Determining the context and scale at which functional traits increase *Nicotiana attenuata* yields

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"I believe we could paint a better world if we learned how to see it from all perspectives, as many perspectives as we possibly could. Because diversity is strength. Difference is a teacher. Fear difference, you learn nothing." – Hannah Gadsby, Nanette

Contents

Introduction

Plants as the foundation for human energy acquisition

In terrestrial habitats, plants dominate the production of carbohydrates through photosynthesis, in which photons from sunlight are converted to these organic, energy-storing compounds (Gough, 2011). Most life on Earth depends on this energy captured by plants (Le Cotty & Dorin, 2012; Thompson *et al*. 2018). To meet their nutritional needs, humans domesticated plants through systematic plantations in the form of agriculture, which is thought to have encouraged the appearance of domestication traits (e.g. changes in genetics that led to high yielding phenotypes; Fuller, Allaby & Stevens, 2010).

In modern times, the percentage of undernourished humans has increased between 2015-2017 and is projected to continue due to increasing climate variability (FAO *et al*. 2017; FAO *et al*. 2018). Arable land is becoming progressively scarce, and therefore increasing the number of acres used for agriculture is not as sustainable solution (FAO, 2011). Instead, increasing productivity, defined as the amount of yield created through photosynthesis (Westlake, 1963), in current agricultural fields is needed to combat further increases in undernourishment.

Current methods for increasing plant productivity

The world population is predicted to reach 9.7 billion by 2050, a quantity that requires doubling the current agricultural production to avoid catastrophic increases in malnourishment (Alexandratos & Bruinsma, 2012; United Nations, Department of Economic and Social Affairs, Population Division, 2019). Similar to chemical reactions, the productivity of agricultural fields depends on rate-limiting steps. Within one field season, the extent to which plants store energy in tissues (i.e. sources of nutrition for other organisms) depends on how much energy they must divert to producing defenses against herbivores (Cipollini *et al*. 2017). Additionally, plant diseases can interrupt the production of carbohydrates by altering photosynthetic machinery (Lu & Yao, 2018) or preventing accumulation through the destruction of storage tissues (Savary *et al*. 2012). Between seasons, the limited turnover of soil nutrients and microbiomes in field plots also limits the productivity of continued agricultural plantings over time (Jacoby *et al*. 2017). Plants also need adequate starting "reagents" in order to produce; in particular, soil nitrogen, phosphate, potassium and water are required for plant growth (Marschner, 1995; Mengel *et al*., 2001). When faced with competition from agriculturally unproductive neighboring plants, such as weeds, access to these nutrients may be reduced in agricultural systems (Lehoczky & Reisinger, 2003).

In order to address the issues of nutrient turnover and availability, farmers heavily fertilize agricultural fields through the application of inorganic products manufactured to supply nitrogen, phosphate and potassium. Since 1960, the use of fertilizers has increased nearly five-fold (FAO Fertilizer and Plant Nutrition Bulletin, 2006). Additionally, pesticides are chemical or biological substances that are applied to agricultural fields to combat pests; for example, farmers today apply herbicides to address weed competition, fungicides against pathogenic fungi, nematicides against nematodes, and so on. Insecticides have been used to combat plant energy losses to herbivores, defined as insects that feed on plant hosts, for at least 4,500 years (Rao *et al*. 2007). While early pesticides were typically biological (e.g. plant litter with weed suppressive tendencies), synthetic chemical pesticides became widespread around the 1940s, only preceding the fertilizer boom by a few decades; both contributed to the immense increase in worldwide agricultural production referred to as the Green Revolution (Warren, 1998; Alston, Babcock and Pardey, 2010; USDA, 2017).

Although fertilizers and pesticides have increased agricultural production, both have negative environmental effects. Some fertilizers cause soil acidification, leading to the release of toxic compounds that can decrease root growth both in agricultural and natural plant populations (Bolan & Hedley, 2003; Dashuan & Shuli, 2015; Bojórquez-Quintal *et al*. 2017). Fertilizers may also leach into ground water and cause algal blooms, producing products poisonous to humans and animals (Howarth, 2008; Rabalais *et al*. 2009). Fertilization was found to be the cause of up to 40% of the negative environmental impact of growing wheat (Goucher *et al*. 2017). In addition, many pesticides pose a direct health risk to humans (Damalas & Eleftherohorinos, 2011). Pesticides have also reduced animal populations (e.g. the bald eagle, *Haliaeetus leucocephalus*, see Helfrich *et al*. 2009; or pollinators, see Mahmood *et al*. 2015). The loss of these animal populations can severely affect the overall health of the ecosystem and the agricultural production related to plant reproduction (Pimentel & Edwards, 1982).

The recent increase in environmental awareness resulted in a regulation boom that has significantly reduced the use of fertilizers and pesticides (Ju *et al*. 2009; MacDonald *et al*. 2011; Sun *et al*. 2012; Liu *et al*. 2015). Given the need to maintain agricultural productivity, solutions originating directly from the plant itself rather than from exogenous sources can reduce environmental contamination resulting from large-scale application of fertilizers and pesticides. Since the early 1980s, genetically engineered agricultural plants have shown potential to reduce fertilizer and pesticide applications and support plant productivity in the face of diverse environmental stressors (Bawa & Anilakumar, 2013).

Genetic modification for herbicide-tolerance and pest-resistance

The majority of pesticide expenditures are on herbicides and insecticides (US EPA, 2017). It is therefore not surprising that most genetically modified (GM) crops are either herbicide-tolerant or pest-resistant varieties (Kamle *et al*. 2017). Herbicide-tolerant lines are genetically modified with transgenes that allow them to tolerate broad-spectrum herbicides, such as glyphosate and glufosinate. Glyphosate and glufosinate interrupt amino acid and phenolic biosynthesis in non-tolerant neighboring weeds, leading to lethal levels of ammonia (Schütte *et al*. 2017), and a reduction in competition for soil nutrients (Seyyedi *et al*. 2016).

Unfortunately, herbicide-tolerant GM crops do enable a continued dependence on pesticides (Economic Research Service, United States Department of Agriculture, 2000), as well as on nitrogen fertilization, which improves herbicide efficacy (Cathcart *et al*. 2004). In contrast, pest-resistant GM crops reduce the need for pesticides, replacing them with plant-expressed

genes that defend the plant against targeted insect families (Economic Research Service, United States Department of Agriculture, 2000).

Though herbicide-tolerant crops still represent the majority of cultivated GM crops, pest-resistant crops seemingly have greater potential to support both environmentally sustainable agriculture and increased productivity (Bonny, 2011; Klümper & Qaim, 2014). Accordingly, the amount of area planted with insect-resistant GM lines has steadily increased since 1996 (Bakhsh *et al*. 2015). However, appropriate tests demonstrating that pest-resistant GM lines increase the productivity of agricultural monocultures while requiring less pesticide inputs are scarce. Arguably, the most ecologically interesting comparison would be between insect-resistant GM lines and lines with their natural – endogenous – defenses intact (Ricroch *et al*. 2011; Li *et al*. 2015). Many agricultural species are known to be deficient in their generalized endogenous defenses due to selection for high-yielding lines during domestication, however, investigations into the performance of these GM tools versus an adaptive defense system, with or without the addition of insecticides, have not yet been conducted (Gaillard *et al*. 2017). In **Chapter 1** of this dissertation, I investigate this line of questioning by comparing plants with and without their endogenous defenses to those also with GM insect resistance, in the presence or absence of herbivory in the field.

Biodiversity as a method for increasing population productivity

Reducing pesticide applications supports higher plant and insect biodiversity in and around agricultural fields (Geiger *et al*. 2010). Biodiversity, or the variety and variability of life in a particular ecosystem, comprises intra- as well as interspecific diversity (Wilson 1988; Magurran 2005). Though biodiversity in the form of weed presence has historically been considered negative, increasing the number of plant species within one agricultural field can benefit the yield of particular crops (i.e. intercropping, see for example Li *et al*. 2009). Charles Darwin suggested as early as 1872 that increased plant diversity would correlate with greater population productivity (Darwin, 1872), but only since the 1990s have studies established a significant role of biodiversity in the productivity of both natural and agricultural ecosystems, known as the biodiversity-productivity phenomenon (see for example Schulze & Mooney 1994; Chapin *et al.* 2000; Hooper *et al.* 2005; Hajjar, Jarvis & Gemmill-Herren 2008; Isbell *et al.* 2015).

In order to become an applicable tool for increasing population productivity, the biodiversity-productivity phenomenon must be reduced to the underlying mechanisms by which this effect can be manipulated. Studies that sought to identify these underlying mechanisms have led to several concepts: the insurance hypothesis, niche differentiation, complementation, competition, and sampling effects (defined in Table 1). To date, these hypotheses have been difficult to falsify due to the complexity of the interspecific interactions on which they are based: each species' specific contribution to ecosystem-level effects is masked by the myriad of divergent phenotypes among species (Chapin III *et al*. 1997; Díaz *et al.* 2013; Pavoine, Marcon & Ricotta 2016). In other words, the particular function of each species that was important for ecosystem productivity could not be defined. Therefore, species-based quantifications of biodiversity (e.g. species number, evenness, proportional abundance; defined in Table 2) were joined by new measures of species functional traits, otherwise known as the functional diversity of populations (contributing concepts defined in Table 2, bottom panel; Purvis & Hector 2000; Petchey & Gaston 2006; Schleuter *et al.* 2010).

TABLE 1 - Biodiversity concepts

Phenomena	**Definition**	**Found in[α]**
insurance hypothesis	increasing biodiversity insures ecosystems against declines in their functioning caused by environmental fluctuations	Yachi & Loreau, 1999 and sources within
niche differentiation	differences in the morphology, physiology, or behavior of species that can influence their abundances, dynamics, and interactions with other species, including the ability of various competing species to coexist	Tilman, 2001
complimentary effect	occurs through either resource partitioning or facilitative interactions between species	Cardinale, Palmer & Collins, 2002; Tilman, Lehman & Thomson 1997
competitive exclusion	two species that use the same limiting resources in the same way in the same space and time cannot coexist and must diverge from each other over time in order for the two species to coexist	Iannelli & Pugliese, 2014
sampling effect	the hypothesis that diversity might influence an ecosystem process because of the greater chance that a given species trait would be present at higher diversity, and the effect of its presence on ecosystem functioning	Tilman, 2001

[α]the scientific works from which these definitions were acquired are not necessarily those that first established the concept

TABLE 2 - Estimating biodiversity: from species to traits

Species Tools	**Definition**	**Found in[α]**
species richness	the number of species in a community	Stirling & Wilsey, 2001
species evenness	relative species abundance in a community	Stirling & Wilsey, 2001 and sources within
proportional abundance	species are weighted by their relative abundance in relevant indices (e.g. the Shannon-Weiner index)	Stirling & Wilsey, 2001; Magurran, 1988
Trait/FD[β] Tools	**Definition**	**Found in[α]**
traits-processes link	ecosystem processes should be predictable from the community weighted mean value of traits with proven links with resource capture, usage, and release at the individual and ecosystems levels	Grime, 1998
functional diversity and indices	the range and value of those species and organismal traits that influence ecosystem functioning (e.g. indices from the *fd* R package)	Tilman, 2001; R package *fd* by Laliberté, Legendre & Shipley, 2014; Sivicek & Taft, 2011
functional group density	diversity in functional groups within communities	Sivicek & Taft, 2011

[α]the scientific works from which these definitions were acquired are not necessarily those that first established the concept
[β]FD = functional diversity

Functional diversity studies attempt to connect functionality on the level of traits, which are usually presented as species-level averages, to outcomes on the level of the ecosystem (Smith, Shugart & Woodward 1997; Walker, Kinzig & Langridge 1999; Petchey & Gaston 2002; Lavorel & Garnier 2002; Hooper *et al.* 2005; Lepš *et al.* 2006). However, this approach largely ignores within-species trait variation, which is also important (Booth & Grime 2003; Lepš *et al.* 2006; Hajjar *et al.* 2008; Pregitzer, Bailey & Schweitzer 2013). Previous literature has shown that varying genotypes within a single species can affect community productivity, structure and resilience (see for example Madritch & Hunter 2002, 2003; Booth & Grime 2003; Hughes & Stachowicz 2004; Crutsinger *et al.* 2006, 2008; Des Roches *et al*. 2018). In fact, intraspecific genetic diversity can influence several ecosystem processes as much as species richness (Schweitzer *et al.* 2004; Crutsinger *et al.* 2006; Hughes *et al.* 2008). Additionally, intraspecific diversity increases the accuracy of predictors for community behavior over those based solely on interspecific trait variation (Norberg *et al.* 2001; Lepš *et al.* 2006). Therefore, intraspecific experiments should be included to investigate the drivers of community-level effects. The ability of ecologically-relevant functional traits to be manipulated within a species, possibly even from a genetic basis, would allow for functionally falsifiable tests of potential intraspecific factors that change population productivity.

Identifying ecologically relevant functional traits for genetic manipulation

Ecologically-relevant traits are those which vary among individuals in natural populations and are both generated by and acted on by selection (Darwin, 1859). To also be functional, the traits must contribute in some aspect to the fitness of the plant (Sherman, 1988). For example, pest resistance, which I investigate in **Chapter 1**, is a functional trait: plants invest energy into defense traits to reduce the loss of productive tissues which in turn energetically support the reproductive efforts of the plant.

Almost all agricultural products collected for human consumption are directly related to the reproductive yields of crops; therefore, functional trait selection is relevant to agricultural productivity (National Research Council (US) Committee on Research Opportunities in Biology, 1989). This includes vegetative reproductive yield. For example, root and tuber crops provide nutrition through below-ground vegetative yield and are asexually propagated from this same yield (Léon, 1976). Therefore, an increase in this yield is an increase in reproductive success, contributing to potential fitness. Many tuber or root crops additionally reproduce sexually; importantly, key regulators of flowering in these crops have been identified as essential for the onset of tuber formation, showing an inherent link between reproduction and the production of agricultural yield in these plants (Hannapel *et al*. 2017). Though ongoing studies seek to define this link, it supports the functional aspect of traits that affect vegetative yield production in vegetatively-propagated crops.

Water-use phenotypes as functional traits

Functional traits increase yield through the protection of carbohydrate supplies and photosynthetic machinery, or by increasing overall carbohydrate production. To increase production, functional traits could address the availability of photosynthetic starting materials: light, carbon dioxide, or water. Currently, regional water availability is changing rapidly due to climate change (Misra, 2014). In an agricultural context, maximizing water-use traits, such as water-use efficiency, is a primary strategy for increasing monoculture productivity per water input (Howell, 2001; De Pascale *et al*. 2011). Additionally, some water-use traits can be markers of drought resistance. Screening for drought resistance traits that help to maintain or

increase yield during unpredictable water shortage events is of great agricultural interest (Nadeem *et al*. 2019; Oladosu *et al*. 2019).

Both of these strategies involve screening for trait extremes among crop lines whose variation in water-use is produced through a variety of methods, from intercrossing with wild relatives to genetic modification (Xu *et al*. 2017). However, lack of focus on the methodology involving water-use traits have led to an abundance of imprecise experimental comparisons of these traits (Gilbert & Medina, 2016). For example, in the field, plants with divergent drought resistant phenotypes use local water resources at different rates (Henry *et al*. 2011), a trait which is accentuated among individual pots in the glasshouse (Nable *et al*. 1999). Glasshouse drought experiments that employ a "dry down" method typically use the following protocol: 1) withhold watering, 2) assume a synchronous drought after a set period of time, and 3) sample all genotypes at once. However, this would compare genotypes that experienced varying amounts of days of drought due to unequal rates of drying down, and therefore, divergent dates of drought onset among genotypes. This error can lead to the improper identification of drought-resistant lines, which may explain the remaining gap between successful glasshouse trials and application of these lines in the field (Yang *et al*. 2010).

Despite this, controlling for water availability among pots to ensure drought synchronization in glasshouse experiments has not yet become standard. This may be due to the fact that the extent to which this interferes with observed results has yet to be experimentally quantified. Additionally, plant drought responses are known to differ significantly depending on the developmental stage at which a drought is applied (Dong *et al*. 2019), yet this is also generally left uncontrolled in agricultural screens. In **Chapter 2** of this dissertation, we describe, test, and quantify the confounding effects of the onset of drought and the developmental stage on common drought resistance markers. We additionally determine which markers have higher specificity to genotypic variation rather than experimental factor variation, in case experimental error cannot be avoided.

Following our evaluation of confounding factors in glasshouse experiments that test water-use traits, I apply these results to advance the current understanding of how biodiversity in water-use traits can increase agricultural productivity. Some plant communities consisting of species varied in water-use traits have been shown to have enhanced water use, possibly through niche complementarity (Peer *et al*. 2004). Variation of tree water-use traits has been shown to alter the photosynthetic rates of neighboring trees, which could influence total population productivity (Bunce *et al*. 1977). However, further mechanistic resolution behind these changes have not yet been determined. In **Chapter 3** of this dissertation, I test the effect of intrespecifically varying individuals with low water-use efficiency on population yield. In glasshouse experiments, I control for equal water availability between pots to deduce the contribution of differing soil water availabilities among pots to observed yield results. Importantly, this study advances the understanding of the mechanism behind how variation in an individual's water-use traits can increase population productivity by investigating the effect of a single gene silenced in low percentages of individuals in population. Additionally, this study narrows the spatial scale at which effects caused by this genetic variation occur.

Development of new methods for screening functional traits

Apart from insect resistance and water-use, another trait of interest in increasing population productivity has been the ability of plants to interact with arbuscular mycorrhizal fungal (AMF) networks (Menge, 1983; Hamel, 1996; Vosátka *et al*. 2012). Approximately 80% of land plants

form symbiotic associations with AMF (Smith & Read, 2008), including many crop species. AMF facilitates the acquisition of mineral nutrients for plant hosts in exchange for carbohydrates and fatty acids (Salvioli di Fossalunga & Novero, 2019). However, this symbiosis has been shown to produce a wide range of effects, ranging from positive to negative results on plant growth and yield, depending on the host and AMF genotypes, species, or environmental factors (Köhl *et al*. 2016; Posta & Hong Duc, 2019). Regardless, AMF have been shown to most consistently benefit plants when facing a range of field abiotic stresses, and its potential for use as a bio-fertilizer continues to be of growing interest to researchers (Begum *et al*. 2019).

Studies which determine the impact of AMF on plant yield require quantification of the extent of association between AMF and plant roots. Historically, methods to quantify this symbiosis have involved observing the roots directly with microscopy and counting the presence of fungal structures such as hyphae, arbuscules and vesicles (Bevege, 1968; McGonigle *et al*. 1990; Utobo *et al*. 2011). These methods have been steadily improved since the 1960s, but these improvements do not remove the biggest problem with current quantification techniques: they are either time consuming, destructive, or both (Deguchi *et al*. 2017). It is thus of interest to develop non-destructive methods for quantifying the AMF-plant symbiosis. Ideally, these methods should allow for the screening of plant-AMF associations in real time and with repeated measures. In **Chapter 4** of this dissertation, we describe the establishment of a new method that employs a leaf molecular marker to quantify AMF-plant symbiosis in a fraction of the time needed for the traditional root staining and microscopy method. We additionally investigate the applicability of this method to a selection of crop species.

Using an ecological model plant to stud agriculturally-relevant functional traits

The wild coyote tobacco *Nicotiana attenuata* is an ecological model plant native to the southwestern United States (Baldwin *et al*. 1994). The environment in which *N. attenuata* grows is a primordial agricultural niche: due to frequent fires it has nitrogen-rich soils, much like nitrogen-fertilized agricultural fields (Baldwin & Morse 1994; Lynds & Baldwin, 1998; Baldwin, 2001). Additionally, it grows primarily in a desert habitat and is subject to decreasing natural freshwater resources (i.e. a lowering ground water table throughout the season; Zavala & Baldwin, 2004), similar to some agricultural niches (e.g. Siebert *et al*. 2007). As a member of the Solanaceae family, *N. attenuata* shares a recent common ancestor with essential crop species such as commercialized tobacco, tomato, potato, eggplant, and bell and chili pepper plants. Functional traits that are identified in *N. attenuata* could be applied to these crop species, making it relevant in an agricultural context.

N. attenuata has been extensively studied in its natural habitat using an established field site in the Great Basin Desert, in southwestern Utah, USA (see for example Kessler & Baldwin, 2001; Kessler, Gase, & Baldwin, 2008; Schuman, Barthel, & Baldwin, 2012; Schuman, Allman & Baldwin, 2015). *N. attenuata* interacts with a myriad of insects in the field, many of which are agriculturally relevant (e.g. with *Tupiocoris spp., Manduca spp., Fusarium spp.*; Lynds & Baldwin, 1998). Caterpillars from the Lepidopteran family (e.g. *Spodoptera spp.*) are the main targets of GM insect resistant crop varieties (Sanahuja *et al*. 2011) and are known to feed on *N. attenuata* in its natural habitat (Steppuhn *et al*. 2004). Thus, *N. attenuata* and its habitat are good candidates for evaluating GM defenses.

Due to its arid natural habitat, drought resistant traits are ecologically-relevant to *N. attenuata*'s growth and yield production (Ré *et al*. 2011; Valim *et al*. 2019). *N. attenuata* lines varied in

drought resistant traits may be attained from its extensive toolbox of lines silenced by RNAi interference (Krügel *et al*. 2002; Gase *et al*. 2011), as well as from a recent multi-parent advanced intercross (MAGIC) population (Ray *et al*. 2019). Therefore, the *N. attenuata* system is ideal for investigating convoluting factors which may impede screenings of lines varied in drought resistant traits. Additionally, *N. attenuata* is known to grow in genetically diverse near monocultures, making this species especially applicable for studies employing the variation of functional traits within populations to increase yield (Lynds & Baldwin, 1998; see example in Schuman *et al*. 2015). Finally, *N. attenuata* is known to associate with AMF both in the glasshouse and field (Groten *et al*. 2015).

Overview of the dissertation

To date, ecological and agricultural research to increase population productivity has been conducted separately. However, model species and techniques from ecological studies could be essential for further improvement of agricultural cultivars. This dissertation attempts to use *Nicotiana attenuata* to investigate how the functional traits of insect resistance, water-use and AMF association can affect population yields in both the glasshouse and the field.

The chapters of this dissertation are organized by the extent to which these functional tools have been used to enhance agricultural productivity. In **Chapter 1,** I conduct an ecologically informed test of a well-established agricultural tool: lines modified for pest-resistance. I evaluate the ability of the extra defenses expressed by these lines to enable their production of biomass and reproductive yield in comparison to plants with only their endogenous defenses. Furthermore, I determine the extent to which herbivory that is not targeted by the genetic modification of these lines changes their productivity.

Drought resistance (**Chapter 2**) and water-use efficiency (**Chapter 3**) are functional traits currently of interest for agricultural screenings. In **Chapter 2** of this dissertation I quantify the extent to which oversights in the experimental design of these screens can impede the selection of drought resistant phenotypes. In **Chapter 3** I explore an alternate method for increasing plant population productivity by varying the water-use efficiency of individuals in population, rather than relying on monocultures of individuals with maximized water-use efficiency. Upon confirming the established ecological relationship between biodiversity and productivity, even within populations of one species, I advance the study of the mechanisms behind this phenomenon by determining a single gene responsible for creating this effect, and narrow down the spatial scale at which this occurs: the above-ground, population scale.

Finally, in **Chapter 4**, I look towards a functional trait that is of rising interest for increasing agricultural productivity: the ability of plants to associate with AMF. Though the use of AMF inoculations as biofertilizers in agricultural fields is already under consideration, screening for agricultural lines that differ in their ability to associate with AMF is still relatively new. Selection for this trait in agricultural cultivars is currently hindered by time-consuming and destructive screening methods. We facilitate the study and screening of plants with varying AMF associations through the establishment of a high-throughput screening method based on a leaf molecular marker.

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Overview of Manuscripts

Manuscript 1

Cry1Ac production is costly for native plants attacked by non-Cry1Ac-targeted herbivores in the field

Erica McGale, Celia Diezel, Meredith C. Schuman and Ian T. Baldwin

Published in *New Phytologist*, doi:10.1111/nph.15207 (2018)

In Manuscript 1, I compared the growth and end-of-season yields of genetically modified *Nicotiana attenuata* expressing a well-known transgene that confers Lepidopteran pest resistance (*Cry1Ac* from *Bacillus thuringiensis*; ovCRY), in comparison to *N. attenuata* plants with (EV) or without (irAOC) their endogenous defenses. The ovCRY line was first characterized in this publication. I confirmed that only ovCRY plants produced Cry1Ac protein. I showed that EV and ovCRY had identical induced metabolic profiles using a principal component analysis, while irAOC plant's profile occupied a distinct data space. I sprayed half the replicates with a neonicotinoid insecticide in order to evaluate their growth and yield with and without herbivore pressure. I tested if the neonicotinoid insecticide altered expected levels of JA-induced defense molecules (e.g. phenolamides and diterpinoid glycosides) and found that it did not. I quantified herbivore damage on all plants and found that damage from non-Cry1Ac-targeted insects was dominant on unsprayed plants throughout the season. I recorded the bolting and flowering times, as well as stalk heights, of all plants throughout the season, and the biomasses and reproductive correlates at season end. Unsprayed ovCRY plants lagged in nearly all growth and yield parameters in comparison to endogenously defended (EV) and undefended (irAOC) plants. Reducing pressure from non-Cry1Ac-targeted herbivory with the spraying treatment decreased ovCRY plant's lag in stalk height, shoot biomass, and flower production, indicating the detrimental role of non-Cry1Ac-targeted herbivory on the productivity of this line.

Erica McGale, Celia Diezel, Dr. Meredith C. Schuman, and Prof. Dr. Ian T. Baldwin contributed to the methods, investigation, and project administration. Erica McGale wrote the software, performed the formal analysis, and wrote the manuscript. Erica McGale and Celia Diezel created the visualizations. Celia Diezel, Dr. Meredith C. Schuman, and Prof. Dr. Ian T. Baldwin edited the manuscript. Dr. Meredith C. Schuman and Prof. Dr. Ian T. Baldwin provided the supervision and funding.

Manuscript 2

Early developmental transitions and drought timing influence drought resistance strategies and confound the quantification of genotypic diversity in drought responses

Erica McGale*, Henrique Valim*, Rayko Halitschke and Ian T. Baldwin

*These authors contributed equally to the manuscript

This manuscript has been revised following review in *Plant, Cell & Environment*, and has been resubmitted to *Plant, Cell & Environment*.

In Manuscript 2, we quantified the extent to which comparisons of drought response traits in varying genotypes can be confounded by asynchronous onset-of-drought timings and developmental stages. We demonstrated that consumption-based controlled watering can cause two genetically modified *Nicotiana attenuata* lines divergent in soil water consumption rates to experience synchronous drought events. Alternatively, if these two genotypes were treated with an uncontrolled "dry down", we showed that the soil water content in pots of one genotype were significantly lower than the other within 24 hours. This indicated that one genotype would reach 0g soil water (i.e. onset of drought) on an earlier date than the other. Within one genotype, we showed that plants which dry down over different numbers of days have significantly different physiological drought response values, even if measured on their respective days of drought onset. We applied controlled droughts within the same genotype before and after bolting, and found that ontogenetic drought responses were significantly different depending on the developmental stage at which the treatment was applied. We used a multi-parent advanced generation intercross (MAGIC) population to select 30 *N. attenuata* recombinant inbred lines (RILs) that varied in their rates of water-consumption and fell into three developmental groups: early, medium or late bolting plants. We droughted these 30 RILs in three scenarios: uncontrolled (Un-Sync), controlled for onset of drought (Sync), and controlled for both onset of drought and plant developmental stage (Dev). We found that we could significantly decrease the variation in onset-of-drought timing among genotypes with the Dev treatment. We measured four physiological drought responses (relative water content, RWC; abscisic acid content, ABA; assimilation rate and stomatal conductance) at two time points and performed a variance decomposition to determine which experimental factors most explained the results: onset-of-drought timing, developmental stage, or genetic variation. We found that developmental stage significantly explained variation in all measured responses. Onset-of-drought timing explained the variation in ABA values. However, genetic variation most significantly explained trait variation in ABA, indicating that this response trait may be most reliable for detecting genetic differences in drought responses.

Erica McGale and Henrique Valim contributed to the methods, investigation, project administration, software, formal analysis, and visualization of the work and wrote the manuscript. Dr. Rayko Halitschke and Prof. Dr. Ian T. Baldwin contributed to revising the visualizations and the text of the manuscript. Prof. Dr. Ian T. Baldwin provided supervision and funding.

Manuscript 3

Determining the scale at which variation in water-use traits and AMF associations change population yields

Erica McGale, Henrique Valim, Deepika Mittal, Jesús Morales Jimenez, Rayko Halitschke, Meredith C. Schuman and Ian T. Baldwin

This manuscript has been revised following review in *eLife*, and has been resubmitted to *eLife*.

In Manuscript 3, I investigated the scale at which variation in a low water-use efficiency trait, created through deficiency in mitogen-activated protein kinase 4 (MPK4), increased population yields. I created populations of *Nicotiana attenuata* in the field with 0, 25, 75 and 100% *MPK4*-deficient plants among empty-vector control plants. 25% irMPK4 populations ("low irMPK4") showed overyielding in biomass and reproductive correlate numbers. I tested the dependence of this overyielding on differences in soil water availability by applying consumption-based controlled watering to glasshouse populations with 0, 17, 50, 83 and 100% irMPK4. I observed overyielding again in low irMPK4 populations (17%), indicating that the effect was independent of differences in soil water availability. I tested whether this effect occurred at the neighbor scale (only in immediate neighbors of *MPK4*-deficient plants) or at the population scale (across all plants of the population) by conducting paired-plant-in-a-pot experiments in the glasshouse. Control plants did not alter their yields differently when planted with control or *MPK4*-deficient neighbors. However, I found that only control plants, and not *MPK4*-deficient plants, alter their yields due to the presence of a neighbor, supporting that control plants are responsible for the overyielding in low irMPK4 populations. I evaluated whether specific neighbor configurations around control plants in the glasshouse populations correlated with the increased yields in low irMPK4 populations, but they did not. The effect appeared to be occurring at the population scale. Using a grafting experiment, I determined that *MPK4* in shoot tissue is exclusively required for an *N. attenuata* plant to change its yield in response to the presence of a neighbor. Additionally, the aforementioned field populations were crossed with *N. attenuata* lines silenced in calcium and calmodulin-dependent protein kinase (*CCaMK*) in order to disconnect them from arbuscular mycorrhizal fungal (AMF) networks in the field. I also created uncrossed populations which were able to associate with the AMF networks. I found that connecting the varied populations to the AMF network abolished low irMPK4 population overyielding in biomass, but not in reproductive correlates. Given that AMF plays a significant role in plant-plant communication and resource acquisition below-ground, this indicated that the reproductive correlate overyielding in low irMPK4 populations likely occurred at the aboveground population scale.

Erica McGale, Dr. Jesús Morales Jimenez and Dr. Meredith C. Schuman contributed to the methods and project administration. Erica McGale, Henrique Valim, Deepika Mittal, Dr. Jesús Morales Jimenez, and Dr. Meredith C. Schuman conducted the investigation. Erica McGale, Henrique Valim, Deepika Mittal, and Jesús Morales Jimenez conducted the formal analysis and contributed towards the visualization of the work. Erica McGale wrote the software and manuscript. All authors revised the manuscript. Dr. Meredith C. Schuman and Prof. Dr. Ian T. Baldwin provided supervision and funding.

Manuscript 4

Blumenols as shoot markers of root symbiosis with arbuscular mycorrhizal fungi

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In Manuscript 4, we identified a leaf molecular marker that positively correlates with the extent of arbuscular mycorrhizal fungi (AMF) symbiosis in plant roots. We potted empty-vector (EV) *Nicotiana attenuata* control plants in pairs with a line silenced in calcium and calmodulin-dependent protein kinase (irCCaMK). Half the pairs were inoculated with the AM fungus *Rhizophagus irregularis*. Untargeted metabolomics conducted on root samples from each sample type (EV without AMF, EV-; irCCaMK-; EV+; irCCaMK+) led to the selection of two features (Compound 1 and 2) that followed a particular STEM clustering pattern. Targeted metabolomics on inoculated wild-type *N. attenuata* showed that the selected compounds were also present in the leaves. I analyzed the compound abundances in the leaves of EV+, irCCaMK+ and EV- plants sampled weekly from two to seven weeks post inoculation (wpi). I found that the compounds tracked with AMF symbiosis in the EV+ plants. I provided field samples to test whether EV would show significantly higher compound amounts than irCCaMK in natural soils; we confirmed this was true. Using samples from the time series experiment, we found that the leaf compounds significantly correlated with the numbers of arbuscules and vesicles in roots, as well as with the root length colonization percentage. We tested whether differing biotic (herbivory, pathogen infection, non-pathogenic bacteria & virus infections) and abiotic (drought) treatments could affect accumulation of Compounds 1 and 2; we found that they did not. Using our dexamethasone (DEX)-inducible pOp6/LhGR system we silenced a single leaf in phytoene desaturase (*PDS*) which would disrupt carotenoid synthesis and therefore the synthesis of our two compounds in leaf tissues. We found their levels to be unaffected, indicating their transport to the leaf likely from the roots. We tested the presence of the compounds, namely hydroxy-blumenol and carboxy-blumenol, in the leaves and roots of several crop species and found their presence exclusively in AMF-treated samples, indicating their value for high-throughput screenings of AMF-associating species in agriculture.

Dr. Ming Wang, Dr. Martin Schäfer, Dr. Dapeng Li, and Dr. Rayko Halitschke contributed to the methods and project administration. Dr. Ming Wang, Dr. Martin Schäfer, Dr. Dapeng Li, Dr. Rayko Hallitschke, Dr. Chuanfu Dong, Erica McGale, Dr. Christian Paetz, Dr. Yuanyuan Song, Dr. Suhua Li, Junfu Dong, Sven Heiling, Prof. Dr. Philipp Franken, Dr. Michael Bitterlich, Prof. Dr. Maria J. Harrison, and Prof. Dr. Uta Paszkowski contributed work and/or samples towards the investigation. Dr. Ming Wang, Dr. Martin Schäfer, Dr. Dapeng Li, Erica McGale and Dr. Christian Paetz contributed software, conducted the formal analysis and created the visualizations. Dr. Ming Wang and Dr. Martin Schäfer wrote the manuscript. All authors revised the manuscript. Dr. Christian Paetz, Dr. Karin Groten, Prof. Dr. Philip Franken, Dr. Michael Bitterlich, Prof. Dr. Maria J. Harrison, Prof. Dr. Uta Paszkowski and Prof. Dr. Ian T. Baldwin provided supervision. Prof. Dr. Ian T. Baldwin provided funding.

CHAPTER **1**

1. Cry1Ac production is costly for native plants attacked by non-Cry1Ac-targeted herbivores in the field

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1.1 Summary

- Plants are the primary producers in most terrestrial ecosystems and have complex defense systems to protect their produce. Defense-deficient, high-yielding agricultural monocultures attract abundant nonhuman consumers, but are alternatively defended through pesticide application and genetic engineering to produce insecticidal proteins such as Cry1Ac (*Bacillus thuringiensis*). These approaches alter the balance between yield protection and maximization but have been poorly contextualized to known yield–defense trade-offs in wild plants.

- The native plant *Nicotiana attenuata* was used to compare yield benefits of plants transformed to be defenseless to those with a full suite of naturally evolved defenses, or additionally transformed to ectopically produce Cry1Ac. An insecticide treatment allowed us to examine yield under different herbivore loads in *N. attenuata*'s native habitat. Cry1Ac, herbivore damage, and growth parameters were monitored throughout the season. Biomass and reproductive correlates were measured at season end.

- Non-Cry1Ac-targeted herbivores dominated on noninsecticide-treated plants, and increased the yield drag of Cry1Ac-producing plants in comparison with endogenously defended or undefended plants. Insecticide-sprayed Cry1Ac-producing plants lagged less in stalk height, shoot biomass, and flower production.

- In direct comparison with the endogenous defenses of a native plant, Cry1Ac production did not provide yield benefits for plants under observed herbivore loads in a field study.

1.2 Introduction

Yield in plants, defined as biomass and reproductive correlate production, can be reduced by trade-offs with the production of plant defense metabolites regulated by, for example, the jasmonic acid (JA), salicylic acid, and auxin pathways (Huot *et al*., 2014). Yield–defense trade-offs are based on the commonly accepted notion that plants have access to a limiting pool of resources from which to invest in both yield and defenses (Herms & Mattson, 1992; Huot *et al*., 2014). Yield measures are often used to approximate plant fitness, but it is not correct to interpret yield–defense trade-offs directly as fitness–defense trade-offs: plant defense traits can be costly in terms of yield, while nevertheless increasing plant fitness in the face of stress (Karban & Baldwin, 1997; Baldwin, 1998; Cipollini *et al*., 2014). Studies of invasive plants indicate that it may be possible to improve both resistance and yield: introduced plants are often larger than their native counterparts (Crawley, 1987; Grosholz & Ruiz, 2003), and there is little evidence to correlate this with chemical defense production as opposed to other influences, including convergent evolution (Colautti *et al*., 2009). In addition, recent work with the model plant *Arabidopsis thaliana* has challenged the simple resource-allocation view of yield–defense trade-offs and indicates that plants control these investments well before resources become limiting, via a combination of phytochrome and jasmonate signaling (Campos *et al*., 2016). One way to limit the costs of defense is by inducing defenses only when they are needed. By contrast, continuously produced constitutive defenses steadily draw from the resource pool and may lead to more yield costs than inducible defenses, which selectively increase upon interaction with herbivores (Tollrian & Harvell, 1999).

In modern agriculture, the growth of defense-deficient crop plants in high-density monocultures can cause increases in abundance and damage from local herbivores (Altieri & Letourneau, 1982; Altieri & Nicholls, 2004), implying a constant need for crops to produce defenses. To avoid the potential yield costs from this defense production, pesticide applications are used so that plants do not need to produce their own defenses, but this has the disadvantage that many pesticides are toxic to humans and other organisms, and use can result in potentially harmful residue in the environment and the food supply (Nicolopoulou-Stamati *et al*., 2016). Another solution, which may be employed in parallel to reduce pesticide inputs, is the use of biotechnology to generate crop plants that express targeted resistance traits against the most abundant or damaging herbivores, generally generating more yield through a reduction of foliage loss (Krattiger, 1997), although realized yield may depend strongly on other environmental factors. Over the last 22 yr, biotech crops have been employed extensively in the Americas, Asia, and Australia, gaining over US$150 billion for farmers and reported benefits for society and the environment (James, 2015). Biotech crops express novel traits derived from other organisms, such as increased insect, herbicide, virus, and drought resistance. Among insect-resistant varieties, *Bacillus thuringiensis* (Bt) crops are most often used (Bakhsh *et al*., 2015). Bt crops are engineered to produce Cry toxins from the soil bacterium Bt that compromise the gut integrity of members of specific insect families, making them highly selective insecticides. One such toxin is Cry1Ac (Cry), effective against the larvae of certain lepidopteran insects (Schnepf *et al*., 1998).

Bt crops have been marketed as a solution to simultaneously reduce pesticide use and pest loads, for an increase in overall productivity per input (Krattiger, 1997). They have increased farmer

profits due to several factors, with yield gains remaining among the most important (Qaim & Zilberman, 2003; Paul *et al*., 2017). The potential yield costs of ectopic, constitutive Cry protein production are reduced by introgressing Cry genes into elite high-yielding plant lines (Perlak *et al*., 2001). Despite careful breeding, yield inconsistencies continue to emerge in meta-analyses of Bt crops, and these analyses indicate that negative yield changes could relate to different planting locations (Qaim & Zilberman, 2003; Finger *et al*., 2011) in which nutrient deficiency, abiotic stress, and Cry1Ac-targeted and nontargeted herbivores are environmental variables that may alter yield–defense relationships. Agricultural plants are normally supplemented with fertilizer and water, reducing the chance that nutrient or abiotic conditions contribute heavily to a yield–defense trade-off. However, interaction of crops with Cry1Ac-targeted and nontargeted herbivores remains an influential factor that is more difficult to control. It is known that Bt crop yields commonly vary with the level of infestation (Areal *et al*., 2013), with greater infestation rates of Cry1Ac-target herbivores often producing clearer yield benefits (Klotz-Ingram *et al*., 1999). However, non-Cry1Ac-targeted herbivores may infest Bt crops more heavily (Marvier *et al*., 2007), and the resulting effects on yield are not well characterized.

The payoff for a plant's investments in defenses can greatly increase with increasing herbivore loads, whereas both constitutive and inducible defenses reduce yields if expressed when herbivore pressure is low (Baldwin, 1998; Machado *et al*., 2016). Herbivore occurrence and abundance vary enormously (up to 100%) by year even on small spatial scales (Smatas *et al*., 2008), and attempting to decipher the effect of herbivore loads in meta-analyses on Bt crops with cross-year evaluations, on multiple fields, within or across countries, is technically challenging. Moreover, these meta-analyses and their study components currently lack isogeneic controls, data on the levels of Cry protein throughout the season, and data on how protein levels correspond with herbivore loads and damage (Dandekar *et al*., 1998). In addition, the consequences of Cry toxin production on endogenous plant defenses have not been reported. The presence and effects of endogenous defenses in post-introgression plants are not straightforward (Rowen & Kaplan, 2016; Whitehead *et al*., 2017), and yield consequences may change depending on their interaction with Cry1Ac production. Exploring the consequences of Cry1Ac production in plants with known endogenous defense profiles and without the complications of introgression could illuminate sources of yield variance.

The anti-herbivore defenses of the wild tobacco *Nicotiana attenuata* are particularly well understood and it is possible to perform clean manipulations in order to evaluate the yield consequences of defense in this ecological model plant (Kessler *et al*., 2004; Schuman *et al*., 2012, 2015; Barazani & Baldwin, 2013; Ullmann-Zeunert *et al*., 2013). We used *N. attenuata* to quantify yield costs of endogenous defenses, in comparison with Cry1Ac production, depending on herbivore community and load – an understanding that is essential for increasing the specific targeting of future agricultural biotech development (Huot *et al*., 2014; Paul *et al*., 2017). *N. attenuata* grows in a post-fire, nutrient-rich environment dominated by intra- rather than interspecific competition, which resembles supplemented monoculture crop fields (Baldwin, 2001; Barazani & Baldwin, 2013), and naturally co-occurring herbivores include both Cry1Ac-targeted Lepidoptera and nontargeted Miridae and Coleoptera (Kessler & Baldwin, 2004; Paschold *et al*., 2007). We used plants with their complete suite of native defenses (empty vector (EV)), as well as a previously characterized line transformed to silence the expression of allene oxide cyclase (irAOC), a key enzyme required for the synthesis of jasmonate hormones. The irAOC line is nearly defenseless but has increased yield, as measured by flower and branch number, when grown in environments without herbivores (Machado *et al*., 2013). In addition, we describe lines engineered to ectopically express Cry1Ac (ovCRY)

and select a line for further study that has levels of known endogenous defense metabolites indistinguishable from those of an EV control line. These lines were grown in a field plot in the Great Basin Desert both with and without a neonicotinoid insecticide (NI) application to reduce herbivore loads from the native herbivore community. These experiments allowed the contextualization of the yield consequences of Cry toxin production to those associated with a plant's endogenous defenses, under differing herbivore loads manipulated by a commonly used insecticide, in a model system without the complications of a prior history of breeding. The results indicate that the effects of Cry1Ac production on yield depend strongly on the biotic community and the effectiveness of concurrent endogenous defense production.

1.3 Materials and Method

Plant material and constructs

The EV control line (pSOL3NC, line number A-04-266-3) was previously described by Bubner *et al*. (2006). The irAOC line (pRESC5AOC, line number A-07-457), silenced via RNAi targeting allene oxide cyclase expression resulting in reduction of downstream JA pathway metabolites, was fully characterized in previous work (Kallenbach *et al*., 2012). The ovCRY lines (pSOL9CRY1) were generated by the transformation of wild-type *N. attenuata* Torr. ex S. Watson seeds from the 'UT' inbred line (31st inbred generation descended from a 1988 natural population at the Desert Inn Ranch in southwestern Utah) using Agrobacterium tumifaciens (strain LBA 4404) as previously described (Krügel *et al*., 2002) with the pSOL9 plasmid harboring the full-length CRY1a transgene in the sense orientation. Plants of the first transformed generation (T1) were screened for a c. 75% resistance ratio on hygromycin, indicating a single transgene insertion. Resistant seedlings were used to collect T2 seeds, which were screened for 100% hygromycin resistance, indicating homozygosity (Fig. S1a). Ten independently transformed T2 plants were further characterized by Southern blots using EcoRV and XbaI restriction enzymes. Six T2 lines were used for glasshouse growth bioassays. One homozygous T2 line showing a single insertion of the correct length with both restriction enzymes was used in field experiments (line number A-09-1129-4 or 1129; 13 in Supporting Information Fig. S1b). The specifics of vector construction, the pSOL9 plasmid, and the transformant screening procedure have been previously described (Gase *et al*., 2011). We used the second transformed generation (T2) of ovCRY and the third generation (T3) of the EV and irAOC lines in this study.

Plant cultivation in the glasshouse and field

Germination on Gamborg's B5 medium and glasshouse growth conditions were as previously described (Krügel *et al*., 2002; Schuman *et al*., 2014), with planting conditions varying only for competition pots (two plants planted 5 cm apart in each 1 l pot). For the field experiments, seedlings were germinated on Gamborg B5 media under illumination from fluorescent lights (GE Plant & Aquarium 40 W and GE Warm White 18 W) under ambient temperatures at the field station. At 2–3 wk after germination, seedlings with four visible leaves were transferred into previously hydrated 50 mm peat pellets (Jiffy 703, www.jiffypot.com) treated with Borax (1 : 100 dilution of a 1.1 g l−1 stock solution) and adapted over 2 wk to the field conditions of high light intensity and low relative humidity by keeping seedlings first in shaded, closed translucent plastic 34-quart boxes, then opening the boxes and subsequently transferring open boxes to partial sunlight in mesh tents. At 59 d (2011) and 39 d (2016) after germination, adapted size-matched seedlings were transplanted into an irrigated field plot at the Lytle Ranch

Preserve, Santa Clara, Utah ('Snow Plot', N37.141283, W114.027620). The import and release of transgenic plants was carried out under Animal and Plant Health Inspection Service (APHIS) import numbers 07-341-101n (irAOC, EV), 10-004-105m (ovCRY), and 10-349-101m (crosses: Notes S1), and release numbers 10-349-102r in 2011, and 16-013-102r in 2016. The NI imidacloprid (Confidor, Bayer) was applied to half of the plants in the field as an aqueous spray (1 g l−1) as described later.

Herbivore screens in the field

At 18, 25, and 33 d post-planting (dpp), 15, 13, and 15 replicates of EV, irAOC, and ovCRY plants respectively from each treatment (sprayed or unsprayed) were examined for Tupiocoris notatus, Epitrix spp., and noctuid damage as a percentage of the total plant canopy. Observations were made by visual estimation after training for herbivore damage-type recognition. The area percentage estimation protocol was previously described (Schuman *et al*., 2015). Damage estimates were made by E.M.

Plant defense induction

To standardize the induction of defense responses in the field, three single rosette leaves (positions −1, 0 and +1) of each plant were wounded with a fabric pattern wheel to produce one row of wounds on each side of the leaf midvein (Diezel *et al*., 2011). Fresh wounds were immediately treated with 6 µl of 1 : 20 (v/v) water-diluted oral secretions (OS) from Manduca sexta. This treatment mimics the defense induction triggered by M. sexta without extensive leaf tissue removal (Ohnmeiss & Baldwin, 1994). The same treatment was repeated 3 d later, and again 5 d after the first induction, to the same leaves. The first set of treatments began after the 18 dpp sampling so the 25 and 33 dpp samples would be induced (see timeline in Fig. 1). In the glasshouse, a single induction of one rosette leaf (position −1) was conducted 24 h before sampling for Cry1Ac.

Cry protein measurement

Enzyme-linked immunosorbent assays were used to quantify levels of the Cry1Ac protein (Agdia Incorporated, Elkhart, IN, USA). Fresh leaf discs (0.02 ± 0.005 g, Sartorius AG balance, accuracy ± 0.0001 g) were flash-frozen, ground, and extracted in a 1 : 10 ratio with the extraction buffer provided by the manufacturer. The absorbance of each well of the plate at 650 nm was determined with a Tecan Sunrise (Tecan, www.tecan.com). This sample absorbance reading was normalized to the internal standard wells in each plate by dividing each absorbance by the average of the standard absorbances. This value was used to determine statistically whether any line had absorbances significantly different from buffer control absorbances. Only lines that differed had their values divided by the mass of the tissue used for extraction and were further analyzed. Tissue from 2016 was collected from two leaves per plant, at one older (+1) and one younger (−1) node than the source–sink transition leaf. Cry1Ac amounts from these leaf positions were compared at 33 dpp. A significant difference existed only in +1 leaves; thus, the +1 leaf was analyzed for all three time-points.

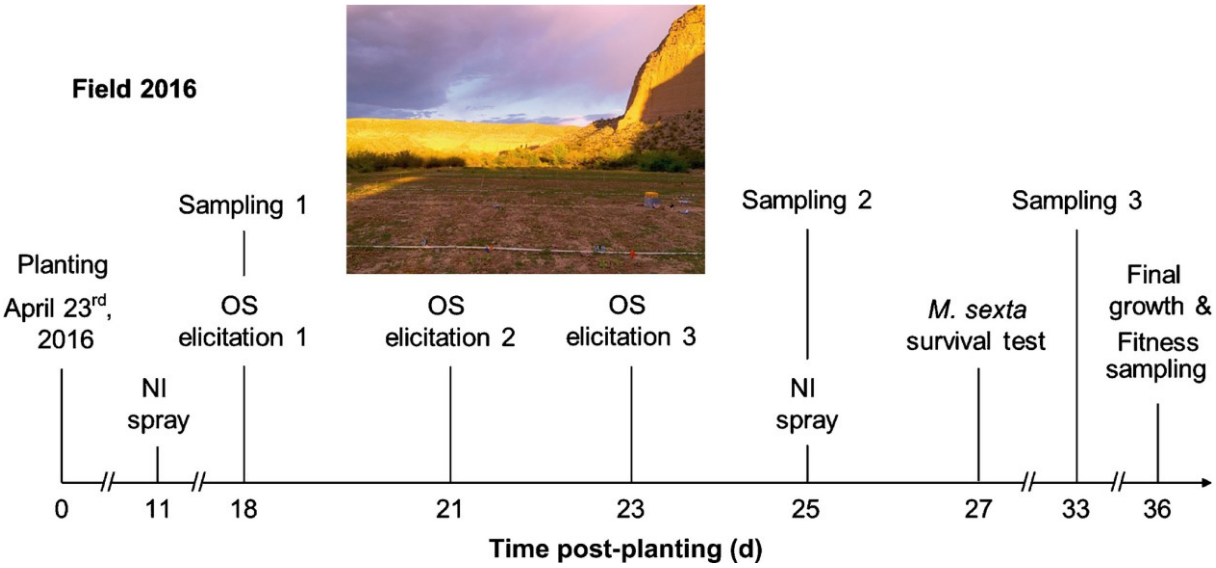

FIGURE 1 - Timeline of 2016 field season planting, treatments, and sampling. Field station at sunset, immediately after the final light leaves the plot, which is the time of day when the neonicotinoid insecticide (NI) applications and *Manduca sexta* oral secretion (OS) elicitations to fresh puncture wounds were conducted. Picture by E. McGale.

Analysis of secondary metabolites

To determine how the secondary metabolite profiles of the ovCRY line compared with the known profiles of the irAOC and EV lines (Kallenbach *et al*., 2012), secondary metabolites were extracted from 0.02 ± 0.005 g (Sartorius AG balance, accuracy ± 0.0001 g) leaf discs harvested at 33 dpp. A principal component analysis (PCA) and a targeted metabolite analysis for defensive metabolites known to be regulated by jasmonate signaling were performed: the hydroxygeranyllinalool diterpene glycosides nicotianoside I and nicotianoside II, and the phenolamide dicaffeoylspermidine.

Samples were extracted and injected into a Dionex UltiMate 3000 U-HPLC system (ThermoFisher, www.thermofisher.com) combined with a Dionex Acclaim C 18 2.2 µm 120 Å 2.1 mm × 150 mm column, as well as a micrOTOF-Q II mass spectrometer (Bruker Daltonik, www.bruker.com) equipped with an electrospray ionization source in positive ion mode, as previously described (Li *et al*., 2016) with only the modification that samples were all extracted in 80% methanol–20% deionized water.

The resulting untargeted data, normalized by sample mass, were converted from netCDF files into an XCMS format using previously published R script (Li *et al*., 2015), edited as previously described to remove contaminants and avoid pseudoreplication (Herden *et al*., 2016), and used for a PCA (see the Statistical analyses subsection).

Nicotianoside I, nicotianoside II, and dicaffeoylspermidine were targeted with the known m/z values of [M + NH4]+ (880.4537), [M + NH4]+ (966.4540), and [M + H]+ (470.2286) respectively in the QuantAnalysis software (Bruker Daltonics).

Glasshouse Manduca sexta *bioassay*

One M. sexta neonate from an in-house colony at the Max Planck Institute for Chemical Ecology in Jena was placed with one leaf disc (2 cm in diameter) of either EV or ovCRY (n = 12) plants in a plastic soufflé cup (Klar Pac). Leaf discs were replaced and survivor rates recorded for six consecutive days.

Field Manduca sexta *bioassays*

A pre-test on unplanted EV and ovCRY seedlings in jiffy plugs and a full test on planted EV, irAOC and ovCRY lines were performed. M. sexta eggs were kindly provided by C. Miles at Binghamton University, Vestal, New York, from an in-house colony. During the pre-test, three M. sexta neonates were placed on each of 14 seedlings (Fig. 2b, 42 M. sexta total for each genotype: EV and ovCRY). The total number of surviving neonates per line was counted for 2 d. Five surviving pre-test M. sexta neonates are pictured for each genotype on the last day.

During the full field test, two M. sexta neonates were placed on a leaf of the unsprayed lines enclosed in a clip cage to exclude predators. Survivors (0–2) were recorded for 15, 9 and 13 replicates of EV, irAOC, and ovCRY plants respectively for 7 d. The leaf was excised and photographed on the last day to estimate feeding damage.

Plant growth and fitness correlate measurements

Plants were recorded as elongating when their stalks were 0.5 cm above the rosette. Plants were recorded as flowering when the first corolla became visible. Rosette diameter was calculated as two times the longest rosette leaf. Elongation was measured as height from the base of the stalk to the highest point of the topmost inflorescence. Plant shoot dry biomass represented all aboveground plant mass, severed below the rosette, placed in a paper bag inside of a plastic box with ventilation holes of 1 cm diameter drilled through the lid, left to dry for 15 d in the sun, and then weighed. Flowers and seed capsules were identified as the pictured components in Fig. 3 and counted individually.

After 35 dpp in the field, wilting disease symptoms occurred on some plants and took an especially high death toll on irAOC and ovCRY plants (Table 2). The apparent health of each plant at the final collection (38 dpp) was recorded on a scale from 4 (healthy) to 0 (dead). Plants were ranked by M.S. according to the scale established in fig. 8(c) of Schuman *et al*. (2012), with a range of 0–4 rather than 1–5. Data collected for plants ranked as 0 were omitted from the figures and statistical analyses of shoot dry biomass (Fig. 4d) and reproductive correlates (Fig. 3) so that these data represent living plants; including rank 0 only increased variance within each treatment group but did not change the statistical relationships between treatment groups.

Statistical analyses

Data from 2011 were analyzed with Statview 5.0 (SAS Institute, Cary, NC, USA). Data were transformed if they did not meet the assumption of homoscedasticity. ANOVA and Student's t-tests were used to extract significances.

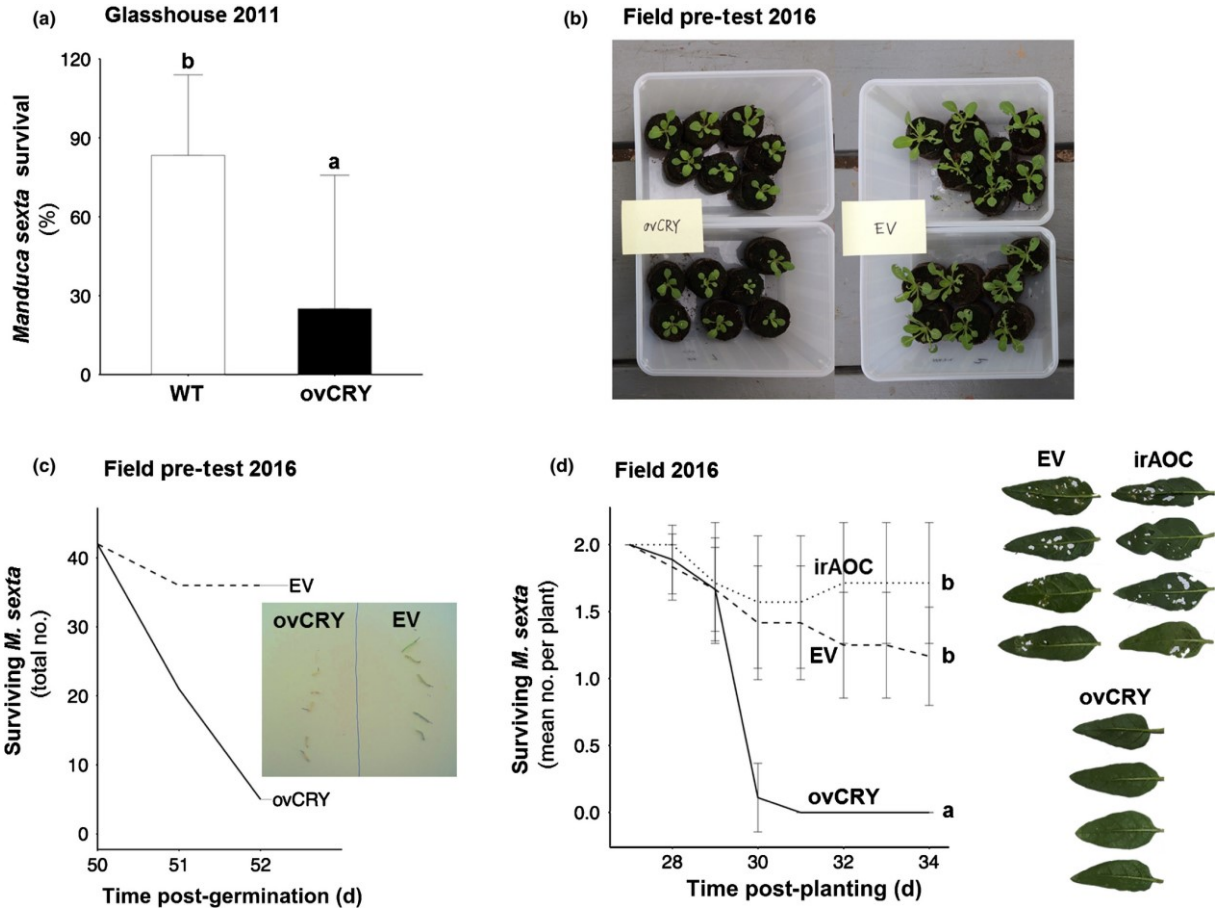

FIGURE 2 - Decreased survival of, and damage from, larvae of a naturally co-occurring lepidopteran species on ovCRY. (a) Mean (+ SE) number of *Manduca sexta* larvae surviving until day 6 as a percentage of individuals placed in soufflé cups that started feeding as neonates on unelicited leaf disks (n = 4) of wild-type (WT, white), and ovCRY (black) plants in the glasshouse. (b) Extent of damage on ovCRY and empty vector (EV) seedlings used in the *M. sexta* larvae survival test in (c). Picture by E. McGale. (c) Total surviving caterpillars of 42 *M. sexta* neonates placed on a total of 14 seedlings of each genotype (as seen in b) as part of a pre-test conducted in the field. Inset: appearance of the last five surviving larvae feeding on ovCRY and EV plants. (d) Mean (± SE) number of surviving caterpillars (of two) placed in a clip cage on one leaf of each plant replicate (n = 15, 9, and 13 for EV, irAOC, and ovCRY respectively) in the field, over a week. Marked significances apply from days 30 to 34, and all lines are not significant from each other from days 27 to 29 post-planting. Pictures (by M. C. Schuman) of the clip-caged leaves are not size comparable, but serve as a reference for damage on day 34 post-planting (the last day of the assay). Letters represent significant differences ($P < 0.05$) in (a, b) ANOVA or (d) Tukey *post-hoc* tests following a significant effect in an ANOVA.

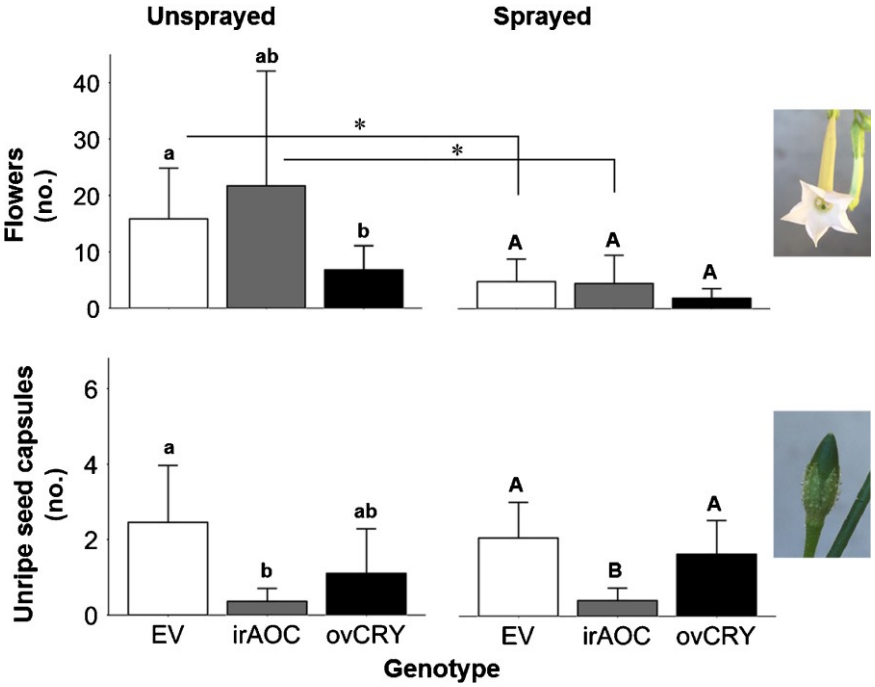

FIGURE 3 - Neonicotinoid insecticide (NI) treatment defines final fitness correlate count differences. Mean (+ SE) number of flowers and unripe seed capsules counted on 1 June 2016 from empty vector (EV), irAOC, and ovCRY plants (unsprayed $n = 15$, 14, and 14 respectively; sprayed $n = 11$, 8, and 5 respectively). Unripe seed capsules removed during the preceding week to prevent ripening were added to the unripe seed capsule counts on this day. Pictures by E. McGale. Letters represent significant differences ($P < 0.05$) in Tukey *post-hoc* tests following a significant effect in an ANOVA, and are compared within NI treatment for each individual correlate. The absence of letters indicates no significant differences within an NI treatment. Asterisks represent significance of the NI treatment within a genotype ($P < 0.05$).

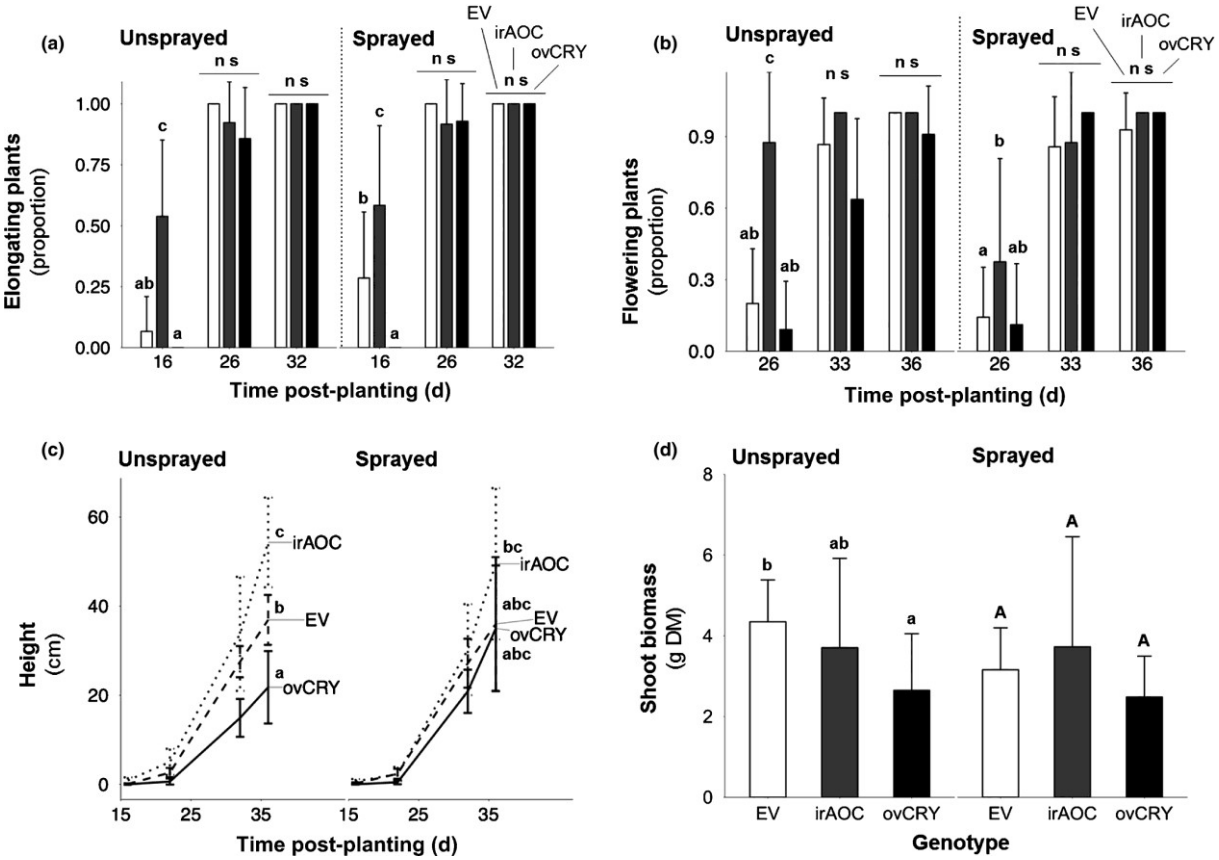

FIGURE 4 - irAOC plants elongate earlier and grow taller, while ovCRY matches empty vector (EV) yet produces significantly less biomass. (a) Proportion (+ SE) of plants from each genotype (n = 15 per neonicotinoid (NI) treatment) that had bolted at the day of measurement. Bolting is defined as a stalk length of > 0.5 cm from the flat rosette center. Significances are compared within each day, across NI treatment panels. (b) Proportion (+ SE) of plants from each genotype (n = 15 per NI treatment) that had flowered at the day of recording. Flowering is defined as having at least one flower with a visible corolla. Significances are compared within each day, across NI treatment. (c) Mean height measurements (± SE) of EV (dashed line), irAOC (dotted line), and ovCRY (full line), sprayed (n = 15, 14, and 14 respectively) or unsprayed (n = 14, 12, and 15 respectively). Significances are only calculated for the final time point (36 d post-planting). (d) Mean dry shoot biomasses (+ SE; DM, dry mass) of EV (white), irAOC (gray), and ovCRY (black) plants from sprayed (n = 11, 8, and 8 respectively) or unsprayed (n = 11, 8, and 5 respectively) replicates. Shoots were collected on 1 June 2016 and weighed on 16 June 2016 after sun drying. Letters represent significant differences ($P < 0.05$) in Tukey *post-hoc* tests following a significant effect in an ANOVA. ns, no significant difference. Letters are compared within their case for each panel.

Data from 2016 were analyzed in R (v.3.3.2; R Core Team, 2016). Data sets were fit to an appropriate model (LM, GLM, GLS, or LME) and each model was checked for normality and homoscedasticity. ANOVA and LSMEANS (lsmeans package) were then used to extract significances and comparisons (after a Tukey post-hoc test) from these data.

Secondary metabolite data from the untargeted XCMS analysis and subsequent curation described earlier were uploaded to MetaboAnalyst (http://www.metaboanalyst.ca) to conduct a PCA (Xia *et al*., 2009, 2012, 2015; Xia & Wishart, 2011a,b, 2016). Pareto data scaling was applied to meet assumptions of normality and homoscedasticity before PCA.

1.4 Results

Neonicotinoid insecticide treatment reduced herbivore damage from non-Cry1Ac-targeted insects

Half of all plants were sprayed with the NI (n = 15 sprayed, S; 15 unsprayed, U, per genotype) to permit assessment of the cost of defense with reduced herbivore loads (Figs 3, 4). Plants were sprayed twice in the evening, once at 11 dpp and once at 25 dpp (timeline, Fig. 1). Three herbivore screens were conducted 18, 25, and 33 dpp (timeline, Fig. 1). Damage from the specialist mirid T. notatus, which was the greatest among damage types recorded in this season, was significantly reduced in sprayed plants of all lines by 25 dpp (Fig. 5, 18 dpp screen: GLS, lsmeans(irAOC, U–S), t = 2.002, P = 0.0464, lsmeans(ovCRY, U–S), t = 2.589, P = 0.0102; 25 dpp screen: quasipoisson GLM, lsmeans(EV, U–S), t = 5.290, P = < 0.0001, lsmeans(irAOC, U–S), t = 5.115, P = < 0.0001, lsmeans(ovCRY, U–S), t = 4.711, P = < 0.0001; 33 dpp screen: quasipoisson GLM, lsmeans(EV, U–S), t = 3.337, P = 0.0008, lsmeans(irAOC, U–S), t = 4.773, P = < 0.0001, lsmeans(ovCRY, U–S) t = 3.406, P = 0.0007). Damage from specialist Epitrix flea beetles peaked c. 18 dpp, and sprayed irAOC and ovCRY plants had significantly less damage (Fig. 5, quasipoisson GLM, lsmeans(irAOC, U–S), t = 2.329, P = 0.0207, lsmeans(ovCRY, U–S), t = 3.074, P = 0.0023). Damage from generalist noctuids, the only herbivores of this set targeted by Cry1Ac, was never large enough to show significant differences between sprayed and unsprayed plants of any genotype. However, the most noctuid damage occurred at 25 dpp; unsprayed irAOC had significantly more damage than unsprayed EV or ovCRY from noctuids at this time (Fig. 5, AOV, lsmeans(irAOC–EV, U), t = −2.451, P = 0.0484, lsmeans(irAOC–ovCRY, U), t = 2.547, P = 0.388). Since we surveyed a variety of specialist and generalist herbivores with varying adaptations to jasmonate defenses and susceptibilities to Cry1Ac, we did not expect to, and did not see, a clear pattern of damage levels by genotype (Fig. 5). Overall, the herbivore load effects that we observed, predominantly on unsprayed plants, resulted mostly from herbivores not targeted by Cry1Ac.

ovCRY plants accumulate Cry1Ac over time when treated with a neonicotinoid insecticide, without altering endogenous defense metabolite production

Cry1Ac protein levels accumulated over time in NI-sprayed ovCRY +1 leaves (line 1129; 18, 25, 33 dpp, Figs 1, 6a) but remained stable or decreased in unsprayed ovCRY +1 leaves (Fig. 6a). The significant increase in sprayed ovCRY +1 leaves occurred from 25 to 33 dpp (Fig. 6a, LM, lsmeans(S, 25–33), t.ratio = −3.751, P = 0.0059), at the same time that amounts dropped to their lowest values in unsprayed plants (15.91 at 33 dpp vs 17.15 at 18 dpp). Leaf selection (−1 vs +1) had an effect on Cry1Ac levels at 33 dpp, where the younger leaf in sprayed and unsprayed plants did not differ significantly in Cry levels (Fig. 6a, inset). In EV and irAOC samples, absorbance readings did not differ significantly from buffer controls (GLS, lsmeans(Blank–EV, 33 dpp, t.ratio = −2.248, P = 0.1141; lsmeans(Blank–irAOC, 33 dpp, t.ratio = −1.751, P = 0.3006).

All plants were elicited three times with M. sexta oral secretions between 18 and 23 dpp (Fig. 1) to promote similar levels of elicitation regardless of herbivore load (Figs 5, 6). We profiled metabolites and conducted a semi-quantitative assessment of specific defensive metabolites known to be regulated by the jasmonate pathway in ovCRY, EV, and irAOC plants, sprayed and unsprayed (Fig. 6b,c). For metabolites known to be regulated by jasmonates

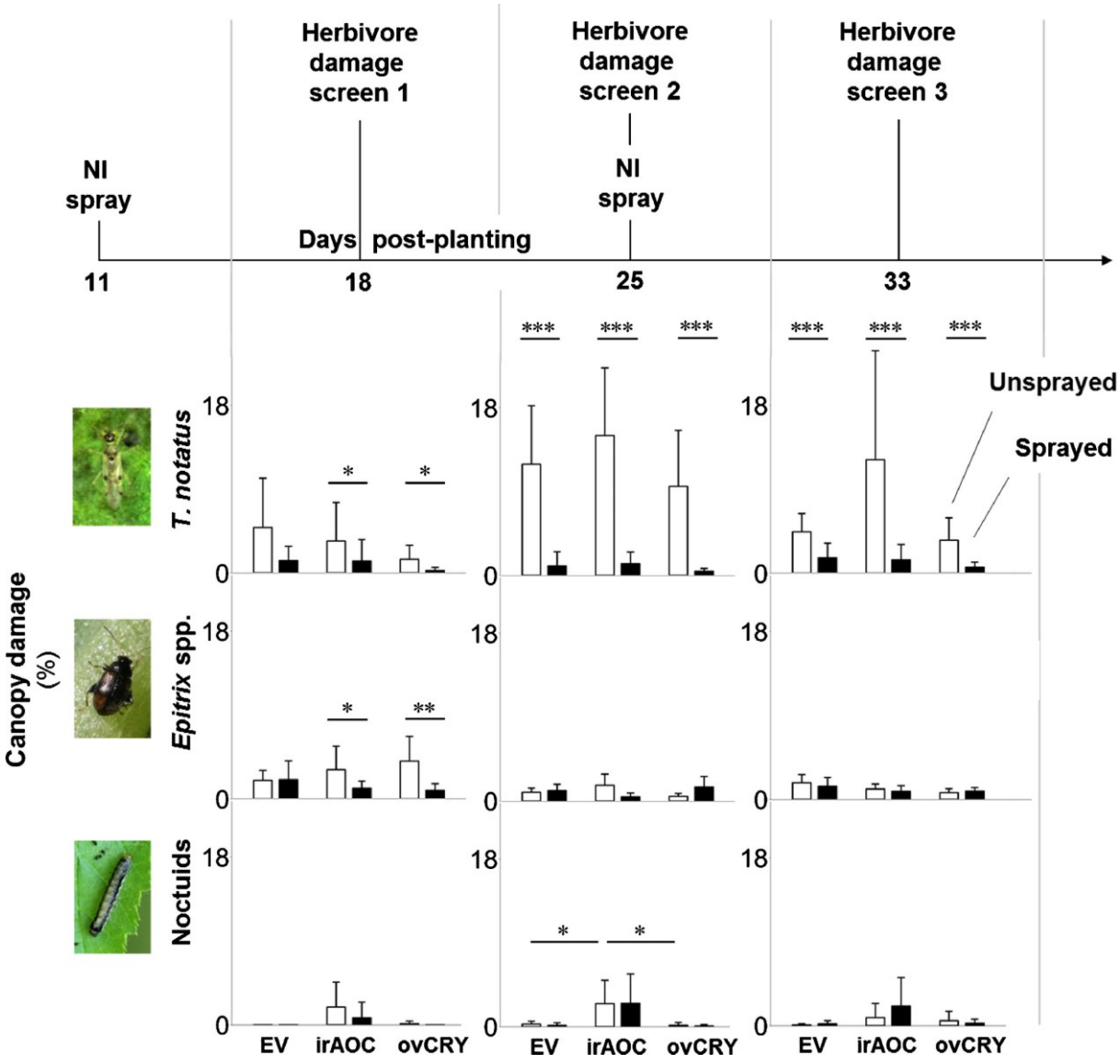

FIGURE 5 - A neonicotinoid insecticide reduces herbivory on empty vector (EV), irAOC, and ovCRY plants. Mean (+ SE) percentage canopy area damaged by *Tupiocoris notatus*, *Epitrix* species, and noctuid larvae, observed on unsprayed (white) and sprayed (black) EV, irAOC, and ovCRY individuals (n = 15, 13, and 15 respectively). Each column represents a different herbivore screening date (18, 25, or 33 d post-planting; see Fig. 1). Pictures of the *T. notatus*, *Epitrix*, and noctuid larvae by, respectively, Christoph Brütting (CC BY 3.0, https://commons.wikimedia.org/w/index.php?curid=23688041), David Nicholls (Ketton Quarry NR, 26 May 2012, http://www.naturespot.org.uk/species/epitrix-atropae), and Andrey Ponomarev (Russland, 21 May 2012, http://www.lepiforum.de/2_forum.pl?md=read;id=89059). Asterisks represent significant differences in Tukey *post-hoc* tests following a significant effect in an ANOVA: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

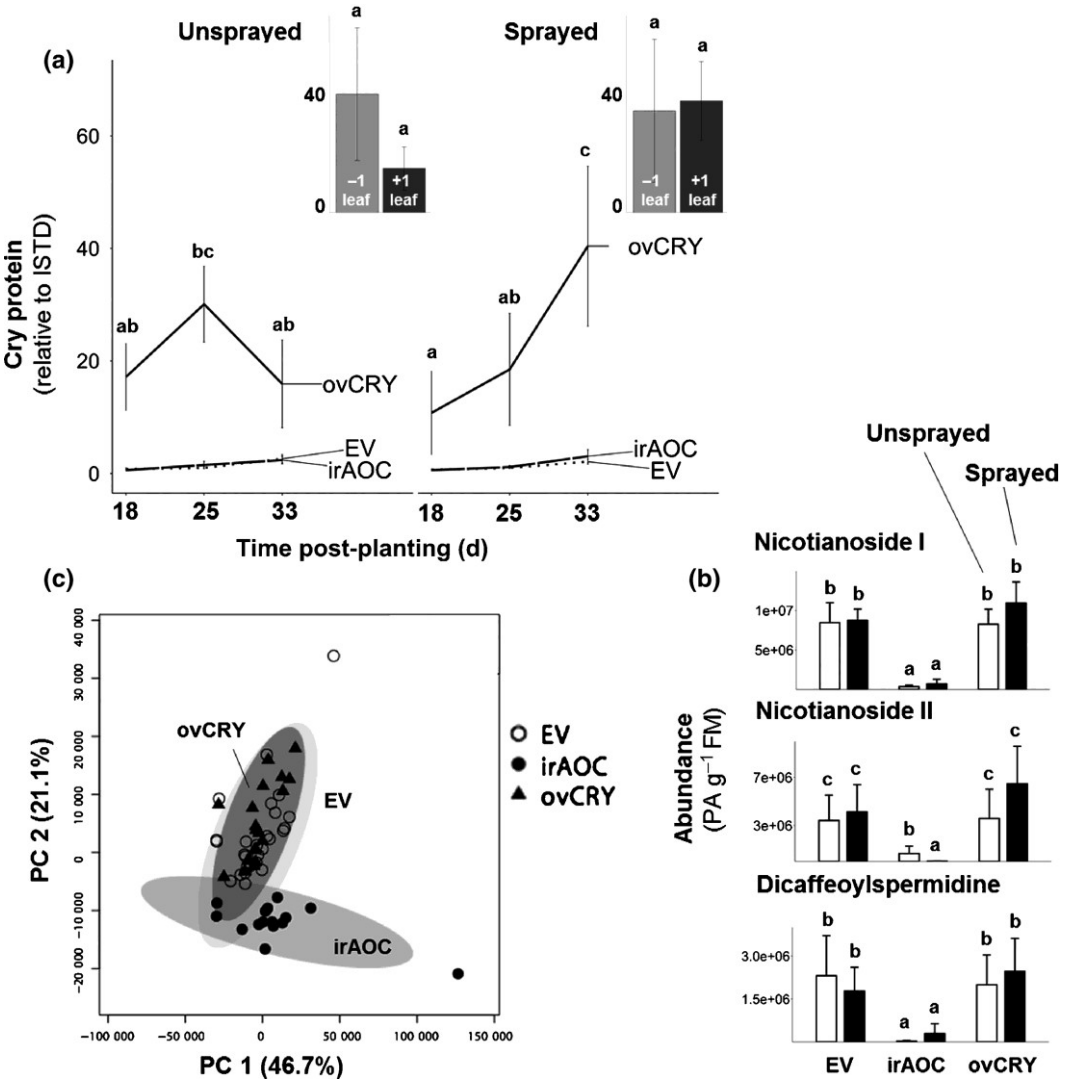

FIGURE 6 - Cry1Ac protein levels vary with time and neonicotinoid protection, while endogenous defenses are not altered in ovCRY; irAOC is defense deficient. (a) Mean (± SE) Cry1Ac protein levels in the +1 leaves (n = 15) of empty vector (EV) (dotted line), irAOC (long dash line), and ovCRY (full line) plants at three different days post-planting (dpp). Insets: mean (+ SE) Cry1Ac protein levels of sprayed and unsprayed developmentally younger (−1) and older (+1) leaves, relative to an internal standard (ISTD) at 33 dpp. (b) Mean (+ SE) accumulation of selected diterpenoid glycosides in +1 leaves of EV, irAOC, and ovCRY plants elicited 1 wk before sampling (three rounds of leaf puncturing and *Manduca sexta* oral secretion applications) in peak area (PA) per gram fresh mass (FM). Unsprayed (white) and neonicotinoid-sprayed (black) plant means are compared. Letters indicate statistical significance between bars within one metabolite. Note: scales are different for each metabolite. (c) Principal component (PC) analysis of untargeted metabolite screens of EV (empty circles), irAOC (filled circles), and ovCRY (triangles) from the 2016 field season. Shaded areas indicate 95% confidence intervals: EV (light grey), irAOC (grey), ovCRY (dark grey). The most distinct data space originates from irAOC plants (lower right), while the data spaces of the EV and ovCRY plants overlap substantially. Letters represent significant differences (P < 0.05) in Tukey *post-hoc* tests following a significant effect in an ANOVA (a, b).

(nicotianoside I, nicotianoside II, dicaffeoylspermidine), levels in irAOC remained near the limit of detection, and were significantly lower than levels in EV and ovCRY, which did not differ between NI treatments (Fig. 6b; statistics in Table 1).

TABLE 1 - Statistics tests and results for Fig. 6b: contrasts of *Nicotiana attenuata* lines within neonicotinoid insecticide (NI) treatment (U, unsprayed; S, sprayed)

Metabolite	Model	*Post hoc*	Contrast	t.ratio	*P*
Nicotianoside I	GLS	Tukey, *lsmeans*	U, EV–irAOC	6.883	< 0.0001
			U, EV–ovCRY	0.130	1.0000
			U, irAOC–ovCRY	−9.383	< 0.0001
			S, EV–irAOC	11.370	< 0.0001
			S, EV–ovCRY	−1.640	0.5762
			S, irAOC–ovCRY	−8.563	< 0.0001
Nicotianoside II	GLS	Tukey, *lsmeans*	U, EV–irAOC	5.320	< 0.0001
			U, EV–ovCRY	−0.643	0.9871
			U, irAOC–ovCRY	−5.567	< 0.0001
			S, EV–irAOC	5.926	< 0.0001
			S, EV–ovCRY	−0.976	0.9234
			S, irAOC–ovCRY	−4.877	< 0.0001
Dicaffeoyl-spermidine	GLS	Tukey, *lsmeans*	U, EV–irAOC	3.558	0.0098
			U, EV–ovCRY	0.398	0.9986
			U, irAOC–ovCRY	−4.387	0.0007
			S, EV–irAOC	3.621	0.0082
			S, EV–ovCRY	−1.076	0.8887
			S, irAOC–ovCRY	−4.119	0.0018

Because the NI treatment did not cause changes in the selected metabolites, the untargeted metabolite profiles of all three lines were analyzed via PCA by genotype. Although the three did not significantly diverge from each other over sampling times, irAOC showed a distinct clustering (lower right region, Fig. 6c), while EV and ovCRY overlapped completely, consistent with the inference that the defensive metabolome of ovCRY differed only in Cry toxin levels from EV.

Cry1Ac production reduces survival of, and damage from, a susceptible specialist herbivore

Cry1Ac production reduced the damage and survival of M. sexta larvae in both glasshouse and field experiments (Fig. 2). The survival of M. sexta neonates on ovCRY leaf discs was less than one-third that on wild-type leaf discs in a glasshouse experiment (25% vs 83%, Fig. 2a, ANOVA, F = 9.8001, P = 0.02031). In the field, three M. sexta neonates were placed each on ovCRY and EV unplanted seedlings (n = 14) and after 2 d only 5/42 larvae survived on ovCRY, whereas 36/42 survived on EV, with visible differences in leaf damage (Fig. 2b,c). At 27 dpp, two M. sexta neonates were placed on a leaf of each flowering *N. attenuata* plant, clip-caged to exclude predators (n = 15 EV, 9 irAOC, 13 ovCRY), and by day 30 no larvae remained alive on ovCRY plants (Fig. 2d, GLM, lsmeans(EV–irAOC, 34 dpp), z.ratio = −1.459, P = 0.3108; lsmeans(EV–ovCRY, 34 dpp), z.ratio = 5.793, P = < 0.0001; lsmeans(irAOC–ovCRY, 34 dpp),

z.ratio = 6.285, P = < 0.0001), consistent with the visibly greater damage on EV and irAOC leaves (pictures, Fig. 2d).

Production of Cry1Ac reduces biomass while that of endogenous jasmonates limits plant growth

A glasshouse growth assay of six independently transformed T2 ovCRY lines was performed to screen for insertion site effects on growth. At 19 d post-germination, three ovCRY lines that had similar Cry1Ac production, as measured by an enzyme-linked immunosorbent assay of seedlings immediately before transplantation, also had similar aboveground wet shoot biomasses (lines 1101, 1131, 1135; Fig. S2a). Lines 1131 and 1135, which produced more Cry1Ac at the seedling stage than a third line (1129), had smaller rosettes than 1129 (Fig. S2b). Lines producing varying amounts of Cry1Ac – 1129, 1099, and 1097 – were potted in individual pots to compare their growth rates with each other and with EV controls (A-04-266-3, '266', Figs S3b, S4). These three were tested for Cry1Ac at 29 dpp and their relative growth at the time appears consistent with a linear trade-off between Cry1Ac protein per gram leaf mass and plant rosette diameter and elongation (Figs S3a,b, S4). Line 1129 was also grown in size-matched pairs in the same pot with line 1099, which produced similar amounts of Cry1Ac at 29 dpp (Fig. S5). Planting size-matched pairs in the same pot amplifies otherwise subtle differences in growth rates that cannot be perceived when plants are grown individually in pots (Glawe *et al*., 2003). Differences in growth again correlated with the amount of Cry1Ac protein per gram leaf mass, as there were no differences between the two lines in growth or Cry1Ac production (Figs S3a, S5). This indicated that growth differences observed in the field study were due to Cry1Ac production.

In the field, elongation and flowering times, stalk height, and aboveground biomass were recorded as measures of plant performance (Fig. 4). A significantly larger proportion of irAOC plants were elongating at 16 dpp than of ovCRY (line 1129) or EV plants for both sprayed and unsprayed plants (Fig. 4a, LM, lsmeans(16 dpp, EV–irAOC, U), t.ratio = −6.230, P = < 0.0001, lsmeans(16 dpp, irAOC–ovCRY, U), t.ratio = 8.504, P = < 0.0001, lsmeans(16 dpp, EV–irAOC, S), t.ratio = −5.460, P = < 0.0001, lsmeans(16 dpp, irAOC–ovCRY, S), t.ratio = 8.309, P = < 0.0001). Within each genotype, there were no significant differences in elongation time by NI treatment. However, sprayed irAOC plants flowered significantly later than unsprayed irAOC plants and no longer differed significantly from sprayed ovCRY (Fig. 4b, LM, lsmeans(26 dpp, irAOC, U–S), t.ratio = 2.956, P = 0.0406, lsmeans(26 dpp, EV–irAOC, U), t.ratio = −4.765, P = 0.0001, lsmeans(26 dpp, irAOC–ovCRY, U), t.ratio = 5.864, P < 0.0001, lsmeans(26 dpp, EV–irAOC, S), t.ratio = −3.303, P = 0.0144, lsmeans(26 dpp, irAOC–ovCRY, S), t.ratio = 2.758, P = 0.0691). NI treatment did not cause a significant change for EV or ovCRY in flowering time.

For unsprayed plants at 35 dpp, EV stalk heights were significantly greater than ovCRY; irAOC was significantly greater than both (Fig. 4c, LM, lsmeans(35 dpp, U, EV–ovCRY), t.ratio = 3.186, P = 0.0341, lsmeans(35 dpp, U, EV–irAOC), t.ratio = −3.052, P = 0.0468, lsmeans(35 dpp, U, irAOC–ovCRY), t.ratio = 5.518, P = 0.0001). Interestingly, sprayed EV, irAOC, and ovCRY did not differ significantly from each other.

ovCRY shoot biomass was significantly lower than that of EV at 44 dpp for unsprayed plants, but not for sprayed plants (Fig. 4d, LME, lsmeans(EV–ovCRY, U), t.ratio = 2.717, P = 0.0245, lsmeans(EV–ovCRY, S), t.ratio = 0.801, P = 0.7042). Shoot dry biomass values for irAOC had a large variance, which eliminated significant differences from EV and ovCRY means, perhaps

due to preferential, and varied, herbivory on these defense-deficient plants (Kallenbach *et al*., 2012). However, similarly to stalk height, irAOC biomass appeared to be greater for unsprayed rather than sprayed plants.

Neonicotinoid insecticide treatment had a larger effect on flowering than both Cry1Ac and endogenous defense production

Reproductive units comprising flowers and unripe seed capsules were counted on plants of each genotype at 38 dpp immediately before biomass harvests (Fig. 3). The NI-sprayed replicates of EV and irAOC had significantly fewer flowers than their unsprayed counterparts (Fig. 3, flowers: LME, lsmeans(EV, U–S), t.ratio = 2.445, P = 0.0177, lsmeans(irAOC, U–S), t.ratio = 2.147, P = 0.0362). Unsprayed EV plants produced significantly more flowers than did ovCRY plants, a difference which disappeared with the NI treatment (Fig. 3, LME, lsmeans(EV–ovCRY, U), t.ratio = 2.858, P = 0.0163, lsmeans(EV–ovCRY, S), t.ratio = 1.536, P = 0.2824). JA pathway components such as allene oxide cyclase are important for fertility, and this was reflected in very low capsule production in irAOC plants (Wasternack *et al*., 2013). Unripe seed capsule counts did not significantly differ between ovCRY and EV in either NI treatment; however, like biomass, means were more similar within the sprayed treatment.

ovCRY and irAOC plants were similarly susceptible to a naturally occurring wilt disease

At the end of the field season, some of the plants showed symptoms of wilt, and infected plants were recorded by genotype. irAOC plants, deficient in endogenous jasmonates, are more susceptible to this native fungal wilt disease (Luu *et al*., 2015). Surprisingly, ovCRY lines were also more susceptible (Table 2). Since the defensive metabolome of ovCRY plants did not differ from EV except in Cry1Ac production (Fig. 6), we speculate that this increase in susceptibility may be associated with the ectopic Cry1A protein production.

1.5 Discussion

Wild plants have sophisticated strategies to balance costs of defense with the ability to produce successful grandchildren; that is, Darwinian fitness. Within one generation, plants additionally employ strategies that balance defense costs with yield, including both production of biomass and seed. Yield–defense trade-offs are not equivalent to fitness defense–trade-offs; and though one may contribute to the other, they do not necessarily correlate (Karban & Baldwin, 1997; Baldwin, 1998; Cipollini *et al*., 2014). However, research on the balance of yield and defense in wild plants may be interesting for their applicability to agricultural objectives (Huot *et al*., 2014). Yield–defense trade-offs in native plants depend on context, such as the occurrence and abundance of certain herbivores (Baldwin, 1998; Machado *et al*., 2016). The complexity of yield–defense trade-offs has been studied in many different systems, including, but not limited to, Solanum nigrum, A. thaliana, *N. attenuata*, and Brassica rapa, and the resulting data have demonstrated overlapping influences on these trade-offs ranging from herbivore abundance and composition to plant light perception and plant defense and hormone plasticity (Baldwin, 2001; Hartl *et al*., 2010; Ballaré, 2014; Berens *et al*., 2017; Züst & Agrawal, 2017). In agriculture, such trade-offs impact profit, which is largely driven by yield. The ectopic production of Cry toxins in plants is increasingly used to reduce costs of protecting crops against targeted lepidopteran pests, and thus increase profits

TABLE 2 - Counts of *Nicotiana attenuata* plants with signs of a native wilt disease at the end of the 2016 field season

Genotype	Dead count		Dead + extensive signs count		Total
	Unsprayed	NI sprayed	Unsprayed	NI sprayed	
EV	0	0	4	4	8
irAOC	3	2	8	7	15
ovCRY	3	1	5	7	12

EV, empty vector; NI, neonicotinoid insecticide.

Counts were recorded 38 d post-planting. Plants were ranked according to the Plant growth and fitness correlate measurements subsection. Dead, rank of 0; dead and plants with extensive signs of the disease, ranks of 0 and 1. Plants of rank 0 were excluded (out of $n = 30$ for each genotype) from dry shoot biomass and fitness correlate data.

for farmers (Krattiger, 1997; Fernandez-Cornejo *et al*., 2014; James, 2015; USDA, 2016), and positive yield reports are an important factor determining continued Bt crop use by farmers under pressure to feed rapidly increasing populations (Ruttan, 1999). However, meta-analyses aggregating data from differing agronomic locations and contexts report mixed yield results for Bt crops (Qaim & Zilberman, 2003; Finger *et al*., 2011; Klümper & Qaim, 2014). *N. attenuata* is an ecological model plant with well-studied anti-herbivore defenses, of which many features are shared with other species (Wu & Baldwin, 2010; Mithöfer & Boland, 2012), and can provide a context in which to study the effects of Cry introduction without the complications of introgression, in a relevant environment under pressure from different herbivores. Mechanisms underlying yield–defense trade-offs are more straightforward to interpret in native plants than in artificially bred and introgressed crop lines (Rowen & Kaplan, 2016; Whitehead *et al*., 2017), although studies on crops are essential for agricultural applications.

We compared yield consequences of Cry1Ac production in *N. attenuata*, with or without insecticide application, in its native habitat. Specifically, we compared Cry1Ac production in addition to endogenous defenses (ovCRY) with an isogenic EV and put this into the context of yield from a line with abrogated endogenous defenses (irAOC). Each genotype was subjected to a lighter or heavier load of herbivores not targeted by Cry1Ac, using applications of an NI to half of the replicates. We report that ovCRY plants produced lower yields than EV plants, as measured by stalk height, dry shoot biomass, and flower production, when not sprayed with the NI. However, NI-sprayed ovCRY no longer lagged behind EV plants in any of the aforementioned yield measures. irAOC plants deficient in endogenous defenses initially grew larger, but they produced highly variable yields that did not differ significantly from those of EV or ovCRY plants for most measures. Interestingly, without additional NI protection, the apparent costs of Cry1Ac (yield differences between EV and ovCRY) were greater than the cost of endogenous defense (yield differences between EV and irAOC) in these plants. Correspondingly, efforts to protect crop plants by ectopic expression of terpene synthase genes, which have been shown to have the potential to attract biological control agents, may result in a reduced, rather than improved, yield. This is due both to physiological costs of suddenly producing a large amount of a single compound in a plant that has not adapted to do so and to ecological costs from a substantially altered herbivore community (Robert *et al*., 2013). This has clear parallels to the observed costs of Cry production.

We observed significantly greater herbivore damage to unsprayed plants throughout the field season, consisting primarily of herbivores not targeted by Cry1Ac, and this was likely responsible for the yield drags of Cry1Ac plants in stalk height, dry shoot biomass, and flower production, in comparison with EV controls. NI-sprayed EV and irAOC plants had fewer flowers than unsprayed plants, potentially indicating a longer term investment in reproductive growth due to reduced stress. It is known that plants, in response to herbivory, may reallocate resources to reproductive sinks at earlier ontogenic stages (Tito *et al*., 2016), which could explain the higher values of reproductive correlate counts for unsprayed plants (Fig. 3, for flowers: EV and irAOC). Although more unsprayed irAOC plants were flowering than EV or ovCRY plants at 26 dpp, flower counts did not reflect this flowering discrepancy between genotypes. Importantly, sprayed irAOC and ovCRY no longer differed in proportion of flowering plants, and the NI spray treatment caused a difference in flowering time only for irAOC, with sprayed plants flowering later (Fig. 4b, 26 dpp) and also yielding fewer flowers (Fig. 3). However, sprayed EV plants also had fewer flowers (Fig. 3) even though there was no difference in elongation or flowering time for EV by spray treatment (Fig. 4a, 16 dpp; Fig. 4b, 26 dpp). Unripe capsule counts are important for the comparison of yield in EV vs ovCRY, but the severe jasmonate deficiency in irAOC plants prevents them from setting capsules (Wasternack *et al*., 2013). There was no correlation between elongation or flowering time and unripe capsule counts. Thus, the differences in fitness correlates in this study do not appear to be explained by differences in the timing of ontogenic transitions, and there is also no simple relationship between these two aspects of plant reproduction in our dataset. These results support the hypothesis that unsprayed plants had a more rapid reproductive maturation unrelated to flowering time, although the NI seemingly did serve to 'level the playing field' between genotypes.

These effects of the NI treatment were likely a direct result of reduced herbivore damage to these plants, since there were no effects of NI treatment on metabolite induction at 33 dpp. The elicitation of endogenous defenses was initially standardized in both sprayed and unsprayed plants with multiple treatments of standardized wounding plus M. sexta oral secretions, a treatment known to strongly elicit jasmonate-mediated defense in *N. attenuata* (Schittko *et al*., 2000). An initial induction can dampen further plant defense metabolite production in response to a variety of herbivore attacks (Viswanathan *et al*., 2007), and this may explain why sprayed and unsprayed plants had similar metabolite profiles despite different damage rates.

Not surprisingly, benefits of Bt crops are clearer when target insects are abundant (Perlak *et al*., 1990; Klotz-Ingram *et al*., 1999). Studies have shown that nontarget insect groups may increase in abundance following introduction of Bt crops (Yu *et al*., 2011), and Bt crops may even be preferred by nontarget herbivores (Marvier *et al*., 2007). We show that herbivory from these nontarget herbivores can have a substantial effect on the growth and yield of Cry-producing plants. In general, we propose that Cry production carries a noticeable cost that may emerge under certain biotic circumstances despite careful breeding. This study presents another extreme example: introducing Cry1Ac production increased susceptibility of *N. attenuata* plants to a native Alternaria–Fusarium fungal pathosystem, (Schuck *et al*., 2014) and this increase in susceptibility was as great as that resulting from the reduction of endogenous JA-mediated defenses (Luu *et al*., 2015; Table 2). Thus, additional resistance traits or targeted pesticide treatments may be critical to maintain or increase yield from Cry1Ac-producing plants. Alternatively, this may indicate that the introduction of a new constitutive defense in a plant, much like the selection for specific defense strategies over time, may require conditions that favor the new trait, such as pressure from a susceptible herbivore community and a concurrent lack of other conflating pressures.

Infection by the native fungal pathogens may have contributed to the breadth of the confidence intervals in the fitness correlate data (Fig. 3), and so we conducted comparisons with data from an earlier field season to exclude this possibility (see Notes S1). This disease was not present in the 2011 field season, and we therefore compared data from three control lines that were present in both 2011 and 2016 field seasons: EV, ovCRY × irAOC, and ovCRY × EV (of which neither produced Cry1Ac protein and thus were not considered further in this study). The significance trends present in the final values of elongation, aboveground dry biomass, and flower counts were consistent between the two field seasons. The regression trend lines of all three genotypes within each field season did not differ significantly from each other, and the elongation rates of EV could not be distinguished between the two years (Figs S6–S8). In fact, confidence intervals in the 2011 season were larger than those in 2016. The data from 2016 thus seem consistent and even more conservative than the 2011 data, especially in flower counts (Fig. S9), so we infer that any significant differences emerging in 2016 were likely to be robust.

We further provide evidence that differences in growth between EV and ovCRY in the 2016 field study were likely due to Cry1Ac production, by comparing the growth of EV to five independently transformed T2 ovCRY lines. Growth of each line was negatively correlated to Cry1Ac accumulation. Additionally, two T2 lines with similar Cry1Ac production at 29 d post potting, grown in competition with each other in the same pot so as to enhance our ability to detect even minor growth differences, were found not to differ in growth. From these results, we infer that the yield drag observed to be associated with Cry1Ac accumulation was not an artifact of the transformation or T-DNA insertion site.

Our study also shows that an NI treatment may stabilize Cry1Ac production in plants throughout the season. Cry toxin amounts in an exogenously overexpressing transformed plant are not exempt from variance when environmental factors cause resource reallocation to occur. For example, there could be fewer raw materials for protein synthesis, or proteins could be degraded to recover amino acids (Dungey & Davies, 1982; Vierstra, 1993), both of which would lead to a decrease of Cry1Ac amounts. In addition, plants may also use promoter methylation to reduce expression of a protein during the course of their development (Weinhold *et al*., 2013). From our observations, Cry toxin amounts in leaves varied greatly throughout the season in unsprayed plants but increased stably in sprayed plants (Fig. 6a). Regardless of NI treatment, ovCRY plants were deadly to the native lepidopteran herbivore M. sexta (Fig. 2d), but under our field conditions this did not yield benefits for unsprayed ovCRY plants in comparison with EV and irAOC plants. The loss of foliar Cry amounts in unsprayed ovCRY indicates that the reason for its yield drag may have been an early reallocation of resources under biotic stress, which did not seem to occur in sprayed plants. We propose that sprayed ovCRY plants benefited from reduced herbivory by having sufficient resources to maintain foliar resources and defenses (both endogenous and Cry) and thus growth, leading to their matching sprayed EV plants in stalk height, dry shoot biomass, flower production, and unripe seed capsule production.

Overall, EV controls consistently grew better and produced more reproductive units than the other two lines, indicating the benefit of a suite of evolved endogenous defenses that are balanced with growth and reproduction in native plants. Efforts to enhance or reintroduce suites of native defenses that would be relevant and nontoxic in crops may thus produce higher and more stable yields than the employment of exogenous single compounds or compound classes. However, Cry toxins are clearly more effective than endogenous defenses against co-evolved herbivores like M. sexta, which have the potential to devastate plants during outbreaks, and

looking to environmental factors which favor the evolution of constitutive defenses may help inform agricultural strategies. Our results show that the potential for re-emerging yield drags caused by Cry production seems to be reduced by appropriately timed pesticide applications with the occurrence of these biotic stresses.

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1.7 Supporting Information

2011 and 2016 field results are consistent

The 2011 and 2016 field experiments shared the genotypes EV, ovCRYxirAOC and ovCRYxEV, interspersed among homozygous genotypes. Neither cross produced Cry1Ac as measured by ELISAs (Fig. S2a), and thus these crosses were not studied further. However in both field seasons, the relationship between these three non-Cry1Ac producing controls was consistent, with ovCRYxirAOC, which is deficient in jasmonates (Fig. S2), having a significantly greater stalk height than its two counterparts (Fig. S3, GLS, lsmeans(ovCRYxirAOC-EV, 2011), t. ratio = -2.260, P = 0.0258, lsmeans(ovCRYxirAOC-ovCRYxEV, 2011), t. ratio = 4.763, P = <.0001; GLS, lsmeans(ovCRYxirAOC-EV, 2016), t. ratio = -3.134, P = 0.0061, lsmeans(ovCRYxirAOC-ovCRYxEV, 2016), t. ratio = 3.422, P = 0.0024). The regression lines of each genotype within each season did not differ significantly from each other (Fig. S3). An overlay of the EV lines from the two field seasons revealed nearly identical growth curves (Fig. S3, inset) but the variability, represented by the confidence intervals, in the stalk height measurements was consistently smaller for the 2016 field season (Fig. S3). The three genotypes furthermore did not differ significantly in aboveground biomass within each year, for plants at similar ontogenic stages (Fig. S4). Correspondingly, there was no significant difference in flower counts in plants of a similar developmental stage in either year, though the number for ovCRYxirAOC tended to be greater than that from ovCRYxEV in 2011 (Fig. S5, 2011: ANOVA, by Genotype, F = 3.0408, P = 0.08254, pairwise t-test(ovCRYxirAOC-ovCRYxEV), P = 0.096; 2016: ANOVA, by Genotype, F = 0.2158, P = 0.8069, pairwise t-test(ovCRYxirAOC-ovCRYxEV), P = 1). Again, the confidence intervals from 2011 were greater than in 2016. From these results, we infer that the 2016 data is not only consistent with the 2011 data, it also appears to be more conservative and likely to only reveal acute differences.

1.8 Supporting Figures

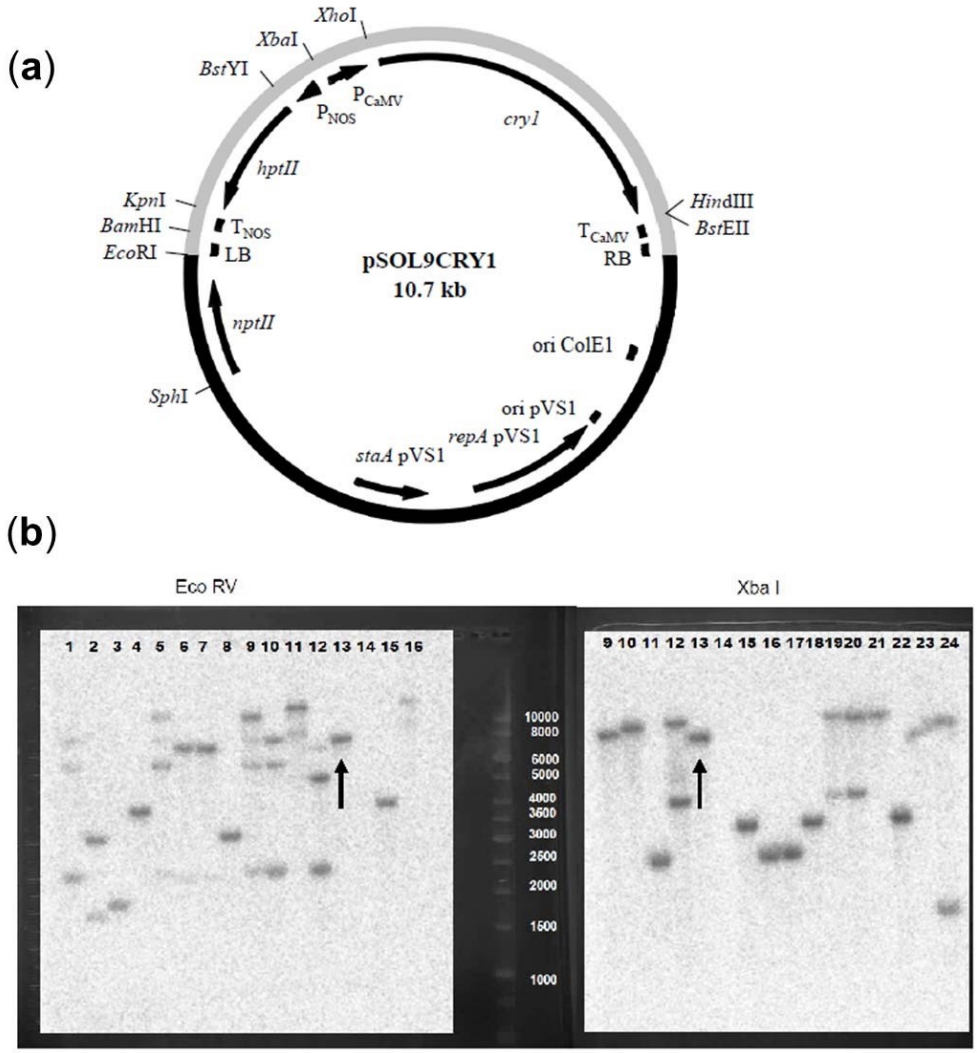

FIGURE S1 - (a) ovCRY transformation construct, pSol9Cry1a, and (b) Southern blot which reveals that each transformed line used harbors a single insertion of the transformation construct (arrows).

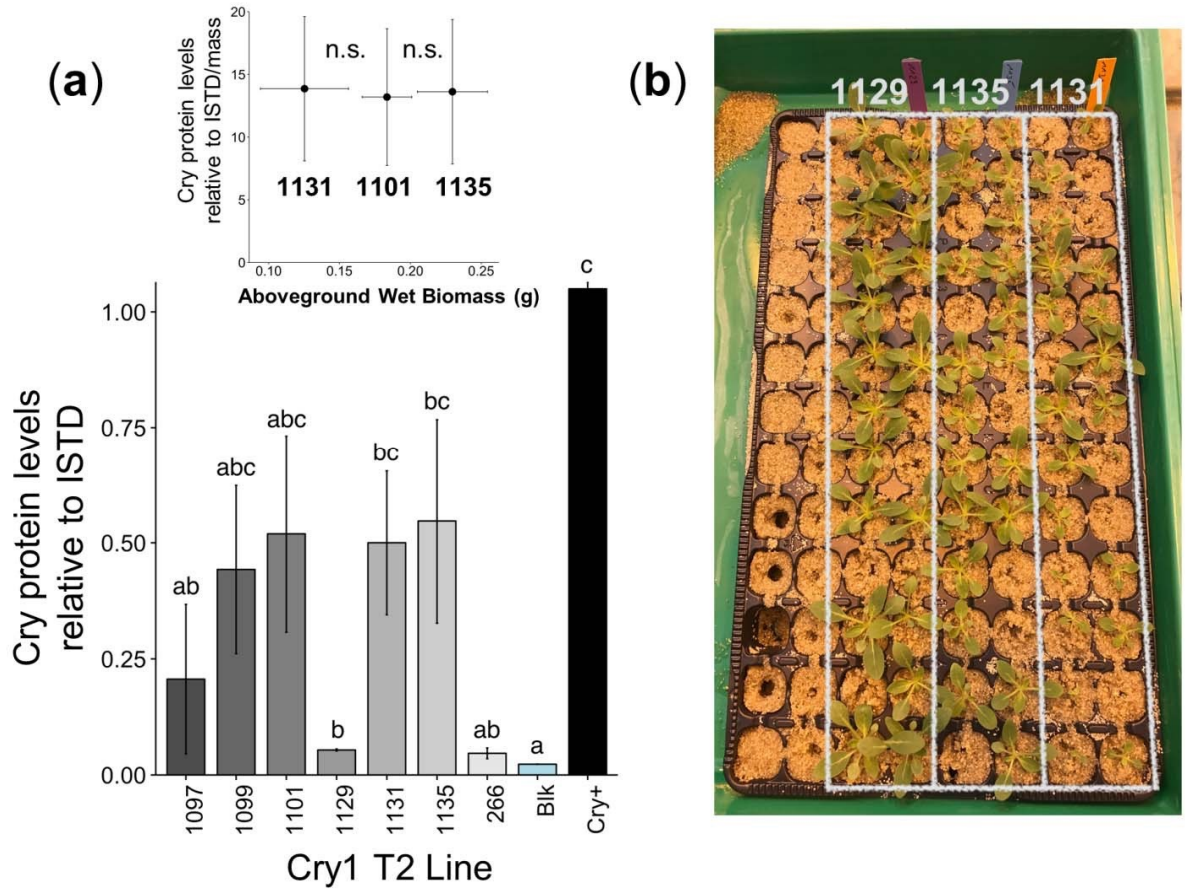

FIGURE S2 - (a) Nineteen-day-old seedling comparison of six T_2 ovCRY lines, including the field-tested ovCRY (1129) and additionally EV (266), show that similar Cry1Ac amounts match with similar seedling biomass and (b) increasing amounts of Cry1Ac are visually inversely correlated with rosette size. Error bars represent 95% confidence intervals. Letters represent significant differences (P<0.05) in Tukey *post-hoc* tests following a significant effect in an ANOVA. n.s. indicates no significant difference. Picture by E.M.

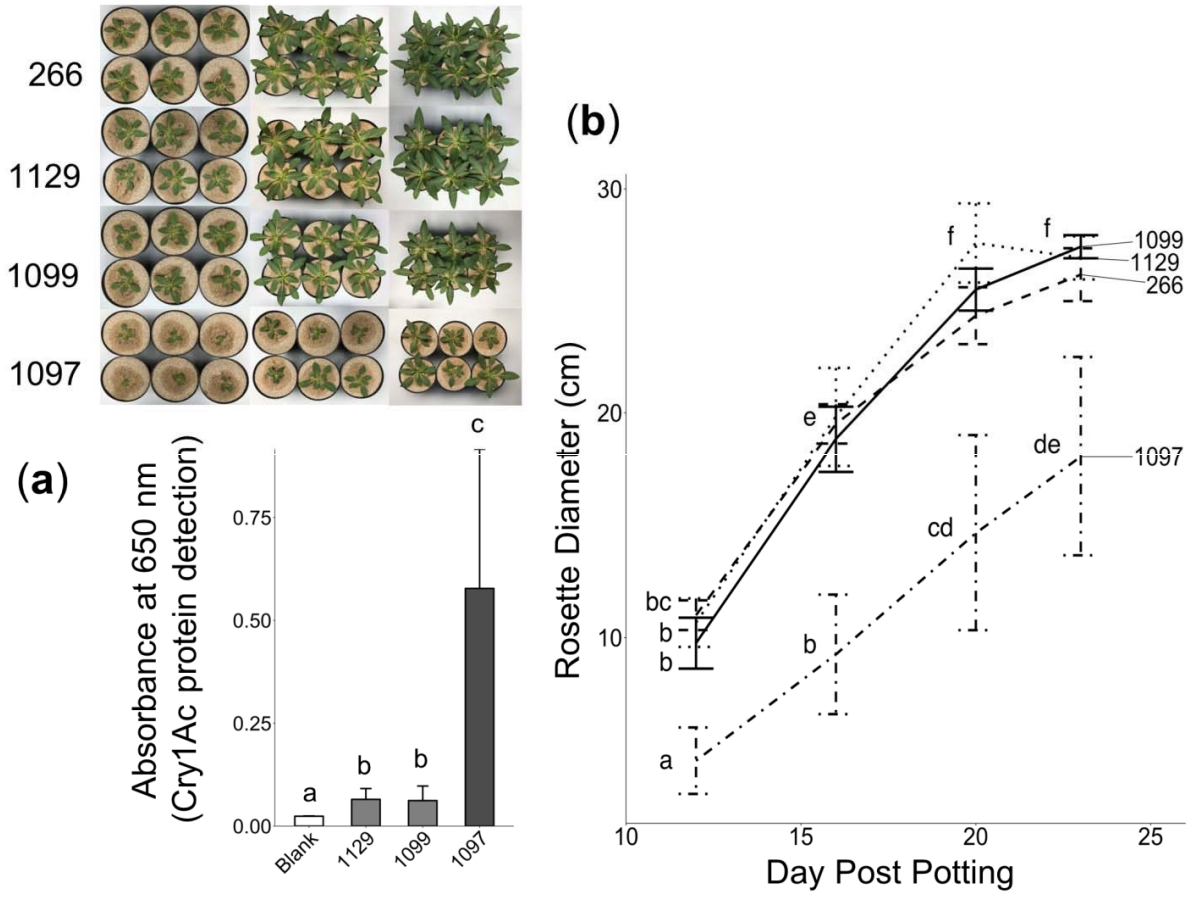

FIGURE S3 - Cry1Ac amounts of three T_2 ovCRY lines indicate a negative correlation between (a) Cry1Ac production and ovCRY's growth lag, shown in (b) rosette diameters and pictures (top left) from a month-long growth assay of field-tested EV (266) and ovCRY (1129), and two additional T_2 ovCRY lines (1097 and 1099). Error bars represent 95% confidence intervals. Letters represent significant differences (P<0.05) in Tukey *post-hoc* tests following a significant effect in an ANOVA. Each column of pictures (by E.M.) respectively show rosettes of each line at 12, 16 and 20 days post potting, which match with the first three time points graphed in panel (b).

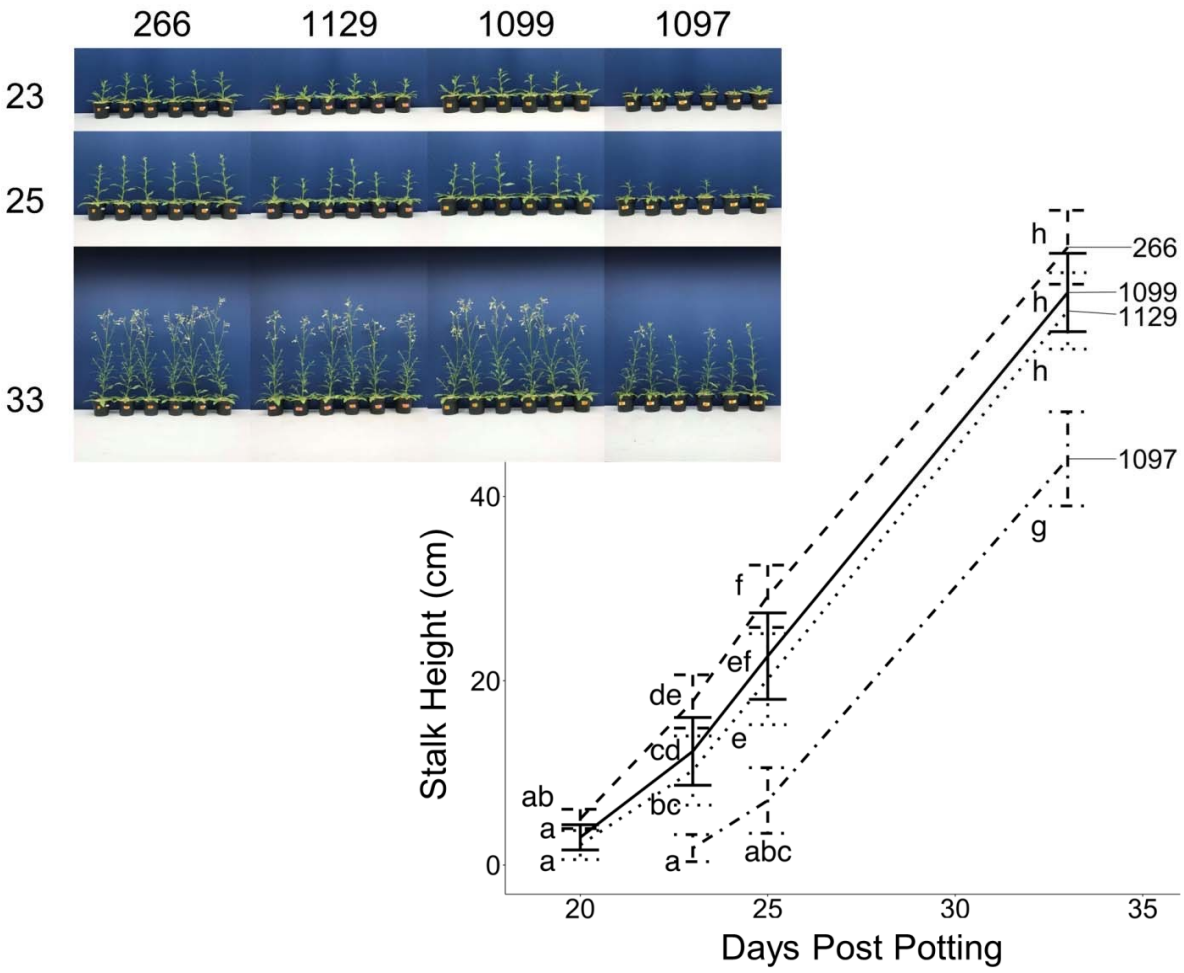

FIGURE S4 - Stalk heights and pictures from a month-long growth assay of field-tested EV, ovCRY, and two additional T2 ovCRY lines that are consistent with the rosette diameter correlation between Cry1Ac production and ovCRY's growth lag. Error bars represent 95% confidence intervals. Letters represent significant differences ($P<0.05$) in Tukey *post-hoc* tests following a significant effect in an ANOVA. Each row of pictures (by E.M.) respectively show elongating plants of each line, with pots presented in the same order, at 23, 25 and 33 days post potting, which match with the last three time points graphed below.

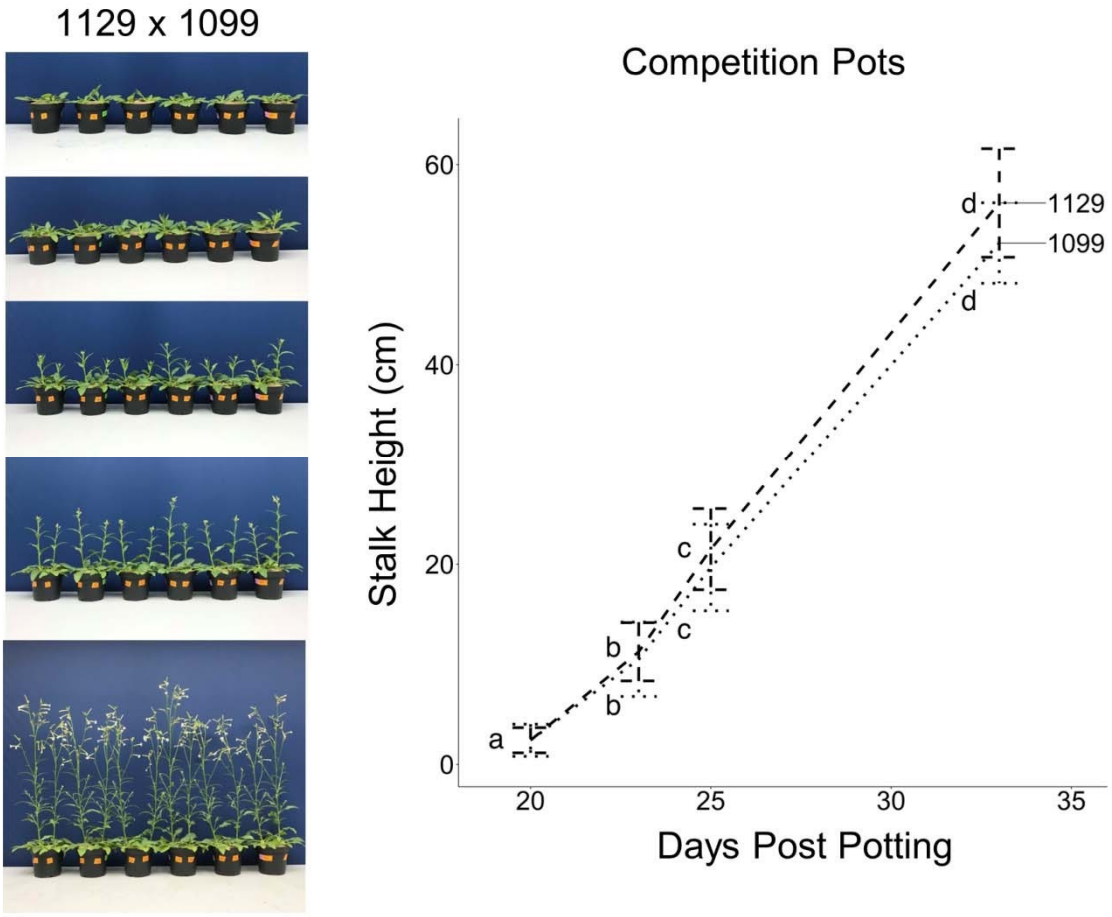

FIGURE S5 - Month-long growth assay of field-tested ovCRY and a line with similar Cry1Ac production planted in size-matched competition pots; no growth differences are revealed in pictures or stalk height values.
Error bars represent 95% confidence intervals. Letters represent significant differences ($P<0.05$) in Tukey *post-hoc* tests following a significant effect in an ANOVA. Starting from the top, the pictures (by E.M.) show the paired plants, with 1129 to the left and 1099 to the right in each pot, at 20, 23, 25 and 33 days post potting, which match all of the graphed time points. Pots are always pictured in the same order.

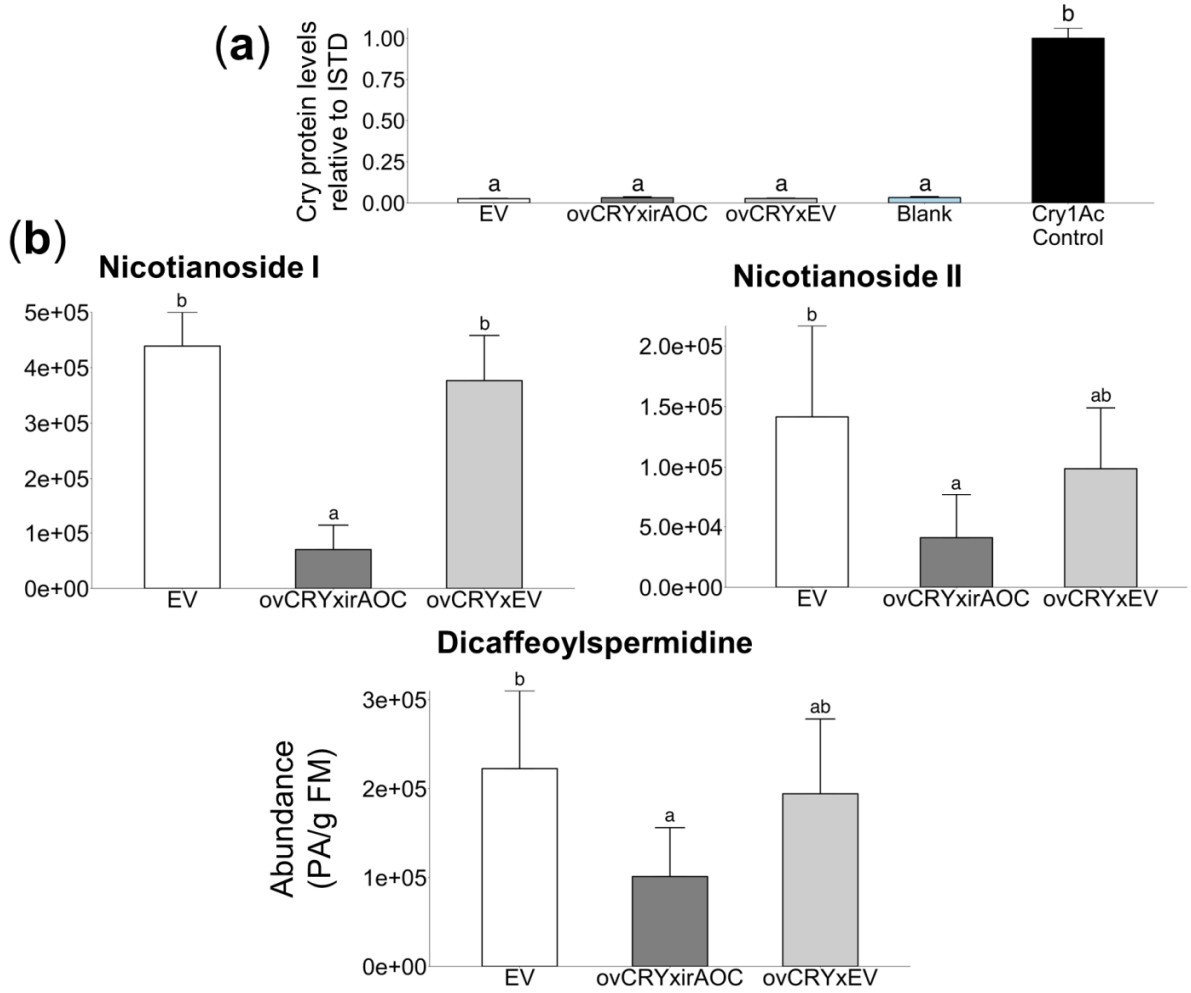

FIGURE S6 - (a) Cry1Ac protein and (b) secondary metabolite (Nicotianoside I, Nicotianoside II, Dicaffeoylspermidine) levels from the 2011 glasshouse show the lack of Cry1Ac abundance in EV and two cross controls, and the defense deficiency of ovCRYxirAOC. Error bars represent 95% confidence intervals. Letters represent significant differences (P<0.05) in Tukey *post-hoc* tests following a significant effect in an ANOVA.

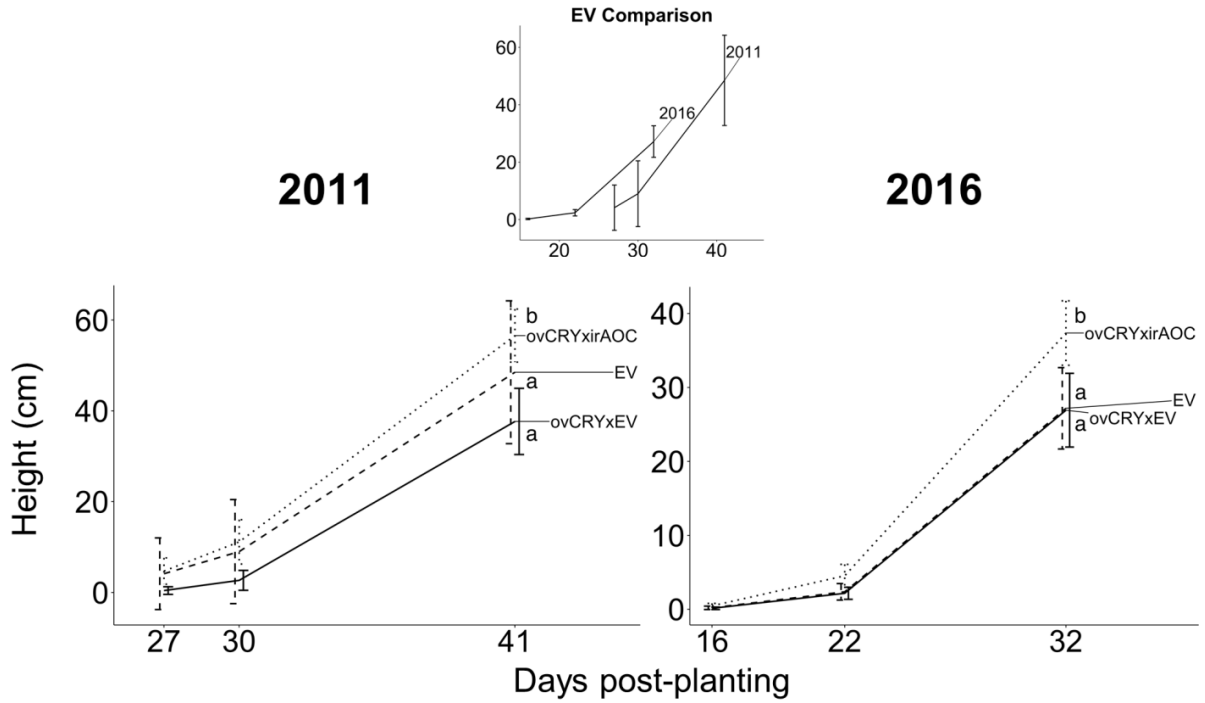

FIGURE S7 - Elongation comparison of EV and two ovCRY cross controls from the 2011 and 2016 field seasons that show consistent growth trends. Error bars represent 95% confidence intervals. Letters represent significant differences (P<0.05) in Tukey *post-hoc* tests following a significant effect in an ANOVA.

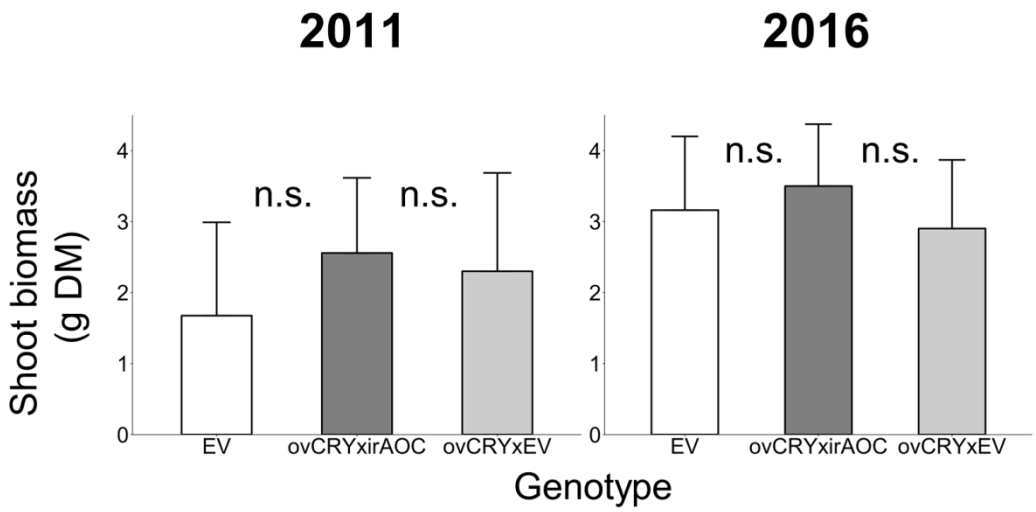

FIGURE S8 - Final dry shoot biomass comparison of developmentally similar EV and ovCRY cross controls in 2011 and 2016 field seasons that show consistent final biomass results. Error bars represent 95% confidence intervals. n.s. indicates no significant difference.

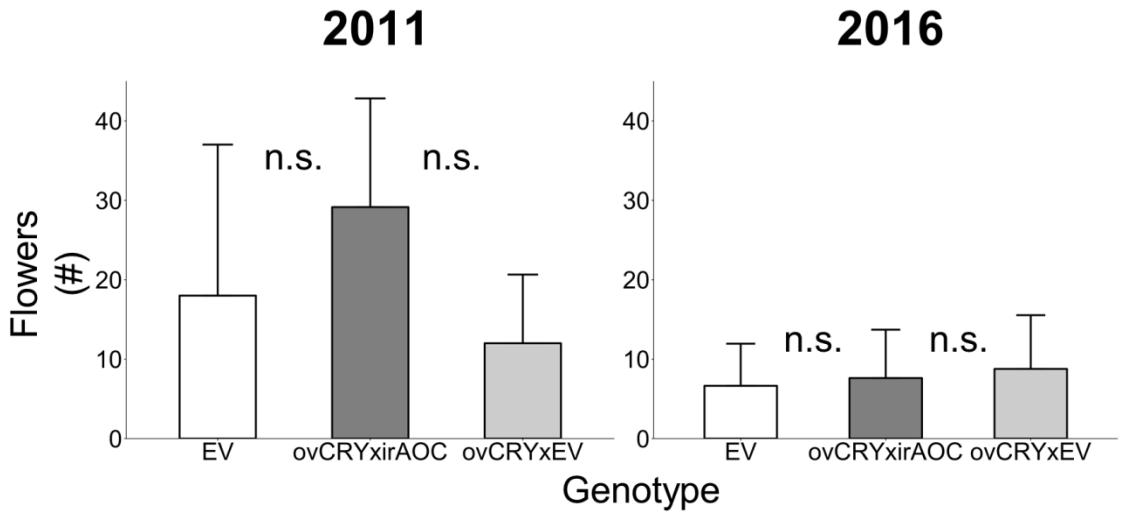

FIGURE S19 - Final flower production comparison of developmentally similar EV and ovCRY cross controls in 2011 and 2016 field seasons that show consistent final flower numbers. Error bars represent 95% confidence intervals. n.s. indicates no significant difference.

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CHAPTER **2**

2. Early developmental transitions and drought timing influence drought resistance strategies and confound the quantification of genotypic diversity in drought responses

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2.1 Abstract

Plant drought resistance is multifaceted, involving complex responses due to changing water conditions. These responses, classified broadly as drought resistance strategies, involve a variety of developmental changes and photosynthetic and hormonal responses. The wild tobacco *Nicotiana attenuata* harbors substantial variation in water-use traits that are typically used as proxies for drought resistance. However, even within one genotype, these traits can be significantly changed if drought treatments are not applied precisely, both in regard to synchronous onset-of-drought timing and the plant developmental stage at which the drought is applied. Here, we use a subset of a multi-parent advanced generation intercross (MAGIC) population of the ecological model species *N. attenuata* to investigate the extent to which the quantification of genetic variation in plant drought resistance strategies is convoluted when undergoing unsynchronized (by timing and developmental stage) versus synchronized drought events. We find that when using equal soil water availability to produce drought events that are synchronized by onset-of-drought timing, the variation in response traits is still more strongly linked to variation in early developmental transitions than to genetic variation. Interestingly, endogenous levels of abscisic acid were more strongly linked to genotypic variation than

variation in the either onset of drought or developmental stage, thereby providing the most accurate and convenient signature of drought responses of the physiological parameters measured.

2.2 Introduction

Deteriorating environmental conditions caused by anthropogenic climate change (Stocker *et al*. 2013) will continue to exacerbate drought events that can severely limit crop yields (Leng & Hall 2019). Drought responses are therefore of increasing interest for crop improvement initiatives (Avramova *et al*. 2015; Bac-Molenaar, Granier, Keurentjes & Vreugdenhil 2015; Obata *et al*. 2015). Despite the immediacy of the topic and increasing attention received, many studies investigating "drought tolerance" do not clearly define this trait (Montalvo-Hernández *et al*. 2008; Ahmad, Devonshire, Mohamed, Schultze & Maathuis 2015; Nemali *et al*. 2015; Arruda *et al*. 2018).

Here, we utilize the paradigm of drought resistance introduced by Ludlow and colleagues (Ludlow, Eds: Kreeb, Richter & Hinckley 1989) and further elaborated by Kooyers (Kooyers 2015), which partitions drought resistance adaptations into three alternative strategies. Briefly, drought escape (DE) involves the 'cashing out' of a plant's resources, curbing vegetative growth to favor reproductive output. In contrast, drought avoidance (DA) involves slowing production under water limitation to maintain leaf water levels and protect the photosynthetic machinery in anticipation of future opportunities for recovery. Finally, drought tolerance (DT) allows a plant to maintain production during a drought event, often through osmotic adjustments via the accumulation of sugars and other osmoprotectants. DT is most relevant for seeds and specific perennial or biannual species, such as resurrection plants (Ingram & Bartels 1996), but may be important in the protection of sensitive tissues such as the shoot apical meristem during severe drought events by protecting these tissues from reactive oxygen species (ROS) induced by osmotic stress (Campo *et al*. 2014; Wilson, Mixdorf, Berg & Haswell 2016; Lee 2018).

Abscisic acid (ABA) is one of the longest-known plant hormones, and the main regulator of drought responses (Daszkowska-Golec 2016). ABA-driven stomatal and photosynthetic responses to desiccation form a key component of DA but can vary among genotypes depending on differences in cellular conditions (McAdam, Sussmilch & Brodribb 2016; Sack, John & Buckley 2017). Mechanisms of DE intimately related to photoperiodic flowering have been elucidated, and are also tightly linked to ABA (Martin-Tryon, Kreps & Harmer 2007; Riboni, Galbiati, Tonelli & Conti 2013). However, a lack of standardized drought treatments across studies, coupled with variations in developmental phenotypes among mutants and knockdown lines, has confounded conclusions. For example, two studies of DE responses in Oryza sativa found drought stress to both delay (Galbiati *et al*. 2016) and promote (Du *et al*. 2018) early flowering. These divergent conclusions highlight the importance of employing consistent and refined drought stress treatments when studying the interaction between drought and drought response traits. Similarly, ecological studies have elucidated the functional differences between DE and DA for herbaceous plants (Franks, Sim & Weis 2007; Estiarte, Bernal, Estiarte & Peñuelas 2011; Franks 2011; Berger *et al*. 2014) but the lack of controlled drought conditions in these studies has hampered the validation of natural variation in drought resistance strategies and our understanding of the physiology (e.g. ABA responses) responsible for this apparent natural variation.

Nicotiana attenuata is an annual wild tobacco species native to western North America (Baldwin, Staszak-Kozinski & Davidson 1994; Baldwin 2001). The species' range is variable, characterized mostly by arid regions of cold and hot deserts. Throughout much of its range, covered by the Great Basin and Mojave deserts, most precipitation occurs in the winter, and conditions become increasingly dry as the growing season progresses (Zavala & Baldwin 2004). Although some studies have examined *N. attenuata*'s dehydration responses (Ré, Dezar, Chan, Baldwin & Bonaventure 2011; Hettenhausen, Baldwin & Wu 2012; Dinh, Baldwin & Galis 2013), only recently have more tightly controlled drought stress conditions been analyzed under field and glasshouse conditions (Valim *et al*. 2019). The substantial genetic and phenotypic variation in *N. attenuata* populations (Bahulikar, Stanculescu, Preston & Baldwin 2004; Li, Baldwin & Gaquerel 2015; Luu *et al*. 2017) also make this species valuable for studies exploring variation in drought resistance strategies. Additionally, the development of two mapping populations, including more recently a multi-parent advanced generation intercross (MAGIC) population of recombinant inbred lines for forward genetic approaches provide us with ideal tools for dissecting this phenotypic variation (Zhou *et al*. 2017; Ray, Li, Halitschke & Baldwin 2019). MAGIC populations allow for the exploration of genomic structure and for locating quantitative trait loci (QTL) of interest for gene discovery (Huang *et al*. 2015), and have already been used in a variety of crop species (Bandillo *et al*. 2013; Huynh *et al*. 2018; Stadlmeier, Hartl & Mohler 2018).

Here, we investigate the genetic variation in drought resistance strategies under conditions of equal and unequal water availability using a subset of recombinant inbred lines (RILs) from a MAGIC mapping population (Ray *et al*. 2019). We analyze a variety of response variables to soil water availability under glasshouse conditions and examine the role of developmental and onset-of-drought timing in the drought response strategies exhibited by individuals in this subset population. The main research questions for this study were:

1. Do watering regime and developmental stage affect the synchronicity of drought stage for different genotypes?
2. If so, does the variance in four commonly used metrics of drought response correspond to the genetic differences of these genotypes, or are genetic differences hidden behind variation due to asynchronous onset-of-drought or developmental stage?

Using a subset of RILs displaying a wide variation in drought responses, we found that both variance in the watering regimes (dry-down vs. controlled daily watering) used to produce a drought treatment and differences across developmental stages of tested plants increased the occurrence of unsynchronized onset-of-drought timings across genotypes. Only when both water availability and developmental stage were controlled for did genotypes enter drought synchronously. Inconsistent drought treatments also created substantial variation within genotypes in several drought response traits. We analyzed whether this variation could still be accounted for in some part by the genetic variation among RILs, but found that the variance in stomatal conductance and assimilation rates were more strongly linked to variation in early developmental transitions and secondly, to variation in onset-of-drought timing due to unsynchronized drought conditions. Of the investigated traits, we found that only ABA levels remained strongly linked to genotypic variation both early in drought and after a prolonged drought period, and hence provide a convenient hormonal signature of drought responses, even if experimental error cannot be avoided. Ultimately, we hope our results provide a strong impetus for a greater focus on synchronizing the drought timing and developmental stage of genotypes in order to evaluate their different drought response phenotypes.

2.3 Results

A synchronized drought treatment is required to compare drought responses among genotypes planted individually in a glasshouse

In order to compare the drought responses at the same sampling time in the glasshouse, plants must be exposed to the same degree of drought occurring in a synchronized manner for all pots; otherwise, two genotypes with different rates of water use will experience the onset of drought at different times (Figure 1). To test the effect of synchronized and un-synchronized drought, we compared two genotypes of *N. attenuata* (designated here as Genotypes X and Y for simplicity) known to have different rates of leaf water loss per day when grown in individual pots in the glasshouse (Table S1; Figure 2B). To conduct the synchronized treatment, we tracked water levels using a gravimetric approach and added water to each pot according to the plant's daily consumption. To achieve synchronized drought in four days, we added four times the previous day's consumption before terminating watering (Figure 2A).

After four days, all pots of each genotype reached 0g of water simultaneously, ensuring that all plants experienced synchronized drought and that a one-time sampling comparing physiological responses to equivalent drought events in each pot would be accurate (Figure 2A). In contrast, when we provided a standard amount of water to each pot, the pots of Genotype X and Y diverged by as much as 8.68g of water per pot within one day (Figure 2B, solid lines). Given this divergence, plants of this treatment would experience drought in an un-synchronized manner, with Genotype Y pots estimated to reach 0g of water in fewer days than those of Genotype X (Figure 2B, dashed lines). Single samplings for example on day 3 would result in a comparison between a genotype that had undergone one day of drought (Genotype X) and a one that had had several days of drought (Genotype Y) which could result in erroneous conclusions (Figure 2C). To compare two genotypes exposed to un-synchronized drought treatments, genotypes would need to be sampled on at least two different days (Figure 2B). Hence in order to meaningfully compare drought responses of different genotypes with one sampling, a synchronized drought event must be ensured.

Un-synchronized drought treatments confound the observation of physiological and ontogenetic drought responses, even within one genotype

To evaluate the extent to which un-synchronized drought treatments can convolute the evaluation of drought responses, we compared the physiological and ontogenetic drought responses of one genotype under different drying schemes and in various developmental stages. Plants of Genotype X either did not undergo a drought scenario, were given controlled amounts of water based on their rates of drying down that allowed them to reach 0g pot soil water availability in one day, or were supplemented with decreasing amounts of water to transition them to 0g pot soil water availability over four days (Figure 2D, left panel). Stomatal conductance values recorded on the day each treatment group reached 0g of water in their pots were significant from measurements of control plants (pooled from both samplings) for the one-day, but not the four-day, treatment group (Figure 2D, left panel). From these results we conclude that varied lengths of drying down can produce confounding results in physiological parameter measurements, even within one genotype.

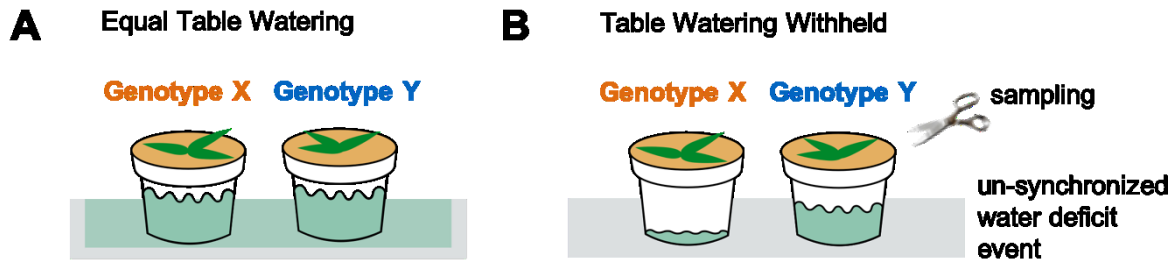

FIGURE 1 - Manipulating water availability in the glasshouse. (A) In the glasshouse, table watering applies soil water resources equally across pots planted with various genotypes. (B) When watering is withheld, differences in water-use between genotypes manifest in un-synchronized water deficit events: a plant with a faster rate of water use, Genotype X, will run out of water before Genotype Y. Simultaneous sampling in this case will erroneously compare a plant undergoing a water deficit event (Genotype X) with plant which is not (Genotype Y).

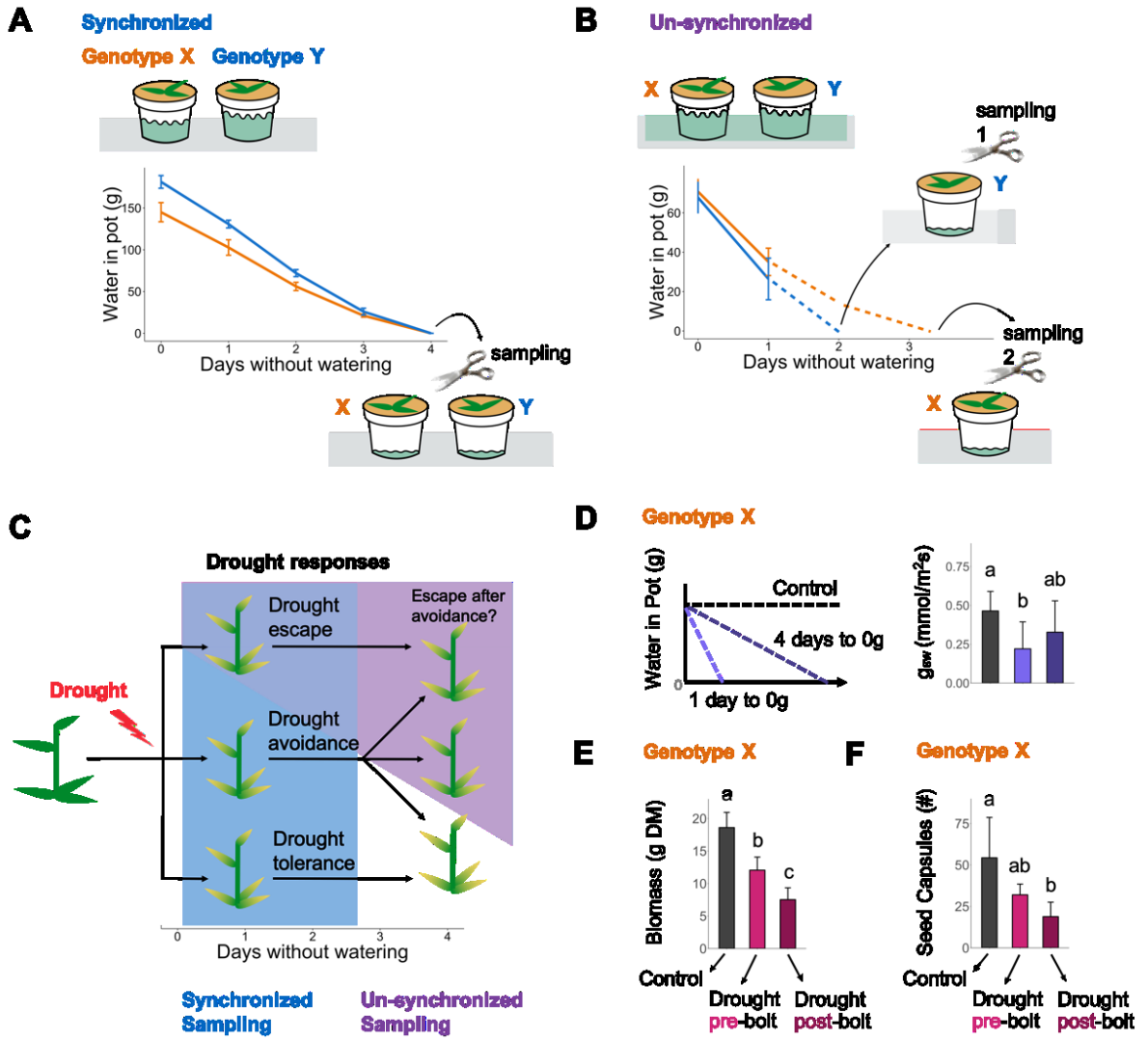

FIGURE 2 - Controlling water consumption per individual in the glasshouse allows for meaningful comparisons between genotypes under similar levels of water deficit stress. (A) By providing individual plants with an amount of water (mean ± CI; Table S1 for Genotype IDs) proportional to their daily consumption, plants will enter a water deficit event simultaneously. (B) Providing the same amount of water to plants with different rates of water use (solid lines, mean ± CI) will lead to un-synchronized water deficit stress (estimated here by dashed lines); unless plants are sampled on different days (Sampling 1 vs. 2), their drought responses will not be comparable. (C) Drought resistance strategies can be divided into three broad categories: drought escape, where a plant attempts to complete its reproductive cycle before drought becomes lethal; drought avoidance, where a plant attempts to decrease rates of water loss; and drought tolerance, where a plant attempts to withstand dehydration stress by osmotic adjustment. Un-synchronized drought treatments can lead to unexpected differences in the drought resistance strategies employed by two different genotypes, due to uneven lengths of water deficit events. (D) Un-synchronized sampling may result in large variations in drought response traits even within one genotype: stomatal conductance (gsw) of Genotype X (mean + CI) is presented without a drought treatment (Control, black) or with one of two drought treatments that either decreased its water availability to 0g in 1 day (light purple) or in 4 days (dark purple). (E) – (F) A drought treatment can significantly change the (E) biomass (mean + CI) and (F) seed capsules (mean + CI) production of plants in comparison to those that did not experience a drought (Control, black). Droughts were applied at different developmental stages (pre-bolt, pink; post-bolt, magenta) in order to test the influence of developmental stage on yield responses due to drought, even within the same genotype (Genotype X).

In a separate glasshouse experiment, Genotype X plants were treated with a controlled drought either before bolting (pre-bolt) or after bolting (post-bolt) and compared to counterparts that did not experience a drought treatment (Figure 2E-F). Both Genotype X plants which received a drought pre-bolt produced significantly lower amounts of biomass than control plants, but post-bolt Genotype X had significantly lower amounts than both its control and pre-bolt drought counterparts (Figure 2E). Concurrently, while Genotype X plants with a pre-bolt drought did not produce significantly different seed capsule numbers than control plants, post-bolt plants did have significantly lower amounts (Figure 2F). From these results we conclude that applying identical drought treatments at different developmental stages can have drastic effects on ontogenetic drought responses, even within one genotype.

Developmental control ensures simultaneous drought onsets in a synchronized drought treatment for genotypes with high developmental variance

To test the effect of developmental stage on the timing of drought onset for a synchronized drought treatment, we compared 30 of the previously described MAGIC-RILs, which differed in their bolting times (Figure S1) under different watering and developmental control regimes. One replicate of the 30 RILs underwent an un-synchronized drought treatment without developmental control (Figure 3A, "Un-sync"), a second replicate underwent a synchronized drought treatment without developmental control (Figure 3A, "Sync"), and a third replicate underwent a synchronized drought treatment with developmental control, where the synchronized drought treatment was only started at the same developmental stage for each plant: within four days of bolting (Figure 3A, "Dev"). The 30 RILs bolted in three distinct groups: early bolting ("Dev-Early"), medium bolting ("Dev-Mid") and late bolting ("Dev-Late"): synchronized droughts was applied within these groups.

As with the un-synchronized treatment for Genotype X and Y (Figure 2B), group Un-sync was watered to the same number of grams of water above their starting (dry) pot weight (Wg) on the day before watering was terminated (Figure 3B, Day 1, left panel). Similarly, as with the synchronized treatment for Genotype X and Y (Figure 2A), group Sync, Dev-Early, Dev-Mid, and Dev-Late had each genotype watered to a different Wg on their respective Day 1 (*Note:* Day 1 occurred three separate days post-germination, for Un-sync/Sync/Dev-Early at 17 days after potting, for Dev-Mid at 20 days after potting, and for Dev-Late at 24 days after potting due to developmental control), according to each pot's daily consumption (Figure 3B, center and right panel). Plants (Un-sync, brown) which started with the same amount of Wg on day 1, quickly diverged in their Wg values due to their varying rates of water use, and therefore the transition to drought (Wg < 0) occurred over three days (from day 4-6, red bar). Plants in the Synchronized treatment (Sync, beige) transitioned into drought over two days (from day 5-6, red bar); however, only when this treatment was paired with developmental control (Dev, blue) did plants transition into a water deficit event on the same day within each bolting group (red bars).

Developmental control groups (Dev-Early, -Mid, and -Late) entered drought on the same day after water termination (Figure 3B, left panel, light blue and blue), and thus samples from the Onset of Drought and Prolonged Drought samplings could be compared. Although Group Dev-Late entered drought one day later after water termination than the other two groups, the entire group entered drought in synchrony (Figure 3B, left panel, dark blue). A variance decomposition analysis for the Onset of Drought sampling was performed to statistically represent these observations (Table 1). When all five groups were included in an analysis of whether variance could be attributed between treatment groups (signifying that treatments

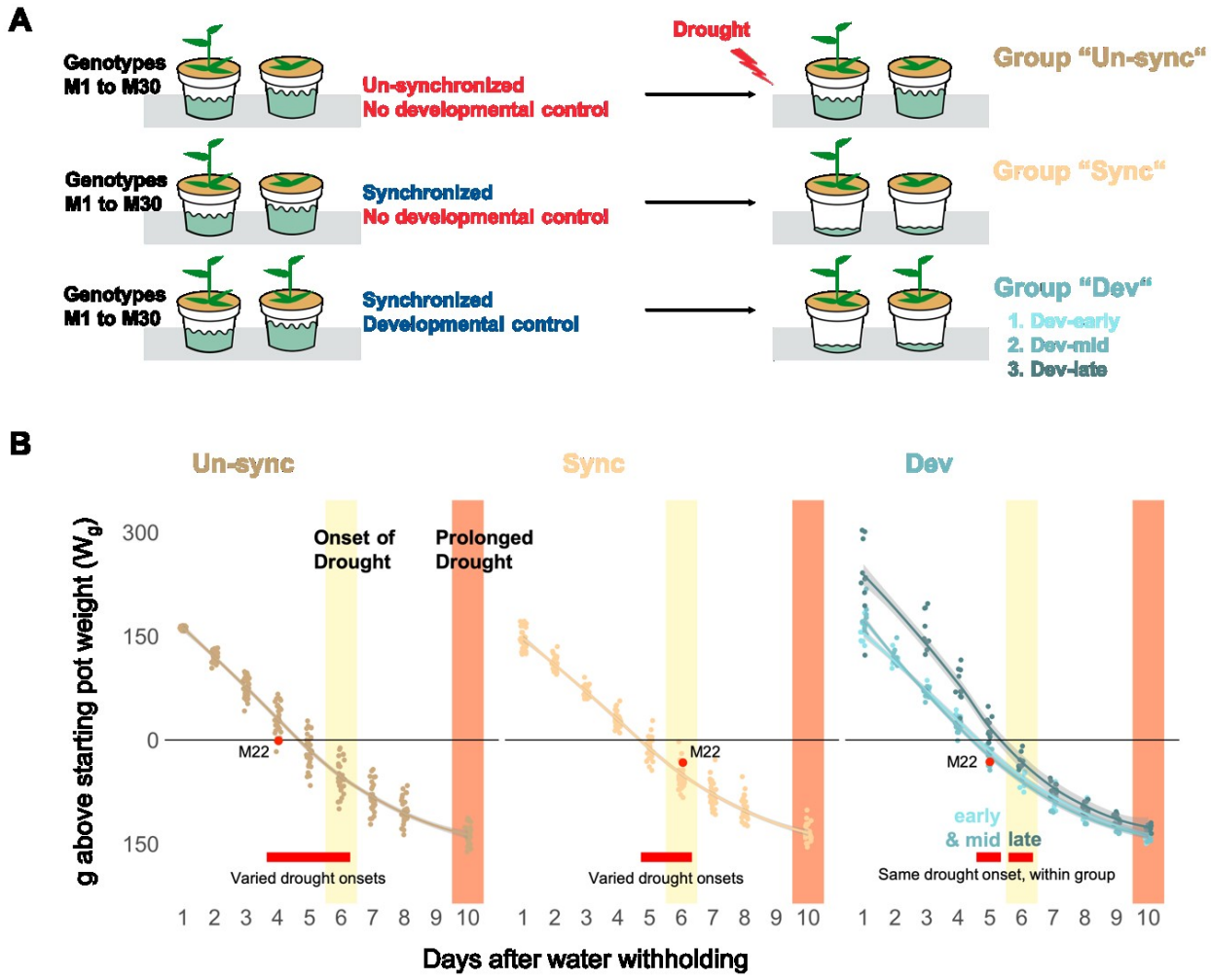

FIGURE 3 - Timing of water deficit stress in a genetically diverse population is less variable when controlling for water use and development. (A) A schematic representation of an experiment comparing 30 intraspecific lines with variable water use and developmental timing (Fig. S2): in Group "Un-sync" (brown), all genotypes were standardized at 40% soil moisture on the day before water withholding (Day 1); in Group "Sync" (beige), plants received water in proportion to their consumption rate on the day before water withholding (for individuals to have a "Synchronized" drought, or 0g above its starting pot weight, Wg, on day 6 of water withholding, 6x individual's daily water consumption rate was provided); in Group "Dev" (blue), plants received consumption-based watering as in Sync (for a Synchronized water deficit event) and were additionally split into three groups of early ("Dev-early", light blue), medium ("Dev-mid", medium blue), and late ("Dev-late", dark blue) bolting plants, ensuring that all plants in Dev underwent drought treatment at the same developmental transition (Developmental control). (B) Comparison of water loss between the three treatments shows that Un-synchronized plants (Un-sync, brown) began at the same amount of Wg on day 1 of treatment, quickly diverged in their Wg values due to their varying rates of water use, and therefore the transition to drought (Wg < 0) occurred over three days (from day 4-6, red bar). Synchronized treatment (Sync, beige) led to a transition to drought over two days (from day 5-6, red bar); however, only when this treatment was paired with developmental control (Dev, blue) did plants transition into a water deficit event on the same day within each bolting group (red bars). Red dots demonstrate the variable range of transition into drought for one line (M2-210) across all three treatments.

account for a larger portion of the variance) or within treatment groups (signifying a high within group variance of transition into drought, attributable e.g. to genetic variation), we found that only 17.5% of variance was explained between groups. An analysis performed on Dev groups revealed that 62.0% of variance was explained between groups. In comparison, when the

variance of just groups Un-sync and Sync was decomposed together, 0% of variance could be explained between groups. From these results we conclude that developmental timing can have a large impact on the application of synchronized drought treatments.

Developmental stage significantly explains variance in plant physiological responses to a drought treatment

To better quantify the importance of synchronized drought and developmental control for producing results reflecting differences among treatments and genotypes rather than differences in soil water availability and development, we used a variance decomposition approach, originally devised for addressing questions of inheritance in quantitative genetics, to uncover simplifying structures in large sets of variables (Fisher 1918; Cheverud & Routman 1995; Lütkepohl 2010; Álvarez-Castro & Yang 2011; Álvarez-Castro & Crujeiras 2019). We performed our analysis on all commonly measured physiological parameters, sampled after withholding water for 2 days (Control, C), 6 days (Onset of Drought sampling), and 10 days (Prolonged Drought sampling), of the 90 MAGIC plants (3 replicates, one in each of the 3 treatment groups in Figure 3). We visualized the results of each physiological parameter by RIL, where the vertical distance among the three replicates represents the variance created by the three different test groups (Figure 4A), either with unsynchronized drought and no developmental control, with synchronized drought and no developmental control, or with control on both (Figure 3A). Each replicate of each RIL was labeled according to the stage of drought, differentiated by the number of days it had been at 0 g of water at each sampling time (Figure 4B-E, left panels, 1-3 days for Onset of Drought; 5-7 days for Prolonged Drought, Drought Stage: DS) and according to the extent to which it had elongated its stalk in centimeters at the time of water termination (Figure 4B-E, center panels, Elongation Stage: ES).

Using repeatability to determine the percentage of variance explained by Drought Stage and Elongation Stage as random effects in our experimental design, and the significance level of these explained portions, we analyzed our data in two parts: one with the variance in results from Control to Onset of Drought and the other from Control to Prolonged Drought (Figure 4B-E, right panels). We found that differences among Elongation Stages significantly explained portions of the variance in all measured physiological parameters except for relative water content in the Onset of Drought sampling, and for all parameters in the Prolonged Drought sampling: relative water content (RWC, Figure 4B, Prolonged Drought: 5.0%, $p = 0.0343$), ABA content (ABA, Figure 4C, Onset of Drought: 5.8%, $p = 0.00213$; Prolonged Drought: 4.1%, $p = 0.0425$), assimilation rate (Figure 4D, Onset of Drought: 26.3%, $p = 3.12e-09$; Prolonged Drought: 19.0%, $p = 8.15e-07$) and stomatal conductance (gsw, Figure 4E, Onset of Drought: 13.3%, $p = 0.000378$; Prolonged Drought: 17.5%, $p = 0.000260$). Differences among Drought Stages only significantly explained portions of variance at the Onset of Drought ABA (Figure 4C, 34.4%, $p = 0.0128$), and under Prolonged Drought gsw (Figure 4E, 5%, $p = 0.0498$). From these results we conclude that developmental stage can significantly affect the results produced when measuring physiological responses to drought and daily variations in soil water availability mainly affect measurements of ABA in early drought stages and measurements of gsw after an extended drought period.

TABLE 1 - Variance decomposition analysis of Figure 3B

Groups	Percent variance explained:	Percent	Significance
Un-sync, Sync, Dev-Early, Dev-Mid, Dev-Late	Among groups	17.5%	0.101
	Within groups	82.5%	
Dev-Early, Dev-Mid, Dev-Late	Among groups	62.0%	0.00394
	Within groups	38.0%	
Un-sync, Sync	Between groups	0%	1
	Within groups	100%	

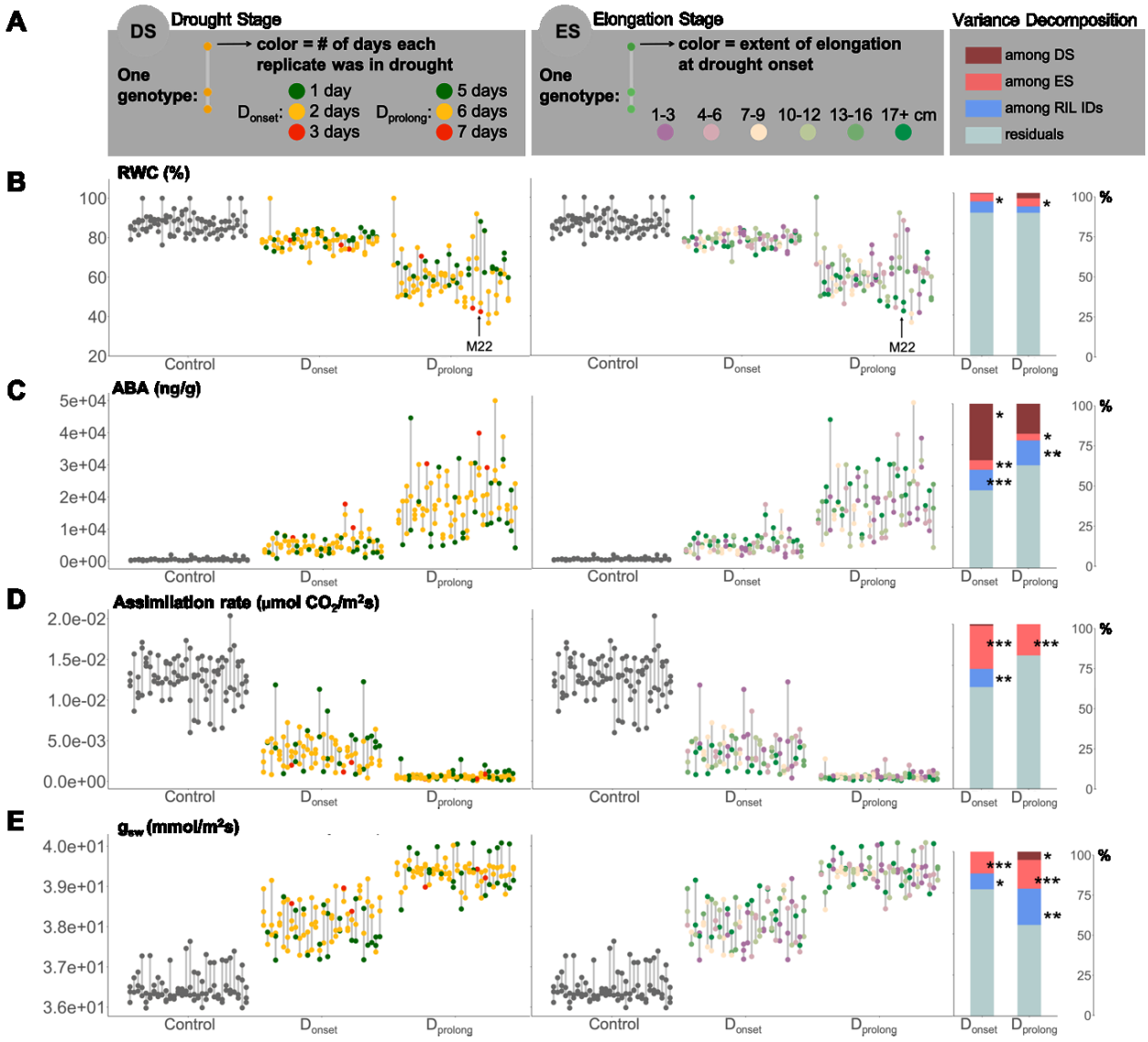

FIGURE 4 - Variance decomposition analysis of common drought response traits in a genetically-diverse population reveals the influence of water deficit stress timing and of development. (A) Common drought response traits were measured in 90 plants (30 genotypes in each watering group in Fig. 3B) of variable water status and developmental stage. Measurements occurred at three time points: before water deficit (Day 2 from Figure 3B; Control), at the expected onset of water deficit (Day 6 from Figure 3B; onset of drought, Donset) and after several days of water deficit stress (Day 10 from Figure 3B: prolonged drought, Dprolong). Left panel: variation in trait results are plotted by genotype (one genotype = one vertical bar), where colors indicate each individual's Drought Stage (DS, a representation of the number of drought days, or days when g above starting pot weight, Wg < 0, which each individual has experienced). Central panel: colors indicate each individual's Elongation Stage (ES, a representation of the developmental stage of each individual at the onset of drought). Right panel: variance decomposition was performed on the total variance of trait results, either from comparing Control and Onset of Drought measurements (Donset) or the Control and Prolonged Drought measurements (Dprolong). Total variance is divided into percentages of variance explained between DS, ES, or remaining in the residuals. Significances of the percentages are presented with asterisks: * = < 0.05, ** = < 0.01, *** = < 0.001. (B) Variance in relative water content (RWC) of leaves was least attributed to DS or ES. Line M2-210, emphasized as red dots across treatments in Fig. 3B, is marked here for reference. (C) Variance of abscisic acid (ABA) content of the leaves was strongly attributed both to DS and ES, while both that of photosynthetic rate (D) and stomatal conductance (E) were strongly attributed to DS in both Donset and Dprolong.

ABA levels best explain variance in RIL drought responses in both onset of drought and prolonged drought treatments

To assess the validity of using RWC, ABA, assimilation rate and gsw for evaluating drought resistant among genetically different populations, we investigated whether variance among RILs (RIL ID) could significantly explain differences in any of these parameters. RWC's variance was not significantly explained by differences among RILs at either the Onset of Drought or under Prolonged Drought (Figure 4B, right panel). ABA variance was both strongly and significantly explained by differences among RILs in both our Onset and Prolonged Drought variance decomposition analyses (Figure 4C, Onset of Drought: 12.5%, $p = 0.000249$; Prolonged Drought: 15.1%, $p = 0.001320$). Variance in assimilation rate was only significantly explained by differences among RILs at the Onset of Drought (Figure 4D, 11%, $p = 0.00506$). Variance in gsw was significantly explained by differences among RILs by only a marginal amount at the Onset of Drought, but was strongly explained by RIL differences under Prolonged Drought (Figure 4E, Onset of Drought: 9.6%, $p = 0.03380$; Prolonged Drought: 22.1%, $p = 0.00379$). From these results we conclude that ABA was the only physiological parameter whose variance could be strongly explained by differences among RILs at both sampling times, at the Onset of Drought and after a Prolonged Drought treatment.

2.4 Discussion

In this study, we evaluated whether drought experimental designs can influence the timing of onset of drought for different genotypes, and whether this difference is timing can cause differences in the interpretability of experimental results. We find that both the watering regime applied and the developmental stage at the onset of drought have strong influences on the timing of the onset of drought for different genotypes, defined here as 0 g of water available in the pot (Wg). We found that tracking water loss per day of individual pots and providing water to each pot according to the plant's daily consumption allowed us to achieve a synchronized drought among genotypes with differing water-use traits (Wg = 0 for all genotypes on the same day, Figure 2A). Further experiments utilizing a subset of a MAGIC-RIL population (Figure S1) revealed that developmental timing can also impact the application of synchronized drought treatments (Figure 3B), and influence most major physiological responses to drought by producing elongation-related variances among samples (Figure 4B-E, right panels). Finally, we observed that ABA was the only physiological parameter whose variance could be strongly explained by differences among RILs (i.e. genetic variation) at both sampling times, at the Onset of Drought and after a Prolonged Drought treatment, and thus provides the most convenient hormonal signature for natural variation in drought responses among the measured physiological variables: relative water content, ABA concentration, assimilation rate, and stomatal conductance (gsw).

We observed that developmental stage had a significant effect on the observed variance of all measured drought response traits at both onset and under prolonged drought (Figure 4B-E). The results of the variance decomposition analysis suggested that developmental stage can significantly affect the results produced when measuring most major plant physiological responses to drought by producing elongation-related variances among samples, but daily variations in soil water availability mainly affect measurements of ABA in early drought stages (Onset of Drought) and measurements of gsw after an extended drought period (Prolonged Drought). Given the large effects observed for individual genotypes between synchronized and un-synchronized watering (Figure 2D), this suggests that controlling for developmental timing may be even more critical when comparing traits associated with drought resistance strategies

across genetically-diverse populations. In order to ensure synchronized drought treatments, termination of watering should be applied to groups of genotypes at the same developmental stage when developmental variation is not a primary factor of interest. However, adjustments may have to be made for plants with late transitions in development (e.g. late bolting) to ensure that their developmental group enters drought on the same day as other developmental groups.

Of the drought response parameters measured, only variance in ABA levels significantly explained differences among RILs (Figure 4C, right panel). These findings indicate that ABA may serve as a genetically-linked response to drought under the right conditions. More work in mapping populations under both field and synchronized watering conditions need to be conducted to further explore the suitability of variation in ABA levels as a marker of drought responses, and to better understand the link between ABA responses and markers of different drought tolerance strategies.

The results reported here are important, given that a large number of studies in the literature not only utilize uncontrolled dry down experimental designs that are applied equally across genotypes, sometimes with known developmental phenotypes, but furthermore utilize measures of drought status that are more strongly affected by variations in the water availability of the individual plant than by genotypic variation. Given the variation in responses to drought between drought escape, drought avoidance, and drought tolerance, which can be elicited by asynchronous drought treatments within the same genotype, it is critical to define not only the wild-type responses in any study system but also to distinguish between low water availability scenarios, which may promote differential water status across genotypes, and true drought responses in plants that reach 0g of water in synchrony. Although there is a high degree of sophistication in the molecular techniques applied in mechanistic drought studies, we propose that more care should be taken to ensure that treatments are applied uniformly in accordance with the desired response to be observed.

2.5 Materials and Methods

Plant materials and MAGIC line selection

All genotypes used in this study are listed in Table S1. Both transgenic lines were derived from the UT-WT genotype, with seeds originally collected from natural populations of *N. attenuata* from the Desert Inn Ranch near Santa Clara, UT, USA (Baldwin *et al*. 1994). Screening of the EV line ("Genotype X," pSOL3NC, line number A-04-266-3) is described by Bubner and colleagues (2006), and screening of the irMPK4 line ("Genotype Y," pRESC5MPK4, line number A-7-163) is described by Hettenhausen and colleagues (2012). Recombinant inbred lines (RILs) from the multi-parent advanced generation intercross (MAGIC) population were generated as described by Ray and colleagues (2019).

The relative water loss of each line used in our study is presented in Table S1. For irMPK4 (Genotype Y), the relative water loss represents rate of transpiration (mmol H20/m2s) in comparison to EV (Genotype X). For the MAGIC-RILs, the relative water loss represents the inverse of the soil moisture (%) of the MAGIC line, a day after receiving a standardized amount of water, in comparison to the UT-WT genotype. These relative water losses were initially used to select the 30 MAGIC lines for use in this study; the resulting lines span the entire range of the phenotype found across a 650-plant population of the MAGIC-RILs in a previous screening. A visual representation of the soil moistures used to calculate the relative water losses of the MAGIC lines is provided in Figure S1A.

Additionally, the 30 MAGIC lines used here were selected for their diversity in the timing of developmental transitions from rosette growth to bolting in a previous screening of the larger MAGIC population. We initially selected lines from two ranges of bolting times: plants that bolted 7.5-10 days and 12.5-14 days before the entire population had bolted (Figure S1A). In this study (Figures 3B, 4), the 30 selected MAGIC lines displayed bolting times that could be clearly separated into three distinct bolting groups of 10 plants each (Figure S1C). This variation was used to control for variation in developmental timing (Figures 3B, 4).

Plant growth and measurements

Seed germination and plant growth in the glasshouse were carried out as described by Krügel and colleagues (Krügel, Lim, Gase, Halitschke & Baldwin 2002) and was undertaken in a glasshouse in Jena, Germany. Soil moisture measurements were taken using a WET-2 soil moisture sensor connected to an HH2 moisture meter (Delta-T Devices, Cambridge, UK) using the standard Organic soil calibration provided by the manufacturer.

Relative water content was calculated as follows: cut leaves were weighed immediately to obtain leaf fresh mass (FM), and placed in individual containers filled with distilled water, abaxial side down for 2 h, after which turgid mass (TM) were obtained. Leaves were subsequently dried in a drying oven for 6 h to obtain dry mass. Relative water content was then calculated using the following equation (Turner, 1981):

$$RWC = (FM - DM / TM - DM) \times 100$$

Assimilation rates and stomatal conductance (gsw, by calculation) were obtained through gas exchange measurements using a LI-COR 6400XT infrared gas analyzer (Lincoln, Nebraska, USA), between 12:00 and 14:00. Biomass was recorded as the fresh shoot mass of each MAGIC line, 70 days post germination (dpg). Fitness correlates were the total count of unripe and ripe seed capsules on each MAGIC plant, 70 dpg.

Sampling was performed at three time points (Control: 2 days, Onset of Drought: 6 days and Prolonged Drought: 10 days after water was withheld) during the experiment, in addition to a final sampling at 70 dpg. Three treatment groups corresponding to an un-synchronized watering regime (Un-sync), a synchronized watering regime (Sync) and control of developmental timing combined with a synchronized watering regime (Dev)) received different levels of watering and developmental control to undergo the ten-day drought treatment. To control for variations in developmental timing, treatment group "Dev" was separated into three subgroups (Dev-Early, Dev-Mid, Dev-Late) according to the bolting times (defined as the first appearance of stem formation from the base rosette) of the 30 selected RILs, and the three samplings of treatment groups Dev-Mid and Dev-Late occurred 3 and 7 days after the other treatment groups (Un-sync, Sync, and Dev-Early). After the drought treatment plants were grown under standard watering conditions until 70 dpg.

ABA extraction and quantification

Abscisic acid (ABA) analysis of leaf material was performed on a UPLC-MS/MS (EvoQ Elite Triple quad-MS; BRUKER DALTONIK GmbH, Bremen, Germany) after extraction in pre-cooled 80% MeOH as described previously (Schäfer, Brütting, Baldwin & Kallenbach 2016; McGale, Diezel, Schuman & Baldwin 2018).

Statistical analysis through variance decomposition

All data were analyzed using R version 3.5.3 (R Core Team 2018) and RStudio version 1.1.463 (Rstudio Team 2016). Variance decomposition analyses were performed through repeatability estimation using the rpt function (rptR package, Stoffel *et al*., 2017). For Figure 3B, the linear mixed effects model for which the repeatability and significance of explained variance analysis was performed was:

lmer(WaterLeft ~ 1 + (1|Group), data = D1)

The variable WaterLeft represents the g of water remaining in the pot, above its starting pot weight (Wg). The only random effect is Group, and it represents treatment groups 1, 2 and 3.1-3 (Figure 3A, see Plant growth and measurements). Data D1 contained all three groups, and the rpt function performed on this Gaussian data estimated the percent of variance explained between groups (Group) versus within groups (residuals). Two additional models with the same structure were run with different data: D1_3 had only groups 3.1-3, and data D1_1_2 had only groups 1 and 2. The analysis was only performed on data collected at the D1 sampling timepoint (Figure 3B, see Plant growth and measurements). The results are presented in Table 1.

For the right panels of Figure 4B-E, the linear mixed effects model for which the repeatability and significance of explained variance analysis was performed was:

lmer(Variable ~ Day + (1|DroughtDays) + (1|ElongationGrp2) + (1|RIL_ID), data = D)

Variable represents either RWC, ABA, assimilation rates, or gsw. D represents either the D1 or the D2 data corresponding to each of the aforementioned variables, across all treatment groups (Figure 3). Day is the only fixed effect, and represents the day of sampling: either C, D1 or D2. DroughtDays is the number of days that each RIL had actually been in drought at the D1 and D2 timepoints. ElongationGrp2 is the range within which the stalk height values occured at D1 or D2. RIL is the ID of the RIL, for which there were three data points which had been tested in each of the three different treatment groups (Figure 3). The variance created by these three test groups was attributed to the random effects either of the RIL ID, to its extent of elongation, or to the number of days it had been in drought. The data followed a Gaussian distribution. The results are displayed in the right panels of Figure 4B-E.

2.6 Acknowledgements

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2.7 Supplemental Figures

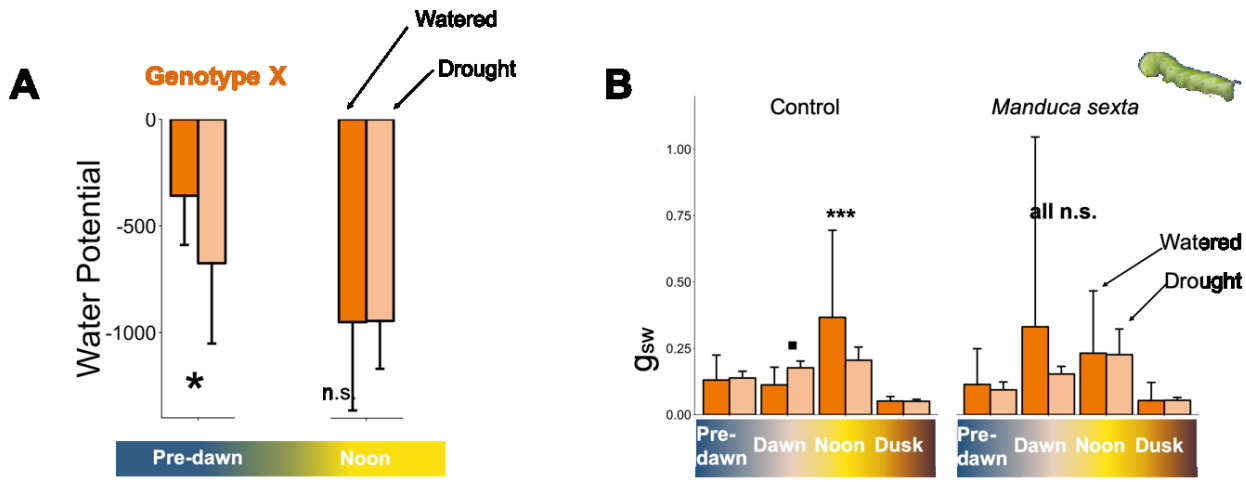

FIGURE S1 - Confounding factors of drought response traits under field conditions. (A) Leaf water potential is well-known to vary diurnally, with water deficit stress being apparent at pre-dawn but not at noon. (B) Stomatal conductance also varies diurnally, with drought responses being most apparent at noon; however, herbivory occurring either naturally or through treatment in the field may confound drought responses.

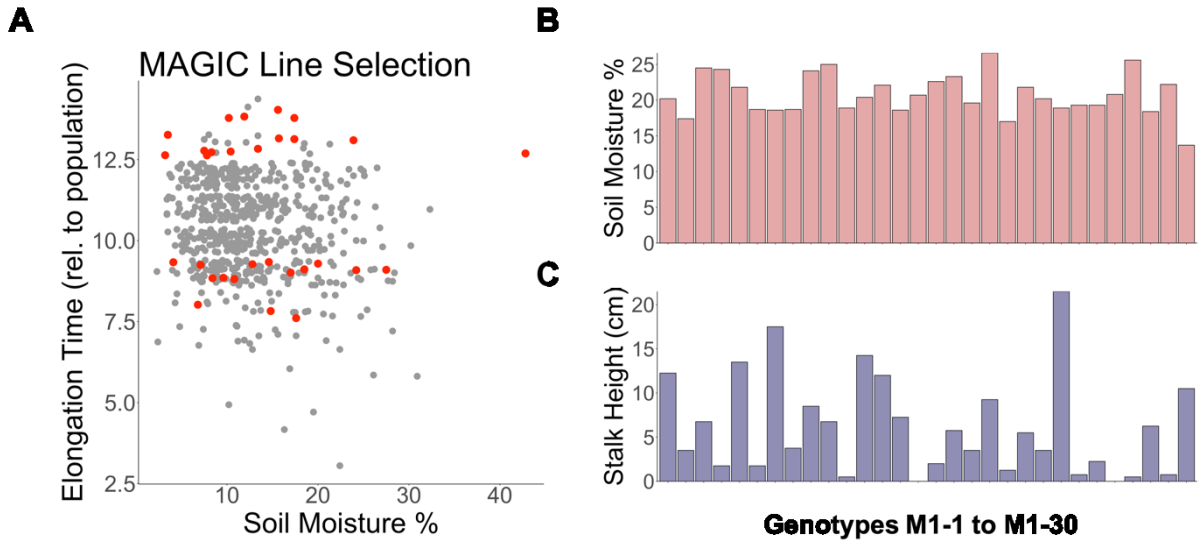

FIGURE S2 - Selection of MAGIC-RIL lines for drought experiment. (A) 30 lines from a large-scale glasshouse screening of 650 MAGIC-RILs were chosen based on their variation across two traits, Soil moisture % and elongation rate. (B) Soil moisture of 30 MAGIC-RILs one day after standardizing soil moisture to 40%. (C) Elongation rate of the 30 MAGIC-RILs before drought treatment, 19 days post-potting.

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CHAPTER **3**

3. Determining the scale at which variation in water-use traits and AMF associations change population yields

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3.1 Abstract

Plant trait diversity is known to influence population yield, but the scale at which this happens remains unknown: divergent individuals might change yields of immediate neighbors (neighbor scale) or of plants across a population (population scale). We use *Nicotiana attenuata* plants silenced in mitogen-activated protein kinase 4 (irMPK4) – with low water-use efficiency – to study the scale at which water-use traits alter intraspecific population yields. In the field and glasshouse, we observed overyielding in populations with low percentages of irMPK4 plants. Paired-plant experiments demonstrated that overyielding emerges at the population scale, unrelated to irMPK4's water-use phenotype. Experimentally altering arbuscular mycorrhizal fungal associations by silencing the Sym-pathway gene NaCCaMK did not affect reproductive overyielding in field-grown plants. Additional micro-grafting experiments revealed that shoot-expressed MPK4 is required to change yield in response to a neighbor, suggesting

that the variation in aboveground MPK4 expression, at the population scale, was responsible for the observed overyielding.

3.2 Introduction

Plant trait diversity is known to increase the productivity and stability of plant populations (Cardinale *et al*., 2012; Isbell *et al*., 2017, 2015; Liang *et al*., 2016; Loreau and de Mazancourt, 2013). Recently, forward genetics research has identified a genetic locus associated with this diversity-productivity relationship (Wuest and Niklaus, 2018). The function of this identified locus has not yet been investigated, likely due to the complexity of plant population experiments. When individuals varying in traits or in loci of interest are planted in pairs, it is possible to conclude that their responses are due to the neighbor (Gibson *et al*., 1999; Harper, 2010). When planted in populations, however, it is unclear whether a plant responds only to properties of its direct neighbors or of the entire population (Gibson *et al*., 1999; Radosevich, 1987). Thus, the scale, or the hierarchical level in biological organization, at which these effects are constrained (Allen and Starr, 1982) has not yet been determined, despite their importance in the study of complex population interactions (Schneider, 2001). Here we define and investigate two spatial scales within populations that can be responsible for changes in total yield (Figure 1): the neighbor scale, where responding individuals (RIs) are constrained to change their growth and yield (quantified in biomass and fitness correlates, i.e. flowers and seed capsules) only in response to direct neighbors that differ in trait expression (divergent plants); and the population scale, where RIs can respond to the total composition of divergent plants in the entire population, creating a change in total population yield in direct proportion to RI abundance (Crawford and Rudgers, 2012; Hughes *et al*., 2008; Smith and Knapp, 2003).

Water-use traits are known to result in changes in total population yield (Caldeira *et al*., 2001; Comas *et al*., 2013; Forrester, 2015; Kimball *et al*., 2014; Marguerit *et al*., 2014; Wang *et al*., 2016; Wu *et al*., 2016), however, the scale at which this occurs (neighbor or population) remains unknown. WUE naturally varies among individuals, both within and among species (Anderegg, 2015; Donovan *et al*., 2007; Heschel *et al*., 2002; Tortosa *et al*., 2016; Yoo *et al*., 2009) and intraspecific variation in WUE traits can be as great as interspecific variation (Messier *et al*., 2010). Yield effects resulting from intraspecific WUE trait variation are of considerable agricultural interest (Dutra *et al*., 2018; Sreeman *et al*., 2018). Interestingly, WUE traits of some trees species alters the photosynthetic parameters and survival of neighboring trees (Bunce *et al*., 1977), suggesting potential neighbor-scale responses that can dramatically influence the yield of populations. However, studies have not pursued how WUE trait variation cause either population-scale or neighbor-scale responses that are responsible for changes in population growth and yield due to the complications that emerge in studying variations in WUE phenotypes.

To adequately study the scale at which RIs respond to variation in WUE of neighbors, one needs to anticipate several factors that would confound the analysis. WUE is calculated as the ratio of carbon assimilation to transpirational water loss, and WUE phenotypes typically result from altered stomatal function that increases plant transpiration. As the frequency of plants with low WUE (high transpiration) increases in a population, the availability of soil water to the population is known to decrease proportionally (Zea-Cabrera *et al*., 2006). RIs may change their growth and yield in response to differences in soil water availability, rather than to the abundance of low-WUE plants in a population. Therefore, controlling for soil water availability independently of the frequency of plants with different WUE traits is

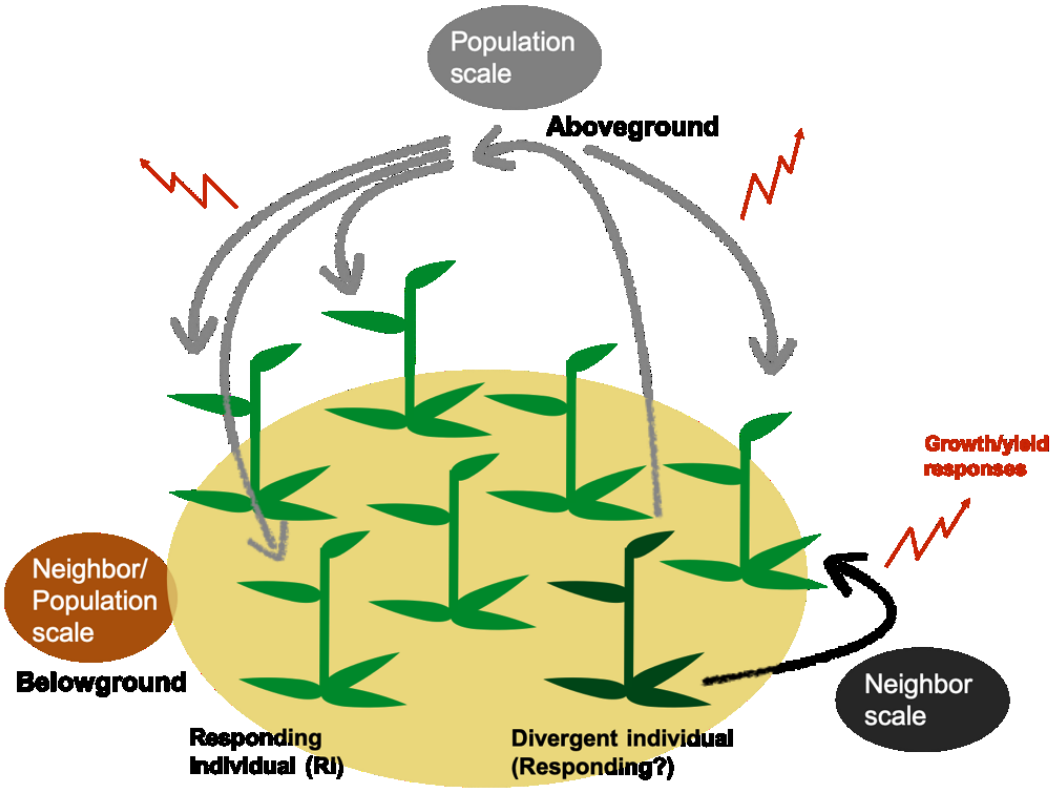

FIGURE 1 - Genetically divergent plants can alter population yields through plant neighbor responses generated at neighbor or population scales. Genetic variation within a population can change population growth and yield by altering individuals' outputs either in localized areas within populations (neighbor scale) or across all plants of a population (population scale). At the neighbor scale (black), a divergent individual (dark green) may elicit responses only in immediate neighbors (responding individuals, RIs). RIs' responses at either spatial scale may include changes in growth and yield, which can cumulatively change a population's growth and yield (red). Responses to divergent individuals could be caused by above- (black, gray) and/or belowground interactions (brown) among plants in population.

essential for the analysis. The ecological relevance of variation in WUE traits is best evaluated in field populations, however standardizing water availability across populations in the field is rarely possible and thus combining inferences from field and glasshouse experiments, where soil water availability can be controlled using gravimetrically controlled watering, provides a useful way forward. Plants that vary in WUE as a result of single-gene manipulations greatly facilitate investigations into the scales at which yield responses are realized in populations. Here we use isogenic plants, silenced in the expression of a single gene that profoundly influences stomatal behavior, to explore the scale at which WUE variation influences population yields.

Mitogen-activated protein kinases (MAPKs) are part of a conserved signaling cascade essential in eukaryotes. The downstream targets of this phosphorylation cascade, such as transcription factors, enable specific plant responses through changes in plant growth and development (Xu and Zhang, 2015). Mitogen-activated protein kinase 4 (MPK4) in *Nicotiana attenuata* and its homologues, MPK12 in *Arabidopsis thaliana* and MPK4/MPK4L in *N. tabacum*, have been implicated in responses to herbivore damage (Gomi *et al*., 2005; Hettenhausen *et al*., 2013; Yanagawa *et al*., 2016), bacterial inoculation (Hettenhausen *et al*., 2012), changes in exogenous and endogenous abscisic acid (ABA) and hydrogen peroxide levels (Des Marais *et al*., 2014; Hettenhausen *et al*., 2012; Jammes *et al*., 2009), vapor pressure deficits (Des Marais *et al*.,

2014) and ozone levels (Gomi *et al*., 2005; Yanagawa *et al*., 2016). Most of these responses involve the regulation of stomatal structure and function: silencing NaMPK4 or NtMPK4/L by RNA interference (Na-irMPK4 and Nt-MPK4/L-IR, respectively) or knocking out AtMPK12 (At-mpk12) results in plants with larger stomata and stomatal apertures, and varying disruptions in stomatal closure (Des Marais *et al*., 2014; Gomi *et al*., 2005; Hettenhausen *et al*., 2012; Marten *et al*., 2008; Yanagawa *et al*., 2016).

The alteration of stomatal phenotypes by MPK4/12 expression strongly influences WUE. Na-irMPK4, Nt-MPK4/L-IR and At-mpk12 all have increased transpiration rates which can be attributed to increased stomatal conductance (Des Marais *et al*., 2014; Gomi *et al*., 2005; Hettenhausen *et al*., 2012; Yanagawa *et al*., 2016). For Na-irMPK4 and At-mpk12, this increase in transpiration rates has been shown to dwarf the associated increases in assimilation rates, resulting in low WUE (Des Marais *et al*., 2014; Hettenhausen *et al*., 2012). However, previous glasshouse studies that tested whether the presence of MPK4/12-derived WUE phenotypes results in individual growth and yield effects in paired-plant-in-a-pot interactions did not control for soil water availability (Des Marais *et al*., 2014; Hettenhausen *et al*., 2012). To our knowledge, no study has investigated whether variation in the abundance of a low WUE trait, generated from the silencing of a single gene, affects population yield; similarly unstudied is the scale at which this might occur. Here, we conduct such a study using experimental *N. attenuata* populations in both the glasshouse and field.

The wild tobacco *N. attenuata* grows in xeric habitats in the western United States, where water limitation and WUE are selective factors throughout the growing season. *N. attenuata* typically grows in genetically diverse populations, in near-monocultures (Baldwin and Morse, 1994; Baldwin *et al*., 1994), and is known to respond differently when paired with genetically-varied intraspecific neighbors: a *N. attenuata* accession collected in Utah (UT) sharing a pot with an accession collected in Arizona (AZ) produces significantly smaller stalks than when sharing a pot with another UT plant (Glawe *et al*., 2003). Additionally, when UT plants are grown in mono- versus mixed-cultures with isogenic lines having genetically modified defense responses, UT plants may suffer less canopy damage from attack by the specialist herbivore Tupiocoris notatus (Adam *et al*., 2018) or more frequent attack by the stem-boring specialist Trichobaris mucorea (Schuman *et al*., 2015).

Nicotiana attenuata plants naturally interact with AMF in the field, establishing networks of connected plants that have the potential to significantly change individuals' responses to neighbors or populations with different WUE phenotypes. AMF are known to change soil water availability and transport among individuals in populations based on each individual's ability to interact with AMF (Egerton-Warburton *et al*., 2007; Reynolds *et al*., 2003; Yang *et al*., 2013). Additionally, AMF have been shown to significantly affect inter-plant interactions in populations, as they provide another medium for inter-plant transfers of nutrients, defense signals and allelopathic chemicals (Ferlian *et al*., 2018; Gorzelak *et al*., 2015; Song *et al*., 2019). Manipulating the ability of a population to interact through an AMF network can provide a means of dramatically altering belowground interactions and narrow the potential causes of population yield changes to either above- or belowground effects (Figure 1). Calcium and calmodulin-dependent kinase (CCaMK) is required for successful plant symbiosis with AMF (Lévy *et al*., 2004), and the abrogation of CCaMK expression provides a valuable tool to disconnect plants from AMF networks in the field (Groten *et al*., 2015). Field plantations of transgenic *N. attenuata* crossed with CCaMK-deficient transgenic lines (irCCaMK) in the plant's native habitat, the Great Basin Desert, allow for the study of population growth and yield effects resulting from trait variation, as well as the scales and tissues in which this

variation occurs. Here, we use a single gene manipulation to create variation in WUE in the background of irCCaMK lines to separate the effects of AMF-mediated interactions in our analysis of the spatial scales at which variation in WUE traits influence population yields.

We investigated the spatial scales at which variation in abundance of low WUE *N. attenuata* plants, generated by the abrogation of MPK4 expression, change total population growth and yield. We used a previously characterized irMPK4 line (Hettenhausen *et al*., 2012) and varied the percentages of this line in field populations with empty-vector (EV) control plants, crossed both with either irCCaMK and EV lines, to manipulate connectivity to the AMF network. We observed increased yields, referred to as overyielding, in populations with low percentages of MPK4-deficient plants ('low-irMPK4'), due primarily to increases in EV plant yield. To exclude soil water availability effects, we grew homozygous irMPK4 and EV lines in glasshouse populations under equal water availability and again observed overyielding in low-irMPK4 populations due to increases in EV yield. We further tested responses at the neighbor scale by growing single plants and mono- and mixed-genotype pairs of EV and irMPK4 under conditions of equal water availability and found no changes in growth or yield between pair types. We analyzed the yield of individuals with different configurations of immediate neighbor genotypes in our glasshouse populations, but these also did not explain changes in individual yields. From these results we conclude that neighbor-scale responses are unlikely to be responsible for the overyielding phenomena. The observation that EV plants change their growth and yield in response to neighbors based on plants grown in single and paired pots, while irMPK4 plants do not, was consistent with the field and glasshouse observations that irMPK4 plants are not RIs. In the glasshouse, changes in EV plants' photosynthetic parameters did not explain the yield increases in low-irMPK4 populations and importantly, EV and irMPK4 plants did not differ in their WUE phenotypes in field populations. Therefore we inferred that irMPK4's WUE phenotype was not responsible for the observed overyielding at the population scale. In experiments with EV shoots micro-grafted to irMPK4 roots, as well as in AMF-connected field populations, MPK4 deficiency in roots was found not to be responsible for the overyielding phenomena. From these results, we infer a novel function of MPK4 in the shoot in mediating *N. attenuata*'s yield response to neighbors, which in low-irMPK4 populations results in reproductive overyielding that results from population-scale interactions.

3.3 Results

irMPK4 x irCCaMK crosses are silenced in MPK4 and abrogated in AMF associations

N. attenuata plants silenced in the expression of MPK4 (irMPK4) have a low water-use efficiency (WUE) phenotype in comparison to empty-vector (EV) control plants in the glasshouse (Figure 2A). The loss of stomatal control increases transpiration rates to levels that surpass the increases in assimilation rates, consequently decreasing WUE, calculated as the ratio of assimilation:transpiration rates (Hettenhausen *et al*., 2012).

Populations of plants growing in the field are commonly interconnected by arbuscular mycorrhizal fungal (AMF) networks that are known to influence access to water and nutrients in the plant rhizosphere (Egerton-Warburton *et al*., 2007; Reynolds *et al*., 2003; Yang *et al*.,

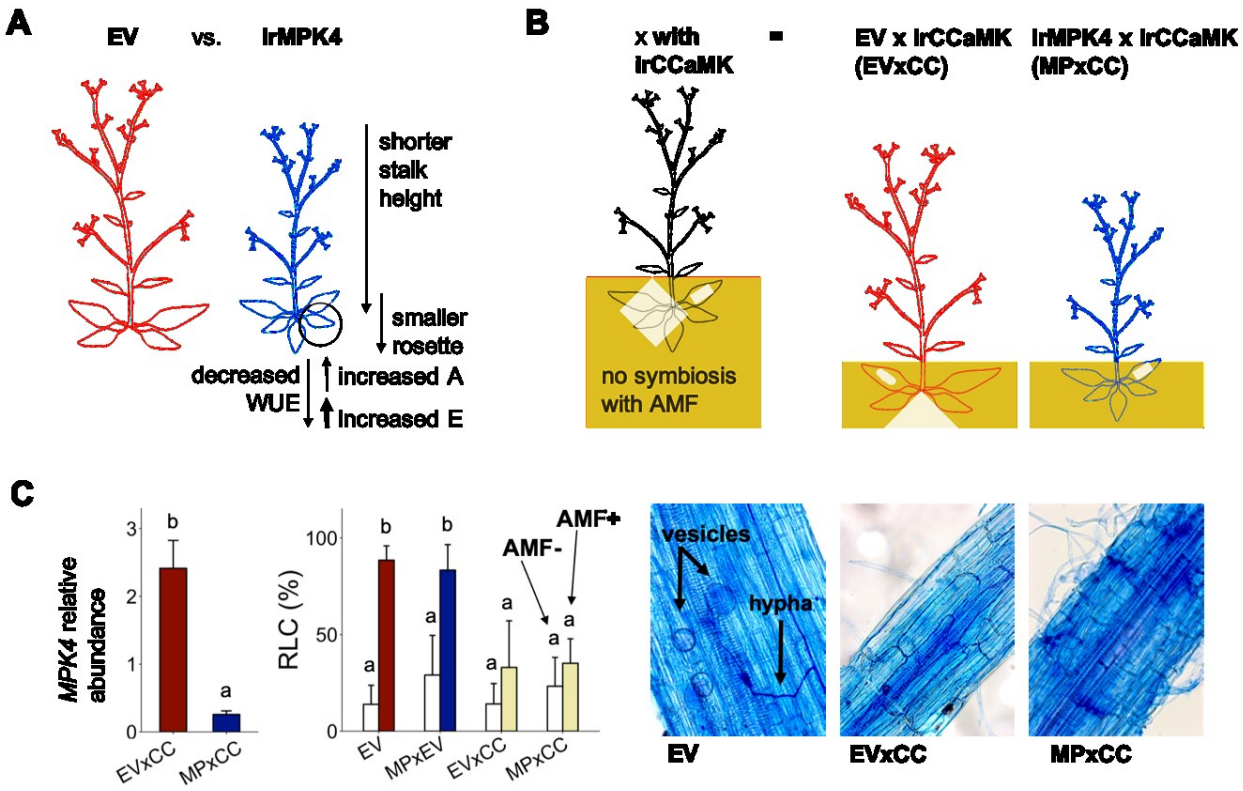

FIGURE 2 - Characterization of EV, irMPK4, irMPK4xEV, EV x irCCaMK and irMPK4 x irCCaMK plants. (A) A schematic summary of the findings of Hettenhausen and colleagues (2012): irMPK4 *Nicotiana attenuata* plants, silenced in mitogen-activated protein kinase 4, have disrupted stomatal control resulting in increased rates of leaf transpiration (E) which surpass the smaller increases in rates of leaf carbon assimilation (A) and therefore decrease water-use efficiency (WUE) in comparison to empty-vector (EV) plants. irMPK4 stalks and rosettes are smaller than those of EV. (B) A schematic demonstrating a method established by Groten and colleagues (2015) to control arbuscular mycorrhizal association in the field: irCCaMK *N. attenuata* plants, silenced in calcium and calmodulin-dependent protein kinase, are crossed with EV and irMPK4 to create EV x irCCaMK (EVxCC) and irMPK4 x irCCaMK (MPxCC) lines hemizygous for each of the transgenes and are not able to associate with arbuscular mycorrhizal fungi (AMF). (C) Mitogen-activated protein kinase 4 (*MPK4*) transcript abundances, calculated relative to a housekeeping gene, in hemizygous MPxCC and EVxCC plants (left panel, mean + CI, n = 9 for EV, 13 for irMPK4) compare with those of homozygous irMPK4 and EV plants (Figure S1). EVxCC and MPxCC roots inoculated with an arbuscular mycorrhizal fungus, *Rhizophagus irregularis* (AMF+), did not show significant increases in comparison to un-inoculated counterparts (AMF-) in root length colonization (RLC; center panel, mean + CI, n = 7 for EVxCC, n = 8 for MPxCC), in contrast to the strong colonization of EV plants (n = 8) and control hemizygous irMPK4 crosses: irMPK4xEV (MPxEV, n = 7-8). Vesicles and hyphae are visible in trypan blue-stained AMF+ EV roots, but not in AMF+ EVxCC and MPxCC roots.

2013), as well as within-population plant neighbor responses (Ferlian *et al*., 2018; Gorzelak *et al*., 2015; Song *et al*., 2019). As silencing the expression of NaCCaMK disconnects plants from AMF networks (Groten *et al*., 2015), we crossed isogenetic, homozygous irCCaMK plants with homozygous EV and irMPK4 lines to generate hemizygous EV x irCCaMK (EVxCC) and irMPK4 x irCCaMK (MPxCC) lines (Figure 2B), which were used for field experiments. The hemizygous crosses retained the levels of MPK4 silencing of the homozygous irMPK4 lines: MPxCC showed an 87% reduction of MPK4 transcript accumulation relative to EVxCC in the field (Figure 2C), whereas irMPK4 had 83% silencing efficiency relative to EV in the glasshouse (Figure S1). To evaluate the abrogation of AMF associations under controlled conditions, we grew the EVxCC and MPxCC crosses in the glasshouse with and without live

AMF inoculum (*Rhizophagus irregularis*) and compared their AMF colonization characteristics to that of EV and a hemizygous irMPK4xEV (MPxEV) control cross. While EV and MPxEV were highly colonized in comparison to non-inoculated controls (Figure 2C, LM, emmeans(EV, AMF-(n = 8) to AMF+(n = 8)), t = -8.894, p = < 0.0001; emmeans(MPxEV, AMF-(n = 7) to AMF+(n = 8)), t = -6.253, p = <0.0001), both EVxCC (emmeans(EVxCC, AMF-(n = 7) to AMF+(n = 7)), t = -2.105, p = 0.4251) and MPxCC (emmeans(MPxCC, AMF-(n = 8) to AMF+(n = 8)), t = -1.417, p = 0.8453) did not differ from un-inoculated controls in root length colonization (RLC). Trypan blue-staining of roots showed the establishment of vesicles and hyphae in EV, but not in EVxCC and MPxCC plants (Figure 2C). From these results, we conclude that the hemizygous crosses retain their MPK4 silencing and do not associate with AMF.

Populations with low percentages of MPK4-deficient plants show overyielding in both the field and glasshouse

In order to evaluate if the percentage of MPK4-deficient plants influences population yield under field conditions, growth and yield of EVxCC and MPxCC individuals in populations with varying percentages of MPxCCs (0, 25, 75, 100%; Figure 3A; Figure S2) were measured and analyzed using de Wit replacement diagrams (Figure 3B-G; Figure S3; de Wit, 1960; Harper, 2010). Increases in yield, referred to as overyielding, were observed in the relative yield totals (RYTs) of 25% MPxCC populations in stalk height (Figure 3C), shoot and root biomass (Figure 3D-E), and unripe and ripe seed capsule values (Figure 3F-G). This overyielding was due only to increases in EVxCC plants: in 25% irMPK4 populations, cumulative EVxCC plant trait values exceeded their predicted values based on their performance in monoculture. MPxCC plant trait values did not differ from their monoculture values in any population type. However, the increase in the cumulative EVxCC trait values in the replacement diagram was not reflected in significant differences between means of EVxCC individuals in 25% MPxCC populations versus in other population types (Figure S3), emphasizing the role of incremental benefits observable at the population-scale rather than in the performance characteristics of each individual in the population.

Plants with low WUE are thought to increase the flow of water-soluble nutrients to the immediate area around their roots as a consequence of excessive transpiration rates (del Amor and Marcelis, 2005; Zea-Cabrera *et al*., 2006). Therefore, we collected soil cores at 5, 15 and 30 cm below the center of each population type and quantified total carbon (Ctotal), nitrogen (N), inorganic carbon (Cinorg), organic carbon (Corg), copper (Cu), iron (Fe), potassium (K), phosphorus (P) and zinc (Zn) concentration (Figure S4). At each depth, there were no significant differences among populations for any nutrient except for Cinorg which was slightly increased at 5 cm depth in the 0% populations (EVxCC monoculture), at 15 cm in 75% MPxCC populations, and at 30 cm in 100% MPxCC populations (Figure S4C). We observed no increases in any inorganic nutrient at any soil depth in the 25% MPxCC populations (Figure S4). Furthermore, the percentage of MPxCC plants in populations did not significantly predict soil moisture at any sampling depth (Figure S5A; R2 = 0.527, F(15, 44) = 5.374, p = 6.097e-06). From these results we conclude that increasing the percentage of MPxCC plants in populations under field conditions leads to a non-additive trend in population yield, unrelated to soil moisture and inorganic nutrient availability, with overyielding occurring in 25% irMPK4 populations.

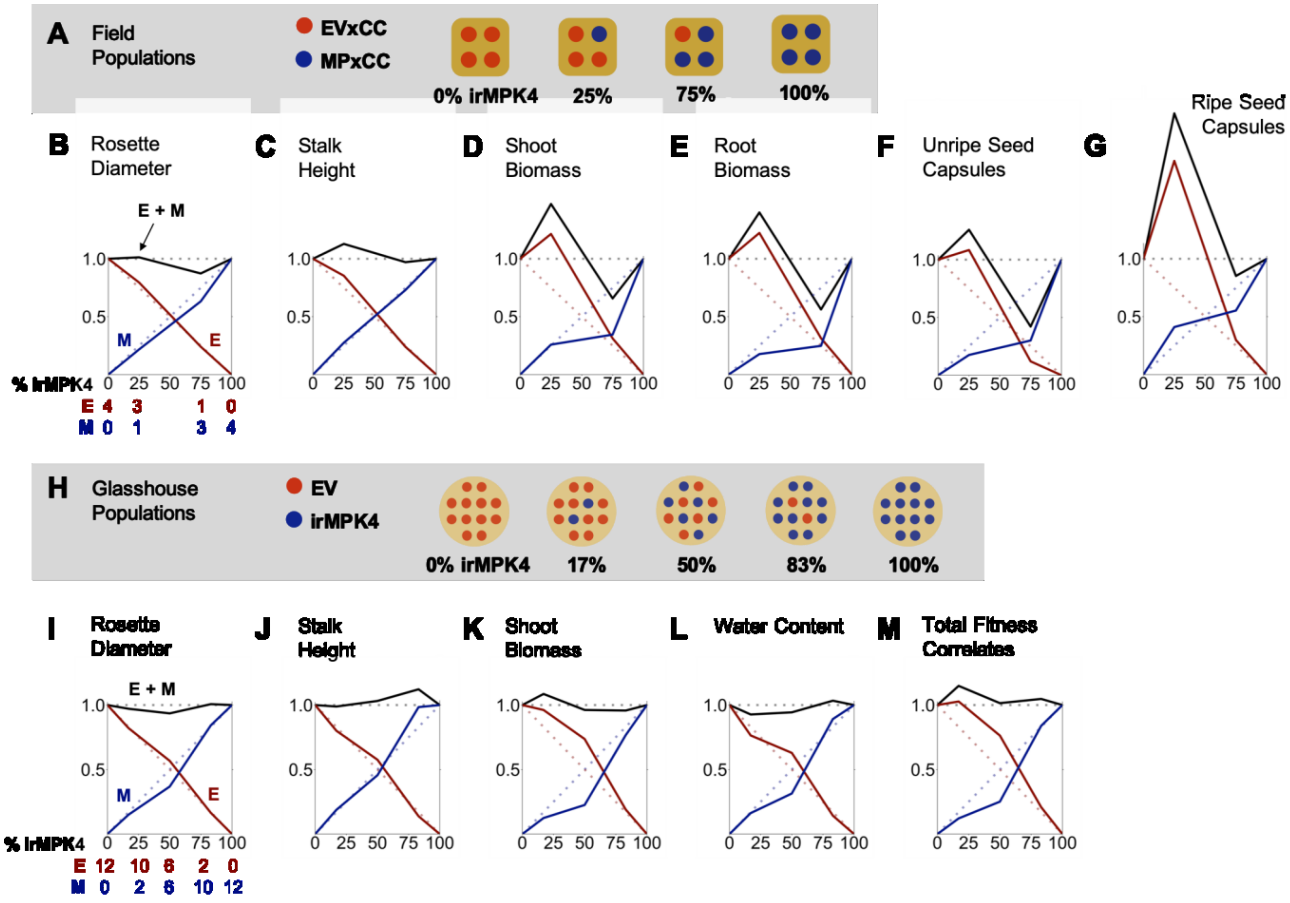

FIGURE 3 - In the field and glasshouse, populations with low percentages of MPK4-deficient plants show overyielding. (A) Field populations of four plants around a central water dripper were planted with varying percentages of EV and irMPK4 (MP) plants crossed with irCCaMK (CC) to abolish interaction with arbuscular mycorrhizal networks: four EVxCC (0%, n = 12), three EVxCC and one MPxCC (25%, n = 20), one EVxCC and three MPxCC (75%, n = 20), or four MPxCC (100%, n = 20) plants (for additional details of the experimental set-up see Figure S2). (B) – (G) Replacement diagrams show relative B) rosette diameters (n = 11-35); C) stalk heights (n = 6-29); D) shoot biomasses (n = 10-31); E) root biomasses (n = 8-31); F) unripe seed capsules (n = 4-14); and G) ripe seed capsules (n = 3-17) of EVxCC (E, red) and MPxCC (M, blue) plants in 0 - 100% irMPK4 field populations. Relative growth and yield for each genotype was calculated as: (trait mean in mixture * # of plants in mixture)/(trait mean in monoculture * 4). Means and error structures are shown in Figure S3. Relative yield totals of the populations (RYT, black) are calculated as E + M. Dotted lines indicate predicted yields from plants in monocultures. (H) Glasshouse populations of 12 plants were planted with varying percentages of irMPK4 plants: 12 EV (0%), 10 EV and 2 irMPK4 (17%), 6 EV and 6 irMPK4 (50%), 2 EV and 10 irMPK4 (83%), or 12 irMPK4 (100%). Each population was watered in proportion to its daily water consumption to ensure equal water availability across all populations (for additional details of the experimental set-up see Figure S5 and *Water treatments* in Materials and Methods). (I) – (M) Replacement diagrams show relative I) rosette diameters (n = 11-35); J) stalk heights (n = 21-41); K) shoot biomasses (n = 21-41); L) water contents (n = 22-41); and M) total reproductive yield measured as counts of fitness correlates (n = 19-44) from EV (E, red) and irMPK4 (M, blue) plants in 0 - 100% irMPK4 glasshouse populations. Relative growth and yield for each genotype was calculated as: (trait mean in mixture * # of plants in mixture)/(trait mean in monoculture * 12). Means and error structures are shown in Figure S7. RYTs (black) are calculated as E + M. Dotted lines indicate predicted yields from monocultures.

To further evaluate whether the water-use phenotype of irMPK4 plants contributed to differences in water and nutrient availability for populations in ways that were undetectable in the field, we created populations in the glasshouse with increasing percentages of irMPK4 (0,

17, 50, 83 and 100%; Figure 3H; Figure S6A) in which we experimentally controlled for water availability among populations (Figure S5B; see Water treatments in Materials and Methods). Replacement diagrams were again used to analyze cumulative growth and yield of EV and irMPK4 plants in varying population types (Figure 3I-M; individual means in Figure S7). The analysis revealed overyielding in shoot biomass and total fitness correlates (reproductive yield) of low-irMPK4 populations (17%; Figure 3K, M), consistent with the field results. Due to the controlled watering schema of the glasshouse experiment, we conclude that this overyielding effect is independent of population water availability.

Overyielding in low-irMPK4 populations does not occur at the neighbor scale

To test if overyielding in low-irMPK4 field and glasshouse populations (Figure 3) resulted from neighbor interactions of EV and irMPK4 plants, we investigated the growth and yield of EV and irMPK4 in monoculture or mixed pairs (Figure 4A), again under conditions of equal water availability (Figure 4B). Analyzing the results of these measurements using replacement diagrams revealed no evidence of overyielding in any of the measured growth and yield parameters for the mixed pairs (Figure 4G-J). We conclude that for EV plants having one irMPK4 neighbor was not sufficient to produce the overyielding response we observed in low-irMPK4 populations.

Varying local configurations of irMPK4 neighbors could also cause neighbor-scale overyielding in EV plants, a property we would not observe in our paired plant experiment. Therefore, in the glasshouse population experiment (Figure 3H), we analyzed growth and fitness measurements of centrally located EV individuals with four direct neighbors. In 0% irMPK4 populations, all four neighbors were EV plants, in 17% irMPK4 populations, two were EV and two were irMPK4 plants, and in 50% and 83% irMPK4 populations, all four were irMPK4 plants. We observed that only in 50% irMPK4 populations, EV plants with four irMPK4 neighbors produced significantly higher growth and yield in comparison to EV plants grown in 0% irMPK4 populations (Figure S7; Table S1). However, 50% irMPK4 populations did not show overyielding (Figure 3I-M), likely because irMPK4 plants simultaneously had significantly smaller rosettes, water contents, and yields compared with 100% irMPK4 monocultures (Figure S7; Table S1). Importantly, EV plants grown in 50% and 83% irMPK4 populations, with the same irMPK4 direct neighbor configuration, did not show consistent changes in growth and yield compared to monocultures. These results are consistent with the inference that overyielding does not occur at the neighbor scale.

MPK4 is necessary for N. attenuata's growth and yield responses to neighbors

To test whether EV and irMPK4 plants respond differently to the presence of a neighbor, we included EV and irMPK4 planted as singles in our paired-pot experiment (Figure 4A). We compared the growth and yield to single plants with individuals in mono- and mixed-culture pairs. Water contents of EV and irMPK4 plants did not differ, whether planted alone or in pairs (Figure 4E; Table 1), indicating equal water availability in the two potting types. EV plants with an EV or irMPK4 neighbor had smaller rosettes, shoot biomass, and reproductive yield than when planted alone (Figure 4C-D, 4F; Table 1). However, this reduction was independent of the neighbor's genotype. In contrast, irMPK4 plants showed no differences in their rosette growth, shoot biomass, or yield when planted in pairs as compared to being grown alone (Figure 4C-F). From these results we conclude that MPK4 is required for *N. attenuata*'s growth and yield responses to a neighbor.

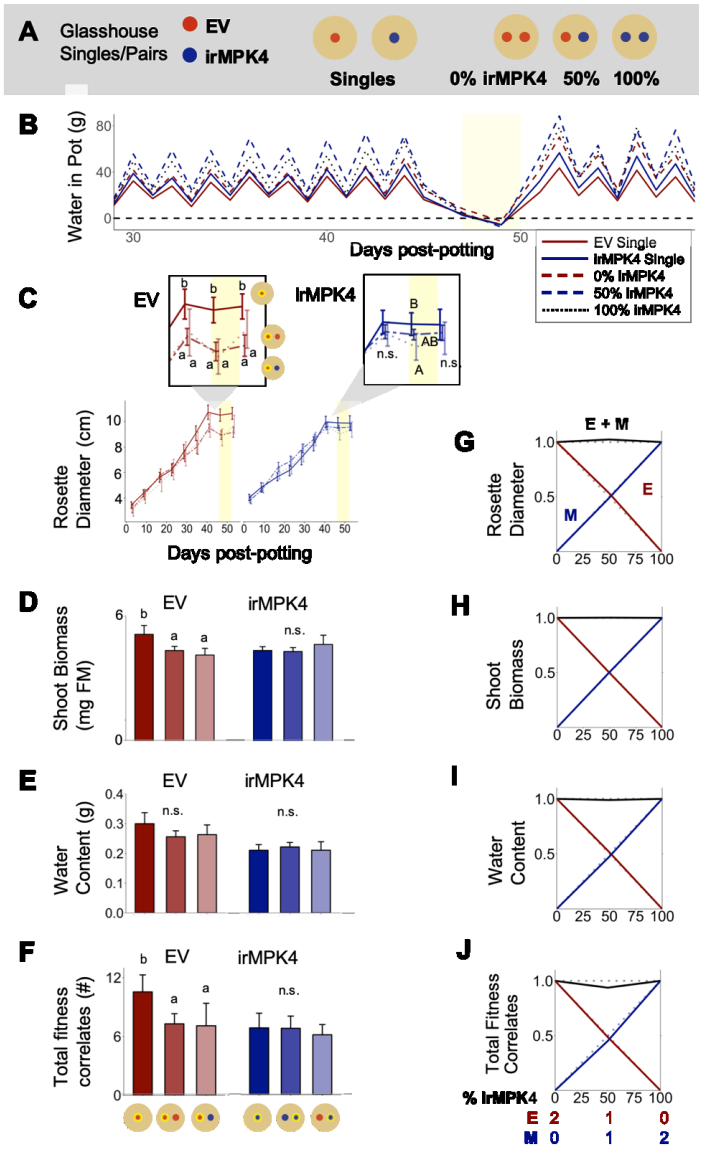

FIGURE 4 - In the glasshouse under equal water availability, EV but not irMPK4 plants have reduced growth in the presence of a neighbor. (A) EV and irMPK4 were planted either as singles or in mono- or mixed-culture pairs. EV monoculture pairs have 0%, mixed-cultures have 50%, and irMPK4 monocultures have 100% irMPK4 plants. (B) All pots were watered based on daily individual consumption to ensure equal water availability (see *Water treatments* in Materials and Methods): mean g of water per pot for each potting type (red, solid: EV Single; red, dashed: 0% irMPK4; blue, solid: irMPK4 Single; blue, dashed: 100% irMPK4; black, dotted: 50% irMPK4) immediately following a watering event (graphical peaks) and immediately preceding the next watering event (graphical troughs) are displayed. Withholding water for two days caused all pots to reach a state of no available water in the pot (yellow shading). (C) – (F) EV (red) and irMPK4 (blue) individual means in each pot type (Single: solid line; 0%/100%: dashed line; 50%: dotted line) for C) rosette diameter (mean cm ± 95% CI, n = 11-24; 3-53 days post potting, dpp), D) shoot biomass (mean + CI, n = 10-22; 71dpp), E) water content (mean + CI, n = 8-22; 71dpp), and F) total reproductive yield measured as counts of fitness correlates (buds, flowers, unripe and ripe seed capsules; mean + CI, n = 9-22; 71dpp). Significant differences are presented within genotypes. Inset of C): Significant differences in EV and irMPK4 rosette diameters among planting types are indicated for the last three time points of the main panel, within each genotype. To evaluate growth effects of the equal water availability (yellow shading), growth values before and after water was withheld are highlighted in the inset. (G) – (J) Replacement diagrams show G) rosette diameters (n = 11-24; 53 dpp); H) shoot biomasses (n = 10-22); I) water contents (n = 8-22); and J) total reproductive yield measured as counts of fitness correlates (n = 9-22) from EV (E, red) and irMPK4 (M, blue) plants in 0 - 100% irMPK4 glasshouse pairs, calculated as (trait mean in mixture*# of plants)/(trait mean in monoculture*2). Relative yield totals (RYT, black) are calculated as E + M. Means and error structures can be found in panels C) – F). Dotted lines indicate no deviations from yields in monocultures.

TABLE 1 - emmeans contrasts of EV individuals in varying potting types for Figure 4C-F[α]

Model	Contrast	Trait	t-value	p-value
LM	EV Single(n = 9) – EV Mono(n = 22)	Water Content	2.378	0.0614
	EV Single(n = 9) – EV Mix(n = 11)	Water Content	1.751	0.2047
LM	EV Single(n = 12) – EV Mono(n = 24)	Rosette Diameter	5.131	<.0001***
	EV Single(n = 12) – EV Mix(n = 12)	Rosette Diameter	4.979	<.0001***
GLS	EV Single(n = 11) – EV Mono(n = 22)	Shoot Biomass	4.196	0.0004***
	EV Single(n = 11) – EV Mix(n = 10)	Shoot Biomass	4.531	0.0002***
LM	EV Single(n = 11) – EV Mono(n = 21)	Total Fitness Correlates	3.848	0.0017**
	EV Single(n = 11) – EV Mix(n = 11)	Total Fitness Correlates	3.323	0.0066**

[α]extracted from linear (LM) or generalized least squares (GLS) models with significant ANOVA results
*p value < 0.05; **p value < 0.01; ***p value < 0.001

EV and irMPK4 photosynthetic phenotypes do not explain overyielding in the glasshouse

To determine if the WUE phenotypes of EV and irMPK4 plants in glasshouse and field populations change with the percentage of MPK4-deficient plants, potentially causing overyielding in low-irMPK4 populations (Figure 3), we measured leaf photosynthetic parameters (assimilation rate, transpiration rate, stomatal conductance) and calculated the WUE of all individuals in both glasshouse and field experiments.

In the glasshouse paired experiment, all measured leaf photosynthetic parameters of EV and irMPK4 plants in single pots were as previously reported (Figure 2A), with irMPK4 plants having significantly higher assimilation rates, transpiration rates, and stomatal conductance than EV plants, and significantly lower WUE (Figure 5A; Table 2). When planted in pairs, EV and irMPK4 plants' assimilation rates, transpiration rates, and stomatal conductance were not significantly different in monoculture (red and blue shadings, respectively) versus in mixed culture (purple shading). EV plants had significantly lower WUE in mixed versus monoculture (Figure 5A; Table 2), whereas irMPK4 plants showed no significant change in WUE across the planting types.

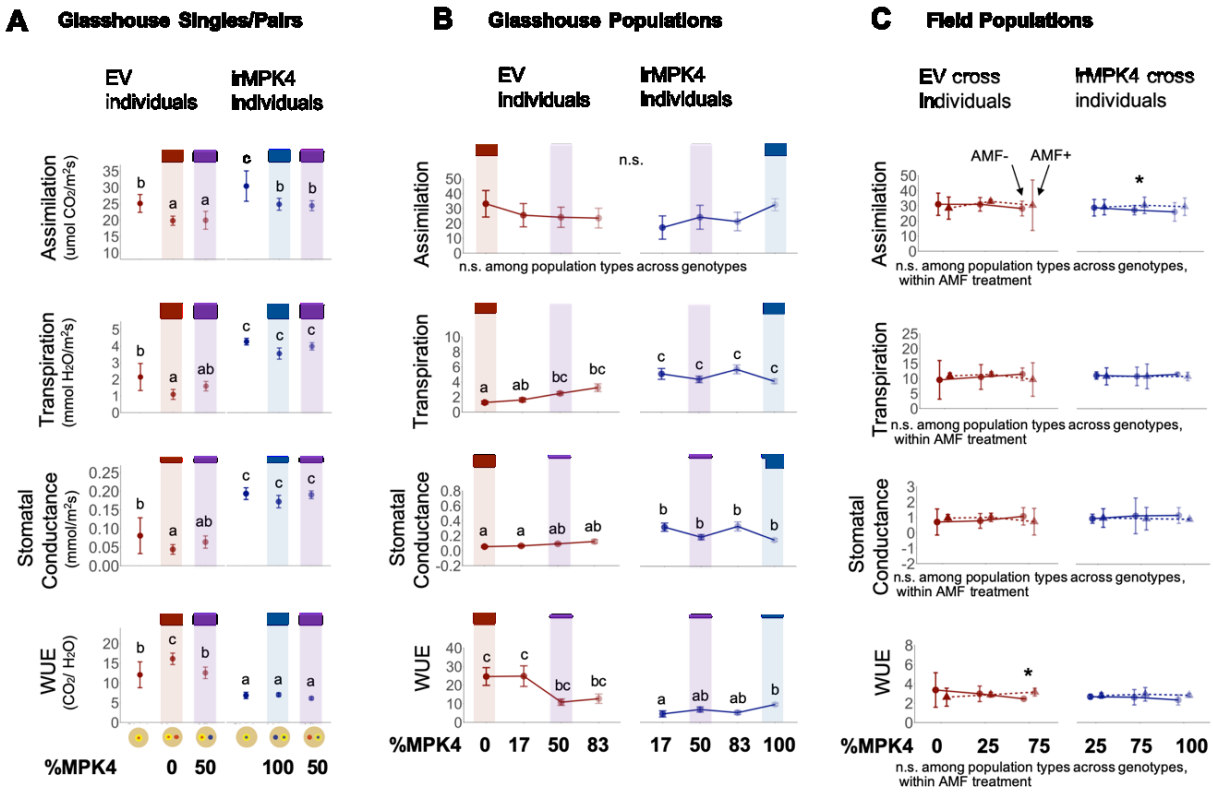

FIGURE 5 - irMPK4 plants have low WUE in glasshouse, but not field experiments, regardless of AMF associations. (A) Assimilation rate, transpiration rate, stomatal conductance and water-use efficiency (WUE; mean ± CI, n = 3-8) of EV (red) and irMPK4 (blue) individuals from each planting type in the paired glasshouse experiment (see Figure 4) at 48 days post potting (dpp). To facilitate comparison of data to the population glasshouse experiment, EV in 0% irMPK4 populations (red shading), irMPK4 plants in 100% irMPK4 populations (blue shading) and both genotypes in 50% irMPK4 populations (purple shading) are highlighted. Significant differences are presented across genotypes. (B) Assimilation rate, transpiration rate, stomatal conductance and WUE (mean ± CI, n = 11-32) of EV (red) and irMPK4 (blue) individuals from each planting type in the population glasshouse experiment (Figure 3H) at 32 dpp. Measurements were taken between 12:00-14:00; additional pre-dawn measurements (4:00-6:00) are included in Figure S8. For comparison to the paired glasshouse experiment, EV and irMPK4 in 0% (red), 50% (purple) and 100% (blue) irMPK4 populations are highlighted. Significant differences are presented across genotypes. (C) Assimilation rate, transpiration rate, stomatal conductance and WUE (mean ± CI, n = 3) of EV (red, circle) and irMPK4xEV (blue, circle) individuals with the ability to associate with arbuscular mycorrhizal fungi (AMF, solid line), or EVxCC (red, triangle) and irMPK4xCC (blue, triangle) individuals without the ability to associate with AMF from the field population experiment. Measurements were performed at 34 dpp on irrigated plants ("Wet", see *Water treatments* in Materials and Methods). Significant differences are presented both across genotypes, within AMF treatments (text below panels), or within the genotype and planting type, between AMF treatments (*: $p<0.05$).

TABLE 2 - emmeans contrasts of EV to irMPK4 individuals planted as singles for Figure 5A[α]

Model	Contrast	Trait	t-value	p-value
LME	EV Single(n = 4) – irMPK4 Single(n = 3)	Assimilation	-3.947	0.0134*
	EV Mono(n = 7) – EV Mix(n = 4)	Assimilation	-0.123	1.0000
	irMPK4 Mono(n = 8) – irMPK4 Mix(n = 4)	Assimilation	0.396	0.9985
LME	EV Single(n = 4) – irMPK4 Single(n = 4)	Transpiration	-8.089	<.0001***
	EV Mono(n = 8) – EV Mix(n = 4)	Transpiration	-3.171	0.0527
	irMPK4 Mono(n = 8) – irMPK4 Mix(n = 4)	Transpiration	-2.776	0.1104
LME	EV Single(n = 3) – irMPK4 Single(n = 4)	SC	-8.089	<.0001***
	EV Mono(n = 8) – EV Mix(n = 4)	SC	-3.171	0.0527
	irMPK4 Mono(n = 8) – irMPK4 Mix(n = 4)	SC	-2.776	0.1104
LME	EV Single(n = 4) – irMPK4 Single(n = 4)	WUE	6.394	0.0001***
	EV Mono(n = 8) – EV Mix(n = 4)	WUE	3.723	0.0205*
	irMPK4 Mono(n = 8) – irMPK4 Mix(n = 4)	WUE	3.203	0.0544

[α]extracted from linear-mixed effect (LME) models with significant ANCOVA results
SC = Stomatal Conductance; WUE = Water-Use Efficiency
*p value < 0.05; **p value < 0.01; ***p value < 0.001

In the glasshouse population experiment, irMPK4 plants in 100% (blue shading) versus 50% (purple shading) irMPK4 populations showed no significant differences in any photosynthetic parameter (Figure 5B), which was consistent with the glasshouse paired experiment. EV plants in 0% (red shading) versus 50% (purple shading) irMPK4 populations were not significantly different from each other in any parameter except for significantly higher transpiration rates of EV plants in 50% irMPK4 populations compared with those in 0% irMPK4 populations (Figure 5B; LMER, EV: emmeans 0%(n = 32)-50%(n = 16), t = -3.744, p = 0.0082). While the results

of statistical comparisons of EV responses (0% versus 50% irMPK4) differ between the pair (Figure 5A) and population (Figure 5B) experiments, the effects on the means for the two experiments remained consistent: with EV transpiration rates increasing (Pairs0% to 50%: +0.49; Populations0% to 50%: +1.22) and WUE decreasing (Pairs0% to 50%: -3.56; Populations0% to 50%: -13.78).

In the glasshouse, EV plants in low-irMPK4 populations did not have significantly different photosynthetic parameter values compared with other population types (Figure 5B). In addition, photosynthetic parameters were measured at a pre-dawn (AM; Figure S8), which included dark-adapted chlorophyll fluorescence measurements (Fv/Fm) reflecting the maximum yield of the photosynthetic system (Signarbieux and Feller, 2011). The AM photosynthetic parameter values of EV plants in low-irMPK4 (17 %) populations also did not significantly differ from EV plants in any other population type (Figure S8).

In the field, irMPK4 photosynthetic parameters become similar to those of EV, regardless of AMF associations

In the field experiment, EVxCC and MPxCC plants that lacked the ability to associate with arbuscular mycorrhizal networks (AMF-), did not differ in any photosynthetic parameters, whether these were compared between genotypes or across population types (Figure 5C). To test if the ability to interact with an AMF network changes patterns of photosynthetic performance, we additionally analyzed photosynthetic parameters of EV and irMPK4xEV (MPxEV) plants that could interact with AMF networks (AMF+). Similar to the irCCaMK crosses (-AMF), EV and irMPK4 plants capable of associating with AMF did not differ across population types and the two genotypes (Figure 5C). We further tested whether the AMF association could change photosynthetic parameters within a planting type. Only irMPK4 plants in 75% irMPK4 populations had marginally higher assimilation rates, and EV plants in 75% irMPK4 populations had a marginally higher WUE (Figure 5C, GLS, irMPK4 in 75% irMPK4: emmeans AMF-(n = 3) - AMF+(n = 3), t = -2.511, p = 0.0363; GLS, EV in 75% irMPK4: emmeans AMF-(n = 3) - AMF+(n = 3), t = -8.148, p = 0.0144). From these field and the previous glasshouse results, we conclude that the WUE phenotype is not likely to have accounted for the greater growth and yield of plants in low-irMPK4 populations.

Shoot MPK4 expression is required for N. attenuata to alter its reproductive yield in response to a neighbor

We tested the effect of tissue-specific MPK4 expression on plant yield responses to a neighbor. To separate the role of irMPK4 expression in shoots from those in roots, we created chimeric plants by micro-grafting EV shoots to irMPK4 roots (heterografts), EV shoots to EV roots (EV homografts) and irMPK4 shoots to irMPK4 roots (irMPK4 homografts; Figure 6A). Because the RNAi silencing signals travel from shoots-to-roots but not vice-versa in *N. attenuata*, micrografting of RNAi lines such as irMPK4 does not permit the investigation of shoot-only MPK4 knockdowns (Fragoso *et al*., 2011). Hetero- and homo-irMPK4 grafts retained similar levels of MPK4 silencing in roots or roots and shoots, respectively (Figure S1). We grew the grafts under conditions of equal water availability, with or without an ungrafted EV neighbor. Photosynthetic parameter profiling of these grafted plants revealed that the heterografts were similar to EV homografts in assimilation, transpiration, stomatal conductance, and WUE (Figure 6B), while the irMPK4 homografts showed significantly higher transpiration rates, and stomatal conductance and lower WUE (Figure 6B; Table 3).

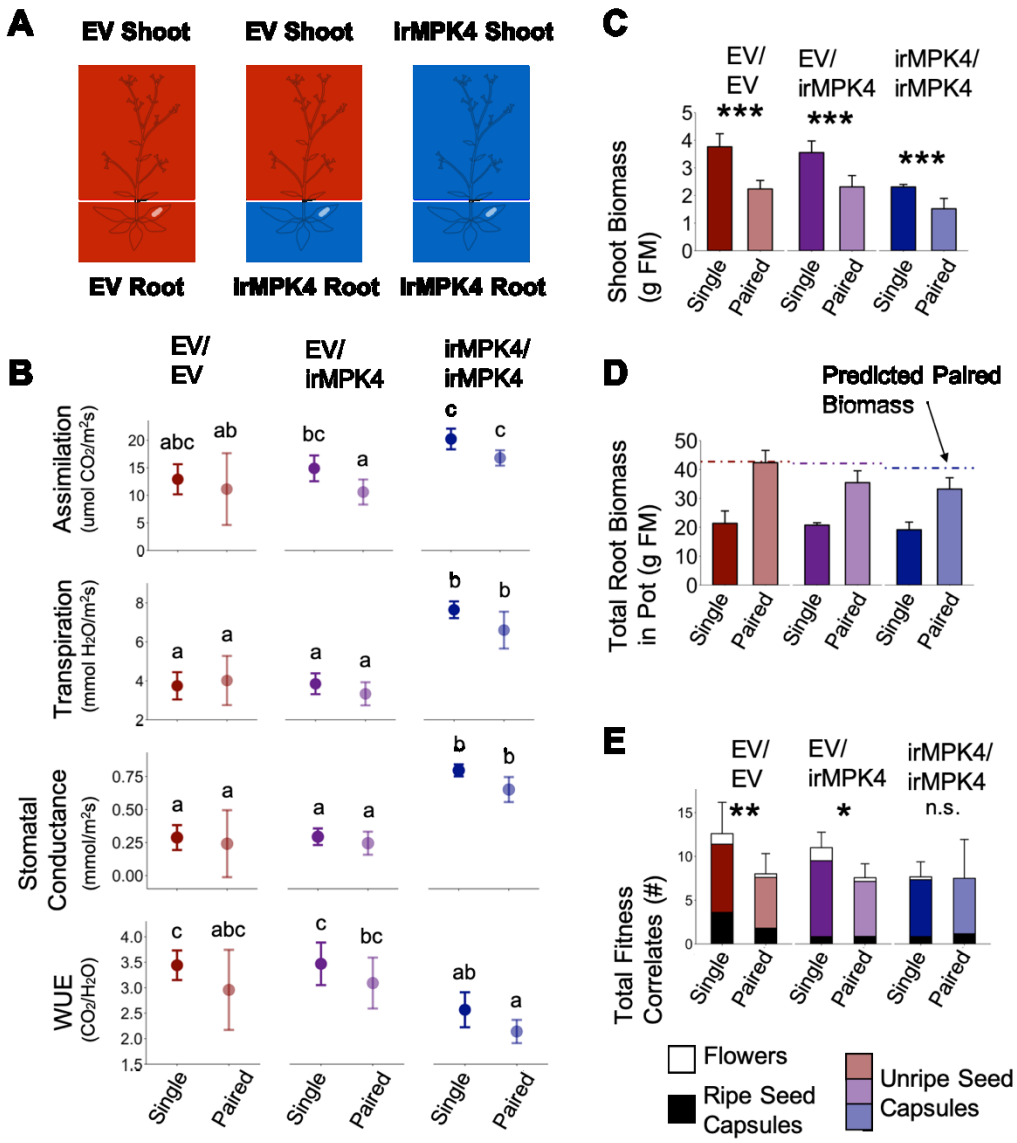

FIGURE 6 - Expression of MPK4 in the shoots mediates changes in *N. attenuata* reproductive output in response to neighbors. (A) EV shoots were micro-grafted onto irMPK4 roots, producing plants deficient in MPK4 in the root but not in the shoot (EV/irMPK4, Figure S1). These were compared to EV/EV and irMPK4/irMPK4 homografts as controls. All three graft types were grown both as singles and in pairs with an ungrafted EV neighbor, under conditions of equal water availability in a glasshouse experiment. (B) Assimilation rates, transpiration rates, stomatal conductance and water-use efficiency (WUE; mean ± CI, n = 3-7) of single and paired plants of each grafting type (EV/EV: red; EV/irMPK4: purple; irMPK4/irMPK4: blue) were measured at 37 dpp (Figure S10A). Significant differences are presented across all graft and potting types. (C) Shoot biomass (mean + CI, n = 4-6) of EV/EV (red), EV/irMPK4 (purple) and irMPK4/irMPK4 (blue) individuals in each potting type (Single and Paired) was recorded at 50 dpp. Significant differences within genotypes are indicated (***: $p<0.001$). (D) Total root biomass in each pot (mean + CI, n = 4-6) was recorded for each potting type at 50 dpp. Dashed lines indicate the total pot root biomass predicted from the summed root biomasses of the respective genotype + EV/EV when planted as single plants in pots. (E) Counts (#) of fitness correlates (mean + CI, n = 5-7) of EV/EV (red), EV/irMPK4 (purple) and irMPK4/irMPK4 (blue) individuals in each pot type were recorded at 50 dpp. Statistical analyses were only performed for the total fitness correlates, although each bar is dissected into its contributing parts: flowers (white), unripe seed capsules (color), and ripe seed capsules (black). Significant differences within genotypes are indicated (*: $p<0.05$; **: $p<0.01$).

TABLE 3 - Statistical emmeans contrasts within planting treatments for Figure 6B[α]

Parameter	Model	Contrast	t value	p value
Assimilation	LM	S: irMPK4/irMPK4(n = 6) to EV/EV(n = 5)	-5.718	0.0001***
		S: irMPK4/irMPK4(n = 6) to EV/irMPK4(n = 7)	-4.537	0.0014**
		P: irMPK4/irMPK4(n = 5) to EV/EV(n = 3)	-3.666	0.0127*
		P: irMPK4/irMPK4(n = 5) to EV/irMPK4(n = 6)	-4.837	0.0007***
Transpiration	LM	S: irMPK4/irMPK4(n = 5) to EV/EV(n = 5)	-10.979	<.0001***
		S: irMPK4/irMPK4(n = 5) to EV/irMPK4(n = 6)	-11.163	<.0001***
		P: irMPK4/irMPK4(n = 4) to EV/EV(n = 4)	-6.506	<.0001***
		P: irMPK4/irMPK4(n = 4) to EV/irMPK4(n = 6)	-9.008	<.0001***
Stomatal conductance	LM	S: irMPK4/irMPK4(n = 4) to EV/EV(n = 5)	-9.429	<.0001***
		S: irMPK4/irMPK4(n = 4) to EV/irMPK4(n = 7)	-9.971	<.0001***
		P: irMPK4/irMPK4(n = 6) to EV/EV(n = 3)	-7.209	<.0001***
		P: irMPK4/irMPK4(n = 6) to EV/irMPK4(n = 7)	-9.079	<.0001***
WUE	LM	S: irMPK4/irMPK4(n = 6) to EV/EV(n = 4)	3.696	0.0109**
		S: irMPK4/irMPK4(n = 6) to EV/irMPK4(n = 7)	4.000	0.0051**
		P: irMPK4/irMPK4(n = 6) to EV/EV(n = 5)	3.240	0.0329*
		P: irMPK4/irMPK4(n = 6) to EV/irMPK4(n = 6)	4.376	0.0019**

[α]extracted from linear (LM) or generalized least squares (GLS) models with significant ANOVA results
S: Singles; P: Paired
*p value < 0.05; **p value < 0.01; ***p value < 0.001

All graft types had shoot biomasses that were significantly reduced when plants were grown in pairs versus planted alone (Figure 6C; Table 4). The total root biomass per pot represented the roots of one plant for single pots, and two plants together for the paired pots. We compared the observed paired-pot root biomasses to a linear prediction of the paired-pot root biomass based on the addition of single-pot root biomasses of the respective graft types in the pair. Pot root biomasses of EV homograft pairs were equal to two times the root biomass of an EV homograft in a single pot. In contrast, the paired heterografts and irMPK4 homografts had smaller root biomasses than were predicted from individually grown plants (Figure 6D).

While both the EV homografts and heterografts displayed significant reductions in reproductive yield in response to an EV neighbor, irMPK4 homografts did not show a significant difference in reproductive yield between single and paired pl ants (Figure 6E; Table 4). From these results we conclude that silencing MPK4 in the roots changes the neighbor-related root biomass production of *N. attenuata*, but MPK4 in the shoots is required to alter reproductive yield in response to neighbors.

Association with an AMF network abolishes biomass, but not reproductive overyielding in field populations with low percentages of MPK4-deficient plants

In order to evaluate if altering belowground interactions affects overyielding in 25% irMPK4 field populations, we compared the growth and yield of EV and MPxEV crosses, having the ability to interact with an AMF network, in field populations with varying percentages of MPxEV (0, 25, 75, 100%) with responses observed in populations with abrogated AMF interactions (Figure 3). Overyielding was observed in unripe and ripe seed capsule counts in 25% MPxEV populations (Figure 7D-E), but not in the shoot and root biomasses RYTs for these same populations (Figure 7B-C). The overyielding in capsules, similar to the response in populations without AMF network associations, occurred as a result of increases in the number of capsules in EV plants, relative to the predicted yield based on their productions in monoculture.

To compare the biomass-to-reproductive-yield associations across populations with and without AMF association, we analyzed the data as allometric trajectories (Weiner, 2004; Wu *et al*., 2003). The presence of the AMF network significantly changed the allometric trajectories of EV individuals in 0% irMPK4 populations: EV plants had a significantly larger allocation to seed capsules per unit biomass than did EVxCC plants (Figure 7F, slopes: EVxCC($n = 10$) = 0.73, EV($n = 7$) = 3.3), and their trajectories started at a higher biomass threshold. In addition to the slope of the allometric trajectory, which indicates plasticity in resource allocation, the R2 value, which indicates the extent to which a plant's trajectory is close to its reproductive potential (Weiner, 2004), also increased from EVxCC to EV plants in 0% irMPK4 populations (Figure 7F; R2: EVxCC($n = 10$) = 0.51, EV($n = 7$) = 0.82). However, the allometric allocations of EV and EVxCC plants did not differ in 25% and 75% irMPK4 populations. MPxEV and MPxCC allometric trends did not differ in the 25% and 100% irMPK4 population type (Figure 7G). The 75% populations were excluded due to a lack of replication at the end of the field season.

We conclude that the loss of biomass overyielding in low-irMPK4 populations with AMF association is due to a change in the allometric trajectory of EV plants to higher biomass levels, which dwarfed EV biomass production in all other populations, without altering seed capsule production relative to the other populations. These results indicate that the overyielding does not require AMF-mediated belowground interactions.

TABLE 4 - Statistical emmeans contrasts within planting treatments for Figure 6C, E[α]

Parameter	Model	Contrast	t value	p value
Shoot Biomass	LM	EV/EV: $S_{(n = 5)}$ - $P_{(n = 5)}$	-7.823	<.0001***
		EV/irMPK4: $S_{(n = 4)}$ - $P_{(n = 6)}$	-6.232	<.0001***
		irMPK4/irMPK4: $S_{(n = 6)}$ - $P_{(n = 6)}$	-4.442	<.0001***
TFC	LM	EV/EV: $S_{(n = 9)}$ - $P_{(n = 11)}$	-2.637	0.0106*
		EV/irMPK4: $S_{(n = 13)}$ - $P_{(n = 12)}$	-3.620	0.0006***
		irMPK4/irMPK4: $S_{(n = 13)}$ - $P_{(n = 10)}$	-0.024	0.9813

[α]extracted from linear (LM) or generalized least squares (GLS) models with significant ANOVA results
TFC: Total Fitness Correlates; S: Singles; P: Paired
*p value < 0.05; **p value < 0.01; ***p value < 0.001

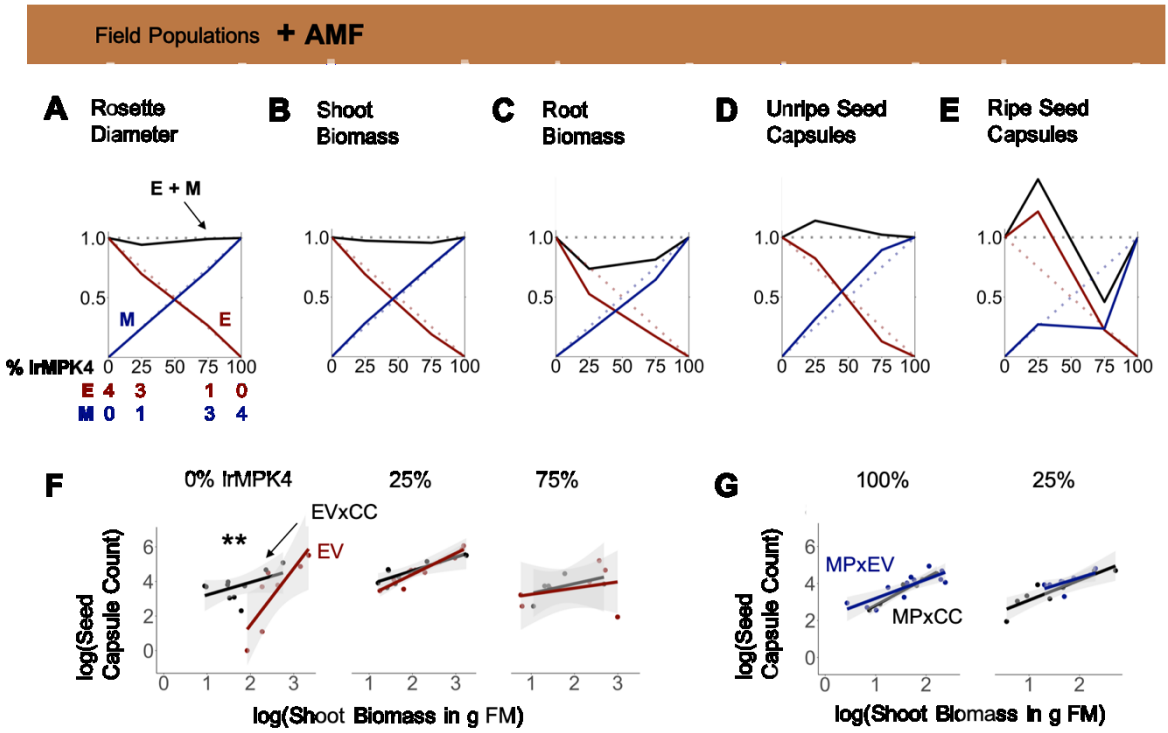

FIGURE 7 - Interaction with arbuscular mycorrhizal fungi (AMF) abolishes overyielding in biomass, but not seed capsule production of populations with low percentages of MPK4-deficient plants. (A) – (E) EV and irMPK4 individuals crossed with EV instead of irCCaMK (EVxEV: EV; irMPK4xEV: MPxEV) can associate with arbuscular mycorrhizal fungi. Field populations were varied in percentages of EV and MPxEV plants (Figure 3A, Figure S2). Replacement diagrams show A) rosette diameters (n = 12-28); B) shoot biomasses (n = 6-12 excl. M in 75% = 2); C) root biomasses (n = 7-12 excl. M in 75% = 2); D) unripe seed capsules (n = 6-14 excl. M in 75% = 2); E) ripe seed capsules (n = 6-16) of EV (E, red) and MPxEV (M, blue) plants in 0 - 100% irMPK4 field populations. Relative growth and yield for each genotype is calculated as (trait mean in mixture*# of plants)/(trait mean in monoculture*4). Relative yield totals (RYT, black) are calculated as E + M. Dotted lines indicate no deviations from yields in monocultures. (F) – (G) Allometric trajectories of F) EV x irCCaMK (EVxCC, black) plants compared to EV (red) plants in 0%, 25% and 75% irMPK4 populations, as well as G) irMPK4 x irCCaMK (MPxCC, black) plants compared to irMPK4xEV (MPxEV, blue) plants in 100% and 25% irMPK4 populations. Asterisks indicate significant differences within population types (lstrends, pairwise comparisons of slopes of fitted lines: *** $p<0.001$, ** $p<0.01$, * $p<0.05$).

3.4 Discussion

Individual-level variation in resource-use traits, such as water-use efficiency (WUE), can change overall population yields (Campitelli *et al.*, 2016; Kenney *et al.*, 2014). This occurs when plants respond to neighbors which are divergent in WUE with changes in growth and reproductive yield. However, the scale within a population's hierarchical organization at which this phenomenon occurs (Allen and Starr, 1982) is not yet known: yield changes in responding individuals (RIs) may be triggered only in immediate neighbors (neighbor scale; Figure 1) or in individuals across a population (population scale). Additionally, interactions may occur at these scales above- or belowground. Our analyses revealed that low abundances of irMPK4 plants intermixed with EV plants result in higher yields for *N. attenuata* populations, both in the glasshouse and the field (Figure 3). This overyielding effect was not caused by differences in soil water availability, which was controlled for in the glasshouse (Figure 3H-M; Figure S7; Figure 4), nor irMPK4's WUE phenotype (Figure 2A), which was not observed in the field

(Figure 5). Interestingly, we find that yield-increasing responses in low-irMPK4 populations likely occurred aboveground, at the population scale (Figure 2B-C; Figure 6).

Given that manipulating plant density is known to change overyielding results in mixed plant population experiments (Stachová *et al*., 2013; Weiner, 1980), it was important to consider that our glasshouse populations (12 plants, 5 cm apart) differed in plant density from our field populations (4 plants, 10 cm apart). However, considering that the overyielding results were consistent between the glasshouse and field, we infer that the overyielding observed in low-irMPK4 populations was not due to population differences in plant density.

Based on results from previous neighbor-effect studies using low-WUE phenotypes, we initially hypothesized that the neighbor scale (Figure 1, black arrow) would be critical for producing individual changes in yield. In paired-plant competition experiments, the *A. thaliana* CVI mutant (low WUE) produced more seeds than Ler (high WUE), although the opposite was true when these two lines were planted individually (Campitelli *et al*., 2016). In addition, the *N. attenuata* irMPK4 line growing in competition with EV caused both genotypes to wilt two days after the termination of watering, but when grown individually, only irMPK4 showed this wilting phenotype (Hettenhausen *et al*., 2012). Despite these previously observed changes in the neighbor responses to plants deficient in the homologous MPK12 or MPK4 genes, in this study we did not see differences between EV plants paired with EV or irMPK4 in any growth or yield parameters (Figure 4). In contrast to the earlier studies, we controlled for water availability in our glasshouse experiments, to exclude that differences in water availability are driving the observed responses. Plants with low WUE can change the soil water availability in certain microenvironments and thus responses of neighbors due to the decreasing water table (Zea-Cabrera *et al*., 2006). While controlling for water availability abolished irMPK4 plant's differential effects on EV neighbors in paired pots, in the field without controlled watering, we did not observe differences in soil moisture among populations with different irMPK4 abundances (Figure S5). Even a watering treatment on a section of the field plot did not cause differentiated rates of drying-down among the different population types (Figure S5). Additionally, we tested the influence of water availability in the glasshouse by controlling for soil water availability among populations (Figure 3H-M). We hypothesized that, with equalized soil water availability, we would not observe the overyielding response in low-irMPK4 populations, but the overyielding remained independent of water availability. Therefore, we infer that the influence of soil water availability on neighbor scale interactions was not related to the observed population overyielding.

Paired-plant-in-a-pot experiments offer a limited range of interactions: individuals can only be observed in 0%, 50% and 100% irMPK4 mixtures (Figure 4). The growth and yield responses of plants at the neighbor scale could depend on the identities of several immediate neighbors, as is the case in populations. Therefore, using a glasshouse population experiment, we compared the growth and yield of EV plants among a range of immediate irMPK4 neighbors (0 irMPK4 in 0% irMPK4 populations, 2 in 17% populations, and 4 in both 50 and 83% populations). We did not observe a correlation between the growth and yield of EV plants and the number of immediate irMPK4 neighbor plants (Figure S7). With these results and the lack of yield effects from our paired-pot experiments, we infer that the responses resulting in the observed population overyielding likely occur at the population rather than at the neighbor scale.

We then investigated the mechanism driving the population-scale response independent of changes in soil water availability. Trees with differing WUE influence photosynthetic

parameters of neighboring trees (Bunce *et al*., 1977). Therefore, we examined the photosynthetic parameters of plants in the field and glasshouse populations. In the field, EV and irMPK4 plants did not differ in their photosynthetic parameters, including WUE (Figure 5C). In the glasshouse populations, irMPK4 showed a decreased WUE phenotype, but the photosynthetic parameters of EV and irMPK4 individuals in low-irMPK4 populations were not different from those in their respective monocultures (Figure 5B). From these results, we infer that the population-scale factor responsible for the low-irMPK4 population overyielding is independent of the WUE phenotype of irMPK4 plants.

Other factors that could cause the observed overyielding in low-irMPK4 populations at the population scale include various plant-plant interactions ranging from niche complementation to the exchange of chemical signals. For example, hydrological niche complementation could explain the increased population yield through a more efficient spatial or temporal use of local water resources in plants exhibiting different water-use traits (Silvertown *et al*., 2015). However, the lack of differences in WUE of irMPK4 plants and soil moisture among the different populations in the field, where overyielding effects were strongest (Figure S5), renders hydrological niche complementation an unlikely explanation. However, other competitive complementation effects could have occurred. EV plants were the only individuals that responded with changes in growth and yield to the presence of a neighbor (Figure 4). EV plants may be able to benefit from certain percentages of non-responsive neighbors such as irMPK4 plants (Figure 4) by increasing their growth and yield relative to their growth when adjacent to responding neighbors. This could lead to the highest population overyielding when EV plants appeared in high frequencies in the population, as is the case in low-irMPK4 populations. However, the specific traits of irMPK4 plants responsible for this effect remain unidentified.

Volatiles can accumulate differently in the headspaces of various plant populations (Schuman *et al*., 2015) and have been shown to be a mechanism by which plants detect and respond to potentially competitive neighbors (Engelberth and Engelberth, 2019; Ninkovic *et al*., 2016; Pierik *et al*., 2013). irMPK4 and EV plants are known to emit distinct volatile profiles in response to herbivory: irMPK4 plants release 5x higher levels of trans-α-bergamotene than EV plants (Hettenhausen *et al*., 2012). Furthermore, root exudates can accumulate belowground in populations and provide information for plants about their neighbors' identity and performance, which may result in plant growth responses (Semchenko *et al*., 2014). However, irMPK4 root exudates have not yet been characterized. Further research on the chemical profiles of irMPK4 and EV plants in field populations is needed to identify potential above- or belowground factors which could cause responses at the population scale.

We explored the influence of the above- or belowground scale on the observed overyielding by experimentally altering arbuscular mycorrhizal fungal (AMF) associations in field populations with varied amounts of MPK4-deficient plants. Although AMF associations have a strong impact on plants' access to soil water and nutrients (Egerton-Warburton *et al*., 2007; Reynolds *et al*., 2003; Yang *et al*., 2013), as well as on belowground plant-plant interactions (Ferlian *et al*., 2018; Gorzelak *et al*., 2015; Song *et al*., 2019), we consistently observed reproductive overyielding in low-irMPK4 populations regardless of the AMF association (Figure 3). While disconnection from the mycorrhizal network does not exclude other belowground interactions as causative factors, our paired grafting experiment revealed aboveground tissue-specific requirements for the response of *N. attenuata* plants to neighbor presence. MPK4 transcript accumulation is required to respond to the presence of a neighbor (Figure 5F, 7E), and the presence of these transcripts in the shoot alone is sufficient to elicit this yield-altering response (Figure 7E). This suggests that MPK4 presence in the shoot mediates the observed population

reproductive overyielding. Naturally occurring alleles of MPK12 in *A. thaliana* have been found to produce varying amounts of MPK12 (Des Marais *et al*., 2014), indicating that there may be unexplored functions of MPK4/12, which could also play a role in mediating increases population yield responses. Because the RNAi silencing signals travel from shoots to roots in *N. attenuata* (Fragoso *et al*., 2011), we were unable to investigate the function of shoot-only MPK4 knockdowns. The use of mutant or natural alleles of MPK4/12 could allow for the analysis of reciprocal mpk12/wt and wt/mpk12 grafts in *A. thaliana*.

The results of this study are consistent with the well-established phenomenon that divergent individuals in a population can increase population productivity (Chapin III *et al*., 1997; Chapin, *et al*., 1998; Hooper *et al*., 2005; Naeem *et al*., 1994; Schulze and Mooney, 1994) and advance our understanding by identifying the scale within populations (neighbor vs. population, above vs. belowground) at which this yield response occurs. Additionally, we identify a single gene, MPK4, which when silenced in low abundances in a population, is responsible for population overyielding. By excluding neighbor-scale effects and controlling resource availability, we demonstrate that MPK4 influences population yield through RIs at the population scale independent of water availability, but further experiments are needed to identify the specific mechanisms mediating this effect. This work contributes to our understanding of how populations may become more productive as a result of greater genetic and functional diversity and suggests that experiments exploring the scales at which these effects occur can identify novel means to increase the productivity of agronomic monocultures.

3.5 Materials and Methods

Plant material and constructs

Characterization of the empty-vector (EV) *Nicotiana attenuata* control line (pSOL3NC, line number A-04-266-3) is described in Bubner *et al* (Bubner *et al*., 2006). The irMPK4 line (pRESC5MPK4, line number A-7-163), silenced in the production of MITOGEN-ACTIVATED PROTEIN KINASE 4 (MPK4) through RNAi targeting MPK4 transcripts, is characterized in Hettenhausen *et al* (Hettenhausen *et al*., 2012, 2013). The irCCaMK line (pSOL8CCAMK, line number A-09-1212-1-4), silenced in the production of CALCIUM AND CALMODULIN-DEPENDENT PROTEIN KINASE (CCaMK) through RNAi targeting CCaMK transcripts, is characterized in Groten *et al* (Groten *et al*., 2015).

EVxirCCaMK (pSOL3NCxpSOL8CCAMK, ‘EVxCC’) and irMPK4xirCCaMK (pRESC5MPK4xpSOL8CCAMK, ‘MPxCC’) crosses were generated by growing homozygous EV (second generation, T2), irMPK4 (T2) and irCCaMK (third generation, T3) in the glasshouse and hand pollinating the styles of EV and irMPK4 emasculated flowers with pollen from the anthers of irCCaMK flowers. Control crosses EVxEV (pSOL3NCxpSOL3NC, ‘EVxEV’) and irMPK4xEV (pRESC5MPK4xpSOL3NC, ‘MPxEV’) with the same paternal genotypes were created by pollination with pollen from EV. Hand-pollinated flowers were tagged with string and resulting seed capsules were collected. The ripe seeds from these crosses provided the seed source for the field population experiment (Figure 3). A characterization experiment in the glasshouse revealed that EVxEV and EVxCC, as well as MPxEV and MPxCC, were not significantly different in water loss rates per day (Figure S9A, Table S1), shoot and root biomass (Figure S9B-C). For all other experiments in the glasshouse, T3 generation EV and irMPK4 homozygous lines were used.

Plant growth conditions

Importation and release of transgenic crosses in the field station (Lytle Ranch, Utah, USA) was carried out under Animal and Plant Health Inspection Service (APHIS) import permit numbers 07-341-101n (EV) and 10-349-101m (EVxirCCaMK, irMPK4xirCCaMK, irMPK4xEV), and release 16-013-102r.

Glasshouse and field germination and growth were described previously (McGale *et al*., 2018), with modifications only in planting design. Field plants were planted in four-plant populations in a square design (Figure 3; Figure S2), with 10 cm between each adjacent neighbor. Plants of the glasshouse population experiment were potted in twelve-plant populations (Figure 3; Figure S6), with 5 cm between each adjacent neighbor. Glasshouse plants in both of the paired experiments (grafted and ungrafted) were also planted 5 cm from their neighbor plants. The planting substrate consisted of a bottom layer of large clay aggregate (Lecaton, 8-16mm diameter, approx. 10% of pot volume), a central layer of small clay aggregate (Lecaton, 2-4mm diameter, approx. 80% of pot volume) and a top layer of fine sand (approx. 10% of pot volume). This substrate provides optimal drainage in the pots for the purposes of water control, and conditions similar to the sandy, clay soil of the natural habitat of *N. attenuata*.

Plant growth and yield measurements

For the field experiment (Figure 3A-G), rosette diameter measurements were extracted from photos taken between 19:00 and 20:00, in which each individual plant was pictured next to a standard metal square (5x5 cm) for scale. Plant stalk height measurements were recorded as the height from the base of the stalk at the ground level to the highest point of the topmost inflorescence. Plant shoot and root dry biomass were measured by placing respective biological matter in a paper bag inside of a plastic box with ventilation holes of 1 cm diameter drilled through the lid and left to dry for 15 days in the sun, before being removed from the bag and weighed. Unripe seed capsules were counted simultaneously for all plants, immediately before harvesting for shoot and root biomass. Due to APHIS regulations, ripening seed capsules were counted and subsequently removed to prevent opening and releasing seeds into the field; the total ripe capsules collected is presented (Figure 3G).

For all glasshouse experiments (Figures 3H-M, 4, 6), rosette diameter was measured directly on the plant. Plant stalk height was measured as in the field. Shoot biomass consisted of all aboveground matter (severed below the rosette), placed inside a bag for drying at 80°C for 2h, after which the plant matter was removed from the bag and weighed. The shoot biomass was also weighed for fresh mass, and the water content of the plant at harvest was reported as the difference between the fresh and dry shoot biomasses. All fitness correlates were counted at harvest, including flowers (counted as flowers when the corolla became visible by pushing through the sepals), unripe and ripe seed capsules, and the total of all of these together was reported (Figure 3M).

Soil moisture and element content

Soil cores were taken from the field by driving a split tube core borer (53 mm, Eijkelkamp, Giesbeek, Netherlands) 30 cm into the ground, and carefully removing it with the core intact. 5 cm pieces of field soil were cut from the core from 0 to 5, 10 to 15, and 25 to 30 cm below ground. Each of these 5 cm thick sections were weighed, left to dry in the sun in UV-excluding boxes similar to those used for the drying of shoot biomass (see Plant growth and yield

measurements), and weighed again when dry (determined to be when the mass fluctuated <0.1 g between days). Soil moisture was calculated for each sample (% soil moisture = (fresh soil mass - dry soil mass / fresh soil mass) * 100), taken from 21 to 30 dpp in the different population types (Figure S5, n = 1 per population).

Soil cores were obtained using the same method at 54 dpp with replication (n = 2-9) to determine the soil content of total, inorganic and organic carbon (Ctotal, Cinorg, Corg, respectively), nitrogen (N), copper (Cu), iron (Fe), potassium (K), phosphorus (P), and zinc (Zn) in each type of population at the end of the season (Figure S4). Samples were dried at 80°C for 6h in a drying oven, sieved and milled for Ctotal determination (elemental analyzer; High TOC, Elementar, Hanau, Germany), Cinorg (loss-on-ignition from elemental analyzer), Corg (Ctotal - Cinorg), and N (elemental analyzer) at the Max Planck for Biogeochemistry in Jena, Germany. Cu/Fe/K/P/Zn concentration were determined by microwave digestion and atomic absorption spectroscopy (Karpiuk *et al*., 2016).

Water treatments

Field populations were watered every week for 1h at dusk (20:00 to 21:00) from a central water dripper (2L/h drip rate) present in each population. After 34 dpp, one section of the plot was no longer watered until the final harvest (Dry), while a small subsection was watered two more times (Wet) in order to obtain gas exchange measurements on sections with varying water treatments at 48 dpp. Soil moistures at 21-30 dpp in these two parts of the plot (see Soil moisture and element content) were analyzed by regression to test if results from both of these parts could be summarized together in Figure 3 (Figure S5). Watering treatment and the interaction with depth or day did not significantly predict soil moisture (Figure S5B, Wet subsection: 'Part2'; day: 'variable'; model fit: R2 = 0.406, F(7, 147) = 16.04, p-value = 1.474e-15). Therefore shoot and root biomass, as well as unripe and ripe seed capsule data collected from full populations in both sections were reported together as one mean (Figure 3).

In the glasshouse, all populations and pairs (grafted and ungrafted) underwent the following regimented watering to control for water availability: after potting, pot were given establishment watering (soil moisture maintained around 20%), allowing root development to the bottom of the pot for a transition from top watering to bottom watering. After three weeks, pots with population types began to show detectable differences in water loss and consumption-based watering began at ecologically relevant soil moistures (Valim *et al*., 2019). This reflected the known decrease in soil moisture throughout the life cycle of *N. attenuata* in the field (Zavala and Baldwin, 2004). For the population and pair experiment, ecologically relevant soil moisture was achieved by daily watering of individual pots to a two-day water supply, calculated as:

$$WM = 2*\text{mean}(WL\text{-}1, WL\text{-}2) + DP$$

WM = pot mass (g) to which the pot needed to be watered
WL-1 = water loss (g) from the previous to the current day
WL-2 = water loss (g) from two days to one day prior
DP = dry pot mass (g of pot with dry substrate, before planting)

The two-day water supply is illustrated for our glasshouse paired experiment (Figure 4B). To allow larger growth and thus accentuate growth differences in plants in the grafted pair experiment (Figure 6), the water supply was raised to five days (WM = 5*mean(WL-1, WL-2) + DP), bringing soil moisture percentages up to 20-30%. The higher soil moisture did not affect the differences in photosynthetic parameters of EV and irMPK4 homografts compared to those reported for the homozygous EV and irMPK4 plants in the paired experiment (Figure 5A, 6B). There was no significant correlation between the amount of water added in our watering

regimes and the amount of water lost (demonstrated two times during watering regime of the grafted experiment, Figure S10B-C).

Leaf turgor and potential effects of controlled watering on diurnal rhythms

Yara ZIM-probes were placed on 2-3 replicates of EV or irMPK4 plants in all glasshouse population types (Figure 3H). The probes consist of two magnets clipped onto both sides of a leaf, of which the lower magnet includes a pressure sensor. All probes are initialized at a clamping pressure between 10 and 30 kPa on a turgescent leaf. The probes allow continuous measurement of leaf turgor pressure throughout an experiment, and we present 48h of continuous monitoring from 00:00 December 4th to 00:00 December 7th (Figure S11).

Absolute pressure values could not be compared quantitatively; in contrast to previously reported measurements (Zimmermann *et al*., 2008) our recordings initialized at different clamping pressures. A comparison of three replicates of irMPK4 in 100% irMPK4 populations showed that variation in peak-trough amplitudes within one genotype/population type (irMPK4 in 100% irMPK4 populations; Figure S11C) exceeds variation between EV and irMPK4 in all other populations (Figure S11A-B). Therefore, we compared the time of day at which changes in turgor pressure values occurred. For all EV and irMPK4 plants in all population types, the diurnal turgor pressure changes (peaks and troughs) do not occur at different times (Figure S11A-B).

Additionally, we observed if our genotypes experience differential diurnal dry-downs or unexpected drought events that may not be captured by our daily pot weighing and watering for our controlled water treatment. This may be reflected in "noisier" curves (increased oscillations within the peaks or troughs of the diurnal leaf turgor changes) or inverted leaf turgor pressure curves around noon (Martínez-Gimeno *et al*., 2017), however, we did not observe any of these qualities across our measured plants. We therefore inferred that our controlled watering treatment was not causing unknown diurnal drying differences among individuals in our glasshouse population types and proceeded with applying it to all glasshouse experiments (Figure 3H-M, Figure 4, Figure 5A-B, Figure 6).

Gas exchange measurements and water-use efficiency calculations

Gas exchange measurements including photosynthesis and transpiration rates, and stomatal conductance (via calculation), were performed using a LI-COR 6400XT infrared gas analyzer (Lincoln, Nebraska, USA), both in the field and the glasshouse between 12:00 and 14:00 (Figure 5).

The LI-6400XT was combined with a Leaf Chamber Fluorometer in the glasshouse to additionally obtain chlorophyll fluorescence measurements after 6h of dark adaptation (lights off at 22:00, measurements from 4:00 to 6:00; Figure S8A). A saturating pulse of light was applied to the dark-adapted leaves to ensure that all photosystem II (PII) energy was released as fluorescence and detected as the Fm value. Fv was calculated from Fm minus F0 (F0 being the base level of fluorescence emitted without the saturating pulse). Fv/Fm represents the maximum quantum yield of PII, which was used as a measure of photosynthesis limitations unrelated to stomata (Signarbieux and Feller, 2011). Photosynthesis and transpiration rates were also acquired concomitantly with Fv/Fm values during this pre-dawn sampling, and stomatal conductance and WUE were calculated (Figure S8B-E).

Water-use efficiency (WUE) was calculated as the ratio of photosynthetic rate (μmol CO2/m2s) to transpiration rate (mmol H2O/m2s), thus resulting in units of carbon dioxide molecules used per 1000 water molecules (Figure 5A, B, C; S8E).

Micro-grafting

Seven-day-old seedlings were micro-grafted as described previously (Fragoso *et al*., 2011), with EV scions grafted to both EV (EV/EV) and irMPK4 (EV/irMPK4) rootstocks, and irMPK4 scions grafted only to irMPK4 (irMPK4/irMPK4) rootstocks (Figure 6). The average grafting success was 90% ($p > 0.05$ between genotypes, ANOVA, Tukey HSD post hoc).

Transcript abundance

RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of total RNA using RevertAid H Minus reverse transcriptase (Fermentas) and oligo (dT) primer (Fermentas). qPCR was performed in a Mx3005P PCR cycler (Stratagene) using 5X Takyon for Probe Assay (No ROX) Kit (Eurogentec), TaqMan primer pairs and double fluorescent dye-labeled probe. *N. attenuata* Sulfite Reductase (ECI) was used as a standard housekeeping gene, and its primer sequences and probe, as well as the MPK4 primer sequences and probes, are as published previously (Wu *et al*., 2007). MPK4 transcript levels were quantified relative to the housekeeping gene as described in Wu *et al* (Figure 2C, S1; Wu *et al*., 2007).

Statistical analysis

All data were analyzed using R version 3.4.2 (RC Team 2017) and RStudio version 1.0.153 (RStudio Team 2016). Replication for experiments is indicated in the figure captions. The replacement diagrams in Figures 3, 4, and 7 do not display statistical significance, but facilitate the visualization of cumulative population overyielding (de Wit, 1960). Statistical means of the data used to produce these diagrams are presented in Figure S3 and S7.

Some pseudoreplication resulted from plants being measured from within the same population or pot throughout our experiments (Figures 3-4, 7, S3, S7). We evaluated whether this effect contributed significantly to changes in our dependent variable using ANOVA comparisons of nested linear mixed effects models (i.e. LME/R models with and without the pseudoreplication as a random effect) as described by Zuur *et al*. (R packages lme4, nlme; Bates *et al*., 2015; Pinheiro and Bates, 2019; Zuur *et al*., 2009). Pseudoreplication was only included as a random effect in the respective LME/R model if it was significant; the model was then fitted for its fixed effects, and was checked for outliers, homoscedasticity and normality (Zuur *et al*., 2009). Pairwise post hoc comparisons of fixed effects were extracted from the model using the R package emmeans (Lenth *et al*., 2019), following their significance in an ANOVA. ANCOVA analyses were not used as the variable representing pseudoreplication (i.e. population or pot number) is inherently non-independent, which violates the assumptions for testing the significance of a co-variate with ANCOVA, but does not violate the assumptions of a random effect in a mixed effects model.

Datasets without significant pseudoreplication were fit to the best suited of either a linear model (LM; RC Team Package stats), a generalized least squares model (GLS, R package nlme) or an LME model, and were checked for outliers, homoscedasticity and normality. Pairwise post hoc

comparisons for fixed effects were extracted as above, or with Tukey HSD tests (R package stats) following their significance in ANOVA.

Regression analyses (Figure 7F, G) were performed using the lstrends function (R package emmeans) and statistical significances were extracted using pairs (RC Team Package graphics).

3.6 Acknowledgements

We thank the glasshouse team at the Max Planck Institute for Chemical Ecology and colleagues in the 2016 field work team for support; the technical staff at the Department of Molecular Ecology, particularly Dr. Klaus Gase, as well as Lucas Cortes Llorca, for advice on genetic material extractions; the International Max Planck Research School (IMPRS) on the Exploration of Ecological Interactions with Chemical and Molecular Techniques and the Young Biodiversity Research Training Group - yDiv for their support of E.M. and H.V.; and the reviewers of the first submission of this manuscript to eLife, whose advice was essential in revising the manuscript.

3.7 Supplemental Tables and Figures

TABLE S1 - emmeans contrasts of EV and irMPK4 individuals for Figure S7[α]

Model	Contrast	Trait	t-value	p-value
LME	EV in 0% irMPK4$_{(n = 40\text{-}44)}$ − EV in 50% irMPK4$_{(n = 21\text{-}22)}$	Rosette Diameter	-2.786	0.0268
		Stalk Height	-2.644	0.0438
		Shoot Biomass	-3.859	0.0009
		Water Content	-2.130	0.1475
		Total Fitness Correlates	-3.825	0.0010
LME	irMPK4 in 100% irMPK4$_{(n = 31\text{-}32)}$ − irMPK4 in 50% irMPK4$_{(n = 22)}$	Rosette Diameter	3.898	0.0006
		Stalk Height	1.387	0.5091
		Shoot Biomass	5.359	<.0001
		Water Content	3.819	0.0010
		Total Fitness Correlates	3.794	0.0011

[α]extracted from linear-mixed effect (LME) models with significant ANCOVA results

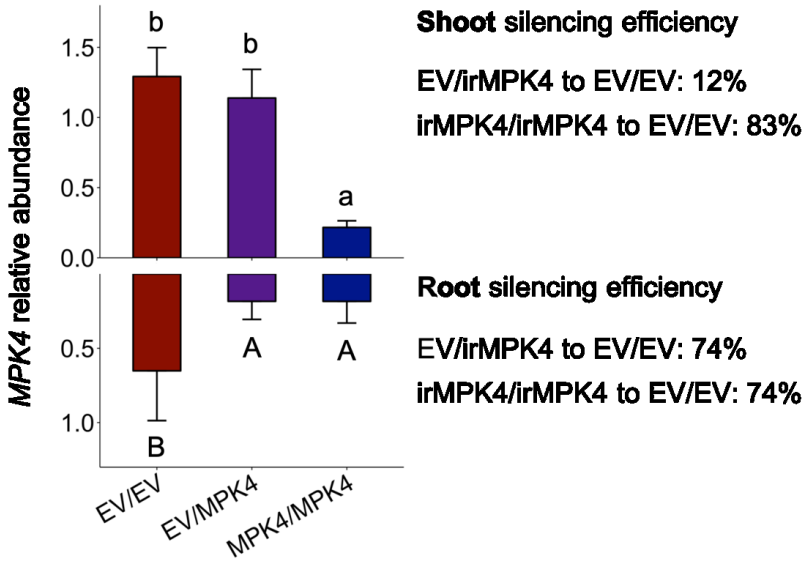

FIGURE S1 - *MPK4* transcript abundance relative to a housekeeping gene (+ 95% CI) and silencing efficiency in homozygous EV and irMPK4 shoots (top) and roots (bottom) which were grafted together for the glasshouse grafting pair experiment (n = 8 for EV/EV, 9 for EV/irMPK4, 6 for irMPK4/irMPK4). Transcript accumulation data is also used to compare to the *MPK4* silencing efficiency in hemizygous EV and irMPK4 crosses (Figure 2C).

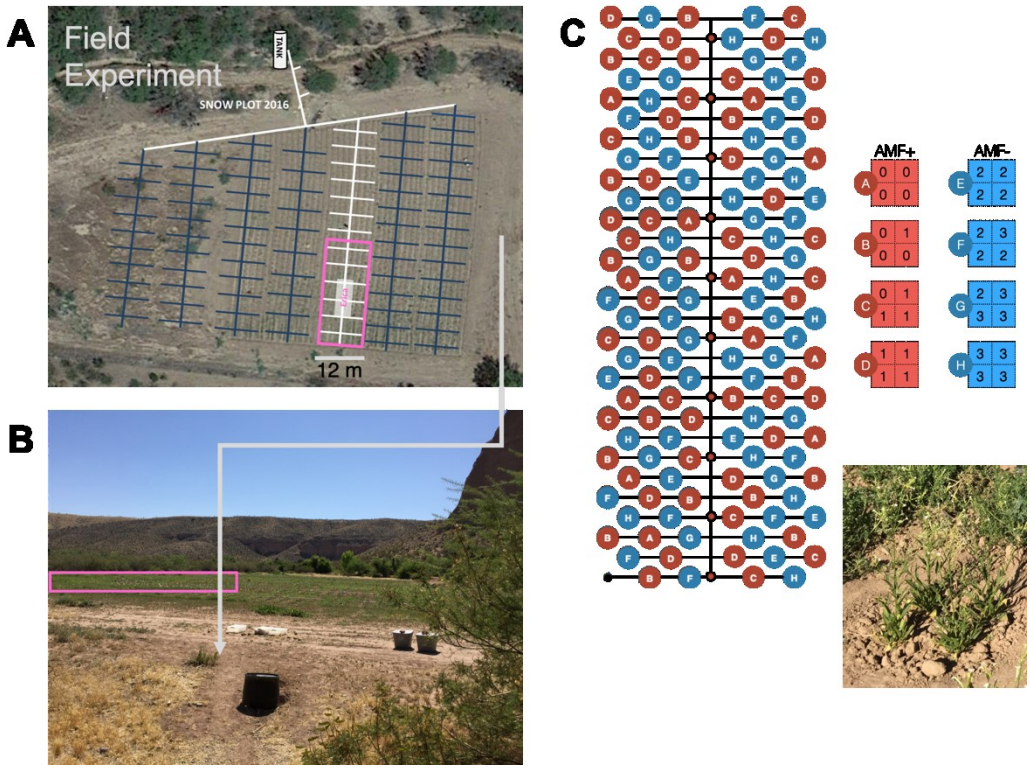

FIGURE S2 - Layout of field population experiment at the Lytle Ranch Reserve field plot ("Snow Plot", Utah) from an A) aerial view (pink, Google Images), from a B) side view (pink, picture by E.M.), and as a C) schematic of all planted populations, with an example population at harvest, 53 days post planting (dpp; inset, picture by E.M).

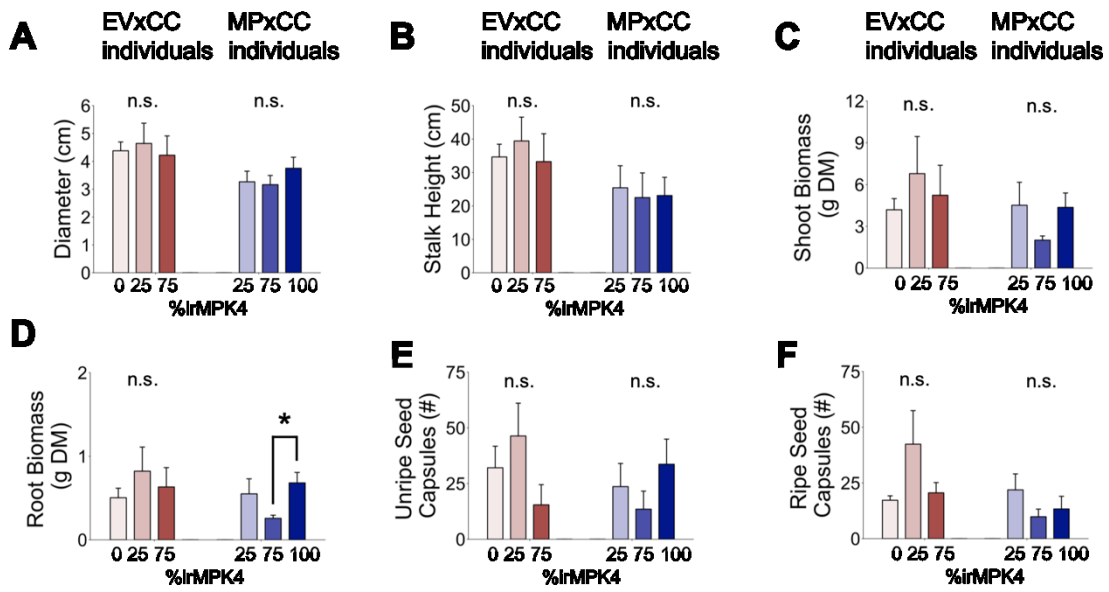

FIGURE S3 - Growth and yield of EV and irMPK4 individuals crossed with irCCaMK (EVxCC and MPxCC, respectively) compared when planted in the varying field population types (see Figure 3A): A) rosette diameter (mean + CI, n = 11-35; 13 dpp), B) stalk height (mean + CI, n = 6-29; 23 dpp), C) shoot biomass (mean + SE, n = 10-31; 46-53 dpp), D) root biomass (mean + SE, n = 8-31; 46-53 dpp), E) unripe seed capsules (mean + SE, n = 4-14; 46-53 dpp) and F) ripe seed capsules (mean + SE, n = 3-17; 46-53 dpp). Significant differences are presented within genotypes (*: $p<0.05$).

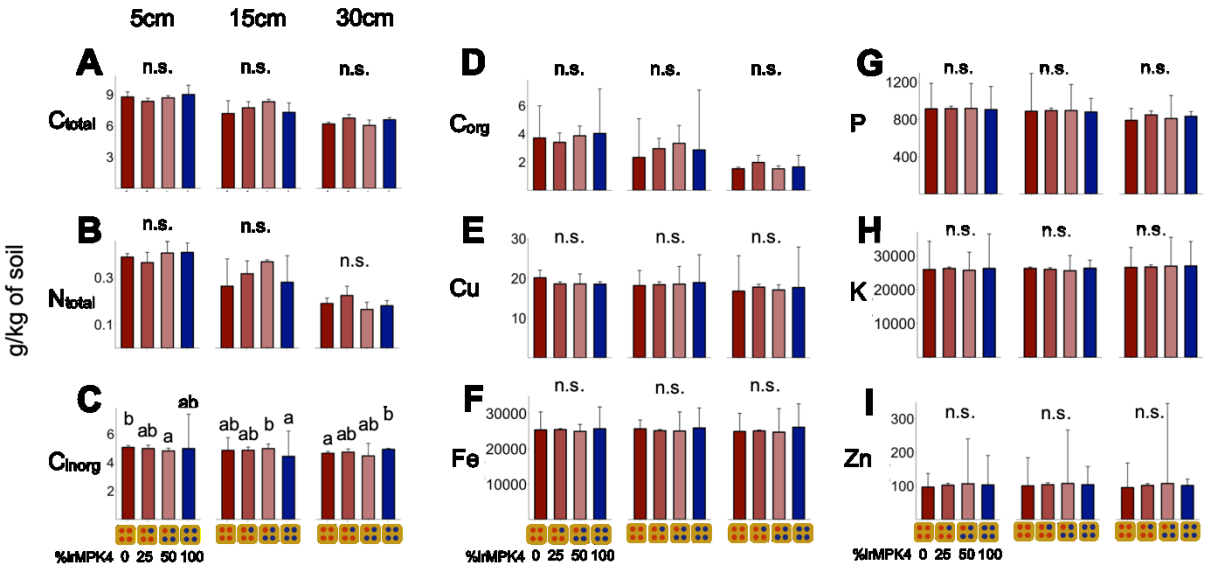

FIGURE S4 - Soil element concentration (mean + CI, y-axis, n = 3) of A) total carbon, B) total nitrogen, C) inorganic carbon, D) organic carbon, E) copper, F) iron, G) phosphorus, H) potassium, and I) zinc, taken from soil cores 5, 15 and 30 cm below the center dripper of each population type (x-axis).

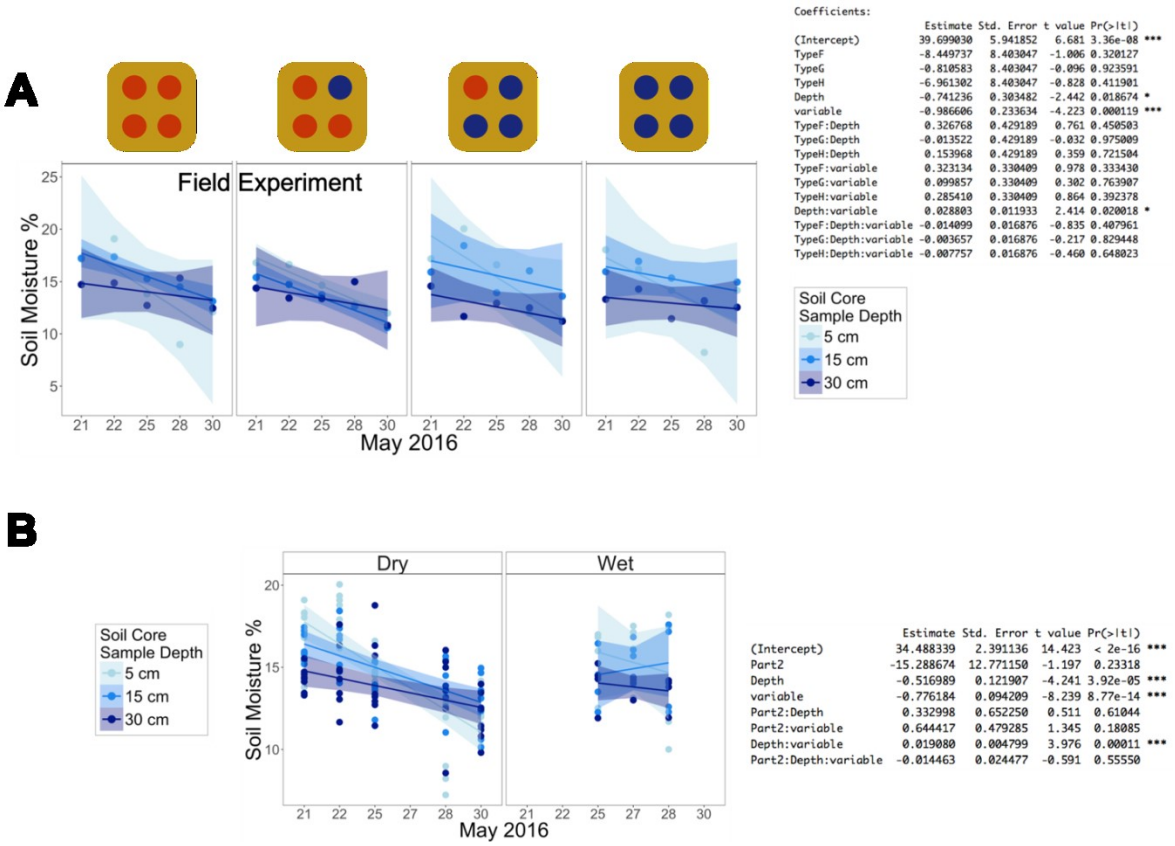

FIGURE S5 - Soil moisture (%) of soil cores taken 5, 15 and 30 cm below the center dripper of each population type over 9 days , either A) cumulatively across the plot or B) divided into the "Dry" and "Wet" subsections (see *Water treatments* in Materials and Methods), with corresponding regression analysis results.

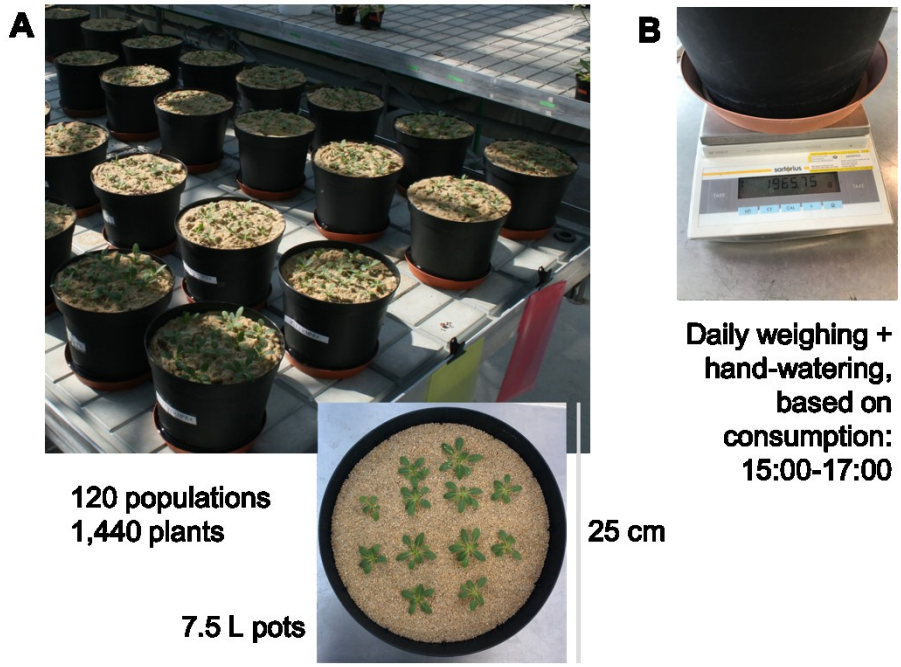

FIGURE S6 - Layout of glasshouse population experiment as pictured from A) above the glasshouse table (picture by D.M.), and above a single example pot (inset, picture by E.M.). Each 7.5L pot was B) weighed daily to determine how much water was lost during the day before as well as provide consumption-based watering based on daily water loss (picture by E.M.).

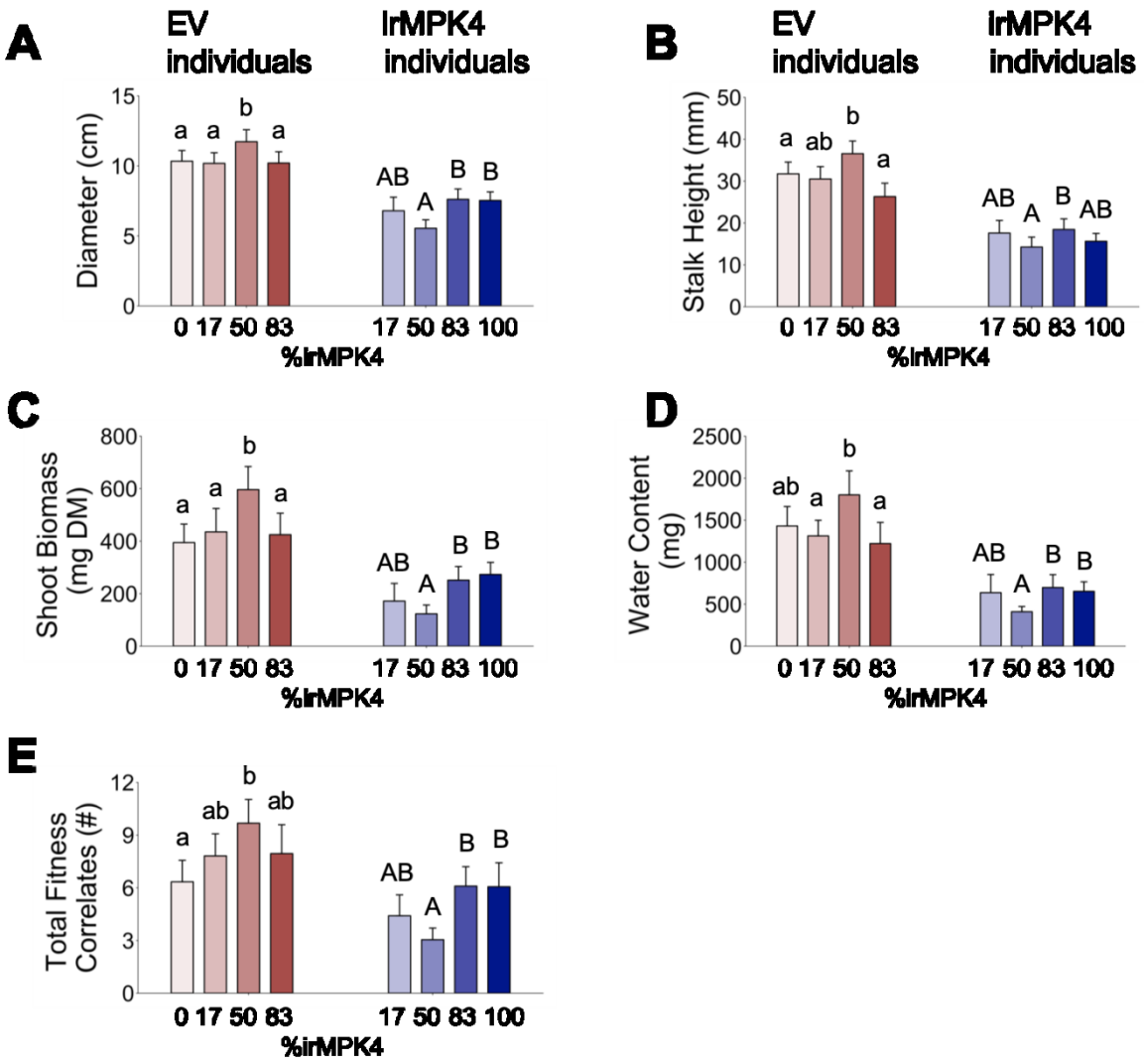

FIGURE S7 - Growth and yield of EV and irMPK4 individuals compared when planted among the varying glasshouse population types (see Figure 3H): A) rosette diameter (mean + CI, n = 11-35; 30 dpp), B) stalk height (mean + CI, n = 21-41; 30 dpp), C) shoot biomass (mean + CI, n = 21-41; 50 dpp), D) water content (mean + CI, n = 22-41; 50 dpp), E) total reproductive yield measured as counts of fitness correlates (buds, flowers, unripe and ripe seed capsules; mean + CI, n = 19-44; 50 dpp). Significant differences are presented within genotypes.

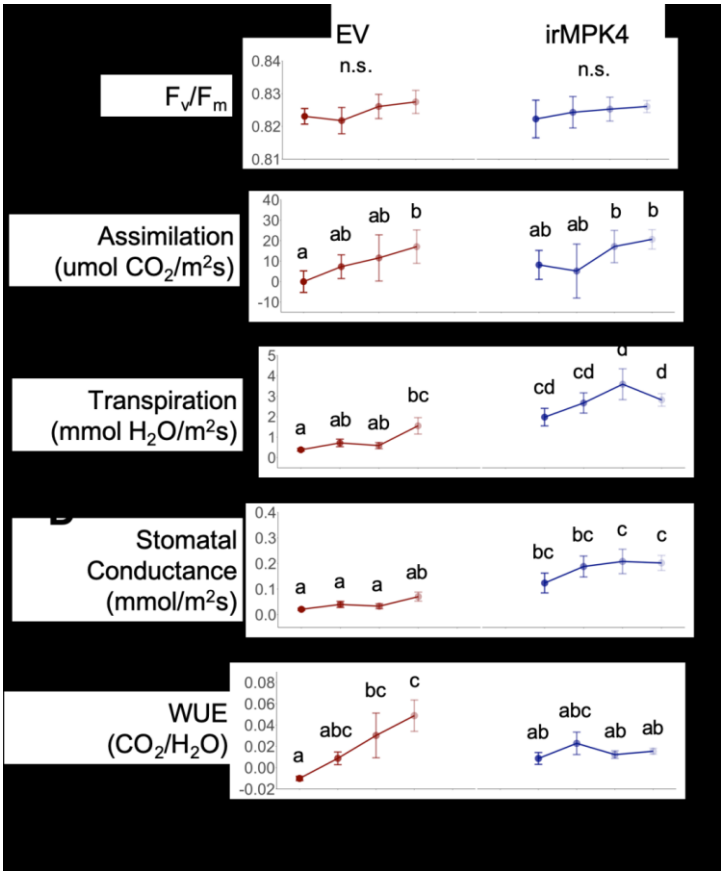

FIGURE S8 - Pre-dawn photosynthetic measurements of EV (red) and irMPK4 (blue) plants in the various planting types of the population glasshouse experiment. A) F_v/F_m, the maximum yield of the photosynthetic systems, B) assimilation rates, C) transpiration rates, D) stomatal conductances, and E) water-use efficiencies (WUEs; mean ± CI, n = 11-32) were measured 32 days post-planting (dpp). Significant differences are presented across genotypes and planting types.

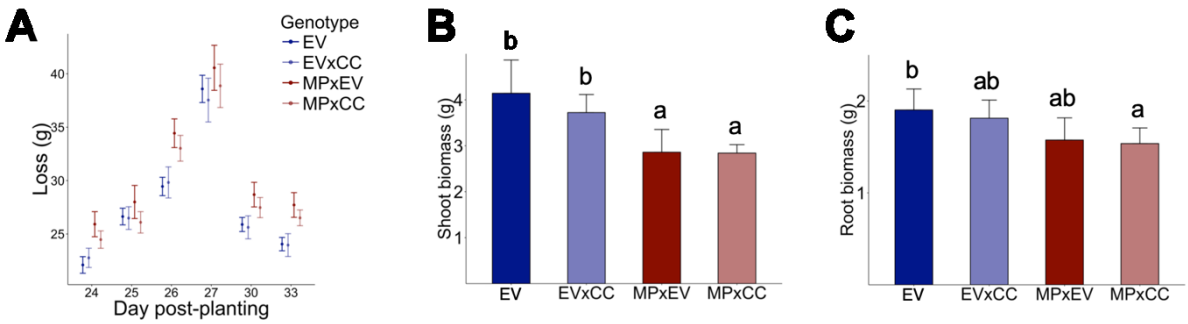

FIGURE S9 - Characterization experiment comparing EV to EV x irCCaMK (EVxCC) and irMPK4xEV (MPxEV) to irMPK4 x irCCaMK (MPxCC) in A) water loss per day (g ± CI, n = 15-16), B) fresh shoot biomass (g + CI, n = 5-7) , and C) fresh root biomass (g + CI, n = 6-7).

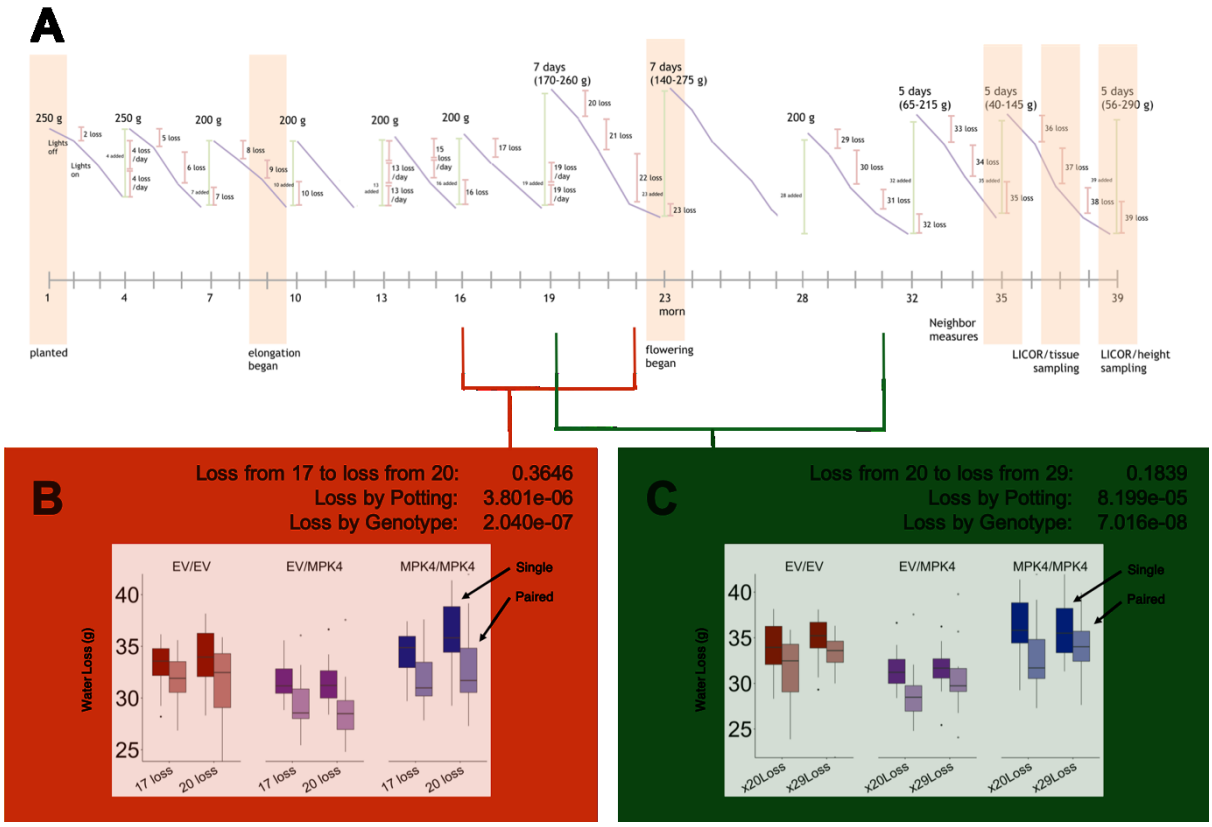

FIGURE S10 - Watering regime of the glasshouse grafted pair experiment: A) the amount of water (g) given to each pot on each day of watering is indicated above the watering event (green). Orange shading highlights key experimental events (developmental changes and sampling times). Two analyses on whether B) increasing the amount of water given to a pot during a watering event changes the water loss per day of the pot (y-axis) from experimental day 17 (given 200 g water) to 20 (given 170-260 g water, x-axis) or whether C) decreasing water given to a pot during a watering event changes the water loss per day of the pot (y-axis) from experimental day 20 (given 170-260 g water) to 29 (given 200 g water, x-axis), are presented. Each analysis is accompanied with respective p-values from an ANOVA conducted on the variables: loss between days, loss between potting type (single: dark colors; paired: faded colors), or loss by genotype of the grafted plant in the pot (red: EV/EV; purple: EV/irMPK4; blue: irMPK4/irMPK4).

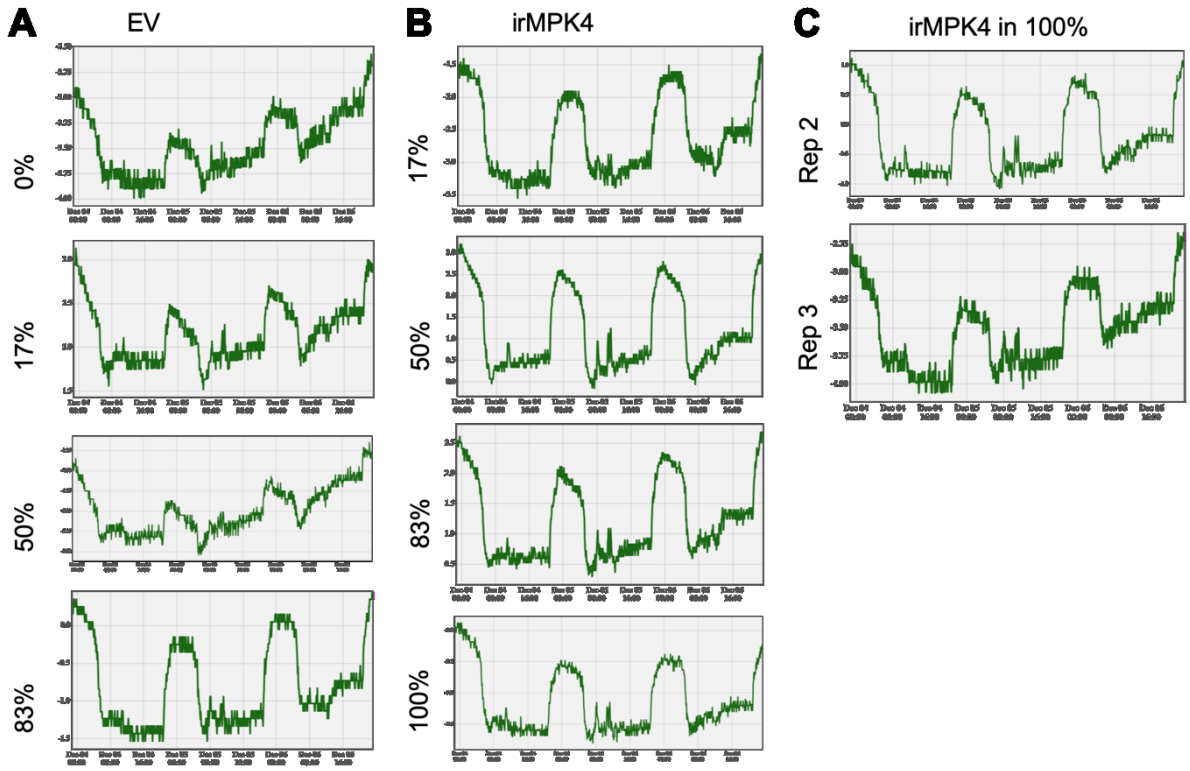

FIGURE S11 - Yara ZIM-probe leaf turgor measurements (mean kPa, representative recordings of 3 replicate measurements are shown). Leaf turgor was recorded over the 48h period from 00:00 December 4th to 00:00 December 7th on A) EV and B) irMPK4 individuals in all glasshouse population types. Note: relative turgor pressure values (y-axis) are not comparable among recordings due to the different clamping pressure at the initialization of recording (see *Leaf turgor and potential effects of controlled watering on diurnal rhythms* in Materials and Methods). C) Two additional replicates of irMPK4 plants in 100% populations demonstrate that within treatment group variance in peak-trough values exceeds among treatment group variance.

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CHAPTER **4**

4. Blumenols as shoot markers of root symbiosis with arbuscular mycorrhizal fungi

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5.1 Abstract

High-through-put (HTP) screening for functional arbuscular mycorrhizal fungi (AMF)-associations is challenging because roots must be excavated and colonization evaluated by transcript analysis or microscopy. Here we show that specific leaf-metabolites provide broadly applicable accurate proxies of these associations, suitable for HTP-screens. With a combination of untargeted and targeted metabolomics, we show that shoot accumulations of hydroxy- and carboxyblumenol C-glucosides mirror root AMF-colonization in *Nicotiana attenuata* plants. Genetic/pharmacologic manipulations indicate that these AMF-indicative foliar blumenols are synthesized and transported from roots to shoots. These blumenol-derived foliar markers, found in many di- and monocotyledonous crop and model plants (*Solanum lycopersicum, Solanum tuberosum, Hordeum vulgare, Triticum aestivum, Medicago truncatula* and *Brachypodium distachyon*), are not restricted to particular plant-AMF interactions, and are shown to be applicable for field-based QTL mapping of AMF-related genes.

5.2 eLife Digest

All plants need a nutrient called phosphorus to grow and thrive. Phosphorus is found in soil, but the supply is limited so plants often struggle to acquire enough of it. To overcome this problem, many plants form friendly relationships (or symbioses) with certain fungi in the soil known as arbuscular mycorrhizal fungi. The fungi colonize plant roots and supply phosphorus and other nutrients in return for sugars and various molecules.

Although many crop plants – including barley and potatoes – are able to form these symbioses, farmers commonly apply fertilizers containing phosphate and other nutrients to their fields to increase the amount of food they produce. Breeding new crop varieties that are better at forming symbioses with the fungi could reduce the need for fertilizers. However, the methods currently available to study these relationships are laborious and time-consuming, typically requiring samples of plant roots to be examined in a laboratory.

Wang, Schäfer *et al*. used an approach called metabolomics to search for molecules in coyote tobacco plants that indicate the plants have formed symbioses with arbuscular mycorrhizal fungi. The experiments found that a group of molecules called blumenols accumulate in the roots and also in the shoots and leaves of plants with these symbioses, but not in the tobacco plants that were not able to associate with the fungi. Experiments in several other plant species including tomato, potato and barley produced similar findings, suggesting that the blumenols

may be a useful and potentially universal indicator of symbioses between many different plants and fungi.

Measuring the levels of blumenols in plant shoots and leaves is much quicker and easier than current methods of identifying fungal symbioses in plant root samples. Therefore, blumenols may be a useful tool for plant breeders who would like to screen large numbers of plants for these symbioses, and breed crops that negotiate better interactions with the beneficial fungi

5.3 Introduction

More than 70% of all higher plants, including crop plants, form symbiotic associations with arbuscular mycorrhizal fungi (AMF) (Brundrett and Tedersoo, 2018). While the fungus facilitates the uptake of mineral nutrients, in particular phosphorous (P) and nitrogen, the plant supplies the fungus with carbon (Helber *et al*., 2011; Bravo *et al*., 2017; Jiang *et al*., 2017; Keymer *et al*., 2017; Luginbuehl *et al*., 2017). The interaction affects plant growth (Rooney *et al*., 2009; Adolfsson *et al*., 2015) and resistance to various abiotic and biotic stresses (Pineda *et al*., 2010; Vannette *et al*., 2013; Chitarra *et al*., 2016; Sharma *et al*., 2017). Although AMF interactions are physically restricted to the roots, they influence whole-plant performance, hence systemic metabolic responses have been anticipated, and searched for, but no general AMF-specific responses have been found (Bi *et al*., 2007; Toussaint, 2007; Schweiger and Müller, 2015). While changes in foliar levels of carbohydrates, proteins, and amino acids, as well as secondary metabolites and phytohormones have been shown to respond to AMF inoculation (Schweiger *et al*., 2014; Aliferis *et al*., 2015; Adolfsson *et al*., 2017), these changes are not specific to AMF interactions and tend to be general responses to various abiotic and biotic stresses. Moreover, these metabolic responses also tend to be taxa-specific, and many are likely indirect consequences of AMF-mediated effects on plant growth and development.

In contrast, large amounts of blumenol-type metabolites accumulate in roots after AMF inoculation. These compounds are apocarotenoids, in particular C_{13} cyclohexenone derivatives, produced by the cleavage of carotenoids. After AMF colonization, a C_{40} carotenoid is cleaved by carotenoid cleavage dioxygenase 7 (CCD7) to produce a C_{13} cyclohexenone and a C_{27} apocarotenoid which is further cleaved by CCD1 to yield a second C_{13} cyclohexenone (Floss *et al*., 2008; Vogel *et al*., 2010; Hou *et al*., 2016). The compounds have been found to accumulate in the roots of AMF-colonized plants in a manner highly correlated with the fungal colonization rate (Fester *et al*., 1999). Other stimuli such as pathogen infection and abiotic stresses, do not induce their accumulations (Maier *et al*., 1997). The AMF-induced accumulation of these compounds is widespread and has been observed in roots of plant species from different families, including mono- and dicotyledons, (*Hordeum vulgare*, Peipp *et al*., 1997); *Solanum lycopersicum* and *Nicotiana tabacum*, Maier *et al*., 2000; e.g., *Zea mays*, Fester *et al*., 2002; *Lotus japonicus* and *Medicago truncatula*, Fester *et al*., 2005; *Ornithogalum umbellatum*, Schliemann *et al*., 2006; *Allium porrum*, Schliemann *et al*., 2008).

Blumenols are classified into three major types; blumenol A, blumenol B and blumenol C (Figure 1A). However, previous studies have reported that only blumenol glycosides containing a blumenol C-based aglycone are positively correlated with mycorrhizal colonization. The aglycone can be additionally hydroxylated at the C11 or carboxylated at the C11 or C12 position (Maier *et al*., 1997; Maier *et al*., 2000). Additionally, 7,8-didehydro versions of blumenol C have been reported (Peipp *et al*., 1997). The glycosylation usually

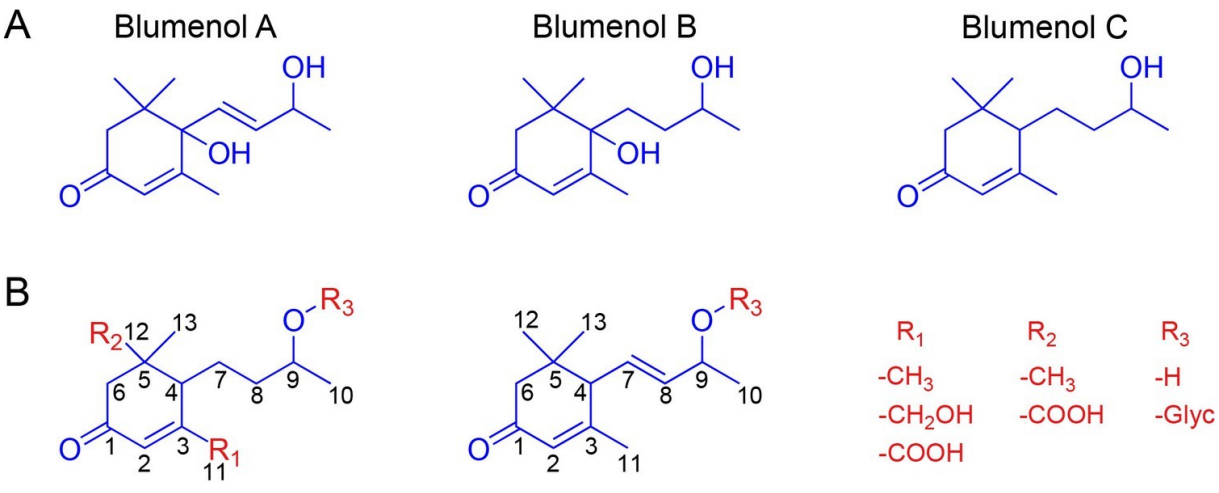


FIGURE 1 - Blumenol core structures and exemplary modifications. (A) Structure of blumenol A, blumenol B and blumenol C. (B) Exemplary blumenol C derivatives. Glyc, glycoside.

occurs as an *O*-glycoside at the C9 position (Strack and Fester, 2006), but glycosylations at the hydroxylated C11 position have also been observed (Schliemann *et al*., 2008). The glycosyl moiety can be a single sugar or combinations of glucose (Glc), rhamnose, apiose, arabinose and/or glucuronic acid, which, in turn can be additionally malonylated or contain a 3-hydroxy-3-methylglutarate decoration (Strack and Fester, 2006; Schliemann *et al*., 2008). The connections among sugar components can also vary (e.g., glucose-(1''→4')-glucose or glucose-(1''→6')-glucose; Maier *et al*., 2000; Fester *et al*., 2002). The particular type of decorations appears to be highly species-specific and it is likely that additional structural variants remain to be discovered. Exemplary structures are shown in Figure 1B.

Interestingly, blumenols such as blumenol A, blumenol A-9-*O*-Glc, blumenol B, blumenol C and blumenol C-9-*O*-Glc, were also reported to occur in the aerial parts of various plant species (Galbraith and Horn, 1972; Bhakuni *et al*., 1974; Takeda *et al*., 1997). However, most of these studies focused on the identification of natural products using large scale extractions (up to several kg of plant material) and were not performed in the context of AMF colonization. Furthermore, some blumenol compounds were also found in plant families that are known to have lost their ability to establish AMF interactions (Brassicaceae: Cutillo *et al*., 2005; Urticaceae: Aishan *et al*., 2010). These reports indicate AMF-independent constitutive levels of particular blumenols in aerial plant parts. Adolfsson *et al*., 2017 analyzed blumenol accumulations together with other metabolites in leaves of plants with and without AMF colonization. None of these studies reported AMF-specific accumulations of blumenols or transcripts specific for their biosynthesis. The concentrations of some blumenol derivatives were even reported to be down-regulated in response to AMF colonization (Adolfsson *et al*., 2017).

The identification of a reliable metabolite marker in aerial plant tissues would be highly useful for AMF research since the characterization of AMF-associations is still laborious and time-consuming, typically requiring destructive root harvesting and microscopic examination or transcript analyses (Vierheilig *et al*., 2005; Parádi *et al*., 2010). To identify readily accessible AMF-indicative shoot metabolites, we hypothesized that a subset of the AMF-induced root metabolites would accumulate in shoots as a result of transport or systemic signaling.

5.4 Results

Blumenols are AMF-indicative metabolic fingerprints in roots

We performed an untargeted metabolomics analysis of root tissues in a transgenic line of *Nicotiana attenuata*, silenced in the calcium- and calmodulin-dependent protein kinase (ir*CCaMK*) and empty vector (EV) plants co-cultured with or without *Rhizophagus irregularis* (Figure 2A). By using ir*CCaMK* plants, unable to establish a functional AMF-association (Groten *et al*., 2015), we were able to dissect the AMF association-specific metabolic responses from those changes that result from more general plant-fungus interactions. Untargeted metabolome profiling of roots using liquid chromatography (LC) coupled to time-of-flight mass spectrometry (qTOF-MS) resulted in a concatenated data matrix consisting of 943 mass features (*m/z* signals detected at particular retention times). A co-expression network analysis was conducted in which nodes represent *m/z* features and edges connect metabolite mass features originating from similar in-source fragmentations and sharing biochemical relationships (Li *et al*., 2015; Li *et al*., 2016). For example, features representing well-known compounds, like nicotine and phenylalanine, were tightly connected (Figure 2B). A STEM clustering pipeline was performed to recognize patterns of metabolite accumulations in the genotype × treatment data matrix [(EV/ir*CCaMK*) × (-/+AMF inoculation)]. As a result, 5 of 8 computed distinct expression patterns were mapped onto the covariance network in Figure 2B (shown in different colors). A tightly grouped cluster of unknown metabolites, highlighted in red (Figure 2B) occupied a distinct metabolic space. Metabolites grouped in this cluster were highly elicited upon mycorrhizal colonization in EV, but not in ir*CCaMK* plants and not found in plants without AMF associations (Figure 2C). The structures of the compounds of this cluster were annotated based on tandem-MS and NMR data. Five metabolites were annotated as blumenols: 11-hydroxyblumenol C-9-*O*-Glc (Figure 2C; Compound 1), 11-carboxyblumenol C-9-*O*-Glc (Figure 2C; Compound 2), 11-hydroxyblumenol C-9-*O*-Glc-Glc (Compound 3), blumenol C-9-*O*-Glc-Glc (Compound 4) and blumenol C-9-*O*-Glc (Compound 5).

To quantify these compounds throughout the plant, we used a more sensitive and specifically targeted metabolomics approach based on LC-triple-quadrupole-MS. The abundance of the five blumenol C-glycosides continually increased with mycorrhizal development (Figure 2—figure supplement 1A) and was highly correlated with the mycorrhizal colonization rate as determined by the transcript abundances of classical arbuscular mycorrhizal symbiosis-marker genes (fungal house-keeping gene, *Ri-tub; plant* marker genes, *Vapyrin*, *RAM1*, *STR1* and *PT4;* Park *et al*., 2015; Figure 2—figure supplement 1B, Data Set 1).

Hydroxy- and carboxyblumenol C-glucoside levels in leaves positively correlate with root colonizations

Compounds 1 and 2 showed a similar AMF-specific accumulation in the leaves, as observed in the roots (Figure 2D). The other analyzed blumenols were not detected in leaves (Compounds 3 and 4; Figure 3—figure supplement 1A) or showed a less consistent AMF-specific accumulation (Compound 5; due to its constitutive background level; Figure 3—

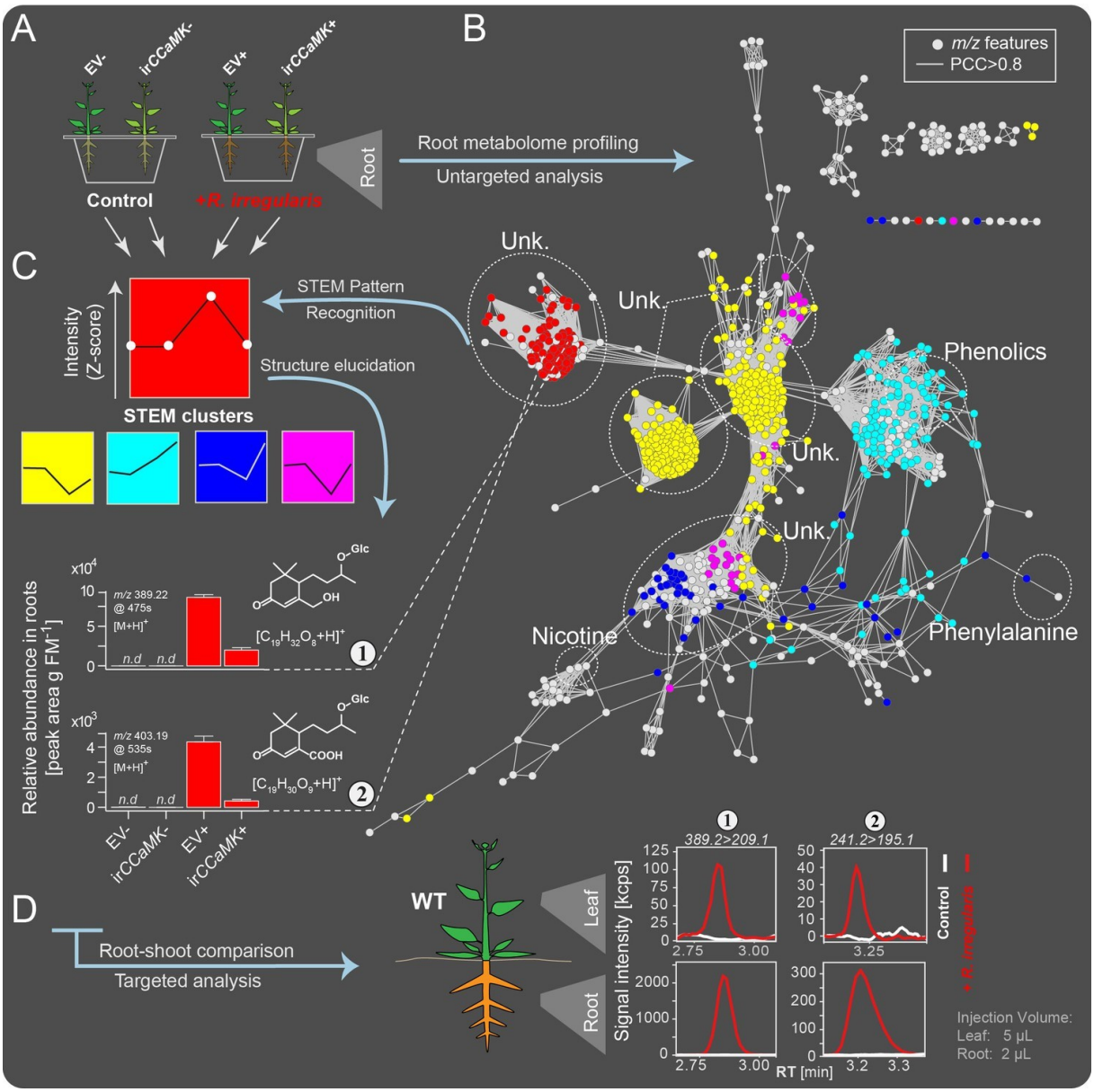


FIGURE 2 - Combined targeted and untargeted metabolomics identified blumenol derivatives as AMF-indicative in-planta metabolic fingerprints in the roots and leaves of *Nicotiana attenuata* plants. (A) Experimental set-up. EV and irCCaMK plants were co-cultured and inoculated with or without *R. irregularis*. Six weeks after inoculation (wpi), root samples were harvested for metabolite profiling. (B) Covariance network visualizing m/z features from UHPLC-qTOF-MS untargeted analysis (n = 8). Known compounds, including nicotine, phenylalanine and various phenolics, and unknown metabolites (Unk.) are enclosed in dashed ellipses. (C) Normalized Z-scores of m/z features were clustered using STEM Clustering; 5 of 8 significant clusters are shown in different colors and mapped onto the covariance network. The intensity variation (mean +SE) of 2 selected features (Compounds 1 and 2) are shown in bar plots (n.d., not detected). (D) Representative chromatograms of Compounds 1 and 2 in roots and leaves of plants with and without AMF inoculation, as analyzed by targeted UHPLC-triple quadrupole-MS metabolomics.

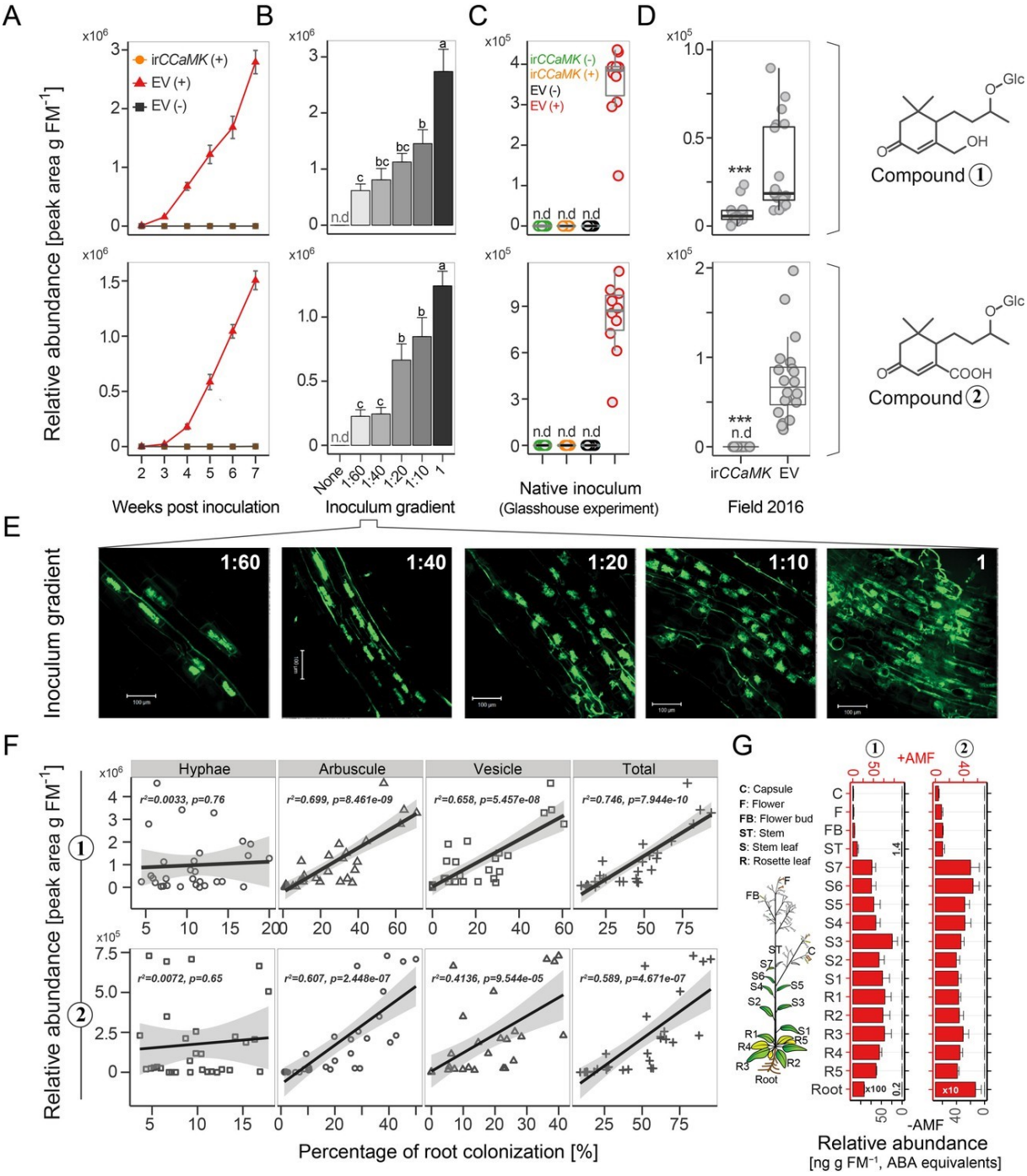


FIGURE 3 - Compounds 1 and 2 are leaf markers of root AMF colonization in *N. attenuata*. (A) Time lapse accumulations of Compounds 1 and 2 in leaves of EV plants with (EV+, red) or without (EV-, black) AMF-inoculation and of irCCaMK plants with AMF-inoculation (irCCaMK+, orange, covered by black) (means ± SE, n ≥ 5). (B) Leaf abundances of Compounds 1 and 2 (five wpi) of plants inoculated with different inoculum concentrations (means + SE, n ≥ 4); different letters indicate significant differences ($p<0.05$, one-way ANOVA followed by Fisher's LSD). (C) Compounds 1 and 2 in leaf samples of EV and irCCaMK plants inoculated with (+) or without (-) AMF inoculum isolated from the plant's native habitat (six wpi); different letters indicate significant differences ($p<0.05$, one-way ANOVA followed by Tukey's HSD, n = 10). (D) Field experiment (Great Basin Desert, Utah, USA): Compounds 1 and 2 in leaf samples of EV (n = 20) and irCCaMK (n = 19) plants sampled eight weeks after planting. (Student's *t-test*: ***$p<0.001$). (E) Representative images of WGA-488 stained roots of plants shown in B) (bar = 100 µm). (F) Leaf Compounds 1 and 2 relative to the percentage of root colonization by hyphae, arbuscules, vesicles and total root length colonization of the same plants (linear regression model). (G) Compounds 1 and 2 in 17 different tissues of plants with (+AMF, n = 3, red bars) or without (-AMF, n = 1, black bars) AMF-inoculation harvested at six wpi.

figure supplement 1A). The identity of Compounds 1 and 2 in the leaves was verified by high resolution qTOF-MS (Figure 3—figure supplement 1B–E).

Next, we determined the correlations between the contents of AMF-indicative foliar Compounds 1 and 2 and root colonization rates. In a kinetic experiment, the amount of both compounds steadily increased in the leaves of plants inoculated with *R. irregularis* (Figure 3A, Figure 3—figure supplement 2). At three wpi, the abundance of compounds 1 and 2 in the leaves was sufficient to reflect the colonization level of the roots. In contrast, the classical AMF-marker-genes, which are usually analyzed in the roots, did not respond in the leaves (Figure 4). In an inoculum-gradient experiment using increasing inoculum concentrations, proportionally higher Compound 1 and 2 levels were observed (Figure 3B), accurately reflecting the differential colonization of roots across treatments (Figure 3E). In addition to inoculation with a single AMF species (*R. irregularis*), we also tested mycorrhizal inoculum originally collected from the plant's native habitat, the Great Basin Desert in Utah, USA, which mainly consists of *Funneliformis mosseae* and *R. irregularis*. EV plants inoculated with this 'natural inoculum' also accumulated Compounds 1 and 2 in leaves, while ir*CCaMK* plants did not (Figure 3C). The analysis of a second independently transformed irCCaMK line confirmed the result that when the association with *R. irregularis* was genetically abrogated, Compounds 1 and 2 failed to accumulate in leaves of plants co-cultured with the AMF (Figure 3—figure supplement 3). When planted into the plant's natural environment in Utah, both EV and ir*CCaMK* plants could be clearly distinguished by their leaf Compound 1 and 2 contents. The signature of Compound 2 provided a better quality marker in these field-grown plants (Figure 3D, Figure 3—figure supplement 4). The foliar contents of these two compounds were highly correlated with the percentage of arbuscules in roots, the core structure of AMF interactions (Figure 3F, Figure 3—figure supplement 2). In contrast, other biotic or abiotic stresses, including herbivory, pathogen infection and drought stress, did not elicit the foliar accumulations of Compounds 1 and 2 (Figure 5). Such stimuli also do not induce blumenol accumulation in roots (Maier *et al*., 1997). An analysis of various plant tissues, including different leaf positions, stem pieces, flowers and capsules revealed that these AMF-specific signatures accumulated throughout the shoot (Figure 3G). Taken together, we conclude that the contents of particular blumenols in aerial plant parts robustly reflect the degree of mycorrhizal colonization in *N. attenuata* plants.

AMF–indicative blumenols in shoots most likely originate from the roots

Blumenols are apocarotenoids originating from a side branch of the carotenoid pathway (Hou *et al*., 2016). Most of the candidate genes for blumenol biosynthesis were upregulated in roots, but not in leaves of *N. attenuata* plants in response to mycorrhizal colonization (Figure 6A, Figure 6—figure supplement 1A). We inferred that these AMF-indicative leaf apocarotenoids are transported from their site of synthesis in colonized roots to other plant parts. This is consistent with the occurrence of blumenols in stem sap (Figure 6—figure supplement 1B) which was collected by centrifuging small stem pieces. To clarify the origins (local biosynthesis vs. transport) of these leaf blumenols, we genetically manipulated the carotenoid biosynthesis of *N. attenuata* plants. To minimize the effects of a disturbed carotenoid biosynthesis on the AMF-plant interaction, we used the dexamethasone (DEX)-inducible pOp6/LhGR system to silence phytoene desaturase (PDS) expression in a single DEX-treated leaf position (Schäfer *et al*., 2013). Treated leaves showed clear signs of bleaching, indicating PDS silencing (Figure 6B, Figure 6—figure supplement 1C), but levels

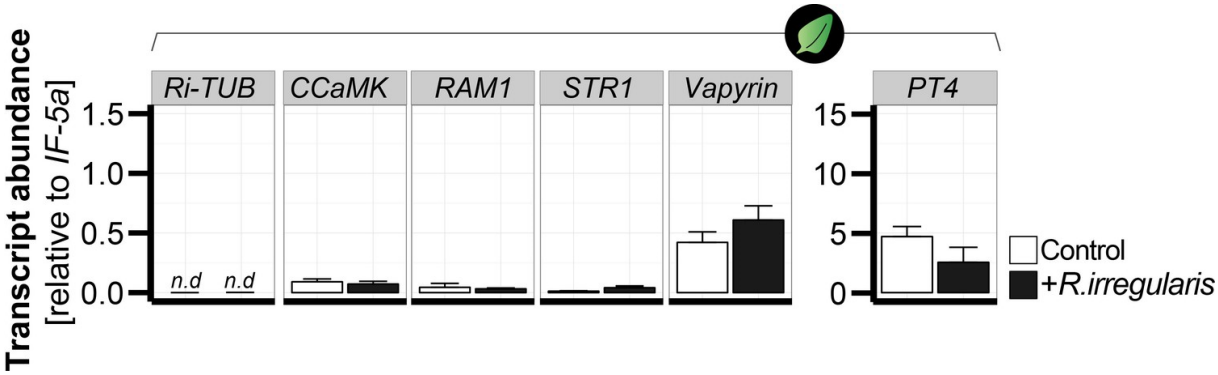
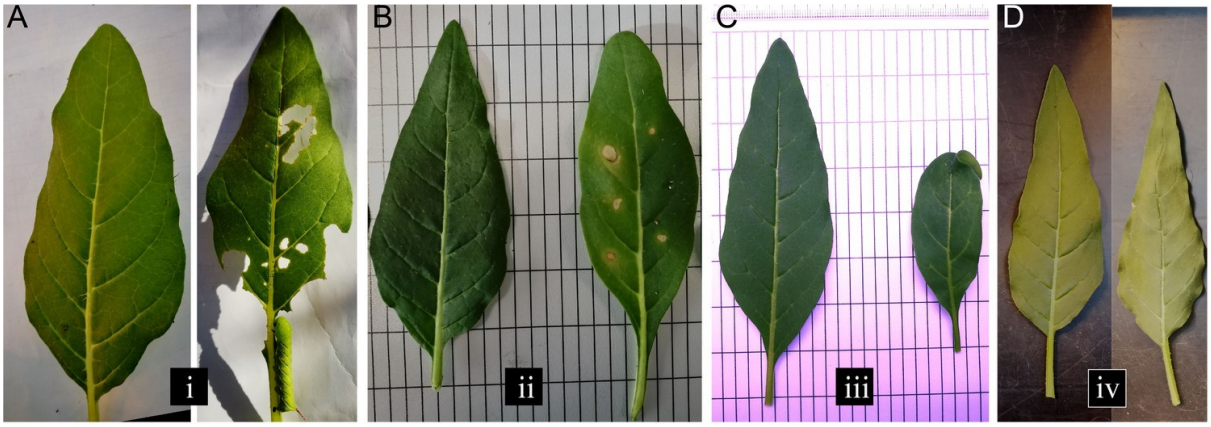
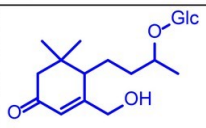


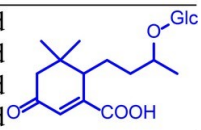
FIGURE 4 - Transcript abundance of classical arbuscular mycorrhizal symbiosis-marker genes do not respond in leaves of mycorrhizal and control *N. attenuata* plants. The transcript abundance (relative to *NaIF-5a*) of classical root marker genes was analyzed in leaves of *N. attenuata* plants in the presence (+*R. irregularis*, black bars) and absence (control, white bars) of root colonization with *R. irregularis*. The marker genes include the *R. irregularis* specific housekeeping gene, *Ri-tub*, as well as the plant-derived marker genes *CCaMK*, *Vapyrin*, *PT4*, *STR1* and *RAM1*. Leaf samples were harvested six wpi and analyzed by qPCR. Data represent means +SE ($n \geq 3$), n.d., not detected.



E

Treatment	Compound 1	Compound 2
i. Herbivory (*M. sexta*)	n.d	n.d
ii. Pathogen infection (*B. cinerea*)	n.d	n.d
iii. Bacteria & virus infection	n.d	n.d
iv. Drought (3 days)	n.d	n.d





n.d, not detected

iii. Infection with *A. tumefaciens* carrying the *Tobacco Rattle Virus*

FIGURE 5 - Different biotic and abiotic stresses do not elicit accumulations of Compounds 1 and 2 in leaves. (A–D) Representative leaves of *N. attenuata* plants subjected to different stresses (right leaf), as well as the untreated controls (left leaf): (A) *Manduca sexta* feeding for 10 days; (B) *Botrytis cinerea* infection for five days. (C) Infection for two weeks with *Agrobacterium tumefaciens* carrying the Tobacco Rattle Virus; (D) Dehydration for three days. For each treatment, four biological replicates were used. (E) Accumulation of Compounds 1 and 2 in treated samples from (A–D). n.d., not detected.

of the AMF-indicative Compounds 1 and 2 were not affected, consistent with their transport from other tissues, likely the highly accumulating roots. As a control, we analyzed the non-AMF-inducible Compound 6, showing constitutive levels in aerial tissues (Figure 6—figure supplement 2). In DEX-treated leaves, Compound 6 concentrations were reduced by nearly 40%, consistent with local production (Figure 6B, Figure 6—figure supplement 1D). To confirm the within-plant transport potential of blumenols, we dipped roots of seedlings into aqueous solutions of Compounds 1 or 2. After overnight incubation, the blumenol derivatives were clearly detected not only in roots, but also in shoots (Figure 6C, Figure 6—figure supplement 1E).

The analysis of AMF-indicative blumenols as HTP screening tool for forward genetics approaches

To test the potential of the foliar AMF-indicative metabolites as a screening tool, we quantified the concentration of Compounds 1 and 2 in leaves of plants of a population of recombinant inbred lines (RILs) of a forward genetics experiment, an experiment which would be challenging with the classical screening tools of root staining or nucleic acid analysis. We focused our analysis on Compound 2 due to the superior quality of its signature in the leaves of field-grown plants (Figure 3—figure supplement 4). The experiment consisted of a population of RILs from a cross of two *N. attenuata* accessions (Utah, UT and Arizona, AZ) (Zhou *et al*., 2017) which differ in their mycorrhizal colonization (Figure 7A–B, Figure

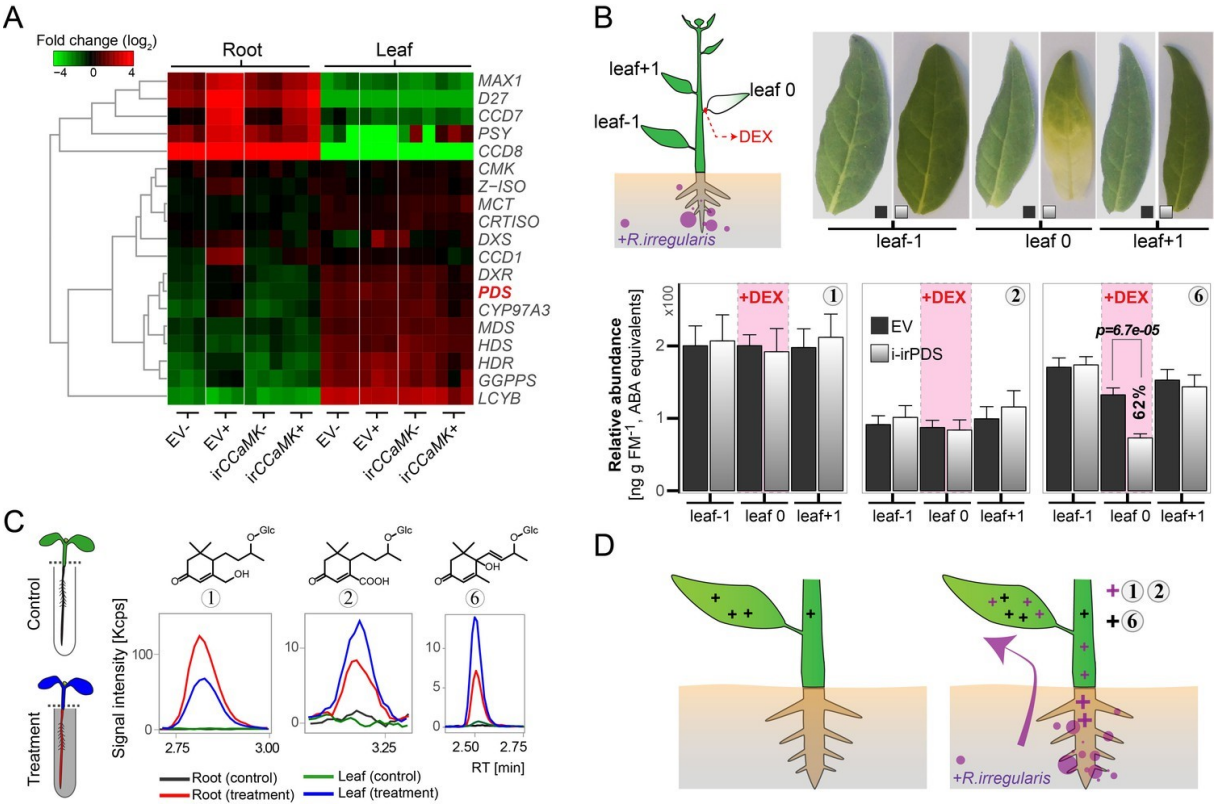


FIGURE 6 - AMF-indicative Compounds 1 and 2 in shoots of mycorrhizal plants originate from the roots. (A) Hierarchical clustering analysis of transcript abundance from RNA-seq of methylerythritol 4-phosphate (MEP) and (apo)carotenoid biosynthetic genes (for details see Figure 6—figure supplement 1A). (B) Compounds 1, 2 (AMF-specific) and 6 (not AMF-specific) in AMF-inoculated i-irPDS and EV plants. On each plant, a single stem leaf (leaf 0) was elicited with 100 µM DEX-containing paste for three weeks; treated and adjacent, untreated control leaves (leaf −1 and leaf +1) were harvested. Representative leaves are shown (bleaching indicates PDS silencing); (means +SE, n ≥ 6). The same leaf positions in i-irPDS and EV plants were compared by Student's *t*-tests. (C) Contents of Compounds 1, 2 and 6 in the roots and shoots of seedlings whose roots were dipped for 1 d into an aqueous solution with (treatment) or without (control) AMF-indicative blumenols. (D) Model of blumenol distribution in plants with (right panel) and without (left panel) AMF colonization. The model illustrates constitutive blumenols (e.g., Compound 6 in *N. attenuata*) and AMF-indicative ones (e.g., Compounds 1 and 2 in *N. attenuata*) and their inferred transport.

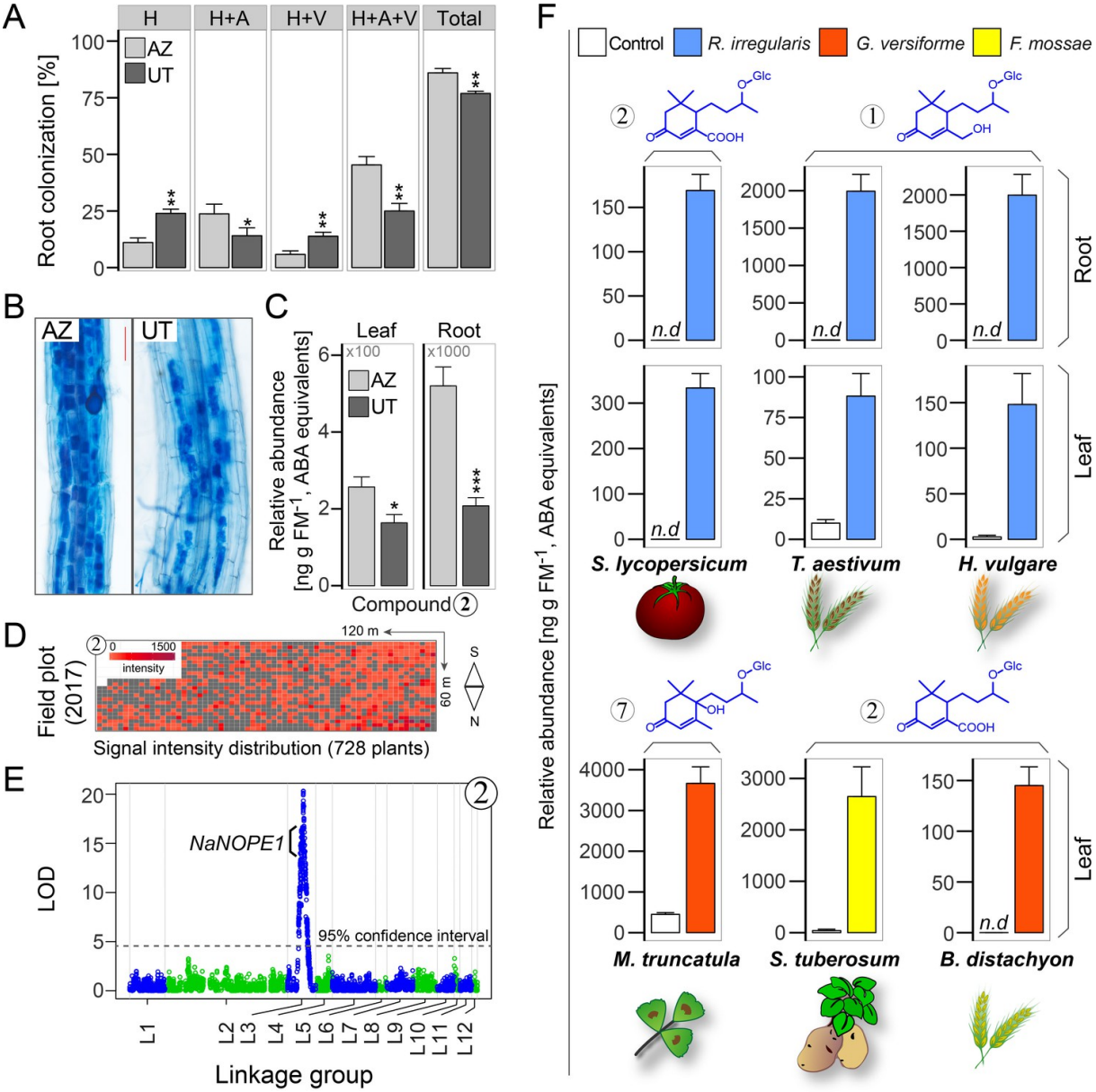


FIGURE 7 - AMF-indicative changes in blumenols in aerial plant parts are valuable research tools providing accurate assessments of functional AMF associations in high-throughput screenings of multiple plant and AMF species. (A) Root colonization analysis in two *N. attenuata* accessions (UT/AZ). H: hyphae; A: arbuscules; V: vesicles; Total: total root length colonization (n = 4; Student's *t*-test, *p<0.05, **p<0.01, ***p<0.001). (B) Representative images of trypan blue stained roots (six wpi; bar = 100 µm). (C) Compound 2 in roots and leaves of UT and AZ plants with and without AMF-inoculation (means +SE, n = 8). (D) Heatmap of the normalized abundance of foliar Compound 2 in plants of a UT-AZ RIL population (728 plants) planted across a 7,200 m2 field plot. (E) QTL mapping analysis of the data from D. The QTL on linkage group three contains *NaNOPE1*, an AMF-associated gene, in addition to others. LOD, logarithm of the odds ratio. (F) Blumenol contents of different crop and model plants with and without AMF inoculation (*S. lycopersicum* (n = 6), *T. aestivum* (n = 10), *H. vulgare* (n = 5): eight wpi; *M. truncatula* (n = 3): seven wpi; *S. tuberosum* (n = 5): six wpi; *B. distachyon* (n = 4): five wpi). Different plant and AMF species were used as indicated (means +SE; n.d., not detected).

7—figure supplement 1) and accumulation of foliar Compound 2 in the glasshouse (Figure 7C). A QTL analysis of 728 plants grown across a 7200 m^2 field plot (Figure 7D) revealed that the abundance of Compound 2 mapped to a single locus on linkage group 3 (Figure 7E), which

harbored a homologue of *NOPE1* (*NIATv7_g02911*) previously shown to be required for the initiation of AMF symbioses in maize and rice (Nadal *et al*., 2017). Transcripts of *NaNOPE1* were more abundant in AZ roots after AMF inoculation, but did not differ significantly in leaves (Figure 7—figure supplement 1B). While clearly requiring additional follow-up work, these results highlight the value of these signature metabolites for HTP screens, which form the basis of most crop improvement programs.

AMF-indicative blumenols in shoots are a widespread response of various plant species to different kinds of AMF

The AMF-specific accumulation of blumenol C-derivatives in roots is a widespread phenomenon within higher plants (Strack and Fester, 2006); however, how general are the observed blumenol changes in aerial parts across different combinations of plants and AMF species? We analyzed *Solanum lycopersicum*, *Triticum aestivum* and *Hordeum vulgare* plants with and without AMF inoculation and again we found an overlap in the AMF-specific blumenol responses in roots and leaves, consistent with the transport hypothesis. Further analyses led to the identification of additional AMF-indicative blumenols in the leaves of *Medicago truncatula*, *Solanum tuberosum* and *Brachypodium distachyon*. We identified various types of blumenols that showed an AMF-specific accumulation in the shoot, including blumenol B (Compound 7), which has not previously been reported in an AMF-dependent context (Figure 7F; Figure 7—figure supplement 2). As reported for roots, the particular blumenol types were species-dependent, but the general pattern was widespread across monocots and dicots in experiments conducted at different research facilities. In tests with diverse fungal species (*R. irregularis, F. mosseae* and *Glomus versiforme),* the observed effects were not found to be restricted to specific AMF taxa (Figure 7F; Figure 7—figure supplement 2). In short, the method is robust.

5.5 Discussion

AMF-interactions are proposed to have played an important role for the colonization of land by plants and still play an important role for a majority of plants by improving the function of their roots and increasing whole-plant performance. Consequently, the investigation of AMF-mediated effects on a host plant's physiology has been an important research field for many decades and characteristic transcriptional and metabolic changes have been observed in the roots of AMF-colonized plants. However, the cumbersome analysis of AMF-interactions, involving destructive harvesting of root tissues and microscopic or transcript analysis, restrains large-scale investigations and commercial applications. AMF interactions were also shown to affect the primary and secondary metabolism in the systemic, aerial tissues of plants; however none of these responses proved to be widespread and sufficiently specific to function as reliable markers (Schweiger and Müller, 2015). Here we describe the discovery of particular blumenols as AMF-indicative markers in leaves and other systemic aerial tissues and illustrate their potential application as tools for research and plant breeding.

Systemic AMF-mediated metabolite changes

Metabolites and metabolite responses are often specific to particular parts and tissues of a plant (Li *et al*., 2016; Lee *et al*., 2017), but it is also known that local responses can spread to other plant parts. Additionally, metabolites do not only accumulate at their place of biosynthesis but can be readily transported throughout the plant (Baldwin, 1989). Therefore, we hypothesized

that local changes in the roots might also be reflected in the systemic aerial tissues, either by signaling or transport. This allowed us to identify specific AMF-indicative blumenols in the shoot (Figure 3) despite the occurrence of other highly abundant and constitutively produced compounds and blumenols that are not indicative of AMF-associations. Interestingly, the confirmation of compound identities in leaf samples with high resolution MS techniques proved to be challenging and required additional sample purification steps. Likely, such matrix effects thwarted the detection of these AMF-indicative, systemic blumenol responses in previous investigations. Under our conditions, AMF-indicative blumenols began mirroring the colonization rates of roots at around three wpi (Figure 3—figure supplement 2). Although microscopic methods can detect first signs of AMF colonization of the roots already after a few days (Brundrett *et al*., 1985), the sensitivity of our method was found to be sufficient to analyze colonization rates observed in the glasshouse and, more importantly, in nature (Figures 3 and 7). The discovery of these AMF-indicative blumenol compounds in diverse plant species interacting with different AMF species (Figure 7, Figure 7—figure supplement 2) further indicates that these responses are widespread. AMF-induced blumenols in the roots have been shown to be quantifiable by various MS and photodiode array based detector setups. However, AMF-induced blumenols occur in many-fold lower amounts in the leaves compared to the roots (e.g., Compound 1 in *N. attenuata* approximately 1/10) and, at these low concentrations, their analysis is likely thwarted by complex matrix effects. Therefore, their analysis requires detection systems with advanced sensitivity and selectivity as is offered by state-of-the-art triple quadrupole technology and enhanced sample preparations (e.g., by solid-phase-extraction based purification and concentration) which were used in the quantitative detection of leaf blumenols described here. In addition to the blumenols, mycorradicin, another biosynthetically related type of apocarotenoids, was reported to accumulate in AMF-colonized roots (Klingner *et al*., 1995) and it would be interesting to investigate if mycorradicin accumulates throughout the plant, as well.

Root-to-shoot transport of AMF-indicative blumenols

Despite the AMF-induced accumulation of blumenols in the shoot, putative candidate genes of the apocarotenoid biosynthesis pathway were only induced in the roots of AMF-inoculated plants (Figure 6A, Figure 6—figure supplement 1A). To exclude other mechanisms (e.g., post-transcriptional regulation) mediating the local production of the blumenol compounds in the leaves, we genetically manipulated the carotenoid pathway in a tissue-specific manner. It is challenging to manipulate blumenols without affecting the AMF-colonization of the plant, since other carotenoid-derived compounds, such as strigolactones, are known to play an important role in this process (Lanfranco *et al*., 2018). To circumvent these problems, we used the LhGR/pOp6 system for chemically inducible RNAi-mediated gene silencing of PDS (Schäfer *et al*., 2013) to impair carotenoid biosynthesis only in a particular leaf of AMF-inoculated plants. Interestingly, only the constitutively produced Compound 6 was reduced in the treated leaves, while the AMF-indicative Compounds 1 and 2 were not affected by our treatment (Figure 6B). This indicated that instead of being locally produced, Compound 1 and 2 are translocated from the roots, an inference consistent with the occurrence of AMF-indicative blumenols in stem sap and the capacity of seedlings to transport blumenols from the root to the shoot from hydroponic solution (Figure 6C, Figure 6—figure supplement 1B,D). It seems likely that the AMF-indicative blumenols are transported in the xylem with the transpiration stream (Figure 6D). The blumenol glucosides (Compounds 1, 2 and 6) are hydrophilic low-molecular weight (402, 388 and 386 Da) compounds that are unlikely to pass membranes without further support, e.g., by transporters. It was recently demonstrated that ATP-binding cassette (ABC) transporters (G-type) are involved in the root-to-shoot transport of ABA, a phytohormone with

a molecular structure related to blumenols, and these transporters resemble the function of other ABCG-type proteins reported to mediate the long-distance transport of cytokinins and strigolactones (Borghi *et al*., 2015). Whether blumenols are transported by similar mechanisms remains an interesting question.

Functional implication of blumenol accumulation and transport

Blumenols were shown to accumulate in large amounts in the roots of various plants after AMF-inoculation (Strack and Fester, 2006) and our data indicate that they are subsequently distributed throughout the plant (Figure 3G). While the conservation of this response in various plants after inoculation with different AMF species (Figure 7F, Figure 7—figure supplement 2) indicates an important functional role in the AMF-plant interaction, this function remains to be explored. Unfortunately, the current knowledge of the biological activity of blumenols only vaguely indicates potential systemic functions of AMF-induced blumenols in shoot tissues. Activity studies on vomifoliol, the aglycone of the not AMF-indicative Compound 6, showed that this compound induces stomatal closure similar to the structurally related abscisic acid (Stuart and Coke, 1975). Additionally, blumenols are known to suppress seed germination and plant growth (Kato-Noguchi *et al*., 2012; Kato-Noguchi *et al*., 2015). Therefore, AMF-induced blumenols could serve as systemic signals that mediate the large-scale adjustments in general physiology that are thought to accompany AMF-interactions. For example, AMF-induced blumenols could be involved in the regulation of differential susceptibility of AMF-inoculated plants to stresses, such as drought or pathogen infection.

AMF-indicative blumenols as tool for research and plant breeding

Even if classical tools for the quantification of AMF-plant interactions can offer superior sensitivity they are labor intensive and highly destructive which limits their application in studies that require high sample throughput, as well as in experiments that require repeated analysis of plants. We propose that the analysis of AMF-indicative blumenols in the shoot provides a convenient, easy-to-use, and minimally destructive tool to interrogate plant-AMF interactions in a HTP manner that allows for forward genetic studies even under field conditions (Figure 7E) and empowers plant breeding programs to produce mycorrhiza-responsive and P-efficient high-yielding lines (van de Wiel *et al*., 2016). Currently, phosphate fertilizer is derived from phosphate rock, a non-renewable resource, which is predicted to be depleted soon (Vaccari and Strigul, 2011). By enabling breeding programs to select crop varieties which have negotiated AMF symbioses that deliver high yields with minimal P inputs, this discovery could help steer the ‘green revolution’ away from intense agricultural inputs and the collateral environmental damage they cause. While some of the ‘green revolution’ crop varieties with gibberellin response defects are potentially more efficient in Pi uptake as a result of their higher root colonization rates by AMF (Floss *et al*., 2013; Foo *et al*., 2013) this serendipitous breeding event underscores the value of explicitly designing crop breeding programs to produce crops that negotiate more favorable AMF associations.

5.6 Materials and Methods

KEY RESOURCES TABLE

Reagent type (species) or resource	Designation	Source or reference	Additional information
Genetic reagent (*N. attenuata*)	A-09-1212-1	Groten *et al*. (2015), DOI: 10.1111/pce.12561	Stably silenced in CCaMK via RNAi
Genetic reagent (*N. attenuata*)	A-09-1208-6	Groten *et al*. (2015), DOI: 10.1111/pce.12561	Stably silenced in CCaMK via RNAi
Genetic reagent (*N. attenuata*)	A-11-92−4 × A-11-325-4	Schäfer *et al*. (2013), DOI: 10.1111/tpj.12301	Chemically-inducible silenced in PDS via RNAi
Genetic reagent (*N. attenuata*)	A-04-266-3	Bubner *et al*. (2006), DOI: 10.1007/s00299-005-0111-4	Empty vector control
Biological sample (*N. attenuata*)	AZ-UT RIL	Zhou *et al*. (2017), DOI: 10.1016/j.cub.2017.03.017	Biparental QTL mapping population

Plant material and AMF inoculation

For our experiments with *Nicotiana attenuata* (Torr. ex S. Wats.), we used plants from the 31st inbred generation of the inbred 'UT' line, ir*CCaMK* [A-09-1208-6 and A-09-1212-1(Groten *et al*., 2015) plants that are stably silenced in *CCaMK* via RNAi, i-ir*PDS* plants (A-11-92−4 × A-11-325-4; Schäfer *et al*., 2013) harboring the LhGR/pOp6 system for chemically-inducible RNAi-mediated gene silencing of phytoene desaturase (PDS) and the respective empty vector (EV) transformed plants (A-04-266-3; Bubner *et al*., 2006) as controls. Details about the transformation and screening of the ir*CCaMK* plants are described by Groten *et al*. (2015) and for the i-ir*PDS* plants by Schäfer *et al*. (2013). Seeds were germinated on Gamborg B5 as described by Krügel *et al*. (2002). The advance intercross recombinant inbred line (RIL) population was developed by crossing two *N. attenuata* inbred lines originating from accessions collected in Arizona (AZ) and Utah (UT), USA (Glawe *et al*., 2003; Zhou *et al*., 2017). Additionally, we used *Solanum lycopersicum* 'Moneymaker', *Hordeum vulgare* 'Elbany 'and *Triticum aestivum* 'Chinese Spring 'plants.

For glasshouse experiments, plants were treated according to Groten *et al*. (2015). In brief, they were transferred into dead (autoclaved twice at 121°C for 30 min; non-inoculated controls) or living inoculum (*R. irregularis*, Biomyc Vital, inoculated plants) diluted 1:10 with expanded clay (size: 2–4 mm). Pots were covered with a thin layer of sand. Plants were watered with distilled water for 7 d and subsequently fertilized every second day either with a full strength hydroponic solution (for 1 L: 0.1292 g $CaSO_4 \times 2H_2O$, 0.1232 g $MgSO_4 \times 7H_2O$, 0.0479 g K_2HPO_4, 0.0306 g KH_2PO_4, 2 mL KNO_3 (1 M), 0.5 mL micronutrients, 0.5 mL Fe diethylene triamine pentaacetic acid) or with a low P hydroponics solution containing only 1/10 of the

regular P-concentration (0.05 mM). Plants were grown separately in 1L pots, if not stated otherwise. In the paired design (Figure 2), ir*CCaMK* plants were grown together with EV plants in 2L pots and the watering regime was changed to ¼ of the regular P-concentration after plants started to elongate. Glasshouse experiments with natural inoculum (Figure 3C) were conducted in a mesocosm system (four boxes, each 2 pairs of EV and ir*CCaMK* plants). Plants were maintained under standard glasshouse conditions (16 hr light, 24–28°C, and 8 hr dark, 20–24°C and 45–55% humidity) with supplemental light supplied by high-pressure sodium lamps (Son-T-Agro).

The field experiments were conducted as described by Schuman *et al*. (2012). Seedlings were transferred to Jiffy pots and planted into a field plot at the Lytle Ranch Preserve in the Great Basin Desert (Utah, USA: N 37.1412, W 114.0275). Field season 2016 (Figure 3D): field experiments were conducted under the US Department of Agriculture Animal and Plant Health Inspection Service (APHIS) import permission numbers 10-004-105m (ir*CCaMK*) and 07-341-101n (EV) and the APHIS release permission number 16-013-102r. EV and ir*CCaMK* plants were planted in communities of six plants, either of the same genotype or with both genotypes in equal number.

Sample collection

During harvests, roots were washed and briefly dried with a paper towel. Subsequently, they were cut into 1 cm pieces and mixed. Plant tissues were shock-frozen in liquid nitrogen immediately after collection, ground to a fine powder and stored at −20°C (short-term storage)/−80°C (long-term storage) until extraction. From the root samples, an aliquot was stored in root storage solution (25% ethanol and 15% acetic acid in water) at 4°C for microscopic analysis.

For stem sap collection, branches of *N. attenuata* plants were cut into 1.5 cm long pieces and placed into small 0.5 mL reaction tubes with a small hole in the tip, which were placed in a larger 1.5 mL reaction tube. The tubes were centrifuged for 15 min at 10 000 × g. The stem sap from the larger reaction tubes was collected and stored at −20°C.

Samples prepared at other laboratory facilities

Medicago truncatula (Figure 7 and Figure 7—figure supplement 2) and *Brachypodium distachyon* (Figure 7 and Figure 7—figure supplement 2) samples were prepared at the laboratory of Prof. Maria Harrison from the Boyce Thompson Institute for Plant Research (Ithaca, NY, USA). *M. truncatula* plants were grown in a growth chamber with a 16 hr light (25°C)/8 hr dark (22°C) cycle. *B. distachyon* plants were grown in growth chamber with a 12 hr light (24°C)/12 hr dark (22°C) cycle. All experiments were carried out in surface sterilized containers, autoclaved growth substrates and with surface sterilized spores and seeds as described previously (Liu *et al*., 2004; Hong *et al*., 2012; Floss *et al*., 2013). The growth substrates were mixtures of play sand (average particle 200–300 µm), black sand (heterogeneous particle size 50–300 µm) and gravel (heterogeneous particle size 300 µm −10 mm) as outlined below. For *M. truncatula*, 2 d-old seedlings were planted into 20.5 cm cones (Cone-tainers) containing a 1:1 mixture of sterile black sand and gravel with 200 surface-sterilized *G. versiforme* spores placed on a layer of play sand positioned 4 cm below the top of the cones. Five seedlings were planted into each cone. Plants were fertilized twice weekly with 20 mL of modified 1/2-strength Hoagland's solution (Millner and Kitt, 1992) containing 100 µM potassium phosphate. Plants were harvested 49 d post planting and tissue frozen in liquid

nitrogen and stored at −80 C. One cone containing five seedlings represents one biological replicate. The harvest date was 3/3/2015. *B. distachyon* seedlings were planted into cones (Cone-tainers) containing a 2:1:1 mixture of black sand:play sand:gravel with 300 surface-sterilized *G. versiforme* spores placed on a layer of play sand positioned 4 cm below the top of the cones. Plants were fertilized twice weekly with 20 mL of modified 1/4-strength Hoagland's solution (Millner and Kitt, 1992) containing 20 µM potassium phosphate. Plants were harvested 35 d post planting and tissue frozen in liquid nitrogen and stored at −80 C. Each cone contained three plants and each biological replicate consisted of a pool of 4 cones. The harvest date was 6/20/2016.

S. lycopersicum 'Moneymaker '(Figure 7—figure supplement 2) and *Solanum tuberosum* 'Wega' (Figure 7) samples were prepared at the laboratory of Prof. Philipp Franken by Dr. Michael Bitterlich from the Leibniz-Institute of Vegetable and Ornamental Crops (Großbeeren/Erfurt Germany). *S. lycopersicum* were transplanted into 10 L open pots containing a sand/vermiculite mixture (sand: grain size 0.2–1 mm; Euroquarz, Ottendorf-Okrilla, Germany, vermiculite: agra vermiculite, Pullrhenen, Rhenen, The Netherlands; 1:1 v:v) and grown in the glass house from March to May (20-28:17 °C day:night, PAR: 300–2000 µmol m^{-2} s^{-1}). Mycorrhizal plants were inoculated with a commercial inoculum either containing *R. irregularis* DAOM 197198 (INOQ, Schnega, Germany) or *F. mosseae* BEG12 (MycAgro Laboratory, Breteniere, France) with 10% of the substrate volume and were harvested after 11 or 6 weeks, respectively. *S. tuberosum* tubers of similar size were planted into 3 L pots filled with the same substrate and grown in a growth cabinet (20:16 °C day:night, 16 hr light, 8 hr dark; PAR: 250–400 µmol m^{-2} s^{-1}, 50% rH). Mycorrhizal plants were inoculated with a commercial inoculum containing *F. mosseae* BEG12 (MycAgro Laboratory, Breteniere, France) with 10% of the substrate volume and were harvested after 6 weeks. Non-mycorrhizal counterparts were inoculated with the same amount of autoclaved (2 hr, 121°C) inoculum and a filtrate. The filtrate was produced for every pot by filtration of 200 mL deionized water through Whatman filter (particle retention 4–7 µm; GE Healthcare Europe GmbH, Freiburg, Germany) containing approx. 200 mL of inoculum. The same amount of deionized water (200 mL) was added to mycorrhizal pots. Plants were irrigated every other day with 400–600 mL nutrient solution (De Kreij *et al*., 1997); 40% of full strength) with 10% of the standard phosphate to guarantee good colonization (N: 10.32 mM; P: 0.07 mM, K: 5.5 mM, Mg: 1.2 mM, S: 1.65 mM, Ca: 2.75 mM, Fe: 0.02 mM, pH: 6.2, EC: 1.6 mS). For the experiment in the glasshouse, additional irrigation was carried out with deionized water until pot water capacity every other day. The pooled bulk leaf sample was dried at 60°C for 48 hr, ground to a fine powder and stored under dry conditions at room temperature until further analyses.

Stress treatments

Herbivory treatments were conducted by placing *Manduca sexta* neonates, originating from an in-house colony, on the plants. After feeding for two weeks, rosette leaves were harvested. As controls, we harvested leaves from untreated plants.

For bacteria and virus infection, plants were inoculated with *Agrobacterium tumefaciens* carrying the Tobacco Rattle Virus. The inoculation was conducted by infiltrating leaves with a bacteria suspension using a syringe. The treatment was conducted as described for virus-induced gene silencing described by Ratcliff *et al*. (2001) and by Saedler and Baldwin (2004). After incubation for three weeks, stem leaves of the treated plants and untreated control plants were harvested.

The fungal infection was done with *Botrytis cinerea*. On each plant, three leaves were treated by applying six droplets, each containing 10 µL of *B. cinerea* spore suspension (10^6 spores mL^{-1} in Potato Extract Glucose Broth, Carl Roth GmbH), to the leaf surface. As control, plants were treated with broth without spores in the same way. Samples were collected after four days incubation.

Drought stress was induced by stopping the watering for four days. Subsequently, stem leaves of the drought-stressed plants and the continuously watered control plants were harvested. In contrast to the other samples of the stress experiment, leaves were dried before analysis to compensate for weight differences caused by changes in the water content.

Sample preparation - extraction and purification

For extraction, samples were aliquoted into reaction tubes, containing two steel balls. Weights were recorded for later normalization. Per 100 mg plant tissues (10 mg in case of dry material), approximately 1 mL 80% MeOH was added to the samples before being shaken in a GenoGrinder 2000 (SPEX SamplePrep) for 60 s at 1150 strokes min^{-1}. After centrifugation, the supernatant was collected and analyzed. For triple-quadrupole MS quantification, the extraction buffer was spiked with stable isotope-labeled abscisic acid (D_6-ABA, HPC Standards GmbH) as an internal standard.

Stem sap was diluted 1:1 with MeOH spiked with D_6-ABA as an internal standard. After centrifugation, the supernatant was collected and analyzed.

The purification of *N. attenuata* leaf extracts for high resolution qTOF-MS was conducted by solid-phase-extraction (SPE) using Chromabond HR-XC 45 µm benzensulfonic acid cation exchange columns (Machery-Nagel) to remove abundant constituents, such as nicotine and phenolamides. After purification the samples were evaporated to dryness and reconstituted in 80% methanol.

Compound identification was conducted by NMR with purified fractions of root and leaf extracts. Compounds 1, 3 and 4 were extracted from root tissues of *N. attenuata* and purified by HPLC (Agilent-HPLC 1100 series; Grom-Sil 120 ODS-4 HE, C18, 250 × 8 mm, 5 µm; equipped with a Gilson 206 Abimed fraction collector). Compounds 2 and 7 were extracted from a mixture of leaf tissues from different plant species (*M. truncatula, Z. mays, S. lycopersicum* and *N. attenuata*). The first purification step was conducted by SPE using the Chromabond HR-XC 45 µm benzensulfonic acid cation exchange columns (Machery-Nagel) to remove hydrophilic and cationic constituents. Additional purification steps were conducted via HPLC (Agilent-HPLC 1100 series; Phenomenex Luna C18(2), 250 × 10 mm, 5 µm; equipped with a Foxy Jr. sample collector) and UHPLC (Dionex UltiMate 3000; Thermo Acclaim RSLC 120 C18, 150 × 2.1 mm, 2.2 µm; using the auto-sampler for fraction collection).

Untargeted MS based analyses

For high resolution mass spectrometry (MS), indiscriminant tandem mass spectrometry (idMS/MS), tandem MS (MS^2) and pseudo-MS^3 were used. Ultra-high performance liquid chromatography (UHPLC) was performed using a Dionex UltiMate 3000 rapid separation LC system (Thermo Fisher), combined with a Thermo Fisher Acclaim RSLC 120 C18, 150 × 2.1 mm, 2.2 µm column. The solvent composition changed from a high % A (water with 0.1% acetonitrile and 0.05% formic acid) in a linear gradient to a high % B (acetonitrile with 0.05%

formic acid) followed by column equilibration steps and a return to starting conditions. The flow rate was 0.3 mL min^{-1}. MS detection was performed using a micrOTOF-Q II MS system (Bruker Daltonics), equipped with an electrospray ionization (ESI) source operating in positive ion mode. ESI conditions for the micrOTOF-Q II system were end plate offset 500 V, capillary voltage 4500 V, capillary exit 130 V, dry temperature 180°C and a dry gas flow of 10 L min^{-1}. Mass calibration was performed using sodium formiate (250 mL isopropanol, 1 mL formic acid, 5 mL 1 M NaOH in 500 mL water). Data files were calibrated using the Bruker high-precision calibration algorithm. Instrument control, data acquisition and reprocessing were performed using HyStar 3.1 (Bruker Daltonics). idMS/MS was conducted in order to gain structural information on the overall detectable metabolic profile. For this, samples were first analyzed by UHPLC-ESI/qTOF-MS using the single MS mode (producing low levels of fragmentation that resulted from in-source fragmentation) by scanning from m/z 50 to 1400 at a rate of 5000 scans s^{-1}. MS/MS analyses were conducted using nitrogen as collision gas and involved independent measurements at the following four different collision-induced dissociation (CID) voltages: 20, 30, 40 and 50 eV. The quadrupole was operated throughout the measurement with the largest mass isolation window, from m/z 50 to 1400. Mass fragments were scanned between m/z 50 to 1400 at a rate of 5000 scans s^{-1}. For the idMS/MS assembly, we used a previously designed precursor-to-product assignment pipeline (Li *et al*., 2015; Li *et al*., 2016) using the output results for processing with the R packages XCMS and CAMERA (Data Set 2).

Additional MS/MS experiments were performed on the molecular ion at various CID voltages. For the fragmentation of the proposed aglycones via pseudo-MS^3, we applied a 60 eV in-source-CID transfer energy which produced spectra reflecting the loss of all sugar moieties.

Structure elucidation by NMR

Purified fractions were completely dried with N_2 gas and reconstituted with MeOH-d_3 prior to analysis by nuclear magnetic resonance spectroscopy (NMR). Structure elucidation was accomplished on an Avance III AV700 HD NMR spectrometer (Bruker-Biospin, Karlsruhe, Germany) at 298 K using a 1.7 mm TCI CryoProbe™ with standard pulse programs as implemented in Bruker TopSpin (Version 3.2). Chemical shift values (δ) are given relative to the residual solvent peaks at δ_H 3.31 and δ_C 49.05, respectively. Carbon shifts were determined indirectly from ^1H-^{13}C HSQC and ^1H-^{13}C HMBC spectra. The data are shown in Table 1 and compared with previously published reference data (Matsunami *et al*., 2010). Blumenol C glucoside and byzantionoside B differ only in the configuration of position C-9; blumenol C glucoside is (9*S*)-configured whereas byzantionoside B has a (9*R*)-configuration. Characteristic ^{13}C-chemical shift differences can thus be found for the positions C-9, C-10 and C-1'. In byzantionoside, C-9 and C-10 were reported to have chemical shifts of δ_C 75.7 and δ_C 19.9, respectively. In contrast, the chemical shifts for the same positions in blumenol C glucoside were reported to be lowfield shifted to δ_C 77.7 and δ_C 22.0, respectively. Experimental chemical shifts of C-9 for the compounds identified in this publication were in the range from δ_C 77.2 to δ_C 78.2, and for C-10 in the range from δ_C 21.6 to δ_C 21.9, respectively. C-1' of byzantionoside was reported to have a chemical shift of δ_C 102.3, while for blumenol C glucoside the chemical shift was δ_C 104.1. The experimental chemical shifts for C-1' of the compounds of this publication are in the range from δ_C 103.8 to δ_C 104.1. Hence the ^{13}C-chemical shift data are completely consistent with the structures being

TABLE 1 - 1H and 13C NMR data for compounds 1–4 and 7. ***Note:*** only data from Compound 1 and 2 are shown here due to space limitations. For the full table, see: https://doi.org/10.7554/eLife.37093.031

No.	**Compound 1**			**Compound 2**		
Pos.	**δ_H**	**mult., J [Hz]**	**δ_C**	**δ_H**	**mult., J [Hz]**	**δ_C**
1	-	-	202.3	-	-	203.1
2	6.06	dd, 1.8/1.8	121.3	6.40	s	128.5
3	-	-	172.4	-	-	172.6
4	1.92	dd, 5.2/5.2	47.8	2.64	m	46.3
5	-	-	37.2	-	-	36.9
6	2.59 2.02	d, 17.5 d, 17.5	48.5	2.03 2.60	d, 17.4 d, 17.4	48.0
7	1.66 1.82	m m	26.8	1.62 1.88	m m	27.6
8	1.63	m	37.1	1.60	m	36.0
9	3.82	dd, 6.2/ 11.7	77.2	3.80	m	77.7
10	1.24	d, 6.2	21.6	1.21	d, 6.1	21.9
11	4.32 4.16	dd, 17.8/1.8 dd, 17.8/1.8	64.9	-	-	160.1
12	1.02	s	28.4	1.01	s	28.4
13	1.12	s	27.5	1.12	s	27.5
1'	4.31	d, 7.9	103.8	4.30	d, 7.9	104.1
2'	3.15	dd, 7.9/9.0	75.0	3.13	dd, 7.9/8.9	75.1
3'	3.33	dd, 9.0/9.0	77.9	3.35	dd, 8.9/8.9	78.0
4'	3.27	dd, 9.0/9.0	71.4	3.27	dd, 8.9/8.9	71.5
5'	3.25	m	77.6	3.25	m	77.6
6'	3.85 3.65	dd, 11.8/2.2 dd, 11.8/5.5	62.5	3.84 3.66	dd, 2.0/12.3 dd, 5.0/12.3	62.6

s, singlet; s br, broad singlet; d, doublet; dd, doublet of doublet; m, multiplet

blumenol C glucosides rather than byzantionoside B. More characteristic differences can be found in the ^{1}H chemical shifts. The methylene shifts for H-7 of byzantionoside were reported to have chemical shifts of δ_H 1.50 and δ_H 1.98 while for blumenol C glucoside the same position

showed chemical shifts of δ_H 1.67 and δ_H 1.81. Experimental ^1H chemical shifts for H-7 of the compounds 1–4 of this publication were found in the range of δ_H 1.62 to δ_H 1.69 and δ_H 1.80 to δ_H 1.88, respectively. Consequently, the NMR data clearly establish the structures to be blumenol C derivatives and not byzantionosides.

Targeted metabolite analysis

For chromatographic separations, a UHPLC (Dionex UltiMate 3000) was used to provide a maximum of separation with short run times. This reduced the interference from other extract components (matrix effects), increased the specificity of the method, and met the requirements of a HTP analysis. The auto-sampler was cooled to 10°C. As a stationary phase, we used a reversed phase column (Agilent ZORBAX Eclipse XDB C18, 50 × 3.0 mm, 1.8 µm) suitable for the separation of moderately polar compounds. Column temperature was set to 42°C. As mobile phases, we used: A, 0.05% HCOOH, 0.1% ACN in H_2O and B, MeOH, the composition of which was optimized for an efficient separation of blumenol-type compounds within a short run time. We included in the method a cleaning segment at 100% B and an equilibration segment allowing for reproducible results across large samples sets. The gradient program was as follows: 0–1 min, 10% B; 1–1.2 min, 10–35% B; 1.2–5 min, 35–50% B; 5–5.5 min, 50–100% B; 5.5–6.5 min, 100% B; 6.5–6.6 min, 100–10% B and 6.6–7.6 min, 10% B. The flow rate was set to 500 µL min^{-1}. Analysis was performed on a Bruker Elite EvoQ triple quadrupole MS equipped with a HESI (heated electrospray ionization) ion source. Source parameters were as follows: spray voltage (+), 4500V; spray voltage (-), 4500V; cone temperature, 350°C; cone gas flow, 35; heated probe temperature, 300°C; probe gas flow, 55 and nebulizer gas flow, 60. Samples were analyzed in multiple-reaction-monitoring (MRM) mode; the settings are described in Table 2.

Method for targeted blumenol analysis in N. attenuata

The compound list was limited to the AMF-indicative markers in *N. attenuata*, Compound 1 and 2, the not AMF-indicative Compound 6 and the internal standard (D_6-ABA). Accordingly, the gradient program was adjusted as follows: 0–1 min, 10% B; 1–1.2 min, 10–35% B; 1.2–3 min, 35–42% B; 3–3.4 min, 42–100% B; 3.4–4.4 min, 100% B; 4.4–4.5 min, 100–10% B and 4.5–5.5 min, 10% B. The MRM settings are given in Table 3.

Determination of the AMF colonization rate

To determine the fungal colonization rates and mycorrhizal structures, root samples were stained and analyzed by microscopy. For WGA-Alexa Fluor 488 staining, roots were first washed with distilled water and then soaked in 50% (v/v) ethanol overnight. Roots were then boiled in a 10% (w/v) KOH solution for 10 min. After rinsing with water, the roots were boiled in 0.1 M HCl solution for 5 min. After rinsing with water and subsequently with 1x phosphate-buffered saline solution, roots were stained in 1x phosphate-buffered saline buffer containing 0.2 mg mL^{-1} WGA-Alexa Fluor 488 overnight in the dark. Zeiss confocal microscopy (LSM 510 META) was used to detect the WGA-Alexa Fluor 488 (excitation/emission maxima at approximately 495/519 nm) signal. Trypan blue staining was performed as described by Brundrett *et al*. (1984) to visualize mycorrhizal structures. For the counting of mycorrhizal colonization, 15 root fragments, each about 1 cm long, were stained with either trypan blue or WGA-488 followed by slide mounting. More than 150 view fields per slide were surveyed with 20x object magnification and classified into five groups: no colonization, only hyphae (H), hyphae with arbuscules (H + A), hyphae with vesicles

TABLE 2 - MRM-settings used for targeted blumenol analysis.

Nr.	Compound name	RT	Q1 [m/z]*, †	Q3 [m/z] ‡, § (CE [V])
1	11-hydroxyblumenol C-Glc¶, **	2.82	+389.22	227.16 (-2.5), **209.15 (-7.5)**, 191.14 (-12.5), 163.10 (-15), 149.10 (-17.5)
2	11-carboxyblumenol C-Glc¶, **	3.22	+403.22	241.16 (-2.5), 223.15 (-7.5), 177.10 (-15), 195.14 (-12.5)
			+241.16 #	223.15 (-5), 177.10 (-15), **195.14 (-10)**
3	11-hydroxyblumenol C-Glc-Glc ¶, **	2.5	+551.27	389.22 (-2.5), 227.16 (-7.5), **209.15 (-10)**, 191.14 (-15), 149.10 (-20)
4	Blumenol C – Glc-Glc ¶, **	3.47	+535.27	373.22 (-2.5), **211.00 (-10)**, 193.10 (-17.5), 135.00 (-22.5), 109.00 (-22.5)
5	Blumenol C - Glc ¶, ††	4.18	+373.22	**211.20 (-6)**, 193.16 (-9), 175.10 (-15), 135.12 (-16), 109.10 (-20)
6	Blumenol A - Glc¶, ††	2.51	- 385.20	**153.10 (14)**
			+387.20	225.15 (-5), 207.14 (-8), 149.10 (-18), 135.12 (-16), 123.08 (-23)
7	Blumenol B - Glc¶, **	2.5	+389.22	227.16 (-5), **209.15 (-7.5)**, 191.14 (-12.5), 153.10 (-17.5), 149.10 (-17.5)
8	Blumenol C – Glc-GlcU¶, ‡‡	3.25 and 3.38	+549.27	373.22 (-2.5), **211.00 (-10)**, 193.10 (-17.5), 135.00 (-22.5), 109.00 (-22.5)
9	11-hydroxylumenol C – Glc-Rha‡‡	2.8	+535.27	389.22 (-2.5), **227.16 (-7.5)**, 209.15 (-10), 191.14 (-15), 149.10 (-20)
10	Blumenol C – Glc-Rha¶, ‡‡	4.1	+519.27	373.22 (-2.5), **211.00 (-10)**, 193.10 (-17.5), 135.00 (-22.5), 109.00 (-22.5)
11	Hydroxyblumenol C-Hex-Pen‡‡	2.5	+521.27	389.22 (-2.5), 227.16 (-7.5), **209.15 (-10)**, 191.14 (-15), 149.10 (-20)
	D_6-ABA††	4.5	- 269.17	**159.00 (10)**

RT: retention time
CE: collision energy
Glc: glucose
GlcU: glucuronic acid
Rha: rhamnose
Hex: hexose
Pen: pentose
*Resolution: 0.7
†[M + H]+ or [M-H]- if not stated differently
‡Resolution: 2
§ Quantifiers are depicted in bold
# [M + H-Glc]+
¶ Verified by high resolution MS
**Verified by NMR
††Optimized with commercial available standards
‡‡Transitions predicted based on structural similar compounds and literature information

TABLE 3 - MRM-settings for the analysis of selected blumenols in *N. attenuata*.

Nr.	Compound name	RT	Q1 [m/z]*, †	Q3 [m/z]‡, § (CE [V])
1	11-hydroxyblumenol C-Glc¶, **	2.82	+389.22	227.16 (-2.5), **209.15 (-7.5)**, 191.14 (-12.5), 163.10 (-15), 149.10 (-17.5)
2	11-carboxyblumenol C-Glc¶, **	3.22	+403.22	241.16 (-2.5), 223.15 (-7.5), 177.10 (-15), 195.14 (-12.5)
			+241.16#	223.15 (-5), 177.10 (-15), **195.14 (-10)**
6	Blumenol A - Glc ¶, ††	2.51	- 385.20	**153.10 (14)**
			+387.20	225.15 (-5), 207.14 (-8), 149.10 (-18), 135.12 (-16), 123.08 (-23)
	D_6-ABA††	4.0	- 269.17	**159.00 (10)**

RT: retention time
CE: collision energy
Glc: glucose
Hex: hexose
Pen: pentose
*Resolution: 0.7
†[M + H]+ or [M-H]- if not stated differently
‡Resolution: 2
§Quantifiers are depicted in bold
#[M + H-Glc]+
¶Verified by high resolution MS
**Verified by NMR
††Optimized with commercial available standards

(V + H), and hyphae with arbuscules and vesicles (A + V + H). The proportions of each group were calculated by numbers of each group divided by total views.

For the molecular biological analysis of colonization rates, RNA was extracted from the roots using the RNeasy Plant Mini Kit (Qiagen) or NucleoSpin RNA Plant (Macherey-Nagel) according to the manufacturer's instructions and cDNA was synthesized by reverse transcription using the PrimeScript RT-qPCR Kit (TaKaRa). Quantitative (q)PCR was performed on a Stratagene Mx3005P qPCR machine using a SYBR Green containing reaction mix (Eurogentec, qPCR Core kit for SYBR Green I No ROX). We analyzed the *R. irregularis* specific housekeeping gene, *Ri-tub* (GenBank: EXX64097.1), as well as the transcripts of the AMF-induced plant marker genes *RAM1*, *Vapyrin*, *STR1* and *PT4*. The signal abundance was normalized to *NaIF-5a* (NCBI Reference Sequence: XP_019246749.1). The primer sequences are summarized in Table 4.

Transcript analysis of the apocarotenoid pathway

The transcript analysis of the (apo)carotenoid pathway was conducted based on RNA-seq (Data Set 3) by using *N. attenuata* roots with or without *R. irregularis* inoculations. The data analysis methods are based on the previously published pipeline of Ling *et al*. (2015). Representative values for transcripts abundances are TPM (Transcripts per kilobase of exon model per million mapped reads).

TABLE 4 - Sequences of primers used for qPCR-based analysis of AMF-colonization rates.

Gene	Forward primer	Reversed primer
NaIF-5a	GTCGGACGAAGAACACCATT	CACATCACAGTTGTGGGAGG
NaRAM1	ACGGGGTCTATCGCTCCTT	GTGCACCAGTTGTAAGCCAC
NaVapyrin	GGTCCCAAGTGATTGGTTCAC	GACCTTCAAAGTCAACTGAGTCAA
NaSTR1	TCAGGCTTCCACCTTCAATATCT	GACTCTCCGACGTTCTCCC
NaPT4	GGGGCTCGTTTCAATGATTA	AACACGATCCGCCAAACAT
NaCCaMK	TTGGAGCTTTGTTCTGGTGGT	ATACTTGCCCCGTGTAGCG
NaNOPE1	ACTTGATGCCATGTTTCAGAGC	TCCAATTCGCGATAAGCTGGT
Ri-TUB	TGTCCAACCGGTTTTAAAGT	AAAGCACGTTTGGCGTACAT

Blumenol transport experiment

To analyze the root-to-shoot transfer potential of blumenols, we placed three *N. attenuata* seedlings, previously germinated on petri dishes with GB5 Agar for approximately 10 days, into 0.5 mL reaction tubes. The roots were placed into the tube, while the shoot projected out of the tube. The tubes were carefully covered with parafilm, which held the seedlings in place and isolated roots from shoots (see Figure 6C). The tubes were filled with tap water supplemented with 0.5% v/v plant extracts enriched in Compounds 1 or 2 (unknown concentration; purified fractions), or a commercial available standard of Compound 6 (25 ng μL^{-1} end concentration; Roseoside; Wuhan ChemFaces Biochemical Co., Ltd.). Compound 1 or 2 were prepared from a mix of leaf tissues from different plant species (*M. truncatula, Z. mays, S. lycopersicum* and *N. attenuata*) by methanol extraction followed by purification by SPE (Chromabond HR-XC column) and HPLC (Agilent-HPLC 1100 series; Phenomenex Luna C18(2), 250 × 10 mm, 5 µm; equipped with a Foxy Jr. fraction collector). As a control, we used tap water supplemented with the respective amounts of MeOH. The seedlings were incubated for one day in a Percival climate chamber (16 hr of light at 28°C, and 8 hr of dark at 26°C). During sample collection, roots and shoots were separated and the roots were rinsed in water (to reduce the surface contamination with the incubation medium). While the shoots were analyzed separately, the roots of all seedlings from the same treatment were pooled. Sample extraction was conducted as described above.

Inducible PDS silencing

For the temporal and spatial restriction of PDS gene silencing, we treated the petiole of the second oldest stem leaf of AMF-inoculated and non AMF-inoculated i-ir*PDS* and EV plants with a 100 µM dexamethasone-containing lanolin paste (1% v/v DMSO). The lanolin paste was prepared and applied as described by Schäfer *et al*. (2013). The treatment started three weeks after potting and was conducted for three weeks. The lanolin paste was refreshed twice per week. On each plant the treated leaf and the adjacent, untreated leaves were harvested for analysis.

QTL analysis

The field experiments for QTL analysis were conducted in 2017. Collected leaf samples were extracted as described with 80% MeOH spiked with D_6-ABA as internal standard and analyzed with the method described under '*Method for targeted blumenol analysis in N. attenuata'*. The peak areas for Compound 2 were normalized by the amount of extracted tissue and internal standard and log-transformed. Samples with missing genotype or phenotype information were removed. In total, 728 samples were used for QTL mapping analysis. For quantitative trait loci (QTL) mapping, we used the AZ-UT RIL population and data analysis described by Zhou *et al*. (2017).

Statistics

Statistical analysis of the data was performed with R version 3.0.3 (http://www.R-project.org/). The statistical methods used and the number of replicates are indicated in the figure legends.

5.7 Acknowledgements

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5.8 Supplemental Figures

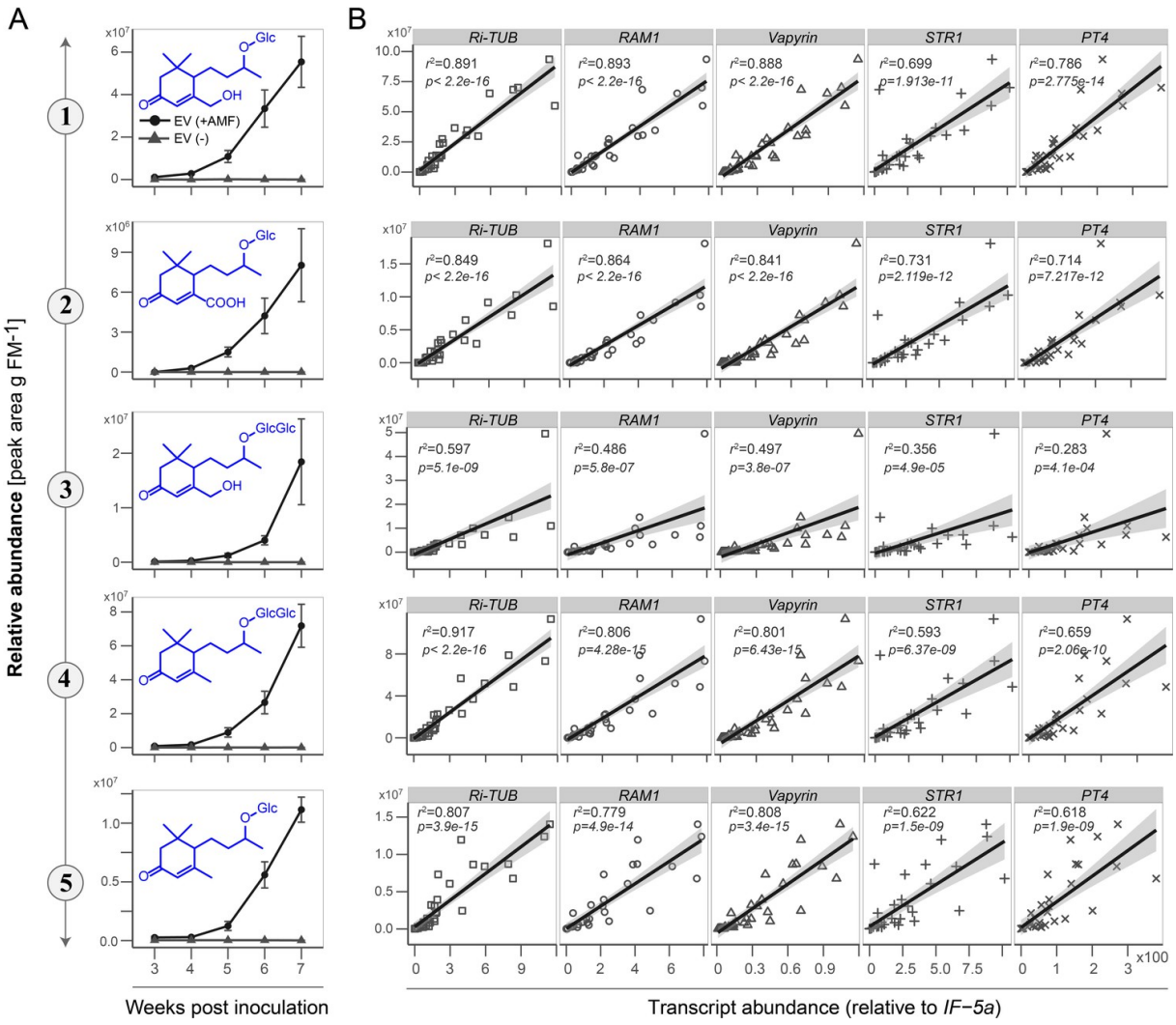

FIGURE 2, FIGURE SUPPLEMENT 1 - Abundance of root blumenol derivatives correlates positively with root AMF colonization. (A) Time lapse accumulations (3–7 weeks post inoculation, n ≥ 3 for each time point) of Compounds 1, 2, 3, 4 and 5 in roots of plants with (EV+, black lines with circles) and without (EV-, grey lines with triangle) AMF inoculation. The experiment was conducted with empty vector (EV) transformed plants. Data are means ±SE. (B) Abundance of Compounds 1, 2, 3, 4 and 5 relative to the transcript abundance of the *R. irregularis* specific housekeeping gene, *Ri-tub* (GenBank: EXX64097.1), as well as to the plant-derived marker genes *RAM1*, *Vapyrin*, *STR1* and *PT4* (Gene ID and transcripts abundance are listed in Data Set 1). The transcript abundance was quantified by q-PCR, relative to *NaIF-5a* (NCBI Reference Sequence: XP_019246749.1). The correlations among blumenol derivatives and the transcript abundances of marker genes were analyzed by linear regression (lm) models.

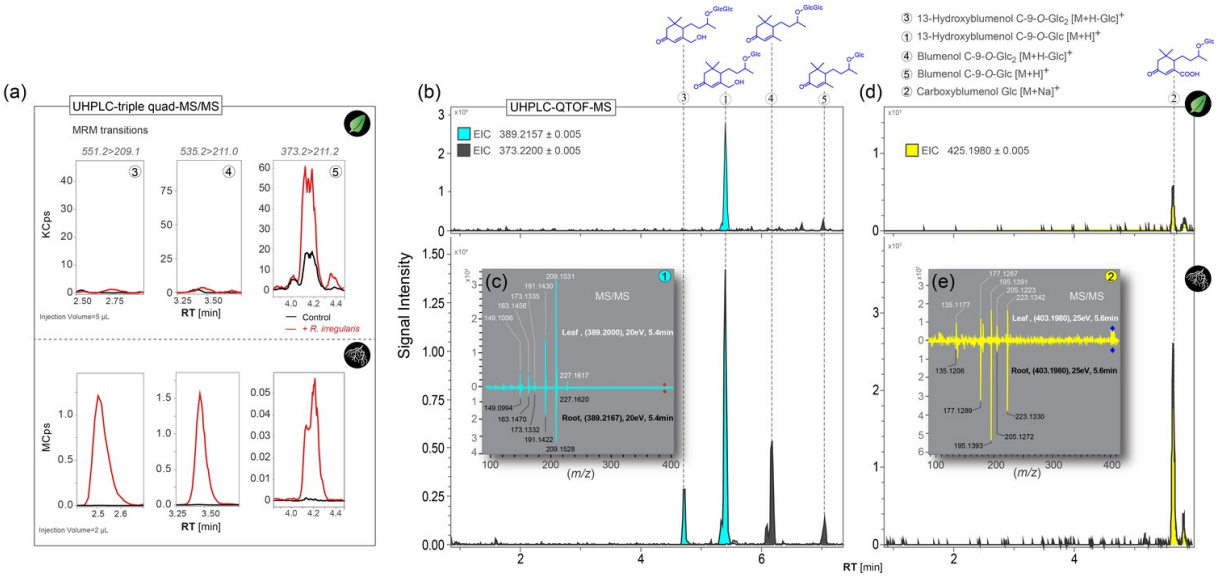

FIGURE 3, FIGURE SUPPLEMENT 1 - AMF-induced accumulation of blumenol derivatives in roots and leaves of *N. attenuata*. (A) Representative chromatograms of targeted tandem MS-based analyses of Compounds 3, 4 and 5 in roots (bottom panel) and leaves (top panel) of *N. attenuata* plants after inoculation with *R. irregularis* (+*R. irregularis*, red line, 6wpi) and in untreated control plants (Control, black line). Experiments were conducted with wild type (WT) plants. The respective precursor-to-product ion transitions are indicated at the top. (B), (D) Representative chromatograms of a high resolution MS-based analysis of Compounds 1, 3, 4 and 5 (B), as well as Compound 2 (D) in roots (bottom panel) and leaves (top panel) of *N. attenuata* plants after inoculation with *R. irregularis*. Extracted ion chromatograms (EIC) are labeled by colors and settings listed at the top. (C), (E) Comparison of fragmentation patterns of Compounds 1 (C) and 2 (E) in both tissues by high resolution tandem MS.

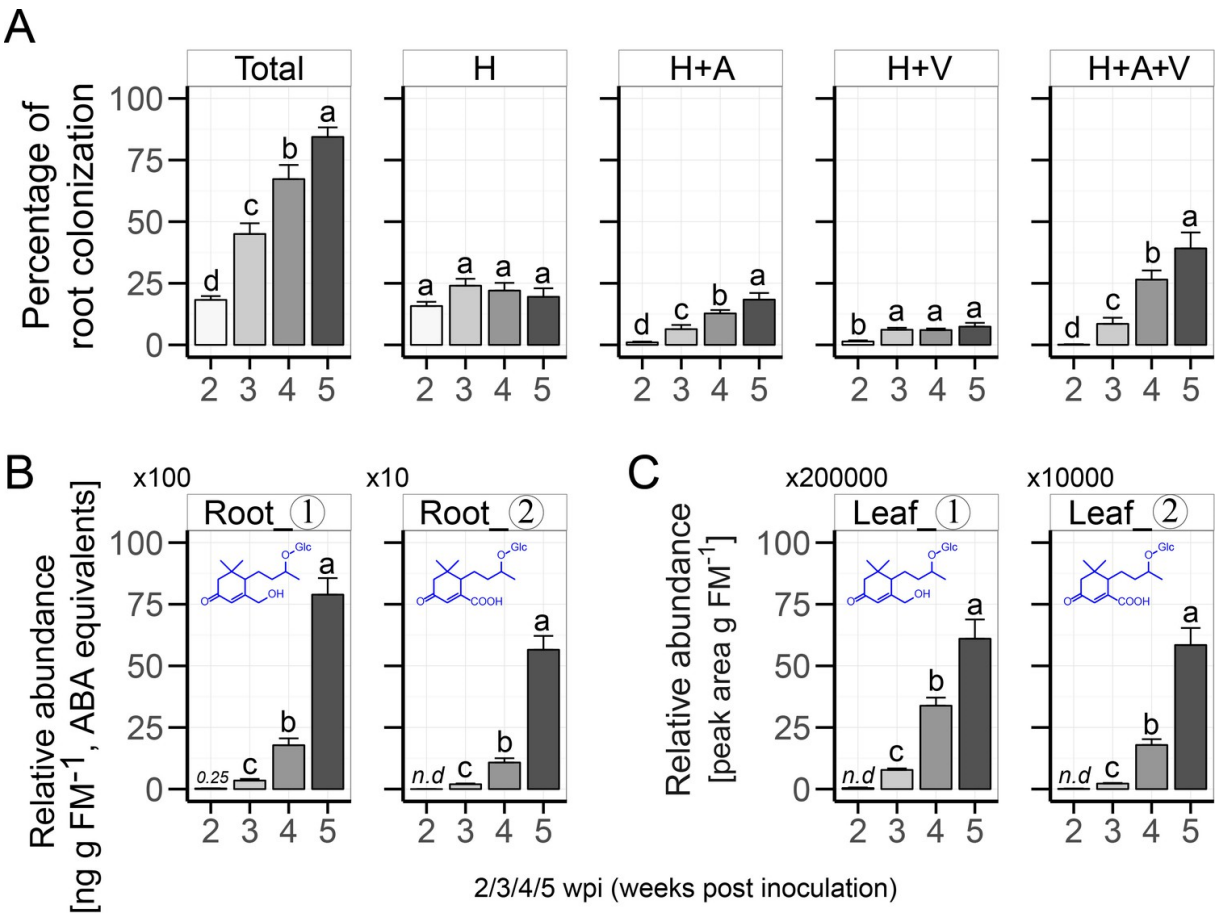

FIGURE 3, FIGURE SUPPLEMENT 2 - Time course analysis of the root colonization by AMF and the corresponding accumulation of Compounds 1 and 2 in roots and leaves of *N. attenuata*. (A) Root colonization in EV plants at different time points after inoculation with *R. irregularis* (2/3/4/5 wpi). H: hyphae; A: arbuscules; V: vesicles; Total: total root length colonization (means +SE; n = 8). (B, C) Abundances of Compounds 1 and 2 in roots (B) and leaves (C) of plants at different time points after inoculation with *R. irregularis* (2/3/4/5 wpi; means +SE; $n \geq 5$). Different letters indicate significant differences ($p<0.05$, one-way ANOVA followed by Tukey's HSD). n.d., not detected.

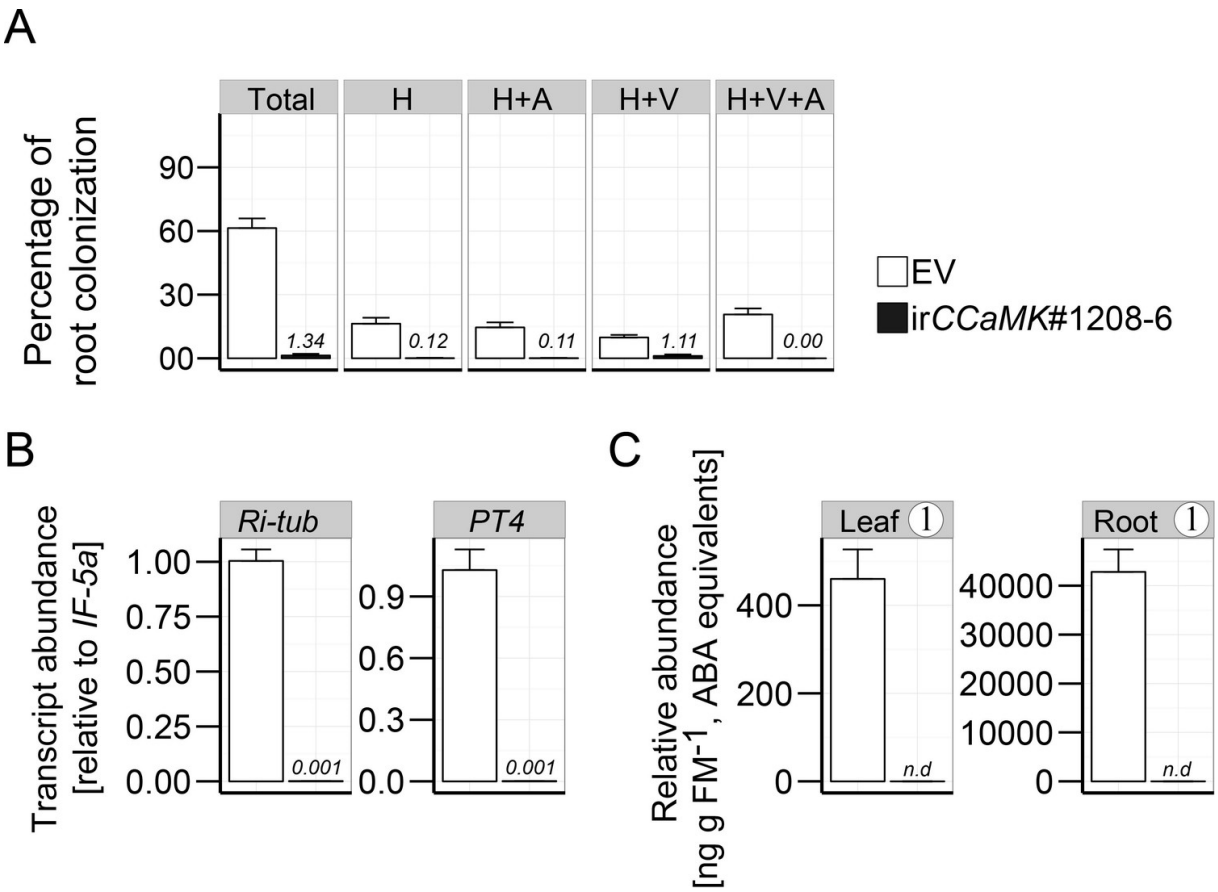

FIGURE 3, FIGURE SUPPLEMENT 3 - Root AMF colonization and abundance of Compound 1 in a second independently transformed irCCaMK line. (A) Root colonization analysis in EV and irCCaMK (A-09-1208-6) plants. H: hyphae; A: arbuscules; V: vesicles; Total: total root length colonization (means +SE; EV, n = 9; irCCaMK; n = 7). (B) Transcript abundance of AMF marker genes in roots of EV and irCCaMK plants inoculated with *R. irregularis* (six wpi; means +SE; n = 6). (C) Compound 1 levels in roots and leaves of EV and irCCaMK plants inoculated with *R. irregularis* (six wpi; means +SE; n = 8).

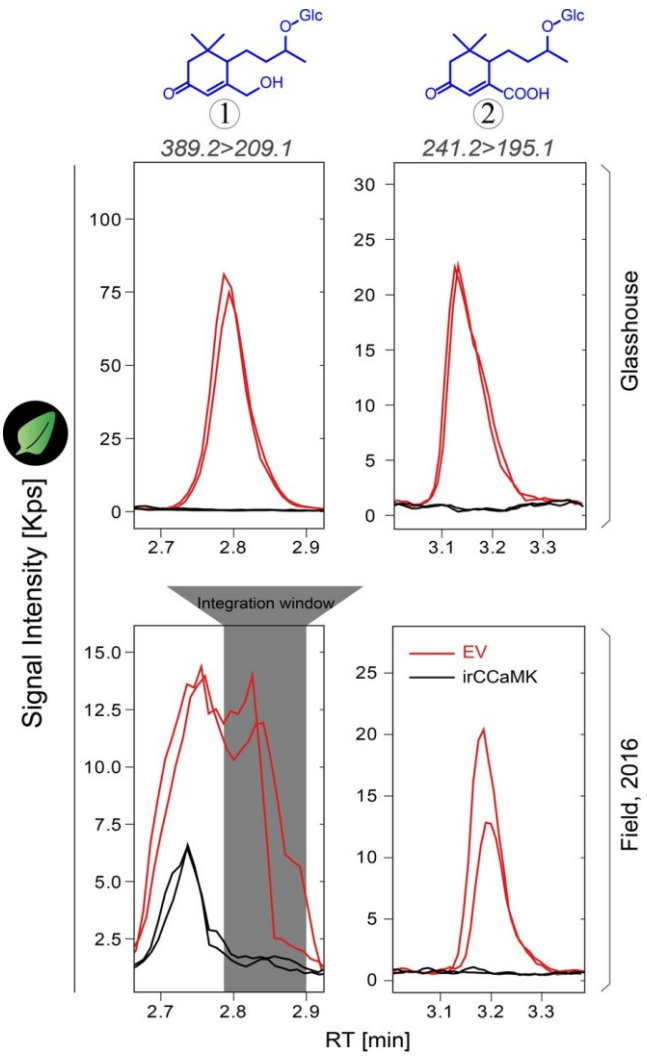

FIGURE 3, FIGURE SUPPLEMENT 4 - Signals from Compound 1 are partially disturbed in field samples, but not for Compound 2. Leaf samples were harvested from glasshouse-(top panel) and field-grown, Utah, 2016 (bottom panel) plants for analysis. Representative chromatograms of two samples of each genotype, EV (red) and irCCaMK (black), are shown. Grey area indicates the peak integration window used for the quantification of Compound 1.

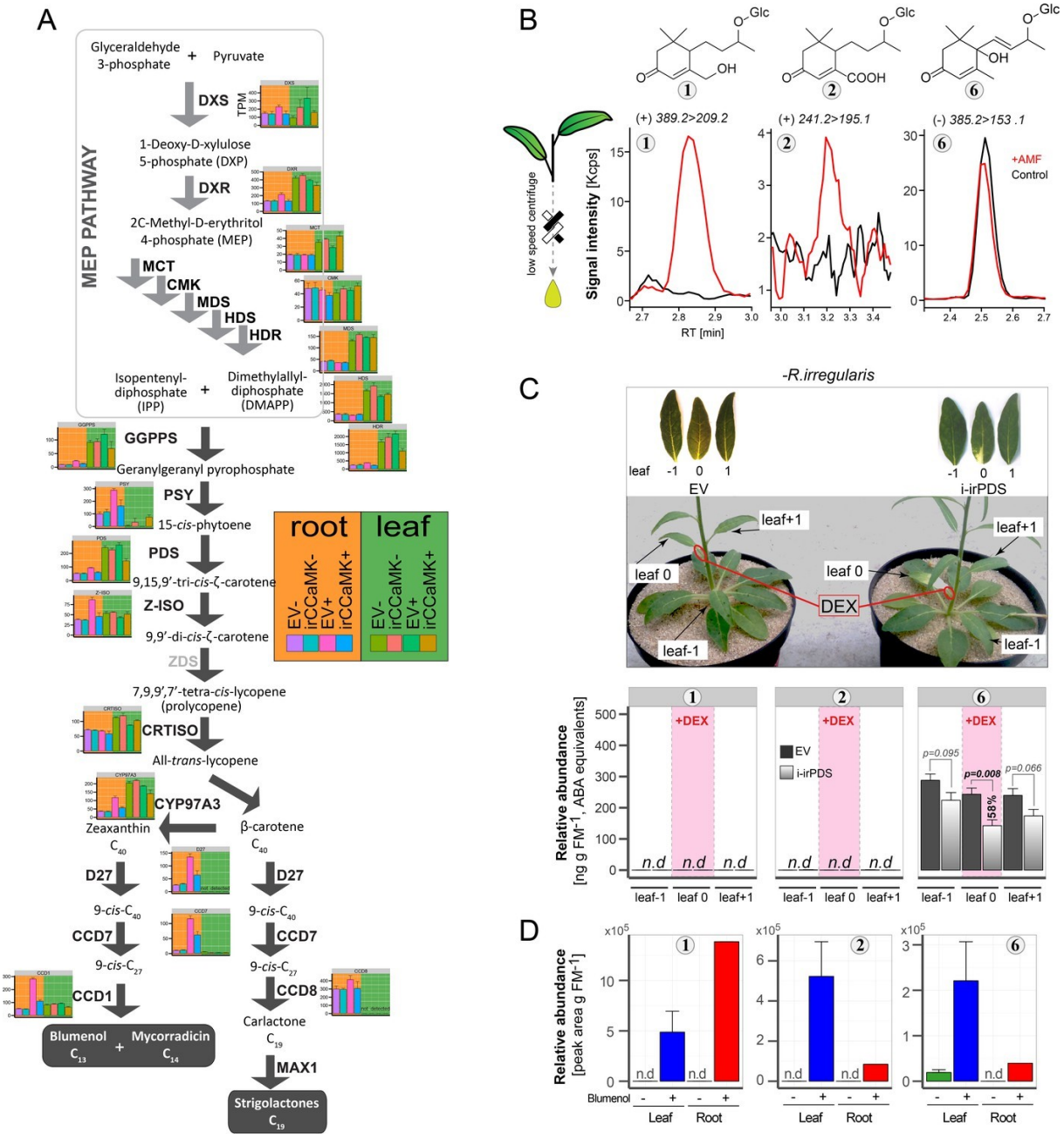

FIGURE 6, FIGURE SUPPLEMENT 1 - Foliar levels of Compounds 1 and 2 are derived from roots. (A) Transcript abundance of MEP and apocarotenoid pathway biosynthetic genes (based on homologies to tomato, *Arabidopsis* and tobacco). Plant materials from the same experimental set-up as in Figure 2A were used for sequencing. Data are means +SE (n = 3) generated by RNA-seq and the abundance of each transcript is expressed in TPM (Transcripts per kilobase of exon model per million mapped reads). Transcripts were analyzed in roots (left panel, orange background) and leaf tissues (right panel, green background) of EV and irCCaMK plants with (EV+ and irCCaMK+ respectively) and without (EV- and irCCaMK- respectively) inoculations with *R. irregularis*. Gene abbreviations; *CRTISO*: carotenoid isomerase; *GGPPs*: geranylgeranyl diphosphate synthase; *PSY*: phytoene synthase; *PDS*: phytoene desaturase; *ZDS*: ζ-carotene de-saturase; *Z-ISO*: ζ- carotene isomerase; *CCD*: carotenoid cleavage dioxygenase; *MAX1*: cytochrome P450-type monooxygenase *CYP711A1*; *DXS*: 1-deoxy-D-xylulose 5-phosphate synthase; *DXR*: 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *MCT*: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase; *CMK*: 4-(cytidine 5′-diphospho)−2-C-methyl-D-erythritol kinase; *MDS*: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *HDS*: 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase; *HDR*: 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; *D27*: carotenoid isomerase. (B) Representative chromatograms from a targeted tandem MS-based analysis of Compounds 1, 2 and 6 in stem sap fluid of *N. attenuata* plants after *R. irregularis* inoculation (+AMF, red line, 6wpi) and of untreated control

plants (Control, black line). The respective precursor-to-product ion transitions are indicated at top. (C) Accumulations of Compounds 1, 2 and 6 in non AMF-inoculated plants after local silencing of the carotenoid biosynthesis in the DEX-treated leaf. The experiment was performed with plants harboring a transformation construct mediating the chemically-inducible silencing of the phytoene desaturase (i-irPDS), as well as with empty vector (EV) plants. On each plant, a single stem leaf (leaf 0) was treated with a 100 µM dexamethasone (DEX) containing lanolin paste for 3 weeks. The adjacent, untreated leaves (leaf −1 and leaf +1) were harvested as controls. Representative leaves are shown (bleaching indicates functional PDS silencing). Data are means +SE (n ≥ 6). For statistical analysis, the samples from the same leaf positions in i-irPDS and EV plants were compared by Student's *t* test. (D) Contents of Compounds 1, 2 and 6 in the roots (red bars) and shoots (blue bars) of seedlings whose roots were dipped into an aqueous solution with or without addition of the respective blumenols. Seedlings were incubated for 1d before analysis. Data are means +SE (shoot, n = 3; root, n = 1). The data originate from the same experiment presented in Figure 6C.

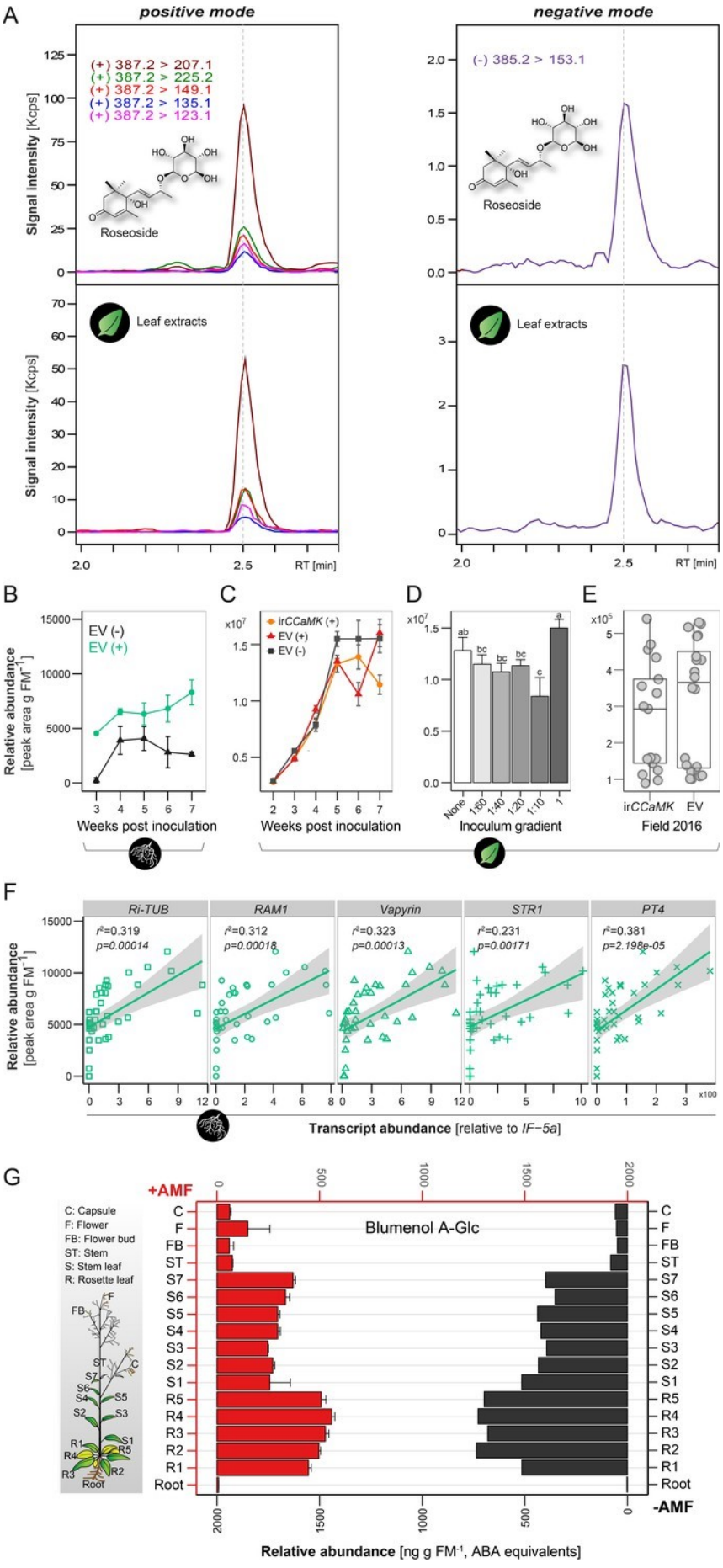
A
positive mode
negative mode
(+) 387.2 > 207.1
(+) 387.2 > 225.2
(+) 387.2 > 149.1
(+) 387.2 > 135.1
(+) 387.2 > 123.1
(-) 385.2 > 153.1
Roseoside
Leaf extracts
Signal intensity [Kcps]
RT [min]
B
EV (-)
EV (+)
Relative abundance [peak area g FM-1]
Weeks post inoculation
C
irCCaMK (+)
EV (+)
EV (-)
Weeks post inoculation
D
Inoculum gradient
None
1:80
1:40
1:20
1:10
1
E
irCCaMK
EV
Field 2016
F
Ri-TUB
RAM1
Vapyrin
STR1
PT4
r²=0.319
p=0.00014
r²=0.312
p=0.00018
r²=0.323
p=0.00013
r²=0.231
p=0.00171
r²=0.381
p=2.198e-05
Transcript abundance [relative to IF-5a]
G
+AMF
-AMF
Blumenol A-Glc
C: Capsule
F: Flower
FB: Flower bud
ST: Stem
S: Stem leaf
R: Rosette leaf
Relative abundance [ng g FM-1, ABA equivalents]

FIGURE 6, FIGURE SUPPLEMENT 2 - Compound 6 is constitutively produced in shoots of *N. attenuata* and not indicative of AMF associations. (A) Representative chromatograms from a targeted tandem MS-based analysis of Compound 6 in leaves of *N. attenuata* (bottom panel) and as comparison, a blumenol A-9-O-glucoside (roseoside) standard (top panel). The precursor-to-product ion transitions are indicated. (B) Time lapse accumulations of Compound 6 in roots of EV plants with (EV+, green line) or without (EV-, black line) AMF-inoculation. Data represent means ±SE ($n \geq 3$). (C) Time lapse accumulations of Compound 6 in leaves of EV plants with (EV+, red line) or without (EV-, black line) AMF-inoculation and of irCCaMK plants with AMF-inoculation (irCCaMK+, orange line). Data represent means ±SE ($n \geq 5$). (D) Comparison of the abundances of Compound 6 in leaves of plants inoculated with different inoculum concentrations, samples were harvested at 5 weeks-post-inoculation (wpi). Data are means +SE ($n \geq 4$). Different letters indicate significant differences ($p<0.05$, one-way ANOVA followed by Fisher's LSD). (E) Field experiment (Great Basin Desert, Utah, USA): leaf samples of EV ($n = 20$) and irCCaMK ($n = 19$) plants were sampled 8 weeks after planting and amounts of Compound 6 were analyzed. For statistical analysis, Student's t test was applied. (F) Abundance of Compound 6 relative to the transcript abundance of the *R. irregularis* specific housekeeping gene, *Ri-tub* (GenBank: EXX64097.1), as well as to the plant derived marker genes *RAM1*, *Vapyrin*, *STR1* and *PT4*.The transcript abundance was quantified by q-PCR, relative to *NaIF-5a* (NCBI Reference Sequence: XP_019246749.1). The correlation between Compound 6 and transcript abundance of marker genes was analyzed by linear regression (lm) models. (G) Distribution of Compound 6 in different plant tissues, as indicated, of plants with (+AMF, $n = 3$, red bars) or without (-AMF, $n = 1$, black bars) AMF-inoculation. Samples were harvested at six wpi.

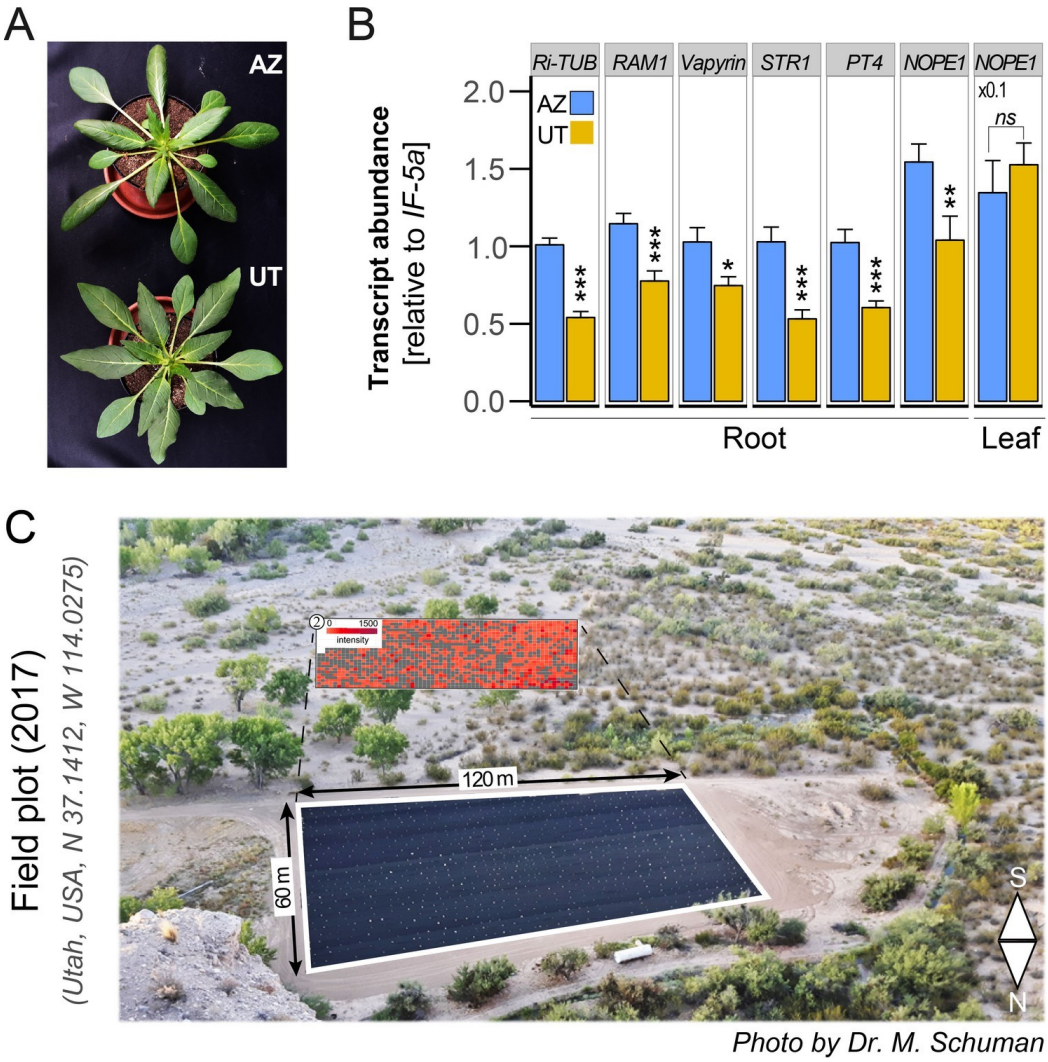

FIGURE 7, FIGURE SUPPLEMENT 1 - Phenotypes of UT and AZ accessions and field plot planting design. (A) Representative *N. attenuata* plants of the UT and AZ accessions in the rosette stages of growth (12 days after potting). (B) Transcripts of marker genes in roots responding to AMF colonization in UT and AZ after six wpi inoculated with *R. irregularis* were quantified by qPCR in the same samples as in Figure 4A–C (n = 8); Student's t test *p<0.05, **p<0.01, ***p<0.001. (C) Field plot of 728 sampled individual plants in Utah, USA, 2017.

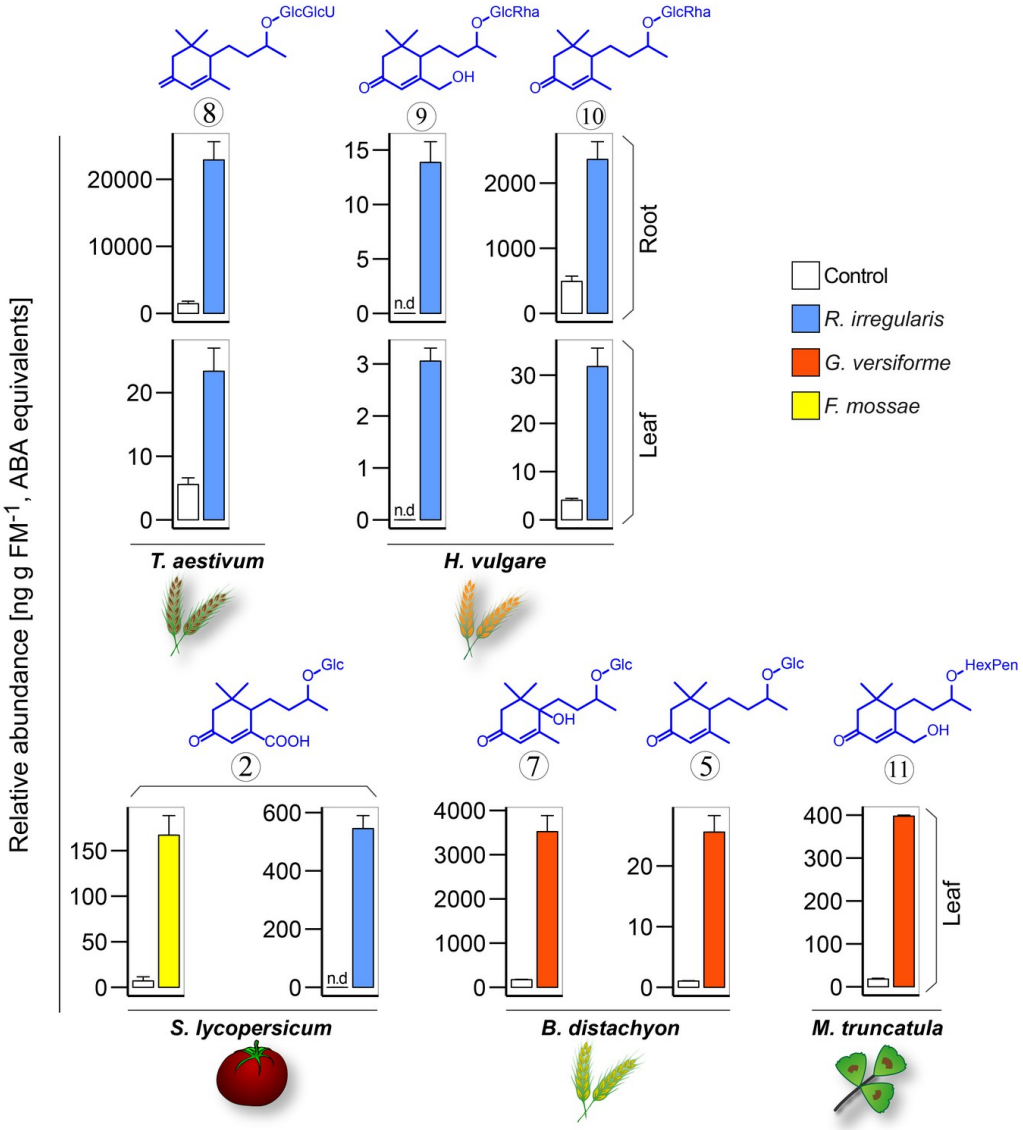

FIGURE 7, FIGURE SUPPLEMENT 2 - AMF-indicative changes in blumenols in aerial plant part are valuable research tools providing accurate assessments of functional AMF associations of multiple plant and AMF species (continued from Figure 7F). Blumenol contents of different crop plants with and without AMF inoculation (*T. aestivum*: eight wpi, n = 5; *H. vulgare*: eight wpi, n = 10; *S. lycopersicum* with *F. mossae*: 6 wpi, n = 5; *S. lycopersicum* with *R. irregularis*: 11 wpi, n = 6; *B. distachyon*: five wpi, n = 4; *M. truncatula*: seven wpi, n = 3). Different plant and AMF species were used, as indicated; means +SE, n.d., not detected.

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Discussion

Overview of the discussion

Advances in agricultural research inherently depend on the fundamental understanding of plant function investigated in plant ecology. In this dissertation, I use an ecological model plant, *Nicotiana attenuata*, to evaluate both established and emerging agricultural practices for their potential to increase population yields, and discuss the results in light of current methods for cultivar enhancement. This includes considering the roles of genetic engineering, intraspecific diversity, and accurate, high-throughput method development for increasing agricultural yields in the face of increasing climate instability.

How is genetic modification relevant to agricultural cultivar improvement?

The studies presented in this dissertation rely on genetically modified (GM) lines of *Nicotiana attenuata*. Following the first successful plant transformation in 1983, genetic modification has become a widespread tool in both ecological and agricultural studies (Gase & Baldwin, 2012; Bawa & Anilakumar, 2013). Along with the rise of GM techniques came an equal and opposite social reaction: to date, GM crops are disproportionally reflected negatively in the media, often paired with overinflated statistics regarding the public's opposition to the technology (Augoustinos *et al*. 2010). Most negative portrayals rely on criticisms of the methods for creating GM lines and largely unfounded claims that these may lead to harmful effects on humans (Maghari & Ardekani, 2011).

The GM lines used in this dissertation were produced through one of two methods: ectopic overexpression (OV) or RNA interference (IR). The OV line utilized in **Chapter 1** was generated by cloning a copy of the *Bacillus thuringiensis* (Bt) *Cry1Ac* gene into the desired plasmid vector and transforming parenchymatous plant cells, which have the ability to undergo both cell division and differentiation (Zaid *et al*. 2001). Using the viral machinery included in the plasmid vector, the gene of interest (goi) is embedded into an unknown location in the genome of each parenchymatous cell and is inherited in all subsequent cells that form new plant tissues. The unknown insertion location has been one source of public concern, given the possibility that the insertion site may disrupt another gene encoded in the genome, which could affect plant function and yield quality, or potentially create by-products that are toxic to humans (Maghari & Ardekani, 2011).

Several protocols exist to address these insertion site concerns. The protocol utilized by the studies in this dissertation involve the extracting of DNA from OV tissues and verification that

only one plasmid insertion exists in the genome through Southern blotting or other DNA sequence counting techniques (e.g. like the NanoString nCounter platform; see Gase *et al*. 2011 and Valim *et al*. 2019). Two or more independent single-insertion transformed lines are then screened for physiological traits throughout their life cycle. If these independent lines display identical phenotypes and overexpression can be confirmed by measuring transcript abundance (e.g. by qPCR), it is likely that they both ectopically produce the extra gene product without divergent effects from plasmid insertion in varying locations (Schwachtje, Kutschbach & Baldwin, 2008). As an alternative to the first step, the location of insertions in the genome can be determined by using a single primer that anneals to the insertion sequence. Conducting a polymerase-chain reaction (PCR) with this primer will amplify part of the insertion region as well as several hundred base pairs either preceding or following the insertion. The PCR product is then sequenced. A basic local alignment search tool (BLAST) can then align to the genome of the transformed organism to determine the location of the insertion (Gase *et al*. 2011). Since this can be time-intensive and expensive, the majority of screens involve the first method of Southern blotting or DNA sequence counting, as was used in the screening of the ovCRY *N. attenuata* line used in **Chapter 1**.

Public concern regarding Cry1Ac-producing GM lines also focuses on the specificity of the Cry1Ac toxins to their target organisms, as well as the use of transgenes from non-plant organisms more generally (e.g. Bt). The Cry1Ac protein is known to specifically bind to a receptor in the gut of Lepidopteran insects and to not have any binding partners within the human digestive system (Schnepf *et al*. 1998). We additionally confirm in **Chapter 1** that *N. attenuata* plants containing Cry1Ac increase mortality in *Manduca sexta* (Lepidoptera, Sphingdae), but this may have been a result of the feeding inhibition of *M. sexta* larvae on Cry1Ac-containing plant material, rather than through binding of the toxin (Ch.1, Fig.2). This reflects previous findings on the toxicity of Cry1Ac to *M. sexta*, where binding to the gut receptor did not appear to be the mechanism through which toxicity was achieved (Jenkins *et al*. 1999).

Despite evidence that Cry proteins interact only with receptors in the gut of Leptidopteran species, as well as reports from major world health groups on the safety of GM crops for consumers, the public remains wary of GM crops. This may be due to their association with herbicide-tolerant crops, for which harmful effects on human health have been reported (Brookes *et al*. 2017). However, for herbicide-tolerant GM lines, impacts on human health did not result solely from the inserted transgene, but rather from the increased use of herbicides, which were shown to affect humans (Brookes *et al*. 2017). Horizontal gene transfer between digested plant tissue and the gut microbiome is another concern, but has been shown to be unlikely (Keese, 2008). In addition, the potential for the allergenic properties of one organism to be transferred to another through a transgene is evaluated before GM crop varieties are released into the market (Goodman *et al*. 2008). Regardless, when considering lines with transgenes from other organisms in the future of agricultural cultivar improvement, the social reception of these lines must also be accounted for, as this may affect the efficacy of the line for its intended purpose (e.g. to reduce undernourishment) as populations may continue to be reticent about GM crop consumption more broadly. Golden rice, which is genetically modified with three transgenes that allow the biosynthesis of beta-carotene in the edible rice endosperm, provides an illustrative example (Ye *et al*. 2000). When ingested by humans, beta-carotene is metabolized into vitamin A, thereby potentially addressing prevalent vitamin A deficiency in global regions where rice is a major food crop. Golden rice was estimated to have the potential to prevent up to one million cases of child mortality or blindness resulting from vitamin A deficiency per year (UNICEF and The Micronutrient Initiative, 2004). However, due to the

poor public reception of golden rice as a GM crop, distribution of this life-saving food crop was greatly hindered (Dubock, 2014).

The other lines used in this dissertation are gene knockdown lines generated by IR (**Chapter 1, 2, 3**). Generation of IR lines involves taking short portions of the targeted genes, inverting them, and repeating them in tandem. These inverted-repeats (IR) are typically 100-300bp long, and are inserted into a plasmid vector. The plasmid vector is then transformed into parenchymatous plant cells as described above. When the IR insert is transcribed, interaction of these double stranded RNA molecules (dsRNA) with RNAse III-like DICER proteins cause dsRNA to be trimmed into short (20-25 bp) interfering RNA complexes (siRNA). These complexes are then integrated by Argonaute proteins (AGOs) into RNA-induced silencing complexes (RISC). During this loading process, one of the strands of RNA in the siRNA is degraded in order to allow the other strand to target messenger RNA (mRNA) from the goi. When the RISC complex binds to a piece of mRNA, the target is degraded by catalytically active AGOs, causing a "knock-down" of both the transcripts and the functional product of the gene (Majumdar *et al*. 2017). Regarding the previous concerns for GM lines, insertion number and effects are tested for IR lines as for OV lines. All IR lines used in Chapters 1-3 of this dissertation were screened in previous works (Bubner *et al*. 2006; Kallenbach *et al*. 2012; Hettenhausen *et al*. 2012; Groten *et al*. 2015). In addition, in contrast to OV lines, no transgene from another organism is inserted in these lines, eliminating concerns related to cross-organism insertions.

OV and IR GM lines allow for directly testing the functional consequences of alterations in the transcription of single gene products (either overexpression or silencing) in field plants, whether planted as individuals (**Chapter 1, 2**), or in mono- or mixed-culture intraspecific populations (**Chapter 3**). This is an important tool for determining specific mechanisms behind plant productivity. Aside from OV and IR GM lines, other, more recent genetic engineering technologies have been developed known as genome editing. In 2012, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (CRISPR/Cas9) protein complexes were applied as a genome-editing tool for the first time (Jinek *et al*. 2012). They are now frequently used in genetically engineering plants with gene knock-ins or knock-outs. CRISPR/Cas9 methods target specific genomic locations for editing using approximately 20 bp guide strands that coordinate the movement of a Cas9 to known cleavage sites, in contrast to the un-site-specific OV or IR methods. Because this method relies on endogenous DNA repair mechanisms, namely by nonhomologous end-joining or homologous recombination, it cannot be distinguished from natural processes of mutations and repairs. In some countries this has decreased public and political anxiety and prevented extensive regulation, an important consideration for widespread implementation (Grohmann *et al*. 2019). However, despite this positive outlook, some studies suggest that guide sequences based on 20bp are not specific enough to prevent off-site targets across the genome (Lin *et al*. 2014). As this technology is relatively new and debates are ongoing about off-target effects (Lin & Wong, 2018), the use of OV and IR technologies continues to be important. Because OV and IR lines are already in use and systematically regulated in agriculture, using OV and IR technologies in this dissertation reflects their current relevance for agricultural improvement.

Increasing plant productivity in the face of climate change

The practice of breeding agricultural cultivars to improve productivity had been in place for thousands of years before agriculturalists began to understand the genetic underpinnings of this process (Dudley, 1997). Traditionally, high-yielding varieties are inbred or outcrossed in order

to increase trait variation and to introgress desirable traits from parental genotypes, with GM methods also being used today (Xu *et al*. 2017). GM technology arrived after the Green Revolution, during which increased fertilizer and pesticide use allowed for exponential growth in agricultural productivity (Warren, 1998; Alston, Babcock, & Pardey, 2010; USDA, 2017). However, the negative environmental impacts of fertilizer and pesticide over-use make these techniques unsustainable (Pimentel & Edwards, 1982; Bojórquez-Quintal *et al*. 2017; Goucher *et al*. 2017). Therefore, some of the first widespread GM agricultural lines were either herbicide-tolerant lines made to reduce the need for fertilizer or insect-tolerant lines intended to replace exogenous pesticide application, both while maintaining productivity (Kamle *et al*. 2017). Today, agricultural cultivars face a new limitation to their productivity: the instability of the world's climate.

Climate change has emerged as one of the greatest challenges facing agricultural productivity in the 21st century (Ray *et al*. 2015). Global warming is projected to increase drought events faced by crops worldwide (Sheffield & Wood, 2007). Drylands currently make up 40% the Earth's land area, but are predicted to increase by 11-23% by 2100 (Huang *et al*. 2016). Additionally, increases in global temperatures caused by carbon dioxide (CO_2) accumulation is predicted to rapidly reduce biodiversity: these conditions favor warming-responsive, fast-growing plants which can quickly take over communities (see for example Gedan & Bertness, 2009). The biodiversity of plants in communities, whether in natural or agricultural ecosystems (e.g. in grasslands between crop plantings), provides essential ecosystem services, such as carbon cycling and the maintenance of soil fertility (Millennium Ecosystem Assessment, 2005). Reductions in ecosystem services lead to ecosystem degradation, which reduces the capacity of an ecosystem to support new organisms, such as future crops (Zari, 2014). A loss in biodiversity is thus a loss in the longevity of arable land.

In addition, climate change is predicted to affect future pest loads due to increased warming events: yearly communities may have high rates of turnover (Nooten *et al*. 2014). An increased variation in pest community composition makes protection of agricultural yield through targeted GM technology more difficult. For example, GM lines modified to produce Cry proteins, and therefore protected from the herbivory of Lepidopteran moth larva, have been shown to increase agricultural yield in years where targeted insects are dominant on the crop (Qaim & Zilberman, 2003). However, in years where non-target insects are dominant, these constitutively produced defenses may only serve as an unnecessary energy cost. Relatedly, field ecological studies have shown that the production of endogenous defenses without herbivore pressure comes at the cost of yield production for plants (Baldwin, 2001). Though many studies have evaluated the impact of GM crops on non-target insects (summarized in Naranjo, 2014), few have presented evidence of the effects of non-target insects on GM crops. Additionally, the productivity of GM crops has never been compared to the endogenous defenses of plants in the face of an unpredictable herbivore load. Therefore, in **Chapter 1** of this dissertation, I compare the growth and yield of *N. attenuata* Cry1Ac OV lines to counterparts with and without the endogenous defenses in the field.

Because of low Lepidopteran pest presence during the field season in which I compared CryAc-production to the endogenous defenses of *N. attenuata* , I was able to perform a yield-cost evaluation of non-target insects (e.g. *Tupiocoris* and *Epitrix*) on Cry1Ac-producing plants. This work demonstrated the slower growth and lower biomass production in Cry producing plants relative to plants producing only their endogenous defenses when under pressure from non-target insects (Ch.1, Figs.3,4). When the plants were protected by a neonicotinoid insecticide, differences in stalk height, shoot biomass, and flower production between Cry-producers and

endogenously defended plants were abolished (Ch.1, Figs.3,4). I considered that a neonicotinoid insecticide spray employed on a nicotine-producing plant species may cause a metabolic reaction that could affect these results. However, the insecticide spray did not cause significant changes of induced metabolite within genotypes (and therefore was excluded in the displayed PCA, see Materials and Methods; Ch.1, Fig.6C,) nor did they change phenolomide or diterpinoid glycoside levels from sprayed to unsprayed plants (Ch.1, Fig.6B).

Given these results, I inferred that endogenously-defended plants would be more productive than Cry-producing lines, with or without pressure from herbivory, due to the ability to modulate their defenses to their specific needs. Although all plants were elicited ("induced") by *Manduca sexta* oral secretions to reliably compare their metabolic profiles, endogenously defended plants could adjust their defense output after treatment. Both non-endogenously-defended and Cry-protein producers were constrained to certain resource allocation strategies, which may have made them less able to adapt to varying biotic pressures. Plants are limited in the amounts of resources and energy they can divert to various functions, including to defense and growth. Without the flexibility to redirect resources, plants may lose the ability to efficiently invest in production of agriculturally-relevant yields. This implies that selecting for traits in agricultural cultivars that provide constitutive responses for plants are not ideal in the face of increased environmental uncertainty due to climate change. Rather than attempting to predict relevant traits for future field seasons, it may be worthwhile to rely on the endogenous capabilities of plants to address a variety of biotic and abiotic factors. Current research efforts could then focus on addressing other pressing limitations for plants, such as the acquisition, retention, and use of local resources.

Both criticism of over-fertilization and the increasing unreliability of precipitation due to climate change have made screens for resource-use trait maximization a priority in breeding programs (Fan *et al*. 2012). For instance, these programs have bred for nitrogen (N)-use efficiency through changes in plant uptake, utilization, and N harvest index (Lammerts van Bueren & Struik, 2017). Alternatively, genotypes with certain microbiome recruitment traits may be able to acquire nitrogen or phosphate at higher rates, as particular fungi (e.g. arbuscular mycorrhizal fungi, AMF; Bonfante & Genre, 2010) and bacteria (e.g. *Acidobacterium capsulatum*, Kielak *et al*. 2016) can facilitate a plant's uptake of these nutrients. In addition, water-use efficiency and drought resistance traits are also the subject of current screens, in order to address increasingly variable regional water availability (Misra, 2014). These screens, however, exhibit similar problems with the insertion of single defense traits: they focus on only individual plant traits, rather than promoting flexible responses in the face of unknown field factors. Approaching the dilemma of future plant productivity from the point of view of selecting for a "Renaissance plant", equipped with optimal traits in multiple categories, may be more suited for future environmental variability. However, many traits participate in trade-offs in the plants (i.e. growth-defense traits; Huot *et al*. 2014), rendering this difficult at the individual level. Alternatively, at the population level, planting together diverse varieties, even within one crop species, could increase the resiliency and productivity of entire populations, as has been shown in the biodiversity-productivity and biodiversity-stability phenomena developed in ecological research (see for example, Hajjar, Jarvis, & Gemmill-Herren, 2008; Isbell *et al*. 2015).

The biodiversity-productivity phenomenom: the future for agriculture?

Most investigations into the mechanisms behind the biodiversity-productivity phenomenon have sought to predict species interactions that could increase cumulative population yields (e.g. niche differentiation; Introduction, Table 1; Zuppinger-Dingley *et al*. 2014). This research vastly advanced the field of biodiversity, but is no longer sufficient: the identification of mechanisms which can be readily manipulated to produce population-level effects is of immediate interest for maintaining agricultural productivity. These mechanisms cannot be easily determined from species-level studies due to myriad divergent traits among species (Chapin III *et al*. 1997). In addition, intraspecific variation can also have large effects on population yield, but is often not considered in these studies (Des Roches *et al*. 2018, and studies within).

In **Chapter 3** of this dissertation, I study the productivity of single-species *N. attenuata* populations due to variation in a genetically-manipulated functional trait. I varied percentages of plants that were silenced through IR in mitogen-activated protein kinase 4 (*MPK4*), among wildtype-like control plants. The IR line was previously characterized to have low water-use efficiency (WUE) phenotype in comparison to the controls (Hettenhausen *et al*. 2012; Ch.3, Fig.2A). Populations with 25% *MPK4*-deficient IR lines had increased cumulative population yields in comparison to all other population types in the field (Ch.3, Figs.3D-G). Though these field populations only consisted of four plants, I was able to replicate this effect in a glasshouse population experiment using 12-plant populations, planted at a different density (Ch.3, Figs.3K,M). Therefore, density and the size of the population, two factors that are typically known to interfere with population yields (Weiner, 1980), did not appear relevant to our result. This indicated potential for *MPK4* variation to be studied in even larger contexts.

The work presented in **Chapter 3** aimed to bridge the gap in understanding from the input of manipulated variation to the output of increased yield. To do so, I provided a detailed analysis of phenotypic changes in the manipulated trait across population type (Ch.3, Fig.5). Second, I attempted to determine the scale, whether among only immediate neighbors, or across full populations, at which genetic variation may be causing increased yield (Ch.3, Figs. 4,S7). Finally, I tested the dependency of the observed effect on aboveground versus belowground tissues in the divergent and control plant (Ch.3, Figs.6,7). Each of these tasks were complicated by either time-intensive measurements or lack of precedent in population experiments. However, each could provide valuable information for future experimental approaches to determine the connection between the variation of a gene or trait and increased population yield.

Based on previous literature demonstrating that trees with divergent photosynthetic phenotypes could change the photosynthetic profiles of their neighbors (Bunce *et al*. 1977), I tested for photosynthetic changes among control plants in varying population types. Interestingly, I discovered that the low WUE phenotype of *MPK4*-deficient lines did not appear in the field (Ch.3, Fig.5C). Despite the absence of the expected phenotype, deficiency in *MPK4* was confirmed in these plants, in comparison to the control plants (Ch.3, Fig.2C). Additionally, the low WUE phenotype was observed in the glasshouse experiments (Ch.3, Fig.2B), but changes in the control plant's photosynthetic phenotypes never reflected the overyielding in 25% populations. We inferred the effect to be dependent on *MPK4*-deficiency, unrelated to WUE. Our results may provide a warning for future applications of biodiversity to increase population productivity: implementing variation from the trait level may not be as reliable as a manipulation from a genetic basis. In fact, a meta-analysis of lab and field phenotypic data showed only modest correlation ($r^2 = 0.26$; Poorter *et al*. 2016). The increasing accessibility of forwards genetics tools can help with determining the genetic bases of trait variation, such as quantitative trait locus (QTL) mapping on structured populations of recombinant inbred lines

(RILs; definition of QTL mapping: Shimoyama *et al*. 2012). Recently, QTL mapping even resolved an effect of plant biodiversity to a single locus (Wuest & Niklaus, 2018). What remains is to test these loci to determine causation.

Single gene manipulations still do not provide an understanding of how the altered genes can cause population effects. The experiments in **Chapter 3** sought to break ground in this area by experimentally evaluating the scale, or level of hierarchical organization (Allen and Starr, 1982), at which low percentages of *MPK4*-deficient plants increase population yields. I evaluated if divergent plants caused changes only in immediate neighbor plants (neighbor scale), or in plants across the entire population (population scale; Ch.3, Fig.1). Paired-plant-in-a-pot experiments revealed that *MPK4*-deficient plants had no unique effect on neighbor control plants (Ch.3, Fig.4). However, paired-plant experiments may be too simple to reveal effects that are relevant in the population context. In ecological experiments, simplification of experimental designs to include only fractions of the influential factors present in natural growth scenarios are known to decrease the potential of observing significant effects (Rillig *et al*. 2019). Therefore, I analyzed the effect of various configuration of immediate plant neighbors in the glasshouse populations on control plant yield. I found no trends that reflected our overyielding result (Ch.3, Fig.S7). Though causation at the population-scale was not directly tested, I was able to exclude the neighbor-scale. In terms of applicability, mechanisms manifesting at the population scale would be advantageous over those at the neighbor scale: agricultural seed mixes could be made with certain percentages of a divergent genotype and distributed randomly across agricultural fields. Neighbor scale dependencies may require a laborious process to ensure neighbor scale structure within populations. Screens for population-scale-specific effects may therefore be essential: based on the work in **Chapter 3** of this dissertation, I suggest that "polling" subsets of plants within biodiversity-productivity test populations to evaluate the influence of direct neighbor configurations could advance the selection of beneficial traits for diversity applications.

Finally, in **Chapter 3**, I sought to further narrow the dependency of observed overyielding effects to above- or belowground tissues. This is typically difficult to elucidate for populations given the complexity of the plant-plant interactions both in the shoots and the roots. Instead of performing individual plant sampling, I disconnected entire populations from AMF networks (by crossing individuals with plants silenced in the Sym-pathway gene calcium and calmodulin-dependent protein kinase, *CCaMK*; Groten *et al*. 2015) and compared them to populations which could associate with the AMF networks (i.e. uncrossed). AMF is known to benefit plants by increasing their nutrient acquisition, as well as by facilitating plant-plant signal transfer belowground (Salvioli di Fossalunga & Novero, 2019; Song *et al*. 2019). Though comparing populations with and without AMF association cannot directly demonstrate a role in belowground tissues in creating the overyielding observed in our 25% populations, it can indicate they have a major influence on the yield outcome. We found that biomass overyielding was abolished by interaction with an AMF network, but that reproductive overyielding was unaffected (Ch.3, Fig.7). We inferred that reproductive overyielding was likely specific to the variation of *MPK4*-deficient plants in 25% populations, as well as to the aboveground tissues, which had remained unmanipulated in this test. Further work would be essential to directly manipulate aboveground interactions of *MPK4*-deficient and control plants. Perhaps the use of a real-time gene silencing method, such as the DEX-inducible, single-leaf silencing system employed in **Chapter 4** of the dissertation (Schäfer *et al*. 2013), could allow for growth and metabolomic measurement of control individuals in *N. attenuata* field populations before and after low percentages of plants are induced to have *MPK4*-deficiency in select above-ground

tissues. This may lead to the identification of potential signaling pathways or molecules involved in the *MPK4*-variance to population yield effect.

Our three main results from **Chapter 3** were intended to apply and evaluate potential tools for narrowing the operating scale of gene to population effects. However, this is far from an exhaustive evaluation of all potential tools that could continue to be developed for this purpose. Importantly, our work emphasizes the value of method development in this area.

Petitioning for more extensive considerations of methodology

Screening promising diversity-yield-related genes, such as *MPK4*, for use in an agricultural context would require new methods to be implemented. Ideally, these lines could be directly field tested, as glasshouse space and resources may be too limited to conduct population tests. Field screens of these lines, however, would be laborious (e.g. quantifying yield from an entire field, potential plant "polling"). Reliable pre-screens to determine the lines of interest for these field tests would therefore be essential to avoid wasted efforts in the field.

For water-use-related traits such as WUE, levels of photosynthetic parameters, and plant drought responses, current screening techniques are not entirely dependable. Many experimental designs do not take into account equal water availability when comparing these traits among genotypes (see for example, Chen *et al*. 2014). Lines that vary in water-use traits will likely vary in their rates of soil water consumption. When watering is withheld, they will therefore expend their local water resources over different periods of time, leading to different days of drought onset per genotype (Nable *et al*. 1999; Henry *et al*. 2011). If sampling occurs simultaneously for all genotypes, then some will have experienced longer droughts than others solely based on their genotype, which may confound sampling results depending on the hypothesis of the experiment (Gilbert & Medina, 2016). Controlling for the onset of drought through soil water availability measurements is not new, and yet is still not standard in agricultural screens. In **Chapter 2** of this dissertation, we hypothesized that this was due to the lack of an experimental determination of the extent to which differences in drought onset can convolute the screening of water-use phenotypes such as drought responses. We therefore sought to provide these results. In addition, we tested the extent to which applying drought events to differing genotypes in varying developmental stages could also confound screenings of water-use phenotypes.

We measured common drought response markers in 30 *N. attenuata* lines, varied in their water-use and developmental traits, in each of three treatments: while undergoing an uncontrolled, onset-of-drought controlled, or onset-of-drought and developmental stage-controlled drought event (Ch.2, Fig.3). We performed a variance decomposition of the cumulative data, in order to see what experimental parameters would be influential in explaining the variation created within the drought response phenotypes. We found that for almost all recorded drought response measurements, developmental stage or onset-of-drought timing explained more variance than the genetic differences among the lines (Ch.2, Fig.4). This indicated that these experiments largely fail to identify differences among genotypes, and would lead to wasted efforts with further tests. Reducing occurrences of error in screenings is essential for minimizing the investment needed to make biodiversity-productivity methods feasible to test.

For other resource-use traits, the time they take to be sampled and processed may be inhibitory to their application in agricultural screens. For example, association of plants with AMF networks can significantly improve plants resource acquisition and use (Salvioli di Fossalunga

& Novero, 2019), but screening for AMF association using traditional microscopy methods is both destructive and time-consuming (Deguchi *et al*. 2017). In **Chapter 4**, we identified a high-throughput, non-destructive leaf molecular marker which correlates with the extent of AMF-association a plant has in the roots: blumenol. This was not a new molecule, but its association with the extent of plant-AMF symbiosis was novel (Ch.4, Fig.3). The time-saving quality of our method was clear: traditionally, staining roots and placing them on microscope slides would require approximately 17 minutes per sample, preceding approximately 40 minutes of microscopic quantification, and resulting in a time investment of one hour per sample. On the other hand, a one-step methanol-based metabolite extraction from leaf tissues could be achieved in approximately 1 minute per sample, and blumenol was analyzed using an 18-minute program on a Bruker Elite EvoQ triple quadrupole MS with a HESI (heated electrospray ionization) ion source. Factoring in several minutes per sample for peak integration, the blumenol marker would allow quantification a plant's association with AMF in 20 minutes sample, of which 18 minutes did not require active participation (i.e. samples running on the machine). In contrast, the traditional method required constant involvement in the process, from staining to structure identification under the microscope. Essentially, using the blumenol marker cuts quantification time by 30-fold.

Such advances are essential for the future implementation of traits like AMF association in agricultural cultivars. Yet, it is important to consider that agricultural screening for the blumenol markers we identified do not necessarily produce the relevant information needed for application. To date, we do not understand the functional aspect of the blumenol molecule. Though our work indicated correlation between the number of arbuscules (centers of nutrient transfer between the plant and the fungus; Gutjahr & Parniske, 2013) in the roots and blumenol amounts, this has not been investigated further. The particular plant-AMF interaction that produces the blumenol marker would provide necessary mechanistic understanding for screenings of this trait.

Developing appropriate methodologies for screening agriculturally-relevant functional traits is often overlooked in the interest of faster results and production. However, a lack of methodological assessment can lead to influential errors due to experimental design, as was demonstrated by **Chapter 2**. Despite the results that this dissertation presents reflecting new tools to increase population yields (**Chapters 3, 4**), perhaps the most important conclusion may be the importance of careful methodological consideration before conducting screenings. Among these considerations, we emphasize the importance of understanding the environmental and experimental contexts that alter the efficacy of screens (**Chapter 1, 2**). Additionally, we encourage the evaluation of the spatial scale at which effects originating from a cultivar may occur (**Chapter 3**) to be able to manipulate it more precisely. Unifying ecological and agricultural techniques can help to achieve these goals; perhaps increased collaboration between the two fields should be emphasized for continued progress towards sustaining both plant, and human, populations.

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Summary

At least 10,000 years ago, humans began domesticating crop plants into consistent, high-yielding food sources. Plants continue to provide 90% of human food energy intake worldwide. However, as human populations increase and arable land becomes scarce or unproductive due to climate instability, plant food sources may no longer be able to sustain human nutritional requirements. Plant populations must become more productive.

This dissertation uses an ecological model plant, *Nicotiana attenuata*, to evaluate the contexts and scales at which plant populations can increase their productivity. I explore the current uses and future potentials of three functional traits that can be selected for, or genetically modified, in crop cultivars to improve agricultural yields. First, I test the efficacy of current agricultural pest-resistance technology in increasing yield. The pest-resistance technology (*Cry1Ac* expression), conferred through genetic modification to *N. attenuata*, did not increase yield in comparison to endogenously defended, or even undefended *N. attenuata* lines. Due to the scarcity of Cry1Ac-targeted insects in this field season, plants with more flexible use of their direct defenses were able to be more productive, demonstrating the benefit of naturally evolved defenses in the face of yearly-inconsistent pests.

Resource-use traits such as plant water-use or association with arbuscular mycorrhizal fungal (AM) networks that facilitate nutrient access are as important to agricultural productivity as pest-resistance. Current screenings for water-use traits among agricultural varieties are insufficient: they do not account for varying rates of soil water consumption or plant development in applying drought treatments, and therefore, do not lead to reproducible results in the field. We use variance decomposition to quantify the extent to which these factors, when left uncontrolled, can significantly change observed results. I then apply the ecologically established biodiversity-productivity phenomenon to attempt to increase population yields by varying the percentage of plants with a low water-use efficiency trait among control plants in *N. attenuata* field populations. Low percentages of this trait caused overyielding. Using both novel and developed methodologies, I advance the understanding of the mechanisms behind this effect by identifying one of its genetic bases, and narrowing the spatial scale and plant tissue at which it occurs. Finally, we develop a method for screening agricultural cultivars for association with AM fungal networks by using a high-throughput leaf molecular marker rather than traditional microscopy methods, which are laborious and destructive. This work emphasizes the benefits of methodological development, which can both improve screenings for agriculturally-relevant functional traits and allow for application of ecologically-informed alternatives to increase population yield (e.g. intraspecific diversity).

Zusammenfassung

Bereits vor mindestens 10 000 Jahren begannen Menschen Pflanzen zu domestizieren und gewannen damit einheitliche und ertragreiche Nahrungsquellen. Auch heute nehmen Menschen 90% ihrer Nahrungsenergie über Pflanzen auf. Allerdings könnten in Zukunft Pflanzen nicht ausreichen, um die menschlichen Nahrungsbedürfnisse zu decken. Die Weltbevölkerung wächst und Klimainstabilität führt zu schrumpfenden Agrarflächen oder sinkenden Erträgen. Pflanzen müssen dementsprechend ertragreicher werden.

In dieser Dissertation wird die Modellpflanze *Nicotiana attenuata* verwendet, um die Zusammenhänge und Ausmaße zu erforschen, in denen Pflanzenpopulationen ihre Leistungsfähigkeit steigern können. Ich untersuche die derzeitige Anwendung sowie zukünftige Potentiale von drei funktionellen Eigenschaften, die in der Züchtung selektiert oder in Pflanzen genetisch modifiziert werden können, um landwirtschaftliche Erträge zu steigern. Zuerst analysiere ich, wie effizient derzeitige landwirtschaftliche Schädlingsresistenztechnologien die Ausbeute erhöhen. Die Expression des *Cry1Ac* als Verteidigungssystem, eingebracht in *N. attenuata* durch genetische Modifikation, führte zu keiner Ertragssteigerung im Vergleich zu Pflanzen mit endogener Verteidigung oder sogar unverteidigten *N. attenuata*-Linien. Da in der Saison des Feldversuchs nur wenige Insekten vorkamen, gegen die das Cry1Ac-System gerichtet ist, reagierten Pflanzen mit endogener Verteidigung plastischer und damit produktiver auf ganzjährlich variierende Herbivorgemeinschaften.

Genauso wichtig wie Schädlingsresistenz sind die Ressourcennutzung sowie die Assoziation mit arbuskulären Mykorrhizapilzen (AM-Pilzen), die den Zugang zu Nährstoffen unterstützen. Derzeitige Untersuchungen zu Wassernutzungseigenschaften verschiedener Agrarsorten sind unzureichend: In Versuchen mit Dürrebehandlung werden Unterschiede im Bodenwasserverbrauch oder die Entwicklung der Pflanzen nicht berücksichtigt und führen dementsprechend nicht zu reproduzierbaren Ergebnissen im Feld. Durch Varianzzerlegung quantifizieren wir, in welchem Ausmaß die einzelnen Faktoren, sollten sie unkontrolliert bleiben, die Ergebnisse signifikant verändern können. Mithilfe des ökologisch etablierten Biodiversitäts-Produktivitäts-Phänomens habe ich versucht die Populationserträge zu erhöhen, indem ich die Anteile von Pflanzen mit geringer Wasserverbrauchseffizienz und Kontrollpflanzen variiert habe. Waren solche Pflanzen zu einem geringen Prozentsatz in *N. attenuata* Feldpopulationen vorhanden, führte dies zu einer Ertragssteigerung. Mittels sowohl neuer als auch etablierter Methoden ist es mir gelungen zum Verständnis der Mechanismen hinter diesem Effekt beizutragen, indem ich eine der zugrundeliegenden genetischen Ursachen identifiziert habe und das räumliche Ausmaß sowie das Pflanzengewebe, in dem dieser Effekt auftritt, eingegrenzt habe. Abschließend entwickelten wir eine Methode, um Kultivare in Agrarpopulationen auf Assoziation mit AM-Pilzen zu untersuchen, bei der im *high-throughput-*

Verfahren ein molekularer Marker im Blatt statt der traditionellen, aufwendigen und schädlichen Mikroskopiemethode verwendet wurde. Diese Arbeit betont die Vorteile von Methodenentwicklung, welche sowohl die Suche nach landwirtschaftlich relevanten Eigenschaften verbessern kann als auch die Anwendung von ökologisch begründeten Alternativen zur Ertragssteigerung ermöglicht (z.B. intraspezifische Diversität).

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Note: inconsistencies in reference formatting have resulted from extraction from differing journal formats. These will be adjusted in the final version.

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Erklärung

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.

Jena, Erica McGale

List of Publications

Scientific Articles

Under Review

1. **McGale, E.**, Valim, H., Mittal, D., Morales Jimenez, J., Hallitschke, R., Schuman, M.C., Baldwin, I.T. Determining the scale at which variation in water-use traits and AMF associations change population yields. *Revised and resubmitted to eLife.* Original version: BioRxiv doi: 10.1101/682674

2. **McGale, E.***, Valim, H.*, Hallitschke, R., Baldwin, I.T. Early developmental transitions and drought timing influence drought resistance strategies and confound the quantification of genotypic diversity in drought responses (*indicates co-first authorship). *Revised and resubmitted to Plant, Cell, & Environment.*

3. Xu, S., Kreitzer, C., **McGale, E.**, Lackus, N. D., Guo, H., Köllner, G., Schuman, M. C., Baldwin, I.T., Zhou, W. Allelic differences of clustered terpene synthases contribute to correlated intra-specific variations of floral and herbivory-induced volatiles in a wild tobacco. *Submitted to New Phytologist.*

4. Valim, H., Dalton, H., Joo, Y., **McGale, E.**, Halitschke, R., Gaquerel, E., Baldwin, I.T., Schuman, M.C. TOC1 in *Nicotiana attenuata* regulates efficient allocation of nitrogen to defense metabolites under herbivory stress. *Intended for submission to New Phytologist.*

2019

1. Valim, H., **McGale E.**, Yon F., Halitschke R., Fragoso V., Schuman, M.C., Baldwin, I.T. (2019). The clock gene TOC1 in shoots, not roots, determines fitness of *Nicotiana attenuata* under drought. Plant Physiology, 181 (1): 305-318. doi: 10.1104/pp.19.00286.

2018

1. **McGale, E.**, Diezel, C., Schuman, M. C., Baldwin, I. T. (2018). Cry1Ac production is costly for native plants attacked by non-Cry1Ac-targeted herbivores in the field. New Phytologist, 219(2), 714-727. doi: 10.1111/nph.15207.

2. Schuman, M. C., Meldau, S., Gaquerel, E., Diezel, C., **McGale, E.**, Greenfield, S., Baldwin, I. T. (2018). The active jasmonate JA-Ile regulates a specific subset of plant

jasmonate-mediated resistance to herbivores in nature. Frontiers in Plant Science, 9: 789. doi: 10.3389/fpls.2018.00787.

3. Wang, M., Schäfer, M., Li, D., Halitschke, R., Dong, C.-F., **McGale, E.**, Paetz, C., Song, Y., Li, S., Dong, J., Heiling, S., Groten, K., Franken, P., Bitterlich, M., Harrison, M., Paszkowski, U., Baldwin, I. T. (2018). Blumenols as shoot markers for root symbiosis with arbuscular mycorrhizal fungi. eLife, 7: e37093. doi: 10.7554/eLife.37093.

2017

4. Zhou, W., Kügler, A., **McGale, E.**, Haverkamp, A., Knaden, M., Guo, H., Beran, F., Yon, F., Li, R., Lackus, N., Köllner, T. G., Bing, J., Schuman, M. C., Hansson, B. S., Kessler, D., Baldwin, I. T., Xu, S. (2017). Tissue-specific emission of (E)-a-bergamotene helps resolve the dilemma when pollinators are also herbivores. Current Biology, 27(9), 1336-1341. doi: 10.1016/j.cub.2017.03.017.

Presentations

1. **McGale E.** (2019). Determining the scale at which variation in WUE traits changes population yields. Talk presented at Institute Symposium, Max Planck Institute for Chemical Ecology, Jena, DE

2. **McGale E.** (2019). Low abundances of irMPK4 plants in population increase total population yield, but only without AMF interactions. Talk presented at 18th IMPRS Symposium, Max Planck Institute for Chemical Ecology, IMPRS, Dornburg, DE

3. **McGale E.** (2018). MPK4 in the roots mediates *Nicotiana attenuata*'s neighbor-related growth responses. Talk presented at Talk, Friedrich-Schiller-Universität, Institute of Ecology and Evolution, Jena, DE (invited speaker)

4. **McGale E.** (2018). NaMPK4 plays a role in *Nicotiana attenuata*'s growth responses to neighbors. Talk presented at 17th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

5. Schuman, M.C., Oh, Y., **McGale E.** (Jan 2018). Introduction to gas exchange, fluorescence, and photosynthesis measurements with IRGA (Infra-Red Gas Analyzer). Talk presented at Institute Seminar, MPI for Chemical Ecology, Jena, DE

6. **McGale E.** (2015). Functional diversity explored by varying the interspecies diversity in *N. attenuata* populations undergoing drought stress. Talk presented at iDiv Annual Conference, Leipzig, DE

Posters

1. Rocha A.C., Chuang L., Wang M., Li D., **McGale E.**, Schäfer M., Groten K., Halitschke R., Baldwin I.T. (2019). Blumenols as fingerprints of AMF colonization in

Nicotiana attenuata roots. Poster presented at Institute Symposium, Max Planck Institute for Chemical Ecology, Jena, DE

2. **McGale E.**, Valim H., Halitschke R., Baldwin I.T. (2018). Testing drought resistance traits in glasshouse experiments: when to control for water use and water availability. Poster presented at Institute Symposium, MPI für Chemische Ökologie, Jena, DE

3. **McGale E.** (2017). Cry-worthy cost of producing an ectopic defense protein. Poster presented at 16th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

4. Wang M., **McGale E.**, Wilde J., Valim H., Groten K., Schuman M.C., Baldwin I.T. (2016). Fine-scale changes with large-scale consequences: Tuning expression of key genes and interactions with mycorrhizal fungi alters plant phenotypes and community dynamics. Poster presented at SAB Meeting 2016, MPI for Chemical Ecology, Jena, DE

5. Valim H., **McGale E.**, Fragoso V., Baldwin I.T., Schuman M.C. (2016). How does the core circadian clock component TOC1 mediate responses to stress in plant root and shoots. Poster presented at 15th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Patents

1. Baldwin, I.T., Halitschke, R., Wang, M., Li, D., Schäfer, M., **McGale, E.**, Heiling, S. (2019). Leaf markers for root colonization by arbuscular mycorrhizal fungi in plants. European Patent Office: PCT/EP2019/054738 filed February 26, 2019.