructor* (Diptera), *G. gallaesolidaginis* (Lepidoptera) and *E. solidaginis* (Diptera) report that insects are able to induce IAA accumulation in galls (Tooker and De Moraes, 2011a, 2011b; Zhu et al., 2010, 2011). Studies on the pteromalid wasp *Trichilogaster acacialongifoliae*, the psyllid *Pachypsylla celtidis*, the tephritid fly *E. solidaginis* and the maize orange leafhopper *Cicadulina bipunctata* report high concentrations of CKs in galls (Dorchin et al., 2009; Mapes and Davies, 2001a, 2011b; Straka et al., 2010; Tokuda et al.,

2013). In leaf-miner insects, studies on *Stigmella argyropeza* Z., *St. argentipedella* Z., *Stigmella* spp. and *Phyllonorycter blancardella* 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***). Evidence for how AUX works in leaf-miners are scarce, but is has been demonstrated that application of AUX on poplar leaves could induce phenotypes which are similar to those found in the tunnels of leaf-miners (La Rue, 1937).

Whether or not insects can be the source of AUX and/or CKs – rather than simply manipulating the plant phytohormonal balance/signalling – found at high levels at the insects feeding sites remains a challenging question to be answered in most systems. The most-convincing evidence comes from a recent study providing the first demonstration of AUX biosynthesis by an insect species using labeled tryptophan (Yamaguchi et al., 2012). A sawfly species (Hymenoptera: *Pontania* sp.) that induces galls on *Salix japonica*, was shown to synthesize and secrete IAA (Yamaguchi et al. 2012). These larvae also contain high CK levels (Yamaguchi et al. 2012). Interestingly, females can also play a role in gall induction or growth by injecting at oviposition fluids that are rich in phytohormones. In sawflies, 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 and Davies 2001b; Dorchin *et al*. 2009; Straka *et al*. 2010; Tooker and De Moraes 2011a, 2011b; 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., 2017a*/Chapter 3**) suggesting their ability to produce and deliver these effectors to the plant (Giron *et al*. 2013; Bartlett and Connor 2014; Tooker and Helms 2014; **Zhang et al., 2017a*/Chapter 3**).

Previous experiments in the *Malus domestica*/*P. 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 demonstrated that these leaf areas are enriched in CKs and that CKs that accumulated in the mined area do not originate from the plant CK-biosynthetic pathway (**Zhang *et al*. 2016/Chapter 2**). Several lines of evidence also showed that insects contain large amounts of CKs and that phytohomones accumulating in the mines most likely originate from the insect itself (Body *et al.* 2013; Zhang et al. 2017a/Chapter 3). 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., 2017a*/Chapter 3**).

The objective of the present study was to conduct an extensive identification and quantification of CKs and AUX in both green and yellow leaves of *M. domestica* infested by *P. mespillela*, a closely related species of *P. blancardella* sharing the same ecological niche but that do not harbor *Wolbachia*. This study was designed to investigate whether or not leaf-mining insects modulate the AUX profile of their host-plant like their gall-inducer counterpart, as we know that gallers can modify AUX levels (Henderson and Bonner, 1952; Purohit et al., 1980; Tooker and De Moraes, 2011a, b) and that gallers and leaf-miners share similar strategies for manipulating the plant (Giron et al. 2016; **Zhang et al. 2017a*/Chapter3**). We also aimed to identify if closely related species sharing the same ecological niche developed 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.

# 4.5. Materials and methods

## 4.5.1. Biological material

*Phyllonorycter mespilella* (Lepidoptera: Gracillariidae) is a moth of the Gracillariidae family. This polyvoltine leaf-mining microlepidopteran of apple trees is closely related to *P. blancardella* (Gutzwiller et al., 2015) and the two species are morphologically cryptic (Dedeine and Giron Unpublished). 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 *P. blancardella* and divided into five instars (Pottingger and Leroux, 1971; Body et al., 2015). The first three third instars (L1-L3) 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 larval 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 (Pottingger and Leroux, 1971; Djemaï et al., 2000; Body *et al*., 2015). Similarly to *P. blancardella* and other leaf-mining species, *P. mespilella* manipulate the host plant to induce 'green island'.

Both green and yellow leaves infested by L4 instars (only one mine per leaf) and unmined (an adjacent neighboring leaf) leaves were simultaneously collected in the field between 09:00 a.m. and 10:00 a.m. in autumn (November) on *M. domestica* apple-trees, in a biologically managed orchard in La Chapelle-aux-Naux, France (47°19'09'' North, 0°25'42'' East). 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).

## 4.5.2. 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) 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, 2004) for COI amplification and wsp81f and wsp691r (Braig et al, 1998) for wsp amplification. COI amplified products were then sequenced in both directions at Eurofins Genomics, Germany. Species identification were performed using the "species"-level identification function of the BOLD ID Engine (Ratnasingham and Hebert, 2007) based upon

aligned COI sequence data and infection status were verified by agarose gel electrophoresis on wsp amplified products.

### 4.5.3. LC/MS analysis of plant hormones

The analysis of plant hormones was carried out following the protocole developed by Dobrev and Kaminek (2002) and Dobrev and Vankova (2012). In brief, for each sample (n = 22 in green leaves and n=12 in yellow leaves for each area: M, U1, U2 and C), an aliquot of 100mg fresh weight was transferred into amicrocentrifuge tube. 500µl of cold extraction buffer (methanol/water/formic acid, 15/10/5, v/v/v, -20oC) were added to each tube as well as a mixture of stable isotope labelled internal standards (10pmol) (see **Table 3**). After incubation for 30min at -20°C, each extract was centrifuged at 17000 rpm and the supernatant collected. A second extraction of the residue was performed for each sample to ensure that all the target compounds are 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 reversed phase–cation exchange SPE column (Oasis-MCX, Waters), in order to remove from the extract as much as possible of interfering substances without losing significant amount of target compounds. Two hormone fractions were sequentially eluted: (1) fraction A eluted with methanol contained AUX, including active forms IAA and phenylacetic acid (PAA), reversible storage forms IAA-Aspartate (IAA-asp) and IAA-Glutamate (IAA-glu) and a catabolite of IAA, 2-oxindole-3-acetic acid (oxIAA). (2) fraction B eluted with 0.35 M $NH_4OH$ in 70% methanol contained CKs, including CK precursors (phosphorylated 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), reversible storage forms (CK glucosides CKGCs): *t*ZOG, *t*ZROG, DZOG, DZROG, *c*ZOG and *c*ZROG) but also 2-methylthio-derivatives (2-Me-SZ and 2-Me-SZR). Fractions were evaporated to dryness in vacuum concentrator and dissolved into 30 µl 10% methanol. An aliquot (10 µl) from each fraction was separately analyzed on HPLC (Ultimate 3000, Dionex) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems) set in selected reaction monitoring mode. Mass spectrometer was run on

electrospray ionization mode, negative for fraction A, and positive for fraction B. Ion source parameters included: ion source voltage -4000 V (negative mode) or +4500 V (positive mode), nebulizer gas 50 psi, heater gas 60 psi, curtain gas 20 psi, heater gas temperature 500°C. Quantification of hormones was done using isotope dilution method with multilevel calibration curves (r2>0.99). Data processing was carried out with the Analyst 1.5 software (Applied Biosystems).

### 4.5.4. Statistical analyses

Statistical analyses were performed using SPSS version 19. Non-parametric statistics (Kruskal-Wallis test followed by all pairwise multiple comparisons) were used as data were not normally distributed even after log-transformation.

## 4.6. Results

### 4.6.1. Insect species identification and detection of endosymbionts

Cloning and sequencing of the corresponding products show that the insects identified are *P. mespillela* which are uninfected with *Wolbachia*.

## 4.6.2. CK content decreases in mined tissues

In total, 17 CKs that belong to four classical types of CKs (*t*Z, *c*Z, iP, DZ type CKs) and their methyltio derivatives were identified in both green and yellow leaves (**Table 4**). Identified CKs include precursors (phosphorylated CK nucleotides (CKNTs): iPNT and *t*ZNT), active forms (CK ribosides (CKRBs): iPR, *t*ZR, DZR and *c*ZR; CK free bases (CKFBs): iP, *t*Z, DZ and *c*Z), reversible storage forms (CK glucosides (CKGCs): *t*ZOG, *t*ZROG, DZOG, DZROG, *c*ZOG and *c*ZROG) but also one 2-methylthio-derivative (2-Me-SZR). *t*Z type is the only type of CKs that is observed in all CK fractions (CKNTs, CKRBs, CKFBs and CKGCs). Overall, the feeding activity of leaf-mining larvae induces a decrease of CKs in mines compared to control leaves (**Figure 14**). Distinct patterns can be observed on green and on yellow leaves. In green leaves, lower amounts are observed for all CKs in mines except for *t*ZR levels that remain constant. Only *t*Z and *t*ZROG levels show a slight trend towards and accumulation in mines. In yellow leaves, there is a global decrease of CKs in mines but the analysis of the CK composition reveals specific patterns for iPR, *t*ZR, and *t*Z that have higher levels than controls in the mine and DZR, DZ and *t*ZROG that remain constant.

## 4.6.3. AUX content decreases in mined tissues

In total, 5 forms of AUX were identified in both green and yellow leaves including two naturally occurring active AUX (IAA and PAA), and three inactive modified AUX forms (IAA-Asp, IAA-Glu and oxIAA) **(Figure 15**). Overall, AUX are largely accumulated in leaf tissues and are 2.6 times and 3.4 times more abundant than CKs in green and yellow mines respectively. However, the feeding activity of the leaf-mining larvae causes a strong decrease of AUX in mined tissues compared with controls (**Figure 15**). Green and yellow leaves show very similar patterns, with constant levels of IAA and IAA-Asp but lower levels of PAA, IAA-Glu and oxIAA in mines compared to controls.

**Table 3.** Cytokinins (CKs) and Auxin (AUX) scanned for by liquid chromatography-positive electrospray ionization tandem mass spectrometry (HPLC- (ESI+)-MS/MS). Deuterated internal standards for CKs identification purchased from OlChemim Ltd. (Olomouc, Czech Republic) and for AUX identification purchased from Cambridge Isotope Laboratories (Massachusetts, U.S.A) were used for the analysis.

| | Isoprenoid cytokinins | Labeled CK standard |
|---|---|---|
| | Nucleotides (CKNTs) | |
| 1. | *Trans*-zeatin riboside-5'-monophosphate (*t*ZNT) | $^{2}H_{5}$-*t*ZRMP |
| 2. | *Cis*-zeatin riboside-5'-monophosphate (*c*ZNT) | |
| 3. | Dihydrozeatin riboside -5'-monophosphate (DZNT) | $^{2}H_{3}$DZRMP |
| 4. | $N^{6}$-isopentyladenosine-5'monophosphate (iPNT) | $^{2}H_{6}$-iPRMP |
| | Ribosides (CKRBs) | |
| 5. | *Trans*-zeatin riboside (*t*ZR) | $^{2}H_{5}$*t*ZR |
| 6. | *Cis*-zeatin riboside (*c*ZR) | |
| 7. | Dihydrozeatin riboside (DZR) | $^{2}H_{3}$-DZR |
| 8. | $N^{6}$-isopentyladenosine (iPR) | $^{2}H_{6}$-iPR |
| | Free bases (CKFBs) | |
| 9. | *Trans*-zeatin (*t*Z) | $^{2}H_{5}$-*t*Z |
| 10. | *Cis*-zeatin (*c*Z) | |
| 11. | Dihydrozeatin (DZ) | $^{2}H_{3}$-DZ |
| 12. | $N^{6}$-isopentyladenine (iP) | $^{2}H_{6}$iP |
| | Glucosides (CKGCs) | |
| 13. | *Trans*-zeatin-O-glucoside (*t*ZOG) | $^{2}H_{5}$-*t*ZOG |
| 14. | *Cis*-zeatin-O-glucoside (*c*ZOG) | |
| 15. | Dihydrozeatin-O-glucoside (DZOG) | $^{2}H_{7}$DZOG |
| 16. | *Trans*-zeatin-O-glucoside riboside (*t*ZROG) | $^{2}H_{5}$-*t*ZROG |
| 17. | *Cis*-zeatin-O-glucoside riboside *c*ZROG | |
| 18. | Dihydrozeatin-O-glucoside riboside (DZROG) | $^{2}H_{7}$DZROG |
| 19. | *Trans*-zeatin-9-glucoside (*t*Z9G) | $^{2}H_{5}$-*t*Z9G |
| 20. | *Cis*-zeatin-9-glucoside (*c*Z9G) | |
| 21. | Dihydrozeatin-9-glucoside (DZ9G) | $^{2}H_{3}$-DZ9G |
| | Methylthiols (2-MeS-CKs) | |
| 22. | 2-Methylthio-*trans*-zeatin (2MeSZ) | $^{2}H_{5}$MeSZ |
| 23. | 2-Methylthio-*trans*-zeatin riboside (2MeSZR) | $^{2}H_{5}$MeSZR |
| 24. | 2-Methylthio-$N^{6}$-isopentyladenine (2MeSiP) | $^{2}H_{6}$MeSiP |
| 25. | 2-Methylthio-$N^{6}$-isopentyladenosine (2MeSiPA) | $^{2}H_{6}$MeSiPR |
| | Auxin | Labeled CK standard |
| 26 | Indole-3-acetic acid (IAA) | |
| 27 | Phenylacetic acid (PAA) | |
| 28 | IAA-Aspartate (IAA-asp) | $^{13}C_{6}$-IAA |
| 29 | IAA-Glutamate (IAA-Glu) | |
| 30 | 2-oxindole-3-acetic acid (oxIAA) | |

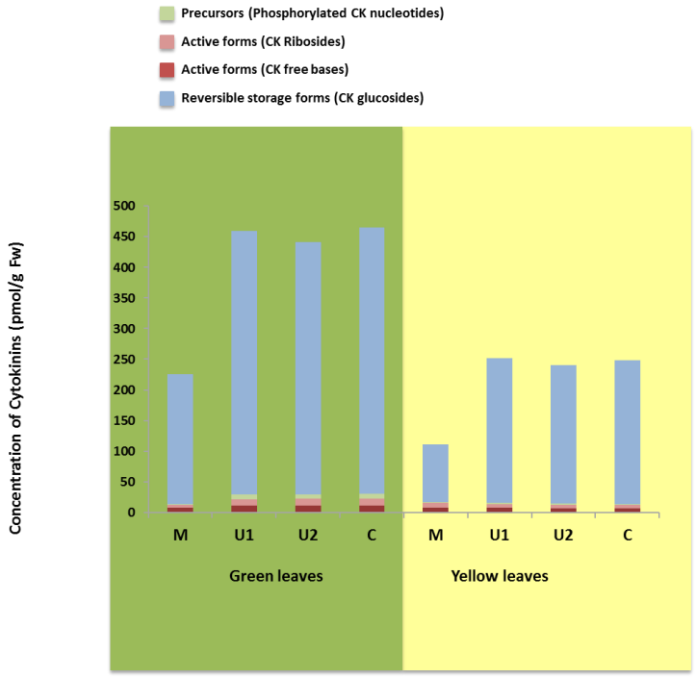



**Figure 14.** Total CKs identified in green and yellow leaves. Levels of four classical types of CKs (phosphorylated CK nucleotides (CKNTs): iPNT and *t*ZNT), active forms (CK ribosides (CKRBs): iPR, *t*ZR, DZR and *c*ZR; CK free bases (CKFBs): iP, *t*Z, DZ and cZ), reversible storage forms (CK glucosides (CKGCs): *t*ZOG, *t*ZROG, DZOG, DZROG, *c*ZOG and *c*ZROG) in mined (M) and unmined plant tissues (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Backgroud color: light green-green leaf tissues; light yellow-yellow leaf tissues.

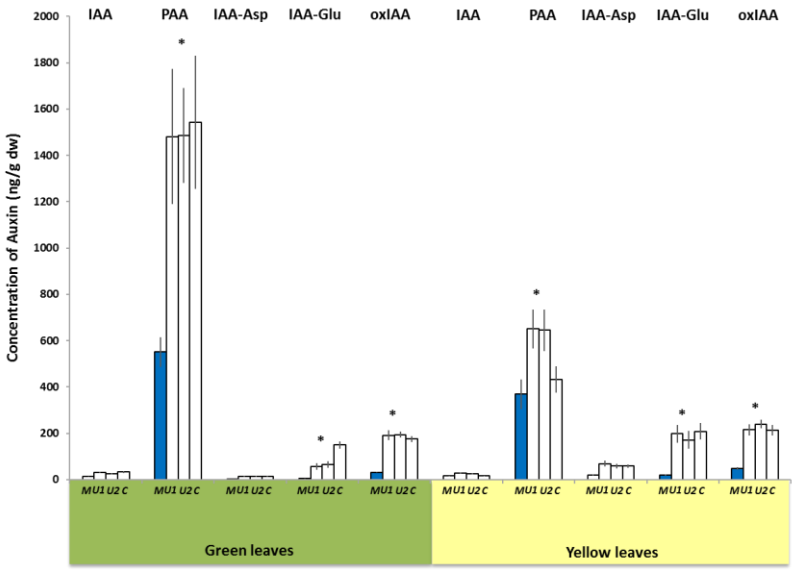



**Figure 15.** Changes in AUX levels in green and yellow leaf tissues of mined (M) and unmined areas (unmined ipsilateral tissues U1, unmined contralateral tissues U2 and control tissues C). Data are presented as mean ± SEM. Statistical differences are shown by asterisks. Blue bars shown significant decreases of AUX concentrations. Indole-3-acetic acid (IAA), Phenylacetic acid (PAA), IAA-aspartate (IAA-Asp), IAA-glutamate (IAA-Glu), oxo-IAA (OxIAA).

**Table 4.** CK concentrations in mined *vs* control leaf areas following attack by *P. mespilella* and *P. blancardella*

| | | Green leaves | | | Yellow leaves | | |
|---|---|---|---|---|---|---|---|
| **CK categories** | **CKs** | **CK concentrations in mines (pmol/g)** | **Levels in mines compared to controls** | | **CK concentrations in mines (pmol/g)** | **Levels in mines compared to controls** | |
| | | ***P. mespillela*** | ***P. mespilella*** | ***P. blancardella*** | ***P. mespilella*** | ***P. mespilella*** | ***P. blancardella*** |
| **CKNTs** | iPNT | 0.51 ± 0.08 | − | 0 | 0.25 ± 0.05 | - | 0 |
| | *t*ZNT | 0.26 ± 0.07 | − | + | 0.34 ± 0.11 | 0 | 0 |
| | DZNT | 0 | | + | 0 | | 0 |
| | *c*ZNT | 0 | | + | 0 | | - |
| **CKRBs** | iPR | 1.19 ± 0.15 | − | + | 1.20 ± 0.26 | + | + |
| | *t*ZR | 2.08 ± 0.36 | 0 | + | 5.05 ± 0.93 | + | + |
| | DZR | 1.22 ± 0.21 | − | + | 1.61 ± 0.24 | 0 | + |
| | *c*ZR | 0.22 ± 0.03 | − | - | 0.31 ± 0.04 | - | - |
| **CKFBs** | iP | 0.48 ± 0.12 | − | + | 0.50 ± 0.22 | 0 | 0 |
| | *t*Z | 5.42 ± 0.60 | 0 | + | 6.12 ± 1.20 | + | 0 |
| | DZ | 0.41 ± 0.09 | − | 0 | 0.54 ± 0.16 | - | 0 |
| | *c*Z | 0.30 ± 0.08 | − | 0 | 0.24 ± 0.08 | - | 0 |
| **CKGCs** | *t*ZOG | 16.40 ± 1.64 | − | + | 10.84 ± 1.63 | - | + |
| | *t*ZROG | 171.66 ± 18.20 | 0 | + | 61.63 ± 7.14 | 0 | + |
| | DZOG | 4.17 ± 0.27 | − | + | 3.52 ± 0.40 | - | + |
| | DZROG | 11.33 ± 0.96 | - | + | 9.95 ± 0.83 | 0 | + |
| | *c*ZOG | 0.40 ± 0.04 | - | 0 | 0.58 ± 0.07 | - | 0 |
| | *c*ZROG | 8.49 ± 0.78 | - | - | 8.41 ± 1.02 | - | - |
| **2-MeS-CKs** | 2-Me-SZ | 0 | | 0 | 0 | | 0 |
| | 2-Me-SZR | 38.22 ± 4.44 | - | + | 49.29 ± 7.19 | - | - |

Data are shown as mean ± S.E.M. CK levels increased in mines compared to controls are shown by +, decreased levels are shown by - and levels not altered in mines compared to controls are shown by 0.
Data on *P. blancardella* obtained from **Zhang et al. 2017a*.**

# 4.7. Discussion

Gall-inducing and leaf-mining insects are iconic examples of plant manipulation. Phytohormones lie at the very core of mechanisms allowing larvae to inhibit the plant immune responses and/or improve nutritional benefits for the parasitic herbivore at the expense of the plant (Giron et al., 2016; Lieutier et al., 2017). More specifically, CKs and AUX have been shown to play a key role in gall and 'green island' induction (Giron et al., 2013; Tooker and Helms, 2014; Giron et al., 2016).

While high concentration of CKs have been found in leaves infected by *P. blancardella* (Giron et al., 2007; Body 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 3 CK active forms (iPR, *t*ZR, tZ) most likely due to the strong decrease of CKs in non-infested leaf areas resulting from the senescing programme rather than a CK accumulation in the mine *per se*. Indeed, iPR and *t*Z levels in mines remain constant when leaves are turning yellow while they strongly decrease in unmined areas. *t*ZR show a slightly different pattern with an increased concentration in mines from yellow leaves compared to mines from green leaves.

Increased levels of iPR, *t*ZR and *t*Z (CK active forms) 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. 2017a***/Chapter 3). For *P. mespilella*, larvae most likely only maintain somehow the CK content of mines, thereby enabling insects to overtake the plant senescing programme. Whether the increased level of *t*ZR in mines from yellow leaves compared to mines from green leaves results from an 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 remain to be established. Second, CKs profiling show distinct patterns between *P. blancardella* and *P. mespilella* (absence of DZ and *c*Z in *P. blancardella*; absence of DZNT, cZNT and 2-MeS-Z in *P. mespilella*. Third, a

time course characterization of CKs in apple leaves attacked by *P. blancardella* shows that mines are enriched in CKs both on green and yellow leaves (**Zhang et al. 2017a***/Chapter 3) while higher levels of iPR, *t*ZR and *t*Z are only observed on yellow leaves for *P. mespilella*. Taken together, these results suggest mechanisms underlying the plant-insect interaction are different between the 2 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 for the plant or for the insect of decreased CK levels in mined areas on green leaves 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 and Mok, 2001; Sakakibara, 2006). It may well be a plant defense response (Giron et al., 2013; Naseem et al., 2015) or an insect-mediated modulation of CKs towards 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 potentially allow for an additional generation of insects (Kaiser et al., 2010).

Bacterial symbionts have been hypothesized to contribute to the production of CKs by *P. blancardella* especially through the synthesis of specific 2-MeS-CKs (**Zhang et al., 2017a***/Chapter 3). 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 failed to induce an accumulation of CKs. Antibiotic treatments previously shown that *Wolbachia*-free *P. blancardella* 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, *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*. Comparisons of life-history traits and population dynamics in *P. mespilella* and *P. blancardella* are expected to show marked differences due to the inability of *P. mespilella* larvae to induce an accumulation of CKs in mines therefore compromising their ability to fully control their nutritional supply (Body et al., 2015) and the plant immune response (Body, 2013) in both green and yellow leaves.

While AUX have been demonstrated to play a key role in plant manipulation by gall-inducing insects (Bartlett and Connor, 2014; Tooker and Helms, 2014) and that larvae can supply the hormones themselves (Yamaguchi et al., 2012), our results show that leaf tissues infected by *P. mespilella* have lower levels of AUX. Both active (PAA) and inactive forms (IAA-Glu and oxIAA) of AUX are in lower concentrations in mines compared to control tissues. IAA, is usually considered as the main AUX in plant tissues (Korasick et al., 2013), but IAA and its amino acid conjugates (IAA-Asp and IAA-Glu) were found in much lower levels than PAA in our system. Interestingly, IAA and the inactive IAA-Asp that can easily be converted into the active form (Campanella et al., 2008) were maintained at a constant level during the infection. This contrasts with results available on gall-inducers despite the fact that gallers and leaf-miners share numerous similarities in mechanisms involved in plant manipulation and insect-induced effects on plants (Giron et al., 2016; Chapter 3).

Because data on AUX modulation by leaf-miners are not available for any other system it can't be excluded that production of AUX is not part of the strategy used by leaf-miners to colonize their host-plant. Feeding strategies used by plant reprogrammers presumably evolved to face similar constraints partially imposed by the endophytic lifestyle shared by leaf-miners and most gall-inducers (Dempewolf et al., 2005; Stone and Schönrogge, 2003) leading to strong evolutionary convergences (Giron et al., 2016). However, it is likely, that mechanisms of plant manipulation can vary among taxa. Alternatively, the absence of AUX modulation may be linked to an inability of *P. mespilella* to produce and deliver AUX to the plant as observed for CKs. How the absence of *Wolbachia* is involved in the metabolic capacities of *P. mespilella* larvae and their inability to control the phytohormonal profile of the mine will require further investigations.

# 4.8. Conclusion

Our study demonstrates that the *Wolbachia*-free leaf-mining moth *P. mespilella* fails to modulate plant AUX and CK levels under variable environmental conditions and accumulation of active CKs is strictly restricted to mines on yellow leaves. An extensive identification and quantification of phytohornomones 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 both green and yellow leaves. This contrasts with results previously observed in the closely related moth species *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 are different between the 2 leaf-miner species and that *P. mespilella* larvae most likely do not produce CKs. Interplay between AUX and CKs and their effects on plant growth and immunity are complex and variable according to their precise spatial distributions within plant tissues (Costacurta and Vanderleyden, 1995; Pernisová et al., 2011; Schaller et al., 2015) and the specific plant-insect/pathogen system (Kazan and Manners, 2009; Naseem et al., 2015). Our understanding of how AUX and 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* and *Oryza sativa* (Kazan and Manners, 2009; Naseem et al., 2015). Too much generalization will neglect the involved complexity in a particular biotic interaction especially when it involved a tripartite relationship between a plant, an insect and bacterial symbionts. As a consequence, elucidating molecular mechanisms underlying the observed decrease of CKs and AUX in leaf tissues attacked by *P. mespilella* and estimating their fitness consequences for the insect will require further investigations. However, converging experimental evidences pointing towards the influence of bacterial symbionts in the ecology and the 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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# Chapter 5. Discussion and conclusion

Phytohormones have long been hypothesized to play a key role in the interactions between plant-manipulating organisms and their host–plants. Recently CKs have reemerged as key players involved in many plant-insect interactions, especially for plant manipulating insects such as gall-inducers and leaf-miners. Mechanistic understanding of how phytohormones operate in these plant reconfigurations and their origin is lacking due to limited information on the molecular and biochemical phytohormonal modulation following attack by plant-manipulating insects.

# 5.1. Cytokinins as a tool for invading the host plant

CKs have been shown to play a central role in plant colonization and exploitation by various plant-associated organisms including both antagonists and mutualists (Frugier et al., 2008; Giron et al., 2013). In such interactions, CKs can be the specific target of biotic invaders to withdraw plant resources for their own benefit. A clear example of this is the implication of CKs in the formation of 'green islands' induced by leaf-miners and various plant pathogens where nutrients are redirected towards the infection site and where host cell death is delayed. As important molecules implicated in plant defence, CKs can also be used to disrupt defensive responses (Beckman and Ingram, 1994). The molecular and biochemical characterization of the phytohormonal reconfiguration of green apple leaves following attack by the leaf-miner *P. blancardella* (infected with the endosymbiotic bacteria *Wolbachia*) was conducted by microarray and LC-MS/MS to provide an extensive characterization of how the leaf-miner *P. blancardella* modulates the major phytohormones and the transcriptional activity of plant cells in leaves of *Malus domestica* (Chapter 2). We found that CKs strongly accumulated in mined tissues. We then conducted an extensive time course characterization of CKs in both attacked green and yellow apple leaves by HPLC-(ESI+)-MS/MS (Chapter 3). It allowed us to show that larvae are able to modulate the CK levels both on green and yellow leaves. While plant modulation has important benefits under a senescing environment by allowing insects to survive adverse conditions it also allows insects to control their nutritional environment and to maintain a nutritional homeostasis even under distinct leaf environments (Body et al. 2013).

We demonstrated that leaf-mining by *P. blancardella* leads to a strong reprogramming of the plant phytohormonal balance associated with increased nutrient mobilization, inhibition of leaf senescence and mitigation of plant direct and indirect defense (Chapter 2). Leaf-mining was indeed associated with enhanced biosynthesis of JA precursors but not the active form, a weak alteration of the SA pathway and a clear inhibition of the ABA pathway. Previous results obtained in this system have demonstrated that insects control the sugar and amino acid content of the mine to create an enhanced nutritional microenvironment (Body et al., 2013; Body 2013). Insects can also inhibit the production of phenolic compounds that are the main secondary metabolites produced by apple trees to fight against insect pests (Body 2013). In an attempt to

investigate the influence of the phytohormonal modulation on the plant indirect defense I investigated how insects potentially modulate leaf abscission through control of ABA synthesis Chapter 2). I also investigated the emission of herbivore-induced plant volatiles (HIPVs) induced by leaf-mining larvae (**ANNEXE 7.2**). HIPVs are known to be produced by attacked plants to mediate the attraction of natural enemies and/or repel herbivores (Dicke and Baldwin, 2010; **Zhang et al., 2013a, b***). Phytohormones control the production of HIPV (Kost & Heil, 2006; Heil & Bueno, 2007). A preliminary experiment identifying and quantifying volatiles of healthy, and attacked green apple leaves showed that 13 different compounds can be found in healthy and infested leaves. This includes green leaf volatiles [hexanal, (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol], esters [(*Z*)-3-hexenyl acetate, methyl salicylate, 3-hexen-1-ol, benzoate], aldehydes [heptanal, benzaldehyde], monoterpenes ($\alpha$-pinene, 3-carene, limonene, ocimene) and sesquiterpenes [(*E*)-$\beta$-caryophyllene, $\alpha$-farnesene]. Compared to what is observed for the vast majority of herbivore-attacked plants, HIPV emission was very limited, most compounds showing no alterations after herbivore attack. Our results show however that both early fluid-feeding and late tissue-feeding infested-leaves emit significantly more $\alpha$-farnesene than healthy leaves, and there is no difference between early- and late-infested leaves. Interestingly, early-instar larvae induce a significant decrease of ocimene compared to control leaves, but this compound increases to the same level as control leaves for late-instar larvae. The same pattern was observed for hexanal. $\alpha$-Farnesene is one of the simplest acyclic sesquiterpenes (Wang et al., 2011) and it was first discovered in apple peels and was found to play a role in plant defense (Huelin & Murray, 1966; Pare & Tumlinson, 1999; Yang et al. 2011). Interestingly, *c*Z-type CKs and sesquiterpenes may both derive from the MVA pathway (Mok & Mok 2001; Aharoni et al., 2006; Sakakibara 2006) and our results strongly suggest late-instar *P. blancardella* larvae inhibit the MVA pathway (**Zhang et al., 2017***). Modulation of CKs trough the MVA pathway may thus explain HIPVs profiles observed. The monoterpene ocimene has also been reported to play a key role in affecting plant defense, for example by stimulating the activity of parasitic insects (Birkett et al., 2000). Additionally, *t*Z type CKs and monoterpenes may both derive from the MEP pathway (Mok & Mok 2001; Aharoni et al., 2006; Sakakibara 2006). During early-infestation, the larvae may suppress ocimene emission allowing for their successful development. Accumulation of *t*Z-type CKs for food maintenance and senescence inhibition as larvae grow may explain the increase in ocimene levels in leaves attacked by tissue-feeding larvae. (**Zhang et al., 2017***). Despite of an increase of ocimene in late-instar infested leaves, amounts were found to be similar with healthy leaves suggesting that insects may still control

somehow the plant defense system. As a green leaf volatile (GLV), hexanal is always connected with plant defense against insects (Pichersky & Gershenzon, 2002; Shiojiri et al., 2006) and is emitted immediately upon tissue damage (Hatanaka, 1993). Interestingly, some GLV are shown to prime JA-mediated plant defense in plants (Engelberth et al., 2004; Frost et al., 2007; Frost et al., 2008) and it is reported that JA and SA play pivotal roles in the regulation of HIPV emission (Venkatesan, 2015). The decrease of hexanal in leaves infected by fluid-feeding larvae is consistent with the hypothesis that insects limit the production and emission of HIPVs thus limiting plant direct and indirect defense. The reason why hexanal levels increase as larvae grow, still need further investigations but may be related to the extended tissue damages induced by tissue-feeding larvae and how larvae modulate SA and JA levles. Overall, the HIPV profiles in our system indicate that plant indirect defense is suppressed (or at least not activated) by larvae especially by fluid-feeding young larvae that have a limited impact on the plant morphology. Whether or not this translates into a limited attraction of natural enemies like parasitoids will require conducting field experiments and functional olfactory tests. Combined with data on plant direct defense (Giron unpublished; Body 2013) these results suggest that insects, through the phytohormonal control of their host-plant, disrupt the global plant defensive system. Future molecular research is now needed to fully understand the exact interactions between volatiles emission and phytohormone modulation especially considering complex crosstalks between the different phytohormones (see chapter 1). Field and laboratory functional studies are also required to have a deeper understanding on the ecological consequences of these results in terms of natural enemies attraction and parasitism/predation rates according to the larval feeding mode.

# 5.2. Origin of cytokinins: phylogenetic espionage and insect endosymbionts

Due to the regulatory role of CKs on plant morphology, plant defence, leaf senescence and source–sink relationships (causing nutrient mobilization towards the infection site), it is not surprising that these phytohormones have been a privileged target of arthropods and pathogens over the course of the evolutionary arms race between plants and their biotic partners. The ability to perceive, interpret and manipulate plant signals is likely to provide insect herbivores or plant pathogens with novel adaptive capacities enabling them to invade new ecological niches (Schultz 2002; Schultz & Appel 2004; Kaiser et al. 2010). Indeed, many signalling molecules involved in plant response to insects and pathogens are phytohormones and a high number of them are similar across kingdoms (Giron et al., 2013). Such similarities set the ground for possible exploitation of signalling pathways by one participant for its own benefit.

There has been considerable debate about the likely origin of CKs in infected leaves, as it is not usually clear whether they are produced by the pathogen/insect or by the plant. Indeed, pathogens and herbivorous insects potentially influence the levels of phytohormones by inducing plant genes involved in CKs biosynthesis, degradation or response, but they can also produce and secrete relevant phytohormones themselves (Jameson 2000; Farnsworth 2004; Robert-Seilaniantz et al. 2007; Walters, McRoberts & Fitt 2008). Accumulation of CKs in mines despite a weak expression of plant CK-related genes (Chapter 2) strongly suggests that insects produce and deliver CKs to the plant as a strategy to manipulate the physiology of the leaf to create a favorable nutritional environment. Additional data about the dynamics of CKs and the spatial distribution of CKs (Chapter 3) further confirm this hypothesis. Indeed, the spatial distribution of CKs within attacked leaves show that plant manipulation is strictly limited to the mine, suggesting the absence of CK translocation from distant leaf areas towards the insect feeding site. CKs are also detected in the highest levels in larvae (chapter 3) reinforcing our hypothesis that CKs accumulating in the mines originate from the insect itself.

Presence of bacteria-specific methylthio-CKs (Chapter 3) is consistent with previous results suggesting that insect bacterial symbionts contribute to the observed phenotype through the synthesis of specific 2-MeS-CKs. Comparison of strategies used by *P. blancardella* and a closely related leaf-miner species *P. mespilella* sharing the same ecological niche but that differ in its *Wolbachia* (Chapter 4) suggests that mechanisms underlying the plant-insect interaction are different between the two leaf-miner species and that *P. mespilella* larvae most likely do not produce CKs. It further provides converging experimental evidences pointing towards the influence of bacterial symbionts in the ability of leaf-mining moths to control the physiology of their host-plant. The evolutionary origin of CKs involved remains to be fully addressed and functional tests are now needed to validate the possible role of the various CKs and the specific role of each partner in this intricate plant-insect-microbe interaction. Our study provides key findings towards the understanding of molecular mechanisms underlying this intricate plant-insect-microbe-interaction and strengthen the idea that insect-associated microbes are active players in plant manipulation to the benefit of the insect host.

## 5.3 Evolutionary convergence between leaf-mining and gall-inducing insects

Feeding strategies used by plant reprogrammers presumably evolved to face similar constraints partially imposed by the endophytic lifestyle shared by leaf-miners and most gall-inducers (Dempewolf et al., 2005; Stone and Schönrogge, 2003). Like insects in closed galls, leaf-miners simultaneously live in and eat their plants with no possibility to escape in case of inadequate food supply (due to plant defensive mechanisms and/or seasonal variations of the plant quality) or attack by natural enemies (Sinclair and Hughes, 2010; Stone and Schönrogge, 2003). The main hypotheses for the adaptive significance of gall-induction (nutrition, microclimate, defense) can also be applied to leaf-miners (Connor and Taverner, 1997; Sinclair and Hughes, 2010). Performance of gall-inducers and leaf-miners is also highly dependent on the oviposition choice of their mother, a characteristic shared with all insects that have relatively sessile developmental stages.

Analyses revealed that major CK types accumulating in mines and larvae are similar to what is observed for most gall-inducers (Chapters 2/3), suggesting that strategies underlying the plant manipulation may be shared between herbivorous insects with distinct life histories. In order to further investigate this hypothesis, I preliminarily explored CK and AUX profiles in the gall-inducing *Mayetiola destructor* developing on the susceptible wheat 'Newton' (*Triticum aestivum* L.) and AUX levels in a leaf-mining system (Chapter 4). For Hessian flies, different parts of wheat (two and five days larvae infesting leaf one, leaf two, root, sheath and meristem – **see Annexe 7.1**) were compared. A total of 21 types of CKs and 3 types of AUX where quantified, including two *N*-glucosides CKs (*t*Z9G, cZ9G) that were not found in *M. domestica* leaves infested by *P. blancardella* or *P. mespilella.* Overall, after a two-days-infestation, Hessian fly larvae don't cause much variation of CKs but induce an increase of AUX in infested-plants compared to controls. Five days after infestation, *t*Z-type cytokinin significantly increase in infested leaves and in roots. Five-days-infestations cause a general increase of CKs and a marginally fluctuation of AUXs. Recent published data show that IAA increases 1 and 3 days after infestation in sheath (Zhu et al., 2010) and slightly in meristems one to seven days after infestation (Tooker and De Moraes, 2011). Our results partly agree with these results and the differences may be explained by the different feeding time, the different genotype of the wheats (susceptible and resistant) and Hessian flies strains (virulent and avirulent). The general increase of CKs five-days after infestation and of AUX two days after infestation demonstrates the importance of dynamic approaches in the study of plant-insect interactions. Combined with an extensive metabolic profiling, time course identification and quantification of phytohornomones are required to reveal plant alterations that are relevant to the ecology of the leaf-mining larvae (Chapter 4). Results obtained in Hessian fly are similar to what is observed for most gall-inducers. Gall-inducers and leaf-miners share some aspects of the plant manipulation especially regarding the modulation of CKs. Whether endosymbiotic bacteria are also key in gall induction and in CK-mediated phenotypes will require further investigations. The role of insect endosymbiotic bacteria in AUX modulation also awaits additional experiments. Interestingly, *P. mespilella* did not modulate AUX but data on leaf-miners are scarce and will need further investigations in order to conclusively demonstrate whether or not AUXs can be key mediators of plant manipulation for leaf-miners.

# 5.4 Conclusion

Understanding the exact role of phytohormones in plant-insect-microbe interactions and how they interact with each other requires an extensive metabolic profiling and time course identification and quantification of phytohornomones. The ‘‘plant response approach” can highlight key candidate plant functions that need to be disrupted for the insect to establish itself and feed successfully and may help to solve mechanisms underlying plant manipulation by gall-inducers and leaf-miners. Looking at similarities in the phytohormonal responses elicited by plant reprogrammers in a diversity of biological systems may help to identify converging mechanisms and the adaptive significance of plant manipulation for insects. Because insect symbionts are key players in many plant-insect interactions, they should be taken into account to elucidate the possible origin of the chemical stimuli involved in insect-induced plant manipulations. The development of new sequencing techniques applicable to small and/or non-model species will undoubtedly accelerate identification of mechanisms underlying plant manipulation and the specific role of each partner involved in plant-insect-microbe interactions. This should pave the way towards the discovery of innovative plant protection strategies.

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# Chapter 7. Annexes

# 7.1. Modulation of Auxins and Cytokinins in a gall-inducing hessian fly

## 7.1.1. Objectives.

Comparing strategies of CK and AUX modulation in leafminers and gall-inducers. Investigate precisely CK and AUX modulation in Hessian fly.

*Working hypothesis*:

## 7.1.2. Method.

We chose susceptible (Newton) wheat with/without hessian fly larvae as biological materials. CKs and AUX of two and five days' larvae infesting leaf one, leaf two, root, sheath and meristem were analysed. Five replicates for each treatment were performed.

## 7.1.3. Resutls.

Generally, after a two-days-infestation, hessian fly larvae don't cause much variation of CKs but cause an increase of AUX in infested-plants compared with control ones while five-days-infestation cause general increase of CKs and a marginally fluctuation of AUX.

# 7.2. Leaf-miner induced volatile profiles in apple tress

## 7.2.1. Objectives.

Estimating the impact of phytohormnal modulation for plant indirect defenses.

*Working hypothesis*

## 7.2.2. Method.

Six 1-2 days old adults were allowed to oviposit on apple shoots with 8 fully developed leaves per plants for one week. The feeding habits of first three instars resulted in leafmines visible on the leaf underside (early-infested leaves). After the third instar hypermetamorphosis occurs, the typically spotted tentiform-shaped mine is formed by last two instars (late-infested leaves). Same size and age healthy apple trees were used as healthy control. Volatiles of healthy, early-infested and late-infested apple leaves with 8 fully developed leaves were collected on June, 2015 and 2016 from 10 a.m. to 10 a.m. (24 hours) using a dynamic headspace method and analyzed by GC-MS/MS. The procedure was similar to that described by **Zhang et al.*** (2013).

## 7.2.3. Results

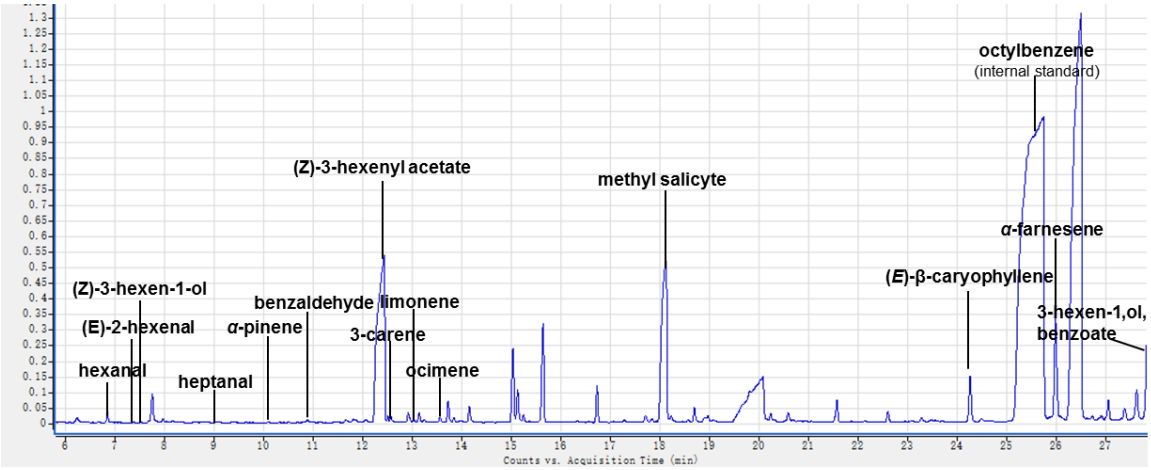


**Figure 16. Main volatile compounds in all leaf-materials.**

Hui ZHANG

# Manipulation des Végétaux par les organismes endophytes

## Dialogue chimique et moléculaire entre les insectes manipulateurs de plantes et leurs plantes hôtes


## Résumé

En raison de leur rôle central dans la physiologie et le développement des plantes, les phythormones ont depuis longtemps été considérées comme des médiateurs chimiques déterminants dans la capacité des insectes à contrôler leur environnement végétal. Les mécanismes permettant aux insectes de manipuler la balance phytohormonale permettant ainsi la régulation des apports nutritifs et la modulation des défenses végétales demeurent néanmoins pour la plupart inconnus, en particulier pour les insectes galligènes et mineurs de feuilles. L'objectif de ma thèse visait à caractériser les capacités de modulation phytohormonale par les insectes manipulateurs de plantes avec un accent particulier sur le lépidoptère mineur de feuille *Phyllonorycter blancardella*. Nous avons ainsi développés une caractérisation spatio-temporelle de la réponse des plantes aux attaques des larves mineuses au niveau moléculaire et biochimique. Une comparaison entre différents systèmes biologiques nous a permis d'évaluer les similitudes entre les stratégies adoptées par les les insectes galligènes et les insectes mineurs de feuilles, d'identifier l'origine possible des phytohormones impliquées dans la manipulation de la plante et le rôle des bactéries endosymbiotiques d'insectes dans ces interactions.


Mot clés : Interactions plantes-insectes, manipulation, phytohormones, cytokinines, mineurs de feuilles, insectes, bactéries symbiotiques d'insectes, *Phyllonorycter blancardella, Phyllonorycter mespilella, Malus domestica, Wolbachia.*


## Résumé en anglais

Because 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. Insects are using phytohormones to manipulate their host plants for their own benefit, regulating nutrient provisioning and plant defenses. However, a mechanistic understanding of how phytohormones operate in plant reconfigurations by plant-manipulating insects, especially by gall-inducing and leaf-minging insects, is lacking. The objective of my Ph.D. was to provide an extensive characterization of how plant-manipulating insects modulate the plant global hormonal balance with a specific focus on the leaf-mining moth *Phyllonorycter blancardella*. We thus developed a time course characterization of plant transcriptomic and biochemical responses following attack by leaf-mining larvae. A comparative analysis between different biological systems allowed us to estimate similarities in strategies developed leaf-mining and gall-inducing insects, to identify the possible origin of phytohormones involved in the plant manipulation and to estimate the role of insect endosymbiotic bacteria in these interactions.


Keywords: Plant-insect interactions, plant manipulation, phytohormones, cytokinins, leaf-miner, insects, insect bacterial symbionts, *Phyllonorycter blancardella, Phyllonorycter mespilella, Malus domestica, Wolbachia*.