이학박사학위논문

Host selection behavior of a herbivorous insect, *Trichobaris mucorea* and defense responses of the host plant, *Nicotiana attenuata*

초식 곤충 바구미의 숙주 선택에 대한 행동학적 특성과 숙주식물 야생 담배의 방어기작

2016 년 8 월

서울대학교 대학원

생명과학부

이지숙

Host selection behavior of a herbivorous insect, *Trichobaris mucorea* and defense responses of the host plant, *Nicotiana attenuata*

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August 2016

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이 논문을 이학박사 학위논문으로 제출함
2016년 6월

서울대학교 대학원
생명과학부
이지숙

이지숙의 이학박사 학위논문을 인준함
2016년 6월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

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Abstract

Plant chemicals play important roles on host selection behaviors of herbivorous insects, especially females are able to recognize plant-producing chemicals to select their host plants and these host-selection behaviors are often consistent with the preference-performance hypothesis; females oviposit on hosts that maximize the performance of their offspring. However, the metabolites used for these oviposition choices and responsible for differences in offspring performance remain unknown for ecologically-relevant interactions. In this study, I examined host selection behaviors of two sympatric sibling species: *Datura* weevil, *Trichobaris compacta* and tobacco weevil, *Trichobaris mucorea* in the field and the glasshouse with transgenic host plants specifically altered in different components of their secondary metabolism. I found that both *Trichobaris* species adults were mainly observed in *Datura wrightii* growing in the Great Basin Desert of southwestern USA, but only *T. mucorea* larvae were colonized in the wild tobacco, *Nicotiana attenuata* growing the same area. In the glasshouse-experiments, both *Trichobaris* females strongly preferred to feed on *D. wrightii* rather than on *N. attenuata*, females of *T. compacta* oviposited only on *D. wrightii* but, *T. mucorea* females preferred to oviposit on *N. attenuata*. These oviposition behaviors increased their offspring performances which are larval survival and growth. Although the half of the *T. compacta* larvae

survived in nicotine-free *N. attenuata* lines but, nicotine did not influence oviposition behaviors of *T. compacta* as well as *T. mucorea*. Jasmonic acid (JA)-induced sesquiterpene volatiles were key compounds influencing *T. mucorea* female oviposition choices, while these sesquiterpenes had no effect on larvae performance. I conclude that there is strong correlation between female oviposition preference and larval performance of two sibling *Trichobaris* species between *D. wrightii* and *N. attenuata* plants. Furthermore I verified that each level of host selection behavior such as female oviposition choice and offspring performance is affected by different plant secondary metabolites. To understand affecting factor of *T. mucorea* larvae performance on host plant defense response, I focused on host plant induced defense metabolite against *T. mucorea* larvae in stem tissue. When leaf-feeding insect herbivores attack, plants activate defense responses in both attacked as well as unattacked systemic leaves. These defense responses are largely regulated by herbivory-induced JA. Little is known about the defense responses of the stems or the role of JA signaling in these responses. I show here how the attack of *T. mucorea* larva, a tobacco stem weevil, triggers a defense response in the stems of *N. attenuata* plants. Female *T. mucorea* adults lay eggs on the stems of *N. attenuata*, and neonates burrow into the stems and feed on the pith. To examine this stem defense, firstly I established an herbivore performance assay, in which *T. mucorea* eggs were experimentally inoculated into *N. attenuata* stems. *T.*

mucorea elicited high levels of JA and jasmonoyl-L-isoleucine in the pith of stem that have been attacked, as happens when chewing insects damage *N. attenuata* leaves. Chlorogenic acid (CGA) levels were highly increased in the pith of attacked stems in a JA-dependent manner, while the level of CGA in the attacked *N. attenuata* leaves is known to be unchanged. To test whether induced CGA confers resistance of the pith, I used a transgenic CGA-free *N. attenuata* plant that could not produce hydroxycinnamoyl quinate CoA transferase (*NaHQT*). In glasshouse and field experiments, *T. mucorea* larvae performed better in these *NaHQT*-silenced plants than on wild-type plants, indicating that the CGA in stems is a direct defense metabolite that plants produce to protect themselves against *T. mucorea* attack.

Keyword: *Trichobaris mucorea*, *Nicotiana attenuata*, plant secondary metabolites, host selection behavior, pith defense, chlorogenic acid

Student Number: 2011-30111

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Chapter 1. Introduction

1.1. Plant-herbivorous insect interaction

Plants have evolved sophisticated mechanisms to cope with the onset of unexpected environmental conditions. Plant stressors can be biotic (e.g. herbivores and pathogens) or abiotic (e.g. drought and cold) (Weis and Abrahamson, 1985). Plant-herbivore interactions have been framed through evolution in an antagonistic way: Plants are eaten by herbivores but have evolved defenses that lower herbivores' performance and ultimately reduce their fecundity (Rosenthal and Berenbaum, 2012). In terms of the amount of plant material consumed, herbivorous insects are the most important biotic stressors of plants and represent one of the most diverse groups of animals on the planet (van der Meijden, 1996; Rosenthal and Berenbaum, 2012).

From the perspective of the insect, developing detoxifying mechanisms that enable tolerance of plants defense metabolites can benefit individual performance, which in turn is reflected in higher reproductive output (Ivie et al., 1983). This defense mechanism both creates a new niche and narrows the range of possible host plants for the insect, often to a single genus. Such insects become specialist herbivores (Dowd et al., 1983; Ali and Agrawal, 2012). Some herbivorous insects even use the plant defense traits to which they have developed a tolerance to defend themselves against predators or as mating signals, in a phenomenon known as concealment (Armitage and Conner, 2000; Kumar et al., 2014). While these herbivores demonstrate a

degree of dietary specialization, on the other end of the spectrum are the generalist herbivores, which can feed on more than one plant family (Ali and Agrawal, 2012). Another important distinction between herbivorous insects is feeding guilds, groups that share a certain feeding behavior that relies on a certain type of host plant tissue or cell (Howe and Jander, 2008). For example, chewing insects use their strong mandibles to feed on plant tissue, causing extensive plant cell damage and loss of tissue (Karban and Baldwin, 1997), while piercing/sucking insects use their mouthparts to penetrate and consume the contents of plant cells or vasculature, inflicting minimal damage on the plant (Price, 1997; Walling, 2000).

Plants have developed a diverse array of strategies to prevent damage by herbivores. Classically, these are categorized according to the mechanism of action: constitutive or inducible, and direct or indirect (Jaenike, 1978; Baldwin and Preston, 1999). Constitutive defenses are constantly activated within the plant, without external stimuli. Inducible defenses are produced or activated in response to external stimuli, such as attacks by herbivores (Kessler and Baldwin, 2002). Inducible defense is considered a cost-efficient strategy because it is employed as needed, making plants defenses (Karban et al., 1997) remarkably plastic and responsive to different grades and types of herbivore (Schwachtje and Baldwin, 2008). Plant direct defenses have negative effects on herbivore growth and fecundity, while plant indirect defenses appeal for aid from, or reward, predators on another

trophic level (We and Baldwin, 2010; Meldau et al., 2012). The costs and benefits of these different actions, and their effect on plants' Darwinian fitness, also define how plants respond to herbivore attack (Kessler and Baldwin, 2002). This interaction also demonstrates the optimal defense hypothesis (ODH), which predicts that within-plant allocation of defenses depends on whether, and how often, a plant organ is attacked, and how valuable that organ is to plant fitness (Kessler and Baldwin, 2002; Erb et al., 2012). While defense traits are beneficial for plants in the presence of herbivores, in the absence of herbivores they are expensive and can reduce plant fitness (Baldwin, 1998). Therefore, inducible defense traits deployed only when necessary can reduce costs compared to constitutive defenses. Inducible defenses are also more flexible, allowing plants to respond in a certain way depending on the type of herbivorous insect and the amount of damage, and other abiotic or biotic coexisting (Kessler and Baldwin, 2001; Awmack and Leather, 2002). A single plant defense trait is therefore able to act on many different levels determined by different herbivore feeding guilds (Erb et al., 2012; Fürstenberg-Hägg et al., 2013).

1.1.1 *Nicotiana attenuata* as an ecological model organism for plant-herbivore interaction

The wild tobacco plant *Nicotiana attenuata* Torr. ex S.Watson, also known as coyote tobacco, is a species in the Solanaceae family. *N. attenuata*

is distributed from northwestern Mexico, east to the Great Basin region and north to southern Canada (Baldwin, 2001; Wünsche et al., 2011). *N. attenuata* is typically 50-150 cm in height with prolate-lanceolate to circular leaf blades 2-10 cm in length and 2-4 cm wide. Its glandular complexion is caused by trichrome spread over the surface of the leaves and stem, which comprise most of the tissue (Goodspeed, 1954). Its natural environment is cold semi-desert at altitudes of 1,000-2,600 m (Goodspeed, 1954). *N. attenuata* germinates in burned habitats, with the largest populations being found 1 to 3 years after fires along roadsides and in dry washes and rocky or sandy ground with outcrops (Baldwin and Preston, 1999). Most *N. attenuata* seeds respond to smoke stimulants, which terminate dormancy and initiate germination in post-fire conditions (Baldwin and Preston, 1999). In this type of germination behavior, seeds germinate synchronously in nitrogen rich soils and are selected for rapid growth when the water supply is high (Lynds and Baldwin, 1998). *N. attenuata* has evolved a short generation time with rapid vegetative growth and prolonged lifetime seed production, as well as highly plastic adaptive responses (Baldwin, 1998; Kessler and Baldwin, 2001; Diezel et al., 2011a).

N. attenuata is an important host plant for herbivores that colonize post-fire environments every year. The plant has evolved efficient induced resistance strategies to cope with a highly unpredictable and varied community of herbivores of different levels of specialization and belonging

to various guilds (Fig. 1-1). More than other taxa, herbivorous insects attack *N. attenuata* on a variety of spatial scales, indicating the plant successful coexistence with herbivores and exemplifying plant-insect interaction on an ecological and molecular-biological level (Baldwin, 2001; Gaquerel et al., 2013). Among these herbivorous insects are the larvae of the specialist tobacco hornworm, *M. sexta*, whose interaction with *N. attenuata* is well documented. Fatty acid-amino acid conjugates in the oral secretions of *M. sexta* larvae activate the JA signaling cascade (Baldwin et al., 2001; Kessler et al., 2004). JA signaling activates JA-responsive transcription factors, leading to the biosynthesis of induced small metabolites (Woldemariam et al, 2011; Fürstenberg-Hägg et al., 2013) such as nicotine and a variety of phenylamides. These decrease herbivore performance (Baldwin, 1999; Steppuhn et al., 2004; Kaur et al., 2010). Nicotine is present constitutively in undamaged *N. attenuata* tissues, but is inducibly increased in response to damage (Baldwin, 1998, 1999). The two major phenolamides found in *N. attenuata* are caffeoyl-putrescine (CP) and dicaffeoyl-spermidine (DCS) (Onkokesung et al., 2012). Both CP and DCS accumulate constitutively in reproductive tissues and are strongly induced by herbivory in leaves, but other major phenylamides chlorogenic acid (CGA) and rutin are constitutive defense metabolites in leaf tissue (Kaur et al., 2010; Onkokesung et al., 2012).

More than two decades of using genetic tools and analytical toolboxes

in the field have demonstrated the ecological consequences of silencing or overexpressing particular genes. Multiple layers of plant defense mechanism in response to herbivore attack have been developed in *N. attenuata* due to the plant's ease of cultivation in vitro, and there is now a well-established protocol for the generation of stable transformed lines (Baldwin et al., 2001; Krügel et al., 2002; Gaquerel et al., 2013). These properties make *N. attenuata* a great model system for the study of plant-insect interaction at the molecular and ecological levels.

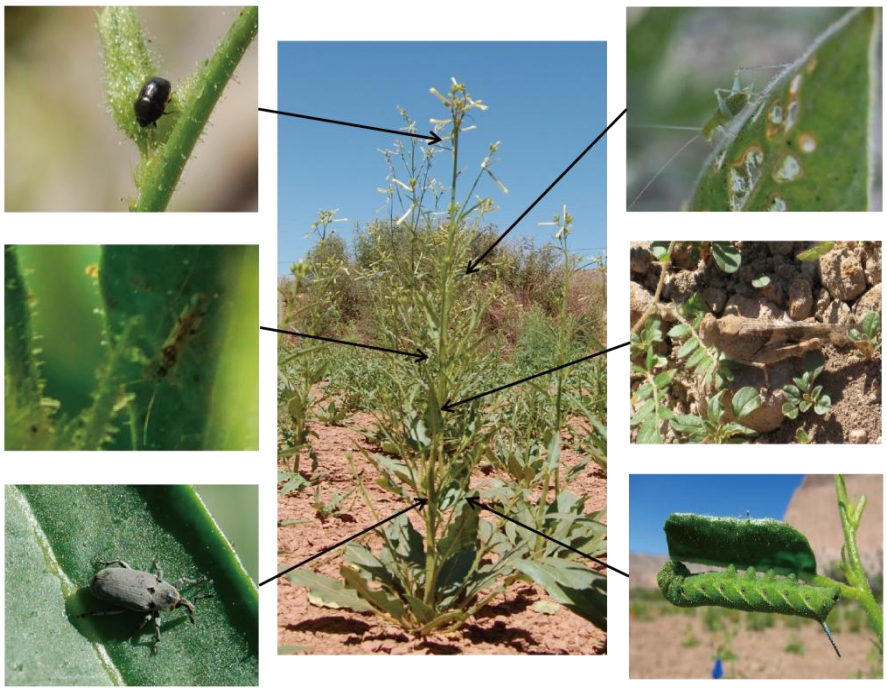

Fig. 1-1. *Nicotiana attenuata* (centre) with a variety of herbivores in native habitat, the Great Basin Desert. On the right, from the top, the tree cricket *Oecanthus* spp, the grasshopper *Epitrix* spp, larvae of the sphingid *Manduca sexta*. On the left side, from the top, the thyreocorid bug *Corimelaena extensa*, the mired *Tupiocoris notatus*, and the adult of the stem borer *Trichobaris mucorea*.

1.1.2. Plant defense mechanism and secondary metabolites

Plant defense mechanisms are activated when components of insects saliva come into contact with plant surfaces during feeding (Wu and Baldwin, 2010). The detection of the presence of a herbivore triggers regulatory responses involving various phytohormones, with the jasmonic acid (JA) pathway playing a dominant role in host resistance by producing toxins or feeding-deterrent compounds (Wu and Baldwin, 2010). JA is synthesized in chloroplast and peroxisomes through the oxylipin pathway (Creelman and Mulpuri, 2002). Free fatty acid (alpha linolenic acid) is converted to OPDA (12-oxo-phytodienoic acid) through serial catalyzing by lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOS). OPDA is converted into JA by three-step oxidation (Wasternack, 2007). Among the various types of JA conjugates, the isoleucine-combined form of JA (JA-Ile) is known to be an active compound in JA signaling (We and Baldwin, 2010; Fürstenberg-Hägg et al., 2013). JA-Ile further binds to COI1 protein (*coronatine insensitive 1*) and activates JA-response transcription factors like MYC2 (Wu and Baldwin, 2010). Finally, transcription factors decrease herbivory by increasing plant inducible secondary metabolite levels. Although JA signaling pathway is highly conserved in the plant kingdom, it is becoming increasingly clear that multiple hormone response pathways interact to translate initial perception events into appropriate responses that increase plant fitness in the presence

of aggression (Erb et al., 2012).

JA activates plant defense signaling in response to herbivorous insect damage; constitutive or induced accumulation of toxic secondary metabolites is observed in the damaged areas of such plants (Howe and Jander, 2008). The most metabolically diverse classes of plant secondary metabolites play a role in plant defense (Aharoni et al., 2005). Alkaloid-type secondary metabolites (e.g., caffeine, nicotine, morphine, and cocaine) are especially well known for the way their metabolic effects act as a defense against herbivorous insects (Steppuhn et al., 2004; Howe and Jander, 2008). Other well-studied classes of secondary metabolites with a defense role are cardenolides, tannins, saponins, glucosinolates, and terpenoid (Ikonen et al., 2002; Howe and Jander, 2008; Rosenthal and Berenbaum, 2012; Fürstenberg-Hägg et al., 2013). Plant secondary metabolites in many plants show multiple synergistic effects against herbivory. A combination of two monoterpenoids of the *Thymus vulgaris* plant are more additive effective against attacks by *Spodoptera litura* than one monoterpernoid (Hummelbrunner and Isman, 2001). Together, nicotine and protein inhibitor (PI) production in wild tobacco plants have a negative effect on *Manduca sexta* feeding (Steppuhn and Baldwin, 2007). In addition, the synergistic effects of plants' metabolic diversity can provide a defense against multiple herbivores of various feeding types. One in-depth study of glucosinolate metabolites demonstrate that a diverse profile of secondary metabolites

provides specificity of defense response (Howe and Jander, 2008). Indole- and aliphatic-glucosinolates of *A. thaliana*, for example, show differing effects on four different feeding leaf-herbivores (Mewis et al., 2005; Halkier and Gershenzon, 2006). Therefore, plant defensive metabolites can act independently or synergistically as direct repellents, antifeedants, and toxins on herbivorous insects, depending on the situation.

1.2. Host selection behavior of herbivorous insects

The relationship between oviposition preference and offspring performance of herbivorous insects is the key to understanding the evolutionary ecology of host selection specificity (Thompson, 1988a). Oviposition preference is defined as the hierarchical ordering of plant species by ovipositing females. In plants of equal mass of several species offered simultaneously, preference would be expressed as the proportion of eggs laid on each of the plant species (Singer, 1986). Performance is used as a composite term for survival and mass at all immature stages—egg, larval and pupal—and larval growth rate, as indicated by adult fecundity and longevity (Jaenike, 1978; Thompson, 1988a). Specificity of host selection in simultaneous trials is able to be defined as the number of plant species on which females oviposition that is given a preference hierarchy, does an individual female in a simultaneous selection trait limit her oviposition to

the most preferred plant species (Thompson, 1988a; Thompson and Pellmyr, 1991).

Much of the theoretical and experimental research on the relationship between maternal choice of host plant for offspring and the offspring performance in the selected host plant has been investigated based on the preference-performance hypothesis (PPH) or optimal oviposition theory (Jaenike 1978; Thompson 1988). This hypothesis is easily explained using the mother knows best metaphor; oviposition preference patterns of herbivorous insects are supposed to correspond to the suitability of the host for offspring development (Jaenike, 1978; Liu et al., 2010).

To recognize their host plants, herbivorous insects require sophisticated detection mechanisms (Bruce et al., 2005). Herbivorous insects select hosts using one of two processes: choosing the host from a distance using olfactory and visual cues; or selecting the host only after contact using gustatory cues (Dethier, 1982; Visser, 1988). This process is regulated by integration, within the insect central nervous system (CNS), of numerous sensory inputs, including olfactory and gustatory semiochemical cues from the host plant, and physical information such as plant color, shape, and texture (Bruce et al., 2005; Carrasco et al., 2015). Collectively, the combination of sensory inputs are experienced by the insect as attraction or deterrence, allowing it to recognize a suitable host plant (Bernays and Chapman, 2007; Carrasco et al., 2015).

Many studies have been done on positive correlation between oviposition preference and larval performance that are consistent with the PPH (Thompson, 1988a; Mayhew, 1997; Knolhoff and Heckel, 2014). An example is the diamond back moth, a major pest of cruciferous crops. This species shows strong positive correlation between oviposition preference and pupal weight in 26 potential host plants (Zhang et al., 2012). However, several cases showed weak linkages between female adult choice and offspring growth, a result described as “bad motherhood” (Valladares and Lawton, 1991; Underwood, 1994; Jallow and Zalucki, 2003; Shikano et al., 2010). However, the general rule is one of positive association, and there is no clear explanation for this apparent contradiction. The neural constraints hypothesis, which posits that the oviposition behavior of insects may be constrained by the ability of their neural systems abilities to interpret complex signals, has been supported by meta-analysis (Gripenberg et al., 2010). Interestingly, recent studies demonstrate that overexpressed transgenic plants of aliphatic- and indolyl-glucosinolates from *Arabidopsis thaliana* negatively affects whitefly oviposition preference and egg survival (Markovich et al., 2013). These studies investigated the relationship between female preference and offspring performance based on specific evidence using molecular tools. But the studied insect-host plant interactions were not ecologically relevant. Therefore, further study is needed to determine how plant chemicals cause inconsistencies between

female preference and offspring performance, and which plant chemical signals are involved.

1.3. Purpose of research

This study focuses on two main areas. The first area is host-plant interaction involving two species of the genus *Trichobaris*. The host selection behavior of the stem-borer weevil species of *Trichobaris* is poorly understood. While *T. compacta,* known as the Datura weevil, and *T. mucorea* are distributed in the same habitat, their actual host range and host selection behavior traits are mostly unknown. As described above, the ecology of the host-selection behavior of female insects has been well studied and it is known that the levels of certain host plant metabolites are positively or negatively correlated with oviposition preferences and offspring performance. However, it remains unclear whether adult females choose the best plants for their offspring by sensing the various metabolites that affect the larval performance. This study examines the preference-performance hypothesis at the molecular level using transgenic *N. attenuata* plants developed as a model system for studying herbivore interaction at the ecological and molecular-biological levels (Baldwin, 2001; Kessler and Baldwin, 2001). The plant was used to investigate the oviposition behavior of two naturally co-occurring sibling *Trichobaris* species and the

consequences of female oviposition preference for larval performance in field and laboratory experiments. The plant produced reduced amounts of the defense hormone jasmonic acid, and nicotine, a toxic alkaloid. It also emitted large numbers of sesquiterpene volatiles.

The second area of study, concentrating on the plant side, involves the defense response of *N. attenuate* against *T. mucorea* larvae. As described above, plants are able to produce specific defense signals and chemicals in response to attacks by herbivores in several different feeding guilds (Karban and Baldwin, 1997; Strauss and Agrawal, 1999; Dicke and Hilker, 2003). Some inducible defenses are triggered by the feeding patterns of herbivores, and some by the type of damage herbivores cause to plants' tissues (Korth and Dixon, 1997). Most research on inducible defenses in plant-herbivore interactions has focused on plant-leaf feeder interactions (Haukioja and Koricheva, 2000; Preisser and Bastow, 2005). Furthermore, when leaf-chewing or -sucking insects attack plants, several toxic metabolites are produced and accumulate in leaf tissues. JA produced by plants plays an important role in eliciting these defense responses in leaves (Kessler and Baldwin, 2002; Erb et al., 2012). However, little is known about the toxic metabolites regulated by JA-signaling against attack from stem-boring insects, which are rarely noticed until the adult insects emerge after the larvae have completed their development inside the stem. Also, while there has been extensive research on the leaf-defense response mechanism of *N.*

attenuata and its use against leaf-chewer *M. sexta* and leaf-hopper *Empoasca* spp. (Van Dam and Hare, 1998; Kessler et al., 2004; Kallenbach et al., 2012; Dinh et al., 2013; Herden et al., 2015), there is a gap in knowledge regarding the plant stem defense response. This study examines pith-specific defense responses in *N. attenuata* to larvae attack by the stem-boring insect *T. mucorea*.

In short, this research has two major goals: to understand host selection specificity in Solanaceae stem-boring insects of the two species of *Trichobaris*; and to describe the plant defense response against stem-boring weevils feeding on the pith part of the stem tissue in the wild tobacco plant *N. attenuate.* The study highlights the importance of ecological context and complexity when studying plant-insect relationships, and provides a unique insight into the ecology of plant defense. Furthermore, this study constitutes a significant advance in the study of plant-insect interactions or relationships, providing key information on the implementation of herbivore resistance breeding strategies in plants.

Chapter 2. Life cycle and biology of tobacco stem weevil, *Trichobaris mucorea* (Coleoptera: Curculionoidea)

2.1. Introduction

Weevils are classified in the superfamily, Curculionidae, which contain more than 60,000 described species and likely four times as many in existence (Marvaldi et al., 2002; Oberprieler et al., 2007). Curculionidae distribute almost all over the world even in harsh environments, e.g. humid tropics, subaquatic, subterrancean, desert, and tundra, because they can feed on virtually all plants (Oberprieler et al., 2007). This family weevil predominantly live inside of plants (endophytic life), e.g. root, flower, seeds, and floral bud (Marvaldi et al., 2002). Especially the genus *Trichobaris* are known as Solanaceae weevil attacking tobacco, tomato, potato, and other solanum cultivated plants in distribution with 12 described species (O'Brien and Wibmer, 1982; Cuda and Burke, 1986; Huerta-Paniagua et al., 2004; Hare, 2005; De-la-Mora et al., 2015).

Trichobaris species also have endophytic life, which oviposited the egg inside of the stem of selected host plants, like other Curculionidae weevils (Diezel et al., 2011b) There has been mainly reported on the mechanisms plant resistance of leaves in response to leaf herbivore in the past, so economic importance of herbivores in other plant organs often underestimated (Erb et al., 2009). *Trichobaris* larvae spend whole life at inside of host plants until become new adult, so they often make severe damages to major crops, e.g. tomato and potato (Cuda and Burke, 1986;

Huerta-Paniagua et al., 2004). *Trichobaris mucorea* LeConte species has known as a tobacco stalk borer (Barber, 1935), therefore *T. mucorea* can cause severe damage to tobacco. However, basic biological information on tobacco stalk weevil (*T. mucorea*) is still limited. Here I present quantitative observations of *T. mucorea* with their host plants in the native habitat, Great Basin Desert, Utah, USA. Also, I examine development, longevity, and behavior traits of *T. mucorea* in the laboratory.

2.2. Methods

2.2.1. Study sites and insect collections

T. mucorea were monitored in and collected from Lytle Preserve in the Great Basin Desert of southwestern Utah, USA. In 2013 May to June, I collected adults from the native *Datura wrightii* population. To check larval development observation, I used remained head capsules or mandibles from past instar. When they became to next stadium, they left head capsule. The first instar head capsule which transformed second instar length and width are 0.4 mm. Next head capsule 0.5 mm in long and width that turned to third instar stage. The third head capsule was 0.6 mm long, 0.7 mm wide (n=5). All the head capsules were light brown and the mandibles were dark brown. The larval exuvium with head capsule were observed when they had pupation. Last head capsule 1.5 mm, 1mm in length and width (n=5). The

larvae were grown at 26℃/24℃ (16hr light /8hr dark) and 65% relative humidity in growth chamber.

2.2.2. Scanning electron microscope (SEM) images of *Trichobaris mucorea*

Pictures were taken of insects collected from the native *N. attenuata* and *D. wrightii* population in 2014. For species identification of *T. mucorea*, male reproductive organ images were taken pictures by Axiocam HRc connected to stereomicroscope SV 11 and captured with AxioVision 4.0 software (Zeiss, Germany). For SEM image analysis, *T. mucorea* were fixed by Karnovsky's fixative at room temperature. The specimen were dehydrated in a graded series of ethanol and affixed to studs with conductive gold paint. Freshly killed insect specimens were mounted dorsal surface in scanning electron microscope (SEM). Antennal part were viewed and photomicrographed with the SEM using an accelerating voltage of 10 kv and a working distance of 8-10 mm.

2.3. Result and discussion

2.3.1. *Trichobaris mucorea* picture and their appearance at the study site

T. mucorea adults can be observed from March when they were coming out from overwintering sites (Diezel et al., 2011b). I have found *T. mucorea* in *D. wrightii* and *N. attenuata* (Fig. 2-1A and B), In native habitat, *T. mucorea* had hole- and surface feeding type, 1 to 5 mm in diameter (see yellow allow in Fig. 2-1A and C). *T. mucorea* larvae have found at Lytle, which is experimental site of *N. attenuata*, in 2014 (n=200) and native *N. attenuata* population in 2015 (n=37). Although infection rates were highly variable each year (Fig. 2-1D), *T. mucorea* infected *N. attenuata* plants were observed for 10 years in the Lytle Preserve, Utah, USA (Diezel et al., 2011b; Schuman et al., 2015). Normally only one larval is found per a plant. However, huge-grown *N. attenuata* (> 1m) had thick side branch, so sometimes there are more than one larval found in a single plant (Fig. 2-1D). At the end of its feeding period is that end of June and beginning of July, larvae excavated a partial hole through the vascular bundles and epidermal layers to the outside of the stem for the exit of the adult. In August the adults emerged from the stems and likely overwinter in dried and senescent *N. attenuata* stems.

Although *T. mucorea* is known as tobacco stalk weevil (Barber, 1935), I barely observed *T. mucorea* adults in *N. attenuata* in the field sites. Most of *T. mucorea* were coexisted with *T. compacta* in *D. wrightii* in the field. But, *T. mucorea* may prefer to oviposit to *N. attenuata* plants. As abundances of *T. mucorea* adults decreased during the evening, I assumed

that *T. mucorea* moved to *N. attenuata* for oviposition during the night. It is also interestingly why only *T. mucorea* choose *N. attenuata* as host plants for larvae and *D. wrightii* for adults. It is likely that *T. mucorea* has some advantages of choosing in *N. attenuata* as a female' oviposition which means insects detoxification of plant secondary metabolites is regarded as important evolutionary process in sympatric speciation (Drès and Mallet, 2002; Thompson, 1988b). Therefore, *T. mucorea* may evolve specific detoxification process of nicotine for escaping from strong competition with *T. compacta*. However, *N. attenuata* is relatively unreliable host plants than *D. wrightii* because *N. attenuata* is annual and post pioneer species (Stanton et al., 2016). Ultimately, *T. mucorea* may have dual strategy of host plants decision for maximizing fitness of each developmental stage. Additionally, it needs to experimental investigate *T. mucorea* host selection traits to know why *T. mucorea* adults observed in both *D. wrightii* and *N. attenuata* plants.

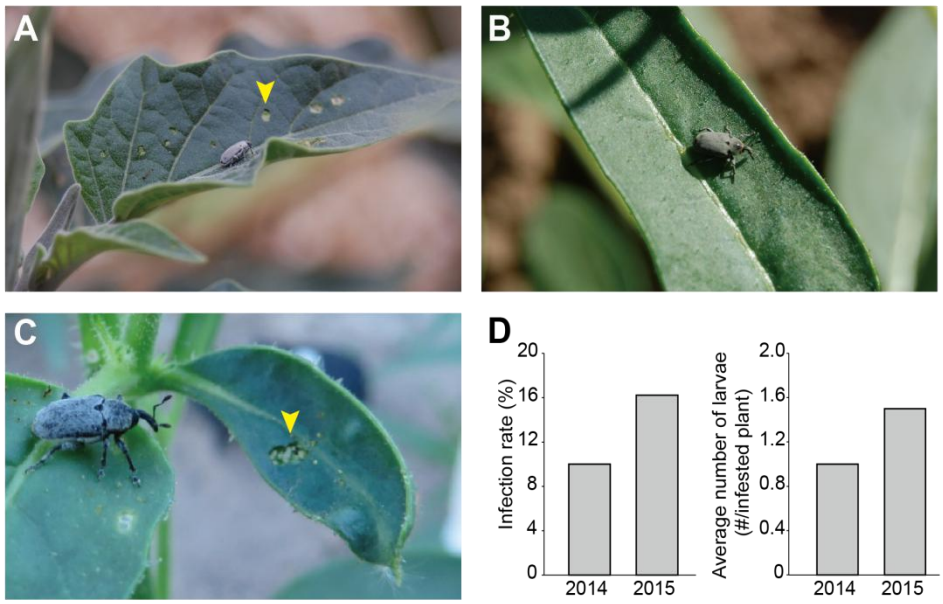

Fig. 2-1. *Trichobaris mucorea* adult in natural populations of *N. attenuata* and *D. wrightii*. (**A-C**) The adult usually feed on leaf tissue, their larva feed on pith part of stem tissue. (**A, C**) Feeding damage by adults on *D. wrightii* and *N. attenuata* (yellow arrow). (**D**) *Trichobaris* larvae infection rate and average number of larvae per infested plants at 2014 and 2015 in native population of *N. attenuata* plants.

2.3.2. Identification of two *Trichobaris* species

Differences in the reproductive mechanism are strongly believed to be of the greatest taxonomic value because it is necessary to co-adapt of standardized genital structures of both sex (Barber, 1935). The male of *Trichobaris* genus genitalia of most of the forms have been studied about identification *Trichobaris* species by extracting median lobe of male reproductive organ (Barber, 1935). The key point is the orifice of the median lobe is partly closed by a pair of sclerotized plates which differ more or less in shape in the different *Trichobaris s*pecies. *T. mucorea* male showed that round and bended median lobe (ml) (side view, Fig. 2-2A), but *T. compacta* (*Datura* weevil) male had keen shape of median lobe (side view, Fig. 2-2B). In addition, a pair of sclerotized plates (op) in *T. mucorea* has not fully cover in all orifice (mo) part, and copulatory organ has oval shape with grooved end area, but orifice of *T. compacta* was fully covered by sclerotized plates (top views, Fig. 2-2A and B).

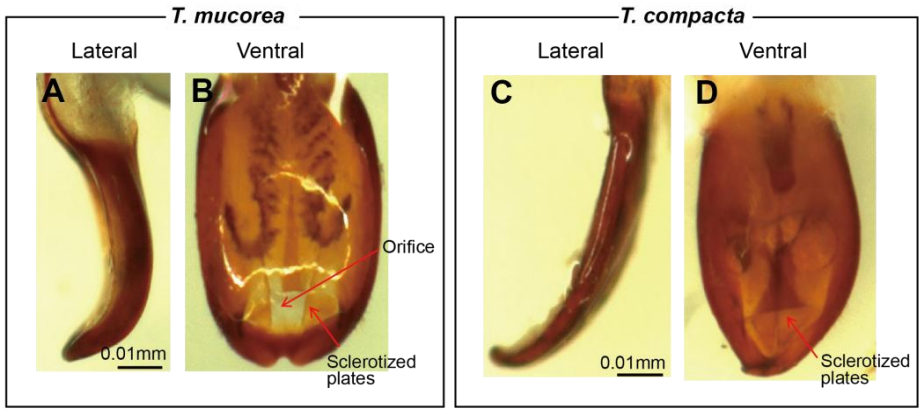

Fig. 2-2. Rigid male reproductive organs of *T. mucorea* compared with sibling weevil, *T. compacta*. (**A, C**) Lateral part of median lobe of two *Trichobaris* species. (**B, D**) Ventral part of median lobe of two *Trichobaris* species.

2.3.3. Male and female differences of *T. mucorea*

Previous literature (Barber, 1935) mentioned that the female and male have different shape of antenna but that was not clear explanation for discrimination, thereby I needed to certain characteristic. I observed that sexual distinctive was able to be as antenna shape using a Scanning Electron Microscope (SEM) images (Fig. 2-3A-F). The antenna shape of male looks keen in tip part with vestiture on the whole antenna which has full covered vestiture (Fig. 2-3A and B). On the other hand, the female has convex shape of tip part in their antenna, and shows three bands that mean no vestiture area (Fig. 2-3D and E). Another key for difference trait was end part of abdominal ventrites in the male. From SEM micrographs images, the male possess a reproductive organ that united to end area compared with female tip part of abdominal ventrites (Fig. 2-3C and F). Moreover, I found that male *T. mucorea* significantly smaller than female *T. mucorea* (Fig. 2-3G); the female was 7.2 ± 0.2 mm long with 2.35 ± 0.1 mm wide (n=100) and the male was 5.8 ± 0.1 mm long with 2 mm ± 0.1 mm wide (n=100).

2.3.4. Oviposition site of *T. mucorea* in *N. attenuata* and *D. Wrightii*

Female *T. mucorea* laid light-yellow eggs on *N. attenuata* basal stem area before the initiation of stem elongation that neonate larvae can easily enter the newly developing pith to complete development stems without making significant growth defect in infested plants. The performance of oviposition was observed that firstly female made a hole through their rostrum in any stem part but, usually I observed basal part of stem. After that female deposited the egg in epidermal tissue which also made cover layer for protected the egg in both *N. attenuata* and *D. wrightii* (see yellow allow in Fig. 2-4A and B).

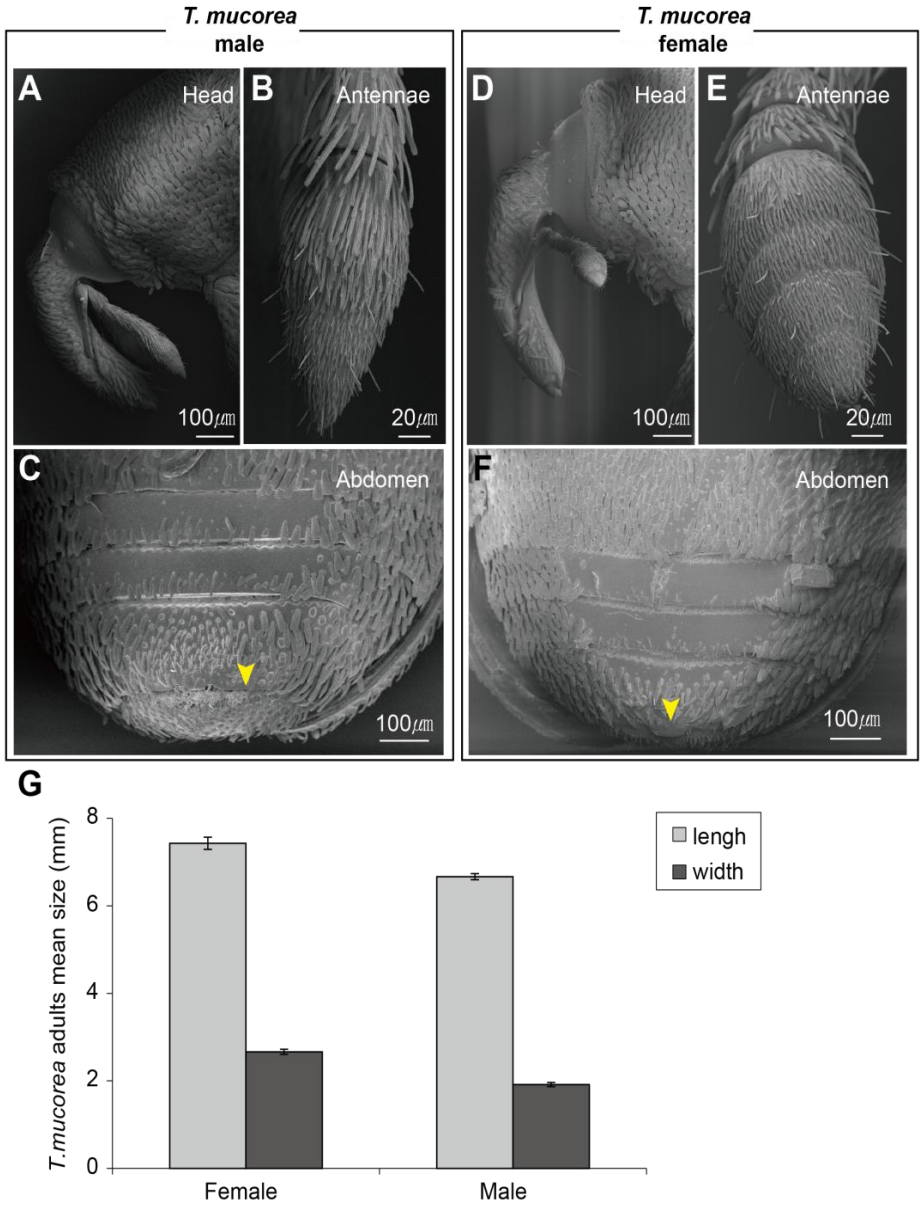
T. mucorea
male
T. mucorea
female
A
Head
B
Antennae
D
Head
E
Antennae
100㎛
20㎛
100㎛
20㎛
C
Abdomen
F
Abdomen
100㎛
100㎛
G
T.mucorea adults mean size (mm)
8
6
4
2
0
lengh
width
Female
Male

Fig. 2-3. Morphological comparison of *T. mucorea* male and female. (**A-F**) Morphological differences were determined through SEM micrographs. (**A, B**) End part antenna of male, it shows that shape is like the tip of brush. (**D, E**) End part antenna of female looks like bulging shape comparted with male. (**C**) Tip part of abdominal ventrites in male; the yellow allow indicates that the male reproductive organ for mating. (**F**) Tip part of abdominal ventrites in female that has pointed shape; the yellow allow indicates that the female reproductive organ for mating. (**G**) Length and width comparisons between *T. mucorea* male and female (n=100).

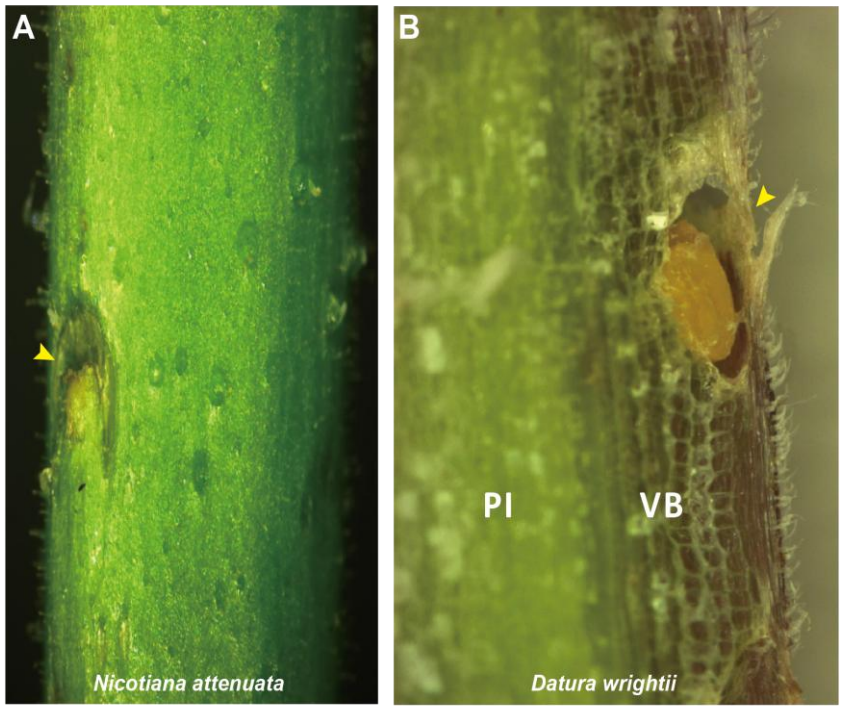

Fig. 2-4. Oviposition traits of *T. mucorea*. (**A**) The egg was deposited in epidermal layer of *N. attenuata* stem. (**B**) The deposited egg was in *D. wrightii* epidermis. The egg was only covered by thin epithermal tissue (yellow arrow) as shown in *N. attenuata* and *D. wrightii*. PI=Pith, VB=Vascular bundle.

2.3.5. Development and longevity of *T. mucorea*

T. mucorea undergo complete metamorphosis. From egg to adult grow over a period of approximately 1~2 months. From laboratory rearing, the larvae development has forth stage based on quantification of molts and head capsule (Fig. 2-5). The eggs of the *T. mucorea* are spherical form; length is 1mm, 0.5mm in diameter. Newly deposited eggs are bright- yellow, when the eggs are not able to hatch turn to dark brown and black. The duration of the egg stage ranged from 2 to 7 days. The mandibles or head capsules of the developing larvae were visible through the egg chorion 5-7 days. The total period of larval development is about 21-34 days. Neonate was translucent yellow, and the head was pale bright brown, 0.5 ± 0.3 mm long, 0.3 ± 0.1 mm wide (n = 10) meanwhile, difficult to find with unaided eyes. The duration of the neonate to first instar stadium took 4-7 days, from first instar to second instar took 6-7 d in the laboratory The First instar was 4.3 ± 0.3 mm long, 1.0 mm wide (n = 10), bright yellow body. The second instar was 5.6 ± 0.1 mm long, 1.5 ± 0.1 mm wide (n = 10). The duration of the first instar to second instar stadium 6-7 days in the laboratory. The third instar was 8.3 ± 0.6 mm long, 2.3 ± mm wide (n=10). The forth instar was 8 mm long, 3 mm wide (n = 10) which was the biggest size than other larvae and pupa. From second instar to third instar took 6-10 days. The duration of the third instar to forth instar stadium was 5-10 days in the laboratory. The first larvae to forth larvae were similar in appearance to each other except

neonate. Full-grown larvae started to pupation. The larvae grew over to reach a pre-pupa stadium, body length of 11 mm and width was 3.5 mm (n = 5). Before pupation the larvae which mean pre-pupa step shrank and became dull. After 3-7 days, the forth instar shrinked to pre-pupa stage and after 4-6days pre-pupa changed to pupa. The pupa was 7.5 ± 0.1 mm long, 2 mm wide (n = 10). After 6-14days, the pupa became an adult. The adult was 6.3 ± 0.3 long, 2.1 mm (n = 10). The adult longevity was approximately 1-3 months.

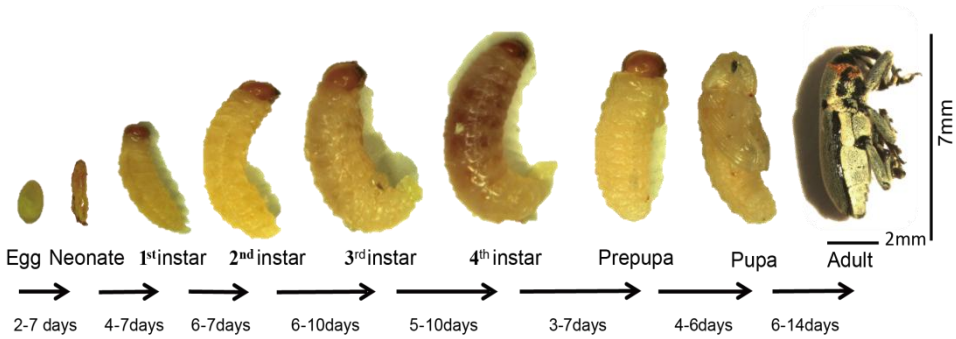

Fig. 2-5. Developmental stages and approximate metamorphosis durations of *T. mucorea*. The figure visualized the developmental process of *T. mucorea* from egg to adult stage. Sizes of pictures represent average size of each developmental stage.

2.3.6. Behavior characteristics of *T. mucorea* larvae

There are unique behaviors of *T. mucorea* larvae in the stem. In case of second or third instars stage, they emit to a droplet from their anal area, oral secretion as well against external stimuli (Fig. 2-6A). Last forth instar larvae stage; they did two distinctive behaviors, one is that the larvae bored outward like gate or window, penetrating the woody secondary xylem but not the cortical and epidermal layers of the stem for apparently easy to later adult emergence (Fig. 2-6B). And second is that the larvae than made a woody fibers chamber before entering a non-feeding period, pupation. This behavior facilitates that the larvae can be safe during pupa stage and until becoming new adults (Fig. 2-6C and D).

Since larvae of *T. mucorea* had endophytic life in the stem, it is unclear why the larvae had anal secretion behavior in response to mechanical stimuli. Although I could not find ant predator and parasitoids of *T. mucorea* in the study site, larva parasite wasp, *Neocatolaccus tylodemae*, was reported in potato stalk weevil, *Trichobaris trinotata* (Cuda and Burke, 1986). In addition, many larval insects have predator avoidance performance to increase their Darwinian fitness (McGee et al., 2009; Combes et al., 2012; Schuman et al., 2012)

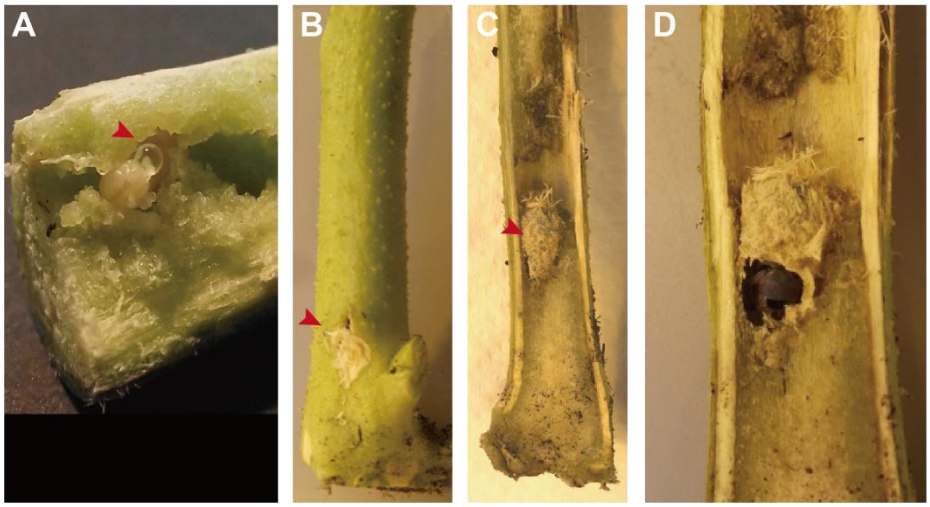

Fig. 2-6. Behavior charactieristics of *T. mucorea*. (**A**) The second and third instar larva emitted a droplet from their anal part. Red arrow indicates anal secretion of the larvae that was colorless and transparent. (**B**) The forth instar larva made a "window" in the stem before pupation to prepare the way to go out when they become adults. Red arrow indicates the exit which is light part compared with stem color. (**C**) The forth instar larva invented a chamber like a nest in order that protect to pupa stadium. Red arrow presents the chamber was made by last instar larva. (**D**) From the forth larva to pupa, *T. mucorea* larvae stayed for until became a new adult.

Chapter 3. Host selection specificity of two *Trichobaris* species[a]

[a] This study has been accepted in *Molecular Ecology.*

3.1 Introduction

At the broad host selection traits of herbivores through adult preference and larval performance level studied as various suggestion of hypothesizes on this relationship which is a crucial issue of the host selection specificity in insect-plant interaction (Thompson, 1988a; Price, 1997). There are theories to explain host plant selection in herbivore; the most common explanation is the preference-performance hypothesis (PPH), also known as optimal oviposition theory (Jaenike, 1978; Thompson, 1988a) predicts that a female insect will preferentially oviposit on host plants that maximize their larvae performance. These hypotheses states that adult preference patterns are supposed to correspond to host suitability for offspring development due to females know to their fitness perform best by oviposition on high quality host plants (Jaenike, 1978). This is particularly the case for insects whose larvae have little or limited mobility to relocate, the female's host plant choice strongly constrains the consequences for the offspring performance which are positive correlation between oviposition preference and offspring performance (Jaenike, 1978; Thompson and Pellmyr, 1991; Zalucki et al., 2002; Bertheau et al., 2009; Clark et al., 2011; Mazaheri et al., 2011; Zhang et al., 2012). However, many studied have been found to make poor or no correlation between adult preference and offspring (Jallow and Zalucki, 2003; Brodbeck et al., 2007; Gripenberg et al.,

Chatzigeorgiou et al., 2010; Shikano et al., 2010). The negative correlation assume that females may select host plants that are optimal for adult nutrition instead of optimal for offspring which mean the female oviposit where they fed host plant for adult benefit (Scheirs and Bruyn, 2002). As many factors are able to interact to determine adult preference and larval performance respectively, the interactions among factors may lead to weak understanding of the preference-performance relationship (Craig et al., 2000). Consequently, there is need to more studies to understand preference-performance linkage including specific evidence related to host selection factors.

Host selection of herbivores is mainly affected by plant secondary metabolites. These compounds may act attractants or deterrents cues that signal the quality of plants for food or their offspring growth, therefore herbivores especially specialist insects choose host plants upon which to feed and oviposit on the basis of plant secondary metabolites (Ostrand et al., 2008; Matsuki et al., 2011). One of the best-studied groups of plant specialized metabolites are the glucosinolates related to host suitability depend on plant defense secondary metabolites as chemical stimulant in Brassicales (Halkier and Gershenzon, 2006). For instance, accumulating high levels of aliphatic- or indolyl-glucosinolates of *Arabidopsis thaliana* plants negatively affected to performance and selectivity of the whitefly, *Bemisia tabaci* with closely related chemical signatures in heterogeneous

habitats (Markovich et al., 2013) and glucosinolates of *Barbarea vulgaris* were active as oviposition stimulants for the diamondback moth, *Plutella xylostella* and other insect specialists of Brassicaceae in determining host plant preference (Marazzi and Städler, 2004, 2005; Renwick et al., 2006; Sarfraz et al., 2006). Another example, tobacco hornworm , *M. sexta* accept to feeding as a indioside D compound of solanaceous host plant (del Campo et al., 2001), *Agelastica alni*, leaf beetle preferred to feed on depending on phenolic content in five different Salix plants (Ikonen et al., 2002), Catechol metabolite of roasted coffee bean is oviposition stimulant for cigarette beetle (Nagasawa et al., 2014) and monoterpenes among volatiles were used as a negative cue for feeding by Christmas beetles to select host trees (Matsuki et al., 2011).

In particular, *T. compacta* reported as *D. wrightti* weevil and *T. mucorea* is *N.attenuata* weevil (Barber, 1935). Although both *T. compacta* and *T. mucorea* distribute same habitat such as Arizona, California, Utah of Southern United States and Mexico (www.sil.si.edu/bcaproject), their accurate host range and host selection traits are poorly understood. And *N. attenuata* was developed as a model system for studying ecological and molecular biological level with herbivores interaction (Kessler and Baldwin, 2001). Therefore it is great system to know *Trichobaris* host selection traits apply with managed *N. attenuata* plants. In this study, I investigated some of the fundamental aspects of *Trichobaris* species host selection behavior

through patterns of adult preference (feeding and oviposition) and larval performance (survival and mass) in two sibling *Trichobaris* species, *T. compacta* and *T. mucorea* species that determined whether female oviposition preference was positively correlated with larval performance. I also investigated specific factors for female oviposition decision and larval performance used different combination of transgenic plants which manipulated candidate secondary metabolites of *N. attenuata* plants.

3.2 Methods

3.2.1. Plants and insects

N. attenuata (Torr. ex Wats.) wild-type (WT) originated from a native population located 25km north of the field station where the current work was performed in southwestern Utah, was stably transformed to alter the expression of the following secondary metabolites and fully characterized previously in isogenic homozygous lines harboring a single transgene insertion: irPMT (108-3 line) and irAOC (457-1 line) plants are reduced to 3-4% of WT nicotine levels (Steppuhn *et al.* 2004) and 0.01% of elicited WT jasmonic acid (JA) levels (Kallenbach *et al.* 2012), respectively, and ovTPS10 plants (10-3 line) that produce 50-fold more (E)-β-farnesene and 100-fold more (E)-α-bergamotene compared to elicited WT plants (Schuman *et al.* 2014). *N. attenuata* seeds of 31st inbred generation were

sterilized and germinated on Gamborg's B5 medium (Duchefa) as described previous study (Krügel et al., (2002). Seedlings were maintained at 26℃/16h light (155μmol $m^{-2}s^{-1}$): 24℃/8h dark cycle. Young seedlings were planted in Teku plastic pots 10 days after germination; after 10 days, plants were transferred to 2L pots. *D. wrightii* (Regel) seeds were obtained from B &T World Seeds (Paguignan, France), and later the seeds were harvested from inbred plants in the glasshouse. *D. wrightii* plants were grown in 2L pots under the same growth conditions as *N. attenuata* plants. All plants were grown in the glasshouse with 16h light (26-28℃) and 8h dark (22-24℃) cycles under Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600 high pressure sodium lights (Philips) with water supplied daily via an automatic watering system.

In 2013, *T. compacta* and *T. mucorea* adults were collected from their natural habitat, the Great Basin Desert in southwestern Utah (D1-D5 sites; Fig. 3-1). With these field collected adults, a laboratory colony was established (Fig. 3-2). *T. mucorea* and *T. compacta* adults were fed *D. wrightii* leaves/floral buds and *Trichobaris* females laid eggs on the petioles of *N. attenuata* or floral buds of *D. wrightii*. Collected eggs were experimentally inoculated into the basal stems in *N. attenuata* or *D. wrightii*. Three weeks later, the egg-inoculated stems were split to collect the larvae (in the WT plants larvae were usually in 2^{nd} or 3^{rd} instars). Each *Trichobaris* larva was transferred to a 15mL tube filled with an artificial diet which

following ingredients were mixed together and autoclaved at 16 psi and 120℃ (1000 ml distilled water, 40 g agar, 30 g casein, 100 g cellulose powder, 5 g Wesson's salts, 30 g wheat germ, 30 g sucrose, 3 g glucose, 3 g trehalose, 200 mg inositol, 1250 mg methyl para-hydroxybenzoate and 900 mg sorbic acid. After autoclaving, 500 mg cholesterol and 2 ml linseed oil were then mixed in. The following were added after cooling to 70℃: 3 g ascorbic acid, 2 g choline chloride, 20 mg niacin, 20 mg calcium pantothenate, 5 mg thiamine, 10 mg riboflavin, 5 mg pyridoxine, 5 mg folic acid, 400 pg biotin, 40pg vitamin B12, 350 mg penicillin, 350 mg streptomycin, and 2 ml 10% formaldehyde. All ingredients were mixed together thoroughly and the diet was poured into plastic petri dishes and left to set) developed for stem weevil larvae (Malone and Wigley, 1990). About one week later, I collected the newly emerged adults from the tubes. All steps were performed in a growth chamber with a 16h light (26℃) and 8h dark (24℃) cycle and 65% humidity (Snijders Scientific, Tillburg, and The Netherlands).

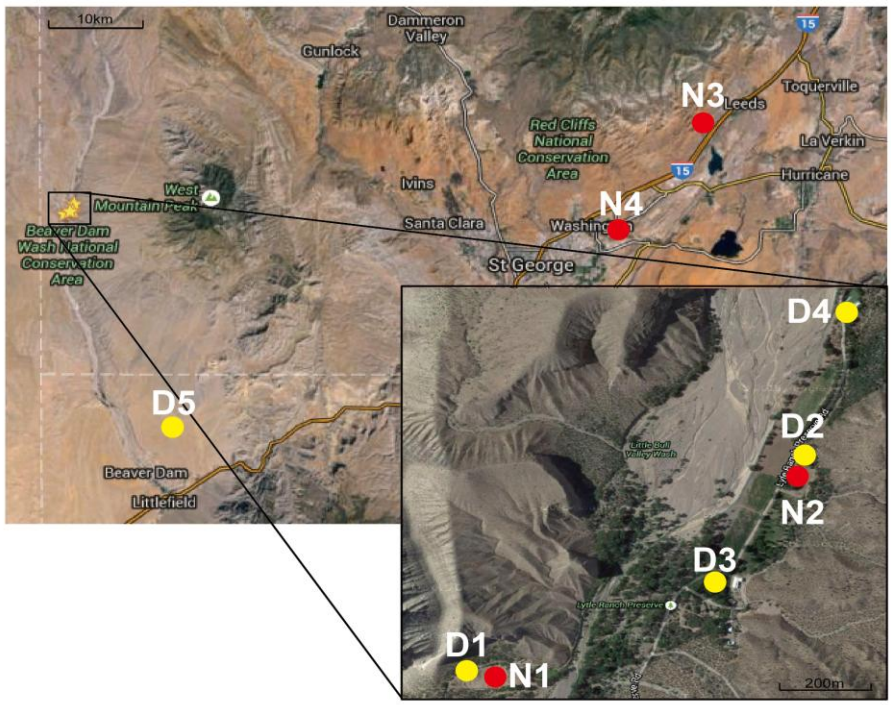

Fig. 3-1. Sites surveyed in the Great Basin Desert of southwestern Utah, USA. *D. wrightii* populations: D1, 37’14”14.09N, 114’02”82.41W, n=81; D2, 37’14”66.91N, 114’01”97.08 n=110; D3, 37’14”36.24N, 114’02”19.9W, n=45; D4, 37’15”03.61N, 114’01”86.07W, n=76; D5, 36’95”56.45N, 113’92”24.54W, n=77. *N. attenuata* populations: N1, 37’14”12.52N, 114’02”76.20W, n=200; N2, 37’14”63.29N, 114’01”97.56W, n=200; N3, 37’13”27.80N, 113’23”49.50W, n=20; N4, 37’07”45.60N, 113’28”52.80W, n=97.

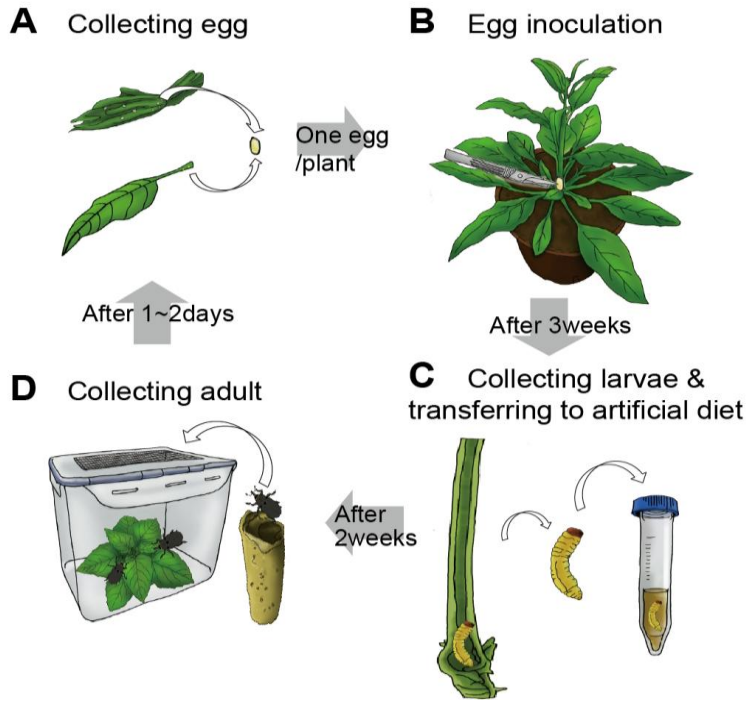

Fig. 3-2. Setup for the culturing of *Trichobaris* species in the laboratory. (**A**) *T. mucorea* and *T. compacta* adults were collected from their native habitat. Eggs of each *Trichobaris* species were collected from *N. attenuata* petioles and *D. wrightii* buds. (**B**) One egg was inoculated into the basal part of each *N. attenuata* stem during the early elongation stage of plant growth. Five to seven days after inoculation, the eggs hatched, and the neonates burrowed into the pith and started feeding. (**C**) After three weeks, egg-inoculated stems were cut carefully, and larvae were extracted and weighed before being transferred to individual 15mL falcon tubes with artificial diet and reared to adults. (**D**) Newly emerged adults were transferred to the colony containers with *D. wrightii* and *N. attenuata* plants for mating and oviposition.

3.2.2. Adult preference experiment

Adult preference was evaluated through feeding and oviposition behavior in a dual choice test conducted in a white mesh cage (47.7 x 47.5 x 93.0 cm, BugDorm, BioQuip, Rancho Dominguez, CA) in a glasshouse at 26℃-28℃ under 16h of light condition by Philips SON-T Agro 400 (Philips, Germany, http://www.lighting.philips.co.uk/) sodium lights. Paired-wise plants were used same development stage (elongation stage) and same height and randomly distributed in a cage. Tested insects which newly emerged females were allowed to mate in single pairs in a same container for two nights before insects released. I used a small plastic cup that placed in middle place of two plants for reducing position effect, and each three gravid females released to the small plastic cup in each cage. After three days, each plant of all experiments was measured consumed area for feeding preference and counted eggs for oviposition preference that females deposited in epidermal tissue which also made cover layer for protection the eggs (Fig. 3-3).

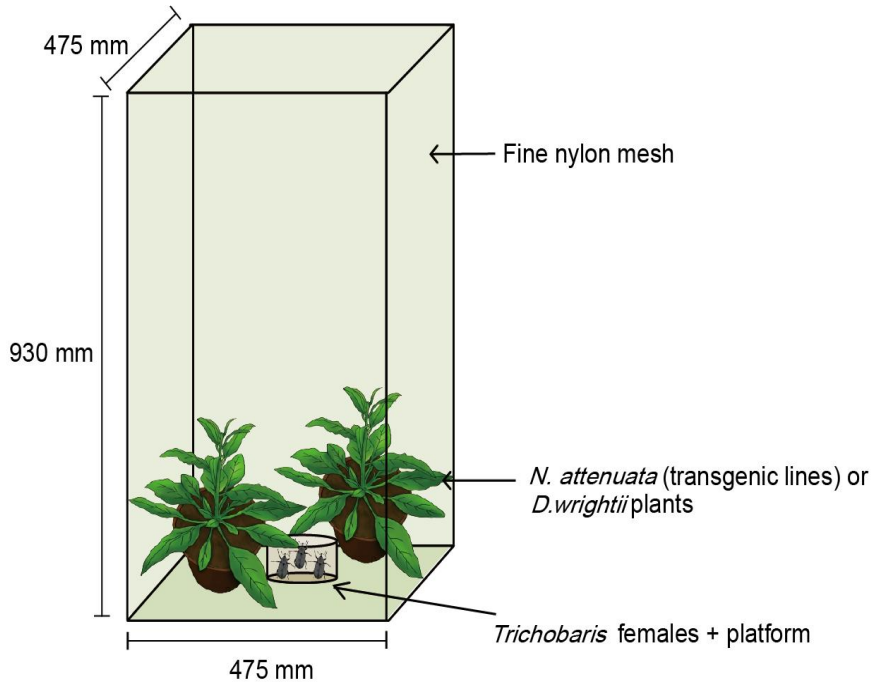

Fig. 3-3. Experimental device for adult preference experiments. Paired-wise plants were used same development stage (elongation stage) of transgenic *N. attenuata* or *D. wrightii* plants and same height and randomly distributed in a cage. A small plastic cup that placed in middle place of two plants for reducing position effect, and each three gravid females released to the small plastic cup in each cage.

3.2.3. Larval performance experiment

To test for larval performance of two *Trichobaris* species, I used egg inoculation method (Fig. 3-4) modified *Trichobaris* species oviposition pattern. First, I collected eggs from *Trichobaris* females laid on newly. The egg slightly abraded the epidermal layers of the basal part of the stem of elongation stage plants that I used larval performance experiment and inserted each *Trichobaris* species egg into each the abraded stem part. After three weeks, we slit stems to collected larvae and measured survival and mass from each plant.

Fig. 3-4. Method for *Trichobaris* egg inoculation into the stems of early elongation-stage *N. attenuata* plants. The epidermal layer of basal part of stem slightly is abraded by a keen knife and insert each egg into the abraded stem part using a forcep (red circle).

3.2.4. Statistical analyses

All measures were conducted with Origin 8 SR1 (OriginLab Cop. Northampton, USA) or the publically available R package (version 3.1.2., http://www.r-project.org/). The total consumed area of each pair of plants for feeding preference and oviposition preference to compare the number the of eggs deposited on each pair of plants with the null hypothesis of preference was compared by a replicated goodness-of-fit *G*-test (Sokal and Rohlf, 1995). Because the *G*-test are additive compared with chi-square test. And the *G*-test can conduct an elaborate experiment in which the *G*-values of different parts of the experiment add up to an overall *G*-value for the whole experiment. In case of survival rate was used chi-square test due to the nominal values was survived and death and it has only one part of the experiments. To show significance of different mean values between two different samples, larval mass was analyzed one-way ANOVA followed by Fisher's least significant difference (LSD).

3.3. Results

3.3.1. Field observation

Abundance of two *Trichobaris* adults was investigated in *Datura* population and *Nicotiana* population where I selected five different *Datura* population and four different *Nicotiana* populations of Great Basin Desert of

Southwestern Utah in 2014 (Fig. 3-1) . Although *T. mucorea* is known as tobacco stem borer of *N. attenuata*, I found most of *T. mucorea* adult in *Datura* population and observed only few *T. mucorea* adults in *Nicotiana* population (Fig. 3-5A) and I collected larvae from infected *N. attenuata* from Lytle plot (N2) population and investigated adult appearance rate that identified newly emerged adults from collected larvae. All of collected larvae became *T. mucorea* adult except died larvae (Fig. 3-5B). Although most of Trichobaris adults observed in *D. wrightii* population, particularly *T. mucorea* adults, I confirmed infected *N. attenuata* by *T. mucorea* which means they would have different cues for adult preference and larva performance of host selection decision between *D. wrightii* and *N. attenuata*.

3.3.2. Adult preference and larva performance of two *Trichobaris* species between *D. wrightii* and *N. attenuata*

To understand two sibling *Trichobaris* species host selection traits, firstly I investigated adult preference and larva performance between *D. wrightii* and *N. attenuata* host plants. I investigated female preference of *T. compacta* and *T. mucorea* between *D. wrightii* and *N. attenuata* using dual choice assay. Feeding preference results of two *Trichobaris* gravid females which released three days, both *T. compacta* and *T. mucorea* females showed that strong preference to *D. wrightii*. Although *T. mucorea* is known as tobacco stem borer, unpredictably the female prefer to feed on *D. wrightii*

(Fig 3-6A, E; $p < 0.01$). This result could come with why *T. mucorea* mainly observed in *Datura* populations not *Nicotiana* populations (Fig. 3-5C). But oviposition preference of two *Trichobaris* female was totally different. Concordantly feeding preference result, *T. compacta* oviposition preference indicated toward *D. wrightii* plants (Fig. 3-6B; $p < 0.001$). In case of *T. mucorea* female preferred to oviposit their egg into *N. attenuata* plants (Fig. 3-6F; $p < 0.001$). Feeding and oviposition preference behavior pattern of *T. compacta* female was same into *D. wrightii* but, *T. mucorea* was different pattern that the female preferred to feed on *D. wrightii* while oviposit into *N. attenuata*. These results said that two *Trichobaris* species presented different preference trend, *T. compacta* female had same host plant preference for feeding and oviposition. *T. mucorea* female showed that different feeding and oviposition choice between *D. wrightii* and *N. attenuata* host plants.

Larval performance was assessed survival and mass from three week continues feeding condition using egg inoculation method which we explained above method and material part. *T. compacta* larva only survived in *D. wrightii* plant (Fig. 3-6C; $p < 0.01$) and *T. compacta* larval mass only presented the larva fed on *D. wrightii* (Fig. 3-6D; $p < 0.01$). In case of *T. mucorea* larva performance indicated that their larva was able to survive in *D. wrightii* but, their survival was much higher in *N. attenuata* (Fig. 3-6G; $p < 0.01$). *T. mucorea* larva mass was not different fed on between *D. wrightii* and *N. attenuata* (Fig. 3-6H; $p = 0.836$). *T. compacta* larva performance

results were consistent with adult preference pattern that the females exclusively preferred to *D. wrightii* plants. In case of *T. mucorea*, oviposition preference result which preferred to *N. attenuata* more correlated with the larva survival in *N. attenuata* than *D. wrightii*. These different host selection preferences and larva performance of two *Trichobaris* species would effect on secondary metabolite of host plants. Therefore I hypothesized among alkaloids; nicotine which is most abundant metabolite of *N. attenuata* would be the strongest influences factor for host selection in two *Trichobaris* species.

3.3.3. Adult preference and larva performance of two *Trichobaris* species between EV and irPMT transgenic plant of *N. attenuata*

To rigorously investigate the role of nicotine in adult oviposition and feeding preferences and larval performance, I conducted choice assays with each of the two *Trichobaris* species with EV plants (empty-vector transformed WT *N. attenuata*) and irPMT plants (silenced in *putrescine N-methyltransferase*, which is a key enzyme required for nicotine biosynthesis; see Fig. 3-7A). I carried on choice test between EV and irPMT (silenced putrescine N-methyltransferase which is nicotine synthesis enzyme) plants to investigate adult preference and larval performance of two *Trichobaris* species. Both two *Trichobaris* species showed that no different feeding

preference between EV and irPMT plants (Fig. 3-7B, F; p = 0.203, p = 0.291 respectively). Unpredictably *T. compacta* female rarely laid eggs on irPMT plants (Fig. 3-7C; p = 0.095). Also *T. mucorea* females did not show any preference between EV and irPMT plants (Fig. 3-7G; p = 0.291). Nicotine was not affected to two *Trichobaris* females feeding and egg-laying decision. While *T. compacta* and *T. mucorea* larval performance showed different patterns comparison with adult preference between EV and irPMT plants. *T. compacta* larval survived more than 50% in irPMT plants (Fig. 3-7D; $p < 0.001$) but, all of *T. compacta* larvae did not survived in EV plants consistent with above larva performance experiment of Datura and Nicotiana plants. Larval mass of *T. compacta* which fed on irPMT plants much higher than the larval fed on EV plants (Fig. 3-7E; $p < 0.001$).In case of *T. mucorea* larva performance, survive rate was not different in both plants (Fig. 3-7H; p = 0.705) same as larval mass pattern (Fig. 3-7I; p = 0.574). Although nicotine was not influencing factor in *T. compacta* female oviposition decision, nicotine affected on *T. compacta* larva performance. However *T. mucorea* female preference and larvae performance, both levels indicated that nicotine was not consideration to oviposition and larval act.

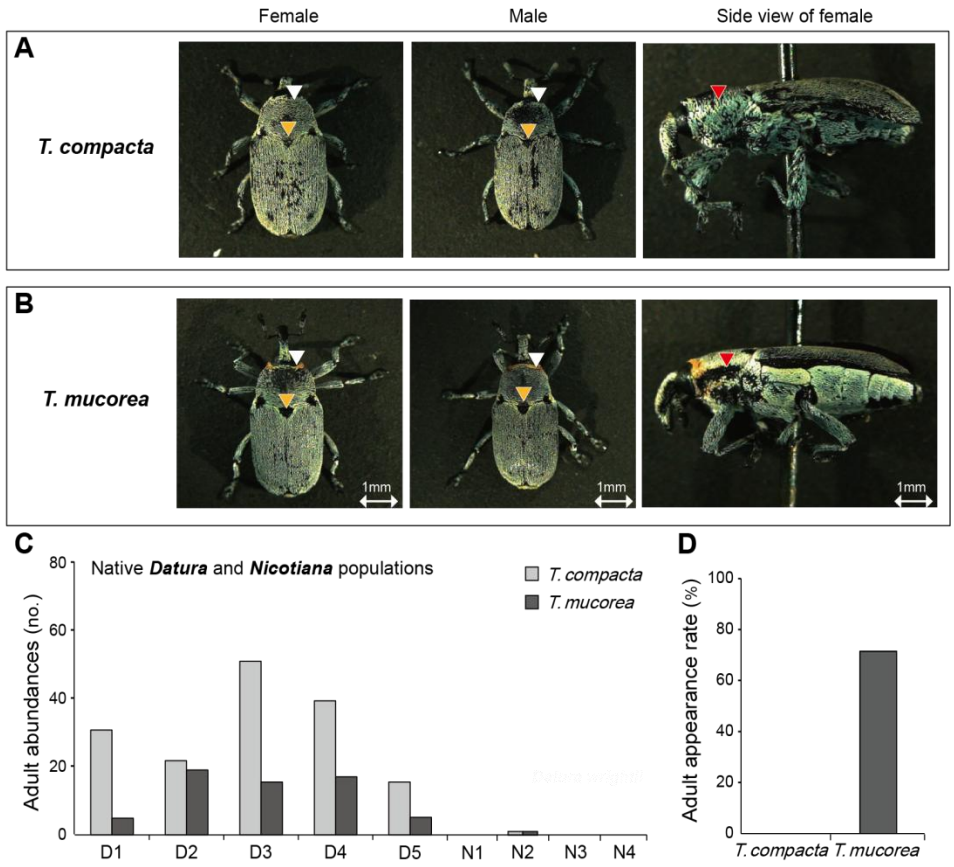
Female
Male
Side view of female
A
T. compacta
B
T. mucorea
1mm
1mm
1mm
C
Native Datura and Nicotiana populations
T. compacta
T. mucorea
Adult abundances (no.)
0
20
40
60
80
D1
D2
D3
D4
D5
N1
N2
N3
N4
D
Adult appearance rate (%)
0
20
40
60
80
100
T. compacta
T. mucorea

Fig. 3-5. Morphological difference and field observation of two sympatric *Trichobaris* species from the Great Basin Desert. (**A**) Dorsal view of *Trichobaris compacta* female, male and lateral view of female. (**B**) Dorsal view of *T. mucorea* female, male and lateral view of female. Three morphological traits highlighted by white, red, and orange inverted triangles distinguish the adults of the two *Trichobaris* species: an orange band on the head part of *T. mucorea* (white inverted triangle) which is absent from *T. compacta*, a black strip on the lateral aspect of *T. mucorea* thorax (red inverted triangle), again absent from *T. compacta*, three black spots (orange inverted triangle) on the dorsal part of *T. mucorea* which is larger than those on *T. compacta*. Larval stages are not readily distinguished. (**C**) Relative abundance of *T. compacta* and *T. mucorea* adults found in *Datura wrightii* and *Nicotiana attenuata* populations (see Fig. 3-1). Both *Trichobaris* species were observed in the five populations of *D. wrightii* but rarely observed in the four *N. attenuata* populations. (**D**) Larvae collected which were 2nd or 3rd instar stage (n=14) from *N. attenuata* plants (n=200) growing in the N2 population (see Fig. 3-1). 10 larvae were successfully reared to adulthood and produced *T. mucorea* adults, and 4 larvae died at the larval stage.

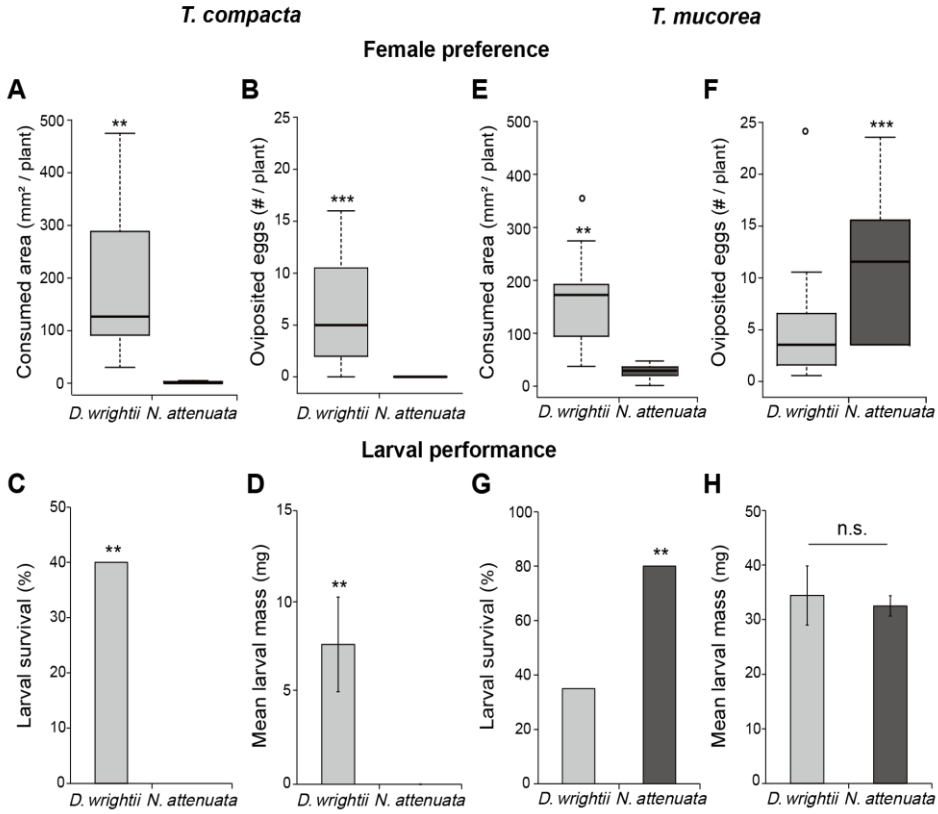
T. compacta
T. mucorea
Female preference
A
B
E
F
Consumed area (mm² / plant)
Oviposited eggs (# / plant)
D. wrightii
N. attenuata
Larval performance
C
D
G
H
Larval survival (%)
Mean larval mass (mg)
n.s.

Fig. 3-6. *T. compacta* and *T. mucorea* adult preference and larval performance in *D. wrightii* versus *N. attenuata* host plants. (**A**) Leaf area consumed by *T. compacta* adult females in choice tests: *T. compacta* adults fed more on *D. wrightii* than on *N. attenuata* plants (*G*-test; **, $p < 0.01$; n=8). (**B**) Numbers of eggs oviposited by *T. compacta*: females oviposited more on *D. wrightii* than on *N. attenuata* plants (*G*-test; ***, $p < 0.001$; n=8). (**C**) Percentages of surviving larvae of *T. compacta* three weeks after egg inoculation: *T. compacta* larvae survived only in *D. wrightii* plants (χ^2-test; **, $p < 0.01$; n=20). (**D**) Mean (± SE) larval mass of *T. compacta* that fed on *D. wrightii* plants (one-way ANOVA; **, $p < 0.01$, n=20). (**E**) Leaf area consumed by *T. mucorea* females: *T. mucorea* adults fed more on *D. wrightii* than on *N. attenuata* plants (*G*-test; **, $p < 0.01$; n=8). (**F**) Numbers of eggs oviposited by *T. mucorea*: females oviposited more into *N. attenuata* stems than into *D. wrightii* plants (*G*-test; ***, $p < 0.001$; n=8). (**G**) Percentages of surviving larvae of *T. mucorea* three weeks after egg inoculation: Survival rates of *T. mucorea* larvae were higher when the larvae fed on *N. attenuata* than on *D. wrightii* plants (χ^2-test; **, $p < 0.01$; n=20). (**H**) Mean (± SE) larval mass of *T. mucorea* that fed on *D. wrightii* and *N. attenuata* plants: There was no significant difference in the larval mass (one-way ANOVA; n.s., not significant; n = 20).

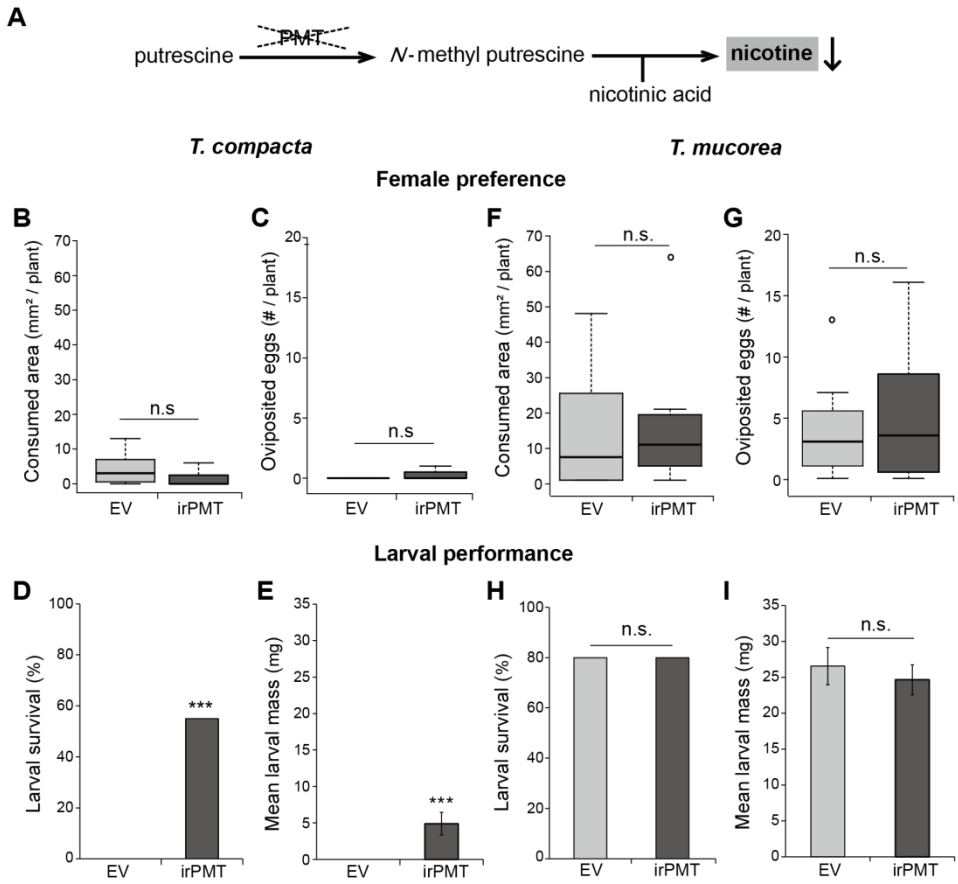
A
putrescine
PMT
N-methyl putrescine
nicotinic acid
nicotine
T. compacta
T. mucorea
Female preference
B
C
F
G
Consumed area (mm² / plant)
Oviposited eggs (# / plant)
n.s
n.s.
EV
irPMT
Larval performance
D
E
H
I
Larval survival (%)
Mean larval mass (mg)
***

Fig. 3-7. Feeding and oviposition preference of *T. compacta* and *T. mucorea* females and larval performance in nicotine-producing versus nicotine-free plants. (**A**) Simplified biosynthetic pathway of nicotine. To generate a nicotine-free plant, one of the key nicotine biosynthesis enzymes, *putrescine N-methyltransferase*, was silenced in *N. attenuata* plants (irPMT; Steppuhn *et al.* 2004). (**B**) Leaf area of EV and irPMT plants consumed by *T. compacta* females. (**C**) Numbers of eggs on EV and irPMT plants oviposited by *T. compacta* females: there were no significant differences in feeding and oviposition preferences (*G*-test; n.s., not significant; n=8). (**D**) Percentages of surviving larvae of *T. compacta* in EV and irPMT plants three weeks after egg inoculation: *T. compacta* larvae survived only in irPMT plants (χ^2-test; $p < 0.001$; n=20). (**E**) Mean (± SE) larval mass of *T. compacta* that fed on irPMT plants (one-way ANOVA; ***, $p < 0.001$; n=20). (**F**) Leaf area consumed of EV and irPMT plants by *T. mucorea* females. (**G**) Numbers of eggs on EV and irPMT plants oviposited by *T. mucorea* females: No differences in feeding and oviposition preference (*G*-test; n.s., not significant; n=8). (**H**) Percentages of surviving larvae of *T. mucorea* in EV and irPMT plants three weeks after egg inoculation: no significant difference in survival rates (χ^2-test; $p = 0.143$; n=20). (**I**) Mean (± SE) larval mass of *T. mucorea* fed on EV and irPMT plants: no significant difference in larval mass (one-way ANOVA; $p = 0.574$; n.s., not significant; n=20).

3.3.4. JA-mediated defense compounds affected on *T. mucorea* adult preference and larva performance in *N. attenuata*

To examine whether JA-induced signaling has an effect on either adult preference or larval performance of *T. mucorea*, I conducted a dual-choice assay with irAOC (*allene oxidase cylase*-silenced) transgenic lines impaired in their ability to synthesize JA (Fig. 3-8A). *T. mucorea* female indicated that significantly feeding preference to irAOC plants (Fig. 3-8B; $p < 0.001$). In contrast, oviposition preference of *T. mucorea,* the females preferred to deposit on EV plants (Fig. 3-8C; $p < 0.05$). Feeding and oviposition preference were opposite direction. Consequently *T. mucorea* female showed that preferably fed on irAOC plants whereas, deposited in EV plants. Larval performance results, there was no significant difference in larval survival between EV and irAOC plants (Fig. 3-8D, $p = 0.450$) but, larvae fed on irAOC plants were grown much bigger than the larvae fed on EV plants which three weeks continuous feeding period after egg inoculation (Fig. 3-8E, $p < 0.001$). Larvae mass was significantly correlated with JA absences.

3.3.5. Volatile cues guide *T. mucorea* females to *N. attenuata* host plant for oviposition

From above results, I confirmed that different feeding and oviposition choice of *T. mucorea* female between EV and irAOC plants. It indicates that attractant cue may exist for egg-laying into their proper host plants. In a previous field study found increased infestation of *T. mucorea* in the *TERPENE SYNTHASE 10* (*TPS10*)-overexpressing plants (ovTPS10), which produce more (*E*)-α-bergamotene (TAB) and (*E*)-β-farnesene (TBF) sesquiterpene volatiles than WT *N. attenuata* plants, and in WT plants that were surrounded by ovTPS10 in small populations (Fig. 3-9A; Schuman *et al.* 2015). Therefore, I hypothesized that *T. mucorea* female oviposition cue would be sesquiterpenes volatiles such as TAB and TBF. From dual-choice assay between EV and ovTPS10 *N. attenuata* plant, there was no feeding preference (Fig. 3-9B; $p = 0.073$) whereas, *T. mucorea* female preferred to oviposit the egg into ovTPS10 plants (Fig. 3-9C; $p < 0.001$). And larva performance of *T. mucorea* showed that there were no different survival and mass between EV and ovTPS10 plants (Fig. 3-9D, E; $p = 0.467$, $P = 0.868$ respectively). Overexpressed TAB and TBF volatiles were affected to oviposition decision in adult preference produced by ovTPS10 plants but, did not change larva performance.

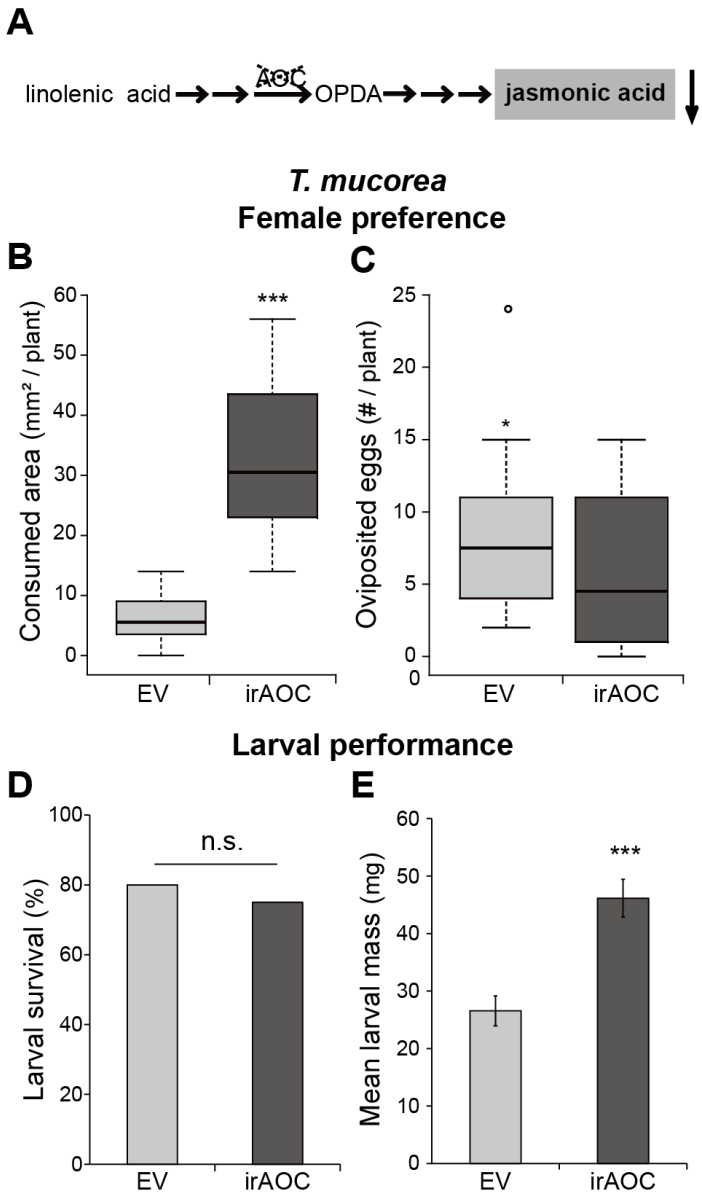
A
linolenic acid
AOC
OPDA
jasmonic acid
T. mucorea
Female preference
B
Consumed area (mm² / plant)
***
EV
irAOC
C
Oviposited eggs (# / plant)
*
EV
irAOC
Larval performance
D
Larval survival (%)
n.s.
EV
irAOC
E
Mean larval mass (mg)
***
EV
irAOC

Fig. 3-8. *T. mucorea* female preference and larval performance in EV versus irAOC plants. (**A**) Simplified biosynthetic pathway of JA. irAOC, silenced the jasmonic acid (JA) biosynthesis gene, *allene oxide cyclase* in *N. attenuata* (Kallenbach et al., 2012). OPDA; 12-oxo-phytodienoic acid. (**B**) Leaf area of EV and irAOC plants consumed by *T. mucorea* females: *T. mucorea* adult fed more on irAOC than on EV plants (*G*-test; ***, $p < 0.001$; n = 14). (**C**) Numbers of eggs on EV and irAOC plants oviposited by *T. mucorea* females: *T. mucorea* oviposited more into EV plants than into irAOC plants (*G*-test; *, $p < 0.05$; n = 14). (**D**) Percentages of surviving larvae of *T. mucorea* in EV and irAOC plants three weeks after egg inoculation: No significant difference in survival rates (χ^2-test; $p = 0450$; n=16). (**E**) Mean (± SE) larval mass of *T. mucorea* fed on EV and irAOC plants: *T. mucorea* larva fed on irAOC plants had significantly higher mass than those fed on EV plants (one-way ANOVA; **, $p < 0.01$; n=16).

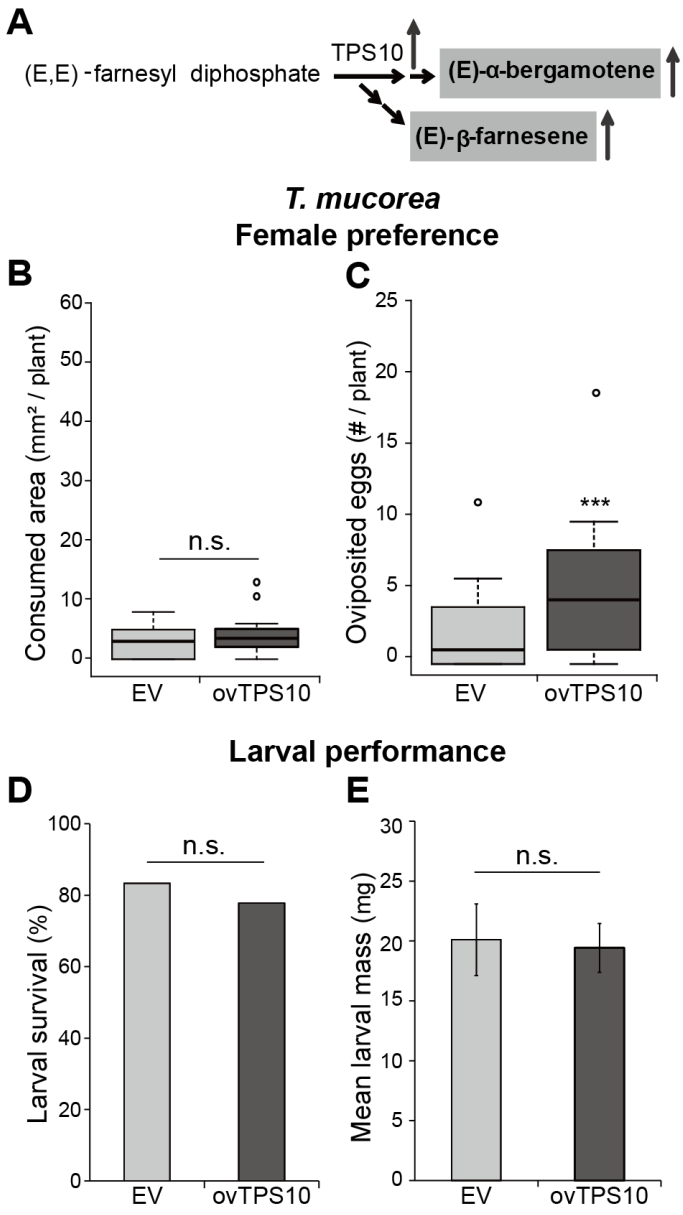
A
(E,E)-farnesyl diphosphate
TPS10
(E)-α-bergamotene
(E)-β-farnesene
T. mucorea
Female preference
B
Consumed area (mm² / plant)
n.s.
EV
ovTPS10
C
Oviposited eggs (# / plant)
***
EV
ovTPS10
Larval performance
D
Larval survival (%)
n.s.
EV
ovTPS10
E
Mean larval mass (mg)
n.s.
EV
ovTPS10

Fig. 3-9. *T. mucorea* adult female preference and larval performance in EV versus ovTPS10 plants. (**A**) Simplified biosynthetic pathway of sesquiterpene (*E*)-α-bergamotene and (*E*)-β-farnesene. ovTPS10, overexpressed of *Zea mays TERPENE SYNTHASE 10* gene (Schuman *et al.* 2014). (**B**) Leaf area of EV and ovTPS10 plants consumed by *T. mucorea* females: No feeding preference of *T. mucorea* (*G*-test; n.s., not significant; n = 14). (**C**) Numbers of eggs on EV and ovTPS10 plants oviposited by *T. mucorea* females: Females oviposited more into ovTPS10 than into EV plants (*G*-test; ***, $p < 0.001$; n = 14). (**D**) Percentages of surviving larvae of *T. mucorea* in EV and ovTPS10 plants three weeks after egg inoculation: no significant difference in survival rates (χ^2-test; $p = 0.467$; n=10). (E) Mean (± SE) larval mass of *T. mucorea* that fed on EV and ovTPS10 plants: no mass difference (one-way ANOVA; $p = 0.868$; n=10).

3.4. Discussion

Our study shows that two *Trichobaris* species preference and larval performance relationship were positively correlated between *D. wrightii* and *N. attenuata* host plants respectively which considered with preference-performance hypothesis. *T. compacta* female preferred to oviposit and feed on *D. wrightii* which was better for their larva performance such as survival and mass. *T. compacta* female was seldom laid the eggs into *N. attenuata* consistent with larvae performance. This may indicate that there was strong correlation between *T. compacta* female choice and larva performance which mean they have been specialized to *D. wrightii* as robust host plant. In case of *T. mucorea*, the female preferred to oviposit on *N. attenuata* that was more proper for their larva survival. However, *T. mucorea* female feeding preference was different with oviposition decision. They preferred to feed on *D. wrightii* than *N. attenuata* plants. This result may explain why most of *T. mucorea* adult observed in *D. wrightii* population, not *N. attenuata* population in the field. It is known that previous studies of vine weevil, *Otiohynchus sulcatus* showed that no correlation between feeding and eggs ovipositing which mean the weevils were not ovipositing where they fed and that leaf nutrition for adult feeding was not affected to their egg-laying behavior (Clark et al., 2011). In addition, a response for adult feeding maximized to nutritionally relevant host plants even though their

offspring performance was disadvantageous (Scheirs et al., 2000; Janz et al., 2005). Next our experiment was nicotine affect to host selection of two *Trichobaris* species using WT *N. attenuata* and nicotine biosynthesis-silenced *N. attenuata* (irPMT). Nicotine is the most abundant alkaloid of *N. attenuata* that is known to be an efficient defense compound against herbivores (Baldwin, 1999; Steppuhn et al., 2004). *T. compacta* larva significantly rescued as high survival rate and mass in nicotine-free plants. However the female did not preferred to oviposit the egg into irPMT plants. This suggests that nicotine was *T. compacta* larva feeding barrier in *N. attenuata*. In case of *T. mucorea*, did not show any preference between EV and irPMT plants and larva performance also presented no difference between them. This result indicates that nicotine was not consideration factor to egg-laying and larval performance of *T. mucorea* that might be due to the larvae has been adapted to nicotine as ability of detoxification.

To know oviposition stimulant of *T. mucorea* female in *N. attenuata* plant, first we tested that the hypothesis that JA mediated compound affected on the female oviposition choice. JA is highly accumulated phytohormone against herbivore attack that mediated induced defense metabolites and it is known that JA deficient by silencing of JA biosynthesis pathway, particularly allene oxide cyclase (AOC) silenced *N. attenuata* plants showed the lowest level of JA among other silenced initial committed step of JA biosynthesis pathway (Kallenbach et al., 2012). *T. mucorea*

female oviposition preference and larval performance were different direction between EV and irAOC plants. In adult preference level, the female preferred to feed on irAOC plants while, preferred to oviposit the egg into EV plants. In larvae level, there was no difference larval survival between EV and irAOC plants but, larva mass was significantly higher fed on irAOC than EV plant. This may indicate that for egg-laying, the female need to some stimulants by JA mediated factors even if their offspring performed much better in irAOC plants. Next experiment for understanding oviposition cues of *T. mucorea* was carried out more specific cures by manipulated volatiles which emitted by plants as guide to insect is able to be host-finding and acceptance behavior (Knolhoff and Heckel, 2014). I used jasmonate-regulated volatiles such as TAB and TBF which was over-expressed TPS10 in *N. attenuata* (Schuman et al., 2014). Previous study of Schuman *et al.* (2015) presented that higher infestation rates of *T. mucorea* in heterogeneous habitat (wild type and ovTPS10 *N. attenuata* plants mixed) than homogeneous habitat (wild type and wild type mixed) that released in the Utah field. Our results were consistent with earlier study (Schuman et al., 2015) showed that *T. mucorea* female preferred to oviposit into ovTPS10 than EV plants. In contrast, there was no differences larval performance between them. This result indicates that TAB and TBF volatiles are affected to oviposition behavior of *T. mucorea*. It suggests that there are different factors applied to egg deposition decision such as TAB and TBF volatile and

larval performance such as nicotine between *Trichobaris* species and their host plants.

Most of present studied about female preference and larval performance relationship focused on various combinations test of host plants which were supported for the PPH (Karungi et al., 2010; Zhang et al., 2012; Akol et al., 2013; Beuzelin et al., 2013). These studies pointed out broadly considered factors of oviposition and offspring performance that assumed morphological and biochemical interaction from wide host plants. Another type of studies showed that closely related specialist and generalist adult oviposition preference and larval performance relationship which were positive or negative correlation according to different host plants (Liu et al., 2012; Friberg et al., 2015). Whereas, this study zoomed in specific factors of host plant secondary metabolites between female egg-laying and larval performance that was manipulated one compound through different transgenic plants that nicotine was two sibling *Trichobaris* larvae host selection barrier for their performance and JA mediated compound especially TAB and TBF volatiles were important cues for *T. mucorea* female oviposition behavior. Even this study used experimentally manipulated and constitutively induced sesquiterpenes which regulated by JA signaling for female oviposition preference, *T. mucorea* female will use for TAB and TBF sesquiterpenes induced by *N. attenuata* for oviposition choice cues in natural habitat. Because *N. attenuata* consistently expose to

abiotic and biotic stress, it can be activate JA signaling.

This study is the first to show that solanaceous weevil *Trichobaris* genus host selection traits in dual choice experiments of various transgenic plants. Two sibling *Trichobaris* species, *T. compacta* and *T. mucorea* host range clearly divided into *D. wrightii* and *N. attenuata* host plants respectively even if *T. mucorea* female preferred to feed on *Datura* plant. This may indicate that each *Trichobaris* species tightly associated with their host plants as a specialist that mean specialization of herbivores was involved adaptation to the specific chemical from actual host, and specialized herbivores may respond to one or few host specific chemicals as an attractant for host selection (Zhang et al., 2012; Markovich et al., 2013). Previous study of *Trichobaris* showed that released JA deficient lines of *N. attenuate* were highly infected by *T. mucorea* in Utah field. This study suggested *T. mucorea* larval performance as survival trend which affected different JA related metabolite (Diezel et al., 2011b). However, the high infection rate did not give an evidence of female preference and larval performance relationship. This study provided the key insight of *Trichobaris* species host selection traits which were two *Trichobaris* larval performance affected by nicotine metabolite from *N. attenuate* including *T. mucorea* larval performance did not considered whether nicotine exist or absence and TAB and TBF volatiles attracted to *T. mucorea* female oviposition in *N. attenuata*. This trend suggests that the oviposition preference and larval

performance traits depending on different metabolites from host plants may indicate to evolve as widely separated unit. In conclusion, this study demonstrates that strong correlation between female oviposition preference and larval performance of two sibling *Trichobaris* species between *D. wrightii* and *N. attenuata* host plants. And each level of host selection behavior such as female oviposition choice and offspring performance is affected by different plant secondary metabolites. For more information of understanding host selection mechanisms of *Trichobaris* species, further experiments would be necessary to specifically find out *T. compacta* female oviposition cues for distinguish *D. wrightii* as actual host plant and *T. mucorea* larval performance which is influenced factor of JA related metabolites regarding to direct defense compound effect in *N. attenuate*.

Chapter 4. Pith defense response of *N. attenuata* against *T. mucorea* larvae

4.1 Introduction

T. mucorea larvae are normally invisible, because they remain inside plant stems until they emerge as adults. *T. mucorea* females lay light-yellow eggs on the base of newly elongating stems of the wild tobacco plant, *N. attenuata*. Larvae tunnel into the center of stems immediately after hatching where they feed on the pith. Near the end of the last larval stage, larvae make a partial exit window completed to the epidermal layer through which the adults will later emerge. Hence unlike other stem-borers, *T. mucorea* completes their entire development in the stem of a single *N. attenuata* plant. Young larvae of a spotted stem borer, *Chilo partellus,* initially feed on the leaves of maize, rice, and sorghum; later the older larvae move into the stems of these plants (Girijashankar et al., 2005). The larvae of a potato stem borer, *Hydraecia micacea,* are able to move from one plant to other plant (Deedat and Ellis, 1983), while the larvae of a soybean stem borer, *Dectes texanus,* generally stay in one plant during the entire larval stage (Hatchett et al., 1975). Some stem-boring insects induce abnormal stem growth: for example, *Eurosta solidaginis* larvae stimulate its host plant, *Solidago altissima*, to produce a ball-shaped gall, which subsequently provides food for the larvae (Abrahamson et al., 2001).

Hence the association between stem borers and their host plants can span the entire spectrum of intimacies found in other plant-herbivore interactions. Given that the loss of only small amounts of stem and the

vascular tissues housed within stems can have devastating fitness consequences for the plant (Ohnmeiss and Baldwin, 2000; Zangerl et al., 1996), it is surprising that we know much more about the defense of leaves than do of stems (Haukioja and Koricheva, 2000; Preisser and Bastow, 2005). How do plants respond to the attack of their stems? Ralph *et al*. (2006) examined large-scale transcriptional changes in a spruce, *Picea sitchensis*, after it was attacked by a stem-boring insect, *Pissodes strobe* (a white pine weevil). Zhou *et al*. (2011) measured the levels of gene expression in rice after it was attacked by the striped stem borer, *Chilo suppressalis*. Both studies revealed that stem-borer attack activated the biosynthesis of genes involved in a well-known plant defense hormone, JA. In addition, many genes involved in the biosynthesis of secondary metabolites were also elicited; these genes, in turn, have the potential to alter the chemistry of pith, which for herbaceous stems, is the tissue that sustains the growth and development of many stem borers. A milkweed specialist weevil, *Rhyssomtus lineaticollis*, modifies the polarity and amount of cardenolides in the pith of *Asclepias syriaca* (Fordyce and Malcolm, 2000). Sitka and Norway spruce trees accumulate more lignin in bark tissues in response to spruce bark beetle attack (Wainhouse et al., 1990). These results suggest that JA signaling elicits the production of secondary metabolites in stems against stem-borer attack, much as it does in leaves against attack from leaf herbivores.

Inducible defenses, as opposed to constitutive defenses, can be produced by plants when needed; inducible defenses are therefore potentially less costly than constitutive defenses (Karban and Baldwin, 1997). Plants are able to elicit specific defense signaling and tailor the chemicals they produce in response to the different types of herbivores that attack them (Karban and Baldwin, 1997; Dicke and Hilker, 2003; Wu and Baldwin, 2010). The specificity of inducible defenses can be triggered by the feeding patterns of herbivores, the herbivores' salivary constituents (oral secretion), and the types tissues that are attacked (Bonaventure et al., 2011). Until now, the mechanisms responsible for the inducible responses elicited by stem-borer attack have gone unstudied.

N. attenuata is an annual plant found in the southwestern United States. Over the last several years, *T. mucorea* larvae, which infest *N. attenuata* plants grown at field station, prefer to feed on *N. attenuata* plants silenced in JA biosynthesis or signaling than on control plants with intact JA signaling and biosynthesis (Diezel et al., 2011b). Pith choice assays have revealed that the frequency of *T. mucorea* infection is correlated with pith palatability in the field (Diezel et al., 2011b). In this study, I advanced our understanding of the inducible responses in *N. attenuata* stems and show that JA signaling elicits the dramatic accumulations of phenolics that are not JA-regulated in leaves. Since *T. mucorea* is normally univoltine on *N. attenuata* in nature and adults oviposit over many weeks in the spring, frequently in the night

when they are difficult to observe. As described previous chapter 3, I also used *T. mucorea* colony and egg inoculation method for further experiment, so that stems of different RNAi lines could be experimentally infested. Next I analyzed pith chemistry in *T. mucorea*-infested plants, identified a highly induced phenolic, developed RNAi lines for the silencing of this phenolic and measured herbivore performance of the larvae in the laboratory and the field on these RNAi lines.

4.2 Methods

4.2.1. Plant growth conditions

N. attenuata of 31st inbred generation seeds, which were originally collected from a native population at a field site located in Utah, USA was used for all experiments. Stably silenced inverted repeat(ir) plants were used to silence jasmonic acid (JA) synthesis (irAOC; Kallenbach *et al.*, 2012), JA conjugation (irJAR4/6; Wang *et al.*, 2008), JA-Ile perception (irCOI1; Paschold *et al.*, 2007), nicotine production (irPMT; Steppuhn *et al.*, 2004) and trypsin protease inhibitors (TPIs) production (irPI; Zavala & Baldwin, 2004). Seeds were sterilized and germinated on Gamborg's B5 medium (Duchefa) as described previously (Krügel et al., 2002). Seedlings were maintained at 26℃/16h of 155μmol $m^{-2}s^{-1}$ light: 24℃/8h of dark cycle. After 10 days, young seedlings were planted individually in Teku plastic

pots containing peat-based substrate. Ten days later, early rosette plants were transferred to soil in 1L pots and grown in glasshouse with a day/night cycle of 16h (26℃-28℃)/8h (22℃-24℃) under supplemental light from Master Sun-T PIA Agro 400 or Master Sun-T PIA Plus 600 high-pressure sodium lamps (Philips) with daily supplied an automatic glasshouse watering system. For chemical analysis in pith of *N. attenuata*, I harvested adjacent upper part (about 5cm) of eaten area by *Trichobaris mucorea* larvae. I also sampled the pith of *N. attenuata* without egg inoculation as a control.

4.2.2. In vitro *T. mucorea* bioassay

1st instar larvae of *T. mucorea*, which were reared for one week in the stems of wild type *N. attenuata* plants was used for study larval performance on artificial diets which was followed recipe of stem weviil from Malone and Wigley study (1990) supplemented with chlorogenic acid (CGA; Sigma Aldrich). Bioassays were conducted with concentrations of 0mM, 0.85mM, and 8.46mM CGA added to the artificial diets. Concentration of 2.8mM CGA is the average level of CGA that is induced in the attacked pith by *T. mucorea* larva attack. A 0.85mM CGA is similar to the level of CGA in leaf of *N. attenuata*. An 8.46mM CGA level is similar to the maximum level of CGA that I measured in the attacked pith. All in vitro assays were performed in a growth chamber with a 16h light (26℃)/8h dark (24℃) cycle and 65% humidity (Snijders Scientific).

4.2.3. Analysis of secondary metabolites

Approximately 100 mg of frozen pith materials were homogenized with three steel beads by Genogrider 2000 (SPEX Certi Prep) at 1200 strokes per min, for 60 seconds, once with 1 mL of the extraction buffer (60% solution 1; 2.3 mL/L of acetic acid, 3.41 g/L ammonium acetate adjusted to pH 4.8 with 1 M NH_4OH, and 40% (v/v) methanol) as described in Heiling *et al.*, (2010). Supernatants were collected after 20 min of centrifugation at 16,100g at 4℃. 1mL of particle-free supernatant (after additional centrifugation) was analyzed by HPLC (Agilent-HPLC 1100 series) using a chromatographic column (Chromolith Fast Gradient RP18e, 50 x 2 mm, Merk) connected to a pre-column (Gemini NX RP18, 3 um, 2 x 4.6 mm, Phenomenex) with a column oven maintained at 40℃. Separated samples were detected with PDA (Photo Diode Array) and ELS (Evaporative Light Scattering, Varian) detectors. The mobile phase comprised of solvent A (0.1% formic acid and 0.1% ammonium hydroxide solution in water (pH 3.5) and solvent B (methanol) was used in a gradient mode (time/concentration, min/%) for A: 0:00/100; 0.50/100; 6.50/20; 10:00/20; 15:00/100 with a flow rate 0.8 mL/min^{-1}. Under these conditions, nicotine eluted at retention time (RT) of 0.5 min (detected by UV absorbance at 260 nm); caffeoylputrescine (CP), chlorogenic acid (CGA), and dicaffeoylspermidine (DCS) eluted at RTs of 2.6, 3.0, and 3.9 min, respectively (detected at 320 nm). Rutin eluted at RT 4.7 min and it was detected at 360 nm. Diterpene glycosides (DTGs)

eluting between RT 7.0 and 8.5 min were detected by the ELS detector. The peak areas were integrated using the Chromeleon chromatographic software (version 6.8; Dionex), and the amounts of metabolites were calculated using serial dilution of external standard mixtures of nicotine, CGA, and rutin.

4.2.4. Phytohormone measurements

Approximately 100mg of frozen materials was homogenized with two steel beads in a Genogrider 2000 (SPEX Certi Prep) at 1,200 strokes min^{-1}. Phytohormones (JA, JA-Ile, SA, and ABA) were extracted by vortexing for 10 min after the addition of ethyl acetate spiked with internal standards: 100ng of [$^{2}H_{2}$] JA and 20ng each of JA-[$^{13}C_{6}$] Ile, [$^{2}H_{4}$] SA, and [$^{2}H_{6}$] ABA. The extracted samples were centrifuged at 16,100g at 4℃ for 20 min, and the upper supernatant were transferred into another new tubes. The samples were evaporated to near dryness in a vacuum concentrator (Eppendorf) at 30℃. The dried samples were dissolved in 500ul 70% (v/v) methanol:water for analysis with the Varian 1200 LC-ESI-MS/MS system as described by Gilardoni *et al.* (2011). The phytohormones were detected in negative ESI mode. Molecular ions [M-H] at mass-to-charge ratio (*m/z*) 209, 322, 137, and 263 (213, 328, 141, and 269), generated from endogenous JA, JA-Ile, SA, and ABA, were fragmented under 12-, 19-, 15- and 9-V collision energy, respectively. The ratios of ion intensities of the respective product ions and internal standards, *m/z* 59 and 63, *m/z* 130 and 136, *m/z* 93 and 97, and *m/z*

153 and 159, were used to quantify endogenous JA, JA-Ile, SA, and ABA, respectively. The resulting amounts of hormones were divided by the exact fresh mass of plant materials used for the extraction of each sample.

4.2.5. Gene expression analyses by RT-qPCR

To analyze transcript levels of *NaPMT*, *NaCHS*, and *NaHQT* genes in attacked pith and unattacked pith, total RNA was extracted using RNeasy Plant mini kit (Qiagen). The synthesis of cDNA was performed with 1µg of total RNA using RevertAid^{TM} H Minus Reverse Transcriptase kit (Fermentas) and oligo-dT primer (Fermentas). Quantitative real-time PCR (qPCR) was carried out with synthesized cDNA from three biological replicated samples using the core reagent kit for SYBR Green I (Ecrogentec) and gene specific primer pairs (*NaHQT*; forward sequence CTCCTTTGCCACCAGGTTA, reverse sequence ATGTCGGGCCACGGA TTAAA, *NaPMT*; forward sequence TCATTGGACCAAGATCGAG, reverse sequence TGGAAATTATGATAATTACTGCAGA, *NaCHS*; forward sequence TTCACGTTTCAAGGCCCAA, reverse sequence TGCTCCATC AGCGAAAAGG, *NtEFa*; forward sequence CCACACTTCCCACATTGC TGTCA, reverse primer sequence CGCATGTCCCTCACAGCAAAAC) on a Stratagene MX3005P PCR. Relative transcript levels were calculated from dilution series of cDNA samples, and normalized by the expression of the tobacco housekeeping gene, *N. tabacum* elongation factor-1α (*NtEF1α*).

4.2.6. Statistical analyses

Data analysis was conducted with Origin 8 SR1 (OriginLab Cop. Northampton, USA) or the publically available R package (version 3.1.2, http://www.r-project.org/). We used one-way ANOVA followed by Tukey's honestly significant difference (HSD) as post hoc test for multiple samples and Fisher's least significant difference (LSD) for larva mass analysis, chi-test for survival rate analysis.

4.3 Results

4.3.1. Induction of JA levels in pith of plants attacked by *T. mucorea* larvae

To analyze pith chemistry, I harvested pith from the egg-inoculated plants that had been attacked and from non-inoculated plants (Fig. 4-1A) and measured the levels of JA and jasmonoyl-L-isoleucine (JA-Ile) in the pith of *N. attenuata* plants three weeks after *T. mucorea* egg inoculation. *T. mucorea* larva attack elicited JA and JA-Ile levels in the pith of *N. attenuata* plants (Fig. 4-1B; $p < 0.01$). Levels of abscisic acid (ABA) were also increased in the pith of plants that had been attacked (Fig. 4-2A; $p < 0.05$), but pith levels of salicylic acid (SA) were unchanged (Fig. 4-2B; $p = 0.679$).

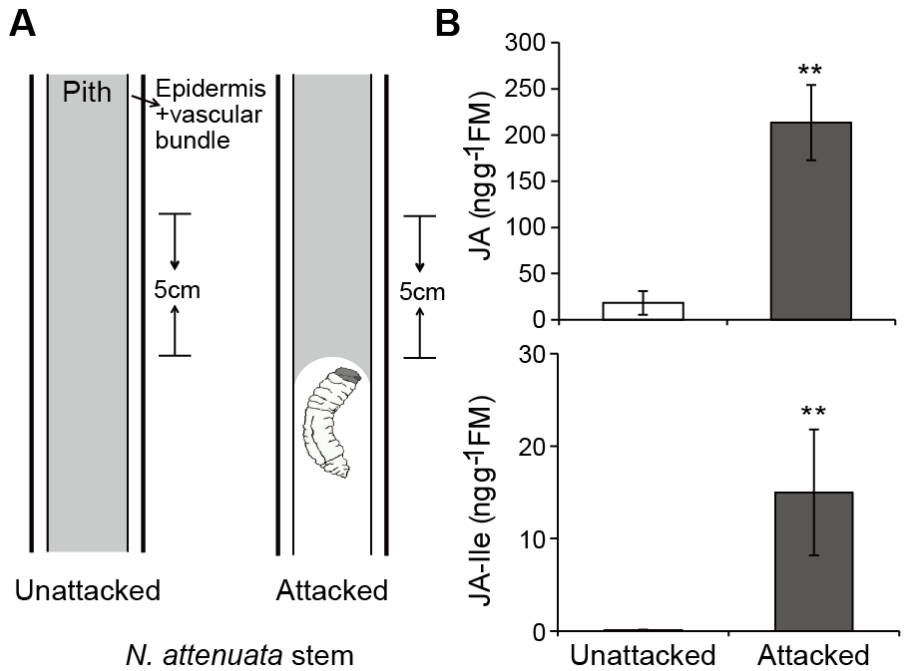

Fig. 4-1. Levels of jasmonic aicd (JA) and jasmonoyl-L-isoleucine (JA-lIe) in unattacked and attacked pith by *T. mucorea* larva. (**A**) Schematic diagram of the pith sampling from unattacked and attacked plants. Three weeks after egg inoculation, we collected attacked pith approximately 5cm in length above the feeding area by *T. mucorea* larva. Unattacked pith was collected from the similar position of non-inoculated stem. (**B**) Mean (±) levels of jasmonic acid (JA) and jasmonoyl-L-isoleucine (JA-Ile) in undamaged and damaged pith. Asterisks indicate significant differences between treatments (one-way ANOVA; **, $p < 0.01$; n=6). FM, fresh mass.

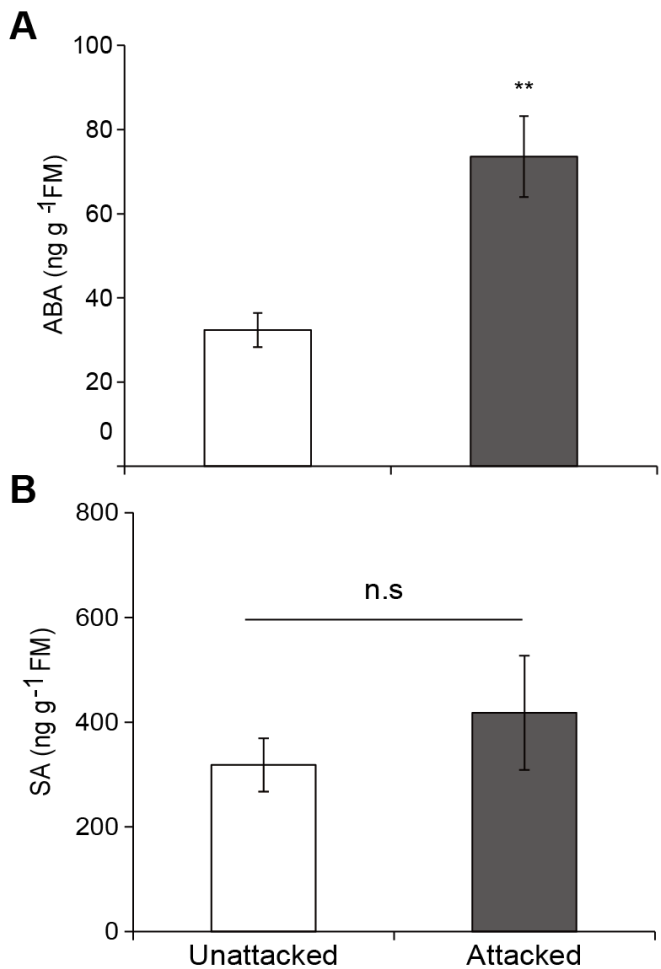

Fig. 4-2. Levels of abscisic acid (ABA) and salicylic acid (SA) in unattacked and attacked pith by *T. mucorea* larva. Mean (± SE) levels of (**A**) abscisic acid (ABA) and (**B**) salicylic acid (SA) in control and attacked pith. ABA levels were significantly increased in the attacked pith. SA levels in attacked pith were similar with that in control pith. (one-way ANOVA; **, $p < 0.01$; n=6). n.s, not significant.

4.3.2. *T. mucorea* performance in transgenic plants silenced in JA signaling and perception

To examine whether JA or JA signaling affects the performance of *T. mucorea* larvae, I inoculated the eggs into several transgenic plants silenced in their JA biosynthesis (irAOC), JA-Ile conjugation (irJAR4x6), or JA-Ile perception (irCOI1). Three weeks after egg inoculation, larval mass and larval developmental stages were measured. I found that *T. mucorea* larvae performed better in JA- or JA signaling-deficient plants than in EV plants (Fig. 4-3A; $p < 0.05$). In addition, most of the larvae fed on these silenced plants had attained the pre-pupal stage by three weeks when stems for split open for examination; at this time, larvae fed on EV plants had only attained the second- or third-instar stages (Fig. 4-3B; $p < 0.05$).

4.3.3. Levels of chlorogenic acid and *NaHQT* transcript in the pith of plants attacked by *T. mucorea* larvae

N. attenuata plants are able to synthesize secondary metabolites for their defense against leaf-chewing insect attacks (Karban and Baldwin, 1997; Halitschke and Baldwin, 2003; Geyter et al., 2012). To establish which biosynthetic pathways of secondary metabolites are induced by stem-boring insects, we first measured the levels of nicotine, rutin, and chlorogenic acid (CGA) in the pith of *N. attenuata* plants. While the levels of nicotine and rutin in the pith of plants that had been attacked were not changed (Fig. 4-

4A; $P = 0.98$ and $p = 0.33$, respectively), CGA levels were highly induced in the attacked pith from three weeks continuous larvae feeding period after egg inoculation (Fig. 4-4A; $p < 0.001$) that did not affect to CGA levels in systemic leaf (Fig. 4-5; $p = 0.995$). Furthermore, I measured the transcript levels of biosynthetic genes for each metabolite. Levels of the hydroxycinnamoyl quinate CoA transferase (*NaHQT*) gene, a key enzyme involved in CGA synthesis, were also induced in the pith of attacked plants by *T. mucorea* larvae (Fig. 4-4B; $p < 0.001$). Levels of the chalcone synthase (*NaCHS*) gene, which is involved in flavonoid biosynthesis, were unchanged in the pith of attacked plants (Fig. 4-4B; $p = 0.792$). While nicotine levels in the pith of attacked plants were similar to those in the pith of controls, transcript levels of putrescine N-methyltrasnferase (*NaPMT*), a key enzyme in nicotine biosynthesis, were strongly induced in the pith of attacked plants (Fig. 4-4B; $p < 0.001$). Other defense-related metabolites in *N. attenuata*, such as caffeoylputrescine, dicaffeoylspermidine, and 17-hydroxyherany llinalool diterpene glycosides were not detected in the pith of attacked and unattacked plants.

In addition, previous study of field demonstrated that silencing nicotine and trypsin proteases inhibitors (TPIs) increases the number of plants infested by *T. mucorea* larvae in the field (Diezel et al., 2011b). Therefore, we examined whether nicotine and TPIs levels affect the larval performance of *T. mucorea*. Eggs from *T. mucorea* were inoculated into nicotine- or TPI-

silenced plants and larval mass was measured three weeks later. The larvae fed on nicotine- or TPI-silenced plants gained similar amount of mass compared to the larvae fed on EV plants (Fig. 4-6), suggesting that nicotine and TPI may influence the oviposition decisions of *T. mucorea* females or play an important role in the pith of field grown plants.

4.3.4. JA-dependent induction of chlorogenic acid in pith

To evaluate if JA signaling regulates the induction of CGA levels in pith, I measured the amounts of CGA in irAOC, irJAR4x6, and irCOI1 lines three weeks after *T. mucorea* egg inoculation. Without egg inoculation, there was no significant difference in CGA contents between EV and JA- or JA signaling-deficient plants (Fig. 4-7; $p = 0.79$). However, CGA levels in the pith of irAOC, irJAR4x6, and irCOI1 lines attacked by *T. mucorea* larvae were significantly lower than CGA levels in the pith of attacked EV plants (Fig. 4-7; $p < 0.001$). These results suggest that JA and JA signaling is essential for CGA induction in the pith of attacked *N. attenuata* plants.

I also compared the global metabolic profiles of pith as analyzed by an untargeted LC-ESI-qTOF analysis from attacked and unattacked EV and irAOC plants. The pith samples were collected same as previous experiments which in three weeks continuous feeding after egg inoculation of *T. mucorea*. Principal component analysis (PCA) revealed that the metabolic profiles of pith from attacked and control EV and irAOC plants

clearly separated into four distinct groups. The relative distance along PC1 (57.6%) or PC2 (41.2%) between attacked and control EV groups was greater than the distance between attacked and control irAOC groups, which is consistent with the observation that larval feeding largely alters pith chemistry in *N. attenuata* plants in a JA-dependent manner (Fig. 4-8).

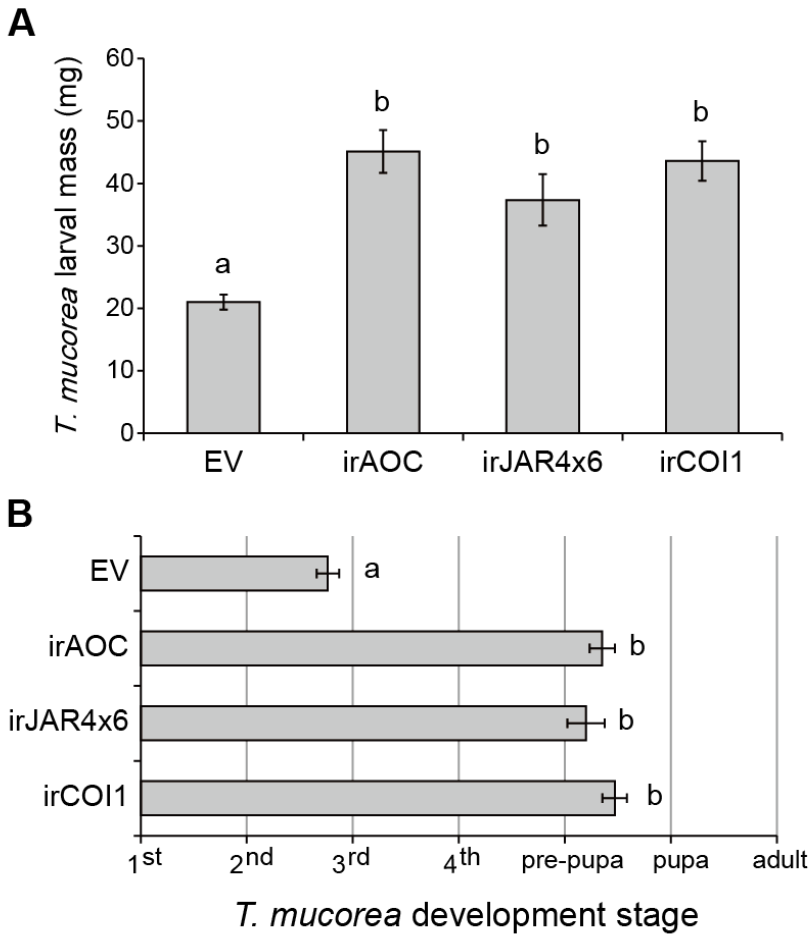
A
T. mucorea larval mass (mg)
60
50
40
30
20
10
0
a
b
b
b
EV
irAOC
irJAR4x6
irCOI1
B
EV
irAOC
irJAR4x6
irCOI1
a
b
b
b
1st
2nd
3rd
4th
pre-pupa
pupa
adult
T. mucorea development stage

Fig. 4-3. *T. mucorea* larvae perform better in the stems of transgenic plants impaired in JA-biosynthesis, JA-Ile conjugation, or JA-perception than in WT plants with intact JA signaling. (**A**) Mean (± SE) mass of larvae developing in the stems of EV (empty-vector transformed wild-type plants), irAOC (*NaAOC*-silenced lines impaired in JA biosynthesis), irJAR4x6 (*NaJAR4* and *NaJAR6*-silenced lines impaired in JA-Ile conjugation), and irCOI1 (*NaCOI1*-silenced lines impaired in JA perception). The larva mass was measured three weeks after the inoculation. Larvae fed on irAOC, irJAR4x6, and irCOI1 did not differ but gained significantly more mass than those on EV plants (one-way ANOVA followed by Fisher's least significant difference test; $p < 0.001$; n=20). (**B**) Developmental stages of larvae fed on EV, irAOC, irJAR4x6, or irCOI1 three weeks after egg inoculation. *T. mucorea* larvae collected from irAOC, irJAR4x6, or irCOI1 had developed faster than those in the stems of EV plants (one-way ANOVA followed by Tukey's honest significant difference(HSD); $p < 0.05$; n=20). Different letters indicate significant differences among treatments.

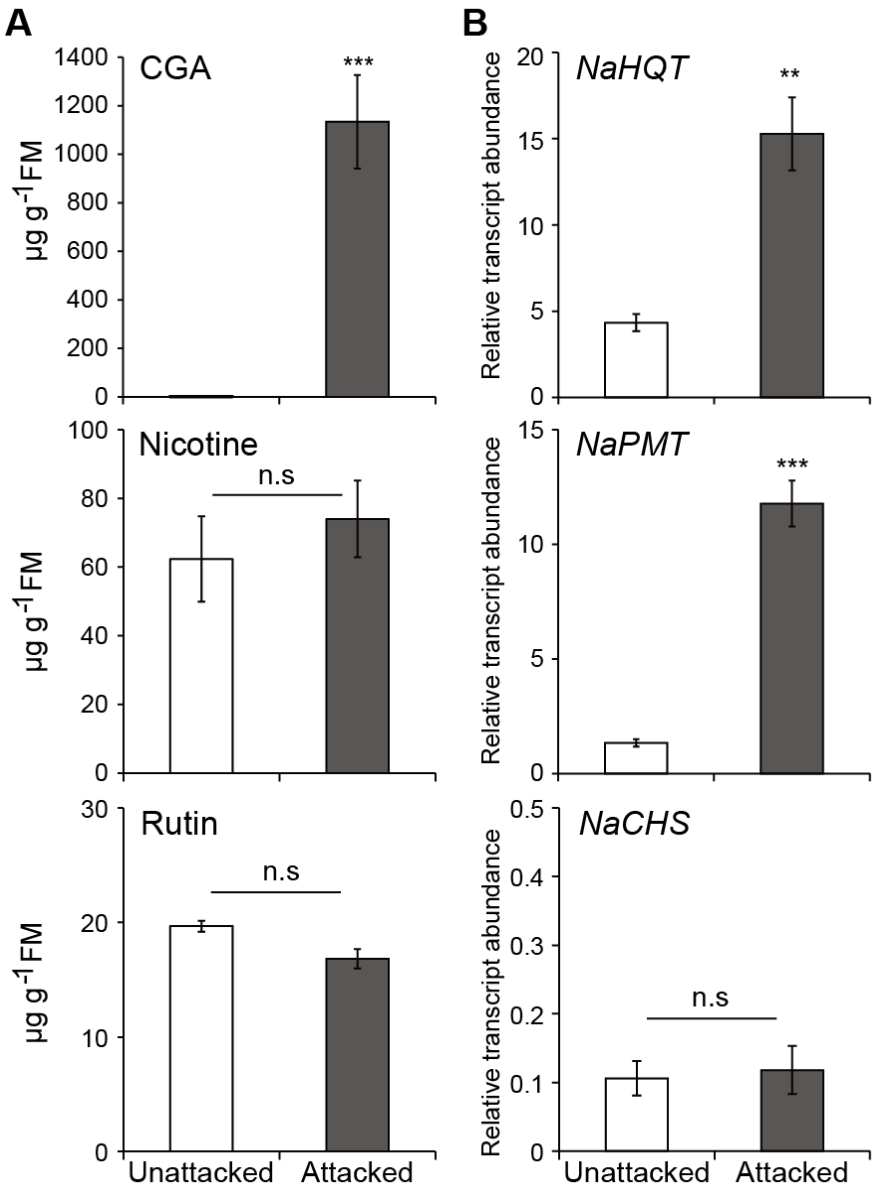
A
B
CGA
***
µg g-1FM
1400
1200
1000
800
600
400
200
0
NaHQT
**
Relative transcript abundance
20
15
10
5
0
Nicotine
n.s
100
80
60
40
20
0
NaPMT
***
15
10
5
0
Rutin
n.s
30
20
10
0
NaCHS
n.s
0.5
0.4
0.3
0.2
0.1
0
Unattacked
Attacked

Fig. 4-4. Levels of chlorogenic acid (CGA) and *NaHQT* transcript in attacked pith by *T. mucorea* larva. (**A**) Mean (± SE) levels of chlorogenic acid (CGA), nicotine, and rutin in control and attacked pith three weeks after egg inoculation. (**B**) Mean (± SE) levels of transcript abundance of *NaHQT* (hydroxcnamoyl-CoA quinate transferase), *NaPMT* (putrescine N-methyltrans ferase), *NaCHS* (chalcone synthase), key biosynthetic enzymes for CGA, nicotine, and rutin, respectively, in unattacked and attacked pith. Asterisks indicate significant differences among treatments (one-way ANOVA; **, $p < 0.01$; ***, $p < 0.001$; n=6). FM, fresh mass; n.s, not significant.

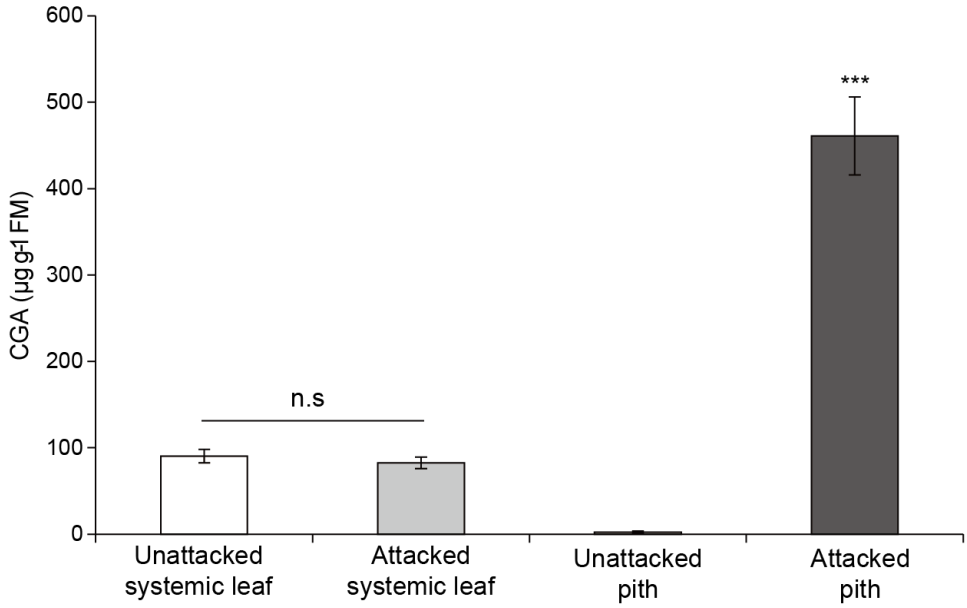

Fig. 4-5. Levels of CGA in systemic leaf and attacked pith by *T. mucorea* larvae. Induced CGA level from attacked pith did not increase to CGA content of systemic leaf which was sampling three weeks continuous feeding period after egg inoculation. At that experiment, I used wild type *N. attenuata* plants which grow in 2L pot (one-way ANOVA; ***, $p < 0.001$; n=6). n.s, not significant.

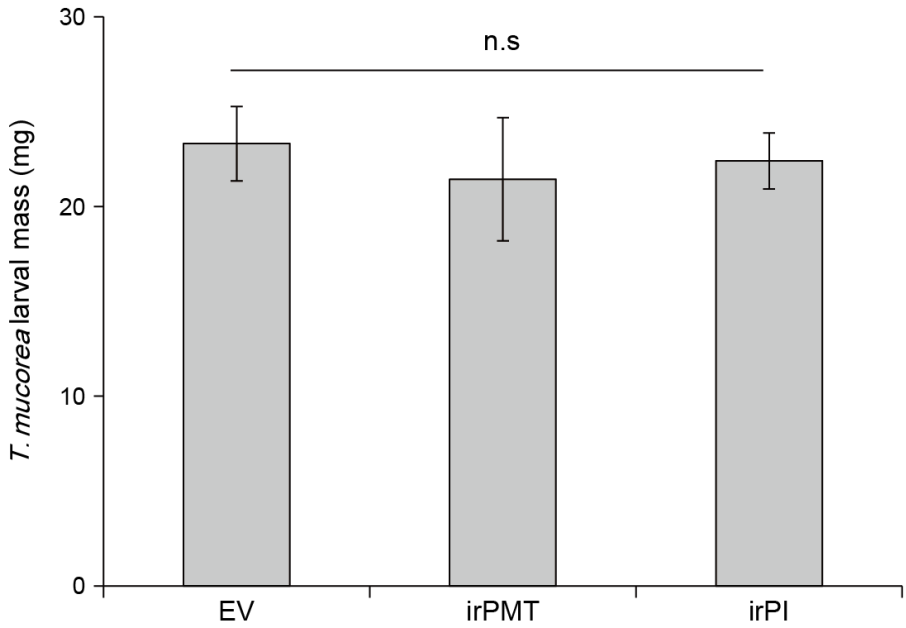

Fig. 4-6. Performance of *T. mucorea* larvae in irPMT, irPI, or EV plants.
Larvae were collected after three weeks of continuous feeding after egg inoculation (one-way ANOVA; Fisher's least significant difference (LSD) test; p = 0.742; n=20). n.s, not significant.

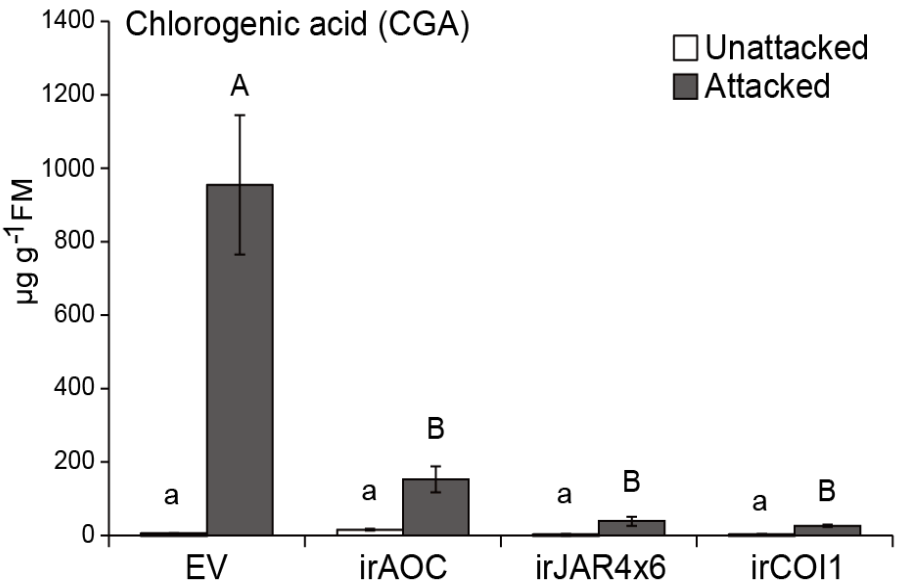
Chlorogenic acid (CGA)
Unattacked
Attacked
µg g-1FM
1400
1200
1000
800
600
400
200
0
A
a
B
a
a
B
a
B
EV
irAOC
irJAR4x6
irCOI1

Fig. 4-7. Accumulated levels of CGA in unattacked and attacked pith of transgenic plants impaired in irAOC, irJAR4x6, irCOI1 and EV plants.
Mean (± SE) concentrations of chlorogenic acid (CGA) accumulated in pith from stems of EV, irAOC, irJAR4x6, and irCOI1 plants. Inducible CGA production in attacked pith of irAOC, irJAR4x6, and irCOI1 plants was significantly lower than that of EV plants. Attacked pith was collected three weeks after egg inoculation, and unattacked pith was collected at the same time. The levels of CGA in attacked pith are elicited by JA-mediated manner. Lowercase letters refer to comparisons among unattacked plants, and capital letters present differences among damaged plants. Different letters indicate significant differences among genotypes in the same treatments (one-way ANOVA followed by Tukey's HSD; $p < 0.001$; n=6).

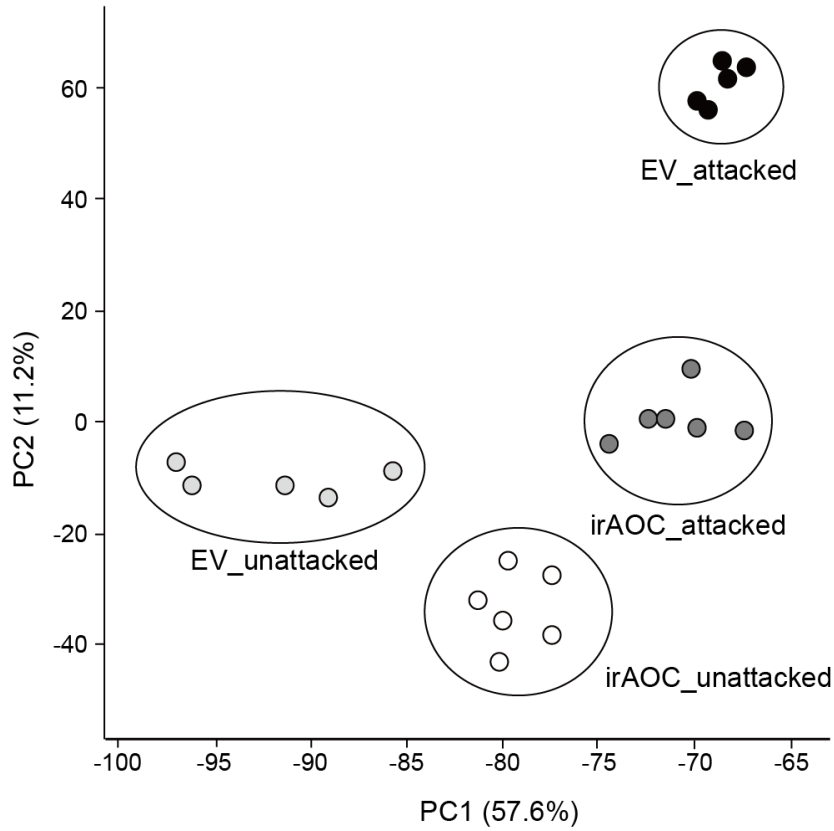
60
40
20
0
-20
-40
PC2 (11.2%)
-100
-95
-90
-85
-80
-75
-70
-65
PC1 (57.6%)
EV_attacked
irAOC_attacked
EV_unattacked
irAOC_unattacked

Fig. 4-8. Untargeted metabolic profile of principal component analysis (PCA) in unattacked and attacked pith of EV and irAOC plants. I measured pith chemistry in EV and irAOC plants with and without egg inoculation. Samples were collected three weeks after continuous feeing after *T. mucorea* egg inoculation. I used 40% methanol-based extraction method to extract pith metabolites and separated the metabolites using Dionex rapid separation liquid chromatography system. The separated metabolites were positively charged by electro spray ionization (ESI) and exact mass to charge ratio of ions was measured with a MicroToF (Time-of-Flight; Bruker Daltonik, Bremen, Germany). Raw data files were converted to netCDF format and processed by XCMS (http://fiehnlab.ucdavis.edu/staff/kind/Metabolomics/Peak_Alignment/xcms/) and CAMERA (http://bioconductor.org/packages/devel/bioc/html/CAMERA.html).

Only peaks that were found in at least 75% of the replicates with absolute intensities higher than 5 megacounts s^{-1} were used in the analysis. PCA was performed using MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp), followed normalization by log transformation and Pareto scaling.

4.3.5. *T. mucorea* larval performance in chlorogenic acid-deficient plants

To evaluate the defensive value of CGA against stem-borer attack, we fed *T. mucorea* first-instar larvae on artificial diets spiked with different amounts of CGA: 0mM, 0.85mM (average CGA levels in the leaf), and 8.46mM (similar to the maximum CGA levels measured in the attacked pith). The mean mass of the fourth-instar larvae differed among treatments (Fig. 4-9; $p < 0.05$). Larvae fed on artificial diet containing 0.85mM and 8.46mM CGA gained significantly less weight than those fed on 0mM CGA (Fig. 4-9; $p < 0.05$).

Next I used the CGA-deficient plants (irHQT-153 and -121) silenced in the *NaHQT* gene, the final enzyme in CGA biosynthesis pathway (Fig. 4-10). And full coding sequence of *NaHQT* shared high similarity with HQT genes in potato and tomato (Fig 4-11). The levels of *NaHQT* transcripts in irHQT leaves were 95% lower than the levels in EV leaves (Fig. 4-12A; $p < 0.001$), a reduction which corresponds with the low levels of CGA in leaves of irHQT-153 and -121 plants (Fig. 4-12B; $p < 0.001$). CGA amounts in the pith of unattacked *N. attenuata* plants were barely detectable (Fig. 4-12C). Attack from *T. mucorea* larvae elicited dramatic increases in CGA levels in the pith of EV plants. But the induced levels of CGA in the pith of irHQT plants were much smaller than the levels in the pith of EV plants (Fig. 4-12C; $p < 0.001$). The amounts of nicotine, rutin, and HGL-DTGs were

similar in between EV and irHQT plants (Fig. 4-13). To examine larval performance on both EV and irHQT plants, *T. mucorea* eggs were inoculated into the basal stem of EV plants and of two irHQT lines. Three weeks after the inoculation, larval mass was measured. The larvae fed on the two irHQT lines gained significantly more mass than did the larvae fed EV plants (Fig. 4-12D; $p < 0.05$), indicating that CGA is a critical defense chemical that plants use to defend their pith against attack from *T. mucorea* larvae.

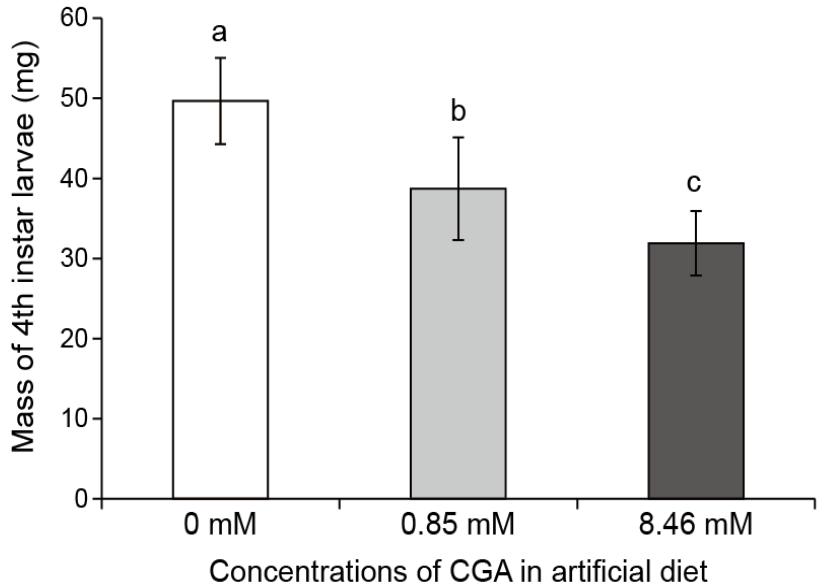

Fig. 4-9. *T. mucorea* larvae mass in different levels of CGA spiked with artificial diet. Mean (± SE) mass of *T. mucorea* larvae fed artificial diet supplemented with different levels of chlorogenic acid (CGA): 0mM, 0.85mM (typical levels of CGA in *N. attenuata* leaves), and 8.46mM (similar to the maximum CGA levels measured in attacked pith). Different letters indicate significant differences among treatments (one-way ANOVA followed by Fisher's least significant difference (LSD) test; $p < 0.05$; n=10).

A

ATGGGAAGTGAAAAAATCATGAAGATTAACATCAAAGAATCAACATTAGTAAAACCATCAAAACCAACACCAACAAAGAG
ACTTTGGAGTTCTAACTTAGATTTAATAGTGGGAAGAATTCATCTTTTAACAGTATATTTTTATAAACCAAATGGATCTT
CAAATTTCTTTGATACAAAAATAATGAAAGAAGCATTAAGTAATGTTCTTGTTTCATTTTATCCAATGGCTGGGAGATTA
GGTAGAGATGAACAAGGAAGAATTGAGATAAATTGTAATGGAGAAGGAGTTTTATTTGTTGAAGCTGAAAGTGATGCTTT
TGTTGATGATTTTGGTGATTTTACTCCAAGTTTGGAACTTAGGAAACTTATTCCTACTGTTGATACTTCTGGTGATATTT
CTACTTTCCCCCTCATCATCTTTCAGGTTACTCGTTTCAAATGTGGTGGAGTTTCACTTGGTGGAGGAGTATTCCACACT
TTATCAGATGGTCTCTCATCCATTCACTTCATCAACACATGGTCCGATATTGCCCGAGGCCTCTCCGTGGCCATCCCGCC
GTTCATTGACCGGACCCTCCTCCGTGCACGGGACCCACCGACATCGTCGTTCGAGCACATTGAGTATCATCCTCCTCCAT
CTCTTATTTCATCATCAAAAACCAAAGAATCCACAAGCCCAAAGCCTAGTACCACAACCATGTTAAAATTCTCTAGTGAC
CAACTTGGGCTTCTAAAGTCCAAGTCCAAACATGAAGGTAGCACATACGAAATCCTCGCGGCCCATATTTGGCGTTGCAC
GTGCAAAGCACGTGCACTGTCCGACGATCAATTGACCAAATTACATGTTGCCACTGATGGTAGGTCTAGACTTTGCCCTC
CTTTGCCACCAGGTTACTTAGGAAATGTTGTGTTCACAGCTACACCAATGGCAAAATCAAGTGAACTTTTACAAGAACCA
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CGAATTACAGCCCGATTTATCGGCTTTAATCCGTGGCCCGACATACTTTGCTAGCCCTAATCTTAATATTAATAGTTGGA
CTAGATTGCCTGTTCATGATTCAGATTTTGGATGGGGAAGACCAATTCATATGGGACCAGCTTGCATTTTATATGAAGGG
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B

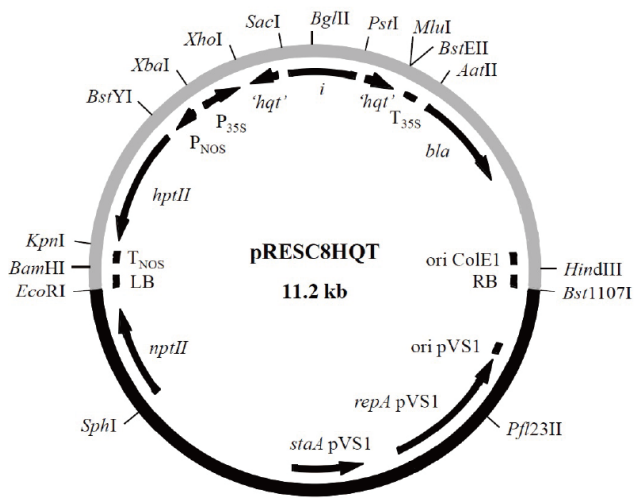
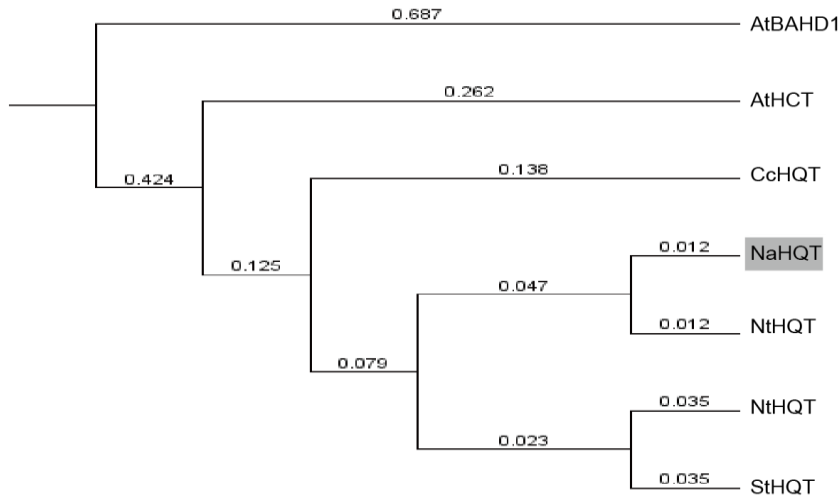


Fig. 4-10. Generation of stable *NaHQT*-silenced *N. attenuata* plants. (**A**) Protein coding sequence of *NaHQT* gene. I used a 331bp region in *NaHQT* gene for gene silencing (red letters). (**B**) The pRESC8HQT vector containing inverted repeat elements of *NaHQT* gene used for *Agrobacterium tumefaciens*-mediated transformation and generation of stably silenced *N. attenuata* irHQT plants.

A


0.687
AtBAHD1
0.262
AtHCT
0.424
0.138
CcHQT
0.012
NaHQT
0.125
0.047
0.012
NtHQT
0.079
0.035
NtHQT
0.023
0.035
StHQT

B

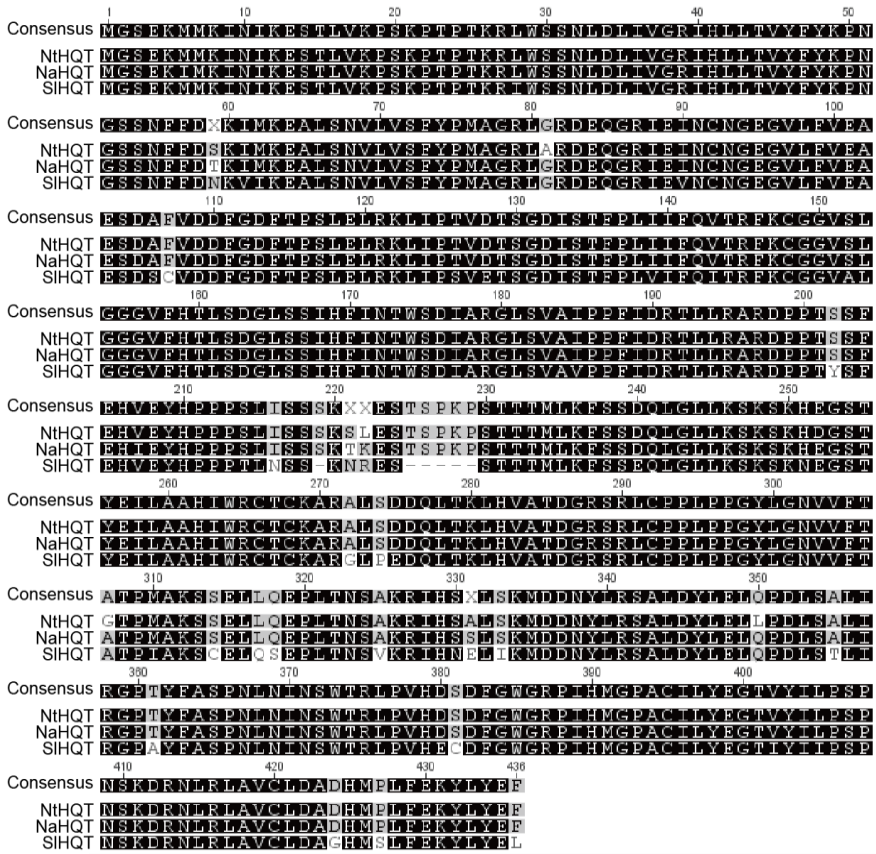

Consensus
NtHQT
NaHQT
SlHQT

Fig. 4-11. Phylogenetic trees and protein alignment of *HQT* genes. (**A**) Full-length amino acid sequence was aligned using the Geneious software. Unweighted Pair Group Method with the Arithmetic mean (UPGMA) was used to make the phylogenetic tree. The numbers represent the number of amino acid substitutions per site by applying the Jukes-Cantor model. *AtBAHD1*, which is another BAHD family acyltransferase, is used for outgroup. NaHQT is highlighted in a grey background. (**B**) Full-length amino acid sequence were aligned using the Geneious software (At, *Arabidopsis thaliana;* Cc, *Coffea canephora;* Na, *Nicotiana attenuata;* Nt, *Nicotiana tabacum;* Sl, *Solanum lycopersicum;* St, *Solanum tuberisum*).

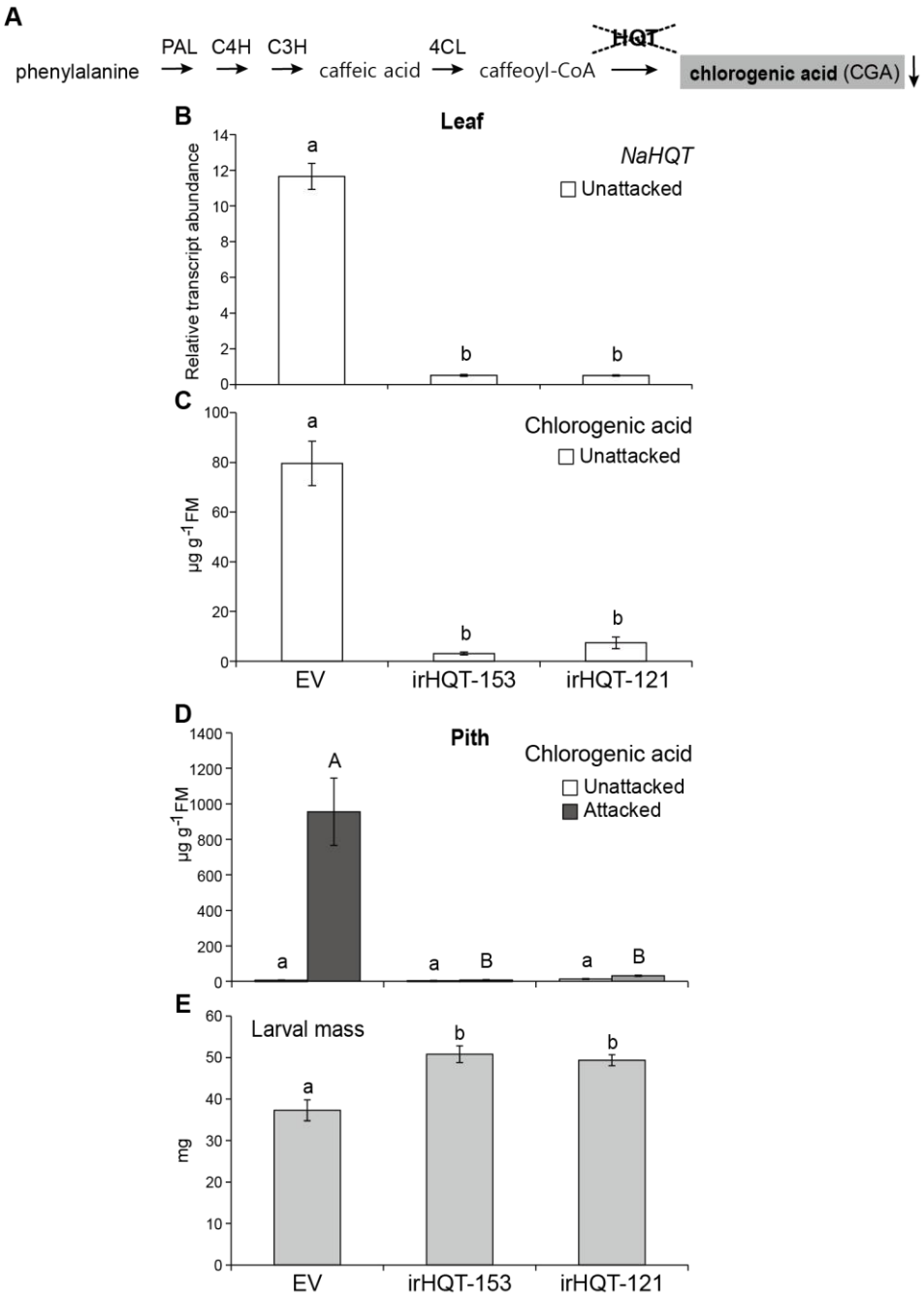

A
phenylalanine
PAL
C4H
C3H
caffeic acid
4CL
caffeoyl-CoA
HQT
chlorogenic acid (CGA)
B
Leaf
NaHQT
Unattacked
Relative transcript abundance
C
Chlorogenic acid
Unattacked
µg g-1FM
EV
irHQT-153
irHQT-121
D
Pith
Chlorogenic acid
Unattacked
Attacked
µg g-1FM
E
Larval mass
mg
EV
irHQT-153
irHQT-121

Fig. 4-12. Silencing efficiency of two transgenic lines, irHQT-153 and -121 plants which silenced key gene of CGA biosynthesis and T. mucorea larvae performance. (**A**) Simplified biosynthetic pathway of CGA in *N. attenuata* plants (Niggeweg et al., 2004). PAL, *phe ammonia lyases*; C4H, *cinnamic acid-4-hydroxylase*; C3H, *cinnamic acid-3-hydroxylase*; 4CL, *4-coumaroyl-CoA:ligase*;HQT, hydroxycinnamoyl-CoA quinate transferase. (**B**) Relative transcript abundance of *NaHQT* (hydroxcnamoyl-CoA quinate transferase), a key enzyme in chlorogenic acid biosynthesis, in EV and two independent *NaHQT*-silenced lines (irHQT-153 and -121). Silencing efficiency of *NaHQT* gene was approximately 95% (one-way ANOVA; $p < 0.05$; n=3). Leaves of rosette-stage EV plants were used for RNA extraction. (**C**) Mean (±SE) levels of CGA in the leaves of EV, irHQT-153, and irHQT-121 plants used for the transcript analysis (one-way ANOVA; $p < 0.001$; n=6). (**D**) Mean (±SE) levels of CGA in unattacked and attacked piths of EV, irHQT-153, irHQT-121 plants three weeks after *T. mucorea* egg inoculation. CGA levels in damaged irHQT plants were significantly lower than those in damaged EV plants (one-way ANOVA followed by Tukey's HSD; $p < 0.001$; n=6). (**E**) Mean (±SE) mass of larvae fed EV, irHQT-153, and irHQT-121 plants three weeks after the egg inoculation. *T. mucorea* larvae fed on irHQT plants gained significantly more mass than those fed on EV plants (one-way ANOVA followed by Fisher's least significant difference (LSD) test; $p < 0.05$; n=20). FM. fresh mass.

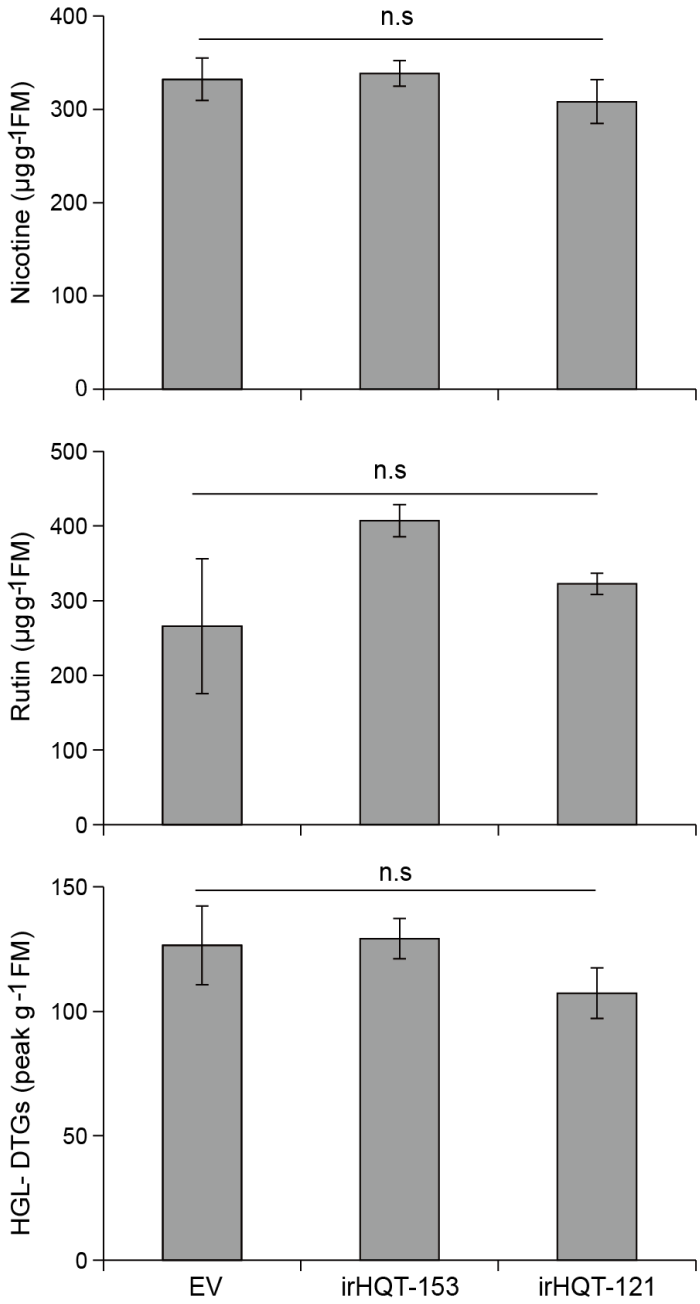

n.s
Nicotine (µgg-1FM)
400
300
200
100
0
n.s
Rutin (µg g-1FM)
500
400
300
200
100
0
n.s
HGL- DTGs (peak g -1 FM)
150
100
50
0
EV
irHQT-153
irHQT-121

Fig. 4-13. Levels of nicotine, rutin, and 17-hydroxyherany llinalool diterpene glycosides (HGL-DTGs) in irHQT plants. Silencing *NaHQT* does not affect the levels of nicotine, rutin, and HGL-DTGs in rosette-stage leaves. Leaves from EV, irHQT-153, and irHQT-121 plants were harvested without *T. muocrea* egg inoculation. Nicotine (one-way ANOVA; $p = 0.246$), rutin (one-way ANOVA; $p = 0.144$), and HGL-DTGs (one-way ANOVA; $p = 0.381$) did not differ in EV, irHQT-153, and irHQT-121 plants. FM, fresh mass; EV, empty-vector transformed wild-type plants; n.s, not significant.

4.3.6. The role of chlorogenic acid in leaf- and stem-defense in the field

To examine whether CGA also functions as a defense compound in the field, we inoculated *T. mucorea* eggs into EV and irHQT-153 plants grown in their native habitat, the Great Basin Desert, Utah, USA in 2014. For this experiment, I selected twenty plants per genotype which were all in the early stage of stalk elongation, the stage when adults would naturally select plants for oviposition. Three weeks after egg inoculation, I counted the number of surviving larvae in the egg inoculated EV and irHQT-153 plants. The survival rate of larvae was significantly higher in irHQT-153 than in EV plants (Fig. 4-14A; $p < 0.05$). When I measured CGA levels in attacked and unattacked pith of EV and irHQT-153 plants grown in the field, attacked pith contained significantly more CGA levels in EV plants compared to CGA levels in irHQT-153 plants (Fig. 4-14B; $p < 0.05$), as was also observed in the glasshouse-grown plants (Fig. 4-12C).

I also monitored herbivore damage from naturally occurring leaf herbivores on EV and irHQT-153 plants that had not been inoculated with *T. mucorea* eggs. As expected, CGA levels in the leaves of EV plants were much higher than that in irHQT-153 plants under unattacked condition (Fig. 4-14C; $p < 0.001$). I calculated the mean percentages of leaf area damaged by mirids (*Tupiocuris* spp. and *Empoasca* spp.), grasshoppers (*Trimerotropis* spp.), Noctuidae (*Spodoptera* spp.), flea beetles (*Epitrix* spp.), and tree

crickets (*Oecanthus* spp.). Interestingly, leaf herbivore damage on EV and irHQT-153 plants was not statistically different (Fig. 4-14D). These results suggest that the induced CGA produced by *N. attenuata* plants is more critical for the plants' defense against stem-boring insects than against leaf herbivores.

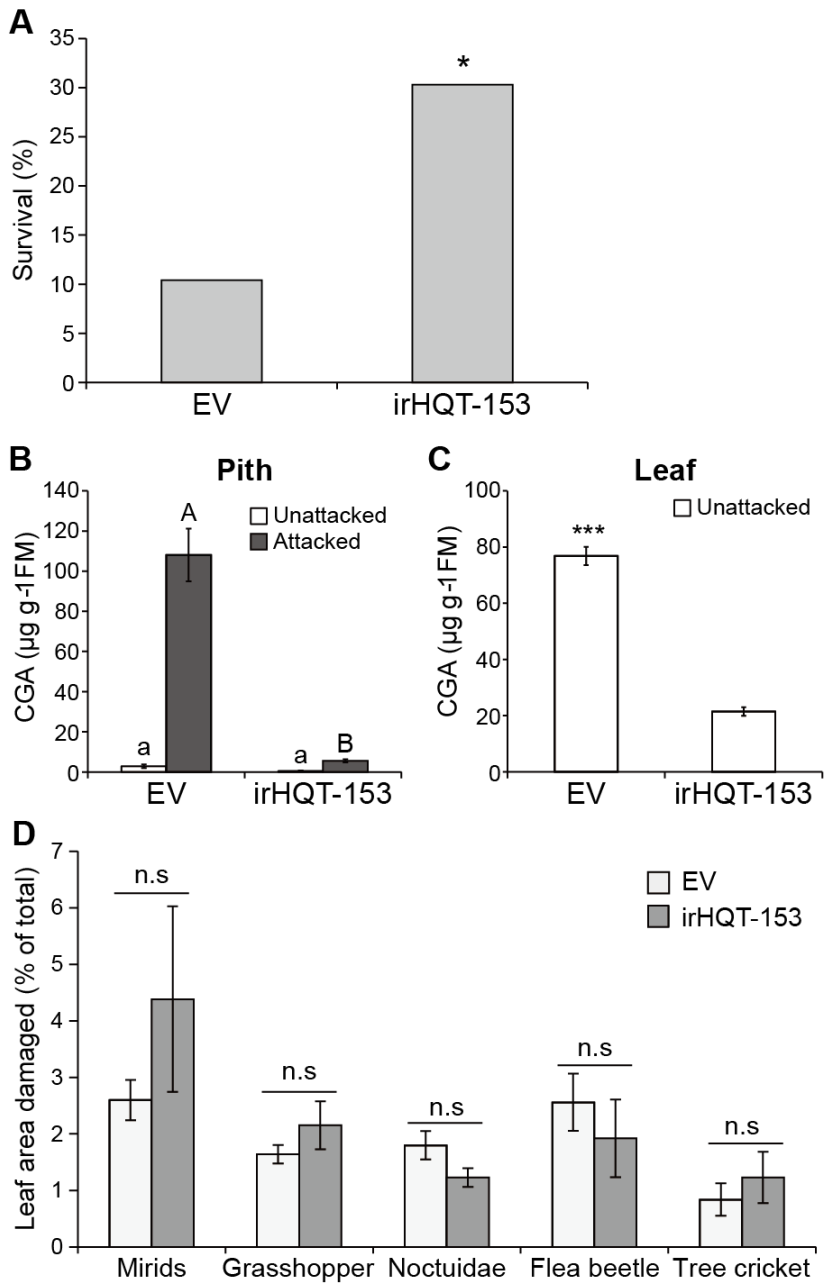

A
Survival (%)
35
30
25
20
15
10
5
0
*
EV
irHQT-153
B
Pith
CGA (µg g-1FM)
140
120
100
80
60
40
20
0
A
a
a
B
Unattacked
Attacked
EV
irHQT-153
C
Leaf
CGA (µg g-1FM)
100
80
60
40
20
0
***
Unattacked
EV
irHQT-153
D
Leaf area damaged (% of total)
7
6
5
4
3
2
1
0
n.s
n.s
n.s
n.s
n.s
EV
irHQT-153
Mirids
Grasshopper
Noctuidae
Flea beetle
Tree cricket

Fig. 4-14. Survival rate of *T. mucorea* when *NaHQT*-silenced plants were planted into the field and influence of native leaf-feeding herbivores in irHQT plants. Size-matched EV and *NaHQT*-silenced (irHQT-153) lines were planted in a randomized design in their native habitat, the Great Basin Desert, Utah, USA in 2014. (**A**) Survival rates of *T. mucorea* larvae experimentally oviposited into the pith of EV or irHQT-153 in the field. The number of survived larvae was counted three weeks after egg inoculation. (**B**) Mean (±SE) levels of CGA in unattacked and attacked piths of EV and irHQT-1 plants grown in the field. CGA levels in attacked EV plants were significantly higher than those in attacked irHQT-153 plants (one-way ANOVA followed by Tukey's HSD; $p < 0.001$; n=6). (**C**) Mean (±SE) levels of CGA in leaves of rosette-stage unattacked EV and irHQT-1 plants. Levels of CGA in irHQT-1 plants grown in the field were significantly lower than in EV plants (one-way ANOVA; $p < 0.001$; n=6). (**D**) Mean (±SE) leaf area consumed by native herbivores. Damage from the native herbivore community was measured as the % of leaf canopy damaged by mirids (*Tupiocuris spp.*) leafhoppers (*Empoasca spp.*), grasshoppers (*Trimerotropis spp.*), noctuid larvae (*Spodoptera spp.*), flea beetles (*Epitrix spp.*), and tree crickets (*Oecanthus spp.*) in EV and irHQT-153 plants. There were no significant differences between EV and irHQT-153 plants (Student *t*-test; n=20). Asterisk indicates significant differences between genotypes (χ^2-test; *, $p < 0.05$; ***, $p < 0.001$; n=17). FM. fresh mass; n.s, not significant.

4.4 Discussion

Plants produce various toxic metabolites to defend themselves against herbivores. These chemicals may reduce herbivore performance, survival rates, or reproductive success. CGA, an ester formed between caffeic acid and (L)-quinic acid, is one of the most abundant soluble phenolic compounds in solanaceous species, which include tomato, potato, tobacco, and eggplant; CGA is also found in apples, artichokes, coffee, pears, plums, and switchgrass (Niggeweg et al., 2004). CGA is oxidized to chlorogenoquinone, an electrophilic chemical which binds to free amino acids and proteins; this reduces the activity of digestive enzymes in insect guts (Felton et al., 1989; Felton and Duffey, 1991). CGA is known to increase the resistance of corn to herbivore attack from the fall armyworm (*Spodoptera frugiperad*), corn earworm (*Helicoverpa zea)*, leaf beetle, and leafhopper (Gueldner et al., 1992; Dowd and Vega, 1996; Ikonen, 2002; Jassbi, 2003). CGA levels are correlated with the resistance of carrots to attack by the *Psila rosae* fly (Ellis, 1999), and CGA also inhibits the growth of the tobacco armyworm, *Spodoptera litura* (Stevenson et al., 1993). In addition, CGA not only affects the primary but also the secondary trophic level. The predator performance of a stinkbug was negatively affected by CGA-fed prey (Traugott and Stamp, 1996). However, tobacco plants of modified *phe-ammonia-lyses* (PAL) enzyme which elevated levels of

phenylpropanoids including a six-fold increase in CGA showed little effect on larval growth of the tobacco hornworm, *M. sexta*, or on the tobacco budworm, *Heliothis virescens*, all folivores (Eichenseer et al., 1998; Johnson and Felton, 2001). Here I show that high levels of CGA in *N. attenuata* stems reduce the growth of *T. mucorea* larvae but have little effect against attack by leaf-chewing or -sucking insects.

CGA production in *N. attenuata* leaves is uncoupled from JA and JA signaling. Methyl jasmonate treatment has little effect on the level of CGA in *N. attenuata* leaves (Oldham and Baldwin, 2001). Treatment with oral secretions from the specialist herbivore *M. sexta* induces high levels of JA and JA-Ile in *N. attenuata* leaves, but this treatment does not induce CGA levels in these tissues (Onkokesung et al., 2012). Similarly, silencing JA biosynthesis or signaling does not alter CGA levels in *N. attenuata* leaves (Paschold et al., 2007; Demkura et al., 2010). However, CGA amounts in the pith of *N. attenuata* are tightly coupled with JA production and JA signaling (Fig. 4). These results suggest that the connection between JA signaling and defense metabolites can vary among different plant tissues. The COI1 protein is the JA-Ile receptor which has been found in several plants (Paschold et al., 2007). The COI1-JA-Ile complex initiates the degradation of proteins from the jasmonate ZIM domain (JAZ) that repress the expression of JA-responsive genes. The *N. attenuata* genome contains at least 12 JAZ proteins; these proteins are involved in several JA-dependent

signaling pathways especially JAZd regulates JA and JA-Ile levels in flowers and JAZh is involved in leaf defense responses when the specialist herbivore *M. sexta* attacks (Oh et al., 2012, 2013). Whereas I did not observe the differences in the larval mass of *T. mucorea* that fed on irJAZd and irJAZh plants compared to those that fed on control plants (Fig. 4-15), it is likely that other JAZ proteins specifically regulate CGA levels in the pith of *N. attenuata*. And other stem defense metabolites against *T. mucorea* larva attack can be exist from larvae mass result between EV and lignin-deficient plants (Fig. 4-16) which silenced major lignin biosynthetic gene, *cinnamyl alcohol dehydrogenase* (*NaCAD*) (Kaur et al., 2012). *T. mucorea* larvae mass when fed on lignin-deficient plants much higher than the larvae fed on control plants. It is likely lignin metabolites also induced from attacked pith part and bring into play as quantitate defense against *T. mucorea* larvae. But other phenolic compound, flavonoid metabolite did not affected on *T. mucorea* larvae mass (Fig. 4-16) using flavonoid-deficient plants that the plant silenced *chalcone synthases* gene (*NaCHAL*) which is key gene of flavonoid biosynthesis (Kessler et al., 2008).

In the field experiment, I examined the roles of CGA for plant defense in the pith and also the leaf of *N. attenuata* plants. Although I observed a high survival rate among *T. mucorea* larvae inoculated into CGA-silenced plants, there was no significant difference in the amount of leaf damage from herbivores between control and CGA-silenced plants. CGA in *N. attenuata*

leaves might be involved in plant defense against abiotic stress rather than biotic stress. For instance, CGA in tomato play an important role for leaf protection from UV-B radiation in the glasshouse (Clé et al., 2008). UV-B radiation also elicits CGA accumulation in *N. attenuata* leaves (Demkura et al., 2010). More experiments, however, are needed to understand the role of CGA in leaves.

Plant tissues that have low nutritive content are predicted to have low levels of defensive compounds (Zangerl and Bazzaz, 1992). Pith is thought to be a less nutritious than leaves, because pith is mainly composed of cellulose and lignin (Fordyce and Malcolm, 2000). Levels of nicotine, rutin, HGL-DTG, and CGA in the pith of unattacked *N. attenuata* were less than those in leaves (Figs. 4-4 and 4-13). Allowing *T. mucorea* larvae to feed on pith, however, dramatically increased levels of CGA in pith. Elicited CGA levels in the attacked pith were similar with those in the leaves of *N. attenuata* plants (Fig. 4-14). This study demonstrates that plants have developed a cost-effective inducible system to protect their tissues from herbivore attack and that JA signaling plays a central role in eliciting responses, but that JA signaling elicits different metabolites in different tissues.

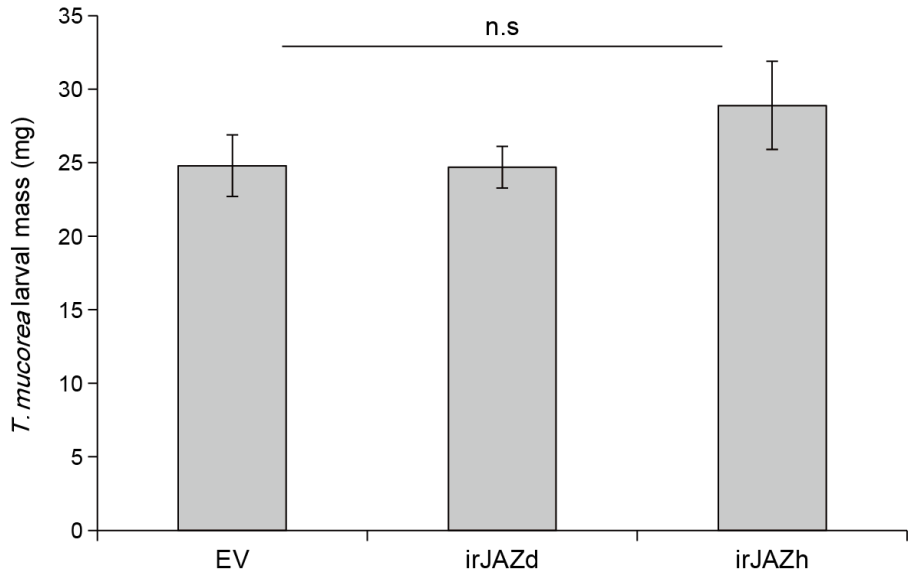


Fig. 4-15. Performance of *T. mucorea* larvae fed irJAZd, irJAZh, or EV plants. Larvae were collected after three weeks of continuous feeding after egg inoculation (one-way ANOVA; Fisher's least significant difference (LSD) test; p = 0.33; n=20). n.s, not significant.

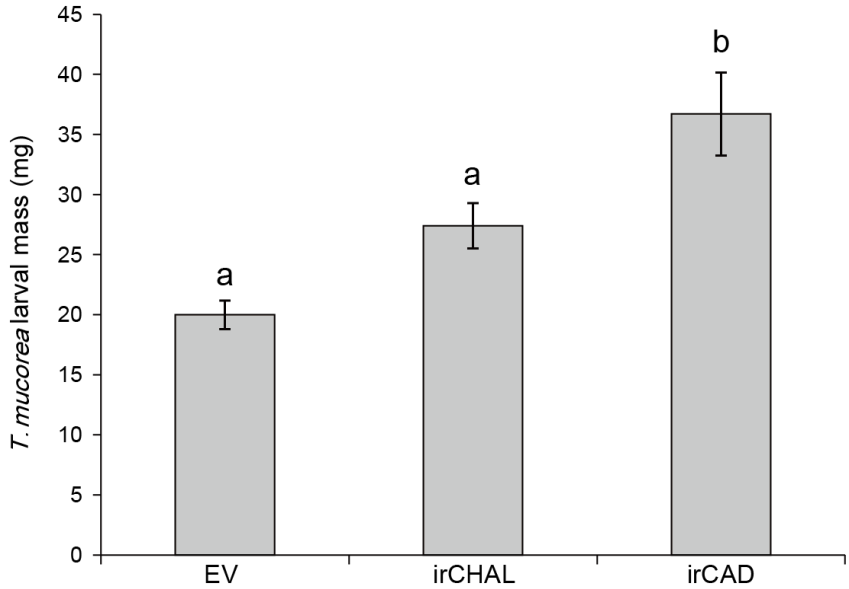


Fig. 4-16. Performance of *T. mucorea* larvae fed irCHAL, irCAD or EV plants. Larvae were collected after three weeks of continuous feeding after egg inoculation. Different letters indicates significant differences between genotypes. (one-way ANOVA; Fisher's least significant difference (LSD) test; $p < 0.05$; n=20).

Chapter 5. General conclusion

While the ecology of host selection behavior of female insects has been well studied, and it is known that the levels of certain host plant metabolites are positively or negatively correlated with oviposition preferences, it remains unclear whether adult females choose the best plants for their offspring by sensing the very metabolites that affect the larval performance. I have examined the preference-performance hypothesis (PPH) that "mother knows best" at the molecular level with transgenic *N. attenuata* plants that produced reduced amounts of the defense hormone jasmonic acid, as well as a toxic alkaloid, nicotine, and emitted large amounts of sesquiterpene volatiles, to understand the oviposition behavior of two naturally co-occurring sibling species, *T. compacta* and *T. mucorea* and the consequences of oviposition choices for larval performance in field and laboratory experiments. *T. compacta* and *T. mucorea* females chose host plants that maximized their offspring's performance. Interestingly, however, the toxic alkaloids that determined larval performance were not involved in the oviposition decisions. Rather, JA-induced sesquiterpenes were the key metabolites in attracting *T. mucorea* females to their host plant, *N. attenuata*, and these sesquiterpenes had no effect on the larval performance of *T. mucorea*. As such, this study significantly advances our understanding of the PPH by demonstrating how semiochemicals used to mediate oviposition decisions are different from those responsible for larval performance. In other words, mother may know what's best for her offspring, but she doesn't

know why her choices are good ones for her offspring's performance.

From an evolutionary perspective, in terms of host selection specificity, the two sibling species of *Trichobaris* have mainly been observed in the *D. wrightii* population despite *T. mucorea* is reported as tobacco weevil. I confirmed that *T. mucorea* can survive in the *D. wrightii* plant. This means *T. mucorea* selected as host plant both *N. attenuata* and *D. wrightii*, whereas *T. compacta* selected only the *D. wrightii* plant as a host. These results may demonstrate that toxic secondary metabolites produced by host plants are expanded to the host range of *T. mucorea*. The two *Trichobaris* species may have a common ancestor and share an ecological niche in *D. wrightii* plants, creating competition within the same habitat. This competition may be caused a host shift on the part of *T. mucorea,* which may acquire detoxification ability from the nicotine alkaloid of *N. attenuata,* leading to a host shift by sympatric speciation. Generally, insect host shifts caused by sympatric speciation involve genetic change (Wheat et al., 2007; Kirsch et al., 2011). Also *T. compacta* and *T. mucorea* face an accurate barrier to mating. Therefore, the two *Trichobaris* species may have genetically divided during host adaptation, that it may be evolved different gene such as CYP gene and P450s enzyme (Feyereisen and Lawrence, 2012; Bass et al., 2013) in order to detoxify host secondary metabolites.

It is well known that induced plant defense response in leaf tissue against attack by leaf-chewing or -sucking herbivores is strongly correlated

with eliciting of JA. However, plant defense strategy in stem tissue against stem-boring herbivores with ecological relevance relationship is poorly understood due to the fact that stem tissue faces less selection pressure, in terms of defense mechanism action, than leaf tissue. Here, I investigated stem-specific defense responses in *N. attenuata* against the stem-boring herbivore *T. mucorea* larvae. I found that the chlorogenic acid (CGA) metabolite was highly induced in pith tissue by *T. mucorea* larvae attack via JA signaling, a defense response that is distinct from the leaf defense strategy in *N. attenuata*. By genetic manipulation and in vitro bioassay of CGA metabolite, I clarified that CGA negatively affects *T. mucorea* performance as a stem defense compound both in the glasshouse and in field conditions. Taken together, this pith defense study highlights the important role that CGA metabolite from stem tissue plays in inducible defense against stem-boring herbivore attack, providing a mechanical, tissue-specific defense strategy against herbivores of various feeding guilds.

As the basis for further research, I investigated inducible defense response in stem tissue against a stem-boring herbivore, and assert that there is a tissue-specific defense in the stem. For proved this story, the research will require further hypotheses about tissue-localized defense. Pith defense responses showed not only different JA-signaling responses, but also localized patterns. To falsify this hypothesis would require measurement of the defense response and comparison with leaf induced defense by *N.*

attenuata against specialist leaf-chewer *M. sexta,* which has been the subject of many other studies. Testing the ecological consequences of tissue-specific defense would require measuring mass changes in the larvae of both the *M. sexta* caterpillar and *T. mucorea,* feeding solely on *N. attenuata* and both *M. sexta* and *T. mucorea* larvae feeding simultaneously on *N. attenuata,* which means its plant-mediated interactions between leaf herbivore, *M. sexta* and stem herbivore, *T. mucorea*. Transcription levels of 13-lipoxygenases (LOX) such as LOX3 and LOX6 will also need to be tested to monitor systemic response transfer or change in pith to leaf. These 13-lipoxygenases are related to local and systemic wound response to jasmonate synthesis (Chauvin et al., 2013) of attacked pith. Ultimately, further study of tissue specific defense will show that a plant knows where it hurts and that a plant's response depends on how smart it is in the location where the attacks occurred. Based on previous results, nicotine is not a factor in *T. mucorea* female oviposition choice or larval performance; it can also be hypothesized that *T. mucorea* larva is able to detoxify nicotine metabolite in the *N. attenuata* host plant. It will be need to analyze *T. mucorea* larva frass which feed on *N. attenuata* plant for nicotine metabolite, and conduct in vitro assay, e.g. *T. mucorea* larva feed on artificial diet spiked with nicotine compared with *T. compacta* frass analysis. The two *Trichobaris* have ability of different toxic metabolite detoxifications from *D. wrightii* and *N. attenuata* plants. Testing will be needed to discriminate genetic level the host selection

behaviors of the larvae of the two *Trichobaris* through gene expression in the gut and hemolymph, to see whether these organs of the larvae have different CYP genes or P450s enzyme expression. In addition, it will be necessary to examine how two *Trichobaris* females select the *D. wrightii* plant for feeding and ovipositing, although *D. wrightii* is hard to manipulate genetically.

References

Abrahamson, W.G., Eubanks, M.D., Blair, C.P., and Whipple, A.V. (2001). Gall flies, inquilines, and goldenrods: a model for host-race formation and sympatric speciation. Am. Zool. *41*, 928–938.

Aharoni, A., Jongsma, M.A., and Bouwmeester, H.J. (2005). Volatile science? Metabolic engineering of terpenoids in plants. Trends Plant Sci. *10*, 594–602.

Akol, A.M., Masembe, C., Isabirye, B.E., Kukiriza, C.K., and Rwomushana, I. (2013). Oviposition preference and offspring performance in phytophagous fruit flies (Diptera: Tephritidae): the african invader, *Bactrocera invadens*. Int. Res. J. Hortic. *1*, 1–14.

Ali, J.G., and Agrawal, A.A. (2012). Specialist versus generalist insect herbivores and plant defense. Trends Plant Sci. *17*, 293–302.

Armitage, C.J., and Conner, M. (2000). Social cognition models and health behaviour: a structured review. Psychol. Heal. *15*, 173–189.

Awmack, C.S., and Leather, S.R. (2002). Host plant quality and fecundity in herbivorous insects. Annu. Rev. Entomol. *47*, 817–844.

Baldwin, I.T. (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proc. Natl. Acad. Sci. *95*, 8113–8118.

Baldwin, I.T. (1999). Inducible nicotine production in native *Nicotiana* as

an example of adaptive phenotypic plasticity. J. Chem. Ecol. *25*, 3–30.

Baldwin, I.T. (2001). An ecologically motivated analysis of plant-herbivore interactions in native tobacco. Plant Physiol. *127*, 1449–1458.

Baldwin, I.T., and Preston, C.A. (1999). The eco-physiological complexity of plant responses to insect herbivores. Planta *208*, 137–145.

Baldwin, I.T., Halitschke, R., Kessler, A., and Schittko, U. (2001). Merging molecular and ecological approaches in plant–insect interactions. Curr. Opin. Plant Biol. *4*, 351–358.

Barber, H.S. (1935). The tobacco and solanum weevils of the genus *Trichobaris* (US Dept. of Agriculture).

Bass, C., Zimmer, C.T., Riveron, J.M., Wilding, C.S., Wondji, C.S., Kaussmann, M., Field, L.M., Williamson, M.S., and Nauen, R. (2013). Gene amplification and microsatellite polymorphism underlie a recent insect host shift. Proc. Natl. Acad. Sci. *110*, 19460–19465.

Bernays, E.A., and Chapman, R.F. (2007). Host-plant selection by phytophagous insects (Springer Science & Business Media).

Bertheau, C., Salle, A., Roux-Morabito, G., Garcia, J., Certain, G., and Lieutier, F. (2009). Preference-performance relationship and influence of plant relatedness on host use by Pityogenes chalcographus L. Agric. For. Entomol. *11*, 389–396.

Beuzelin, J.M., Wilson, L.T., Showler, A.T., Meszaros, A., Wilson, B.E., Way, M.O., and Reagan, T.E. (2013). Oviposition and larval development of a stem borer, *Eoreuma loftini*, on rice and non-crop grass hosts. Entomol. Exp. Appl. *146*, 332–346.

Bonaventure, G., VanDoorn, A., and Baldwin, I.T. (2011). Herbivore-associated elicitors: FAC signaling and metabolism. Trends Plant Sci. *16*, 294–299.

Brodbeck, B.V, Andersen, P.C., Oden, S., and Mizell, R.F. (2007). Preference-performance linkage of the xylem feeding leafhopper, *Homalodisca vitripennis* (Hemiptera: Cicadellidae). Environ. Entomol. *36*, 1512–1522.

Bruce, T.J.A., Wadhams, L.J., and Woodcock, C.M. (2005). Insect host location: a volatile situation. Trends Plant Sci. *10*, 269–274.

del Campo, M.L., Miles, C.I., Schroeder, F.C., Mueller, C., Booker, R., and Renwick, J. a (2001). Host recognition by the tobacco hornworm is mediated by a host plant compound. Nature *411*, 186–189.

Carrasco, D., Larsson, M.C., and Anderson, P. (2015). Insect host plant selection in complex environments. Curr. Opin. Insect Sci. *8*, 1–7.

Chatzigeorgiou, A.C., Papadopoulos, N.T., and Prophetou-Athanasiadou, D.A. (2010). Effect of cotton cultivars on the oviposition preference of pink bollworm (Lepidoptera: Gelechiidae). J. Pest Sci. (2004). *83*, 289–296.

Chauvin, A., Caldelari, D., Wolfender, J., and Farmer, E.E. (2013). Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded *Arabidopsis thaliana* leaves: a role for lipoxygenase 6 in responses to long-distance wound signals. New Phytol. *197*, 566–575.

Clark, K.E., Hartley, S.E., and Johnson, S.N. (2011). Does mother know best? The preference-performance hypothesis and parent-offspring conflict in aboveground-belowground herbivore life cycles. Ecol. Entomol. *36*, 117–124.

Clé, C., Hill, L.M., Niggeweg, R., Martin, C.R., Guisez, Y., Prinsen, E., and Jansen, M.A.K. (2008). Modulation of chlorogenic acid biosynthesis in *Solanum lycopersicum*; consequences for phenolic accumulation and UV-tolerance. Phytochemistry *69*, 2149–2156.

Combes, S.A., Rundle, D.E., Iwasaki, J.M., and Crall, J.D. (2012). Linking biomechanics and ecology through predator–prey interactions: flight performance of dragonflies and their prey. J. Exp. Biol. *215*, 903–913.

Craig, T.P., Itami, J., Shantz, C., Abrahamson, W.G., Horner, J., and Craig, J. (2000). The influence of host plant variation and intraspecific competition on oviposition preference and offspring performance in the host races of *Eurosta solidaginis*. Ecol. Entomol. *25*, 7–18.

Creelman, R.A., and Mulpuri, R. (2002). The oxylipin pathway in *Arabidopsis*. Arab. B. e0012.

Cuda, J.P., and Burke, H.R. (1986). Reproduction and development of the potato stalk borer (Coleoptera: Curculionidae) with notes on field biology. J. Econ. Entomol. *79*, 1548–1554.

Van Dam, N.M., and Hare, J.D. (1998). Biological activity of *Datura wrightii* glandular trichome exudate against *Manduca sexta* larvae. J. Chem. Ecol. *24*, 1529–1549.

Deedat, Y.D., and Ellis, C.R. (1983). Damage caused by potato stem borer (Lepidoptera: Noctuidae) to field corn. J. Econ. Entomol. *76*, 1055–1060.

De-la-Mora, M., Piñero, D., and Núñez-Farfán, J. (2015). Phylogeography of specialist weevil *Trichobaris soror*: a seed predator of *Datura stramonium*. Genetica *143*, 681–691.

Demkura, P.V., Abdala, G., Baldwin, I.T., and Ballare, C.L. (2010). Jasmonate-dependent and -independent pathways mediate specific effects of solar ultraviolet B radiation on leaf phenolics and antiherbivore defense. Plant Physiol. *152*, 1084–1095.

Dethier, V.G. (1982). Mechanism of host-plant recognition. Entomol. Exp. Appl. *31*, 49–56.

Dicke, M., and Hilker, M. (2003). Induced plant defences: from molecular biology to evolutionary ecology. Basic Appl. Ecol. *14*, 3–14.

Diezel, C., Allmann, S., and Baldwin, I.T. (2011a). Mechanisms of optimal defense patterns in *Nicotiana attenuata*: flowering attenuates

herbivory-elicited ethylene and jasmonate signalingF. J. Integr. Plant Biol. *53*, 971–983.

Diezel, C., Kessler, D., and Baldwin, I.T. (2011b). Pithy protection: *Nicotiana attenuata*'s jasmonic acid-mediated defenses are required to resist stem-boring weevil larvae. Plant Physiol. *155*, 1936–1946.

Dinh, S.T., Baldwin, I.T., and Galis, I. (2013). The HERBIVORE ELICITOR-REGULATED1 gene enhances abscisic acid levels and defenses against herbivores in *Nicotiana attenuata* plants. Plant Physiol. *162*, 2106–2124.

Dowd, P.F., and Vega, F.E. (1996). Enzymatic oxidation products of allelochemicals as a basis for resistance against insects: effects on the corn leafhopper *Dalbulus maidis*. Nat. Toxins *4*, 85–91.

Dowd, P.F., Smith, C.M., and Sparks, T.C. (1983). Detoxification of plant toxins by insects. Insect Biochem. *13*, 453–468.

Drès, M., and Mallet, J. (2002). Host races in plant–feeding insects and their importance in sympatric speciation. Philos. Trans. R. Soc. London B Biol. Sci. *357*, 471–492.

Eichenseer, H., Bi, J.L., and Felton, G.W. (1998). Indiscrimination of *Manduca sexta* larvae to overexpressed and underexpressed levels of phenylalanine ammonia-lyase in tobacco leaves. Entomol. Exp. Appl. *87*, 73–78.

Ellis, P.R. (1999). The identification and exploitation of resistance in carrots

and wild Umbelliferae to the carrot fly, *Psila rosae* (F.). Integr. Pest Manag. Rev. *4*, 259–268.

Erb, M., Lenk, C., Degenhardt, J., and Turlings, T.C.J. (2009). The underestimated role of roots in defense against leaf attackers. Trends Plant Sci. *14*, 653–659.

Erb, M., Meldau, S., and Howe, G.A. (2012). Role of phytohormones in insect-specific plant reactions. Trends Plant Sci. *17*, 250–259.

Felton, G.W., and Duffey, S.S. (1991). Protective action of midgut catalase in lepidopteran larvae against oxidative plant defenses. J. Chem. Ecol. *17*, 1715–1732.

Felton, G.W., Donato, K., Del Vecchio, R.J., and Duffey, S.S. (1989). Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. J. Chem. Ecol. *15*, 2667–2694.

Feyereisen, R., and Lawrence, I.G. (2012). 8–insect CYP genes and P450 enzymes. Insect Mol. Biol. Biochem. 236–316.

Fordyce, J.A., and Malcolm, S.B. (2000). Specialist weevil, *Rhyssomatus lineaticollis*, does not spatially avoid cardenolide defenses of common milkweed by ovipositing into pith tissue. J. Chem. Ecol. *26*, 2857–2874.

Friberg, M., Posledovich, D., and Wiklund, C. (2015). Decoupling of female host plant preference and offspring performance in relative specialist

and generalist butterflies. Oecologia *178*, 1181–1192.

Fürstenberg-Hägg, J., Zagrobelny, M., and Bak, S. (2013). Plant defense against insect herbivores. Int. J. Mol. Sci. *14*, 10242–10297.

Gaquerel, E., Stitz, M., Kallenbach, M., and Baldwin, I.T. (2013). Jasmonate signaling in the field, part II: insect-guided characterization of genetic variations in jasmonate-dependent defenses of transgenic and natural *Nicotiana attenuata* populations. Jasmonate Signal. Methods Protoc. 97–109.

De Geyter, N., Gholami, A., Goormachtig, S., and Goossens, A. (2012). Transcriptional machineries in jasmonate-elicited plant secondary metabolism. Trends Plant Sci. *17*, 349–359.

Gilardoni, P.A., Hettenhausen, C., Baldwin, I.T., and Bonaventure, G. (2011). *Nicotiana attenuata* LECTIN RECEPTOR KINASE1 suppresses the insect-mediated inhibition of induced defense responses during *Manduca sexta* herbivory. Plant Cell *23*, 3512–3532.

Girijashankar, V., Sharma, H.C., Sharma, K.K., Swathisree, V., Prasad, L.S., Bhat, B. V, Royer, M., San Secundo, B., Narasu, M.L., and Altosaar, I. (2005). Development of transgenic sorghum for insect resistance against the spotted stem borer (*Chilo partellus*). Plant Cell Rep. *24*, 513–522.

Goodspeed, T.H. (1954). The genus *Nicotiana*. Chron. Bot. *16*, 102–135.

Gripenberg, S., Morriën, E., Cudmore, A., Salminen, J., and Roslin, T. (2007). Resource selection by female moths in a heterogeneous environment: What is a poor girl to do? J. Anim. Ecol. *76*, 854–865.

Gripenberg, S., Mayhew, P.J., Parnell, M., and Roslin, T. (2010). A meta-analysis of preference-performance relationships in phytophagous insects. Ecol. Lett. *13*, 383–393.

Gueldner, R.C., Snook, M.E., Widstrom, N.W., and Wiseman, B.R. (1992). TLC screen for maysin, chlorogenic acid, and other possible resistance factors to the fall armyworm and the corn earworm in *Zea mays*. J. Agric. Food Chem. *40*, 1211–1213.

Halkier, B.A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Annu. Rev. Plant Biol. *57*, 303–333.

Hare, J.D. (2005). Biological activity of acyl glucose esters from *Datura wrightii* glandular trichomes against three native insect herbivores. J. Chem. Ecol. *31*, 1475–1491.

Hatchett, J.H., Daugherty, D.M., Robbins, J.C., Barry, R.M., and Houser, E.C. (1975). Biology in Missouri of *Dectes texanus*, a new pest of soybean. Ann. Entomol. Soc. Am. *68*, 209–213.

Haukioja, E., and Koricheva, J. (2000). Tolerance to herbivory in woody vs. herbaceous plants. Evol. Ecol. *14*, 551–562.

Heiling, S., Schuman, M.C., Schoettner, M., Mukerjee, P., Berger, B., Schneider, B., Jassbi, A.R., and Baldwin, I.T. (2010). Jasmonate and

ppHsystemin regulate key malonylation steps in the biosynthesis of 17-hydroxygeranyllinalool diterpene glycosides, an abundant and effective direct defense against herbivores in *Nicotiana attenuata*. Plant Cell *22*, 273–292.

Herden, J., Meldau, S., Kim, S., Kunert, G., Joo, Y., Baldwin, I.T., and Schuman, M.C. (2015). Shifting *Nicotiana attenuata*'s diurnal rhythm does not alter its resistance to the specialist herbivore *Manduca sexta*. J. Integr. Plant Biol.

Howe, G.A., and Jander, G. (2008). Plant immunity to insect herbivores. Annu. Rev. Plant Biol. *59*, 41–66.

Huerta-Paniagua, R.A., Bautista-Martinez, N., Bravo-Mojica, H., Carrillo-Sanchez, J.L., and Diaz-Gomez, O. (2004). Altitude distribution of *Trichobaris championi* Barber (Coleoptera: Curculionidae) and field observations about its biology. Agrociencia *38*, 97–106.

Hummelbrunner, L.A., and Isman, M.B. (2001). Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). J. Agric. Food Chem. *49*, 715–720.

Ikonen, A. (2002). Preferences of six leaf beetle species among qualitatively different leaf age classes of three Salicaceous host species. Chemoecology *12*, 23–28.

Ikonen, A., Tahvanainen, J., and Roininen, H. (2002). Phenolic secondary

compounds as determinants of the host plant preferences of the leaf beetle *Agelastica alni*. Chemoecology *12*, 125–131.

Ivie, G.W., Bull, D.L., Beier, R.C., Pryor, N.W., and Oertli, E.H. (1983). Metabolic detoxification: mechanism of insect resistance to plant psoralens. Science *221*, 374–376.

Jaenike, J. (1978). On optimal oviposition behavior in phytophagous insects. Theor. Popul. Biol. *14*, 350–356.

Jallow, M.F.A., and Zalucki, M.P. (2003). Relationship between oviposition preference and offspring performance in Australian *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Aust. J. Entomol. *42*, 343–348.

Janz, N., Bergström, A., and Sjögren, A. (2005). The role of nectar sources for oviposition decisions of the common blue butterfly *Polyommatus icarus*. Oikos *109*, 535–538.

Jassbi, A.R. (2003). Secondary metabolites as stimulants and antifeedants of *Salix integra* for the leaf beetle Plagiodera versicolora. Zeitschrift Für Naturforsch. C *58*, 573–579.

Johnson, K.S., and Felton, G.W. (2001). Plant phenolics as dietary antioxidants for herbivorous insects: a test with genetically modified tobacco. J. Chem. Ecol. *27*, 2579–2597.

Kallenbach, M., Bonaventure, G., Gilardoni, P.A., Wissgott, A., and Baldwin, I.T. (2012). *Empoasca* leafhoppers attack wild tobacco

plants in a jasmonate-dependent manner and identify jasmonate mutants in natural populations. Proc. Natl. Acad. Sci. USA *109*, E1548–E1557.

Karban, R., and Baldwin, I.T. (1997). Induced responses to herbivory (University of Chicago Press).

Karban, R., Agrawal, A.A., and Mangel, M. (1997). The benefits of induced defenses against herbivores. Ecology *78*, 1351–1355.

Karungi, J., Lubanga, U.K., Kyamanywa, S., and Ekbom, B. (2010). Oviposition preference and offspring performance of *Crocidolomia pavonana* (Lepidoptera: Pyralidae) on different host plants. J. Appl. Entomol. *134*, 704–713.

Kaur, H., Heinzel, N., Schöttner, M., Baldwin, I.T., and Gális, I. (2010). R2R3-NaMYB8 regulates the accumulation of phenylpropanoid-polyamine conjugates, which are essential for local and systemic defense against insect herbivores in *Nicotiana attenuata*. Plant Physiol. *152*, 1731–1747.

Kaur, H., Shaker, K., Heinzel, N., Ralph, J., Gális, I., and Baldwin, I.T. (2012). Environmental stresses of field growth allow cinnamyl alcohol dehydrogenase-deficient *Nicotiana attenuata* plants to compensate for their structural deficiencies. Plant Physiol. doi:10.1104/pp.112.196717.

Kessler, A., and Baldwin, I.T. (2001). Defensive function of herbivore-

induced plant volatile emissions in nature. Science *291*, 2141–2144.

Kessler, A., and Baldwin, I.T. (2002). Plant responses to insect herbivory: the emerging molecular analysis. Annu. Rev. Plant Biol. *53*, 299–328.

Kessler, A., Halitschke, R., and Baldwin, I.T. (2004). Silencing the jasmonate cascade: induced plant defenses and insect populations. Science *305*, 665–668.

Kessler, D., Gase, K., and Baldwin, I.T. (2008). Field experiments with transformed plants reveal the sense of floral scents. Science *321*, 1200–1202.

Kirsch, R., Vogel, H., Muck, A., Reichwald, K., Pasteels, J.M., and Boland, W. (2011). Host plant shifts affect a major defense enzyme in Chrysomela lapponica. Proc. Natl. Acad. Sci. USA *108*, 4897–4901.

Knolhoff, L.M., and Heckel, D.G. (2014). Behavioral assays for studies of host plant choice and adaptation in herbivorous insects. Annu. Rev. Entomol. *59*, 263–278.

Korth, K.L., and Dixon, R.A. (1997). Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. Plant Physiol. *115*, 1299–1305.

Krügel, T., Lim, M., Gase, K., Halitschke, R., and Baldwin, I.T. (2002). *Agrobacterium*-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. Chemoecology *12*, 177–183.

Kumar, P., Rathi, P., Schöttner, M., Baldwin, I.T., and Pandit, S. (2014). Differences in nicotine metabolism of two *Nicotiana attenuata* herbivores render them differentially susceptible to a common native predator. PLoS One *9*, e95982.

Liu, Z., Scheirs, J., and Heckel, D.G. (2010). Host plant flowering increases both adult oviposition preference and larval performance of a generalist herbivore. Environ. Entomol. *39*, 552–560.

Liu, Z., Scheirs, J., and Heckel, D.G. (2012). Trade-offs of host use between generalist and specialist Helicoverpa sibling species: adult oviposition and larval performance. Oecologia *168*, 459–469.

Lynds, G.Y., and Baldwin, I.T. (1998). Fire, nitrogen, and defensive plasticity in *Nicotiana attenuata*. Oecologia *115*, 531–540.

Malone, L.A., and Wigley, P.J. (1990). A practical method for rearing argentine stem weevil, *Listronotus bonariensis* (Coleoptera: Curculionidae) in the laboratory. New Zeal. Entomol. *13*, 87–89.

Marazzi, C., and Städler, E. (2004). *Arabidopsis thaliana* leaf-surface extracts are detected by the cabbage root fly (*Delia radicum*) and stimulate oviposition. Physiol. Entomol. *29*, 192–198.

Marazzi, C., and Städler, E. (2005). Influence of sulphur plant nutrition on oviposition and larval performance of the cabbage root fly. Agric. For. Entomol. *7*, 277–282.

Markovich, O., Kafle, D., Elbaz, M., Malitsky, S., Aharoni, A.,

Schwarzkopf, A., Gershenzon, J., and Morin, S. (2013). *Arabidopsis thaliana* plants with different levels of aliphatic- and indolyl-glucosinolates affect host selection and performance of *Bemisia tabaci*. J. Chem. Ecol. *39*, 1361–1372.

Marvaldi, A.E., Sequeira, A.S., O'Brien, C.W., and Farrell, B.D. (2002). Molecular and morphological phylogenetics of weevils (Coleoptera, Curculionoidea): do niche shifts accompany diversification? Syst. Biol. *51*, 761–785.

Matsuki, M., Foley, W.J., and Floyd, R.B. (2011). Role of volatile and non-volatile plant secondary metabolites in host tree selection by christmas beetles. J. Chem. Ecol. *37*, 286–300.

Mayhew, P.J. (1997). Adaptive patterns of host-plant selection by phytophagous insects. Oikos 417–428.

Mazaheri, A., Khajehali, J., and Hatami, B. (2011). Oviposition preference and larval performance of *Aeolesthes sarta* (Coleoptera: Cerambycidae) in six hardwood tree species. J. Pest Sci. (2004). *84*, 355–361.

McGee, M.R., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., and Schoenfuss, H.L. (2009). Predator avoidance performance of larval fathead minnows (*Pimephales promelas*) following short-term exposure to estrogen mixtures. Aquat. Toxicol. *91*, 355–361.

van der Meijden, E. (1996). Plant defence, an evolutionary dilemma:

contrasting effects of (specialist and generalist) herbivores and natural enemies. In Proceedings of the 9th International Symposium on Insect-Plant Relationships, (Springer), pp. 307–310.

Meldau, S., Erb, M., and Baldwin, I.T. (2012). Defence on demand: mechanisms behind optimal defence patterns. Ann. Bot. *110*, 1503–1514.

Mewis, I., Appel, H.M., Hom, A., Raina, R., and Schultz, J.C. (2005). Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiol. *138*, 1149–1162.

Nagasawa, A., Kamada, Y., Kosaka, Y., Arakida, N., and Hori, M. (2014). Catechol - an oviposition stimulant for cigarette beetle in roasted coffee beans. J. Chem. Ecol. *40*, 452–457.

Niggeweg, R., Michael, A.J., and Martin, C. (2004). Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat. Biotechnol. *22*, 746–754.

O'Brien, C.W., and Wibmer, G.J. (1982). Annotated checklist of the weevils (Curculionidae sensu lato) of North America, Central America, and the West Indies (Coleoptera: Curculionoidea). Mem. Am. Entomol. Inst. *34*, 1–382.

Oberprieler, R.G., Marvaldi, A.E., and Anderson, R.S. (2007). Weevils, weevils, weevils everywhere. Zootaxa *1668*, 491–520.

Oh, Y., Baldwin, I.T., and Galis, I. (2012). NaJAZh regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in *Nicotiana attenuata* plants. Plant Physiol. *159*, 769–788.

Oh, Y., Baldwin, I.T., and Galis, I. (2013). A jasmonate ZIM-domain protein NaJAZd regulates floral jasmonic acid levels and counteracts flower abscission in *Nicotiana attenuata* plants. PLoS One *8*, e57868.

Ohnmeiss, T.E., and Baldwin, I.T. (2000). Optimal defense theory predicts the ontogeny of an induced nicotine defense. Ecology *81*, 1765–1783.

Oldham, N.J., and Baldwin, I.T. (2001). Rapid HPLC Screening of Jasmonate-induced increases in tobacco alkaloids , phenolics , and diterpene glycosides in *Nicotiana attenuata*. 3553–3558.

Onkokesung, N., Gaquerel, E., Kotkar, H., Kaur, H., Baldwin, I.T., and Galis, I. (2012). MYB8 controls inducible phenolamide levels by activating three novel hydroxycinnamoyl-coenzyme A: polyamine transferases in *Nicotiana attenuata*. Plant Physiol. *158*, 389–407.

Ostrand, F., Wallis, I.R., Davies, N.W., Matsuki, M., and Steinbauer, M.J. (2008). Causes and consequences of host expansion by *Mnesampela privata*. J. Chem. Ecol. *34*, 153–167.

Paschold, A., Halitschke, R., and Baldwin, I.T. (2007). Co(i)-ordinating defenses: NaCOI1 mediates herbivore-induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in

avoiding defenses. Plant J. *51*, 79–91.

Preisser, E.L., and Bastow, J.L. (2005). Plant damage from and defenses against “cryptic” herbivory: a guild perspective. J. Plant Interact. *1*, 197–210.

Price, P.W. (1997). Insect ecology (John Wiley & Sons).

Ralph, S.G., Yueh, H., Friedmann, M., Aeschliman, D., Zeznik, J.A., Nelson, C.C., Butterfield, Y.S.N., Kirkpatrick, R., Liu, J., and Jones, S.J.M. (2006). Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale . Plant. Cell Environ. *29*, 1545–1570.

Renwick, J.A.A., Haribal, M., Gouinguené, S., and Städler, E. (2006). Isothiocyanates stimulating oviposition by the diamondback moth, *Plutella xylostella*. J. Chem. Ecol. *32*, 755–766.

Rosenthal, G.A., and Berenbaum, M.R. (2012). Herbivores: their interactions with secondary plant metabolites: ecological and evolutionary processes (Academic Press).

Sarfraz, M., Dosdall, L.M., and Keddie, B.A. (2006). Diamondback moth-host plant interactions: implications for pest management. Crop Prot. *25*, 625–639.

Scheirs, J., and Bruyn, L. De (2002). Integrating optimal foraging and

optimal oviposition theory in plant–insect research. Oikos *96*, 187–191.

Scheirs, J., Bruyn, L.D., and Verhagen, R. (2000). Optimization of adult performance determines host choice in a grass miner. Proc. R. Soc. B Biol. Sci. *267*, 2065–2069.

Schuman, M.C., Barthel, K., and Baldwin, I.T. (2012). Herbivory-induced volatiles function as defenses increasing fitness of the native plant *Nicotiana attenuata* in nature. Elife *1*, e00007.

Schuman, M.C., Palmer-Young, E.C., Schmidt, A., Gershenzon, J., and Baldwin, I.T. (2014). Ectopic terpene synthase expression enhances sesquiterpene emission in *Nicotiana attenuata* without altering defense or development of transgenic plants or neighbors. Plant Physiol. *166*, 779-797.

Schuman, M.C., Allmann, S., and Baldwin, I.T. (2015). Plant defense phenotypes determine the consequences of volatile emission for individuals and neighbors. Elife *4*, e04490.

Schwachtje, J., and Baldwin, I.T. (2008). Why does herbivore attack reconfigure primary metabolism? Plant Physiol. *146*, 845–851.

Shikano, I., Akhtar, Y., and Isman, M.B. (2010). Relationship between adult and larval host plant selection and larval performance in the generalist moth, *Trichoplusia ni*. Arthropod. Plant. Interact. *4*, 197–205.

Singer, M.C. (1986). The definition and measurement of oviposition

preference in plant-feeding insects. In Insect-Plant Interactions, (Springer), pp. 65–94.

Sokal, R.R., and Rohlf, F.J. (1995). Biometry: the principles and practice of statistics in biological research. (W.H. Freeman and Co.).

Stanton, M.A., Preβler, J., Paetz, C., Boland, W., Svatoš, A., and Baldwin, I.T. (2016). Plant-mediated pheromone emission by a hemipteran seed feeder increases the apparency of an unreliable but rewarding host. New Phytol. *211*, 113-125.

Steppuhn, A., and Baldwin, I.T. (2007). Resistance management in a native plant: nicotine prevents herbivores from compensating for plant protease inhibitors. Ecol. Lett. *10*, 499–511.

Steppuhn, A., Gase, K., Krock, B., Halitschke, R., and Baldwin, I.T. (2004). Nicotine's defensive function in nature. PLoS Biol. *2*, 1074–1080.

Stevenson, P.C., Anderson, J.C., Blaney, W.M., and Simmonds, M.S.J. (1993). Developmental inhibition of *Spodoptera litura* (Fab.) larvae by a novel caffeoylquinic acid from the wild groundnut, *Arachis paraguariensis* (Chod et Hassl.). J. Chem. Ecol. *19*, 2917–2933.

Strauss, S.Y., and Agrawal, A.A. (1999). The ecology and evolution of plant tolerance to herbivory. Trends Ecol. Evol. *14*, 179–185.

Thompson, J.N. (1988a). Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. Entomol. Exp. Appl. *47*, 3–14.

Thompson, J.N. (1988b). Coevolution and alternative hypotheses on insect/plant interactions. Ecology *69*, 893–895.

Thompson, J.N., and Pellmyr, O. (1991). Evolution of oviposition behavior and host. Annu. Rev. Entomol. *36*, 65–89.

Traugott, M.S., and Stamp, N.E. (1996). Effects of chlorogenic acid-and tomatine-fed caterpillars on the behavior of an insect predator. J. Insect behavior *9*, 461-476.

Underwood, D.L.A. (1994). Intraspecific variability in host plant quality and ovipositionaI preferences in *Eucheira socialis* (Lepidoptera: Pieridae). Ecol. Entomol. *19*, 245–256.

Valladares, G., and Lawton, J.H. (1991). Host-plant selection in the holly leaf-miner: does mother know best? J. Anim. Ecol. 227–240.

Visser, J.H. (1988). Host-plant finding by insects: orientation, sensory input and search patterns. J. Insect Physiol. *34*, 259–268.

Wainhouse, D., Cross, D.J., and Howell, R.S. (1990). The role of lignin as a defence against the spruce bark beetle *Dendroctonus micans*: effect on larvae and adults. Oecologia *85*, 257–265.

Walling, L.L. (2000). The myriad plant responses to herbivores. J. Plant Growth Regul. *19*, 195–216.

Wang, L., Allmann, S., Wu, J., and Baldwin, I.T. (2008). Comparisons of LIPOXYGENASE3-and JASMONATE-RESISTANT4/6-silenced plants reveal that jasmonic acid and jasmonic acid-amino acid

conjugates play different roles in herbivore resistance of *Nicotiana attenuata*. Plant Physiol. *146*, 904–915.

Wasternack, C. (2007). Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann. Bot. *100*, 681–697.

Weis, A.E., and Abrahamson, W.G. (1985). Potential selective pressures by parasitoids on a plant-herbivore interaction. Ecology 1261–1269.

Wheat, C.W., Vogel, H., Wittstock, U., Braby, M.F., Underwood, D., and Mitchell-Olds, T. (2007). The genetic basis of a plant–insect coevolutionary key innovation. Proc. Natl. Acad. Sci. USA *104*, 20427–20431.

Woldemariam, M.G., Baldwin, I.T., and Galis, I. (2011). Transcriptional regulation of plant inducible defenses against herbivores: a mini-review. J. Plant Interact. *6*, 113–119.

Wu, J., and Baldwin, I.T. (2010). New insights into plant responses to the attack from insect herbivores. Annu. Rev. Genet. *44*, 1–24.

Wünsche, H., Baldwin, I.T., and Wu, J. (2011). Silencing NOA1 elevates herbivory-induced jasmonic acid accumulation and compromises most of the carbon-based defense metabolites in *Nicotiana attenuata.* J. Integr. Plant Biol. *53*, 619–631.

Zalucki, M.P., Clarke, A.R., and Malcolm, S.B. (2002). Ecology and behavior of first instar larval Lepidoptera. Annu. Rev. Entomol. *47*,

361–393.

Zangerl, A.R., and Bazzaz, F.A. (1992). Theory and pattern in plant defense allocation. Plant Resist. to Herbiv. Pathog. 363–391.

Zangerl, A.R., Rutledge, C.E. (1996). The probability of attack and patterns of constitutive and induced defense : a test of optimal defense theory. Am. Nat. 599–608.

Zavala, J.A., and Baldwin, I.T. (2004). Fitness benefits of trypsin proteinase inhibitor expression in *Nicotiana attenuata* are greater than their costs when plants are attacked. BMC Ecol. *4*, 11.

Zhang, P.J., Lu, Y.B., Zalucki, M.P., and Liu, S.S. (2012). Relationship between adult oviposition preference and larval performance of the diamondback moth, *Plutella xylostella*. J. Pest Sci. *85*, 247–252.

Zhou, G., Wang, X., Yan, F., Wang, X., Li, R., Cheng, J., and Lou, Y. (2011). Genome-wide transcriptional changes and defence-related chemical profiling of rice in response to infestation by the rice striped stem borer *Chilo suppressalis*. Plant Physiol. *143*, 21–40.

국문초록

식물의 2차 대사물질은 초식곤충의 숙주 선택을 위한 행동에 중요한 역할을 한다. 특히, 어미곤충은 적절한 숙주 선택을 위해 식물이 생산하는 화학물질을 인식한다고 알려져 있다. 초식곤충이 숙주식물을 선택하는 행동학적 특성은 어미곤충은 자신의 애벌레가 가장 잘 자랄 수 있을 숙주 식물을 선택해서 산란을 한다는 가설로 주로 설명된다. 그러나 현재까지 숙주 식물의 2차 대사물질이 어미곤충의 산란을 위한 선택과 애벌레의 적합성에 각기 어떤 영향을 미치는 가에 대한 연구가 생태적으로 긴밀하게 연결된 식물-곤충의 관계에서 진행되지 않았다. 따라서 본 연구는 흰독말풀 (*D. wirghtii)*을 숙주로 한다고 알려진 *T. compacta*와 야생담배 (*N. attenuata*)를 숙주로 한다고 알려진 *T. mucorea*를 이용하여 연구를 진행하였다. 특히, 이 2종의 바구미는 같은 분포지역을 공유하는 근연종이다. 식물의 2차 대사물질에 따른 바구미의 숙주 선택의 행동학적 특성을 알아보기 위하여, 특정 2차대사 물질이 다르게 생산되는 유전자변형 식물을 이용하였으며, 실험은 온실과 실제 생육지에서 실시하였다. 첫째로 두 *Trichobaris* 종의 성체 모두 미국의 남서부에 위치한 그레이트베이슨사막에서 자생하는 흰독말풀 개체군에서 관찰되었지만 야생담배 개체군에서는 드물게 발견 되었다. 온실실험을 위해서 자연 서식지에서 두 *Trichobaris* 종의 성체를 수집하여 먼저 흰독말풀과 야생담배 사이에서 먹이 선호도와 산란 선호도를 조사하였다. 그 결과, 두 *Trichobaris* 어미곤충 모두 야생담배보다는 흰독말풀을

먹이로 선호했지만, 흥미롭게도 *T. compacta*와 *T. mucorea* 어미곤충은 야생담배에 산란 선호도를 보였다. 이러한 선호도는 각 *Trichobaris* 애벌레의 생존과 성장과도 양의 상관관계를 나타내었다. 두 종의 *Trichobaris*의 숙주선택이 나뉘는 것이 식물의 2차 대사산물에 의한 결과라는 가설을 검증하기 위해, 먼저 야생담배가 가지고 있는 2차 대사산물 중에서 가장 풍부한 알칼로이드 성분인 니코틴을 합성하는 유전자를 발현시키지 못하게 하여 니코틴생성이 아주 소량만 되는 야생담배 (irPMT)와 정상의 야생담배와의 산란 선호도와 애벌레의 성장을 분석하였다. 두 *Trichobaris* 어미곤충 모두 니코틴의 유무에 산란 선호도가 변하지 않았지만, *T. compacta* 애벌레의 경우는 니코틴 생성이 적은 야생담배에서 생존과 성장이 더 높게 나타났다. 하지만, *T. mucorea* 애벌레는 니코틴의 유무와는 전혀 상관이 없었다. *T. mucorea*의 숙주 선택의 영향을 미치는 요인을 알아보기 위해서 재스몬 산 (Jasmonic acid, JA)-의존적으로 생성되는 식물의 2차 대사산물의 합성을 낮춘 식물 (irAOC) 과 정상의 야생담배에서 *T. mucorea*의 산란선호도와 애벌레의 성장을 측정하였다. 흥미롭게도 재스몬 산 의존적인 식물의 2차 대사물들에 의해 산란 선호도는 촉진되었고, 애벌레의 성장은 줄어들었다. 더 나아가 재스몬 산 의존적이고 휘발성 2차 대사산물인 세스퀴테르펜이 많이 분비되는 형질전환 된 야생담배 (ovTPS10) 와 정상의 야생담배와의 어미곤충의 선호도와 애벌레 성장의 적합성을 측정하였다. 흥미롭게도 세스퀴테르펜이 많이 분비되는 야생담배에 *T. mucorea* 어미곤충이 더 많은 알을 낳았지만 자식인

애벌레의 생존과 성장에는 전혀 영향을 미치지 않았다. 결론적으로 위 결과는 숙주 선택에 영향을 주는 식물의 2차 대사산물은 어미곤충이 인식하는 물질과 애벌레에 영향을 미치는 물질이 서로 다르게 작용한다는 것을 시사한다.

앞선 결과에서 재스몬산 의존적인 식물의 2차 대사산물이 애벌레의 성장에 미친다는 것을 보였다. 이를 기반으로 *T. mucorea* 애벌레가 숙주 식물을 공격할 때, 식물이 분비하는 방어물질이 무엇인지 알아보는 연구를 수행하였다. *T. mucorea* 애벌레는 야생담배의 줄기 중 에서도 중앙에 위치한 수(pith) 라는 부위를 먹는다. 애벌레에 의해서 공격받은 식물의 수 부위를 샘플링 하여 식물호르몬을 측정한 결과 식물의 방어물질 분비에 관여하는 호르몬인 JA와 JA-Ile가 정상식물의 수에 비해 높은 농도를 유지하였다. 2차 대사산물을 측정한 결과, 특이하게도 클로로겐산(chlorogenic acid, CGA)만이 특이적으로 증가했으며, 클로로겐산의 합성에 관여하는 유전자(*NaHQT*)의 발현도 대조군 식물과 비교하여 유의하게 높게 나타났다. 클로로겐산이 *T.mucorea*에 대한 식물줄기의 방어물질로 작용한다는 가설을 검증하기 위해, 첫째로 인위적으로 합성한 클로로겐산 (실제로 공격 당한 줄기에서 유도되는 양)을 인공적으로 만든 먹이에 섞어 애벌레의 성장을 알아본 결과 아무것도 넣지 않은 인공먹이에서 보다 성장이 저해 되는 결과를 나타내었다. 둘째로 클로로겐산의 합성 유전자(*NaHQT*)의 발현을 억제시키고 클로로겐산이 정상 야생담배보다 90%이상 적게 분비되는 형질전환 된 야생담배를 *T. mucorea* 애벌레가 먹었을 때 생존과

성장이 상당히 높아 진다는 것을 알 수 있었다. 이는 야생담배가 분비하는 클로로겐산이 *T. mucorea* 애벌레의 성장을 저해하는 방어물질로 작용한다는 것을 시사한다. 더 나아가 줄기에서의 클로로겐산은 잎의 방어특성과는 다르게 JA에 의존적으로 분비되었다. 이 결과를 통해서 *T. mucorea* 애벌레는 야생담배가 분비하는 클로로겐산 2차대사물질에 의해 영향을 받고 이 클로로겐산은 잎과는 다르게 줄기에서 조직특이적인 방어기능을 한다는 것을 시사한다.

주요어: 줄기곤충, 야생담배, 식물 2차 대사물질, 숙주선택행동, 줄기방어 클로로겐산

학번: 2011-30111