Defense mechanisms of the wild tobacco plant, *Nicotiana attenuata*, against its native pathogens

DISSERTATION

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Van Thi Luu, M.Sc.

Born on 15.09.1986 in Hai Duong, Viet Nam.

Friedrich-Schiller-Universität

Max Planck Institute for Chemical Ecology

Gutachter:

1. Prof. Dr. Ian T. Baldwin (Jena)
2. PD Dr. Kerstin Voigt (Jena)
3. Dr. Habil. Patrick Schweizer (Gatersleben)

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Table of contents

Chapter 1: Introduction

1. Plant-environment interactions

The health of green plants is of vital importance to most forms of life on Earth, as they provide the energy and carbon skeletons most organisms depend on. In nature, plants interact with many different abiotic and biotic factors. These interactions are complex and directly influence plant health (Agrios, 2005).

Abiotic factors which have a significant influence on plant health include light, temperature, water status, wind, soil nutrients, and air pollutants. These environmental factors support plant live, however, non-infectious diseases can occur on plants when those factors are out of the plant's favorable range. For example, excess light can cause sun scorching, while lack of sufficient light can cause pale leaves, leaf dropping or lower flower production (Lucas, 2009). Drought can affect water-use efficiency and photosynthesis rates through CO_2 assimilation and respiration, leading to a reduction in plant growth and development or even complete crop failure (Farooq *et al.*, 2009). Since non-infectious diseases caused by abiotic stresses are not transmitted from diseased to healthy plants, they can be controlled by creating favorable environmental conditions for plant growth (Agrios, 2005).

Biotic factors such as plant competitors and herbivores also strongly influence plant health. Plant competitors can act directly as parasites or antagonists within a mixed population. For example, the parasite *Orobanche* attacks roots and drains water and nutrients from the host and can cause a significant production loss in sunflower, tobacco, and tomato (Musselman, 1980). Plant competitors can also be intraspecfic if co-existing plants of the same species compete for resources, affecting biomass production, flowering, and survival (Ungar, 1992; Wang *et al.*, 2005). Plants can also be attacked by various insect herbivores belonging to diverse taxa and functional groups, ranging from sucking to chewing insects and from generalists to specialists. Some insect herbivores, such as aphids and leaf hoppers, spend only a period of their life cycle on plants, while other insects, such as leaf miners and gall-forming sawflies, spend their entire life cycle on the host plant (DeLong, 1971; Dixon, 1977; Cornell, 1990; Hespenheide, 1991). Insect herbivores can cause huge losses in plant productivity; however, they

can also have mutualistic interactions with plants under certain conditions. For instance, the hawkmoth *Manduca sexta* often pollinates *Solanaceous* plants, but also lays eggs on the same plants that later develop into herbivorous larvae (Adler & Bronstein, 2004). Similarly, Yucca moths pollinate flowers and oviposit their eggs into the floral ovary, so that their developing larvae have enough Yucca seeds as a food resource (Kula *et al.*, 2014).

In nature, plants interact with a complex microbial community. Plant-microbe interactions are commonly categorized as being pathogenic, commensalistic, or mutualistic (Newton *et al.*, 2010). These plant-microbe interactions have a strong impact on plant health and development. Beneficial microbes, such as mycorrhizal fungi and rhizobia, associate with roots and provide plants with mineral nutrients and fixed nitrogen, respectively, in exchange for carbon (Parniske, 2008; Bonfante & Anca, 2009). Pathogenic microbes can be fungi, bacteria, and viruses, which depend entirely upon an external supply of organic compounds from plants. Some of them actively kill plants and drain nutrients from dead tissues; others can develop a more mutualistic relationship with their hosts, and obtain nutrients from living plants. Therefore, the effect of microbial pathogens on plants can vary from death, to severe damage, or to the diversion of nutrients (Lucas, 2009). The interactions between plants and microbes can also be context-dependent, shifting along the functional spectrum depending on environmental conditions or the life cycle of the microbe or the plant (Newton *et al.*, 2010). Some microbes are plant-beneficial only on particular hosts and under particular conditions or stresses, such as drought or pathogen infestation (Gaiero *et al.*, 2013).

In brief, plant health is constantly under attack by both abiotic and biotic stresses in nature. Unlike animals, plants are sessile organisms and cannot run away from these stresses. In order to survival, plants must cope with the particular environment in which they grow. Plants that are not able to do so, show abnormal growth and/or dysfunction of normal activity and are defined as diseased plants (Agrios, 2005). Hence, plant disease results from interactions between the environment, the host and the pathogen.

2. Plant disease caused by fungal pathogens

Among microbial pathogens, fungi are the most common cause of plant diseases (Agrios, 2005). It is estimated that there are about 250,000 species of higher plants, but six times as many (1.5 million) species of fungi (Lattanzio *et al.*, 2006). Hence, all plants are attacked by at least one fungal species, and each of the pathogenic fungi can also attack one or more plant species. Some fungi, known as biotrophs, can grow and multiply only in close association with their host plants. Others, known as necrotrophs, promote the destruction of host cells to obtain their nutrients. Hemibiotrophs, on the other hand, require a host plant for a part of their life but can complete their life cycle on dead organic matter (Agrios, 2005).

Fungal pathogens are dispersed in various ways, including directly through the air or soil and indirectly through disease vectors. Many fungi, for example, *Septoria tritici*, take advantage of rain drops which splash fungal spores from wet surfaces (Eyal, 1999). In contrast, soil-borne fungi, such as *Fusarium* spp., are passively dispersed as a result of soil erosion and flooding as well as other agriculture-related practices (Strausbaugh & Maloy, 1986; Dita & Martínez-de la Parte MSc, 2014). While it is true that vectors such as insects feeding on the plant play a key role in transmitting viruses, they are also essential for dispersal of many fungi, such as *Ophiostoma novo-ulmi* (Webber, 2000; Jacobi *et al.*, 2007).

Fungal pathogens usually enter the plant through wounds caused by abscission scars, lateral root formation or vector bites. Another path of entry is via natural openings such as stomata, lenticels, glands or nectaries. Some pathogens can even directly penetrate through the cuticle, epidermis or other vulnerable sites (Agrios, 2005). To increase its success in invading plants, some pathogens are able to produce enzymes to degrade the plant cell wall (Juge, 2006). These enzymes include pectate lyases, acetyl esterases, xylanases, and a variety of endoglucanases that cleave cellulose, xyloglucan, and other glucans (Lebeda *et al.*, 2001). Once inside a plant, pathogens exhibit various types of growth in order to obtain nutrients from host tissues. They can grow subcuticularly, intercellularly, vascularly, intracellularly or partially intracellularly with haustoria into the host cells (Lucas, 2009). *Fusarium oxysporum* and *Verticillium albo-atrum*, for example, both grow through the apical region of the root to reach the developing xylem. The fungi then grow through vessels and pass from cell to cell via pit

pairs. These fungi can also use plant transpiration to spread their microconidia (Yadeta & Thomma, 2014).

Infected plants usually show various disease symptoms, such as an increase in respiration due to enhanced degradative metabolism (Agrios, 2005). Diseased plants also show reduced photosynthesis rates due to the degradation of chlorophyll (chlorosis) and a translocation of nutrients towards the pathogen instead of young plant tissue, leading to reduced shoot or root growth (curly leaf, abnormal shoots or roots) (Lucas, 2009). Some infected plants are affected in water use, leading to wilt syndrome or increased transpiration. Overall, diseased plants are affected in their growth, development, and Darwinian fitness.

3. Defense mechanisms of plants against pathogens

In order to withstand pathogens, plants have evolved diverse means of survival, including various avoidance, tolerance, and defense mechanisms. Disease avoidance happens when a susceptible host does not coincide with the pathogen in either space or time (Burdon, 1987). For example, the early germination of *Avena barbata* in New South Wales or early flowering of wheat in Israel is important to reduce the effects of crown rust disease (Burdon *et al.*, 1983; Dinoor & Eshed, 1984). When plants are under attack by pathogens, they also have the ability to tolerate either direct damage by pathogen-derived compounds or indirect damage through their own immune system, therefore limiting the impact of pathogens on host health, performance, and fitness (Schafer, 1971; Hartleb *et al.*, 1997). For example, the oat cultivar Otter has higher infection rates of the fungus *Puccinia coronata*, but maintains similar yields to the cultivar Cherokee (Politowski & Browning, 1978). The anti-pathogen defense mechanisms in plants are classified into constitutive (passive) or inducible (active) defenses depending on whether they are pre-existing features or are turned on after microbial attack (Lucas, 2009).

3.1. Constitutive defense

Constitutive defenses are defensive mechanisms that are continuously presented in plants and protect plants against pathogens. This constitutive defense includes anatomical features such as the cuticle, bark, the thickness of plant cell walls and the structure of stomata, all of which are

effective obstacles to penetration by pathogenic fungi (Blanchette & Biggs, 2013). For instance, the thickness and structure composition of grapevine cuticles are essential factors in the penetration efficiency of the fungal pathogen *Botrytis cinerea* (Hill *et al.*, 1981; Commenil *et al.*, 1997). The resistance of a host to pathogens has been found to be related to the number, spatial arrangement, structure, and operation of stomata (Royle, 1976). *Pinus* species resistant to white pine blister rust, caused by *Cronartium ribicola*, has less stomata than more susceptible species (Spaulding, 1925). Constitutive defenses also involve various plant secondary metabolites, such as phenols, alkaloids, glycosides, saponins, tannins, and resins (Lucas, 2009). These chemicals are commonly stored in vacuoles or organelles, but they can also be concentrated in the outer cell layer of the plant (Osbourn, 1996). For instance, phenolic compounds such as catechol and protocatechuic acid have been found on outer scales of onion bulbs and play an important role in onion resistance to smudge disease caused by *Colletotrichum circinans* (Walker & Link, 1935). Another phenolic compound, chlorogenic acid, was also found in potato tubers resistant to common scab *Streptomyces scabies* (Johnson & Schaal, 1952). Some of those chemicals can be stored as non-toxic precursors that are converted into toxic forms following tissue damage or pathogen attack (Osbourn, 1996). *Brassicaceae* crops contain glucosinolates that can be degraded to form isothiocyanate volatiles, which inhibit the growth of several fungal pathogens such as *Fusarium oxysporum* (Smolinska *et al.*, 2003). In general, biotrophic fungi try to avoid the release of constitutive antibiotic compounds by minimizing the damage to the host, while necrotrophic fungi cause a substantial release of these compounds (Osbourn, 1996; Lattanzio *et al.*, 2006). These plant secondary metabolites can either directly affect fungal pathogens through antifungal activity or indirectly by the inactivation of fungal extracellular hydrolases, which are needed for fungal establishment in the plant (Hartleb *et al.*, 1997). For example, the antifungal activity of saponin is associated with its ability to complex with fungal membrane sterols and cause pore formation (Fenwick *et al.*, 1991). Tannins and related phenolic compounds inhibit extracellular hydrolases (cellulase, pectinase, laccase, xylanase, etc.) produced by invading pathogens, thus preventing their rapid development in the plant (Scalbert, 1991; Lattanzio *et al.*, 2006).

Among those constitutive defense mechanisms are plant trichomes, which are produced by around 30% of all vascular plants, and are well-known as physical barriers against the feeding

and movement of herbivores (Glas *et al.*, 2012); moreover, reports also suggest that they might be important for anti-pathogen defenses. Calo *et al.* (2006) demonstrate that trichome density affects the establishment and spreading of *Botrytis cinerea* infection in *Arabidopsis.* Similarly, a study by Chattopadhyay *et al.* (2011) also found that trichome density and resistance against powdery mildew are positively correlated in mulberry. Plant trichomes not only act as physical barriers, but are also known as places where various secondary metabolites are produced and stored (Weinhold *et al.*, 2011; Glas *et al.*, 2012). The main classes of secondary chemicals produced in trichomes are terpenoids, flavonoids, methyl ketones, and *O*-acyl sugars (*O*-AS) (Gang *et al.*, 2001; Fridman *et al.*, 2005; Treutter, 2006; Gershenzon & Dudareva, 2007; Glas *et al.*, 2012). Most trichome secondary metabolites have been studied regarding their functions in defense against insect herbivory (Weinhold & Baldwin, 2011; Weinhold *et al.*, 2011; Glas *et al.*, 2012). For example, the sesquiterpene (E)-β-farnesene released from glandular trichomes of the wild potato (*Solanum berthaultii*) repells aphids (*Myzus persicae*) (Gibson & Pickett, 1983). Phaseoloidin, a homogentisic acid glucoside, was shown to be excreted from trichomes of *Nicotiana attenuata* and reduce the larval growth of the specialist larvae *Manduca sexta* and the generalist larvae *Spodoptera littoralis* (Weinhold *et al.*, 2011). Interestingly, many secondary metabolites have been considered to be "generalized defense compounds" that affect the performance of both pathogens and herbivores. In *Plantago lanceolata*, iridoid glycosides increase resistance to the generalist herbivore *Spodoptera exigua* and the biotrophic fungal pathogen *Diaporthe adunca* (Biere *et al.*, 2004). Chlorogenic acid, a widespread phenolic compound among vascular plants, is an antimicrobial agent (Almeida *et al.*, 2006) as well as a deterrent to many insects (Hay *et al.*, 1992; Vermerris & Nicholson, 2008; del Campo *et al.*, 2013). These studies suggest that trichome secondary metabolites involved in anti-herbivore defenses might also play a role in anti-fungal defenses of plants.

O-AS are a group of compounds that consists of aliphatic acyl groups esterified to the hydroxyl groups of glucose or sucrose. Most of *O*-AS are mono-, di- or tri-acyl sugars depending on the number of acyl groups esterified to the sugar backbone via O-acylation (Puterka *et al.*, 2003). It can be found in the trichomes of many *Solanaceous* species including *Solanum*, *Nicotiana*, *Datura* and *Petunia* (Chortyk *et al.*, 1997; Kroumova & Wagner, 2003) and can be accumulated in enormous amounts in *Solanum pennellii* (up to 20% of leaf dry mass) (Fobes *et*

al., 1985). *O*-AS have been shown to play an important role in the plant defense against insect herbivores. For examples, *O*-AS can deter or repel aphids, beet armyworm, and leafminers (Goffreda *et al.*, 1989; Hawthorne *et al.*, 1992; Juvik *et al.*, 1994; Puterka *et al.*, 2003). However, it is unknown whether *O*-AS are generalized defense compounds involved in defense against both herbivores and pathogens.

3.2. Induced defense

The constitutive production of defensive metabolites is costly and requires an allocation of resources which cannot be invested in other plant functions (Tollrian & Harvell, 1999). Hence, it is in many cases more beneficial for the plant not to invest in the production of secondary metabolites constitutively, but rather as they are needed during pathogen attack. This mechanism of induced defense is defined as defense responses of plants that are only activated after pathogen challenge. The process of induced defense begins with the recognition of the pathogen's presence via their elicitors by the host plant. These elicitors include both nonspecific and specific elicitors. Nonspecific elicitors are released by various pathogens, and include compounds such as toxins, fatty acids, carbohydrates, glycoproteins, peptides or extracellular microbial enzymes. Specific elicitors, on the other hand, are produced by certain pathogens and are only recognized by a specific host plant (Agrios, 2005). After elicitor recognition and signal transduction, defensive genes are activated, leading to changes in the level of defensive proteins and secondary metabolites in the plant (Hartleb *et al.*, 1997). In induced defense, the success of pathogen infection depends on how quickly the plant can recognize the presence of a pathogen and how quickly it can activate the signaling cascade and mobilize its resources for defenses (Agrios, 2005).

Induced defenses of a plant can be enhanced by resistance-inducing agents (inducers). These inducers can be of a biotic nature, including beneficial, non-pathogenic or pathogenic microbial organisms. They can also be of an abiotic nature, such as microbial and plant metabolites or exogenous chemicals (e.g. fungicides) (Kloepper *et al.*, 1992; Hartleb *et al.*, 1997). When the inducers are necrotrophic pathogens, the induced defense response is called systemic acquired resistance (SAR). When the inducers are non-pathogenic or beneficial microbial organisms, so-called biocontrol agents (BCAs), the induced defense is named induced

systemic resistance (ISR). ISR and SAR are dissimilar in their induction, signaling and level of defense elicited in the host plant. The defense level produced by SAR is high, long-lasting, and covers a broad spectrum, while induced defense generated by ISR is less strong and depends on the host plant genotype as well as the presence of BCAs (Vallad & Goodman, 2004; Pieterse *et al.*, 2014). BCAs, besides directly triggering ISR in plants, can also indirectly enhance plant resistance via the production of anti-pathogen metabolites or the competition for nutrients e.g. through the production of siderophores. They can also alter the plant's microbiome, enhance nutrient uptake or root development in plants (Raaijmakers & Mazzola, 2012; Pieterse *et al.*, 2014; Lorito & Woo, 2015). In the last 40 years, research on BCAs has yielded a growing list of commercially available products, and the BCAs of choice are *Bacillus* spp., *Pseudomonas* spp. and *Trichoderma* spp. (Kloepper *et al.*, 2004; Meziane *et al.*, 2005; Raaijmakers *et al.*, 2010; Lorito & Woo, 2015). However, the anti-pathogen activity of BCAs is often inconsistent and has a narrow spectrum (Thomashow *et al.*, 2007; Weller & Thomashow, 2015). Some BCAs can induce systemic resistance under *in vitro* conditions but not under field conditions; it may also perform well in one location or during one field season but not during in the next. This inconsistency in the performance of BCA is due to a wide range of biotic and abiotic factors that impact root and foliage colonization as well as the expression of genes involved in biocontrol and/or the activity of biocontrol metabolites (Ownley *et al.*, 2003; Pierson III & Pierson, 2010; Zamioudis *et al.*, 2013). Recently, there has been an increase in the number of reports that, rather than a single microorganism, a group or consortia of microorganisms can provide more efficient, reliable and consistent anti-pathogen activity under diverse soil and environmental conditions (Stockwell *et al.*, 2011; Xu *et al.*, 2011; Sarma *et al.*, 2015). For example, a combination of *Trichoderma*, fluorescent *Pseudomonas* and *Glomus* suppressed *Fusarium* wilt incidence by more than 50% compared to a single application of *Glomus* in tomato under field conditions (Srivastava *et al.*, 2010). Similarly, applying *P. aeruginosa* PJHU15 together with *T. harzianum* TNHU27 and *B. subtilis* BHHU100 enhanced the defense responses of pea plants against *S. sclerotiorum* in comparison to the individual BCA microbes (Jain *et al.*, 2013). Since microbes in natural habitats live in communities and are recruited by the plant, it is assumed that each microbial component provides a specific benefit to plants (Sarma *et al.*, 2015).

4. *N. attenuata* as a model plant system to study plant-pathogen interaction

The use of wild plants is important in studies of plant pathology, and especially of wild plants which are phylogenetically related or have characteristics in common with crop plants. They can be a source of new resistant genes as well as new pathogen types which can be highly useful in crop protection (Burdon & Jarosz, 1989). Wild plant pathosystems have been used as models to study the origin of pathogen emergence, the role of plant disease in plant population dynamics, and disease forecast based on pathogen dynamics (Burdon *et al.*, 1987; Lebeda *et al.*, 2008; Stukenbrock & McDonald, 2008). In contrast to crop plants, which have been domesticated along the human agricultural history and specifically selected for certain traits that might interfere with plant defense systems, wild plants maintain their defense systems and actively interact with the evolution of their native pathogens. Identification and characterization of those natural anti-fungal defense mechanisms will empower our ability to sustain crop production in the ever-changing environment and have become one major goal in studying plant-fungal pathogen interactions. Hence, using wild plant pathosystems to study plant-pathogen interactions has gained increasing attention in recent years; however, compared with crop-pathosystems activities, the research focus on wild plant pathosystems has been rather restricted.

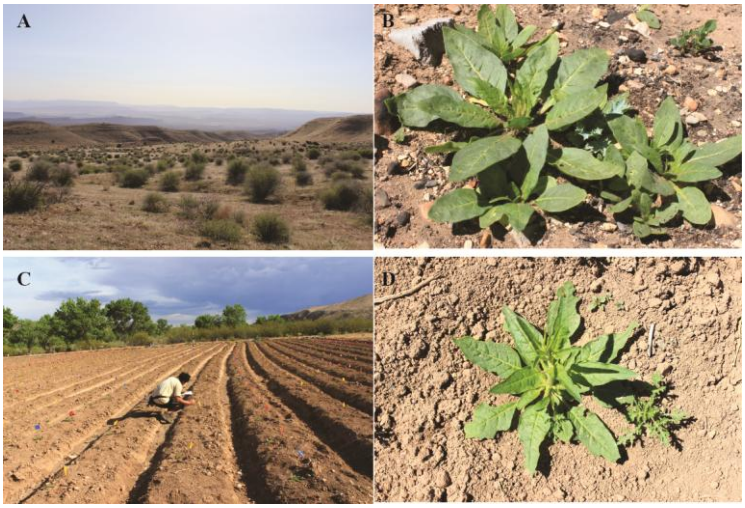

Figure 1. (A) Great Basin Desert, Utah, United States. **(B)** Ecological model plant, the wild tobacco *N. attenuata* germinates in post fire environment. **(C)** New field plot located in its native habitat. **(D)** *N. attenuata* growing in field plot (Photos Van T. Luu).

Nicotiana attenuata Torrey exWatson is a wild tobacco plant that grows natively in post-fire environments of the Great Basin Desert, United States (Baldwin & Morse, 1994). This plant germinates synchronously and forms monoculture-like populations. In contrast to crop plant populations which are often monocultures of singly genotype, native *N. attenuata* populations display a high degree of genetic diversity due to long-lived seed banks. This genetic diversity allows the plant to respond efficiently to various biotic stresses such as herbivory, pathogen attack, and selective pressures of intraspecific competition in order to survive and reproduce (Baldwin, 2001).These qualities make *N. attenuata* a very interesting model plant not only for studies of plant interactions with native herbivores, but also for plant- native pathogen interactions. However, compared to the tremendous accumulated knowledge on *N. attenuata* interactions with its native herbivores, the study of this plant's interaction with native pathogens is rare due to low disease incidence in wild plant populations.

In 2011, a fungal disease outbreak was reported for the first time in native *N. attenuata* populations, and the identification of *Alternaria* spp. and *Fusarium* spp. fungal isolates led to the establishment of a wild pathosystem for *N. attenuata* (Schuck *et al.*, 2014). Taking advantage of this, in my study, the *N. attenuata* wild pathogen system was used to study different defense mechanisms gained by *N. attenuata* to defend itself against its native pathogens.

5. Scope of the thesis

The scope of this thesis is to study different defense mechanisms of the wild tobacco plant, *N. attenuata*, against its native pathogens. My thesis consists of two manuscripts. In **manuscript I**, I studied the constitutive defense mechanism of *N. attenuata* against two native fungal pathogens. In particular, I investigated the role of *O*-acyl sugars (*O*-AS), trichome-specific metabolites, in constitutive defenses against fungal pathogens *Alternaria* and *Fusarium*. I did not only focus on the ecological functions of *O*-AS in defense against pathogens in *N. attenuata*, but also showed their involvement in direct defenses against the specialist herbivore *Manduca sexta*. In this study, I aimed to address the following questions:

- How many *O*-AS are present in *N. attenuata*?
- How large is the natural variation of *O*-AS in *N. attenuata*?
- Do *O*-AS play a role in defense against the native pathogens *Alternaria* and *Fusarium*, and the specialist herbivore *Manduca sexta* in both *in vitro* and *in vivo*?
- What is the potential mechanism of toxicity of *O*-AS hydrolyzed products?

In **manuscript II**, I studied the enhanced defense mechanism of *N. attenuata* against some of its native pathogens, which involves their association with native bacterial and fungal biocontrol agents. I made use of a sudden-wilt disease, which emerged due to continuous planting of *N. attenuata* for 15 years in an experimental field plot, leading to crop failure. In this manuscript, I focused on the following questions:

- What are the causal agents of this sudden wilt disease?
- Can the resistance of *N. attenuata* to sudden wilt disease be enhanced through root-associated microbes (bacteria and fungi) or any other resistance-inducing agents (external chemical treatment through soil amendment and fungicide)?
- Which influence do disease resistance-inducers have on plant performance?
- Do disease resistance-inducers work collectively or individually?

Chapter 2: Manuscript Overview

Manuscript I

***O*-acyl sugars protect the wild tobacco *Nicotiana attenuata* from both native fungal pathogens and a specialist herbivore**

Van Thi Luu, Alexander Weinhold, Chhana Ullah, Stefanie Dressel, Matthias Schoettner, Klaus Gase, Emmanuel Gaquerel, Shuqing Xu and Ian T. Baldwin

Manuscript submitted to Plant Physiology

In this manuscript, I characterized natural variation in *O*-AS among 26 accessions and examined their influence on two native fungal pathogens, *Fusarium brachygibbosum* U4 and *Alternaria* sp. U10, and the specialist herbivore, *Manduca sexta.* At least 15 different *O*-AS structures belonging to three classes were found in *N. attenuata* leaves. A three-fold quantitative variation in total leaf *O*-AS was found among the natural accessions. Experiments with natural accessions and crosses between high- and low-*O*-AS accessions revealed that total *O*-AS levels were associated with resistance against herbivores and pathogens. Removing *O*-AS from the leaf surface increased *M. sexta* growth rate and plant fungal susceptibility. *O*-AS supplementation in artificial diets and germination medium reduced *M. sexta* growth and fungal spore germination, respectively. Finally, silencing the expression of a putative branched-chain alpha-ketoacid dehydrogenase E1 beta subunit encoding gene (*NaBCKDE1B*) in the trichomes reduced total leaf *O*-AS by 20-30% and increased susceptibility to *Fusarium* pathogens. I conclude that *O*-AS function as direct defenses to protect plants from attack from both native pathogenic fungi and a specialist herbivore, and infer that their diversification is likely shaped by the functional interactions among these biotic stresses.

Author contributions: **V.T.L**., A.W., K.G., E.G., S.X. and I.T.B. designed the study. **V.T.L**., A.W., C.U., S.D., M.S. and K.G. carried out experiments. **V.T.L**., A.W., C.U., S.D., M.S. and K.G. analyzed data; **V.T.L**, A.W., C.U., S.D., M.S., K.G., E.G., S.X. prepared a draft which I.T.B. revised. Overall **V. T. L** contributed 70% to the manuscript.

Manuscript II

Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping

Rakesh Santhanam*, **Van Thi Luu***, Arne Weinhold*, Jay Goldberg, Youngjoo Oh and Ian T. Baldwin

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In this **manuscript II**, a sudden-wilt disease that emerged during continuous cropping of *N. attenuata* was used to study the enhanced defense mechanism of *N. attenuata* against its native pathogens. I found that the fungal phytopathogens *Alternaria* spp. and *Fusarium* spp. were highly abundant in the roots of diseased plants and likely contributed to the sudden wilt disease. *In vitro* tests using different disease resistance-inducers including fungicide, bacterial, and fungal treatments reduced *N. attenuata* seedling mortality. Those disease resistance-inducers together with a biochar soil amendment were tested in two field plot with more than 900 plants. Only inoculation with native bacterial isolates significantly attenuated the disease incidence in the field without altering plant performance. The consortium of five bacteria (*Arthrobacter nitroguajacolicus* E46, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176), but not individual members, provided the most effective protection for *N. attenuata* from the sudden wilt disease. These data indicate that the enhanced defense of *N. attenuata* against the native fungal pathogens can be triggered by its root-associated bacteria.

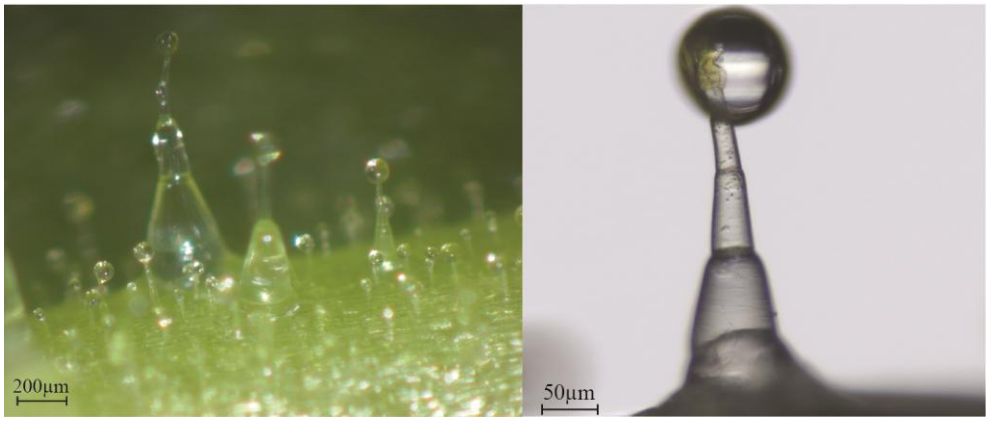
Author contributions: **V.T.L**, R.S and A.W contributed equally to this work. R.S., **V.T.L**., A.W. and I.T.B. designed research; R.S., **V.T.L**., A.W., J.G., Y.O. and I.T.B. performed research; I.T.B. contributed new reagents/analytic tools; R.S., **V.T.L**., A.W., J.G., Y.O. and I.T.B. analyzed data; and R.S., **V.T.L**., A.W. and I.T.B. wrote the manuscript. Overall **V. T. L** contributed 40% to the manuscript.

Chapter 3: Manuscript I

_O_-acyl sugars protect the wild tobacco _Nicotiana attenuata_ from both native fungal pathogens and a specialist herbivore

Van Thi Luu, Alexander Weinhold, Chhana Ullah, Stefanie Dressel, Matthias Schoettner, Klaus Gase, Emmanuel Gaquerel, Shuqing Xu and Ian T. Baldwin

Manuscript submitted to Plant Physiology



N. attenuata trichome (Photos Van T. Luu)

Abstract

O-acyl sugars (*O*-AS) are abundant trichome-specific metabolites that function as indirect defenses against herbivores of the wild tobacco *Nicotiana attenuata;* whether they also function as generalized direct defenses against herbivores and pathogens remains unknown. We characterized natural variation in *O*-AS among 26 accessions and examined their influence on two native fungal pathogens, *Fusarium brachygibbosum* U4 and *Alternaria sp*. U10, and the specialist herbivore, *Manduca sexta.* At least 15 different *O*-AS structures belonging to three classes were found in *N. attenuata* leaves. A three-fold quantitative variation in total leaf *O*-AS was found among the natural accessions. Experiments with natural accessions and crosses between high- and low-*O*-AS accessions revealed that total *O*-AS levels were associated with resistance against herbivores and pathogens. Removing *O*-AS from the leaf surface increased *M. sexta* growth rates and plant fungal susceptibility. *O*-AS supplementation in artificial diets and germination medium reduced *M. sexta* growth and fungal spore germination, respectively. Finally, silencing the expression of a putative branched-chain alpha-ketoacid dehydrogenase E1 beta subunit encoding gene (*NaBCKDE1B*) in the trichomes reduced total leaf *O*-AS by 20-30% and increased susceptibility to *Fusarium* pathogens. We conclude that *O*-AS function as direct defenses to protect plants from attack from both native pathogenic fungi and a specialist herbivore, and infer that their diversification is likely shaped by the functional interactions among these biotic stresses.

Keywords: trichomes, acyl sugars, sugar esters, *Nicotiana attenuata* (tobacco), *Alternaria*, *Fusarium*, *Manduca sexta*

Introduction

In nature, plants are often under attack by multiple enemies including pathogens and herbivores. The coevolutionary interactions between plants and their natural enemies are thought to have resulted in the extraordinary diversity of plant secondary metabolites (Ehrlich & Raven, 1964). More than 200,000 secondary metabolites are known from plants (Altman, 1997; Haslam, 1998; Wink, 2011) and both presence/absence polymorphisms as well as quantitative variations in the concentration of individual compounds are commonly found (Moore *et al.*, 2014). While many secondary metabolites are involved in defense against either pathogens or insect herbivores, some provide protection against both and are considered as "generalized defense compounds" (Biere *et al.*, 2004). For example, iridoid glycosides increase the resistance to both the generalist herbivore *Spodoptera exigua* and the biotrophic fungal pathogen *Diaporthe adunca* (Biere *et al.*, 2004). For these generalized defense compounds, diffuse rather than pairwise coevolutionary interactions are likely to occur among plants and their enemies (Biere *et al.*, 2004). Balancing selection is thought to act on these traits together with other selective forces such as abiotic stresses and inherent tradeoffs with growth fitness costs (Moore *et al.*, 2014). Consequently, the quantitative variation of a given secondary metabolite is thought to be constrained in a given plant tissue (Moore *et al.*, 2014). For example, iridoid glycosides, despite their high putative metabolic production costs (Gershenzon, 1994), substantial natural variation in iridoid glycoside concentrations has been reported in *Plantago lanceolata*, ranging from 0 to 10% dry mass (Marak *et al.*, 2003).

Approximately 30% of all vascular plants produce glandular trichomes which provide physical barriers to insect and pathogen attack and function as production and storage places for various secondary metabolites (Calo *et al.*, 2006; Weinhold *et al.*, 2011; Glas *et al.*, 2012). Terpenoids, phenylpropenes, flavonoids, methyl ketones and *O*-acyl sugars (*O*-AS) are all known to be produced in trichomes (Gang *et al.*, 2001; Fridman *et al.*, 2005; Treutter, 2006; Gershenzon & Dudareva, 2007; Glas *et al.*, 2012). *O*-AS consist of aliphatic acyl groups esterified to the hydroxyl groups of glucose or sucrose. Most *O*-AS harbor mono-, di- or tri-acyl sugars and acyl groups are esterified to the sugar backbone via *O*-acylation (Puterka *et al.*, 2003). In *Solanum lycopersicum*, the *O*-acylation requires the sequential action of BAHD acyltransferase 1 (AT1) to form the first mono-acyl sugar, and other BAHD acyltransferases

(AT2, 3, 4) to add additional aliphatic acids or acetyl groups to the sucrose backbone (Kim *et al.*, 2012; Schilmiller *et al.*, 2012; Fan *et al.*, 2016). The branched- or straight-chained aliphatic acids incorporated in *O*-AS are derived from branched-chain amino acids (i.e., Val, Leu, and Ile) (Walters & Steffens, 1990). In tobacco and petunia, the elongation of aliphatic acids involves α-ketoacid elongation (Kroumova & Wagner, 2003). Branched-chain keto acid molecules are activated as acyl-CoA esters by the branched-chain keto acid dehydrogenase (BCKD) protein complex and these acyl-CoA esters are used for *O*-acylation to produce *O*-AS (Slocombe *et al.*, 2008). *O*-AS are found in trichomes of many *Solanaceous* species including *Solanum*, *Nicotiana*, *Datura* and *Petunia* (Chortyk *et al.*, 1997; Kroumova & Wagner, 2003). They accumulate in large amounts in *Solanum pennellii* (approaching 20% leaf dry mass) (Fobes *et al.*, 1985). Trichome specialized metabolites are thought to be particularly evolutionarily variable (Sallaud *et al.*, 2009; Schilmiller *et al.*, 2009; Gonzales-Vigil *et al.*, 2012), as seen among different *S. pennellii* or *S. habrochaites* natural accessions, in which *O*-AS levels vary in total amounts, the proportion of sucrose or glucose backbones and the types of aliphatic acid esters to the sugar backbones (Shapiro *et al.*, 1994; Kim *et al.*, 2012). However, the biotic selection pressures that shape these patterns of natural variation remain unknown.

O-AS play important roles in plant defense against insect herbivores. It has been shown that *O*-AS can deter or repel aphids, beet armyworm and leaf miner (Goffreda *et al.*, 1989; Hawthorne *et al.*, 1992; Juvik *et al.*, 1994; Puterka *et al.*, 2003). *O*-AS were also known to be excellent emulsifiers and surfactants that readily adhere to arthropod cuticles and thereby can immobilize or suffocate arthropods (Puterka *et al.*, 2003; Wagner *et al.*, 2004). In *Datura wrightii*, a mixture of *O*-AS were found to be responsible for the delayed development of *Manduca sexta* larvae (Van Dam & Hare, 1998) and moderately deterrent to the feeding of the tobacco flea beetles (*Epitrix hirtipennis*) and a weevil, (*Trichobaris compacta*) (Hare, 2005). The function of *O*-AS in defense against pathogens is much less well studied despite a long history of investigations. In 1971 Kato & Arima reported that *Escherichia coli* were inhibited by synthetic sucrose monolaurate, and later this activity was extended to other gram-positive bacteria (Kato & Shibasaki, 1975). In 1986, Marshall & Bullerman showed that sucrose fatty acid ester emulsifiers had antimycotic activity against several fungal species including *Penicillium*, *Alternaria*, *Cladosporium* and *Aspergillus*. However, none of these studies was conducted with natural *O*-

AS. In 1993, Chortyk *et al.* studied plant-derived *O*-AS produced by several *Nicotiana* species and demonstrated their antibiotic activities against several Gram-positive and Gram-negative bacteria. Based on these results, we hypothesized that natural *O*-AS would have a wide range of anti-pathogen activities.

O-AS are found in high concentrations in the trichomes of *Nicotiana attenuata*, a wild tobacco plant that grows in the Great Basin Desert of the United States (Roda *et al.*, 2003; Weinhold & Baldwin, 2011; Weinhold *et al.*, 2011). This plant is well-known for its fire-chasing germination behavior and growth in the immediate post-fire environment where it faces the pressures of highly variable herbivore and pathogen challenges (Baldwin, 2001). Herbivores from more than 20 different taxa including both generalists (*S. litura*) and specialists (*M. sexta*) attack the plant (Baldwin, 2001). *O*-AS have been shown to function as indirect defenses against the specialist herbivore *M. sexta* (Roda *et al.*, 2003; Weinhold & Baldwin, 2011). Neonate larvae frequently consume the exudates of glandular trichomes as their first meal and later-stage larvae consume entire trichomes as they ingest shoot materials. When *O*-AS enter the high pH environment of the larval midgut, the branched-chain aliphatic acids (BCAA) are de-esterified to give their bodies and frass a distinctive odour that attracts ground foraging predators, such as the omnivorous ant (*Pogonomyrmex rugosus*) (Weinhold & Baldwin, 2011). However, it is unknown whether *O*-AS produced by *N. attenuata* can also directly affect *M. sexta* growth and development and thereby also function as a direct defense. *N. attenuata* is also under attack from native fungal pathogens (Schuck *et al.*, 2014; Luu *et al.*, 2015; Santhanam *et al.*, 2015b). *Alternaria* and *Fusarium* species are known to infect *N. attenuata* plants in both native and cultivated plantations (Schuck *et al.*, 2014; Santhanam *et al.*, 2015b). The jasmonic acid (JA) signaling pathway has been shown to play an important role in the defense of this plant against *Fusarium* species (Luu *et al.*, 2015). However, whether the trichomes of *N. attenuata* and their secondary metabolites, such as *O*-AS, play a role in defense against these native fungal pathogens remains unknown.

Here we demonstrate the generalized function of *N. attenuata*'s *O*-AS in direct defenses against both a native herbivore and two native pathogens. We characterized the composition of *N. attenuata*'s *O*-AS by UHPLC/Q-TOF-MS analysis and identified 15 different *O*-AS belonging to three structural classes. Among 26 natural accessions, total leaf *O*-AS levels varied

by 3-fold. By analyzing this natural variation and F_2 crosses between high- and low- *O*-AS ecotypes, we found *O*-AS in *N. attenuata*'s leaves to be associated with resistance to both, fungal pathogens (*F. brachygibbosum* U4 and *Alternaria sp*. U10) and the herbivore (*M. sexta*). Manipulating *O*-AS contents *in vivo* by washing leaves or silencing the expression of a putative branched-chain alpha-ketoacid dehydrogenase E1 beta subunit encoding gene (*NaBCKDE1B*) in trichomes revealed that *O*-AS functions in plant defenses against fungal pathogens. The *in vitro* experiment confirmed that *O*-AS and their BCAA substitutions are detrimental to both herbivores and pathogens. From these results, we conclude that *O*-AS in *N. attenuata* function as direct defenses and infer that these defensive functions shape their natural variation.

Results

At least 15 different *O*-AS are found in *N. attenuata*

To estimate the abundance of *O*-AS in *N. attenuata*, we isolated an *O*-AS-enriched fraction which after examination by UHPLC/TOF-MS was found to be dominated by a complex mixture of *O*-AS (Fig. S1). Other leaf metabolites such as nicotine, 17-hydroxygeranyllinalool diterpene glycosides (DTGs) were removed completely with different fractioning steps. We isolated 1.5 mg *O*-AS per gram fresh mass of plant tissues (mg/gFM). The concentrations of these *O*-AS are comparable to the alkaloid nicotine which is present at 1 mg/gFM concentrations in the plant tissue, and DTGs (2.5 mg/g FM) (Snook *et al.*, 1997). This makes *O*-AS one of the most abundant group of secondary metabolites in *N. attenuata*.

To analyze the *O*-AS structures, a crude mixture was separated by preparative HPLC, resulting in 30 fractions containing different *O*-AS. The separation did not resolve all *O*-AS; some fractions contained more than one *O*-AS, and not all were present in a single fraction. We obtained at least 14 fractions containing 3 or fewer *O*-AS that were suitable for further MS^2 experiments.

The single fractions were injected into an UHPLC/Q-TOF-MS for MS^2 experiments. We selected electrospray ionization conditions that favored the formation of single sodium adducts and performed MS^2 experiments on the sodium adducts of the molecular ions $(M+Na)^+$. The CID-MS^2 spectra clearly showed two different kinds of neutral losses, which could be attributed

to the loss of a hexose (m/z 162.0528 $C_6H_{10}O_5$) and an acetylated hexose (m/z 204.0633 $C_8H_{12}O_6$) (Fig. 1A). In addition, the fragment peaks of an acetylated hexose (m/z 205.0707 $C_8H_{13}O_6^+$) and that of the sodium adduct of a non-acetylated hexose (m/z 185.0420 $C_6H_{10}O_5Na^+$) were also observed in the spectra (Fig. 1A). The identity of the hexose could not be explained by the masses of the neutral losses. Since previous reports have shown that the core molecule of *O*-AS is a sucrose molecule (Simonovska *et al.*, 2006), the observed peaks likely corresponded to either fructose or glucose.

The sodium adducts of tri- or tetra-ester hexoses e.g. m/z 469.2403 (AS4/AS10) or m/z 469.2050 (AS12) were found (Fig. 1A). Since we did not find peaks reflecting a mixed substitution pattern on both sugar moieties of the sucrose core molecule, we inferred that the observed losses corresponded to either acetylated or non-acetylated fructose and that the BCAAs were esterified to only one of the sugars, most likely the glucose as described previously by Arrendale *et al.* (1990).

From these esterified sugars, there were three neutral losses of different BCAAs observable at m/z 116.0837 $C_6H_{12}O_2$, m/z 102.0681 $C_5H_{10}O_2$, and m/z 88.0525 $C_4H_8O_2$. The first mass corresponds to 3-methyl and 4-methyl pentanoic acid and the second mass to either 2-methyl or 3-methyl butanoic acid, as reported previously (Weinhold & Baldwin, 2011). The loss of $C_4H_8O_2$ corresponded to either 2-methyl propanoic acid or butanoic acid. To confirm these inferences about the fatty acid substitutions, we saponified the *O*-AS from a crude plant extract and subjected them to GC-FID analysis. By comparisons with the retention indices of authentic standards, we identified the four major fatty acids described previously (Weinhold & Baldwin, 2011). In addition, we identified 2-methyl-propanoic acid, which we predicted from the neutral losses of m/z 88.0525 ($C_4H_8O_2$) in the MS^2 spectra (Fig. 1B).

In summary, the MS^2 experiments revealed 15 different *O*-AS in *N. attenuata* (Fig. 1C) which were classified into three classes: class 2 (SE-2) (Fig. S2, Table S3), class 3 (SE-3) (Fig. S3, Table S4) and class 4 (SE-4) (Fig. S4, Table S5) according to the scheme of Arrendale *et al.* (1990); Ding *et al.* (2006). Class 2 is the largest class, with six compounds (AS1-AS6) all lacking acetylations of the glucose or fructose moieties, which makes them (tri-*O*-acyl)-α-D-glucopyranosyl-β-D-fructofuranosides. Class 3 contains an acetylated fructose moiety resulting

in five tri-*O*-acyl-α-D-glucopyranosyl-(*O*-acetyl)-β-D-fructofuranosides (AS7-AS11). Class 4 contains four compounds (AS12-AS15) that are characterized by the acetylation of the fructose and glucose moieties and represent (*O*-acetyl-tri-*O*-acyl)-α-D-glucopyranosyl-(*O*-acetyl)-β-D-fructofuranosides.

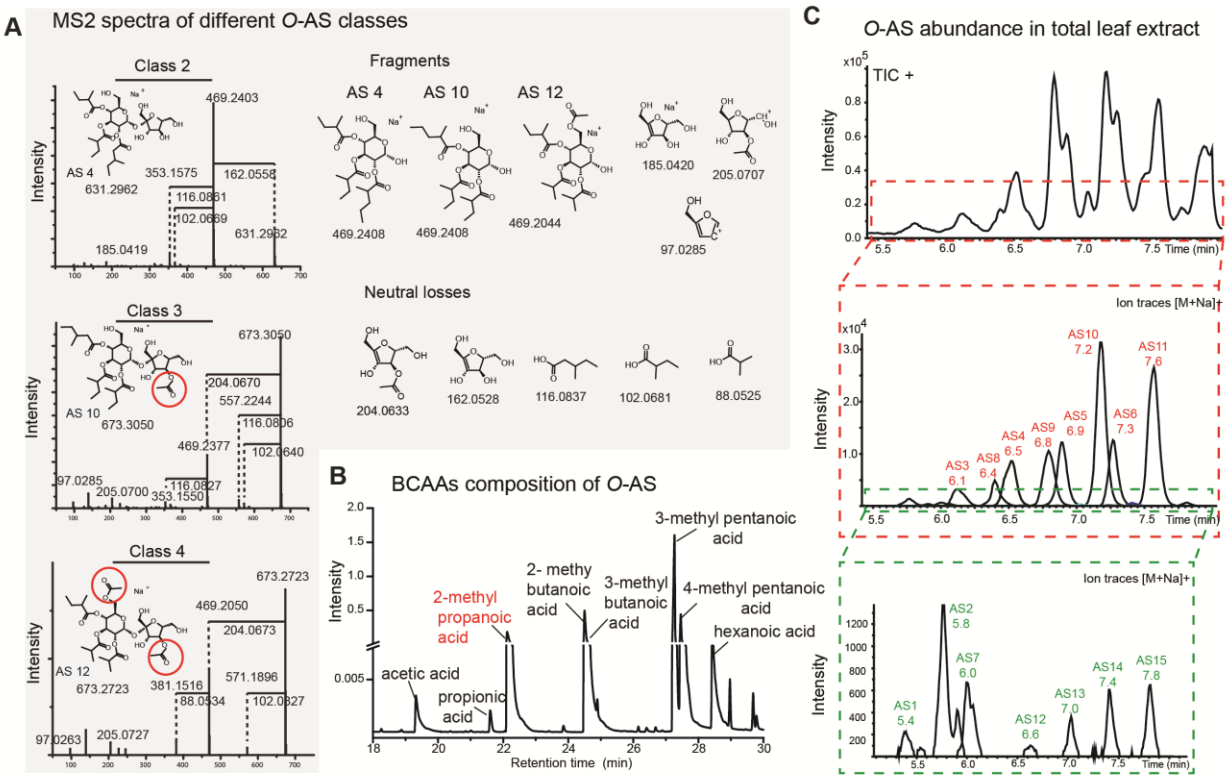


Figure 1: **The *O*-acyl sugars composition of *N. attenuata***. **A**. MS^2 spectra of three *O*-AS sharing the same fragment ion 469 (nominal mass) but belonging to three different structural classes, namely classes 2, 3 and 4. Main fragment peaks and neutral losses are annotated. The position of the fatty acids could not be verified and may vary. MS^2 spectra of all *O*-AS are shown in Fig. S2, S3, S4. **B**. GC-FID analysis of a saponified crude extract of *N. attenuata O*-AS revealed five major branched-chain aliphatic acids (BCAAs): 2-methyl propanoic acid (in red), 2-methyl butanoic acid, 3-methyl butanoic acid, 3-methyl pentanoic acid and 4-methyl pentanoic acid. Three less abundant acids were identified as acetic acid, propionic acid and hexanoic acid. **C**. Total ion current of a positive mode UHPLC/TOF-MS run (TIC+) from a whole leaf extract with single ion traces for the sodium adducts $[M+Na]^+$ of 15 different *O*-AS. Peak annotations correspond to the MS^2 spectral information shown in Tables S4, S5, S6**.**

_O_-AS levels vary three-fold among 26 _N. attenuata_ natural accessions

We examined the natural variation in total *O*-AS contents of leaves from different *N. attenuata* accessions using UHPLC/TOF-MS by summing normalized peak areas of all 15 *O*-AS, and expressing the totals relative to UT, the well characterized inbred line. Among the 26 accessions, total *O*-AS varied three-fold in their quantities, ranging from 0.48 to 1.44-fold of that found in UT (Fig. 2A). The accession A85 had the highest total *O*-AS content with 44% more than that found in UT, while A83 and A84 had the lowest total *O*-AS content with 45-50% of the UT levels of *O*-AS. Interestingly, both high- and low-content accessions were collected from a same region (Table S1), indicating that *O*-AS contents are highly variable within native *N. attenuata* populations.

Furthermore, we evaluated the variation in *O*-AS composition among the 26 accessions. A heatmap showing a hierarchical clustering of the 26 accessions and 15 *O*-AS was created using the Metaboanalyst 3.0 online software www.metaboanalyst.ca (Xia *et al.*, 2015). The peak area/gFM data was standardized using Auto scaling. The levels of each individual *O*-AS varied significantly among the different accessions (Fig. 2B). For instance, A83, A84 and UT had similar peak areas of AS6 but different peak areas of AS5, AS6, AS10, AS11 (Fig. 2B, Fig. S5). Based on this variation, the 26 natural accessions were classified into 3 major groups: A, B and C. The UT was grouped together with the high-*O*-AS accessions (group C) such as A179, A176, A85. The A83, A84 were grouped together with other low-*O*-AS accessions such as A422, A421, A278 in group A. The 15 *O*-AS were classed into 2 groups: group 1 contained *O*-AS class 3 and 4, group 2 contained all *O*-AS class 1, 2 *O*-AS class 2 and 1 *O*-AS class 4 (Fig. 2B). These results indicate that the *O*-AS clustering based on cross-individual expression patterns do not overlap with the *O*-AS classes, suggesting the variation in individual *O*-AS differs from the variation in *O*-AS classes.

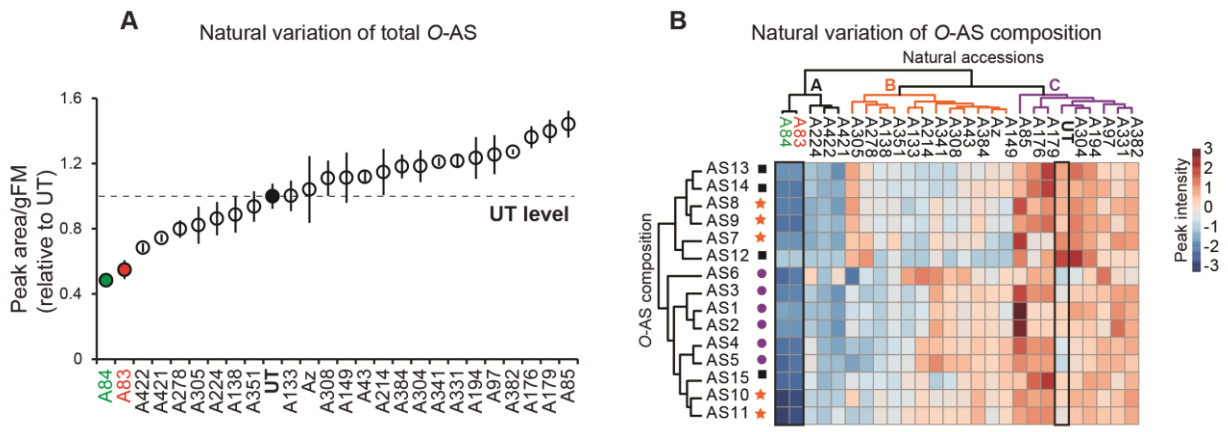


Figure 2. **The natural variation in *O*-acyl sugars levels among 26 natural accessions**. **A**. Relative abundance of total *O*-AS among different accessions. Three biological replicates for each natural accession were used to estimate *O*-AS contents. The average peak area per gram fresh mass (peak area/gFM) was normalized to that of the UT accession. **B**. Heatmap showing the hierarchical clustering of 26 accessions and the 15 *O*-AS based on peak area/gFM data in MetaboAnalyst (distance measure using Euclidean, and clustering algorithm using complete linkage). The degree of peak intensity for each individual *O*-AS is denoted by a different color from blue (low) to red (high). The *O*-AS class 2, 3 and 4 are indicated by a dot (●), star (★) and square (■) respectively.

Natural accessions with lower levels of *O*-AS are more susceptible to native herbivore and fungal pathogens

Because the genes responsible for *O*-AS composition are not yet elucidated in *N. attenuata*, it was not possible to genetically manipulate specific groups of the *O*-AS chemotype to investigate their ecological functions. Hence, we took advantage of the natural variations in the total *O*-AS pools produced in *N. attenuata* leaves to examine their overall defensive function. We selected two natural accessions A83 and A84 which had low levels of *O*-AS compared to UT (Fig. 2A) for herbivore and pathogen bioassay. Newly hatched *M. sexta* larvae were allowed to feed on A83, A84 and UT plants. We found that the *M. sexta* larvae that had fed on A83 and A84 plants had larger masses compared to those that fed on UT plants (Fig. S6A). A83 and A84 differed from UT in not only *O*-AS levels but also in other traits such as having a higher trichome density, broader leaves, smaller stem diameters and were delayed in bolting and

flowering times (Fig. S7). Thus, the observed difference in *M. sexta* performance may have resulted from traits in addition to *O*-AS contents.

Because jasmonic acid (JA) signaling is crucial for *N. attenuata*'s herbivore resistance (Baldwin, 1998), we examined the JA signaling pathway in A83 and A84 after mimicking herbivore attack. Oral secretions (OS) of *M. sexta* were immediately applied to leaf puncture wounds, phytohormones including JA and JA-Ile were measured at 1 h; nicotine, DTGs and trypsin proteinase inhibitors (TPIs) were measured at 48 h after elicitation. The two natural accessions were not compromised in most of JA signaling associated compounds compared to UT (Fig. S6B). JA was induced significantly higher in A83 plants compared to UT (*t*-test, P=0.04) while JA-Ile was significantly higher in both A83 and A84 (*t*-test, P=0.007 for A83 and P=0.008 for A84). These two accessions also had significantly higher levels of TPI compared to UT after OS elicitation (*t*-test, A83: P =0.001, A84: P=0.01). A83 had higher levels of constitutive and induced DTGs level compared to UT (*t*-test, P=0.02). While A83 showed no significant difference in nicotine induction, A84 had 38.9% lower levels than UT (Fig. S6B). Interestingly, *O*-AS levels of A83, A84 and UT were not changed by OS-elicitation and the two accessions had lower constitutive and induced total *O*-AS levels compared to UT (Fig. S6C). We conclude that the two natural accessions have lower *O*-AS contents but stronger inductions of the JA signaling pathway after OS elicitation.

To access the herbivore and pathogen susceptibility of the two natural accessions while minimizing the side effect of induced defenses, we used detached leaves to feed *M. sexta* larvae and inoculate with native fungal pathogens. *M. sexta* fed on A83, A84 detached leaves attained greater masses than those fed on UT plants after 12 days of feeding (DOF) (*t*-test, A83: P=0.028, A84: P=0.037) (Fig. 3A). Both *F. brachygibbosum* U4 (*Fusarium*) and *Alternaria* sp. U10 (*Alternaria*) caused significantly larger necrotic lesions on A83 and A84 than on UT detached leaves at 5 days post inoculation (DPI) (*t*-test, P<0.001 for both *Fusarium* and *Alternaria*) (Fig. 3B, C). From these results we conclude that the two accessions A83 and A84 are more susceptible to *M. sexta* and fungal pathogens than UT. In conclusion, the natural accessions A83, A84 are more susceptible to herbivore and pathogen attack while containing lower levels of total leaf *O*-AS but higher levels of herbivore-induced defenses, with the exception of nicotine.

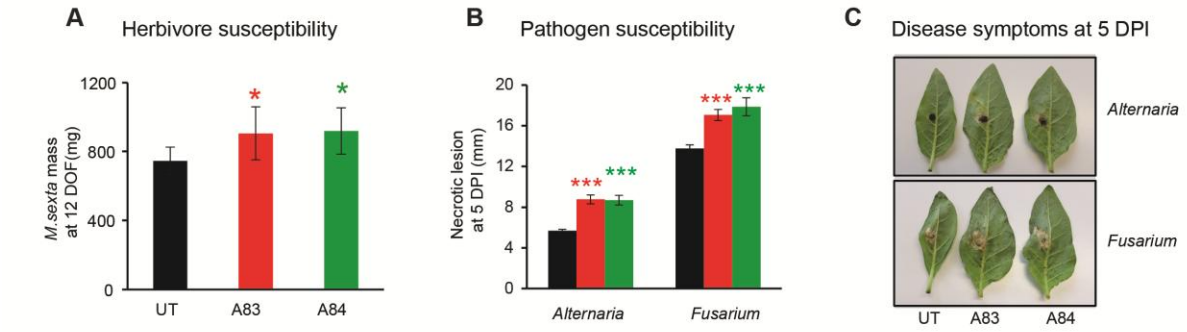


Figure 3.**Two low-*O*-acyl sugars accessions were more susceptible to a native herbivore and pathogens**. **A**. *M. sexta* larvae fed on A83 (red bar) and A84 (green bar) plants gained more masses than those fed on UT plants after 12 days of feeding (DOF). **B**. *Fusarium* and *Alternaria* infection resulted in larger necrotic lesions on leaves of A83 and A83 than UT after 5 days post inoculation (DPI). **C**. Necrotic lesions caused by *Alternaria* and *Fusarium* on detached leaves of UT, A83 and A84 at 5 DPI. The error bars represent standard errors (n=30). Asterisks indicate significant differences between A83 (red asterisks) or A84 (green asterisks) and UT at a given treatment (*t* test, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$).

F_2 crosses reveal that the overall *O*-AS pool is associated with resistance against both fungal pathogens and herbivores

While the two natural accessions A83 and A84 were more susceptible to a herbivore and pathogens, they also differed in morphologies as well as defense related traits, pointing to genetic differences. To segregate their genetic backgrounds via natural recombination, we created F_2 genetic crosses between UT and A84. Thirty independent crosses were made and a pooled population of F_2 individuals was created (Fig. 4A). We measured *O*-AS contents among 162 F_2 individuals and selected 30 individuals which had similar *O*-AS levels to UT (UT-like) and 30 individuals which had similar *O*-AS levels to A84 (A84-like) for herbivore and pathogen bioassay. *M. sexta* larvae that had fed on detached leaves of A84-like gained more masses than those that fed on UT-like at 9 DOF (*t*-test, P<0.001) (Fig. 4B). The caterpillars also developed faster on A84-like than on UT-like as they reached higher instar stages at 9 DOF (*G*-test, P=0.004). This indicates that the A84-like group is more susceptible to *M. sexta* than the UT-like group. Furthermore, both *Fusarium* and *Alternaria* challenges caused larger necrotic lesions on

A84-like than on UT-like plants at 5 DPI (*t*-test, *Fusarium*: P=0.011, *Alternaria*: P=0.015) (Fig. 4C), indicating that A84-like individuals are more susceptible to these fungal pathogens than UT-like individuals. Since the 30 individuals in each group were segregated in their genetic backgrounds but similar in *O*-AS contents, we conclude that the level of total *O*-AS in leaf is associated with defenses against both fungal pathogens and herbivore.

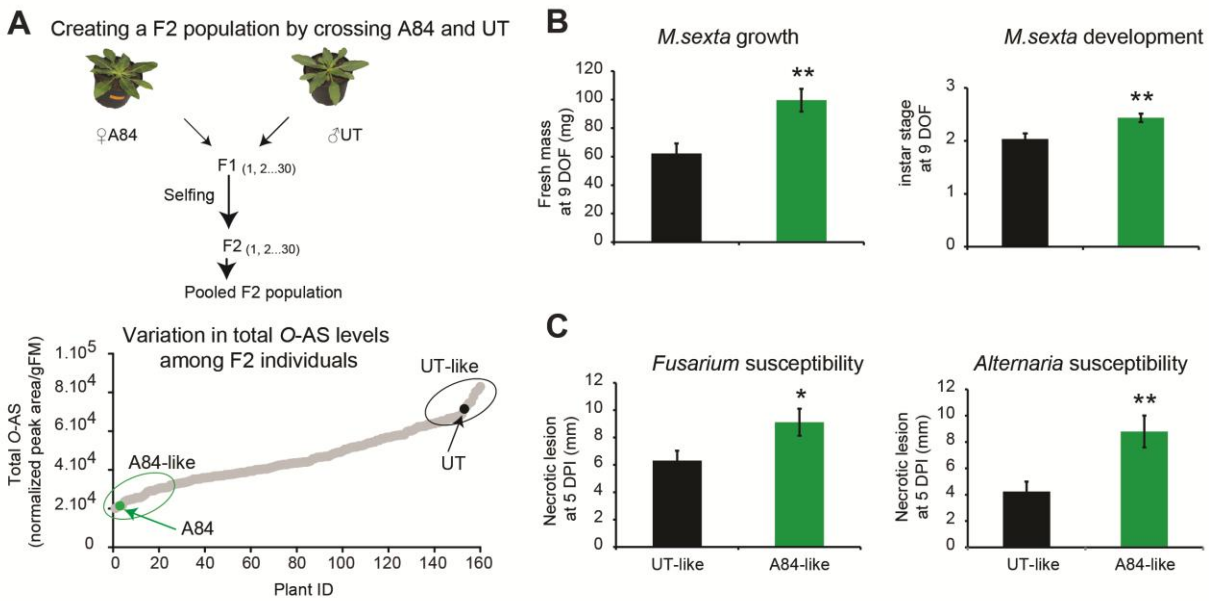


Figure 4. **The *O*-acyl sugars levels were associated with growth rates of *M. sexta* and fungal susceptibilities. A**. Diagram showing how the genetic crosses for the F_2 population was created, from which two groups of plants (n=30) were selected based on their *O*-AS contents. **B**. *M. sexta* larvae that fed on UT-like plants showed reduced masses and delayed development in comparison with those that fed on A84-like plants after 9 days of feeding (DOF). **C**. *Fusarium* and *Alternaria* caused larger necrotic lesion on A84-like plants than on UT-like plants after 5 days post inoculation (DPI). The error bars indicates standard error. Asterisks indicate significant differences between A84-like and UT-like plants (*t*-test for all but *G*-test for *M. sexta* development, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$).

Removing *O*-AS from leaf surfaces increased caterpillar growth and fungal necrotic lesions

To further explore the involvement of *O*-AS in *N. attenuata*'s defense against insect and pathogens, we manipulated the leaf *O*-AS content by removing them from the leaf surfaces via

leaf washing. To test whether washing with water was a suitable method to remove *O*-AS, leaf discs were punched from UT leaves and sonicated in water for 10 min. The first and second wash was analyzed separately. We observed that this washing method removed the trichome droplets without destroying the trichome structure (Fig. 5A). The first water wash is sufficient to remove most of the leaf surface metabolites (Fig. 5B). Compared to whole leaf extracts (Fig. S1), the nicotine peak intensity in the first water wash was relatively low, indicating that washing by water removed mainly *O*-AS. Using the same washing technique, washes of A83 and A84 leaves showed lower total *O*-AS compared to those of UT (Fig. S8), suggesting that this method is also suitable for comparing total *O*-AS among different genotypes. To be able to wash a large number of leaves for feeding and pathogen assays, we simply soaked leaves with an excess of water for 30 seconds. The washed leaves contained only 25% of their total *O*-AS by this method (Fig. 5C).

To evaluate whether removing *O*-AS from leaf surface alters plant defenses, we performed herbivore and pathogen bioassays on *N. attenuata* washed leaves. We found that *M. sexta* larvae that fed on washed leaves were significantly heavier than those that fed on unwashed leaves (*t*-test, $P<0.001$) (Fig. 5D). Necrotic lesions caused by *Fusarium* or *Alternaria* were significantly larger on washed leaves in comparison to unwashed leaves (*t*-test, *Fusarium*: $P<0.001$, *Alternaria*: $P=0.017$). These data demonstrate that removing *O*-AS from leaf surfaces increases *N. attenuata* susceptibility to herbivore and fungal pathogens.

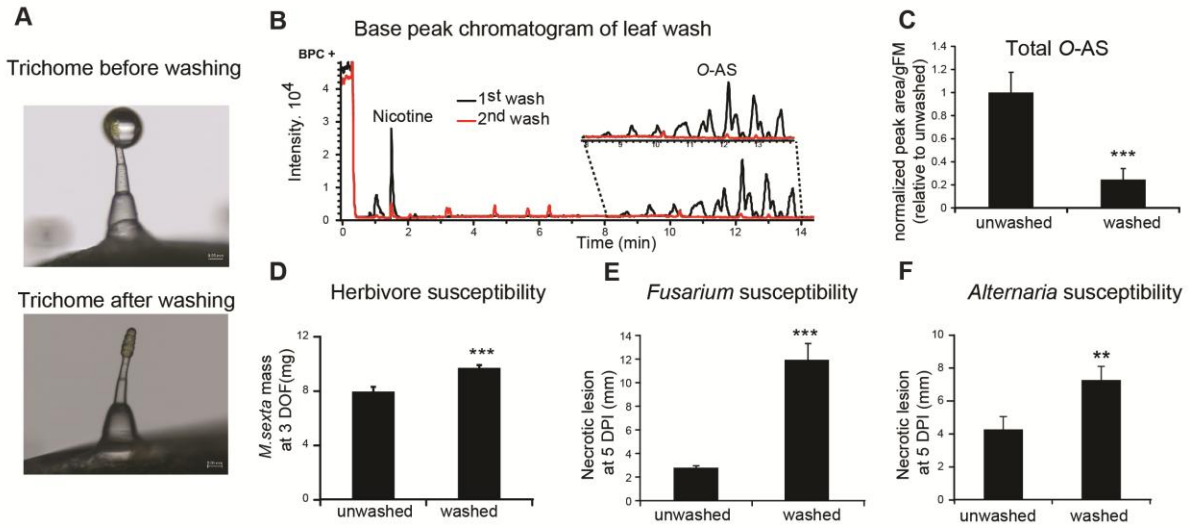


Figure 5. Removing *O*-acyl sugars from leaf surfaces increased herbivore and fungal susceptibility. **A**. Image of a *N. attenuata* trichome before and after washing with water. Pictures were taken with Axio Zoom.V16 stereo microscope at 180X magnification. **B**. Base peak chromatogram (BPC+) of a positive mode UHPLC/TOF-MS analysis revealed that leaf washing can efficiently remove most of leaf trichome *O*-AS. 1st wash and 2nd wash are indicated by black and red lines, respectively. **C**. Washing by water removed about 75% of the total leaf *O*-AS. **D**. *M. sexta* fed on washed leaves gained more mass than those that fed on unwashed leaves (n=30). **E**&**F**. Larger necrotic lesion on washed leaves compared to unwashed leaves caused by *Fusarium* (**E**) and *Alternaria* (**F**) at 5 days post inoculation (DPI) (n=10). The error bars represent standard error. Asterisks indicate significant differences between washed and unwashed in a given treatment (Student's-*t* test, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$).

Silencing *NaBCKDE1B* in trichomes led to 20-30% reduction in total leaf *O*-AS and increased susceptibility to *Fusarium* fungal pathogen

To genetically manipulate the *O*-AS content of *N. attenuata in vivo*, we searched for candidate genes that may control *O*-AS biosynthesis in this plant. The branched-chain keto-acid dehydrogenase (BCKD) complex is known to control the production of BCAAs which are used for *O*-AS production (Fig. 6A) (Slocombe *et al.*, 2008). Using virus-induced gene silencing,

Slocombe *et al.* (2008) demonstrated that silencing a gene encoding an E1 beta subunit of this enzyme complex (*NbBCKDE1B*) reduced total *O*-AS in *N. benthamiana* by 70%. In the *N. attenuata* genome, we identified a homologue of this gene with a length of 1092bp (Fig. S9), named as *NaBCKDE1B*. This gene had significantly lower transcript abundance in trichomes of A83, but not in A84, in comparison to UT trichomes (*t*-test, P=0.004) (Fig. S10). Because both A83 and A84 contain less *O*-AS in comparison to UT, this result suggested the potential involvement of *NaBCKDE1B* and other unknown genes in controlling *O*-AS level in *N. attenuata*.

To reduce total *O*-AS level in *N. attenuata*'s leaves, we used RNAi gene silencing via *Agrobacterium* transformation to silence *NaBCKDE1B* gene. Because *O*-AS are known to be synthesized in trichomes (Kandra & Wagner, 1988; Kroumova & Wagner, 2003) and to avoid the pleiotropic effect of silencing this gene ectopically, we aimed to silence this gene specifically in trichomes. A trichome specific promoter from *S. lycopersicum* (SlAT2 promoter), described by Schilmiller *et al.* (2012), was used to drive expression of a GFP-beta-glucuronidase (GUS) fusion protein in *N. attenuata* plants (Fig. S11A). We checked three independent T_1 lines (A-14-175, 181 and 182) for the GUS expression and found that in 2 independent T_1 lines (A-14-181 and 182) the GUS signals were specifically localized in the tip cells of trichomes (Fig. S11B), which indicated that the SlAT2 promoter could drive trichome-specific expression of RNAi constructs in *N. attenuata*.

Silencing of the *NaBCKDE1B* gene using SlAT2 trichome specific promoter was carried out using an inverted-repeat (ir) RNAi construct of *NaBCKDE1B* (Fig. S12B). 12 independent T_0 lines were used for screening. Two independently irBCKDE1B T_2 transformed lines (A-15-132-10 and A-15-147-2) were found to harbor a single T-DNA insertion (Fig. S12A). They also had more than a 78% reduction of *NaBCKDE1B* transcript abundance in trichomes (Fig. 6B) and more than a 40% in leaves (Fig. 6C). The transformed plants had similar morphology (Fig. S12D) and trichome density as wild type (WT) plants (Fig. S12C). These results indicate that the specific silencing of this gene in *N. attenuata* trichomes does not dramatically influence overall plant physiology. However, total *O*-AS in these two transgenic lines was reduced by only 20-30% in leaf washes (Fig. 6D) and in the whole leaf (Fig. 6E) while the proportion of each *O*-AS was unaffected (Fig. S12F). This data suggests that specific silencing of *NaBCKDE1B* in

trichomes does not result in a major change in total *O*-AS in leaves of *N. attenuata*. As consequence, although, there was a trend for *M. sexta* larvae to attain greater masses when fed on the two irBCKDE1B lines as well as larger necrotic lesions caused by *Alternaria* on these lines, no significant difference was found (Fig. 6F, H, Fig. S13). In the case of *Fusarium*, there was a significant increase in necrotic lesion caused by this fungus on irBCKDE1B lines compared to those on WT at 8 DPI (*t*-test, $P<0.001$ for both 132-10 and 147-2) (Fig. 6G). This result indicates that specific silencing of *NaBCKDE1B* in trichomes increases susceptibility to *Fusarium*.

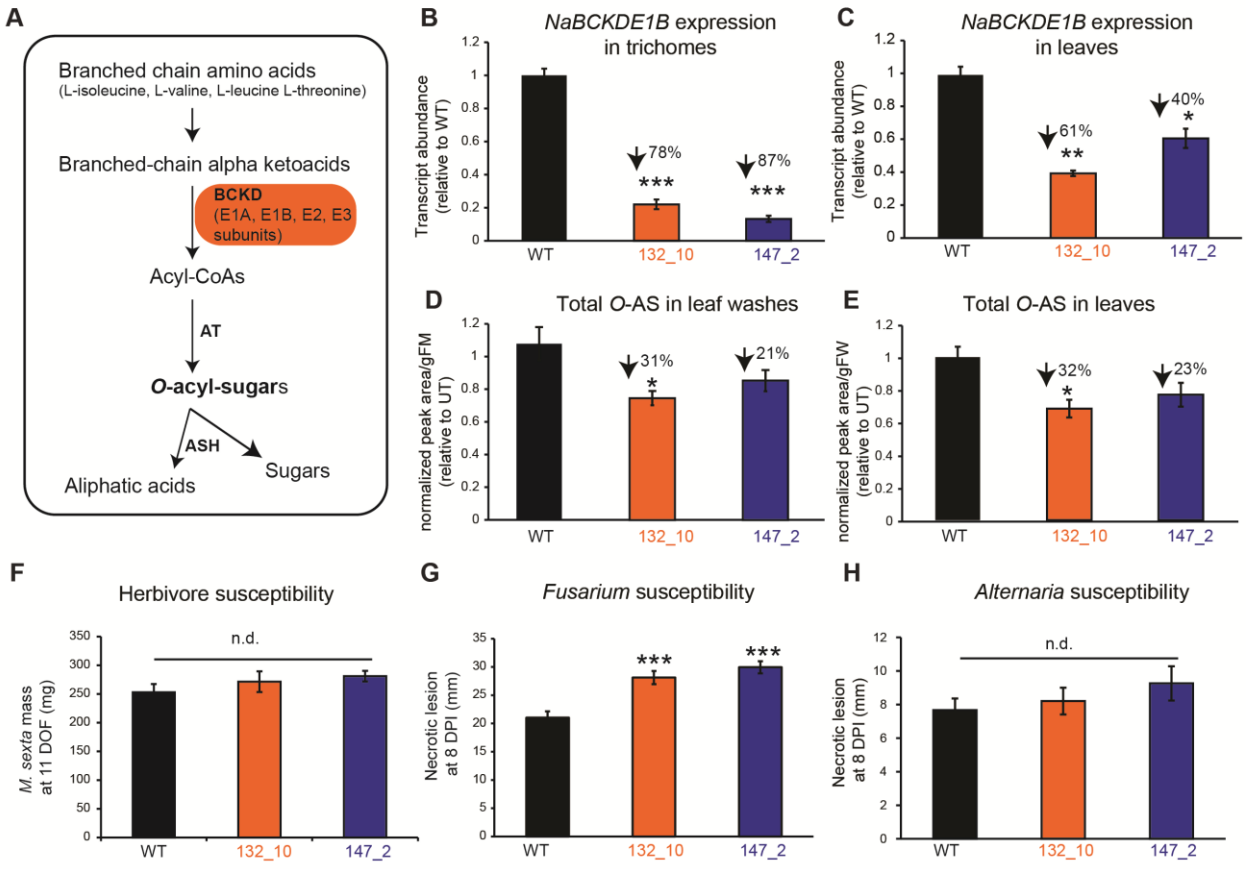


Figure 6. Silencing *NaBCKDE1B* in trichomes reduced total leaf *O*-AS by 20-30% and increased susceptibilities to *Fusarium*. A. Diagram shows the simplified model of *O*-AS biosynthesis in *Solanaceous* plants based on current knowledge. BCKD, branched-chain alpha-ketoacid dehydrogenase with E1A, E1B, E2 and E3 subunits; AT, acyltransferases; ASH, acylsugaracyl hydrolases. **B&C** Trichome-specific silencing of *NaBCKDE1B* resulted in 78-87% and 40-61% reductions of its transcript abundance in trichomes and whole leaves, respectively (n=6 for gene expression in leaf, n=3 (2 pooled samples per replicate) for gene expression in trichomes). **D&E**. Trichome specific silencing of *NaBCKDE1B* reduced *O*-AS content to 21-31% and 23-32% in leaf washes (n=6) and total leaf extracts (n=6), respectively. **F**. *M. sexta* larvae that fed detached leaves of WT and irBCKDE1B plants grew similarly (n=30). **G&H.** Necrotic lesions caused by *Fusarium* and *Alternaria* on irBCKDE1B and WT detached leaves at 8 and 10 days post inoculation (DPI), respectively (n=30). Error bars represent standard error. Asterisks indicate significant differences between WT and individual irBCKDE1B lines (*t* test, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$).

Adding *O*-AS or a mixture of the 4 main BCAAs to artificial diet or medium reduced *M. sexta* growth, *Fusarium* and *Alternaria* spore germination and mycelium growth

To test whether *O*-AS has direct detrimental effects on both herbivore and pathogens, a crude extract of *O*-AS from *N. attenuata* plant was added to artificial diets for *M. sexta* and germination medium for *Fusarium* and *Alternaria*. To mimic the normal *O*-AS concentrations found in plants (1.5mg/gFM), we produced artificial diets with the *O*-AS concentration of 1.2mg/g diet. For spore germination, the concentration of *O*-AS was tested within a range of 0.187 mg/mL to 3mg/mL to see concentration-dependent effects. As a result, *M. sexta* larvae that fed *O*-AS contained artificial diets showed significantly reductions in their masses compared to those that fed on *O*-AS free artificial diets (*t*-test, P=0.002 at 12 DOF) (Fig. 7A). *Fusarium* germination and hyphal growth were strongly inhibited by *O*-AS which was already seen at the lowest concentration tested, 0.375 mg/mL (ANOVA, $F_{4,88}$=244.51, P<0.001). This inhibition effect increased with increasing *O*-AS concentrations in the germination medium (Fig. 7B, Fig. S14). For *Alternaria*, *O*-AS at the concentration of 0.375 significantly reduced their hyphal length (ANOVA, $F_{4, 612}$=85.62, P<0.001) but not the percentage of germinated spores. Higher *O*-AS concentrations (1.5mg/mL) were required to significantly reduce number of *Alternaria* germinated spores (ANOVA, $F_{4,111}$=12.53, P<0.001). These results indicate that spore germination and growth of *Fusarium* are more sensitive than *Alternaria* to *O*-AS. In summary, we conclude that *N. attenuata O*-AS strongly inhibit caterpillar growth as well as fungal germination and growth.

In *N. attenuata*, *O*-AS consist of BCAAs esterified to a sucrose core molecule. To get insights into the mechanisms of their toxicity, we tested the effect of BCAAs on *M. sexta* growth as well as fungal pathogen germination and growth. A mixture of the 4 main BCAAs produced in *N. attenuata* including 2-methyl butanoic acid, 3-methyl butanoic acid, 3-methyl pentanoic acid and 4-methyl pentanoic acid was created with proportion of each BCAAs reflecting their proportion in the *O*-AS crude extract and what is released from *M. sexta*'s bodies after *O*-AS ingestion as described by Weinhold and Baldwin (2011). To compare its effect with *O*-AS, a BCAA mixture of 1.2 mg/g diet was added to the artificial diets and a range of concentrations (from 0.187 to 3 mg/mL) was added into fungal germination medium. Interestingly, this mixture of BCAAs showed a strong effect on *M. sexta* growth (*t*-test, P<0.001 at 12 DOF) (Fig. 7C).

Moreover, *Alternaria* and *Fusarium* hyphal length were significantly inhibited at the lowest concentration tested, 0. 187 and 0.375 mg/mL, respectively (ANOVA, *Alternaria*: $F_{5,149}$=83.03, P<0.001, *Fusarium*: $F_{4,125}$=59.67, P<0.001) (Fig. 7D, Fig. S15). Spore germination of *Alternaria* and *Fusarium* was significantly reduced at concentrations of 0.375 and 0.75 mg/mL, respectively (ANOVA, *Alternaria*: $F_{5,79}$=121.01, P<0.001, *Fusarium*: $F_{4,65}$=1639.2, P<0.001) (Fig. 7D). These results indicate that the mixture of 4 main BCAAs are detrimental to *M. sexta*, *Fusarium* and *Alternaria*, suggesting the toxicity of *O*-AS toward herbivore and the fungal pathogen is due to their BCAA substitutions.

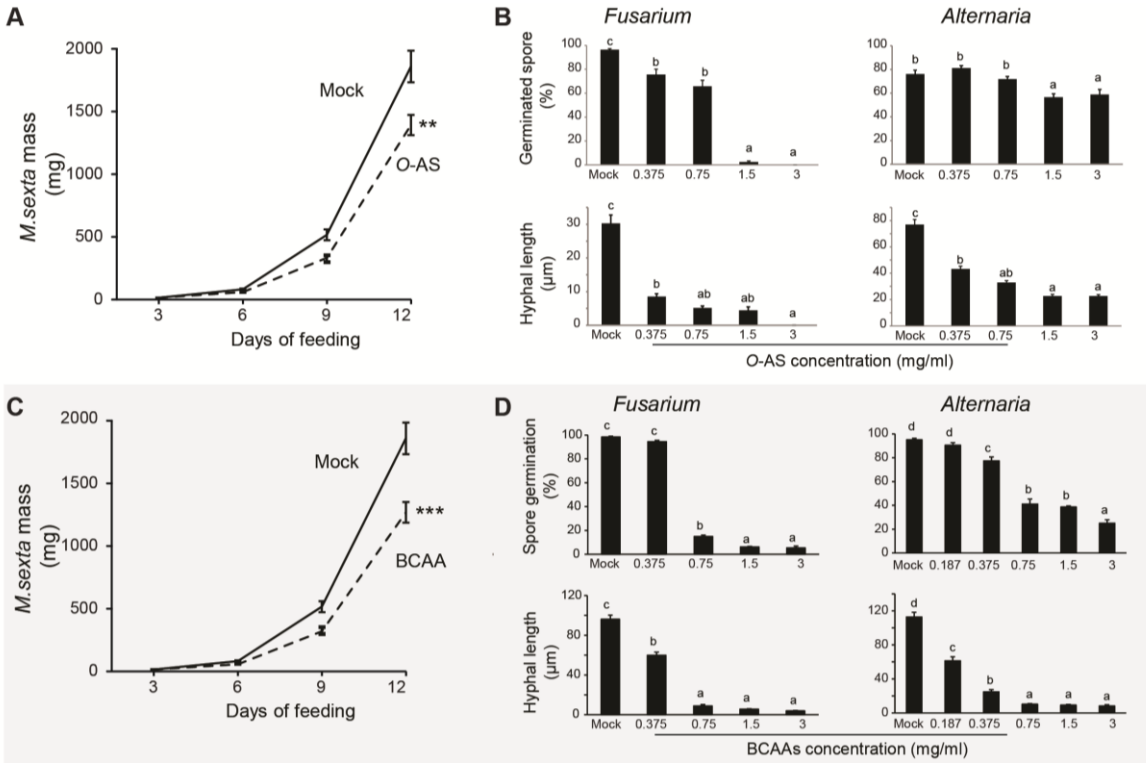


Figure 7. Both *O*-acyl sugars crude extracts and a mixture of the 4 BCAAs suppressed *M. sexta* growth and fungal pathogen germination *in vitro*. A&C. *M. sexta* larvae grow slower when they fed on artificial diets supplemented with *O*-AS crude extracts or a mixture of the 4 main BCAAs in comparison to those fed a mock diet. Solid lines indicate mock diet, dashed lines indicate diet amended with *O*-AS crude extract or the mixture of 4 BCAAs. Asterisks indicate significant differences between two treatments (*t* test, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$). **B&D**. Spore germination and hyphal length of *Fusarium* and *Alternaria* fungal on plain agar supplemented with an *O*-AS crude extract or a mixture of 4 BCAAs with different concentrations. Different letters (a-b) indicate significantly different data groups determined by one-way ANOVA with Bonferroni *post-hoc* test, $P \leq 0.05$. In all panels, error bars indicate standard errors.

Discussion

In this study, we characterized 15 *O*-AS from 3 structural classes whose concentrations varied three-fold among natural accessions in *N. attenuata* and demonstrated their generalized function in defense against a native herbivore and two fungal pathogens. The contents of *O*-AS in *N. attenuata*'s natural accessions were associated with plant resistance to two fungal pathogens and a specialist herbivore. *In vivo* manipulating *O*-AS contents via leaf washing or silencing *NaBCKDE1B* gene in trichomes revealed a crucial role of *O*-AS in defenses against native fungal pathogens. *In vitro* experiments confirmed that *O*-AS and their BCAA substitution were detrimental to *M. sexta* and fungal pathogens. Hence, we provide evidence for the direct defense function of *O*-AS in *N. attenuata* against a specialist herbivore and native pathogens, which complements the previously established functions as indirect defenses (Weinhold and Baldwin 2011).

In *N. attenuata*, total *O*-AS varied up to three-fold among 26 natural accessions, which is similar to that reported from *S. pennellii* (2-to 4-fold) (Shapiro *et al.*, 1994), but less than that reported from *S. habrochaites* (8-fold) (Kim *et al.*, 2012). We also found that accessions collected from the same location have different *O*-AS contents and compositions. For instance, the accession A85 had 44% more while A83 and A84 contained 45-50% less total *O*-AS than UT, all of which were collected from the same geographic region. This large variation in *O*-AS levels within *N. attenuata* populations is consistent with the metabolomic variation within population previously reported by Li *et al.* (2015) and likely results from the high genetic variation within *N. attenuata* populations which in turn results from their long-lived seed banks and their fire-chasing behavior (Bahulikar *et al.*, 2004).

The structural variation found among the 26 natural accessions can be organized into three different groups that differ in the acetylations of the glucose/fructose components of the sucrose core. The *O*-AS clustering based on cross-individual expression patterns did not overlap with these *O*-AS classes, indicating that the variation in individual *O*-AS differed from the variation in *O*-AS classes. Interestingly, in *S. habrochaites*, the variation of *O*-AS classes results from variation in a single gene encoding acyltransferase 2 (Kim *et al.*, 2012). For *N. attenuata*, the mechanisms responsible for the variation in *O*-AS individual or class remain unknown. As a

consequence, the ecological function of the different classes remains elusive, but one could speculate that a larger number of acidic substituent increases the lipophilicity of a particular compound. Since *O*-AS are important leaf surface chemicals exuded by trichomes, increased lipophilicity might be advantageous for the exudation and surface adherence process. Furthermore, *O*-AS are thought to destroy the membranes of soft-bodied insects and cause their death by desiccation (Puterka *et al.*, 2003). According to this scenario, a greater lipophilicity could increase the defensive function of *O*-AS. Indeed, the mixture of the 4 main BCAAs including 2-methyl butanoic acid, 3-methyl butanoic acid, 3-methyl pentanoic acid and 4-methyl pentanoic acid alone are detrimental to *M. sexta*, suggesting that BCAAs which are released after *O*-AS digestion are detrimental compounds. We also observed a toxic effect on both *Fusarium* and *Alternaria*. For the pathogens, BCAAs are well-known to disrupt the cell membrane, inhibit the myristoylation of proteins, inhibiting b-oxidation, triacylglycerol synthesis, sphingolipid synthesis as well as showing topoisomerase activity as reviewed by Pohl *et al.* (2011). To gain more knowledge about the function of individual *O*-AS, fraction-guided bioassays can be used. A prerequisite for such an approach will be the purification of individual *O*-AS, which unfortunately was not possible with the reverse phase HPLC method used in this study. Because *O*-AS represent a highly lipophilic compound class, normal phase HPLC system may provide the separation required to fractionate single compounds for use in bioassays.

To examine the *in vivo* function of *O*-AS, we manipulated the *O*-AS contents by washing the leaf surface with water. About 75% of the total *O*-AS in leaves was removed, and so was nicotine. We do not know yet whether *O*-AS and nicotine function synergistically to enhance plant defense. However, we observed that A84 plants had not only lower *O*-AS contents but also lower nicotine inductions after simulated herbivore attack. We speculate that nicotine-*O*-AS synergisms are responsible for the greater *M. sexta* larvae growth on the A84 plants compared to those fed on A83 or UT plants. The washing treatment could also damage trichomes and leaf surfaces, resulting in wound responses that increase flavonols content which may influence herbivore and pathogen susceptibility (Malhotra *et al.*, 1996; Faini *et al.*, 1997; Roda *et al.*, 2003). Therefore, the differences in herbivore and pathogen susceptibility cannot be solely attributed to the difference in *O*-AS contents between washed and unwashed leaves.

To provide a cleaner manipulation of *O*-AS contents *in vivo*, we stably silenced the expression of a putative branched-chain alpha-ketoacid dehydrogenase E1 beta subunit encoding gene (*NaBCKDE1B*) in *N. attenuata*. To avoid the pleiotropic effect of silencing this gene throughout plant via its function in branched chain amino acids catabolism in different tissues (Peng *et al.*, 2015), we specifically silenced this gene in *N. attenua*'s trichomes, which are known to be the location of *O*-AS biosynthesis in *Solanaceous* species (Kandra & Wagner, 1988; Kroumova & Wagner, 2003). In *N. attenuata*, staining *O*-AS with Rhodamine B also showed that *O*-AS are highly accumulated in trichomes (Weinhold & Baldwin, 2011). Therefore, we expected that silencing a gene controlling *O*-AS levels in trichomes would reduce total leaf *O*-AS. We obtained two independent transgenic lines with more than 78% reduction of *NaBCKDE1B* transcript abundance in trichomes and no influence on trichome density and the plant morphology. However, the specific silencing of *NaBCKDE1B* in trichomes only resulted in a 20-30% reduction of the total *O*-AS in *N. attenuata*'s leaves. The use of the SlAT2 promoter in our silencing construct may explain the anemic reductions in total *O*-AS levels. This promotor specifically drives gene expression in the tip cell of trichomes, which in *N. attenuata*'s trichomes contain 2-6 cells (Fig. S11). Silencing *NaBCKDE1B* only in the trichome tip cells may have minor effects on total *O*-AS levels if *O*-AS are also synthesized in the other trichome cells. In addition, *O*-AS precursors may be transported from leaf mesophyll cells to trichomes to provide BCAA precursors for *O*-AS synthesis in *NaBCKDE1B*-silenced trichomes. Slocombe *et al.* (2008) demonstrated that transiently silencing *NbBCKDE1B* by virus induce silencing led to a 70% reduction in the total *O*-AS in *N. benthamiana*, consistent with our hypothesis on the existence of a transport system of *O*-AS precursors throughout the plant. To test this hypothesis, an *in vitro* synthesis of *O*-AS using isolated trichomes or detached leaves and C^{14} labeled precursors coupled with the non-destructive *O*-AS analysis by NMR-spectroscopy could be done. However the elucidation of the *O*-AS biosynthesis pathway and the genes involved in different steps of *O*-AS biosynthesis would be required for such an experiment.

Recently, the *S. lycopersicum O*-AS metabolic network has been successfully reconstructed *in vitro* where *O*-AS assembly begins by adding a five-carbon acyl chain to the pyranose ring of sucrose by acyltransferase 1 (AT1) and followed by the addition of a second acyl chain by AT2 (Fan *et al.*, 2016). AT3 adds the third acyl chain to create tri-acylsucrose

(Schilmiller *et al.*, 2015) and finally, AT4 adds an acetyl group to the pyranose ring of a tri-acylsucrose acceptor (Kim *et al.*, 2012; Schilmiller *et al.*, 2012). The synthesized *O*-AS can be hydrolyzed by acyl sugar acylhydrolases 1 and 2 (ASH1 and ASH2) (Schilmiller *et al.*, 2016). All of these newly discovered genes involving in *O*-AS biosynthesis could potentially influence the total *O*-AS content in the plant, in particular the first committed step mediated by AT1 and the hydrolysis step of ASH1 and ASH2. In addition, the more upstream genes involving BCAA production, elongation and activation such as genes encoding the 3 subunits of the branched-chain keto-acid dehydrogenase (BCKD) complex, threonine deaminase (TD), acetolactate synthase (ALS), isopropyl malate synthase (IPMS), isopropyl malate dehydrogenase (IPMD), and isopropylmalate dehydratase (IPDS) (Slocombe *et al.*, 2008) could also be considered for their contributions to total *O*-AS content in the plant. Our gene expression analysis of *N. attenuata* homologes encoding 1 beta (E1B) and 2 alpha (E1A) subunit of BCKD (*NaBCKDE1B*, *NaBCKDE1A*_1 and *NaBCKDE1A_2*), acyltransferases (*NaAT1* and *NaAT2*), acyl sugar acylhydrolases (*NaASH1* and *NaASH2*) in the trichomes of two low *O*-AS containing natural accession A83 and A84 (Fig. S10) suggested that not only genes involved in *O*-AS synthesis (*NaBCKDE1B*, *NaBCKDE1A_2*, *NaAT1* and *NaAT2*), but also genes involved in *O*-AS degradation (*NaASH2*) potentially control the total *O*-AS content in *N. attenuata*. Hence, a detail *O*-AS metabolic network for *N. attenuata* would enhance our understanding of *O*-AS biosynthesis and provide a more efficient means of manipulating *O*-AS contents in this plant.

Although silencing *NaBCKDE1B* expression in trichomes led to only a 20-30% reduction in total leaf *O*-AS, this modest reduction resulted in a significantly higher susceptibility of the transgenic lines to *Fusarium*, but not *Alternaria*. From these results we infer that *Fusarium* is more sensitive to *O*-AS compared to *Alternaria*. In our *in vitro* test, we observed that *Fusarium* spore germination could be inhibited at the low *O*-AS concentration of 0.375 mg/mL, while to inhibit *Alternaria*, higher concentration of 1.5mg/mL was required. Based on our *O*-AS extraction results, the *O*-AS concentration in *N. attenuata* is normally about 1.5mg/gFM. The 20-30% reduction of this concentration results in a difference of 0.3-0.45 mg/gFM which is equal to 0.3-0.45mg/mL *O*-AS used for fungal inhibition test. This *O*-AS range is already sufficient for the reduction in fungal spore germination and growth of *Fusarium*, but not *Alternaria in vitro*. A further analysis of the interaction of these fungal pathogens and *O*-AS could answer the question

of why *Fusarium* is more sensitive to *O*-AS than *Alternaria*. In nature, fungal spores can be dispersed via air and rainfall to land on the leaf surface, where they wait to germinate (Timmer, Lavern W *et al.*, 2003) ; hence spores are in direct contact with excreted *O*-AS on the leaf surface. We speculate that fungal spores can be fully covered by *O*-AS due to their small size which makes their germination exquisitely sensitive to *O*-AS. Recently, it is shown that trichomes can serve as entry points for *Fusarium* infection (Nguyen *et al.*, 2016), hence not only excreted *O*-AS but also *O*-AS retained inside trichomes could act as the first layer of defense against fungal pathogen infections.

In contrast to the small size of fungal pathogens, chewing herbivores such as *M. sexta* have large bodies and hence reduced exposures to *O*-AS. We observed that the effect of *O*-AS in the artificial diet on *M. sexta*'s mass can be already seen after 6 days of feeding. Thus, we hypothesize that the early stage of caterpillars is more sensitive to *O*-AS and *O*-AS may act as lipophilic exudates that enhance the penetration of other toxic agents into caterpillar via skin contact. To grow, chewing insects need to consume large amounts of leaf tissue. While silencing *NaBCKDE1B* in trichomes led to a reduction of *O*-AS in the leaf, we do not know if silencing this gene also alters leaf nutrient contents that compromise larval performance on the transgenic lines. In addition, the sensitivity of *M. sexta* to *O*-AS may also be concentration-dependent and a reduction of 0.3-0.45mg/gFM may not be sufficient to result in significant differences in caterpillar growth.

In native environments, *N. attenuata* seeds remain dormant for the long between-fire intervals and germinate when the smoke signal is sensed and leaf litter is removed or pyrolized (Baldwin & Morse, 1994), thus, the location of *N. attenuata* populations as well as their herbivores and pathogens are unpredictable. This unpredictability likely accounts for *N. attenuata's* remarkable plasticity in herbivore and pathogen defense strategies. Here we show that *N. attenuata*'s *O*-AS can be considered as “generalized defense compounds” that play roles in defense against both native pathogens and herbivores. We also found substantial variation among 26 natural accessions, in both total amounts and compositions. Hence, we suggest that *N. attenuata O*-AS natural variation results from selection pressures from both herbivores and pathogens. Furthermore, the variation in *O*-AS production can also be shaped by other selective forces such as abiotic stresses, competition effects or ecological fitness tradeoffs. Remaining

questions regarding fitness cost are the cost of *O*-AS production and why *N. attenuata* produces *O*-AS if BCAAs are actually the basis of their resistance to both pathogens and herbivores. Weinhold and Baldwin (2011) demonstrated that trichome secretions were frequently the first meal of neonate caterpillars and the sucrose core of *O*-AS could provide a sweet enticement for their ingestion so that larvae could be olfactory tagged for predation, as an indirect defense. Esterification of BCAAs to sugars could also function as a stable delivery mechanism for these highly volatile BCAAs. Answering these questions would help us understanding how the balancing selection is generated which likely accounts for the great diversity of *O*-AS concentrations and compositions found in natural accessions.

Materials and methods

Plant, caterpillar and fungal materials and growth conditions

We used 26 *Nicotiana attenuata* Torr.ex S. Watson natural accessions which were collected over the last 20 years in the Southwestern United States (Table S1). The accessions UT, A83, A84 were collected from the same region (UT: 37°19'36.26"N, 113°57'53.05"W; A83 and A84: 37°19'34"N, 113°57' 38"W). The UT accession used as a control for comparison was inbred for 30 generations and A83, A84 were inbred for 5 generations under glasshouse conditions in Jena, Germany. Seed germination and growth condition are the same as described by Krugel *et al.* (2002).

Manduca sexta eggs were from our in-house colony. *M. sexta* eggs were kept in a growth chamber (Snijders Scientific, Tilburg, the Netherlands) for 16 h light at 26°C, 8 h dark at 24°C and 65% relative humidity until the larvae hatched. Newly hatched neonates were used for all feeding experiments.

Phytopathogens used in this study are *Fusarium brachygibbosum* Padwick U4 (*Fusarium*) and *Alternaria sp*. U10 (*Alternaria*) which were originally isolated from diseased plants grown in native population in Utah, USA (Schuck *et al.*, 2014) and shown to be a suitable system for studying *N. attenuata*'s defense against native pathogens (Luu *et al.*, 2015). The fungus was cultured and maintained as described by Luu *et al.* (2015).

_O_-AS isolation and characterization

To isolate *O*-AS from *N. attenuata* plants, we used a protocol similar to Van Dam and Hare (1998). All plant parts except the flowers were harvested. After extraction, the glue-like, brownish yellow residue was obtained and kept under argon at 4°C until further use for bioassay and for MS^2 experiment. A small portion was dissolved in 40% methanol and analyzed by ultra-high performance liquid chromatography/time-of-flight mass spectrometry (UHPLC/TOF-MS) to verify the extraction. See Supplemental methods for more details.

To fractionate the *O*-AS for MS^2 experiment, extracted *O*-AS were dissolved in acetonitrile at a concentration of 1mg/mL. An Agilent 1100 HPLC system equipped with a DAD detector was used and the fractions were collected with a Foxy fraction collector (Isco) in 20 mL glass reaction tubes (Schott). 40 fractions of 30 s were cut starting 5 min after injection. The fractions were transferred to scintillation vials and the solvent was evaporated in a vacuum centrifuge (Eppendorf). The single fractions were then analyzed for their content by injection into an UHPLC/TOF-MS system with conditions described in Weinhold and Baldwin (2011). See Supplemental methods for more details.

For MS^2 experiments, 1µL of each fraction was separated using a Dionex RSLC system (Dionex, Sunnyvale, USA) with a Dionex Acclaim RSLC 120 C-18 column (150 x 2.1 mm, 2.2 µm). MS detection was carried out with an ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) (BrukerDaltonik, Bremen, Germany) operated in a positive electrospray mode ionization. Mass calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid). Elemental formula and masses were calculated with the ACD/Labs 12 ChemSketch calculating tool (ACD/Labs, Frankfurt, Germany). See Supplemental methods for more details.

The analysis of *O*-AS branched chain aliphatic acid (BCAA) composition was done using a Varian 3800 gas chromatograph equipped with a ZB-Wax-plus column (30m x 0.25mm x 0.25µm, Restek) and a flame ionization detector (GC-FID) (Agilent). The identities of the BCAA's were verified by the injection of authentic standards at a concentration of 50ng/µL.

Retention indices were calculated in reference to an alkane standard mixture (C8-C20, Sigma-Aldrich). For more details, see Supplemental methods.

_O_-AS relative comparison analysis

To cross-compare *O*-AS levels among different accessions or genotypes, we extracted leaf or trichome *O*-AS using a method described by Gaquerel *et al.* (2010) with some modifications. After extraction, 1 µL supernatants were separated using a UPLC Dionex RSLC system with a Dionex Acclaim RSLC 120 C-18 column (150 x 2.1 mm, 2.2 µm). MS detection was carried out with the UHPLC/TOF-MS system operated in positive electrospray mode. Typical instrument settings were described in Gilardoni *et al.* (2011) with some modification. The peak areas were integrated using extracted ion traces for the sodium adduct [M+Na]+ of each individual *O*-AS in the QuantAnalysis software version 2.0 SP1 (BrukerDaltonics). The amount was normalized with sucrose monolaurate (Sigma) as an internal standard and the fresh mass of the tissue. Total *O*-AS was calculated by summing the normalized peak areas of all 15 *O*-AS. The 15 *O*-AS together with their indicating m/z values and retention times (RT) are listed in Table S2. See Supplemental methods for more details.

To compare *O*-AS in leaf wash among different genotypes, leaf discs of 18mm diameter was punched from *N. attenuata* fully developed leaf at rosette stage and placed into 50 mL Falcon tube. 10 mL of miliQ water containing sucrose monolaurate as an internal standard was used to wash the leaf disc by sonication for 10 min. The leaf wash was filtered through Whatman paper filter, 2mL of filtered wash was evaporated using rotary evaporator connected with a CentriVapconcentration systems (Labconco), dissolved in 100 µL of 40% methanol and 1µL of this extract were analyzed by UHPLC/TOF-MS with conditions as described above.

Extraction and analysis of phytohormones, secondary metabolites and trypsin protein inhibitors

To identify whether A83 and A84 are compromised in the JA signaling pathway in response to herbivory, we evaluated the induction of JA, JA-Ile phytohormones and its associated secondary metabolites and proteins such as nicotine and 17-hydroxygeranyllinalool diterpene glycosides (DTGs) and trypsin proteinase inhibitors (TPIs) after mimicking *M. sexta* attack. Rosette leaves were wounded using a fabric pattern wheel with three rows of puncture onto each side of the midvein and directly applying 20 µL of a 1: 5 (v/v) milliQ water-diluted *M. sexta* oral secretions solution (WOS). The intact plants were used as control. The treated leaves were harvested at 1 h after the treatment for phytohormone measurement and at 48 h after induction for secondary metabolites and protein measurement. 5-6 biological replicates were used for each treatment.

Phytohormone extraction and quantification were carried out as described in Gilardoni *et al.* (2011). JA and JA-Ile were quantified by comparing its peak area with the peak area of its respective internal standard as described in Wu *et al.* (2007) and calculated per gram fresh mass (µg/gFM). For more details, Supplemental methods.

Nicotine and DTGs were extracted as described above for *O*-AS extraction. Analysis of these metabolites was done using an HPLC-DAD as previously described by Keinanen *et al.* (2001) with some modification (see Supplemental methods for details). The peak areas were integrated using the Chromeleon nicotine in plant tissue was calculated using external dilution series of standard mixtures of nicotine. The peak areas were quantified to estimate total DTGs contents and normalized it to tissue fresh mass. The method was described previously by Kaur *et al.* (2010)

The qualitative determination of TPI activities was performed by radial diffusion assay relative to protein content as previously described Van Dam *et al.* (2001).

Caterpillar and fungal bioassays

M. sexta feeding assay was performed by placing newly hatched *M. sexta* neonates on the fully expanded leaves either on intact or detached leaves from 30 day-old plants. *M. sexta* mass was recorded to access the performance of larvae.

For *M. sexta* feeding on artificial diet, the artificial diet was prepared as described by Grosse-Wilde *et al.* (2010). While the diet was still liquid and cooling (about 50°C), 600 mg of extracted *O*-AS were dissolved in 5 mL dichloromethane (DCM) and added into 500 g diet to achieve a final concentration of 1.2 mg/g diet which is similar concentration of *O*-AS *in planta* (1.5 mg/gFM, in this study). As a control, 5 mL of pure DCM was added into the medium. The diets were heated and stirred on a hot plate until the DCM had evaporated. The diet was placed in plastic boxes and kept at 5°C until use. To supplement the artificial diet with a mixture of branched- chain aliphatic acids (BCAAs), 600 µL mixture of 4 main BCAAs including 2-methyl butanoic acid (19.26 µL), 3-methyl butanoic acid (63.69 µL), 3-methyl pentanoic acid (493.05 µL), and 4-methyl pentanoic acid (23.99 µL) was prepared. This mixture was added directly to the artificial diet and mixed thoroughly. Freshly hatched *M. sexta* larvae were transferred to plastic containers containing pieces of artificial diet. 30 plates per diet type and 2-3 larvae per plate were fed *ad libitum* in a climate chamber (50% relative humidity, 26°C during days and 24°C during nights under 12 h of light). Larval mass was measured every 3 days until 12 days.

To test the fungal susceptibility of different plant accessions or genotypes, we used detached leaf assay which was described by Schuck *et al.* (2014). After 5, 8 and 10 days post inoculation (DPI), the smallest and largest diameter of the necrotic lesion were measured using a caliper. Average of necrotic lesion was calculated and presented as an indicator of plant susceptibility to pathogens.

To test the effect of *O*-AS on fungal spore germination and mycelial growth *in vitro*, 30 mg of extracted *O*-AS and 30 µL of BCAAs mixture were dissolved in 1 mL DCM and added in 10 mL of spore germination medium which was comprised of 1.2% plant agar and 10 mM $MgCl_2$. The control medium contained only 1 mL of DCM. The medium was heated in a water bath until the DCM is evaporated. The medium was diluted to attain a final concentration of 3,

1.5, 0.75 and 0.375mg/mL for *O*-AS and 3, 1.5, 0.75, 0.375 and 0.187 mg/mL for BCAAs. Fungal spores were harvested from 14 day-old cultures as described by Luu *et al.* (2015). Approximately 2.5 mL of spore germination medium was spread onto microscopic glass slides and allowed to solidity. Then 10 µL of spore suspension (concentration 10^4) placed on the solid germination medium and spread with an inoculation loop. Eight glass slides were prepared for each treatment and each of them was kept in a sterile Petri dish (9 cm diameter) with moist Whatman paper (1 mL sterile water added) to maintain the high humidity required for spore germination. The Petri dishes were incubated in a dark chamber at 25°C and the spore germination was monitored every hour under an inverted light microscope (Axiovert 200, Carl Zeiss Microscopy GmbH, Germany) coupled with a camera (AxioVision). Spore germination rate was determined after 6 and 12 h for *Alternaria* and *Fusarium*, respectively. Three microscopic fields per slide were photographed randomly and considered as technical replicates. The percentage of germinated spores was calculated based on numbers of spore germinated divided by a total number of spores observed per microscopic field. Hyphal length of the germinated spore in each microscopic field was measured by ImageJ software (Wayne Rasband, National Institutes of Health, USA).

Trichome-specific silencing of *N. attenuata BCKDE1B* using *Agrobacterium tumefaciens*

Comparing publicly available sequences at NCBI for gene encoding branched-chain alpha-ketoacid dehydrogenase E1 beta subunit (*BCKDE1B*) from *Arabidopsis* and other closely related *Solanaceous* species including *S. pennellii* and *N. tabacum* with the *N. attenuata* 454 transcriptome (Gase & Baldwin, 2012) and our in-house genome database, we identified 1 homologue (*NIATv7_g34895)* in *N. attenuata*.

To elucidate the role of this *NIATv7_g34895* gene in controlling *O*-AS levels in *N. attenuata*, we used RNAi-mediated gene silencing with stable *Agrobacterium* transformation developed by Krugel *et al.* (2002). To avoid the pleiotropic effects of silencing this gene on plant performance, we specifically silenced this gene in trichomes using a trichome-specific promoter, *S. lycopersicum* SlAT2, as previously discovered by Schilmiller *et al.* (2012) was used to drive expression of a GFP-beta-glucuronidase (GUS) fusion protein in *N. attenuata* plants. *N. attenuata* 30th inbred plants were transformed using the LBA4404 strain of *Agrobacterium*

tumefaciens as described by Krugel *et al.* (2002). Three independent T_1 transgenic lines (A-14-175, A-14-181, A-14-182) were selected based on hygromycin resistance and all showed GUS staining in the tip cell of trichomes, regardless of what type of tissue they were located on (i.e., hypocotyl, cotyledon, stem, leaf, etc.). No GUS staining was seen in other parts of the plant.

To generate irBCKDE1B plants, we cloned a 351 bp fragment of *NaBCKDE1B* gene (see Supplemental methods for the sequence) as an inverted repeat construct into pRESC8TRCAS transformation vector containing a hygromycin (*hptII*) resistance gene as selection marker and the *S. lycopersicum* SlAT2 promoter to specifically silence this gene in trichome. *N attenuata* 30th inbred plants were transformed using the LBA4404 strain of *A. tumefaciens* as described in Krugel *et al.* (2002). Homozygous transgenic lines were selected by screening of T2 generation seeds that showed hygromycin resistance, and T-DNA insertions were confirmed by Southern blot hybridization, using genomic DNA from selected lines and ^{32}P-labeled PCR fragment of the *hptII* gene as a hybridization probe (Fig. S12A) according to Gase *et al.* (2011). Quantitative real-time PCR was used to select the best silenced transgenic lines: irBCKDE1-132-10, 147-2. Detail method for RNA extraction, cDNA synthesis, quantitative real-time PCR and primers used are provided in Supplemental methods.

GUS staining

Histochemical staining with X-gluc (5-bromo-4-chloro-3-indolyl glucuronide) was done using the protocol described by Jefferson *et al.* (1987). GUS staining was observed under ZEISS Stereomicroscope SV 11 with 4X.

Trichome density determination

Trichome density was determined by counting trichomes on the adaxial and abaxial sides of leaf discs of three different laminar positions that span the length of the leaf under a Zeiss Stereomicroscope Axio Zoom.V16 (Carl Zeiss) at 32X magnification. Trichome densities were calculated as the number of trichomes per mm^2 leaf area.

Trichome harvesting method

Trichomes were harvest by cutting stem and branches into small pieces with about 5 cm, placed into 50 mL Falcon tube and frozen in liquid N_2. Frozen tissues were shaked thoroughly for 30 seconds and dislodged trichomes were collected into 2 mL Eppendorf tube and ground for RNA extraction.

Statistical analysis

Statistics were performed using Excel (Microsoft, http://www.microsoft.com) and the SPSS software version 17.0 (www.spss.com). Statistical significance was evaluated using one-way analysis of variance (ANOVA) at a 0.05 level and means were compared by the Bonferroni *post-hoc* test. For analysis of differences in plant performance, the *t* test was used with the two-tailed distribution of two sets of samples. The number of replicates (*n*) used in each experiment is detailed in the figure captions.

Accession numbers

The coding sequences have been submitted to GenBank/EMBL data libraries as locus tags A4A49_34895 (NIATv7_g34895), A4A49_19321 (NIATv7_g19321), A4A49_12742 (NIATv7_g12742), A4A49_04553 (NIATv7_g04553), A4A49_42350 (NIATv7_g42350), A4A49_11346 (NIATv7_g11346) and A4A49_41468 (NIATv7_g41468).

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Literature Cited

Altman A (1997) Agricultural biotechnology. CRC Press

Arrendale RF, Severson RF, Sisson VA, Costello CE, Leary JA, Himmelsbach DS, Vanhalbeek H (1990) Characterization of the sucrose ester fraction from *Nicotiana glutinosa*. Journal of Agricultural and Food Chemistry **38:** 75-85

Bahulikar RA, Stanculescu D, Preston CA, Baldwin IT (2004) ISSR and AFLP analysis of the temporal and spatial population structure of the post-fire annual, *Nicotiana attenuata*, in SW Utah. BMC Ecology **4:** 12

Baldwin IT (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proceedings of the National Academy of Sciences of the United States of America **95:** 8113-8118

Baldwin IT (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. Plant Physiology **127:** 1449-1458

Baldwin IT, Morse L (1994) Up in Smoke: II. Germination of *Nicotiana attenuata* in response to smoke-derived cues and nutrients in burned and unburned soils. Journal of Chemical Ecology **20:** 2373-2391

Biere A, Marak HB, van Damme JMM (2004) Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? Oecologia **140:** 430-441

Calo L, Garcia I, Gotor C, Romero LC (2006) Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a trichoderma alpha-1,3-glucanase. Journal of Experimental Botany **57:** 3911-3920

Carolina H. Pohl JLFKaVST (2011) Antifungal free fatty acids: a review. Science against microbial pathogens: communicating current research and technological advances. Microbiology Book Series - Number 3 A. Méndez-Vilas, **:** 61–71

Chortyk OT, Kays SJ, Teng Q (1997) Characterization of insecticidal sugar esters of *Petunia*. Journal of Agricultural and Food Chemistry **45:** 270-275

Chortyk OT, Severson RF, Cutler HC, Sisson VA (1993) Antibiotic activities of sugar esters isolated from selected *Nicotiana* species. Bioscience Biotechnology and Biochemistry **57:** 1355-1356

Ding L, Xie FW, Zhao MY, Xie JP, Xu GW (2006) Rapid characterization of the sucrose esters from oriental tobacco using liquid chromatography/ion trap mass spectrometry. Rapid Communications in Mass Spectrometry **20:** 2816-2822

Ehrlich PR, Raven PH (1964) Butterflies and plants: a study in coevolution. Evolution **18:** 586-608

Faini F, Labbe C, Salgado I, Coll J (1997) Chemistry, toxicity and antifeedant activity of the resin of *Flourensia thurifera*. Biochemical Systematics and Ecology **25:** 189-193

Fan PX, Miller AM, Schilmiller AL, Liu XX, Ofner I, Jones AD, Zamir D, Last RL (2016) In vitro reconstruction and analysis of evolutionary variation of the tomato acylsucrose metabolic network. Proceedings of the National Academy of Sciences of the United States of America **113:** E239-E248

Fobes JF, Mudd JB, Marsden MPF (1985) Epicuticular lipid-accumulation on the leaves of *Lycopersicon pennellii* (Corr.) D'arcy and *Lycopersicon esculentum* Mill. Plant Physiology **77:** 567-570

Fridman E, Wang JH, Iijima Y, Froehlich JE, Gang DR, Ohlrogge J, Pichersky E (2005) Metabolic, genomic, and biochemical analyses of glandular trichomes from the wild tomato species *Lycopersicon hirsutum* identify a key enzyme in the biosynthesis of methylketones. The Plant Cell **17:** 1252-1267

Gang DR, Wang JH, Dudareva N, Nam KH, Simon JE, Lewinsohn E, Pichersky E (2001) An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. Plant Physiology **125:** 539-555

Gaquerel E, Heiling S, Schoettner M, Zurek G, Baldwin IT (2010) Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in *Nicotiana attenuata* leaves during simulated herbivory. Journal of Agricultural and Food Chemistry **58:** 9418-9427

Gase K, Weinhold A, Bozorov T, Schuck S, Baldwin IT (2011) Efficient screening of transgenic plant lines for ecological research. Molecular ecology resources **11:** 890-902

Gershenzon J (1994) Metabolic costs of terpenoid accumulation in higher plants. Journal of Chemical Ecology **20:** 1281-1328

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nature Chemical Biology **3:** 408-414

Gilardoni PA, Hettenhausen C, Baldwin IT, Bonaventure G (2011) *Nicotiana attenuata* LECTIN RECEPTOR KINASE1 suppresses the insect-mediated inhibition of induced defense responses during *Manduca sexta* herbivory. The Plant Cell **23:** 3512-3532

Glas JJ, Schimmel BCJ, Alba JM, Escobar-Bravo R, Schuurink RC, Kant MR (2012) Plant glandular trichomes as targets for breeding or engineering of resistance to herbivores. International Journal of Molecular Sciences **13:** 17077-17103

Goffreda JC, Mutschler MA, Ave DA, Tingey WM, Steffens JC (1989) Aphid deterrence by glucose eEsters in glandular trichome exudate of the wild tomato, *Lycopersicon pennellii*. Journal of Chemical Ecology **15:** 2135-2147

Gonzales-Vigil E, Hufnagel DE, Kim J, Last RL, Barry CS (2012) Evolution of TPS20-related terpene synthases influences chemical diversity in the glandular trichomes of the wild tomato relative *Solanum habrochaites*. Plant Journal **71:** 921-935

Grosse-Wilde E, Stieber R, Forstner M, Krieger J, Wicher D, Hansson BS (2010) Sex-specific odorant receptors of the tobacco hornworm *Manduca sexta*. Frontiers in Cellular Neuroscience **4**

Hare JD (2005) Biological activity of acyl glucose esters from *Datura wrightii* glandular trichomes against three native insect herbivores. Journal of Chemical Ecology **31:** 1475-1491

Haslam E (1998) Practical polyphenolics: from structure to molecular recognition and physiological action. Cambridge University Press

Hawthorne DJ, Shapiro JA, Tingey WM, Mutschler MA (1992) Trichome-borne and artificially applied acylsugars of wild tomato deter feeding and oviposition of the leafminer *Liriomyza trifolii*. Entomologia Experimentalis Et Applicata **65:** 65-73

Jefferson RA, Kavanagh TA, Bevan MW (1987) Gus fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. Embo Journal **6:** 3901-3907

Juvik JA, Shapiro JA, Young TE, Mutschler MA (1994) Acylglucoses from wild tomatoes alter behavior and reduce growth and survival of *Helicoverpa zea* and *Spodoptera exigua* (Lepidoptera, Noctuidae). Journal of Economic Entomology **87:** 482-492

Kandra L, Wagner GJ (1988) Studies of the site and mode of biosynthesis of tobacco trichome exudate components. Archives of Biochemistry and Biophysics **265:** 425-432

Kato A, Arima K (1971) Inhibitory effect of sucrose ester of lauric acid on the growth of *Escherichia coli*. Biochemical and Biophysical Research Communications **42:** 596-601

Kato N, Shibasaki I (1975) Comparison of antimicrobial activities of fatty acids and their esters. Journal of Fermentation Technology **53:** 793-801

Kaur H, Heinzel N, Schottner M, Baldwin IT, Galis 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 Physiology **152:** 1731-1747

Keinanen M, Oldham NJ, Baldwin IT (2001) Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. Journal of Agricultural and Food Chemistry **49:** 3553-3558

Kim J, Kang K, Gonzales-Vigil E, Shi F, Jones AD, Barry CS, Last RL (2012) Striking natural diversity in glandular trichome acylsugar composition is shaped by variation at the acyltransferase2 locus in the wild tomato *Solanum habrochaites*. Plant Physiology **160:** 1854-1870

Kroumova AB, Wagner GJ (2003) Different elongation pathways in the biosynthesis of acyl groups of trichome exudate sugar esters from various solanaceous plants. Planta **216:** 1013-1021

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

Li DP, Baldwin IT, Gaquerel E (2015) Navigating natural variation in herbivory-induced secondary metabolism in coyote tobacco populations using MS/MS structural analysis. Proceedings of the National Academy of Sciences of the United States of America **112:** E4147-E4155

Luu VT, Schuck S, Kim SG, Weinhold A, Baldwin IT (2015) Jasmonic acid signalling mediates resistance of the wild tobacco *Nicotiana attenuata* to its native *Fusarium*, but not *Alternaria*, fungal pathogens. Plant Cell and Environment **38:** 572-584

Malhotra B, Onyilagha JC, Bohm BA, Towers GHN, James D, Harborne JB, French CJ (1996) Inhibition of tomato ringspot virus by flavonoids. Phytochemistry **43:** 1271-1276

Marak HB, Biere A, Van Damme JMM (2003) Fitness costs of chemical defense in *Plantago lanceolata* L.: Effects of nutrient and competition stress. Evolution **57:** 2519-2530

Marshall DL, Bullerman LB (1986) Antimicrobial activity of sucrose fatty acid ester emulsifiers. Journal of Food Science **51:** 468-470

Moore B, Andrew RL, Kulheim C, Foley WJ (2014) Explaining intraspecific diversity in plant secondary metabolites in an ecological context. New Phytologist **201:** 733-750

Nguyen TTX, Dehne HW, Steiner U (2016) Maize leaf trichomes represent an entry point of

infection for *Fusarium* species. Fungal Biology **120:** 895-903

Peng C, Uygun S, Shiu SH, Last RL (2015) The impact of the branched-chain ketoacid dehydrogenase complex on amino acid homeostasis in *Arabidopsis*. Plant Physiology **169:** 1807-1820

Puterka GJ, Farone W, Palmer T, Barrington A (2003) Structure-function relationships affecting the insecticidal and miticidal activity of sugar esters. Journal of Economic Entomology **96:** 636-644

Roda AL, Oldham NJ, Svatos A, Baldwin IT (2003) Allometric analysis of the induced flavonols on the leaf surface of wild tobacco (*Nicotiana attenuata*). Phytochemistry **62:** 527-536

Sallaud C, Rontein D, Onillon S, Jabes F, Duffe P, Giacalone C, Thoraval S, Escoffier C, Herbette G, Leonhardt N, Causse M, Tissier A (2009) A novel pathway for sesquiterpene biosynthesis from Z,Z-farnesyl pyrophosphate in the wild tomato *Solanum habrochaites*. The Plant Cell **21:** 301-317

Santhanam R, Luu VT, Weinhold A, Goldberg J, Oh Y, Baldwin IT (2015) Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping. Proceedings of the National Academy of Sciences of the United States of America **112:** E5013-E5020

Schilmiller AL, Charbonneau AL, Last RL (2012) Identification of a BAHD acetyltransferase that produces protective acyl sugars in tomato trichomes. Proceedings of the National Academy of Sciences of the United States of America **109:** 16377-16382

Schilmiller AL, Gilgallon K, Ghosh B, Jones AD, Last RL (2016) Acylsugar acylhydrolases: carboxylesterase-catalyzed hydrolysis of acylsugars in tomato trichomes. Plant Physiology **170:** 1331-1344

Schilmiller AL, Moghe GD, Fan PX, Ghosh B, Ning J, Jones AD, Last RL (2015) Functionally divergent alleles and duplicated loci encoding an acyltransferase contribute to acylsugar metabolite diversity in *Solanum* trichomes. The Plant Cell **27:** 1002-1017

Schilmiller AL, Schauvinhold I, Larson M, Xu R, Charbonneau AL, Schmidt A, Wilkerson C, Last RL, Pichersky E (2009) Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. Proceedings of the National Academy of Sciences of the United States of America **106:** 10865-10870

Schuck S, Weinhold A, Luu VT, Baldwin IT (2014) Isolating fungal pathogens from a dynamic disease outbreak in a native plant population to establish plant-pathogen bioassays for the ecological model plant *Nicotiana attenuata*. Plos One **9**

Shapiro JA, Steffens JC, Mutschler MA (1994) Acylsugars of the wild tomato *Lycopersicon pennellii* in relation to geographic-distribution of the species. Biochemical Systematics and Ecology **22:** 545-561

Simonovska B, Srbinoska M, Vovk I (2006) Analysis of sucrose esters-insecticides from the surface of tobacco plant leaves. Journal of Chromatography A **1127:** 273-277

Slocombe SP, Schauvinhold I, McQuinn RP, Besser K, Welsby NA, Harper A, Aziz N, Li Y, Larson TR, Giovannoni J, Dixon RA, Broun P (2008) Transcriptomic and reverse genetic analyses of branched-chain fatty acid and acyl sugar production in *Solanum pennellii* and *Nicotiana benthamiana*. Plant Physiology **148:** 1830-1846

Snook ME, Johnson AW, Severson RF, Teng Q, White RA, Sisson VA, Jackson DM (1997) Hydroxygeranyllinalool glycosides from tobacco exhibit antibiosis activity in the tobacco budworm [*Heliothis virescens* (F)]. Journal of Agricultural and Food Chemistry **45:** 2299-2308

Timmer LW, Peever TL, Solel Z, Akimitsu K (2003) *Alternaria* diseases of citrus-novel pathosystems. Phytopathologia Mediterranea **42:** 99-112

Treutter D (2006) Significance of flavonoids in plant resistance: a review. Environmental Chemistry Letters **4:** 147-157

Van Dam NM, Hare JD (1998) Biological activity of *Datura wrightii* glandular trichome exudate against *Manduca sexta* larvae. Journal of Chemical Ecology **24:** 1529-1549

Van Dam NM, Horn M, Mares M, Baldwin IT (2001) Ontogeny constrains systemic protease inhibitor response in *Nicotiana attenuata*. Journal of Chemical Ecology **27:** 547-568

Wagner GJ, Wang E, Shepherd RW (2004) New approaches for studying and exploiting an old protuberance, the plant trichome. Annals of Botany **93:** 3-11

Walters DS, Steffens JC (1990) Branched chain amino acid metabolism in the biosynthesis of *Lycopersicon pennellii* glucose esters. Plant Physiology **93:** 1544-1551

Weinhold A, Baldwin IT (2011) Trichome derived *O*-acyl sugars are a first meal for caterpillars that tags them for predation. Proceedings of the National Academy of Sciences of the United States of America **108:** 7855-7859

Weinhold A, Shaker K, Wenzler M, Schneider B, Baldwin IT (2011) Phaseoloidin, a homogentisic acid glucoside from *Nicotiana attenuata* trichomes, contributes to the plant's resistance against *Lepidopteran* herbivores. Journal of Chemical Ecology **37:** 1091-1098

Wink M (2011) Annual plant reviews, biochemistry of plant secondary metabolism, Vol 40. John Wiley & Sons

Wu J, Hettenhausen C, Meldau S, Baldwin IT (2007) Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. The Plant Cell **19:** 1096-1122

Xia J, Sinelnikov IV, Han B, Wishart DS (2015) MetaboAnalyst 3.0-making metabolomics more meaningful. Nucleic acids research **43:** W251-W257

Supplemental Figures

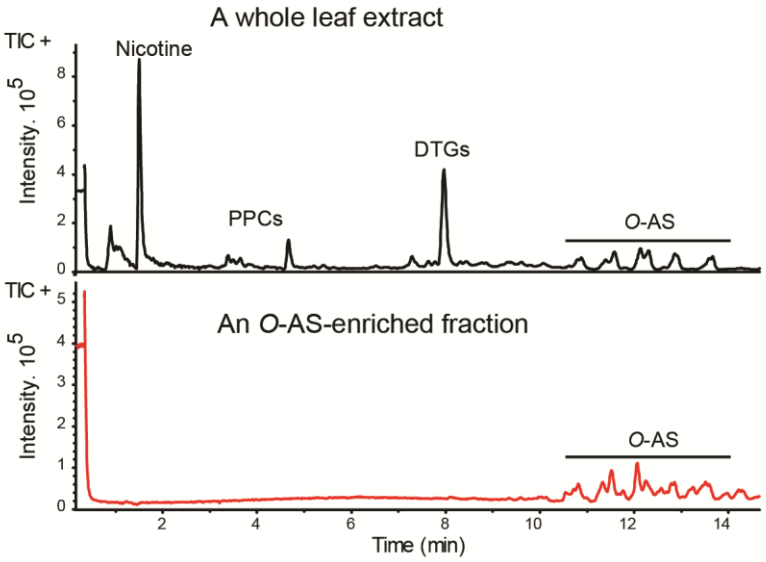

Figure S1. Total ion chromatogram of a positive mode UHPLC/TOF-MS run (TIC+) of a whole leaf extract which is compared with an *O*-acyl sugars (*O*-AS)-enriched fraction. The leaf extract is dominated by nicotine, phenylpropanoid polyamine conjugates (PPCs), 17-hydroxygeranyllinalool diterpene glycosides (DTGs), and *O*-AS, while the *O*-AS-enriched fraction was dominated by a mixture of *O*-AS.

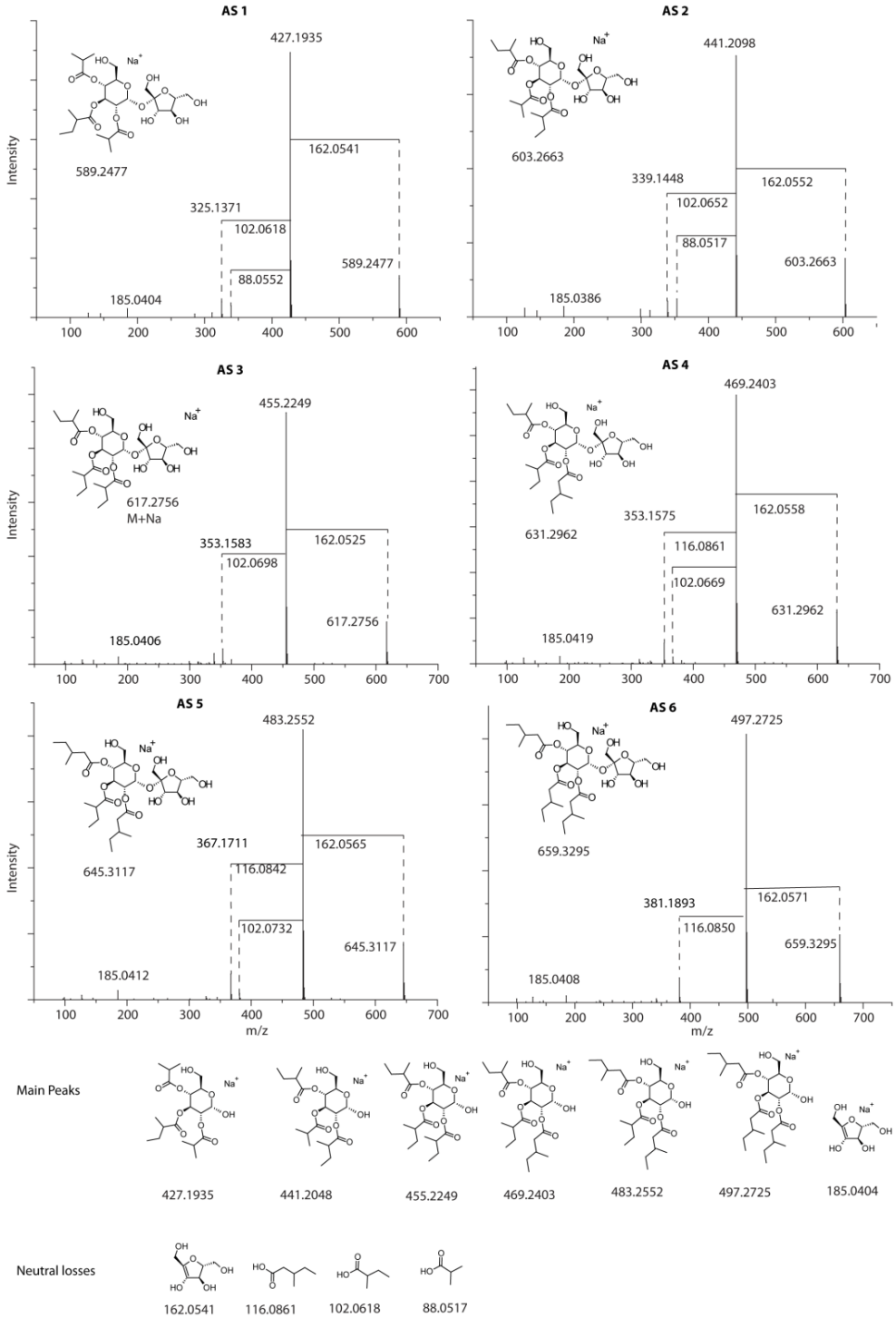

Figure S2: MS^2 spectra of class 2 *O*-acyl sugars and the annotation of the main fragment peaks (Main peaks) and neutral losses. The position of the branched- chain aliphatic could not be verified and may vary.

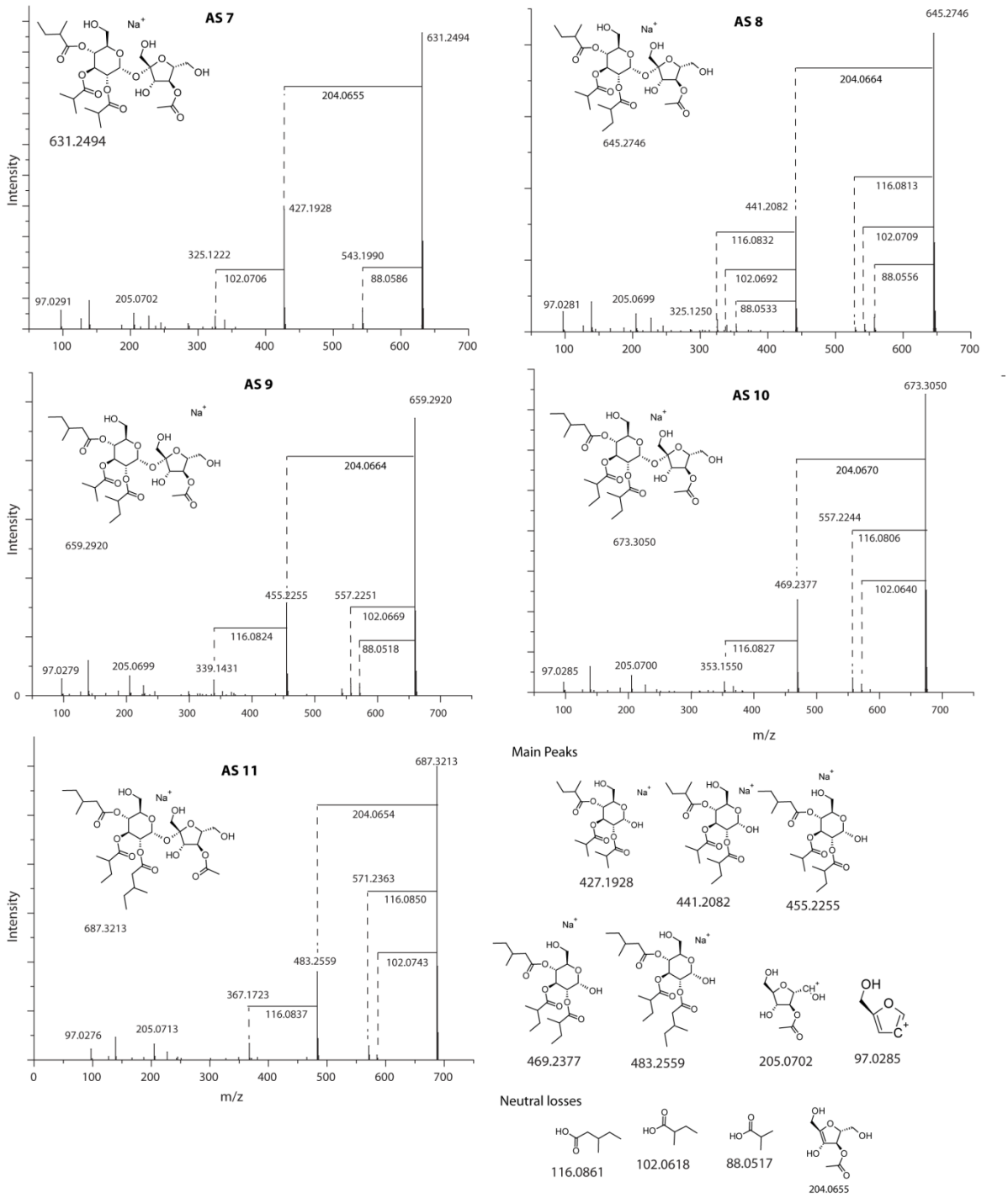

Figure S3: MS^2 spectra of class 3 *O*-acyl sugars and the annotation of the main fragment peaks (Main peaks) and neutral losses. The position of the branched- chain aliphatic could not be verified and may vary.

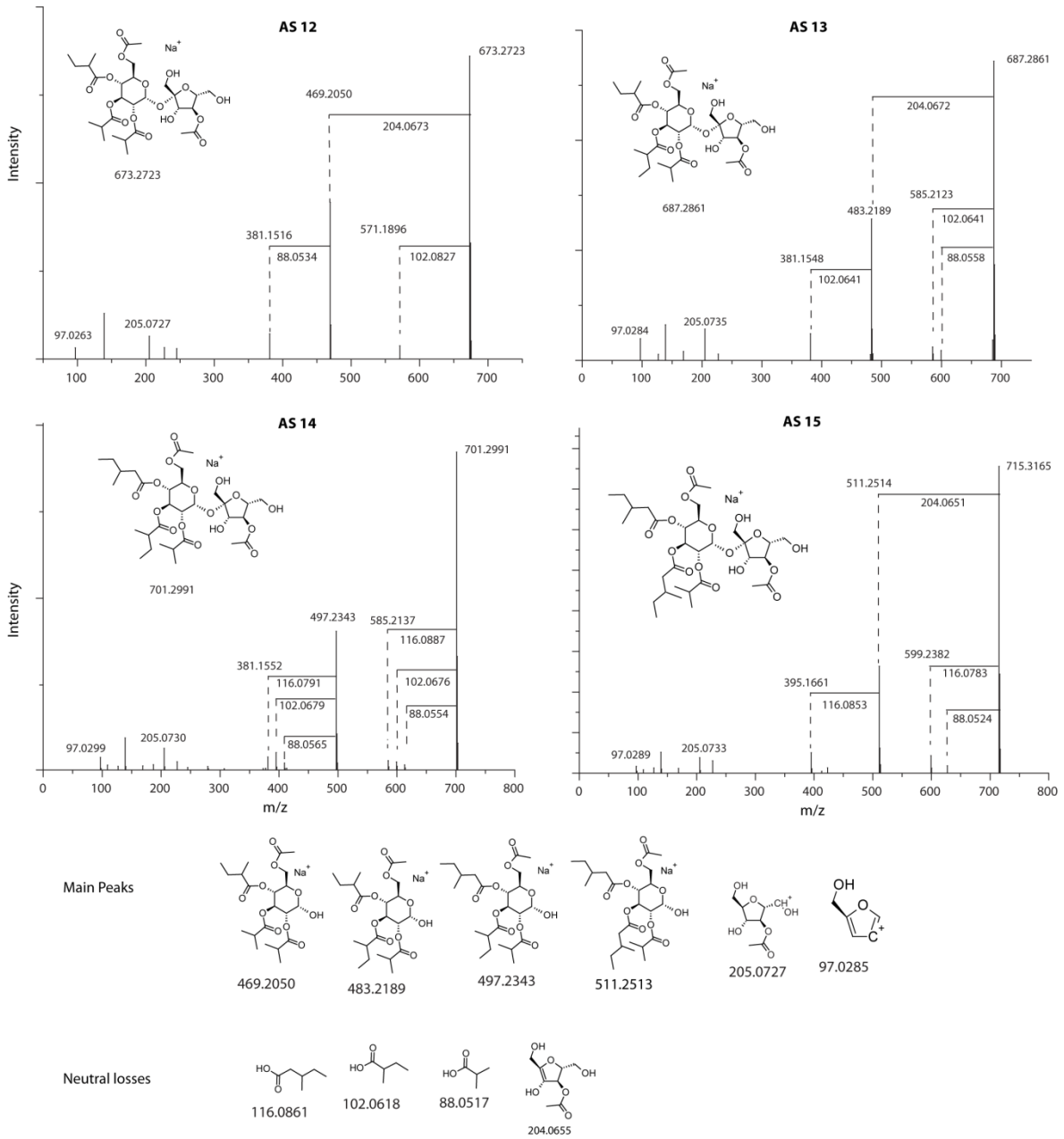

Figure S4: MS^2 spectra of class 4 *O*-acyl sugars and the annotations of the main fragment peaks (Main peaks) and neutral losses. The position of the branched- chain aliphatic could not be verified and may vary.

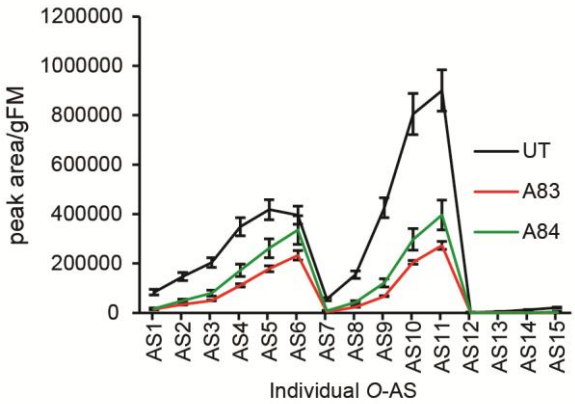

Figure S5. *O*-acyl sugars composition of A83, A84 and UT plants. Peak area per gram fresh mass (peak area/gFM) of each individual *O*-AS revealing the overall reduction of all 15 *O*-AS in A83 and A84 compared to UT.

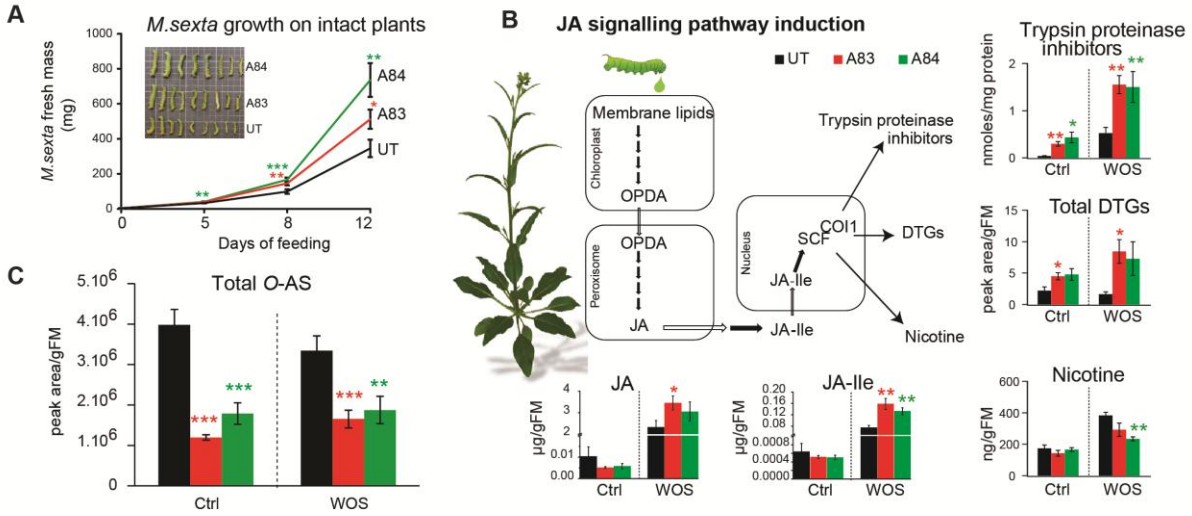

Figure S6. **Two low *O*-AS accessions are not compromised in JA signaling but more susceptible to the specialist herbivore**. **A.** *M. sexta* larvae fed on A83 (red line) and A84 (green line) plants grew faster than those fed on UT plants. The error bars represent standard errors (n=30). **B**. The JA-induced defenses in two low *O*-AS accessions are intact. A simplified herbivore-induced JA signaling and the defensive responses it activates in *N. attenuata* are shown in the middle and five measured traits are shown as bar plots. µg/gFM: µg per gram of fresh mass. Ctrl: control, WOS: wound leaf plus *M. sexta*'s oral secretion. Black bars represent UT while red and green bars represent A83 and A84 plants, respectively. The error bars represent standard error (n= 5- 6). **C**. *O*-AS are not induced after herbivory. In all panels, asterisks indicate significant differences between A83 (red asterisks) or A84 (green asterisks) and UT at a given treatment or a given time point (*t* test, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$).

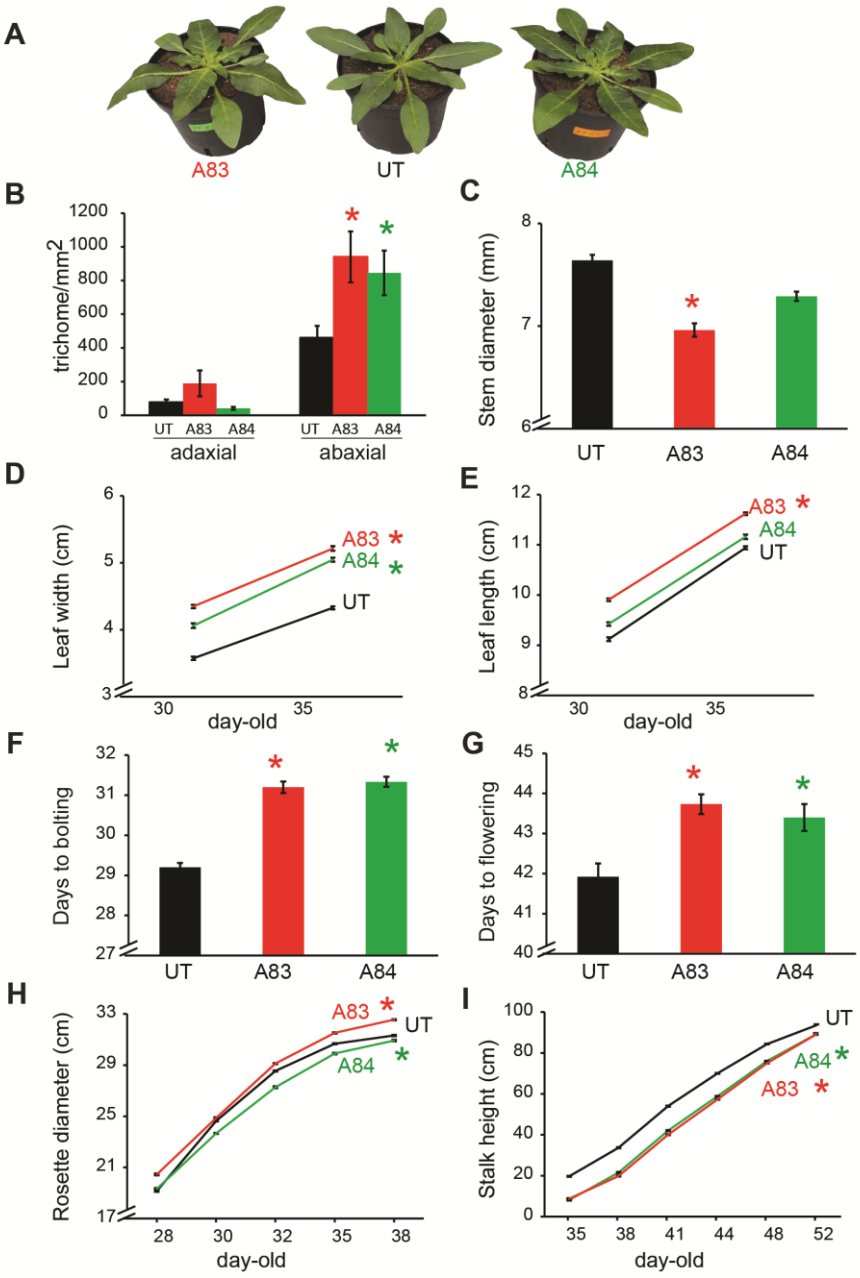

Figure S7. Morphological differences between UT, A83 and 84 plants. **A**. Representative pictures of UT, A83 and A84 plants at the rosette-stage of growth (30 day-old plants). **B**. Trichome density on the leaves of UT, A83 and A84 plants (n=5). Stem diameter (**C**), leaf width (**D**), leaf length (**E**), the number of days to bolting (**F**), the number of days to flowering (**G**), rosette diameter (**H**) and stalk height (**I**) of UT compared to that of A83 and A84. Asterisks indicate significant differences between A83 (red asterisks) or A84 (green asterisks) and UT at a given treatment (*t* test, *: P ≤0.05). Note the breaks in the Y-axis.

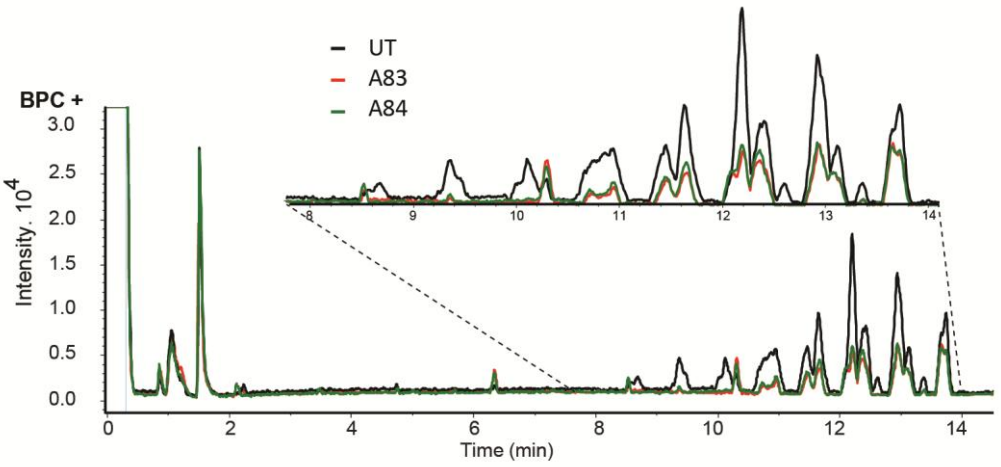

Figure S8. A83 and A84 show less *O*-acyl sugars in the water wash compared to UT. Base peak chromatogram of a positive mode UHPLC/TOF-MS analysis (BPC+) from UT (black line), A83 (red line), A84 (green line) of a 1st water leaf wash.

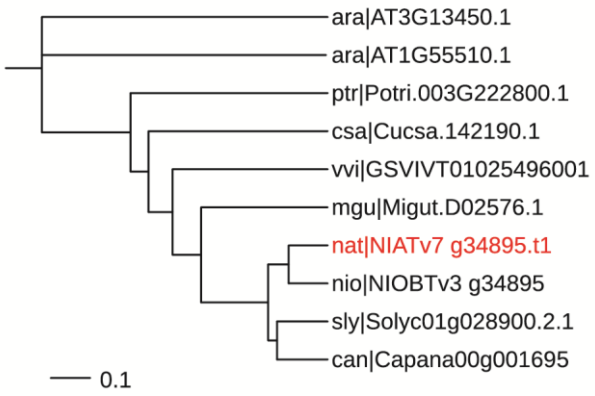

Figure S9. Phylogenetic relationship of genes encoding branched-chain alpha-keto acid dehydrogenase E1 beta subunit among *N. attenuata* and other closely related species. The tree was constructed using the neighbor-joining method based on the whole gene sequences using MEGA5 software. Sequences were aligned using ClustalW algorithm implemented in the MegAlign software. The numbers on the scale indicates distances with relative units. Species names: ara| *Arabidopsis thaliana* (*At*); ptr| *Populus trichocarpa* (*Potri*.); csa| *Cucumis sativus* (*Cucsa*); vvi| *Vitis vinifera* (*Vi*); mgu| *Mimulus guttatus* (Migut); nat| *Nicotiana attenuata* (*NIAT*); nio| *Nicotiana obtusifolia* (*NIOBT*); sly| *Solanum lycopersicum* (*Sl*); can| *Capsicum annuum* (*Capana*)

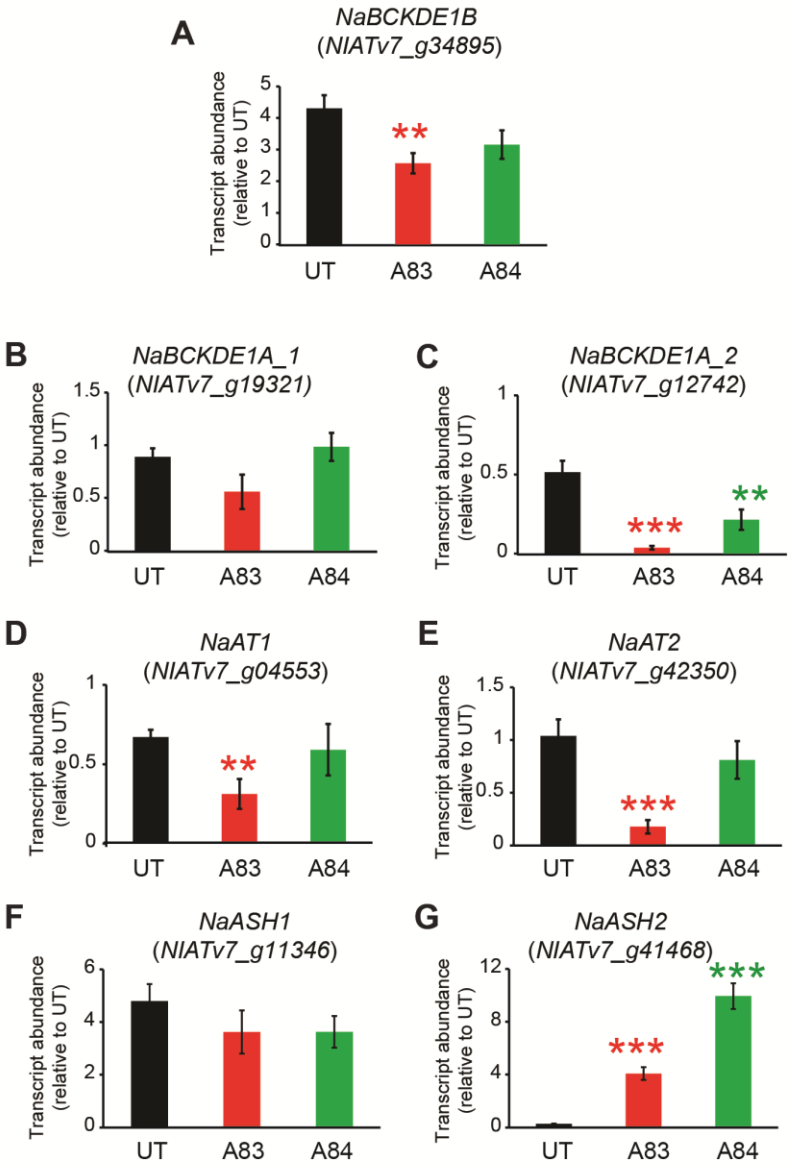

Figure S10. Gene expression in trichomes of UT in comparison to A83 and A84. Relative expression of *N. attenuata* genes encoding putative branched-chain alpha-ketoacid dehydrogenase *NaBCKDE1B* (**A**), *NaBCKDE1A* (**B**, **C**), acyltransferases *NaAT1* (**D**), *NaAT2* (**E**), acylsugar acyl hydrolase *NaASH1* (**F**), *NaASH2* (**G**) in trichomes between A83, 84 compared to UT (n=5-6). Relative gene expression was normalized to the expression of the house keeping gene *N. attenuata* actin 7. Asterisks indicate significant differences between A83 (red asterisks) or A84 (green asterisks) and UT at a given treatment (*t* test, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$).

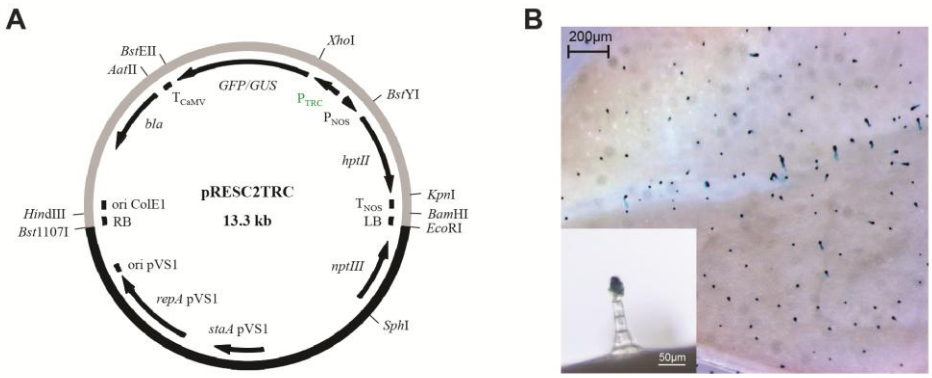

Figure S11. Expression specificity of *S. lycopersicum* trichome specific promoter (SlAT2) in *N. attenuata*. **A**. The pRESC2TRC series binary plant transformation vectors used for expressing SlAT2::GFP/GUS in *N. attenuata*. The1495bp of the trichome-specific promoter from *S. lycopersicum* SlAT2 gene was inserted into pRESC2TRC vector with the hygromycin (*hptII*) resistance gene as a selection marker. Abbreviations: LB / RB, left / right border of T-DNA; PNOS / TNOS, promoter / terminator of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens*; PTRC, trichome specific promoter from *Solanum lycopersicum* SlAT2; T35S, 35S terminator from cauliflower mosaic virus; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301 (AF234297); *nptII*,amino glycoside phosphotransferase class II; ori, origin of replication. **B**. SlAT2::GUS is expressed in tip cells of both type C and D trichome of *N. attenuata*. Images obtained using a ZEISS stereomicroscope SV 11 with 4x and Axio Zoom.V16 Stereo microscope at 180X magnification.

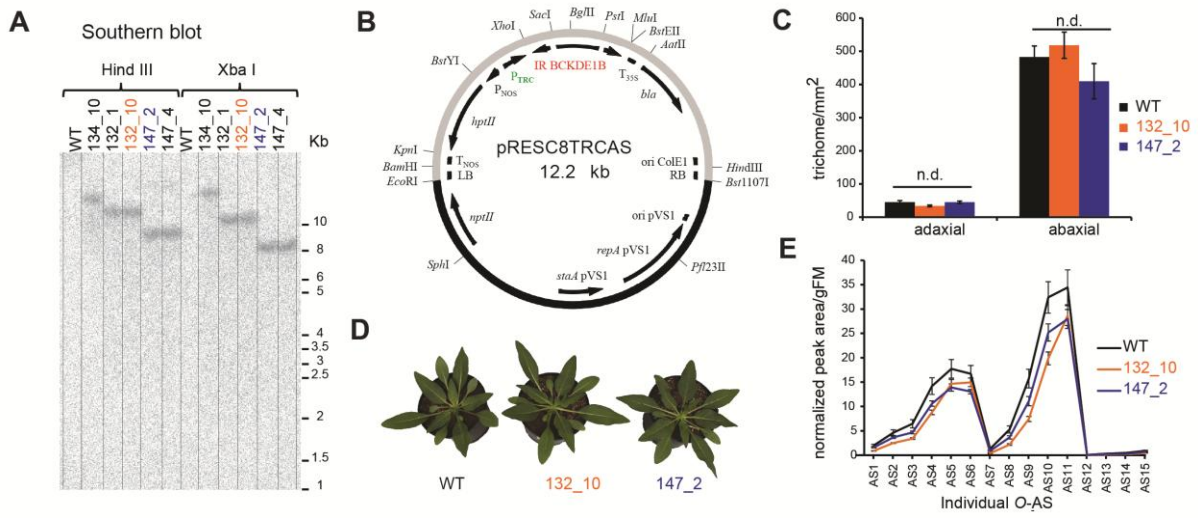

Figure S12. Creating irBCKDE1B transgenic lines using *Agrobacterium*-transformation. **A**. Two *Agrobacterium*-transformed lines irBCKDE1B-132-10 and irBCKDE1B-147-2 were subjected to Southern blot analysis using genomic DNA digested with HindIII and XbaI restriction enzyme and hptII-radiolabeled probe. Both lines showed single insertions of T-DNA fragment into the genome. **B**. The pRESC8TRCAS series binary plant transformation vectors used for silencing BCKDE1B encoding gene (*NIATv7_g34895*).A 351bp of NaBCKDE1B gene was inserted into pRESC8TRCAS vector as an inverted-repeat construct with hptII used as plant selection marker gene. Abbreviations: LB / RB, left / right border of T-DNA; PNOS / TNOS, promoter / terminator of the nopaline synthase gene from the Ti plasmid of *Agrobacterium tumefaciens*; PTRC, trichome specific promoter from *Solanum lycopersicum* SlAT2; T35S, 35S terminator from cauliflower mosaic virus; *hptII*, hygromycin phosphotransferase gene from pCAMBIA-1301 (*AF234297*); i, intron 3 of *Flaveriatrinerviapdk* gene for pyruvate, orthophosphate dikinase; *nptII*,amino glycoside phosphotransferase class II; ori, origin of replication. **C**. Trichome density of two independently transformed homozygous irBCKDE1B lines and wild-type (WT) plants (n=5). **D** The similarity of the morphology of two *Agrobacterium*-transformed lines irBCKDE1B-132-10 and irBCKDE1B-147-2 at the rosette-stage of growth. **E**. Individual *O*-AS in 2 irBCKDE1B independent lines. Error bars represent standard error. Asterisks indicate significant differences between WT and individual irBCKDE1B lines (*t* test, ***: $P \leq 0.001$, **: $P \leq 0.01$,*: $P \leq 0.05$).

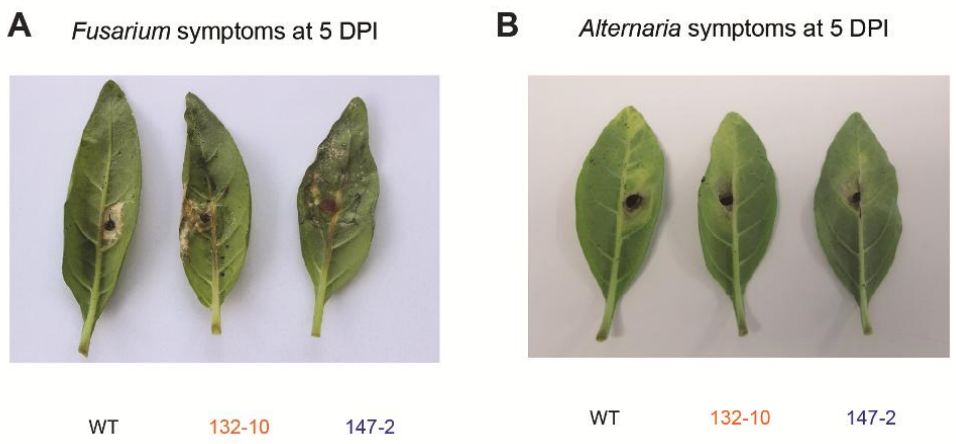

Figure S13. Detached leaf assay on irBCKDE1B transgenic lines. Necrotic lesions caused by *Fusarium* (**A**) and *Alternaria* (**B**) and on detached leaves of WT, 132-10 and 147-2 at 5 days post inoculation (DPI **)**

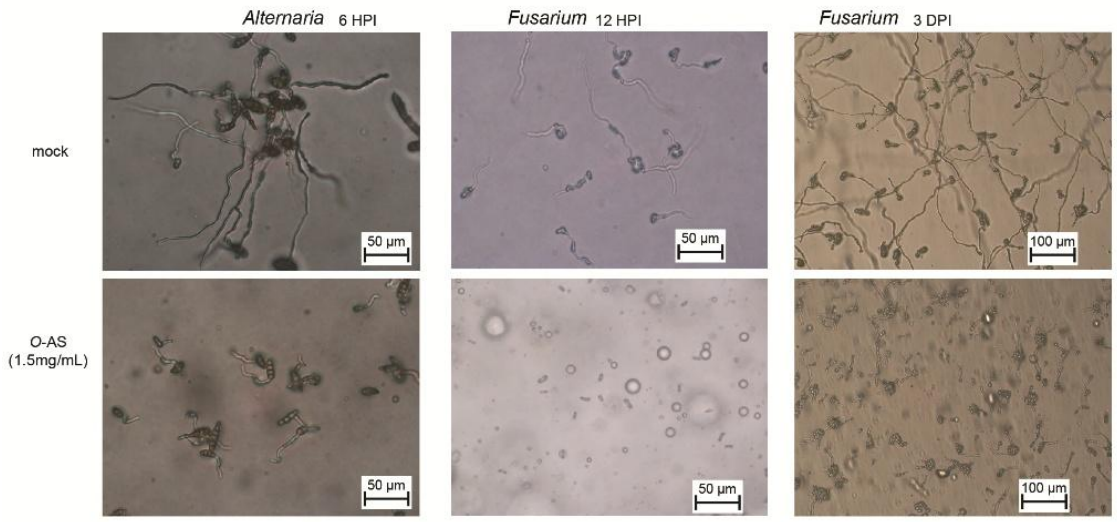

Figure S14. Effect of *O*-acyl sugars on fungal pathogens. Spore germination and hyphal length of *Alternaria* and *Fusarium* fungal on plain agar supplemented with *O*-AS concentrations 1.5 mg/mL in comparison with mock treatment at 6 and 12 h post inoculation (HPI) or 3 days post inoculation (DPI). Pictures were taken under the light microscope Axio Observer.D1 with 400 X magnification.

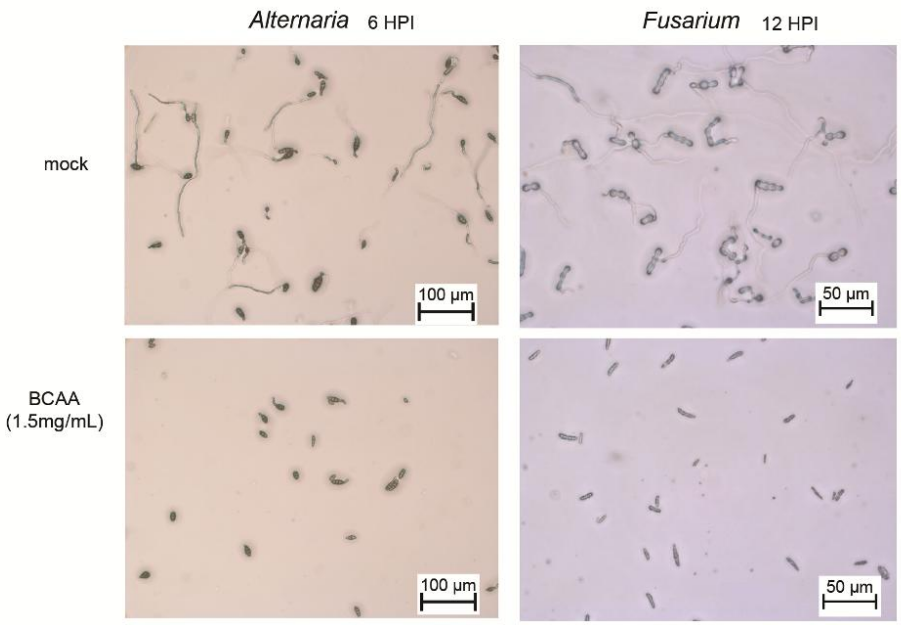

Figure S15. Effect of branched-chain aliphatic acids (BCAAs) on fungal pathogens. Spore germination and hyphal length of *Alternaria* and *Fusarium* fungal on plain agar supplemented with BCAAs at concentrations of 1.5 mg/mL in comparison with mock treatment at 6 and 12 h post inoculation (HPI). Pictures were taken under the light microscope Axio Observer.D1 with 400 X magnification.

Supplemental Tables

Table S1: GPS coordinates of 26 natural accessions

No. of accession	Accession	Latitude	Longitude
1	A84	37°19'35.48"N	113°57'38.28"W
2	A83	37°19'35.48"N	113°57'38.28"W
3	A422	35°12'44.80"N	111°28'24.80"W
4	A421	35°12'31.10"N	111°28'8.38"W
5	A278	37°16'16.22"N	114° 7'39.67"W
6	A305	37°45'19.61"N	118°35'41.82"W
7	A224	37°19'33.89"N	113°57'54.16"W
8	A138	37° 8'19.58"N	114° 1'35.10"W
9	A351	37°17'9.10"N	114° 7'31.50"W
10	UT	37°19'36.26"N	113°57'53.05"W
11	A133	37° 6'12.50"N	113°49'36.60"W
12	Az	35°13'8.62"N	111°28'26.03"W
13	A308	37°13'5.50"N	113°48'24.25"W
14	A149	35°12'56.07"N	111°27'41.29"W
15	A43	37°17'9.10"N	114° 7'31.50"W
16	A214	37°13'15.83"N	113°48'20.86"W
17	A384	37°14'27.05"N	113°49'36.71"W
18	A304	37°20'22.52"N	114° 2'40.86"W
19	A341	37° 9'45.30"N	114° 0'58.52"W
20	A331	37°13'15.83"N	113°48'20.86"W
21	A194	37°20'22.52"N	114° 2'40.86"W
22	A97	37°21'35.24"N	113°56'38.68"W
23	A382	37°14'27.05"N	113°49'36.71"W
24	A176	37°16'38.65"N	113°53'35.18"W
25	A179	37°21'1.04"N	113°57'5.17"W
26	A85	37°19'35.48"N	113°57'38.28"W

Table S2. The list of m/z and retention times (RT) used to identify and measure *O*-acyl sugars (*O*-AS). *O*-AS annotation was done as described by Kim *et al.* (2012) which includes sucrose backbone (S for sucrose), the number of acyl chains, the total number of carbons for acyl chains and the expected length of each acyl chain. For instance, S3:13(4,5,4): acyl sucrose with 3 acyl chains of 4, 5 and 4 carbons, so in total 13 carbons.

***O*-acyl sugars**	***O*-AS annotation**	**m/z**	**m/z width**	**RT(min)**	**RT width**
AS1	S3:13(4,5,4)	589.24	0.05	8.6	0.5
AS2	S3:14 (5,4,5)	603.26	0.05	9.4	0.5
AS3	S3:15 (5,5,5)	617.27	0.05	10.1	0.5
AS4	S3:16 (6,5,5)	631.29	0.01	10.9	0.5
AS5	S3:17 (6,5,6)	645.31	0.01	11.7	0.5
AS6	S3:18 (6,6,6)	659.32	0.01	12.3	0.5
AS7	S4:15 (2,4,4,5)	631.25	0.05	9.9	0.5
AS8	S4:16 (2,5,4,5)	645.27	0.01	10.7	0.5
AS9	S4:17 (2,5,4,6)	659.29	0.01	11.3	0.5
AS10	S4:18 (2,5,5,6)	673.3	0.01	12	0.5
AS11	S4:19 (2,6,5,6)	687.32	0.01	12.9	0.5
AS12	S5:17 (2,4,4,5)	673.27	0.01	11.1	0.5
AS13	S5:18 (2,4,5,5,2)	687.28	0.01	11.8	0.5
AS14	S5:19 (2,4,5,6,2)	701.29	0.01	12.6	0.5
AS15	S5:20 (2,4,6,6,2)	715.31	0.01	13.4	0.5

Table S3: Branched chain aliphatic acids (BCAAs) compositions of *N. attenuata O*-acyl sugars (*O*-AS). Retention times (RT) and indices (RI) of authentic standards are compared to those found in the extract of saponified *O*-AS. The most abundant BCAAs are highlighted (bold).

		RT	**RI**	**RT**	**RI**
	Acid	Single Standards		extracthydrolysis	
1	aceticacid	19.386	1456	19.319	1453
2	propionicacid	21.538	1543	21.603	1546
3	**2-methyl propanoicacid**	22.281	1574	22.124	1567
4	**2-methy butanoicacid**	24.679	1678	24.501	1670
5	**3-methyl butanoicacid**	24.678	1678	24.501	1670
6	**3-methyl pentanoicacid**	27.393	1802	27.265	1796
7	**4-methyl pentanoicacid**	27.601	1813	27.46	1806
8	hexanoicacid	28.536	1859	28.426	1853

Table S4: MS^2-spectral data showing the annotation of class 2 *O*-acyl sugars elemental formulas, their calculated monoisotopic masses, the elemental formula of fragment ions, and their annotations, intensity and m/z values compared to the calculated m/z values.

ID	Monoisotopic mass El Formula	Elemental Formula Fragments	Annotation	Intensity	m/z Fragment	m/z Calc	Δppm
AS1	566.2926 $C_{25}H_{42}O_{14}$	$C_{25}H_{42}O_{14}Na^+$	$[M+Na]^+$	14.68	589.2477	589.2467	1
		$C_{19}H_{32}O_9Na^+$	$[(M -C_6H_{10}O_5)+Na]^+$	100.00	427.1935	427.1938	0.3
		$C_{15}H_{24}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_4H_8O_2)+Na]^+$	4.36	339.1383	339.1414	3.1
		$C_{14}H_{22}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	5.88	325.1317	325.1257	6
		$C_6H_{10}O_5Na^+$	$[(M -C_{19}H_{32}O_9)+Na]^+$	3.33	185.0404	185.042	1.6
AS2	580.2731 $C_{26}H_{44}O_{14}$	$C_{26}H_{44}O_{14}Na^+$	$[M+Na]^+$	100.00	603.2663	603.2623	4
		$C_{20}H_{34}O_9Na^+$	$[(M -C_6H_{10}O_5)+Na]^+$	33.88	441.2048	441.2095	4.7
		$C_{15}H_{24}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_4H_8O_2)+Na]^+$	7.52	353.1583	353.1571	1.2
		$C_{15}H_{24}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	6.58	339.1448	339.1414	3.4
		$C_6H_{10}O_5Na^+$	$[(M -C_{20}H_{34}O_9)+Na]^+$	4.36	185.0404	185.042	1.6
AS3	594.2887 $C_{27}H_{46}O_{14}$	$C_{27}H_{46}O_{14}Na^+$	$[M+Na]^+$	16.82	617.2756	617.2779	2.3
		$C_{21}H_{36}O_9Na^+$	$[(M -C_6H_{10}O_5)+Na]^+$	100.00	455.2249	455.2251	0.2
		$C_{17}H_{28}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_4H_8O_2)+Na]^+$	1.83	367.1745	367.1727	1.8
		$C_{16}H_{26}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	6.13	353.1538	353.157	3.2
		$C_{15}H_{24}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	4.30	339.1365	339.1414	4.9
		$C_6H_{10}O_5Na^+$	$[(M -C_{21}H_{36}O_9)+Na]^+$	2.88	185.0406	185.042	1.4
AS4	608.3044 $C_{28}H_{48}O_{14}$	$C_{28}H_{48}O_{14}Na^+$	$[M+Na]^+$	18.98	631.2962	631.2926	3.6
		$C_{22}H_{38}O_9Na^+$	$[(M -C_6H_{10}O_5)+Na]^+$	100.00	469.2403	469.2408	0.5
		$C_{17}H_{28}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	2.54	367.1625	367.1727	10.2
		$C_{16}H_{26}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	7.66	353.1575	353.157	0.5
		$C_6H_{10}O_5Na^+$	$[(M -C_{22}H_{38}O_9)+Na]^+$	2.71	185.0419	185.042	0.1
AS5	622.3201 $C_{29}H_{50}O_{14}$	$C_{29}H_{50}O_{14}Na^+$	$[M+Na]^+$	20.23	645.3117	645.3093	2.4
		$C_{23}H_{40}O_9Na^+$	$[(M -C_6H_{10}O_5)+Na]^+$	100.00	483.2553	483.2564	1.1
		$C_{18}H_{30}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_5H_{10}O_2)+Na]^+$	2.33	381.1848	381.1883	3.5
		$C_{17}H_{28}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	9.52	367.1711	367.1727	1.6
		$C_6H_{10}O_5Na^+$	$[(M -C_{23}H_{40}O_9)+Na]^+$	3.42	185.0412	185.042	0.8
AS6	636.3249 $C_{30}H_{52}O_{14}$	$C_{30}H_{52}O_{14}Na^+$	$[M+Na]^+$	25.23	659.324	659.3249	0.9
		$C_{24}H_{42}O_9Na^+$	$[(M -C_6H_{10}O_5)+Na]^+$	100.00	497.2725	497.2721	0.4
		$C_{18}H_{30}O_7Na^+$	$[(M -C_6H_{10}O_5 - C_6H_{12}O_2)+Na]^+$	9.24	381.1893	381.1883	1
		$C_6H_{10}O_5Na^+$	$[(M -C_{24}H_{42}O_9)+Na]^+$	2.54	185.0408	185.042	1.2

Table S5: MS^2-spectral data showing the annotations of class 3 *O*-acyl sugars elemental formulas, their calculated monoisotopic masses, the elemental formula of fragment ions, their annotations, intensity and m/z values compared to the calculated m/z values.

ID	Monoisotopic mass El Formula	Elemental Formula Fragments	Annotation	Intensity	m/z Fragment	m/z Calc	Δppm
AS7	608.2680 $C_{27}H_{44}O_{15}$	$C_{27}H_{44}O_{15}Na^+$	$[M+Na]^+$	100.00	631.2554	631.2572	1.8
		$C_{23}H_{36}O_{13}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	5.99	543.199	543.2048	5.8
		$C_{19}H_{32}O_9Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	40.35	427.1928	427.1939	1.1
		$C_{14}H_{22}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	4.27	325.1222	325.1258	3.6
		$C_8H_{13}O_6^+$	$[M -C_{19}H_{31}O_9Na]^+$	5.32	205.0702	205.0706	0.4
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	6.32	97.0291	97.0285	0.6
AS8	622.2837 $C_{28}H_{46}O_{15}$	$C_{28}H_{46}O_{15}Na^+$	$[M+Na]^+$	100.00	645.2746	645.2729	1.7
		$C_{24}H_{38}O_{13}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	4.83	557.219	557.2204	1.4
		$C_{23}H_{36}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	2.55	543.2058	543.2048	1
		$C_{22}H_{34}O_{13}Na^+$	$[(M- C_6H_{12}O_2)+Na]^+$	1.48	529.1925	529.1891	3.4
		$C_{20}H_{34}O_9Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	38.54	441.2082	441.2095	1.3
		$C_{16}H_{26}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	1.57	353.1538	353.157	3.2
		$C_{15}H_{24}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	2.22	339.1365	339.1414	4.9
		$C_{14}H_{22}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	4.11	325.125	325.1258	0.8
		$C_8H_{13}O_6^+$	$[M -C_{20}H_{33}O_9Na]^+$	6.09	205.0699	205.0706	0.7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	6.79	97.0281	97.0285	0.4
AS9	636.2993 $C_{29}H_{48}O_{15}$	$C_{29}H_{48}O_{15}Na^+$	$[M+Na]^+$	100.00	659.292	659.2885	3.5
		$C_{25}H_{40}O_{13}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	2.92	571.2342	571.2361	1.9
		$C_{24}H_{38}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	3.64	557.2251	557.2204	4.7
		$C_{23}H_{36}O_{13}Na^+$	$[(M- C_6H_{12}O_2)+Na]^+$	2.38	543.2059	543.2048	1.1
		$C_{21}H_{36}O_9Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	33.45	455.2255	455.2251	0.4
		$C_{17}H_{28}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	1.14	367.1748	367.1727	2.1
		$C_{16}H_{26}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	1.41	353.1542	353.157	2.8
		$C_{15}H_{24}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	5.64	339.1431	339.1414	1.7
		$C_8H_{13}O_6^+$	$[M -C_{21}H_{35}O_9Na]^+$	7.08	205.0699	205.0706	0.7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	6.08	97.0279	97.0285	0.6

Table S5 continued:

ID	Monoisotopic mass El Formula	Elemental Formula Fragments	Annotation	Intensity	m/z Fragment	m/z Calc	Δppm
AS10	650.315 $C_{30}H_{50}O_{15}$	$C_{30}H_{50}O_{15}Na^+$	$[M+Na]^+$	100.00	673.305	673.3041	0.9
		$C_{25}H_{40}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	2.72	571.2365	571.2361	0.4
		$C_{24}H_{38}O_{13}Na^+$	$[(M- C_6H_{12}O_2)+Na]^+$	4.80	557.2244	557.2204	4
		$C_{22}H_{38}O_9Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	31.03	469.2377	469.2408	3.1
		$C_{17}H_{28}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	1.95	367.1767	367.1727	4
		$C_{16}H_{26}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	3.46	353.155	353.157	2
		$C_8H_{13}O_6^+$	$[M -C_{22}H_{37}O_9Na]^+$	5.67	205.07	205.0706	0.6
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3.36	97.0276	97.0285	0.9
AS11	664.3306 $C_{31}H_{52}O_{15}$	$C_{31}H_{52}O_{15}Na^+$	$[M+Na]^+$	100.00	687.3213	687.3198	1.5
		$C_{26}H_{42}O_{13}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	1.69	585.2495	585.2517	2.2
		$C_{25}H_{40}O_{13}Na^+$	$[(M- C_6H_{12}O_2)+Na]^+$	4.84	571.2363	571.2361	0.2
		$C_{23}H_{40}O_9Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	30.06	483.2559	483.2565	0.6
		$C_{17}H_{28}O_7Na^+$	$[(M -C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	5.62	367.1723	367.1727	0.4
		$C_8H_{13}O_6^+$	$[M -C_{23}H_{39}O_9Na]^+$	5.41	205.0713	205.0706	0.7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3.72	97.0285	97.0285	0

Table S6: MS^2-spectral data showing the annotations of class 4 *O*-acyl sugars elemental formulas, their calculated monoisotopic masses, the elemental formula of fragment ions, and their annotations, intensity and m/z values compared to the calculated m/z values.

ID	Monoisotopic mass El Formula	Elemental Formula Fragments	Annotation	Intensity	Fragment Mass	Calc	Δppm
AS 12	650.2786 $C_{29}H_{46}O_{16}$	$C_{29}H_{46}O_{16}Na^+$	$[M+Na]^+$	100.00	673.2723	673.2678	4.5
		$C_{25}H_{38}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	3.31	585.2072	585.2153	8.1
		$C_{24}H_{36}O_{14}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	4.42	571.1896	571.1997	10.1
		$C_{21}H_{34}O_{10}Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	51.73	469.205	469.2044	0.6
		$C_{17}H_{26}O_8Na^+$	$[(M -C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	8.37	381.1516	381.1519	0.3
		$C_8H_{13}O_6^+$	$[M -C_{21}H_{33}O_{10}Na]^+$	7.53	205.0727	205.0706	2.1
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3.72	97.0263	97.0285	2.2
AS 13	664.2942 $C_{30}H_{48}O_{16}$	$C_{30}H_{48}O_{16}Na^+$	$[M+Na]^+$	100.00	687.2861	687.2835	2.6
		$C_{26}H_{40}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	3.36	599.2303	599.231	0.7
		$C_{25}H_{38}O_{14}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	4.52	585.2123	585.2153	3
		$C_{22}H_{36}O_{10}Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	47.27	483.2189	483.2201	1.2
		$C_{17}H_{26}O_8Na^+$	$[(M -C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	8.99	381.1548	381.1519	2.9
		$C_8H_{13}O_6^+$	$[M -C_{22}H_{35}O_{10}Na]^+$	10.54	205.0735	205.0706	2.9
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	7.30	97.0284	97.0285	0.1
AS 14	678.3098 $C_{31}H_{50}O_{16}$	$C_{31}H_{50}O_{16}Na^+$	$[M+Na]^+$	100.00	701.2973	701.2991	1.8
		$C_{27}H_{42}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	1.58	613.2455	613.2467	1.2
		$C_{26}H_{40}O_{14}Na^+$	$[(M - C_5H_{10}O_2)+Na]^+$	2.58	599.2348	599.231	3.8
		$C_{25}H_{38}O_{14}Na^+$	$[(M- C_6H_{12}O_2)+Na]^+$	2.90	585.2137	585.2153	1.6
		$C_{23}H_{38}O_{10}Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	43.62	497.2343	497.2357	1.4
		$C_{19}H_{30}O_8Na^+$	$[(M -C_8H_{12}O_6 - C_4H_8O_2)+Na]^+$	2.15	409.1778	409.1832	5.4
		$C_{18}H_{28}O_8Na^+$	$[(M -C_8H_{12}O_6 - C_5H_{10}O_2)+Na]^+$	5.48	395.1664	395.1676	1.2
		$C_{17}H_{26}O_8Na^+$	$[(M -C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	4.11	381.1552	381.152	3.2
		$C_8H_{13}O_6^+$	$[M -C_{23}H_{37}O_{10}Na]^+$	6.83	205.0703	205.0706	0.3
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	3.96	97.0299	97.0285	1.4
AS 15	692.3225 $C_{32}H_{52}O_{16}$	$C_{32}H_{52}O_{16}Na^+$	$[M+Na]^+$	100.00	715.3165	715.3148	1.7
		$C_{28}H_{44}O_{14}Na^+$	$[(M - C_4H_8O_2)+Na]^+$	2.46	627.2641	627.2623	1.8
		$C_{26}H_{40}O_{14}Na^+$	$[(M- C_6H_{12}O_2)+Na]^+$	5.74	599.2382	599.231	7.2
		$C_{24}H_{40}O_{10}Na^+$	$[(M -C_8H_{12}O_6)+Na]^+$	34.86	511.2514	511.2513	0.1
		$C_{18}H_{28}O_8Na^+$	$[(M -C_8H_{12}O_6 - C_6H_{12}O_2)+Na]^+$	6.68	395.1661	395.1676	1.5
		$C_8H_{13}O_6^+$	$[M -C_{24}H_{39}O_{10}Na]^+$	5.07	205.0733	205.0706	2.7
		$C_5H_5O_2^+$	$[C_8H_{13}O_6 - C_2H_4O_2 - HCHO - H_2O]^+$	2.24	97.0289	97.0285	0.4

Literature Cited

Kim J, Kang K, Gonzales-Vigil E, Shi F, Jones AD, Barry CS, Last RL (2012) Striking natural diversity in glandular trichome acylsugar composition is shaped by variation at the acyltransferase2 locus in the wild tomato *Solanum habrochaites*. Plant Physiology **160:** 1854-1870

Supplemental Methods

O-AS isolation and characterization

To isolate *O*-AS from *N. attenuata* plants, we used a protocol similar to that of Van Dam and Hare (1998). All plant parts except the flowers were harvested. The stem was cut into small pieces (approx. 10cm). Around 1 kg of tissue was combined in a 5 L glass beaker (Schott) and soaked in 3 L of chloroform for 1h of stirring. Afterwards, the plant tissue was removed and the solvent was dried over sodium sulfate (anhydrous Sigma-Aldrich). The chloroform was removed in a rotary evaporator and the residue was resolved in acetonitrile (ACN) (VWR International) and sonicated. The ACN phase was partitioned 3 times against n-hexane (VWR International) (ACN: n-Hexane 1:2). The hexane phase was discarded and the ACN was removed in a rotary evaporator. The residue was solved in dichloromethane (DCM) (VWR) and partitioned 3 times against 1N tartaric acid (Sigma-Aldrich) 2 times against distilled water. Afterward, the DCM was removed and the glue-like, brownish yellow residue was kept under argon at 4°C until further use for bioassay and for MS^2 experiment. A small portion was dissolved in 40% methanol and analyzed by ultra-high performance liquid chromatography/ time-of-flight mass spectrometry (UHPLC/TOF-MS) to verify the extraction.

To fragment the *O*-AS for MS^2 experiment, extracted *O*-AS was dissolved in acetonitrile to a concentration of 1mg/mL. We used an Agilent 1100 HPLC system equipped with a DAD detector. Separation was achieved on a preparative Luna 5n C18 column (250 x 10 mm, 5µm, Phenomenex) connected to a Luna 5n C-18 guard column (50 x 10 mm, 5 µm) with a mixture of deionized water (0.1 % (v/v) formic acid + 0.1% (v/v) ammonia) (solvent A) and methanol (solvent B) at a flow rate of 3 mL/min. We used an isocratic gradient with 80 % of solvent B for 20 min and then increased to 95% of solvent B in 5 min. The post-run time was 7 min. We collected fractions with a Foxy fraction collector (Isco) in 20 mL glass reaction tubes (Schott). 40 fractions of 30 s were cut starting 5 min. after injection. The fractions were transferred to scintillation vials and the solvent was evaporated in a vacuum centrifuge (Eppendorf). The single fractions were then analyzed for their content by injection into an UHPLC/TOF-MS system (BrukerDaltonik, Bremen, Germany) with conditions described in Weinhold and Baldwin (2011).

For MS^2 experiments, 1µL of each fraction was separated using a Dionex RSLC system (Dionex, Sunnyvale, USA) with a Dionex Acclaim RSLC 120 C-18 column (150 x 2.1 mm, 2.2 µm). The following binary gradient was applied: 0 to 1 min isocratic 90% A (deionized water, 0.1% (v/v) acetonitrile (Baker, HPLC grade), and 0.05% formic acid), 10% B (acetonitrile and 0.05%formic acid); 1 to 9 min linear gradient to 80% B; isocratic for 2 min. The flow rate was 400 µL/min. MS detection was carried out with an ultra-high performance liquid chromatography/quadrupole time-of-flight mass spectrometry (UHPLC/Q-TOF-MS) (BrukerDaltonik, Bremen, Germany) operated in positive electrospray mode. Typical instrument settings were as follows: capillary voltage, 4500 V; dry gas temperature, 180 °C; dry gas flow, 10 L/min. Ions were detected from m/z 50 to 1400 at a repetition rate of 1 Hz. The instrument was operated in autoMS/MS mode at various CID voltages from 5 to 75 eV for sodium adducts. Mass calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid). Elemental formula and masses were calculated with the ACD/Labs 12 ChemSketch calculating tool (ACD/Labs, Frankfurt, Germany).

Analysis of *O*-AS acid composition

One mg of a crude *O*-AS mixture, obtained as described above, was saponified by adding 1 mL of a 0.2 M aqueous potassium hydroxide solution (Sigma-Aldrich). The solution was sonicated and kept in a sealed vial for 24 h. The mixture was neutralized by adding 1 mL of a 0.2 M hydrochloric acid solution and then partitioned against 1.5 mL dichloromethane (VWR). One µL of the dichloromethane phase was then injected into a Varian 3800 gas chromatograph equipped with a ZB-Wax-plus column (30m x 0.25mm x 0.25µm, Restek) and a flame ionization detector (GC-FID) (Agilent). The injector temperature was set to 230°C and the flow was 1 mL/min (constant flow). The oven was kept at 40°C for 5 min, then heated to 185°C at a rate of 5°C/min and finally with a rate of 30°C/min to 250 °C. The FID was operated at 250°C with 25 mL/min make up gas flow and 30 mL/min hydrogen and 300 mL/min compressed air flow. The identities of the carboxylic acids were verified by the injection of authentic standards at a concentration of 50ng/µL. Retention indices were calculated in reference to an alkane standard mixture (C8-C20, Sigma-Aldrich).

_O_-AS relative comparison analysis

To cross-compare *O*-AS levels among different accessions or genotypes, we extracted leaf or trichome *O*-AS using a method described by Gaquerel *et al.* (2010). Briefly, approximately 100 mg of ground leaf materials (without the midvein) or 50mg trichome materials were homogenized with 2 steel beads by GenoGrinder 2000 (SPEX SamplePrep) at 1100 strokes per minute for 30 seconds and then extracted with 1 mL extraction solution (50 mM acetate buffer, pH 4.8, containing 40% methanol spiked with sucrose monolaurate (Sigma) with final concentration of 10 ng/µL as an internal standard. The extraction was done using GenoGrinder 2000 (SPEX SamplePrep) with 1100 strokes per min for 15 minutes. After centrifugation at 13,200 rpm for 20 min at 4°C, the supernatant was collected and centrifuged again. 100 µL of the supernatant was transferred to a HPLC vial and 1 µL supernatants were separated using a HPLC Dionex RSLC system (Dionex, Sunnyvale, USA) with a Dionex Acclaim RSLC 120 C-18 column (150 x 2.1 mm, 2.2 µm). The following binary gradient was applied: 0.5 min isocratic 90% A (deionized water, 0.1% (v/v) acetonitrile (Baker, HPLC grade), and 0.05% formic acid), 10% B (acetonitrile and 0.05%formic acid); 13 min linear gradient to 80% B; isocratic for 1.5 min. The flow rate was 400 µL/min. MS detection was carried out with the UHPLC/TOF-MS system (Bruker Daltonik, Bremen, Germany) operated in positive electrospray mode. Typical instrument settings were described by Gilardoni *et al.* (2011) with some modification: capillary voltage, 4500 V; dry gas temperature, 200 °C; dry gas flow, 10 L/min. Ions were detected from m/z 50 to 1400 at a repetition rate of 2 Hz. Mass calibration was performed using sodium formate clusters (10 mM solution of NaOH in 50/50% v/v isopropanol/water containing 0.2% formic acid). The peak areas were integrated using extracted ion traces for the sodium adduct [M+Na]+ of each individual *O*-AS in the QuantAnalysis software version 2.0 SP1 (Bruker Daltonics).The amount of each *O*-AS in plant tissue was normalized internal standard and the fresh weight of the tissue. Total *O*-AS was calculated by summing the normalized peak area of all 15 *O*-AS. The annotated 15 *O*-AS together with its m/z value and retention time (RT) is listed in Table S2.

Extraction and analysis of phytohormones, secondary metabolites

Phytohormone extraction was carried out as described previously by Gilardoni *et al.* (2011). Briefly, 0.1 g of frozen leaf tissue was homogenized with a Genogrinder 2000 (BTC and OPS Diagnostics). One milliliter of ethylacetate spiked with [9,10-$^{2}H_2$]-dihydro-JA and [$^{13}C_6$]-JA-Ile was added to the samples. After vortexing, the samples were centrifuged for 20 min at 12,000g (4°C). The organic phase was collected and evaporated to dryness, which were subsequently reconstituted in 300 mL of 70% (v/v) methanol/water for analysis on a Bruker Elite EvoQ Triple quad-MS equipped with a HESI (heated electrospray ionization) ion source using the MRM transitions described in (Schäfer *et al.*, 2016). Each phytohormone (JA, JA-Ile) was quantified by comparing its peak area with the peak area of its respective internal standard as described in Wu *et al.* (2007). Phytohormone levels were quantified per gram fresh mass (µg/gFM).

Nicotine and hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) were extracted as described above for *O*-AS extraction. Analysis of these metabolites was done using an HPLC-DAD method described by Keinanen *et al.* (2001) with some modification. Briefly, after extraction, 1µL of the supernatants were injected into an Agilent 1100 HPLC (Agilent HPLC 1100 Series, Palo Alto, CA) installed with a Chromolith FastGradient RP-18 (5032 mm; Merck, Darmstadt, Germany) endcapped 50 x 2 mm HPLC column (Lot No. HX802433 Merck, Darmstadt, Germany) attached to a Gemini NX RP18, 3µm, 2 x 4.6 mm precolumn (Phenomenex, Aschaffenburg, Germany) with a column oven set at 40°C. The mobile phase consisted of a mix 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. Under these conditions, nicotine eluted at a retention time (RT) of 0.5 min (detected by UV absorbance at 260 nm. HGL-DTGs were detected by evaporative light scattering detector (ELSD) after HPLC separation at RT from 7.15 to 8.31 min. The peak areas were integrated using the Chromeleon nicotine in plant tissue was calculated using external dilution series of standard

mixtures of nicotine. The peak areas were quantified to estimate total HGL-DTGs contents and normalized it to tissue fresh mass. The method was described previously by Kaur *et al.* (2010)

Ir-construct sequence of *NaBCKDE1*

GAAGATGTTGGTTTCGGTGGTGTCTTTCGTTGCACTACTGGATTAGCTGACCGATTTG
GAAAACAGAGAGTTTTTAACACTCCTTTATGTGAGCAGGGCATAGTTGGATTTGCTA
TTGGTCTGGCTGCAATGGACAATCGAGCTATAGCAGAAATTCAATTTGCAGATTATA
TTTTTCCTGCTTTCGATCAGATCGTCAATGAAGCTGCGAAATTCAGATATAGGAGTG
GTAATCAGTTCAACTGCGGAGGCTTAACTATAAGAGCACCTTATGGAGCTGTTGGAC
ATGGCGGGCATTACCACTCACAATCCCCTGAATCTTTCTTCTGCCATGTTCCTGGTAT
AAAGGTG

RNA extraction, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from approximately 50 mg of frozen leaf or trichome tissue with Trizol (Thermo scientific, 15596-026), followed by DNase-I treatment (Thermo Scientific) according to the manufacturer's instructions. The cDNA was synthesized from 2 µg of total RNA using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, K162). Quantitative real-time PCR was conducted with synthesized cDNA using the Takyon™ qPCR Kits for SYBR® assays (Eurogentec) and gene-specific primer pairs using Mx3005P PCR cycler (Stratagene). Relative gene expression was calculated from calibration curves obtained by analysis of dilution series of cDNA samples, and the values were normalized by the expression of housekeeping gene *N. attenuata* actin 7. All reactions were performed using the following qPCR conditions: initial denaturation step of 95°C for 3 min, followed by 40 cycles each of 95°C for 10 s, 60°C for 20s and 72°C for 40s, followed by melting curve analysis of PCR products.

Primer sequences for SYBR-qPCR

NaBCKDE1B_For: 5'- GTATAAAGGTGGTCATCC-3'
NaBCKDE1B_Rev: 5'- GCAACATATAATCATCTTCA-3'

Na_Actin 7_For: 5'- TTCTTCGTCTGGACCTTGCT-3'
Na_Actin 7_Rev: 5'- ATCATGGATGGCTGGAAGAG-3'

NaBCKDE1A_1_For: 5'- CAATACATTATGGCTCTAAC -3'
NaBCKDE1A_1_Rev: 5'- GTCCATTTTCAGAGAATAAG -3'

NaBCKDE1A_2_For: 5'- GACCCAGTAACTAGATTCAG -3'
NaBCKDE1A_2_Rev: 5'- GTAAATACATGCTTAATTGG -3'

NaAT1_For: 5'- CTTATTCATCCAAGCAGTA -3'
NaAT1_Rev: 5'- AAGATAGTACCTCTTCTGG -3'

NaAT2_For: 5'- GTTCATCCAAAAGTTTTAC -3'
NaAT2_Rev: 5'- TCACAGCATGGACTAATG -3'

NaASH1_For: 5'- GAACTTTATGGCATAGTTG -3'
NaASH1_Rev: 5'- GTAGTAAACTAAGACGGGTAG -3'

NaASH2_For: 5'- GTTTTCTCTAAAGACGTCAC -3'
NaASH2_Rev: 5'- TGACGTAACAGTGATTCC -3'

Literature Cited

Gaquerel E, Heiling S, Schoettner M, Zurek G, Baldwin IT (2010) Development and validation of a liquid chromatography-electrospray ionization-time-of-flight mass spectrometry method for induced changes in *Nicotiana attenuata* leaves during simulated herbivory. Journal of Agricultural and Food Chemistry **58:** 9418-9427

Gilardoni PA, Hettenhausen C, Baldwin IT, Bonaventure G (2011) *Nicotiana attenuata* LECTIN RECEPTOR KINASE1 suppresses the insect-mediated inhibition of induced defense responses during *Manduca sexta* herbivory. The Plant Cell **23:** 3512-3532

Kaur H, Heinzel N, Schottner M, Baldwin IT, Galis 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 Physiology **152:** 1731-1747

Keinanen M, Oldham NJ, Baldwin IT (2001) Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. Journal of Agricultural and Food Chemistry **49:** 3553-3558

Schäfer M, Brütting C, Baldwin IT, Kallenbach M (2016) High-throughput quantification of more than 100 primary- and secondary-metabolites, and phytohormones by a single solid-phase extraction based sample preparation with analysis by UHPLC–HESI–MS/MS. Plant Methods **12:** 30

Van Dam NM, Hare JD (1998) Biological activity of *Datura wrightii* glandular trichome exudate against *Manduca sexta* larvae. Journal of Chemical Ecology **24:** 1529-1549

Weinhold A, Baldwin IT (2011) Trichome derived *O*-acyl sugars are a first meal for caterpillars that tags them for predation. Proceedings of the National Academy of Sciences of the United States of America **108:** 7855-7859

Wu J, Hettenhausen C, Meldau S, Baldwin IT (2007) Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. The Plant Cell **19:** 1096-1122

Chapter 4: Manuscript II

Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping

Rakesh Santhanam*, **Van Thi Luu***, Arne Weinhold*, Jay Goldberg, Youngjoo Oh and Ian T. Baldwin

*: equal contribution

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Diseased *N. attenuata* in the field (Photos Arne Weinhold)

PNAS PLUS

Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping

Rakesh Santhanam[1], Van Thi Luu[1], Arne Weinhold[1], Jay Goldberg, Youngjoo Oh, and Ian T. Baldwin[2]

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Jena 07745, Germany

Edited by Jeffery L. Dangl, Howard Hughes Medical Institute and The University of North Carolina at Chapel Hill, Chapel Hill, NC, and approved August 6, 2015 (received for review March 23, 2015)

Plants maintain microbial associations whose functions remain largely unknown. For the past 15 y, we have planted the annual postfire tobacco *Nicotiana attenuata* into an experimental field plot in the plant's native habitat, and for the last 8 y the number of plants dying from a sudden wilt disease has increased, leading to crop failure. Inadvertently we had recapitulated the common agricultural dilemma of pathogen buildup associated with continuous cropping for this native plant. Plants suffered sudden tissue collapse and black roots, symptoms similar to a *Fusarium–Alternaria* disease complex, recently characterized in a nearby native population and developed into an in vitro pathosystem for *N. attenuata*. With this in vitro disease system, different protection strategies (fungicide and inoculations with native root-associated bacterial and fungal isolates), together with a biochar soil amendment, were tested further in the field. A field trial with more than 900 plants in two field plots revealed that inoculation with a mixture of native bacterial isolates significantly reduced disease incidence and mortality in the infected field plot without influencing growth, herbivore resistance, or 32 defense and signaling metabolites known to mediate resistance against native herbivores. Tests in a subsequent year revealed that a core consortium of five bacteria was essential for disease reduction. This consortium, but not individual members of the root-associated bacteria community which this plant normally recruits during germination from native seed banks, provides enduring resistance against fungal diseases, demonstrating that native plants develop opportunistic mutualisms with prokaryotes that solve context-dependent ecological problems.

Fusarium | microbiome function | plant disease resistance | *Nicotiana attenuata* | *Alternaria*

Eukaryotes maintain many complex relationships with the microbes they host, which can be so abundant and diverse that they frequently are considered a eukaryote's second genome. The complex relationships mediated by microbial associates are being revealed rapidly, thanks to the advances in sequencing, microbial culturing techniques, and the reconstitution of associated microbial communities in gnotobiotic systems (1, 2), even if some of these putative functional roles may need to be evaluated more critically (3).

When plants germinate from their seed banks, they typically acquire a selection of the diverse fungi and bacteria that exist in native soils, and a subset of this community becomes root-associated. The best characterized are the bacterial microbiomes of *Arabidopsis thaliana*. Approximately half of the bacterial community in the plant root is representative of the soil flora; the remainder is a conserved core consisting of a smaller number of bacterial lineages from three phyla: Actinobacteria, Proteobacteria, and Bacteroidetes (2, 4). Because these bacterial communities occur in nondiseased plants, they are thought to represent commensalistic or possibly mutualistic associations.

Root-associated microbes could benefit plants in many ways, and a recent review (5) highlighted the parallel functional roles of the microbiomes of the human gut and those of plant roots. The best-characterized beneficial functions for plants are (*i*) the plant growth-promoting rhizobacteria (PGPR), which promote growth by a variety of direct and indirect means that include increasing nutrient availability, interfering with ethylene (ET) signaling, and preventing diseases (6), and (*ii*) the bacteria that elicit induced systemic resistance (ISR) (7) by activating jasmonic acid (JA) and ET signaling (8). PGPR and ISR have been studied in a variety of cultivated and model plants, usually with model microbes (5), but little is known about their ecological context or whether they increase the growth and fitness of native plants. Whether PGPR and ISR functions occur among the well-characterized root-associated bacterial communities of *Arabidopsis*, either collectively or individually, also remains unknown.

The well-described agricultural phenomenon of disease-suppressive soils that harbor microbiomes that suppress particular soil-borne pathogens (9) illustrates the complexity of the dynamics involved. Native soils have a certain degree of pathogen-suppressive ability, frequently seen when a crop is grown continuously in a soil, suffers an outbreak of a disease, and subsequently becomes resistant to the disease (5). Perhaps the mechanisms involved are best understood in a root disease of wheat caused by *Gaeumannomyces graminis* var *Tritici* infections, known as "take-all" disease.

Significance

Plant roots associate with the diverse microbial community in soil and can establish mutualistic relationships with microbes. The genetic characterization of the plant microbiome (total microbiota of plants) has intensified, but we still lack experimental proof of the ecological function of the root microbiome. Without such an understanding, the use of microbial communities in sustainable agricultural practices will be poorly informed. Through continuous cropping of a seed-sterilized native plant, we inadvertently recapitulated a common agricultural dilemma: the accumulation of phytopathogens. Experimental inoculations of seeds with native bacterial consortium during germination significantly attenuated plant mortality, demonstrating that a plant's opportunistic mutualistic associations with soil microbes have the potential to increase the resilience of crops.

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Data deposition: Sequences for LK020799–LK021108 and LN556288–LN556387 have been deposited in the European Nucleotide Archive database and KR906683–KR906715 in The National Center for Biotechnology Information.

[1]R.S., V.T.L., and A.W. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: Baldwin@ice.mpg.de.

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ECOLOGY

After many years of continuous wheat cropping with several disease outbreaks, the disease suddenly wanes, apparently because of the build-up of antagonistic *Pseudomonas* spp. (9). Whether any of these interactions also occur in native plants remains unknown.

Nicotiana attenuata, a native annual tobacco of North America, germinates from long-lived seed banks to grow in the immediate postfire environment (10). When *N. attenuata* seeds germinate from their seed banks, they acquire a root-associated microbiome from their native soils which has been characterized by pyrosequencing and culture-dependent approaches (11–14). The composition of the root-associated microbiome is not influenced by a plant's ability to elicit JA signaling (14), but ET signaling, as mediated by the ability both to produce and to perceive ET, plays a decisive role in shaping the "immigration policy" for the root-associated microbiome (12). A certain *Bacillus* strain, B55, was isolated from the roots of an ET-insensitive *N. attenuata* plant (35S etr-1) and was able to rescue the impaired-growth and high-mortality phenotype of ET-insensitive plants under field conditions (15). Beneficial effects were attributed to B55's ability to reduce sulfur and produce dimethyl disulfide, which *N. attenuata* uses to alleviate sulfur deficiencies. This rescue provided one of the first demonstrations that the soil bacteria recruited by plants during germination can form opportunistic mutualistic relationships with their host based on the host plant's ecological context. Here we provide a second example that involves protection against a sudden wilt disease, which accumulated in a field plot after consecutive planting of *N. attenuata* seedlings.

Results and Discussion

Emergence of the Sudden Wilt Disease. For the past 15 y, we have planted the wild tobacco *N. attenuata* continuously in a field plot at Lytle Ranch Preserve, located in the plant's native environment of the Great Basin Desert, Utah. Seeds were germinated on sterilized medium, and young plants were first transferred to Jiffy peat pellets, to acclimate them to the environmental conditions, before they were planted in the field plot (Movie S1).

We observed the sporadic occurrence of a sudden wilt disease 8 y ago, which first affected elongated plants, causing them to wilt and die rapidly. In addition to the wilting symptoms, the normally white roots became black, and the two symptoms together (wilting plus black roots) were considered diagnostic of a plant being affected by the sudden wilt disease (Fig. S1). Plant mortality increased gradually over the years, and plants began to show symptoms at earlier developmental stages. By the end of the field season 2012, more than half (584 of 1,069) of the *N. attenuata* plants on the original (hereafter, "Old") plot, including different transgenic lines, showed these wilting symptoms and died; this value likely underestimates the actual death rate, because plants replaced during the early establishment stage (during the first 10 d after planting) were not included in this count. The sudden wilt disease seems to be specific for *N. attenuata*, because other plants or weeds growing on the plot were unaffected (Fig. S1). Interestingly, *Nicotiana obtusifolia*, which also is native to the Great Basin Desert, seemed to be less affected during the 2012 field season, because only 2 of 12 *N. obtusifolia* plants on the Old plot died. The emergence of the sudden wilt disease recapitulates a common agricultural dilemma that results from the accumulation of plant pathogens after continuous cropping and reuse of the same area for several years (16, 17). To avoid this problem, crop rotation is nearly as old as agriculture itself and entails the use of different crops in succession to interrupt the disease cycle of plant pathogens (18, 19). Because crop rotation was not an option for our research program, we compared the effectiveness of different disease-control methods, including biocontrols, fungicide treatment, and soil amendments, for *N. attenuata* planted in the Old plot.

***Alternaria* and *Fusarium* Fungal Phytopathogens Were Abundant in the Roots of Diseased Plants.** To identify and work with the microbial culprits of the sudden wilt disease, we isolated bacteria and fungi from the roots of diseased *N. attenuata* plants grown in the Old plot. A total of 36 fungal and 70 bacterial isolates were retrieved from the roots of diseased plants (Fig. 1 and Dataset S1). Based on the sudden wilt symptoms and the literature, we expected to find the bacterial plant pathogen *Ralstonia solanacearum*, because its ability to cause wilting symptoms in solanaceous plants is well known (20). Among 70 bacterial isolates, the only potential plant pathogens were *Pseudomonas tremae* and *Curtobacterium flaccumfaciens* (21), but both were recovered at low frequencies (≤2%). In contrast, isolates of plant pathogenic fungi of the *Fusarium* and *Alternaria* genera were abundant (Fig. 1). *Fusarium oxysporum* was the most abundant (25%), followed by *Fusarium solani* (22%) and different *Alternaria* species, which together represented ∼21% of the isolates.

Wilt diseases in solanaceous plants can be caused by various pathogens, such as *Fusarium* wilt (*F. oxysporum*) or bacterial wilt (*R. solanacearum*) (22, 23). Because *Fusarium* spp. and *Alternaria* spp. were isolated in abundance from diseased roots, we considered them to be the potential causal agents of the sudden wilt disease. The repeated planting of *N. attenuata* violated the natural disease-avoidance strategy of the plant's normally ephemeral, fire-chasing populations and likely led to an accumulation of pathogens. Moreover because our experimental procedures use sterile medium for germination and a preadaption period in Jiffy peat pellets, the roots' contact with the bacterial community in the native soil in the field occurs weeks after germination, and one of the strong inferences of this study is that the recruitment of beneficial microbes occurs soon after germination. Hence, these plants may lack the opportunity to recruit microbes from the surrounding soil at an early stage of their development and therefore lack the appropriate microbial community required for pathogen resistance. Whether plants acquire bacteria during the early stage of growth in Jiffy pellets is not known, but if they do, then these bacterial recruits are unable to protect the plants against the wilt disease. Furthermore, previous work (14) demonstrated that isogenic field-grown *N. attenuata* plants harbor highly divergent bacterial root communities that likely reflect spatial differences in soil microbial communities; from this variability we infer that microbes acquired during growth in the Jiffy pellets do little to shape the plant bacterial community that is retained throughout growth in the field (14). An additional vulnerability factor that likely contributed to the accumulation of specialized pathogens (24) is that our plantation populations are de facto genetic monocultures, in stark contrast with the high genetic diversity of native populations, which likely is a result of the long-lived seed banks and the differential recruitment of different cohorts into populations after fires (25, 26).

In Vitro Tests of Fungicide, Bacterial, and Fungal Treatments Reduced *N. attenuata* Seedling Mortality. A native fungal outbreak was used to develop an in vitro pathosystem for *N. attenuata* with native isolates (13). In this study, we used this pathosystem to test different strategies of minimizing the occurrence of the sudden wilt disease in the field.

For the in vitro tests, we used two fungal isolates: *Fusarium oxysporum* U3, isolated from the roots of diseased *N. attenuata* plants from the Old plot, and *Alternaria* sp. U10 from the established pathosystem described in ref. 13. With these fungi we examined biocontrol strategies and fungicide application that could provide resistance. Biocontrols are beneficial microbes that protect plants from microbial pathogens (27). For the biocontrol treatments, we used four native fungal isolates, *Chaetomium* sp. C16, C39, and C72 and *Oidodendron* sp. Oi3, which were isolated from diseased plants but were reported to be potential biocontrol agents (28, 29), and six native bacterial isolates (*Arthrobacter nitroguajacolicus* E46, *Bacillus cereus* CN2, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176), which had been

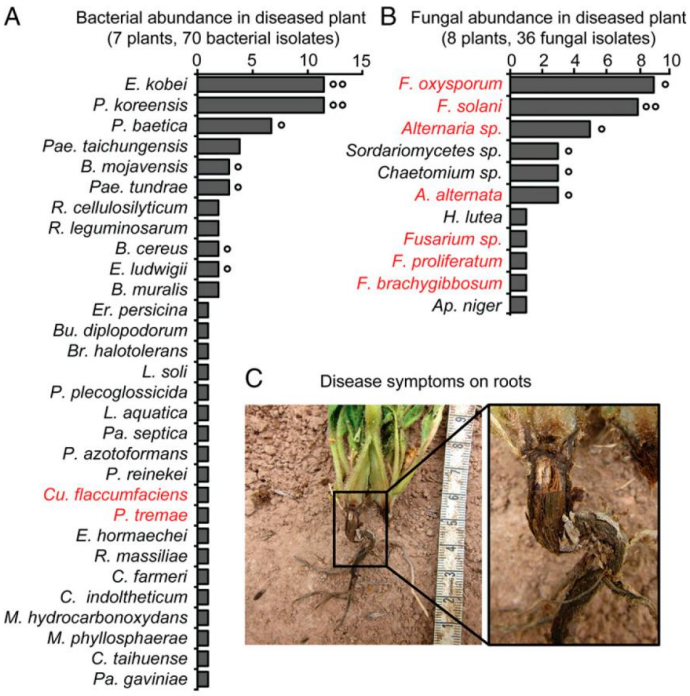

Fig. 1. Abundance of bacteria and fungi isolated from the roots of diseased *N. attenuata* plants. Abundance of culturable bacteria and fungi isolated from native field-grown plants exhibiting the sudden wilt disease symptoms. Potential plant pathogens are in red font. (*A*) Only two potential bacterial pathogens (*C. flaccumfaciens* and *P. tremae*) were found in the 70 members of the bacterial community retrieved from the roots of seven diseased plants. (*B*) In contrast, potential fungal pathogens (*Alternaria* and *Fusarium*) were abundant among the 36 culturable isolates of the fungal community from the roots of eight diseased plants. Isolates, which were found in two or more or four or more plants are indicated by (°) and (°°), respectively. Bacterial genus acronyms: B, *Bacillus*; Br, *Brevibacterium*; Bu, *Budvicia*; C, *Chryseobacterium*; Ci, *Citrobacter*; Cu, *Curtobacterium*; E, *Enterobacter*; Er, *Erwinia*; L, *Leifsonia*; M, *Micrococcus*; P, *Pseudomonas*; Pa, *Pantoea*; Pae, *Paenibacillus*; R, *Rhizobium*. Fungal genus acronyms: A, *Alternaria*; Ap, *Aspergillus*; F, *Fusarium*; H, *Hypocrea*. (*C*) Symptoms of the sudden wilt disease in field-grown *N. attenuata* plants included black coloration of the roots. For details see Fig. S1.

isolated from the roots of healthy *N. attenuata* plants from the same field location (12, 14). The selection of these bacterial isolates was based on in vitro plant growth-promoting effects on *N. attenuata* (15, 30); the isolates had been reported as biocontrol agents in the literature (28, 29, 31).

The treatment of the seeds with fungicide significantly reduced seedling mortality when seedlings were challenged with *Fusarium* sp. U3 and *Alternaria* sp. U10 [U3 $t_{(1,8)} = 2.52$, $P < 0.03$; U10 $t_{(1,8)} = 8.23$, $P < 0.0001$, *t*-test] (Fig. S2). The treatment of the seeds with bacteria was most effective when all six strains were mixed, which significantly reduced mortality from both fungal pathogens [U3 $F_{7,32} = 6.6$, $P < 0.0001$; U10 $F_{7,32} = 9.1$, $P < 0.0001$, ANOVA, least significant difference (LSD)] (Fig. S2). Fungal isolates showed inconsistent effects, and some appeared to have negative effects on plant growth. Two fungal isolates, *Chaetomium* sp. C72 and *Oidodendron* sp. Oi3, were selected for field experiments because they reduced seedling mortality in seedlings inoculated with *Fusarium* sp. U3 ($F_{4,13} = 11.961$, C72, $P < 0.0001$; Oi3, $P < 0.05$, ANOVA, LSD) (Fig. S2) without negatively affecting subsequent seedling growth.

In summary, we selected the mixed bacterial inoculation, two fungal isolates (C72 and Oi3), and the fungicide for large-scale tests in the diseased Old plot. The use of biocontrol strains recently has become a popular alternative to conventional chemical treatments. However, biocontrol bacterial strains that can protect plants from phytopathogens under in vitro conditions frequently are less successful under glasshouse conditions and even might be detrimental under field conditions; this context dependence makes the screening of potential biocontrol candidates challenging (32). The use of bacterial or fungal isolates native to the host plant may increase the success rate in screening experiments, because these microbes are likely to be better adapted to their host and its associated environmental conditions than are generalist strains retrieved from culture collections (33). In agriculture, the use of such locally adapted isolates has been shown to decrease the incidence of *Fusarium* wilt disease in peanut plant (34).

Inoculation with Native Bacterial Isolates Significantly Attenuates Disease Incidence in the Field Without Slowing Plant Growth. For the field experiments in 2013, we included soil amendment as a third disease-control strategy and combined these strategies to produce seven different treatment groups: control, bacteria, fungi, fungicide 1×, fungicide 5×, charcoal, and charcoal plus fungicide (combined treatment) (Fig. 2). Because the germination of *N. attenuata* seeds is elicited by smoke, which initiates growth in burned soil, we simulated this soil condition by adding shredded charcoal as a soil amendment at the time of planting (Fig. S3). The application of pyrolyzed plant material (biochar) is a common farming practice that has been shown to have several beneficial effects on plants, increasing crop yields and mitigating

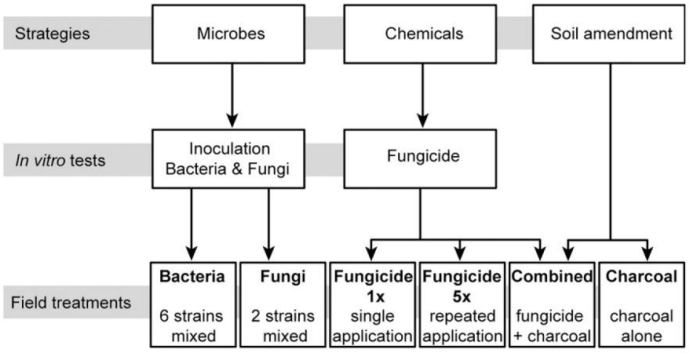

Fig. 2. Workflow of the three main strategies and the treatments used for 2013 field experiment. Of the three main strategies pursued to curb the spread of the disease in the field, the inoculation with microbes (bacteria or fungi) and fungicide treatment were first evaluated under in vitro conditions in the laboratory (Fig. S2). The mixed inoculation with six bacterial isolates, two fungal isolates, and the treatment with a commercially available fungicide in vitro reduced the mortality of *N. attenuata* seedlings infected with native isolates of fungal pathogens (*Fusarium* sp. and *Alternaria* sp.), and these treatments were selected for the field experiments. The combination of the strategies resulted in seven different treatments (including control treatment) that were deployed for the 2013 field experiment. All treatments were applied to *N. attenuata* seedlings before or during their planting into the field. The repeated fungicide treatment (fungicide 5×) was reapplied four times at 1-wk intervals after planting.

disease symptoms (35, 36). For a slow release of the fungicide, we combined the charcoal and fungicide treatments and presoaked the charcoal with the fungicide solution (combined treatment).

A total of 735 *N. attenuata* plants from the seven treatment groups were planted in the Old plot. As a control experiment, 261 plants were randomly assigned to the seven treatment groups and planted into the New plot; the two plots are located about 900 m apart, and the New plot had been used only during the previous two growing seasons without any signs of the sudden wilt disease (Fig. S3). The plants from the seven treatment groups were planted in a block in a randomized design (Fig. S4). In the Old plot, the first dead plants were observed quite early. Because these plants were still small, with a rosette diameter of about 5 cm, the black coloration of the roots was not always visible. In such cases, the cause of death could not be assigned to the sudden wilt disease and was categorized as "only wilting symptoms" (Fig. S4). Most of the plants with only wilting symptoms were observed in three treatment groups (fungi, charcoal, and combined) and contributed to the overall high mortality of these groups (Fig. S4). Three days later (15 d post planting, dpp), the majority of the newly dying plants showed the characteristic black roots (Fig. S1), as did the great majority of plants that subsequently died (Fig. S4).

The treatments fungicide 1×, fungicide 5×, and charcoal showed no significant mortality reduction compared with the control treatment, and the fungi and combined treatments, even at 15 dpp, showed elevated mortality rates compared with the controls (Fig. 3). Only the plants inoculated with the mixed bacteria showed a consistently attenuated death rate with a statistically significant reduction compared with the control plants at 15 and 18 dpp p ($P < 0.05$, G test) (Fig. 3). Over the 22-d observation period, the increase in plant mortality showed two peaks at 15 and 30 dpp (Fig. S4). At the end of the observation period, 219 of 735 plants (26.7%) on the Old plot had died, and 20.2% showed all the symptoms of the sudden wilt disease (Fig. 3 and Fig. S4). As in the previous season, *N. obtusifolia* plants showed lower mortality than *N. attenuata* and seemed to be substantially more resistant to the sudden wilt disease (Fig. S4). In contrast, none of the plants from the seven treatment groups on the New plot died or showed symptoms of the sudden wilt disease, even though all treatments and planting procedures were performed identically on the Old and New plots.

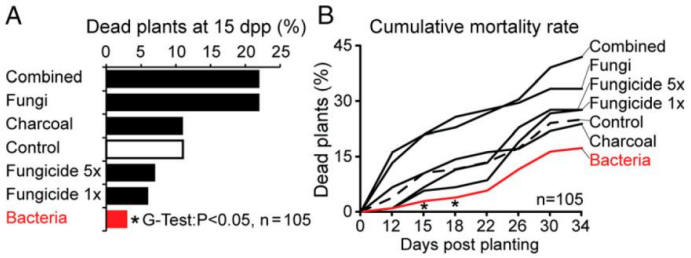

Fig. 3. Efficiency of the different treatments in reducing the mortality of field-grown *N. attenuata* plants (2013 field season). Plants in the different treatment groups (fungi, charcoal, fungicide 5×, fungicide 1×, bacteria, and combined charcoal + fungicide) were planted together with control plants in a randomized design on the Old (diseased) field plot (see Materials and Methods). (*A*) Plant mortality at 15 dpp was significantly reduced in the bacterially treated group compared with the control plants (G test: $P < 0.05$, $n = 105$ plants per group). (*B*) The increase in plant mortality was observed every 3 or 4 d for a 22-d observation period. The plants receiving the bacterial treatment had the lowest overall mortality rate. For details of the spatial distribution of plants and the rate of change in mortality see Fig. S4.

In addition to mortality, we quantified the growth (rosette diameter and stalk height) of all plants from both field plots. The combined and fungi-treated plants had the highest mortality rates and the strongest reductions in growth on both field plots (Fig. 4) (Old plot, rosette diameter, $F_{6,515} = 10.13$; $P < 0.0001$, fungal and combined $P < 0.05$, ANOVA, LSD; stalk height, $F_{6,515} = 23.66$, $P < 0.0001$ fungal and combined $P < 0.05$, ANOVA, LSD at 34 dpp. New plot, rosette diameter, $F_{6,254} = 4.09$, $P = 0.0006$, fungal and combined $P < 0.05$, ANOVA, LSD; stalk height, $F_{6,254} = 14.36$, $P < 0.0001$, fungal and combined $P < 0.05$, ANOVA, LSD). The remaining treatments (charcoal, fungicide 1×, and fungicide 5×) did not reduce mortality and these plants were distinctly smaller on the Old plot (Fig. 4). The mixed bacteria treatment did not reduce plant growth on either field plot and was the only treatment that consistently reduced plant mortality. We conclude that although the bacteria mixture provided a biocontrol effect against the pathogen, it did not significantly increase plant growth (Old plot, rosette diameter, $F_{6,515} = 10.13$. $P < 0.0001$, bacteria $P = 0.9$, ANOVA, LSD, stalk height; $F_{6,515} = 23.66$, $P < 0.0001$, bacteria $P = 0.9$, ANOVA, LSD; for New plot data, see Table S1).

Bacteria Inoculation Did Not Influence Other Plant Performance Traits. Because our group has studied plant–herbivore interactions with plants germinated under sterile conditions, we were interested in understanding if the bacterial inoculation would alter the expression of traits known to be involved in *N. attenuata's* defense responses to attack from its native herbivore communities. We quantified the constitutive and herbivore-induced levels of phytohormones, secondary metabolites, and volatiles as well as plant biomass, reproductive output, and herbivore damage from the native herbivore community in bacterially inoculated plants. None of the 32 parameters analyzed indicated differences between the bacteria-treated and control plants (Table S1), demonstrating that the bacterial inoculations specifically influenced pathogen resistance but not traits essential for herbivore resistance.

Consortium of Bacteria Provide the Protection. A combination of multiple biocontrol strains can provide improved disease control over the use of single organisms (31). Therefore, under in vitro conditions we examined the effect of bacterial consortia, each lacking a particular strain that had proved effective during the 2013 field season (Fig. S5). Because of regulatory reasons, one strain (CN2), which was classified as a potential S2 strain in Germany, had to be excluded from further experiments (*SI Materials and Methods*), reducing the mix to five isolates. Consortia lacking the isolates K1, E46, or A176 (mix minus K1, mix minus E46, and mix minus A176) were significantly less effective in reducing mortality in seedlings inoculated with *Alternaria* sp. U10 than the mix of all five strains ($F_{6,48} = 34.9$, $P < 0.0001$, ANOVA, LSD), indicating that these strains are essential for the protective effect. Deleting the other two strains (mix minus B55 and mix minus A70) did not change seedling mortality, indicating that these bacteria alone could not protect plants effectively from the sudden wilt disease (Fig. S5). Based on these results, the consortia were split into subgroups including either two (B55 + A70) or three bacteria (K1 + A176 + E46), and these subgroups were evaluated in another field trial in 2014. Consistent with the results from the in vitro experiments, the inoculation with three bacteria (K1 + A176 + E46) or the mixture of all five bacteria (K1 + A176 + E46 + B55 + A70) reduced mortality rates in the field by 36 and 52%, respectively (Fig. 5). The inoculation with only two strains (B55 and A70) had no effect. This result indicates that the protection is not explained purely by a founder effect in which rapid root colonization blocks a niche from being colonized by other microbes, including

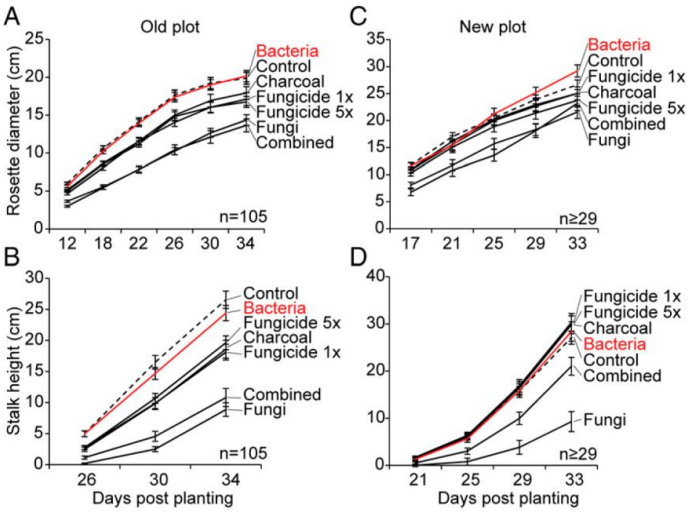

Fig. 4. Growth parameters of plants in the different treatment groups in two field plots. *N. attenuata* plants from the different treatment groups (bacteria, charcoal, fungicide 1×, fungicide 5×, fungi, and combined treatment with charcoal + fungicide) were planted together with control plants in 2013 into two field plots (Old and New), and their growth parameters (rosette diameter and stalk height) were quantified. (*A* and *B*) Mean rosette diameter and stalk height of the different treatment groups compared with control plants (dotted line) grown in the Old (diseased) plot (± SEM; $n = 105$ plants per group). (*C* and *D*) Mean rosette diameter and stalk height of plants from the different treatment groups compared with control plants (dotted line) grown in the New plot (± SEM; $n \geq 29$ plants per group). A comprehensive characterization of 32 traits known to be important for insect resistance and general ecological performance, including hormone levels and defense parameters (Table S1), was conducted on plants grown in the New plot to evaluate the effect of bacterial inoculation on traits not directly related to fungal pathogen resistance.

pathogens. The strongest mortality reduction in the field was achieved when these two strains were included in the bacterial mixture (Fig. 5), indicating that they do contribute important synergistic effects to the other strains of the consortium. Because of the lower replicate number (about half as many plants as in 2013), the 2014 results were not statistically significant ($P > 0.05$, $n = 45$, G test). Mixtures of commercial biocontrol strains sometimes combine multiple mechanisms of action to enhance the consistency of disease control (37). These synergistic mechanisms include the many different forms of antibiosis, biofilm formation, and founder effects as well as mechanisms that function indirectly through the host by eliciting systemic resistance (e.g., ISR) (7, 38).

Persistence of Biocontrol Bacteria in Late-Stage Plants. For effective suppression of pathogens under competitive natural conditions, biocontrol strains need to be excellent colonizers and persist as root endophytes (39, 40). In the development of commercial biocontrol agents, the focus has long been on *Pseudomonas* and *Bacillus* taxa because of their efficient root-colonizing capacity and their direct pathogen antagonistic characteristics associated with the production of lytic enzymes and antibiotics (41). From our mixed inoculations that included native *Pseudomonas* and *Bacillus* taxa, four of six strains were reisolated from the surface-sterilized roots of 2013 field-grown flowering-stage plants harvested 34 dpp. While the *Bacillus* isolates (K1 and CN2) dominated the bacterial root isolates, *Pseudomonas* strains (A176 and A70) were recovered at lower frequencies (Fig. S6). Furthermore, an additional test of the robustness of the bacterial association was performed with a second inbred ecotype of *N. attenuata*, originally collected from Arizona, which was preinoculated with the five isolates at the seed germination stage and planted in the New plot along with the Utah ecotype. All five isolates could be reisolated at the end of the growing season (Fig. S7). Because the Arizona ecotype had been planted only on the New plot, and no plants were lost to the sudden wilt disease, we performed in vitro assays to evaluate the disease-suppressive effect of the bacterial consortium for this ecotype. The consortium of five bacterial isolates also reduced the mortality rate of a Arizona ecotype [$t_{(1,20)} = 17.682$, $P < 0.001$, t-test] (Fig. S7). This result indicates that the consortium of isolates provides protection to a second *N. attenuata* ecotype. The reproducibility and persistence of the members of the mixed bacterial consortium in two *N. attenuata* ecotypes planted over two field seasons demonstrates that these native bacterial taxa establish stable associations with *N. attenuata* roots at germination which persist throughout growth under field conditions.

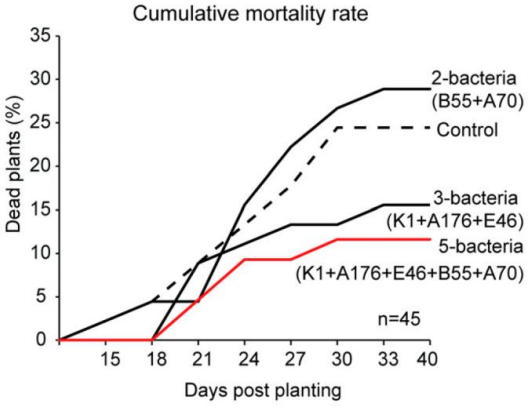

Fig. 5. Reproducibility of the disease-suppression effect of bacterial consortia in the 2014 field season. Based on the results of in vitro tests (Fig. S5) we parsed the bacterial consortia into two groups of two (B55 + A70) or three (K1 + A176+ E46) bacteria and compared these groups with the mixture of the five isolates (K1 + A176 + E46 + B55 + A70) in protecting inoculated seedlings from the sudden wilt when planted into the Old plot. Preinoculated plants were planted together with control plants in a randomized design on the Old (diseased) plot (2014 field season; see *Materials and Methods*). Inoculation with three bacteria (K1 + A176 + E46) or five bacteria (K1 + A176 + E46 + B55 + A70) reduced plant mortality by 36% and 52%, respectively, compared with control plants at 40 dpp ($n = 45$ plants per group). The inoculation with two bacterial strains (B55 + A70) had no significant effect in reducing the rate of death compared with noninoculated control plants.

Opportunistic Mutualisms and the Opportunities They Afford Agriculture. Soil arguably harbors the world's most diverse microbial communities, and when seeds germinate from their seed banks in native soils, they have the opportunity to recruit particular microbial taxa from these marketplaces of potential microbial partners (42). Microbial interactions are commonly categorized as being pathogenic, commensalistic, or mutualistic, as if these traits were fixed features of host and microbial taxa, but most are likely to be context dependent, shifting along the functional spectrum depending on environmental conditions or during the life cycle of the microbe or the plant (43). Root microbiomes are notoriously diverse (2, 4), and some of the diversity may arise from particular microbes being of benefit only to particular hosts under particular conditions or stresses, such as drought or pathogen infestation (44). As shown in this study and others (31), beneficial microbial communities can be acquired from the soil at an early stage during germination and establish beneficial associations that last throughout the entire life cycle of the plants. If plants lack such an early colonization, as in our previous planting procedure, they are exposed suddenly to the field microbiota during planting. Allowing the plant to interact with bacteria either on agar plates or during the Jiffy stage may fill empty niches of the root environment and allow plants to cope better with soil-derived pathogens.

To understand the mechanisms by which a consortium of microbes is recruited soon after germination and maintained in a context-specific manner will require a better understanding of the chemical signals that plants release as they germinate and the opportunities that differences in root morphology and growth afford microbes for colonization. Although organic acids [e.g., malic acid (45)] and certain secondary metabolites [e.g., benzoxazinoids (46)] have been found to mediate the recruitment of particular microbes under in vitro conditions, untargeted metabolomics and genomic approaches are sorely needed to evaluate the processes that are involved when plants are grown under real-world conditions. Crops, likewise, could benefit from location-specific consortia, depending on the region and type of soil in which they are grown.

These opportunistic mutualisms that plants develop with their root-associated microbes have great potential to increase the resilience of crop yields to the ever-changing landscape of abiotic and biotic stresses in agriculture, as many others have argued (31, 33, 47, 48). This work demonstrates that native plants use this strategy, and considerably more attention needs to be focused on the issue for crop plants. Have crop plants lost such abilities, and do they differ from their wild ancestors regarding their root-associated microbiota (49)? Certainly we should reconsider agricultural practices, such as the use of nonspecific antimicrobial seed treatments, that could thwart this important recruitment process. Moreover each plant species likely benefits from recruiting a specialized consortium of bacteria, which needs to be evaluated separately for each plant system. Likewise, evidence of phytoprotective roles of microbes from in vitro experiments should be evaluated under agricultural conditions, because certain microbes (e.g., those used in our fungal treatment) could prove to be

detrimental under field conditions. Progress is being made in rapidly querying, in a high-throughput manner, the ability of the diverse soil microbial communities from around the globe to synthesize antimicrobial secondary metabolites (50). We infer from the research reported here that native plants have been querying the soil microbial community throughout evolutionary history to help them solve context-specific challenges, and we need to empower our crop plants to do the same.

Materials and Methods

Plant Material and Culture Conditions. Wild-type *N. attenuata* Torr. Ex S. Watson seeds of the "Utah" ecotype were collected originally from a population at the DI (Desert Inn, 37.3267N, 113.9647W) ranch in Utah in 1989. For all in vitro and field experiments, wild-type seeds of the 31st inbred generation were surface sterilized and germinated on Gamborg's B5 plates (Duchefa) as previously described (51). Seeds of the "Arizona" ecotype were used in the 22nd inbred generation.

Isolation of Bacteria and Fungi from Field-Grown Plants. Field-grown *N. attenuata* plants at rosette and elongated stages that displayed symptoms of the sudden wilt disease were used for the isolation of potential plant pathogenic bacteria as described in ref. 12. Isolation of potential pathogenic fungi was carried out as described in ref. 13. Identification of bacterial and fungal isolates was performed as previously described (13, 14). The reisolation of the preinoculated bacteria was performed likewise using surface-sterilized roots of healthy plants to enrich endophytic bacteria. For detailed information, see *SI Materials and Methods*.

Plant Treatments in the Field. Field experiments were conducted at a field station at the Lytle Ranch Preserve in Utah. For the 2013 field season, seeds were inoculated with the mixed bacterial solution (*Arthrobacter nitroguajacolicus* E46, *Bacillus cereus* CN2, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176) or two native fungal isolates (*Chaetomium* sp. C72 and *Oidodendron* sp. Oi3). For the fungicide treatment, Jiffy pots (Jiffy 703, jiffygroup.com) were soaked with 15 mL of 1% fungicide solution (Landor; Syngenta) one night before planting. For the charcoal treatment, ~100 g of charcoal was added to the soil surrounding each plant before planting. For the combined treatment the charcoal was presoaked with 25 mL of 5% fungicide solution (Landor; Syngenta). Size-matched plants of each treatment group were planted in a randomized design (735 on the Old plot and 261 on the New plot). For the repeated fungicide (fungicide 5×) treatment, plants were watered weekly with 50 mL of 1% fungicide solution. For the 2014 field season, bacterial consortia consisting of two (B55 + A70), three (K1 + A176 + E46), or five (K1 + A176 + E46 + B55 + A70) bacteria were used for seed inoculation, and 180 plants from the different treatments were planted into the Old plot. See *SI Materials and Methods* for additional experimental details; Table S1 lists the 32 ecological traits used to characterize bacterially inoculated plants planted into the New plot.

Nucleotide Sequence Accession Numbers. The sequencing data for LK020799–LK021108 and LN556288–LN556387 have been deposited in the European Nucleotide Archive and KR906683–KR906715 in The National Center for Biotechnology Information. Also see Dataset S1.

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1. McFall-Ngai M, et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci USA* 110(9):3229–3236.
2. Lundberg DS, et al. (2012) Defining the core *Arabidopsis thaliana* root microbiome. *Nature* 488(7409):86–90.
3. Hanage WP (2014) Microbiology: Microbiome science needs a healthy dose of scepticism. *Nature* 512(7514):247–248.
4. Bulgarelli D, et al. (2012) Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488(7409):91–95.
5. Berendsen RL, Pieterse CMJ, Bakker PAHM (2012) The rhizosphere microbiome and plant health. *Trends Plant Sci* 17(8):478–486.
6. Glick BR (2012) Plant growth-promoting bacteria: Mechanisms and applications. *Scientifica (Cairo)* 2012:963401.
7. Pieterse CMJ, et al. (2014) Induced systemic resistance by beneficial microbes. *Annu Rev Phytopathol* 52(1):347–375.
8. Kloepper J, Ryu C-M (2006) Bacterial endophytes as elicitors of induced systemic resistance. *Microbial Root Endophytes,* SE-3, Soil Biology, eds Schulz BE, Boyle CC, Sieber T (Springer, Berlin), pp 33–52.
9. Weller DM, Raaijmakers JM, Gardener BBM, Thomashow LS (2002) Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annu Rev Phytopathol* 40(1):309–348.
10. Baldwin IT (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. *Plant Physiol* 127(4):1449–1458.
11. Long HH, Schmidt DD, Baldwin IT (2008) Native bacterial endophytes promote host growth in a species-specific manner; phytohormone manipulations do not result in common growth responses. *PLoS One* 3(7):e2702.
12. Long HH, Sonntag DG, Schmidt DD, Baldwin IT (2010) The structure of the culturable root bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception. *New Phytol* 185(2): 554–567.
13. Schuck S, Weinhold A, Luu VT, Baldwin IT (2014) Isolating fungal pathogens from a dynamic disease outbreak in a native plant population to establish plant-pathogen bioassays for the ecological model plant *Nicotiana attenuata*. *PLoS One* 9(7):e102915.
14. Santhanam R, Groten K, Meldau DG, Baldwin IT (2014) Analysis of plant-bacteria interactions in their native habitat: Bacterial communities associated with wild tobacco are independent of endogenous jasmonic acid levels and developmental stages. *PLoS One* 9(4):e94710.
15. Meldau DG, Long HH, Baldwin IT (2012) A native plant growth promoting bacterium, *Bacillus sp.* B55, rescues growth performance of an ethylene-insensitive plant genotype in nature. *Front Plant Sci* 3(June):112.
16. Schippers B, Bakker AW, Bakker PAHM (1987) Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. *Annu Rev Phytopathol* 25(1):339–358.
17. Oyarzun P, Gerlagh M, Hoogland AE (1993) Relation between cropping frequency of peas and other legumes and foot and root rot in peas. *Neth J Plant Pathol* 99(1): 35–44.
18. Curl EA (1963) Control of plant diseases. *Bot Rev* 29(4):413–479.
19. Krupinsky JM, Bailey KL, McMullen MP, Gossen BD, Turkington TK (2002) Managing plant disease risk in diversified cropping systems. *Agron J* 94(2):198–209.
20. Katawczik M, Mila AL (2012) Plant age and strain of *Ralstonia solanacearum* affect the expression of tobacco cultivars to Granville wilt. *Tob Sci* 49:8–13.
21. Bull CT, et al. (2012) Letter to the editor list of new names of plant pathogenic bacteria (2008-2010) Prepared by the International Society of Plant Pathology Committee on the Taxonomy of Plant Pathogenic Bacteria (ISPP-CTPPB). *J Plant Pathol* 94(1):21–27.
22. Larkin RP, Fravel DR (2001) Efficacy of various fungal and bacterial biocontrol organisms for control of Fusarium wilt of tomato. *Plant Dis* 82(9):1022–1028.
23. Chen Y, et al. (2013) Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. *Environ Microbiol* 15(3):848–864.
24. Stockwell CA, Hendry AP, Kinnison MT (2003) How does biodiversity influence the ecology of infectious disease? *Trends Ecol Evol* 18(2):94–101.
25. Schuman MC, Heinzel N, Gaquerel E, Svatos A, Baldwin IT (2009) Polymorphism in jasmonate signaling partially accounts for the variety of volatiles produced by *Nicotiana attenuata* plants in a native population. *New Phytol* 183(4):1134–1148.
26. Kessler D, et al. (2012) Unpredictability of nectar nicotine promotes outcrossing by hummingbirds in *Nicotiana attenuata*. *Plant J* 71(4):529–538.
27. Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. *Appl Environ Microbiol* 71(9):4951–4959.
28. Gindrat D, Gordon-Lennox G, Walther D, Jermini M (1992) Biocontrol of sugarbeet damping-off with Chaetomium globosum: Promises and questions. *Biological Control of Plant Diseases*, SE - 26, NATO Advanced Science Institute Series, eds Tjamos EC, Papavizas GC, Cook RJ (Springer, New York), pp 197–201.
29. Alexander BJR, Stewart A (2001) Glasshouse screening for biological control agents of *Phytophthora cactorum* on apple (*Malus domestica*). *N Z J Crop Hortic Sci* 29(3): 159–169.
30. Santhanam R, Baldwin IT, Groten K (2015) In wild tobacco, *Nicotiana attenuata*, variation among bacterial communities of isogenic plants is mainly shaped by the local soil microbiota independently of the plants' capacity to produce jasmonic acid. *Commun Integr Biol* 8(2):e1017160.
31. Compant S, Clément C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. *Soil Biol Biochem* 42(5):669–678.
32. Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* 63(c):541–556.
33. Köberl M, et al. (2013) *Bacillus* and *Streptomyces* were selected as broad-spectrum antagonists against soilborne pathogens from arid areas in Egypt. *FEMS Microbiol Lett* 342(2):168–178.
34. Tonelli ML, Taurian T, Ibáñez F, Angelini J, Fabra A (2010) Selection and in vitro characterization of biocontrol agents with potential to protect peanut plants against fungal pathogens. *J Plant Pathol* 92(1):73–82.

35. Lehmann J, et al. (2011) Biochar effects on soil biota – A review. *Soil Biol Biochem* 43(9):1812–1836.
36. Sohi SP (2012) Carbon storage with benefits. *Science* 338(6110):1034–1035.
37. Jetiyanon K, Kloepper JW (2002) Mixtures of plant growth-promoting rhizobacteria for induction of systemic resistance against multiple plant diseases. *Biol Control* 24(3): 285–291.
38. Niu D-D, et al. (2011) The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. *Mol Plant Microbe Interact* 24(5):533–542.
39. Lugtenberg BJJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annu Rev Phytopathol* 39(1):461–490.
40. Doornbos RF, Loon LC, Bakker PAHM (2011) Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. *Agron Sustain Dev* 32(1): 227–243.
41. Figueiredo M, Seldin L, Araujo F, Mariano R (2011) Plant growth promoting rhizobacteria: Fundamentals and applications. *Plant Growth and Health Promoting Bacteria*, SE-2, Microbiology Monographs, ed Maheshwari DK (Springer, Berlin), pp 21–43.
42. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P (2012) Structure and functions of the bacterial microbiota of plants. *Annu Rev Plant Biol* 64:807–832.
43. Newton AC, Fitt BDL, Atkins SD, Walters DR, Daniell TJ (2010) Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. *Trends Microbiol* 18(8):365–373.
44. Gaiero JR, et al. (2013) Inside the root microbiome: Bacterial root endophytes and plant growth promotion. *Am J Bot* 100(9):1738–1750.
45. Rudrappa T, Czymmek KJ, Paré PW, Bais HP (2008) Root-secreted malic acid recruits beneficial soil bacteria. *Plant Physiol* 148(3):1547–1556.
46. Neal AL, Ahmad S, Gordon-Weeks R, Ton J (2012) Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PLoS One* 7(4):e35498.
47. Meldau DG, et al. (2013) Dimethyl disulfide produced by the naturally associated bacterium *bacillus* sp B55 promotes *Nicotiana attenuata* growth by enhancing sulfur nutrition. *Plant Cell* 25(7):2731–2747.
48. Bakker M, Manter D, Sheflin A, Weir T, Vivanco J (2012) Harnessing the rhizosphere microbiome through plant breeding and agricultural management. *Plant Soil* 360(1-2): 1–13.
49. Bulgarelli D, et al. (2015) Structure and function of the bacterial root microbiota in wild and domesticated barley. *Cell Host Microbe* 17(3):392–403.
50. Charlop-Powers Z, Owen JG, Reddy BVB, Ternei MA, Brady SF (2014) Chemical-biogeographic survey of secondary metabolism in soil. *Proc Natl Acad Sci USA* 111(10): 3757–3762.
51. Krügel T, Lim M, Gase K, Halitschke R, Baldwin IT (2002) Agrobacterium-mediated transformation of *Nicotiana attenuata*, a model ecological expression system. *Chemoecology* 12(4):177–183.
52. Kelman A (1954) The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology* 44(12):693–695.
53. El-Nakeeb MA, Lechevalier HA (1963) Selective isolation of aerobic *Actinomycetes*. *Appl Microbiol* 11(July):75–77.
54. Diezel C, von Dahl CC, Gaquerel E, Baldwin IT (2009) Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. *Plant Physiol* 150(3):1576–1586.
55. Schuman MC, Barthel K, Baldwin IT (2012) Herbivory-induced volatiles function as defenses increasing fitness of the native plant *Nicotiana attenuata* in nature. *eLife* 1:e00007. Available at dx.doi.org/10.7554/eLife.00007. Accessed August 15, 2015.
56. Kallenbach M, et al. (2014) A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. *Plant J* 78(6):1060–1072.
57. Wu J, Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. *Annu Rev Genet* 44(1):1–24.
58. Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. *PLoS Biol* 2(8):E217.
59. Heiling S, et al. (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(1): 273–292.
60. Oh Y, Baldwin IT, Gális I (2012) NaJAZh regulates a subset of defense responses against herbivores and spontaneous leaf necrosis in *Nicotiana attenuata* plants. *Plant Physiol* 159(2):769–788.

Supporting Information

Santhanam et al. 10.1073/pnas.1505765112

SI Materials and Methods

Isolation of Bacterial and Fungal Pathogens from Diseased *N. attenuata* Plants. Elongated and rosette field-grown *N. attenuata* plants displaying symptoms of the sudden wilt disease were shipped to the laboratory facility in Germany within 2 d of excavation. The isolation of potential plant pathogenic bacteria was performed as described in ref. 12 using nutrient agar (Sigma) and Casamino acid-peptone-glucose agar adapted for the isolation of *R. solanacearum* (52) supplemented with antifungal agents, cyclohexamide and nystatin, both at 25 μg/mL. Isolation of potential pathogenic fungi was carried out as described in ref. 13 on potato dextrose agar (PDA; Fluka) and water agar (53) supplemented with the antibacterial agents streptomycin and penicillin, both at 20 μg/mL. Bacterial and fungal genomic DNA extraction, amplification of 16S rDNA, and internal transcribed spacer and direct sequencing and identification were performed as described in ref. 14 for bacterial isolates and ref. 13 for fungal isolates.

In Vitro Seedling Mortality Assays. Two plant-protection strategies, (microbial and chemical), were evaluated with an in vitro assay for the protection they afford to *N. attenuata* seedlings against two different fungal pathogens: *Alternaria* sp. U10, which was isolated from a native population of diseased *N. attenuata* plants in Utah (13), and *Fusarium oxysporum* U3, which was isolated from the roots of diseased plants from the Old plot in 2012.

Potential fungal and bacterial biocontrol isolates were native root-associated isolates from field-grown *N. attenuata* plants (14). Six native bacterial isolates, *Arthrobacter nitroguajacolicus* E46, *Bacillus cereus* CN2, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176, were chosen because they had promoted plant growth in a previous experiment (30) or had been reported in the literature to be potential biocontrol strains. The four native fungal isolates, *Chaetomium sp*. C16, C39, and C72 and *Oidodendron* sp. Oi3, were isolated from diseased plants of the Old field plot; these isolates were not reported in the literature as being pathogenic but had potential biocontrol effects (28, 29). We tested these strains with seedlings challenged with *Fusarium* and *Alternaria* fungal pathogens in vitro. *Bacillus cereus* CN2 was used only in the 2013 experiments because its classification as an S2 strain by the German authorities prohibited its further use.

Potential biocontrol bacterial and fungal isolates were maintained on nutrient agar and PDA medium, respectively. Surface-sterilized *N. attenuata* seeds were incubated for 12 h with mixed bacterial cultures by combining equal concentrations of all six bacterial isolates (E46, CN2, B55, K1, A70, and A176) from fresh individual bacterial cultures grown overnight to a working concentration of 10^{-4} cfu/mL in sterile H_2O. For the partitioned consortia experiments, five different mixes were made by excluding single individual isolates from the consortia: e.g., mix minus K1 treatment consisted of four different bacteria but not K1 (A70 + A176 + B55 + E46). In this fashion, the following different bacterial consortia were made: mix minus A70, mix minus A176, mix minus B55, and mix minus E46. Arizona ecotype seeds also were treated with the consortium of five bacterial isolates. For each potential biocontrol fungal strain, mycelium was harvested from two 14-d-old fungal plates and dissolved in 1 mL of sterile H_2O to incubate sterile *N. attenuata* seeds for 5 min. For the fungicide treatment, surface-sterilized seeds were treated for 5 min with 1 mL of undiluted fungicide solution (Landor; Syngenta) containing fludioxonil (25 g/L), difenoconazol (20 g/L), and tebuconazol (5 g/L). Bacteria-, fungi-, and fungicide-treated seeds were germinated on Gamborg's B5 plates (GB5; Duchefa). After 7 d, germinated seedlings were inoculated with fungal pathogens by placing 1-cm-diameter plugs from 14-d-old PDA in the center of seedling plates (see Fig. S2*A* for the experimental set-up) and were incubated in a growth chamber (22 °C, 65% humidity, 16 h light). Dead seedlings were counted 26 d postinoculation from 10 replicate plates, and the mean percentage of seedling mortality was calculated.

Plant Treatments in the Field. For the field experiments, wild-type seeds were germinated on Gamborg's B5 plates (Duchefa) and preinoculated with the mixed bacterial solution or with fungal mycelium, as described above. About 16 d after germination, seedlings were transferred into 50-mm Jiffy peat pots (Jiffy 703, jiffygroup.com) prehydrated with tap water and were placed under shaded conditions for more than 2 wk to adapt the seedlings to the strong sun and relative low humidity of the Great Basin Desert. Well-grown and adapted seedlings were transplanted into the Old plot (54). A detailed description of the field-planting procedure is provided in the supplemental movie (Movie S1).

For the 2013 field season, shortly before planting, Jiffy pots were watered with 10 mL of mixed bacterial or fungal solution. The mixed bacterial cultures were generated by scraping the separately cultivated strains (E46, CN2, B55, K1, A70, and A176) from well-grown nutrient agar plates and diluting them in tap water. The bacterial strains were mixtures of equal volumes pooled from the appropriate strains; all solutions were cloudy, indicating a visually estimated very high OD. The mixture of two potential biocontrol fungal isolates (*Chaetomium* sp. C72 and *Oidodendron* sp. Oi3) was prepared in a similar manner by scrapping fungal mycelium from 10 fully grown PDA plates, equally pooled, and diluted in water. For the fungicide treatment, Jiffy pellets were soaked with 15 mL of 1% fungicide solution (Landor; Syngenta) one night before planting. For the charcoal treatment, Royal Oak All-Natural Hardwood Lump Charcoal (Walmart) was chipped into small pieces, and ~100 g were added to the soil surrounding each plant before planting. For the combined treatment (charcoal and fungicide), 100 g of chipped charcoal were presoaked with 25 mL of 5% fungicide solution (Landor; Syngenta) before planting (Fig. S3 *A* and *B*). The treatments were designed to be easily applicable (for a future scale-up) and directly performed during germination (bacteria and fungi), during the Jiffy stage (bacteria, fungi, and fungicide 1×), or during planting (charcoal and combined) (Fig. S3); only the repeated fungicide (fungicide 5×) treatment was performed after planting also. Size-matched plants were planted in a randomized design into the field plot on the Lytle Ranch Preserve, Utah. The experiment was conducted on two separate field plots; of the 819 plants planted in the Old (diseased) field plot, 735 plants were randomly assigned to the seven different treatment groups, as were 261 plants in a New plot (control). The two field plots are ~900 m apart and are separated by a river (Fig. S3*E*). During planting, each plant was fertilized with 5 g of Osmocote Smart-Release Plant Food (19-6-12 N-P-K) (Scotts-Sierra Horticulture) suitable for slow nutrient release over 4 mo. The plants in the repeated fungicide (fungicide 5×) treatment were watered every seventh day with 50 mL of 1% fungicide solution poured into a hole located 8–10 cm from the plant. Growth parameters and plant mortality were recorded every 3 or 4 d for a period of 22 d.

For the 2014 field experiment, three bacterial consortia consisting of two (B55 + A70), three (K1 + A176 + E46), or five (K1 + A176 + E46 + B55 + A70) bacteria were tested. Only the seeds were treated with the bacterial mixtures, and in total 180 plants (n = 45 for each group) were planted into the Old field plot (diseased) as described above. The mortality of all plants was recorded every 3 d for 22 d. To evaluate further the reproducibility of the bacterial association with roots, in the 2014 field season, *N. attenuata* inbred genotypes originally collected from Arizona and Utah were treated with a mixture of five bacterial isolates and were planted at the New field plot as mentioned above.

Phenotypic Characterization of Bacterially Treated Plants in the Field. A selection of 32 morphological and chemical traits (Table S1) known to be important in mediating *N. attenuata's* ecological performance were measured in 7–21 pairs of control plants and plants treated with the mixture of six native root-associated bacteria that had been planted in the New field plot. Replicate values given in Table S1 reflect the number of pairs of plants used in a given analysis.

Leaf chlorophyll content was measured in the largest nonsenescent leaf using a Minolta SPAD-502 (Konica Minolta Sensing, Inc.). Plant growth and reproduction parameters and herbivore damage were assayed once, as previously described (55), before the plants were destructively harvested for biomass measures.

Measurements of foliar and floral volatiles were conducted using polydimethylsiloxane (PDMS) tubes to absorb volatiles from headspace samples as previously described (56). Herbivore-induced plant volatiles were elicited by wounding leaves followed by the immediate application of *Manduca sexta* oral secretions (W+OS) (25), and ventilated polyethylene terephthalate (PET) containers were immediately placed around the wounded leaves and around similar mature, nonsenescent control leaves. PDMS tubes were placed in the PET containers for 1 h immediately after treatment and then were removed and stored in tightly closed amber 1.5-mL GC vials (56); a new PDMS tube was placed in the PET container and left there until 48 h after treatment, at which time it also was removed and stored. To measure floral volatiles, a PDMS tube was placed inside a ventilated PET container enclosing a single, newly opened flower as previously described (56) and was exposed to the floral headspace for 12 h, from 20:00–8:00 the following day; then the PDMS tube was removed and stored as described (8), and a new PDMS tube was placed in the headspace, incubated from 8:00 until 20:00, and stored. PDMS samples were kept in sealed vials until thermal desorption (TD)-GC-quadrupole MS (QMS) analysis was performed as described (56). TD-GC-QMS analysis was performed on a TD-20 thermal desorption unit (Shimadzu, www.shimadzu.com) connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu). An individual PDMS tube was placed in an 89-mm glass TD tube (Supelco, www.sigmaaldrich.com), desorbed, and analyzed as described (56). Compounds were identified by comparison of spectra and Kovats retention indices against libraries (Wiley, National Institute of Standards and Technology, eu.wiley.com/WileyCDA/WileyTitle/productCd-1118615964.html), and, where possible, by comparison with pure standards.

The phytohormones JA and JA-Ile and secondary metabolites nicotine, chlorogenic acid, caffeoyl putrescine, dicaffeoyl spermidine, rutin, and hydroxygeranyllinalool diterpene glycosides (HGL-DTGs) were induced by W+OS treatment (57–59). The first stem leaf was collected as a control sample before the W+OS treatment, and the second and third stem leaves were collected 1 or 48 h after the W+OS treatment for phytohormone or secondary metabolite analysis, respectively. Approximately 100 mg of frozen leaf samples were homogenized in liquid nitrogen and extracted, and the phytohormone and secondary metabolite concentrations were quantified as described in ref. 60. A Varian 1200 LC-ESI-MS/MS system (Varian) was used for phytohormones analysis, and HPLC (Agilent-HPLC 1100 series) coupled to a photodiode array and evaporative light scattering detector (HPLC-PDA-ELSD) was used for the secondary metabolite analysis.

Variables measured in microbially treated and untreated plants were compared using individual t tests. Wilcoxon pairwise tests were used when a variable did not meet the assumptions of parametric tests. All statistics were performed in R-Studio (R Core Team, 2012). P values were not corrected for multiple tests.

Bacterial Reisolation from Field-Grown Control and Preinoculated Plants. Root-associated bacteria were reisolated from surface-sterilized roots as described above using control and bacterially preinoculated plants harvested from both field plots in the 2013 field season (seven roots for each treatment from the Old field plot and three roots for each treatment from the New field plot). In the 2014 field season, the roots of five plants were analyzed when the plants had reached the early flowering stage. Bacterial plates were incubated at 28 °C for 4 d, and colonies were picked, subcultured, purified, and identified by 16S rDNA sequencing as described above.

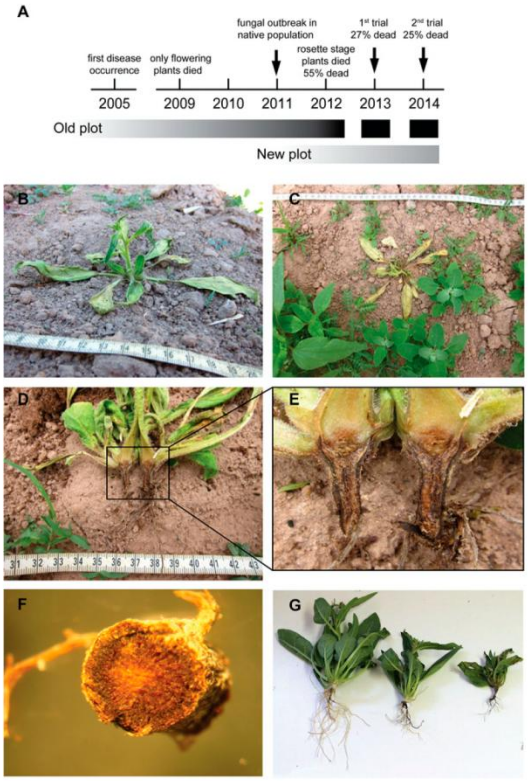


Fig. S1. Symptoms of the sudden wilt disease. (*A*) Disease symptoms first occurred only sporadically in our field plot in elongated and flowering plants and later were also observable in rosette-stage plants. Regular field experiments on the Old plot were ended in 2012 because of the unacceptably high plant mortality. Total plant mortality was recorded during the last three field seasons. (*B*) Sudden wilt disease symptoms characterized by dry or flaccid leaves developed within 1 or 2 d in *N. attenuata* plants. (*C*) The wilting was specific to *N. attenuata*; surrounding plants were not affected, and the surrounding soil usually was still moist. (*D–F*) The signature characteristic of a diseased plant was the development of black roots; the discoloration was visible on the outside as well as in longitudinal sections. The occurrence of wilting together with the black roots was diagnostic of a plant being affected by the sudden wilt disease. (*G*) Plants with differently pronounced disease levels were observed during the 2014 field season and illustrate the course of the disease. Wilting first was observed only in elongated plants (here with mild symptoms and mainly white roots) but during the last three field seasons appeared in younger plants also (here with marked symptoms and completely black roots).

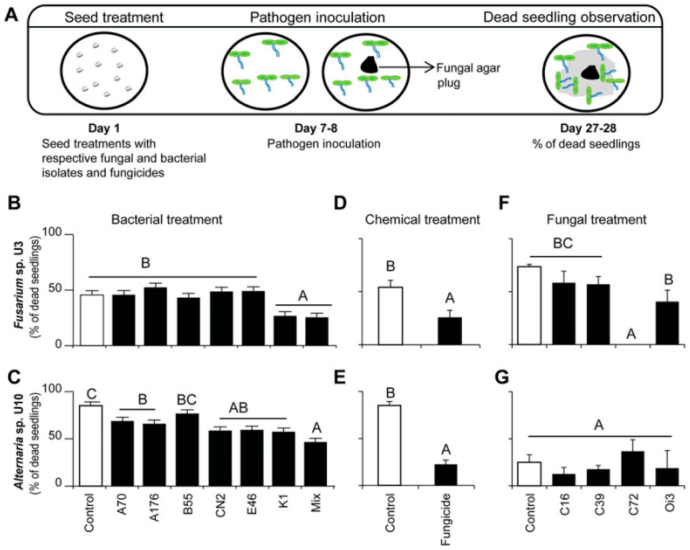


Fig. S2. Inoculation with a mixture of native bacteria, a fungicide, and two native fungal isolates reduced seedling mortality under in vitro conditions. (*A*) Schematic of the in vitro experimental setup. Seedling mortality was observed in separate infection assays using two fungal pathogens that previously had been isolated from diseased *N. attenuata* plants in a native population and characterized: *Fusarium* sp. U3 and *Alternaria* sp. U10 (3). (*B* and *C*). Evaluation of six native bacterial isolates for potential biocontrol abilities. The seeds were inoculated with individual cultures of *P. azotoformans* A70, *P. frederiksbergensis* A176, *B. megaterium* B55, *B. cereus* CN2, *A. nitroguajacolicus* E46, or *B. mojavensis* K1 or with a mixture of all strains (*SI Materials and Methods*). The mixed inoculation of all six strains had the strongest effects against *Fusarium* sp. U3 and *Alternaria* sp. U10 and was selected as the treatment for the field experiments. (*D* and *E*) The fungicide seed treatment (Landor; Syngenta) significantly reduced seedling mortality of *N. attenuata* seedlings infected with fungal pathogens. (*F* and *G*) Native fungal isolates were tested for possible biocontrol abilities. The seeds were inoculated with *Chaetomium* sp. (C16, C39, and C72) or *Oidodendron* sp. (Oi3) before being infected with fungal pathogens. The C72 and Oi3 treatments were chosen for field experiments because they reduced the mortality of seedlings inoculated with *Fusarium* sp. U3. Bars represent mean seedling mortality (± SEM, $n = 10$ plates); the different letters above the bars indicate significant differences in a one-way ANOVA with Fisher's protected least significant difference (PLSD) test; $P < 0.05$.

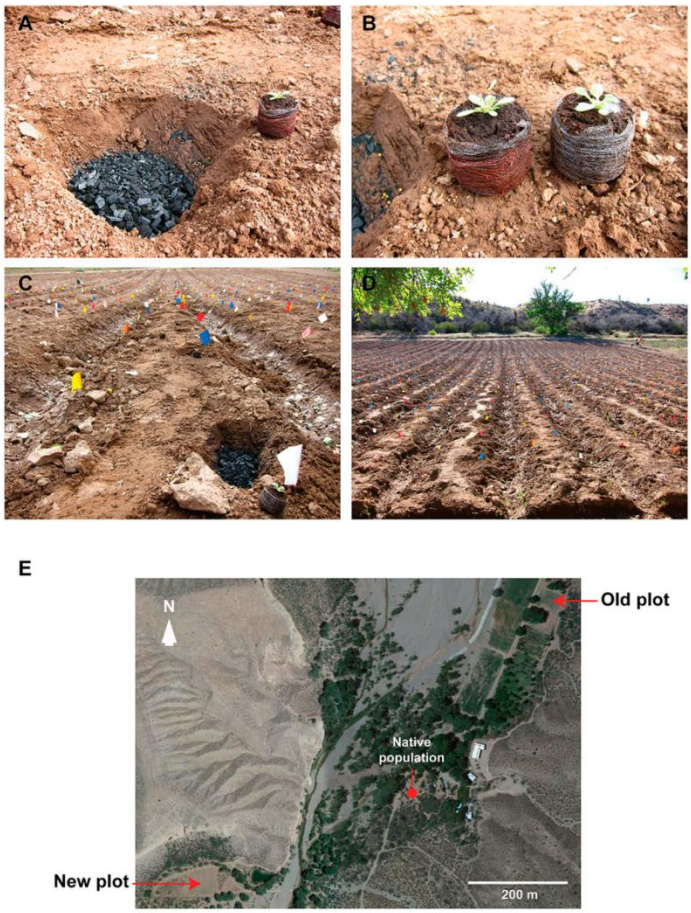


Fig. S3. Planting of the treatment groups during the 2013 field season. (*A*) Before planting, charcoal was added to the charcoal and combined treatment groups. (*B*) Jiffy pots were treated before planting; the applied fungicide solution can be seen by the red color. (*C* and *D*) Treatment groups were planted in a randomized design on the lower section of the Old field plot (N 37.1463 W 114.0198), and, as a control experiment, also on the New plot (N 37.1412 W 114.0275). (*E*) Google Maps view of Old and New field plots ~900 m apart.

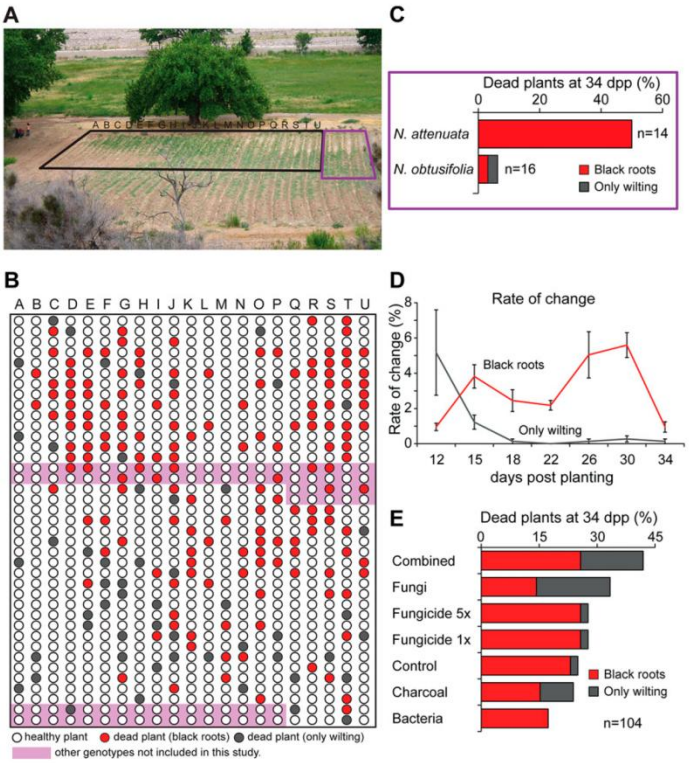


Fig. S4. Overview of the Old field plot and the rate of change in plant mortality during the 2013 field season. (*A*) The Old field plot during the 2013 season. The rectangle indicates the lower area of the plot where plants had been planted. This portion of the plot was selected because the greatest number of diseased plants was found in this area during the 2012 growing season. (*B*) Schematic illustrating the spatial distribution of plants (from the rectangle shown in *A*), distinguishing plants with the sudden wilt symptoms (wilting and black roots) and plants with only wilting symptoms. The occurrence of the sudden wilt disease was not distributed equally throughout the plot. (*C*) Mortality rate of *N. obtusifolia* planted together with *N. attenuata* in an adjacent block of a separate experiment. (*D*) The development of plant mortality is shown as the rate of change (percentage dead plants at one observation minus dead plants at previous observation). The error bars reflect the differences among the treatments. Plants with only wilting symptoms were observed mainly at the early time points. (*E*) Percentage of dead plants at 34 dpp showing black roots or only wilting symptoms. Most plants with only wilting symptoms were found in the fungi, charcoal, and combined (charcoal + fungicide) treatments.

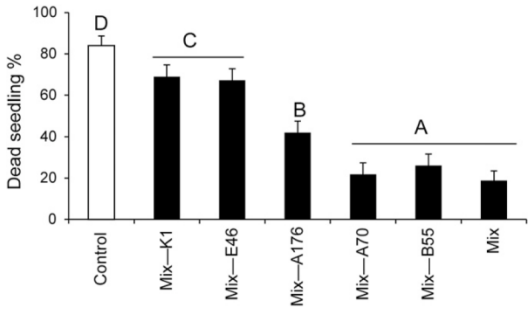


Fig. S5. In comparison with the effects of seedling inoculation with a mixed bacterial consortium consisting of five taxa, the individual absences of three bacterial isolates (K1, E46, and A176) significantly increased seedling mortality under in vitro conditions. Different mixed bacterial consortia were evaluated for potential biocontrol abilities against *Alternaria* sp. U10. Mixed bacterial consortia lacking one bacterial isolate (e.g., mix minus K1, mix minus E46, and mix minus A176) significantly increased seedling morality. Based on these results, the three-bacteria mixed consortium (K1 + A176 + E46) and two-bacteria mixed consortium (B55 + A70), along with the mixture of all five bacteria, were selected as treatments for the 2014 field experiments. Bars represent mean seedling mortality (± SE; n = 7–10); the different letters above the bars indicate significant differences in a one-way ANOVA with Fisher's PLSD test; $P < 0.05$. For the experimental set-up see Fig. S2*A*.

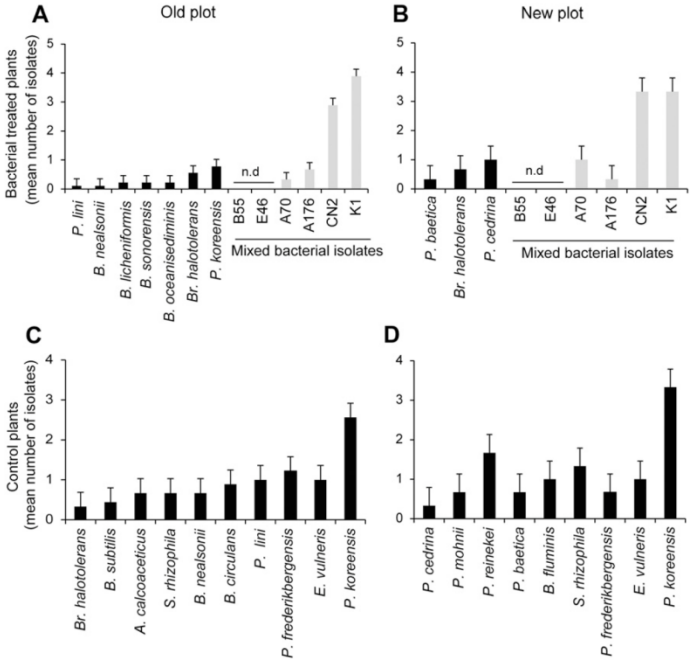


Fig. S6. Reisolation of the bacteria from healthy field-grown plants at the flowering stage demonstrated the persistence of the inoculated bacterial taxa. In 2013, healthy plants from the control and bacterial treatment groups were harvested at the early flowering stage from both field plots, and the culturable bacterial consortium were isolated. (*A* and *B*) Four of the six native bacterial taxa used in the bacterial mix (seed and Jiffy treatment) persisted throughout growth under field conditions and were reisolated from the roots of plants from both field plots. The inoculated roots showed strong colonization by *B. cereus* CN2 and *B. mojavensis* K1. The bars represent the mean number of isolates (± SEM; Old field plot: n = 7 roots, 70 isolates; New field plot: n = 3 roots, 30 isolates). (*C* and *D*) The control plants showed natural colonization by *P. frederiksbergensis*, which was also used in the bacterial mixture as A176. Bacterial genus acronyms: A, *Acinetobacter*; B, *Bacillus*; Br, *Brevibacterium*; *E*, *Escherichia*; P, *Pseudomonas*; S, *Stenotrophomonas*.

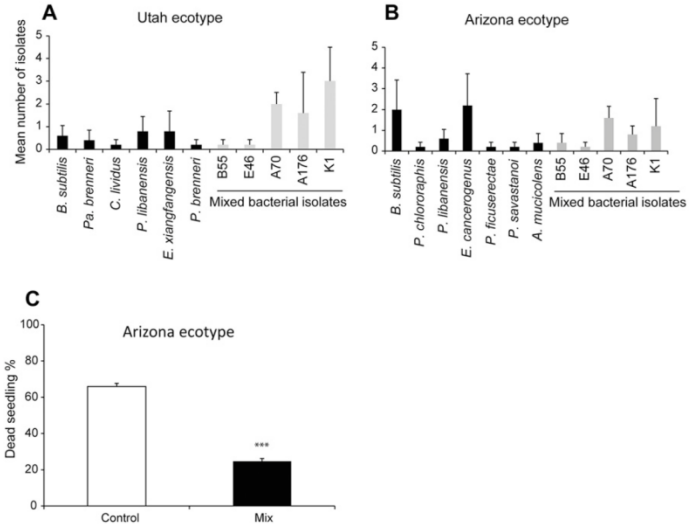
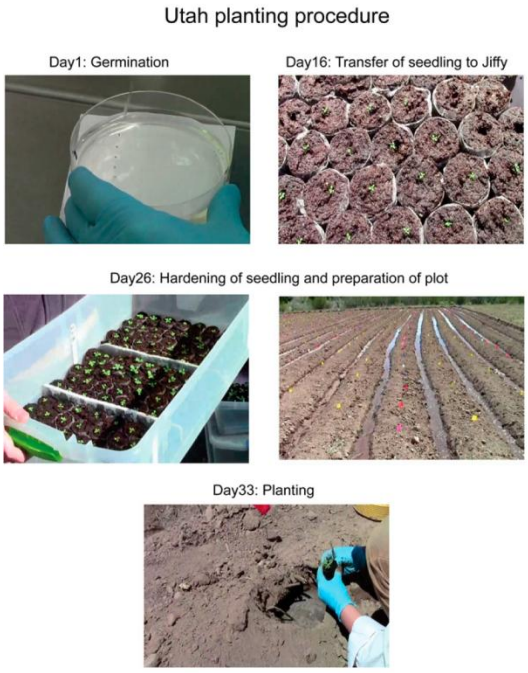


Fig. S7. Reproducibility of the reisolation of the mixed bacterial consortium from Utah and Arizona genotypes in the 2014 field season and the protection effect of the consortium for the Arizona ecotype. Bacterially treated Utah and Arizona genotypes were harvested at the early flowering stage from the New field plot, and the culturable bacterial consortium were isolated. (*A* and *B*) All five native bacterial taxa used in the bacterial inoculation treatment (seed treatment only) persisted throughout growth under field conditions of both ecotypes. The persistence of the inoculated bacteria taxa within both ecotypes demonstrates the consistency of the bacterial association with *N. attenuata*. The bars represent the mean number of isolates (± SEM; $n = 5$ roots, 50 isolates). Bacterial genus acronyms: A, *Achromobacter*; B, *Bacillus*; C, *Ciceribacter*; E, *Enterobacter*; P, *Pseudomonas*; Pa, *Pantoea*. (*C*) Under in vitro conditions, the consortium of five mixed bacterial isolates significantly reduced the mortality of the *N. attenuata* Arizona ecotype inoculated with *Alternaria* sp. U10 (± SEM; $n = 11$, $P < 0.001$, *t*-test).

Table S1. Traits important for *N. attenuata*'s ecological performance and insect resistance were compared between bacterially treated and control plants grown in the New field plot during the 2013 field season

Variable	Test	*n*	Test statistic	*P* value
Plant-growth parameters				
Root length	*t* test	21	−0.162	0.872
Shoot length	*t* test	21	0.222	0.826
Root/shoot ratio	*t* test	21	−0.454	0.653
Rosette diameter	*t* test	21	0.318	0.753
Stem length	*t* test	21	−0.313	0.756
Number of branches	*t* test	21	−0.267	0.791
Number of buds	*t* test	21	−0.976	0.335
Number of flowers	Wilcoxon	21	204.500	0.696
Number of seed capsules	Wilcoxon	21	201.000	0.522
Chlorophyll	*t* test	12	−1.258	0.222
Herbivore damage				
Grasshopper damage	Wilcoxon	21	187.500	0.342
Mirid damage	Wilcoxon	21	186.000	0.338
Noctuid damage	Wilcoxon	21	174.000	0.238
Flea beetle damage	Wilcoxon	21	230.000	0.727
Tree cricket damage	Wilcoxon	21	231.000	0.697
Flower volatiles				
Benzyl acetone flower volatile	Wilcoxon	16	106.000	0.415
Leaf volatiles 1 h after W+OS				
3(Z)-hexen-1-ol	Wilcoxon	16	30.000	0.491
2(E)-hexen-1-ol	Wilcoxon	16	30.000	0.491
Putative alpha-pinene	Wilcoxon	16	22.500	0.886
3(Z)-hexenyl acetate	Wilcoxon	16	23.000	0.950
3(Z)-hexenyl isobutanoate	Wilcoxon	16	32.000	0.345
3(Z)-hexenyl butanoate	Wilcoxon	16	24.500	1.000
3(Z)-hexenyl 2-methylbutanoate	*t* test	16	−0.907	0.383
3(Z)-hexenyl valerate	*t* test	16	−0.879	0.400
Putative sesquiterpene oxide	Wilcoxon	16	25.000	0.950
Leaf volatiles 48 h after W+OS				
1-Hexanol	*t* test	16	0.884	0.396
3(Z) hexen-1-ol	Wilcoxon	16	45	0.19
Putative alpha-pinene	Wilcoxon	16	29.5	0.81
3(Z)-hexenyl isobutanoate	Wilcoxon	16	36	0.721
3(Z)-hexenyl butanoate	*t* test	16	0.336	0.742
Putative alpha-terpineol	*t* test	16	1.442	0.174
3(Z)-hexenyl 2-methylbutanoate	Wilcoxon	16	40	0.442
3(Z)-hexenyl valerate	Wilcoxon	16	38	0.574
Alpha-duprezianene	Wilcoxon	16	44	0.227
Putative sesquiterpene oxide	*t* test	16	2.185	0.048
Phytohormones at W+OS (0 h)				
JA	*t* test	7	−0.266	0.7945
JA-Ile	*t* test	7	−0.171	0.867
ABA	*t* test	7	−1.49	0.162
Phytohormones 1 h after W+OS				
JA	*t* test	7	0.068	0.9473
JA-Ile	*t* test	7	2.042	0.0659
ABA	*t* test	7	0.011	0.991
Secondary metabolites at W+OS (0 h)				
Nicotine	*t* test	7	0.442	0.6661
Caffeoylputrescine	*t* test	7	0.426	0.6792
Chlorogenic acid	*t* test	7	0.132	0.8973
Dicaffeoyl spermidine	*t* test	7	0.124	0.9032
Rutin	*t* test	7	−0.325	0.7506
HGL-DTGs	*t* test	7	−0.325	0.7507
Secondary metabolites 48 h after W+OS				
Nicotine	*t* test	7	0.247	0.8094
Caffeoylputrescine	*t* test	7	0.545	0.5977
Chlorogenic acid	*t* test	7	1.513	0.1585
Dicaffeoyl spermidine	*t* test	7	1.521	0.1566
Rutin	*t* test	7	−0.759	0.4624
HGL-DTGs	*t* test	7	−0.759	0.4624



Movie S1. Utah field-planting procedures.

Movie S1

Dataset S1. Fungal and bacterial isolates retrieved from diseased roots

Dataset S1

Chapter 5: Discussion

Plants form the foundation for most of all food chains on the planet. In contrast to animals, plants are largely immobile and must cope with the particular environment in which they grow. To gain Darwinian fitness, plants need to defend themselves against attacks from various pathogens and herbivores, cope with abiotic stresses, and also compete with other autotrophs for resources. *Nicotiana attenuata* plants germinate in a post-fire and desert environment; hence they face varied and continuous attack from natural enemies including both pathogens and herbivores (Baldwin, 2001). Herbivores from more than 20 different taxa, including both generalists and specialists, attack *N. attenuata* (Baldwin, 2001). *Fusarium brachygibbosum* U4 (*Fusarium*) and *Alternaria* sp. U10 (*Alternaria*) were found to infect *N. attenuata* plants in both native and cultivated field (Schuck *et al.*, 2014; Luu *et al.*, 2015; Santhanam *et al.*, 2015b). In addition, environmental conditions are ever-changing, and there is no single optimal solution for plants to protect themselves from the diverse assemblage of biotic stresses. As a consequence, plants harbor multiple defense strategies against natural enemies (Van Dam, 2009; Carmona & Fornoni, 2013; Turley *et al.*, 2013). In this thesis, I used *N. attenuata* as a model plant to study defense solutions of wild plants against their native pathogens. I found that *N. attenuata* plants have evolved a complex defense system against their native fungal pathogens. This defense system includes the ability of *N. attenuata* to produce *O*-AS as both constitutive and generalized defense strategies. It also includes the ability of *N. attenuata* to generate a great variation in *O*-AS contents and compositions among natural accessions. Furthermore, the complex defense system of *N. attenuata* also includes the association of this plant with its root microbes to enhance the plant's defense against fungal pathogens. In the following sections, I will discuss these pathogen defense mechanisms utilized by *N. attenuata* in detail.

1. *O*-AS function as constitutive defense compounds of *N. attenuata* against fungal pathogens

O-AS are trichome secondary metabolites which can be accumulated in enormous amounts, with up to 20% of leaf dry weight in *Solanum pennellii* (Fobes *et al.*, 1985) and 1.5

mg/gFM in *N. attenuata* (**manuscript I**). *O*-AS were found to be constitutively produced in trichomes of many *Solanaceous* species including *Solanum*, *Nicotiana*, *Datura*, and *Petunia* (Chortyk *et al.*, 1997; Kroumova & Wagner, 2003). In nature, fungal spores are dispersed via air and rain to land on the leaf surface, where they wait to germinate (Timmer, L.W. *et al.*, 2003); hence fungal spores are in direct contact with excreted *O*-AS on the leaf surface. Fungal spores may be fully covered by *O*-AS due to their small size, which makes their initial germination and growth particularly sensitive to *O*-AS. In fact, adding *O*-AS to fungal germination medium reduced spore germination and growth of both *Fusarium brachygibbosum* U4 and *Alternaria* sp. U10 (**manuscript I**). Recently, it was shown that trichomes can serve as entry points for *Fusarium* infection (Nguyen *et al.*, 2016), and thus not only excreted *O*-AS but also *O*-AS retained inside trichomes could act as the first layer of defense against fungal pathogen infections. In **manuscript I**, experiments with natural accessions and crosses between high- and low-*O*-AS accessions revealed that total *O*-AS levels were associated with resistance against both fungal pathogens *Alternaria* and *Fusarium*. Removing *O*-AS from the leaf surface increased the size fungal necrotic lesions on *N. attenuata* detached leaves. Trichome-specific silencing of a putative branched-chain alpha-ketoacid dehydrogenase E1 beta subunit encoding gene (*NaBCKDE1B*) led to a reduction in 20-30% of total leaf *O*-AS, and increased susceptibility to *Fusarium* pathogens. Hence, **manuscript I** provides evidence for the direct defense function of *O*-AS in *N. attenuata* against native pathogens.

O-AS consist of branched chain aliphatic acids (BCAAs) esterified to the hydroxyl groups of sucrose core molecule (Puterka *et al.*, 2003). BCAAs are well-known to disrupt the cell membrane of pathogens, inhibiting the myristoylation of proteins, β-oxidation, triacylglycerol synthesis, and sphingolipid synthesis, as well as showing topoisomerase activity as reviewed by Pohl *et al.* (2011). The BCAAs hydrolyzed from *O*-AS may be responsible for *O*-AS toxicity to fungal pathogens. Indeed, the mixture of 4 main BCAAs in *N. attenuata*, including 2-methyl butanoic acid, 3-methyl butanoic acid, 3-methyl pentanoic acid, and 4-methyl pentanoic acid, showed direct inhibition effects on both *Fusarium* and *Alternaria* spore germination and growth. Interestingly, the 20-30% reduction of total leaf *O*-AS due to silencing *NaBCKDE1B* expression in trichomes led to a significantly higher susceptibility of the transgenic lines to *Fusarium*, but not *Alternaria*. These results suggest that *Fusarium* is more

sensitive to *O*-AS compared to *Alternaria*. In the *in vitro* test, I observed that *Fusarium* spore germination could be inhibited at lower *O*-AS concentrations (0.375 mg/mL), while to inhibit *Alternaria*, higher concentrations (1.5mg/mL) were required. It is still an open question why *Fusarium* spores are more sensitive to *O*-AS than *Alternaria* spores. One reason could be because *Alternaria* is more well-known as leaf pathogen, while *Fusarium* is more a root pathogen (Roncero *et al.*, 2003; Thomma, 2003). The specialization of *Alternaria* to infect leaves has been developed along plant-pathogen evolutionary, which results in an adaptation of this fungus to leaf surface metabolites via the thick, melanized cell wall (Rehnstrom & Free, 1996; Thomma, 2003). However, more analyses of the interaction of these fungal pathogens and *O*-AS are required to answer this question.

Although the fitness costs of *O*-AS production have not been studied yet and will have to be investigated in further studies, it is assumed that constitutive production of these secondary metabolites is costly for the plant and requires an allocation of resources which cannot be invested in other plant functions, such as growth and reproduction (Baldwin, 1998; Tollrian & Harvell, 1999; Strauss *et al.*, 2002). In nature, plants are constantly under attack from not only pathogens, but also from herbivores. Hence, it is more beneficial for plants to invest their resources in the production of secondary metabolites that can be involved in defense against both pathogens and herbivores (Krischik *et al.*, 1991; Biere *et al.*, 2004).

2. *O*-AS function as generalized defense compounds of *N. attenuata* against both pathogens and herbivores

In *N. attenuata*, *O*-AS have been shown to function as indirect defenses against the specialist herbivore *M. sexta*. *O*-AS-fed *M. sexta* larvae emit a distinct odor of branched-chain aliphatic acids from their bodies and frass that attracts their natural enemies (*Pogonomyrmex rugosus*) (Weinhold & Baldwin, 2011). In **manuscript I**, I showed that the total levels of *O*-AS were associated with *M. sexta*'s growth rates. Removing *O*-AS from the leaf surface increased *M. sexta* growth on *N. attenuata* detached leaves, and artificial diet supplemented with *O*-AS significantly reduced *M. sexta* growth and development. Thus, *O*-AS not only function as constitutive defenses against the fungal pathogens *Fusarium* and *Alternaria*, and as indirect

defenses against *M. sexta* (Weinhold & Baldwin, 2011), but also as a direct defense against *M. sexta*.

The mechanism for the detrimental effects of *O*-AS on herbivores may involve the membrane toxicity of BCAAs, similar to their effect on pathogens. The supplementation of 4 main BCAAs in artificial diet significantly reduced *M. sexta* growth and development. However, pathogens and herbivores may also respond differently to *O*-AS or their degradation products. Results from **manuscript I** show that compared to the fungal pathogen *Fusarium*, *M. sexta* growth is less affected by *O*-AS. The reduction in 20-30% of total leaf *O*-AS via trichome-specific silencing of the gene *NaBCKDE1B* did lead to increased performance of *Fusarium*, but not of *M. sexta* larvae. Another hypothesis for the detrimental effects of *O*-AS on *M. sexta* is that they may act as lipophilic exudates that enhance the penetration of other toxic agents into caterpillars through the skin. If this is true, it could help to explain the lower effect of *O*-AS on *M. sexta* performance relative to fungi such as *Fusarium*, as *M. sexta* have large bodies in contrast to the small size of fungal spores, hence reducing exposure to *O*-AS contact. Further studies of the *O*-AS digestion process by *M. sexta* as well as its detoxification mechanisms will enhance our understanding of how *O*-AS influence *M. sexta* growth and development.

In conclusion, *O*-AS production can be considered as a generalized defense strategy of *N. attenuata* against both native herbivores and pathogens. Having this generalized defense strategy helps plants to utilize their resources; however, it is not always the best defense strategy of plants. A disadvantage of having the generalized defense strategy is that plants face the risk of developing pathogens or herbivores resistant to their generalized defense compounds, resulting in an evolutionary arms race between plants and natural enemies. In particular, the increase in one defense strategy of plants places strong selective pressures on natural enemies to also increase their virulence and ability to overcome the plant defense (Anderson *et al.*, 2010). Furthermore, the generalized defense strategy is also difficult to be maintained in plant defense system. Especially, when different attacking organisms (herbivores or pathogens) have different sensitivity to generalized defensive compounds, plants may need to develop a more specific response to each attacker (Krischik *et al.*, 1991). Therefore, to strengthen the generalized defense strategy, *N. attenuata* plants have evolved to exhibit a great phenotypic plasticity of this defense among different plant individuals.

3. *O*-AS variation among *N. attenuata* natural accessions

The need to contend with various environmental challenges has resulted not only in the extreme diversity of chemicals and their functions among different plant species but also in the phenotypic plasticity of individual plants within one species (Baldwin, 2001; Moore *et al.*, 2014). Hence, each individual plant will reconfigure its phenotype in response to biotic and abiotic environmental challenges. Similarly, variation of herbivore or pathogen communities has been shown to influence plant defense strategies (Kliebenstein & Rowe, 2008; Turley *et al.*, 2013). This variation of pathogen or herbivore communities was thought to be a selective agent driving the evolution of plant secondary metabolites (Krischik *et al.*, 1991). In native environments, *N. attenuata* faces a highly dynamic community of native herbivores and pathogens. As *N. attenuata* seeds remain dormant for long between-fire intervals and germinate when smoke signals are sensed and leaf litter is removed or pyrolized (Baldwin & Morse, 1994), the location of *N. attenuata* populations as well as their herbivores and pathogens are unpredictable. This unpredictability likely accounts for *N. attenuata's* remarkable plasticity in herbivore and pathogen defense strategies. In **manuscript I**, I show that *N. attenuata*'s *O*-AS can be considered as generalized defense compounds that plays a role in defense against both native pathogens and herbivores. I also found substantial variation among 26 natural accessions in both total amounts and the composition of *O*-AS. I suggest that *N. attenuata O*-AS natural variation results from the selection pressure of both herbivores and pathogens.

The mechanisms responsible for the variation in composition of *O*-AS in *N. attenuata* remain unknown. As a consequence, the ecological function of the various classes remains elusive, but one could speculate that they may have specialized defense functions against certain herbivores or pathogens, as shown for other plant secondary metabolites (Moore *et al.*, 2014). To gain more knowledge about the function of individual *O*-AS, bioassay-guided fractionation can be used. A prerequisite for such an approach will be the purification of individual *O*-AS, which unfortunately was not possible with the reverse phase HPLC method used in this study. Because *O*-AS represent a highly lipophilic compound class, normal phase HPLC systems may provide the separation required to fractionate single compounds which can be used in bioassays.

In conclusion, *N. attenuata* has high plasticity in *O*-AS production among different individuals in order to cope with highly variable herbivore and pathogen communities. The plasticity of defense strategies in a plant species is driven by the genetic variation of that species, as well as how the genotype determines the phenotype within the constraints imposed by other traits (Moore *et al.*, 2014). It is known that *N. attenuata* has high genetic variation, which translates into high phenotypic variation between and within populations (Bahulikar *et al.*, 2004; Schuck *et al.*, 2014; Li *et al.*, 2016). This high genetic variation results from long-lived seed banks which recruit *N. attenuata* seeds from different generations and locations to the same population at a certain time (Baldwin, 2001). In a previous study, this genetic variability was suggested as an explanation for the plasticity of pathogen resistances and the coexistence of individuals with different defense strategies within a wild population (Schuck *et al.*, 2014). In contrast to wild plant populations where genetic variation is often high, crop plants are designed to have a mono-genotype or mono-culture in order to create homogenous harvests. This is one of many reasons that field crops often possess low phenotypic plasticity and high susceptibility to pathogens and herbivores. In **manuscript II**, the isogenic or *de facto* genetic uniformity of planted *N. attenuata* lines is likely one of the factors that contributed to the accumulation of specialized pathogens as well as to the plant's high susceptibility to sudden wilt disease. Increasing intraspecific diversity in crop plants would increase plasticity in defenses against herbivores and diseases, hence, reduce yield loss due to pathogenic disease or herbivory.

Although chemical diversity helps to reduce the ability of natural enemies to overcome plant defense, however, it is not always beneficial for plants given the cost for maintaining inactive compounds (Biere *et al.*, 2004; Moore *et al.*, 2014). The ecological fitness cost associated with production of secondary metabolites is even more pronounced when plants are interacting with other abiotic and biotic factors in their ecological niche, such as drought stresses, intraspecific competitors or mutualistic partners (Weiner & Thomas, 1986; Kessler & Baldwin, 2007). Hence, the cost of maintaining inactive compounds may sometimes have caused them to be lost, and strong directional selection may cause the accumulation of more effective bioactive compounds. Previous studies have shown that effective defenses can sometimes arise from quantitative without qualitative diversity. For instance, chemical defenses of *Plantago lanceolata* against the specialist herbivore *Junonia coenia* are based upon only two iridoid glycosides and

two phenylpropanoid glycosides (Adler *et al.*, 1995). Furthermore, some defense compounds might exhibit some potential autotoxicity for the plant (Baldwin & Callahan, 1993). To reduce all those disadvantages of producing defensive compounds, plants develop associations with other co-occurring partners, especially microbes, in order to enhance their defenses against natural enemies.

4. *N. attenuata* associations with native microbes enhance disease resistance

When *N. attenuata* plants germinate from their seed banks, they typically associate with diverse fungi and bacteria that exist in native soils, and a subset of this community becomes root-associated (Long *et al.*, 2010; Santhanam *et al.*, 2015a). Among those, certain microbes can promote plant growth and boost mineral availability for *N. attenuata* (Meldau *et al.*, 2012; Santhanam *et al.*, 2015a). Other microbes such as *Fusarium* and *Alternaria* fungi can be pathogenic for *N. attenuata* plants in their native environments (Schuck *et al.*, 2014; Luu *et al.*, 2015). However, most of plant-microbe interactions are likely to be context-dependent, shifting along the functional spectrum (pathogenic, commensalistic, or mutualistic) depending on environmental conditions or during the life cycle of the microbe or the plant (Newton *et al.*, 2010). In **manuscript II**, I showed that *N. attenuata* root-associated microbes can enhance disease resistance in the plant.

Sudden wilt disease emerged from the continuous planting of *N. attenuata* for 15 years in an experimental field located in the plant's native environment. The common agricultural dilemma of pathogen buildup associated with continuous cropping was therefore inadvertently recapitulated for this native plant. Plants suffered sudden tissue collapse and black roots, symptoms similar to a *Fusarium-Alternaria* disease complex previously characterized in a nearby native population (Schuck *et al.*, 2014). Based on classical isolation techniques, cloning, and sequencing, I found that the fungal phytopathogens *Alternaria* spp. and *Fusarium* spp. were highly abundant in the roots of diseased plants and likely contributed to the sudden wilt disease. These results indicate that the repeated planting of *N. attenuata* undermines the natural disease-avoidance strategy of the plant's normally ephemeral, fire-chasing populations, and likely led to an accumulation of pathogens. Similarly to crop plants, various plant protection methods were suggested, including plant rotation, fungicide treatment, soil amendment, and biocontrol. Crop

rotation entails the use of different crops in succession in order to interrupt the disease cycle of plant pathogens (Curl, 1963; Krupinsky *et al.*, 2002). In fact, due to the marker demand of certain crops, farmers often select the same crop for growing in their fields. Crop rotation is also not an option for plants that used in research programs such as *N. attenuata*. Therefore, in **manuscript II**, I only compared the effectiveness of different disease-control methods, including fungal or bacterial biocontrols, fungicide and a biochar soil amendment for *N. attenuata*. A field trial with more than 900 plants in two field plots revealed that only inoculation with a mixture of native bacterial isolates significantly reduced disease incidence and mortality in the infected field plot.

The five native bacterial isolates (*Arthrobacter nitroguajacolicus* E46, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70, and *Pseudomonas frederiksbergensis* A176) were originally isolated from the roots of healthy *N. attenuata* plants in the same field where the fungal disease appeared. They are therefore likely to be better adapted to their host and its associated environmental conditions. The reproducibility and persistence of the inoculated bacteria in *N. attenuata* planted over two field seasons demonstrates that these native bacteria establish stable associations with *N. attenuata* roots which persist throughout the plant's growth under field conditions. The enhanced defense of *N. attenuata* that results from association with native bacteria works most effectively when all five bacterial strains are mixed, indicating that the association of *N. attenuata* with a consortium of bacteria that works collectively to enhance the plant's disease resistance. In addition to enhanced pathogen resistance, no other effects of bacterial biocontrol agents on plant performance (growth, biomass, reproductive outputs) or traits essential for herbivore defenses was observed. This demonstrates the specificity of the bacterial inoculation to pathogen resistance and a less costly relationship between *N. attenuata* and its native bacteria.

The mechanism through which this consortium of five bacteria enhances disease resistance in *N. attenuata* remains unknown and need to be investigated in future studies. It is hypothesized that bacterial consortium may elicit induced systemic resistance in *N. attenuata* plants, activating the production of anti-pathogen secondary metabolites. Since the enhanced resistance was most obvious when five bacterial isolates were mixed, they may also exhibit other synergistic mechanisms, such as antibiotic production, biofilm formation, or founder effects.

Sometimes, a mixture of biocontrol strains combines multiple mechanisms of action to enhance the consistency of disease control (Sarma *et al.*, 2015). Thus, diversity in biocontrol mechanisms offered by each microbial component may help in strengthening disease suppression (Sarma *et al.*, 2015). The combination of multiple actions from a biocontrol consortium can also reduce the ability of the pathogen to quickly overcome plant defenses, similarly to a plant having multiple resistance genes. Furthermore, it also increases the efficiency, reliability and consistency of anti-pathogen activity under diverse soil and environmental conditions (Stockwell *et al.*, 2011). However, since this defense strategy involves multiple biocontrol agents, the anti-pathogen activity of a consortium depends on the interaction between different biocontrol agents that may change under certain conditions. Hence, artificially mixed microbial combinations may lead to increased, similar or even reduced pathogen suppression (Xu *et al.*, 2011). In our case, when two fungal isolates, *Chaetomium* sp. C72 and *Oidodendron* sp. Oi3 were mixed, plant mortality due to sudden wilt disease even increased in the field (**manuscript II**). This suggests that microbial partners can also exhibit suppressive effects on other partners (Sarma et al., 2015).

In nature, beneficial microbial communities can be acquired from the soil at an early stage during germination and establish beneficial associations that last throughout the entire life cycle of the plants (Long *et al.*, 2010; Edwards *et al.*, 2015; Santhanam *et al.*, 2015a). It is unknown how those bacteria are recruited and maintained in a context-specific manner by *N. attenuata* in nature. Beneficial bacteria may generate signals that distinguish them from other soil microbes so that they can be recognized by the plant. *N. attenuata* plants may also tailor the content of their root exudates, such as amino acids, sugars, fatty acids and organic acids, to selectively recruit certain bacteria. Although some organic acids and secondary metabolites, such as flavonoids and strigolactones, have been found to mediate the recruitment of microbes under *in vitro* conditions (Steinkellner *et al.*, 2007; Rudrappa *et al.*, 2008; Neal *et al.*, 2012), untargeted metabolomic and genomic approaches are needed to evaluate the processes that are involved when plants are grown under real-world conditions.

5. Conclusion and future perspectives

This dissertation work unravels different defense mechanisms of the wild tobacco plant *N. attenuata* against its native pathogens and proves the importance of using wild plant

pathosystems to study plant- microbe interaction in an ecologically relevant context. By studying interaction of *N. attenuata* and its native pathogens, this study shows that *N. attenuata* has evolved a complex defense system for an effective defense against fungal pathogens.

Manuscript I demonstrates the direct defense function of *O*-AS in *N. attenuata* against a specialist herbivore and native pathogens, a function which complements their previously established function as indirect defenses. This result has a significant implementation in plant breeding or engineering of resistance to pathogens and herbivores. Further studies need to focus on the elucidation of the *O*-AS biosynthesis pathway and the genes involved in different steps of *O*-AS biosynthesis to provide a more efficient means of manipulating *O*-AS contents in crop plants. This study also suggests that the defensive functions of *O*-AS likely shape their natural variation and raises many open questions regarding to their fitness costs such as the cost of *O*-AS production and why *N. attenuata* produces *O*-AS if BCAAs are actually the basis of their resistance to both pathogens and herbivores, topics which require further study. Answering these questions would help us understanding how the balancing selection is generated which likely accounts for the great diversity of *O*-AS concentrations and compositions found in natural accessions.

Manuscript II shows that native plants can use opportunistic mutualistic relationships with their root-associated microbes to enhance their defenses against native fungal pathogens. Further research needs to be focused on similar issue for crop plants, including whether they lost the ability to develop opportunistic mutualistic relationships with their microbial partners, whether crop plants differ from their wild ancestors in their root-associated microbiota, which mechanisms crop plants use to govern recruitment and activity of recruited microbes, and which synergistic mechanisms are behind the bacterial consortia protection effect. Answering these lingering questions may open up opportunities to utilize this phenomenon in the increase of crop productivity, especially in the ever-changing landscape of abiotic and biotic stresses in agriculture.

Summary

In nature, the wild tobacco *N. attenuata*, like all plant species, is threatened by various natural enemies including fungal pathogens. In order to survive and maintain Darwinian fitness, the plant possesses a sophisticated defense system that is the result of a long evolutionary history and is still continuously challenged by a changing environment. To identify and characterize the anti-fungal mechanisms maintained in the plant's defense system and that actively interact with their native environment, I used the natural pathosystem of *N. attenuata*.

I studied *O*-acyl sugars (*O*-AS), trichome-specific metabolites that are highly abundant in several *Solanaceous* species including *N. attenuata*. To examine their role in constitutive defenses against two native fungal pathogens, *Fusarium brachygibbosum* U4 and *Alternaria* sp. U10, I combined several approaches, ranging from using natural variation to *in vitro* and *in vivo* manipulation of *O*-AS contents in *N. attenuata*. Experiments with natural accessions and crosses between high- and low-*O*-AS accessions revealed that total *O*-AS levels were associated with the plant's resistance to the tested pathogens. Removing *O*-AS from the leaf surface increased fungal susceptibility and their supplementation in the germination medium reduced fungal spore germination. Finally, silencing the expression of a putative branched-chain alpha-ketoacid dehydrogenase E1 beta subunit-encoding gene (*NaBCKDE1B*) in the trichomes reduced total leaf *O*-AS by 20-30% and increased susceptibility to *Fusarium* pathogens. These results demonstrate that *N. attenuata* has developed a constitutive defense strategy against the native fungal pathogens, *Fusarium* and *Alternaria*, via the production of *O*-AS. Interestingly, using the same approaches, I found that *O*-AS were also involved in direct defense against the native herbivore *Manduca sexta*. This indicates a generalized defense function of *O*-AS against both pathogens and herbivores, as well as the ability of *N. attenuata* to harbor a generalized defense strategy against multiple natural enemies. Furthermore, *O*-AS content and composition were found to vary substantially among 26 *N. attenuata* natural accessions, indicating the ability of this plant species to maintain high phenotypic plasticity in order to cope with the evolution of resistance in pathogens and herbivores.

The emergence of sudden wilt disease in field-grown plants created a unique opportunity to investigate mechanisms for enhanced defense in *N. attenuata*. Based on classical isolation

techniques, cloning, and sequencing, I found that the fungal phytopathogens *Alternaria* spp. and *Fusarium* spp. were highly abundant in the roots of diseased plants and likely contributed to sudden wilt disease. *In vitro* tests using fungicide, bacterial and fungal treatments reduced *N. attenuata* seedling mortality. Among 4 tested root-associated fungi, *Chaetomium* sp. C72 and *Oidodendron* sp. Oi3 were shown to have beneficial effects. A combination of five native bacterial isolates (*Arthrobacter nitroguajacolicus* E46, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70 and *Pseudomonas frederiksbergensis* A176) was most effective in reducing plant mortality. Different protection strategies including fungicide treatment and inoculation with native root-associated bacterial and fungal isolates, together with a biochar soil amendment, were tested on two different field plots for two field seasons with more than 900 plants. Only inoculation with a consortium of native bacterial isolates significantly attenuated the disease incidence in the field. These data indicate that the defense of *N. attenuata* against native fungal pathogens can be enhanced by root-associated bacteria, indicating the ability of this plant to develop opportunistic mutualistic relationships with its own root microbiome under high stress conditions. Interestingly, enhanced disease resistance was most obvious in the field when the plant is associated with a consortium of bacteria instead of a single bacterial isolate. This suggests that combined effects of multiple biocontrol agents help the plant to stabilize its defenses under more complex environmental condition.

In summary, *N. attenuata* has evolved a complex defense system for an effective defense against fungal pathogens. This system includes the ability to produce *O*-AS as a constitutive and generalized defense strategy. It also involves the ability of *N. attenuata* to generate high phenotypic plasticity in *O*-AS content and composition among natural accessions. Furthermore, this complex defense system contains the ability of *N. attenuata* to associate with the root microbiome in order to enhance its defense against fungal pathogens. Understanding the function, complexity and interaction of different defense strategies maintained by the plant under real-world conditions will empower our ability to sustain crop production in the challenge of climate changes.

Zusammenfassung

In der Natur ist der wilde Tabak *N. attenuata*, wie alle Pflanzenarten, von verschiedenen natürlichen Feinden, einschließlich pilzlichen Pathogenen, bedroht. Um zu überleben und ihre darwinistische Fitness zu erhalten, besitzen Pflanzen ein ausgeklügeltes Abwehrsystem, das aus einer langen Evolutionsgeschichte resultiert und immer wieder von veränderten Umweltbedingungen herausgefordert wird. Um die Abwehrmechanismen gegen Pilze zu identifizieren und zu charakterisieren, die im Verteidigungssystem der Pflanze aufrechterhalten wurden und aktiv mit ihrer natürlichen Umgebung interagieren, verwendete ich ein natürliches Pathosystem von *N. attenuata*.

Ich habe *O*-Acyl-Zucker (*O*-AS), Metabolite, die spezifisch in Blatthaaren (Trichomen) in zahlreichen Nachtschattengewächsen, einschließlich *N. attenuata*, vorkommen, untersucht. Um ihre Rolle in der konstitutiven Verteidigung gegen zwei einheimische Pilzpathogene, *Fusarium brachygibbosum* U4 und *Alternaria* sp. U10 näher zu analysieren, kombinierte ich verschiedene methodische Ansätze, die von der Untersuchung natürlicher Variation bis zu *in vitro*- und *in vivo*-Manipulation von *O*-AS-Gehalten in *N. attenuata* reichten. Experimente mit natürlichen Gehalten und Kreuzungen zwischen Linien mit hohem und niedrigem *O*-AS Gehalt zeigten, dass die Gesamt-*O*-AS-Werte mit der Resistenz der Pflanze gegenüber den getesteten Pathogenen assoziiert waren. Das Entfernen von *O*-AS von der Blattoberfläche erhöhte die Pilzanfälligkeit; Keimungsmedium, das mit *O*-AS angereichert war, hemmte die Keimung der Pilzsporen. Schließlich konnte durch verringerte Expression einer putativen verzweigten alpha-Ketoacid-Dehydrogenase-E1-beta-Untereinheit, die das Gen (NaBCKDE1B) in den Trichomen kodiert, der *O*-AS-Gehalt des gesamten Blatts um 20-30% reduziert werden. Dies erhöhte die Anfälligkeit für *Fusarium*-Pathogene. Diese Ergebnisse zeigen, dass *N. attenuata* eine konstitutive Verteidigungsstrategie gegen die nativen Pilzpathogene *Fusarium* und *Alternaria* mittels der Produktion von *O*-AS entwickelt hat. Interessanterweise fand ich mit den gleichen experimentellen Ansätzen heraus, dass die *O*-AS auch in die direkte Verteidigung gegen den in der Heimat von *N. attenuata* vorkommenden Pflanzenfresser *Manduca sexta* beteiligt waren. Diese Ergebnisse legen eine allgemeine Verteidigungsfunktion von *O*-AS in der Abwehr sowohl gegen Pathogene als auch gegen Herbivoren nahe. Damit implizieren sie, dass *N. attenuata* eine allgemeine Verteidigungsstrategie gegen mehrere natürliche Feinde zu beherbergt. In weiteren

Untersuchungen fand ich heraus, dass sowohl die *O*-AS-Gehalte als auch deren Zusammensetzung in 26 verschiedenen, natürlichen *N. attenuata* Accessions grundlegend variieren. Dies deutet darauf hin, dass diese Pflanzenart eine hohe phänotypische Plastizität erzeugen kann, um der Resistenzentwicklung der Pathogene und Pflanzenfresser besser vorzubeugen.

Das Auftreten der „Plötzlichen Welkekrankheit" (sudden wilt disease) von *N. attenuata*-Pflanzen, die auf unserem Versuchsfeld in Utah wuchsen, bot eine einmalige Gelegenheit, Mechanismenfür eine erhöhte Verteidigung gegenüber Pathogenen zu untersuchen. Auf der Grundlage von klassischen Isolierungstechniken, Klonierung und Sequenzierung fand ich heraus, dass die Krankheitsverursacher vermutlich *Alternaria* spp. und *Fusarium* spp. sind. Die Pilzpathogene waren in den Wurzeln der kranken Pflanzen reichlich vorhanden und trugen wahrscheinlich zu der plötzlichen Welkekrankheit bei. *In-vitro*-Tests unter Verwendung von Fungiziden, Bakterien- und Pilzinokulation reduzierten die Sterblichkeit von *N. attenuata*-Keimlingen. Von 4 getesteten Wurzel-assoziierten Pilzen, zeigten *Chaetomium* sp. C72 und *Oidodendron* sp. Oi3 positive Effekte. Eine Kombination aus fünf einheimischen bakteriellen Isolaten (*Arthrobacter nitroguajacolicus* E46, *Bacillus megaterium* B55, *Bacillus mojavensis* K1, *Pseudomonas azotoformans* A70 und *Pseudomonas frederiksbergensis* A176) war der wirksamste Ansatz, um die Pflanzensterblichkeit zu senken. Verschiedene Schutzstrategien, einschließlich Fungizidbehandlung und Inokulation mit einheimischen, Wurzel-assoziierten Bakterien- und Pilzisolaten zusammen mit einem Bodenverbesserer aus Biokohle, wurden auf zwei Versuchsfeldern für zwei Feldsaisons mit mehr als 900 Pflanzen getestet. Nur die Inokulation mit nativen Bakterienisolaten verminderte das Auftreten der Krankheit im Feld signifikant. Diese Daten zeigen, dass die Verteidigung von *N. attenuata* gegen einheimische pilzliche Pathogene durch die mit ihrer Wurzel assoziierten Bakterien verbessert werden kann. Dies deutet auf die Fähigkeit dieser Pflanze hin, opportunistisch-mutualistische Beziehungen mit ihrem eigenen Wurzelmikrobiom unter Stressbedingungen zu entwickeln. Interessanterweise war die erhöhte Krankheitsresistenz im Feld am deutlichsten zu sehen, wenn die Pflanze mit einem Konsortium von Bakterien – und nicht nur mit einem einzigen Bakterienstamm – inokuliert wurde. Dies deutet darauf hin, dass kombinierte Effekte von mehreren Strategien, die an der Biokontrolle beteiligt sind, der Pflanze helfen, ihre Verteidigung unter natürlichen Umweltbedingungen zu stabilisieren.

Zusammenfassend hat *N. attenuata* ein komplexes Abwehrsystem für eine wirksame Verteidigung gegen Pilzpathoge entwickelt. Dieses System schließt ihre Fähigkeit, *O*-AS als konstitutive und allgemeine Verteidigungsstrategie zu produzieren, ein. Es beinhaltet auch die Fähigkeit von *N. attenuata*, eine hohe phänotypische Plastizität in *O*-AS-Gehalten und Zusammensetzungen unter natürlichen Bedingungen zu erzeugen. Darüber hinaus umfasst dieses komplexe Abwehrsystem die Fähigkeit von *N. attenuata*, sich mit dem Wurzelmikrobiom zu assoziieren, um die Abwehr der Pflanzen gegen Pilzpathogene zu verstärken. Ein besseres Verständnis der Funktion, Komplexität und Interaktion zwischen verschiedenen Verteidigungsstrategien, die in der Pflanze unter natürlichen Bedingungen erhalten geblieben sind, kann uns dabei helfen, die Pflanzenproduktion unter den Herausforderungen des weltweiten Klimawandels zu erhalten.

References

(Introduction & Discussion)

Adler LS, Bronstein JL (2004) Attracting antagonists: does floral nectar increase leaf herbivory? Ecology **85:** 1519-1526

Adler LS, Schmitt J, Bowers MD (1995) Genetic variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the specialist herbivore *Junonia coenia* (Nymphalidae). Oecologia **101:** 75-85

Agrios GN (2005) Plant pathology, Ed 5th. Elsevier Academic Press, San Diego

Almeida AAP, Farah A, Silva DAM, Nunan EA, Gloria MBA (2006) Antibacterial activity of coffee extracts and selected coffee chemical compounds against enterobacteria. Journal of agricultural and food chemistry **54:** 8738-8743

Anderson JP, Gleason CA, Foley RC, Thrall PH, Burdon JB, Singh KB (2010) Plants versus pathogens: an evolutionary arms race. Functional plant biology : FPB **37:** 499-512

Bahulikar RA, Stanculescu D, Preston CA, Baldwin IT (2004) ISSR and AFLP analysis of the temporal and spatial population structure of the post-fire annual, *Nicotiana attenuata*, in SW Utah. BMC ecology **4:** 12

Baldwin IT (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proceedings of the national academy of sciences of the United States of America **95:** 8113-8118

Baldwin IT (2001) An ecologically motivated analysis of plant-herbivore interactions in native tobacco. Plant physiology **127:** 1449-1458

Baldwin IT, Callahan P (1993) Autotoxicity and chemical defense: nicotine accumulation and carbon gain in solanaceous plants. Oecologia **94:** 534-541

Baldwin IT, Morse L (1994) Up in Smoke: II. Germination of *Nicotiana attenuata* in response to smoke-derived cues and nutrients in burned and unburned soils. Journal of chemical ecology **20:** 2373-2391

Biere A, Marak HB, van Damme JMM (2004) Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? Oecologia **140:** 430-441

Blanchette R, Biggs A (2013) Defense mechanisms of woody plants against fungi. Springer Science & Business Media

Bonfante P, Anca I-A (2009) Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annual review of microbiology **63:** 363-383

Burdon J (1987) Diseases and plant population biology. CUP Archive

Burdon J, Jarosz A (1989) Wild relatives as sources of disease resistance. Cambridge University Press, Cambridge, UK

Burdon J, Oates J, Marshall D (1983) Interactions between *Avena* and *Puccinia* species. I. The wild hosts: *Avena barbata* Pott Ex Link, *A. fatua* L, *A ludoviciana* Durieu. Journal of applied ecology**:** 571-584

Burdon JJ, Purcell AH, Lindow SE (1987) Diseases and plant-population biology Science **238:** 221-221

Calo L, Garcia I, Gotor C, Romero LC (2006) Leaf hairs influence phytopathogenic fungus infection and confer an increased resistance when expressing a trichoderma alpha-1,3-glucanase. Journal of experimental botany **57:** 3911-3920

Carmona D, Fornoni J (2013) Herbivores can select for mixed defensive strategies in plants. New phytologist **197:** 576-585

Chattopadhyay S, Ali KA, Doss SG, Das NK, Aggarwal RK, Bandopadhyay TK, Sarkar A, Bajpai AK (2011) Association of leaf micro-morphological characters with powdery mildew

resistance in field-grown mulberry (*Morus* spp.) germplasm. AoB plants **2011**

Chortyk OT, Kays SJ, Teng Q (1997) Characterization of insecticidal sugar esters of *Petunia*. Journal of agricultural and food chemistry **45:** 270-275

Commenil P, Brunet L, Audran J-C (1997) The development of the grape berry cuticle in relation to susceptibility to bunch rot disease. Journal of experimental botany **48:** 1599-1607

Cornell HV (1990) Survivorship, life history, and concealment: a comparison of leaf miners and gall formers. American naturalist**:** 581-597

Curl EA (1963) Control of plant diseases by crop rotation. The botanical review **29:** 413-479

del Campo ML, Halitschke R, Short SM, Lazzaro BP, Kessler A (2013) Dietary plant phenolic improves survival of bacterial infection in *Manduca sexta* caterpillars. Entomologia experimentalis et applicata **146:** 321-331

DeLong DM (1971) The bionomics of leafhoppers. Annual review of entomology **16:** 179-210

Dinoor A, Eshed N (1984) The role and importance of pathogens in natural plant communities. Annual review of phytopathology **22:** 443-466

Dita MA, Martínez-de la Parte MSc E (2014) Technical manual prevention and diagnostic of *Fusarium* wilt (Panama disease) of banana caused by *Fusarium oxysporum* f. sp. cubense tropical race 4 (TR4).

Dixon AFG (1977) Aphid ecology: life cycles, polymorphism, and population regulation. Annual review of ecology and systematics **8:** 329-353

Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V (2015) Structure, variation, and assembly of the root-associated microbiomes of rice. Proceedings of the national academy of sciences of the United States of America **112:** E911-E920

Eyal Z (1999) The *Septoria tritici* and *Stagonospora nodorum* blotch diseases of wheat.

European journal of plant pathology **105:** 629-641

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) Plant drought stress: Effects, mechanisms and management. *In* E Lichtfouse, M Navarrete, P Debaeke, S Véronique, C Alberola, eds, Sustainable agriculture. Springer Netherlands, Dordrecht, pp 153-188

Fenwick G, Price K, Tsukamoto C, Okubo K (1991) Saponins. Toxic substances in crop plants**:** 285-327

Fobes JF, Mudd JB, Marsden MPF (1985) Epicuticular lipid-accumulation on the leaves of *Lycopersicon pennellii* (Corr.) D'arcy and *Lycopersicon esculentum* Mill. Plant physiology **77:** 567-570

Fridman E, Wang JH, Iijima Y, Froehlich JE, Gang DR, Ohlrogge J, Pichersky E (2005) Metabolic, genomic, and biochemical analyses of glandular trichomes from the wild tomato species *Lycopersicon hirsutum* identify a key enzyme in the biosynthesis of methylketones. The plant cell **17:** 1252-1267

Gaiero JR, McCall CA, Thompson KA, Day NJ, Best AS, Dunfield KE (2013) Inside the root microbiome: bacterial root endophytes and plant growth promotion. American journal of botany **100:** 1738-1750

Gang DR, Wang JH, Dudareva N, Nam KH, Simon JE, Lewinsohn E, Pichersky E (2001) An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. Plant physiology **125:** 539-555

Gershenzon J, Dudareva N (2007) The function of terpene natural products in the natural world. Nature chemical biology **3:** 408-414

Gibson R, Pickett J (1983) Wild potato repels aphids by release of aphid alarm pheromone.

Glas JJ, Schimmel BCJ, Alba JM, Escobar-Bravo R, Schuurink RC, Kant MR (2012) Plant glandular trichomes as targets for breeding or engineering of resistance to herbivores. International journal of molecular sciences **13:** 17077-17103

Goffreda JC, Mutschler MA, Ave DA, Tingey WM, Steffens JC (1989) Aphid deterrence by glucose eEsters in glandular trichome exudate of the wild tomato, *Lycopersicon pennellii*. Journal of chemical ecology **15:** 2135-2147

Hartleb H, Heitefuss R, Hoppe H-H (1997) Resistance of crop plants against fungi. G. Fischer

Hawthorne DJ, Shapiro JA, Tingey WM, Mutschler MA (1992) Trichome-borne and artificially applied acylsugars of wild tomato deter feeding and oviposition of the leafminer *Liriomyza trifolii*. Entomologia experimentalis et applicata **65:** 65-73

Hay M, Steinberg P, Rosenthal G, Berenbaum M (1992) Herbivores: their interactions with secondary plant metabolites, Vol 2. Academic press, INC.

Hespenheide HA (1991) Bionomics of leaf-mining insects. Annual review of entomology **36:** 535-560

Hill G, Stellwaag-Kittler F, Huth G, Schlosser E (1981) Resistance of grapes in different developmental stages to *Botrytis cinerea*. Journal of phytopathology **102:** 328-338

Jacobi WR, Koski RD, Harrington TC, Witcosky JJ (2007) Association of *Ophiostoma novo-ulmi* with *Scolytus schevyrewi* (Scolytidae) in Colorado. Plant disease **91:** 245-247

Jain A, Singh A, Singh S, Singh HB (2013) Microbial consortium-induced changes in oxidative stress markers in pea plants challenged with *Sclerotinia sclerotiorum*. Journal of plant growth regulation **32:** 388-398

Johnson G, Schaal LA (1952) Relation of chlorogenic acid to scab resistance in potatoes. Science **115:** 627-629

Juvik JA, Shapiro JA, Young TE, Mutschler MA (1994) Acylglucoses from wild tomatoes alter behavior and reduce growth and survival of *Helicoverpa zea* and *Spodoptera exigua* (Lepidoptera, Noctuidae). Journal of economic entomology **87:** 482-492

Kessler D, Baldwin IT (2007) Making sense of nectar scents: the effects of nectar secondary

metabolites on floral visitors of *Nicotiana attenuata*. The plant journal **49:** 840-854

Kliebenstein DJ, Rowe HC (2008) Ecological costs of biotrophic versus necrotrophic pathogen resistance, the hypersensitive response and signal transduction. Plant science **174:** 551-556

Krischik VA, Goth RW, Barbosa P (1991) Generalized plant defense: effects on multiple species. Oecologia **85:** 562-571

Krischik VA, Goth RW, Barbosa P (1991) Generalized plant defense: effects on multiple species. Oecologia **85:** 562-571

Kroumova AB, Wagner GJ (2003) Different elongation pathways in the biosynthesis of acyl groups of trichome exudate sugar esters from various solanaceous plants. Planta **216:** 1013-1021

Krupinsky JM, Bailey KL, McMullen MP, Gossen BD, Turkington TK (2002) Managing plant disease risk in diversified cropping systems. Agronomy journal **94:** 198-209

Kula AAR, Castillo DM, Dudash MR, Fenster CB (2014) Interactions between a pollinating seed predator and its host plant: the role of environmental context within a population. Ecology and evolution **4:** 2901-2912

Lattanzio V, Lattanzio VM, Cardinali A (2006) Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. Phytochemistry: Advances in research **661:** 23-67

Lebeda A, Petrželová I, Maryška Z (2008) Structure and variation in the wild-plant pathosystem: *Lactuca serriola-Bremia lactucae*. *In* A Lebeda, PN Spencer-Phillips, BM Cooke, eds, The downy mildews: Genetics, molecular biology and control. Springer Netherlands, pp 127-146

Li D, Baldwin I, Gaquerel E (2016) Beyond the canon: Within-plant and population-level heterogeneity in Jasmonate signaling engaged by plant-insect interactions. Plants **5:** 14

Long HH, Sonntag DG, Schmidt DD, Baldwin IT (2010) The structure of the culturable root

bacterial endophyte community of *Nicotiana attenuata* is organized by soil composition and host plant ethylene production and perception. New phytologist **185:** 554-567

Lucas J (2009) Plant pathology and plant pathogens. John Wiley & Sons

Luu VT, Schuck S, Kim SG, Weinhold A, Baldwin IT (2015) Jasmonic acid signalling mediates resistance of the wild tobacco *Nicotiana attenuata* to its native *Fusarium*, but not *Alternaria*, fungal pathogens. Plant cell and environment **38:** 572-584

Meldau DG, Long HH, Baldwin IT (2012) A native plant growth promoting bacterium, *Bacillus* sp. B55, rescues growth performance of an ethylene-insensitive plant genotype in nature. Frontiers in plant science **3:** 112

Moore B, Andrew RL, Kulheim C, Foley WJ (2014) Explaining intraspecific diversity in plant secondary metabolites in an ecological context. New phytologist **201:** 733-750

Musselman LJ (1980) The biology of *Striga*, *Orobanche*, and other root-parasitic weeds. Annual review of phytopathology **18:** 463-489

Neal AL, Ahmad S, Gordon-Weeks R, Ton J (2012) Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. PLos one **7:** e35498

Newton AC, Fitt BD, Atkins SD, Walters DR, Daniell TJ (2010) Pathogenesis, parasitism and mutualism in the trophic space of microbe–plant interactions. Trends in microbiology **18:** 365-373

Newton AC, Fitt BD, Atkins SD, Walters DR, Daniell TJ (2010) Pathogenesis, parasitism and mutualism in the trophic space of microbe–plant interactions. Trends in microbiology **18:** 365-373

Nguyen TTX, Dehne HW, Steiner U (2016) Maize leaf trichomes represent an entry point of infection for *Fusarium* species. Fungal biology **120:** 895-903

Osbourn AE (1996) Preformed antimicrobial compounds and plant defense against fungal

attack. The plant cell **8:** 1821

Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nature reviews microbiology **6:** 763-775

Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM, Bakker PAHM (2014) Induced systemic resistance by beneficial microbes. Annual review of phytopathology **52:** 347-375

Pohl C, Kock J, Thibane V (2011) Antifungal free fatty acids: a review. Science against microbial pathogens: communicating current research and technological advances. Microbiology Book Series - Number 3 A. Méndez-Vilas, **:** 61–71

Politowski K, Browning JA (1978) Tolerance and resistance to plant-disease - epidemiological study. Phytopathology **68:** 1177-1185

Puterka GJ, Farone W, Palmer T, Barrington A (2003) Structure-function relationships affecting the insecticidal and miticidal activity of sugar esters. Journal of economic entomology **96:** 636-644

Rehnstrom AL, Free SJ (1996) The isolation and characterization of melanin-deficient mutants of *Monilinia fructicola*. Physiological and molecular plant pathology **49:** 321-330

Roncero MIG, Hera C, Ruiz-Rubio M, Maceira FIG, Madrid MP, Caracuel Z, Calero F, Delgado-Jarana J, Roldan-Rodriguez R, Martinez-Rocha AL, Velasco C, Roa J, Martin-Urdiroz M, Cordoba D, Di Pietro A (2003) *Fusarium* as a model for studying virulence in soilborne plant pathogens. Physiological and molecular plant pathology **62:** 87-98

Royle D (1976) Structural features of resistance to plant diseases. *In* Biochemical aspects of plant-parasite relationships, Phytochemical society symposium series, pp 161-193

Rudrappa T, Czymmek KJ, Paré PW, Bais HP (2008) Root-secreted malic acid recruits beneficial soil bacteria. Plant physiology **148:** 1547-1556

Santhanam R, Baldwin IT, Groten K (2015) In wild tobacco, *Nicotiana attenuata*, variation among bacterial communities of isogenic plants is mainly shaped by the local soil microbiota independently of the plants' capacity to produce jasmonic acid. Communicative & integrative biology **8:** e1017160

Santhanam R, Luu VT, Weinhold A, Goldberg J, Oh Y, Baldwin IT (2015) Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping. Proceedings of the national academy of sciences of the United States of America **112:** E5013-E5020

Sarma BK, Yadav SK, Singh S, Singh HB (2015) Microbial consortium-mediated plant defense against phytopathogens: Readdressing for enhancing efficacy. Soil biology and biochemistry **87:** 25-33

Scalbert A (1991) Antimicrobial properties of tannins. Phytochemistry **30:** 3875-3883

Schafer JF (1971) Tolerance to plant disease. Annual review of phytopathology **9:** 235-&

Schuck S, Weinhold A, Luu VT, Baldwin IT (2014) Isolating fungal pathogens from a dynamic disease outbreak in a native plant population to establish plant-pathogen bioassays for the ecological model plant *Nicotiana attenuata*. Plos one **9**

Smolinska U, Morra M, Knudsen G, James R (2003) Isothiocyanates produced by Brassicaceae species as inhibitors of *Fusarium oxysporum*. Plant disease **87:** 407-412

Spaulding P (1925) A partial explanation of the relative susceptibility of the white pines to the white pine blister rust (*Cronartium ribicola*, Fischer),

Srivastava R, Khalid A, Singh U, Sharma A (2010) Evaluation of arbuscular mycorrhizal fungus, fluorescent *Pseudomonas* and *Trichoderma harzianum* formulation against *Fusarium oxysporum* f. sp. *lycopersici* for the management of tomato wilt. Biological control **53:** 24-31

Steinkellner S, Lendzemo V, Langer I, Schweiger P, Khaosaad T, Toussaint J-P, Vierheilig H (2007) Flavonoids and strigolactones in root exudates as signals in symbiotic and pathogenic

plant-fungus interactions. Molecules **12:** 1290-1306

Stockwell V, Johnson K, Sugar D, Loper J (2011) Mechanistically compatible mixtures of bacterial antagonists improve biological control of fire blight of pear. Phytopathology **101:** 113-123

Strausbaugh CA, Maloy OC (1986) *Fusarium* scab of irrigated wheat in central Washington. Plant disease **70:** 1104-1106

Strauss SY, Rudgers JA, Lau JA, Irwin RE (2002) Direct and ecological costs of resistance to herbivory. Trends in ecology & evolution **17:** 278-285

Stukenbrock EH, McDonald BA (2008) The origins of plant pathogens in agro-ecosystems. Annual review of phytopathology **46:** 75-100

Thomma BPHJ (2003) *Alternaria* spp.: from general saprophyte to specific parasite. Molecular plant pathology **4:** 225-236

Timmer LW, Peever TL, Solel Z, Akimitsu K (2003) *Alternaria* diseases of citrus - Novel pathosystems, Vol 42

Treutter D (2006) Significance of flavonoids in plant resistance: a review. Environmental chemistry letters **4:** 147-157

Turley NE, Godfrey RM, Johnson MT (2013) Evolution of mixed strategies of plant defense against herbivores. New phytologist **197:** 359-361

Ungar IA (1992) The effect of intraspecific competition on growth, reproduction, and survival of the halophyte *Spergularia marina*. International journal of plant sciences **153:** 421-424

Van Dam N (2009) How plants cope with biotic interactions. Plant biology **11:** 1-5

Vermerris W, Nicholson R (2008) Isolation and identification of phenolic compounds. *In* Phenolic compound biochemistry. Springer, pp 151-196

Walker JC, Link KP (1935) Toxicity of phenolic compounds to certain onion bulb parasites. Botanical gazette **96:** 468-484

Wang LW, Showalter AM, Ungar IA (2005) Effects of intraspecific competition on growth and photosynthesis of *Atriplex prostrata*. Aquatic botany **83:** 187-192

Webber JF (2000) Insect vector behavior and the evolution of Dutch elm disease. *In* CP Dunn, ed, The Elms: Breeding,conservation, and disease management. Springer US, Boston, MA, pp 47-60

Weiner J, Thomas SC (1986) Size variability and competition in plant monocultures. Oikos**:** 211-222

Weinhold A, Baldwin IT (2011) Trichome derived *O*-acyl sugars are a first meal for caterpillars that tags them for predation. Proceedings of the national academy of sciences of the United States of America **108:** 7855-7859

Weinhold A, Shaker K, Wenzler M, Schneider B, Baldwin IT (2011) Phaseoloidin, a homogentisic acid glucoside from *Nicotiana attenuata* trichomes, contributes to the plant's resistance against *Lepidopteran* herbivores. Journal of chemical ecology **37:** 1091-1098

Xu XM, Jeffries P, Pautasso M, Jeger MJ (2011) Combined use of biocontrol agents to manage plant diseases in theory and practice. Phytopathology **101:** 1024-1031

Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Van Thi Luu

Jena, December 13, 2016

Erklärung über laufende und frühere Promotionsverfahren

Hiermit erkläre ich, dass ich keine weiteren Promotionsverfahren begonnen oder früher laufen hatte. Das Promotionsverfahren an der Biologisch-Pharmazeutischen Fakultät ist mein erstes Promotionsverfahren überhaupt.

Van Thi Luu

Jena, December 13, 2016

Curriculum vitae

Van Thi Luu

Born 15th September 1986, Hai Duong, Vietnam

Nationality: Vietnamese Email: van@schepler.de

Language Proficiency: Vietnamese (mother language), English (fluent), German (basic)

Education

- 2012- present: Ph.D candidate at Max Planck Institute for Chemical ecology, Jena, Germany
- 2010- 2012: Master student of Molecular Cellular Developmental Biology of Plant, Faculty of Molecular Biosciences, Heidelberg University, Heidelberg, Germany
- 2004 – 2008: Diploma student of Biotechnology, Faculty of Agronomy, Hanoi University of Agriculture, Vietnam.
- 2001 – 2004: Studied at Nam Sach senior high school, Namsach, Haiduong, Vietnam.

Research Experience

+ *Microbiology:*

- Bacterial, fungal isolation, propagation and identification using specific primers, 16S sequencing and metabolomics method: *Pyricularia oryzae* causes fungal blast in rice, *Xanthomonas oryzae pv. oryzae* which causes bacterial leaf blight in rice; *Fusarium* and *Alternaria* fungal pathogens caused a sudden wilt disease on wild tobacco plants.
- Fungal, bacterial inoculation and symptom quantification for *Pyricularia oryzae, Xanthomonas oryzae* pv. *oryzae. Fusarium brachygibbosum, Fusarium solani. Alternaria* species.
- Identification of fungal morphology using microscopy.
- Screening of biocontrol agents for “sudden wilt disease” in wild tobacco (*N. attenuata*)

+ *Molecular biology:*

- Genomic DNA extraction, RNA extraction, plasmid isolation.
- Gene cloning (primer design, PCR amplification, DNA isolation, cloning into E.coli).
- Reverse transcription PCR to generate cDNA from mRNA and quantitative RT-PCR using SYBR Green
- SDS-PAGE for proteins separation (membrane protein of baker's yeast)
- Gene transformation by infiltration, DNA-PGE-Calcium transfection and Electro-transfection into protoplasts.
- Gene sequencing, alignment and blast using NCBI.

+ *Plant genetic and plant breeding*

- Molecular marker assisted selection (MAS) in plant breeding (ex. Identification of aromatic genes, genes controlling amylose content, resistant genes to bacterial leaf blight in rice).
- QTLs mapping for genes controlling nectar production, seed germination in *Nicotiana attenuata* plants using DNA-seq

+ *Analytical chemistry*

- Phytohormone extraction and quantification by using UPLC-MS
- Secondary metabolites extraction and quantification by using HPLC, MicroTOF-Q
- Metabolomics of pathogen infected plant tissues using UPLC-MicrOToF-MS

+ *Plant biotechnology*

- *Arabidopsis thaliana*, *Nicotiana attenuate* seed germination, cultivation
- Rice pollen culture to generate haploid diploid plants.
- *Arabidopsis* protoplasts isolation.

Publications

- **Van Thi Luu**, Alexander Weinhold, Chhana Ullah, Stefanie Dressel, Matthias Schoettner, Klaus Gase, Emmanuel Gaquerel, Shuqing Xu and Ian T. Baldwin. *O*-acyl sugars protect the wild tobacco *Nicotiana attenuata* from both native fungal pathogens and a specialist herbivore. Submitted to Plant physiology.
- Santhanam, R., **Luu, V. T**., Weinhold, A., Goldberg, J. K., Oh, Y., Baldwin, I. T. (2015). Native root-associated bacteria rescue a plant from a sudden-wilt disease that emerged during continuous cropping. Proceedings of the National Academy of Sciences of the United States of America, 112(36), E5013-E5020. doi:10.1073/pnas.1505765112.
- **Luu, V. T.,** Schuck, S., Kim, S.-G., Weinhold, A., Baldwin, I. T. (2015). Jasmonic acid signaling mediates resistance of the wild tobacco *Nicotiana attenuata* to its native *Fusarium*, but not *Alternaria*, fungal pathogens. Plant, Cell and Environment, 38(3), 572-584. doi:10.1111/pce.12416.
- Schuck, S., Weinhold, A., **Luu, V. T**., Baldwin, I. T. (2014). Isolating fungal pathogens from a dynamic disease outbreak in a native plant population to establish plant-pathogen bioassays for the ecological model plant *Nicotiana attenuata*. PLoS One, 9(7): e102915. doi:10.1371/journal.pone.0102915.

Oral Presentations

- **Luu, V. T.** (2016). Trichome *O*-acyl sugar protects wild tobacco plant against its native fungal pathogens. Talk presented at XIV Meeting of the Working Group Biological Control of Fungal and Bacterial Plant Pathogens, Berlin, DE
- **Luu, V.T.** (2014). Is *N.obtusifolia* more resistance to fungal pathogen than *N. attenuata*? Talk presented at 6th ILRS Symposium, International Leibniz Research School for Microbial and Biomolecular Interactions, Jena, DE

Poster Presentations

- Karimi Dorcheh E., **Luu V.T.**, Santhanam R., Li X., Kumar R.N., Baldwin I.T. (2016). *Nicotiana attenuata*-microbiome interaction. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE
- **Luu V.T.**, Baldwin I.T., Kim S., Kim S., Xu S. (2016). Plant trichome metabolites involved in constitutive defenses against herbivore and fungal pathogens. Poster presented at 15th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE
- Santhanam R., **Luu V.T.**, Weinhold A., Groten K., Baldwin I.T. (2016). Native root-associated bacteria protect their host plant from a fungal sudden-wilt disease via synergistic mechanism. Poster presented at 15th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE
- **Luu V.T.**, Baldwin I.T., Kim S.G., Kim S.T., Xu S. (2015). Using forward genetics to identify the genetic basis of trichome O-acyl sugars in *Nicotiana attenuata*. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE
- **Luu V.T.**, Baldwin I.T. (2015). Mechanism behind the resistance of wild tobacco (Nicotiana species) to its native fungal pathogen (*Alternaria alternata*). Poster presented at Student Conference on Microbial Communication (MiCom), Jena, DE
- **Luu V.T.**, Baldwin I.T., Kim S.G., Xu S. (2015). Identifying genetic basis underlying natural variations of trichome O-acyl sugars in *Nicotiana attenuata*. Poster presented at 8th ILRS Symposium, International Leibniz Research School for Microbial and Biomolecular Interactions, Jena, DE
- Santhanam R., **Luu V.T.**, Weinhold A., Baldwin I.T. (2014). Opportunistic root-associated bacteria protect *N. attenuata* from a wilt disease which arose from continuous cropping. Poster presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE
- **Luu V.T.**, Schuck S., Kim S.G., Baldwin I.T. (2014). Defense of a native tobacco (*N. attenuata*) against its wild fungal pathogen *Alternaria* and *Fusarium*: the role of JA signaling pathway. Poster presented at 7th ILRS Symposium, International Leibniz Research School for Microbial and Biomolecular Interactions, Jena, DE

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- Funding from Vietnamese Ministry of Education and Training for Master study in Heidelberg University, Germany, 10. 2010-10.2012.
- Funding from International Leibniz Research School (ILRS) for Ph.D project at Max Planck Institute for Chemical Ecology, Jena, Germany, 10. 2012-10. 2015.
- Funding from Max Planck Institute for Chemical Ecology to continue Ph.D project, 10. 2015-present.

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