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# THÈSE

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## Antoine GUIGUET


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# Origine évolutive et bases moléculaires du mode de vie galligène chez les Gracillariidae

**THÈSE dirigée par :**

| | | |
|---|---|---|
| **GIRON David** | Directeur de Recherche | CNRS/Université de Tours |
| **LOPEZ-VAAMONDE Carlos** | Chargé de Recherche | INRA d'Orléans |
| **OHSHIMA Issei** | Maître de Conférences | Université préfectorale de Kyoto |

**RAPPORTEURS :**

| | | |
|---|---|---|
| **CONNOR Edward** | Professeur | Université d'État de San Francisco |
| **SUGIO Akiko** | Chargé de Recherche | INRA de Rennes |

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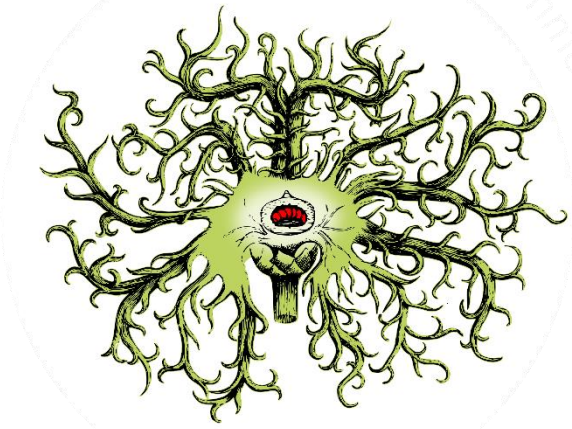
**JURY :**

| | | |
|---|---|---|
| **CASAS Jérôme** | Professeur | Université de Tours |
| **CONNOR Edward F.** | Professeur | Université d'État de San Francisco |
| **GIRON David** | Directeur de Recherche | CNRS/Université de Tours |
| **GUIVARC'H Nathalie** | Professeur | Université de Tours |
| **LOPEZ-VAAMONDE Carlos** | Chargé de Recherche | INRA d'Orléans |
| **NYMAN Tommi** | Chargé de Recherche | Inst. Norvégien de Rech. Bioéco. |
| **OHSHIMA Issei** | Maître de Conférences | Université Préfectorale de Kyoto |
| **SUGIO Akiko** | Chargé de Recherche | INRA de Rennes |

*[Les Galles] sont toutes des productions monstrueuses, mais en les voyant on n'a pas cette espèce d'horreur qu'on a quelquefois, ou ce dégoût qu'on a presque toujours lorsque l'on considère les productions monstrueuses des animaux ; elles peuvent même fournir un agréable spectacle à qui parcourt toutes leurs variétés de figures.*

*[Galls] are all monstrous productions, but seeing them we do not have that kind of horror we sometimes have, or that disgust we almost always feel when we consider the monstrous productions of animals; they can even provide a pleasant spectacle to which runs through all their varieties of figures.*

*René-Antoine Ferchault de Réaumur,*
*in Mémoires pour servir à l'histoire des insectes Tome III, 1740*



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# Résumé de la thèse

## Contexte scientifique

Les galles induites par les insectes représentent une forme avancée de manipulation du développement et de la physiologie des plantes. La capacité d'induire une galle est répandue parmi les ordres d'insectes herbivores, et on compte parmi les insectes possédant cette capacité d'importants ravageurs agronomiques. Cependant, les mécanismes moléculaires impliqués dans la formation des galles d'insectes restent mal connus tandis que les processus évolutifs conduisant à l'apparition du mode de vie galligène chez les insectes restent débattus. Ces éléments de contexte scientifique sont développés dans le **chapitre 1**.

## Objectifs de la thèse

L'objectif général de ma thèse est d'étudier l'évolution (**objectif 1**) et les bases moléculaires (**objectif 2**) de l'induction des galles chez les insectes. Pour y parvenir, ma thèse s'est concentrée sur les Gracillariidae, une famille d'insectes de l'ordre des lépidoptères dont la plupart des espèces sont mineuses de feuilles au stade larvaire. Plus spécifiquement, mon travail a porté sur deux espèces japonaises, *Borboryctis euryae* Kumata et Kuroko et *Calopitlia cecidophora* Kumata, que l'on a sélectionné en raison de leur mode de vie atypique parmi les Gracillariidae.

## Systèmes d'étude

***Borboryctis euryae*** a pour plante-hôtes *Eurya emarginata* Makino et *E. japonica* Thunberg, deux arbustes à feuilles persistantes de la famille des Pentaphylacacées. Le développement larvaire de cette espèce est caractéristique de la sous-famille des Acrocercopinae et se déroule en cinq stades au cours desquels l'insecte connait un changement de mode de vie. Les premier et deuxième stades forment une mine linéaire sous-épidermique, puis le troisième stade crée une mine élargie dans laquelle l'insecte complète son développement larvaire. Cependant, contrairement aux autres Acrocercopinae, on observe un renflement de la mine au cours du développement larvaire de *B. euryae*, suggérant qu'il s'agit d'une espèce galligène.

***Caloptilia cecidophora*** Kumata est associé à *Glochidion acuminatum* Müller Argoviensis, *G. obovatum* Siebold & Zucc. et *G. rubrum* Blume (Phyllanthaceae). Le mode de vie de *C. cecidophora* diffère largement de celui des autres espèces du même genre. Chez la grande majorité de *Caloptilia*, le premier stade larvaire forme une mine linéaire sous-épidermique, le deuxième creuse une mine en tache, le troisième forme une mine tentiforme et les stades larvaires restants quittent la mine pour former un enroulement de feuille dans lequel ils complètent leur développement jusqu'à pupaison. Par opposition, *C. cecidophora* induit la formation d'une galle prenant la forme d'un renflement de feuille au sein duquel se trouve une chambre larvaire. Tout le développement larvaire a lieu à l'intérieur de la galle. Cependant, le processus d'initiation de la galle *C. cecidophora* restait inconnu et il a été suggéré que les premiers stades larvaires étaient d'abord mineurs de feuilles avant d'induire la galle.

Ces deux espèces ont en commun de présenter potentiellement une transition du mode de vie mineur de feuille au mode de vie galligène au cours de leur développement. Cette caractéristique est rare chez les insectes induisant des galles, pour qui l'induction de la galle coïncide généralement avec le début de l'interaction avec leur plante hôte. Si elles sont confirmées, les stratégies alimentation mixtes de ces deux espèces représenteraient une opportunité pour répondre aux questions concernant la trajectoire évolutive conduisant à l'évolution de l'induction de la galle chez ces insectes et permettraient de développer des approches comparatives afin de déchiffrer les mécanismes moléculaires de l'induction de la galle.

L'étude présentée dans le **chapitre 2** porte sur la nature histologique du renflement des mines de feuilles induit par *B. euryae*, sur la valeur nutritive du tissu néoformé (appelé cal) pour les larves et sur le rôle de l'insecte pour l'induction de ce développement tissulaire anormal. À cette fin, nous avons combiné des expériences sur des insectes élevés sur le terrain et en laboratoire. Les résultats ont montré que la prolifération de cal est induite par les larves de stade quatre et que le cal représente un apport nutritif indispensable au larves de ce même stade. Le suivi phénologie de *B. euryae* a révélé que la durée du stade quatre pouvait être variable au sein de la population étudiée et qu'elle pouvait atteindre plusieurs mois, suggérant que la prolifération de cal nutritif entretenue par les larves de stades quatre leur permet d'ajuster leur durée d'exploitation de leur plante hôte.

Le **chapitre 3** présente une étude histologique des premières étapes de l'induction de la galle par *C. cecidophora* afin de vérifier si les premiers stades sont capables ou non d'induire des symptômes de galle. À cette fin, nous avons élevé des insectes en serre sur des plantes en pot et avons décrit les premières étapes du développement larvaire. À l'aide de techniques histologiques, nous avons étudié les processus morphogénétiques conduisant à la formation de la galle. Nous avons comparé ce mode de vie à celui d'une espèce sympatrique et étroitement apparentée qui ne provoque pas de galle, *C. ryukyuensis*, qui se développe sur *G. lanceolatum* Hayata et *G. zeylanicum* (Gaertner). Les résultats ont permis de confirmer l'existence d'une transition du mode de vie mineur de feuille au mode de vie galligène au cours des premiers stades de développement de *C. cecidophora*. En outre, la comparaison du développement larvaire des deux espèces a fourni des arguments pour discuter de la nature adaptative des galles.

Le **chapitre 4** présente une étude visant à explorer le processus évolutif ayant conduit à l'apparition du mode de vie galligène dans le genre *Caloptilia*. En utilisant des échantillons prélevés sur le terrain, nous avons reconstruit une phylogénie des *Caloptilia* associés aux plantes de la famille des Phyllantaceae. Grâce à des échantillonnages sur le terrain et à des collaborations, nous avons obtenu trois espèces induisant des galles non décrites ainsi que six autres *Caloptilia* non décrites au mode de vie enrouleur de feuille. Les deux phylogénies, réalisées respectivement à partir de cinq gènes et à partir d'éléments ultra-conservés, supportent la monophylie des espèces *Caloptilia* galligènes. La comparaison de la morphologie des galles ainsi que la comparaison de la distribution des plante-hôtes parmi les *Caloptilia* associées aux Phyllanthaceae nous ont permis de discuter des possibles contraintes évolutives et avantages adaptatifs du mode de vie galligène.

Le **chapitre 5** présente une étude visant à identifier les gènes possiblement impliqués dans l'induction de la galle de *C. cecidophora*. Nous avons séquencé les transcriptomes de chaque stade larvaire et appliqué une double approche comparative. Tout d'abord, nous avons comparé l'expression des gènes des stades mineurs de feuille avec des stades induisant des galles afin de sélectionner des gènes surexprimés lors de l'induction de la galle. Ensuite, nous avons comparé les gènes surexprimés chez *C. cecidophora* avec le transcriptome de *C. ryukyuensis*, une espèce apparentée et non-galligène. L'analyse des résultats a fourni une liste d'effecteurs candidats pour la formation de la galle.

La **discussion générale** présente l'apport de cette thèse à la compréhension globale des interactions entre les insectes manipulant les plantes et leurs plantes-hôtes. Il résume nos principales conclusions et aborde la question de l'existence d'un continuum de manipulateurs de plantes, ainsi que des conséquences et des perspectives fournies par ce cadre conceptuel.

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# Annex

Annex 1. Guiguet, A., Dubreuil, G., Harris, M.O., Appel, H.M., Schultz, J.C., Pereira, M.H., and Giron, D. (2016). Shared weapons of blood- and plant-feeding insects: Surprising commonalities for manipulating hosts. J. Insect Physiol. 84, 4–21. ..... 160

# Chapter 1.

# General Introduction

Plant galls, one of the great curiosities of the insect world, exert a spontaneous fascination on naturalists since ancient times. While the trophic interactions between insects and plants are first understood as the destructive action of the former on the latter, galls are the proof that conversely, this relationship sometimes leads to the formation of a new plant structure that is absent without the insect. This "fruit" whose product is not a seed but a "fly" has not ceased to question as it was obvious that it seemed to contravene the normal order of relations between living beings. Despite the description of insects in galls since the Antiquity by Hippocrates (460-377 BC), Theophrastus (371-286 BC), and Pliny the Elder (23-79 AD), it was not until the 17th century that naturalists started to consider that insects are the cause of gall formation. While the emergence of insects from oak galls was previously understood as the result of a spontaneous generation, three of the most eminent scientists of their time, Marcello Malpighi (1628-1694) father of the microscopic anatomy, Antoni van Leeuwenhoek (1632-1723) father of microbiology, and Jan Swammerdam (1630-1680) a renowned anatomist, simultaneously showed that gall development followed the oviposition of an insect (Leeuwenhoek and Hoole, 1800; Malpighi, 1675; Swammerdam, 1758). This discovery marked the foundation of the scientific study of galls, also called Cecidology.

Three centuries later, the study of insect galls is still of interest for many biologists as many aspects of this phenomenon remain poorly understood. The challenge of understanding molecular, ecological and evolutionary aspects of gall induction has never been greater than today as gall-inducing insects comprise some of the most devastating pests of global agriculture. Among them, the Hessian fly (*Mayetiola destructor*) and the Asian rice gall midge (*Orseolia oryzae*) represent major threats for wheat and rice yields respectively. The phylloxera (*Daktulosphaira vitifolia*) remains an incurable pest compared to other diseases of grapevine, despite of one century of study of this species. At the same time, human transport and global warming impact gall-inducer range expansion and favor biological invasions. For example, the recent introduction into Europe of the Asian Chestnut gallwasp (*Dryocosmus kuriphilus*) and the Australian blue gum gall-inducing chalcid wasp (*Leptocybe invasa*) outside of their native ranges have caused massive infestations on Chestnut and gum trees (*Eucalyptus* spp.) in Europe. At a wider scale, given that most interactions between plant and herbivorous insects are ruled by mutual manipulation processes, the understanding of one of the most complex form of manipulation of plant physiology and development by an insect can help us to shed light on the ecology and evolution of plant-insect interactions.

In this introduction, I review current knowledge on the evolution of the gall-inducing lifestyle and the mechanisms underlying gall formation. In the first part, I define the object of study, characterizing it in its diversity and introducing the terminology. In the second part, I summarize the state of knowledge about the evolution of the gall-inducing habit and, in particular, the evolutionary pathways leading to gall-formation and the adaptive value of this life-style. In the third part, I review the hypothesis about mechanisms of gall induction and their experimental support. Finally, I detail the main objectives of my thesis and the experimental approaches used to address them.

# 1. Gall induced by Insects: definitions and diversity

## 1.1. Definitions of galls

The first problem in studying galls lies in the definition of this term, as there is no widely accepted definition (Williams, 1994). The term "gall" has several putative origins and the most probable is from latin with the term "galla" that designated the oak galls. Galla itself comes from the indo-european "gel" meaning "round", probably referring to the spherical shape of some *Andricus* galls on oak (Redfern and Askew, 1992). The etymology being old and from a vernacular origin, attempts to scientifically define this term are far posterior to the word invention. One of the first propositions was made by de Réaumur who extended his definition of galls to other insect-induced outgrowths (de Réaumur, 1740).

*[galls are] outgrowths, tuberosities which rise on different parts of the plants or trees, which owe their birth to insects which have grown in their interior.*

It is now known that insects are not the only organisms to distort the development of their host plant. Comparable structures are induced by mites, nematodes, fungi and bacteria (Meyer and Maresquelle, 1983). In some cases, the manipulation of plant development has been found to lead to an inhibition of growth around the attacked zone. The following definition of galls extended the case beyond insects to include all forms of plant parasitism and to include all forms of manipulation of plant development.

*Galls are abnormal growths formed from tissues of a plant or other host, due to the parasitic activity of another organism.*(Redfern et al., 2002)

The development of microscopy and histological studies of gall development allowed a definition based on cellular alterations involved in gall induction.

*Galls are plant tissues or organs formed by hyperplasia (increased cell number) and/or hypertrophy (increased cell size) induced by parasitic or pathogenic organisms* (Dreger-Jauffret and Shorthouse, 1992; Mani, 1964)

The rigorous application of this extensive definition led to consider galls as any alteration of the plant development such as plant defensive tissue reactions following scars for example. In order to exclude the wounding responses resulting from a simple defensive reaction of the plant, and to take into account the nutritional role of the galls, I decided to adopt the following definition of galls in the present work:

*[Galls are] plant tissues or organs formed by hyperplasia (increased cell number) and/or hypertrophy (increased cell size) induced by parasitic or pathogenic organisms and that play a role in their nutrition.*

## 1.2. Gall-inducer as an herbivorous feeding guild

Phytophagous insects comprise one fourth of the biota (Grimaldi and Engel, 2005), and there is a remarkable diversity of associations between them and their host plants (Schoonhoven et al., 2005; Slansky et al., 1987). They can be grouped in different categories based on food preference (specialist vs. generalist), feeding habits (sap-feeder vs. tissue chewer) and/or spatial localization (root vs. shoot herbivores) (Schoonhoven et al., 2005). Herbivorous insects can also be divided in two categories: the **ectophagous**, also called exophagous or free-living, whose life takes place on their host plant, and the **endophagous** that live inside it. Several other categories can be used to define the plant-insect interaction. Foliage feeders that fold leaves using silk to create a protective shelter are named **leaf-rollers** when one leaf is rolled and **leaf-tiers** when several are attached together. Endophagous feeding guilds are defined by the type of plant organ they attack and if their gallery is superficial (miners) or deep (borers) in plant tissues. The most common life-styles are the **leaf-miners** and the **stem borers**, to which can be added feeding habits that are less common like stem-miners, seed-borers, fruit-miners or flower-miners.

The gall-inducing lifestyle is often linked to the endophagous herbivores since most **gall-inducers** live inside plant tissues. However, this feeding habit hardly fit the criterion of those two categories. A significant number of insects that induce galls are not internalized within plant tissues or only partially (Figure 1.1A). Several clades of gall-inducers, like the nematine sawflies (Nyman et al., 2000) and the gall-inducing aphids (Kutsukake et al., 2012), comprise both endophagous and ectophagous species.

Given the gall-inducers and the leaf-miners are the central life-styles studied in the present thesis, it is necessary to introduce their diversity and the vocabulary used for their description. It also requires to describe the diversity of insect species that adopt these two feeding habits, with a particular focus on the taxonomic groups of species studied in this thesis.

## 1.3. Gall-inducer and Leaf-miner classification

Insect galls vary from minute tissue alterations for *M. destructor* (Harris et al., 2006a) to large multicellular outgrowth like the *Diplolepis* galls on rose (Shorthouse and Leggo, 2002). Their structure are generally predictable in size and shape for a given insect species, and share common traits that allowed their classifications. Küster proposed a classification system based on histological descriptions of gall development (Küster, 1911). This classification distinguished the **kataplasmas**, which involve tissue modifications without new differentiation, from the **prosoplasmas**, which are more complex structures with differentiated tissues. Largely

used during the twentieth century, a more familiar terminology based on gall shape is now preferred (Dreger-Jauffret and Shorthouse, 1992) (Figure 1.1A).

*Pit galls* and *blister galls*: slight depression induced of one side of the leaf blade, along with the epidermis split in the case of blister galls.

*Roll* and *fold galls*: roll or fold of the leaf margin.

*Pouch galls*: large invagination in the leaf blade with a remaining opening.

*Covering galls*: invagination in the leaf blade or of the stem surface that encloses the insect galler.

*Bud* and *rosette galls*: bud alteration ranging from simple leaf primordia enlargement for the former to clusters of modified leaves for the latter.

*Mark galls*: stem swelling (or more rarely leaf swelling) induced by an insect that is already enclosed at the beginning of the gall initiation, thanks to egg deposition within the plant or after the insect burrows inside.

Although convenient because intuitive, this classification had the disadvantage to present many categories overlap (Redfern and Askew, 1992). There were several attempts to determine narrower morphological types in order to quantify their prevalence in nature but this terminology still requires a standardization to be widely used (dos Santos Isaias et al., 2014).

The classification of leaf-miners suffers from less difficulties. To follow the typology described by Hering (Hering, 1951) the diversity of leaf-mining forms is expressed according to two criteria: the tissue in which the larva has been feeding and the direction of feeding (Figure 1.1B). Three main groups of mines can then be identified: the **serpentine mines**, or ophionomes, when the larva moves linearly, the **blotch mines**, or stigmatonomes, when the larval gallery grows like a patch, and the **tentiform mines**, or ptychonome, which is a modification of the blotch mine in which the epidermis of one side is folded by the larva in order to create a cavity (Figure 1.1B). If the species of the host plant is added to the combination of these criteria (most leaf-miner being specialists; e.g. Lopez-Vaamonde et al., 2003), this information describes a wide variety of forms and is often sufficient to identify the leaf-miner species.

## 1.4. Galls induced by insects

Another approach to grasp the diversity of galls is to describe the variety of insects having the ability to induce this phenotype on their host plant. In the following overview, I summarize insect taxa showing a gall-inducing habit and then focuses on the taxa that were studied in the present thesis.

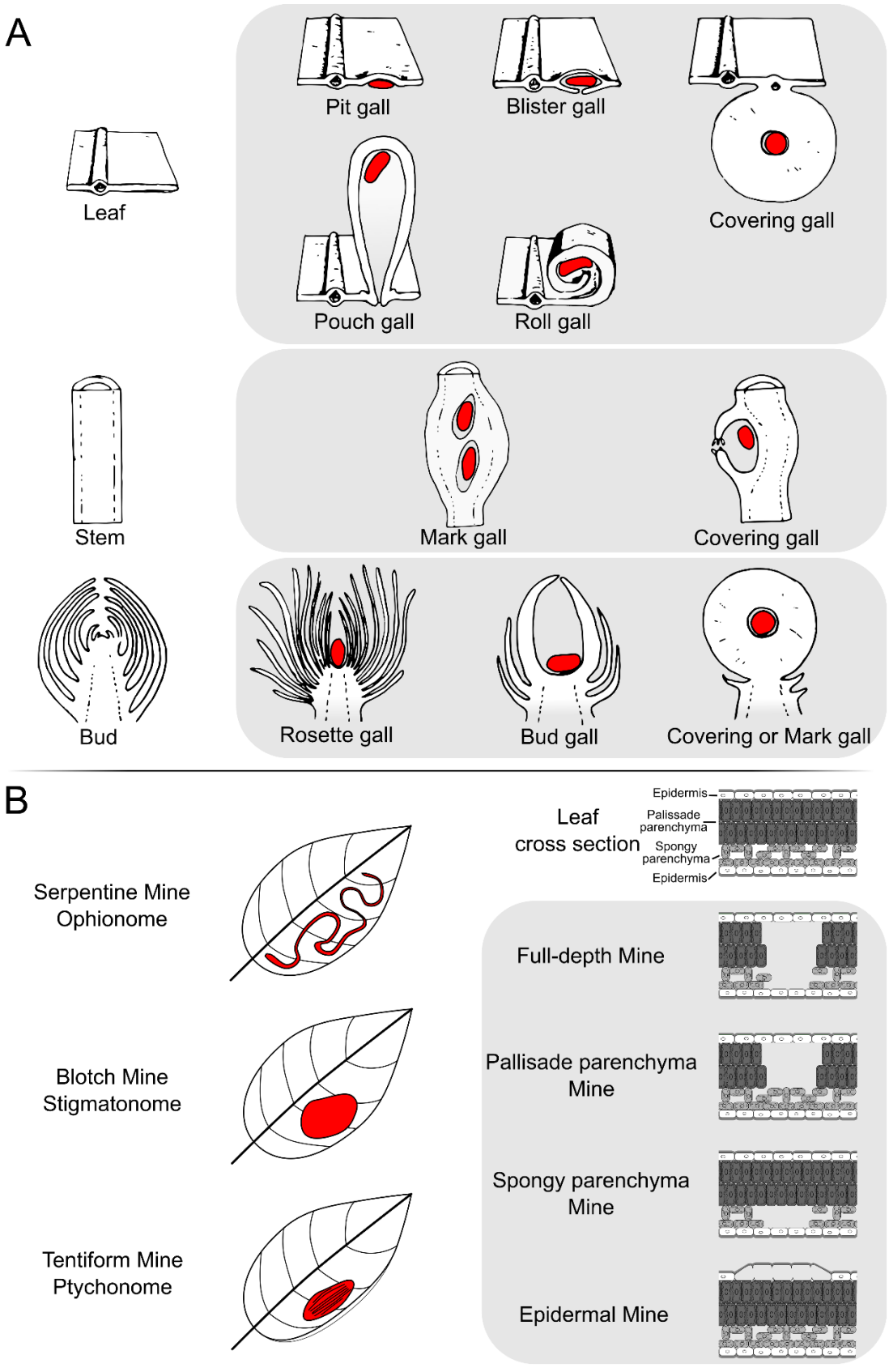



Figure 1.1. Schematic drawings of the main types of galls on leaf, stem and bud (A) adapted from (Dreger-Jauffret and Shorthouse, 1992) and of the main types of leaf-mines (B) adapted from (Hering, 1951). In (A), the gall-inducer is represented in red. In (B), the left panel shows three possible shapes of leaf-mines (in red), and the right panel shows the histology of the damages made by leaf-miners.

Gall-inducing insects have been estimated to represent 2% of the total number of described insect species (Buhr, 1965). Thus this life-style can be considered as rare among insects. However, the prevalence of this feeding habit is probably highly underestimated. Based on studies assessing the number of gall-inducing insects per plant, their global diversity has been extrapolated to be between 21,000 to 211,000 species (Espírito-Santo and Fernandes, 2007). Recently, an assessment of insect diversity based on metabarcoding revealed an unexpected diversity among gall midges (Cecidomyiidae), estimating that this dipteran family could be the most megadiverse group of insects with an estimated diversity of over one million of species (Hebert et al., 2016).

The gall-inducing habit occurs in six orders: Thysanoptera, Hemiptera, Hymenoptera, Coleoptera, Diptera and Lepidoptera (Dreger-Jauffret and Shorthouse, 1992) (Figure 1.2). Among them, the order Diptera contains the more gall-inducing species with Gall midges (Cecidomyiidae), being the most diverse group of gall-inducing insects. They comprise several major agronomic pests like *Sitodiplosis mosellana* and *M. destructor* on wheat and *O. oryzae* on rice. The second largest group is the Cynipid family (Hymenopteran), which contains more than one thousand gall-inducing species that are mostly associated with Fagaceae and in particular *Quercus* (Ronquist et al., 2015).

## 1.5. Galls induced by Lepidopterans

Lepidoptera represents the largest diversification of herbivorous insects, and perhaps not surprisingly, also contains the highest diversity of endophagous habits, including many types of borers, concealed leaf feeders, leaf-miners, gall-inducers and inquilines (Powell et al., 1998). In fact, endophagy arose early in the evolution of Lepidoptera and was a key waypoint in the radiation of the rest of the group (Menken et al., 2010; Powell et al., 1998). Compared to other insect orders, the gall-inducing habit is relatively rare among Lepidopterans. Almost all of the 160,000 described lepidopteran species are herbivores during their larval stage (Mitter et al., 2017), but only 179 are known to be gall-inducers, to which it can be added 173 unidentified lepidopteran galls (Miller, 2005). Even considering a later estimate adding 25 undescribed gall-inducing lepidopteran (Nishida and Miller, 2005), the proportion of gall-inducing species remains low in this group. To date, the gall-inducing habit has never been reported in Macrolepidopteran. Half of described species are distributed in only 20 families, all belonging to the microlepidopterans (Miller, 2005).

In addition to the rarity, the status of galls in Lepidoptera has been questioned. Lepidopteran galls are generally stem swelling around the larval gallery (Miller, 2005), and these abnormal plant structures were long been considered as simple wounding reactions rather than manipulations of plant development (Buhr, 1965; Joannis, 1922). The lack of consensus regarding the gall definition and the lack of knowledge on induction mechanisms does not settle this debate yet at this stage.

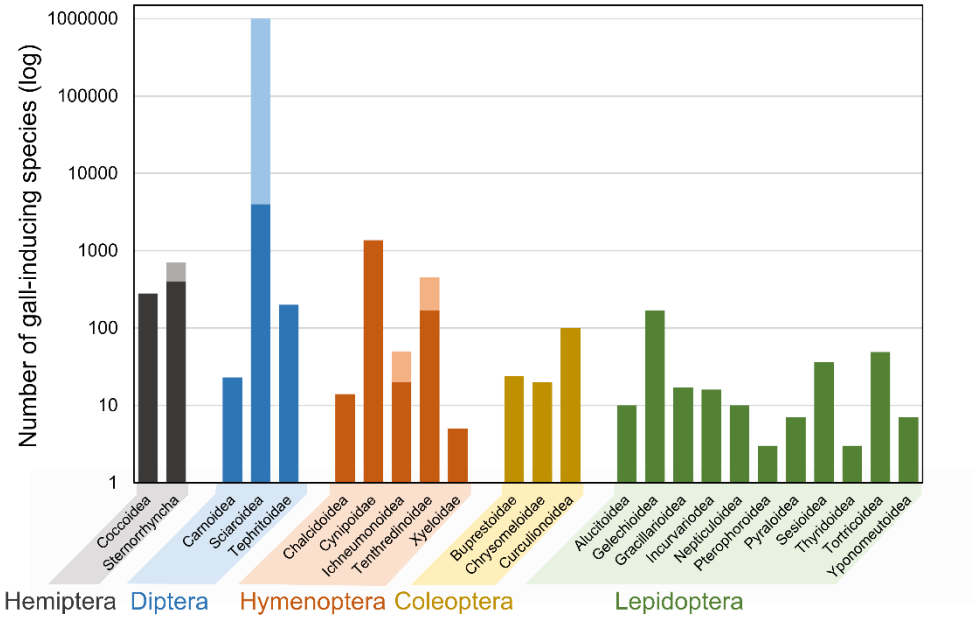



Figure 1.2. Number of gall-inducing species in insects, grouped by super-families. Lighter colors show highest estimates (Hebert et al., 2016; Miller, 2005; Raman et al., 2005).

However, clear examples of galls can be found in some clades of Lepidopteran in which the gall-inducing life-style is abundant: the superfamily Gelechioidea, the family Cecidosidae and the genus *Mompha*. Most of the gall-inducing Lepidopteran belong to the Gelechioidea (Miller, 2005). In this superfamily, the *Gnorimoschema* associated with Asteraceae are well studied (Miller, 2000). This genus comprises 18 species, all inducing galls on stems (Nishida and Miller, 2005). In particular, *Gnorimoschema gallaesolidaginis* associated with *Solidago* spp. has been a model system for its ecology (Heard and Kitts, 2012; Nason et al., 2002; Tooker and De Moraes, 2011a) and, to a lesser extent, to investigate the molecular interaction with its host plant (Beck, 1953; Tooker and De Moraes, 2011a). The Cecidosidae family comprises 18 species, all inducing complex galls whose appearance is similar to the Cynipid galls, to the point that some were mistakenly associated with this group in the past (Moreira et al., 2017; Silva et al., 2018). Finally, the genus *Mompha* would comprise 72 gall-inducing species (Nishida and Miller, 2005).

## 1.6. Galls induced by Gracillariidae

The family Gracillariidae (Lepidoptera), is one of the most diverse group of internal-feeding micromoths, comprising about 2000 described species belonging to more than 100 genera (Kawahara et al., 2017). The leaf-mining is the dominant life-style in this family but some species mine other organs like stems, fruits and petals or are leaf-rollers. It also contains a dozen of gall-inducing species in the wider estimates (Nishida and Miller, 2005).

Among them, *Aristaea* sp. induces a blister-like gall on the leaf veins of *Aster* (Asteraceae), *Caloptilia murtfeldtella* induces a stem gall on *Penstemon* (Plantaginaceae), and *C. cecidophora* induces a leaf-gall on *Glochidion* (Phyllanthaceae) (Miller, 2005). *Epicephala*

*corruptrix* also induces galls, on fruit of *G. obovatum* (Phyllanthaceae) and three undescribed *Epicephala* species on fruits of *Phyllanthus* species (Phyllanthaceae) (Kawakita and Kato, 2016; Kawakita et al., 2015).

Other cases of gall-inducing Gracillariidae are mentioned in the literature and still require confirmation. Meyrick described several species as being gall-inducers that were not observed since their description: for example *Gracillaria hercoscelis* that was described as inducing leaf-gall on an undetermined plant (Meyrick, 1939) and *Acrocercops habroscia* that was collected in galls on *Calophyllum inophyllum* (Clusiaceae) leaves (Meyrick and Clarke, 1916). In addition, some species like *Borboryctis euryae* and *Acrocercops macaria* induce an inflated blotch mine which is sometimes considered as a gall (Ahn, 2012; Turner, 1913). Finally a Gracillariidae, probably misidentified as an *Epicephala*, has been found to induce the proliferation of cells in its leaf-mine that can be considered as a gall (New, 1981).

# 2. Evolution of gall formation in insects

Quoted several times in the Origin of Species (Darwin, 1869), galls are presented by Darwin as examples of the great developmental plasticity of plants, showing that the morphology of living organisms can "[undergo] great modifications, independently of the gradual accumulation of slight inherited modifications". Subsequently, they were even at the center of a debate between supporters and opponents of the Darwinian theory, the latter seeing in the negative impact of galls on plants an objection to natural selection (Cockerell, 1890; Hollis, 1889; Mivart, 1889; Romanes, 1889).

Low frequency of the gall-inducing life-style among herbivorous combined with the numerous and distant taxa bearing gall-inducing species, strongly suggest that the gall-inducing habit has appeared many times independently in insects. However, evolutionary pathways leading to the acquisition of this ability to manipulate plant development remain uncertain. The huge diversity of gall morphology among species also questioned not only the possible underlying mechanisms involved but also the adaptive significance of this phenotype.

## 2.1. Pathways of gall evolution in insects

Based on Kuster's classification of galls, Wells summarized for the first time evolutionary hypotheses about the origin of galls (Wells, 1921) (Figure 1.3A). Thanks to descriptions of the gall development and with the help of the von Baer Laws (Baer, 1828), he suggested possible evolutionary pathways leading to the formation of galls and the evolution of their forms in each of the known orders of gall-inducing insects. However, these scenarios were not supported by phylogeny, and are now obsolete.

Progresses in the phylogenetic analyses of gall-inducing insects and more detailed descriptions of the development of galls allowed by new technologies provided better supported evolutionary scenarios. Price proposed a two-pathway evolutionary scenario (Price et al., 1987). All gall-inducing insects would have evolved from ancestral free-feeding herbivores, either through plant-mining or through sedentary surface-feeding (Figure 1.3B). Under this scenario, the plant-miners examples provided were fruit flies and lepidopteran stem-borers, whereas proposed sedentary surface-feeders were aphids, psyllids, thrips, sawflies and gall midges.

Two main objections can be addressed to the Price scenario. First, the phylogeny of gall midges suggests that their ancestral life-style would have been fungus feeders rather than plant feeders. Indeed, basal groups of Cecidomyiidae are mycetophagous and some gall midges have retained an association with symbiotic fungus (Roskam, 2005). Moreover, several fungal genes that play a role in the interaction with the host plant were horizontally transferred into Cecidomyids (Cobbs et al., 2013). These results strongly suggest that the mycetophagy has been a preadaptation of the gall induction for Cecidomyiids. Secondly, in the Cynipidae family, another large group of gall-inducers, is excluded from the scenario since their ancestral state is unlikely to be surface-feeder or plant-miner according to phylogeny (Ronquist et al., 2015).

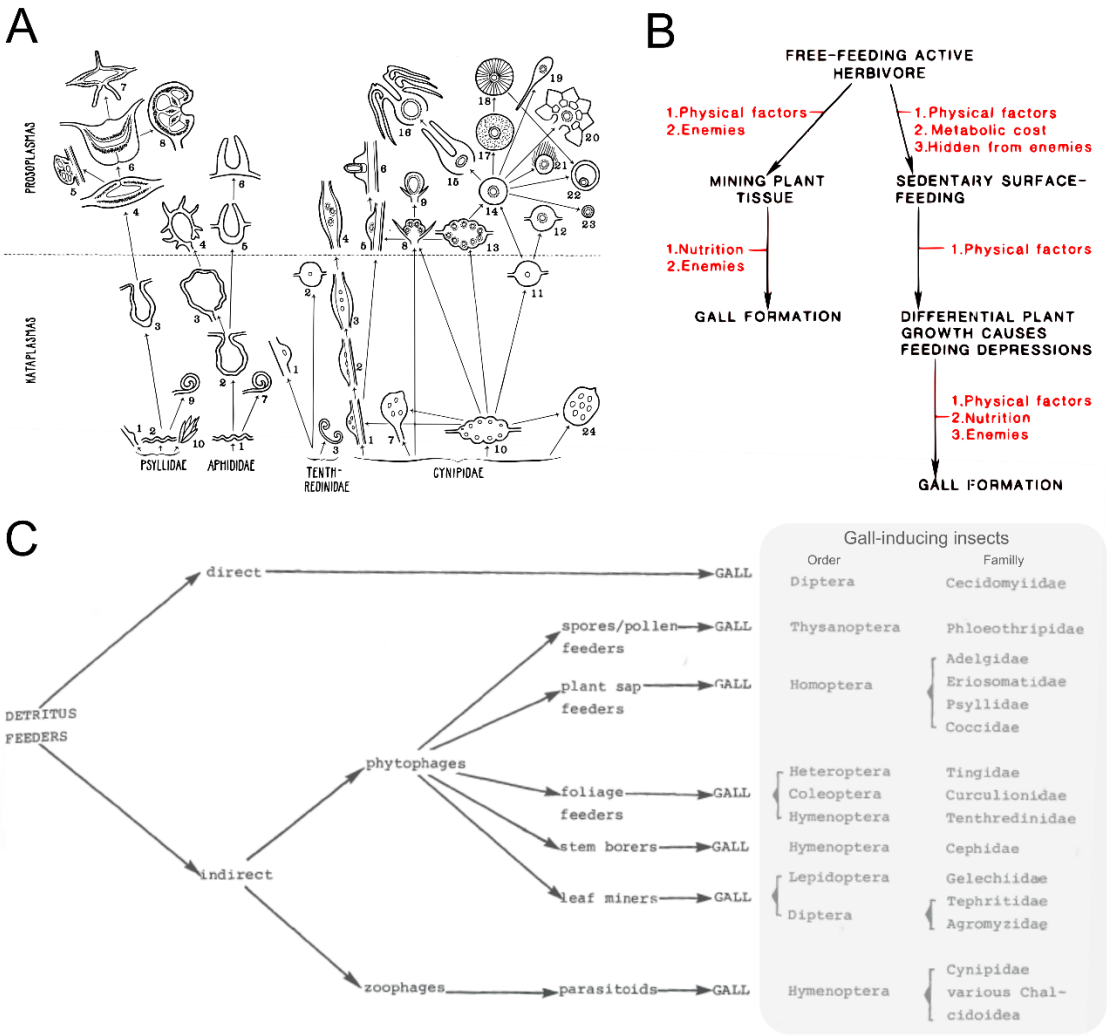



Figure 1.3. Evolutionary pathways proposed for gall induction. Diagram adapted from Wells (1921) presenting evolutionary scenari for gall forms in four gall-inducing groups, with a transition from kataplasmas to protoplasmas (A). The two routes of evolution of gall-induction from free-feeding hactive herbivores proposed by (Price et al., 1987), with possible selective factors (in red) (B). Evolutionary pathways proposed by (Roskam, 1992), with mention of insect families that may have followed them (on the right) (C).

More recently, a more complete evolutionary pathway hypothesis has been proposed (Roskam, 1992) (Figure 1.3C). Under this scenario, gall-inducing would have evolved from plant-mining (leaf-miners and stem-borers), from ectophagous lifestyles (foliage-feeders, sap-feeders and pollen-feeders), from fungus-feeders or from parasitoids. Although more detailed, these pathways are diversely supported by experimental data. The parasitoid pathway received a strong support from phylogenetic analyses in the cases of Cynipidae (Ronquist et al., 2015), Chalcidoidea (La Salle, 2005) and Braconidae (Zaldívar-Riverón et al., 2014). An origin from foliage-feeders in Tenthredinidae is also confirmed by the phylogeny (Nyman et al., 2000). In comparison, the origin from leaf-miners is generally weakly supported. Most gall-inducing Gelechiidae make stem-galls, which rather suggests an origin from stem-borers (Miller, 2005). Gall-inducing fruit flies, may have evolved from ovary-miners (Korneyev et al., 2005). Based on phylogeny, an origin of gall-induction from leaf-miners is likely for the *Mompha* genus

(Sohn et al., 2016). The fact that majority of Agromyzidae (Diptera) are leaf-miners and that gall-inducing species are rare in the familly, suggesting that leaf-mining is a possible ancestral state (Dempewolf, 2005). A similar hypothesis can be drawn for Gracillariidae (Lepidoptera) comprising mostly leaf-miners.

## 2.2. Adaptive significance of galls

Three main hypotheses have been proposed about the adaptive value of insect galls (Stone and Schönrogge, 2003): *i)* the nutrition hypothesis, *ii)* the natural-enemy hypothesis, and *iii)* the microenvironment hypothesis.

### *The nutritive hypothesis*

In its simplest form, the nutrition hypothesis states that feeding on gall tissues provides enhanced nutrition compared to other available plant tissues, resulting in a high performance of the gall-inducer compared to other feeding guilds (Price et al., 1987). The broader definition includes examples where plant manipulation does not lead to an apparent increase of nutrients, but rather a control of the nutrient levels in galls to match the nutritional requirements of gall-inducing insects (Hartley and Lawton, 1992).

This is the adaptive hypothesis that received the most experimental support through four main approaches:(1) histochemical, biochemical or metabolomics analyses of gall nutrient content compared to ungalled tissues; (2) experimental manipulation of host plant nutrition to determine whether the gall-inducer controls gall nutrient levels; (3) investigation of the fitness consequences of the artificial manipulation of gall-nutrient levels on gall-inducers ;and (4) performance of a non-gall-inducing insects when feeding on galls.

There is a considerable amount of research showing an enhancement of the nutritional quality within galls (reviewed in Giron et al., 2016). In many gall-inducing insects, the insect feeding site is turned into mobilizing sinks that accumulates nutrients to feed the larvae. Among them, gall-inducing aphids were probably the most studied. The use of carbon-14 labelling revealed that gall-inducing aphids induce an accumulation of carbon-compounds, most likely sugars, into the gall tissues (Compson et al., 2011; Nabity et al., 2013). Gall midges have been found to manipulate carbon:nitrogen ratios (C:N) with various trends according to the species. *Daphnephila taiwanensis* and *D. sueyenae* on *Machilus thunbergii* increase the C:N ratio in the gall (Huang et al., 2014), whereas attacked by the Hessian fly on wheat (Zhu et al., 2008) and infestation by Asian rice gall midge on rice (Agarrwal et al., 2016) decrease the C:N ratio. Gall-wasps have also been found to induce a carbohydrate accumulation in the gall tissues (Dorchin et al., 2006; Fay et al., 1993; Stone et al., 2002). For organisms feeding on plants, enhanced nutrition may also implies a control over plant defenses and both plant primary and secondary metabolisms can be affected (REF). Cynipids and sawflies have been found to improve their food source quality by decreasing the defensive compounds in the gall compared to other plant tissues (tannins & phenols) (Ikai and Hijii, 2007; Nyman and Julkunen-Tiitto, 2000).

However high levels of nutrients not always benefit insects, and accumulation of primary metabolites are not sufficient to demonstrate the nutritive hypothesis (Hartley, 1998; Hartley and Lawton, 1992). A first criterion is that nutrient levels within the gall must be controlled by the gall-inducer. This was demonstrated in gall-inducing aphids, who maintain the nutritional quality of their feeding site even when the plant nutrition is modulated by addition of synthetic fertilizers (Dardeau et al., 2015; Purdy and Miller, 2013). Similarly, the nutritional environment of the thistle gall fly, *Urophora cardui*, remains stable and favorable to the insect despite of the alteration of the plant nutrition (Gange and Nice, 1997). Such nutritional homeostasis to match the nutritional requirements of insects is key in the strategy developed by plant-manipulators (Body et al., 2013). A second line of evidence in favor of the nutrition hypothesis is a better performance of gall-inducers on gall tissues. Such an increase of insect survivorship has first been measured in Cynipids where an artificial enhancement of nitrogen levels in galls impaired insect survivorship (Hartley and Lawton, 1992). A similar manipulation of the gall nutritional status also resulted in a decrease of thistle gall fly performance (Gange and Nice, 1997). In gall-inducing aphids, *Tetraneura* species associated with gall tissues with higher concentrations of total amino acids produced larger numbers of offspring (Suzuki et al., 2009). However, measurements of insect performance on galls may confound adaptation to the gall with the increased nutritional value of galls (Miller and Raman, 2018). Following this criticism, a high performance of non-gall-inducing insects on gall tissues would be a stronger argument in favor of the nutrition hypothesis. Several free-living aphids belonging to non-gall-inducing species have been found to perform better on aphid galls (Forrest, 1971; Kennedy, 1951; Koyama et al., 2004). The endophagous caterpillar of the *Hellinsia glenni* moth grew better and had a higher emergence rate when transplanted from stem to gall tissues (Diamond et al., 2008). Finally, opportunistic beetles perform better on gall than on other plant tissues (Blair et al., 2010).

### *The enemy hypothesis*

The Enemy hypothesis states that the gall protects the inducing insect from its natural enemies and pathogens. Most galls being enclosed environments, all attacks must cross the gall tissues, and morphological features making it difficult to reach the gall-inducer should be adaptive. Confirmation of this hypothesis ideally requires to compare the mortality of a gall-inducer with its non-gall-inducing ancestor, which is impossible for long-established gall-inducer lineages (Stone and Schönrogge, 2003). This direct evidence for the enemy hypothesis being impossible to obtain, studies looked for indirect support by (1) comparing the mortality rate of gall-inducing and non-gall-inducing species, and by (2) correlating gall morphology traits with changes of insect mortality rates.

The comparison of the mortality of gall-inducers with those of other feeding guilds has been assessed in only few cases. Gall-inducing sawflies have been shown to be attacked by fewer parasitoid species and experience lower mortalities than do free-living forms (Price and Pschorn-Walcher, 1988). This effect has also been tested in tortricids, among which gall-

inducers are rare, and galls appeared to provide a better protection against parasitoid colonization than any other larval feeding habit (Mills, 1993). Gall-inducing also affects the shape of enemy communities. Among sawflies, some parasitoid groups were totally excluded by the gall-inducing life-style, especially those lacking ovipositors like tachinids (Price and Pschorn-Walcher, 1988). However, these results cannot be generalized to all gall-inducing insects. While concealment of endophagous insects provide them with a better protection against enemies compared to ectophagous (Cornell and Hawkins, 1995), differences of mortality due to natural enemies between gall-inducing and non-gall-inducing endophagous insects is equivocal. Meta-analyses of mortality across herbivorous feeding habits show that gall-inducers tend to be better protected against parasitoids than leaf-miners (Hawkins et al., 1997), whereas other indicate that survival rate of leaf-miners and gall-inducers are similar (Cornell, 1990). Other analyses tend to suggest that parasitoid richness in gall-inducers is equivalent to ectophagous insects and higher than other endophagous groups (Hawkins, 1988; Hawkins and Lawton, 1987). Therefore, the adaptive value of gall protection against enemies is probably not supported as a long-term hypothesis.

The fact that galls do not represent an efficient defense against enemies in general can be the result of enemies' counter-adaptations. Identifying such counter-adaptations in natural enemies represents an indirect evidence for the enemy hypothesis. Interestingly, mortality curves of herbivorous insect can also provide indirect evidence for the enemy hypothesis. Whereas leaf-miner survival rate decreases linearly during its development, the mortality of gall-inducers shows a peak at earlier stages of development (Cornell, 1990; Craig et al., 1990). This "window of vulnerability" in first steps of insect development is presumably due to the yet undeveloped gall structures and therefore supports the protective value of galls.

Gall forming herbivores exhibit considerable variations in gall morphology and this diversity is ideal to test whether gall shape has an impact on enemy attacks. Many studies tested the "Gall diameter hypothesis", testing if larval accessibility to natural enemies is controlled by gall size (Price and Clancy, 1986). For example, cynipid larvae found deepest in the gall are least attacked by parasitoids (Jones, 1983). In *Eurytoma gigantea*, individuals inducing larger galls are generally favored by selection (Weis et al., 1992). *Urophora cardui* survival against parasitoid also increases with the number of larval chambers, and then gall size (Zwölfer and Arnold-Rinehart, 1994). More recently, a negative correlation between parasitoid attack rates and gall size was demonstrated in *Diplolepis roase* (László and Tóthmérész, 2013), supporting the gall diameter hypothesis. However, the same authors found that a larger gall size makes them more vulnerable to bird attacks (László et al., 2014). In addition, studies on gall midges did not support the gall diameter hypothesis (Rossi et al., 1992; Waring and Price, 1989) suggesting that other gall traits need to be considered such as toughness, hairiness and stickiness that can be negatively correlated with parasitism rates (Bailey et al., 2009). Additional traits can also provide indirect defenses against enemies. The surface of some cynipid galls is covered by extrafloral nectaries allowing for the recruitment of ant guards (Fernandes et al., 1999; Seibert, 1993; Washburn, 1984). Gall-inducers can also affect plant volatile emissions, reducing for example volatile organic compounds used by parasitoids to localize infested plants. This has been shown in the gall-inducing aphid *Tetraneura nigriabdominalis* (Takei et al., 2015),

the tephritid fly *E. solidaginis* (Tooker et al., 2008), as well as in psyllid galls (Damasceno et al., 2010). Gall-inducers such as the aphid *Slavum wertheimae* can also induce the emission of repelling odors to browsing mammals (Rostás et al., 2013). Finally, gall-shape and color has been proposed to be a form of aposematism, meaning the external appearance of the gall would prevent herbivores to damage the gall (Inbar et al., 2010; Yamazaki, 2016).

### *The microenvironment hypothesis*

According to the microenvironment hypothesis, or harsh environment hypothesis, the gall provides better physical conditions compared to the external environment (Price et al., 1987). This adaptive hypothesis has been suggested by the fact that, at a global scale, the fauna of gall-inducing insect is richer in areas where the hygrothermal stress is higher (Price et al., 1998). At a regional scale, gall-inducing insects has been found to be more abundant and to survive better in xeric habitats compared with mesic habitats (Fernandes and Price, 1992). Similarly, the dry and hot environment of the Amazonian upper canopy host among the highest diversity of gall-inducing insects (Julião et al., 2014). However, this trend is not always clear and may vary among regions. For instance, Ribeiro and Basset (2016) also found in Australia a positive gall establishment and survivorship in the upper canopy, but the authors insisted on the fact that this diversity relied most on a low number of "super-host" plants. Still in Australia, no correlation has been found between the number of gall-inducing species and rainfall (Blanche, 2000). Interestingly, this study identified an increasing trend of the number of gall-inducing species per plant according to a decrease of rainfall, suggesting that dryness favor the occurrence of "super-host" (Blanche, 2000). Finally, the study of South African fauna did not revealed any link between hygrothermal stress and gall abundance (Veldtman and McGeoch, 2003).

Some studies intended to test this hypothesis at the species level. For instance, the gall midge *Mikiola fagi* has been found to be more abundant at forest edges, suggesting a preference for a dryer environment (Kelch et al., 2016). Fay et al., (1993) proposed that the manipulation of water fluxes in the plant by the cynips *Antistrophus silphii* may result in controlled humidity within the gall, which may be a protection against stress during drought. The most convincing evidence of the microenvironment hypothesis has been obtained for the cynips *Andricus quercuscalifornicus*, which maintains humitidy levels near saturation within the gall chamber which results in a positive effect on larva survival (Miller et al., 2009). However, the gall morphology sometimes misleadingly suggests a high level of protection from environmental conditions. Thus, the supposed greater resistance to desiccation expected for the galls on *Metrosideros polymorpha*, due to the abundant trichomes encountered at their surface, has been contradicted by the experiment (Amada et al., 2017).

# 3. Mechanisms of gall induction by insects

Long before the first studies on its chemical composition, insect secretions were postulated to cause the formation of plant galls. In the second half of the fifteenth century, Malpighi hypothesized the existence of an "ichor", a substance injected in the plant by the female when ovipositing and responsible for the gall formation (Malpighi, 1675). The idea that insect salivary glands could be implicated in the production and secretion of cecidogenous substances came in the nineteen century (Lacaze-Duthiers, 1853). Darwin even proposed that the "[…] *poisonous secretion of the insect causes the growth of the gall*" and "[…] *probably acts on one or two cells alone, which, being abnormally stimulated, rapidly increase by a process of self-division*" (Darwin, 1869). We now know that salivary secretions and/or oviposition fluids are the origins of substances responsible for the manipulation of plant development by insects (Giron et al., 2016). However, both the nature and the mode of action of these chemical inducers remain unclear.

Experimental attempts to identify "cecidogenous substances" first tried to artificially induce gall symptoms. Although application of extracts from gall-forming insects succeeded to induce gall symptoms (e.g. Beck, 1953; Molliard, 1917), experiments failed to identify the chemicals involved. More recently, a mix of two phytohormones, auxin and cytokinins (CKs), was sufficient to induce an abnormal plant growth similar to a gall (Bartlett and Connor, 2014).

Identification of effectors of gall induction faces three major challenges (Guiguet et al., 2016; Stone and Schönrogge, 2003):

- Gall-inducers use signal molecules that are chemically similar to those normally used in plant development, which would make them difficult to identify.

- Gall-inducers effects on their host plant are expected to trigger a cascade of reactions. Separating the primary impact on plant morphogenesis from secondary effects is a major challenge.

- Experiments require insects in sufficient numbers at different development stages and to have access to their salivary secretions or salivary glands. To date the only gall-inducing species to be routinely reared in laboratories is the Hessian fly *M. destructor*.

Phytohormones are key orchestrators of plant physiological processes. As such, they were suspected to be involved in gall formation. However, other effectors may also be involved and new genomic tools may facilitate their discovery.

## 3.1. Galls and phytohormones

Auxin is the first class of plant growth hormones that was discovered (Enders and Strader, 2015). These substances modulate plant development at extremely low concentration and were rapidly suspected to be involved in gall formation. Boysen-Jensen, who carried out

the pioneer experiment leading to the discovery of auxin, was the first to suggest that phytohormones could be involved in gall induction by insects (Boysen Jensen, 1948). He compared the action of *Mikiola fagi* on beech with the effect of indole-3-acetic acid (IAA), the main auxin in higher plants, on the development of tobacco leaves. Results obtained suggested that these phytohormones were most likely present in insects secretions (Boysen Jensen, 1948). Seventy years later, the levels of auxin and other plant hormones were measured in many insect galls. Abundance and distribution of growth hormones (excluding hormones involved in plant defense such as jasmonic acid and salicylic acid) in plants and insects provided indications about their putative role in gall development (Tooker and Helms, 2014) (Table 1).

### *Auxin*

Auxins regulate many aspects of plant development (Zhao, 2010) explaining why they were suspected to be involved in the physiological processes leading to gall formation. Measures revealed accumulations of IAA in most tested galls. Needle galls of the gall midge *Janetiella coloradensis* on *Pinus edulis* show 17 times more auxin activity than normal needles (Byers et al., 1976). Compared to ungalled tissues, IAA concentration in galls where twice higher for an *Eucalyptus* psyllid (Khattab and Khattab, 2005), up to fifty times higher for the gall midge *Rhopalomyia yomogicola* on the Japanese Mugwort (Tanaka et al., 2013), from two to seven fold higher in the eucalyptus gall wasp *L. invasa* (Li et al., 2017), in high concentration in wheat infested by the Hessian fly whereas it was undetectable in uninfected tissues (Zhu et al., 2010) and even more than a hundred times higher in the case of the pteromalid wasp *Trichilogaster acaciaelongifoliae* on *Acacia longifolia* (Dorchin et al., 2009). The convergence of these results with those obtained about the gall-inducing bacteria *Agrobacterium tumefasciensens*, which show that the increase of auxin concentration in crown galls plays a key role in the crown gall formation (Yamada, 1993), strengthen the hypothesis of an auxin implication in insect gall development.

These high concentrations of auxin in gall tissues raised the question of the origin of those molecules. Two hypotheses proposed that the accumulating auxin has a plant origin. First, Hartley (1999) proposed that, like in the literature about galls induced by *Rhizobium* and *Agrobacterium,* phenolic compounds could mediate the interaction between the plant and the gall-inducer and cause the increase in IAA abundance. It has been confirmed since that flavonoids inhibit auxin transport at the site of rhizobial infection (Brown et al., 2001) and that this inhibition was required in the formation of indeterminate nodules (Wasson et al., 2006). However this effect cannot be generalized as it is not involved in the development of *Rhizobium* determinate nodules and its role in crown gall formation has been infirmed (Bhattacharya et al., 2010). Data in gall-inducing insects are lacking to test this hypothesis. Second, Miles (1999) hypothesized that auxin accumulation in gall tissues resulted from an inhibition of IAA-oxidase, the system that degrades the plant hormone, due to low oxygen availability. To support the assumption, the author mention that stomatal occlusion can cause “eruptive galls” on leaves. Despite of its interest, this hypothesis fails to explain the process of gall initiation, when plant tissues do not suffer from possible oxygen restrictions.

Table 1.1. Dynamics of growth regulating phytohormones in galls and gall-inducing insects. Green-island inducing insects are mentioned in green, while gall-inducers are mentioned in black. For plant tissues: orange and up arrow: higher concentration in gall tissues, blue and down arrow: lower concentration in gall tissues. **Comparison between interior and exterior parts of the gall.For insects: x evidence of absence, *evidence of biosynthesis by the insect, IAA: auxin, CK: cytokinins, ABA: abscisic acid, GA: gibberelins, BR: brassinosteroids.

| Insect | Host-plant | Insect | | | | Gall VS ungalled tissue | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IAA | CK | ABA | BR | IAA | CK | ABA | GA | BR | |
| Hemiptera - Cicadellidae | | | | | | | | | | | |
| Cicadulina bipunctata | *Zea mays* | | | | | ↓ | ↑ | ↑ | ↓ | | Tokuda et al. 2013 |
| Hemiptera - Psyllidae | | | | | | | | | | | |
| psyllid (?) | *Eucalyptus obliqua* | | | | | ↑ | N.S. | ↑ | ↓ | | Khattab et Khattab 2005 |
| *Pachypsylla celtidismamma* | *Celtis occidentalis* | | CK | ABA | | | ↓ | N.S. | | | Straka et al. 2010 |
| *Stenopsylla nigricornis* | *Symplocos lucida* | IAA | CK | ABA | | N.S. | N.S. | N.S. | | | Kai et al. 2017 |
| Hemiptera - Heteroptera | | | | | | | | | | | |
| *Copium teucrii* | *Teucrium polium* | | | | | ↑ | ↑ | ↑ | ↓ | | Zalat et al. 2000 |
| Sternorrhyncha | | | | | | | | | | | |
| *Tetraneura nigriabdominalis* | *Ulmus davidiana* | IAA* | CK | | | ↓ | ↓ | | | | Takei et al. 2015 |
| *Daktulosphaira vitifoliae* | *Vitis ssp.* | | | | | ↓ | ↑ | | | | Eitle et al. 2017 |
| Lepidoptera - Gelechiidae | | | | | | | | | | | |
| *Gnorimoschema gallaesolidaginis* | *Solidago altissima* | IAA | | ABA | | ↑** | | ↓ | | | Tooker et de Moraes 2011a |
| Lepidoptera - Gracilariidae | | | | | | | | | | | |
| *Phyllonorycter blancardella* | *Malus domestica* | | CK | | | | ↑ | | | | Giron et al. 2007 |
| *Phyllonorycter mespillela* | *Malus domestica* | | CK | | | | ↑ | | | | Zhang et al. 2018 |
| Lepidoptera - Nepticulidae | | | | | | | | | | | |
| *Ectoedemia argyropeza* | *Poppulus tremula* | | CK | | | | ↑ | | | | Engelbrecht 1969 |
| *Ectoedemia argentipedella* | *Betula pendula* | | CK | | | | ↑ | | | | Engelbrecht 1969 |
| Diptera - Cecidomyiidae | | | | | | | | | | | |
| *Wachtlietla persicariae* | *Polygonum amphibium* | | | | | ↑ | | | | | Dieleman 1969 |
| *Hartigiola annulipes* | *Fagus sylvatica* | | | | | | ↑ | | | | Engelbrecht 1971 |
| *Mikiola fagi* | *Fagus sylvatica* | | | | | | ↑ | | | | Engelbrecht 1971 |
| *Janetiella coloradensis* | *Pinus edulis* | | | | | ↑ | | | ↑ | | Byers et al. 1976 |
| *Mayetiola destructor* | *Triticum aestivum* (susc.) | | | | | ↑ | | | | | Zhu at al. 2010 |
| *Mayetiola destructor* | *Oryza sativa* (non host) | | | | | | | ↓ | | | Zhu at al. 2011 |
| *Mayetiola destructor* | *Triticum aestivum* (res.) | | | | | | | ↓ | | | Zhu at al. 2011 |
| *Mayetiola destructor* | *Triticum aestivum* | | | | | N.S. | | | | | Tooker et de Moraes 2011b |
| *Asteromyia carbonifera* | *Solidago altissima* | | | ABA | | | | | | | Heath et al. 2013 |
| *Rhopalomyia yomogicola* | *Artemisia princeps* | IAA* | | | | ↑ | ↑ | | | | Tanaka et al. 2013 |
| Diptera - Chloropidae | | | | | | | | | | | |
| *Lipara lucens* | *Phragmites australis* | | | | | ↓ | | ↑ | | | De Bruyn et al. 1998 |
| Diptera - Tephritidae | | | | | | | | | | | |
| *Eurosta solidaginis* | *Solidago altissima* | | CK | | | | N.S. | | | | Mapes and Davies 2001a |
| *Eurosta solidaginis* | *Solidago altissima* | IAA | | | | N.S. | | | | | Mapes and Davies 2001b |
| Hymenoptera - Chalcididae | | | | | | | | | | | |
| chalcid wasp (?) | *Erythrina latissima* | | CK | | | | | | | | van Staden & Davey (1978) |
| *Leptocybe invasa* | *Eucalyptus grandis* × *E. camaldulensis* | | | | | ↑ | ↑ | N.S. | ↑ | | Li et al. 2017 |
| *Leptocybe invasa* | *Eucalyptus exserta* | | | | | ↑ | ↑ | ↑ | ↑ | | Li et al. 2017 |
| Hymenoptera - Cynipidae | | | | | | | | | | | |
| *Cynips quercusfolii* | *Quercus robur* | IAA | | | | ↑ | | | | | Kaldewey 1965 |
| *Dryocosmus kuriphilus* | *Castanea* sp. | | | | | ↑ | ↓ | | | | Yokota et al. 1974 |
| *Dryocosmus kuriphilus* | *Castanea crenata* | | CK | | | | | | | | Ohkawa 1974 |
| *Dryocosmus kuriphilus* | *Castanea* sp. | | | | ~~BR~~ | | | | | ↑ | Arima et al. 1983 |
| *Dryocosmus kuriphilus* | *Castanea mollissima* | IAA | CK | ~~ABA~~ | | ↑ | ↑ | ↑ | | | Wood and Payne 1988 |
| Hymenoptera - Pteromalidae | | | | | | | | | | | |
| *Trichilogaster acaciaelongifoliae* | *Acacia longifolia* | IAA | CK | | | | | | | | Dorchin et al. 2009 |
| Hymenoptera - Tenthredinae | | | | | | | | | | | |
| *Pontania proxima* | *Salix* sp. | | | | | | ↑ | | | | Leitch 1994 |
| *Pontania proxima* | *Salix* sp. | | | | | | ↑ | | | | McDermott et al. 1996 |
| *Pontania* sp. | *Salix japonica* | IAA* | CK | | | N.S. | ↑ | | | | Yamaguchi et al. 2012 |

An alternative hypothesis is that auxins would be secreted by insects in the plant. Extracts of *Eurosta solidaginis* maggots contain IAA and induce abnormal growth when injected in *Solidago altisima* stem (Beck, 1953). High levels of IAA has also been measured in the body of many gall-inducing insects (Dorchin et al., 2009; Kai et al., 2017; Mapes and Davies, 2001a; Takei et al., 2015; Tanaka et al., 2013; Tooker and De Moraes, 2011a; Wood and Payne, 1988; Yamaguchi et al., 2012). In galls of *G. gallaesolidaginis* on *S. altissima*, the levels of IAA tend to be higher in galled versus ungalled tissues and were significantly higher in the interior part of the gall compared to the exterior (Tooker and De Moraes, 2011a). These results are consistent with an IAA secretion by the caterpillar within the gall chamber. The ability of gall-forming insects to synthesize IAA from tryptophan has been demonstrated for *Pontania* (Yamaguchi et al., 2012), *Rhopalomyia yomogicola* (Tanaka et al., 2013) and *Tetraneura nigriabdominalis* (Takei et al., 2015). A novel biosynthetic pathway of auxin has even been described in sawfly and silkworm (Suzuki et al., 2014; Yokoyama et al., 2017). Transcriptomes of *Trioza* sp. galls on *Metrosideros polymorpha* (Myrtaceae) (Bailey et al., 2015), of galls of *Leptocype invasa* on *Eucalyptus* (Oates et al., 2015) and of galls of phylloxera *D. vitifolia* on the wild grapevine *Vitis riparia* (Schultz et al., 2018) all reveal an over-expression of genes involved in auxin response. This is consistent with auxin synthesis and secretion by these gall-inducing insects.

Altogether these results strongly support the hypothesis that auxins could be a cecidogenous substance of insect origin. However, the measurements of auxin concentrations in some galls tend to mitigate the central role given to this phytohormone in gall development. Indeed, several studies point that IAA levels are unchanged in gall versus intact tissues (Kai et al., 2017; Mapes and Davies, 2001a; Tooker and De Moraes, 2011b; Yamaguchi et al., 2012) or even decrease in galls (De Bruyn et al., 1981; Takei et al., 2015; Tokuda et al., 2013; Wood and Payne, 1988). The role of auxin could potentially differ according to plant-insect systems and particularly according to the impact of gallers on host plant growth. Indeed, *M. destructor* (Dipteran) induce a nutritive tissue without activating cell divisions (Harris et al., 2006a) and the growth of younger leaves is stunted in infested wheat (Harris et al., 2006b). Similarly, *Cicadulina bipunctata* (Cicadellidae) causes stunting of plant growth (Tokuda et al., 2013), *Lipara lucens* (Diptera) stop the stem growth (De Bruyn et al., 1981), and *Stenopsylla nigricornis* induces a leaf folding by inhibiting leaf growth at its feeding site (Kai et al., 2017). Finally, most of cited studies here used mature galls instead of galls in their early stage when the induction occurs (Miller and Raman, 2018), and used full galls instead of focusing on the tissue surrounding the insect, which implies that they could have missed phytohormonal changes occurring at finer spatial and temporal scales.

### *Cytokinins*

Contrary to auxins, in which one molecule, IAA, generates the majority of auxin effects in plant, CKs contain four main active molecules that occur in nature: trans-zeatin, cis-zeatin, dihydrozeatin and isopentenyl adenine (Sakakibara, 2006). Early research on these phytohormones used a synthetic cytokinin, 6-furfuryladenine also called kinetin. Named according to their promoting effect on cytokinesis (i.e. the partition of a cell into new cells)

(Amasino, 2005), CKs interact with IAA to regulate plant growth and developmental processes (Schaller et al., 2015). The joined effects of CKs and IAA being known to control the crown gall development (Akiyoshi et al., 1983; Gohlke and Deeken, 2014), and the physiological effects of CKs on cell division led to the investigation of the role of CKs in the gall induction by insects.

A combined increase of IAA and CKs similar to what occurs in the crown gall is observed in galls induced by the eucalyptus gall wasp (Li et al., 2017) and by *Rhopalomyia yomogicola* (Tanaka et al., 2013). In the case of the gall induced by the tephritid fly *E. solidaginis* on the goldenrod *S. altissima*, IAA and CK levels increase significantly when expressed as weight per stem length but not when expressed on a weight per weight basis (Mapes and Davies, 2001a, 2001b). However, CKs accumulate whereas IAA is depleted in the gall of *Dryocosmus kuriphilus* (Wood and Payne, 1988), in the leaf-hopper gall on maize (Tokuda et al., 2013) and in *Pontania* galls on *Salix* (Leitch, 1994; McDermott et al., 1996; Yamaguchi et al., 2012). Cytokinins level has also been found to be lower in gall tissues compared to ungalled tissues but only in the case of the psyllid *Pachypsylla celtidismamma* (Straka et al., 2010) and the aphid *Tetraneura nigriabdominalis* (Takei et al., 2015). Thus, even if interaction with IAA in gall formation is unclear, the accumulation of CKs is a frequent pattern suggesting a central role in gall development. Again, a too large time and special scales of gall sampling in the cited studies could explain at part of the variation among experiments.

The main hypothesis regarding the origin of the CKs in gall tissues is a biosynthesis and a secretion by the insect. High CK activity has been first identified in leaf-mining larvae that induce a "green island" phenotype in their mine, meaning the mined tissue stays photosynthetically active during leaf senescence (Engelbrecht et al., 1969). The high concentration of zeatin in salivary glands suggested a secretion by the insect (Engelbrecht, 1971). Similar high concentrations of CKs have been recorded in the body of many plant manipulators: in the other green-island inducers (Giron et al., 2007; Zhang et al., 2018), in aphid gallers (Takei et al., 2015), in psyllids gallers (Kai et al., 2017; Straka et al., 2010), in Chalcip wasp (Van Staden and Davey, 1978), in *Eurosta solidaginis* (Mapes and Davies, 2001b), in the chestnut gall wasp (Ohkawa, 1974; Wood and Payne, 1988), and in *Pontania* (Yamaguchi et al., 2012). These numerous examples plead for a synthesis of CKs by insects. In some insects, CK biosynthesis could be carried out by bacterial symbionts, in particular *Wolbachia* (Giron and Glevarec, 2014; Kaiser et al., 2010). Currently no direct demonstration of synthesis and secretion of CKs in gall-inducing insects are available.

### *Abscisic acid*

Besides of auxin and CKs that have long been hypothesized to play a role in the manipulation of host plants by insects, dynamics of other phytohormones have only recently received interest. Known as the "stress hormone", the abscisic acid (ABA) is involved in regulation of responses to abiotic and biotic stresses, including modulation of plant growth (Davies, 2004). ABA is also directly promoting root growth (McAdam et al., 2016).

Several studies measured high ABA levels in insect galls. High concentration of ABA has been found in galls of *Cicadulina bipunctata* (Tokuda et al., 2013), *Lipara lucens* (De Bruyn et al., 1981), *Leptocybe invasa* (Li et al., 2017), the psyllid galler tested by Khattab and Khattab (2005) and *Dryocosmus kuriphilus* (Wood and Payne, 1988). This general trend is consistent with results obtained in the crown gall that also show an increase of ABA consecutively to *Agrobacterium* infection (Gohlke and Deeken, 2014). Interestingly, the level of this plant hormone has been found to stay constant (Tokuda et al., 2013) or to decrease (Zhu et al., 2011) in the case of resistant plant varieties. On the contrary, ABA concentration was lower in the galls of the eucalyptus on a highly susceptible variety than a less susceptible one (Li et al., 2017). This is consistent with a role of ABA in the defensive response to biotic stress and ABA accumulation could result from the hydric stress caused by insect feeding (Tokuda et al. 2013). If the role of exogenous ABA in manipulating plant defenses in the gall is privileged (Tooker and Helms, 2014), it may also alter plant development.

Interestingly several gall-inducing insects have been found to contain high concentrations of ABA including the psyllids *Pachypsylla celtidismamma*,*Stenopsylla nigricornis* (Kai et al., 2017; Straka et al., 2010) and *Gnorimoschema gallaesolidaginis* (Tooker and De Moraes, 2011a). A high concentration of ABA along with other carotenoids in the gall midge *Asteromyia carbonifera* highly suggested a biosynthesis of ABA (Heath et al., 2013a). Gall midges acquired carotenoid biosynthesis enzymes by horizontal gene transfer from fungi (Cobbs et al., 2013), which could allow them to produce ABA. As many gall midges have obligate fungal symbionts, an ABA secretion by the larva could facilitate fungal growth (Heath et al., 2013b). This raises the yet unsolved question of the accumulation or a possible biosynthesis of ABA in gall-inducing insects.

### *Gibberellins*

The class of Gibberelin (GA) is associated with the promotion of germination, flowering and growth, and thus act as an antagonist of ABA (Weiss and Ori, 2007). GAs are diterpenoid acid and if 126 GA have been found in nature, the main bioactive GAs are $GA_1$, $GA_3$, $GA_4$, and $GA_7$ (Yamaguchi, 2008). Isolated from the fungal pathogen *Gibberella fujikuroi* and named after it, this phytohormone class is known to be involved in the alteration of plant growth by pathogens. However attempts to measure GA in galls are scarce. High levels of Gas have been recorded in the galls of *Janetiella coloradensis* (Byers et al., 1976) and the eucalyptus gall wasp (Li et al., 2017). On the contrary, the level of $GA_3$ decreases in the psyllid gall on *Eucalyptus* (Khattab and Khattab, 2005) and $GA_1$ and $GA_4$ decline under the action of *Cicadulina bipunctata* (Tokuda et al., 2013). Recently, the analysis of the transcriptome of *D. vitifolia* galls on the wild grapevine revealed a downregulation of GA responsive genes (Schultz et al., 2019). To our knowledge, GAs have never been measured within the insect body.

### *Brassinosteroids*

Finally, a potential role of brassinosteroids (BRs) in gall induction has received little interest. Nevertheless, these sterol derivatives are powerful growth-promoting phytohormones (Müssig, 2005). Increasing evidence suggest BRs have an important function in defense response against herbivorous insects (Erb et al., 2012; Lee et al., 2018). Interestingly, the study of the chestnut gall played an important role in the research on BRs ; it allowed to isolate and characterize one BR, castasterone, demonstrating for the first time the presence of BRs in other plants than *Brassica napus* (Yokota et al., 1982). Arima et al. (1984) found that BR content in galls tend to be higher than in intact leafves and similar to young shoot. They also show that BRs tend to decrease during gall ageing (Arima et al., 1984). Larvae contained only traces of BR activity, suggesting all BRs measured had a plant origin. This is the only example of a measurement of BR activity in insect galls. Given that naturally occurring BRs have a metabolic function in insects (Zullo and Adam, 2002), they should be taken into account in future research of cecidogenous substances.

Additional references were used to build the Table 1: Dieleman, 1969; Eitle et al., 2017; Kaldewey, 1965; Yokota et al., 1974; Zalat et al., 2000.

## 3.2. Genomic studies of galls: the cecidome

Until the last decade, phytohormones concentrated the majority of the research on the mechanisms of gall induction. Facing the challenges previously mentioned of finding new effectors involved in gall-induction and identifying their targets, the research on galls focused on described signaling pathways. In this context, the opening of the genomic era provided new opportunities to investigate the gall-insect system and allowed to consider capturing the **cecidome**, defined as “the entire set of genes that mediate the interaction between the plant and gall-inducer” (Nabity, 2016). Increasing number of such studies were published in recent years (Figure 1.4, Table 2) (as reviewed in Oates et al., 2016).

To date the greater insights on molecular interactions between a gall-inducer and its host plant came from the research on the Hessian-fly wheat system. Firstly, transcriptomic approaches for the first time revealed that a high percentage of hessian fly transcripts (40%) were actually secreted, which represented one of the most complex salivary gland secretomes for a herbivorous insect (Chen et al., 2008). Then, genome sequencing came to confirm the complexity of Hessian fly secretions, showing that more than 7% of its genome was estimated to encode putative effector proteins (Zhao et al., 2015). Among them, the protein family SSGP-71 codes for E3 ligase-mimicking effectors that may hijack plant proteasome to defeat plant immunity, and represents the largest known arthropod gene family (Zhao et al., 2015). In addition, the analysis of Hessian fly genome also revealed a protein phosphatase 2C domain as a potential effector, which may impact plant stress signal transduction (Zhao et al., 2016). This system is now considered as the model species for gall-induction (Harris et al., 2014).

Table 1.2. Genomic studies of gall and leaf-mine systems. Green: leaf-ming insects. SG: Salivary glands

| Insect | Host-plant | Method | Reference |
|---|---|---|---|
| Hemiptera - Psyllidae | | | |
| *Trioza* sp. | *Metrosideros polymorpha* | Insect + Gall transcriptomes | Bailey et al. 2015 |
| Sternorrhyncha | | | |
| *Daktulosphaira vitifoliae* | *Vitis ssp.* | Insect Genome | Delmotte et al. 2013 |
| *Daktulosphaira vitifoliae* | *Vitis ssp.* | Gall transcriptome | Nabity et al. 2013 |
| *Daktulosphaira vitifoliae* | *Vitis ssp.* | Insect (Full) transcriptome | Rispe et al. 2016 |
| *Daktulosphaira vitifoliae* | *Vitis ssp.* | Insect (Full) transcriptome | Zhao et al. 2017a |
| *Daktulosphaira vitifoliae* | *Vitis ssp.* | Insect (Full) transcriptome | Zhao et al. 2017b |
| *Daktulosphaira vitifoliae* | *Vitis ssp.* | Gall transcriptome | Schultz et al. 2019 |
| *Phloeomyzus passerinii* | *Populus x canadensis* | Insect (SG) Transcriptome | Portillo Lemus et al. 2018 |
| Phylloxerid species | various plants | Insect (Full) transcriptomes | Zhao et Nabity 2017a |
| Phylloxerid species | various plants | Insect (Full) transcriptomes | Zhao et Nabity 2017b |
| *Schlechtendalia chinensis* | *Rhus chinensis* | Insect (Full) transcriptome | Wang et al. 2016 |
| *Schlechtendalia chinensis* | *Rhus chinensis* | Gall transcriptome | Wang et al. 2017 |
| *Schlechtendalia chinensis* | *Rhus chinensis* | Gall transcriptome | Chen et al. 2018 |
| *Tamalia spp.* | *Arctostaphylos spp.* | Insect (Full) transcriptomes | Miller et al. 2015 |
| Lepidoptera - Gracilariidae | | | |
| *Phyllonorycter blancardella* | *Malus domestica* | Leaf-mine transcriptome | Zhang et al. 2016 |
| Lepidoptera - Lyonetiidae | | | |
| *Leucoptera coffeella* | *Coffea arabica* | Leaf-mine transcriptome | Cardoso et al. 2014 |
| Diptera - Cecidomyiidae | | | |
| *Mayetiola destructor* | *Triticum aestivum* | Gall transcriptome | Liu et al. 2007 |
| *Mayetiola destructor* | *Triticum aestivum* | Insect (SG) Transcriptome | Chen et al. 2008 |
| *Mayetiola destructor* | *Triticum aestivum* | Insect (Gut) Transcriptome | Zhang et al. 2010 |
| *Mayetiola destructor* | *Triticum aestivum* | Insect Genome | Zhao et al. 2015 |
| *Mayetiola destructor* | *Triticum aestivum* | Insect Genome | Zhao et al. 2016 |
| *Orseolia oryae* | *Oryza sativa* | Insect (Full) transcriptome | Sinha et al. 2012 |
| *Orseolia oryae* | *Oryza sativa* | Gall transcriptome | Rawat et al. 2013 |
| *Sitodiplosis mosellana* | *Triticum aestivum* | Insect (SG) Transcriptome | Al-jbory at al. 2018 |
| ? | *Litsea acuminata* | Gall transcriptome | Shih et al. 2018 |
| Diptera - Agromyzidae | | | |
| *Liriomyza huidobrensis* | *Arabidopsis thaliana* | Leaf-mine transcriptome | Zhang et al. 2012 |
| Diptera - Tephritidae | | | |
| *Procecidochares utilis* | *Eupatorium adenophorum* | Insect (Full) Transcriptome | Gao et al. 2014 |
| *Procecidochares utilis* | *Eupatorium adenophorum* | Insect (Gut) Transcriptome | Li et al. 2018 |
| Hymenoptera - Chalcididae | | | |
| *Leptocybe invasa* | *Eucalyptus grandis* | Gall transcriptome | Oates et al. 2015 |
| Fig wasps | *Ficus obtusifolia* | Insect + Gall metatranscriptome | Martinson et al. 2015 |
| Hymenoptera - Cynipidae | | | |
| *Biorhyza palida* | *Quercus robur* | Insect + Gall transcriptomes | Hearn 2013 |

Studies of non-model species also showed interesting insights of the cecidome. Firstly, genomic studies revealed how the gall-inducers reprogram the plant physiology and development. Thus, the gall metabolism appeared to be deeply modified compared to normal tissues, presenting an over expression of carbohydrate metabolism (Martinson et al., 2015) and, more generally, being characterized by a source-sink change (Nabity et al., 2013; Shih et al., 2018). Coupled with a reduced expression of photosynthesis-related, some galls can be considered as shifting from

autotrophic to heterotrophic (Nabity et al., 2013; Shih et al., 2018). *D. vitifoliae* has also been shown to alter water and mineral transport (Nabity et al., 2013). At the same time of metabolic shifts, genes controlling plant development also present a differential expression, informing of the tissue identity of the newly formed gall. For instance, the over-expression of the biotin carboxyl carrier protein and other markers of seed tissues suggest the tissues of gall of the cynips *Biorhyza pallida* may be identified as a seed organ by the plant (Hearn, 2013). Recently, the transcriptome of the gall of *D. vitifoliae* revealed transcriptional similarities with carpel gene expression, in accordance to the phenotypical resemblance between galls and flowers (Schultz et al., 2019).

Secondly, transcriptomes of gall systems showed how plant defenses are reorganized after insect attack. The phylloxerid *D. vitifoliae* has been found to alter defenses in grapes leaves by decreasing gene expression for non-mevalonate and terpenoid synthesis and increasing gene expression in shikimate and phenylpropanoid biosynthesis (Nabity et al., 2013); A similar alteration of the terpene profile has been recorded in *L. invasa* galls on *Eucalyptus* (Oates et al., 2015). Chen et al. (2018) described the molecular basis of tannin accumulation in *Rhus* galls. The activation of flavonoids synthesis observed in fig galls (Martinson et al., 2015) re-opened the hypothesis of the role of phenolic compounds in insect gall development (Hartley, 1999). It also suggested that the gall-inducing insect tolerates these toxic compounds. Regarding leaf-miners, transcriptomic studies showed an up-regulation of the jasmonate pathway that implies an activation of plant defenses, suggesting a lower level of manipulation of plant defenses than in gall-inducers (Cardoso et al., 2014; Zhang et al., 2016, 2012).

Thirdly, non-model gall-inducing insects have also been the object of transcriptomic studies. In total, few studies looked for effectors secreted by non-model gall-inducing insects to date (Hearn, 2013; Martinson et al., 2015; Portillo Lemus et al., 2018). Notably, the analysis of the cecidome of the *B. pallida*/*Quercus* system suggested new hypotheses for gall induction, among which we can cite a possible role of chitinases that may induce cell re-differentiation by modifying host arabinogalactans (Hearn, 2013). Other studies investigated digestion capacity of gall-inducing insects. Several species show high expression levels of detoxification genes suggesting counter adaptation to plant chemical defense (Li et al., 2018; Sinha et al., 2012; Wang et al., 2016; Zhao et al., 2017a). Alternatively, transcriptomic techniques were used to investigate molecular evolution of gall-inducing species, like the speciation of *Tamalia* (Miller et al., 2015), or the evolution of the carotenoid function (Zhao and Nabity, 2017a) and of amino acid transporters in the phylloxeriids (Zhao and Nabity, 2017b).

From a methodological point of view, most of these studies used a comparative approach in their experimental design. Regarding the research on plant response to gall-inducers, the studies focusing on the physiological changes occurring during gall formation logically compared gene expression in galls with healthy tissue (Nabity et al., 2013), whereas those investigating the molecular bases of incompatibility of plant-insect interactions compared susceptible versus resistant plants e.g. (Liu et al., 2007; Oates et al., 2015). Comparison points are less obvious when working on the insect side of the interaction, and this probably constrained the choice of species to study. Studies looking for effectors of gall-induction in non-model species either

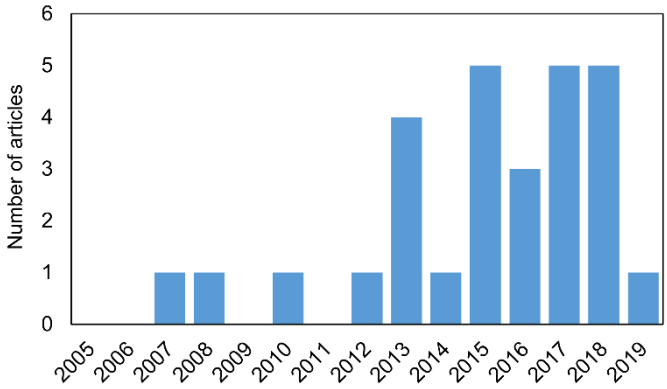



Figure 1.4. Number of genomic studies on gall systems published per year.

performed intra-or inter-specific comparisons. For intra-species comparisons, some compared two developmental stages with one supposedly involved in gall-induction (Hearn, 2013), other compared two life forms of the same species with one being gall-forming (Rispe et al., 2016). Regarding inter-species comparisons, the compared species were either closely related (Miller et al., 2015) or more distant (Portillo Lemus et al., 2018), and some studies compared multiple related species (Zhao and Nabity, 2017a, 2017b). Finally, the research focusing on resistance and virulence mechanisms compared the gene expression of virulent biotypes with that of avirulent biotypes (Rawat et al., 2012; Zhao et al., 2015), and of insect grown on susceptible plant versus insects grown resistant plant (Cardoso et al., 2014; Sinha et al., 2012).

Additional references were used to build the Table 2: Delmotte et al., 2013; Gao et al., 2014; Zhao et al., 2017b.

# Conclusion

In conclusion, despite the fact that galls and the insects that induce them have been the object of a considerable amount of research, many questions remain open. Firstly, we saw that the gall-inducing live-style can take many forms and that insects that have this feeding habit are highly diverse. However, some gall-inducing species remain overlooked in some groups of insects, like Lepidopterans. Secondly, I summarized knowledge about gall evolutionary pathways and adaptive value of the gall-inducing life-style. The former pointed that the origin of gall-induction is still under debate, notably about their origin in leaf-miner insects. The latter showed that if the nutritive hypothesis and the enemy hypothesis are both strongly supported, in contrast the support for the microenvironment hypothesis is weaker. Moreover, we saw that no hypothesis is universally supported and that adaptive value of the gall phenotype changes according to the ecological context. Finally, the review of the current knowledge about the mechanisms of gall induction by insects showed that, while the research effort has focused mostly on signaling molecules until recently, the mechanism of action and origin of phytohormones in gall formation remains unclear. In this context, the application of next generation sequencing to study the molecular mechanisms of the interaction between gall-inducing insects and their host-plant has already started to yield encouraging results.

In this thesis, I studied two gall-inducing micromoths belonging to the family Gracillariidae (Lepidoptera). Using principally histological techniques I described their life-style and the developmental process leading to the gall formation in both cases. Focusing on one species along with an extensive sampling of related species, I reconstructed the phylogeny in order to infer the evolution of gall in a genus with a unique ecology. Finally, using an intra- and inter-species transcriptomic comparative approach relying on the particular life-style and the phylogenetic framework of the studied species, I looked for effectors possibly involved in gall induction.

# Thesis outline

## Objectives of this study

The overall aim was to investigate the evolution (**Objective 1**) and molecular bases (**Objective 2**) of gall induction in insects. To achieve this, this thesis focused on Gracillariidae, an important family of insects in the order Lepidoptera that exhibits a great variety of feeding strategies (Nakadai and Kawakita 2016; Kawahara et al. 2017) More specifically, I focused on two Japanese species, *Borboryctis euryae* Kumata et Kuroko and *Calopitlia cecidophora* Kumata, that were selected for their interesting larval feeding behaviors.

Objective 1: To infer the evolution of gall induction in insects. This objective aims at determining where and when did the gall-inducing habit evolved in a group of insects largely dominated by leaf-miners and leaf-rollers.

Objective 2. To identify mechanisms of gall induction in insects. This objective aims at identifying key histological plant alterations and insects secreted salivary effectors in biological models with contrasting, and sometimes transitional feeding strategies.

## Study systems

***Borboryctis euryae*** grows on *Eurya emarginata* Makino and *E. japonica* Thunberg, two evergreen plants from the family Pentaphylacaceae (Kumata et al., 1988) (Figure 1.5A). *Borboryctis euryae* belongs to the subfamily Acrocercopinae and, in several aspects, its larval feeding behavior corresponds to the standard type in this subfamily (Kawahara et al., 2017). First and second instars make a sub-epidermal serpentine mine and the third instar creates a blotch mine. Then the larva hypermetamorphosis, which leads to a transition from fluid-feeding to tissue-feeding mouthparts (Scoble, 1992; Body et al., 2015), occurs between third and fourth instars. The larvae spend their fourth and fifth instars in this blotch mine, feeding on parenchyma tissues. *Borboryctis euryae* distinguishes from other Acrocercopinae at the fourth instar where the blotch mine is inflated. When opened, the 4$^{th}$ instar larval chamber shows traces of callus proliferation that some authors consider as a gall (Ahn, 2012).

***Caloptilia cecidophora*** Kumata is associated with *Glochidion acuminatum* Müller Argoviensis, *G. obovatum* Siebold & Zucc. and *G. rubrum* Blume (Phyllanthaceae) (Kawakita et al., 2010; Kumata, 1966) (Figure 1.5B). The life-style of *C. cecidophora* largely differs from other species of the genus. In the great majority of *Caloptilia*, the first and the second instars form serpentine mines, second instars form blotch mines, third larval instars form a tentiform mine, and remaining larval instars leave the mine and form a leaf-roll and pupate in it or outside. On the contrary, *C. cecidophora* induces the formation of a gall on the leaf, along the vein, that looks similar to some sawflies leaf-galls (Kumata, 1966). All the larval development takes place inside the gall. The gall initiation process was unclear and it has been suggested that larvae first make a leaf-mine before inducing the gall (Kumata, 1982).

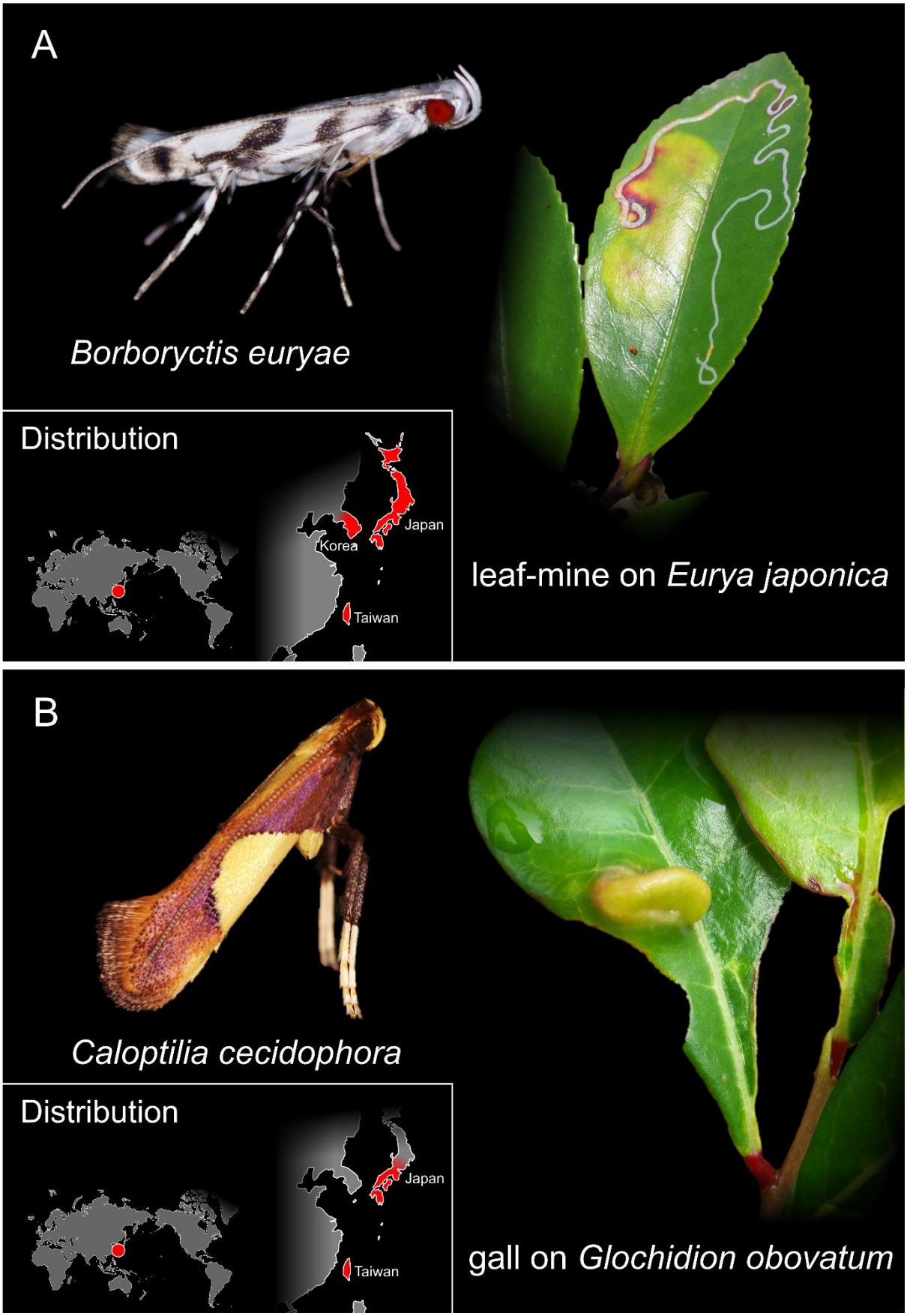



Figure 1.5. Species studied in this thesis.

These two species have in common to potentially represent a transition from leaf-mining to gall-inducing during their larval development. This characteristic is rare, if not unique, among gall-inducing insects that generally start inducing their gall from the beginning of their interaction with their host plant. If confirmed, these mixed feeding behaviors would be suitable to address questions about the evolutionary pathway of gall induction and to develop comparative approaches to decipher molecular mechanisms of gall induction.

**Chapter 2** details the investigation on the histological nature of the leaf-mine swelling induced by *B. euryae*, on the nutritive value of the neoformed tissue for the larvae and on the role of the insect for the induction of this abnormal tissue development. For this purpose, we combined experiments on field and laboratory reared insects. The study on *B. euryae* reopened the old debate about the significance of callus formation in leaf-mines. Situated in the "no-man's land between leaf-mine and galls", the proliferation of undifferentiated cells in insect galleries on leaves was often considered as the result of physical conditions in the mine rather than the consequences of an active manipulation of plant development (Hering, 1951).

**Chapter 3** shows a histological study of early steps of *C. cecidophora* gall induction in order to test whether early instars are able or not to induce gall symptoms. For this purpose, we reared insects in a greenhouse on potted plants and described the early steps of larval development. Using histology techniques, we investigated the morphogenetic processes leading to gall formation. We compared this life-style to that of a closely related species that does not induce a gall, *C. ryukyuensis* associating with *G. lanceolatum* Hayata and *G. zeylanicum* (Gaertner). With a comparison of the larval development of both species, paralleling *C. cecidophora* and *C. ryukyuensis* provided arguments to discuss the adaptive nature of galls.

**Chapter 4** presents a study that aimed to explore more deeply the evolutionary process that led *Caloptilia* species to induce a leaf-gall. Using field-collected specimens we reconstructed a phylogeny of Phyllantaceae-associated *Caloptilia*. Thanks to field sampling and collaborations, we obtained several undescribed gall-inducing species as well as other undescribed leaf-rolling *Caloptilia* associated with Phyllanthhaceae. The description of gall morphologies and the inferred phylogenies allowed to explore the origin of the gall-inducers as well as phylogenetic constraints on gall evolution.

**Chapter 5** presents a study that aimed to identify genes possibly involved in gall induction in *C. cecidophora*. We sequenced the transcriptomes of each larval instar and applied a double comparative approach. First, we compared gene expression of leaf-mining with gall-inducing instars in order to select genes that are upregulated at the occurrence of gall induction. We also compared genes that are over-expressed in the gall-inducing *C. cecidophora* with genes expressed in the non-gall-inducing sister species *C. ryukyuensis*. Expression patterns provided a list of possible candidate effectors of gall formation.

The **general discussion** presents the contribution of this thesis to the overall understanding of interactions between plant manipulating insects and their host-plants. It summarizes our main findings and addresses the question of the existence of a continuum of plant manipulators and the consequences and perspectives that are provided by this conceptual framework.

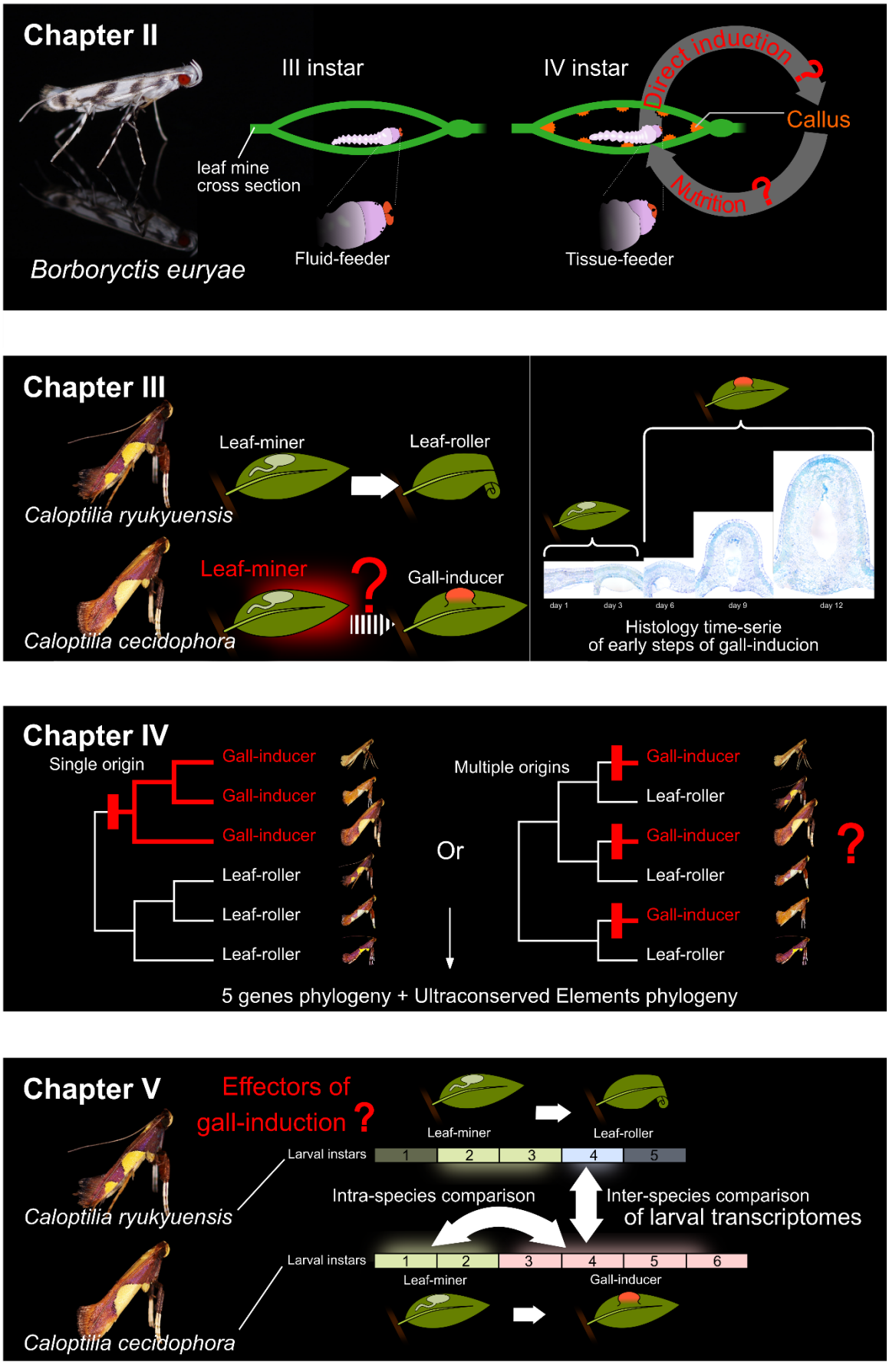



Figure 1.6. Graphical abstract.

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# Chapter 2.

# Inside the horn of plenty: leaf-mining micromoth manipulates its host plant to obtain unending food provisioning

# Résumé du Chapitre 2

Les feuilles représentent la principale ressource nutritive pour les insectes herbivores et leurs performances sont principalement fonction de leur qualité nutritionnelle (Awmack and Leather, 2002). Les insectes peuvent adopter différentes stratégies alimentaires pour optimiser l'acquisition de nutriments. Parmi celle-ci on observe les mineurs de feuille qui se nourrissent de manière sélective de tissus de haute qualité nutritionnelle et les inducteurs de galle qui induisent le développement d'un nouveau tissu présentant une valeur nutritionnelle accrue. Certains mineurs de feuilles peuvent par ailleurs manipuler leur environnement nutritionnel, notamment en maintenant l'activité photosynthétique dans la mine alors que la feuille entre en senescence (Body et al., 2013). Cependant, ces insectes n'affectent, *a priori*, pas le développement de la plante et les ressources nutritives de la mine restent en quantité limitées. Des cas de prolifération de tissu dans les mines de feuilles, appelés cals, ont néanmoins été décrits (Hering, 1951), mais le rôle direct de l'insecte dans la formation de cellules végétales supplémentaires et la fonction nutritionnelle de ce tissu n'ont jamais été établis.

Cette première étude visait à étudier la formation de cal dans les mines réalisées par les larves du lépidoptère mineur *Borboryctis euryae* (Lepidoptera: Gracillariidae) dans les feuilles d'*Eurya japonica* (Pentaphylacaceae), afin de préciser l'intérêt nutritif du cal pour l'insecte et rôle de l'insecte dans la formation de ce tissu.

Le développement larvaire de *B. euryae* se déroule en cinq stades : les premier et deuxième stades creusent une mine serpentiforme dans l'épiderme de la feuille, le troisième établit une mine élargie à l'intérieur du parenchyme dans laquelle l'insecte va compléter son développement larvaire jusqu'au stade cinq (Figure 2.1A). Un renflement de la mine est observable à partir du stade quatre. L'étude histologique de la mine de *B. euryae* a tout d'abord confirmé la présence de cal et a montré que son développement coïncide avec le quatrième stade de développement larvaire (Figure 2.2). La quantité de cal par mine a été estimée à 12,7mg en moyenne, pour une densité de surface de 0,11mg/mm$^2$ (Figure 2.3).

Pour tester l'intérêt nutritif du cal pour la larve d'insecte, nous avons provoqué une inhibition de la formation de call en détachant les feuilles minées de l'arbre, puis nous avons observé la capacité de l'insecte à finaliser son développement dans ces conditions. Des feuilles minées par des larves de premier, deuxième, troisième et quatrième stade ont été détachées puis maintenues dans des tubes au contact d'une solution hydrique. Aucune des larves dont la mine avait été détachée aux premier, deuxième et troisième stades ne parvient à se développer au-delà du stade quatre (Figure 2.4). Par opposition, 92% des larves dont la mine avait été détachée au stade quatre ont atteint le stade pupe, et 66% ont atteint le stade adulte (Figure 2.4). L'étude histologique des feuilles minées détachées au troisième stade larvaire a permis de corréler la mortalité des larves de quatrième stade avec une formation réduite de cal (Figure 2.5). Pour montrer que cette absence de cal était la cause de cette mortalité, nous avons ensuite réalisé un transfert de larves de quatrième stade provenant de mines détachée au troisième stade (présentant une absence de formation de cal) dans une mine détachée au quatrième stade

(présentant du cal déjà formé). Ce transfert a permis de restaurer une survie de 66% des larves jusqu'à l'âge adulte, ce qui démontre que le cal représente une ressource nutritive nécessaire au développement des larves de quatrième stade.

Pour tester le rôle de l'insecte dans la formation du cal, nous avons décrit l'histologie de mines dont les larves avaient été préalablement tuées et retirées au stade trois, précédant la formation de cal. L'absence de cal observée a permis de conclure à un rôle actif de l'insecte dans la formation du cal (Figure 2.5). La description histologique de mines dont les larves avaient été tuée à plusieurs intervalles de temps après leur transition de stade trois à quatre a montré que la quantité de cal formé était corrélée au temps passé par la larve de stade quatre dans la mine (Figure 2.6), ce qui suggère une stimulation chimique de la part de l'insecte.

La veille phénologique que nous avons réalisée de Juillet 2015 à Novembre 2017 a révélé une expansion et une variation considérables de la durée du quatrième stade (Figure 2.1). Nous proposons l'hypothèse de la «corne d'abondance» selon laquelle le cal induit par les larves mineuses fournirait une alimentation continuellement renouvelable, ce qui permettrait un temps de développement larvaire flexible. Ceci représente le premier exemple d'un mineur de feuilles manipulant le développement de plantes à son avantage, à la manière d'un inducteur de galle. Nous proposons de nommer ce mode de vie «mine-galle».

## Article N°1

# Inside the horn of plenty: leaf-mining micromoth manipulates its host plant to obtain unending food provisioning

Antoine Guiguet[1,2]*, Akihisa Hamatani[2,#a], Taisuke Amano[2], Seiji Takeda[3,4], Carlos Lopez-Vaamonde[1,5], David Giron[1], Issei Ohshima[2]

[1]Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS/Université de Tours, UFR Sciences et Techniques, Tours, France

[2]Department of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto 606-8522, Japan

[3]Cell and Genome Biology, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

[4]Biotechnology Research Department, Kyoto Prefectural Agriculture Forestry and Fisheries Technology Center, Kitainayazuma Oji 74, Seika, Soraku–gun, Kyoto 619–0244, Japan

[5]INRA, UR0633 Zoologie Forestière, Orléans, France

[#a]Current Address: Department of Agriculture and Fisheries, Shiga Prefecture, Shiga, Japan

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# Abstract

Leaves represent the main resource for herbivorous insects and their performances are mainly a function of leaf nutritional quality. Two feeding strategies are known to optimize the exploitation of leaf resources: leaf-miners that selectively feed on tissues of high nutritional quality and gall-inducers that induce the development of a new tissue showing an enhanced nutritional value. Some leaf-miners are known to also manipulate their nutritional environment, but do not affect plant development. Cases of callus proliferation in leaf-mines have been reported, however, the direct role of the insect in the formation of additional plant cells and the nutritional function of this tissue have never been established. Using an experimental approach, we show that leaf-mining larvae of micromoth, *Borboryctis euryae* (Lepidoptera: Gracillariidae), that grow on *Eurya japonica* (Pentaphylacaceae), actively induce callus proliferation within their leaf-mine at the fourth instar. We experimentally demonstrated that, at this developmental stage, the larva feeds exclusively on this newly formed tissue and feeding of the tissue is essential for completing larval stage. Phenological census revealed considerable expansion and variation of fourth instar duration caused by the continuous production of callus. We propose here the "cornucopia" hypothesis which states that the newly produced callus induced by the leaf-mining larvae provides virtually unending nourishment, which in turn allows flexible larval development time. This represents the first example of a leaf-miner manipulating plant development to its benefit, like a gall-inducer. We propose to name this life style "mine-galler".

# Introduction

Plant quality is one of the main drivers in phytophagous insect performance (Awmack and Leather, 2002). The quality of plants for herbivores is mostly defined by the nutritional content and the physical and chemical defenses. In turn, plant nutritional quality is influenced by several environmental factors such as soil fertility, light condition, temperature, water deficit and seasonality (Scheirs et al., 2002; Uyi et al., 2018). In addition, the low quality of host plants can be an adaptive trait selected upon the high selective pressure exerted by insects. Many plants contain secondary metabolites that represent a defensive strategy, making them unsuitable for most herbivores (Bennett and Wallsgrove, 1994). Moreover, sublethal effects of low nutritional quality or low digestibility of plant tissues are also considered as defenses against herbivory. In particular, the slow-growth high-mortality hypothesis predicts that prolonged larval development, due to a low quality of the host-plant, increases the mortality of herbivores from their natural enemies (Moran and Hamilton, 1980). Although the general validity of this hypothesis is still debated (Chen and Chen, 2018; Murphy et al., 2018), it could explain the strategies developed by some insect species to decrease the impact of whole plant quality on their developmental time. In particular, two feeding strategies are generally considered as adaptation to optimize the nutritional quality of the exploited plant tissues: gall-inducing and leaf-mining.

Leaf-mining and gall-inducing are two endophagous life-styles differing by the insect capacity to manipulate the physiology of the host plant: whereas leaf-miners consume plant tissue by dwelling inside it, gallers induce growth and differentiation of new plant tissues. In this newly formed structure, gall-inducing insects are often able to induce the formation of a highly differentiated tissue, called nutritive tissue, which shows both an enhanced nutritive quality and contains less chemical defenses (Meyer and Maresquelle, 1983). On the contrary, leaf-miners are defined as feeding only on pre-existing tissues. One of the hypothesis about the adaptive value of this life-style predicts that miners select tissues with high nutrient content and low chemical and structural defense content (Connor and Taverner, 1997). This so-called selective-feeding hypothesis has been verified in some cases (Scheirs et al., 2001), but some leaf-miners remain sensitive to decrease in host plant quality suggesting limits to this strategy (Uesugi, 2015).

This binary view defining the gallers versus miners, however, is challenged by the ability of some leaf-miners to manipulate their nutritional environment. For example, the green-island phenotype induced by *Phyllonorycter* species on apple tree leaves allows maintenance of plant tissue quality in a degenerating context (Body et al., 2013). Several insects are also able to induce a cell proliferation inside leaf-mines that may play a role in their nutrition, like for gallers (Gertz, 1918). However, contrary to galls, the role of the leaf-miner in the development of this undifferentiated tissue, called callus, within the mine and its nutritive significance for the insect are not well understood. It has been suggested that the callus growth is caused by the physical conditions inside the mine, in particular a high moisture level, rather than the result of a direct effect of the insect on the plant (Hering, 1951). On the other hand, it has been shown that callus formation could be stimulated by larval frass deposition, but without testing the nutritive role

of this newly formed tissue (Jeanneau, 1971). Some studies found that the larva feeds on the callus but with no evidence that the observed cell growth is directly induced by the insect (Hesse, 1972). Thus, to date, the insect role in callus formation in the mine is still unclear and the nutritive value of this newly formed tissue for the insect remains to be established.

Here we study the Japanese leaf-mining micromoth *Borboryctis euryae* Kumata and Kuroko (Lepidoptera, Gracillariidae), whose larvae feed on *Eurya japonica* (Pentaphylacaceae), also called "hisakaki". As indicated by its genus name ("borbos" means "swollen", and "oryctis" means "mine"), the gallery formed inside a leaf by its larva is inflated. First, we verified that the mine inflation was caused by a callus proliferation using a histological approach. The larval stage corresponding to the initiation of the mine inflation (callus) was identified, and the callus (mass) were also quantified. Then, we designed manipulative experiments to address the following questions: (i) Does the callus growth turn the mined-leaf into a sink organ, like gall development does? (ii) Does the insect feed on the callus, and if yes, is it a facultative or an obligatory feeding source (like a gall)? (iii) Does *B. euryae* larvae directly induce the callus development in the mine, or is it indirectly stimulated by the physical conditions inside the mine? Finally, the link between the feeding behavior of *B. euryae* larvae and its developmental phenology is discussed to assess the potential benefits of callus proliferation within the leaf-mine.

# Material and Methods

## *Field sampling*

All insect samples were collected from or observed in the wild forest in Kibogaoka Cultural Park, (35˚05' N, 136˚08' E; altitude 180-230 m) or Yukinoyama Historical Park (35˚07' N, 136˚15' E; altitude 110-130 m), Shiga prefecture, Japan. Both study areas are secondary forests of both deciduous and evergreen vegetation, dominated by konara (*Quercus serrata*, Fagaceae), soyogo (*Ilex pedunculosa*, Aquifoliaceae), Chinese sumac (*Rhus javanica*, Anacardiaceae) and hisakaki (*Eurya japonica*, Pentaphylacaceae), the host plant of *B. euryae*. The climate of the study site is a temperate type having hot and humid summers and moderately cold and dry winters (see S1 Table and S1 Fig for details).

## *Laboratory rearing*

To assess the dynamics of the mine inflation, we established a lab rearing of *B. euryae*. Leaf-mines of late larval instars (fourth and fifth) were collected and reared them into adults in the laboratory as described in (Ohshima, 2005). A single pair of male and female adults was introduced into a centrifuge tube (118-mm long, 28-mm diameter) containing a young leaf. After starting oviposition, each female was transferred to a mesh bag or a mesh cage wrapping twigs with young leaves of potted *E. japonica* in the greenhouse at Kyoto Prefectural University, Kyoto, Japan. Since females lay their eggs at night, oviposition was checked the

next morning and recorded the development of mines every day after hatching. Changes of larval instars were recorded by counting the molting of the head-capsules of previous developmental stages.

### *Histological study of the early steps of the mine inflation*

To verify if the mine inflation is due to the proliferation of callus, histological cross-sections of mines collected on the field were made before and after appearance of inflation symptoms. Therefore, cross-sections were made at third instar and early fourth instar (one day after molting) when the mine is still flat, and at fourteen days after beginning of fourth instar, when the mine inflation is clearly visible. Cross-section of the mine of a fifth instars was made to compare the feeding of this instar mode with previous development stages (for description of the method, see section below "*General procedure of leaf-mine histology"*).

### *Phenological census of B. euryae larvae*

To assess the number of generations per year and the duration of each stage of larval development, we carried out a phenology census of a wild population of *B. euryae*. Three *E. japonica* trees in the Kibogaoka Cultural Park were selected for the monitoring of phenological dynamics of *B. euryae* larvae in nature (Figure 2.S1). Trees less than two meters high were selected in order to allow us to inspect every leaf. The number of each instar larvae was recorded from June 2015 to October 2017 with approximately half-month intervals.

### *Estimation of callus mass*

To estimate the mass of callus present in inflated mines of fourth instar, 47 leaves mined by fourth instar larvae and 49 intact leaves with similar size were collected from two trees. The collected mined and intact leaves were weighed and measured their length and width. The length and width of each mine were also measured in order to estimate their surface areas. The leaf and mine shapes were respectively approximated by two circular segments and by an ellipse (Figure 2.S2). The larvae were weighed at about 0.5 mg. Given that it represents less than 1% of plant tissues of the mine, the effect of larval presence on mined-leaf mass can be neglected. The leaf area density of mined and intact leaves was calculated by dividing their mass by their calculated surface. The average callus mass per mine was estimated by multiplying the difference between leaf area density of mined and intact leaves with the average mine surface.

### *Exp. 1: Detached leaves experiment*

To test whether *B. euryae* larval development requires plant resources that are not contained in the mined-leaves, contrary to classical leaf-miners, mined-leaves of every instar were detached and the development and the viability of larvae were recorded. Ten *E. japonica* trees were selected for this experiment (Figure 2.S1). Leaves mined by first, second, third or fourth instar

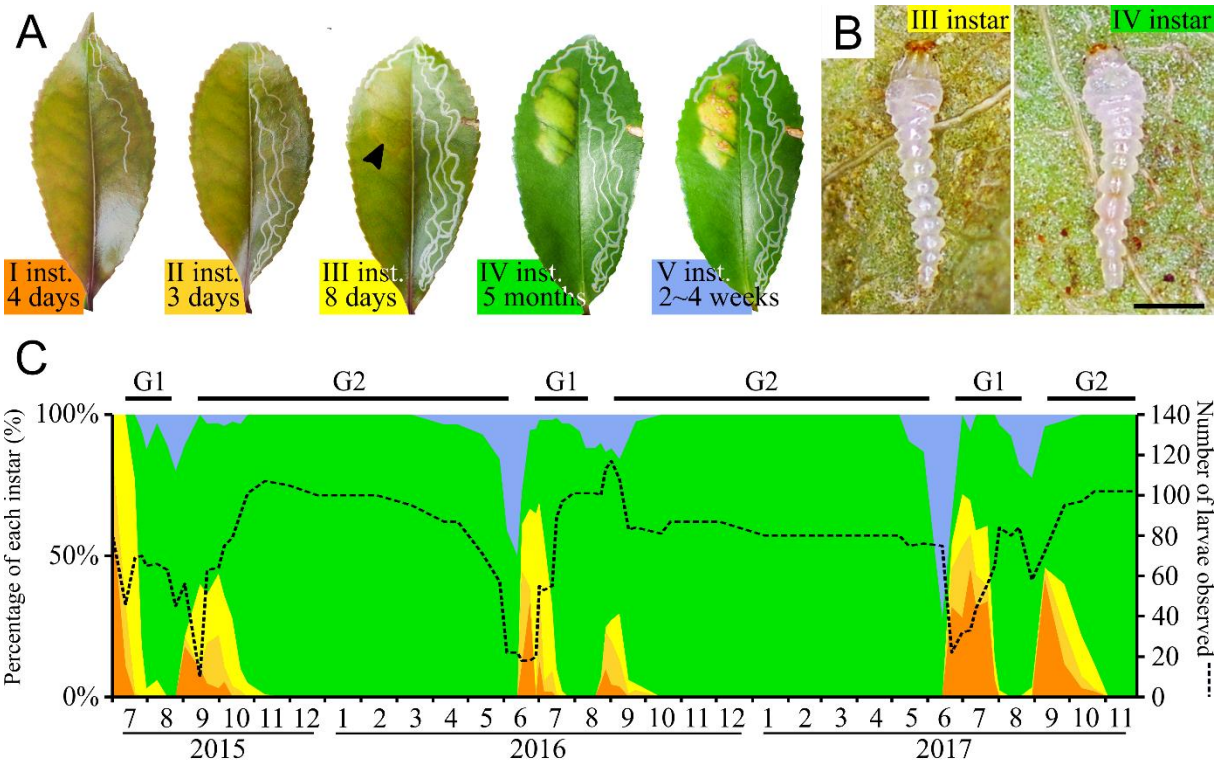



Figure 2.1. Life history and phenology of *Borboryctis euryae*. (A) The leaf-mines made by the five larval instars of *B. euryae* on *Eurya japonica*. First and second instars, respectively in dark and light orange and lasted 4 and 3 days, make a serpentine mine in the adaxial epidermis. The third instar (yellow) burrow a blotch mine (black arrow), visible by a brightening of leaf color and lasted 8 days. The fourth instar (green) is associated with an inflation and a progressive yellowing of the blotch mine. It lasted about five months in our rearing. The last stage (blue) lasted from 2 to 4 weeks and was linked with the apprearance of brown patches on the mine. (B) The morphology of third and fourth instar larvae. The mandibula shape, visible as the red structure on larva head, designate by its elongation a fluid-feeding habit for the third instar whereas its compactness for the fourth instar correspond to a tissue-feeder mode. Scale: 500µm. (A) B. euryae phenology. The left axis show the percentage of each instar recorded at each time point (same color code as in (A). The right axis corresponds to number of larvae at each count (dashed line). Instar abundances show the occurrence of two generation each year (G1 and G2) and the fact that fourth instar percentage do not reach zero at the end of G1 indicate that a part of the population is univoltine.

larvae were detached from the ten trees. Five mined leaves per tree were collected for each instar, and each detached leaf hosted only a single larva ($n_{instar}$=50, Figure 2.S3.). Detached leaves were immediately transferred to the laboratory. Mined leaves were maintained in a centrifuge tube (118-mm long, 28-mm diameter) and a 2% sucrose solution was added to the leaf stalk that were wrapped with wiping paper to keep the leaves fresh (Ohshima, 2005). Each larva was reared and the stage of death or adult emergence was recorded. Larvae that were parasitized by natural enemies (wasps) were removed from the data. All rearings and subsequent experiments were conducted at 25±1 ˚C under photoperiod of 16-h light: 8-h dark. This experiment was conducted from August to September in 2015 and from July to October in 2016.

To verify whether detached mined-leaves at third instar affect callus development that normally occurs after molting into fourth instar, histological cross-sections of fourth instar mines that had been detached at the third instar were made (n=3) (for description of the method, see section below "*General procedure of leaf-mine histology"* first paragraph).

### *Exp. 2: Larval translocation experiment*

We aimed to test whether the absence of callus development in leaf-mines detached before the larvae reach the fourth instar (see Results) is sufficient to explain the larval mortality in leaf-mines (see Results). For this purpose, we conducted a translocation experiment. The experiment consisted of letting larvae in mined leaves detached at the third instar, grow until reaching the fourth instar, and of transferring them into vacant fourth instar mines freshly detached (Figure 2.S3.). Third instar mines collected from the wild were reared in the laboratory as the detached leaf experiment, and then each reared mine was opened when the larva reached the fourth instar and carefully extracted the larva with a small brush to translocated into a vacant fourth instar mine. In order to prepare the vacant destination mines, mature fourth instar mines were collected from the wild at each translocating day. The fourth instar mines were made vacant by making a small aperture in the mine with a surgical scalpel and removing the fourth instar larva with forceps just before the translocation (Figure 2.S3.). The only difference from the leaf-mines detached before inside larvae molted into the fourth instar is the presence of callus in the mine in which the larva was transferred, thus a recovery of a normal viability into adult would mean that callus is necessary to fourth instar development. As a control, to evaluate the effect of transfer on larval viability, fourth instars directly collected in the study site were translocated into fresh vacant fourth instar mines. The viability until adulthood was recorded in both conditions and was compared with of the viability in the detached leaves experiment (Exp. 1). This experiment was conducted from June to October 2016. Mines containing translocated larvae were reared in the laboratory as the detached leaf experiment.

### *Exp. 3: Larval ablation experiment*

To test whether the fourth instar larva directly induces callus development, late third instars were killed before they molt in to the next stage and the mine-structure was observed fourteen days later. Because an inflation is normally noticed after this time, an absence of callus formation would be interpreted as evidence of a direct role of the fourth instar larva in the induction of tissue proliferation. Larvae were killed with a thin needle. Histological cross-sections were performed on mines of late third instars, fourteen days after killing larvae (n=3). As a control, cross-sections of fourth instar mines fourteen days after they molted from third instar (n=3) were performed (for description of the method, see section below "*General procedure of leaf-mine histology"* first paragraph).

In order to test whether the induction of callus development is the result of a short event at the beginning of the fourth instar or a continuous process during this developmental stage, we tested if callus amounts are correlated with the time elapsed after beginning of fourth instar or with the duration of the interaction with the fourth instar larva. For this purpose, larvae inside mines were killed at three, six and ten days after molting into the fourth instar and, in each case, a histological cross-section was made fourteen days after molting into fourth instar (for description of the method, see section below "*General procedure of leaf-mine histology"* second paragraph). If callus formation is caused by a single early event, the amount of callus is expected to be the same in each condition, if depletion due to larval feeding is negligible, or to

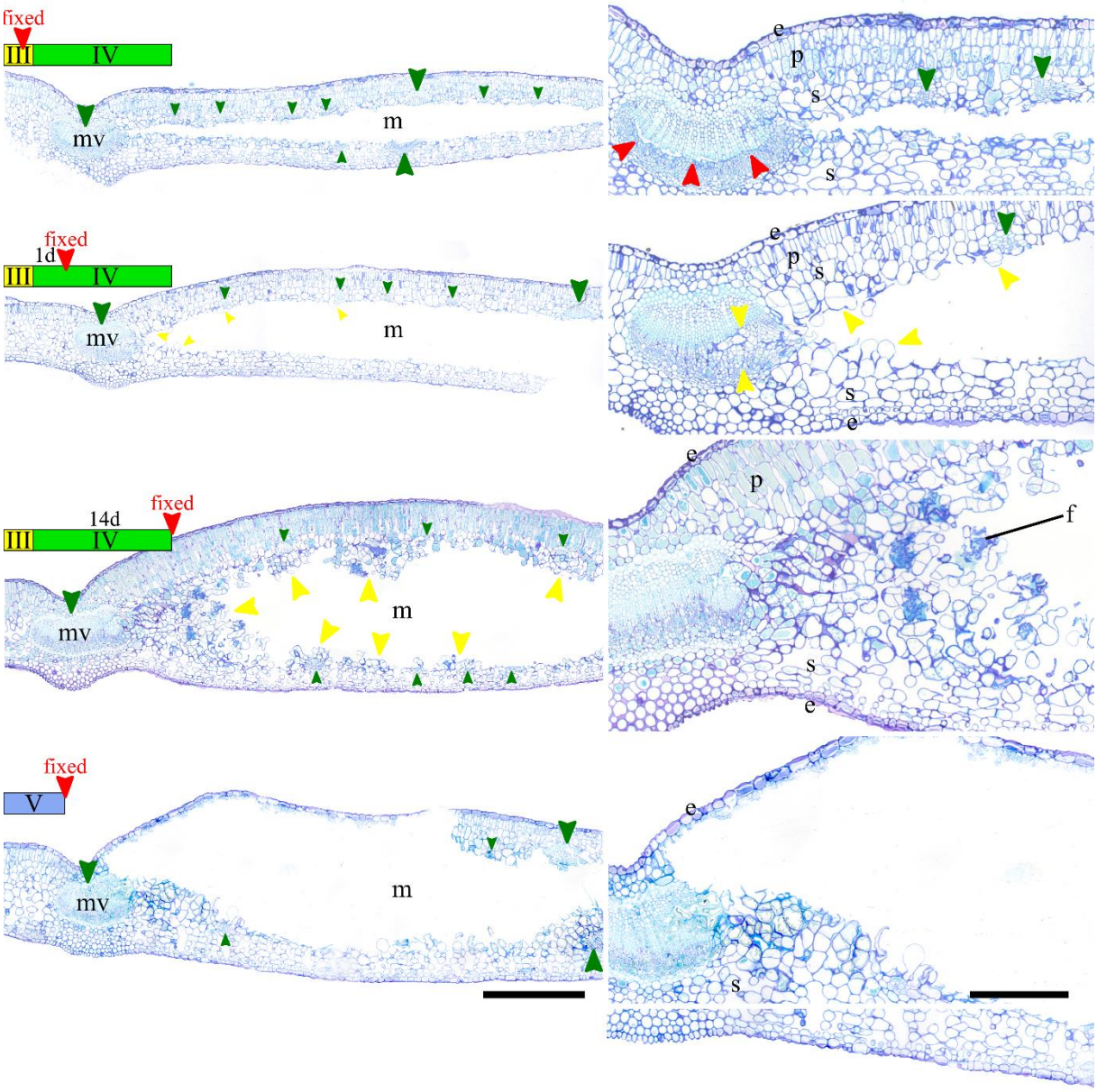



Figure 2.2. Histology of *Borboryctis euryae* leaf-mines on *Eurya japonica* in normal conditions. Cross-sections of mined leaves (left) and detail (right). From top to bottom, fixed at third instar, at one day after beginning of fourth instar, t fourteen days after beginning of fourth instar, at fifth instar. Legend: e: epidermis, p: palisade parenchyma, s: spongy parenchyma, f: larva frass, mv: mid-vein, m: mine, large green arrow: primary and secondary vein, small green arrow: other vein, yellow arrow: callus, red arrow: cut inside the mid-vein. Scale: 500µm (left), 200µm (right). Staining: Toluidine Blue O.

decrease as the duration of fourth instar increases, if insect feeding has a significant effect on the amount of callus. On the contrary, a positive correlation with the duration of fourth instar would be interpreted as evidence of a continuous induction process through the fourth instar larval life.

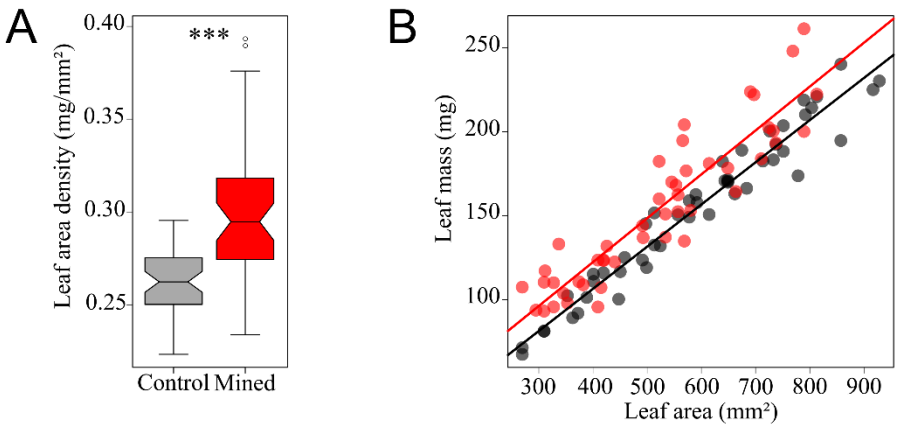



Figure 2.3. Estimation of the mass of callus in *Borboryctis euryae* leaf-mines on *Eurya japonica*. (A) Comparison of leaf-area density between leaves with fourth instar mines (red) and intact leaves (black), and (B) evolution of their mass in function of leaf area.

Larvae were killed by stabbing with a tiny needle from outside mines. As a negative control larvae were killed at the end of third instar and left mines for fourteen days on host plants before fixation. Callus quantity was estimated by dividing the area of callus on light micrographs by the mine width. This experiment was conducted in autumn 2017.

### *General procedure of leaf-mine histology*

Cross sections of about 2 mm width and 4 mm length were cut and were fixed for 4 h with 2.5% paraformaldehyde and 0.4% glutaraldehyde in 0.1 M McIlvaine citrate-phosphate buffer, pH 7.0. After dehydration in a graded series of ethanol, samples were embedded in medium grade LR White resin (London Resin Company Ltd, UK). Semi-thin sections (1 µm thick) were collected with a diamond knife (Diatome, Biel, Switzerland) installed on an ultracut R microtome (Leica, Rueil-Malmaison, France), placed on slides, fixed by heating at 120°C for 2 min and stained with Toluidine Blue O 0,1% (w/v in water) (Sigma-Aldrich, T0394).

Cross sections for the ablation experiment were cut and fixed with the same protocol but were included in paraffin. Section of 10µm were made with a rotary microtome (369 Yamato Kohki, Japan) and stained with Toluidine Blue O 0,1% (w/v in water) (Sigma-Aldrich, T0394).

Images were assembled using MosaicJ plugin of ImageJ (Thévenaz and Unser, 2007) and treated with Photoshop and Lightroom (Adobe) in order to adjust contrast and white balance, and for cropping.

### *Statistical analyses*

Leaf area densities were compared using Welch's *t*-test. The variations of leaf mass as a function of leaf area in mined and unmined leaves were compared using an analysis of covariance (ANCOVA). Results of the detached experiment were analyzed with the generalized linear model (GLM) using binomial probability distributions. The effect of variable among the ten particular "trees" and of detaching "instars" was tested on a response variable "viability

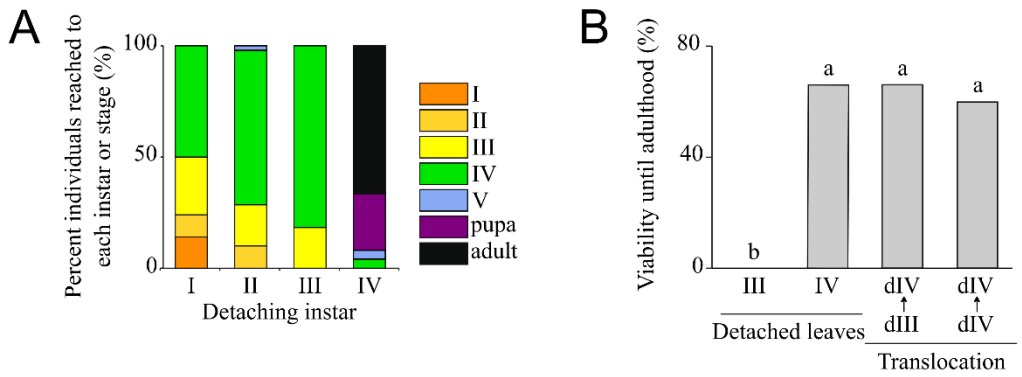



Figure 2.4. Effect of detaching *Eurya japonica* leaves mined by *Borboryctis euryae* on larval viability and callus development. (A) Last developmental stage reached after detaching a mined leaf, as a function of larval instar at leaf detachment, in percentage (Exp. 1: detached leaves experiment). (B) Viability until adulthood when mined leaf is detached at third and at fourth instar, and when larva is transferred at third and at fourth instar into a fourth instar mine, in percentage (Exp. 2: translocation experiment).

until adulthood". The differences in percentages of survival ratio until adulthood in the translocation experiments were analyzed using Fisher's exact test, and the sequential Bonferroni correction (Rice, 1989) was applied to the *P*-values to keep the significance level at 0.05 throughout the multiple comparisons. The corrected *P*-values were indicated as *P* (*adjusted*). For the larval ablation experiment, a Spearman's rank correlation coefficient between the increase of callus area and the duration of fourth instar was calculated. All statistical tests were carried out using the R package version 3.2.3 (R Developmental Core Team, 2016).

# Results

## *B. euryae larval development*

Eggs hatched three days after oviposition. Development time for first, second and third instars were four, three and eight days respectively (Figure 2.1A). Whereas first and second instars made a serpentine mine, the third instar expanded its gallery into a blotch mine. Mine inflation appeared at the fourth instar and concurrently with the hypermetamorphosis that involved large morphological changes with a shift from fluid-feeder to tissue-feeder mouthparts (Figure 2.1B) (Body et al., 2015). The blotch mine gradually inflated and turned to pale yellowish color about eleven days later. The fourth instar contrasted with other larval instar by its duration. During our greenhouse rearing, it lasted about five months before molting into fifth instar. This last fifth developmental stage took between two and four weeks (the imprecision was due to the difficulty to assess the instar transition due to the thickness of the mine tissues). It wass marked by the appearance of brown patches on the adaxial surface of the mine. Then the larva made a hole to exit the mine and spined a cocoon.

### *Phenology census*

Field observations revealed two occurrences of first-instar larvae each year, from mid-June to July and from mid-August to September, indicating that *B. euryae* has two generations per year (Figure 2.1C). However, about half of fourth instars did not molt into fifth instar during summer 2016 and 2017, suggesting half of the population is univoltine. All larvae reached fourth instar before November and overwintered at this stage. The size of insect population remained stable during the three years of recording, with about 100 individuals during winter period.

### *Histological study of the early steps of the mine inflation*

Third instar mine sections showed that the gallery is cut longitudinally through the superior half of the spongy parenchyma (Figure 2.2). As a consequence, large veins were sectioned in their middle and smaller ones were cut in their inferior part. A cut was done inside the mid-vein along the cambial layer. The parenchyma did not show any trace of feeding, which was consistent with the fluid-feeder mouthparts observed on third instar larvae. In the young fourth instar mine, the cut inside the primary vein was filled by hypertrophied cambial cells in division towards the mine gallery. Several cells showed mitotic activity close to the vascular system. At fourteen days after beginning of the fourth instar, an intense mitotic activity was present close to the mid-vein, and secondarily at the vicinity of each vascular element. As this growing mass parenchyma cells showed no apparent organization, this tissue can be considered as a callus. The presence of frass containing undigested cell wall confirmed the fourth instar is a tissue feeder. The absence of parenchyma destruction and the presence in the callus of broken cells indicated that the fourth instar exclusively feeds on the callus at this stage. On the contrary, the cross-section of fifth instar mine revealed patches of destruction of adaxial parenchyma, including palisade parenchyma, which showed that this developmental stage was feeding on those tissues. Those “feeding windows” corresponded with the brown patches noticed on the adaxial side of the mine (Figure 2.1A).

### *Estimation of callus mass*

The area density of mined leaves was superior to the unmined ($t$ = -6.2166, df = 62.656, $p$-value = 4.612e-08) (Figure 2.3A). The coefficients between leaf area and leaf mass were similar between mined and unmined leaves, and there was no significant interaction between leaf area and the two conditions (mined or unmined) (ANCOVA, for leaf area × condition, $P$ = 0.577) (Figure 2.3B). This suggests that the callus mass per mine is constant and is not a function of leaf area. The difference of intercept provided an estimation of the callus mass: 12.7mg per mine. After dividing by the average mine surface the callus amounts in the mine was estimated to represent an area density of 0.11mg/mm², which corresponded to an increase of 45% compared to control leaf area density.

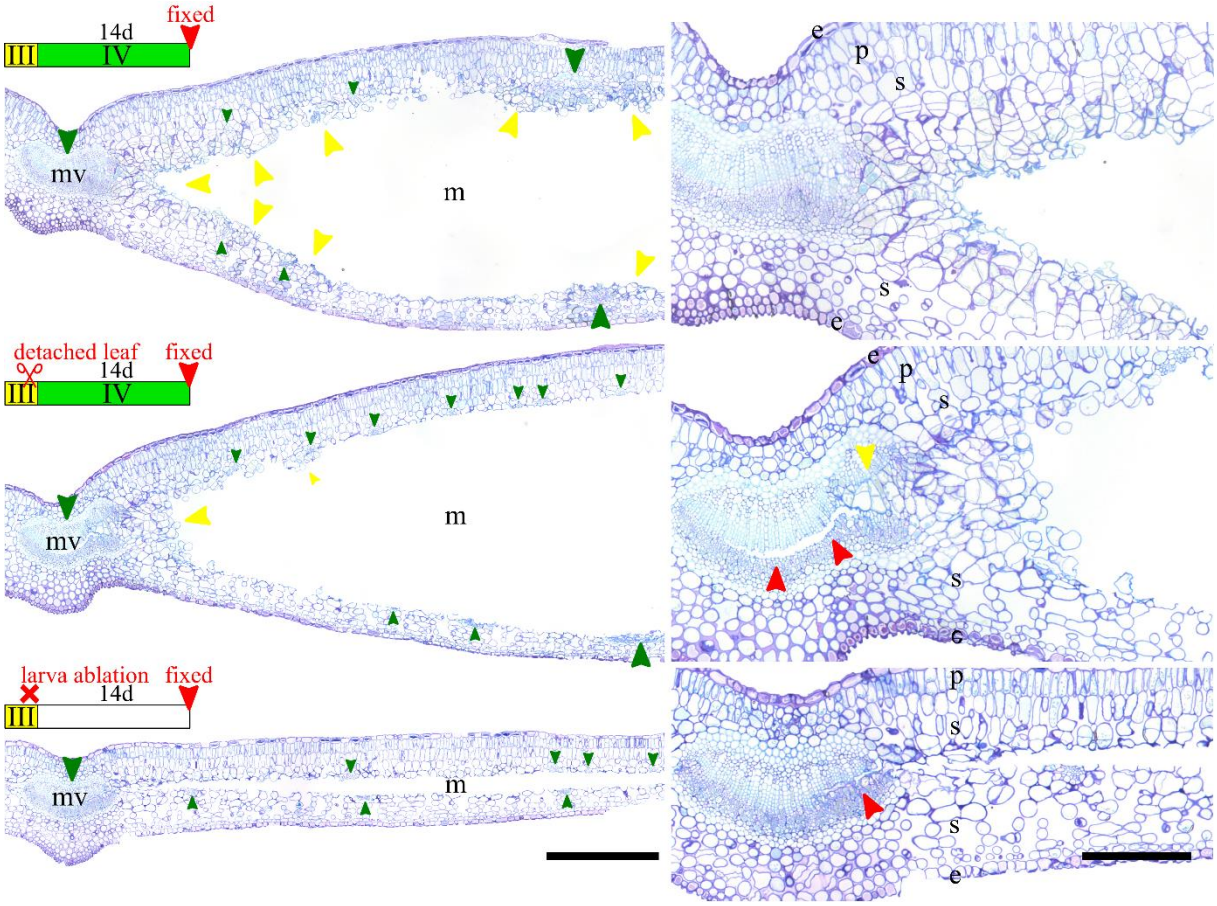



Figure 2.5. Histology of *Borboryctis euryae* leaf-mine on *Eurya japonica* after detaching the leaf and killing the larva. Cross-sections of mined leaves (left) and detail (right). From top to bottom, Fourth instar mine fixed at fourteen days after beginning of fourth instar (control), at fourteen days after beginning of fourth instar and after detaching the mined leaf at third instar (Exp. 1: detached leaves experiment), at fourteen days after killing the larvae at late third instar (Exp. 3: larval ablation experiment). Legend: e: epidermis, p: palisade parenchyma, s: spongy parenchyma, mv: mid-vein, m: mine, large green arrow: primary and secondary vein, small green arrow: other vein, yellow arrow: callus, red arrow: cut inside the mid-vein. Scale: 500µm (left), 200µm (right). Staining: Toluidine Blue O.

### *Exp. 1: Detached leaves experiment*

No larvae succeeded to develop into pupae when mined leaves were detached at first, second and third instars, and we did not notice any inflation of the detached leaves even when the inside larvae developed into fourth instar (Figure 2.4A). All larvae died before fifth instar, except one in a mined leaf detached at second instar that died just after molting into fifth instar. In contrast, in the case of leaves detached at fourth instar, 92% of the larvae successfully pupated and 66% could survive until adulthood. Significant effect of the detached instars was detected on the reached instars or stages (GLM, $X^2 = 63.312$, $P = 1.764e\text{-}15$) and on the viability until adulthood (GLM, $X^2 = 105.439$, $P < 2.0e\text{-}16$). There was no influence of the ten fixed trees on the reached instars or stages (GLM, $X^2 = 0.065$, $P = 0.7994$) and on the viability until adulthood (GLM, $X^2 = 0.064$, $P = 0.7998$). All detached leaves remained green until the end of insect life and did not present any sign of decaying.

### *Exp. 1: Histology of detached third instar leaf-mine*

Callus proliferation was present in the leaf-mine, especially around the midvein (Figure 2.5). However, its abundance was considerably reduced compared to the control. In addition, no callus was observed around other vascular elements except in the vicinity of the midvein, and it filled only partially the midvein cut.

### *Exp. 2: Larval translocation experiment*

Contrary to those that were not translocated, where no one survived until pupation, 66% of larvae translocated into mines containing callus became adult, which represents statistically significant increase of viability (Fisher's exact tests, $P$ = 6.683e-14 < *P (adjusted)*) (Figure 2.4B). Their viability was neither statistically different from those translocated between two mines containing callus (Fisher's exact tests, $P$ = 0.679 > *P (adjusted)*), nor from those reared from mined leaves detached at fourth instar (Fisher's exact tests, $P$ = 1 > *P (adjusted)*).

### *Exp. 3: Histology of leaf-mines after larval ablation*

Sections revealed an absence of callus formation when larvae were killed at third instar (Figure 2.5). The leaf-mine's histology was similar in every aspect with that of third instar mines (Figure 2.2). In contrast, the callus increased for the section where larvae were killed after molting into fourth instar. Callus was mainly observed around main veins and increased until the ten days treatment in which high volumes of callus surrounded every vascular element (Figure 2.6A). Although the present small dataset did not allow us to conclude about the dynamics of callus formation, the correlation between the time spent by the fourth instar in the mine and the amount of callus in it was significant ($r^2$ = 0.9630609, $P$ = 0.008475) (Figure 2.6B).

## Discussion

Our results provide new insight into the function and the origin of gall-like structures observed in some leaf-mines. The presence of cell proliferation in some leaf-mines used to be interpreted as a wound reaction, probably stimulated by moisture condition in the mine (Hering, 1951). Here we present the first case for which we can exclude physical conditions in the mine and tissue regeneration as the direct causes of callus formation, given that no callus develops in the absence of the fourth instar larvae. Furthermore, the newly formed tissues are usually considered as byproducts of leaf-miner feeding that do not count in their nutrient supply (Hering, 1951). On the contrary, our results show that callus represents the unique food source of the fourth instar larvae and, as a consequence, is essential for *B. euryae* larval development. Finally, a direct role of the insect in the induction of this proliferative tissue, although suspected (Hering, 1951; Jeanneau, 1971), has never been demonstrated before this study.

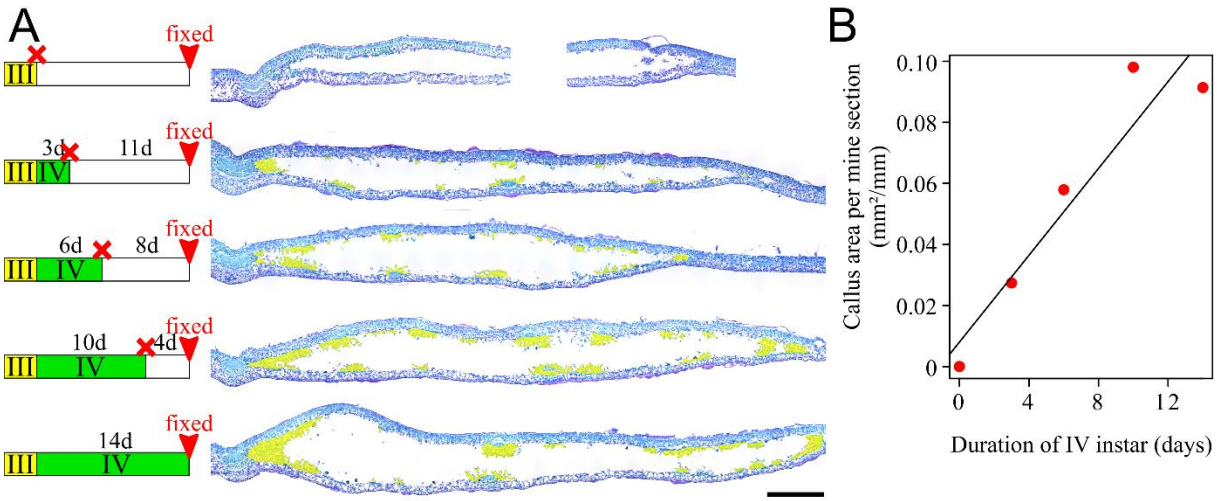



Figure 2.6. Increase of callus tissue in the mine as a function of the duration of the interaction with fourth instar larva. (A) Cross-sections of mined leaves at fourteen days after beginning of fourth instar and when larva is killed three, six or ten days after beginning of fourth instar. Callus is highlighted in yellow. Scale: 1mm. Staining: Toluidine Blue O. (B) Callus area per mine section measured on histology sections.

Therefore, the characteristics of *B. euryae* life-habit place it in the so-called "no man's land" between leaf-miners and gall-inducers (Hering, 1951). Thus, it supports that leaf-miners and gall-inducers are part of a continuum of plant manipulation (Giron et al., 2017). We propose to name this intermediate feeding strategy "mine-galling", a term coined by (New, 1981). Mine-gallers are defined as an endophagous herbivore that start and ends their interaction with their host plant by mining tissues, and induce the formation of a callus that is necessary for its subsistence. They differ from strict gall-inducers by the absence of induction of a new plant organ formation, like neovascularization and tissue differentiation. If those criteria are met, endophagous herbivores already mentioned as having callus in their galleries could be considered as mine-gallers. In particular, several other microlepidopteran may meet our definition (Hesse, 1972; New, 1981; van Nieukerken et al., 2016). We recently found other cases also belonging to the subfamily Acrocercopinae (Gracillariidae) as *Borboryctis* (unpublished data). We note that these insects have in common to make mines inside the mesophyll of sclerophyllous leaves or to bore through petioles, which suggests that callus development may require a confined environment with low evaporation, although those physical conditions are not directly involved in callus induction.

As mine-gallers share with the leaf-miners that induce a green-island phenotype the ability to enhance their nutritional environment (Body et al., 2013), they may have some mechanisms of plant manipulation in common. It has been established that these insects maintain a photosynthetic activity in their mines in autumn by secreting cytokinins (Giron et al., 2007). In addition, cytokinins and auxins are known to be produced by many gall-inducers (Tooker and Helms, 2014) and are essential for successful *in vitro* callus induction (Perianez-Rodriguez et al., 2014). Future work should assess whether secreted phytohormones play a role in callus induction by *B. euryae*. The fact that callus development is not resulting from a single induction event but from continuous stimulation is compatible with regular phytohormone injection by the insect.

The nutritional quality of callus is also unclear. We show that it is essential for larval development from the fourth instar onwards. The fourth larval stage is the longest and more variable in duration, it feeds exclusively on callus and dies if callus is not produced. As a possible explanation, the callus may contain less toxic compounds like caffeine, known to be present in *E. japonica* leaves (Ashihara et al., 2017), than the mesophyll cells. Supplementary studies are needed to compare the metabolome and intracellular organelles of callus and those of the mesophyll.

Mine-gallers may constitute a transitional form in the evolution of gall-inducers from leaf-miners, an evolutionary path that is still poorly understood (Price et al., 1987). The lack of a robust phylogeny prevents us from inferring possible origins of this life-style in the case of *B. euryae*. However, our observations give elements to discuss its adaptive significance. Histological cross-sections of fourth instar mines reveal that callus formation at the edges of the mine, and particularly within the mid vein, cause the mine swelling (Figure 2.2). Now, creating a three-dimensional space in their mines is a common strategy in Gracillariidae, usually by making a tentiform mine (Hering, 1951), and could represent a way to avoid parasitoids (29). Given that the stiffness of *E. japonica* evergreen leaves could make it impossible to fold a tentiform mine like other Acrocercopinae, the mine-gall life-style may constitute an alternative to increase the mine volume in order to avoid or limit parasitoids attack, in addition to a nutritional enhancement. Indeed, of 200 larvae used in the detached experiment, only two larvae parasitised in the *B. euryae* population, and this parasitism rate is drastically lower than that of other Acrocercopine species (Ohshima et al., 2015).

Without excluding this adaptive hypothesis, the phenological census analysis allows us to formulate another one. Our results show that *B. euryae* overwinter as fourth instar inside the mine, which consequently can last up to eight months. Moreover, we found that a substantial part of the population was not bivoltine but univoltine, which means that maximal fourth instar duration could reach up to eleven months. Similarly, having different voltinism in one population is also known in some gall-inducing species (Redfern and Hunter, 2005; Yukawa et al., 2016). These long durations for a leaf-miner instar may be allowed by the continuous regeneration of the callus induced by the insect. Like a cornucopia, the potentially unending nourishment provided by the mine to the fourth instar may constitute a considerable adaptive value, allowing *B. euryae* to have flexible larval development time. Contrary to other gall adaptive hypotheses that suppose that gall adaptive value may come from the better living conditions they provide compared to non-galling systems, i.e. a better nutrition quality, a better protection against enemies, or against environmental conditions (Stone and Schönrogge, 2003), the “cornucopia hypothesis” assumes that the maintenance of favorable living condition provides an adaptive value to this life-style. This value may consist in buffering the impact of the variation of environmental conditions (Yukawa et al., 2016) or mitigating parasitoid impact on populations (Redfern and Hunter, 2005).

As a broader perspective, this system is particularly suitable for comparative approaches. Contrary to most gall-inducing insects for which plant manipulation starts with their interaction with their host plant, galls appear at a specific time of *B. euryae* development in the leaf. This

characteristic allows intra-species comparison of gene expressions, a powerful method that could help to find factors of gall-induction.

## Acknowledgements

We thank Kibogaoka Cultural Park and Yukinoyama Historical Park for their permission to conduct field work and sampling, Masayuki Kobayashi for his help for collecting samples, and Atsushi Kawakita and Sylvain Pincebourde for their comments and suggestions. We acknowledge Françoise Laurans, Véronique Lainé-Prade, Takehiro Masumura and Tomoka Miyahara for their help in the histological preparation.

# Supplementary Figures and Tables

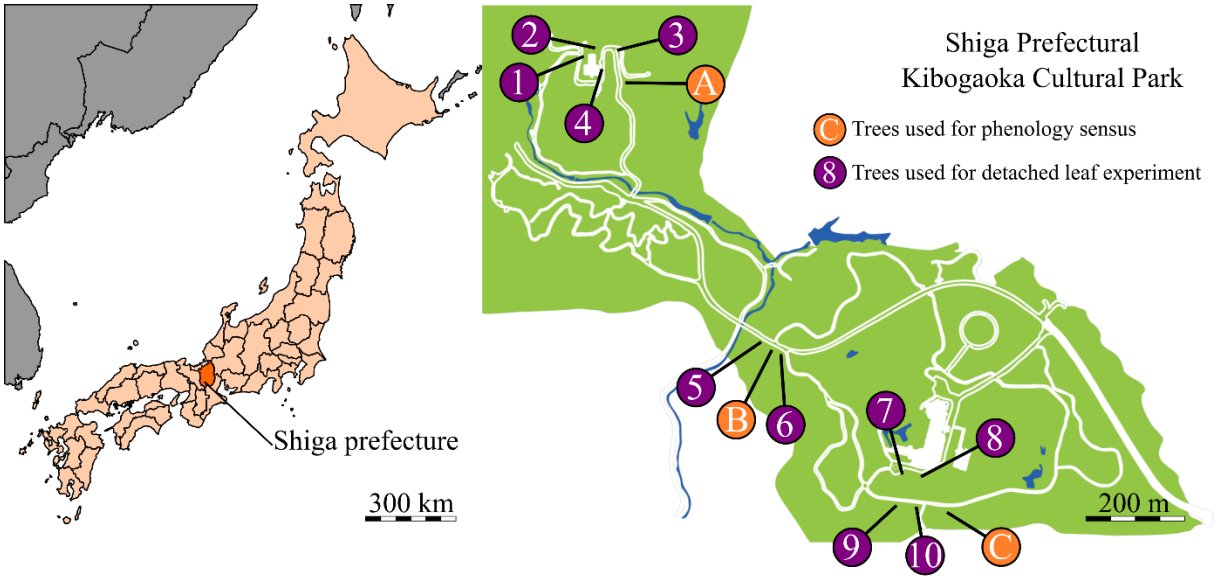
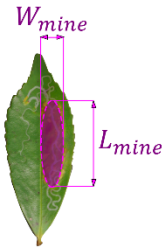



Figure 2.S1. Location of the study site. Orange circles show trees used in phenology census and purple circles show trees used for detached leaf and larval translocation experiments.

Estimation of mine area $A_{mine} = \pi \cdot \frac{L_{mine}}{2} \cdot \frac{W_{mine}}{2}$

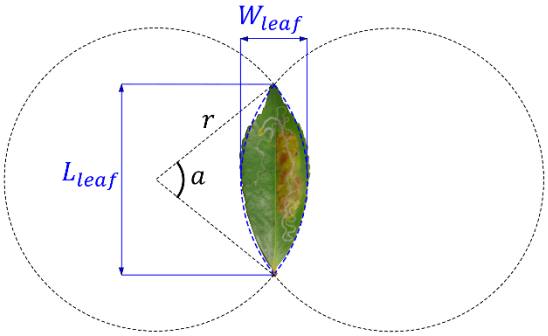



Estimation of the leaf area

1) Calculation of the radius r

$$r^2 = \left(\frac{L_{leaf}}{2}\right)^2 + \left(r - \frac{W_{leaf}}{2}\right)^2$$

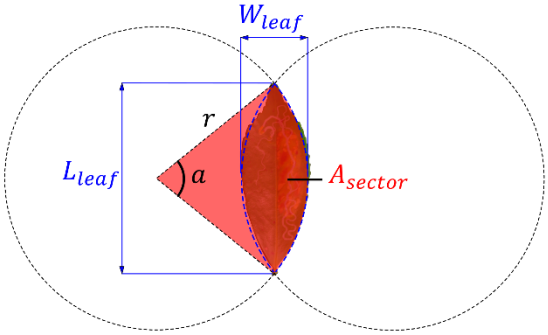
$$\boxed{r = \frac{{L_{leaf}}^2 + {W_{leaf}}^2}{4W_{leaf}}}$$




2) Calculation of the angle a

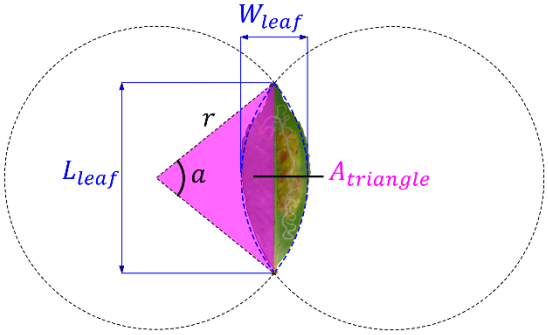
$$\sin\left(\frac{a}{2}\right) = \frac{L_{leaf}}{2r}$$

$$\boxed{a = 2\sin^{-1}\left(\frac{L_{leaf}}{2r}\right)}$$




3) Calculation of the area of the circular sector

$$A_{sector} = \pi \cdot r^2 \cdot \frac{a}{360}$$




4) Calculation of the area of the triangle

$$A_{triangle} = \frac{1}{2} \cdot L_{leaf} \cdot r \cdot \cos\left(\frac{a}{2}\right)$$

5) Estimation of the leaf area

$$\boxed{A_{leaf} = (A_{sector} - A_{triangle}) \times 2 = \left(\pi \cdot r^2 \cdot \frac{a}{360} - \frac{1}{2} \cdot L_{leaf} \cdot r \cdot \cos\left(\frac{a}{2}\right)\right) \times 2}$$

Figure 2.S2. Geometrical estimate of leaf and leaf-mine area.

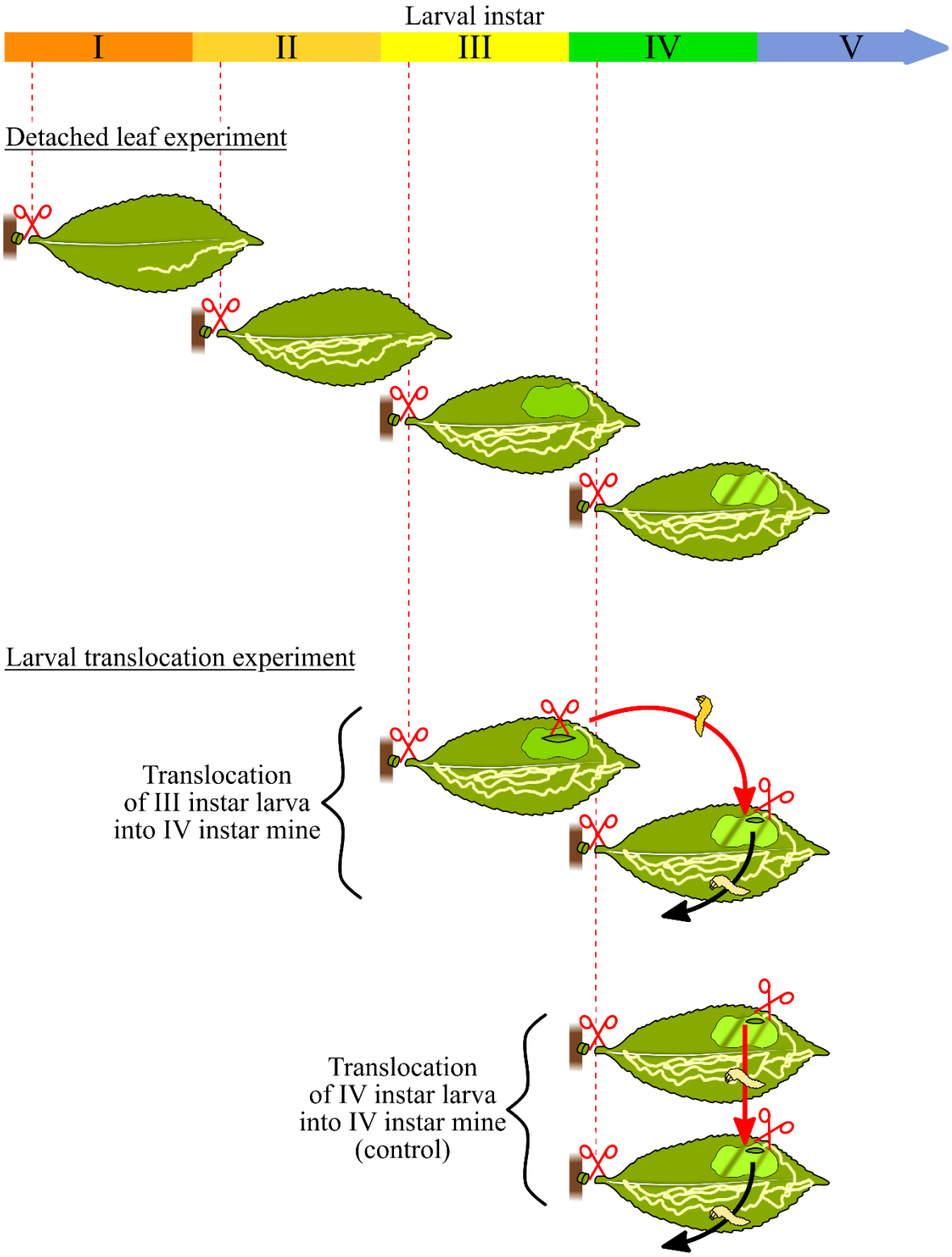



Figure 2.S3. Plan of detached leaves and larval translocation experiments (Exp. 1 and Exp. 2).

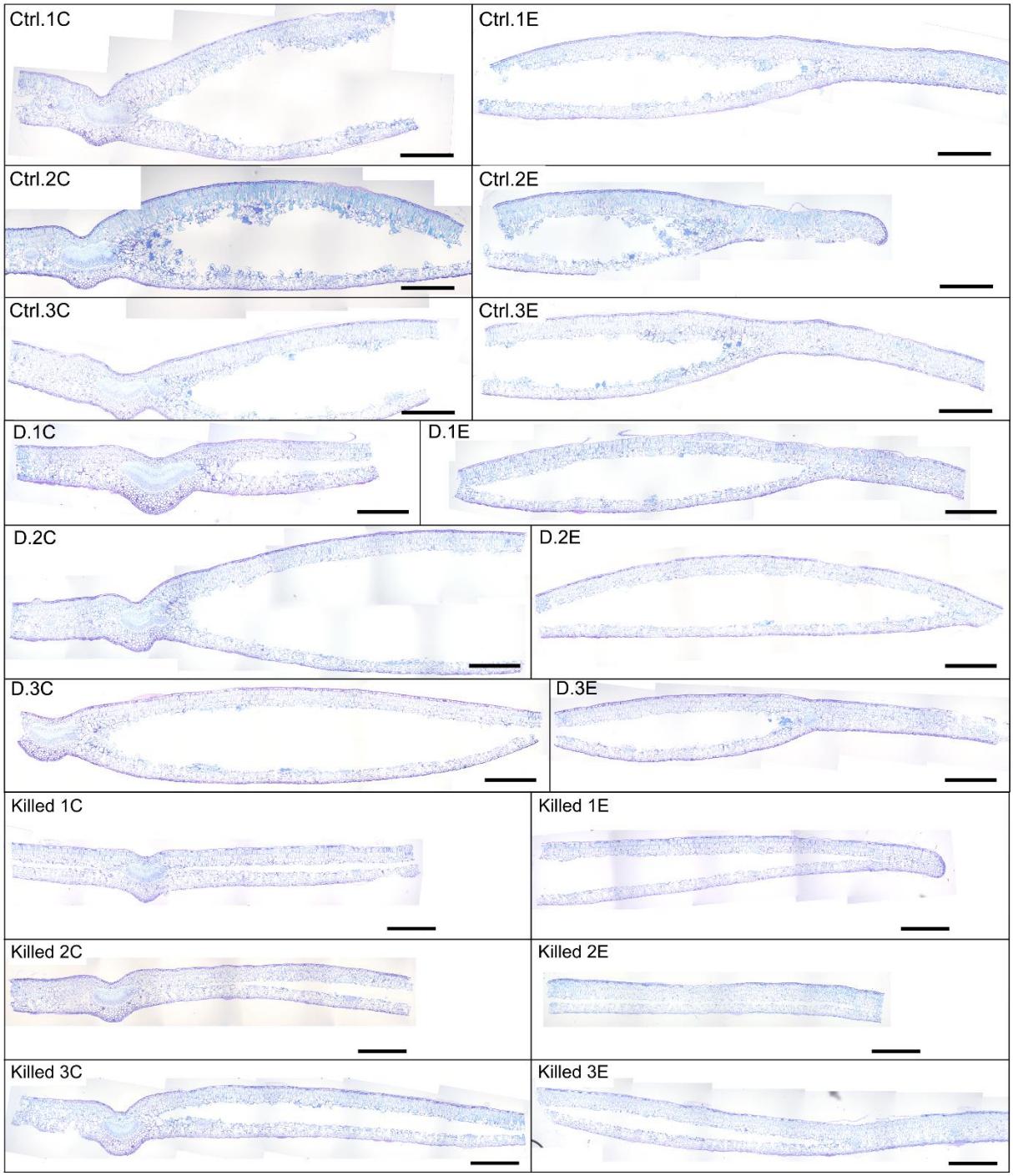



Figure 2.S4. Replicates of the leaf-mines cross-sections of the detached leaf experiment (Exp. 1) and the larva ablation experiment (Exp. 3). Cross-sections of leaf-mines at fourteen days after beginning of fourth instar (Ctrl.), at fourteen days after beginning of fourth instar on a detached leaf (D.), and at fourteen days after killing the larvae at late third instar (Killed). Each mine has been cut close to midvein (C) at the mine edge (E). Scale: 500µm. Staining: Toluidine Blue O.

Table 2.S1. Meteorological data of the study site in 2015 to 2017. All data derived from the observation point of Japan Meteorological Agency in Higashiomi town, which is the nearest observation point to the study site.

2015

| Month | Rainfall (mm) | Temperature (℃) | | | | |
|---|---|---|---|---|---|---|
| | Total | Mean | | | Maximum | Minimum |
| | | per day per hour | highest per day | lowest per day | | |
| Jan | 183 | 3.1 | 6.9 | -0.2 | 10.8 | -4.9 |
| Feb | 61.5 | 4.2 | 8.5 | 0.4 | 13.7 | -2.4 |
| Mar | 150.5 | 7.6 | 13.6 | 2.3 | 24.2 | -2.5 |
| Apr | 133 | 13.7 | 19.2 | 8.5 | 28.8 | 2.9 |
| May | 86.5 | 19.2 | 25.8 | 13.1 | 31.8 | 7 |
| Jun | 214.5 | 21.1 | 25.8 | 17.3 | 32.1 | 12.1 |
| Jul | 205.5 | 25.6 | 30.6 | 22 | 37 | 18.1 |
| Aug | 163.5 | 26.7 | 32.3 | 22.6 | 37.6 | 19.4 |
| Sep | 216 | 21.3 | 26 | 17.6 | 30.3 | 12.7 |
| Oct | 30 | 16 | 22.6 | 10.1 | 26.3 | 4.9 |
| Nov | 148 | 12.5 | 17.3 | 8.1 | 23.8 | 1.5 |
| Dec | 84.5 | 7.3 | 12 | 2.9 | 20.1 | -2.5 |

2016

| Month | Rainfall (mm) | Temperature (℃) | | | | |
|---|---|---|---|---|---|---|
| | Total | Mean | | | Maximum | Minimum |
| | | per day per hour | highest per day | lowest per day | | |
| Jan | 95.5 | 3.9 | 8.8 | -0.5 | 15.5 | -5.3 |
| Feb | 85.5 | 4.7 | 10.4 | 0 | 20.6 | -4.8 |
| Mar | 76 | 8.1 | 14.2 | 2.5 | 21.5 | -2.2 |
| Apr | 136.5 | 14 | 19.8 | 8.2 | 27.4 | 0.2 |
| May | 91.5 | 19.1 | 25.1 | 13.6 | 31 | 8 |
| Jun | 255 | 21.7 | 26.6 | 17.7 | 32 | 10.4 |
| Jul | 122.5 | 26 | 31.4 | 22 | 35.9 | 19.5 |
| Aug | 101 | 27.3 | 33.4 | 22.8 | 36.6 | 18.9 |
| Sep | 273.5 | 23.9 | 28.6 | 20.3 | 33.4 | 14.6 |
| Oct | 139.5 | 17.9 | 23.3 | 13 | 31.4 | 4.8 |
| Nov | 83.5 | 10.6 | 15.7 | 5.9 | 20.9 | 1.1 |
| Dec | 91.5 | 6.5 | 11.4 | 1.7 | 19 | -1.9 |

2017

| Month | Rainfall (mm) | Temperature (℃) | | | | |
|---|---|---|---|---|---|---|
| | Total | Mean | | | Maximum | Minimum |
| | | per day per hour | highest per day | lowest per day | | |
| Jan | 132 | 2.9 | 7.4 | -1 | 13.2 | -5.4 |
| Feb | 92 | 3.1 | 7.4 | -0.8 | 13.1 | -3.5 |
| Mar | 87 | 6.3 | 12.1 | 1 | 19 | -3.3 |
| Apr | 96 | 12.9 | 19 | 6.9 | 27.3 | -0.3 |
| May | 67.5 | 18.8 | 25.5 | 12.9 | 31.2 | 6.5 |
| Jun | 142.5 | 20.6 | 26.4 | 15.4 | 30.9 | 9.5 |
| Jul | 127.5 | 26.8 | 32.1 | 23.2 | 34.9 | 19.5 |
| Aug | 146 | 26.8 | 32.1 | 22.9 | 35.7 | 18.5 |
| Sep | 112.5 | 21.7 | 27.1 | 16.8 | 30.7 | 12 |
| Oct | 532.5 | 16.5 | 20.3 | 13.1 | 29.1 | 5.7 |
| Nov | 53.5 | 9.5 | 15.3 | 4.4 | 21.5 | -0.2 |
| Dec | 44.5 | 4 | 9.2 | -0.1 | 14.1 | -3.2 |

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# Chapter 3.

# Origin of gall-inducing from leaf-mining in *Caloptilia* micromoths (Lepidoptera, Gracillariidae)

# Résumé du Chapitre 3

La capacité d'induire des galles a évolué à plusieurs reprises indépendamment dans six ordres d'insectes et est particulièrement commune chez les hyménoptères, les diptères et les hémiptères (Dreger-Jauffret and Shorthouse, 1992). Les chemins évolutifs conduisant à l'apparition du mode de vie galligène au cours de l'évolution des insectes restent discutés. Deux voies d'évolution parallèles des galles chez les insectes herbivores ont été proposées (Price et al., 1987) : une première supposant qu'une sédentarisation aurait progressivement induit une dépression au niveau du site de nourrissage, et une second proposant qu'une internalisation au sein des tiges de plantes serait préalable à l'acquisition de la capacité d'induire une galle. La voie « sédentaire » a été démontrée dans le cas des tenthrèdes (Nyman et al., 2000), des thrips (Crespi et al., 1997) et des pucerons (Stern, 1995), tandis que les mouches téphritides et certaines chenilles de lépidoptères sont proposées comme exemples de la voie « endophage » (Price et al., 1987). Dans ce chapitre nous étudions la possibilité d'une évolution du mode de vie galligène à partir d'une autre voie évolutive prenant son origine chez une autre catégorie d'insectes endophages, les mineurs de feuille.

Notre étude a eu pour objet la description du mode de vie larvaire de *Caloptilia cecidophora* (Lepidoptera, Gracillariidae), une espèce du Sud du Japon induisant des galles sur *Glochidion obovatum*, *G. rubrum* et *G. acuminatum* (Phyllanthaceae) (Fig. 1). Au sein du clade s'espèces associées à des Phyllanthaceae, *C. cecidophora* est la seule espèce décrite comme induisant une galle (Nakadai and Kawakita, 2016). Les larves de *Caloptilia* connaissent en général quatre modes de vie successifs au cours de leur développement : le premier stade creuse une mine linéaire, le deuxième stade établit une mine élargie, le troisième stade rassemble les extrémités de la mine au moyen de soies pour former ce que l'on nomme une mine tentiforme, et enfin le quatrième stade quitte la mine tentiforme pour réaliser un enroulement de la feuille dans lequel la larve continuera à se développer au cinquième et dernier stade (Kumata et al., 2013) (Figure 3.S1). Au contraire, le seul mode de vie connu des larves de *C. cecidophora* est le mode de vie galligène. Cependant, il a été suggéré que les premiers stades de développement de cette espèce pourraient être mineurs de feuilles tout comme les autres *Caloptilia* (Kumata, 1966). Notre étude avait pour objectif de tester cette hypothèse.

Nous avons comparé le mode de vie et le développement larvaire de *C. cecidophora* à ceux de *C. ryukyuensis*, l'espèce décrite lui étant la plus étroitement apparentée ne provoquant pas de galles. Nous avons établi une série chronologique de coupes histologiques de la galle de *C. cecidophora* tous les trois jours après éclosion de l'œuf. Les résultats ont montré que la larve était mineuse de feuille jusqu'au sixième jour suivant l'éclosion, date à laquelle les symptômes de galle (hypertrophie et divisions cellulaires) commencent à apparaitre (Figure 3.1). Une expérience d'ablation de la larve à trois jours post-éclosion a permis de supprimer l'apparition de ces symptômes, démontrant que l'induction de la galle ne survient pas avant trois jours post-éclosion. L'initiation de la formation de la galle coïncide avec le troisième stade de développement larvaire, qui se caractérise par un changement de pièces buccales par rapport aux stades précédents (Figure 3.S2). Au contraire, la mine de *C. ryukyuensis* au même stade

présente une absence de symptômes de galles (Figure 3.S3). L'ensemble de ces résultats confirme l'existence d'une transition de mode de vie au cours du développement larvaire de *C. cecidophora*, depuis un mode de vie mineur vers un mode de vie galligène. Ceci suggère que le mode de vie mineur de feuille constituerait l'état ancestral du mode de vie galligène de *C. cecidophora* (Figure 3.5).

Enfin, la comparaison de la croissance larvaire de *C. cecidophora* et de *C. ryukyuensis* a montré une profonde différence de développement des larves entre ces deux espèces, *C. cecidophora* présentant une absence de croissance du premier au troisième stade associée à une durée de développement réduite ainsi qu'un stade de développement supplémentaire, ce alors que les adultes des deux espèces sont de même taille (Figure 3.4). Cela suggère que la phase de développement précédant la formation de la galle constitue une « fenêtre de vulnérabilité » pour les prédateurs (Figure 3.4) qu'il faut minimiser dans sa durée, un phénomène déjà observé chez d'autres espèces galligènes (Cornell, 1983, 1990; Craig et al., 1990).

## Article N°2

# Origin of gall-inducing from leaf-mining in *Caloptilia* micromoths (Lepidoptera, Gracillariidae)

Antoine Guiguet[1,2], Issei Ohshima[2], Seiji Takeda[3], Françoise Laurans[4], Carlos Lopez-Vaamonde[1,5], David Giron[1]

[1]Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS Université de Tours, UFR Sciences et Techniques, Tours, France

[2]Department of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

[3]Cell and Genome Biology, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

[4] BioForA, INRA, ONF, 45075, Orléans, France

[5]INRA, UR0633 Zoologie Forestière, Orléans, France,

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# Abstract

In insects, the gall-inducing life-style has evolved independently many times. Several evolutionary pathways leading to this lifestyle have been proposed. While there is compelling evidence supporting surface-feeders and stem-borers as ancestral states of insect gall-inducers, an evolutionary pathway from leaf-miners remains hypothetical. Here we explored this question by comparing the developmental processes of two micromoths, a gall-inducer *Caloptilia cecidophora* (Lepidoptera, Gracillariidae), and its non-gall-inducing relative *C. ryukyuensis.* Like other *Caloptilia*, the first and second instars of *C. cecidophora* are leaf-miners and the gall is initiated inside the leaf-mine by the third instar, thus suggesting leaf-mining as an ancestral, plesiomorphic state in this case. This is the first example of an insect species switching from leaf-mining to gall-inducing during larval development. The first two leaf-mining instars of *C. cecidophora* exhibit an absence of growth and a reduced time duration compared to *C. ryukyuensis*. The shortening of the duration of leaf-mining stages is apparently compensated in *C. cecidophora* by a larger egg size than *C. ryukyuensis*, and an additional larval instar during the gall phase.

# Introduction

In the class Insecta, the gall-inducing habit has evolved many times independently in six orders and is especially common among Hymenopterans, Dipterans and Hemipterans (Raman et al., 2005). Gall induction is defined as a plant growth response to stimuli produced by an inducing organism, generated through cell growth (hyperplasia) and/or cell enlargement (hypertrophy), and resulting in a phenotype specific to the gall-inducer species (Meyer and Maresquelle, 1983). The phenotypic specificity distinguishes gall induction from non-specific foreigner-induced plant growth responses such as callus formation (Guiguet et al., 2018). It has been hypothesized that the gall-inducing habit evolved either from the sedentary surface feeding habit or from stem boring (Price et al., 1987). Research efforts have been concentrated on the 'sedentary' route, where it has been shown that sawflies (Nyman et al., 2000), thrips (Crespi et al., 1997) and aphids (Stern, 1995) that induce galls may have evolved from sedentary surface feeders. Regarding the other scenario, origins from the stem-boring habit, tephritid flies and in some caterpillars are proposed as examples (Price et al., 1987).

In addition to those two evolutionary pathways, we propose a third whereby gall induction evolved from the leaf-mining habit. Finding transitional states having gall-inducing characters in leaf-miner groups would support this third hypothesis. One such gall-inducing character is hyperplasia, which has been reported in some leaf-mines (Hering, 1951, Dempewolf, 2005). In addition to the low number of cells generally produced, the nutritive role of the newly formed tissue for the insect and the role of the insect in its formation is debated (Hering, 1951, Dempewolf, 2005). Recently, we established the nutritive role of callus present in the leaf-mines of the micromoth *Borboryctis euryae* (Lepidoptera, Gracillariidae) and the active role played by the insect in the induction of this phenotype (Guiguet et al., 2018). However, given that callus cells form an undifferentiated tissue, this induced phenotype cannot be considered as a phenotype specific to the gall-inducer species. Therefore, the existence of callus in leaf-mines on its own cannot be considered as evidence that leaf-mining is an ancestral state of gallinduction.

Here, we explore the habits of the East Asian micromoth *Caloptilia cecidophora* (Lepidoptera, Gracillariidae) whose larva induces galls on *Glochidion obovatum, G. rubrum* and *G. acuminatum* (Phyllanthaceae) (Figure 3.1). In the *Caloptilia* clade that feed on Phyllanthaceae, *C. cecidophora* is the only species that has been described as gall-inducer (Kumata, 1966). *Caloptilia* larvae typically have four successive styles of leaf-mining during larval development. Serpentine mines are created by first and early second instars, blotch mines by late second instars and three-dimensional tentiform mine by third instar. The remaining larval instars leave the mine and create a shelter by forming a leaf-roll (Kumata et al., 2013) (Figure 3.S1). In *C. cecidophora*, however, late instar larvae make a gall instead of a leaf-roll, and it has been suggested that early larval stages of *C. cecidophora* might be leaf-miners (Kumata, 1966).

We compared *C. cecidophora* larval development with that of its most closely related non-gall-inducing species, *C. ryukyuensis*. We propose that a gall-induction inside the mine provides

support for direct evolution from leaf-mining, whereas a gall induction outside of the mine, which requires departure of the larva from the leaf-mine gallery, provides support for the external feeding life-style being the ancestral state. To test these ideas, we established a time series of the histological changes that occur in mined leaves during the early stages of larval development. We aborted young larvae to test whether gall symptoms would appear in their absence. In addition, we compared the size of *C. cecidophora* and *C. ryukyuensis* at each developmental stage, in order to test the relationship between larval life-styles and patterns of larval growth.

# Methods

## *Field collection and laboratory rearing*

*Caloptilia cecidophora* galls on *Glochidion obovatum* were collected in Tomogashima Island (Wakayama, Japan; 34.28N, 135.00E) and maintained in a laboratory growth chamber (25°C, 75% humidity, LP-1PH, NK system, Osaka, Japan) at Kyoto Prefectural University in Kyoto, Japan. Adults were sexed and transferred to mesh cages (40x40x40cm) held in a greenhouse (25°C, 70±10% humidity). Each cage contained one or two pairs of moths and tissue paper soaked with a 2% sucrose solution, which provided as a food for adults (Ohshima, 2005). A branch of a potted *G. obovatum* bearing young leaves was placed in the cage. *C. cecidophora* females will only lay eggs on very fresh leaves. Oviposition was checked daily. When eggs were found, the twig was replaced. All rearing was conducted at Kyoto Prefectural University, Japan.

We also collected mines of a closely related (Kawakita et al., 2010) non-gall-inducing species, *C. ryukyuensis*, on *G. zeylanicum* and *G. lanceolatum* in Ishigaki Island (Okinawa, Japan; 24.32-51N, 124.07-27E) and reared them on *G. zeylanicum* in Kyoto Prefectural University using the same protocol described above for *C. cecidophora*.

## *Histological analysis of gall ontology*

We made a time series of the initial stages of the gall development by sampling attacked leaves every three days, starting when the egg hatched and continuing until the gall with its larval chamber had been formed. To analyze the internal structure of a mature gall, we sectioned field collected galls occupied by fifth instars. To test whether galls are induced by third instar larvae, we killed second instar larvae three days post hatching (dph3) and then sampled the leaf at dph6. We killed the larvae with a thin needle. Larval instars were determined by counting the number of head-capsule moults of previous instars (Kumata, 1978, 1985). Head capsules were collected inside the leaf-mines and identified by binocular microscope and transmitted light.

Cross-sections (about 2 mm wide) were cut with a surgical scalpel and fixed for 4 h with 2.5% paraformaldehyde and 0.4% glutaraldehyde in 0.1 M McIlvaine citrate-phosphate buffer, pH 7.0. After dehydration in a graded series of ethanol, samples were embedded in medium grade

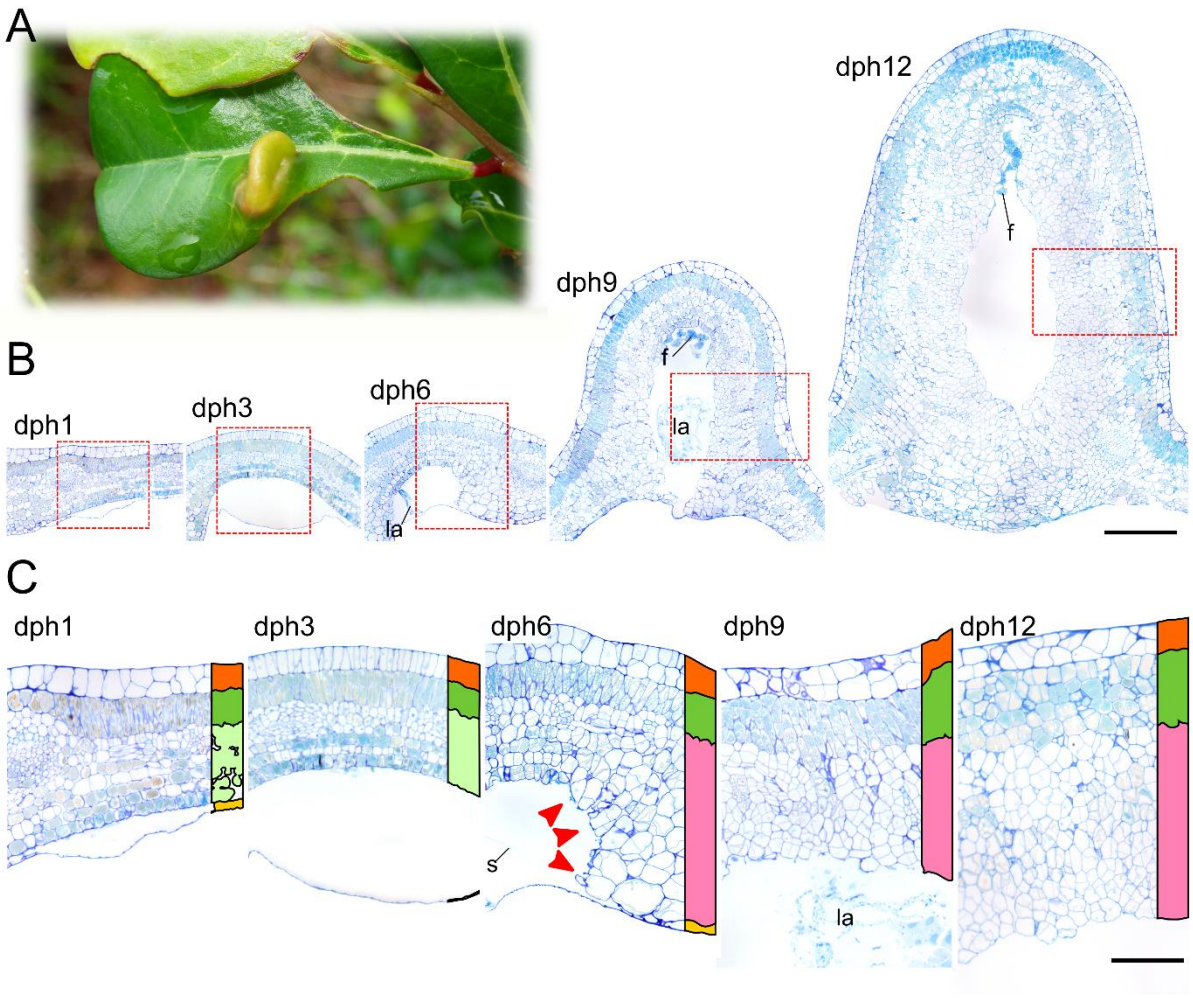



Figure 3.1. Gall initiated on *Glochidion obovatum* leaves attacked by *Caloptilia cecidophora* (A). Time series of histological sections of *C. cecidophora* gall initiation. (B) Cross sections of *G. obovatum* leaves attacked by *C. cecidophora* were made at 1 day post-hatching (dph1), at dph3, at dph6, at dph9, and at dph12. Red rectangles correspond to the zone detailed in (C). Legend, orange: upper epidermis, green: palisade parenchyma, light green: spongy parenchyma, yellow: lower epidermis, pink: altered parenchyma showing hypertrophied cells and an activation of cell division, s: silk, la: larvae, f: frass. Red arrows point cell division and hypertrophia orientated in direction to insect chamber. Scale: 500µm for (B), 100µm for (C). Staining: Toluidine Blue O.

LR White resin (London Resin Company Ltd, UK). Semi-thin sections (1 µm thick) were cut with a diamond knife (Diatome, Biel, Switzerland), or a triangular glass knife for the experiment in which the larva has been removed, installed in an ultracut R microtome (Leica, Rueil-Malmaison, France), placed on slides, fixed by heating at 120°C for 2 min and stained with Toluidine Blue O 0,1% (w/v in water) (Sigma-Aldrich, T0394). Images were assembled using MosaicJ plugin of ImageJ (Thévenaz and Unser, 2007). Photoshop (Adobe) was used to adjust contrast and white balance, remove background, and for cropping.

### *Assessment of insect development*

We compared the immature growth patterns of *C. cecidophora* with its closely related non-gall-inducing species, *C. ryukyuensis*, by measuring eggs ($N_{C.cecidophora}$=20 $N_{C.ryukyuensis}$=11), larvae (N comprised between 6 and 22, for details see Table 3.S1) and pupae ($N_{C.cecidophora}$=10

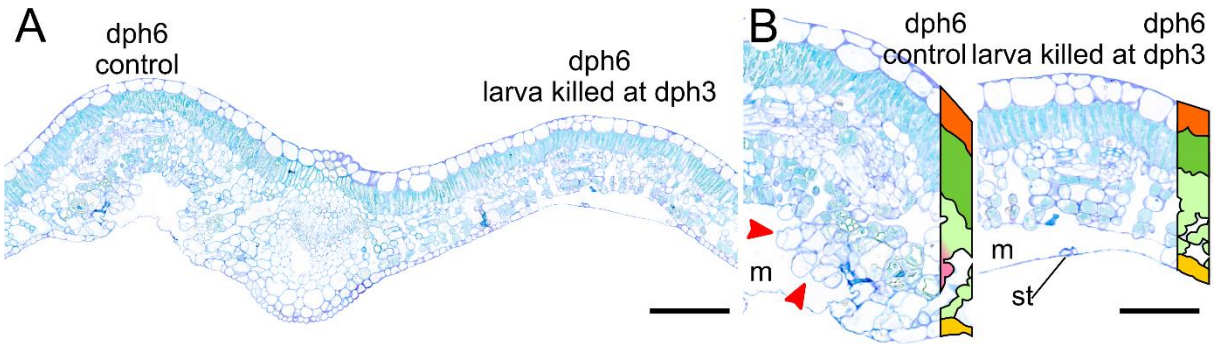



Figure 3.2. Histological sections of *Caloptilia cecidophora* gall after larva abortion. Cross sections of *Glochidion obovatum* leaves attacked by *C. cecidophora* were made at 6 day post-hatching (dph6) after killing the larva at dph3. Legend, m: mine, st: stomata, for remaining see figure 1. Scale: 500µm for (A), 100µm for (B). Staining: Toluidine Blue O.

$N_{C.ryukyuensis}$=7). After inducing oviposition of field-collected pairs of both species, we measured egg width and length. After hatching, we measured head-capsule width of first and second instars. Later larval instars and pupae of *C. cecidophora* and *C. ryukyuensis* were directly collected from the field (Tomogashima and Ishigaki Islands, respectively). We measured head-capsule with the micrometer of a light microscope (Leica M205C). In addition, we measured the duration of both first and second instars and filmed each stage of larval development for both species (Pentax K3, with binocular microscope Leica S6E).

The shape of the egg area was approximated being an ellipse. Area was calculated using length and width measurements. Egg areas, head-capsule widths and pupa lengths were compared using Welch's *t*-tests. For the comparisons of larval head capsules among instars, we applied the sequential Bonferroni correction (Rice, 1989) to *p*-values to keep the significance level at 0.05 throughout the tests. The corrected *p*-values are indicated as *p(adjusted)*. All statistical analyses were conducted using RStudio version 1.1.453 (RStudio, Boston, USA) with R 3.3.1 (R Developmental Core Team 2016).

Larval head morphology before and after gall induction began, in particular mouthparts of the second and third instars of *C. cecidophora*, was analyzed using scanning electron microscopy. Larvae were dehydrated in ethanol overnight, mounted on scanning electron microscopy stubs, coated with gold, and observed with a scanning electron microscope (JEOL DATUM, JSM-5800LV) at an accelerating voltage of 20 kV. Images were assembled using MosaicJ plugin of ImageJ. Colors were added using Inkscape (v0.92, www.inkscape.org).

# Results

## *Gall ontology*

First and second instars of *C. cecidophora* mine along the lower side of the leaf (Figure 3.S1, Movie 3.S2). This is also the case for *C. ryukyuensis* and other *Caloptilia* (Figure 3.S1, Movie 3.S3). Analysis of the time series of cross-sections of attacked leaves (Figure 3.1) revealed that the first instar makes a gallery inside the lower epidermis at one day-post hatching (dph1). This

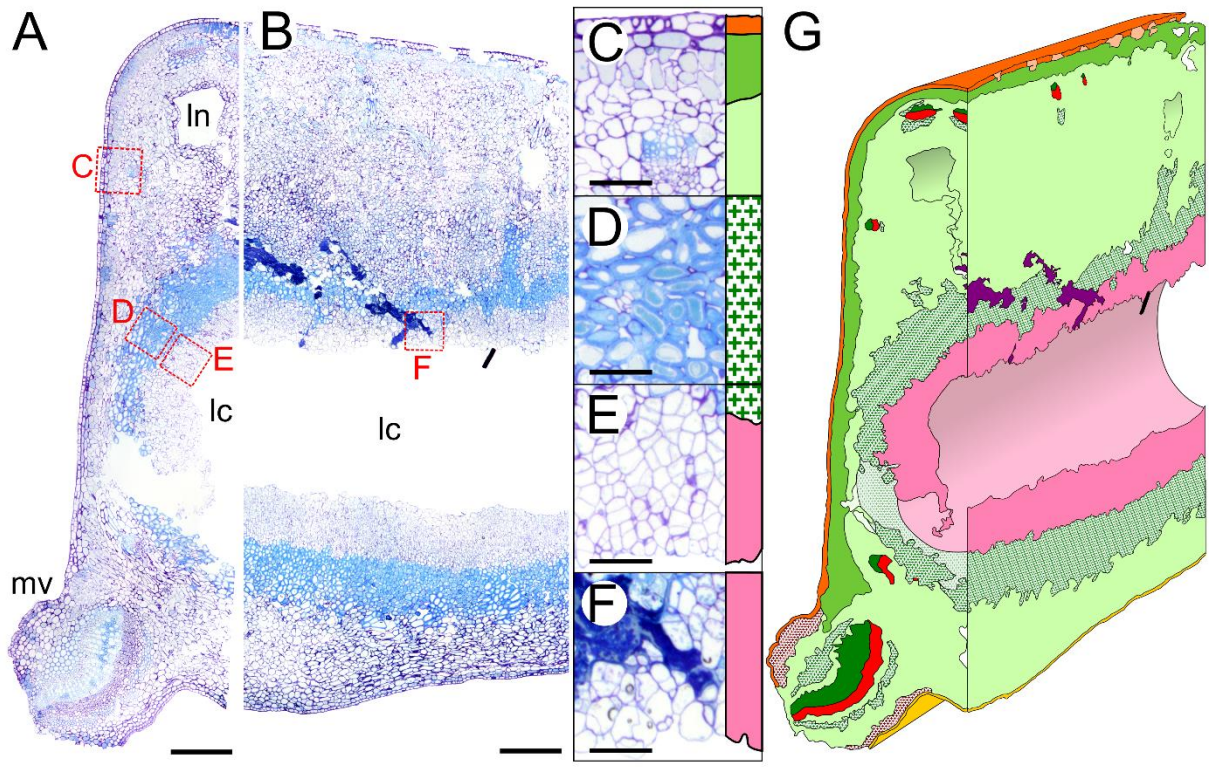



Figure 3.3. Histological sections and 3D modeling of *Caloptilia cecidophora* gall at late larval instar. (A) Transversal section. (B) Sagittal section. (C) Detail of gall epidermis. (D) Detail of the sclerenchyma. (E) Detail of nutritive tissue. (F) Detail of insect frass trapped in nutritive tissue. (G) 3D modeling of a mature gall based on histology sections. Legend, dark green: xylem, red: phloem, pink: nutritive tissue, green cross: scerenchyma, red cross: colenchyma, purple: frass, lc: larva chamber, ln: lacuna, mv: midvein, for remaining see figure 3.1. Scale: 500µm for (A) and (B), 100µm from (C) to (F). Staining: Toluidine Blue O.

mine is enlarged into a blotch mine by the second instar, which begins dph3. The absence of any tissue alteration shows that those two instars are true leaf-miners.

The third instar begins at dph4. Two days later, at dph6, the mine induced by *C. cecidophora* differs from the types of mines induced by other *Caloptilia* species by the formation of hypertrophied cells showing periclinal divisions (i.e. cell divisions whose division plan is parallel to the leaf surface) in the spongy parenchyma located at the edges of the mine (Figure 3.1). Cell hypertrophy and hyperplasia - activation of cell divisions- occurred after the insect reaches the third instar. This evidence suggests that gall induction is initiated by the third instar. Such tissue alterations are not observed in third instar mines of *C. ryukyuensis*. (Figure 3.S5).

To test whether gall induction coincides with the beginning of the third instar, we killed the late second instar larva and observed the effect on the development of the leaf region attacked by the insect. Three days after killing the late second instar larva, no alteration was observed in the spongy parenchyma cells adjacent to the larva, contrary to the control leaf-mine in which the same tissue exhibits cell hyperplasia and hypertrophy (Figure 3.2), demonstrating that gall induction requires the third instar larva.

The closure of the gall at the lower side starts with third instar using silk to fold up the leaf cuticle of the lower epidermis (Movie 3.S2). Gall closure is completed at dph12 by the coming together of the newly formed tissues (Figure 3.1). Frass accumulates in the upper region of the

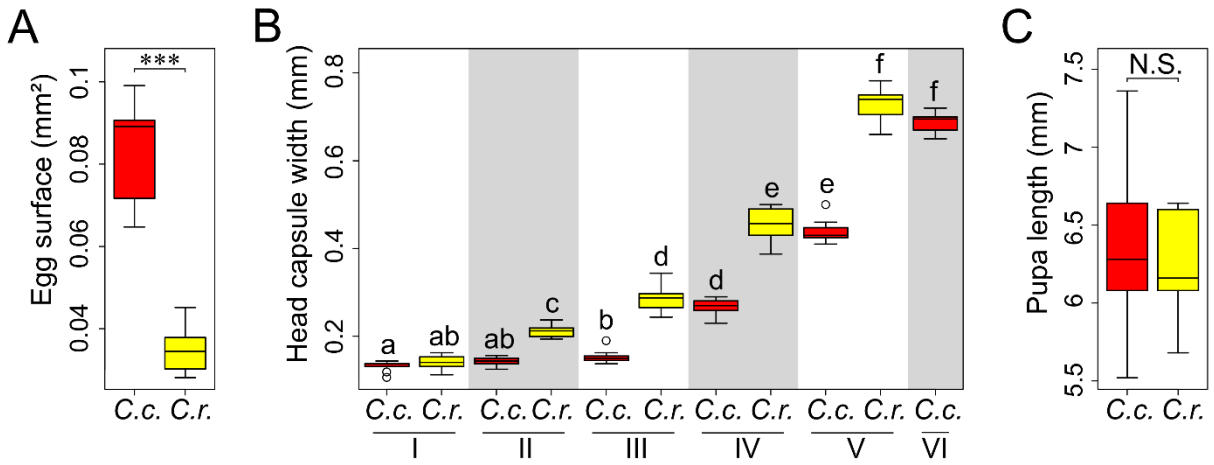



Figure 3.4. Boxplots showing insect size along development of *Caloptilia cecidophora* (*C.c.*, in red) and its closely related non-gall-inducing species *C. ryukyuensis*. (*C.r.*, in yellow) (A) Egg area. (B) Head capsule width of each larva instar, instars followed by a different letter have significantly different size (see Table S8). (C) Pupal length (***: <0.001, N.S.: no significant difference).

gallery from dph9. The presence of frass within the tissue of the same region at latter stage of gall development (Figure 3.3) provides evidence that the dense parenchyma that lines the larva merges also at the upper part to form an insect chamber.

In mature galls (Figure 3.3), the insect chamber is lined with two kinds of tissues that are absent in the normal leaf: a sclerenchyma, and a dense parenchyma that presents traces of feeding. In addition, we found an antero-posterior asymmetry in the sagittal section of mature galls (Figure 3.S6). Sclerenchyma is absent in the anterior part but present in the posterior, where it co-occurs with frass. At the end of larval development and before pupation, the dense parenchyma is entirely eaten in the anterior region of the gall. A "window" of intact upper epidermis cuticle through which the adult moth escapes is appeared at the anterior surface (Figure 3.S7).

### *Larval development*

It took three days for *C. cecidophora* larvae to moult to the third instar (n=8), whereas *C. ryukyuensis* took five days (n=9). Eggs of *C. ryukyuensis* are smaller than those of *C. cecidophora* ($t$ = 16.075, df = 28.542, $p$-value = 7.72e-16) (Figure 3.4A). The first larval instars of both species have similar head capsule widths (Figure 3.4B, Table 3.S8). *C. ryukyuensis* larval body size increases significantly at each instar, whereas the body size of *C. cecidophora* larvae remains the same through the third instar (Figure 3.4B). Larval size starts to increase at the fourth instar. It continues to grow during the fifth instar. *C. cecidophora* possess a sixth instar, an additional developmental stage in comparison with the five larval instars of *C. ryukyuensis* and other *Caloptilia* (Kumata, 1982). This extra sixth instar shows similar head capsule width the fifth instar of *C. ryukyuensis*. Pupae of both species show similar body length ($t$ = 0.37783, df = 14.996, $p$-value = 0.7109).

In both species, the head morphology of the second larval instar is as follows: 1) flat prognathous head, 2) maxillae, labial palpi and 3) spinneret are absent and mandibles are flattened plates (Movies 3.S2 and 3.S3, Figure 3.S4). These features are consistent with fluid-

feeding behaviour in caterpillars (Body et al., 2015). In both species, third instar larvae show mandibles that conform to the normal lepidopteran chewing type (Movies 3.S2 and 3.S3, Figure 3.S4). This indicates that larval hypermetamorphosis in *C. cecidophora* occurs between the second and third instar, as in many other *Caloptilia* species (Kumata, 1978), but in this case, happens concurrently with the switch to feeding on induced gall tissue.

# Discussion

Our results establish the existence of a leaf-mining stage during the first two larval instars of the gall-inducing micromoth *C. cecidophora.* This is the first example of an insect switching from leaf-mining to the induction of a complex gall. Gall-like structures have previously been described in leaf-mines but differ from what are described here because they only involve a limited formation of callus (Guiguet et al., 2018; Hering, 1951). Our results provide evidence for a transitional state between leaf-miners and gall-inducers. This suggests that leaf-miners offers a third evolutionary pathway to the gall-inducing habits of insects. Price previously proposed two evolutionary pathways, one from sedentary herbivory and another from boring herbivory (Kumata, 1966).

We show that the gall stage evolves from a tentiform mine and that the induction of the gall occurs at the third instar (Figure 3.5). Like most other *Caloptilia* species, the third instar larva draws together the spongy parenchyma of the mine edges using silk (Movie 3.S2). However, in the case of *C. cecidophora,* activation of cell division at the mine edges leads to their fusion, which progressively creates a closed cavity inside the altered spongy parenchyma. The gall closure mechanism is a mix of a physical process, the lower epidermis folding involving the use of silk, and an uncharacterized chemical process, activation by the larva of leaf cell hypertrophy and proliferation through hyperplasia (Figure 3.5). To our knowledge, a gall ontology that begins with mechanical folding of the leaf by the insect has never been described before in arthropod-induced galls. Furthermore, this shows that folding of a three-dimensional tentiform mine might constitute a preadaptation to gall induction, thus supporting that induction of leaf depression can precede leaf gall evolution (Kumata, 1966).

The gall-inducting habit is associated with a significant reduction in the duration of the leaf-mining larval stage. In *C. cecidophora* the first two leaf-mining instars last only three days compared to five days for its closest relative *C. ryukyuensis*. The larva of *C. ryukyuensis* builds what appears to be a longer serpentine mine in the first instar. In contrast, the larva of *C. cecidophora* builds a mine whose dimensions are reduced to what appears to be the minimal surface required for the creation of a tentiform mine.

In addition, this shortening of early larval development duration noticed in *C. cecidophora* is associated with a difference of growth pattern in comparison to *C. ryukyuensis* (Figure 3.4). Whereas *C. ryukyuensis* has a continuous growth, *C. cecidophora* larvae keep the same size from the first to the third instar. *C. cecidophora* larval growth begins when the gall starts to develop. A similar pattern is seen in gall-inducing cynipids, for which larvae remain very small until putatively defensive gall morphologies have developed (Stone and Schönrogge, 2003). As

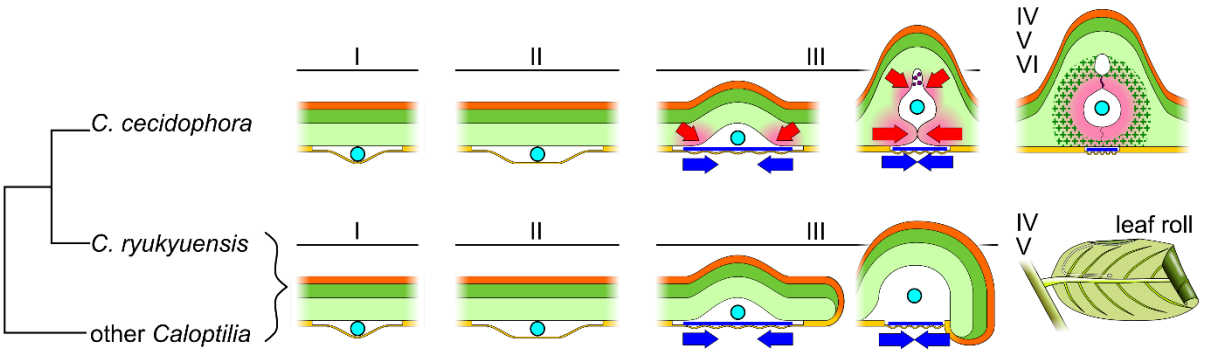



Figure 3.5. *Caloptilia cecidophora* interaction with its host plant compared to *C. ryukyuensis* and other *Caloptilia*. Every first and second instars are epidermis miners in this genus. Like other *Caloptilia*, *C. cecidophora* third instar folds its bloch mine into a tentiform shape by weaving silk threats on lower epidermis; then, the mine folding is the result of mechanical forces (blue arrows). However, contrary to other species of the genus, *C. cecidophora* activates tissue growth at its feeding sites, inducing morphogenetic movements (red arrows) that progressively seal the two joined upper leaf sides. Secondary morphogenesis occurs at upper part of the larval chamber, trapping frass and creating a lacuna. Late stages of this species stay in the gall until pupation, whereas other *Caloptilia* leave the tentiform mine to build a leaf-roll

a result, the larval growth accumulates a one-instar delay compared to *C. ryukyuensis*. *C. cecidophora* makes up for this early absence of growth by adding a sixth instar that does not occur in other *Caloptilia* species. This peculiar growth pattern compared to its non-gall-inducing sister species suggests an intense selective pressure to reduce the duration of the early leaf-mining stages. It supports the hypothesis that early stages of gall formation, when the insect does not benefit yet from gall protection, represent a "window of vulnerability" to enemy attacks (Cornell, 1983, 1990; Craig et al., 1990).

The internal structure of *C. cecidopho*ra galls with a layer of neovascularized nutritive tissue surrounded by sclerenchyma (Figure 3.3), which confers mechanical resistance to the gall shows the ability of *C. cecidophora* to induce tissue differentiation. This internal organization in two layers is similar to some of the most complex galls of cynipids or cecidomyiids (Stone and Schönrogge, 2003). This convergent internal structure suggests that there might be limited ways of inducing galls due to plant physiological constraints.

Gall induction did not proceed after we killed the second instar leaf-mining larva. This suggests that the cecidogenous substance is only secreted from the third larval instar. This differs from other gall-inducers for which gall induction begins at the start of insect's interaction with the host plant either as a feeding larva (e.g. cecidomyiids and aphid) or as an ovipositing adult (e.g. cynipids). This particularity may represent an opportunity to better understand the mechanism of gall induction and to identify chemical effectors involved. Omics and chemical investigations are facilitated by comparative approaches (Oates et al., 2016). The mixed feeding habit of *C. cecidophora* and its phylogenetic proximity with non-gall-inducing relatives allow such approaches. In addition, we developed a rearing protocol to produce large number of adults from eggs in greenhouses. These lab colonies can be used to set up experiments in controlled conditions, making *C. cecidophora* an excellent model to investigate fundamental questions about the physiology and evolution of gall induction.

## Acknowledgments

We thank Akihisa Hamatani and Taisuke Amano for their help for collecting, rearing and measuring insects, as well as Véronique Lainé-Prade, Takehiro Masumura and Tomoka Miyahara for preparing some samples for histology. We thank Marion Harris for insightful comments on a previous version of this manuscript. We thank Anantanarayanan Raman, Rosy dos Santos Isaias, Graham Stone, Odette Rohfritsch and especially Atsushi Kawakita for their comments and suggestions. We thank J-C. Simon, and G. Dubreuil for fruitful discussions.

## Supplementary Figures and Tables

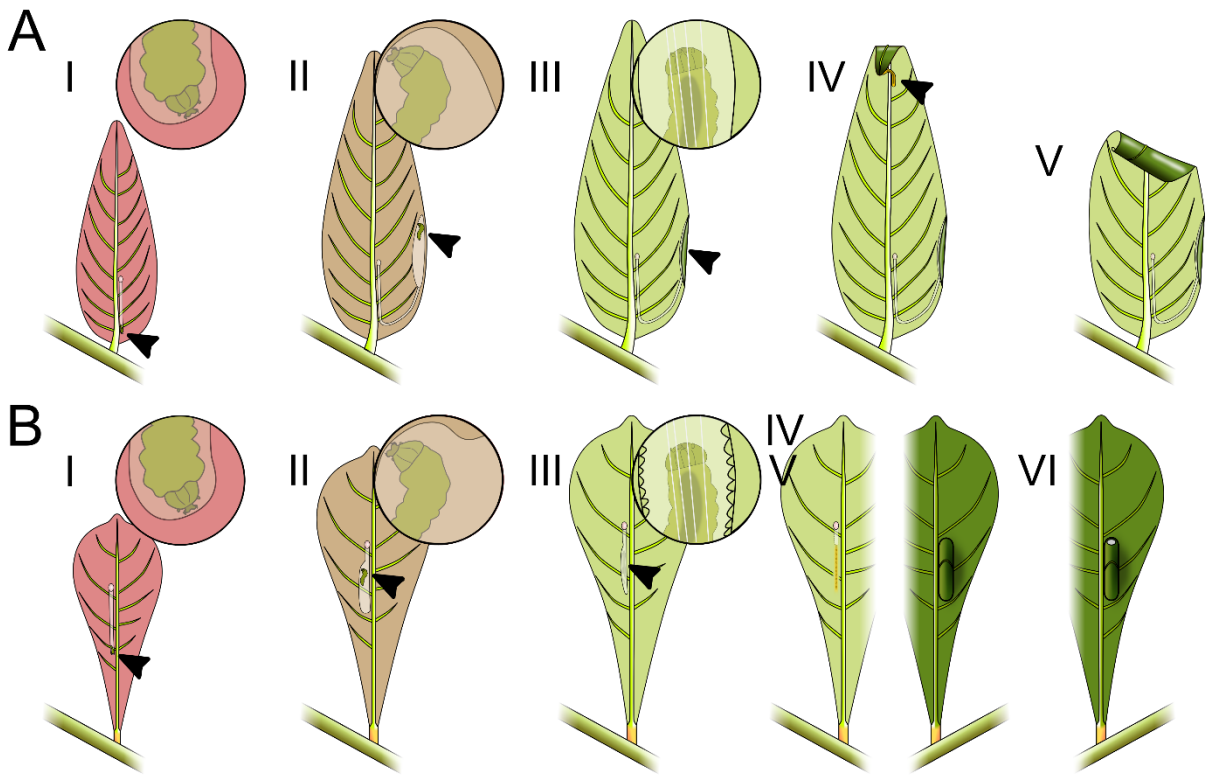



Figure 3.S1. *Caloptilia ryukyuensis* life-style (A) compared to *Caloptilia cecidophora* life-style (B). Both species make a serpentine mine at first instar, a blotch mine at second instar and a tentiform mine at third instar. *C. cecidophora* starts to induce a gall inside this mine and will stay inside it until sixth instar and pupation, whereas *C. ryukyuensis* leaves its mine at fourth instar to build a leaf-roll in which it will complete its larval development.



Movie 3.S2. Movie of *Caloptilia cecidophora* development on *Glochidion obovatum*. (https://www.youtube.com/watch?v=AuBRkgHMGr0)



Figure 3.S3. Movie of *Caloptilia ryukyuensis* development on *Glochidion lanceolatum*. (https://www.youtube.com/watch?v=r4XZMuMOAJM)

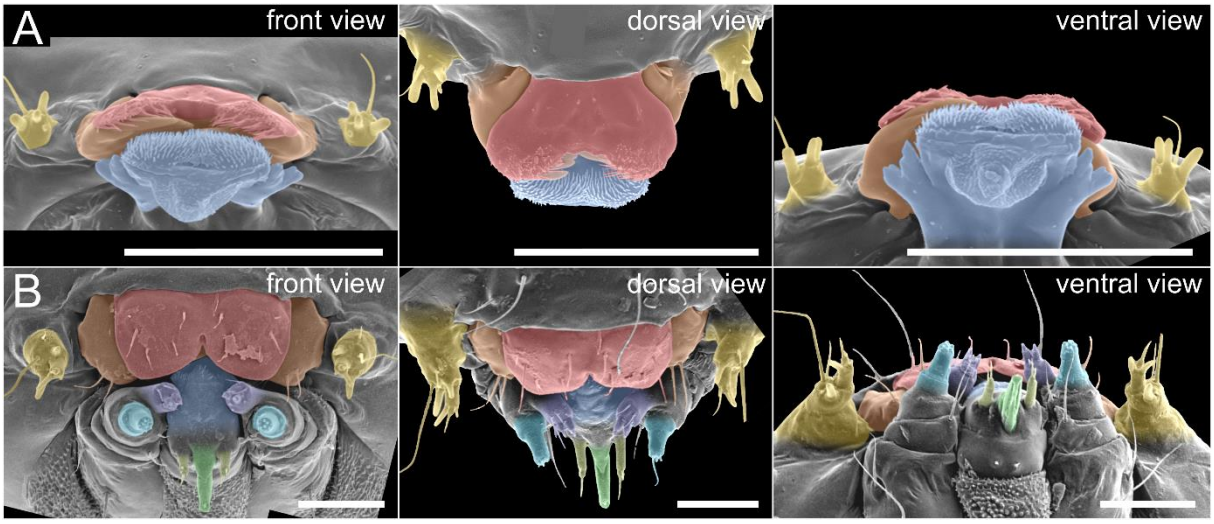



Figure 3.S4. Mouthparts of *Caloptilia cecidophora* second (A) and third instars (B) as seen in scanning electron microscopy. Legend, red: labrum, yellow: antenna, orange: mandibles, purple: galea, dark blue: labium, light blue: maxillar palpi, dark green: labial palpi, light green: spinneret. Scale: 100µm.

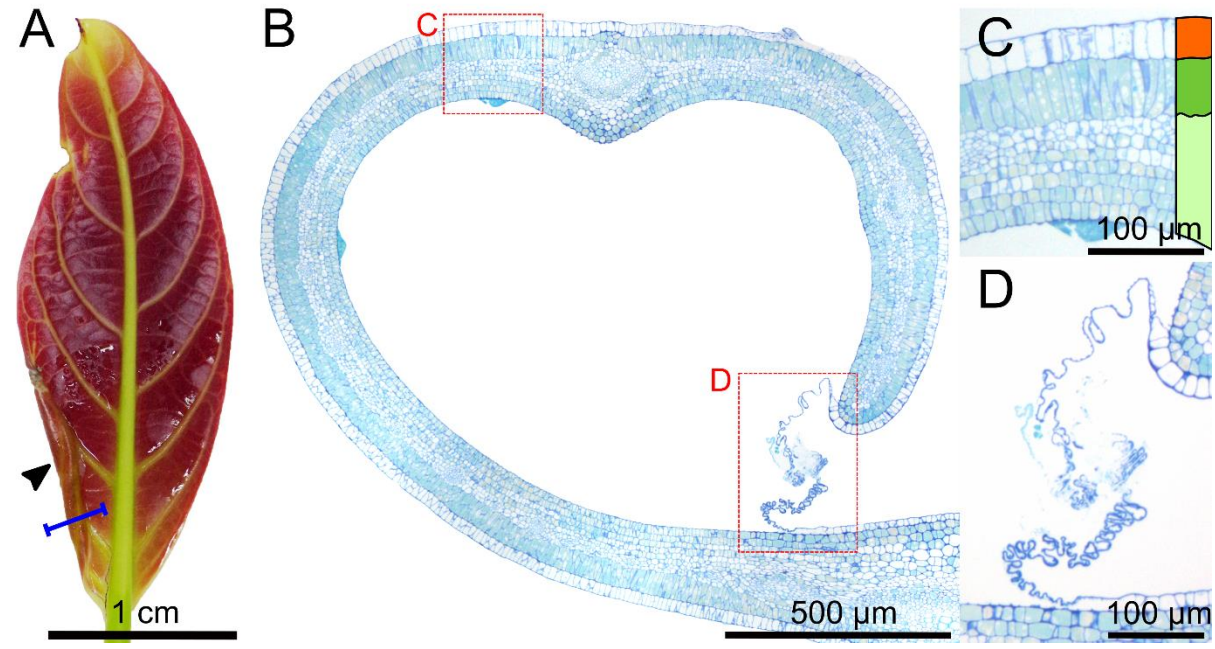



Figure 3.S5. Tentiform mine of *Caloptilia ryukyuensis* third instar, indicated with black arrow on *G. lanceolatum* (A). Blue line indicates the region cross-sectioned. (B) Histology section of the tentiform mine. (C) Detail of mined leaf. (D) Detail of folded upper epidermis cuticle. Legend, see figure 3.1. Staining: Toluidine blue.

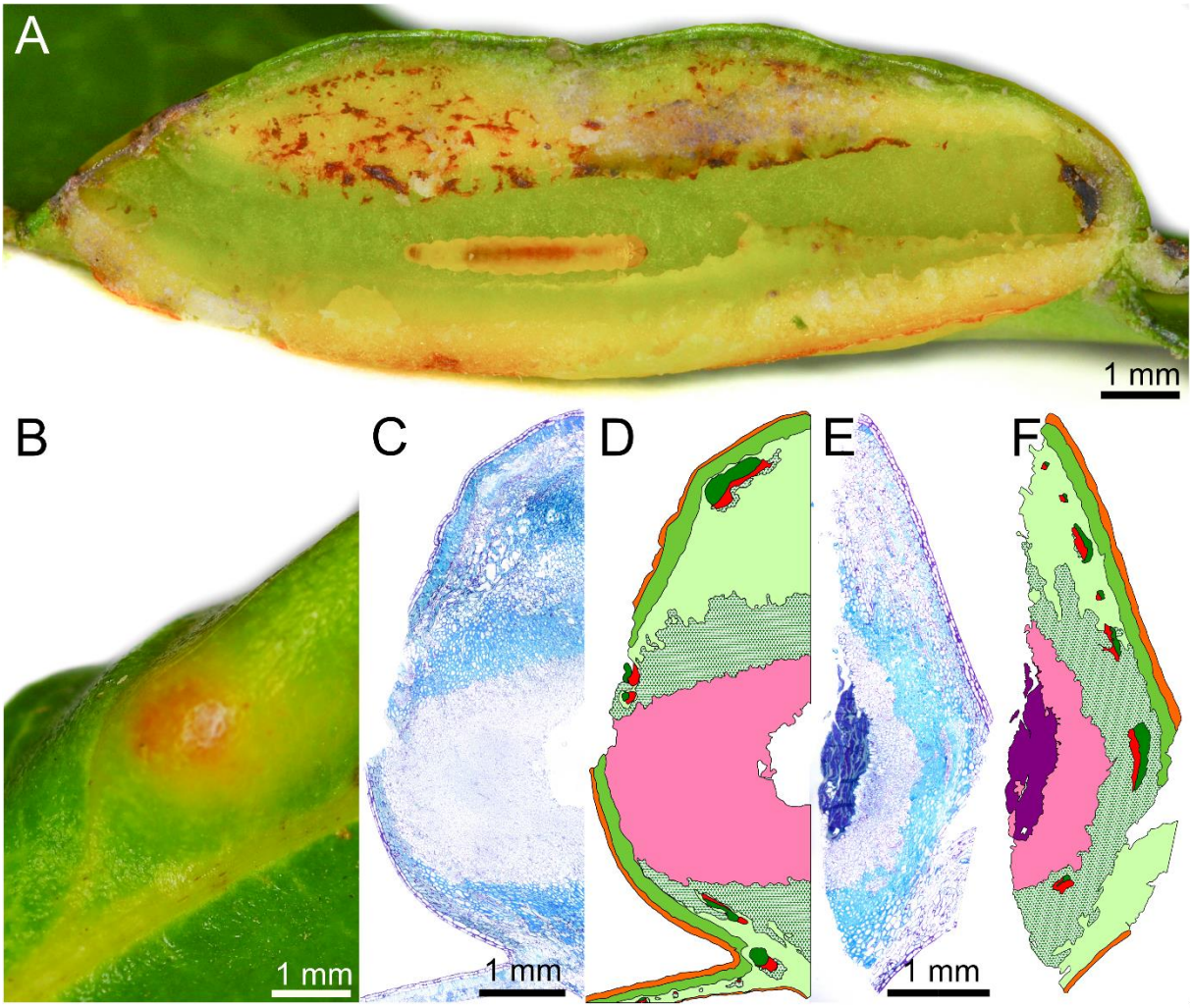



Figure 3.S6. (A) Sagittal section of *Caloptilia cecidophora* late instar gall on *Glochidion obovatum*. (B) Detail of the anterior part of the gall. (C) Histology sagittal section of the anterior part and (D) the interpretation drawing. (E) Histology sagittal section of the posterior part and (F) the interpretation drawing. Legend, see figure 3.1 and 3.3.

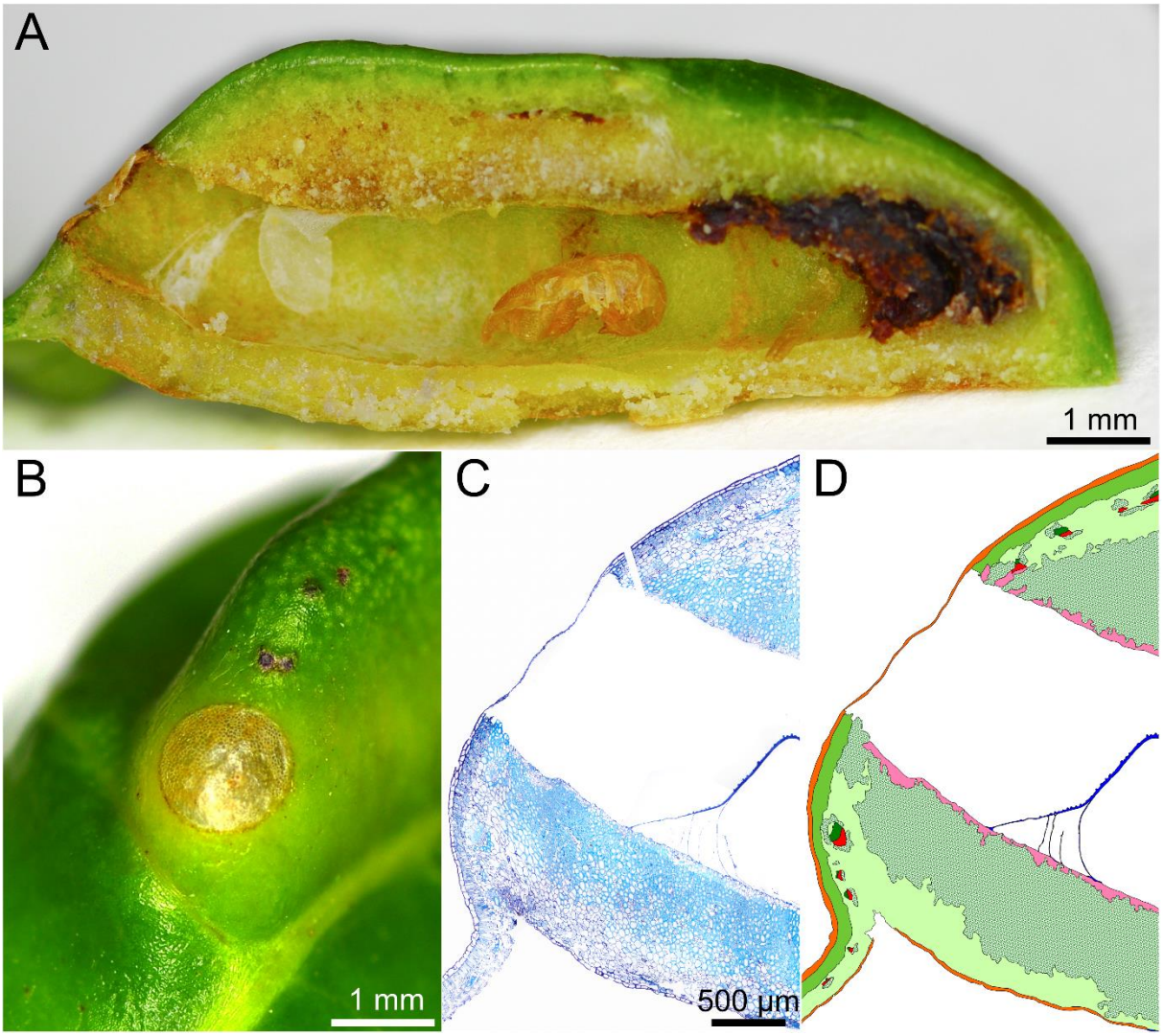



Figure 3.S7. (**A**) Sagittal section of *Caloptilia cecidophora* gall at pupa stage on *Glochidion obovatum*. (**B**) Detail of the anterior part of the gall. (**C**) Histology sagittal section of the anterior part and (**D**) the interpretation drawing. Legend, dark blue: silk, for remaining see figure 3.1 and 3.3.

Table 3.S1. Welch's t-test statistics for the comparison of *Caloptilia cecidophora* (Cc) and *C. ryukyuensis* (Cr) head capsule widths. Numbers indicate the instars. Bold *p*-values are smaller than *p(adjusted)*)

| N | *p* \ *t* | Cc1 | Cr1 | Cc2 | Cr2 | Cc3 | Cr3 | Cc4 | Cr4 | Cc5 | Cr5 | Cc6 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | Cc1 | | -1.1385 | -2.1398 | -15.217 | -4.2468 | -19.795 | -23.81 | -34.065 | -35.752 | -54.05 | -50.749 |
| 8 | Cr1 | 0.2752 | | -0.41456 | -10.966 | -1.9745 | -16.775 | -18.445 | -30.768 | -31.747 | -50.249 | -47.163 |
| 12 | Cc2 | 0.0499 | 0.687 | | -16.495 | -2.6701 | -20.142 | -26.365 | -34.889 | -37.056 | -55.398 | -51.938 |
| 14 | Cr2 | **4.00E-11** | **2.17E-07** | **1.44E-14** | | 15.248 | -10.218 | -11.136 | -26.86 | -27.976 | -48.316 | -44.928 |
| 22 | Cc3 | **0.000943** | 0.07861 | 0.01308 | **2.05E-14** | | -19.243 | -25.701 | -34.319 | -36.522 | -55.063 | -51.566 |
| 16 | Cr3 | **8.76E-16** | **1.46E-13** | **1.13E-14** | **1.09E-09** | **9.40E-14** | | 2.522 | -15.864 | -15.468 | -36.33 | -33.412 |
| 20 | Cc4 | **2.20E-16** | **4.48E-11** | **2.20E-16** | **1.53E-12** | **2.20E-16** | 0.0185 | | -20.266 | -20.528 | -42.244 | -38.954 |
| 18 | Cr4 | **2.20E-16** | **2.20E-16** | **2.20E-16** | **2.20E-16** | **2.20E-16** | **2.35E-16** | **2.20E-16** | | 1.455 | -20.109 | -17.513 |
| 11 | Cc5 | **4.16E-16** | **2.20E-16** | **3.16E-14** | **2.56E-13** | **1.52E-13** | **2.09E-13** | **1.59E-12** | 0.1574 | | -22.446 | -19.709 |
| 10 | Cr5 | **1.32E-15** | **2.20E-16** | **4.26E-14** | **6.40E-14** | **1.15E-13** | **2.20E-16** | **4.27E-14** | **3.88E-15** | **4.25E-14** | | 2.3539 |
| 6 | Cc6 | **6.66E-10** | **4.02E-11** | **6.99E-09** | **8.58E-09** | **1.31E-08** | **3.88E-11** | **6.34E-09** | **2.72E-10** | **1.35E-09** | 0.03522 | |

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# Chapter 4.

# Evolution of gall-induction and host-plant relationships in Phyllanthaceae-feeding *Caloptilia* (Lepidoptera: Gracillariidae)

# Résumé du Chapitre 4

Le mode de vie galligène est apparu de nombreuses fois de façon indépendante parmi les insectes, conduisant parfois à des radiations adaptatives (Dreger-Jauffret and Shorthouse, 1992). A titre d'exemple, les Cynipides (Hymenoptera) et les Cécidomyiides (Diptera) regroupent chacun plusieurs milliers d'espèces (Gagné, 1989; Ronquist et al., 2015). A l'inverse, les insectes galligènes montrent souvent l'exemple contraire car la plupart sont regroupés dans des lignées isolées contenant un faible nombre d'espèces, ce qui suggère au contraire que ce mode de vie constituerait une impasse évolutive (Day et al., 2016). Par exemple, parmi les 24 familles de lépidoptères comprenant des espèces galligènes, seulement quatre contiennent plus de dix espèces galligènes (Miller, 2005). Le mode de vie galligène pourrait avoir des effets négatifs à long terme sur les performances des insectes. En effet, certaines caractéristiques favorisant la radiation adaptative des insectes galligènes, comme une spécialisation élevée de l'hôte (Price, 2005), pourraient également représenter des contraintes évolutives menant à une impasse évolutive (Day et al., 2016).

Les lignées contenant un faible nombre d'espèces induisant des galles constituent de bons modèles pour étudier les facteurs conduisant à une radiation adaptative plutôt qu'à une impasse évolutive. Le but de cette étude était d'établir une phylogénie des microlépidoptères du genre *Caloptilia* (Gracillariidae) associés aux plantes de la famille des Phyllanthaceae. A ce jour, sept espèces ont été décrites comme étant associées avec des Phyllanthaceae des genres *Bridelia*, *Flueggea* et *Glochidion* (De Prins and De Prins, 2018). Parmi ces espèces, seul *C. cecidophora* est galligène, les autres sont rouleuses de feuilles. Cette position phylogénétique isolée serait en faveur d'une impasse évolutive. Cependant, d'anciennes descriptions suggèrent au contraire l'existence de nombreuses autres espèces galligènes (Docters van Leeuwen, 1922; Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926) (Figure 4.1). La confirmation de cette diversité d'espèces galligènes dans le genre *Caloptilia* et preuve qu'elles forment un groupe monophylétique constituerait un signe de radiation adaptative.

Une succession de campagnes de terrain de 2007 à 2017, sur une aire géographique allant de l'Asie de l'Est à la Polynésie française (Figure 4.2), nous a permis de collecter 46 spécimens de *Caloptilia* associés à des Phyllanthaceae et regroupées dans 16 espèces dont quatre sont galligènes (Figure 4.3, 4.4 et 4.5, Table 4.S1). Nous avons réalisé deux phylogénies moléculaires, une première à partir de cinq gènes, comprenant un gène mitochondrial et quatre gènes nucléaires, et une seconde utilisant le séquençage d'éléments ultra-conservés (Ultra-conserved elements). Les deux méthodes établissent de façon concordante la monophylie des espèces galligènes (Figure 4.6 et 4.7), suggérant une origine évolutive unique de ce mode de vie au sein du genre *Caloptilia*. Cependant, à ce stade, les résultats ne permettent pas de conclure à une radiation adaptative, faute d'estimation de la vitesse de spéciation.

L'observation comparée de la morphologie des galles des quatre espèces galligènes a montré que toutes sont fermées à l'exception de l'espèce la plus basale, dont la galle présente un canal

reliant la chambre larvaire à l'extérieur permettant à la larve d'exporter ses excréments hors de la galle (Figure 4.8).

Enfin, l'étude des associations à leur plant-hôte montre qu'aucune des espèces rouleuses de feuille ne partage de plante-hôte avec une espèce au même mode de vie tandis que les quatre espèces galligènes partagent leur plante hôte avec des *Caloptilia* rouleurs de feuille (Figure 4.9). Cela suggère que le mode de vie galligène pourrait permettre une partition de niches alimentaires et d'échapper ainsi à l'exclusion compétitive entre espèces exploitant la même plante hôte. La compétition pourrait être ainsi considérée comme une force évolutive ayant favorisée l'émergence du mode de vie galligène.

## Article N°3

# Evolution of gall-induction and host-plant relationships in Phyllanthaceae-feeding *Caloptilia* (Lepidoptera: Gracillariidae)

Antoine Guiguet[1, 2], Issei Ohshima[2], Atsushi Kawakita[3], Astrid Cruaud[4], Jean-Yves Rasplus[4], David Hembry[5], Min Wang[6], David Giron[1*], Carlos Lopez Vaamonde[1, 7*]

[1]Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS/Université de Tours, UFR Sciences et Techniques, Tours, France

[2]Department of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

[3]The Botanical Gardens, Graduate School of Science, The University of Tokyo, Tokyo, Japan

[4]CBGP, INRA, CIRAD, IRD, Montpellier SupAgro, Université de Montpellier, Montpellier, France

[5]Department of Entomology, Cornell University, 2130 Comstock Hall, Ithaca, NY 14853, USA

[6]Department of Entomology, South China Agricultural University, Guangzhou 510642, Guangdong, China

[7]Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS/Université de Tours, UFR Sciences et Techniques, Tours, France, INRA, UR0633 Zoologie Forestière, Orléans, France

*equal contribution

# Abstract

Gall-inducting has evolved repeatedly and independently in different groups of insects, sometimes leading to large adaptive radiations (Price, 2005). However, the majority of gall-inducing lineages contain a reduced number of species, which could be due to an evolutionary dead-end rather than to a starting radiation. Taxa including both gall-inducing and non-galler species could be good models to study the first stages of gall evolution. They can also be used to study whether the evolution of gall-induction leads to adaptive radiation. In this study, we focus on micromoths of the *Caloptilia* genus (Lepidoptera, Gracillariidae) associated with Phyllanthaceae plants, among which one species is known as a gall-inducer. Old literature suggested the existence of a large number of other gall-inducing undescribed species in this genus in south east Asia. If the monophyly of gall-inducers was were supported, it would suggest the possibility of an overlooked adaptive radiation. We collected three gall-inducing and six non-gall-inducing undescribed morpho-species associated with host-plants of the *Glochidion* genus (Phyllanthaceae). We used those samples to infer two molecular phylogenies of *Caloptilia* associated with Phyllanthaceae: 1) a phylogeny based on five genes which are commonly used in insect phylogenetics and 2) a phylogeny based on ultraconserved elements (UCEs). Both phylogenetic reconstructions show that gall-inducers form a well-supported monophyletic group, but deeper nodes were resolved only by the UCEs. The UCEs phylogeny strongly supported the monophyly of Phyllanthaceae- and *Glochidion*-associated taxa. The gall-inducer clade is placed at the most derived position of the *Glochidion*-associated clade, demonstrating that gall-induction had a single origin and most likely evolved from non-galling species on *Glochidion*. This would suggest that gall induction could be a key innovation which would have opened up a new ecological niche for *Glochidion*-associated *Caloptilia*. The inferred phylogenetic trees also uncover the series of gall structure evolution. The most basal gall-inducing species makes a gall with a small hole that allows the larva inside the gall to dispose its feces, contrary to other completely closed galls that are induced by more derived species. This would suggest that opened galls might be ancestral in gall-inducing *Caloptilia* due to constraints on waste disposal, which could be a common trait in the evolution of galls in other insect groups.

# Introduction

Gall-inducing has evolved many times independently through evolutionary time (Meyer and Maresquelle, 1983). In insects, gall induction has evolved in at least six different orders, leading to several radiations (Dreger-Jauffret and Shorthouse, 1992; Price, 2005). Indeed, several groups of gall-inducing insects include genera with many species, suggesting that speciation has been rapid, without morphological changes extensive enough to justify placement in different genera: e.g. sawflies (Hymenoptera: Tenthredinidae) (Nyman et al., 2000, 2006), oak gall wasps (Hymenoptera: Cynipidae) (Ronquist and Liljeblad, 2001; Stone et al., 2009), gall-inducing aphids (Hemiptera: Aphididae) (Abbot and Withgott, 2004; Inbar et al., 2004), and gall midges (Diptera: Cecidomyiidae) (Gagné, 1989). Within these examples gall midges are considered as one of the largest radiations among insects with thousands of undescribed species (Hebert et al., 2016). Conversely, *gall*-inducing insects often provide the opposite example, and most lineages of gall-inducting insects contain a reduced number of species, suggesting a concept of evolutionary dead-end that limits the capacity for future change or diversification (Day et al., 2016). For instance, over the 24 Lepidopteran families containing gall-inducing species, only four of them contain more than 10 species having this life-habit (Miller, 2005). The gall-inducing life-style may have long term negative effects on insect performance, and some characteristics favoring adaptive radiation of gall-inducing insects, like a high host specialization, (Price, 2005), may also represent evolutionary constraints leading to an evolutionary dead-end (Day et al., 2016).

Lineages containing a low number of gall-inducing species represent good models to study the factors leading to an adaptive radiation instead of an evolutionary dead-end. Here, we focused on species of gall-inducing microlepidoptera in the genus *Caloptilia* (Lepidoptera, Gracillariidae), which are associated with Phyllantaceae plants. There are only 7 species of *Caloptilia* known to feed on plants of Phyllanthaceae grouped in the genus *Bridelia*, *Flueggea* and *Glochidion* (De Prins and De Prins, 2018). *Caloptilia cecidophora* is the only described gall-inducing species within the Phyllantaceae-associated *Caloptilia* (Chapter 3). This species is distributed in south Japan and Taiwan and induces galls on the leaves of *Glochidion obovatum*, *G. rubrum* of *G. acuminatum*. Seven other *Caloptilia* species, which are not gall-inducers but leaf-miners like most species in this genus, are also associated with Phyllanthaceae, including four feeding on *Glochidion* (De Prins and De Prins, 2018). Kawakita et al., (2010) and Nakadai and Kawakita (2016) suggested that Phyllanthaceae-feeding *Caloptilia* form a monophylletic group. Interestingly, contrary to most gall-inducing lepidopteran whose gall is generally limited to the swelling of a bored-stem, *C. cecidophora* induces a leaf-gall with a complex histological structure that is comparable to Cynipid or Cecidomyid galls (Chapter 3). Thus, this isolated phylogenetic position would be in favor of an evolutionary dead-end scenario.

However, a close study of the old literature suggests hidden diversity among gall-inducing Phyllantaceae-associated *Caloptilia*. Descriptions of plant galls in south-east Asia and in current Indonesia show that at least ten *Glochidion* species that are not known as being *C. cecidophora* host-plants bear lepidopteran-induced leaf-galls that possess a similar morphology

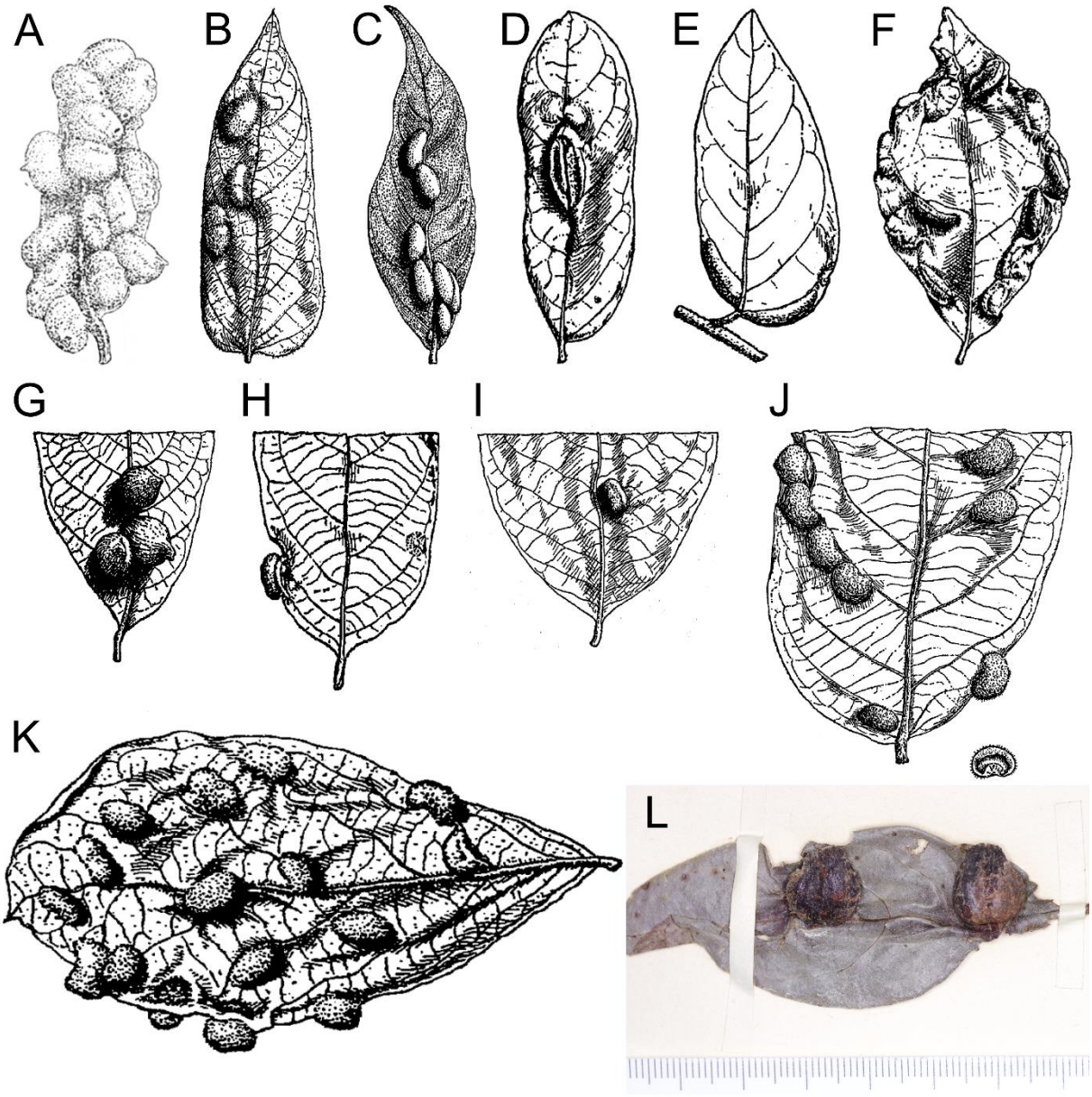



Figure 4.1. Lepidopteran leaf-galls on *Glochidion* leaves described by Docters Van Leeuwen. The plant species are *G. hongkongensis* (A), *G. molle* (B), *G. cyrtostylum* (C), *G. obscurum* (D, E), *G.rubrum* (F), *G. zeylanicum* (G, H), *G. novoguiense* (I), *G. insigne* (J), and *G. zeylanicum* (L). All drawings are reproduced from Docters van Leeuwen-Reijnvaan and Docters van Leeuwen (1926), except the drawing A (Docters van Leeuwen, 1922). Photo L: MNHN

(Docters van Leeuwen, 1922; Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926) (Figure 4.1). If this overlooked diversity is confirmed and if those species form a monophyletic group, it could be considered as a sign of adaptive radiation in this clade.

The main aim of the present study is to elucidate the evolution of the gall-induction habit and host plant use in the *Caloptilia* associated with Phyllanthaceae. Kawakita et al., (2010) and Nakadai and Kawakita (2016) inferred the phylogeny of some *Caloptilia* species associating with *Glochidion*, but very few species were included in their analyses. Here, we study 16 species of *Caloptilia* feeding on Phyllanthaceae, including four gall-inducing species. This intensive taxon sampling allows us to test (1) whether the gall-induction habit evolved multiple

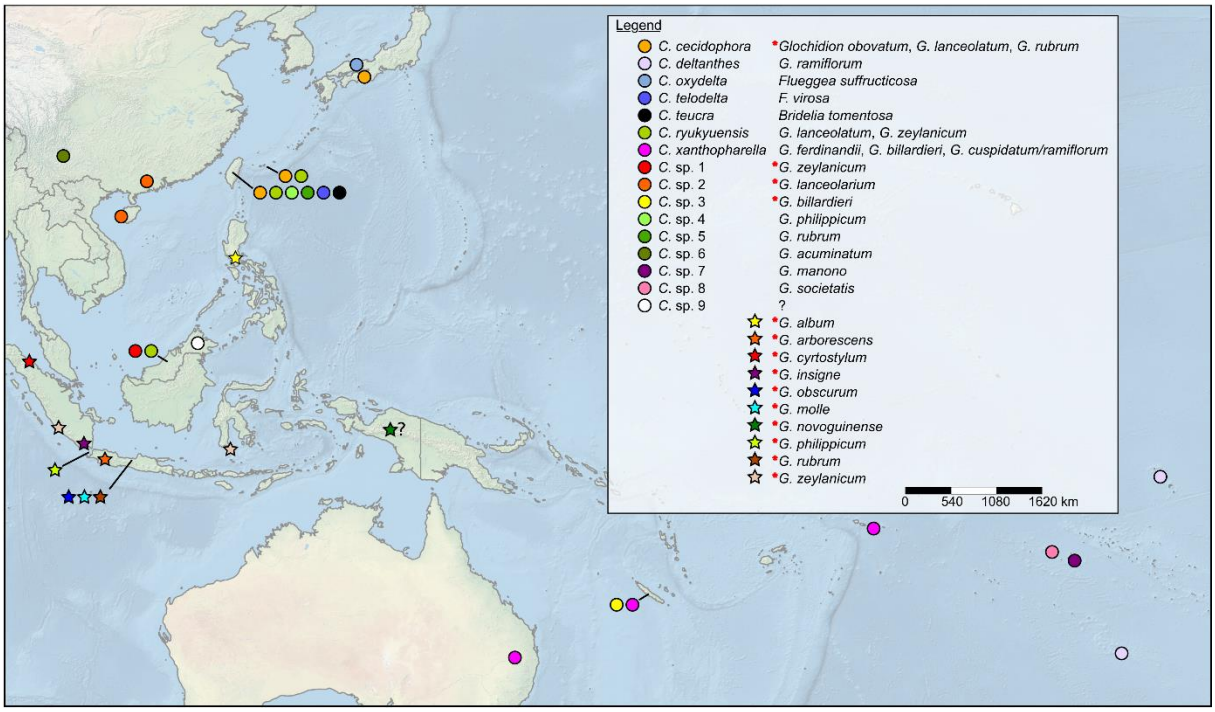



Figure 4.2. Geographical distribution of *Caloptilia* associated with Phyllanthaceae. Circles represent location of samples used in the phylogeny. Stars correspond to the lepidopteran galls on *Glochidion* that are putatively caused by *Caloptilia* species by (Docters van Leeuwen, 1922; Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926; Uichanco, 1919). Red asterisk mark gall-inducing species.

times or have a single origin, (2) whether Phyllanthaceae-feeding *Caloptilia* form a monophyletic group and (3) whether there are cospeciation signals between *Caloptilia* moths and Phyllanthaceae trees. First, we used DNA sequence data from four nuclear genes and one mitochondrial gene, which are often used in insect phylogenetics, with a broader taxonomic sample of 16 species. Secondly, we used a phylogenomic approach using ultraconserved elements (UCEs) (Cruaud et al., 2018; Faircloth et al., 2012).

# Material and Methods

## *Sampling*

Insects were obtained by rearing moths from extensive field collections. Leaves with fully developed mines and galls were collected from trees, across south East Asia from 2007 to 2017 (Figure 4.2). In total, we used 33 adult moths that were for most of them reared from mines or galls, or collected by light-trapping for some specimens, and 13 larvae directly collected within a leaf-mine, a leaf-roll or a gall (Figure 4.3, 4.4 and 4.5, Table 4.S1). To obtain adults, leaves with galls/mines containing mature larvae were kept in plastic boxes (200ml) at constant conditions (22 °C, 55% RH, L:D 18:6 h photoperiod) following Ohshima's (2005) protocol. Voucher specimens have been deposited at the Kyoto Prefectural University. Sixteen ingroup species, and one and five outgroup species were selected for molecular phylogenetics based on five genes and the phylogenomics based on UCEs, respectively Tables 4.S2 and 4.S3. Number of analyzed samples per morpho-species were ranging from one to five. The present ingroup

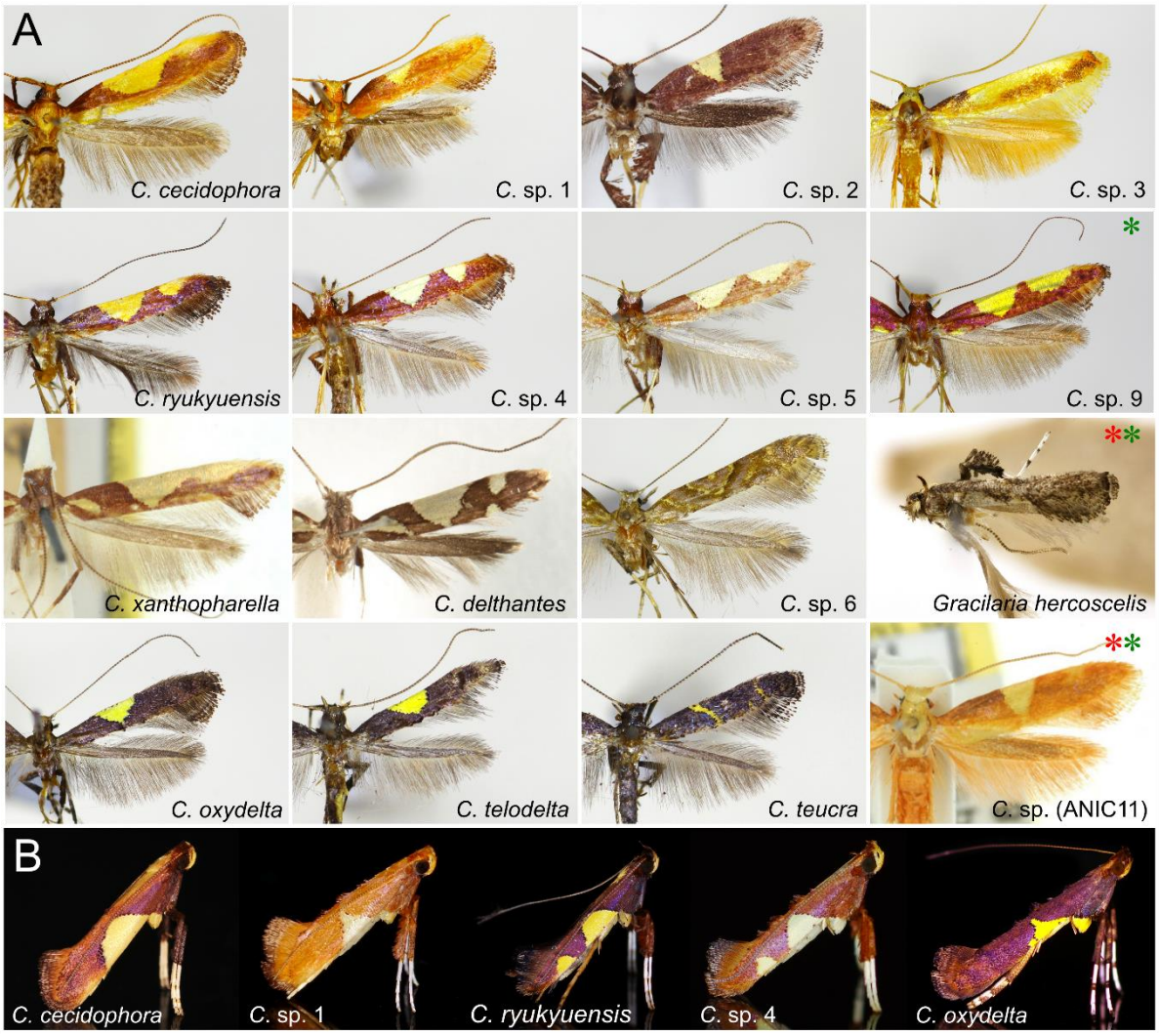



Figure 4.3. Adult wing pattern (A) and resting posture (B) of *Caloptilia* associated with Phyllanthaceae. Red asterisks mark species not included in the phylogeny. Green asterisks mark species with unknown host-plant association.

includes the seven species described as having Phyllanthaceae-association, two undescribed species mentioned in (Kawakita et al., 2010), and putative *Caloptilia* leaf-gallers on *Glochidion* reported by Docters van Leeuwen, (1922), Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, (1926) (Figure 4.1). Sampling localities and years are summarized in Table 4.S1. We used both dried and ethanol preserved samples for the present analyses (Table 4.S2 and 4.S3).

### *DNA extraction, Polymerase Chain Reaction (PCR) amplifications and sequencing of common five genes*

DNA extractions were performed with the Qiagen DNeasy plant mini kit (Qiagen, Valencia, CA, and U.S.A.) following the manufacturer's protocols. Different types of insect parts were used, depending on the abundance, the significance and the state of conservation of the specimens available. According to samples we used, Total DNA was extracted from larval bodies or adult abdomens (see Table 4.S1). For specimens corresponding to undescribed

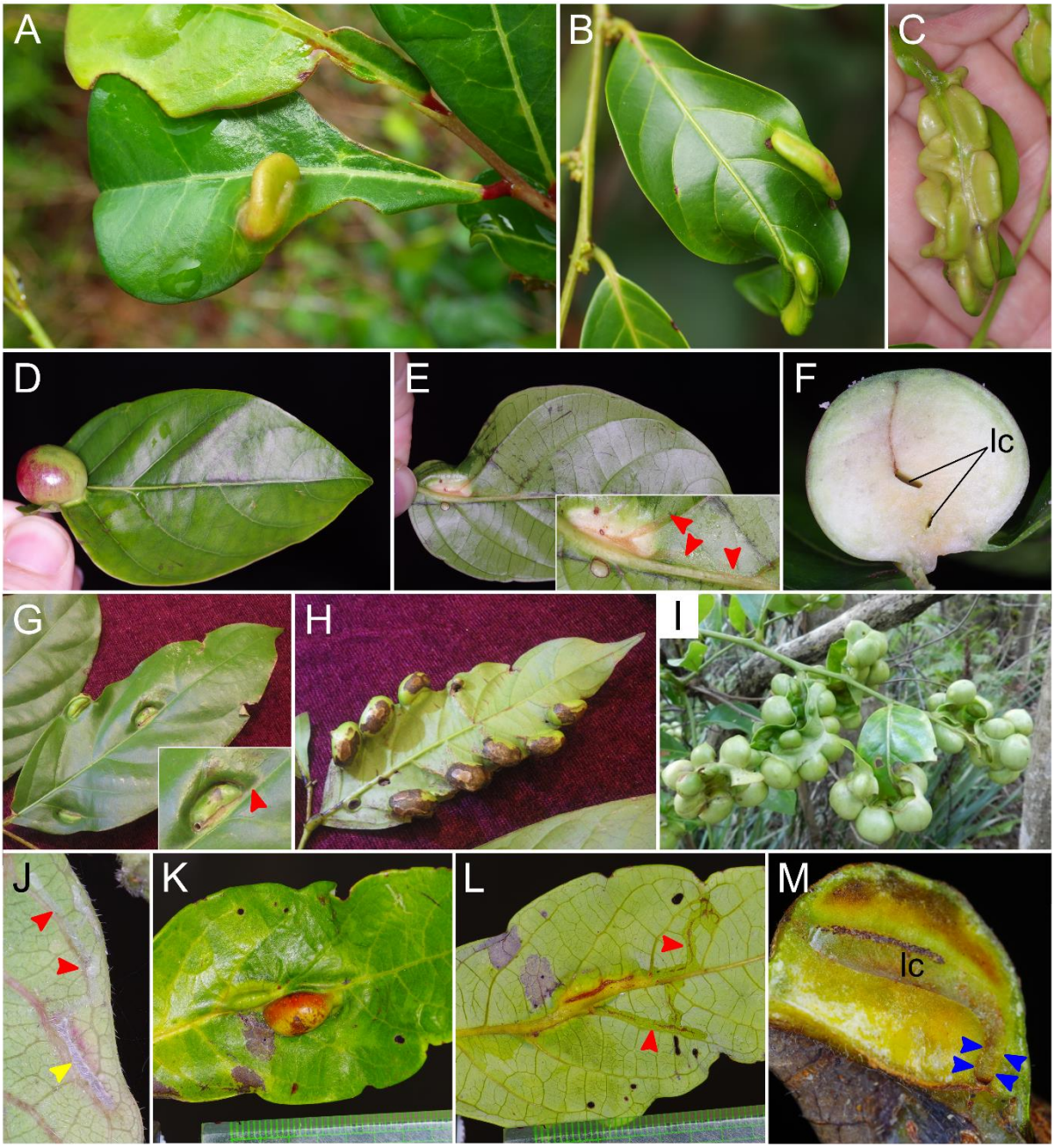



Figure 4.4. Galls induced by *Caloptilia* on Phyllanthaceae. *Caloptilia cecidophora* gall on *Glochidion obovatum* (A), on *G. lanceolatum* (B) and on *G. rubrum* (C). *Calloptilia* sp. 1 gall on *G. zeylanicum* adaxial side (D), abaxial side (E) and cross section (F). *Caloptilia* sp. 2 gall on *G. lanceolarium* adaxial side (G), abaxial side (H), gall abundance on host plant (I). *Calloptilia* sp. 3 on *G. billardieri* adaxial side (J) abaxial side (K) and gall cross section (L). Legend: first and second instars mine: red arrows; third instar mine: yellow arrow; cleaning channel: blue arrows; lc: larval chamber.

species, .non-destructive abdomen extractions were performed so that genitalia preparations could be combined with DNA extraction. We amplified the mitochondrial gene cytochrome oxidase *c* subunit (*COI*, 658 bp), the nuclear arigin kinase (*ArgK*, 573 bp), the elongation factor-1 alpha (*EF-1α*, 541 bp), the carbamoylphosphate synthase domain protein (*CAD*, 614 bp) and the histone H3 (*H3*, 329 bp) (see Table 4.S4 for primer sequence and PCR condition) by polymerase chain reaction (PCR). Amplified PCR products were purified using PCR Purification Kit (Qiagen) or EconoSpin (BIO-BIK) with 50µ volume of the final elusion buffer.

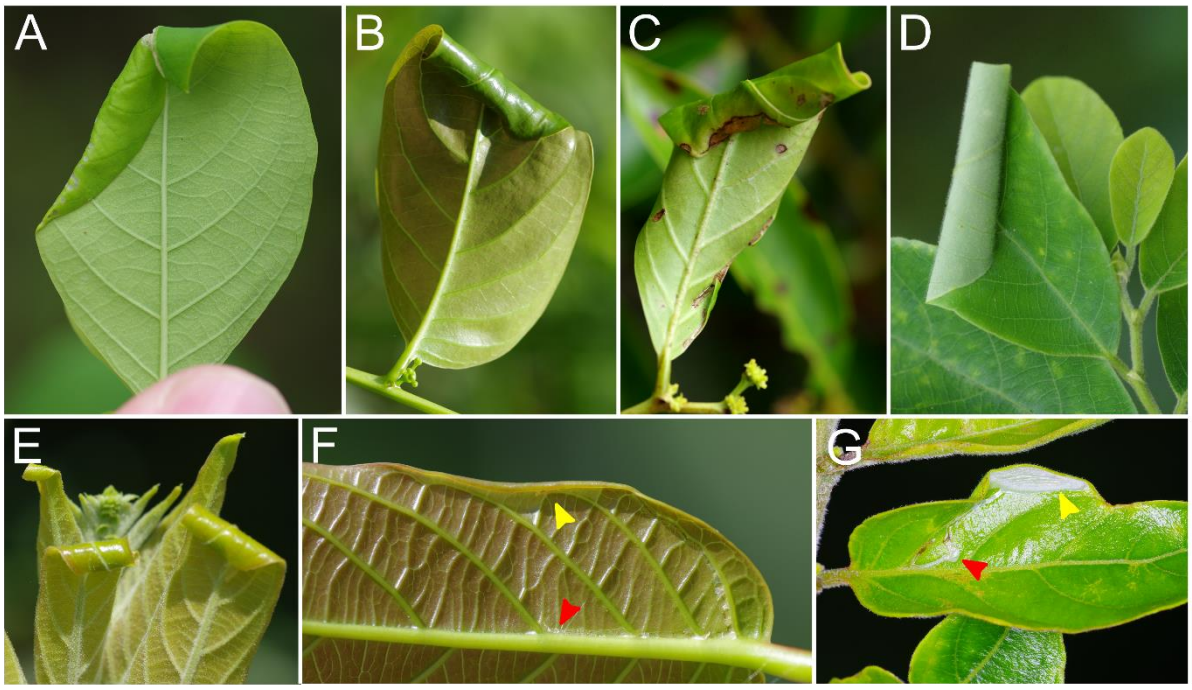



Figure 4.5. Leaf-rolls of Caloptilia on Phyllanthaceae. Caloptilia telodelta on Flueggea virosa (A). Caloptilia ryukyuensis on Glochidion zeylanicum (B) and on G. lanceolatum (C, F). Caloptilia teucra on Bridelia tomentosa (D). Caloptilia sp 4 on G. phillipicum (E, G). Detail of the leaf-mine of C. ryukyuensis on abaxial side (F) and of C. sp. 4 on adaxial side (G). Legend: first and second instars mine: red arrows; third instar mine: yellow arrows.

A single-stranded PCR were performed with Big Dye Terminator v3.1 Cycles Sequencing Kit (Termo Fisher Scientific) for each purified product and then sequencing was conducted by 3730xl DNA Analyzer (Termo Fisher Scientific) according to the manufacture's protocol in Eurofin Genomics Sequencing Center (Tokyo, Japan).

### *Sequence alignment and Phylogenetic Analysis*

A phylogenetic tree was constructed with the five concatenated sequences (*COI*, *ArgK*, *EF-1α*, *CAD* and *H3*). Sequences were aligned manually and concatenated using the program Mesquite (version 3.04. 2015) (Maddison and Maddison, 2016). The phylogenetic tree was inferred by using the maximum likelihood (ML) method implemented in MEGA X (Kumar et al., 2018). The best fit model was chosen based on the Akaike information criterion (AIC) by MEGA and the General Time Reversible (GTR) model (Nei and Kumar, 1998) was selected. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0,3577)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 33,31% sites). The ML tree was searched with SPR (level 5) branch swapping using neighbor-joining tree as a starting point. To assess confidence of each clade, bootstrap analysis was performed using 1000 replicates for each dataset. The analysis involved 30 operational taxonomic units (OTUs). All positions containing gaps and missing data were eliminated in the ML analysis.

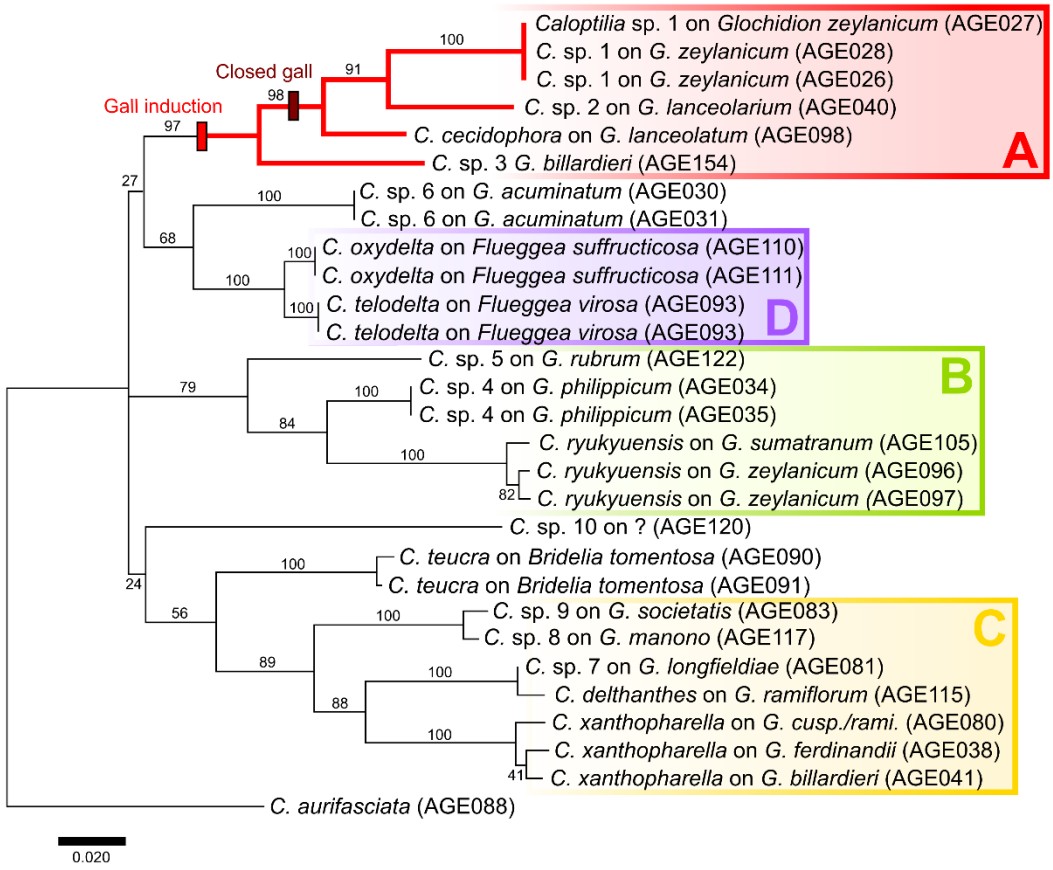



Figure 4.6. Maximum likelihood tree based 5 genes for the *Caloptilia* associated with Phyllanthaceae. The genes used were one mitochondrial (COI) and four nuclear (Hex3, EF1a, CAD and ArgK). Node labels represent the bootstrap.

### *Phylogenomic analysis*

We enriched each pool using the 14,363 probes targeting 1,381 conserved loci, designed for lepidopteran specimens by Faircloth (2017) using MYbaits kits (MYcroarray, Inc.). We followed the protocol explained by Cruaud et al. (2018) except we did not made a whole genome amplification. Phylogenetic tree was estimated from the concatenated, un-partitioned data set using a Maximum Likelihood (ML) approach as implemented in RAxML.

### *Internal arrangement of galls and frass accumulation*

Galls of Phyllanthaceae-associated *Caloptilia* were dissected to observe internal arrangement and frass management. *Caloptilia cecidophora* on *Glochidion obovatum* were sampled in Tomogashima Island (Wakayama, Japan; 34.28N, 135.00E), histology sections were prepared by the method described in Guiguet et al. (2019). Galls of *Caloptilia* sp. 2 and *C.* sp. 3 were cut sagittally in the field and observed by naked eye.

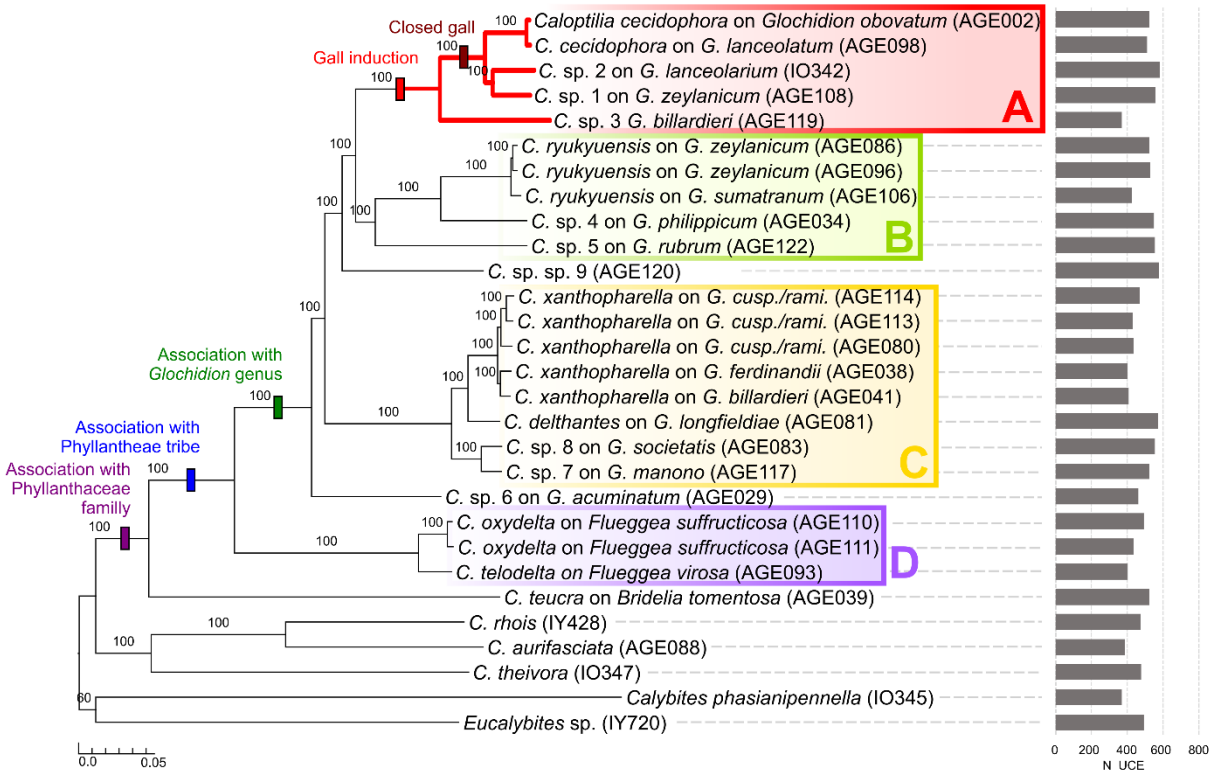



Figure 4.7. Randomized Axelerated Maximum Likelihood tree based ultraconserved elements (UCEs) for the *Caloptilia* associated with Phyllanthaceae. Node labels represent the bootstrap. Histograms shows the distribution of the number of UCEs analyzed for each sample.

# Results

## *Caloptilia phylogeny based on five genes*

We obtained DNA sequences for 16 *Caloptilia* species and one outgroup taxa. The tree with the highest log likelihood (-3447.87) is shown in Figure 6. Maximum likelihood analysis of the concatenated five-gene dataset allowed to depict four well supported monophyletic groups, where bootstrap values were larger or equal to 79% (Figure 6). The first group with a bootstrap value 97% contains the four gall-inducing species, *C. cecidophora*, *C.* sp. 1, *C.* sp. 2 and *C.* sp. 3, and is named "**group A**". The second group (bootstrap value 79%) contains three leaf-rolling species distributed in East Asia, *C. ryukyuensis*, *C.* sp. 4 and *C.* sp. 5, and is named "**group B**". The third group (bootstrap value 89%) contains four Oceanian leaf-rolling species, *C. xanthopharella*, *C. delthantes*, *C.* sp. 8 and *C.* sp. 9, and is named "**group C**". Despite they are genetically close, samples AGE083 and AGE117 were separated in two different species (*C.* sp. 7 and *C.* sp. 8). Given the bad state of conservation of the samples, it did not allowed us to attest if they belong to the same species based on morphological arguments. The fourth group with a 100% support contains the two species associated with *Flueggea* plants, *C. oxydelta* and *C. telodelta*, and is named "**group D**".

The ML analysis is not conclusive about the phylogeny of host-plant relationships because of the low statistical support of the basal nodes. While groups A, B and C are associated with *Glochidion*, monophyly of these clades was not supported. However, monophyly of *Flueggea*-associated species was well supported.

### *Caloptilia phylogeny based on UCE*

Maximum likelihood analysis of the UCEs dataset led to a well resolved phylogeny with high bootstrap supports even for the basal clades of *Caloptilia* associated with Phyllanthaceae (Figure 7). Monophyly of Phyllanthaceae-associated species is strongly supported with 100% bootstrap value. Within the Phyllanthaceae-associated clade, the *Bridelia*-associated species (*C. teucra*) was branched off at the most basal part, and within the remaining Phyllanthaceae-associated clade, the *Flueggea*-associated clade was placed at the most basal position. The number if loci per sample ranged between 369 and 584 (average = 485), (details shown in Table 4.S3).

## Discussion

### *Evolution of gall-induction*

The phylogenies presented here suggest that gall-inducing evolved from leaf-mining and that the gall-inducing habit has a single origin within the clade of leaf-rolling *Caloptilia* associated with *Glochidion* (Figures 6 and 7). In non-gall-inducing species, the larva makes a mine within the leaf epidermis at the first and second instars, then at the third instar folds the blotch mine made by the previous instars in what is called a tentiform mine (Kumata, 1982) (Figure 5). At the fourth instar, the larva leaves the tentiform mine and migrates to the edge of the same or a neighboring leaf to build a leaf-roll, in which the larva will feed on, molt into a fifth instar and pupate (Kumata, 1982) (Figure 5). In a previous study, based on the histology of the gall development, we demonstrated that *C. cecidophora* shares the same life-style until the third instar, although a *C. cecidophora* larva induces an abnormal tissue growth in the walls of the tentiform mine (Chapter 3) leading to a mine-gall structure.

Gall morphology of the three other gall-inducing *Caloptilia* used in the present study shows several common traits with *C. cecidophora*. Although they vary in their size, all of these galls consist of a linear larval chamber (straight or curved) surrounded by tissues that proliferated from the limb that protrudes on one side of the leaf (Figure 4), a morphology that can be classified among the “covering galls” in the classification established by Dreger-Jauffret and Shorthouse (1992). In addition, we directly observed the first leaf-mining instars of *C.* sp. 3 that precedes the gall formation (Figure 4J) and we reported traces of similar early leaf-mining stages in *C.* sp. 1 and *C.* sp. 2 (Figure 4E and 4G), which also corresponds to the life-habit of *C. cecidophora*. Together, these observations support the evolutionary scenario of an evolution of the gall-inducing habit from the third larval instar that makes a tentiform mine, which in

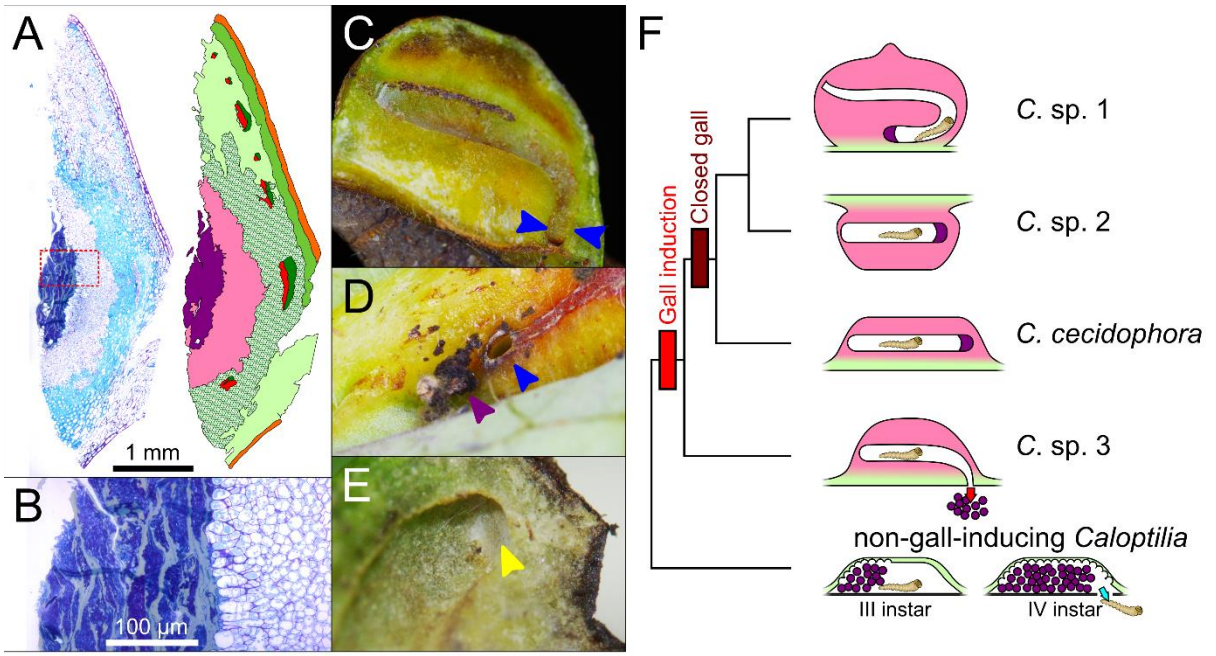



Figure 4.8. Waste management in gall-inducing *Caloptilia*. Histology of the frass accumulation within the gall of *C. cecidophora* and an interpretation drawing (A). Closer view of the histology of the frass accumulated in the galls of *C. cecidophora* (B). Cross section of *C.* sp. 3 gall (C). External view of the exit of the cleaning channel with disposed feces in the gall of *C.* sp. 3 (D). Internal view of the exit of the cleaning channel showing the silk opercula in the gall of *C.* sp. 3 (E). Summary of the evolution of feces management in the gall-inducing *Caloptilia* (F). Schemes represent the internal structure of the gall (gall tissues in pink) or of the tentiform mine of non-gall-inducing *Caloptilia* and show how frass is disposed (frass in purple). Legend, blue arrow: cleaning channel; purple arrow: feces, yellow arrow: silk opercula. Legend of the interpretation drawing (A), orange: epidermis; green: palisade parenchyma; light green: spongy parenchyma; dark green: xylem, red: phloem; green cross: sclerenchyma; pink: nutritive tissue; purple: frass.

other terms means an origin of gall-inducing from the leaf-mining habit in *Caloptilia*. However, we noticed one clear difference in the life-habit of *C.* sp. 3 compared to other gall-inducing *Caloptilia*. Although other species accumulate their frass at one extremity of the linear larval chamber (Figure 8A and 8B), the larval chamber of *C.* sp. 3 is free from any form of feces. Instead, feces are tied by silk and regularly moved out of the gall through a channel linking the larval chamber with the external environment (Figure 8C and 8D). After throwing away their frass, larvae close the cleaning channel with a silk opercula (Figure 8E). As a consequence, the gall of *C.* sp. 3 is opened, whereas the gall of other *Caloptilia* are closed. Considering the strongly supported basal position of *C.* sp. 3 within the gall-inducing clade in the UCE phylogeny (Figure 7), its life-style is likely to be the ancestral state of gall-inducing *Caloptilia* (Figure 8F).

Waste disposal is one of the main constraints of the gall-inducing habit. While the protection against natural enemies and climatic variations are thought to be provided by the galls to their inhabitants (Price et al., 1987), such protection is expected to be more efficient if the insect chamber is separated from the outside. However, an enclosed environment leads to an over accumulation of insect feces or other forms of waste within the insect feeding and developing area. To solved this problems, it has been shown in several groups of gall-inducing insects that the ancestral forms of galls were opened (Nyman et al., 2000; Kutsukake et al., 2012). The ability to induce closed galls evolved secondarily, involving adaptation to dispose waste

produced by the insect (Nyman et al., 2000; Kutsukake et al., 2012;). As an example, closed galls evolved several times in gall-inducing aphids, although they continuously secrete large quantities of honeydew (Kutsukake et al., 2012). Whereas in open galls of aphids, waste is moved out by individuals that are specialized in this task (Benton and Foster, 1992), and the inner surface of closed galls is specialized for absorbing the water contained in the honeydew (Kutsukake et al., 2012).

Like the aphids inducing open galls, *C.* sp. 3 actively disposes their waste outside of the gall. Similar to aphids living within closed galls, the three other *Caloptilia* species inducing closed galls appear to be adapted to reduce their amount of waste accumulation within the larval chamber. The histology section of frass accumulation within the gall of *C. cecidophora* (Figure 8A and 8B) reveals that its feces are highly compacted. Although the detailed mechanisms of this frass reduction are still unknown, it may have played an important role in the evolution of closed galls.

### *Evolution of host-plant associations*

Our study shows that species groups associating with either *Glochidion*, *Flueggea* or *Bridelia* within the Phyllanthaceae-associated clade are clearly distinguished suggesting that host-plant use in *Caloptilia* is phylogenetically conserved (Figure 7). The fact that *Glochidion*-associated clades are placed at most derived position suggests that there was a substantial time lag between the colonization of Phyllanthaceae and *Glochidion*. Further, the gall-inducing species comprise one of the most derived clades in the *Glochidion*-associated clade, meaning that evolution of gall induction also took considerable time lag after host shifting to *Glochidion*. Thus, the present Phyllanthaceae-associated *Caloptilia* system could provide unique opportunities to compare required times between normal host shifting and acquisition of a novel gall induction larval habit.

Host-plant associations show that most of studied *Caloptilia* have a narrow host-range: out of the 13 tested species, only three have more than three host plant species whereas 11 have only one. The majority of species having a single host plant could be due to sampling bias. However, similar results have been obtained in the *Acer* feeding *Caloptilia*, in which, among 13 tested species, four are associated with more than five host-plant species whereas nine are associated with one or two host plants (Nakadai and Kawakita, 2016). The existence of a minority of generalists and majority of specialists has been shown to be the rule in Gracillariidae and *Caloptilia* is not an exception (Lopez-Vaamonde et al., 2003, 2006). Interestingly, with four species of host-plants, *C. cecidophora* is the species having the wider host range, which contradicts the fact that gall-inducers are known to be generally highly host specific (Stone and Schönrogge, 2003).

Ant-other interesting result is the fact that three of the four gall-inducing species share host-plants with leaf-mining species whereas there is no case of species sharing both host plant and the same life-style within Phyllantaceae-associated *Caloptilia* (Figure 9A). Such cases of leaf-rolling and gall-inducing species co-occuring on the same plant have been often observed in

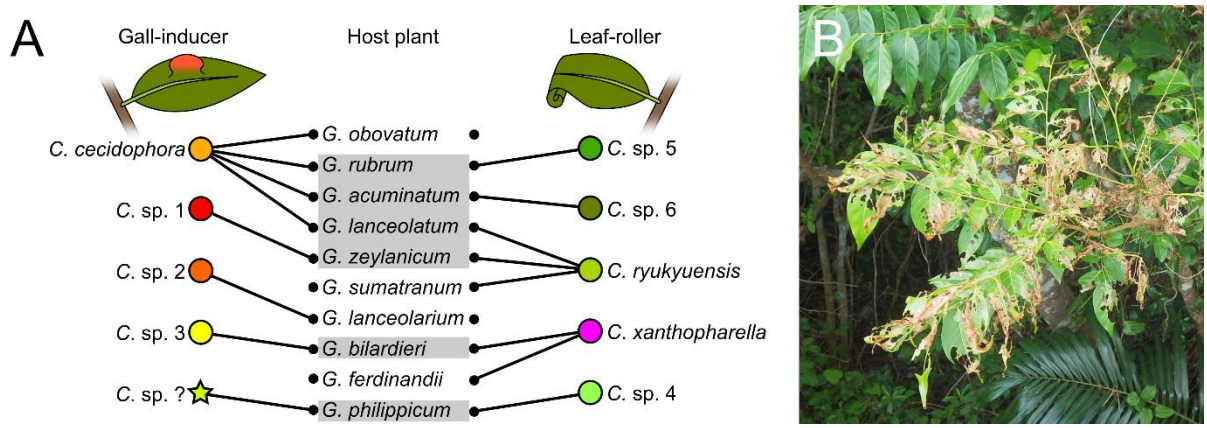



Figure 4.9. Host-plant association of gall-inducing *Caloptilia* indicating leaf-rolling *Caloptilia* that share the same host plant (A) and photo of *Glochidion zeylanimum* with leaves heavily damaged by herbivores (B).

the wild (*C. ryukyuensis* and *C. cecidophora* on *G. lanceolatum*, Taiwan, Guiguet, personal observation). We can also add to this a fourth gall-inducing species, that is likely to be from the *Caloptilia* genus according to the gall description (Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926), and that shares its host-plant *G. philippicum* with *C.* sp4 (Figure 9). Therefore, both leaf-miners and gall-inducers seem to be able to share the same host plant and avoid competitive exclusion by having different life-styles. Indeed, despite that gall-inducers and leaf-miners both exploit the same resource (lower epidermis of young leaves) in early instars, they rapidly occupy different ecological niches within the same host-plant. This might suggest that niche partitioning to avoid competition may have been a strong evolutionary force leading to gall evolution. This result is in conflict with the host-use of *Acer* associated *Caloptilia* whose host ranges of leaf-rolling species are mostly overlapping (Nakadai and Kawakita, 2017). One possible reason is that young leaves of *Acer* represent a super abundant resource that should not generate any conflict of use whereas this resource could be rarer in the case of *Glochidion*. Indeed, many insect herbivores are associating with *Glochidion* trees and their leaves are frequently heavily damaged, which considerably reduces the available resources for late instars of *Caloptilia*. This herbivore pressure could even has been a selective agent for insects protected by galls compared to leaf-rolls (Figure 9B).

## *Biogeography*

Our study confirms that Australasian *Caloptilia* associated with *Glochidion* (Group C) are related to *Glochidion*-associated *Caloptilia* in East/South-East Asia as hypothesized by Hembry et al., (2013a), thereby demonstrating that this host-association has been conserved during the colonization of oceanic islands by *Caloptilia*. In contrast to *Glochidion*-associated *Epicephala* moths that colonized the remote Pacific islands repeatedly (Hembry et al., 2013b), *Caloptilia* associated with *Glochidion* in the Pacific Islands appear to result from a single colonization event of the region. This insect-plant community thus has reassembled itself repeatedly on Pacific islands through biogeographically idiosyncratic processes. However, we also find that *Caloptilia* in the Society Islands and Austral Islands are closely related to each other and to a species from Australia and western Pacific archipelagos (Samoa and New Caledonia), a

common biogeographic pattern in the biota of eastern Pacific archipelagos (Hembry and Balukjian, 2016).

Our results do not allow to conclude that an adaptive radiation has taken place and further work is required to answer this question. First, further sampling could reveal more gall-inducing *Caloptilia* species. Among the ten *Glochidion* species described to host *Caloptilia*-like galls (Docters van Leeuwen, 1922; Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926) (Figure 1), most were found in locations that were not visited during this study (Figure 2). In addition, one barcoded *Caloptilia* collected in Australia (*C.* sp. ANIC11, Figure 3), also corresponds to the morphotype of gall-inducing *Caloptilia*. Interestingly, one gall-inducing *Caloptilia* has been described in Fidji, (*Gracillaria*) *hercoscelis* (Meyrick, 1939) (Figure 3), with no indication on the host plant but the wing pattern suggests it may belong to the group A. Sampling in Fidji would be relevant given this archipelago hosts a high diversity of *Glochidion*, 18 species including 17 endemic (Smith, 1981). Secondly, we aim to generate a time calibration of the UCE phylogeny in order to estimate the divergence time of gall-inducing *Caloptilia*. A rapid diversification of gall-inducing species would allow to conclude to an adaptive radiation of this group, which would strengthen that the gall-induction is a key innovation which has opened up new ecological opportunities for *Glochidion*-associated *Caloptilia*.

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# Supplementary Figures and Tables

Table 4.S1. Insect individuals used for DNA extractions.

| N° | Species | Body part | Location |
|---|---|---|---|
| AGE-002 | *Caloptilia cecidophora* | Larvae | Japan (Tomogashima) |
| AGE-098 | *Caloptilia cecidophora* | Larvae | Taiwan |
| AGE-026 | *Caloptilia* **sp.1** | Adult (Abdomen) | Malaysia (Sarawak) |
| AGE-027 | *Caloptilia* **sp.1** | Larvae | Malaysia (Sarawak) |
| AGE-028 | *Caloptilia* **sp.1** | Larvae | Malaysia (Sarawak) |
| AGE-108 | *Caloptilia* **sp.1** | Larvae | Malaysia (Sarawak) |
| AGE-040 | *Caloptilia* **sp.2** | Adult (Abdomen) | China (Hainan) |
| IO-342 | *Caloptilia* **sp.2** | Adult (Abdomen) | China (Ghanzhou) |
| AGE-119 | *Caloptilia* **sp.3** | Adult (Abdomen) | New Caledonia |
| AGE-154 | *Caloptilia* **sp.3** | Adult (Abdomen) | New Caledonia |
| AGE-086 | *Caloptilia ryukyuensis* | Adult (Abdomen) | Japan (Amami) |
| AGE-096 | *Caloptilia ryukyuensis* | Adult (Abdomen) | Taiwan |
| AGE-097 | *Caloptilia ryukyuensis* | Adult (Abdomen) | Taiwan |
| AGE-105 | *Caloptilia ryukyuensis* | Larvae | Taiwan |
| AGE-106 | *Caloptilia ryukyuensis* | Larvae | Taiwan |
| AGE-034 | *Caloptilia* **sp.4** | Adult (Abdomen) | Taiwan |
| AGE-035 | *Caloptilia* **sp.4** | Larvae | Taiwan |
| AGE-089 | *Caloptilia* **sp.5** | Adult (Abdomen) | Taiwan |
| AGE-122 | *Caloptilia* **sp.5** | Adult (Abdomen) | Taiwan |
| AGE-029 | *Caloptilia* **sp.6** | Larvae | China (Yunnan) |
| AGE-030 | *Caloptilia* **sp.6** | Larvae | China (Yunnan) |
| AGE-031 | *Caloptilia* **sp.6** | Larvae | China (Yunnan) |
| AGE-038 | *Caloptilia xanthopharella* | Adult (Abdomen) | Australia (West) |
| AGE-041 | *Caloptilia xanthopharella* | Adult (Abdomen) | New Caledonia |
| AGE-080 | *Caloptilia xanthopharella* | Adult (Abdomen) | American Samoa |
| AGE-113 | *Caloptilia xanthopharella* | Adult (Abdomen) | American Samoa |
| AGE-114 | *Caloptilia xanthopharella* | Adult (Abdomen) | American Samoa |
| AGE-081 | *Caloptilia delthantes* | Adult (Abdomen) | French polynesia (Rapa) |
| AGE-115 | *Caloptilia deltanthes* | Adult (Abdomen) | French polynesia (Rapa) |
| AGE-084 | *Caloptilia* **sp.7** | Adult (Abdomen) | French polynesia (Tahiti) |
| AGE-117 | *Caloptilia* **sp.7** | Adult (Abdomen) | French polynesia (Tahiti) |
| AGE-083 | *Caloptilia* **sp.8** | Adult (Abdomen) | French polynesia (Huahine) |
| AGE-120 | *Caloptilia* **sp. 9** | Adult (Abdomen) | Malaysia (Sabah) |
| AGE-110 | *Caloptilia oxydelta* | Adult (Abdomen) | Japan (Niimi) |
| AGE-111 | *Caloptilia oxydelta* | Adult (Abdomen) | Japan (Niimi) |
| AGE-112 | *Caloptilia oxydelta* | Adult (Abdomen) | Japan (Niimi) |
| AGE-092 | *Caloptilia telodelta* | Adult (Abdomen) | Taiwan |
| AGE-093 | *Caloptilia telodelta* | Adult (Abdomen) | Taiwan |
| AGE-039 | *Caloptilia teucra* | Larvae | Taiwan |
| AGE-090 | *Caloptilia teucra* | Adult (Abdomen) | Taiwan |
| AGE-091 | *Caloptilia teucra* | Adult (Abdomen) | Taiwan |
| IY428 | *Caloptilia rhois* | Adult (Abdomen) | |
| AGE-088 | *Caloptilia aurifasciata* | Adult (Abdomen) | Taiwan |
| IO-347 | *Caloptilia theivora* | Adult (Abdomen) | |
| IO-345 | *Calybites phasianipennella* | Adult (Abdomen) | |
| IY-720 | *Eucalybites sp.* | Adult (Abdomen) | |

Table 4.S2. Genes sequenced per individual. Legend, F: Forward strand sequence only; R: Reverse strand sequence only; dark grey: outgroup.

| N° | Species | Host plant | Life-habit | Gene | | | | |
|---|---|---|---|---|---|---|---|---|
| AGE-098 | *Caloptilia cecidophora* | *Glochidion lanceolatum* | galler | *COI* | *H3* | | *CAD (R)* | *ArgK (R)* |
| AGE-096 | *Caloptilia ryukyuensis* | *Glochidion zeylanicum* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-097 | *Caloptilia ryukyuensis* | *Glochidion zeylanicum* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD (R)* | *ArgK* |
| AGE-105 | *Caloptilia ryukyuensis* | *Glochidion sumatranum* | leaf-roller | *COI* | *H3* | | *CAD (R)* | *ArgK (F)* |
| AGE-030 | *Caloptilia* **sp.6** | *Glochidion acuminatum* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-031 | *Caloptilia* **sp.6** | *Glochidion acuminatum* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-034 | *Caloptilia* **sp.4** | *Glochidion phillipicum* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-035 | *Caloptilia* **sp.4** | *Glochidion phillipicum* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD (R)* | *ArgK* |
| AGE-111 | *Caloptilia oxydelta* | *Flueggea suffructicosa* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD (R)* | *ArgK (R)* |
| AGE-112 | *Caloptilia oxydelta* | *Flueggea suffructicosa* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK (R)* |
| AGE-092 | *Caloptilia telodelta* | *Flueggea virosa* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-093 | *Caloptilia telodelta* | *Flueggea virosa* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-090 | *Caloptilia teucra* | *Bridelia tomentosa* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-091 | *Caloptilia teucra* | *Bridelia tomentosa* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-026 | *Caloptilia* **sp.1** | *Glochidion zeylanicum* | galler | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-027 | *Caloptilia* **sp.1** | *Glochidion zeylanicum* | galler | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-028 | *Caloptilia* **sp.1** | *Glochidion zeylanicum* | galler | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-040 | *Caloptilia* **sp.2** | *Glochidion lanceolarium* | galler | *COI* | *H3* | | *CAD* | *ArgK* |
| AGE-038 | *Caloptilia xanthopharella* | *Glochidion ferdinandii* | leaf-roller | *COI* | *H3* | | *CAD* | |
| AGE-041 | *Caloptilia xanthopharella* | *Glochidion billardieri* | leaf-roller | *COI* | *H3* | | *CAD* | *ArgK* |
| AGE-080 | *Caloptilia xanthopharella* | *Glochidion ramiflorum/cuspidatum* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-084 | *Caloptilia* **sp.7** | *Glochidion manono* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-083 | *Caloptilia* **sp.8** | *Glochidion societatis* | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |
| AGE-081 | *Caloptilia deltanthes* | *Glochidion longfieldiae* | leaf-roller | *COI* | *H3* | | *CAD* | |
| AGE-115 | *Caloptilia deltanthes* | *Glochidion* sp. | leaf-roller | *COI* | *H3* | | *CAD* | |
| AGE-089 | *Caloptilia* **sp.5** | *Glochidion rubrum* | leaf-roller | *COI* | *H3* | | *CAD* | *ArgK* |
| AGE-154 | *Caloptilia* **sp.3** | *Glochidion billardieri* | galler | *COI* | *H3* | | *CAD* | |
| AGE-120 | *Caloptilia* **sp. 9** | ? | leaf-roller | *COI* | *H3* | | | |
| AGE-088 | *Caloptilia aurifasciata* | ? | leaf-roller | *COI* | *H3* | *Ef1α* | *CAD* | *ArgK* |

Table 4.S3. Ultra-conserved elements sequenced per individual. Legend, dark grey: outgroups.

| N° | Species | Host plant | Life-habit | Nbr of UCEs |
|---|---|---|---|---|
| AGE-002 | *Caloptilia cecidophora* | *Glochidion obovatum* | galler | 523 |
| AGE-098 | *Caloptilia cecidophora* | *Glochidion lanceolatum* | galler | 514 |
| IO-342 | *Caloptilia* **sp.2** | *Glochidion lanceolarium* | galler | 584 |
| AGE-108 | *Caloptilia* **sp.1** | *Glochidion zeylanicum* | galler | 561 |
| AGE-119 | *Caloptilia* **sp.3** | *Glochidion billardieri* | galler | 371 |
| AGE-086 | *Caloptilia ryukyuensis* | *Glochidion zeylanicum* | leaf-roller | 524 |
| AGE-096 | *Caloptilia ryukyuensis* | *Glochidion zeylanicum* | leaf-roller | 528 |
| AGE-106 | *Caloptilia ryukyuensis* | *Glochidion sumatranum* | leaf-roller | 428 |
| AGE-034 | *Caloptilia* **sp.4** | *Glochidion phillipicum* | leaf-roller | 552 |
| AGE-122 | *Caloptilia* **sp.5** | *Glochidion rubrum* | leaf-roller | 557 |
| AGE-120 | *Caloptilia* **sp. 9** | ? | leaf-roller | 578 |
| AGE-038 | *Caloptilia xanthopharella* | *Glochidion ferdinandii* | leaf-roller | 400 |
| AGE-041 | *Caloptilia xanthopharella* | *Glochidion billardieri* | leaf-roller | 409 |
| AGE-080 | *Caloptilia xanthopharella* | *Glochidion ramiflorum/cuspidatum* | leaf-roller | 437 |
| AGE-113 | *Caloptilia xanthopharella* | *Glochidion ramiflorum/cuspidatum* | leaf-roller | 433 |
| AGE-114 | *Caloptilia xanthopharella* | *Glochidion ramiflorum/cuspidatum* | leaf-roller | 472 |
| AGE-081 | *Caloptilia delthantes* | *Glochidion longfieldiae* | leaf-roller | 573 |
| AGE-083 | *Caloptilia* **sp.8** | *Glochidion societatis* | leaf-roller | 557 |
| AGE-117 | *Caloptilia* **sp.7** | *Glochidion manono* | leaf-roller | 526 |
| AGE-029 | *Caloptilia* **sp.6** | *Glochidion acuminatum* | leaf-roller | 463 |
| AGE-110 | *Caloptilia oxydelta* | *Flueggea suffructicosa* | leaf-roller | 492 |
| AGE-111 | *Caloptilia oxydelta* | *Flueggea suffructicosa* | leaf-roller | 435 |
| AGE-093 | *Caloptilia telodelta* | *Flueggea virosa* | leaf-roller | 404 |
| AGE-039 | *Caloptilia teucra* | *Bridelia tomentosa* | leaf-roller | 524 |
| IY428 | *Caloptilia rhois* | | leaf-roller | 476 |
| AGE-088 | *Caloptilia aurifasciata* | *Toxicodendron* sp. | leaf-roller | 391 |
| IO-347 | *Caloptilia theivora* | *Camelia sinensis* | leaf-roller | 479 |
| IO-345 | *Calybites phasianipennella* | | leaf-roller | 369 |
| IY-720 | *Eucalybites sp.* | | leaf-roller | 493 |

Table 4.S4. Primers used in this study and amplification profiles.

| Locus | Primer name | Direction | Sequence | Reference |
|---|---|---|---|---|
| COI | LCO 1490 | Forward | 5'-GGTCAACAAATCATAAAGATATTGG-3' | Folmer at al. 1994 |
| | HCO2198 | Reverse | 5'-TAAACTTCAGGGTGACCA AAAAATCA-3' | Folmer at al. 1994 |
| H3 | HexAF | Forward | 5'-ATGGCTCGTACCAAGCAGACVGC-3' | Svenson & Whiting 2004 |
| | HexAR | Reverse | 5'-ATATCCTTRGGCATRATRGTGAC-3' | Svenson & Whiting 2004 |
| ArgK | ArgK-F7 | Forward | 5'-AACTGGGGWGAYGTYGAGACYCTYGG-3' | Nakadai & Kawakita 2016 |
| | ArgK-R8 | Reverse | 5'-GCACCTGCAGGTGGTACTTG-3' | Nakadai & Kawakita 2016 |
| CAD | CAD-f222 | Forward | 5'-GGDTCWCAAGCWGTDAARGCDATGC-3' | Nakadai & Kawakita 2016 |
| | CAD-r222 | Reverse | 5'-CCDAGDGGRTCAACRTTYTCCAT-3' | Nakadai & Kawakita 2016 |
| EF1α | ef1af2 | Forward | 5'-CCCATTTCKGGCTGGCAYGGAGA-3' | Kawakita et al. 2004 |
| | efiar3 | Reverse | 5'-GACTTKCCRGTACGRCGGTC-3' | Nakadai & Kawakita 2016 |

| | Hot start | Denature | Anneal | Extention | Final extend | Cycles |
|---|---|---|---|---|---|---|
| COI | 94°C (5 min) | 94°C (30 s) | 50°C (30 s) | 72°C (45 s) | 72°C (5 min) | 40 |
| H3 | 94°C (3 min) | 94°C (30 s) | 45°C (30 s) | 65°C (45 s) | 65°C (3 min) | 40 |
| ArgK | 94°C (5 min) | 94°C (30 s) | 50°C (30 s) | 72°C (1 min) | 72°C (7 min) | 30 |
| CAD | 94°C (5 min) | 94°C (30 s) | 50°C (30 s) | 72°C (1 min) | 72°C (7 min) | 30 |
| EF1α | 94°C (5 min) | 94°C (30 s) | 50°C (30 s) | 72°C (1 min) | 72°C (7 min) | 30 |

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# Chapter 5.

# Intra- and inter-specific comparative transcriptomic approaches to reveal candidates effectors of gall induction in the micromoth *Caloptilia cecidophora* (Gracillariidae, Lepidoptera)

# Résumé du Chapitre 5

Alors que les insectes induisant des galles exercent un contrôle important sur le développement et la physiologie de leur hôte, les mécanismes moléculaires à la base de ces processus de manipulation sont encore mal compris. Une façon d'aborder cette question est de se concentrer sur les effecteurs produits par les insectes et susceptibles d'affaiblir le système immunitaire de la plante et/ou de moduler les processus cellulaires. La nouvelle génération de technologies de séquençage a ouvert des opportunités pour mieux comprendre les interactions moléculaires complexes entre les insectes inducteurs de galles et leur plante hôte (Oates et al., 2016), et pour identifier l'ensemble des gènes impliqués dans l'interaction entre la plante et les insectes inducteurs de galle («cécidome» - Nabity et al., 2016). Un nombre croissant d'étude applique ainsi des méthodes transcriptomiques ou génomiques pour identifier les mécanismes moléculaires impliqués de l'induction des galles (e.g. Nabity et al., 2013; Hearn, 2013 ; Bailey et al., 2015; Chen et al., 2018; Schultz et al., 2019).

D'un point de vue méthodologique, la plupart de ces études ont utilisé une approche transcriptomique comparative dans leur design expérimental. En ce qui concerne la recherche sur la réponse des plantes aux inducteurs de galle, les approches comparatives entre l'expression des gènes dans les tissus de la galle et dans les parties saines de la même plante ont permis d'identifier les processus physiologiques clés impliqués dans l'induction de la galle (e.g. Nabity, 2016). Les points de comparaison sont moins évidents lorsque l'on étudie les insectes galligènes, ce qui a probablement limité le choix des espèces à étudier. Certaines études ont opté pour des comparaison intra-espèce en sélectionnant deux stades de développement dont l'un joue un rôle plus important que l'autre dans la formation de la galle (Hearn, 2013), d'autres ont choisi une comparaison inter-espèce en sélectionnant une espèce apparentée non galligène (Miller et al., 2015).

Notre étude a eu pour objet d'utiliser le mode vie larvaire atypique et le contexte phylogénétique de l'espèce galligène *Caloptilia cecidophora* (Lepidoptera, Gracillariidae) afin d'identifier des effecteurs impliquée dans l'induction de la galle. Pour cela, nous avons appliqué une approche de transcriptomique à la fois intra- et inter-espèce, en comparant l'expression des gènes aux stades mineurs de feuille avec les stades galligènes et en comparant le transcriptome de *C. cecidophora* avec celui de *C. ryukyuensis*, une espèce apparentée et non galligène.

Des larves des six stades de développement de *C. cecidophora* et des stades deux, trois et quatre de *C. ryukyuensis* ont été collectées pour séquençage ARN (Illumina NextSeq 500) et pour un assemblage *de novo* des transcriptomes des deux espèces (Trinity). Nous avons utilisé ensuite trois stratégies distinctes pour l'analyse des résultats (Figure 5.1). #1 L'induction constituant selon certains auteurs un processus limité aux premiers instants de l'interaction avec la plant-hôte (Miller and Raman, 2018), nous avons extrait les gènes présentant un pic d'expression au stade trois lorsque débute l'induction de la galle, en excluant les gènes communs au transcrits assemblés de l'espèce non-galligène *C. ryukyuensis* (Figure 5.4, Table 5.2). #2 L'activation du développement de la galle pouvant également être due à une stimulation continue, nous avons

partitionné les profils d'expression des gènes de *C. cecidophora* exprimés de façon différentielle, au moyen d'une carte auto-adaptative, afin de sélectionner les gènes surexprimés durant les stades galligènes. Nous avons ensuite exfiltré des gènes candidats qui étaient également exprimés chez *C. ryukyuensis* (Figure 5.5). #3 Par une approche gène candidat, nous avons recherché au sein des deux transcriptomes des orthologues de l'enzyme BmIAO1, récemment identifiée comme étant responsable de la synthèse d'auxine dans les chenilles de Bombyx mori (Takei et al., 2019). L'auxine est en effet une phytohormone suspectée de jouer un rôle clé dans la formation des galles (Tooker and Helms, 2014) (Figure 5.6).

Les résultats des approches #1 et #2 permettent d'établir une liste de 56 gènes candidats potentiellement impliqués dans la formation de la galle de *C. cecidophora*. On trouve parmi eux des enzymes anti-oxydantes déjà identifiées comme des facteurs de virulence d'organismes manipulateurs de plantes (Dubreuil et al., 2011; Guiguet et al., 2016) et qui pourrait permettre à l'insecte galligène de résister au stress oxidatif suggéré comme étant nécessaire à la formation des galles par certains auteurs (Oliveira et al., 2016). L'approche #2 a permis également d'identifier deux chitinases, des classes d'enzymes fortement exprimées chez les galligènes de Cynipidae (Hymenoptera) et suspectées d'activer une dédifférentiation cellulaire dans les tissus végétaux (Hearn, 2013). Enfin l'approche #3 a permis d'identifier dans les deux espèces des séquences orthologues de BmIAO1, dont l'expression est compatible avec une production accrue d'auxine chez *C. cecidophora* durant les stades galligènes. Des analyses supplémentaires sont cependant nécessaires pour confirmer ces résultats préliminaires.

## Article N°4

# Intra- and inter-specific comparative transcriptomic approaches to reveal candidates effectors of gall induction in the micromoth *Caloptilia cecidophora* (Gracillariidae, Lepidoptera)

Antoine Guiguet[1, 2], Seisuke Kimura[3, 4], Tomoaki Sakamoto[5], Carlos Lopez-Vaamonde[1, 6], David Giron[1*], Issei Ohshima[2*]

[1]Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS/Université de Tours, UFR Sciences et Techniques, Tours, France

[2]Department of Life and Environmental Sciences, Kyoto Prefectural University, Kyoto, Japan

[3]Department of Bioresource and Environmental Sciences, Kyoto Sangyo University, Kamigamo-Motoyama, Kita-Ku, Kyoto, 603–8555, Japan.

[4]Center for Ecological Evolutionary Developmental Biology, Kyoto Sangyo University, Kamigamo-Motoyama, Kita-Ku, Kyoto, 603–8555, Japan.

[5]Faculty of Life Sciences, Kyoto Sangyo University, Motoyama, Kamigamo, Kita-Ku, Kyoto, 603–8555, Japan

[6]Institut de Recherche sur la Biologie de l'Insecte, UMR 7261, CNRS/Université de Tours, UFR Sciences et Techniques, Tours, France, INRA, UR0633 Zoologie Forestière, Orléans, France

*equal contribution

# Abstract

While gall-inducing insects exert a high level of control over their host development and physiology, the molecular mechanisms underlying these manipulation processes are still poorly understood. One way to address this question is to focus on effectors produced by insects and susceptible to undermine the plant immune system and modulate the cellular processes. To find candidate effectors involved in gall induction, we analyzed the transcriptome of the micromoth *Caloptilia cecidophora* (Lepidoptera, Gracillariidae) that induces galls on *Glochidion obovatum* (Phyllanthaceae). This species was selected for its peculiar biology and phylogeny. The larval development of *C. cecidophora* involves a transition from leaf-mining to gall-inducing allowing for the intraspecific comparison of larvae with distinct feeding habits. In addition, *C. ryukyuensis*, a closely related non-gall-inducing species both sympatric and growing of host plant of the same genus, allows for an interspecific comparison of same-aged larvae with distinct feeding habits. We performed RNA-seq and *de novo* assembly for larvae of both species, at each of the six instars of *C. cecidophora* and at second, third and fourth instars of *C. ryukyuensis*, corresponding to the feeding habit transition in *C. cecidophora*. Three independent methods were used to analyze the transcriptomic data. First, we isolated genes showing a peak of expression at the initiation of the gall induction (third instar), and 505 genes were significantly upregulated in third instar compared to the second and the fourth instars. Among them 22 were specific in *C. cecidophora* transcripts and successfully annotated. Secondly, we selected genes with expression patterns matching gall-induction using a principal component analysis and a self-organizing map. Ortholog comparisons revealed 36 genes showing an opposite expression pattern between the two species. Finally, we looked for candidate genes based on literature and compared their expression patterns between the two species and among instars. All together, we obtained a list of 58 genes coding for possible effectors. Preliminary results point toward the production of anti-oxidant enzymes and chitinases and suggest the implication of secreted auxin in gall formation.

# Introduction

Galls are formed by redifferentiation of host plant tissues leading to alteration of plant development. They are often characterized by an activation of cell development (hyperplasy) and cell growth (hypertrophy) mediated by microbes, nematodes or arthropods (Redfern and Askew, 1992; Review on gallers). Gall-inducing characterizes less than 0.001 to 2% of described insects (Buhr, 1965; Espírito-Santo and Fernandes, 2007) but is encountered in six orders, namely Thysanoptera, Hemiptera, Hymenoptera, Coleoptera, Diptera and Lepidoptera (Dreger-Jauffret and Shorthouse, 1992). Several of them are considered as major agronomic pests, like cereal gall midges (Harris et al., 2003), or harmful invasive species, like the chestnut gall wasp or the blue gum chalcid (Csóka et al., 2017). To date, little is known about the molecular mechanism underlying gall-induction by insects limiting our understanding of the ecology and evolution of these plant-insect interactions and impairing the development of novel, robust management strategies.

The next-generation sequencing era opened opportunities to better understand complex molecular interactions between gall-inducing insects and their host-plant (Oates et al., 2016), and to identify the entire set of genes that mediate the interaction between the plant and gall-inducers (‘cecidome’ - Nabity et al., 2016). While data start to be gathered on various biological models, eg. Phylloxera and grape wine (Schultz et al., 2019), Gall wasps and oak trees (Hearn, 2013;), the Hessian fly (Mayetiola destructor) developing on wheat (Tritcum aestivum) is the only galling species where the genome sequencing allowed identifying putative insect effector-encoding genes (Zhao et al., 2016). Several studies analyzed *de novo* assembled transcriptome of gall systems (Nabity et al., 2013; Bailey et al., 2015; Chen et al., 2018; Schultz et al., 2019) but mostly focused on gall transcriptome rather than on the gall-inducers (see for an exception Cambier et al. submitted).

From a methodological point of view, most of these studies used a comparative approach in their experimental design. Regarding the research on plant response to gall-inducers, comparative approaches between gene expression in gall tissues and in healthy parts of the same plant allowed for the identification of key physiological processes involved in gall induction (Nabity, 2016). Other studies investigating the molecular bases of incompatibility of plant-insect interactions compared susceptible versus resistant plants e.g. (Liu et al., 2007; Oates et al., 2015). Comparison points are less obvious when working on the insect side of the interaction, and this probably constrained the choice of species to study. Studies looking for effectors of gall-induction in non-model species either performed intra-or inter-specific comparisons. For intra-species comparisons, some compared two developmental stages with one supposedly involved in gall-induction (Hearn, 2013), other compared two life forms of the same species with one being gall-forming (Rispe et al., 2016). Regarding inter-species comparisons, the compared species were either closely related (Miller et al., 2015) or more distant (Portillo Lemus et al., 2018), and some studies compared multiple related species (Zhao and Nabity, 2017a, 2017b).

In the present study we aim to apply a similar comparative strategy on an insect switching from leaf-mining to gall-inducing over the course of its development (Chapter 3). The *Caloptilia* genus (Lepidoptera, Gracillariidae) comprises more than 300 species that are in general specialist herbivores (Nakadai and Kawakita, 2016). At larval stages, most are leaf-miners at early instars (first, second and third) and leaf-rollers at late instars (fourth and fifth). *Caloptilia cecidophora* is an exception in the *Caloptilia* genus because larvae induce galls on their host-plant (Kumata, 1966). Distributed in south Japan and Taiwan (Figure 5.1A), insect induces galls on leaves of *Glochidion obovatum*, *G. acuminatum*, *G. rubrum* and *G. lanceolatum* (Phyllanthaceae). First and second larval instars of *C. cecidophora* are leaf-miners implying a transition from a non-galling to a gall-inducing habit during the larval development (Chapter 3). In addition, the dozen of *Caloptilia* species associated with *Glochidion* form a monophyletic group (Chapter 4). Gall-induction evolved within this clade from leaf-mining, with the ability to stimulate an abnormal development of leaf-tissues initiated at the third instar (Chapters 3 and 4).

In this study, we aimed to identify effectors involved in gall induction and produced by *C. cecidophora* larvae. The transition from leaf-mining to gall-inducing allowed us to compare the gene expression of larvae before and after the initiation of the gall formation. The phylogeny of Phyllanthaceae associated *Caloptilia* allowed us to select the most closely related non-gall-inducing species *C. ryukyuensis* (Chapter 4). Comparison of gene expression in *C. cecidophora* and in *C. ryukyuensis* allowed us to filter out gene expression variation linked to larval development rather than to gall induction.

# Methods

## *Insect material*

Fourth, fifth and sixth instars of *Caloptilia cecidophora* were collected on *Glochidion obovatum* in Tomogashima island in summer 2016 (Wakayama, Japan; 34.28N, 135.00E). To obtain first, second and third instars that are difficult to find in the field, we established a lab rearing. Mature galls collected in Tomogashima Island were placed in growth chamber (25°C, 70% humidity) until adult emergence. Emerging adults were sexed and one or two pairs of moths were transferred into a mesh box (40x40x40cm) placed in the greenhouse (25°C, 70±10% humidity). Rearing boxes contained a wiping paper soaked with 2% sucrose solution as a food for adults. Because *C. cecidophora* females lay their eggs only on very fresh leaves, a branch of a potted *G. obovatum* with young leaves was placed in the mesh box through a mesh-sealed hole. Oviposition was checked daily and twigs replaced with new intact twigs once eggs were laid on leaves. All rearing experiments were conducted in Kyoto Prefectural University, Japan.

Third and fourth instars of *C. ryukryuensis* were collected on *G. zeylanicum* and *G. lanceolatum* in Ishigaki island in spring 2017 (Okinawa, Japan; 24.32-51N, 124.07-27E). Third and fourth instars were collected on the field. Leaf-mines and leaf-rolls were collected in the field and

reared until adult emergence. Adults were reared as above on potted *G. zeylanicum* in order to obtain second instars.

### *RNA extraction*

Total RNA (RNeasy Plant Mini Kit, QIAGEN) was extracted from three independent pools of full larvae at each instars for *C. cecidophora*, and at second, third and fourth instar for *C. ryukyuensis*. In addition, we made respectively two and one supplementary pools for the third instar of *C. cecidophora* and *C. ryukyuensis*, in order to have more replicates for the developmental stage corresponding to the gall-induction in *C. cecidophora*. For *C. cecidophora*, we pooled 20 larvae for first and second instars, 10 larvae for the third instar and 5 for remaining instars. For RNA concentration was measured using the Qubit RNA Assay Kit in Qubit 2.0 Fluorometer (Life Technologies, CA). The quantity of RNA in samples after extraction ranged from 300 ng to more than 1000 ng. RNA quality was examined with a Bioanalyzer (Agilent Technologies, Santa Clara, CA).

### *RNA sequencing and de novo assembly*

Sequencing was conducted on the Illumina NextSeq 500 platform in Kyoto Sangyo University, Kyoto, Japan. RNA-seq libraries were prepared using TruSeq Stranded Total RNA LT kit (Illumina) following the manufacturer's protocol. All libraries were sequenced by paired-end or single-end sequencing protocols (Table S1). Reference sequences for *C. cecidophora* were generated with paired-end reads including all instars (Table S1), because some genes are likely to express only in specific instars. Reference sequences for *C. ryukyuensis* were assembled with paired-end reads from 2$^{nd}$ instars (Table S1). Reads were assembled into transcriptome contigs using Trinity (Grabherr et al., 2011), with following settings "--trimmomatic --SS_lib_type RF" to trim adapter and low quality sequences from raw reads and to specify strand-specific RNA-seq reads. The minimum assembled contig length in our study was 200 bp. Blastx searches of obtained contigs against *Bombyx mori* protein sequence database (NCBI *Bombyx mori* Annotation Release 102) was conducted to find homologous known gene sequences. Gene ontology (GO) information was mapped to each contig based on the results of blastx against non-redundant protein sequences (nr) and InterProScan with Blast2GO (Gotz et al., 2008).

### *Data analysis*

Three different strategies were used to analyze the obtained data. First, because the gall induction phase is limited to the early moment following the first gall-symptoms (Miller and Raman, 2018), we considered genes in *C. cecidophora* that were overexpressed at the third instar compared to the second and the fourth. In order to extract transcripts that were linked to gall induction, highly expressed transcripts specifically in the third instar were selected, and then transcripts whose orthologous sequences were detected in *C. ryukyuensis* were excluded. Second, assuming that effectors maintaining gall tissues must be produced by larvae continuously even after the initiation of gall induction, we also detected genes showing an

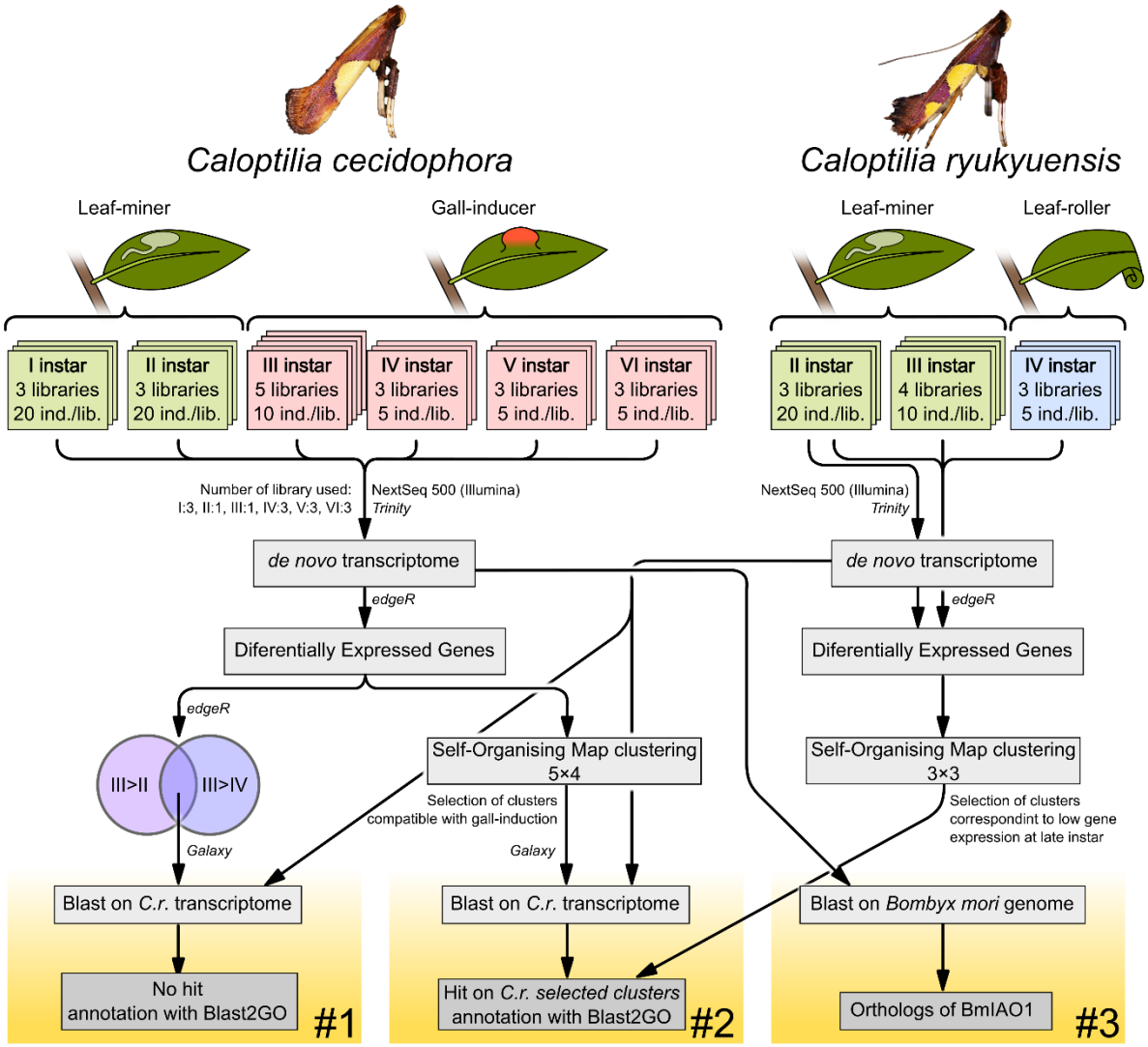



Figure 5.1. Flow diagram of the experimental procedure used to identify putative effectors of gall-induction from *C. cecidophora*.

increased expression during the third and fourth instars or third to fifth instars by using a self-organizing map (SOM) clustering. Third, we used a targeted approach by identifying candidate genes that are likely to be involved in gall induction (e.g. phytohormone related genes), candidate transcripts were extracted according to GO information. We compared expression patterns of the candidate transcriptomes among instars.

*Gene expression profiling with RNA-seq data* (**Approach #1**)

Raw reads were mapped using BWA (Li and Durbin, 2010) (http://bio-bwa.sourceforge.net). Contigs from *de novo* assembly were used as reference sequences for mapping. Transcript expression profiles and DEGs were defined with EdgeR GLMs (Robinson et al., 2010) implemented in R version 3.2.1 (Ref.). Libraries were subjected to trimmed mean of M-values (TMM) normalization in EdgeR. Multi-dimensional scaling was performed by calculating log-fold changes between instars and using DEGs to compute distances in EdgeR with the

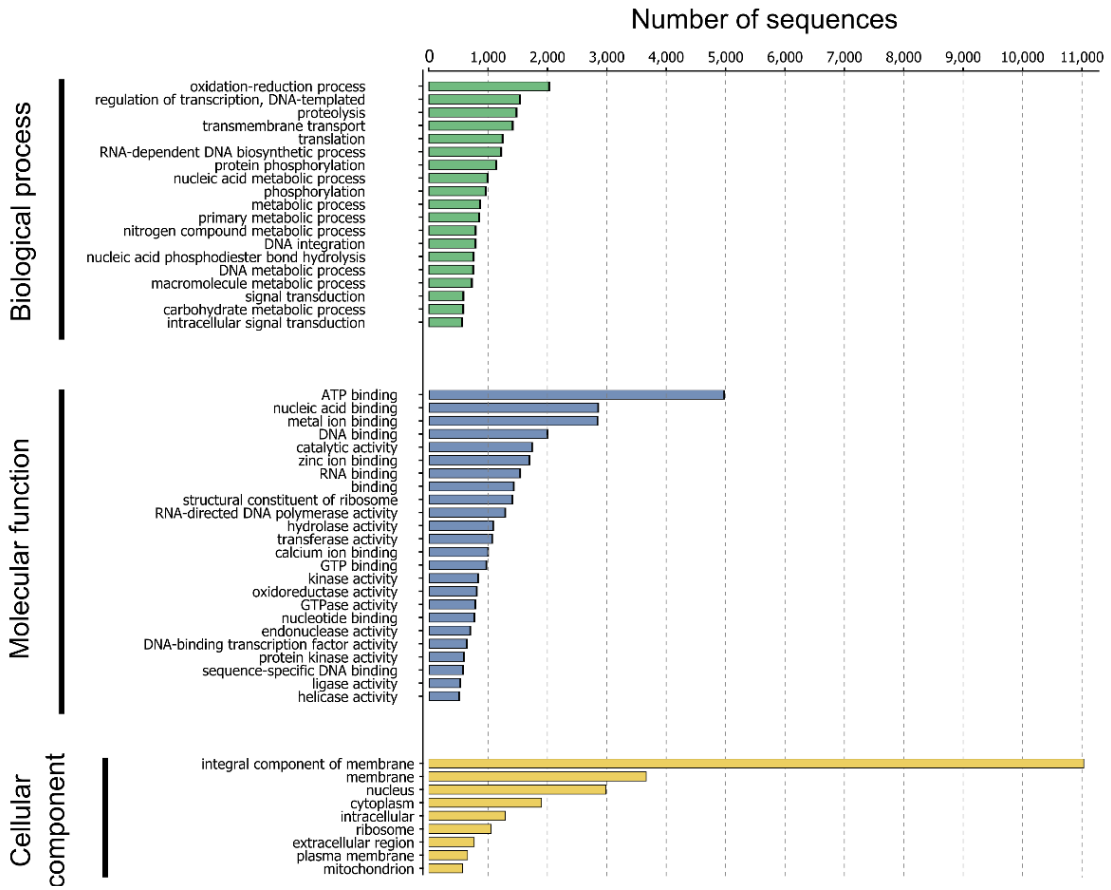



Figure 5.2. Gene ontology assignment for the transcriptome of *Caloptilia cecidophora*. Green: biological process, Blue: molecular function, Yellow: cellular component. These assignments were generated in Blast2GO.

Table 5.1. Transcriptome sequencing and summary statistics of de novo assembling.

| number/length | *C. cecidophora* | *C. ryukyuensis* |
|---|---|---|
| Number of reads from Nextseq (2x150bp, PE) | 512,072,089 | 211,338,082 |
| Total mRNA number | 269,556 | 87,992 |
| Average length of mRNA | 826.81 | 1,063.78 |
| Median | 377 | 557 |
| $N_{50}$ | 1,662 | 1,938 |
| Total gene number | 164,053 | 42,679 |
| Average length of genes | 597.88 | 944.70 |
| Median | 320 | 434 |
| $N_{50}$ | 887 | 1,874 |

"plotMDS" function. Differential expression was calculated using a generalized linear model (GLM) at the gene level with species and instars as factors. The threshold for DEGs was a false discovery rate (FDR) of < 0.01. Genes both less expressed at second and fourth instars than at third instar were extracted from *C. cecidophora* DEGs. Then, the extracted DEGs were

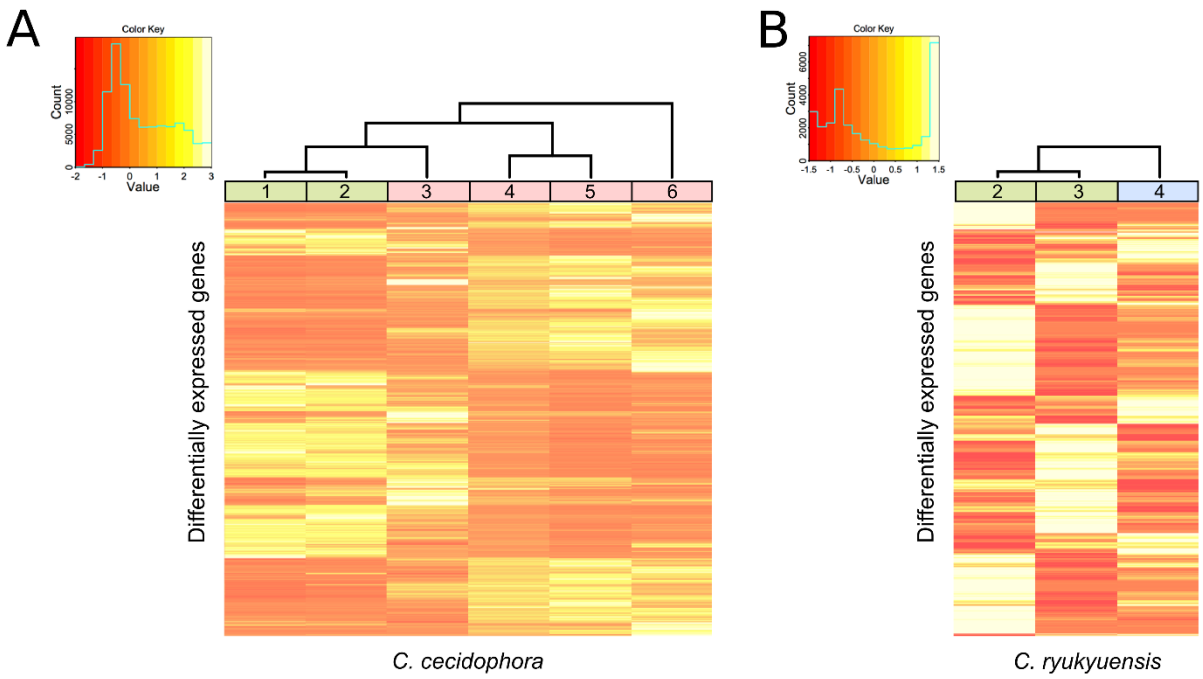



Figure 5.3. Expression profiles of genes that are differentially expressed during the larval development of *C. cecidophora* and *C. ryukyuensis*. (A) Heatmap of differentially expressed genes between the six instars of *C. cecidophora*. (B) Heatmap of differentially expressed genes between the second, third and fourth instars of *C. ryukyuensis*. Numbers correspond to larval instars, green to leaf-miners, pink to gall-inducers and blue to leaf-rollers.

submitted to blastn searches (e-values < 0.001) against the *de novo* assembled sequences of *C. ryukyuensis* to eliminate DEGs whose orthologous sequences were found in *C. ryukyuensis*.

*Self-Organizing Map clustering* (**Approach #2**)

We applied a gene-expression clustering method (Chitwood et al., 2013) on *C. cecidophora* DEGs and *C. ryukyuensis* DEGs defined by EdgeR anova-like test (lowly expressed genes were also filtered based on a minimum sum of 18 counts over all samples). Scaled expression values were used for multilevel 5 × 4 and 3 × 3 rectangular SOM clusters for *C. cecidophora* and for *C. ryukyuensis*, respectively (Figure 5.1) (Kohonen, 1982; Wehrens and Buydens, 2007). We applied 1000 training interactions during clustering, and gene clusters were based on the final assignment of genes to winning units. To focus only on gene-expression patterns instead of expression magnitude, expression values were mean-centered and variance-scaled separately between instars for both species. This procedure allows us to find genes that vary in expression patterns between the two species. Network graphics in Gephi (Bastian et al., 2009) were used to visualize the assignment of genes involved in each SOM cluster between species.

*Literature-based targeted gene approach* (**Approach #3**)

Gall-induction processes are mediated by plant growth regulators (Tooker and Helms, 2014). Among the various phytohormones suspected to be involved in the molecular mechanisms of gall induction by insects, the indole-3-acetic acid (IAA), also called auxin, plays a major role; high concentrations of IAA have been measured in the body of several gall-inducing insects (e.g. Yamaguchi et al., 2012) and a novel plant-independent IAA biosynthesis pathway has

been identified in insects (Suzuki et al., 2014; Takei et al., 2015; Yokoyama et al., 2017). Recently, BmIAOx, the enzyme catalyzing the last step of IAA biosynthesis has been identified in *B. mori* (Takei et al., 2019). We looked for orthologous genes of this enzyme in the transcriptomes of *C. cecidophora* based on the Blastx searches on *B. mori* protein sequence database.

# Results

## *Transcriptome sequencing, de novo assembly and defining differentially expressed genes*

384,060,343 reads were used for *C. cecidophora* and 61,170,034 for *C. ryukyuensis de novo* assemblies (Table 1). The reads were assembled into 269,556 transcript contigs, showing $N_{50}$ and average lengths of 1,662 bp and 826.81 bp for *C. cecidophora*, and into 87,992 transcript contigs showing $N_{50}$ and average lengths of 1938 bp and 1063.78 bp for *C. ryukyuensis* (Table 1). According to their $N_{50}$ values, we considered the *de novo* assembly of both species as enough quality for our present study. Based on these transcript contigs, Trinity generated 164,053 genes for *C. cecidophora*, with $N_{50}$ and average lengths of 887 bp and 597.88 bp respectively, and 42,679 genes for *C. ryukyuensis*, with $N_{50}$ and average lengths of 1874 bp and 944.7 bp (Table 1). After annotation, the most predominant GO terms under the "biological process" category were as follows: oxidation-reduction process (GO: 0055114), regulation of transcription (GO: 0006355), and proteolysis (GO: GO: 0006508), followed by transmembrane transport (GO: 0055085). Under "molecular function," ATP binding (GO: 0005524), nucleic acid binding (GO: 0003676) and metal ion binding (GO: 0046872) were the most enriched terms. Under the "cellular component" category, integral component of membrane (GO: 0016021), membrane (GO: 0016020), and nucleus (GO: 0005634) were the most prominent (Figure 5.2). EdgeR defined 15,409 DEGs among larval instars of *C. cecidophora* and 10,167 DEGs among the second, third and fourth instars of *C. ryukyuensis*. The heatmap (Figure 5.3A) shows that expression in *C. cecidophora* larvae were similar between first and second instars (leaf-mining stages), and between fourth and fifth instars (gall-maturation stages), but that the third instar (gall initiation stage) demonstrated an intermediate pattern between the two groups. The sixth instar of *C. cecidophora* exhibited a different pattern from those of fourth and fifth instars

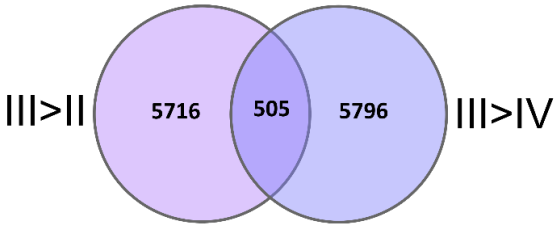



Figure 5.4. Results of approach #1. Venn diagram of *C. cecidophora* genes overexpressed in third instars compared to second and fourth instars.

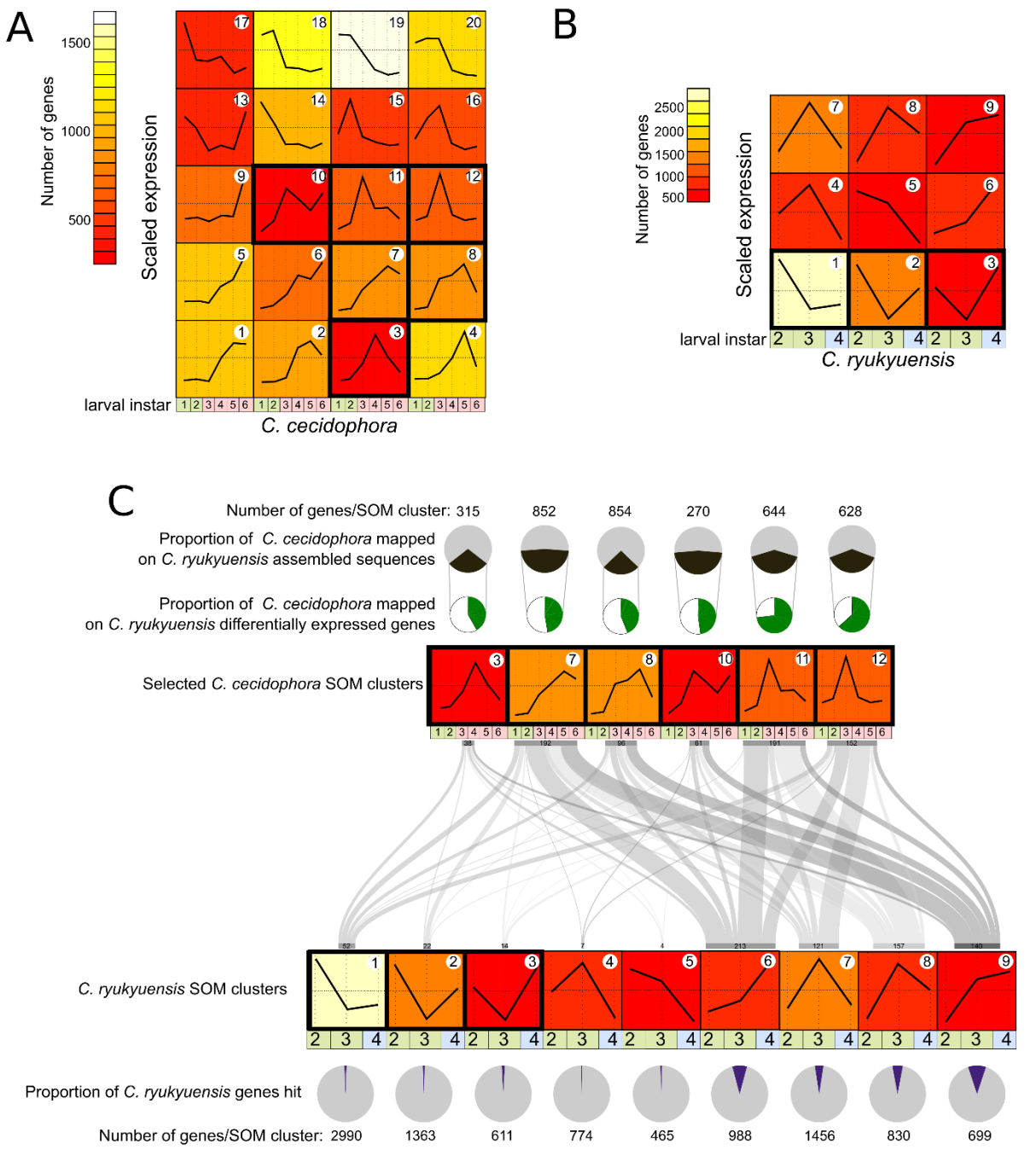



Figure 5.5. Results of approach #2. (A) Five by four SOM clustering of gene expression in differentially expressed genes (DEGs) of *C. cecidophora*. In bold squares, selected *C. cecidophora* clusters after filtering out clusters with expression patterns incompatible with gall-induction. Numbers correspond to larval instars, green to leaf-miners, pink to gall-inducers and blue to leaf-rollers. (B) Three by three SOM clustering of gene expression in DEGs *C. ryukyuensis*. In bold squares, selected *C. ryukyuensis* clusters with a decrease of gene expression at the third instar. (C) Sankey diagram of the result of the Blastn of the SOM cluster 3, 7, 8, 10, 11 and 12 of *C. cecidophora* on *C. ryukyuensis* DEGs clustered by SOM. Upper panel shows the number of gene per *C. cecidophora* cluster with the proportion of genes mapped on *C. ryukyuensis* assembled sequences and DEGs. Lower panel shows the number of genes per *C. ryukyuensis* cluster, with the proportion of genes hit by *C. cecidophora* genes belonging to the selected clusters.

(Figure 5.3A). The heatmap of *C. ryukyuensis* (Figure 5.3B) showed clear differences of expression patterns between second, third and fourth instars. According to the hierarchical clustering, the expression pattern of the third instar is closer to the second than to the fourth instar, which is similar to the results obtained in *C. cecidophora*.

### *Gene expression profiling with RNA-seq data (Approach #1)*

We found 6,221 genes that were significantly upregulated in third than in second instars, and 6,301 genes that were significantly upregulated in third than in fourth instars (Figure 5.4A). Among these genes, 505 genes were significantly upregulated in both comparisons (Figure 5.4A). The homology search using blastn of these 505 genes against *C. ryukyuensis* assembled sequences showed that 378 genes were specific to *C. cecidophora* (Figure 5.4B). The Blast2GO analysis of the 378 *C. cecidophora* specific genes revealed that only 22 genes were successfully annotated on the GO database (Figure 5.4B, Table 5.2) corresponding to the final list of candidate effectors obtained through approach #1.

### *Self-Organizing Map clustering (Approach #2)*

*Caloptilia cecidophora* DEGs were partitioned into 20 clusters with a five by four SOM clustering (Figure 5.5A, Figure 5.S1). Given the lower number of instars analyzed for *C. ryukyuensis*, DEGs of this species were partitioned into 9 clusters with only a three-by-three

Table 5.2. Annotated genes resulting from the Approach #1.

| Cc unigene | Annotation | Blast e-value |
|---|---|---|
| TRINITY_DN36608_c0_g1_i1 | general odorant-binding protein 69a-like | 1.31E-55 |
| TRINITY_DN36608_c0_g1_i2 | general odorant-binding protein 69a-like | 2.16E-56 |
| TRINITY_DN41192_c0_g1_i1 | retrovirus-like pol polyprotein | 1.88E-99 |
| TRINITY_DN41598_c0_g2_i1 | heat shock protein 68-like | 0 |
| TRINITY_DN42214_c0_g1_i1 | major heat shock 70 kDa protein Ab-like | 0 |
| TRINITY_DN43903_c7_g1_i1 | endonuclease-reverse transcriptase | 2.96E-41 |
| TRINITY_DN44326_c5_g1_i1 | Zinc metalloproteinase nas-14 | 3.65E-94 |
| TRINITY_DN44326_c5_g1_i2 | Zinc metalloproteinase nas-14 | 2.02E-128 |
| TRINITY_DN47163_c8_g1_i1 | gag-pol polyprotein | 4.38E-13 |
| TRINITY_DN47685_c3_g1_i7 | ATP-dependent DNA helicase pif1-like | 1.31E-42 |
| TRINITY_DN48268_c3_g1_i6 | aldo-keto reductase AKR2E4-like | 4.61E-19 |
| TRINITY_DN48789_c5_g1_i4 | catalase-like | 1.63E-151 |
| TRINITY_DN48789_c5_g1_i7 | catalase-like | 7.30E-153 |
| TRINITY_DN49457_c1_g1_i3 | cell surface protein precursor | 1.28E-27 |
| TRINITY_DN49457_c1_g1_i7 | hypothetical chloroplast RF1 | 4.00E-09 |
| TRINITY_DN49457_c1_g1_i8 | hypothetical chloroplast RF1 | 1.45E-09 |
| TRINITY_DN49457_c1_g5_i1 | cell wall anchor protein | 1.62E-14 |
| TRINITY_DN50798_c0_g1_i5 | hypothetical protein ALC57_08299 | 5.26E-96 |
| TRINITY_DN50955_c8_g1_i7 | RNA-directed DNA polymerase from mobile element jockey-like | 5.08E-33 |
| TRINITY_DN52711_c3_g1_i1 | serine/threonine-protein kinase BRSK2-like | 2.63E-22 |
| TRINITY_DN56113_c0_g1_i1 | general odorant binding protein 2 | 4.47E-86 |
| TRINITY_DN74715_c0_g1_i1 | odorant binding protein | 5.72E-20 |

SOM clustering analysis (Figure 5.5B and Figure 5.S2). In *C. cecidophora*, clusters from 1 to 15 show genes with a higher expression during either gall-inducing instars (third to sixth instars). Only, clusters 3, 7, 8, 10, 11 and 12 show upregulation at the third instar, and this could be considered as signal that these clusters contain possible effectors for gall-induction.

Among *C. ryukyuensis* clusters, only the 1, 2 and 3 show a decrease of expression at third instar. In total 3,563 genes were involved in the six selected clusters of *C. cecidophora*, and within them, 730 blasted on C. ryukyuensis differentially expressed genes, and 88 blasted of *C. ryukyuensis* selected clusters. Blast2GO analysis of the *C. cecidophora* specific genes revealed

Table 5.3. Annotated genes resulting from the Approach #2.

| Cc unigene | Annotation | value |
|---|---|---|
| TRINITY_DN35105_c0_g1_i1 | heat shock protein 83 | 0 |
| TRINITY_DN42442_c0_g1_i1 | arginine esterase | 3.13E-76 |
| TRINITY_DN43422_c5_g1_i1 | trypsin, alkaline C-like | 9.21E-33 |
| TRINITY_DN43838_c2_g1_i2 | ATP-binding cassette sub-family A ABCA2 | 0 |
| TRINITY_DN43838_c2_g1_i3 | ATP-binding cassette sub-family A ABCA2 | 1.16E-147 |
| TRINITY_DN43838_c2_g1_i4 | ATP-binding cassette sub-family A ABCA2 | 0 |
| TRINITY_DN43838_c2_g1_i5 | ATP-binding cassette sub-family A ABCA2 | 0 |
| TRINITY_DN43950_c8_g1_i1 | 5-hydroxytryptamine receptor 3C | 3.03E-53 |
| TRINITY_DN44716_c0_g3_i1 | heat shock protein 68-like | 0 |
| TRINITY_DN45480_c13_g1_i2 | catalase-like | 9.87E-156 |
| TRINITY_DN45625_c6_g1_i1 | acidic mammalian chitinase-like | 1.75E-90 |
| TRINITY_DN46622_c8_g7_i1 | cytochrome P450 307a1 | 0 |
| TRINITY_DN46857_c10_g1_i1 | organic cation transporter protein-like | 0 |
| TRINITY_DN46857_c10_g1_i1 | organic cation transporter protein-like | 0 |
| TRINITY_DN46917_c2_g2_i1 | peroxiredoxin-6 | 2.09E-120 |
| TRINITY_DN47930_c7_g1_i3 | 4-coumarate--CoA ligase 1-like | 2.94E-103 |
| TRINITY_DN47930_c7_g1_i6 | 4-coumarate--CoA ligase 1-like | 0 |
| TRINITY_DN47930_c7_g1_i9 | 4-coumarate--CoA ligase 1-like | 0 |
| TRINITY_DN48411_c1_g1_i1 | organic cation transporter protein-like | 0 |
| TRINITY_DN48411_c1_g1_i4 | organic cation transporter protein-like | 0 |
| TRINITY_DN48411_c1_g1_i6 | organic cation transporter protein-like | 0 |
| TRINITY_DN48411_c1_g1_i7 | organic cation transporter protein-like | 0 |
| TRINITY_DN48411_c1_g1_i8 | organic cation transporter protein-like | 2.17E-34 |
| TRINITY_DN48537_c7_g1_i2 | putative inorganic phosphate cotransporter | 0 |
| TRINITY_DN49836_c2_g1_i1 | probable cytochrome P450 305a1 | 2.57E-144 |
| TRINITY_DN50740_c5_g1_i5 | probable chitinase 2 | 3.78E-139 |
| TRINITY_DN50817_c2_g2_i2 | delta(24)-sterol reductase-like | 0 |
| TRINITY_DN50878_c4_g1_i1 | scavenger receptor class B member 1-like | 0 |
| TRINITY_DN50878_c4_g1_i2 | scavenger receptor class B member 1-like | 0 |
| TRINITY_DN50878_c4_g1_i3 | scavenger receptor class B member 1-like isoform X2 | 0 |
| TRINITY_DN50878_c4_g1_i6 | scavenger receptor class B member 1-like | 0 |
| TRINITY_DN50995_c1_g2_i1 | UDP-glucuronosyltransferase 2B10-like | 7.29E-157 |
| TRINITY_DN51519_c4_g1_i1 | UDP-glucuronosyltransferase 2B1-like | 3.65E-171 |
| TRINITY_DN51519_c4_g1_i2 | UDP-glucuronosyltransferase 2B1-like | 1.48E-169 |
| TRINITY_DN52330_c2_g1_i2 | membrane-bound alkaline phosphatase-like | 2.18E-15 |
| TRINITY_DN53052_c15_g2_i2 | trypsin, alkaline C-like | 2.96E-72 |

that only 36 genes were successfully annotated, and the final list of candidate effectors obtained through approach #2 were shown in Table 5.3.

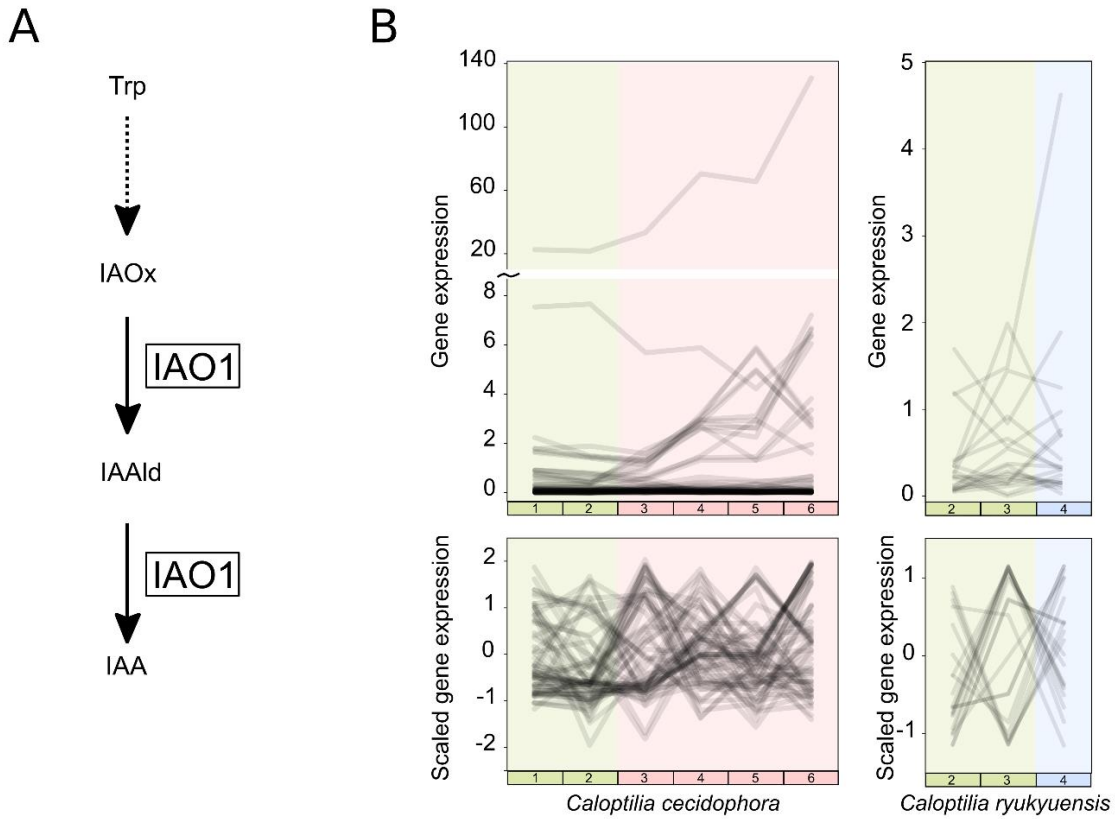
### *Literature-based targeted gene approach (Approach #3)*

The blastn of the sequence of BmIAO1 (XM_004925903.3), the enzyme involved in the auxin synthesis (Takei et al., 2019) (Figure 5.6A), against the *de novo* assembled sequences of *C. cecidophora* and *C. ryukyuensis* detected seven transcripts on *C. cecidophora* but none on *C. ryukyuensis* (Figure 5.6C). In addition, blastx search of *B. mori* "indole-3-acetaldehyde oxidase-like" genes against *C. cecidophora* and *C. ryukyuensis* detected 67 and 20 transcripts, respectively. In the *C. cecidophora* orthologs, one outlier showed an increased expression pattern from the third instar and a peak at the sixth instar (Figure 5.6B). Except one ortholog showing a peak of expression at fourth instar, no clear trends were found among *C. ryukyuensis* orthologs (Figure 5.6B).

## Discussion

There is no unique way to compare two datasets. In the present study, we decided to follow three analytical strategies corresponding to three assumptions non-mutually exclusive regarding the processes of gall-induction: the induction occurs in a short period of time (#1), the induction is a continuous process persisting on the gall maturation phase (#2), and gall induction is mediated by auxin secreted by insects (#3). Despite of the difficulty to identify sequenced genes that is inherent of every work on non-model species, these preliminary analyses allowed to identify several candidate effectors. This study also confirms that SOM is an efficient method to correlate gene expression patterns with the gall phenotype (Nakayama et al., 2018).

With more than 2,000 genes, the oxidation-reduction process category dominated the biological processes identified in *C. cecidophora* transcriptome. The first analysis approach revealed two catalases as candidate effectors (Table 5.2) and the second analysis approach revealed one catalase and one peroxiredoxin (Table 5.3), both enzymes being involved in the oxidation of hydrogen peroxide (Schonbaum and Chance, 1976; Sharapov et al., 2014). Considered as a reactive oxygen species (ROS), the hydrogen peroxide ($H_2O_2$) is a central element of the inflammatory response of animals (Wittmann et al., 2012) and of the hypersensitive response of plants (Levine et al., 1994), both being a defensive reaction to damage. In these processes, the $H_2O_2$ release in the inter-cellular space plays at the same time a role of direct defense against herbivores and a role of danger signal messenger allowing plants to adopt an appropriate physiological response to the attackers (Zebelo and Maffei, 2015). The hyper-sensitive response is involved in the resistance of plants against several gall-inducers (Cornelissen et al., 2002; Höglund et al., 2005). A rapid accumulation of ROS, called oxidative burst, is observed in wheat (*Triticum aestivum*) at the attack site during incompatible interaction with the gall midge *Mayetiola desctuctor* (Liu et al., 2010).




C

```
TRINITY_DN41246_c0_g2_i1    ----------------------------------------------------------------------------------------------------    0
TRINITY_DN41246_c0_g2_i2    ----------------------------------------------------------------------------------------------------    0
TRINITY_DN49610_c3_g2_i12   ----------GCAACCGTATCCATCAATCAAGGCGATGCAACCGTAACCATAGTCCATGGTGGTGTCGAAATGGGGCAAGGTATAAACACTAAAGCCATGCAAATTTGCGCT    102
TRINITY_DN49610_c3_g2_i10   ----------GCAACCGTATCCATCAATCAAGGCGATGCAACCGTAACCATAGTCCATGGTGGTGTCGAAATGGGGCAAGGTATAAACACTAAAGCCATGCAAATTTGCGCT    102
TRINITY_DN49610_c3_g2_i5    ----------GCAACCGTATCCATCAATCAAGGCGATGCAACCGTAACCATAGTCCATGGTGGTGTCGAAATGGGGCAAGGTATAAACACTAAAGCCATGCAAATTTGCGCT    102
TRINITY_DN49610_c3_g2_i4    ----------GCAACCGTATCCATCAATCAAGGCGATGCAACCGTAACCATAGTCCATGGTGGTGTCGAAATGGGGCAAGGTATAAACACTAAAGCCATGCAAATTTGCGCT    102
TRINITY_DN49610_c3_g2_i1    ----------GCAACCGTATCCATCAATCAAGGCGATGCAACCGTAACCATAGTCCATGGTGGTGTCGAAATGGGGCAAGGTATAAACACTAAAGCCATGCAAATTTGCGCT    102
BmIAO1                      AGCGCTGGTATAACCGGAGCAACCGTATCCATCAATCAAGGCGATGCAACCGTAACCATAGTCCATGGTGGTGTCGAAATGGGGCAAGGTATAAACACTAAAGCCATGCAAATTTGCGCT    3000

TRINITY_DN41246_c0_g2_i1    ----------------------------------------------------------    0
TRINITY_DN41246_c0_g2_i2    ----------------------------------------------------------    0
TRINITY_DN49610_c3_g2_i12   CA----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i10   CA----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i5    CA----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i4    CA----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i1    CA----------------------------------------------------------    104
BmIAO1                      CATTTGCTCAAAATTTCCATGGATAAAATCCAAGTCAAAGCAACTAATACGATAGCTTTC    3060

TRINITY_DN41246_c0_g2_i1    ----------------------------------------------------------AGAGGTAGAAATTGACGTGTTGACTGGAGAACATGAAATCCTTCGAGTTGATCTTATCGAGGAT    64
TRINITY_DN41246_c0_g2_i2    ----------------------------------------------------------------------------------------------------    0
TRINITY_DN49610_c3_g2_i12   ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i10   ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i5    ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i4    ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i1    ----------------------------------------------------------------------------------------------------    104
BmIAO1                      GGATTACGCAGTGCCTCTAATTTTGAGCCTTATGATGTTTTTGGAGCTACATTAGCAGAGGTAGAAATTGACGTGTTGACTGGAGAACATGAAATCCTTCGAGTTGATCTTATCGAGGAT    3360


TRINITY_DN41246_c0_g2_i1    ----GTAGGAACTTCAGTAAACCCTGAAATCGATATTGGACAAATCGAAGGTGCTTTCGT----GTAGGAACTTCAGTAAACCCTGAAATCGATATTGGACAAATCGAAGGTGCTTTCGT    120
TRINITY_DN41246_c0_g2_i2    ----------------------------------------CAAATCGAAGGTGCTTTCGT----------------------------------------CAAATCGAAGGTGCTTTCGT    20
TRINITY_DN49610_c3_g2_i12   ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i10   ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i5    ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i4    ----------------------------------------------------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i1    ----------------------------------------------------------------------------------------------------    104
BmIAO1                      ----GTAGGAACTTCAGTAAACCCTGAAATCGATATTGGACAAATCGAAGGTGCTTTCGT----GTAGGAACTTCAGTAAACCCTGAAATCGATATTGGACAAATCGAAGGTGCTTTCGT    3416
                            ****                                    ****

TRINITY_DN41246_c0_g2_i1    CATGGGTTTGGGTTATTGGACCTGTGAACAATTGGTCTACAA----------    162
TRINITY_DN41246_c0_g2_i2    CATGGGTTTGGGTTATTGGACCTGTGAACAATTGGTCTACAA----------    62
TRINITY_DN49610_c3_g2_i12   ----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i10   ----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i5    ----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i4    ----------------------------------------------------------    104
TRINITY_DN49610_c3_g2_i1    ----------------------------------------------------------    104
BmIAO1                      CATGGGTTTGGGTTATTGGACCTGTGAACAATTGGTCTACAACTCCACTTCTGGCCAACT    3476
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Figure 5.6. Results of approach #3. (A) IAA biosynthesis pathway in *Bombyx mori* as described by Takei et al. (2019). (B) Expression profile of predicted indole-3-acetic acid oxidase 1 genes, in *C. cecidophora* on the left panel (67 genes), in *C. ryukyuensis* on the right panel (20 genes). Numbers correspond to larval instars, green to leaf-miners, pink to gall-inducers and blue to leaf-rollers. (C) Sequence alignment of BmIAO1 (XM_004925903.3) and the seven Blastn hits on C. cecidophora assembled sequences.

Peroxiredoxins (PRXs) have been found to be a part of the anti-oxidant arsenal of several animal parasites and were identified in the salivary secretions of several herbivorous insects (Guiguet et al., 2016). PRXs are present in the salivary gland transcriptome or proteome of the aphids *Acyrthosiphon pisum* and *Megoura viciae* (Cooper et al., 2011), and the caterpillars

*Helicoverpa armigera* and *Spodoptera exigua* (Afshar et al., 2013; Celorio-Mancera et al., 2011). Interestingly, this enzyme is also found in the transcriptome and proteome of the Hessian fly *M. destructor* (Chen et al., 2008) and is a virulence factor of the plant parasitic and gall-inducer nematode *Meloidogyne incognita* (Dubreuil et al., 2011). Catalase activity has been found in salivary secretions of several moth species (*Helicoverpa zea*, *Spodoptera exigua*, *Manduca sexta*, and *Heliothis virescens*) and was suggested to reduce inducible plant defenses by catalyzing $H_2O_2$ oxidation (Felton and Duffey, 1991). Similarly, *M. destructor* has evolved a complex antioxidant mechanism to overcome the wheat hypersensitive response, and catalases take an important part in them (Mittapalli et al., 2007). Thus, catalases and the peroxiredoxin identified among genes that are overexpressed during gall-inducing instars may play a role in counteracting the hypersensitive response.

The ROS accumulation in galls has also been suggested to be involved in gall induction (Oliveira et al., 2016). According to this explanatory model, the prolonged hypersensitive response would lead to an accumulation of phenolic compounds that, in turn, triggers an increase level of the growth promoting phytohormone auxin (Oliveira et al., 2016). The higher expression of anti-oxidant proteins in *C. cecidophora* compared to *C. ryukyunesis* suggests a higher resistance against oxidative stress in this species, and is compatible with the phenolic hypothesis. Interestingly, *C. cecidophora* does not produce effectors that will act downstream of the $H_2O_2$ production such as apyrases and calreticulins (Guiguet et al., 2016). So *C. cecidophora* presumably does not block the plant early signaling allowing for the plant-production of ROS that is required for the gall induction but gallers invest in antioxidant mechanisms to prevent any ROS-associated damage to the insect.

The list of candidate effectors obtained by the second approach also contains two chitinases, a "probable chitinase 2" and an "acidic mammalian chitinase-like" (Table 5.3). Insect chitinases are usually considered are defensive mechanisms of insects against fungal pathogens (Hamid et al., 2013). Interestingly, chitinases genes are highly expressed in the larvae of the oak gall cynipid *Biorhyza palida* during gall induction (Hearn, 2013). These chitinases may modify arabinogalatan proteins in the matrix of plant tissues and therefore trigger an embryogenesis-like dedifferentiation and stimulate cell divisions in plant tissues (Hearn, 2013). Indeed arabinogalactans protein contain cleavage sites hydrolysable for chitinases and control somatic embryogenesis in plants (van Hengel et al., 2001). Chitinase that are overexpressed by *C. cecidophora* during gall-induction stages could play a similar role.

The third analysis strategy identified orthologous sequences of BmIAO1, in both *C. cecidophora* and *C. ryukyuensis* transcriptomes suggesting an ability of these insects to synthesize auxin like in *B. mori* (Takei et al., 2019). The increased expression of the most expressed ortholog from third to fourth instars is compatible with a role of IAA in gall-

formation, with a specific action at the gall-maturation phase. However, the significance of the peak of expression at the sixth instar while galls no longer develop remains to be established. Presence of high levels of IAA has already been measured in several gall-inducing insects (Mapes and Davies, 2001; Tooker and De Moraes, 2011), sometimes with evidence of IAA synthesis by the insect (Takei et al., 2015; Tanaka et al., 2013; Yamaguchi et al., 2012). Whether the higher expression of genes involved in IAA biosynthesis in larvae translate into significant concentrations of IAA in the insect body that could be delivered to the plant need further investigations. An ability of *C. cecidophora* to produce biologically active IAA would strengthen the general belief that IAA play an important role in gall-induction by insects.

This study constitutes a preliminary analysis towards the identification of possible candidate effectors involved in gall induction. Further work is now needed to fully exploit the dataset. This study should now also be combined with 1) RT-qPCR to confirm the expression pattern of selected candidates; 2) targeted transcriptomic and proteomic approaches on salivary glands to confirm that candidate effectors are secreted and delivered to the plant; and 3) functional tests on candidate effectors, by CRISP-R silencing for example, to identify targeted plant physiological processes.

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# Supplementary Figures and Tables

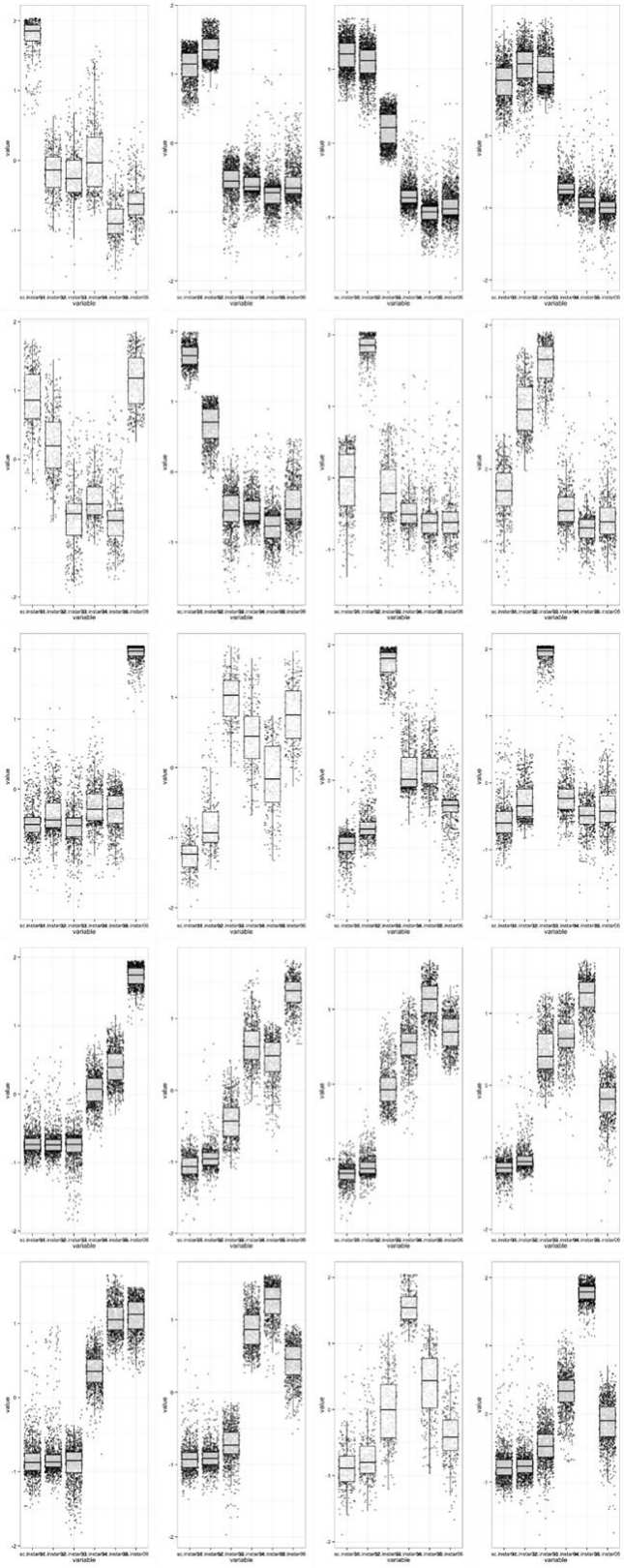


Figure 5.S1. Scaled expression during larval development of *Caloptilia cecidophora*.

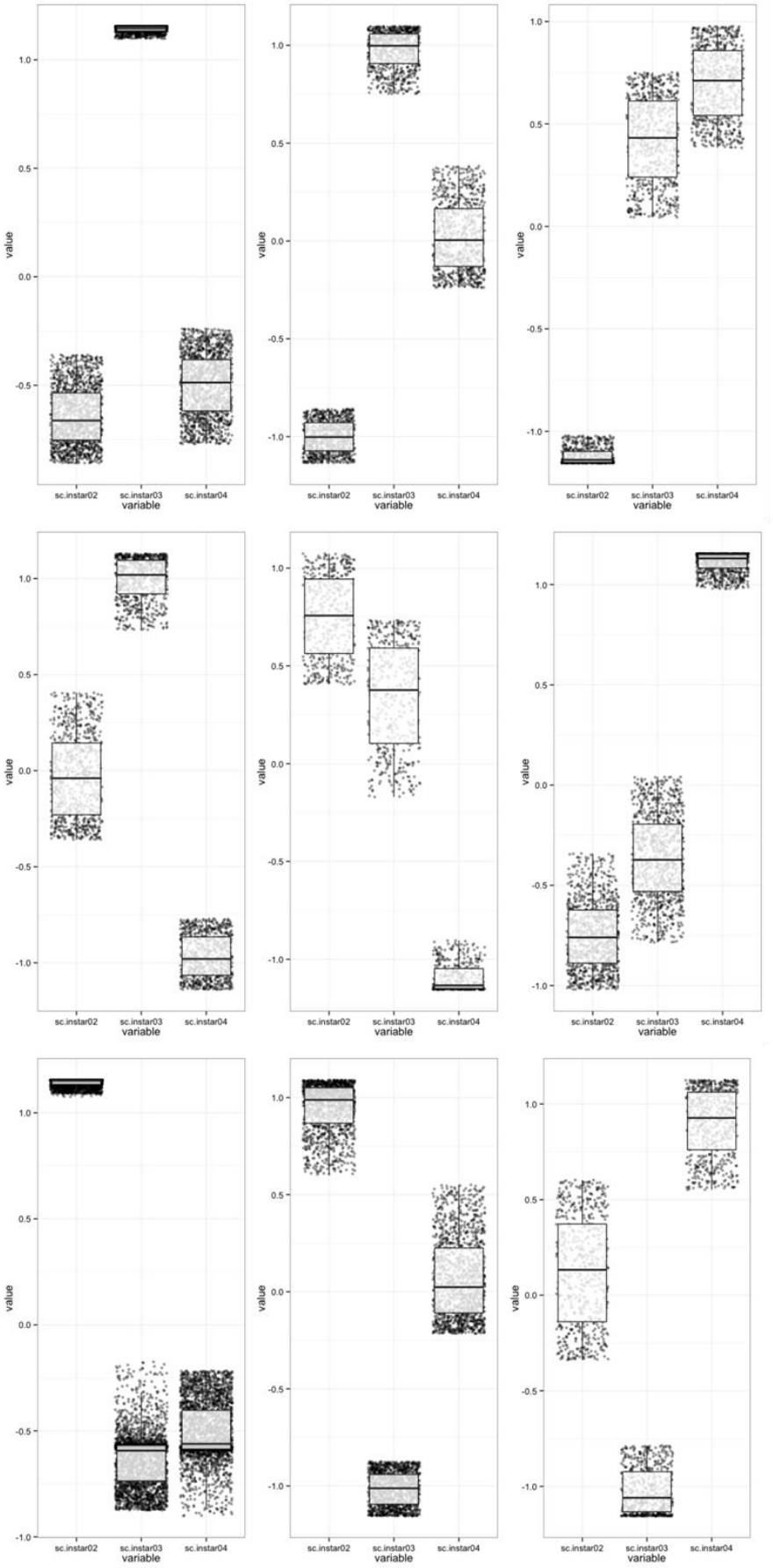



Figure 5.S2. Scaled expression between second, third and fourth instars of *Caloptilia ryukyuensis*.

Table 5.S1. Summary of library information.

| Sample | species | instar | Number of larvae used | single or pair | used for assembly |
|---|---|---|---|---|---|
| AGE-013 | *C. cecidophora* | 1st | 20 | paired | yes |
| AGE-018 | *C. cecidophora* | 1st | 20 | paired | yes |
| AGE-046 | *C. cecidophora* | 1st | 20 | single | |
| AGE-014 | *C. cecidophora* | 2nd | 20 | paired | yes |
| AGE-047 | *C. cecidophora* | 2nd | 20 | single | |
| AGE-068 | *C. cecidophora* | 2nd | 20 | single | |
| AGE-019 | *C. cecidophora* | 3rd | 10 | paired | yes |
| AGE-044 | *C. cecidophora* | 3rd | 10 | single | |
| AGE-045 | *C. cecidophora* | 3rd | 10 | single | |
| AGE-055 | *C. cecidophora* | 3rd | 10 | single | |
| AGE-070 | *C. cecidophora* | 3rd | 10 | single | |
| AGE-009 | *C. cecidophora* | 4th | 5 | paired | yes |
| AGE-010 | *C. cecidophora* | 4th | 5 | paired | yes |
| AGE-011 | *C. cecidophora* | 4th | 5 | paired | yes |
| AGE-005 | *C. cecidophora* | 5th | 5 | paired | yes |
| AGE-006 | *C. cecidophora* | 5th | 5 | paired | yes |
| AGE-007 | *C. cecidophora* | 5th | 5 | paired | yes |
| AGE-020 | *C. cecidophora* | 6th | 5 | paired | yes |
| AGE-021 | *C. cecidophora* | 6th | 5 | paired | yes |
| AGE-022 | *C. cecidophora* | 6th | 5 | paired | yes |
| AGE-071 | *C. ryukyuensis* | 2nd | 10 | paired | yes |
| AGE-072 | *C. ryukyuensis* | 2nd | 10 | paired | yes |
| AGE-073 | *C. ryukyuensis* | 2nd | 10 | paired | yes |
| AGE-050 | *C. ryukyuensis* | 3rd | 5 | single | |
| AGE-048 | *C. ryukyuensis* | 3rd | 5 | single | |
| AGE-049 | *C. ryukyuensis* | 3rd | 5 | single | |
| AGE-051 | *C. ryukyuensis* | 3rd | 5 | single | |
| AGE-075-2 | *C. ryukyuensis* | 4th | 3 | single | |
| AGE-078-1 | *C. ryukyuensis* | 4th | 3 | single | |
| AGE-078-2 | *C. ryukyuensis* | 4th | 3 | single | |

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# Chapter 6.

# General Discussion

In biology, classification of observed phenomena meets an epistemological necessity. Plant-insect interactions are diverse (Menken et al., 2010; Schoonhoven et al., 2005) and can be categorized according to the degree of food specialization (specialists *vs.* generalists), the targeted nutritional tissues (sap- *vs.* fluid- *vs.* tissue-feeder), or also the location of the insects in or on the plant (endo- *vs.* ectophagous). The distinction between ectophagous and endophagous herbivores on the one hand, and among the second group, the differentiation of stem-borers, leaf-miners and gall-inducers on another hand is necessary to study the ecology and evolution of insect-plant interactions. At a finer scale, Hering (1951) suggested a classification of leaf-mines based on insect orders and the histology of leaf damage, and Küster (1911) grouped galls according to the degree of differentiation of plant tissues while Dreger-Jauffret and Shorthouse classified them according to the mode of gall formation (Dreger-Jauffret and Shorthouse, 1992) (Chapter 1).

However, while classification undoubtedly sheds light on evolution of organisms and often illustrates their ecology, it may also restrict the scientific community into a biased view of existing relationships between living organisms. Understanding the studied phenomenon often requires questioning existing classifications, and focusing on cases which do not fit within fixed categories. For instance, in paleontology, transitional fossils, sharing traits with both an ancestral and a derived descendant group (Freeman and Herron, 2014), represent a consistent evidence to strengthen or reject an evolutionary hypothesis. These fossils remind us that taxonomic divisions are human constructs that arrange in discrete categories a continuum of variable forms and traits.

Such a questioning of existing classifications has helped, for example, to better understand complex interactions that occur between plant and fungi. Organisms (and specifically microorganisms) have long been divided into two main categories: parasites that develop at the cost of their host, and mutualists that benefit from their host but also provide some rewards in return. While early studies on mycorrhiza clearly identified them as beneficial for the plant, further work has shown that some could be considered as having a negative impact on the plant, blurring lines between mutualism and parasitism and leading to the concept of mutualism-parasitism continuum of mycorrhizal functions (Johnson et al., 1997). If the existence of this continuum is still discussed, it helped to better consider ecological and evolutionary questions raised by the transitions between mutualistic and parasitic states (Johnson and Graham, 2013).

The main aim of this thesis was to study biological, evolutionary and molecular aspects of transitional states between the leaf-mining and the gall-inducing habits in micromoths of the family Gracillariidae. Through a combination of histology and experimental manipulations, we demonstrated that *Borboryctis euryae* induces a “mine-gall”, sharing properties of leaf-miners and gall-inducers at the same time (Chapter 2). In a second part, we showed that *Caloptilia cecidophora* successively adopts both life-styles during its larval development and that gall-inducing evolved from leaf-mining (Chapter 3). Then using a combination of traditional fieldwork with cutting-edge phylogenomic techniques we investigated the evolutionary history of the origin of the gall-inducing habit in the *Caloptilia* genus (Chapter 4). Finally, by taking advantage of the life-style transition occurring along *C. cecidophora* larval development and of

the recent evolutionary transition to gall-inducing in *Caloptila*, we developed a comparative transcriptomic approach in order to find candidate effectors involved in gall induction (Chapter 5).

In this last chapter, I discuss how this project advances our understanding of plant insect interactions, and highlight the importance to consider plant-insect interactions beyond descriptive categories. In particular, I propose to study insects-plant interactions as a continuum of manipulation forms.

# 1. Intermediate life-styles suggest a continuum of plant manipulation in insects

## 1.1. Mine-gall: an overlooked life strategy

As shown in the second chapter, *B. euryae* employes an intermediate feeding strategy between leaf-mining and gall-inducing, with larvae not only mining leaves but also inducing the formation of a callus tissue on which they feed. The formation of this nutritive tissue within the leaf-mine is a consequence of the interaction between the insect and its host-plant as larvae are required for its induction. Contrary to other leaf-miners, "mine-galling" insects strictly require this additional tissue for a successful development. This life-style also differs with most gall-inducing organisms by the fact that the insect-induced newly generated tissue is made of undifferentiated cells. The term "mine-gall" was first introduced by New (1981) to designate the inflated mine of another Gracillariidae, described as an *Epicephala* sp., on *Acacia* leaves. Like *B. euryae*, the larvae bore a serpentine mine through the epidermis and then create a blotch mine within the spongy parenchyma in which they induce a proliferation of callus (Figure 6.1A and 1B). Based on field observations, the author hypothesized that the insect is grazing the callus, at least occasionally to meet its nutritional requirements.

Several species described in the literature or only observed (by us or by colleagues) appear to have a similar "mine-galling" life-habit, suggesting that this intermediate life-history trait may be common among the Gracillariidae. Simply described as a gall, the mine made by *Parectopa leucocyma* larvae on leaves of *Agathis australis* show a similar phenotype (Wise, 1962) (Figure 6.1C). Likewise, the species *Acrocercops habroscia* and *A. haplocosma* were described as gall-inducers (respectively Meyrick and Clarke, 1912 and Meyrick, 1936), but the literature description (Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926) (Figure 6.1D) and field observations (Figure 6.1E) of galls probably induced by a related species on the same host-plant suggested a mine-galling habit. *Acrocercops macaria* may also induce a mine-gall, its gallery on leaves of several Rutaceae being described as inflated (Common, 1990) (Figure 6.1F). We have recently discovered a similar species on *Citrus sp.* in New Caledonia (Figure 6.1G). Another undescribed species of the Acrocercopinae subfamilly has been identified making mine-galls on *Clusia cooperi* (Hanson and Nishida, 2014) and *Clusia flava* (Nishida, unpublished) (Figure 6.1H and 6.1I). Our field work in French Guiana revealed the existence of at least two other mine-gallers, on *Clusia blattophila* and *Clusia fockeana* (Figure 6.1J and 6.1K). Also in French Guiana, we have discovered a mine-gall induced by an *Eucosmophora* sp. on *Micropholis egensis* (Figure 6.1L). In addition, Kenji Nishida reported another case on *Maesa montana* that may belong to the subfamily Acrocercopinae (Figure 6.1M), and Atsushi Kawakita found several mine-galls probably induced by species of the same subfamilly on *Phyllanthus* (Figure 6.1O and 6.1P). Finally, *Phyllonorycter didymopa*, *P. loxozona* and *P. madagascariensis* induce gall-like swellings on *Dombeya* leaves that may be considered as

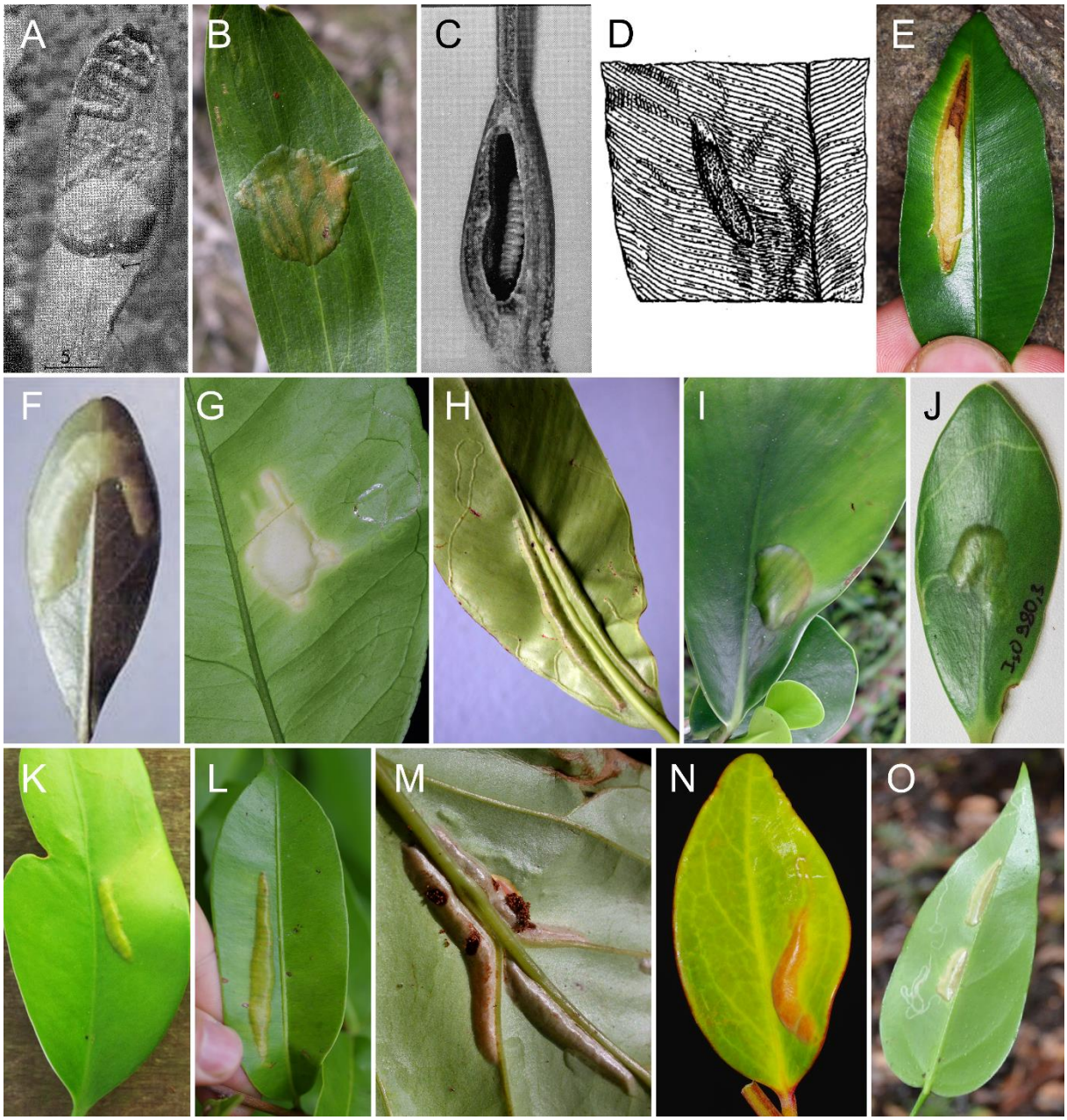



Figure 6.1. Mine-galls of Gracillariidae. (A) *Epicephala* sp. on *Acacia longifolia* (from New, 1981) from Australia, (B) Gracillariidae mine on *Acacia* sp. (photograph: Kenji Nishida) from Australia, (C) *Parectopa leucocyma* on *Agathis australis* (from Wise, 1962) from New Zealand, (D,E) mine-gall on *Callophyllum inophyllum*, probably attributed to *A. haplocosma* (illustration from (Docters van Leeuwen-Reijnvaan and Docters van Leeuwen, 1926)) from Java (Indonesia), (F) *Acrocercops macaria* on Rutaceae (reproduced from Common, 1990) from Australia, (G) Acrocercopinae species on *Citrus* from New Caledonia, (H) Acrocercopinae species on *Clusia cooperi* from Costa Rica (photograph: Kenji Nishida), (I) Acrocercopinae species on *Clusia flava* (photograph: Kenji Nishida) from Costa Rica, (J) Acrocercopinae species . on *Clusia blattophila* from French Guiana, (K) Acrocercopinae species on *Clusia fockeana* from French Guiana, (L) *Eucosmophora* sp. on *Micropholis egensis* from French Guiana, (M)Acrocercopinae species on *Aegiphila anomala* (photograph: Kenji Nishida) from Thailand, (N,O) Acrocercopinae species on *Phyllanthus* sp. from New Caledonia(photograph: Atsushi Kawakita).

mine-galls (De Prins and Kawahara, 2012). So mine-galls could be widespread among the Gracillariidae, particularly in the Acrocercopinae subfamily. They were also in majority encountered in a tropical area, suggesting that tropical climate favour mine-galls.

Mine-galls may be not be restricted to Gracillariidae and have evolved in other groups of microlepidopteran leaf-miners. For instance, several Nepticulidae that induce callus formation in their mine could fit the mine-gall definition. Thus, the midrib of *Periploca laevigata* leaves swells under the mining activity *Muhabbetana nigrifasciata* and the proliferative tissue seems to feed the insect before it starts to mine the limb (Hesse, 1972; Klimesch, 1972). Interestingly, authors used the term "Gallen-minen" or Minen-gallen" to describe the leaf-mine phenotype (Hesse, 1972; Klimesch, 1972). The recently described *Stigmella gallicola* also induces the inflation of the midrib of *Hampea appendiculata* leaves and complete its larval development inside it (van Nieukerken et al., 2016). Several *Ectoedemia* species mine inside the petiole of *Populus* leaves leading to callus formation in the gallery (Hering, 1951), however this newly formed tissue may not be involved in insect nutrition (Wilkinson and Scoble, 1979). In Heliozelidae, *Heliozela resplendella* induces the swelling of the midrib on *Alnus* (Hering, 1951), *H. sericiella* causes the inflation of the petiole on *Quercus* (Prota, 1963) but with no evidence of feeding on callus. Thus, the few other microlepidopteran than Gracillariidae to be mine-galler principally make their mine within the petiole or the main vein of the leaf.

The existence of mine-galls in other insect orders is uncertain. Although similar structures are described Dipterans, like in *Liriomyza trifolii* (Martens and Trumble, 1987), *Phytomyza ilicis* (Jeanneau, 1971) and *P. robustella* (Dempewolf, 2005; Spencer, 1969), the callus is probably not used as a nutritive source in these species. Regarding the Cecidomyiidae family, species like *Monarthropalpus buxi* (Brewer et al., 1984) or belonging to the *Cystiphora* genus (Maresquelle and Meyer, 1965) also live in a leaf-mine but their effect on leaf tissue do not seem to trigger a proliferation of undifferentiated cells.

Overall, all those examples show the relevance of using the term "mine-gall" as it describes an overlooked feeding strategy not only in Gracillariids but probably in all groups of leaf-mining insects. Moreover, this suggests that the space between leaf-miners and gall-inducers, presented as "no-man's land" by Hering (1951), may actually contain numerous species.

## 1.2. Intermediate life-styles result from an active manipulation of the plant by the insect

One of the key questions covered in my thesis is to test the hypothesis that the tissue proliferation observed in mine-galls is induced by the micromoth larvae. The idea that insects play an important role in callus formation was first dismissed by authors so far. Instead, microenvironmental conditions within the mine, like humidity, were suggested to be potentially the cause of callus formation (Hering, 1951). Indeed, leaves bearing mine-galls appear to share common physical properties. Although belonging to different botanical families with different

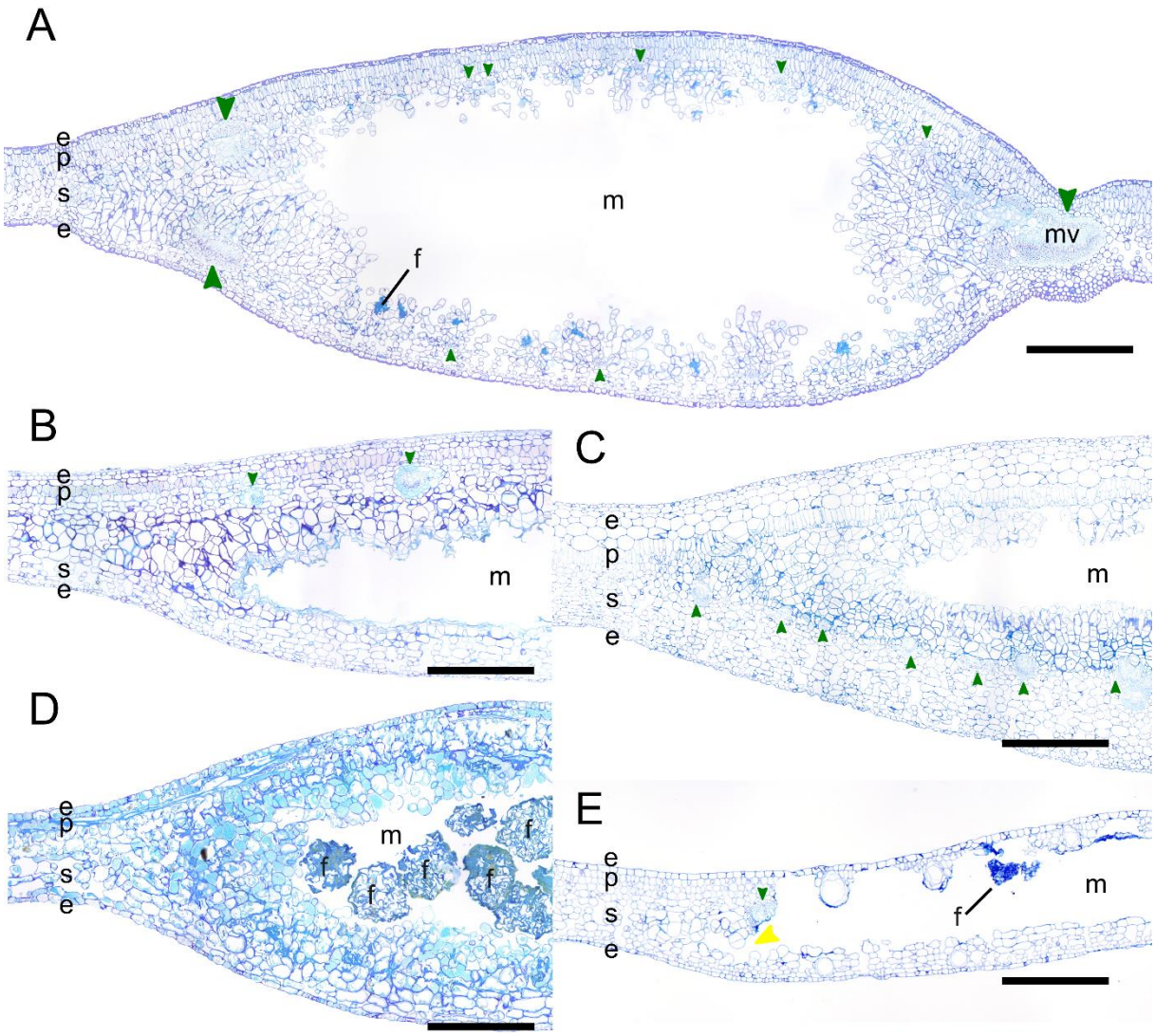



Figure 6.2. Histology of mine-galls. Cross sections of mine-galls of (A) *Borboryctis euryae* on *Eurya japonica*, (B) Acrocercopinae species on *Clusia fockeana*, (C) Acrocercopinae species on *Clusia blattophila*, (D) *Eucosmophora* sp. on *Micropholis egensis*, (E) Acrocercopinae species *Mellicope triphylla*. Legend: e: epidermis, p: palisade parenchyma, s: spongy parenchyma, m: mine, large green arrow: primary and secondary vein, small green arrow: other vein, yellow arrow: callus in the mine gall on *M. triphylla*. Scale: 100 µm. Staining: Toluidine Blue O.

metabolisms, most host-plants have in common to be sclerophyllous. Mine-galls are only observed in hard and thick leaves such as those of Clusiaceae and Araucariaceae.

Moreover, mine-galls are not formed in the epidermis layer like normal mines but inside the limb. The gallery is mined in the spongy parenchyma (Figure 6.2A, 6.2B, 6.2D and 6.2E) or at the interface between spongy and palisade parenchyma (Figure 6.2C). Tissue proliferation has different origins and can be initiated in sectioned vascular bundles (*B. euryae*, Figure 6.2A) ; *Epicephala* sp., New, 1981), or in the spongy parenchyma (Acrocercopinae mine-galls on *Clusia* and *Micropholis*, Figure 6.2B, 6.2C and 6.2D). Since air humidity in the mine is decreased with the transpiration process occurring through stomata (Pincebourde and Casas, 2006), having a mine in the middle of the limb may result in a higher air humidity than epidermal mines. As an exception, mine-galls of Nepticulidae and Heliozelidae (except *H.*

*aesella*) are found within the mid-rib or in the petiole, parts of the leaf that may be even more protected against desiccation.

However, our results on *B. euryae* contradict the assumption of a passive induction of callus proliferation, and show that callus do not grow in the absence of interaction with the alive fourth instar larva and that the quantity of callus is proportional with the duration of this interaction (Chapter 2). Therefore, cellular divisions of the callus are not caused by the physical condition in the mine but are stimulated by the direct effect of the insect in the host plant, probably mediated by salivary secretions *in planta*. Phytohormones are good candidates for this role, and among them cytokinins (CK) are likely to be involved. With auxin, CKs are the main phytohormones involved in regulation of plant development (Werner et al., 2001). CKs also play a key role in regulating plant-insect interactions (see Giron et al., 2013 for review). In leaf-miners, *Phyllonorycter blancardella* (Gracillariidae), *E. argentipedella* and *E. argyropeza* (Nepticulidae) induce an accumulation of CKs in the mined tissue that is responsible of the formation of the "green-island" phenotype (Engelbrecht, 1968; Giron et al., 2007). The fact that high concentrations of CKs found in the salivary glands of the larvae of these insects support the hypothesis of a synthesis of CKs by the insect and a secretion *in planta* (Engelbrecht et al., 1969; Zhang et al., 2017). Interestingly, before mining the limb and triggering the formation of a green-island, *E. argyropeza* larva induces callus proliferation in the petiole (Hering, 1951). In addition to cellular characteristics of the callus, and given that CKs are known to stimulate growth of callus *in vitro* (Miller et al., 1956; Perianez-Rodriguez et al., 2014), secreted CKs could be the underlying cause of this newly formed tissue.

## 1.3. Are intermediate life-styles "red herrings"?

The concept of host manipulation by parasites is central in parasitology, and implicitly assumes that the modification of the host phenotype benefits to the parasite (Hughes et al., 2012). I discuss here the potential benefits for the insect of the mine-gall phenotypes, and highlight the risk of exaggerating the significance of its adaptive advantage. Likewise, I also discuss the case of green-islands as another case of alleged plant manipulation by insects and of their putative adaptive significance.

### *Potential benefits for insect nutrition…*

In Chapter 2 our results show that the renewal of nutritive resources, through callus formation, allows a flexible larval development time in *B. euryae* as the resource is virtually inextinguishable. The same benefit could be found in other mine-gallers. For instance, the constant survivorship of *Phytomyza ilicola* in *Ilex* mines despite high rates of leaf abscission (Kahn and Cornell, 1989) could be due to renewal of callus tissues observed in its mines (Ellis, 2000). However, in most cases the nutritive value of callus formation in mine-gallers is unclear. In several examples mentioned by Hering (1951), callus grows in abandoned parts of the mine which excludes the insect from feeding on it (Hering, 1951). In addition, *E. argyropeza* do not feed on the callus that grows in the petiole mine (Hering, 1951). In dipteran, callus feeding is

only occasional in *Liriomyza* (Kang, 1996). These newly formed tissues may even have a detrimental effect on the insect. Ellis suggested that the filling of the mine due to overgrowth of callus could be the main mortality source of *Phytomyza ilicola* after parasitoid and birds (Ellis, 2000).

The maintenance of photosynthesis in leaf-mines in the case of green-islands has also been suggested to provide an adaptive benefit to the insect that induce it. The green-island phenotype is thought to be a way for the larvae to escape leaf senescence. Leaf senescence is a strategy used by plants against herbivorous insects being an important source of mortality in various groups of insects such as Diptera, Lepidoptera and Coleoptera (other cases mentioned in Connor and Taverner, 1997). Thus, extending leaf photosynthetic activity would be a way for the insect to escape the mortality caused by leaf senescence. This hypothesis has been tested in the case of the green-island inducer *Phyllonorycter maestingella*, in which leaf senescence is a negligible cause of mortality compared to what is observed in other herbivores (Pritchard and James, 1984). In addition, green-islands expand the availability of the food resources and can even allow the completion of a supplementary generation per year (Body et al., 2013; Giron et al., 2007; Hering, 1951). Overall, these arguments represent a weak support for an adaptive value of green-islands. The phylogeny of *Phyllonorycter* shows numerous loses of the green-island trait, suggesting a low adaptive value of the green-island phenotype for the insect (Gutzwiller et al., 2015).

### *…or non-adaptive secondary effect of other manipulation processes?*

A similar discussion of the adaptive role of a conspicuous phenotype resulting from plant manipulation by a parasite already occurred in the case of pathogen-induced green-islands. In this case, the extending exploitation of plant resources was also supposed to increase the parasite fitness. Walters et al. (2007) suggested with a simple model that green-islands are on the contrary "an epiphenomenon arising from the infection process and leaf senescence". They refer to this phenomenon as a "red herring" of the infection process, a "piece of information which is misleading, or is a distraction from the real question"(Oxford English Dictionary). Similarly, the processes of host manipulation generating the green-island and the mine-gall phenotypes may target other plant physiological functions, like the inhibition of indirect defense (Zhang et al., 2016), which could be more important for the insect fitness. Thus, the fact that *Phyllonorycter blancardella* larva collected on green leaves also contains high levels of CK supports the idea that their secretions *in planta* have another function than maintaining photosynthesis on senescing leaves (Zhang et al., 2017).

It is increasingly acknowledged that manipulative parasites alter simultaneously multiple characteristics of their host's phenotype, and that host manipulation should be considered as a multidimensional process (Cézilly and Perrot-Minnot, 2010; Thomas et al., 2010). The extreme pleiotropy in phytohormone effects on plants suggests that their secretion by the insect *in planta* would affect multiple physiological processes at the same time. CKs have been identified as mediating a wide range of plant-microbe-insect interactions and is consequently a good candidate to explore the possible multidimensionality of plant manipulation. Direct synthesis

of CKs *in planta* has been found in bacteria, fungi, parasitic plants, nematodes and insects to manipulate not only plant development but also plant defenses, host susceptibility and nutrient allocation (reviewed in Giron et al., 2013; Spallek et al., 2018). Those effects on plants occurring sometimes simultaneously, it is not possible in the current state of knowledge to distinguish the plant phenotypes that would confer an adaptive advantage to this manipulation from those which do not.

In summary, we propose that both mine-galls and green-islands may represent the tip of the iceberg of host plant manipulation occurring in leaf-miners (Figure 6.3). These mechanisms would occur in many leaf-mining species (if not most), but would generate these conspicuous phenotypes only above a specific threshold. This threshold would consist in physical conditions within the leaf-mines in the case of mine-galls, and in leaf physiological conditions for green-islands. Therefore, this idea implies that some effectors of plant manipulation may be shared by a wide community of leaf-miners.

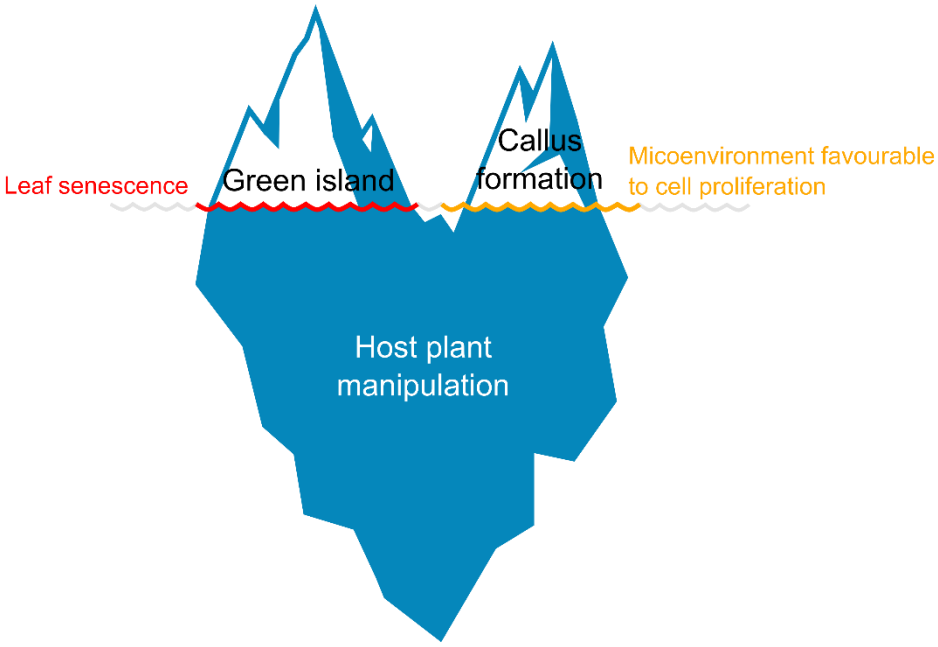



Figure 6.3. Green-island and mine-gall phenotypes as the tip of the iceberg of host plant manipulation by leaf-mining insects.

# 2. A continuum of shared weapons between insect herbivores

## 2.1. Phytohormone biosynthesis by insects may be the rule, not the exception

As we saw in the first chapter (General introduction), many studies have looked for phytohormone production in gall-inducing insects. However, few studies have looked for phytohormones in other herbivores. Studies that looked for the presence of phytohormones in non-herbivore animals are almost inexistent. Below a review recent literature suggesting that phytohormones may actually occur in many insect species and maybe other organisms.

### *Cytokinins*

Recently, Brütting et al. (2018) published the first unambiguous demonstration of CK transfer from an insect (*Tupiocoris notatus)* into a plant *(Nicotiana attenuata)*. CKs injected by the insect into the plant appear to be ineffective in reducing or inhibiting plant defenses but increase food quality (Brütting et al., 2018). Interestingly, the insect is not associated with any external conspicuous plant phenotype that would suggest such a manipulation process, therefore manipulation could be more widely present than previously thought among herbivorous insects.

These results raise the more general question about the ability of insects to produce phytohormones like CKs. There is now growing evidence that microorganisms are important 'hidden players' in insect-plant interactions (Giron et al., 2017). Insect symbionts can directly affect these interactions by providing insects with key nutrients or by interfering with the plant to modulate food provisioning to insects and plant defenses (Giron et al., 2017). While a symbiont-mediated production of CKs in *T. notatus* has not been tested (Brütting et al., 2018), data collected on the leafminer *Phyllonorycter blancardella* demonstrates that insect-associated bacterial symbionts are involved in CK biosynthesis (Giron and Glevarec, 2014; Kaiser et al., 2010). Given that CKs can be produced through a tRNA pathway, they could be virtually synthesized in all organisms (Persson et al., 1994). Interestingly, CKs appear to have an extremely wide spectrum of biological activities on mammals (reviewed in S. Drenichev et al., 2016), but despite of multiple evidence of trans-kingdom functionality of CKs, their biosynthesis in animals is not established (Robischon, 2015). However, a CK biosynthetic pathway similar to plants has been recently discovered in apicomplexan parasites of mammals (Andrabi et al., 2018), showing that CK biosynthesis exists in other kingdoms than plants, fungus and bacteria, and in organisms that do not interact with plants. For instance, CK secretion has been identified in several nematodes, including the detritus feeding nematode *Caenorhabditis elegans* (Siddique et al., 2015).

### *Auxin*

Similarly to CKs, auxins have been recently found to be synthesized by animals. All insects tested by Suzuki et al. (2014), from free living herbivores like *Bombyx mori* and *Aphis gossypii*, to non-herbivore species like *Drosophila melanogaster*, *Musca domestica* and *Apis melifera*, contain significant amounts of auxin. The authors further described a novel auxin biosynthesis pathway in silkworm that may exist in other insects (Suzuki et al., 2014; Yokoyama et al., 2017). The recent description of the key enzyme involved in this biosynthesis (Takei et al., 2019) motivated us to look for its presence in the transcriptome of *C. cecidophora* (Chapter 5). In general, the presence of auxin in non-herbivorous insects and the description of a novel biosynthetic pathway suggests that auxin may have a physiological role in insects than goes beyond plant manipulation. A recent study shows that auxin biosynthesis can be encounter in other non-herbivorous animals, with the evidence of endogenous production of auxin in *Caenorhabditis elegans* (Zhou et al., 2019).

In summary, the high concentration of phytohormones measured in gall-inducers or green-island inducers misleadingly suggested that phytohormone biosynthesis and secretion was specific to these insects. Recent studies show, on the contrary, that other herbivores produce these molecules and use them in their relationship with their host-plant. Beyond these results, that auxin and CKs may be commonly biosynthesized in many organisms. Therefore phytohormones may be important players in the arsenal of effectors shared by most insect herbivores to beat plant defenses and more widely to affect plant physiology.

## 2.2. Toward a continuum of plant manipulation forms by insects

Other elements of the collection of effectors that may be widely used by insects have been identified. For instance, in a recent paper I showed that three protein functions may be commonly encountered in herbivorous secretions: ATPases like apyrases, calcium-biding proteins like calreticulins, and hydrogen peroxide reducing enzymes like peroxiredoxins (Guiguet et al., 2016). These effectors have in common to be found both in secretions of blood- and plant-feeding arthropods and to probably target early stages of the immune response of both animal and plant hosts. Another effector identified in a large number of herbivorous insects is the glucose oxidase (GOX). Shown as suppressing several levels of plant defenses (as reviewed in Giron et al., 2016), this enzyme has been identified in the saliva of many caterpillar species (Eichenseer et al., 2010) as well as in an aphid (Harmel et al., 2008).

Given that some molecular effectors of insect virulence may be found in many species whereas other are restricted to a particular species, they can be ranked according to their occurrence in insects (Figure 6.4A). At one end of the spectrum, effectors expressed in most herbivores if not all, like perhaps auxin and CKs, at the other end, effectors that are specific to one species, as those involved in the gene-for-gene interaction that result of the adaptation to one particular host-plant (Stuart, 2015). In between, some effectors are specifically present in some herbivore

clades, like for the GOX (Eichenseer et al., 2010) or some effector gene families that are specific to aphids (Boulain et al., 2018). This vision I propose here would allow to order herbivores along a continuum of manipulation mechanisms, from the most simple involving a low number of effector molecules generally found in most insects, to the most complex using a diverse and specific repertoire of effectors (Figure 6.4B). Interestingly, the current state of knowledge does not allow us to prejudge the position of species in this continuum of complexity according to their life-styles. For instance, gall-inducing species, generally considered as possessing the most complex molecular dialog with their host plant, may actually use in some cases a reduced number of effectors to induce their gall, as it has been shown that secretion of auxin and CKs could be sufficient to induce this phenotype (Bartlett and Connor, 2014). This new conceptual framework allows to go beyond the classical partition of herbivorous insects as a "*scala naturae*", ranging from foliage feeders supposed to have only simple molecular interactions to gall-inducers that are seen as the most complex forms of interaction (Figure 6.4C).

The words "manipulation" or "hijacking" are often used to describe the way insects affect the physiology and the development of their host plants. These terms, that imply a high degree of control, actually refer to molecular processes that vary in their complexity, and it may be interesting to be able to distinguish insects that simply inhibit some basic immune responses of the plant from those that deeply reprogram plant physiology. A simple count of secreted proteins or other molecules demonstrated as being effectors could be good proxy of the complexity of the manipulation process induced by an insect (Figure 6.4B). To go further, the importance of effectors should be taken into account in the classification. Some are probably master-controllers, whereas others lose their effects in some plant, like the GOX in some caterpillars (Eichenseer et al., 2010). Thus, the quantification of the manipulation complexity may rely on an effector count that would be weighted according to the effector importance (Figure 6.4A).

Applied on the topic developed in this thesis of the intermediate life-styles between leaf-miners and gall-inducers, this conceptual framework may help to understand the evolution of gall-induction. As shown above, phytohormones biosynthesized by insects are to date the best candidates to function as effectors for gall induction and phytohormone production may be much more widespread among insects than previously thought. Therefore, we could hypothesize that phytohormone production could be a preadaptation to gall evolution. To illustrate this hypothesis, we can consider the example of the species of the genus *Ectoedemia* (Nepticuliidae) that are associated with *Populus* (Figure 6.5). As previously mentioned, a CK production has been found to correlate with the green-island phenotype in these species (Engelbrecht, 1968). However, another correlation can be done, between the CK production and the callus proliferation observed in the mine made at early stages in the petiole of the same species (Hering, 1951). This callus proliferation received a low consideration since it apparently does not feed the insect (Hering, 1951). However one species in this clade is an exception as *E. populella* completes all its larval development in the petiole and induces the formation of a spherical gall from the initial gallery (Frost et al., 2012). Interestingly, the derived position of this life-style in the phylogeny of the *Ectoedemia*/*Populus* group (Doorenweerd et al., 2015) strengthens the idea that the gall-induction life-style derivated from a mining life-style in petiole

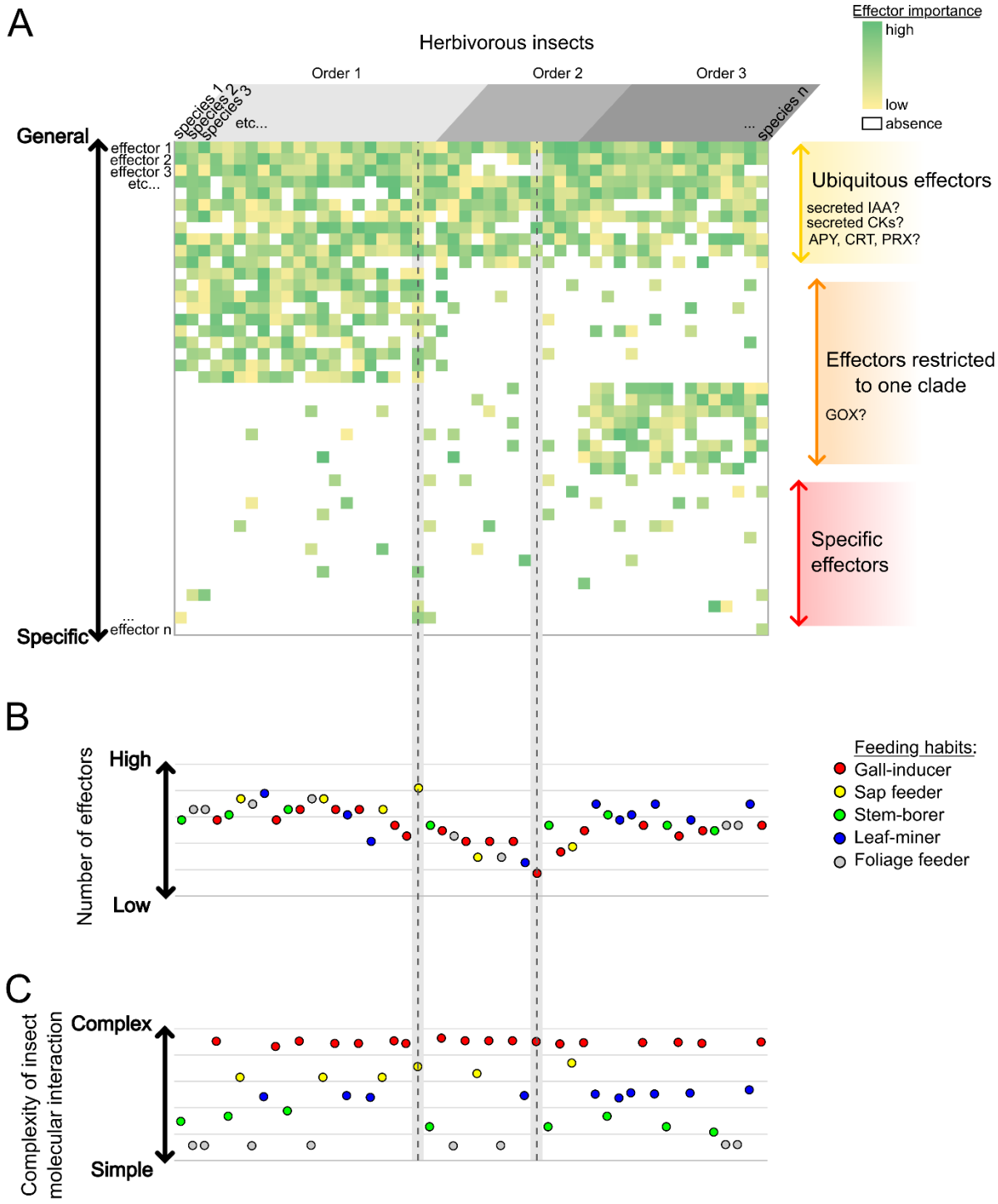



Figure 6.4. Illustration of the concept of continuum of plant manipulation in insects. (A) Each herbivorous insect has a repertoire of secreted effectors. Effectors distribution range from those shared by most insects to effectors that are species specific. Secreted auxin (IAA), cytokinins (CK), apyrases (APY), calreticulins (CRT) or pereoxyredoxins (PRX) could be part of the ubiquitous effectors. The glucose oxidase (GOX) belong to the group of effectors that are restricted to a clade, for instance the Lepidoptera in the case of the GOX. Effector importance in the insect arsenal varies as a function of its effects on the plant. Herbivorous insects could be classified from the simplest to the more complex effector arsenal, based on the number of effectors they secrete (B). This would be an alternative to the intuitive and probably erroneous way to link life-styles with an ability to manipulate plants (C). Dashed lines show the correspondences between the graphs for two species, a sap-feeding species and a gall-inducing species.

containing callus. Thus, the evolution of gall-induction in *E. populella* may result of an amplification of callus growth, a phenotype previously non-adaptive. It could be interesting to test whether this also involves an increase of CK production. In other terms, callus growth, a probable emergent property of the manipulation of other levels of plant physiology through secreted CKs, could have been a preadaptation to gall-induction in *E. populella*. A similar scenario may have occurred in other gall-inducing insects like *Caloptilia*, and to test that hypothesis more studies quantifying the production of phytohormones are needed.

Overall, to improve our knowledge of the effector landscape we need more interspecies comparisons between insect effector arsenals. Studies in this domain are still rare. The study by Eichenseer et al. (2010) of the GOX activity in 23 families of Lepidoptera for a total of 85 species is a model that could be replicated for other effectors. The analysis of the relationship between host range and GOX revealed that highly polyphagous species were more likely to show relatively high levels of GOX compared to species with more limited host ranges. Since the advent of the genomic era, both insect and plant genomes and transcriptomes are being published at an increasing rate and facilitate comparisons of effector repertoires at a genomic level (Boulain et al., 2018; Guiguet et al., 2016; Thorpe et al., 2016). This represents a promising way to decipher the molecular interplay between insect and plants and how this molecular dialogue has evolved.

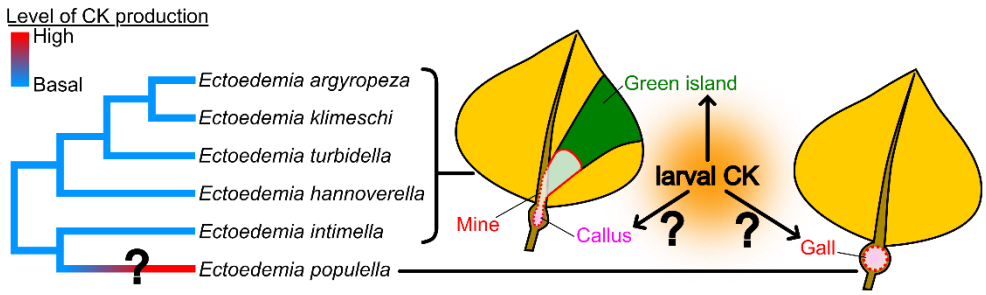



Figure 6.5. Cytokinins are probably involved in the evolution of gall-induction in the *Ectoedemia*/*Populus* group. Cytokinins (CK) are known to be involved in green-island formation in several *Ectoedemia* and CKs could be involved in the callus development observed in the petiole mine made by early larval instars. CKs could be also involved in gall-induction in *E. populella*, possibly associated to an increased CK production compared to other *Ectoedemia*. Phylogeny adapted from Doorenweerd et al. (2015).

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# Annex

Antoine GUIGUET

# Origine évolutive et bases moléculaires du mode de vie galligène chez les Gracillariidae


## Résumé

L'objectif de ma thèse visait à étudier les processus évolutifs ayant conduit à l'évolution du mode de vie galligène et à rechercher des effecteurs impliqués dans l'induction des galles dans la famille des Gracillariidae (Lepidoptera) avec un accent particulier sur deux espèces, *Borboryctis euryae* et *Caloptilia cecidophora*. Nous avons ainsi démontré que ces deux espèces présentent la particularité de posséder un mode de vie intermédiaire entre mineur de feuille et inducteur de galle. Le tissu prolifératif présent dans la mine de *B. euryae* s'apparente en effet à une galle et les larves de *C. cecidophora* connaissent une transition du mode de vie mineur de feuille à galligène au cours de leur développement. Des campagnes de terrain ont permis de découvrir de nouvelles espèces de *Caloptilia* inductrices de galles, et leur étude phylogénétique a montré qu'elles forment un groupe monophylétique. Enfin, exploitant la transition de mode de vie de *C. cecidophora* ainsi que son contexte phylogénétique, nous avons appliqué une approche de transcriptomique comparative intra- et inter-espèce afin de rechercher des effecteurs candidats impliqués dans la formation de galle.


Mots clés : Interactions plantes-insectes, manipulation, galles, mineurs de feuilles, insectes, histologie, phylogénie, transcriptomique, effecteurs, Gracillariidae, *Caloptilia cecidophora*, *Borboryctis euryae*.


## Résumé en anglais

The aim of my thesis was to study the evolutionary processes that led to the evolution of the gall-inducing lifestyle and to look for effectors involved in the induction of galls in the Gracillariidae family (Lepidoptera) with a particular focus on two species, *Borboryctis euryae* and *Caloptilia cecidophora*. We have demonstrated that these two species have a particular intermediate life-style between leaf-miner and gall-inducer. The proliferative tissue in the *B. euryae* mine is similar to a gall and the larvae of *C. cecidophora* undergo a transition from leaf-miner to gall-inducer during their development. Field work has uncovered new gall-inducing *Caloptilia* species, and their phylogenetic study has shown that they form a monophyletic group. Finally, exploiting the transition of feeding habit of *C. cecidophora* as well as its phylogenetic context, we applied a comparative intra- and inter-species transcriptomic approach to search for candidate effectors involved in gall induction.


Keywords: Plant-insect interactions, manipulation, galls, leaf miners, insects, histology, phylogeny, transcriptomics, effectors, Gracillariidae, *Caloptilia cecidophora*, *Borboryctis euryae*.