Long-term effects of the circadian clock on plant fitness in the face of abiotic and biotic stress

DISSERTATION

zur Erlangung des akademischen Grades

Doctor rerum naturalium

(Dr.rer.nat.)

vorgelegt

dem Rat der Biologisch-Pharmazeutischen Fakultät

der Friedrich-Schiller-Universität Jena

von

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geboren am 16. Oktober 1991 in Araras

MAX-PLANCK-GESELLSCHAFT

Max-Planck-Institut für chemische Ökologie

Exploration of Ecological
Interactions with Molecular
and Chemical Techniques

Gutachter:

Beginn der Promotion:

Eingereicht am:

Tag der Verteidigung:

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Introduction

The importance of timing for plant responses to stress

Now that increasingly accessible -omics tools have revealed the intricate inner world of plant signaling and metabolism, it is hard to imagine that plants were considered as essentially passive synthesizers thirty years ago, with plant secondary metabolites being perhaps most famously referred to as "flotsam and jetsam on the metabolic beach" (Haslam, 1986). Despite the vast catalogue of plant secondary metabolites made by botanists and biochemists throughout the twentieth century, it was entomologists who, by the middle of the century, had begun to appreciate the ecological significance of secondary metabolites for insect specialization and survival on particular host plants, and therefore their importance for plant defense (Fraenkel, 1959; Hartmann, 1996, 2007).

From the plant's perspective, this vast chemical toolkit is essential for survival. Given that plants deal with a variety of stresses that place limitations on their fitness, and yet are sessile organisms, the most likely manner of "escape" available to them is across temporal rather than spatial scales (D.F Rhoades, 1979), and through chemical rather than mechanical means. Plants' panoply of induced and constitutive secondary metabolites, and the regulation in timing and quantity of their production, thus provide plants with their only means of remaining alive long enough to safeguard the next generation in the face of herbivore attack and environmental extremes from which they cannot escape (Ian T Baldwin, 2017).

Plants' rhythmic responsiveness to stimuli, such as stomatal sensitivity to light, have long been known and measured experimentally, presaging the description of an endogenous timekeeping mechanism (F. Darwin, 1898). The circadian clock is a conserved timekeeping mechanism that enables plants, like other organisms, to maintain maximum responsiveness at particular periods of the day in their fight for survival (Harmer, Panda, & Kay, 2001). Studies have elucidated mechanisms by which the interlocking pieces of the circadian clock and its peripheral systems interact to enable responses to a variety of stresses (Seo & Mas, 2015; Sharma & Bhatt, 2015). In contrast, the functional role of the clock in optimizing performance under particular day length regimes has been demonstrated (Dodd *et al.*, 2005), which is consistent with the observed variation in circadian rhythm across latitudes (Greenham *et al.*, 2017).

Despite a wealth of insights into the circadian clock's involvement in stress signaling (detailed below, *the circadian clock machinery and its outputs*), few studies have explored the functional role of the circadian clock in stress responses. While some studies have explored the role of diurnal rhythms and circadian inputs on herbivore feeding (Goodspeed, Chehab, Min-Venditti, Braam, & Covington, 2012; Herden *et al.*, 2016) or on the diurnal regulation of abscisic acid-mediated stomatal aperture control (H. G. Lee, Mas, & Seo, 2016; Legnaioli, Cuevas, & Mas, 2009; Seo *et al.*, 2009), there is a paucity of evidence for the fitness consequences of altered circadian rhythms, with physiological performance often standing in for true functional evidence of fitness effects. Related to this lack of functional evidence is a general lack of focus on the intersection between the clock's role in regulating developmental transitions and responses to stressful conditions in nature, with the few studies that explore this interplay focusing on photoperiodic mechanisms (see e.g. Izawa *et al.*, 2011). By investigating the role of the circadian clock in shaping the responses of the desert annual *Nicotiana attenuata* to biotic and abiotic

stresses, the work presented in this dissertation attempts to fill some gaps in our understanding of the circadian clock's function in enhancing plant fitness under stressful conditions.

Abiotic and biotic challenges faced by *Nicotiana attenuata*

N. attenuata is a wild tobacco growing across western North America, from southern Canada to northern Mexico, particularly in the cold deserts of the Great Basin (Lynds & Baldwin, 1998; Wells, 1959). It is largely found in disturbed environments, such as after fires, and its germination is stimulated by smoke cues in soil (Ian T. Baldwin, Staszak-Kozinski, & Davidson, 1994). The fire-chasing strategy of *N. attenuata* often leads to large-scale germination in near monocultures and subsequently to intense levels of intraspecific competition. *N. attenuata* plants are known to display high phenotypic plasticity in response to herbivory, not only in their defense chemistry (Lynds & Baldwin, 1998; van Dam & Baldwin, 2001) but also in flower production and subsequent pollinator recruitment (D. Kessler, Diezel, & Baldwin, 2010). Given the variable but mostly dry climate conditions throughout a majority of its range in combination with this competitive environment, it is likely that *N. attenuata* displays phenotypic plasticity in its responses to abiotic conditions, although this remains largely untested aside from in response to nitrogen availability.

Drought resistance in annual plants is often categorized as drought avoidance (DA) or drought escape (DE), with DE engaging rapid developmental responses that complete a plant's life cycle before drought proves fatal, and DA engaging rapid physiological and biochemical responses that reduce water loss to prevent dehydration-related damage, ensuring survival before resuming growth after drought alleviation (Kooyers, 2015). While both DE and DA responses may lead to increased fitness, evidence from model systems where drought shortens the growing season, similarly to *N. attenuata,* suggests that DE responses resulting in earlier flowering and rapid completion of the life cycle are more beneficial for Darwinian fitness than the phenotypic plasticity that comprises DA responses (Franks, 2011).

N. attenuata also displays high levels of phenotypic plasticity when it comes to biotic interactions with herbivores and pollinators in nature. A well-known example is the interaction between *N. attenuata* and its key pollinator and simultaneously, specialist herbivore, *Manduca sexta,* the tobacco hornworm moth (D. Kessler, Gase, & Baldwin, 2008). Although the majority of *N. attenuata's* flowers are night-opening, benefiting Lepidopteran nectar foragers like *M. sexta*, a small number of its flowers open in the morning, when nectar foraging and pollination is done by hummingbirds, including *Archilochus alexandri* (D. Kessler *et al.*, 2010; Yon *et al.*, 2017).

Aside from trade-offs between outcrossing and herbivory, *N. attenuata* displays a variety of plastic responses to herbivory itself. Herbivore attack induces both direct chemical defenses that directly target an herbivore's ability to feed or survive on the plant, as well as volatile indirect defenses that recruit predators to remove the offending herbivore (Halitschke, Keßler, Kahl, Lorenz, & Baldwin, 2000). Nicotine and trypsin proteinase inhibitor (TPI), two direct defenses, increase plant fitness under field conditions in the face of herbivory (Steppuhn, Gase, Krock, Halitschke, & Baldwin, 2004; Stitz, Baldwin, & Gaquerel, 2011; Zavala & Baldwin, 2004), while indirect defenses have been recently shown to also increase fitness under field conditions (Schuman, Barthel, & Baldwin, 2012). Aside from nicotine and TPI, *N. attenuata* also produces

several other classes of direct defenses, among them two abundant phenolamides, caffeoylputrecine and di-caffeoyl spermidine (Kaur, Heinzel, Schöttner, Baldwin, & Gális, 2010; Onkokesung *et al.*, 2012). These defenses have been shown to be highly specific to wounds treated with oral secretions of *M. sexta,* a specialist herbivore (Halitschke & Baldwin, 2003; A. Kessler & Halitschke, 2007; Stork, Diezel, Halitschke, Gális, & Baldwin, 2009) and to vary between plants elicited by generalist and specialist herbivore oral secretions (Voelckel & Baldwin, 2004). Given that induced defenses can be highly costly from a fitness perspective in the absence of herbivory (van Dam & Baldwin, 2001; Voelckel, Schittko, & Baldwin, 2001; Zavala & Baldwin, 2004; Zavala, Patankar, Gase, & Baldwin, 2004) and that plants produce a great number of differentially-induced direct defenses, tuning the allocation of resources between different direct defenses is likely to have a significant effect on fitnes, although this remains largely untested.

Plasticity in the allocation of nitrogen between defenses and growth

The extent to which plant allocation of resources between growth and defense constitute a trade-off is of great importance to a variety of theories of plant defense (Herms & Mattson, 1992; Doyle McKey, 1974; Meldau, Erb, & Baldwin, 2012). Given that tobacco plants have been demonstrated to not recover nicotine-invested nitrogen pools for growth, even under nitrogen-limited conditions (Ian T. Baldwin & Ohnmeiss, 1994), the specific allocation of nitrogen to defense compounds must be partitioned efficiently between the synthesis of secondary metabolites, and growth and development, but also among individual plant secondary metabolites that are more or less effective against specific herbivores such as *M. sexta. N. attenuata* plants in which nitrogen allocation has been investigated using ^{15}N-pulse labeling were found to allocate larger amounts of their nitrogen pools to nicotine if induced with methyl jasmonate, a general inducer of plant chemical defenses (Ian T Baldwin, Gorham, Schmelz, Lewandowski, & Lynds, 1998). On the other hand, plants elicited with *M. sexta* oral secretions have been shown to not increase their nitrogen allocation to nicotine pools, but rather to traffic most new nitrogen into other defenses that may be more effective against *M. sexta* such as the phenolamides caffeoylputrescine and di-caffeoyl spermidine (Ullmann-Zeunert *et al.*, 2012, 2013).

The circadian clock machinery and its outputs: dealing with external cues and stresses

The plant circadian clock

The circadian clock provides a means for plants to contextualize responses to the environment, and both physiological and genomic evidence the adaptive significance of the plant circadian clock (Green, Tingay, Wang, & Tobin, 2002; Hofmann, 2012; Izawa, 2012; Lou *et al.*, 2012). *TIMING OF CAB EXPRESSION 1* (*TOC1*) is an essential component of the central repressilator loop of the circadian clock in most plant species, repressing and being repressed by *LATE ELONGATED HYPOCOTYL (LHY)* (Alabadí *et al.*, 2001; Nohales & Kay, 2016; Pokhilko *et al.*, 2012). While *A. thaliana* has a paralog to *LHY*, *CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1)*, which displays a partially redundant function to *LHY* (McClung, 2010; Mizoguchi *et al.*, 2002), other species, including *N. attenuata,* lack this paralog (Okada *et al.*, 2009; Takata *et al.*, 2009; Yon *et al.*, 2012; Zdepski *et al.*, 2008).

Abscisic acid and plant responses to drought: a circadian connection

The connection between the circadian clock and drought responses via regulation of abscisic acid (ABA) has long been implied by transcriptomic analyses. In one report analyzing several datasets, 722 transcripts belonging to high- and low-amplitude rhythmic genes were queried for their responsiveness to a variety of phytohormones, with ABA-responsive transcripts being significantly overrepresented within this set of rhythmically expressed genes (Mizuno & Yamashino, 2008). *TOC1* has been directly implicated in plants' responses to drought by binding to the promoter region of the ABA receptor *ABAR* (Legnaioli *et al.*, 2009), and TOC1's rhythmicity is in turn strongly affected by ABA levels via the transcription factor *MYB96*, which affects a variety of ABA-dependent functions including response to drought stress (H. G. Lee *et al.*, 2016; K. Lee, Lee, Yoon, Kim, & Seo, 2015; Seo & Park, 2010; Seo *et al.*, 2009).

The role of the circadian clock in shaping jasmonic acid signaling responses

The critical role of timing more generally, and of the circadian clock specifically, has been demonstrated in a variety of plant systems. In *Arabidopsis thaliana,* timing has been shown to be critical to successfully fending off herbivore loads: plants grown with diurnal cycles out-of-sync with larvae of the herbivore *Trichoplusa ni* lose more leaf area than plants in sync with *T. ni*, and out-of-sync herbivores grow more than three times the size of their in-sync counterparts (Goodspeed *et al.*, 2012). Both plants with arrhythmic circadian clocks and plants deficient in jasmonic acid (JA) production lose this in-sync advantage against *T. ni,* demonstrating the necessity of both JA-mediated defense responses and their correct timing via the circadian clock even under diurnal conditions (Goodspeed *et al.*, 2012).

The rhythmicity of JA basal levels, however, is unlikely to be the primary measure of how well-defended plants are at any particular moment in time: for many systems, JA is induced well beyond its basal levels after herbivory. Interestingly, the magnitude of induction is itself under circadian regulation, with lima bean leaf JA levels being induced to levels 2-3 times higher at night when compared to daytime induction (Arimura *et al.*, 2008). Downstream targets of JA signaling, such as the JA receptor COI1, also have highly rhythmic expression patterns in both *A. thaliana* and *N. attenuata*, partially revealing the mechanism by which the magnitude of JA induction changes so drastically throughout the day (Kim, Yon, Gaquerel, Gulati, & Baldwin, 2011; Shin, Heidrich, Sanchez-Villarreal, Parker, & Davis, 2012).

Herbivory responses in N. attenuata: the interplay of JA and ethylene

In *N. attenuata*, the herbivore-elicited JA burst induces the production of a panoply of secondary metabolites of known defensive function, among them the alkaloid nicotine (Halitschke & Baldwin, 2003; Steppuhn *et al.*, 2004). Although nicotine is a highly effective defense against generalist herbivorous insects in nature, it is less useful as a defense against *M. sexta,* a specialist, despite *M. sexta's* preference for feeding on transgenic lines with abrogated nicotine production (Steppuhn *et al.*, 2004).

Developmental timing also plays a crucial role in defense responses, and it is known that levels of defense-related specialized metabolites vary across different tissues and throughout plant development as plants manage their changing energy needs from vegetative to reproductive growth (Meldau *et al.*, 2012; Züst & Agrawal, 2017). One example of this is the changing magnitude of the induction of plant responses before and after flowering in the ethylene and

jasmonate bursts induced by simulated herbivory (Diezel, Allmann, & Baldwin, 2011). Fascinatingly, these responses can be restored by decapitating the shoot apical meristem. Removal of floral meristems shifts the plant from flowering stage, characterized by the beginning of senescence and the trafficking of existing resources to the floral meristem, back to the vegetative stage, characterized by high rates of carbon fixation for both growth and defense, recalling the old adage of plant developmental biology, “stage, not age.”

Overview of the dissertation

The circadian clock is implicated in a huge and ever-increasing number of plant phenotypes and responses. In **Chapter 1**, the role of the circadian clock in shaping plant volatile emissions is used as a case study to outline the movement from mechanistic bases towards the functional consequences of circadian phenotypes under natural conditions, which will be the overall focus of this dissertation. Aside from illustrating the expected role of the circadian clock in shaping diurnal emissions of plant volatiles and the functional consequences of these rhythms, **Chapter 1** further investigates how well-established diurnal phenotypes can manifest at longer temporal scales by reviewing the state of knowledge of ontogenetic patterns in plant volatile emissions.

Chapter 2 investigates the role of developmental stage in plant responses to drought in a multi-parent mapping population of *N. attenuata* plants. By using a subset of individuals from this mapping population, the variation in several drought response traits is found to be strongly linked to variation in early developmental transitions. Furthermore, unequal soil water availability strongly confounded variation in traits related to drought resistance strategies, an effect that is highly relevant to quantify and minimize given that many drought studies use uncontrolled watering and dry-down events to investigate mechanisms of drought response.

In **Chapter 3,** the role of the core circadian clock component *TOC1* in mediating *N. attenuata*’s responses to drought stress is investigated from an ontogenetic perspective. Silencing *TOC1* expression is known to affect plants’ water use phenotype by decreasing water loss through more responsive stomatal closure, and to directly interact with elements of the abscisic acid signaling machinery, an important pathway of plant perception and response to drought stress. Although *N. attenuata* plants abrogated in *TOC1* expression display the same physiological changes as *toc1 A. thaliana* mutants, these changes are not consistent with an increase in the fitness of these plants. Rather, *NaTOC1*-silenced plants were found to display decreased production of reproductive correlates and a shift in allometric trajectories under drought conditions.

Although *TOC1* has not been previously implicated in herbivory responses, another member of the circadian clock machinery, ZTL, has been shown to directly modulate JA responses, with severe consequences for nicotine production. In **Chapter 4,** the role of *TOC1* on *N. attenuata’s* defense chemistry is investigated. Changes in secondary metabolite production for *TOC1*-silenced plants are not purely consistent with the ZTL-mediated JAZ-MYC2 module described in Li *et al.*, and *TOC1*-silenced plants display further alterations in the ethylene-mediated specificity of herbivory responses. Further analyses reveal that *TOC1*-silenced plants allocated nitrogen resources differentially between two important and biosynthetically related classes of defense compounds, nicotine and the phenolamides dicaffeoylputrescine and di-caffeoyl spermidine.

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Overview of Manuscripts

Note: Formatting and terminology in original manuscripts has been maintained based on journal guidelines, and there may be minor differences among chapters.

Chapter 1:

Temporal dynamics of plant volatiles: mechanistic bases and functional consequences

Meredith C. Schuman, Henrique A. Valim and Youngsung Joo

Published in *Deciphering Chemical Language of Plant Communication* (Chapter 1), James D. BlandeRobert Glinwood (Eds.), in the series *Signaling and Communication in Plants.* Springer Publishing, Cham, Switzerland (2016).

In Chapter 1, we review the literature on the role of the circadian clock in shaping plant volatile emissions, and outline the movement from mechanistic bases towards the functional consequences of circadian phenotypes under natural conditions. This work highlights several points investigated throughout this dissertation, including examining the functional consequences of circadian rhythms at ontogenetic and developmental scales alongside diurnal scales. The exploration of the interplay between plant volatile biosynthesis and emission is also relevant for the discussion of secondary metabolites that comprise part of the plant's chemical defense profile that will be involved in Chapter 4, and thus provides an in-depth review of current plant defense theory and its testability by using the evidence for plant volatile compounds and how their production and phenology are affected by the circadian clock as a case study.

Meredith C. Schuman reviewed the literature and created first drafts of manuscript visualizations. Henrique Valim and Youngsung Joo reviewed the literature and wrote the manuscript and edited visualizations.

Chapter 2:

Early developmental transitions 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 Chapter 2, we examine methods of applying drought stress under glasshouse conditions using a subset of a multiparent advanced generation intercross (MAGIC) population. Because a rigorous application of drought stress is essential to interpreting the results of Chapter 3, and because there has not been a concerted development of drought methods for the desert annual

Nicotiana attenuata, we apply various drought strategies and measure the variance in four physiological parameters that are commonly utilized as measures of drought stress in other plant systems: photosynthetic assimilation, stomatal conductance, leaf relative water content, and accumulation of the plant hormone abscisic acid (ABA). We find that, for a subset of genotypes from this MAGIC population with diverse water use and developmental phenotypes, the timing of the onset of drought (defined here as when plants reach zero grams of water, as measured by a gravimetric approach) varies less when plants are provided with individual watering based on their water loss per day, and that developmental timing of the drought event further reduces the variation in how long different genotypes must dry down before onset of drought. Among the four measured physiological parameters, we find that even differences of one to two days and variations in developmental stage contribute more to the variance between genotypes than genotypic differences. ABA accumulation was more strongly linked to genotypic variation and provided a convenient measure of plant drought responses.

Erica McGale and Henrique Valim planned and performed experiments, analyzed the data and wrote the manuscript. Rayko Halitschke provided advice on visualization and edited the manuscript. Professor Ian T. Baldwin motivated and supervised the study and edited the manuscript.

Chapter 3:

The clock gene TOC1 in shoots, not roots, determines fitness of *Nicotiana attenuata* under drought

Henrique F. Valim, Erica McGale, Felipe Yon, Rayko Halitschke, Variluska Fragoso, Meredith C. Schuman and Ian T. Baldwin

Published in *Plant Physiology, 181*(1) pp. 305-318, doi: 10.1104/pp.19.00286 (2019)

In Chapter 3, I investigate the function of the circadian clock component *TIMING OF CAB EXPRESSION1 (TOC1)* in the desert annual *N. attenuata* under both glasshouse and field conditions. Silencing *TOC1* in *N. attenuata* led to decreased water loss and increased water use efficiency, similarly to results reported in *Arabidopsis thaliana.* Interestingly, however, analysis of the functional consequences of *TOC1* silencing under drought conditions revealed that *TOC1*-silenced (irTOC1) plants produced less fitness correlates than their well-watered counterparts, unlike empty vector (EV) plants, which maintained similar levels of fitness correlates under both drought and well-watered conditions. We investigated the contribution of *TOC1* in roots and in whole plants to transcriptomic responses, and found a leaf co-expression module strongly linking red and far-red light signaling responses to *TOC1* silencing in shoots only. Further experiments revealed that the differences mediated by *TOC1* cannot be explained by altering red and far-red light signaling alone, and furthermore that they cannot be explained by other circadian clock components or a general developmentally delayed phenotype shared by irTOC1 plants.

Experiments under field conditions reveal that whole-plant irTOC1 plants fail to increase the conversion of biomass to seed capsule production, unlike both empty vector and root-only *TOC1* silenced plants.

Henrique F. Valim planned and performed experiments, analyzed data, performed micrografting of seedlings, and wrote the manuscript. Erica McGale planned and performed experiments, analyzed data, and edited the manuscript. Felipe Yon planned and performed experiments under circadian conditions and contributed to data analysis of circadian experiments, and edited the manuscript. Rayko Halitschke analyzed data and edited the manuscript. Variluska Fragoso performed micrografting of seedlings, contributed to data analysis of micrografting experiments, and edited the manuscript. Meredith C. Schuman and Professor Ian T. Baldwin planned experiments, analyzed data, contributed funding, supervised the study and edited the manuscript.

Chapter 4:

Chapter 4 TOC1 in *Nicotiana attenuata* regulates efficient production of nitrogen-rich defense metabolites under herbivory stress

Henrique Valim, Heidi Dalton, Youngsung Joo, Erica McGale, Rayko Halitschke, Emmanuel Gaquerel, Ian T. Baldwin* and Meredith C. Schuman*

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Intended for submission to *New Phytologist*

In Chapter 4, I explore the role of the circadian clock component *TIMING OF CAB EXPRESSION1 (TOC1)* in mediating defense chemistry in *N. attenuata* after induction by oral secretions of the specialist herbivore *Manduca sexta*. Because previous literature has reported a role for another circadian clock component, ZEITLUPE (ZTL) in mediating *N. attenuata* nicotine but not phenolamide accumulation, we explored the effect of silencing either ZTL or TOC1 on nicotine and phenolamide accumulation. Interestingly, irTOC1 lines displayed increased nicotine accumulation after elicitation, unlike irZTL plants, which accumulate less nicotine relative to EV plants. irTOC1 plants also accumulated significantly less phenolamides than EV plants, leading to a different ratio between these two nitrogen-rich defenses. We used a nitrogen (^{15}N) pulse-labeling approach to investigate allocation of nitrogen between nicotine and two highly abundant phenolamides, caffeoylputrescine and dicaffeoyl spermidine, and found that irTOC1 plants allocate less nitrogen to phenolamides under control conditions, but that elicitation disrupts this shift, despite not compensating for the shifted levels of defense chemistry accumulation after elicitation. Given the role of ethylene in dampening nicotine increases after elicitation by *M. sexta* in various *Nicotiana* spp., we used an ethylene agonist, 1-methylcyclopropene, to investigate whether irTOC1 plants have an altered ethylene signaling feedback loop. We find that blocking ethylene receptors prior to elicitation increases EV nicotine

accumulation levels to irTOC1 levels, but does not affect differences in phenolamide accumulation, as would be expected for an abrogation of ethylene signal transduction during herbivory, although wild-type levels of ethylene production suggest that this is not a general ethylene insensitivity phenotype. Finally, we examine the functional consequences under field conditions of silencing *TOC1* in whole plants and in roots only, given that nicotine is largely synthesized in roots of *Nicotiana* spp. We find that although root-only *TOC1* silencing may increase nicotine levels similarly to whole-plant silencing without the decreases in phenolamide accumulation, these changes do not alter fitness outcomes for root-only *TOC1*-silenced plants.

Henrique Valim planned and performed experiments, analyzed data and wrote the manuscript. Heidi Dalton, Youngsung Joo and Erica McGale planned and performed experiments, analyzed data and edited the manuscript. Rayko Halitschke analyzed data and edited the manuscript. Emmanuel Gaquerel planned and supervised experiments with Heidi Dalton, analyzed data, supervised the study and edited the manuscript. Ian T. Baldwin planned experiments, supervised the study, contributed funding and edited the manuscript. Meredith C. Schuman planned experiments, supervised the study and edited the manuscript.

Confirmation of the doctoral candidate's contributions:

Jena, Prof. Dr. Ian T. Baldwin

1. Temporal dynamics of plant volatiles: mechanistic bases and functional consequences

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Chapter 1 in *Deciphering Chemical Language of Plant Communication*. Eds. James D. Blande and Robert Glinwood. Signaling and Communication in Plants. Springer Publishing, Cham, Switzerland (2016). doi: 10.1007/978-3-319-33498-1_1

Abstract

Plant volatiles comprise thousands of low-molecular weight, hydrophobic molecules that are classified as 'secondary' (specialized) metabolites, but are closely related to 'primary' (general) metabolites such as fatty acids, amino acids, sterols and carotenoids. In addition to having important physiological functions, these specialized small molecules have a large influence on plants' ecological interactions. By emitting particular blends of volatiles, plants can provide detailed information about their current physiological and ecological states and even manipulate other organisms. In fact, the timing of volatile biosynthesis and emission may be as critical to function as the amount and composition of volatile blends. Here, we critically review the known and hypothesized effects of phenological changes in plant volatile emission, their regulation and importance for function.

1.1 Introduction

In life, time is of the essence. This is no mere cliché but rather an ecologically sound generalization: nearly all multicellular eukaryotes, as well as photosynthetic prokaryotes—the cyanobacteria—possess internal clocks that permit the coordination of their metabolism and activity with diurnal cycles of abiotic factors such as light, temperature and moisture (Bell-Pedersen *et al.* 2005; Yerushalmi and Green 2009). Perhaps as an emergent property of the coordination between individual organisms and abiotic cycles, circadian clocks also allow organisms to coordinate with each other's diurnal activity patterns [Wang *et al.* 2011; Goodspeed *et al.* 2012 (but see Jander 2012); Zhang *et al.* 2013]. Ontogenetic events also determine timing

and prioritization of phenotypes due to, e.g. developmental necessity, adaptation to environmental changes or the transition from vegetative growth to reproduction. And while timing is important in general for ecology, it is crucial in the production and emission of plant volatiles.

1.1.1 A Brief Introduction to Plant Volatiles

Volatile compounds are small molecules (generally < 300 kDa) which are sufficiently lightweight and low polarity to have high vapour pressures under normal environmental conditions (reviewed in Dudareva *et al.* 2006; Baldwin 2010). These molecules may come from any of several biosynthetic pathways that are closely linked to pathways or products of general metabolism, i.e. fats and other lipids, amino acids and proteins (reviewed in Dudareva *et al.* 2006; Goff and Klee 2006; Baldwin 2010). The biosynthetic classes of plant volatiles and their known structures and functions are described in detail in Table 1.1, and example structures are shown in Fig. 1.1. In addition to the compounds shown, large amounts of methanol (μmol min^{-1}) can be produced from the demethylation of pectin in cell walls, and this process is induced by wounding and herbivory (von Dahl *et al.* 2006).

Plant volatiles have important roles within plant tissues in physiology, signalling and defence. When emitted through the cuticle, stomata or wounded tissue or from specialized structures (reviewed in Widhalm *et al.* 2015), they may be perceived by a host of other organisms as well as by remote parts of the plant (Heil and Silva Bueno 2007; reviewed in Baldwin 2010; Dicke and Baldwin 2010). The composition of volatile blends can convey detailed information about the physiological and ecological status of plants—such as the presence of open flowers, attack by herbivores, infection by microbes and production of ripe fruit—which may be used by microbes, animals and other plants, both detrimental and beneficial (reviewed in Dicke and Baldwin 2010). The timing of both production and emission of floral and vegetative volatiles is thus essential to their function in within-plant signalling, as well as in orchestrating interactions with other organisms, and may determine their potential for exploitation by enemies.

In this chapter, we provide an overview of what is currently known about the importance of timing in plant volatile biosynthesis and emission; the roles of plant volatiles we briefly refer to in this overview are elaborated throughout this book. The word importance has no precise definition in biology. We use importance to refer to the biological reasons underlying timing: why and how the production and emission of plant volatiles is timed in particular ways. Biologically, why and how can be precisely defined as distinct levels of analysis at which biological phenomena can be investigated: ‘why’ can be answered either in terms of functional outcomes or evolutionary history, and ‘how’ may be answered in terms of physiological or ontogenetic events (Tinbergen 1963; Sherman 1988).

Here, we focus on a functional level of analysis, including mechanistic and evolutionary dimensions when appropriate to provide a more complete biological picture. We begin from the

Table 1.1 Biosynthetic classes of plant volatiles and their biosynthesis, proposed functions and structural variety

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[a]	Number of known structures
Fatty acid derivatives	Jasmonates	Jasmonates are oxylipins synthesized from 16:3 and 18:3 fatty acids dioxygenated at C13 by 13-lipoxygenase (13-LOX) (Wasternack 2007)	Methyl jasmonate is among the main components of the scent of the jasmine flower, where it was first discovered (Demole et al. 1962). It is a volatile plant hormone which may be involved in plant–plant interactions (Karban et al. 2000; Preston et al. 2001; Kessler et al. 2006), as may the volatile (Z)-jasmone (Birkett et al. 2000)	Methyl jasmonate 302.9 (*Z*)-Jasmone 292	Four stereoisomers
	Green leaf volatiles	GLVs are synthesized via the cleavage of 13-LOX products by hydroperoxide lyase (HPL) to yield hexan-1-al (from 18:2 fatty acids) or (*Z*)-3-hexen-1-al (18:3). (*Z*)-3-Hexen-1-al can be isomerized to (*E*)-2-hexen-1-al spontaneously or by an isomerase; the hexenals can be converted to alcohols by alcohol dehydrogenases, and the alcohols can be esterified (Matsui et al. 2006)	Green leaf volatiles make up the 'cut grass' smell typical of wounded plant tissue (Hatanaka et al. 1987), contribute to the odour of fruits and flowers(Dudareva et al. 2006) and are emitted from roots and are important recognition and flavour components for animal consumers (Halitschke et al. 2004). GLVs may contribute to plant defence as antimicrobials (Deng	(*Z*)-3-Hexen-1-al 127.3 (*Z*)-3-Hexen-1-ol 156.5 (*Z*)-3-Hexen-1-yl acetate 174.2	At least 32 known from plants: four aldehydes (hexan-1-al, (*Z*)-3-hexen-1-al, (*E*)-2-hexen-1-al, and (*E*)-3-hexen-1-al) which provide substrate for four alcohols and at least 24 esters (acetates, propionates, butyrates, isobutyrates, valerates and isovalerates)

(continued)

Table 1.1 (continued)

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[a]	Number of known structures
			et al. 1993) or antifungals (Shiojiri et al. 2006a), direct (Vancanneyt et al. 2001) and indirect (Shiojiri et al. 2006a) anti-herbivore defences, and between (Baldwin et al. 2006; Paschold et al. 2006)-and within-plant (Frost et al. 2008) cues or signals		
	Nine-carbon volatile aldehydes, alcohols and esters	Nine-carbon derivatives are synthesized from 9-lipoxygenase (9-LOX) products of 18:2 and 18:3 fatty acids cleaved at the ninth carbon by HPL and, like GLVs, include aldehydes, alcohols and esters. Some HPLs specifically cleave 9- or 13-hydroperoxides, whereas others cleave both 9- and 13-hydroperoxides. 9-HPL products from 18:2 fatty acids contain one double bond, and those from 18:3 fatty	9-HPL products and their derivatives are odour and flavour components of flowers and fruits (Vancanneyt et al. 2001) and may be involved in seed development in almond (Mita et al. 2005). 9C aldehydes have antifungal properties (Matsui 2006)	(*E*,*E*)-3,6-Nonadienal 201.8 (*E*,*E*)-3,6-Nonadienol 214.7 (*E*,*E*)-3,6-Nonadienyl acetate 247.4	At least 15: five aldehydes which can be converted to five alcohols, which can be esterified; only the acetate esters are well represented in literature

		acids contain two double bonds (De Domenico et al. 2007)			
Terpenoids	Terpene hydrocarbons: Most volatile terpenoids have a five- (hemiterpene), ten- (monoterpene), or 15- (sesquiterpene) carbon skeleton	Terpene hydrocarbons are synthesized from the 5-carbon precursor isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) produced via one of two pathways in plants: the 2-C-methyl-derythritol 4-phosphate (MEP) pathway in plastids or the mevalonic acid (MVA) pathway in the cytosol. Generally, hemiterpenes and monoterpenes are synthesized in the plastid and sesquiterpenes in the cytosol; some sesquiterpenes may be synthesized in the mitochondrion from cytosolic substrate (Rodríguez-Concepción 2006; Kappers et al. 2005). Emission is usually light-dependent (Lerdau and Gray 2003)	Terpene hydrocarbons are components of flower, fruit, green tissue and root odours. Many are allelopathic (Junya Mizutani 1999) or may act in plant defence as antimicrobials or antifungals (Cowan 1999; Khosla and Keasling 2003), direct (cytochrome P450-inducing) (Brattsten 1983) and indirect (Degenhardt et al. 2003) antiherbivore defences or attract pollinators (Schiestl 2010). Most react with atmospheric ozone (Calogirou 1999) and could be involved in plant oxidative stress responses (Vickers et al. 2009)	Isoprene 34.1 Monoterpenes ca. 140–180 (Harborne 1973) Sesquiterpenes: >200 (Harborne 1973)	Isoprene is the only hemiterpene. At least 1000 different mono- and approximately 5000 different sesquiterpenes are known (Seigler 2008). Most are mono- or polycyclic
	Some terpenoids have an irregular number of carbons (8–18); these are called homoterpenes or	The homoterpene trans, trans-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E*,*E*)-	(*E*,*E*)-TMTT and (*E*)-DMNT are herbivore-induced volatiles in many plants and can attract	(*E*,*E*)-TMTT 293.2 (*E*)-DMNT 195.6	(*E*,*E*)-TMTT and (*E*)-DMNT are the only homoterpenes known to be widespread in plants.

(continued)

Table 1.1 (continued)

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[a]	Number of known structures
	apocarotenoids depending on their origin and are derived from the cleavage of larger terpenoids	TMTT] (C16) is derived from the diterpene geranyllinalool (C20) in the plastid (Herde et al. 2008), and the homoterpene trans-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] (C11) is derived from the sesquiterpene (3S)-trans-nerolidol (C15) in the cytosol (Boland and Gäbler 1989) by oxidative degradation, possibly catalyzed by cytochrome P450 enzymes (Dudareva et al. 2006). Apocarotenoids (C8-C18) are cleaved from carotenoids in the plastid by carotenoid cleavage oxygenases (CCOs) (Auldridge et al. 2006; Walter et al. 2010)	parasitoids and predators to plants with feeding herbivores (Dudareva et al. 2006). Apocarotenoids are flavour and odour components of flowers, fruit and green tissue (Camara and Bouvier 2004). In flowers, apocarotenoids increase apparency both to pollinators and predators and may be attractive or repellent; in fruit, they are associated with ripening (Bouvier et al. 2005). Some have antifungal properties (Maffei 2010)		Additionally, three different homoterpenes are emitted from elm leaves following oviposition by the elm leaf beetle *Xanthogaleruca* luteola (Wegener and Schulz 2002), and other structures are known in insects
	Oxidized terpenes and derivatives	Terpenoid hydrocarbons may be further modified by, e.g. cytochrome P450 enzymes, and the	Oxidized terpenes and derivatives are also components of flower, fruit, green tissue and root	Boiling points are higher than the corresponding terpene hydrocarbons	Thousands

		products may be oxidized by dehydrogenases, esterified by acyltransferases, or reduced. Some terpene synthase enzymes incorporate a molecule of CO_2 to produce oxidized terpenoids as their initial product (Dudareva et al. 2006). Terpene alcohols may also be glycosylated, but the glycosides are not volatile	odours (Dudareva et al. 2004). They have similar ecological and physiological roles to those of terpene hydrocarbons but are more often directly toxic (Khosla and Keasling 2003)		
Phenylpropanoids and benzenoids	Acid, aldehyde and alcohol derivatives of L-phenylalanine	L-phenylalanine is converted to trans-cinnamic acid via L-phenylalanine ammonia lyase (PAL). Further conversion of trans-cinnamic acid to other phenylpropanoids is shared with the lignin biosynthetic pathway through the steps of monolignol biosynthesis. Benzenoids originate from the same biosynthetic pathway, but the side chain of transcinnamic acid is enzymatically shortened	Common in floral scents (Vogt 2010); source of pungent flavour in black pepper and chili peppers (capsaicinoids). Methyl salicylate is a component of an herbivore-induced blend attractive to some predators and parasitoids (Van Poecke et al. 2001; Ament et al. 2004)	ca. 180–325 (Oyama-Okubo et al. 2005)	ca. 20 % of all known plant volatiles (Qualley and Dudareva 2008)

(continued)

Table 1.1 (continued)

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[a]	Number of known structures
		by two carbons. L-phenylalanine derivatives with a C2 side chain compete with phenylpropanoids and benzenoids for substrate and are synthesized via different pathways (Dudareva et al. 2006)			
Amino acids and derivatives	Acids, aldehydes, alcohols, esters, nitrogen- and sulphur-containing VOCs derived from amino acids other than L-phenylalanine	Amino acids are deaminated or transaminated to form α-keto acids, which are carboxylated and may subsequently be reduced, oxidated or esterified. Amino acids may also be precursors for acyl coA molecules used in esterification reactions catalyzed by alcohol acyltransferases (Dudareva et al. 2006). Ethylene is derived from methionine and thus belongs in this category	Branched-chain amino acid (Leu, Ile, Val) derivatives are common in fruit. Amino-acid derived esters are found in flowers and fruits (Dudareva et al. 2006). Putrid sulphur-containing compounds, likely derived from methioinine (Dudareva et al. 2006), may serve as direct defences (Berkov et al. 2000)	Ethylene −103.7 3-Methylbutan-2-ol 113.6 Butyl acetate 126.6	

[a]Royal Society of Chemistry. acc. July 2010. ChemSpider: http://www.chemspider.com/

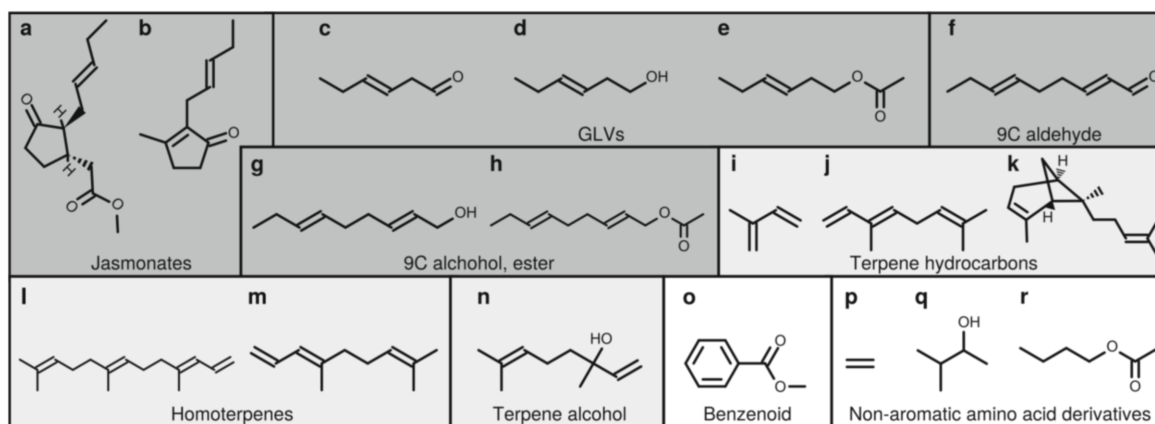

Fig. 1.1 Example structures from the biosynthetic classes of plant volatiles described in Table 1.1: the jasmonates methyl jasmonate (**a**) and (Z)-jasmone (**b**); GLVs (Z)-3-hexen-1-al (**c**), (Z)-3- hexen-1-ol (**d**) and (Z)-3-hexen-1-yl acetate (**e**); 9C compounds (E,E)-3,6-nonadienal (**f**), (E,E)- 3,6-nonadienol (**g**) and (E,E)-3,6-nonadienyl acetate (**h**); terpene hydrocarbons isoprene (**i**), monoterpene β-ocimene (**j**) and sesquiterpene (E)-α-bergamotene (**k**); homoterpenes (E,E)- 4,8,12-trimethyltrideca-1,3,7,11-tetraene [(E,E)-TMTT] (**l**) and (E)-4,8-dimethyl-1,3,7-nonatriene [(E)-DMNT] (**m**); monoterpene alcohol linalool (**n**); benzenoid methyl benzoate (**o**); and non-aromatic amino acid derivatives ethylene (**p**), 3-methylbutan-2-ol (**q**) and butyl acetate (**r**). Classes with the same type of biosynthetic precursors (fatty acids, IPP/DMAPP, amino acids) have identical background shading. Methanol, produced abundantly by demethylation of pectin in plant walls, is not shown

assumption that observed traits are adaptive and reject this view only if evidence does not support it. There is abundant evidence against the alternative assumption that plant volatiles are waste products, released from plants more as a result of their physiochemical properties than of physiological mechanisms, which may opportunistically accrue functions (Niinemets *et al.* 2004; Peñuelas and Llusia 2004; Rosenstiel *et al.* 2004). This is reminiscent of the more than 60-year-old theory that specialized plant metabolites generally are 'flotsam and jetsam on the metabolic beach', comprising waste products which may be opportunistically co-opted for functional roles, a theory which has not been supported for any plant specialized metabolite biosynthetically, physiologically, evolutionarily or functionally studied so far (Hartmann 2007). In fact, even the emission of plant volatiles through cuticles, formerly thought to occur passively, is likely to be actively regulated in order to avoid toxic concentrations of volatiles in membranes (Widhalm *et al.* 2015).

1.1.2 Why Are Plant Volatiles Synthesized and Emitted in Certain Tissues at Certain Times?

1.1.2.1 Function

In order to synchronize with abiotic and biotic factors, organisms need to have a sense of timing. Rhythmic behaviour can efficiently increase fitness by optimizing plant metabolism to abiotic circumstances dictated by the timing of sunrise and sunset (reviewed in Yerushalmi and Green

2009). Since different times of day bring different abiotic conditions and thus biotic communities, each plant has its own 'specific timing' to maximize its fitness (Raguso *et al.* 2003). This is realized not only in visually apparent behaviours like flower opening and leaf movement but also in the emission of plant volatiles, which often show distinct diurnal or nocturnal patterns in different tissues. Plants produce different amounts and combinations of volatiles over time, and these volatile blends have different physiological and ecological functions including:

- Within-plant signalling, which also leads to 'eavesdropping' on other plants (Baldwin and Schultz 1983; Rhoades 1983; Heil and Silva Bueno 2007; reviewed in Heil and Karban 2010)
- Pollinator attraction (Kessler *et al.* 2008) • Seed dispersal (Bolen and Green 1997; Luft *et al.* 2003; Goff and Klee 2006; Klee and Giovannoni 2011)
- Deterrence of herbivores, although they can also be co-opted by herbivores as host location cues (Kessler and Baldwin 2001; De Moraes *et al.* 2001; Halitschke *et al.* 2008; reviewed in Bruce *et al.* 2005) and feeding stimulants (Halitschke *et al.* 2004; Meldau *et al.* 2009)
- Attraction of predators and parasitoids to defend against herbivores (Dicke 1986; De Moraes *et al.* 1998; Kessler and Baldwin 2001; Rasmann *et al.* 2005; Schuman *et al.* 2012; reviewed in Dicke and Baldwin 2010; McCormick *et al.* 2012)
- Mediating interactions with microbes (reviewed in Junker and Tholl 2013)
- Allelopathic inhibition of neighbours (Inderjit *et al.* 2009; reviewed in Glinwood *et al.* 2011)
- Tolerance of abiotic stress (reviewed in Holopainen and Gershenzon 2010)

These roles can be viewed on a spectrum from internal to external, in terms of whether plant volatiles act within or between plant cells, or after emission into the environment, and are usually localized to particular tissues (Fig. 1.2).

1.1.2.2 Evolution

From the evolutionary perspective, the answer to 'why' plants synthesize and emit particular volatiles, at particular times and from particular tissues or structures, is the evolutionary trajectory resulting in the currently observed phenotype. Evolutionary analyses usually study the appearance and mutation of biosynthetic enzymes (reviewed in Pichersky *et al.* 2006), though they may also focus on structures for biosynthesis, storage or emission (reviewed in Lange 2015). The enzymes of GLV and jasmonate biosynthesis are ubiquitous in plants, originating in the green algae (reviewed in Andreou *et al.* 2009; Lange 2015), and the mevalonate and methylerythritol phosphate pathways providing substrate for terpenoid volatiles can be found in bacteria (reviewed in Rodríguez-Concepción *et al.* 2013; Lange 2015). In contrast, many volatile end products are limited to particular plant lineages, indicating rapid diversification of downstream biosynthetic enzymes (reviewed in Pichersky *et al.* 2006). However volatiles from most biosynthetic classes are emitted from vegetative, floral and root tissues in higher plants

(reviewed in Dudareva *et al.* 2004) (although roots are more difficult to analyse and thus less well studied).

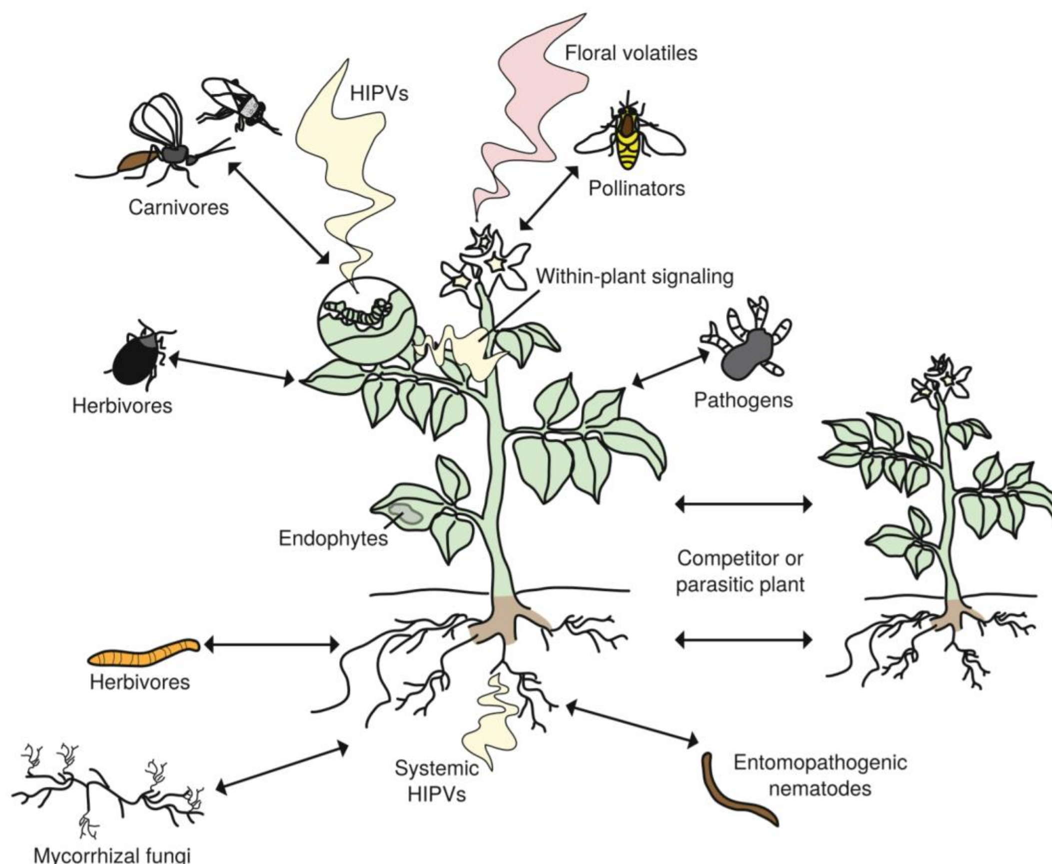

Fig. 1.2 Functional roles of plant volatiles. Modified with permission from Dicke and Baldwin (2010)

The enzymes of plant volatile biosynthesis are derived from general metabolism. For example, the diverse family of terpene synthases is derived from an ancestral ent-Kaurene synthase (Trapp and Croteau 2001, reviewed in Chen *et al.* 2011). The enzymes of jasmonate and GLV biosynthesis carry out the controlled degradation of reactive free fatty acids; substrate for phenylpropanoid and benzenoid volatiles comes from intermediate products of lignin biosynthesis, and other volatiles derive from salvage and degradation pathways of amino acids via α-keto acids (reviewed in Dudareva *et al.* 2006). Thus volatile metabolites are more or less closely linked to the dynamics, regulation and rhythms of general metabolism.

1.1.3 How Is the Induced, Diurnal and Ontogenetic Timing of Volatile Biosynthesis and Emission Regulated?

The roles of physiology and ontogeny in plant volatile synthesis and emission are depicted in Fig. 1.3.

1.1.3.1 Physiology

Physiology provides the immediate mechanisms. The close relationship of plant volatiles to general metabolic pathways dictates precursor availability according to diurnal or circadian control (Pokhilko *et al.* 2015), hormonal signalling and flux channelling (reviewed in Dudareva *et al.* 2006; Nagegowda 2010; Vranová *et al.* 2012). Direct volatile biosynthetic enzymes may be regulated by any or all of these cues, and many of the best-studied examples are of herbivory-induced volatile emission (reviewed in Howe and Jander 2008). Emission, like biosynthesis, is also tightly regulated—even the diffusion of plant volatiles across membranes and cuticles is likely to be an actively regulated process (Widhalm *et al.* 2015). Other than diffusion, emission may occur through stomata (Seidl-Adams *et al.* 2014), wounds (De Domenico *et al.* 2007), or specialized tissues such as flowers (reviewed in Muhlemann *et al.* 2014) or glandular trichomes (e.g. Schuurink 2007; but see Hare 2007). Emission rates are influenced by internal signalling (reviewed in Howe and Jander 2008) and other wounding-related processes (e.g. De Domenico *et al.* 2007) as well as temperature, humidity and light levels, both due to direct physical effects on volatility and—likely more importantly—to the effects of these factors on the physiological mechanisms of emission (e.g. Grote *et al.* 2014).

1.1.3.2 Ontogeny

Ontogeny describes the second mechanistic or the ‘how’ level at which we may investigate the regulation of plant volatile biosynthesis and emission, comprising in part the regulatory physiological system of each life stage and tissue of the plant. To a large extent, however, ontogenetic changes may accompany the development of new tissues and transitions to different life stages, e.g. from vegetative to reproductive (Diezel *et al.* 2011). Perhaps due in part to overlap in the emission profiles of different tissues or to the hormonal signalling functions of some volatiles, vegetative volatiles may interfere with the function of floral volatiles and vice versa (von Dahl *et al.* 2007; Diezel *et al.* 2011; Kessler *et al.* 2011; Desurmont *et al.* 2015). Ontogenetic changes in volatile emission may serve to avoid such interference. Furthermore, both within tissues and across life stages, the development of specialized cells and tissues may be required for the biosynthesis and emission of some volatiles, placing physiological limitations on ontogenetic timing (e.g. Bate *et al.* 1998; reviewed in Dudareva *et al.* 2004; Rodríguez-Concepción *et al.* 2013).

1.2 The Importance of Timing in Plant Volatile Emission

In the following sections, we describe how several well-studied phenomena mediated by plant volatiles can be understood on the timescales of induction, diurnal and circadian rhythms and changes over ontogeny. We focus on functions but also address their regulation.

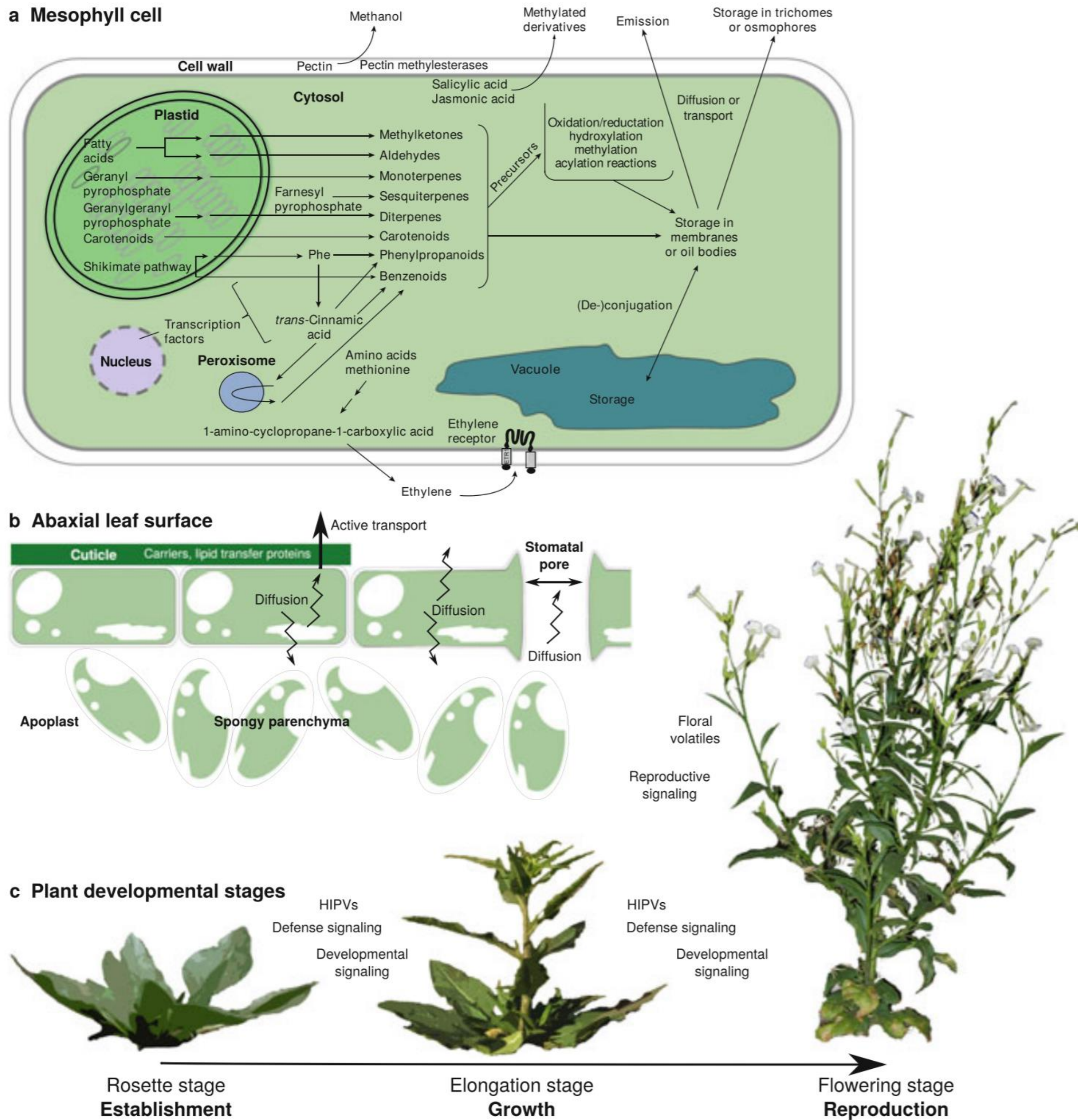

Fig. 1.3 Mechanisms of plant volatile biosynthesis and emission. (**a**) Physiological mechanisms, modified with permission from Baldwin (2010). (**b**) Depiction of possible paths for volatiles— once synthesized, released from conjugates or intracellular storage—to diffuse (over cell walls, through stomata) or be actively transported (through cuticles) to the headspace, using the abaxial leaf surface as an example. (**c**) Vegetative and reproductive stages of the ecological model plant *Nicotiana attenuata* visualized as changes to shoots and changing roles of plant volatiles. Roots and seeds are not shown as less is known about the structural changes or dynamic volatile emissions of these tissues. Plants modified from Schuman *et al.* (2014)

1.2.1 Plant Volatiles as Induced Defences and Defence Signals

It is well known that biosynthesis and emission of specific plant volatiles are induced by stress in every higher plant studied so far (reviewed in Holopainen and Gershenzon 2010), resulting in predictable volatile blends which can serve as reliable cues or signals: cues benefit the receiver with an unspecified effect on the sender, while signals benefit the sender and the receiver, with 'benefit' defined biologically as increase in Darwinian fitness (Greenfield 2002; Allison and Hare 2009). Stress-induced plant volatile blends may be highly specific to particular plant genotypes (e.g. Wu *et al.* 2008; Delphia *et al.* 2009; Schuman *et al.* 2009; reviewed in Wu and Baldwin 2010) and stress events (reviewed in Holopainen and Gershenzon 2010), including specificity to particular herbivores (reviewed in McCormick *et al.* 2012) and numbers of herbivores (e.g. Shiojiri *et al.* 2010). The ubiquity and specificity of stress-induced plant volatile emission indicates the importance of these compounds in structuring ecological communities, from effects on pollinators, herbivores, predators and microbes, to impact on global climate (reviewed in Kessler and Halitschke 2007; Arneth and Niinemets 2010; Dicke and Baldwin 2010; Holopainen and Gershenzon 2010). Herbivore-induced plant volatiles (HIPVs) are among the best-studied induced phenomena in the field of plant– herbivore interactions and have served as highly specific phenotypic read-outs in studies of herbivore elicitors (reviewed in Howe and Jander 2008).

1.2.1.1 Functions of Herbivore-Induced Plant Volatiles (HIPVs)

As semiochemicals, HIPVs can attract parasitoids of herbivores (De Moraes *et al.* 1998) and insectivorous carnivores (Dicke 1986; Kessler and Baldwin 2001) and deter herbivore oviposition (Kessler and Baldwin 2001; De Moraes *et al.* 2001), all of which may enhance plant fitness by increasing the apparency of herbivores to predators (Feeny 1976). However, HIPVs and other plant volatiles also act as host location cues for herbivores (e.g. Halitschke *et al.* 2008; reviewed in Bruce *et al.* 2005; Dicke and Baldwin 2010). In *N. attenuata*, the release of HIPVs reduces herbivore loads by 50 % or more on the emitting plants (Kessler and Baldwin 2001; Allmann and Baldwin 2010; Schuman *et al.* 2012) allowing GLV-emitting plants to produce twice as many flowers, buds and seed capsules as non-emitters in the presence of predators (Schuman *et al.* 2012). HIPVs can also contribute to defence signalling within plants, for example, eliciting the production of hormones and gene transcripts involved in defence and eliciting or priming defence traits (Heil and Silva Bueno 2007; Frost *et al.* 2008; reviewed in Heil and Karban 2010). Likely as a side effect of their ability to respond to their own HIPVs, plants can also elicit or prime defence responses after 'eavesdropping' on HIPVs from neighbours (reviewed in Baldwin *et al.* 2006; Heil and Karban 2010; Scala *et al.* 2013; and see Chap. 7); responses may be specific to plant genotype and interaction (e.g. Li *et al.* 2012). Neighbour volatiles have also been shown to alter biomass allocation and growth in barley (Ninkovic 2003; Kegge *et al.* 2015).

1.2.1.2 Regulation of HIPVs

HIPV emission is regulated by the same signalling systems that elicit other induced defence responses in plants (Fig. 1.3). For example, it has been shown that different elicitors in the regurgitant of *Manduca sexta* (tobacco hornworm) larvae elicit specific HIPVs in *Nicotiana attenuata* (wild coyote tobacco) (Gaquerel *et al.* 2009). Furthermore, in *N. attenuata*, mitogen-activated protein kinase (MAPK) signalling (Meldau *et al.* 2009), jasmonate signalling (Halitschke and Baldwin 2003; Schuman *et al.* 2009; Oh *et al.* 2012; Woldemariam *et al.* 2012, 2013), abscisic acid signalling (Dinh *et al.* 2013) and WRKY transcription factors (Skibbe *et al.* 2008) all have been shown to regulate HIPV emission. Some isoprenoids have been shown to react with and quench reactive oxygen species (ROS) (reviewed in Vickers *et al.* 2009; Holopainen and Gershenzon 2010). ROS are a component of early signalling events that induce stress hormone signalling, including abscisic acid and jasmonate signalling (reviewed in Maffei *et al.* 2007; Wu and Baldwin 2010), and are likely to be involved in the induction of HIPVs and other stress-responsive volatiles.

Interestingly, in *Arabidopsis* (*Arabidopsis thaliana*), it has been shown that HIPV emission is dependent on jasmonates but not on the known active form jasmonoyl isoleucine (JA-Ile) (Fonseca *et al.* 2009), indicating that a different jasmonate may activate genes controlling HIPV emission, while JA-Ile regulates other defence responses (Van Poecke and Dicke 2003; Wang *et al.* 2008). In *Phaseolus lunatus* (lima bean), synthetic JA-Ile analogues similar in structure with coronatine, the highly active jasmonate mimic produced by the biotrophic plant pathogen *Pseudomonas syringae*, strongly elicit HIPV biosynthesis as long as the carbonyl group on the 5-membered ring is intact and regardless of modifications to the amino acid moiety (Krumm *et al.* 1995). Application of methyl jasmonate has also been shown to elicit volatile emission in other wild and domesticated plants including *Solanum peruvianum* (Peruvian wild tomato) (Kessler *et al.* 2011), *Datura wrightii* (sacred Datura) (Hare 2007), *N. attenuata* (Halitschke *et al.* 2000), *P. lunatus* (Ozawa *et al.* 2000) and *Gossypium hirsutum L.* (cotton) (Rodriguez-Saona *et al.* 2001). The volatile emission elicited by jasmonate treatment often partially, but not fully, overlaps with the profiles elicited by feeding of different herbivores (e.g. Dicke *et al.* 1999; Kessler and Baldwin 2001).

1.2.2 Diurnal and Circadian Rhythms of Stress-Induced Volatiles

Volatiles are generally emitted beginning from seconds to hours following induction, and the emission induced by a single event may last as little as minutes or as long as days (see, e.g. Loughrin *et al.* 1994; von Dahl *et al.* 2006; Allmann and Baldwin 2010; Schaub *et al.* 2010; Danner *et al.* 2012; Jardine *et al.* 2012; Kallenbach *et al.* 2014). Longer emission cycles may display diurnal rhythms (e.g. Loughrin *et al.* 1994; Arimura *et al.* 2008) (Fig. 1.4), which may allow plants to synchronize volatile emission temporally with biotic factors, e.g. herbivores, predators and parasitoids. This could increase plant fitness, because many insects also have their own predictable rhythmic behaviour. For example, circadianregulated jasmonate accumulation

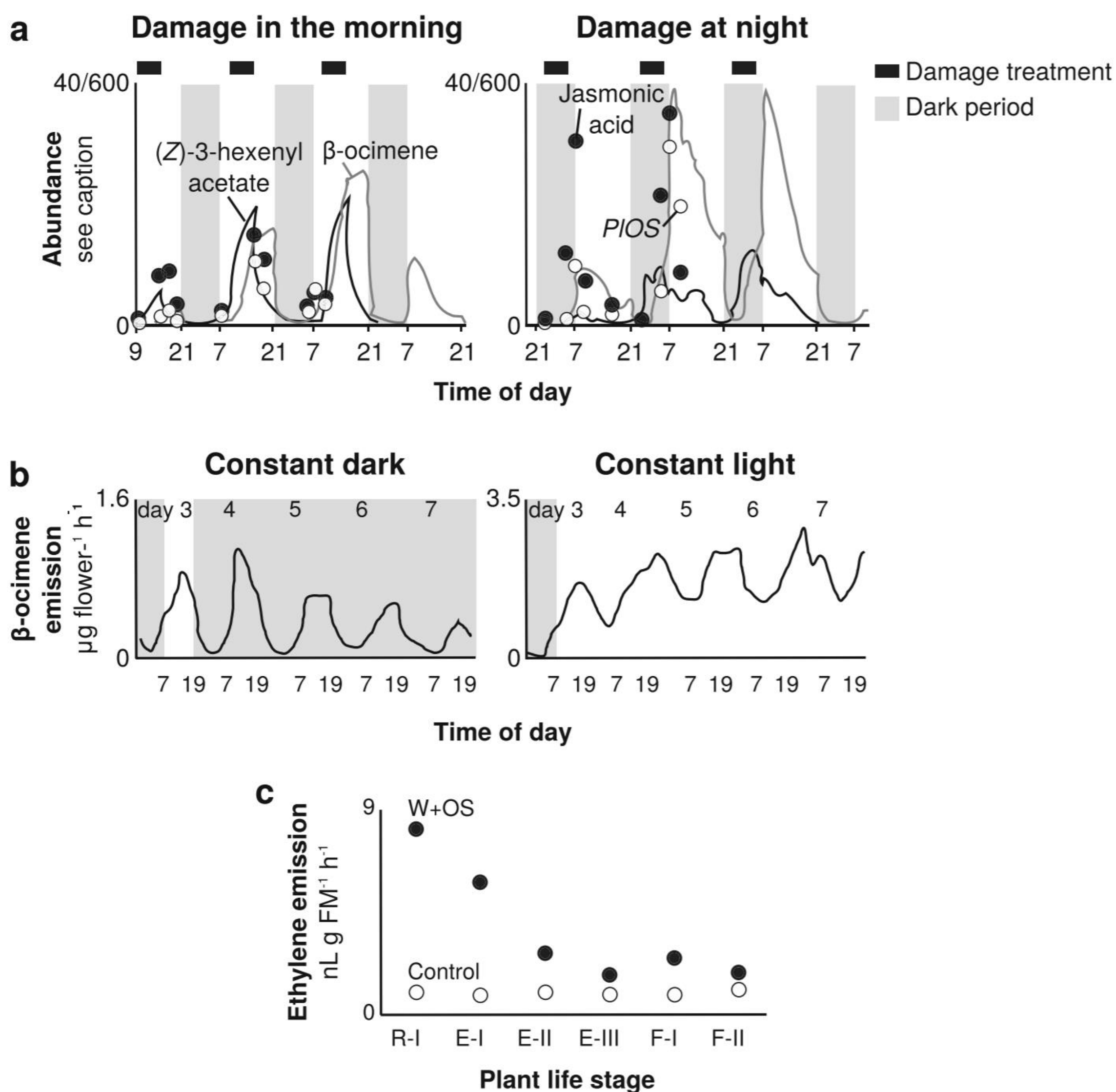

Fig. 1.4 Examples of phenological changes in plant volatile emission. (**a**) The monoterpene β-ocimene is emitted from Phaseolus lunatus leaves during light periods following simulated herbivore damage (MecWorm treatment), while the GLV (Z)-3-hexenyl acetate is emitted immediately upon damage regardless of light or dark period (volatiles shown on the scale of 0– 40 ng g FM^{-1} min^{-1}). Jasmonic acid and PIOS (β-ocimene synthase) transcript accumulation reflect dynamics of β-ocimene emission (jasmonic acid scale: 0–600 ng g FM^{-1}, *PlOS* transcripts relative to *PlACT1* transcripts). Scales in the left and right panel are the same, indicating that jasmonate accumulation and β-ocimene emission are greater after night-time damage. Drawn from data in Fig. 3 of (Arimura *et al.* 2008). (**b**) Circadian oscillation in the emission of β-ocimene from flowers of *Antirrhinum majus* (snapdragon) cv Maryland true pink. Overall, more β-ocimene is emitted under constant light conditions (note difference in scale between *left* and *right panels*). Drawn from data in Fig. 2 of (Dudareva *et al.* 2003). (**c**) Ethylene emission induced after treatment of mature; non-senescent *Nicotiana attenuata* leaves with mock herbivory (wounding and *Manduca sexta* oral secretions, W + OS) is attenuated as plants age; control, untreated leaf at same position. Stages: *R-I* rosette; *E-I* beginning elongation, first inflorescence visible but not fully developed; *E-II* and *E-III* elongated, consecutive days, buds develop rapidly but do not yet open; *F-I* first corolla elongation; *F-II* first fully opened flowers. Drawn from Fig. 1 in (Diezel *et al.* 2011)

can enhance plant resistance to herbivores with synchronized feeding activity (Goodspeed *et al.* 2012; but see Jander 2012). Also, nocturnal volatiles produced by host plants repelled oviposition of nocturnal moths (De Moraes *et al.* 2001; Allmann *et al.* 2013), and determined the behaviour of *Mythimna separata* (Northern armyworm) larvae (Shiojiri *et al.* 2006b).

1.2.2.1 Rhythmic Volatile Emission in Response to Biotic and Abiotic Stress

Most diurnal plant behaviours are synchronized to daily rhythms of abiotic factors like light and temperature, denoted 'zeitgeber' (time givers). In particular, many isoprenoids and terpenoids show strong diurnal rhythms because 75 % of carbon for isoprenoid synthesis originates from photosynthesis, and the methylerythritol phosphate (MEP) pathway has strong diurnal and circadian rhythms (Schnitzler *et al.* 2004; Dudareva *et al.* 2005; Pokhilko *et al.* 2015). In many tree species, monoterpene production positively correlates with light intensity and temperature (Tingey *et al.* 1980; Harley *et al.* 2014; Jardine *et al.* 2015). It should be noted that the lower volatility of sesquiterpenes (Table 1.1) can lead to experimental artefacts, e.g. resulting from their temperature-dependent adsorption to and re-release from collection cuvettes, which can obfuscate emission patterns (Schaub *et al.* 2010). However herbivore-induced emission of the monoterpene β-ocimene in *P. lunatus* leaves showed a diurnal rhythm, while emission levels strongly decreased under artificial dark treatment (Arimura *et al.* 2008) (Fig. 1.4). Although many volatile emission rhythms strongly diminish or disappear without environmental cues, some volatiles retain oscillation under free-running conditions, demonstrating circadian regulation (Hsu and Harmer 2013). Isoprene emission, for example, has a strong circadian rhythm, presumably to protect plants from heat stress and maintain photosynthesis and also to mitigate the effects of oxidative stress in response to abiotic stress-mediated ROS production (reviewed in Sharkey *et al.* 2008; Loreto and Schnitzler 2010). Stress-induced plant volatile emission and its responsiveness to diurnal and seasonal zeitgeber contribute significantly to atmospheric pollution and global climate (Arneth and Niinemets 2010).

1.2.2.2 Are There Examples of Circadian Regulation of HIPVs?

So far no leaf HIPVs have been reported to have circadian-regulated emissions. Perhaps synchronization via the circadian clock is more common either when dictated by abiotic factors as discussed above or in mutualistic interactions. The evolutionary strategy between plants and herbivores is a diffuse arms race: each side responds to selection pressure for counter-adaptation from the other side (Fox 1981). For example, plants are thought to diversify their production of defensive metabolites in response to herbivore adaptation to older defences (Speed *et al.* 2015). So if plants have developed rhythmic traits to synchronize with herbivore behaviour, the herbivore may experience selection pressure to change the behaviour and escape the synchronization. Thus we would predict it is uncommon to find cases of circadian-mediated synchronization between HIPVs and herbivore activity, e.g. as direct defence responses. However, if two species have mutualistic interactions, both sides could benefit from synchronization, and this may be one way in which mutualistic interactions increase the stability

of communities (Georgelin and Loeuille 2014). If there are cases of the circadian regulation of HIPVs, these might originate in physiological roles of these volatiles, if the resulting rhythms are also compatible with their defensive roles. For example, a rhythm dictated by the oxidative stress produced by photosynthesis may also be compatible with the activity patterns of certain herbivores or their natural enemies, or insects may learn to recognize typical, physiologically dictated and thus tightly conserved rhythms of plant volatile emission.

1.2.3 Circadian Timing of Floral Volatile Emission

It is perhaps not surprising that floral volatiles provide the best-known examples of robustly regulated, rhythmic plant volatile emission. Many flowering plants require assistance from pollinators to transfer pollen between flowers, and species with self-compatible flowers often nevertheless benefit from out-crossing mediated by pollinators (reviewed in Rosas-Guerrero *et al.* 2014). Flowers thus produce attractant volatiles for pollinators to enhance chances of out-crossing (Kessler *et al.* 2008). Many plants that require insect pollinators have evolved very specific floral traits for this purpose (reviewed in Raguso 2004). They also produce particular floral volatiles at specific times to synchronize with the activity of their pollinators (reviewed in Muhlemann *et al.* 2014). *N. attenuata* produces benzyl acetone during the night to attract nocturnal moths, *M. sexta* and *Hyles lineata* (whiteline sphinx) (Kessler *et al.* 2008). Whereas *Petunia* spp. (petunia) flowers mainly produce benzenoids to attract night pollinators (Hoballah *et al.* 2005); *Antirrhinum majus* (snapdragon) cv. Maryland true pink flowers emit larger amounts of methyl benzonate during the day to attract bees (Kolosova *et al.* 2001).

Circadian regulation has been shown for many floral volatiles. Bee-pollinated *A. majus* flowers produce the monoterpene myrcene, (E)-β-ocimene and linalool specifically during the day, a rhythm maintained under constant light conditions (Dudareva *et al.* 2003) (Fig. 1.4) and also emit more methyl benzoate during the day (Kolosova *et al.* 2001), while moth-pollinated *Nicotiana suaveolens* (Australian tobacco) and *Petunia* cv Mitchell flowers produce benzenoid volatile compounds at night, a rhythm maintained under constant dark conditions (Kolosova *et al.* 2001). Recently, evidence that the circadian clock directly regulates floral volatiles was reported in *Petunia hybrida* and *N. attenuata* (Yon *et al.* 2016; Fenske *et al.* 2015). Both studies showed that *late elongated hypocotyl* (LHY), a morning element of the circadian clock, is a main regulator in the peak timing of floral volatile emission. These findings suggest that the role of the circadian clock in flowers may be conserved in the Solanaceae. LHY may also transcriptionally regulate isoprene synthase to produce strong circadian rhythms of isoprene emission in grey poplar (Loivamäki *et al.* 2007). Circadian rhythms of plant volatiles are the consequence of circadian regulation of substrate flux and biosynthetic genes at transcriptional and enzymatic levels (Kolosova *et al.* 2001; Dudareva *et al.* 2005; Fenske *et al.* 2015; Pokhilko *et al.* 2015). In addition, circadian emission patterns of plant volatiles may also be influenced by the circadian regulation of emission mechanisms such as stomatal opening or transport through membranes (Lehmann and Or 2015; Widhalm *et al.* 2015) (Fig. 1.3).

1.2.4 Ontogenetic Changes in Plant Volatiles: Theory and Observation

1.2.4.1 Application of Plant Defence Theory to Ontogenetic Patterns

The composition of specialized metabolites in plants, including volatiles, varies not only in response to stress and diurnal events but also throughout lifecycles, across plant life histories and by type of herbivore pressure exerted on plants (Barton and Koricheva 2010). Plant defence theories have long sought to explain this variation with different degrees of success, and because many studies of variation in plant volatile emission are based on plant defence theory, it is important to mention them here. The most commonly employed have been optimal defence (OD) theory (McKey 1974; Rhoades and Cates 1976; McKey 1979; Rhoades 1979), apparency theory (Feeny 1976) and growth-differentiation balance (GDB) theory (Loomis 1932; Loomis 1953; Herms and Mattson 1992). Among these, GDB has been least effective in predicting ontogenetic changes described in literature. The view of ontogenetic changes in GDB is largely mechanistic but oversimplified, treating growth and differentiation as general processes at a level of understanding achieved in the first half of the twentieth century (Loomis 1932, 1953) and considering light and nutrient availability while ignoring other abiotic and biotic stimuli (Stamp 2003). As one study of defensive monoterpenes described, GDB is '...a sourcedriven model that does not, in its simplest form, consider changes in the need (demand) for growth or differentiation products' (Lerdau *et al.* 1994).

Apparency and OD theory have been more useful, despite not focusing on ontogeny, because many hypotheses related to the ontogenetic distribution of plant volatiles can also be posed as functional hypotheses. Further, apparency and OD theory provide some testable functional hypotheses, while the hypotheses posed by GDB theory mix mechanistic and functional levels of analysis and are thus not testable (Sherman 1988; Baldwin 1994; Stamp 2003). Functional studies largely treat ontogenetic stages as black boxes, with no exploration of the mechanistic background for differences which make ontogenetic stages distinctive, though not discrete. In contrast, the mechanistic literature is mostly restricted to developmental biology, though there are studies of the development of tissues, specialized storage and secretory structures which cross the boundary between developmental biology and the study of plant defence (reviewed in Dudareva *et al.* 2004), and studies of developmental regulation of biosynthetic enzymes in floral volatile emission (e.g. Pichersky *et al.* 1994; Bate *et al.* 1998; Dudareva *et al.* 2000). The literature on functions of plant volatiles over ontogeny is our focus here because we feel a critical discussion of the functional literature is lacking.

In a meta-analysis of 116 studies reporting ontogenetic patterns in plant defence traits across 153 plant species interacting with 30 herbivore species, Barton and Koricheva (2010) found that patterns over ontogeny in plant response variables (concentrations of secondary metabolites, measures of physical defence and tolerance) depended on plant life form, type of herbivore and type of response, and thus a generalization about patterns in defence over ontogeny could not be

supported. The plant-age hypothesis (Bryant *et al.* 1992) explicitly applies predictions of OD theory to ontogenetic changes in plants, ‘predicting that that extrinsic factors, namely, selection by herbivores, lead to high levels of defence in juveniles, followed by decreases as plants mature and become less susceptible to the fitness reductions of these attacks’ (Barton and Koricheva 2010). Yet within a life stage, OD predicts that younger tissues should always induce higher direct and indirect defences, if they make a larger contribution to the plant fitness (reviewed in Meldau and Baldwin 2013). Of all tissues, then, reproductive tissues and seed capsules should be the best defended, representing the culmination of the plant’s labours, as the rest of the plant senesces and concentrates metabolites in the reproductive organs (reviewed in Schippers *et al.* 2015). There is an apparent conflict between the predictions that ageing plants reduce defence and that reproductive tissues be well defended.

1.2.4.2 Functional Analyses of Ontogenetic Patterns, Informed by Mechanism

Tissue-based variations may be better explained by changes in plant volatile signalling functions, for example, in response to flowering and senescence (Stout 1996; Desurmont *et al.* 2015; Schippers *et al.* 2015), which are not predicted by plant defence theories. In field studies, inducibility of HIPVs has been observed to drastically decrease in reproductive-stage plants relative to plants undergoing only vegetative growth, for both *Glycine max* (soya bean) and *D. wrightii* (Rostás and Eggert 2008; Hare 2010). Critically, ‘rejuvenation’ by extensive trimming in *D. wrightii* (Hare 2010) partially restored HIPV production, indicating that the presence of reproductive tissues, not plant age, was responsible for HIPV attenuation. Changing ‘functional priorities’ for the volatiles in a plant’s headspace may cause interference during flowering. In *B. rapa*, flowering also abolishes the elicitation of HIPVs from leaves, which causes a 20–30 % decrease in the attractiveness of plants to parasitoids in the face of infestation by the specialist cabbage butterfly (*Pieris brassicae*), likely with fitness consequences for the plant. Vegetative-stage plants perfumed with floral volatiles were also less attractive to parasitoids (Desurmont *et al.* 2015). In the other direction, HIPV emission induced by herbivory or methyl jasmonate application to *S. peruvianum* leaves reduced visitation of flowers by pollinating bees, reducing seed set. In contrast, removal of leaves from flowering plants (mimicking defoliation by herbivores) did not significantly reduce seed set unless at least 80 % of leaf tissue was removed (Kessler *et al.* 2011). Volatile emission from *G. max* after feeding by *Spodoptera frugiperda* (fall armyworm) caterpillars was also shown to be tenfold as great per gram of biomass in vegetative-stage plants, in comparison to reproductive-stage plants, and even within reproductive-stage plants, elicitation of HIPVs was 250-fold as great in leaves as in seed pods (Rostás and Eggert 2008). Differences in blend composition among leaves and seed pods could point to reasons why these large and perhaps unexpected differences exist, but these qualitative differences were not acknowledged or further explored. This is surprising given the body of literature covering the importance of blend composition for insect perception of volatile cues (reviewed in Bruce and Pickett 2011) and the emerging literature indicating its importance for plant responses to volatiles (reviewed in Ueda *et al.* 2012).

Likely depending on their roles in defence versus within-plant signalling, individual HIPVs often do not meet predictions of OD. In *N. attenuata*, mid-aged leaves (fully expanded, non-senescent) emitted the largest amounts of the sesquiterpene (E)-α-bergamotene after induction (Halitschke *et al.* 2000), and (E)-α-bergamotene's inducibility does not decrease after flowering (Schuman *et al.* 2014). However in *G. max*, which produces both isomers of α-bergamotene, the inducibility of both decreases markedly to below detection after flowering; in the vegetative stage, *G. max* has also been shown to produce more total volatiles in younger than older leaves, as predicted by OD (Rostás and Eggert 2008). Whether the individual α-bergamotene isomers play different functional roles corresponding to differences in their regulation across species is unknown. The drastic decrease in herbivore-induced ethylene from leaves in flowering *N. attenuata* plants (Diezel *et al.* 2011) (Fig. 1.4) is posited to be due to ethylene's changing role from regulation of defence in the vegetative stage to regulation of pollen acceptance and flower senescence in the flowering stage (von Dahl *et al.* 2007; Bhattacharya and Baldwin 2012). In *N. attenuata*, emission of the moth-attracting floral volatile benzylacetone (Kessler *et al.* 2008) remains abundant from flowers up until the night of pollination, followed by a sharp reduction in benzylacetone emission and strong post-pollination ET bursts of up to ~300 nL g^{-1} h^{-1} that correlate strongly with pollination success based on the paternal genotype (Bhattacharya and Baldwin 2012). As for HIPVs in *D. wrightii* (Hare 2010), removal of inflorescences restores herbivore-inducible ethylene emission in N. attenuata (Diezel *et al.* 2011).

1.3 Conclusion

Volatile production and emission are dynamic characteristics of plants' responses to their environment over diurnal and ontogenetic time. Figure 1.5 provides a schematic integrating functions of plant volatiles over ecological and temporal scales. Tissue-based variations may lie at the heart of changing priorities for the plant, which allocates defences to different tissues based on specific 'goals' that change over ontogeny and include growth, defence and ultimately pollination and seed dispersal. Some plant volatiles may have co-evolved with specific ecological interaction partners like predators and pollinators, and the production of specific volatile blends may mirror the importance of particular interactions at that life stage. Plant volatiles also serve hormonal functions as the plant transitions through life stages. The unique roles of plant volatiles in defence and development as signals (for the plant) or cues (for other organisms) makes it difficult to apply plant defence theories (e.g. Ninkovic 2003; Pierik *et al.* 2004; Karban 2007; Rostás and Eggert 2008; Renne *et al.* 2014; Mirabella *et al.* 2015), which in any case often produce conflicting predictions (compare for example Barton and Koricheva 2010; and Meldau and Baldwin 2013). We suggest that further research is best guided by attempts to understand these complex phenomena at their functional, evolutionary, physiological and ontogenetic levels.

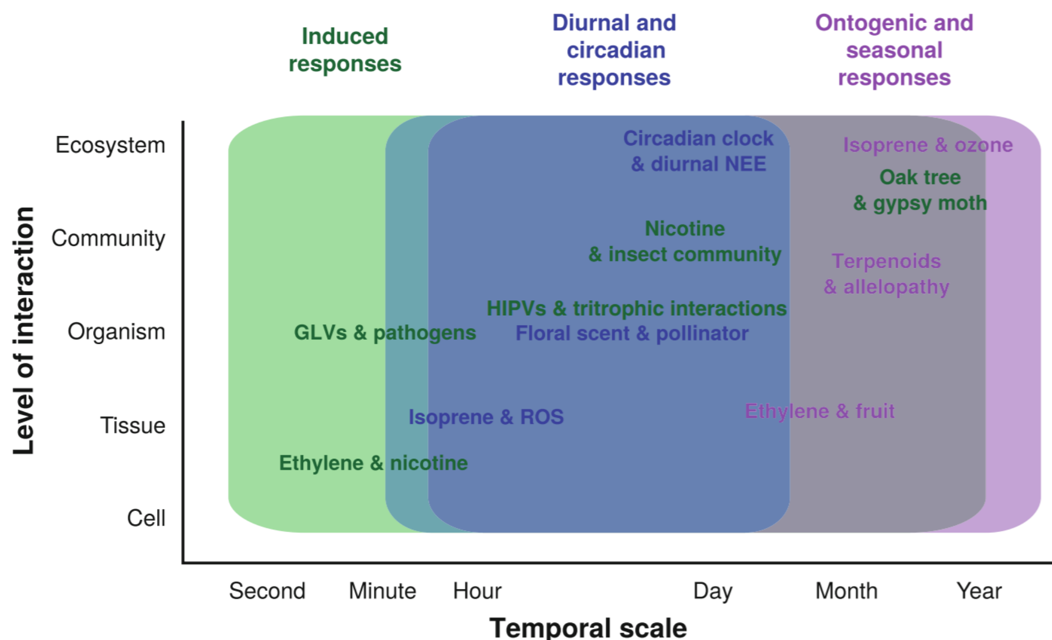

Fig. 1.5 A conceptual scheme of the functions of plant volatiles over ecological and temporal scales

Acknowledgements

The authors acknowledge the Max Planck Society; the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, funded by the German Research Foundation (FZT 118); ERC Advanced Grant to Ian T. Baldwin number 293926; and the Global Research Lab program (2012055546) from the National Research Foundation of Korea for funding.

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

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Submitted to *Plant, Cell & Environment*

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.

2.1. 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.

2.2. 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 H_20/m^2s) 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 (g_{sw}, 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 (W_g). 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 g_{sw}. 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 occurred 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.

2.3. Results

2.3.1. 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).

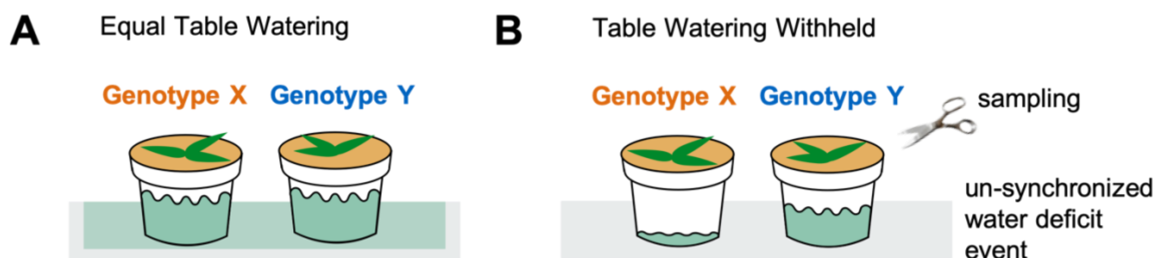

Figure 1: Manipulating water availability in the glasshouse. (A) In the glasshouse, table watering applies soil water resources equally across pots planted with various genotypes. (B) When watering is withheld, differences in water-use between genotypes manifest in un-synchronized water deficit events: 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).

After four days, all pots of each genotype reached 0 g 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 8.68 g 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 0 g 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.

2.3.2. 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

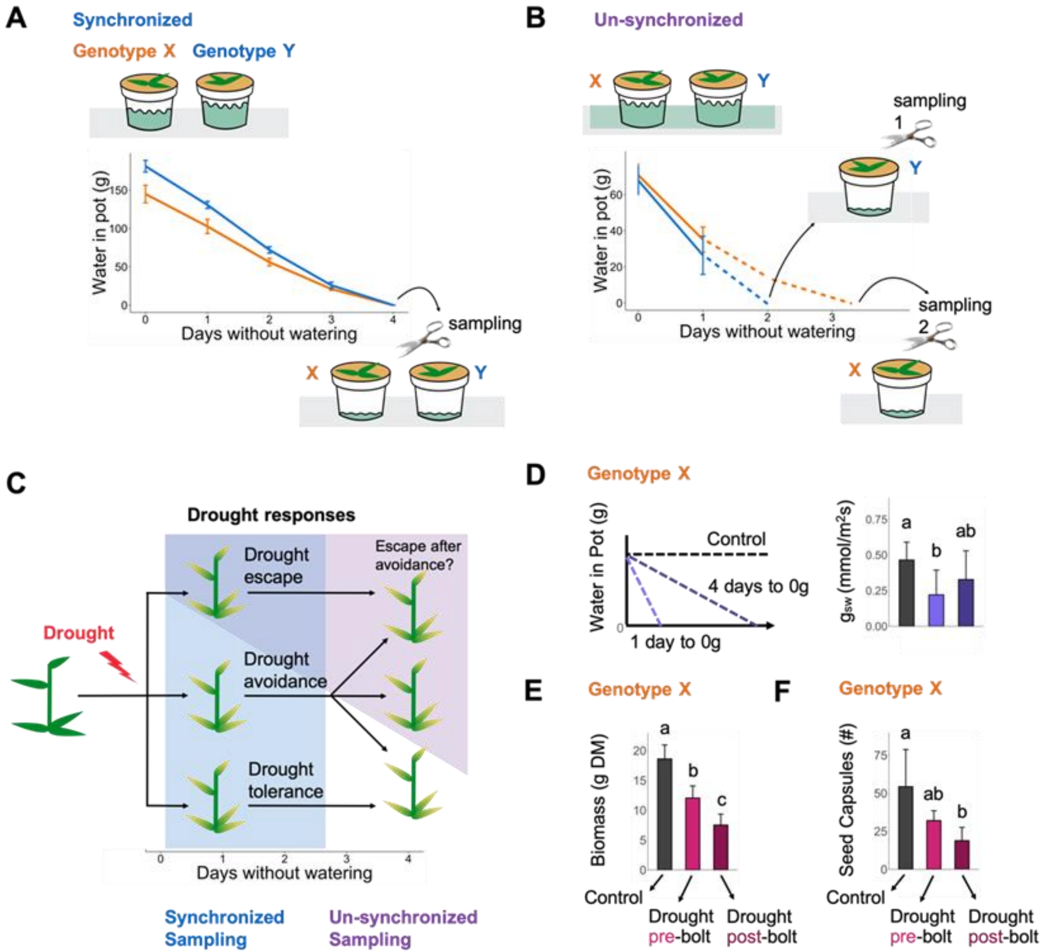

Figure 2: Controlling water consumption per individual in the glasshouse allows for meaningful comparisons between genotypes under similar levels of water deficit stress. (A) By providing individual plants with an amount of water (mean ± CI; Table S1 for Genotype IDs) proportional to their daily consumption, 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).

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 0 g pot soil water availability in one day, or were supplemented with decreasing amounts of water to transition them to 0 g 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.

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.

2.3.3. 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”): a synchronized drought 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 (W_g) 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 W_g on their respective Day 1 (note:

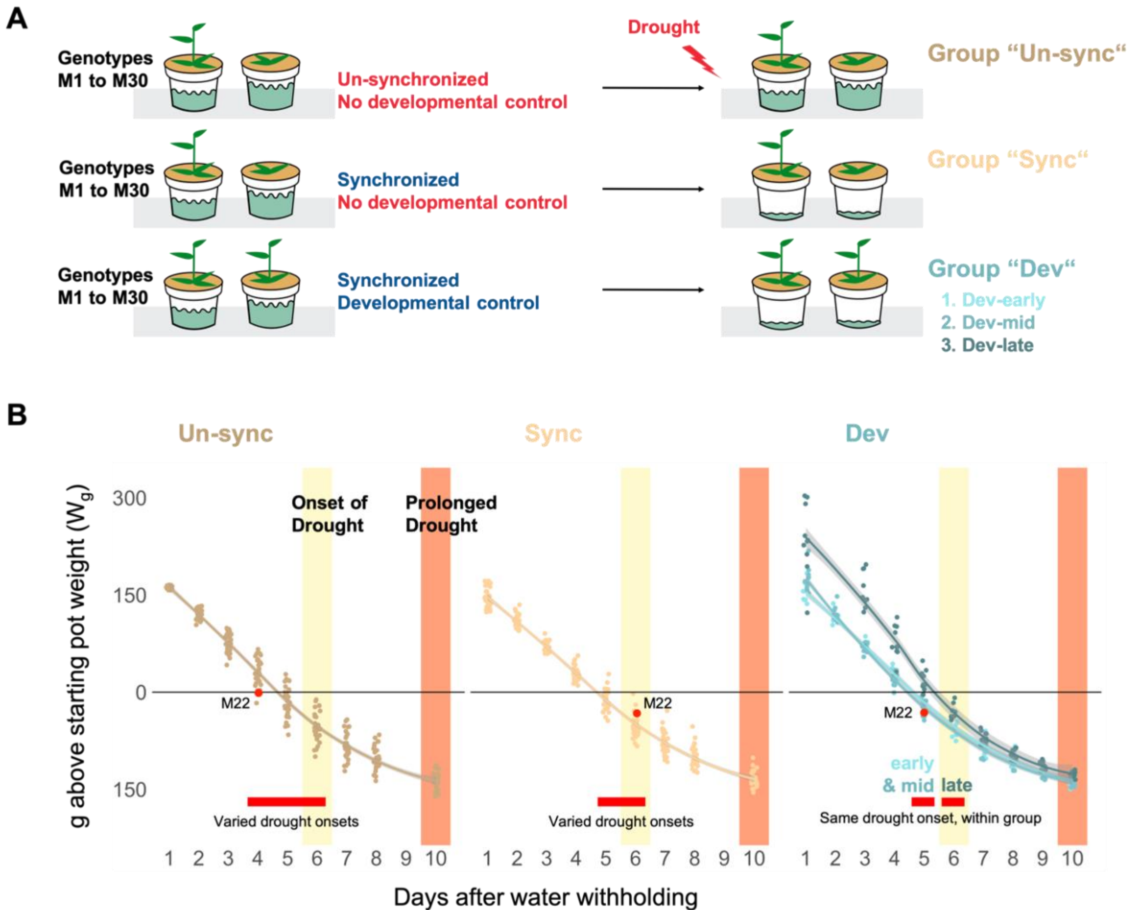

Figure 3: Timing of water deficit stress in a genetically diverse population is less variable when controlling for water use and development. (A) A schematic representation of an experiment comparing 30 intraspecific lines with variable water use and developmental timing (Fig. S2): in Group "Un-sync" (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, W_g, 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 W_g on day 1 of treatment, quickly diverged in their W_g values due to their varying rates of water use, and therefore the transition to drought ($W_g < 0$) 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.

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 W_g on day 1, quickly diverged in their W_g values due to their varying rates of water use, and therefore the transition to drought ($W_g < 0$) 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 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.

2.3.4. 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 (g_{sw}, 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 g_{sw} (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

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%	

mainly affect measurements of ABA in early drought stages and measurements of g_{sw} after an extended drought period.

2.3.5. 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 g_{sw} 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 g_{sw} 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 (W_g). 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 ($W_g = 0$ 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

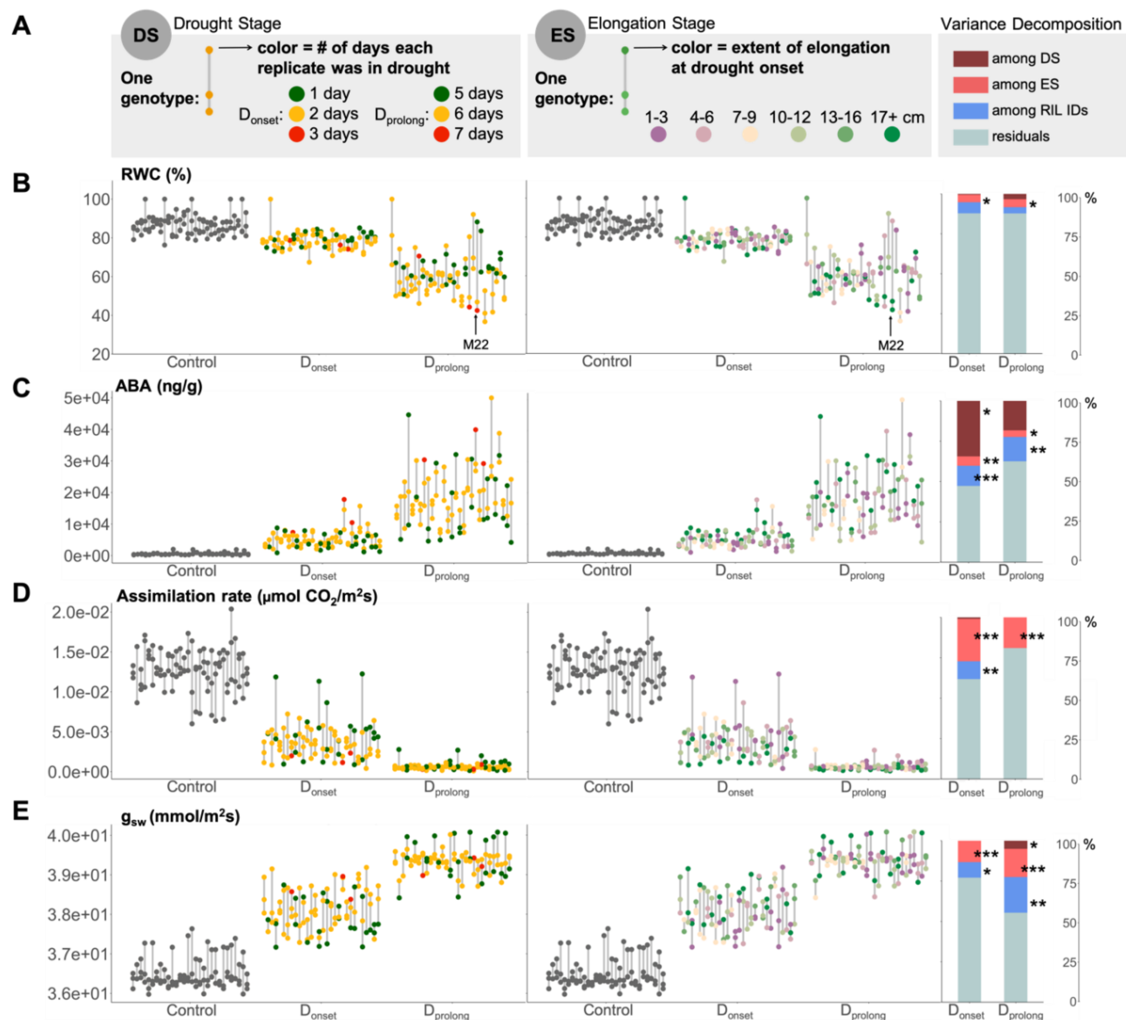

Figure 4: Variance decomposition analysis of common drought response traits in a genetically-diverse population reveals the influence of water deficit stress timing and of development. (A) Common drought response traits were measured in 90 plants (30 genotypes in each watering group in Fig. 3B) of variable 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, D_{onset}) and after several days of water deficit stress (Day 10 from Figure 3B: prolonged drought, $D_{prolong}$). 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, $W_g < 0$, 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 (D_{onset}) or the Control and Prolonged Drought measurements ($D_{prolong}$). 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 (g_{sw}, E) were strongly attributed to DS in both D_{onset} and $D_{prolong}$.

variables: relative water content, ABA concentration, assimilation rate, and stomatal conductance (g_{sw}).

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 g_{sw} 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.

Aknowledgements

We thank the glasshouse department at the Max Planck Institute for Chemical Ecology and the glasshouse screening team of 2017 for support; Prof. Holger Schielzeth for advice on variance decomposition analysis; the technical staff at the department of Molecular Ecology, particularly Dr. Klaus Gase and Ms. Eva Rothe for the MAGIC seeds; and both 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 H.V. and E.M. The authors declare no conflict of interest.

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Supplemental files

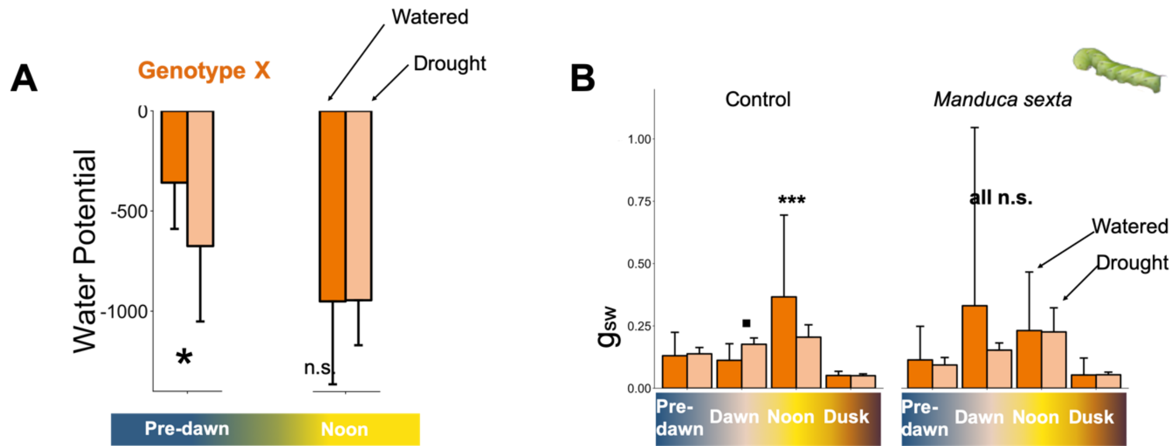

Figure S1: Confounding factors of drought response traits under field conditions. (A) Leaf water potential is well-known to vary diurnally, with water deficit stress being apparent at pre-dawn but not at noon. (B) Stomatal conductance also varies diurnally, with drought responses being most apparent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

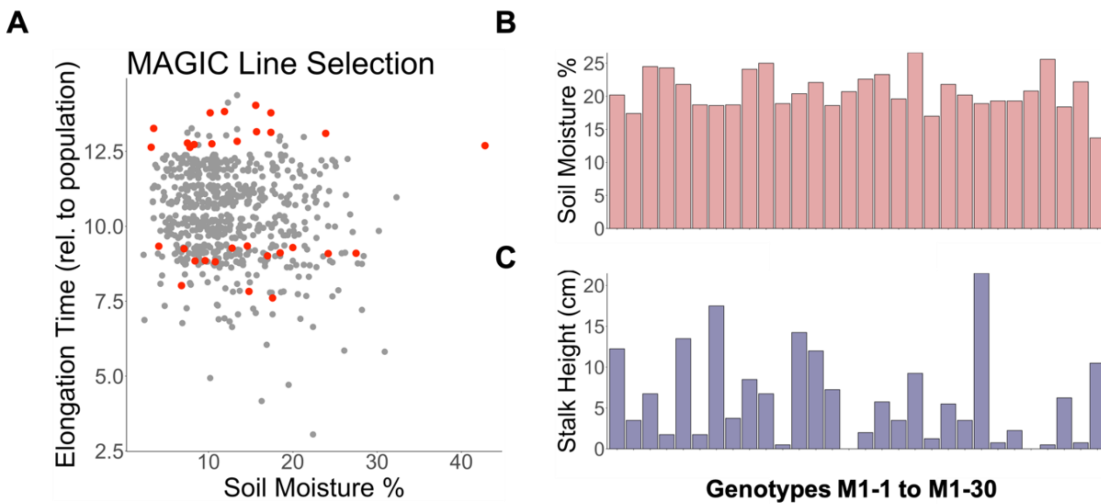

Figure S2: Selection of MAGIC-RIL lines for drought experiment. (A) 30 lines from a large-scale glasshouse screening of 650 MAGIC-RILs were chosen based on their variation across two traits, Soil moisture % and elongation rate. (B) Soil moisture of 30 MAGIC-RILs one day after standardizing soil moisture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

3. The clock gene TOC1 in shoots, not roots, determines fitness of *Nicotiana attenuata* under drought

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Published in *Plant Physiology* (2019). Sep;181(1):305-318. doi: 10.1104/pp.19.00286

Abstract

The highly conserved core circadian clock component TIMING OF CAB EXPRESSION 1 (TOC1) contextualizes environmental stress responses in plants, for example by gating abscisic acid (ABA) signaling and suppressing thermoresponsive growth. Selective interaction of TOC1 with PHYTOCHROME B (PHYB) under far-red-enriched light suggests a connection between circadian gating of light responses and sensitivity to ABA, an important regulator of growth and stress responses, including under drought. However, the fitness consequences of TOC1 function, particularly in the root, are poorly understood. Here, we used the desert annual, *Nicotiana attenuata*, to investigate the function of TOC1 in shoots and roots for maintaining fitness under drought, in both field and glasshouse experiments. Despite marked decreases in leaf water loss, *TOC1*-deficient (irTOC1) lines failed to maintain fitness in response to drought stress as measured by total seed capsule production. Restoring *TOC1* transcript levels in shoots via micrografting was sufficient to restore wild-type drought responses under field conditions.

Microarrays identified a co-expression module in leaves strongly linking red and far-red light signaling to drought responses in a *TOC1*-dependent manner, but experiments with phytochrome-deficient lines revealed that the effects of *TOC1* deficiency under drought cannot be attributed to changes in red/far-red light perception alone. Taken together, these results elucidate the sophisticated, tissue-dependent role of the circadian clock in maintaining fitness in the face of long-term abiotic stresses such as drought.

3.1. Introduction

Plants are sessile, and as a consequence, their fitness depends on the ability to adapt to environmental changes. The circadian clock provides a powerful mechanism for plants to contextualize these adaptive responses to the environment. Both physiological (Green *et al.*, 2002; Izawa, 2012) and genomic evidence (Hofmann, 2012; Lou *et al.*, 2012) indicate the adaptive significance of the plant circadian clock. Much of this evidence is consistent with the importance of the clock in timing responses to abiotic stress (Kant *et al.*, 2008; Mizuno and Yamashino, 2008). *TIMING OF CAB EXPRESSION 1* (*TOC1*) is an essential component of the central repressilator loop of the circadian clock in most plant species, repressing and being repressed by *LATE ELONGATED HYPOCOTYL* (*LHY*) (Alabadi *et al.*, 2001; Pokhilko *et al.*, 2012; Nohales and Kay, 2016). While *Arabidopsis thaliana* has a paralog to *LHY*, *CIRCADIAN CLOCK-ASSOCIATED 1* (*CCA1*), which displays a partially redundant function to LHY (Mizoguchi *et al.*, 2002; McClung, 2010), other species, including *Nicotiana attenuata*, lack this paralog (Zdepski *et al.*, 2008; Okada *et al.*, 2009; Takata *et al.*, 2009; Yon *et al.*, 2012). Because of its central role in regulating a variety of endogenous processes, the circadian clock likely helps to maintain plant fitness in the face of stress (Greenham and McClung, 2015; Seo and Mas, 2015).

Drought is a particularly complex stress, for which it has been difficult to link molecular mechanisms to phenotypes and fitness outcomes (Des Marais, 2008). Mechanistically, drought responses are directly connected to the clock through TOC1. In *A. thaliana*, *TOC1* binds directly to the promoter of the *magnesium-protoporphyrin IX chelatase H subunit (ABAR/CHLH/GUN5)*, controlling its circadian expression, and the time-of-day dependent induction of TOC1 by ABA is reciprocally regulated by ABAR (Legnaioli *et al.*, 2009). While the function of ABAR as an ABA receptor has been called into question (Muller and Hansson, 2009), more recent work has shown specific binding of ABA to ABAR, likely through a domain in the C-terminal half of the protein (Wu *et al.*, 2009). The reciprocal gated induction of ABA by *TOC1* is regulated by the transcription factor *MYB96* (Lee *et al.*, 2016b). The flowering-promoting circadian gene *GIGANTEA (GI)* provides a connection between ABA-dependent responses related to flowering and the clock by stabilizing the central florigen activator *CONSTANS (CO)* in an ABA- and photoperiod-dependent manner (Riboni *et al.*, 2013). *GI* in turn is a positive regulator of *TOC1* and a member of the clock's "evening loop," although the physical evidence for this connection is still lacking (Martin-Tryon *et al.*, 2007).

Variation in circadian clock rhythms among plant tissues has been described (Endo *et al.*, 2014), but the functions of this tissue-level specificity have not been investigated. The circadian clock has been described as tissue-specific, but not tissue-autonomous (James *et al.*, 2008); it has more recently been shown that the plant clock has a hierarchical organization, with the shoot apex clock playing a dominant role in synchronizing distal organ clocks (Takahashi *et al.*, 2015). However, despite the molecular evidence indicating shoot dominance, *A. thaliana* roots and shoots have different rhythmic properties and respond independently to light signals via light transmitted through the stem (Lee *et al.*, 2016a; Nimmo, 2018) and roots can be directly entrained by low-intensity light even in antiphase to attached shoots (Bordage *et al.*, 2016). It is unclear how these tissue-independent responses can functionally emerge from hierarchical, tissue-specific circadian clocks. The combination of both root- and shoot-specific responses to drought (Montero-Tavera *et al.*, 2008), and root- and shoot-specific circadian clock variation (James *et al.*, 2008), implies a potential fitness benefit of root- and shoot-specific variation in circadian clock-mediated drought responses. Whether tissue-specific variation in the circadian clock confers an advantage to plants facing drought remains unknown and is one of the motivations for this work.

TOC1 is also directly involved in suppressing thermoresponsive growth in *A. thaliana* by inhibiting the phytochrome interacting factor PIF4 (Zhu *et al.*, 2016). In addition, selective interactions of TOC1 with PHYTOCHROME B (PHYB) under the far-red light conditions that characterize the end-of-day, when *TOC1* is expressed, further suggest a connection between circadian gating of light responses and sensitivity to ABA (Yeom *et al.*, 2014). Given that *phyB* mutants display decreased stomatal sensitivity and impaired transcriptional responses to exogenous ABA (González *et al.*, 2012), we explored the links between *TOC1*-mediated ABA signaling and light sensing to reveal the ecological significance of these mechanistic interactions.

Despite our deepening molecular understanding of the circadian regulation of drought responses in particular tissues, the significance of these response mechanisms for plant fitness merits more realistic investigation. Rigorous fitness quantification requires the integration of responses on both physiological and developmental time scales in a whole-plant analysis. Allometric, or size-dependent, partitioning of resources between tissue-specific vegetative biomass and final reproductive effort is the gold standard for studying plant fitness responses that occur over developmental time (Weiner, 2004; Younginger *et al.*, 2017). Allometric analyses are essential for understanding life history strategies such as the timing of the switch to reproductive growth from vegetative growth for annual plants (Weiner *et al.*, 2009), and have yet to be used to explore the role of circadian clock components in whole-plant drought responses. Ecologically realistic and sophisticated drought manipulation techniques are also required to study drought responses that occur over both developmental and physiological time scales. In order to avoid erroneously comparing physiological responses of different genotypes in asynchronous developmental stages, drought treatments must be performed at developmentally matched times (e.g. at time of bolting) rather than at the same absolute time post-germination, following the

dictum "stage, not age". Likewise, erroneous comparisons of genotypes with unequal water status must be avoided (e.g. a drought-stressed genotype and a genotype yet to arrive at a similar stage of drought). Here, we combine appropriate watering techniques for field and glasshouse experiments with techniques for exploring tissue-specific analyses of single genes, to explore the circadian clock's function in maintaining plant fitness under stressful conditions.

To explore this function, we have utilized the ecological model species *N. attenuata*, an annual wild tobacco species native to western North America (Baldwin *et al.*, 1994; Kessler and Baldwin, 2001; Dinh *et al.*, 2013). The species' range is characterized by arid regions of cold and hot deserts (Köppen BWh; see Peel *et al.*, 2007) as well as less xeric regions with both hot and dry summers but wet winters (Köppen Csa, Csb) and cold summer climates at higher elevations (Köppen Dsa, Dsb). Given this range and the many available lines of transgenic plants, N. attenuata provides a useful model species to explore the significance of *TOC1* and phytochrome-mediated light signaling for fitness under field conditions.

Here, we explore the consequences of silencing *TOC1* on fitness in the face of drought using micrografting and experimentally-controlled drought scenarios in both field and glasshouse experiments. As previously reported, *TOC1*-silenced plants displayed altered physiological responses to drought, including decreased leaf water loss and increased water-use efficiency. However, we found that *TOC1*-silenced plants incurred severe fitness disadvantages under synchronized drought stress and a controlled watering regime. Under field conditions, *TOC1* function in shoots was sufficient for wild-type allometric drought responses. Transcriptomic analyses of whole-plant or root-only *TOC1*-silenced plants revealed a co-expression module in leaves strongly linking red and far-red light signaling to drought responses. Analysis of transgenic lines silenced in phytochrome expression as well as other developmental regulators and circadian clock components revealed that altering red/far-red light perception alone is not sufficient to explain the fitness consequences of *TOC1* silencing, or transcriptional responses in the face of drought stress, while ectopic overexpression of *LHY* alone phenocopied irTOC1's drought response effects. These results reveal a role for *TOC1* in contextualizing developmental as well as physiological responses for maintaining fitness in the face of drought.

3.2. Results

3.2.1. *N. attenuata TOC1* is required to maintain fitness under controlled drought conditions

Previously, we identified and characterized the core circadian clock genes, among them *TOC1*, in *N. attenuata* (Yon *et al.*, 2012). NaTOC1 displayed high (49% overall) protein sequence similarity to AtTOC1, with both the N-terminal REC domain and the C-terminal CCT motif being conserved, and yeast-two-hybrid assays showed protein interactions between *NaTOC1* and *NaZTL*. *NaTOC1*-silenced plants also displayed late-flowering phenotypes under long day conditions, consistent with the literature (Yon *et al.*, 2012). In agreement with reports in *A.*

thaliana, we observed shortened periods of transcript accumulation in irTOC1 seedlings from two independently silenced lines under constant light of the marker gene *NaROMT* (*resveratrol O-methyltransferase*), which exhibits a circadian-regulated phenotype in EV plants (Supplemental Fig. S1A, B) (Millar *et al.*, 1995; Strayer *et al.*, 2000). The transcript abundance of two other *PRR* genes was not affected in irTOC1 lines (Supplemental Fig. S1C, D), and BLAST searches against the *N. attenuata* genome and transcriptome revealed that the sequence used for RNAi targeting *NaTOC1* does not overlap by more than 18 bp with any region outside of the *NaTOC1* CDS, making off-target effects of the RNAi construct unlikely, as RNAi sequences have been shown to require a 21-nt match or greater for the formation of secondary siRNAs (Schwab and Voinnet, 2010).

We performed a glasshouse experiment comparing irTOC1 and empty vector (EV) plants in order to measure control of water loss as well as developmental responses to drought. We used a gravimetric watering system to maintain a similar water status for EV and irTOC1 plants, to ensure that both genotypes experienced soil water deficit status simultaneously (Fig. 1A). Plants were treated in the rosette stage simultaneously (Supplemental Fig. S2). irTOC1 plants showed increased water use efficiency (Fig. 1B, WUE = assimilation rate divided by transpiration) and significantly reduced leaf water loss after leaf detachment in response to drought, while EV plants did not (Fig. 1C; Control–Drought comparisons by time point per genotype, GLS, lsmeans, Tukey post-hoc). Despite this strong evidence of enhanced physiological tolerance of drought, irTOC1 plants subjected to drought stress failed to maintain fitness (final seed capsule production) in comparison to well-watered irTOC1 control plants, while EV plants showed no change in their fitness correlates in response to the same standardized drought treatment (Fig. 1D). Despite the expected late-flowering phenotype irTOC1 plants displayed (Fig. 1E), neither genotype elongated or flowered earlier under drought conditions, and the drought event occurred when both genotypes were in the rosette stage of growth (Supplemental Fig. S2A and S2B). Because *N. attenuata* seeds may lie dormant for up to more than 150 years in between fires (Preston and Baldwin, 1999), extrapolating to Darwinian fitness would require measuring long-term seed viability under realistic seed bank conditions. Seed capsule production has been frequently used as a measure of relative fitness for *N. attenuata* plants (Karban and Baldwin, 1997; Zavala and Baldwin, 2004; Stitz *et al.*, 2011; Yon *et al.*, 2017) given that seeds per capsule and % viable seeds have been shown to be unaffected by different treatments in populations under native conditions (Baldwin, 1998). In a separate experiment, we did not observe significant effects of an early drought event (similar to Fig. 1) on seed number or seed mass per capsule (Supplemental Fig. S3). Although we did not examine seed viability effects of the drought treatment, we infer that the decrease in capsule number accurately reflects differences in

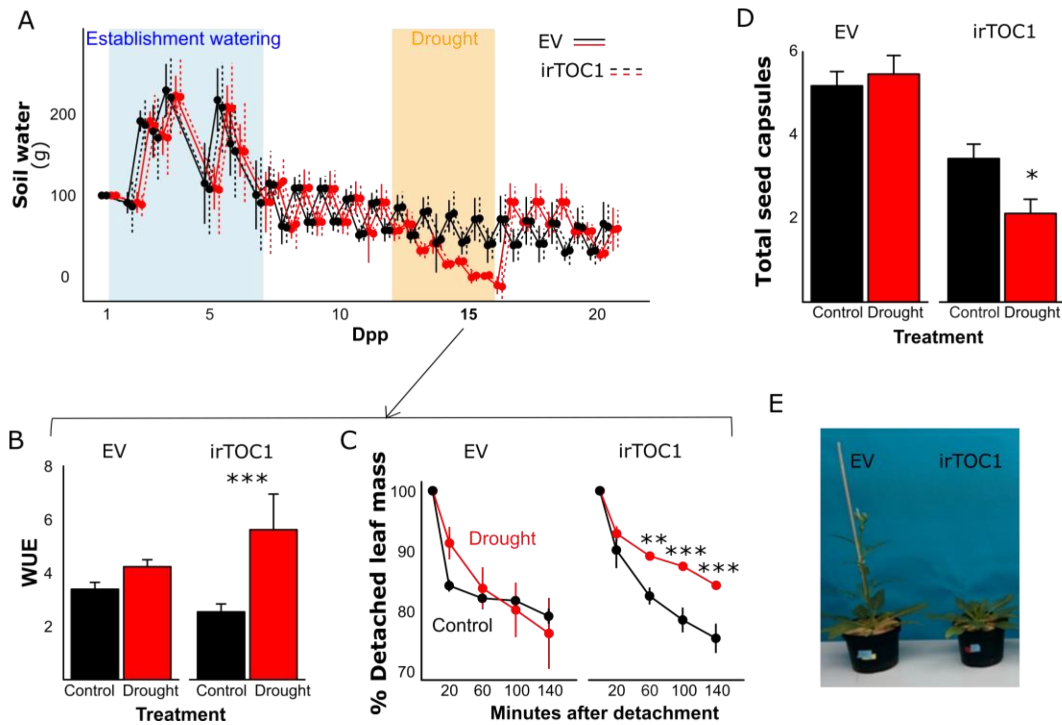

Figure 1: Decreases in leaf water loss of *TOC1*-deficient lines (irTOC1) do not increase plant fitness in response to drought stress. (A) After a period of establishment watering lasting roughly one week, empty vector (EV) and irTOC1 lines were grown using a controlled watering scheme and exposed to a drought period before watering resumed. Plant physiological responses were measured at the end of the drought, at 15 days post potting (dpp). irTOC1 plants under drought conditions had increased water use efficiency (WUE, photosynthetic assimilation rate/transpiration rate) (B) and lower leaf water loss (C) than EV, but had lower reproductive output than EV or (D) control irTOC1 plants after re-watering followed by senescence and dry down. (E) Representative image of irTOC1 developmental delay, bolting 7 days after EV plants. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Control-Drought, multiple comparisons of means, Tukey contrasts following a significant effect in an ANOVA.) Data are plotted as mean values, and error bars represent SEM. Per genotype and treatment, 12 biological replicates were used for (A) and (D), and four biological replicates for (B) and (C).

seed set due to drought exposure, given that more extreme manipulations (50% leaf removal and 2x 500 μg methyl jasmonate application to induce a strong wounding response) failed to induce changes in % viable seeds (Baldwin, 1998). Given these results, we conclude that *TOC1* deficiency confers severe fitness disadvantages under drought despite the seemingly advantageous physiological responses of *TOC1*-deficient plants.

Given the developmental delay observed in *TOC1*-deficient plants, we dissected the role of *TOC1* in drought responses on both developmental and physiological time scales by generating plants with *TOC1*-deficient roots, and wild-type TOC1 function in shoots, via micrografting (Fragoso *et al.*, 2011). A preliminary experiment confirmed that grafting irTOC1 roots to EV shoots generated plants with *TOC1*-silenced roots and with wild-type levels of *TOC1* in shoots (ET, Supplemental Fig. S4A) that were developmentally indistinguishable from those of plants with grafted EV shoots and EV roots (EE, Supplemental Fig. S4B and S4C), in contrast to the substantial developmental delay of plants having both roots and shoots silenced in *TOC1* expression (TT: Supplemental Fig. S4C).

To more rigorously examine the fitness consequences of both whole-plant (TT) as well as root-only (ET) silencing of *TOC1*, we constructed an ecologically appropriate drought scenario for N. attenuata under field conditions (Lytle Preserve, UT, USA), represented schematically in Fig. 2A to illustrate bolting times for each genotype and the timing of water limitation. After an initial establishment phase of three weeks in which plants were watered once per week for one hour at dusk with a drip irrigation system (2L/h flow rate), plants were subjected to slowly decreasing water availability by completely shutting off irrigation lines. Given that most *N. attenuata* lateral roots grow within the first 5 cm of soil under field conditions (Ferrieri *et al.* 2017), we tracked the rate of dry down across the plot with soil core measurements from representative locations at depths of 5, 10, 15, and 30 cm over regular time intervals (Fig. 2B). Final root harvesting confirmed that the bulk of field rooting volume was found within the first 15 cm of soil (Supplemental Fig. S5B). Soil moisture levels around drought-treated plants decreased by around 20%, with the smallest change occurring at 10 cm below the surface (14% decrease to 14.6% soil moisture) and the largest occurring at 30 cm (26% decrease to 11.5% soil moisture). To evaluate whether the drought treatment produced water stress uniformly in all drought-treated plants, we measured leaf water potential at dawn at the end of the experiment. The consistently significant differences between control and drought plants across all three genotypes confirmed that our drought treatment objectives were achieved (Supplemental Fig. S5).

We quantified seed capsule production and dry biomass to determine each genotype's ability to maintain its fitness in the face of decreasing water availability at the end of the growing season. Under drought conditions, EE and ET produced larger amounts of capsules than TT plants by day 54 of the experiment (Fig. 2C). While EE and ET plants maintained similar above- and belowground dry biomass under drought conditions, well-watered TT plants attained

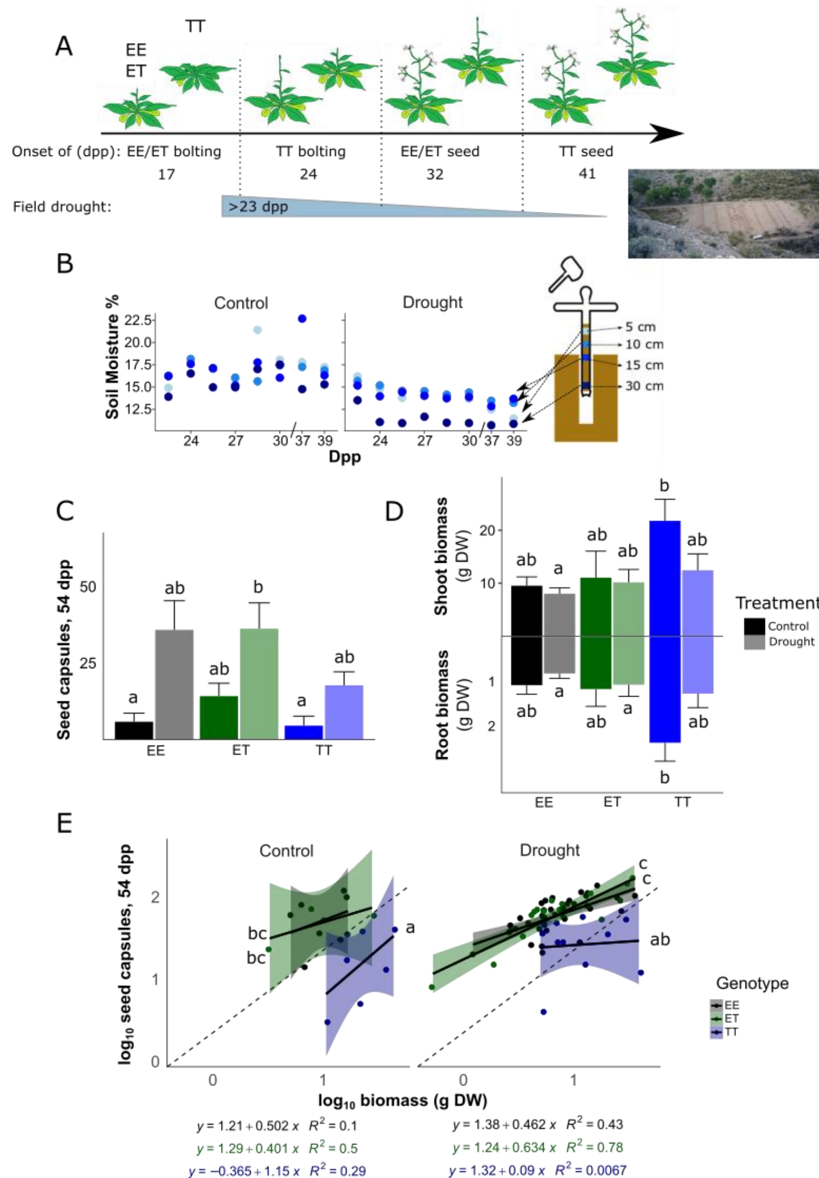

Figure 2: *TOC1* expression in shoots rescues biomass to seed capsule conversions under drought conditions in the field. (A) Experimental setups for fitness measures under both field and glasshouse conditions. Dpp: days post potting. EE: EV shoots/EV roots grafts. ET: EV shoots/irTOC1 shoots. TT: irTOC1 shoots/irTOC1 roots. Water status manipulations were monitored using soil moisture (B) and leaf water potential (Figure S5), which indicated that all three genotypes experienced similar drought stress conditions. (C) Ultimately, TT plants produced somewhat fewer seed capsules under drought conditions than EE and ET plants. (D) TT plants were significantly larger under control conditions in the field, and had somewhat larger roots. (E) TT plants failed to increase conversion of biomass to seed capsules under drought conditions, unlike EE and ET plants. Darker bars represent control samples, while lighter bars represent drought-treated samples. Dashed lines in (E) represent a 1:1 conversion on a log10 scale. (*** p< 0.001, ** p< 0.01, * p< 0.05, pairwise comparisons, multiple comparisons of means, Tukey contrasts following a significant effect in an ANOVA). Data are plotted as mean values, and error bars represent SEM. Per genotype and treatment, 11-15 biological replicates were used for (C), (D), and (E).

significantly greater biomass, roughly twice that of their drought counterparts and of EE and ET plants (Fig. 2D).

The limited effect on fitness correlates and strong effect on final biomass for TT plants under drought implied a change in the relationship between these two parameters. We therefore investigated whether seed capsule production followed an expected allometric pattern (i.e. whether it was dependent on vegetative growth); for annual herbaceous plants like N. attenuata, this pattern is expected to be positive (Weiner *et al.*, 2009). There was a weaker correlation between biomass and seed capsule production for each genotype under control conditions (Fig. 2E, EE R2 = 0.10; ET R2 = 0.50; TT R2 = 0.29). However, EE and ET plants subjected to a controlled dry down period displayed a stronger relationship between biomass and seed capsules (EE R2 = 0.43, ET R2 = 0.78). TT plants under water deficit, on the other hand, showed an even weaker correlation than their well-watered counterparts (R2 = 0.0067). The allometric relationships between biomass and seed capsule production resolved into statistically significant groups, between drought-treated EE and ET plants (c) and all control plants as well as drought-treated TT plants (EE and ET control: bc; TT control: a; TT drought: ab).

3.2.2. Plants silenced in *TOC1* in both shoots and roots show different responses in co-expression modules under drought conditions

In order to better understand the strong fitness effect of *TOC1* silencing under drought conditions, we performed whole-transcriptome microarray analyses on leaves and roots of EE, ET, and TT plants. Following grafting, plants were grown for 13 days after potting, after which control plants remained well-watered and drought plants had water withheld for 7 days. Samples were harvested at the end of this week (Fig. 3A). As with all following experiments, plant water status was monitored using leaf angle at midday, following two preliminary experiments. The first showed that midday leaf angles in *N. attenuata* track the volumetric soil moisture contents and leaf relative water contents (Supplemental Fig. S6), measures of plant water availability. The second showed that when leaf angles reached about 45-50 degrees at midday a strong increase in leaf ABA accumulation and a strong shift in ABA-responsive transcripts were observed (Supplemental Fig. S7). Co-expression module discovery, gene set enrichment analysis (GSEA) and over-representation analyses (ORA) were performed within treatment and tissue groups using the R package *CEMiTool* (Russo *et al.*, 2018).

Figure 3B shows the results of the GSEA, performed using the algorithm *fgsea* (Sergushichev 2016). For each circle, Normalized Enrichment Scores (NES) are calculated that represent the likelihood that the genes in each module are randomly distributed throughout a ranked list of genes (in this case, the z-score normalized expression levels) for each genotype (Subramanian *et al.*, 2005). These values are represented by color and size, with red representing higher and blue lower activity across the module, while the size of each circle corresponds to the absolute value of each NES. The NES and p-values for each module and genotype within each of the four sets (treatment and tissue groups) can be found in Supplemental Table S1. Modules were selected for

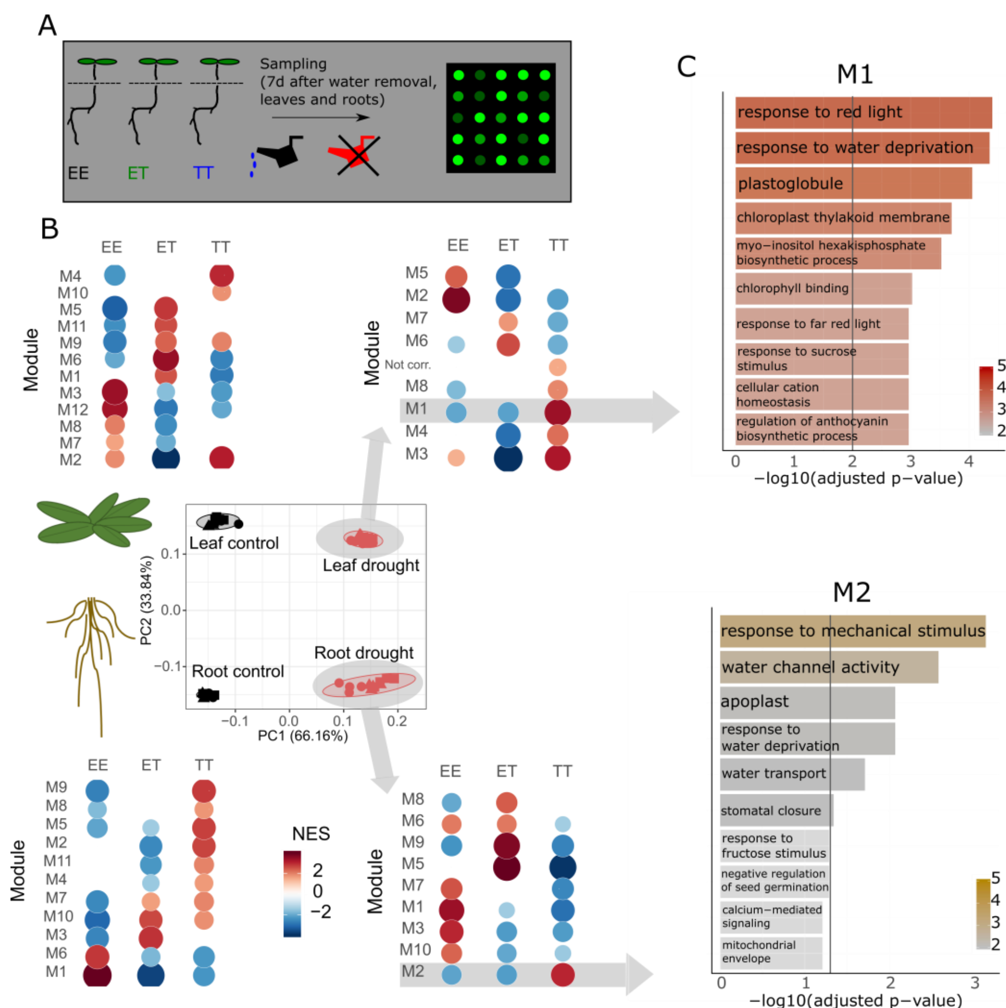

Figure 3: Microarray analysis of plants under control and drought conditions reveals a co-expression module in drought leaves strongly linking drought responses to red and far-red light response. (A) Seedlings (10 d post germination) were grafted to produce three distinct lines: control empty vector (EV) shoot/EV root homografts (EE plants), *TOC1*-deficient (irTOC1) shoot/irTOC1 root homografts (TT plants), and EV shoot/irTOC1 root heterografts (ET plants). Seedlings were then transferred to 1 L pots and grown under normal glasshouse conditions for 13 days before water removal. Leaf and root samples from four biological replicates were then harvested for microarray hybridization. (B) Principal component analysis separated samples according to tissues and treatments, and gene set enrichment analysis was performed using the R package *CEMiTool* within each treatment and tissue sample set separately. Colors represent higher (red) and lower (blue) activity across each module, while the size of each circle corresponds to the absolute NES value. NES values for modules that have an adjusted p-value > 0.05 are removed. (C) Analysis of co-expression modules revealed one module in drought leaves, M1, having a significant overrepresentation of genes related to red and far-red responses, as well as to response to water deprivation, and one module in drought roots, M2, having a significant overrepresentation of genes related to response to water deprivation, water channel activity, and water transport. Both modules were strongly differentially expressed in TT plants relative to EE and ET plants. Bar graphs and colors represent the -log10 adjusted p-value of the enrichment between genes for each GO term. Vertical gray lines represent an adjusted p-value of 0.01. 3-4 biological replicates per genotype, tissue and treatment.

those that were differentially expressed in TT in drought leaves and roots relative to both EE and ET genotypes (Fig. 3B). Two modules that fit this criterion were found, and ORA of these modules were enriched in gene ontology (GO) terms with the most significant adjusted p-values of the enrichment: one co-expression module in drought-treated leaves, M1, had a strong overrepresentation of genes related to red and far-red light responses, as well as water-deprivation responses, while one co-expression module in drought-treated roots, M2, had a strong overrepresentation of genes involved in water-deprivation responses, water channel activity, and water transport (Fig. 3C).

3.2.3. *TOC1*-silenced drought responses are not explained by impaired red/far-red light perception

Given that the R/FR module is differentially expressed in irTOC1 shoots, as well as the evidence for TOC1 interacting directly with PHYB and the phytochrome interacting factors PIF3 and PIF4 (Yeom *et al.*, 2014; Soy *et al.*, 2016; Zhu *et al.*, 2016), we asked whether manipulating this module upstream of TOC1 would produce similar drought response phenotypes to those of irTOC1 plants (Fig. 4A). In seedlings, phyB mutants have constitutively elongated hypocotyls under high R:FR ratios as well as under constant R light, while phyA mutants display constitutively elongated hypocotyls under low R:FR ratios as well as under constant FR light (Smith *et al.*, 1997). Thus, *PHYA* is thought to primarily mediate responses to FR, while *PHYB* primarily mediates responses to R light (Li *et al.*, 2011). We therefore examined the fitness correlates and transcriptional responses of plants silenced in *PHYB1*, *PHYB2* (partially redundant homologs of *NaPHYB*, see Fragoso *et al.*, 2017), *PHYA1*, or both *PHYB1* and PHYB2 simultaneously. To control for the developmental effects of silencing phytochrome and *TOC1* expression that may have affected our fitness measurements, this time we performed drought treatments at the same developmental stage, with each genotype undergoing drought at the onset of elongation (Fig. 4B). Leaf angle was again used as a nondestructive measure to track plant water status (Supplemental Fig. S8C), and plant water status of drought plants was compared to control plants, showing a strong and consistent reduction in relative water content (RWC) of 60-80% across all genotypes at sampling time (Supplemental Fig. S8D).

All genotypes experienced a significant decrease in total seed capsules and biomass production at the end of the experiment under drought conditions (60 days post-potting). However, comparing ratios of seed capsules or biomass of control plants to drought plants revealed a much stronger effect of drought for irTOC1 plants, with a 6- or 5-fold decrease under drought, respectively, indicating that similar results in other experiments did not result from differences in developmental timing. The irPHYB1, irPHYB2, irPHYA, and irPHYB1 x irPHYB2 lines all experienced similar 2-fold decreases under drought, which were also observed in EV plants (Fig. 4C-D). We examined the transcript abundances of *TOC1*-linked response genes at *TOC1* peaking time in EV plants, 19:00 under LD conditions, including *GI*, *ABAR*, and *NaPIFs*, and found similar expression patterns in phytochrome-silenced lines and EV plants, while these genes displayed differential abundance patterns in irTOC1 plants (Fig. 4E-M).

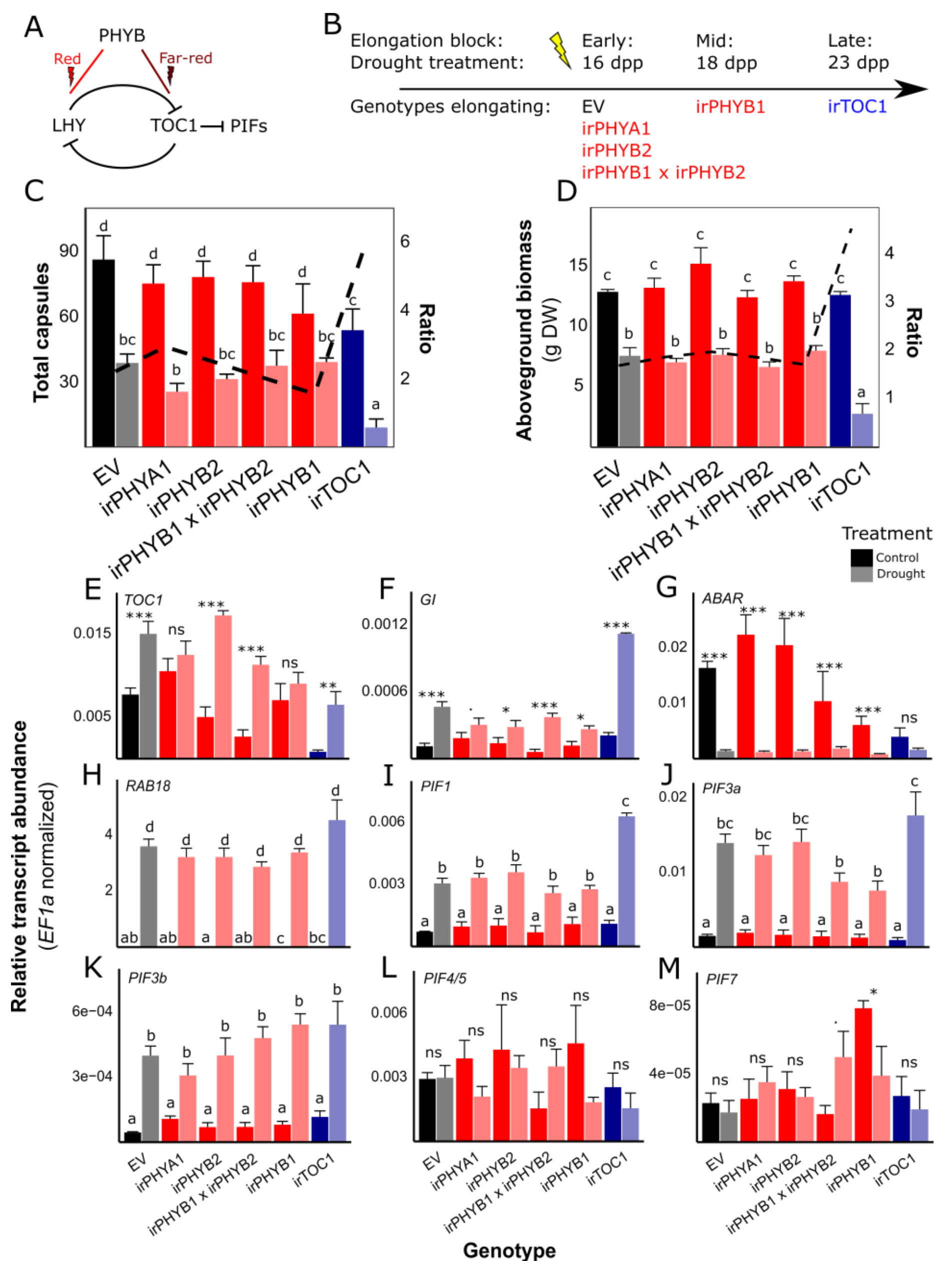

Figure 4: irTOC1 drought responses are not explained by impaired far-red/red light perception. (A) Schematic representation of known interactions between TOC1, LHY, PHYB, and PIFs. (B) Experimental setup for testing drought responses of phytochrome-deficient lines. Due to the developmental effects of silencing *PHYB1* and *TOC1*, genotypes were subjected to drought treatment at the same developmental stage (beginning of elongation) in three blocks. The earliest elongating genotypes ("Early") elongated at 16 days post potting (dpp), a second group ("Mid") elongated at 18 dpp, and a third ("Late") elongated at 23 dpp. (C-D) Unlike EV and phytochrome-deficient lines, irTOC1 experienced a drastic reduction of fitness correlates when subjected to drought relative to control EV levels. (E-M) Transcriptional analysis of TOC1-associated transcripts under drought. Contrasts were made between Control-Drought treatments for each genotype; where all pairwise comparisons were significant, groupings have been added to emphasize differences across genotypes. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, all contrasts relative to EV controls, multiple comparisons of means, Tukey contrasts following a significant effect in an ANOVA). Data are plotted as mean values, and error bars represent SEM. 4-5 biological replicates per genotype and treatment.

3.2.4. Impaired photoperiodic flowering control is not sufficient to explain drought responses and developmental phenotypes in *TOC1*-silenced plants

Given the lack of irTOC1-like drought response phenotypes of phytochrome-deficient lines, we asked if this phenotype is driven by irTOC1's developmental delay, or by other disruptions in the circadian clock mechanism. We examined the transcriptional responses and fitness correlates under control and drought conditions of two lines with developmental delays in photoperiodic flowering (*irCRYP1a* and *irFT3*). Cryptochromes act as blue and ultraviolet-A light receptors; plants with *cry1* (*CRYP1a* in *N. attenuata*) mutant alleles demonstrate late flowering under certain conditions, while plants with gain-of-function mutations in *cry1* exhibit early flowering and higher transcript abundance of the floral promoters *CONSTANS* and *Flowering Locus T* (*NaFT3*) (Exner *et al.*, 2010). We furthermore used lines silenced in and ectopically overexpressing LHY, the other central component of the plant circadian clock (irLHY and ovLHY, respectively). We performed drought treatments, as described in the last section, in a developmentally standardized stage when plants were at the onset of elongation, splitting these genotypes into three blocks according to the timing of elongation between the earliest elongating genotypes, EV and irLHY ("Early"), a second group consisting of irCRYP1a and irFT3 ("Mid"), and a third group consisting of ovLHY and irTOC1 ("Late", Fig. 5A) and again used leaf angle as a nondestructive measure of plant water status (Supplemental Fig. S8C), which was confirmed at sampling by RWC measurements (Supplemental Fig. S8D). As this experiment was performed in parallel to the experiments in Fig. 4, the same EV and irTOC1 controls were used.

Plants from all genotypes experienced a significant decrease in total seed capsules and biomass production at the end of the experiment under drought conditions. While EV, irCRYP1a, irFT3, and irLHY plants showed a similar 2-fold reduction under drought, the decrease in ovLHY was stronger and similar to the drought responses of irTOC1 plants (20- or 8-fold decrease, respectively, Fig. 5B-C). The transcript abundance of the *TOC1*-linked response genes *GI*, *ABAR*, and *NaPIF1* followed similar expression patterns in irTOC1 and ovLHY plants (Fig. 5E, F, and H). The transcript abundance of other NaPIFs either showed similar drought responses across most genotypes (*NaPIF3a* and *NaPIF3b*, Fig. 5I and J), no response to drought in EV, ovLHY, or irTOC1 (*NaPIF7*, Fig. 5L) or a significant response in irTOC1 and irCRYP1a plants only (*NaPIF4/5*, Fig. 5K). These results suggest that impaired photoperiodic flowering mechanisms are not sufficient to phenocopy the drought responses of irTOC1 plants, and that only ovLHY plants exhibit impaired drought responses consistent with those observed in irTOC1 plants.

3.3. Discussion

Here we demonstrate that silencing *TOC1* incurs a severe fitness disadvantages for *N. attenuata* under drought conditions in both the glasshouse and the field. In the glasshouse, these fitness disadvantages occur despite improved physiological responses to drought, such as higher

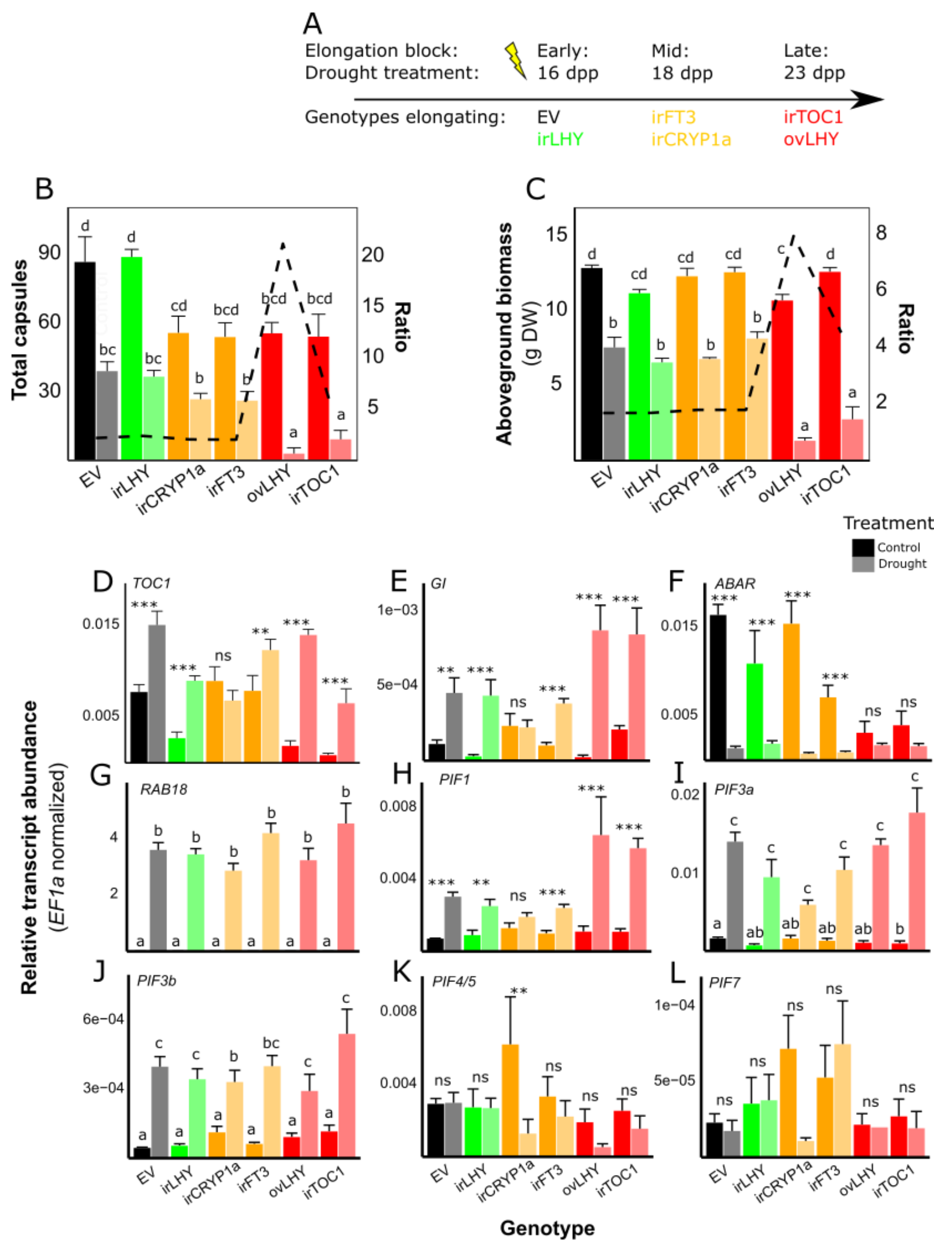

Figure 5: ovLHY phenocopies irTOC1 drought responses and developmental delay. (A) Experimental setup for testing drought responses of phytochrome-deficient lines. Due to the developmental effects of silencing *PHYB1* and *TOC1*, genotypes were subjected to drought treatment at the same developmental stage (beginning of elongation) in three blocks. The earliest elongating genotypes ("Early") elongated at 16 days post potting (dpp), a second group ("Mid") elongated at 18 dpp, and a third ("Late") elongated at 23 dpp. (B-C) irTOC1 and ovLHY experienced a drastic reduction of fitness correlates when subjected to drought, and experienced similar developmental delays. (D-L) Transcriptional analysis of *TOC1*-associated transcripts under drought. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, all contrasts relative to EV controls, multiple comparisons of means, Tukey contrasts following a significant effect in an ANOVA). Data are plotted as mean values, and error bars represent SEM. 3-5 biological replicates per genotype and treatment.

WUE (Fig. 1B) and lower rates of leaf water loss (Fig. 1C). We analyzed the performance of micrografted plants under field conditions through an ecologically realistic drought scenario in order to explore the tissue-specific effects of *TOC1* silencing. Unimpaired *TOC1* function in shoots was sufficient for both wild-type developmental responses (Supplemental Fig. S4) as well as wild-type allometric relationships between whole-plant biomass and seed capsule production under drought conditions in the field (Fig. 2E), as judged by comparison to EV controls which have a wild-type phenotype (Schwachtje *et al.*, 2008). Whole-transcriptome analysis yielded a co-expression module suggesting a function for *TOC1*-mediated R and FR light signaling in drought responses of shoots, but not roots (Fig. 3C). Given that the fitness outcomes were not changed by abrogating R/FR light sensing directly (Fig. 4C-D), the effect of *TOC1* silencing appears to be downstream of phytochromes A, B1, and B2. Further screening of clock- and developmentally-shifted lines (irLHY, ovLHY, irFT3, and irCRYP1a) showed that only a line overexpressing *LHY* displayed the same fitness outcomes and expression pattern of key *TOC1*-linked response genes as were displayed in irTOC1 plants; since *LHY* represses *TOC1*, this may be due to reduced *TOC1* transcripts in the ovLHY line. However, crosses of irTOC1 and ovLHY, or a line bearing a double construct, could determine whether the phenotypic effects observed in both lines are additive; if they are, that would imply that the drought responses of irTOC1 and ovLHY, while yielding similar outcomes, do not stem from the same abrogation of clock function via *TOC1*.

Despite reduced levels of *TOC1* at the chosen sampling time, (Fig. 5D), irLHY lines did not display the same drought-induced changes in transcript abundance of key *TOC1*-linked response genes or share the same fitness outcomes. This may be due to phase and amplitude differences in diurnal *TOC1* expression resulting from silencing and overexpression of *LHY*; previous work has implied a shift in phase during LD conditions for *N. attenuata* irLHY lines (Joo *et al.*, 2017). It is very likely that the circadian rhythm is shifted in the various lines examined in Figs. 4 and 5; indeed, we observe a shift in the rhythm of irTOC1 plants under LD conditions (Supplemental Fig. S1A). How the rhythmicity of the circadian clock is disrupted by drought stress under LD conditions remains unclear. This question cannot be resolved satisfactorily with our single time-point transcript abundance analyses, although kinetic experiments conducted under similar drought conditions with *N. attenuata* clock-shifted lines (irTOC1, irLHY, and ovLHY) could elucidate the differences between *TOC1* transcript abundance dynamics and their resultant effects on drought-related gene expression.

Similar to our findings, impaired *TOC1* expression enhances physiological performance under drought stress conditions in *A. thaliana*, such as greater seedling dehydration survival through altered ABA signaling (Legnaioli *et al.*, 2009). Despite the fitness effects suggested by these short-term performance tests, the fitness consequences of impaired *TOC1* expression in mature plants or throughout development are a novelty from this study, which has not been previously explored in *A. thaliana*, particularly in the context of drought responses. More fine-grained fitness analyses of other clock-manipulated plants have shown that, even if circadian clock-

impaired plants perform similarly to wild-type varieties under normal conditions, differences arise in a sensitized background. For example, while the fertility of *GIGANTEA*-deficient *Oryza sativa (osgi)* was similar to wild type under typical transplanting dates in the field, later transplanting dates yielded lower fertility of *osgi* relative to wild type (Izawa *et al.*, 2011). These developmental effects under field conditions for *osgi* mutants, as well as the developmental effects of *TOC1* silencing in *N. attenuata* and the clear fitness effects observed after seed set (Fig. 1E) highlight the importance of a more rigorous approach for the analysis of fitness under field conditions. Aside from the dormancy issues stemming from *N. attenuata's* long-lived seed bank (up to 100 years or more) that complicate measuring the long-term viability of seeds, seed collection in our field experiments is further complicated by the need to prevent the distribution of ripe seeds from transgenic lines for regulatory compliance. Further tests of the effect of *TOC1* silencing and drought treatment on seed viability would require a long-term seedbank experiment buried under natural conditions. Our analysis of seed number and seed mass per capsule showed that both parameters are affected in EE plants under late drought conditions (Supplemental Fig. S3). However, under the two experimental conditions employed in this study, namely Early Drought conditions (wherein all plants underwent drought at EV bolting time, employed in Fig. 1) and under developmentally-timed drought conditions (wherein each genotype underwent drought at their respective bolting time asynchronously, employed in Figs. 4 and 5), genotypes did not demonstrate differences in these two parameters, and thus seed capsule number likely demonstrates an accurate relative estimate of fitness under these experimental conditions.

Allocation has often been analyzed as reproductive effort (RE), defined as reproductive biomass divided by total biomass. However, RE is more rigorously analyzed as allometric (size-dependent) relationships of reproductive output (e.g. number of seed capsules or reproductive biomass) regressed against vegetative biomass (Weiner *et al.*, 2009). With an allometric analysis, two key points to consider are plasticity in resource allocation, which can be defined as a change in an allometric trajectory (i.e. the slope of the regression), and optimal allocation theory, which predicts that plants will cluster more closely to the allometric regression (i.e. greater R2 values) as they reach their reproductive potential after vegetative growth (Weiner, 2004). Under field conditions, we observed that the allometric trajectory between biomass and seed capsule production was strongly changed under drought conditions for all three genotypes (Fig. 2E). R2 values increased under drought conditions for EE and ET plants, implying an earlier transition to reproductive growth than their control counterparts, while TT plants fail to undergo this transition. This plasticity in resource allocation is thus dependent on shoot, but not root *TOC1* expression. Interestingly, we observed different responses of seed capsule production (Fig. 2C and 4C) and biomass (Fig. 2D and 4D) under field and glasshouse conditions. This difference may be due in part to variation in rooting volume between field- and glasshouse-grown plants: field-grown TT plants under control watering displayed twice the root and shoot biomass of EE and ET plants. Differences in blue light between our field and glasshouse setups may have also influenced growth rates, with reduced blue light availability under glasshouse conditions leading to lower stomatal conductance and photosynthetic capacity (Hogewoning *et al.*, 2010).

The allometric analysis from the field experiments, as well as the microarray analysis, point to a shoot-specific role of *TOC1* in drought responses. In root drought samples, the only transcriptional module with clear functional implications for drought, M2, was differentially expressed between EE/ET and TT plants, implying that a shoot-derived signal may be sufficient for drought responses (Fig. 3B). Although other modules displayed differential expression between EE and ET/TT, these modules had less clearly discernible functional implications for drought responses (Supplemental Table S2). Micrografting of RNAi lines such as irTOC1 does not permit the investigation of shoot-only *TOC1* knockdowns, as the RNAi silencing signals travel from shoots to roots (Fragoso *et al.*, 2011). Lines harboring mutant toc1 alleles would allow for the analysis of reciprocal *toc1*/EV and EV/*toc1* grafts; conversely, heterografts of the ovLHY line which phenocopied irTOC1 drought responses (ovLHY/EV and EV/ovLHY) could be used to evaluate the inference regarding the role of shoot *TOC1* silencing in the abrogation of drought responses. Prior to such experiments, crosses between ovLHY and irTOC1 should be used to determine whether the drought-related phenotypes of these lines are additive, which would change the conclusions that could be drawn from reciprocal grafting of ovLHY.

We infer from the results presented here that R/FR-related drought responses are mediated in a TOC1-dependent manner in the shoots of *N. attenuata* (Fig. 3C, Fig. 4I). TOC1 is known in A. thaliana to mediate rhythmic growth and gate thermoresponsive growth by direct interactions with PIF3 and PIF4, respectively (Soy *et al.*, 2016; Zhu *et al.*, 2016). TOC1 is also known to directly interact with other PIFs (Yamashino *et al.*, 2003) (including PIL5, also known as PIF1), which is also implied in N. attenuata by the increased transcript abundance of PIF1 in irTOC1¬ and ovLHY under drought (Fig. 6H). TOC1-PIF1 may form an analogous gating mechanism to TOC1-PIF3 and TOC1-PIF4 for drought-responsive growth or developmental signals. This proposition could be tested by examining whether NaTOC1 and NaPIF1 interact directly by yeast two hybrid analysis, as well as by using virus-induced gene silencing (VIGS) of NaPIF1 in wild-type and TOC1-deficient backgrounds to evaluate whether VIGS-PIF1 irTOC1 plants display wild-type transcript abundances of TOC1-related marker genes. If all irTOC1-background plants displayed similar transcript abundances of these marker genes, a TOC1-PIF1 interaction is unlikely to form a drought-related gating mechanism analogous to TOC1-PIF3 and TOC1-PIF4 in *A. thaliana*.

3.4. Conclusion

By combining careful drought treatments and an allometric analyses under field conditions with an RNAi-driven screening of the circadian clock, this work reveals the fitness implications of shoot *TOC1* deficiency under water limitation, and shows that *TOC1* expression in the root does not contribute to *TOC1*-dependent fitness responses in biomass to seed capsule conversions. These data provide a functional test, under field conditions, of the hierarchical organization of root and shoot circadian clocks under drought stress. Further transcriptomic analysis and screening of transgenic lines provide evidence that R/FR-related drought responses are mediated

in a *TOC1*-dependent manner in the shoots of *N. attenuata* downstream of phytochromes and other R/FR light perception machinery.

3.5. Methods

Plant materials and constructs

Lines were derived from seeds originally collected from natural populations of *Nicotiana attenuata* from the Desert Inn Ranch near Santa Clara, UT, USA (Baldwin *et al.*, 1994). Seed germination and plant growth in the glasshouse were carried out as described by Krügel and colleagues (Krügel *et al.*, 2002) and was undertaken in a glasshouse in Jena, Germany. Screening of the EV line (pSOL3NC, line number A-04-266-3) is described by Bubner and colleagues (Bubner *et al.*, 2006). Screening of *TOC1*-silenced (ir) lines (pSOL8_16844, A-11-205-4) via RNAi is described by Yon and colleagues (Yon *et al.*, 2012), with additional screening described in Supplemental Fig. S1A-C. Screening of ovLHY, irCRYPa, and irFT3 lines is described in Supplemental Table S3; the additional screening of the ovLHY lines is described in Supplemental Fig. S1D. BLAST searches against the *N. attenuata* genome and transcriptome revealed that the sequence used for RNAi targeting *NaTOC1* does not overlap by more than 18 bp with any region outside of the *NaTOC1* CDS, making off-target effects of the RNAi construct unlikely, as RNAi sequences have been shown to require more than 21-nt of contiguous exact matches for the formation of secondary siRNAs (Schwab and Voinnet, 2010). The irTOC1-205 line was used for all experiments described in this study. Importation and release of transgenic plants were carried out under Animal and Plant Health Inspection Service (APHIS) import permit numbers 07-341-101n (EV) and 11-350-102m (irTOC1) and release permit number 16-013-102r. Field growth conditions were described by McGale and colleagues (Mcgale *et al.*, 2018). Briefly, seedlings were germinated on Gamborg's B5 media under illumination from fluorescent lights (GE Plant & Aquarium 40 W and GE Warm White 18 W) at ambient temperatures at the field station. One week after germination, seedlings were grafted (see below, **Micrografting**, and Fragoso and colleagues (Fragoso *et al.*, 2011)). One to two weeks after grafting, seedlings with four visible leaves were transferred into previously hydrated 50-mm peat pellets (Jiffy 703, www.jiffypot.com) treated with Borax to provide boron, an essential micronutrient (1:100 dilution of a 1.1 g L-1 stock solution) and adapted over two weeks to the field conditions of high light intensity and low relative humidity by keeping seedlings first in shaded, closed translucent plastic 34-quart boxes (Sterilite), then opening the boxes, and subsequently transferring open boxes to partial sunlight in mesh tents (Tatonka). Adapted size-matched seedlings were transplanted into an irrigated field plot at the Lytle Ranch Preserve, Santa Clara, Utah, in April 2016.

Water limitation treatments

Glasshouse plants were grown in sand and kept at 18-35o C under 16:8 h of light:dark cycles, with the light period lasting from 6:00 to 22:00. For the first glasshouse drought experiment

(Figure 1), plants were potted and individual baselines measured of the combined pot, plant, and soil mass; this mass was used as a baseline of 0 g of water. Thereafter, gravimetric water content was measured per pot and per day, and plants were watered with 2x the previous day's water use. Treated plants were not watered on those days, and allowed to dry down to 0 g of water in excess of initial water-holding capacity per pot, before watering re-commenced. Plants were sampled on the day that 0 g of water was reached.

For the second glasshouse drought experiment (Figs. 4 and 5), plants were watered using table flood irrigation, and treated plants were removed from water for 7-9 days to induce drought stress. Leaves at the +1 nodal position (youngest fully extended leaves) were sampled without the midvein, and whole roots were homogenized for root sampling. All tissue was stored at -80°C before RNA or phytohormone extraction. Plant water status was monitored using leaf angle at midday, following two preliminary experiments. The first showed that midday leaf angles in *N. attenuata* track the volumetric soil moisture contents and leaf relative water contents (Supplemental Fig. S6), measures of plant water availability. The second showed that when leaf angles reached about 45-50 degrees at midday a strong increase in leaf ABA accumulation and a strong shift in ABA-responsive transcripts were observed (Supplemental Fig. S7). Relative water content was calculated using the equation displayed in Supplemental Fig. S8B: 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 (DM, Turner 1981).

In the field (Fig. 2), plants were watered using a drip irrigation system approximately once a week, as needed to maintain soil moisture levels, according to soil moisture measurements made in representative locations throughout the field plot using a 53 mm diameter by 40 cm working length split tube sampler (Fig. 2B; Product Number 04.17, Eijkelkamp Soil & Water, Giesbeek, Netherlands). The split tube sampler was used on most days throughout the latter half of the field season to quantify soil water status. Soil core samples were taken from 5, 10, 15, and 30 cm below the surface, weighed, and allowed to dry in closed, aerated boxes outside (air moisture of ~17-20%) for 3-5 days until soil reached a stable weight, and then re-weighed. Water-limited plants were disconnected from the drip irrigation system 23 days post planting, after an initial establishment phase, and allowed to dry down naturally until the conclusion of the experiment.

Gas exchange and water use efficiency (WUE) calculations

Gas exchange and chlorophyll fluorescence measurements were conducted with plants in the early elongating stage of growth with the first fully-extended stem leaves. Measurements were conducted using a LI-6400XT infrared gas analyzer (Li-Cor Bioscience, Lincoln, NE, USA), with an integrated fluorometer in the leaf chamber, and WUE was calculated as assimilation rate divided by transpiration.

Leaf water potential

Leaf water potential was measured using a PMS Model 615 Pressure Chamber Instrument (PMS Instrument Company, Albany, OR). Briefly, the lowest stem leaves of fully mature plants were removed at dawn (5:30-6:30 h) for measurements using the chamber under field conditions near the end of the growing season, when drought treatment water levels were at their lowest.

Micrografting

Seven-day-old seedlings were micro-grafted as described by Fragoso and colleagues (Fragoso *et al.*, 2011). EV (E) and irTOC1 (T) genotypes were used as scions or rootstocks yielding EE, TT, and ET grafts - where the first letter refers to the scion genotype while the second letter, to the genotype of the rootstock. The average grafting success was 52% under glasshouse conditions ($p > 0.05$ between genotypes, ANOVA followed by Tukey HSD post-hoc) and 59% under field conditions ($p < 0.05$ among genotypes: 68% EE, 49% ET, and 54% TT, ANOVA followed by Tukey HSD post-hoc).

Transcript abundance

150 mg of leaf or 300 mg of root tissue were harvested, and RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Unless otherwise displayed (e.g. transcript abundance kinetics in Supplemental Fig. S1), all tissues for transcript abundance were harvested at the *TOC1* peaking time in EV plants, 19:00 under LD conditions, 13 hours into the light cycle. Total RNA was quantified using a NanoDrop (Thermo Scientific, Wilmington, USA) and cDNA was synthesized from 500 ng of total RNA using RevertAid H Minus reverse transcriptase (Fermentas) and oligo (dT) primer (Fermentas). Reverse transcription quantitative PCR (RT-qPCR) was performed in a Mx3005P PCR cycler (Stratagene) using SYBR GREEN1 kit (Eurogentec). The N. attenuata actin gene homolog NIATv7_g21364 and the N. attenuata elongation factor 1a (EF1a) were used as a standard housekeeping gene for normalization. The sequences of primers used for RT-qPCR are provided in Supplemental Table S4. All RT-qPCR data were normalized using the delta-Ct method.

ABA extraction and quantification

Phytohormone analysis of leaf material was performed on a UPLC-MS/MS (EvoQ Elite Triple quad-MS; 296 BRUKER DALTONIK GmbH, Bremen, Germany) following extraction in pre-cooled acidified methanol and column purification as described by Schäfer and colleagues (Schäfer *et al.*, 2016). All tissues for phytohormone analysis were harvested at the TOC1 peaking time for EV plants, 19:00, under LD conditions.

Microarray analysis

Six biological replicates for each treatment and genotype were used for RNA isolation. Total RNA was isolated with TRIzol reagent and labeled cRNA with the Quick Amp labeling kit (Agilent). Each sample was hybridized on Agilent single color technology arrays (60k 60-mer oligonucleotide microarray designed for N. attenuata transcriptome analysis,

http://www.agilent.com, GEO accession number GPL13527). Agilent microarray scanner (G2565BA) and Scan Control software were used to obtain intensity of the spots. All microarray data with each probe name were deposited in the NCBI GEO database. The initial normalization and extraction of low-expressed probes was performed using the R package limma (Ritchie *et al.*, 2015), followed by gene set enrichment analysis and over-representation analysis of samples within tissues and treatments using the R package CEMiTools (Russo *et al.*, 2018).

Statistical analyses

All data were analyzed using *R* version 3.4.2 (RC Team 2017) and *RStudio* version 1.0.153 (RStudio Team 2016). Datasets were fit to LM, GLS, or GLM models after outlier removal and homoscedasticity and normality tests had been applied before model reduction. Pairwise post hoc comparisons were made using the R package *LSMeans* (Lenth, 2016) or else using Tukey HSD tests after significant results in a two-way ANOVA.

Root image analysis

Pictures of roots were taken at the end of the field experiment when harvesting biomass. Pictures were analyzed using the Fiji distribution of the imaging software *ImageJ* (Schindelin *et al.* 2012; Schneider, Rasband & Eliceiri 2012). Scale bars were applied using the 'Analyze' -> 'Set Scale' functionality to measure a standardized paper label in each image (3.1 cm x 1.5 cm).

Accession numbers

Microarray information is available under GEO accession number GPL13527. Sequence data from this work can be found in GenBank under the following accession numbers: NaTOC1 (LOC109210941), NaLHY (LOC109231023), NaPRR5 (LOC109223559), NaPRR9 (LOC109231058), NaGI (LOC109234610), NaFT3 (LOC109243482), NaROMT (LOC109207600), NaABAR (LOC109229433), NaRAB18 (LOC109220601), NaPIF1 (LOC109230623), NaPIF3a (LOC109236817), NaPIF3b (LOC109234067), NaPIF4 (LOC109228555), NaPIF7 (LOC109210563), NaPHYB1 (LOC109232144), NaPHYB2 (LOC109216344), NaPHYA (LOC109226699), NaCRYP1a (LOC109214267).

Acknowledgments

We thank the glasshouse department at the Max Planck Institute for Chemical Ecology and the field team in 2015 and 2016 for support, especially C. Diezel for help with grafting; Brigham Young University for the use of the Lytle Ranch Preserve field station in Utah, USA, and APHIS for constructive regulatory oversight; the technical staff at the department of Molecular Ecology for providing seeds; Y. Oh and L. Cortes-Llorca for generating and performing initial screening and sampling of irCRYP1a and irFT3 lines used in these experiments; O. Kolle and H. Bruelhilde for lending Scholander chambers and D. Veit for assistance with gas handling in the glasshouse; M. Wang, L. Cortes-Llorca and Y. Joo for helpful discussions; and both 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 H..V. and E.M.

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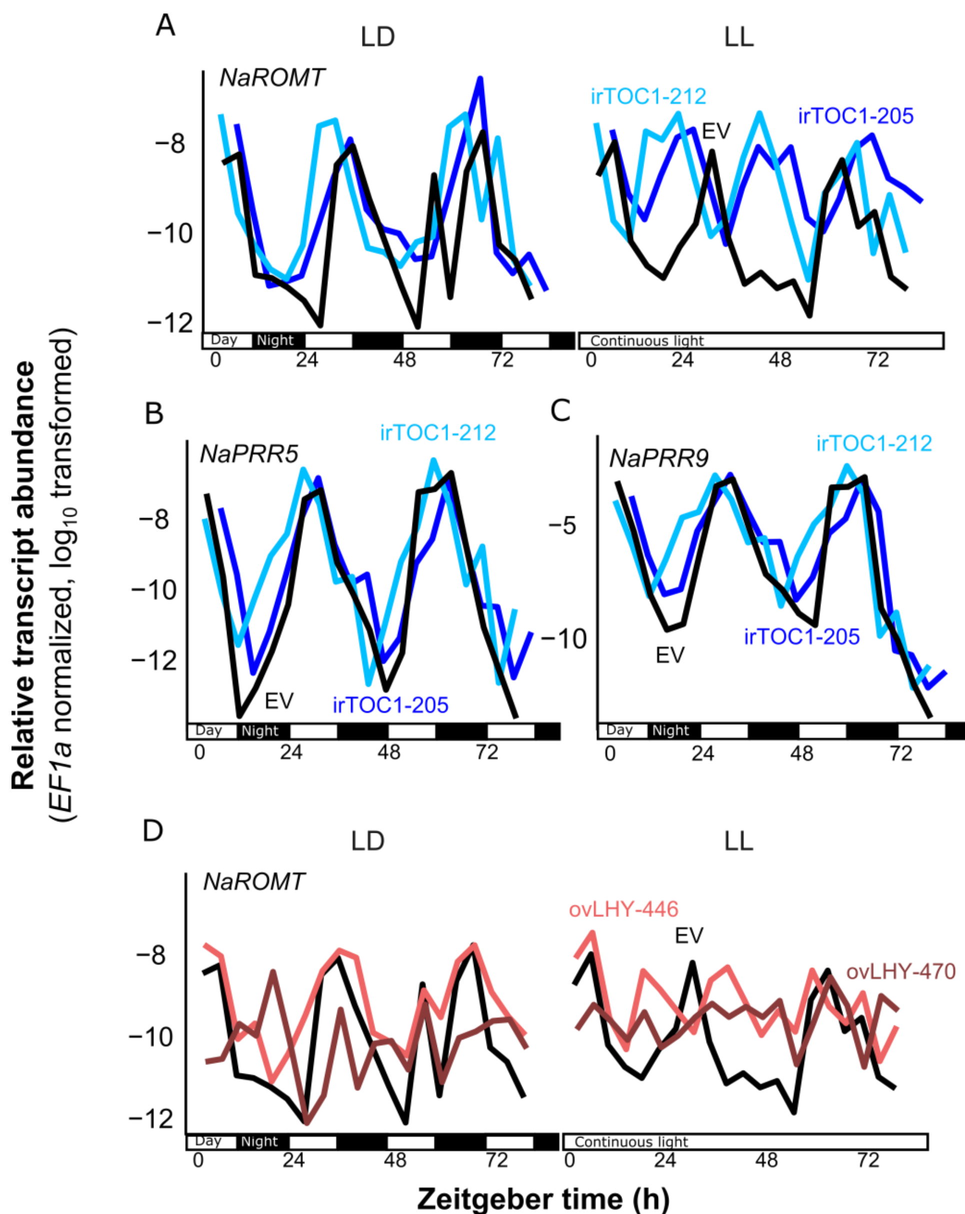
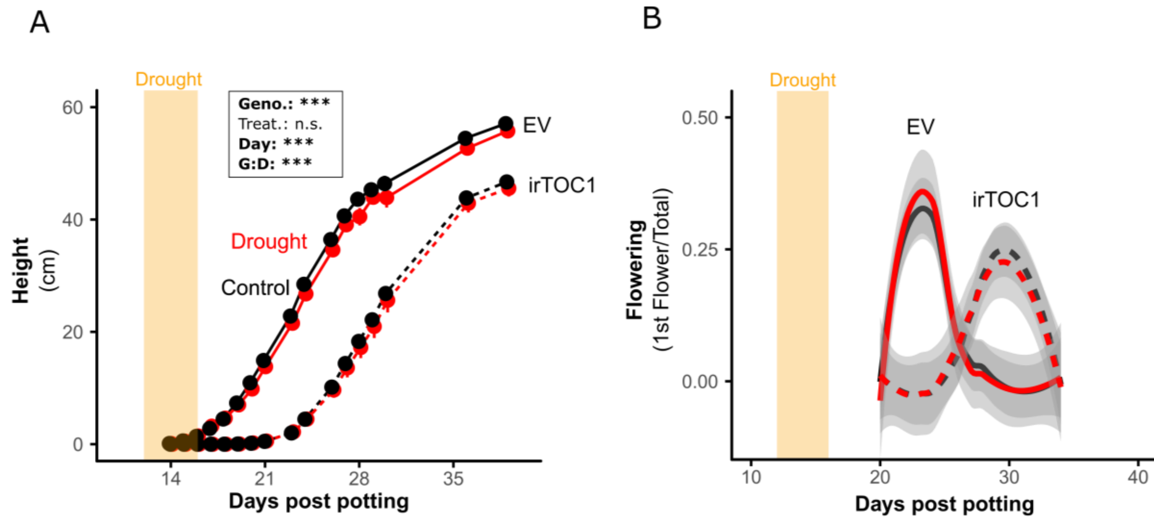
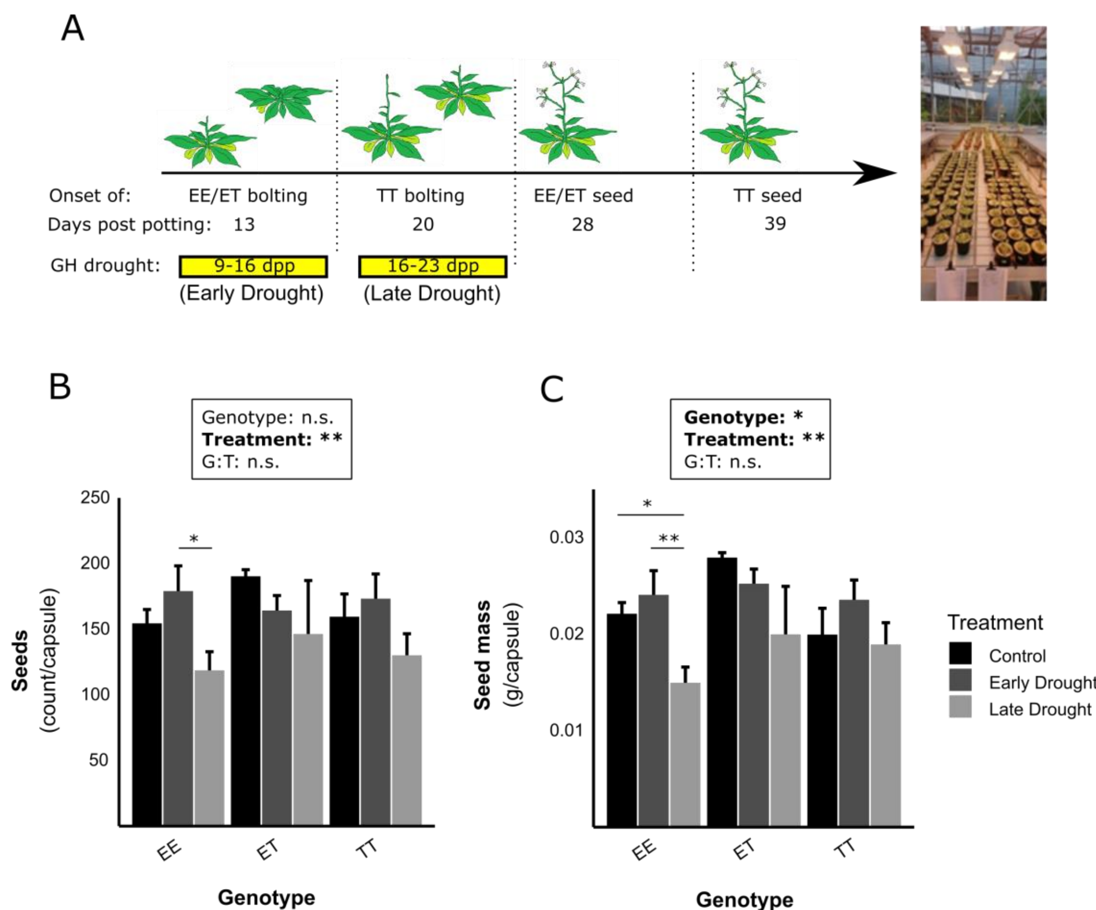
Supplemental files



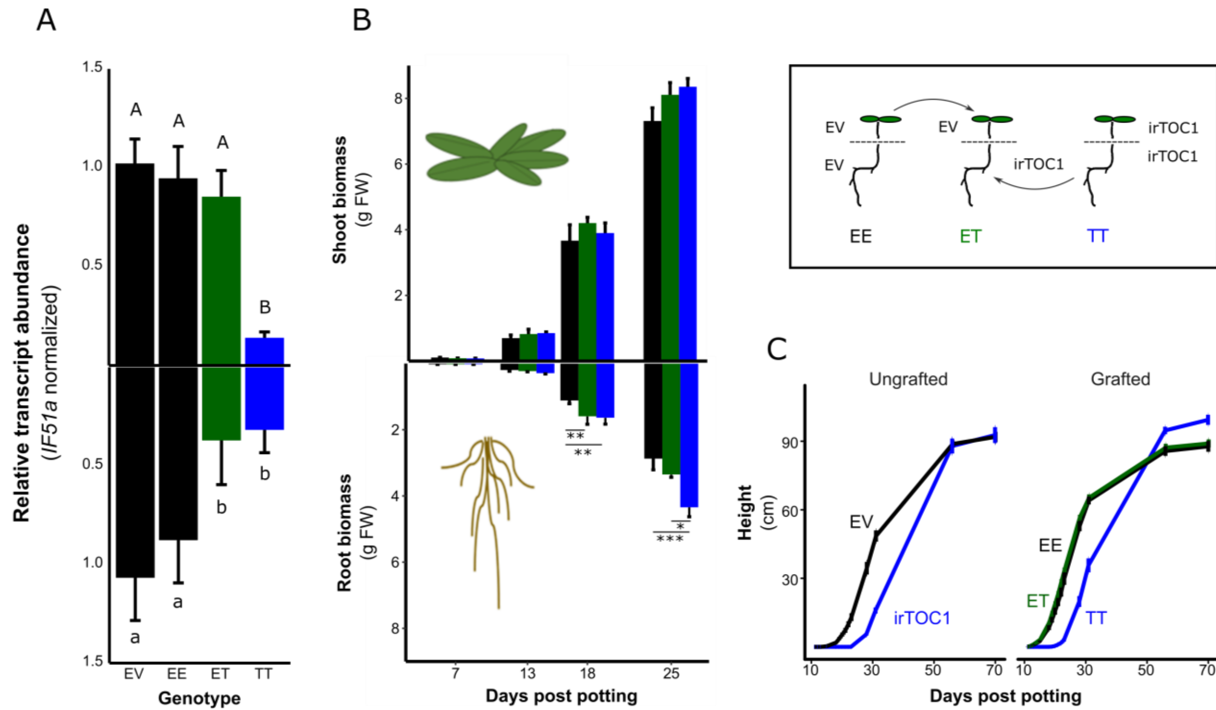
Supplemental Figure S1: *NaTOC1*-silenced plants exhibit clock-silenced phenotypes specific to *TOC1*. (A) irTOC1 plants from two independently transformed lines exhibit shorter phase transcription of the marker gene *NaROMT (resveratrol O-methyltransferase)* which exhibits a circadian-regulated phenotype in EV plants. (B) *PRR5* and (C) *PRR9* transcription levels are not affected by *TOC1*-targeted ir-construct. (D) overexpression *LHY* plants from two independently transformed lines show a loss of rhythmicity phenotype under circadian conditions of the marker gene *NaROMT*.



Supplemental Figure S2: Synchronized drought did not affect flowering or elongation times of irTOC1 or EV plants. Although both elongation (A) and peak flowering time (B) were 7 days later for irTOC1 plants relative to EV plants, drought did not display a significant effect. Flowering was measured as a ratio of the number of plants flowering for the first time on a particular day over the total number of plants for each genotype and treatment. Error bars represent SEM, biological replicates of 15-16 per treatment, day, and genotype.

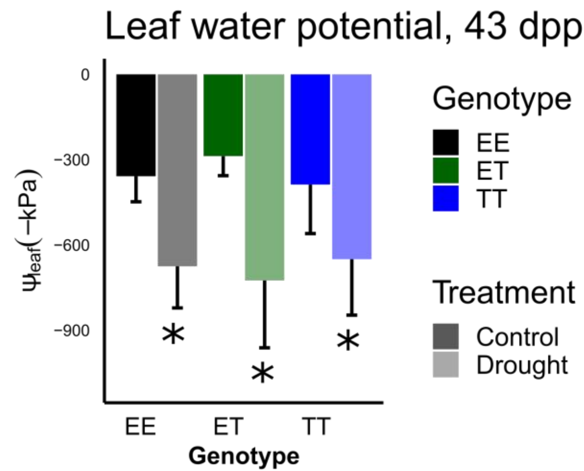


Supplemental Figure S3: Effect of *TOC1* silencing in whole plants or roots only and of drought event timing on seed number and seed mass. (A) EE, ET, and TT plants were subjected to droughts at two developmentally relevant time points, corresponding to the onset of EE and ET elongation (Early Drought) and to TT elongation (Late Drought). (B) Genotype did not have a significant effect on seed counts per capsule, while drought treatment affected seed counts similarly across genotypes. (C) Genotype and drought treatment both displayed a significant effect on seed mass per capsule, with ET plants displaying slightly higher masses under control conditions, and EE plants displaying slightly lower masses under late drought conditions. When considering the early drought events used in Fig. 1 or the developmentally-timed drought events used in Figs. 4 and 5 (i.e. Early Drought for EE and ET, and Late drought for TT), the effects of drought treatment were not significant for either seed count of mass per capsule. (*** $p< 0.001$, ** $p< 0.01$, * $p< 0.05$, pairwise comparisons, multiple comparisons of means, Tukey contrasts following a significant effect in an ANOVA). Error bars represent SEM, 6 biological replicates per genotype and treatment.

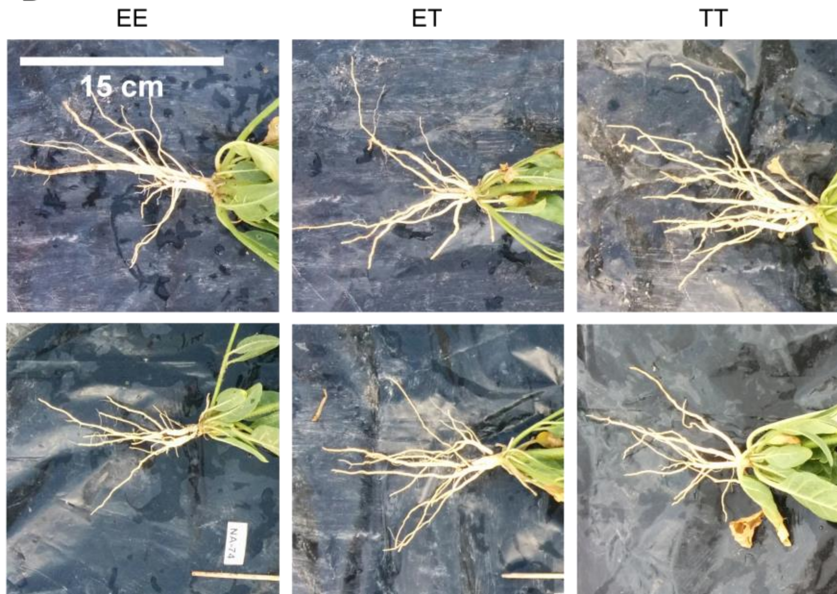


Supplemental Figure S4: EV/irTOC1 micrografting yields plants silenced in *TOC1* in roots, but not shoots. (A) TOC1 transcript abundance of EE, ET, and TT roots and shoots compared to ungrafted EV plants. (B) Shoot and root biomass over 4 timepoints of EE, ET, and TT plants. (n=4 for each timepoint, tissue type, and genotype) (C) Elongation of grafted EE, ET, and TT plants and ungrafted EV and irTOC1 plants. (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, pairwise comparisons, multiple comparisons of means, Tukey contrasts following a significant effect in an ANOVA). Error bars represent SEM, 3-8 biological replicates per genotype for relative transcript abundance, 4 biological replicates per time point and genotype for biomass, 5-12 biological replicates per time point for elongation measurements.

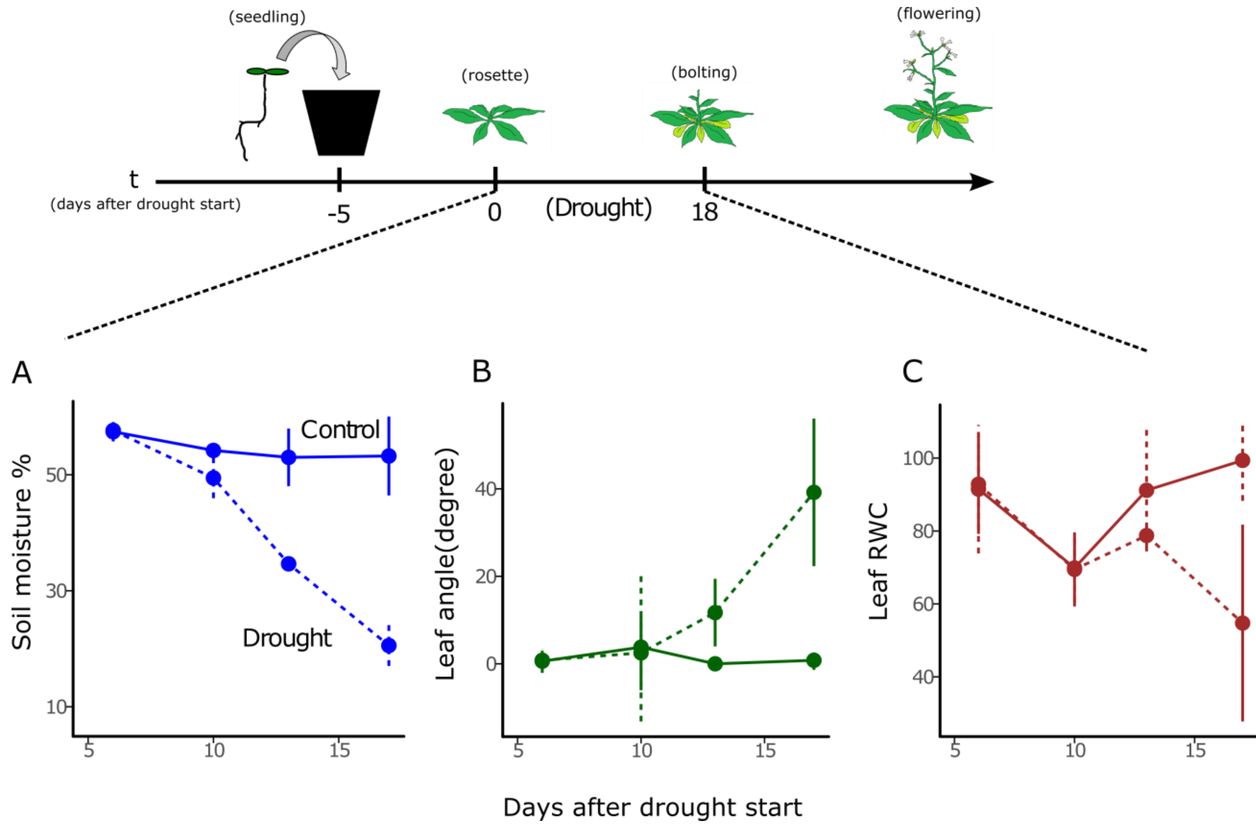
A



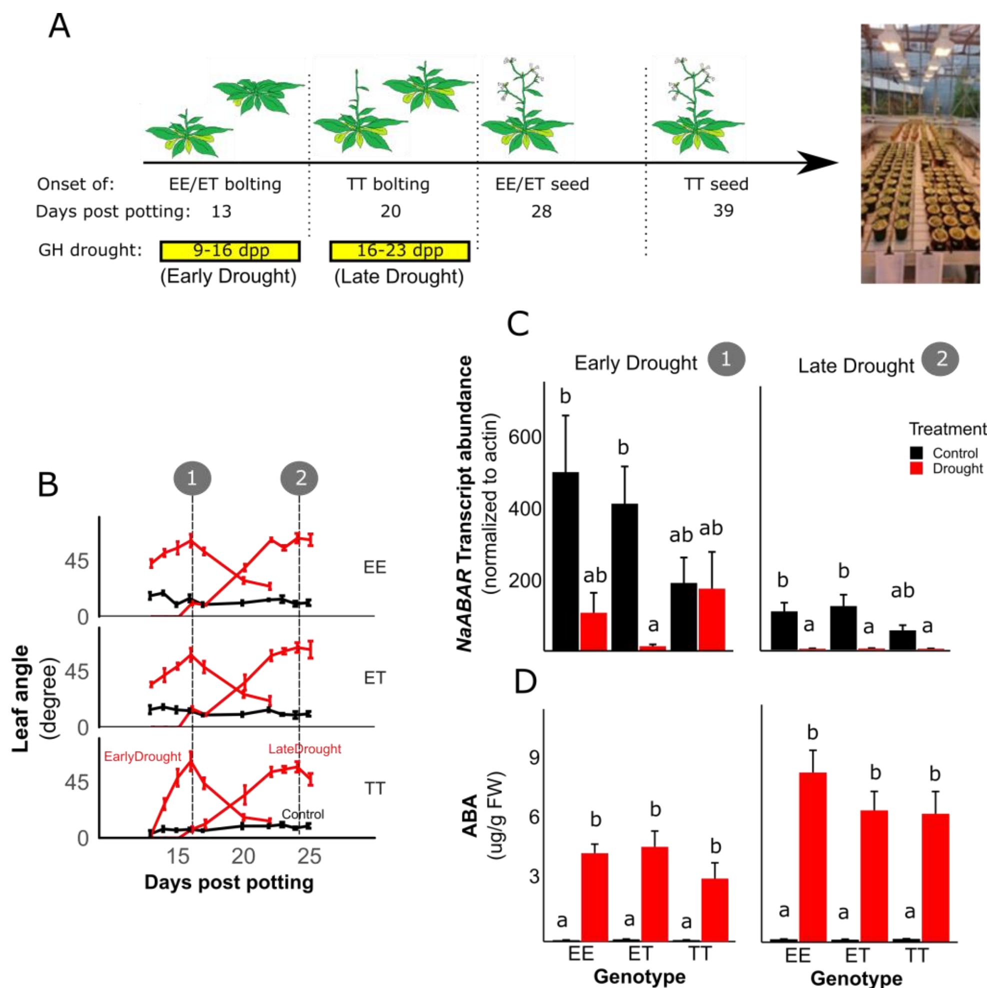
B



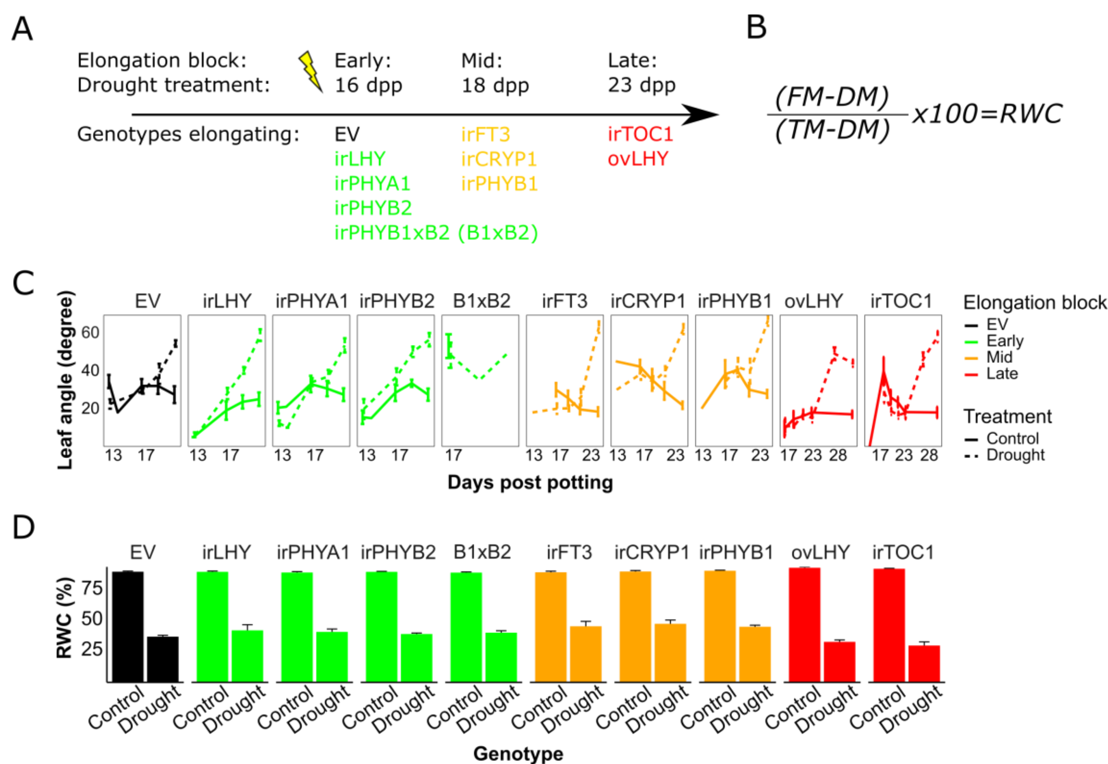
Supplemental Figure S5: Leaf water potential and root morphology under field conditions. (A) EE, ET, and TT plants experienced similar levels of drought stress under field conditions, as measured by leaf water potential. (B) Representative pictures of EE, ET, and TT roots. The white labels present in each picture (visible in the lower left-hand image) were used to generate the scale bars. Error bars represent SEM, 4-6 biological replicates per genotype and treatment.



Supplemental Figure S6: Relationship between water status and leaf angle under glasshouse drought conditions. Analysis of volumetric soil moisture (A), leaf angle (B), and leaf relative water content (RWC, C) in seedlings exposed to drought in glasshouse conditions. Error bars represent SEM, biological replicates of 5-6 per treatment and day for each measurement.



Supplemental Figure S7: The effects of *TOC1* silencing on transcriptional responses of plants exposed to drought in the glasshouse are dependent on developmental time. (A) In order to examine the effect of drought stress on transcriptional and phytohormone responses, EE, ET, and TT plants were subjected to droughts at two developmentally relevant time points, corresponding to the onset of EE and ET elongation (Early Drought) and to TT elongation (Late Drought). (B) Leaf angle was used as a proxy for determining sampling time for transcriptional responses in the glasshouse for experiments in figures 3, 4, 5, and S3. Numbered circles (1 and 2) label time of sampling for Absicic acid (ABA) and transcriptional responses. (C) EE and ET transcriptional responses to drought, measured using the ABA- and *TOC1*-responsive gene *NaABAR*, vary among different developmental time windows. (D) Magnitude of accumulation for the phytohormone abscicic acid (ABA) varied depending on the developmental timing of drought stress, but all plants experienced drastic and significant increases at sampling time, when leaf angles were at 45 degrees or more for each genotype. (*** $p< 0.001$, ** $p< 0.01$, * $p< 0.05$, pairwise comparisons, multiple comparisons of means, Tukey contrasts following a significant effect in an ANOVA). Error bars represent SEM, 5-6 biological replicates per genotype and treatment.



Supplemental Figure S8: Leaf angle and relative water content (RWC) of drought-stressed lines analyzed in Figures 4 and 5. (A) Experimental setup for testing drought responses. Genotypes were subjected to drought treatment at the same developmental stage: beginning of elongation. (B) Leaf relative water content (RWC) calculation. FM: fresh mass of leaf; DM: dry mass of leaf; TM: turgid mass of leaf. (C) Leaf angles of each line increased predictably after drought start, as in Supplemental Figures S6 and S7. (D) RWC at the final time point of each drought, when plants were sampled for transcript analysis. Error bars represent SEM, 5 biological replicates per genotype, treatment and timepoint for leaf angle and 4-5 biological replicates per genotype and treatment for RWC.

Supplemental Table S1: ***CEMiTool*** **analysis gene set enrichment analysis (GSEA) results.** Values represent Normalized enrichment scores (NES) for each module within each treatment and tissue combination. p-values for NES scores are represented by color. Path. = pathway, N.C. = Not Correlated to a module.

p-value:	> 0.05	< 0.05	< 0.01	< 0.001

LeafControl

Path.	EE	ET	TT
M1	-0.66	2.17	-2.42
M10	-1.27	-0.70	1.57
M11	-2.10	2.25	-1.20
M12	2.88	-2.46	-1.77
M2	1.61	-3.43	2.70
M3	2.94	-1.46	-1.95
M4	-2.02	-1.08	2.59
M5	-2.80	2.44	1.05
M6	-1.76	2.98	-2.28
M7	1.38	-1.72	0.67
M8	1.75	-2.15	0.91
M9	-2.39	2.03	1.71

LeafDrought

Path.	EE	ET	TT
M1	-2.08	-2.15	3.39
M2	3.73	-3.05	-2.19
M3	1.42	-3.96	3.22
M4	1.10	-3.00	2.22
M5	2.40	-2.93	0.82
M6	-1.44	2.60	-1.96
M7	1.40	1.77	-2.03
M8	-1.77	0.70	1.94
N.C.	-1.30	0.48	1.52

RootControl

Path.	EE	ET	TT
M1	4.07	-3.77	-2.41
M10	-3.21	2.72	1.88
M11	1.17	-2.37	2.03
M12	0.64	-0.87	0.55
M2	-0.44	-2.62	2.75
M3	-2.68	2.96	-0.96
M4	-0.66	-1.52	1.75
M5	-2.16	-1.45	2.79
M6	2.90	-1.85	-2.37
M7	-2.62	1.69	1.99
M8	-1.79	-0.66	1.80
M9	-2.76	0.58	2.86

RootDrought

Path.	EE	ET	TT
M1	3.28	-1.35	-2.84
M10	2.24	-1.98	-1.41
M2	-2.03	-2.13	2.91
M3	2.90	-2.16	-2.15
M4	-0.94	-0.60	1.07
M5	0.94	3.81	-3.74
M6	1.99	2.00	-1.32
M7	2.42	0.83	-2.48
M8	-1.95	2.32	-0.91
M9	-2.27	3.55	-2.55

Supplemental Table S2: *CEMiTool* overrepresentation analysis (ORA): gene ontology (GO) annotations and p-value results. GeneRatio denotes the proportion of genes in the module enriched for a given pathway out of all the genes in the module enriched for any given pathway. BgRatio denotes the proportion of genes in a given pathway out of all the genes in the gene ontology file. Count denotes the number of genes in the module that are enriched for each pathway. P-values are calculated using the hypergeometric distribution and adjusted for multiple comparisons, and q-values are calculated for FDR control.

Note: Due to space limitations, top five GO terms (by adjusted P-values) for each module and tissue/treatment.

Control Leaf

Module	ID	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
M1	pyrimidine ribonucleotide biosynthetic process	pyrimidine ribonucleotide biosynthetic process	14/375	127/24278	1.10E-08	7.83E-06	7.21E-06	14
M1	RNA methylation	RNA methylation	16/375	185/24278	3.30E-08	1.17E-05	1.08E-05	16
M1	protein refolding	protein refolding	5/375	10/24278	2.02E-07	4.78E-05	4.40E-05	5
M1	protein import into nucleus	protein import into nucleus	11/375	119/24278	2.45E-06	0.000434686	0.000400126	11
M1	protein import into chloroplast stroma	protein import into chloroplast stroma	5/375	19/24278	8.33E-06	0.001181045	0.001087147	5
M2	photosynthesis, light harvesting	photosynthesis, light harvesting	7/268	26/24278	1.02E-08	6.08E-06	5.60E-06	7
M2	structural constituent of ribosome	structural constituent of ribosome	15/268	392/24278	3.40E-05	0.010170585	0.00936541	15
M2	water transport	water transport	11/268	232/24278	5.82E-05	0.011626587	0.010706145	11
M2	apoplast	apoplast	16/268	490/24278	0.0001215	0.014189754	0.013066394	16
M2	ribosome biogenesis	ribosome biogenesis	14/268	390/24278	0.00012169	0.014189754	0.013066394	14
M3	response to UV-B	response to UV-B	10/242	117/24278	2.82E-07	0.000145051	0.000131001	10
M3	negative regulation of catalytic activity	negative regulation of catalytic activity	8/242	80/24278	1.36E-06	0.000348553	0.00031479	8
M3	serine-type endopeptidase activity	serine-type endopeptidase activity	8/242	104/24278	9.82E-06	0.001681694	0.001518793	8
M3	response to auxin stimulus	response to auxin stimulus	12/242	290/24278	3.75E-05	0.004823251	0.004356039	12
M3	regulation of anthocyanin biosynthetic process	regulation of anthocyanin biosynthetic process	4/242	21/24278	5.04E-05	0.005185072	0.004682811	4
M4	myo-inositol hexakisphosphate biosynthetic process	myo-inositol hexakisphosphate biosynthetic process	7/232	59/24278	1.49E-06	0.000832288	0.000775968	7
M4	starch catabolic process	starch catabolic process	5/232	29/24278	7.52E-06	0.002104529	0.001962118	5
M4	response to karrikin	response to karrikin	9/232	187/24278	8.28E-05	0.015452935	0.014407247	9
M4	sucrose metabolic process	sucrose metabolic process	13/232	416/24278	0.00019892	0.026521165	0.0247265	13
M4	response to UV	response to UV	4/232	32/24278	0.0002368	0.026521165	0.0247265	4
M5	cytokinesis by cell plate formation	cytokinesis by cell plate formation	12/113	184/24278	6.36E-11	2.10E-08	1.82E-08	12
M5	cell proliferation	cell proliferation	10/113	170/24278	7.25E-09	1.20E-06	1.04E-06	10
M5	histone phosphorylation	histone phosphorylation	7/113	66/24278	2.44E-08	2.01E-06	1.74E-06	7
M5	nucleosome	nucleosome	7/113	66/24278	2.44E-08	2.01E-06	1.74E-06	7
M5	spindle assembly	spindle assembly	6/113	47/24278	8.17E-08	5.39E-06	4.68E-06	6
M6	nucleosome	nucleosome	12/122	66/24278	5.84E-16	2.08E-13	1.80E-13	12
M6	nucleosome assembly	nucleosome assembly	12/122	81/24278	7.87E-15	1.41E-12	1.21E-12	12
M6	DNA replication initiation	DNA replication initiation	9/122	56/24278	9.41E-12	1.12E-09	9.67E-10	9

M6	protein heterodimerization activity	protein heterodimerization activity	11/122	126/24278	4.07E-11	3.63E-09	3.14E-09	11
M6	cell proliferation	cell proliferation	12/122	170/24278	6.26E-11	4.47E-09	3.86E-09	12
M7	response to carbon dioxide	response to carbon dioxide	3/75	13/24278	7.92E-06	0.001647379	0.001142161	3
M7	jasmonic acid mediated signaling pathway	jasmonic acid mediated signaling pathway	7/75	267/24278	1.89E-05	0.001962386	0.001360561	7
M7	chlorophyll catabolic process	chlorophyll catabolic process	4/75	73/24278	7.78E-05	0.004266599	0.002958118	4
M7	regulation of stomatal movement	regulation of stomatal movement	4/75	74/24278	8.20E-05	0.004266599	0.002958118	4
M7	carbonate dehydratase activity	carbonate dehydratase activity	3/75	30/24278	0.00010826	0.00450377	0.003122553	3
M8	protein disulfide oxidoreductase activity	protein disulfide oxidoreductase activity	7/72	91/24278	9.93E-09	2.24E-06	1.71E-06	7
M8	electron carrier activity	electron carrier activity	10/72	346/24278	7.44E-08	7.16E-06	5.49E-06	10
M8	cell redox homeostasis	cell redox homeostasis	7/72	126/24278	9.55E-08	7.16E-06	5.49E-06	7
M8	carboxylesterase activity	carboxylesterase activity	5/72	70/24278	2.08E-06	0.000116744	8.96E-05	5
M8	electron transport	electron transport	9/72	461/24278	8.76E-06	0.000394189	0.000302442	9
M9	linoleate 13S-lipoxygenase activity	linoleate 13S-lipoxygenase activity	2/36	13/24278	0.00016504	0.011718037	0.007991545	2
M9	anchored to membrane	anchored to membrane	3/36	91/24278	0.00033255	0.011805667	0.008051308	3
M9	oxylipin biosynthetic process	oxylipin biosynthetic process	2/36	53/24278	0.00280909	0.066481892	0.045339763	2
M9	lipid binding	lipid binding	2/36	69/24278	0.0047118	0.083634485	0.057037603	2
M9	peroxidase reaction	peroxidase reaction	2/36	147/24278	0.0200516	0.097346128	0.06638876	2
M10	seed development	seed development	2/30	54/24278	0.00202959	0.18241796	0.151247768	2
M10	oxidoreductase activity	oxidoreductase activity	2/30	60/24278	0.00249888	0.18241796	0.151247768	2
M10	copper ion binding	copper ion binding	3/30	284/24278	0.00509094	0.187765284	0.155681382	3
M10	electron transport	electron transport	3/30	461/24278	0.01888777	0.187765284	0.155681382	3
M10	proteolysis	proteolysis	3/30	487/24278	0.0218028	0.187765284	0.155681382	3
M11	response to toxic substance	response to toxic substance	2/19	17/24278	7.84E-05	0.004780194	0.002557136	2
M11	methyl jasmonate esterase activity	methyl jasmonate esterase activity	1/19	10/24278	0.00779995	0.050883938	0.027220053	1
M11	methyl salicylate esterase activity	methyl salicylate esterase activity	1/19	10/24278	0.00779995	0.050883938	0.027220053	1
M11	procambium histogenesis	procambium histogenesis	1/19	10/24278	0.00779995	0.050883938	0.027220053	1
M11	copper-exporting ATPase activity	copper-exporting ATPase activity	1/19	11/24278	0.00857677	0.050883938	0.027220053	1
M12	lipid binding	lipid binding	5/31	69/24278	2.57E-08	1.90E-06	1.35E-06	5
M12	lipid transport	lipid transport	5/31	95/24278	1.29E-07	4.78E-06	3.40E-06	5
M12	chitin binding	chitin binding	3/31	10/24278	2.25E-07	5.55E-06	3.94E-06	3
M12	chitin catabolic process	chitin catabolic process	3/31	17/24278	1.27E-06	2.34E-05	1.67E-05	3
M12	cell wall macromolecule catabolic process	cell wall macromolecule catabolic process	3/31	29/24278	6.73E-06	9.97E-05	7.09E-05	3

Drought Leaf

Module	ID	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
M1	response to red light	response to red light	18/602	152/24278	4.60E-08	4.02E-05	3.61E-05	18
M1	response to water deprivation	response to water deprivation	29/602	380/24278	1.02E-07	4.48E-05	4.03E-05	29
M1	plastoglobule	plastoglobule	12/602	75/24278	3.05E-07	8.88E-05	7.99E-05	12
M1	chloroplast thylakoid membrane	chloroplast thylakoid membrane	26/602	353/24278	9.18E-07	0.000200538	0.000180419	26
M1	myo-inositol hexakisphosphate biosyn. process	myo-inositol hexakisphosphate biosyn. process	10/602	59/24278	1.72E-06	0.000300148	0.000270035	10
M2	carbonate dehydratase activity	carbonate dehydratase activity	8/427	30/24278	3.58E-08	2.54E-05	2.12E-05	8
M2	Reg. of hydrogen peroxide metabolic process	Reg. of hydrogen peroxide metabolic process	16/427	175/24278	9.02E-08	2.95E-05	2.46E-05	16

M2	protein targeting to membrane	protein targeting to membrane	23/427	360/24278	1.25E-07	2.95E-05	2.46E-05	23
M2	regulation of plant-type hypersensitive response	regulation of plant-type hypersensitive response	23/427	370/24278	2.03E-07	3.61E-05	3.01E-05	23
M2	jasmonic acid mediated signaling pathway	jasmonic acid mediated signaling pathway	18/427	267/24278	1.37E-06	0.000194888	0.000162443	18
M3	nucleosome	nucleosome	10/194	66/24278	1.20E-10	6.05E-08	5.65E-08	10
M3	nucleosome assembly	nucleosome assembly	10/194	81/24278	9.66E-10	2.43E-07	2.27E-07	10
M3	protein heterodimerization activity	protein heterodimerization activity	10/194	126/24278	7.26E-08	1.22E-05	1.14E-05	10
M3	photosynthesis, light harvesting	photosynthesis, light harvesting	5/194	26/24278	1.78E-06	0.000223297	0.000208414	5
M3	iron ion binding	iron ion binding	9/194	287/24278	0.00052705	0.053020733	0.049486757	9
M4	response to karrikin	response to karrikin	5/92	187/24278	0.00073519	0.153860068	0.138588193	5
M4	response to UV-B	response to UV-B	4/92	117/24278	0.00103262	0.153860068	0.138588193	4
M4	chlorophyll binding	chlorophyll binding	2/92	20/24278	0.00258152	0.199970196	0.180121512	2
M4	cell periphery	cell periphery	2/92	24/24278	0.00371327	0.199970196	0.180121512	2
M4	positive regulation of seed germination	positive regulation of seed germination	2/92	24/24278	0.00371327	0.199970196	0.180121512	2
M5	respiratory burst involved in defense response	respiratory burst involved in defense response	5/73	118/24278	2.87E-05	0.004234193	0.003173123	5
M5	ATP catabolic process	ATP catabolic process	6/73	204/24278	3.48E-05	0.004234193	0.003173123	6
M5	response to chitin	response to chitin	7/73	408/24278	0.00022569	0.011711011	0.008776283	7
M5	plant-type hypersensitive response	plant-type hypersensitive response	3/73	44/24278	0.00031614	0.011711011	0.008776283	3
M5	aryl-alcohol dehydrogenase (NAD+) activity	aryl-alcohol dehydrogenase (NAD+) activity	2/73	10/24278	0.00039508	0.011711011	0.008776283	2
M6	sucrose metabolic process	sucrose metabolic process	8/56	416/24278	4.81E-06	0.000841299	0.000647737	8
M6	starch metabolic process	starch metabolic process	7/56	417/24278	4.71E-05	0.004120268	0.003172296	7
M6	enzyme inhibitor activity	enzyme inhibitor activity	3/56	42/24278	0.00012519	0.007302997	0.005622759	3
M6	developmental growth involved in morphogenesis	developmental growth involved in morphogenesis	2/56	11/24278	0.0002836	0.011473702	0.008833888	2
M6	pectinesterase activity	pectinesterase activity	3/56	58/24278	0.00032782	0.011473702	0.008833888	3
M7	anchored to membrane	anchored to membrane	4/28	91/24278	3.53E-06	0.000250608	0.000193204	4
M7	plant-type cell wall	plant-type cell wall	4/28	299/24278	0.00036562	0.009067117	0.006990216	4
M7	pectin catabolic process	pectin catabolic process	2/28	27/24278	0.00044225	0.009067117	0.006990216	2
M7	aspartyl esterase activity	aspartyl esterase activity	2/28	29/24278	0.00051082	0.009067117	0.006990216	2
M7	enzyme inhibitor activity	enzyme inhibitor activity	2/28	42/24278	0.00107331	0.014994613	0.011559969	2
M8	photosynthesis, light harvesting	photosynthesis, light harvesting	6/19	26/24278	2.18E-14	1.07E-12	5.96E-13	6
M8	light-harvesting complex	light-harvesting complex	2/19	13/24278	4.50E-05	0.000771952	0.000431166	2
M8	protein disulfide oxidoreductase activity	protein disulfide oxidoreductase activity	3/19	91/24278	4.73E-05	0.000771952	0.000431166	3
M8	cell redox homeostasis	cell redox homeostasis	3/19	126/24278	0.00012446	0.00152463	0.000851566	3
M8	electron carrier activity	electron carrier activity	3/19	346/24278	0.00234718	0.023002375	0.012847728	3
Not.Correlated	electron transport	electron transport	4/33	461/24278	0.00339526	0.16902683	0.130476851	4
Not.Correlated	negative regulation of catalytic activity	negative regulation of catalytic activity	2/33	80/24278	0.0052988	0.16902683	0.130476851	2
Not.Correlated	cation transport	cation transport	2/33	85/24278	0.00596102	0.16902683	0.130476851	2
Not.Correlated	serine-type endopeptidase activity	serine-type endopeptidase activity	2/33	104/24278	0.00880065	0.16902683	0.130476851	2
Not.Correlated	single-organism metabolic process	single-organism metabolic process	2/33	104/24278	0.00880065	0.16902683	0.130476851	2

Control Root

Module	ID	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count

M1	peroxidase activity	peroxidase activity	25/478	123/24278	1.70E-18	1.10E-15	9.95E-16	25
M1	peroxidase reaction	peroxidase reaction	25/478	147/24278	1.50E-16	4.84E-14	4.37E-14	25
M1	response to nitrate	response to nitrate	27/478	184/24278	4.28E-16	9.25E-14	8.34E-14	27
M1	nitrate transport	nitrate transport	27/478	191/24278	1.12E-15	1.81E-13	1.63E-13	27
M1	cellular response to iron ion starvation	cellular response to iron ion starvation	19/478	89/24278	8.68E-15	1.13E-12	1.01E-12	19
M2	apoplast	apoplast	22/453	490/24278	0.00015224	0.060982019	0.056468677	22
M2	xyloglucan:xyloglucosyl transferase activity	xyloglucan:xyloglucosyl transferase activity	5/453	31/24278	0.00025184	0.060982019	0.056468677	5
M2	cellular glucan metabolic process	cellular glucan metabolic process	5/453	32/24278	0.00029393	0.060982019	0.056468677	5
M2	response to water deprivation	response to water deprivation	18/453	380/24278	0.00031556	0.060982019	0.056468677	18
M2	sesquiterpene biosynthetic process	sesquiterpene biosynthetic process	3/453	10/24278	0.00070237	0.088268852	0.081735984	3
M3	response to chitin	response to chitin	36/178	408/24278	2.00E-28	6.77E-26	5.64E-26	36
M3	respiratory burst involved in defense response	respiratory burst involved in defense response	21/178	118/24278	2.28E-23	3.86E-21	3.21E-21	21
M3	defense response to fungus	defense response to fungus	25/178	314/24278	8.07E-19	9.12E-17	7.59E-17	25
M3	jasmonic acid mediated signaling pathway	jasmonic acid mediated signaling pathway	20/178	267/24278	9.63E-15	8.16E-13	6.79E-13	20
M3	regulation of plant-type hypersensitive response	regulation of plant-type hypersensitive response	20/178	370/24278	4.24E-12	2.87E-10	2.39E-10	20
M4	cysteine biosynthetic process	cysteine biosynthetic process	21/87	251/24278	3.57E-23	7.89E-21	4.40E-21	21
M4	plastoglobule	plastoglobule	14/87	75/24278	9.04E-21	9.99E-19	5.57E-19	14
M4	chloroplast thylakoid membrane	chloroplast thylakoid membrane	21/87	353/24278	4.53E-20	3.34E-18	1.86E-18	21
M4	photosystem II assembly	photosystem II assembly	17/87	183/24278	1.20E-19	6.64E-18	3.70E-18	17
M4	photosynthesis, light harvesting	photosynthesis, light harvesting	10/87	26/24278	1.04E-18	4.58E-17	2.55E-17	10
M5	defense response	defense response	6/53	133/24278	4.48E-07	7.94E-05	6.85E-05	6
M5	chitin catabolic process	chitin catabolic process	3/53	17/24278	6.54E-06	0.000578523	0.000498875	3
M5	cell wall macromolecule catabolic process	cell wall macromolecule catabolic process	3/53	29/24278	3.45E-05	0.002034433	0.001754343	3
M5	chitin binding	chitin binding	2/53	10/24278	0.00020807	0.009050806	0.007804739	2
M5	response to biotic stimulus	response to biotic stimulus	5/53	260/24278	0.00025567	0.009050806	0.007804739	5
M6	peroxidase activity	peroxidase activity	8/71	123/24278	2.81E-09	5.37E-07	4.67E-07	8
M6	heme binding	heme binding	10/71	281/24278	9.12E-09	7.32E-07	6.37E-07	10
M6	peroxidase reaction	peroxidase reaction	8/71	147/24278	1.15E-08	7.32E-07	6.37E-07	8
M6	response to oxidative stress	response to oxidative stress	9/71	282/24278	1.34E-07	6.42E-06	5.59E-06	9
M6	protein dimerization activity	protein dimerization activity	7/71	159/24278	4.23E-07	1.62E-05	1.41E-05	7
M7	pectin catabolic process	pectin catabolic process	3/47	27/24278	1.93E-05	0.00188266	0.001337995	3
M7	aspartyl esterase activity	aspartyl esterase activity	3/47	29/24278	2.40E-05	0.00188266	0.001337995	3
M7	enzyme inhibitor activity	enzyme inhibitor activity	3/47	42/24278	7.40E-05	0.003710705	0.002637175	3
M7	peroxidase activity	peroxidase activity	4/47	123/24278	9.45E-05	0.003710705	0.002637175	4
M7	peroxidase reaction	peroxidase reaction	4/47	147/24278	0.00018796	0.00471063	0.003347816	4
M8	phosphotransferase activity, alcohol group as acceptor	phosphotransferase activity, alcohol group as acceptor	2/48	17/24278	0.00051079	0.074575426	0.065596265	2
M8	oxidoreductase activity	oxidoreductase activity	5/48	419/24278	0.00139078	0.101527148	0.089302899	5
M8	electron transport	electron transport	4/48	461/24278	0.01292641	0.271655704	0.238947338	4
M8	protein disulfide oxidoreductase activity	protein disulfide oxidoreductase activity	2/48	91/24278	0.01401536	0.271655704	0.238947338	2
M8	phosphatidylethanolamine binding	phosphatidylethanolamine binding	1/48	10/24278	0.01959961	0.271655704	0.238947338	1

M9	carbonate dehydratase activity	carbonate dehydratase activity	6/49	30/24278	2.82E-11	3.94E-09	2.58E-09	6
M9	one-carbon metabolic process	one-carbon metabolic process	6/49	62/24278	2.78E-09	1.94E-07	1.27E-07	6
M9	response to carbon dioxide	response to carbon dioxide	4/49	13/24278	1.03E-08	4.82E-07	3.15E-07	4
M9	photosynthesis, light reaction	photosynthesis, light reaction	6/49	89/24278	2.52E-08	8.82E-07	5.77E-07	6
M9	regulation of multi-organism process	regulation of multi-organism process	6/49	93/24278	3.29E-08	9.20E-07	6.02E-07	6
M10	cell wall macromolecule metabolic process	cell wall macromolecule metabolic process	3/32	41/24278	2.14E-05	0.002464434	0.001782062	3
M10	response to hypoxia	response to hypoxia	3/32	66/24278	9.00E-05	0.003606715	0.002608059	3
M10	xylem development	xylem development	3/32	67/24278	9.41E-05	0.003606715	0.002608059	3
M10	1-aminocyclopropane-1-carboxylate oxidase activity	1-aminocyclopropane-1-carboxylate oxidase activity	2/32	13/24278	0.0001301	0.003740247	0.002704618	2
M10	ethylene biosynthetic process	ethylene biosynthetic process	3/32	90/24278	0.00022604	0.005198893	0.003759383	3
M11	sucrose transmembrane transporter activity	sucrose transmembrane transporter activity	3/32	12/24278	4.54E-07	4.72E-05	4.16E-05	3
M11	sucrose transport	sucrose transport	3/32	22/24278	3.15E-06	0.000163748	0.000144191	3
M11	nicotinate nucleotide metabolic process	nicotinate nucleotide metabolic process	2/32	20/24278	0.00031508	0.01092271	0.009618175	2
M11	cellular cation homeostasis	cellular cation homeostasis	2/32	104/24278	0.00829028	0.213049631	0.187604432	2
M11	peptidoglycan biosynthetic process	peptidoglycan biosynthetic process	1/32	13/24278	0.01700417	0.213049631	0.187604432	1
M12	zinc ion transmembrane transport	zinc ion transmembrane transport	3/23	13/24278	2.11E-07	2.05E-05	1.38E-05	3
M12	zinc ion transmembrane transporter activity	zinc ion transmembrane transporter activity	3/23	15/24278	3.35E-07	2.05E-05	1.38E-05	3
M12	iron ion transmembrane transporter activity	iron ion transmembrane transporter activity	2/23	13/24278	6.65E-05	0.002705955	0.001821091	2
M12	cellular response to iron ion	cellular response to iron ion	2/23	24/24278	0.00023396	0.007135793	0.004802346	2
M12	trichoblast differentiation	trichoblast differentiation	2/23	28/24278	0.00031969	0.007800345	0.005249585	2

Drought Root

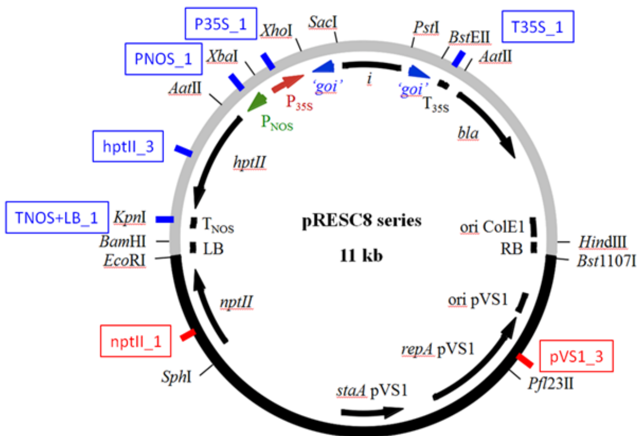
Module	ID	Description	GeneRatio	BgRatio	pvalue	p.adjust	qvalue	Count
M1	nucleosome	nucleosome	20/820	66/24278	2.79E-14	2.55E-11	2.30E-11	20
M1	cell proliferation	cell proliferation	30/820	170/24278	9.83E-14	4.49E-11	4.04E-11	30
M1	nucleosome assembly	nucleosome assembly	20/820	81/24278	2.00E-12	6.09E-10	5.48E-10	20
M1	DNA replication initiation	DNA replication initiation	16/820	56/24278	2.92E-11	6.49E-09	5.84E-09	16
M1	regulation of DNA replication	regulation of DNA replication	21/820	104/24278	3.55E-11	6.49E-09	5.84E-09	21
M2	response to mechanical stimulus	response to mechanical stimulus	7/251	53/24278	1.19E-06	0.000720824	0.000668314	7
M2	water channel activity	water channel activity	6/251	47/24278	8.66E-06	0.002615411	0.002424884	6
M2	response to water deprivation	response to water deprivation	14/251	380/24278	4.59E-05	0.008510047	0.00789011	14
M2	apoplast	apoplast	16/251	490/24278	5.64E-05	0.008510047	0.00789011	16
M2	water transport	water transport	10/251	232/24278	0.00016086	0.019431609	0.018016061	10
M3	xyloglucan:xyloglucosyl transferase activity	xyloglucan:xyloglucosyl transferase activity	6/178	31/24278	9.03E-08	2.06E-05	1.85E-05	6
M3	cellular glucan metabolic process	cellular glucan metabolic process	6/178	32/24278	1.10E-07	2.06E-05	1.85E-05	6
M3	hydrolase activity, hydrolyzing O-glycosyl compounds	hydrolase activity, hydrolyzing O-glycosyl compounds	8/178	106/24278	1.17E-06	0.000145078	0.000130195	8
M3	plant-type cell wall	plant-type cell wall	11/178	299/24278	1.39E-05	0.001291537	0.001159049	11
M3	trichoblast differentiation	trichoblast differentiation	4/178	28/24278	4.98E-05	0.00371846	0.003337012	4
M4	chloroplast thylakoid membrane	chloroplast thylakoid membrane	60/192	353/24278	2.33E-63	8.30E-61	5.57E-61	60
M4	photosystem II assembly	photosystem II assembly	45/192	183/24278	6.36E-55	1.13E-52	7.60E-53	45
M4	rRNA processing	rRNA processing	49/192	263/24278	1.94E-53	2.31E-51	1.55E-51	49

M4	cysteine biosynthetic process	cysteine biosynthetic process	42/192	251/24278	1.37E-43	1.22E-41	8.18E-42	42
M4	plastid organization	plastid organization	29/192	71/24278	6.32E-43	4.50E-41	3.02E-41	29
M5	secondary cell wall biogenesis	secondary cell wall biogenesis	14/116	55/24278	5.31E-21	1.78E-18	1.55E-18	14
M5	glucuronoxylan metabolic process	glucuronoxylan metabolic process	13/116	178/24278	3.25E-12	3.62E-10	3.15E-10	13
M5	xylan biosynthetic process	xylan biosynthetic process	13/116	178/24278	3.25E-12	3.62E-10	3.15E-10	13
M5	hydroquinone:oxygen oxidoreductase activity	hydroquinone:oxygen oxidoreductase activity	4/116	29/24278	1.07E-05	0.000766379	0.000667045	4
M5	lignin biosynthetic process	lignin biosynthetic process	6/116	105/24278	1.14E-05	0.000766379	0.000667045	6
M6	regulation of anthocyanin biosynthetic process	regulation of anthocyanin biosynthetic process	3/88	21/24278	5.84E-05	0.010449333	0.008664252	3
M6	alpha-amylase activity	alpha-amylase activity	2/88	10/24278	0.00057359	0.031310559	0.02596171	2
M6	chitin binding	chitin binding	2/88	10/24278	0.00057359	0.031310559	0.02596171	2
M6	response to jasmonic acid stimulus	response to jasmonic acid stimulus	5/88	202/24278	0.00084975	0.031310559	0.02596171	5
M6	response to UV-B	response to UV-B	4/88	117/24278	0.0008746	0.031310559	0.02596171	4
M7	ethylene biosynthetic process	ethylene biosynthetic process	4/93	90/24278	0.00040082	0.094592624	0.080585152	4
M7	sucrose alpha-glucosidase activity	sucrose alpha-glucosidase activity	2/93	13/24278	0.00110159	0.100727122	0.085811242	2
M7	glucosidase II complex	glucosidase II complex	2/93	15/24278	0.00147553	0.100727122	0.085811242	2
M7	1-aminocyclopropane-1-carboxylate biosynthetic process	1-aminocyclopropane-1-carboxylate biosynthetic process	2/93	18/24278	0.00213405	0.100727122	0.085811242	2
M7	1-aminocyclopropane-1-carboxylate synthase activity	1-aminocyclopropane-1-carboxylate synthase activity	2/93	18/24278	0.00213405	0.100727122	0.085811242	2
M8	response to hypoxia	response to hypoxia	2/28	66/24278	0.0026286	0.134258841	0.091861312	2
M8	alpha,alpha-trehalose-phosphate synthase (UDP-forming) activity	alpha,alpha-trehalose-phosphate synthase (UDP-forming) activity	1/28	11/24278	0.01261606	0.134258841	0.091861312	1
M8	developmental growth involved in morphogenesis	developmental growth involved in morphogenesis	1/28	11/24278	0.01261606	0.134258841	0.091861312	1
M8	polyamine-transporting ATPase activity	polyamine-transporting ATPase activity	1/28	11/24278	0.01261606	0.134258841	0.091861312	1
M8	transition metal ion binding	transition metal ion binding	1/28	13/24278	0.01489334	0.134258841	0.091861312	1
M9	secondary cell wall biogenesis	secondary cell wall biogenesis	3/40	55/24278	0.00010242	0.010940829	0.008819722	3
M9	glucuronoxylan metabolic process	glucuronoxylan metabolic process	4/40	178/24278	0.00020774	0.010940829	0.008819722	4
M9	xylan biosynthetic process	xylan biosynthetic process	4/40	178/24278	0.00020774	0.010940829	0.008819722	4
M9	superoxide metabolic process	superoxide metabolic process	2/40	16/24278	0.00031301	0.012363769	0.009966796	2
M9	superoxide dismutase activity	superoxide dismutase activity	2/40	24/24278	0.00071394	0.022560436	0.018186627	2
M10	lipid binding	lipid binding	5/45	69/24278	1.79E-07	3.20E-05	2.51E-05	5
M10	lipid transport	lipid transport	5/45	95/24278	8.90E-07	7.97E-05	6.23E-05	5
M10	cuticle development	cuticle development	4/45	47/24278	1.73E-06	0.000103381	8.09E-05	4
M10	carpel development	carpel development	3/45	37/24278	4.42E-05	0.001979732	0.001548394	3
M10	very long-chain fatty acid metabolic process	very long-chain fatty acid metabolic process	3/45	46/24278	8.54E-05	0.003058317	0.002391979	3

Supplemental Table S3: Characterization of ovLHY, irFT3, and irCRYP1a lines. (A) T-DNA copy number was determined using the nCounter Copy Number Variation Assay (NanoString Technologies). Green color denotes lines target sites and lines with the same copy number as our reference line, irAGO121. Lines in bold were used for further experiments. (B) Silencing efficiency of irFT3 and irCRYP1a lines. (C) Locations in pRESC8 series of probes used for T-DNA copy number determination. Singl.: single copy callibrator. N.C.: negative control.

A		Ref.	ovLHY						irCRYP1a					Ref.	irFT3								
	Target site	AGO121_01	446-3	446-4	456-3	456-4	**470-3**	**470-4**	**303-3**	**303-4**	306-6-3	306-6-4	306-7-2	AGO121_01	**24-4**	**24-7**	26-7	27-9	30-6	31-6	32-10	37-1	37-8
overread	nptIII_3	0	0	0	0	0	**0**	**0**	**0**	**0**	0	0	0	0	**0**	**0**	1.5	3	0	1.5	3	0	0
overread	nptII_1	0	2	2	1	2	**0**	**0**	**0**	**0**	1	1	1	0	**0**	**0**	0	0	0	0	0	0	0
overread	pVS1_3	0	4	4	0	1	**0**	**0**	**0**	**0**	0	0	0	0	**0**	**0**	2	2	0	2	3.6	0	0
pRESC8	P35S_1	1	2	2	1	2	**1**	**1**	**1**	**1**	1	1	1	1	**1**	**1**	1.6	1	1	1	1	2	2
pRESC8	PNOS_1	1	2	2	1	2	**1**	**1**	**1**	**1**	1	1	1	1	**1**	**1**	2	1	1	1	1	2	2
pRESC8	T35S_1	1	2.5	2.5	1	2	**2**	**2**	**1**	**1**	1	1	1	1	**1**	**1**	2	1	1	1	1	4	3
pRESC8	TNOS+LB_1	1	2	2	1	2	**1**	**1**	**1**	**1**	1	1	1	1	**1**	**1**	2	2.5	1	1	1	2	2
pRESC8	hptII_3	1	2	2	1	2	**1**	**1**	**1**	**1**	1	1	1	1	**1**	**1**	2	2	1	1	1	2	2
Singl.	AOC_2	1	1	1	1	1	**1**	**1**	**1**	**1**	1	1	1	1	**1**	**1**	1	1	1	1	1	1	1
Singl.	S-RNase-2_1	1	1	1	1	1	**1**	**1**	**1**	**1**	1	1	1	1	**1**	**1**	1	1	1	1	1	1	1
Singl.	sulfite_2	1	1	1	1	1	**1**	**1**	**1**	**1**	1	1	1	1	**1**	**1**	1	1	1	1	1	1	1
N.C.	sat-1_1	0	0	0	0	0	**0**	**0**	**0**	**0**	0	0	0	0	**0**	**0**	0	0	0	0	0	0	0
B						% silencing (Norm. to EF1a)			94.46		81.37				99.71			97.69	99.44				

C



Supplemental Table S4. Primer sequences.

Gene	Forward	Reverse
Actin (NIATv7_g21364)	5'-GGTCGTACAACTGGTATTGTG	5'-CCGTGACCTAACTGATAACCT
EF1a (NIATv7_g65928)	5'-CCACACTTCCCACATTGCTGTCA	5'-CGCATGTCCCTCACAGCAAAAC
NaTOC1 (NIATv7_g15373)	5'-ATCGTAGAACGGCAGCACTT	5'-TCACAAACTGTCCCCTCACA
NaPRR5 (NIATv7_g21743)	5'- CACGTCTGGAGGAGAAGAGC	5'- AACGCACGCCTTATAACCAC
NaPRR9 (NIATv7_g13930)	5'-ACGGCCATTCTGATGAAAG	5'-AACCAGAGCTGTTGCGTTG
NaLHY (NIATv7_g29102)	5'-CACTCTTTTCAAGGAAGGTG	5'-GTCGAAGGTGTTACAAGAGC
NaROMT (NIATv7_g32262)	5'- TCGTTGTGCAATTCAGTTGAGC	5'- TGCAGGAGGAAGTTTCAGCT
NaFT3 (NIATv7_g06970	5'-GCCTTCCCAAGTTGTTAACCA	5'-GAATATCAGTGACCAACCAATGGA
NaGI (NIATv7_g10893)	5'- ACACTACAACCGCCCGATTT	5'- CCATTCAACCTCAGGCCCAT
NaCryp1 (NIATv7_g24217)	5'-GGGCTCCTGAAGAAGAAGGT	5'-TTTTGCGCGATGATCTCTAA
NaABAR (NIATv7_g01880)	5'-GGGCAATCAAAAAGTCCATT	5'-TAGGCAATGTCCTCACAAGC
NaRAB18 (NIATv7_g40056)	5'- ACTCATCAGGGTGGTACTGGA	5'-GAGCTGTCGGATCGACGAAG
NaPIF1 (NIATv7_g28756)	5'-AGTCTGCAATGGCGGAAG	5'-GACAGCTTGTTCGGAAGGAA
NaPIF3a (NIATv7_g09791)	5'- GAAGAGGTGTTGAAGGGTTCC	5'-CTACTGGACTGGCCTTGCAT
NaPIF3b (NIATv7_g10692)	5'- CCCAAACTTTGAGCAATTGTG	5'-TGCTGGACTGACTTTGCATC
NaPIF4 (NIATv7_g38454)	5'- TGCTGCAGAAGTGCATAACC	5'-CCCATCCCCATTCCTATTCT
NaPIF7 (NIATv7_g30912)	5'- GGAATCCAAGGCATCAAAGA	5'-TGTTGGTTTTGTTTCCGACA

4. TOC1 in *Nicotiana attenuata* regulates efficient production of nitrogen-rich defense metabolites under herbivory stress

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Manuscript prepared for submission to *New Phytologist*

Summary

- The plant circadian clock is essential for contextualizing plant responses to different environmental signals. Plants use temporal information to respond to herbivore threats, but many of the specific roles of circadian clock components in these responses, and their contribution to fitness, remain unknown.
- We investigate the role of the central clock regulator TIMING OF CAB EXPRESSION 1 (TOC1) in the defense responses of *Nicotiana attenuata* to the specialist herbivore *Manduca sexta* under both field and glasshouse conditions. We also utilize a ^{15}N pulse-labeling approach to quantify nitrogen incorporation into pools of three defense compounds, caffeoylputrescine (CP), dicaffeoyl spermidine (DCS) and nicotine.
- Nitrogen incorporation is decreased in CP and DCS and increased in nicotine pools in irTOC1 plants under control conditions, and these differences are abolished after simulated herbivory. Differences between EV and irTOC1 plants in nicotine but not phenolamide production are abolished by treatment with the ethylene agonist 1-methylcyclopropene, and these changes in defense chemistry occur under field conditions and correspond to reduced fitness for induced irTOC1 plants.

- These results suggest that the circadian clock contributes to plant fitness both by balancing the production of metabolically expensive nitrogen-rich defense compounds and by mediating the allocation of resources between vegetative biomass and reproduction.

4.1. Introduction

Because they are both sessile organisms and at the base of most food chains, plants are pressured to produce effective defenses in the face of herbivory. Several theories have been developed in an attempt to quantify plant defense responses as a function of different factors, in order to explain and predict patterns observed under natural conditions, with varying success (see Schuman & Baldwin, 2016). Many of these theories attempt to determine trade-offs between growth and defense (Herms & Mattson, 1992), in allocation of limited resources (Bryant, Chapin III, & Klein, 1983; Chapin III, Schulze, & Mooney, 1990), or between tissues of different fitness value (D McKey, Janzen, & 5.5, 1979; Doyle McKey, 1974; David F Rhoades, 1979). Generating testable predictions from these theories has proven challenging largely because they address different levels of analysis, from mechanism to function, and because the proposed mechanisms are not grounded in plant physiology. Furthermore, most plant defense theories do not consider the definition of defense, namely the contribution of a trait to the Darwinian fitness of plants under attack (Karban & Baldwin, 1997; Massad, Dyer, & Vega C., 2012; Schuman & Baldwin, 2016; Stamp, 2003).

An important and largely unexplored component is the importance of timing to the function of plant defense. Herbivory occurs unpredictably over the course of a plant's lifetime, and plant defense responses are highly plastic and often specifically induced by herbivory (Lynds & Baldwin, 1998; Meldau *et al.*, 2012; Wagner & Mitchell-Olds, 2018; Wu & Baldwin, 2010). Nonetheless, herbivore feeding behavior can be diurnally rhythmic, while plant defenses are likely constrained by the circadian clock in order to maximize resource use throughout the day (Goodspeed *et al.*, 2012; Goodspeed, Liu, *et al.*, 2013). The plant circadian clock is an endogenous mechanism that touches upon nearly every aspect of plant metabolism (Greenham & McClung, 2015; Nohales & Kay, 2016; Sanchez & Kay, 2016). As such, individual components of the plant circadian clock have been shown to modulate responses to a variety of external stimuli and stresses, including herbivory (Joo *et al.*, 2018; Seo & Mas, 2015; Sharma & Bhatt, 2015; Takase, Mizoguchi, Kozuka, & Tsukaya, 2013; C. Zhang *et al.*, 2019).

Despite strong evidence for the circadian clock's role in modulating responses, there is less evidence regarding its significance for maintaining plant fitness under natural, diurnal environments. The circadian clock component GIGANTEA (GI) has been shown to maximize the fitness of *Oryza sativa* sown at variable times under field conditions (Izawa, 2012; Izawa *et al.*, 2011), while the central circadian clock component TIMING OF CAB EXPRESSION 1 (TOC1), which modulates a variety of growth and stress responses (Legnaioli *et al.*, 2009; Somers, Webb, Pearson, & Kay, 1998; Soy *et al.*, 2016; Zhu, Oh, Wang, & Wang, 2016), has

also been shown to enhance fitness under drought stress in field conditions (Valim *et al.*, 2019). Although there have been few studies directly tying circadian clock components to fitness outcomes under biotic stress, the circadian clock is known to regulate responses to herbivory. Basal levels of the phytohormone jasmonic acid (JA), involved in regulating downstream responses to biotic stresses, is under circadian regulation in *Arabidopsis thaliana* (Goodspeed, Chehab, Covington, & Braam, 2013). JA-induced responses to herbivory are under diurnal rather than circadian regulation in other plant species, with darkness affecting JA accumulation and feeding performance in lima beans (*Phaseolus lutatus*; see Arimura *et al.* 2008). In the desert annual *Nicotiana attenuata,* performance of the herbivore *Manduca sexta* is similarly dominated by diurnal rather than circadian patterns (Herden *et al.*, 2016), and several JA-induced secondary metabolites are under diurnal regulation (Gulati, Baldwin, & Gaquerel, 2014; Joo *et al.*, 2018; Kim *et al.*, 2011).

JA-induced herbivory responses can be highly specific, and are mediated by interactions with other phytohormone pathways. In the wild tobacco species *N. sylvestris* and *N. attenuata,* oral secretions of *M. sexta* dampen the JA-elicited increase in nicotine observed after elicitation with mechanical wounding or the application of exogenous JA (Kahl *et al.*, 2000; McCloud & Baldwin, 1997). Fatty acid conjugates (FACs) in the oral secretions of *M. sexta* are necessary and sufficient to dampen this nicotine response, and function by eliciting an ethylene burst (Halitschke *et al.*, 2001; Kahl *et al.*, 2000; Von Dahl *et al.*, 2007). Ethylene signaling suppresses JA-induced nicotine in *Nicotiana* spp. by suppressing expression of *ethylene response factor* (*ERF*) transcription factors that bind to the promoter regions of nicotine biosynthesis genes such as *putrescine N-methyltransferase* (PMT), and the ethylene burst also leads to large-scale reconfiguration of transcriptomic responses to herbivory (T. Shoji, Nakajima, & Hashimoto, 2000; Tsubasa Shoji, Kajikawa, & Hashimoto, 2010; Voelckel & Baldwin, 2004). Ethylene responses are under circadian regulation in *A. thaliana,* and *TOC1* silencing alters the rhythmicity of ethylene emissions (Thain *et al.*, 2004). Nonetheless, it is unknown whether these functional benefits are directly mediated by the circadian clock.

Although nicotine provides *Nicotiana* spp. with a potent defense in nature (Steppuhn *et al.*, 2004), plants elicited by the nicotine-tolerant specialist *M. sexta* do not increase nicotine production, and subsequently experience lower fitness costs of elicitation than plants elicited by exogenous JA or when ethylene signaling is blocked, leading to high nicotine accumulation after elicitation (Voelckel *et al.*, 2001). This may be because plants mobilize their limited resources to more effective defenses, given nicotine's high metabolic cost, or because nicotine may be co-opted as a defense against parasitism by *M. sexta* larvae (Ian T. Baldwin & Ohnmeiss, 1994; Thorpe & Barbosa, 1986). *N. attenuata* contains many secondary metabolites of defensive value, with the phenolamides caffeoylputrescine (CP) and dicaffeoyl spermidine (DCS) being particularly abundant and effective at limiting *M. sexta* performance (Kaur *et al.*, 2010). CP and DCS are highly abundant in *M. sexta*-elicited plants, are correlated with JA-Ile content in leaves of natural accessions (Gaquerel, Gulati, & Baldwin, 2014).

The transcriptional activation of *MYB8*, the key regulator of phenolamide biosynthesis, requires a functioning JA biosynthetic pathway (Onkokesung *et al.*, 2012). Silencing *MYC2*, the primary transcription factor at the interface of JA biosynthesis and its downstream signaling, does not lead to decreases in CP and DCS after elicitation, perhaps implying an alternate JA-induced pathway from the COI1-mediated JAZ/MYC2 module that regulates nicotine biosynthesis (Chini *et al.*, 2007; Sheard *et al.*, 2010; Thines *et al.*, 2007; Woldemariam *et al.*, 2013). Nicotine biosynthesis is also directly regulated independently of COI1 by an interaction of the circadian clock component ZTL and a JAZ/MYC2 module, and phenolamide levels were not strongly affected by silencing *ZTL* (Li *et al.*, 2018). Given that both phenolamides and nicotine are highly abundant, nicotine-rich, and thus presumably metabolically expensive secondary metabolites at the intersection of JA and ethylene signaling, and given that efficient maintenance of *N. attenuata's* nitrogen household is critical to its survival in highly competitive intraspecific environments (Ian T Baldwin *et al.*, 1998; Lynds & Baldwin, 1998; Ullmann-Zeunert *et al.*, 2012, 2013), we sought to examine the role of the circadian clock in the regulation of these defense metabolites.

In this study, we find that abrogating *TOC1* expression via RNAi-mediated silencing leads to broad changes in plant responses to simulated herbivory. We observed that *TOC1* silencing leads to delays in jasmonate-dependent signaling after elicitation with *M. sexta* oral secretions consistent with previous findings linking the circadian clock component *ZTL* to the jasmonate-mediated regulation of nicotine biosynthesis in *N. attenuata*. Interestingly, we also observed significant decreases in production of CP and DCS, and increased rather than decreased production of nicotine. We performed ^{15}N labeling experiments to ask whether nitrogen incorporation is decreased in phenolamides and increased in nicotine pools, and observed that nitrogen introduced as ^{15}N is preferentially incorporated into nicotine pools under control conditions in irTOC1 plants, while allocation becomes equally distributed between phenolamides and nicotine after elicitation with *M. sexta* oral secretions. These findings are consistent with previous evidence that herbivory disrupts the rhythmicity of the clock (Joo *et al.*, 2018), and that nicotine pools in other *Nicotiana* species are homeostatic and do not function as sources of nitrogen.

Given the specificity of nicotine over-production in irTOC1 plants compared to irZTL plants, we asked whether ethylene signaling may be compromised in irTOC1 plants. Although the ethylene burst is not different in irTOC1 plants, differences between EV and irTOC1 plants in nicotine production are abolished by treatment with 1-methylcyclopropene, an ethylene signaling receptor blocker, pointing to an attenuation of the ethylene signaling feedback loop. This effect was specific to nicotine, and differences in phenolamide accumulation remained largely unchanged. Finally, we find that irTOC1 plants under field conditions experience severe fitness disadvantages associated with inverted pools of phenolamides and nicotine, and that restoring *TOC1* function in shoots only via a seedling micrografting approach is sufficient to decouple differential phenolamide and nicotine accumulation and the subsequent fitness disadvantages

under field conditions, leading to plants with high levels of nitrogen-rich defense compounds and uncompromised fitness relative to EV plants. These results highlight a tissue-specific roles for *TOC1* in the production of metabolically expensive nitrogen-rich defense compounds via an interplay between JA and ethylene signaling.

4.2. Materials and Methods

Plant materials and constructs

Lines were derived from seeds originally collected from natural populations of *Nicotiana attenuata* from the Desert Inn Ranch near Santa Clara, UT, USA (Ian T. Baldwin *et al.*, 1994). 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. Screening of the EV line (pSOL3NC, line number A-04-266-3) is described by Bubner and colleagues (Bubner, Gase, Berger, Link, & Baldwin, 2006). Screening of TOC1-silenced (ir) lines (pSOL8_16844, A-11-205-4) via RNAi is described by Yon and colleagues (Yon *et al.*, 2012), with additional screening described in Valim and colleagues (Valim *et al.*, 2019). Unless specified in figure captions, the irTOC1-205 line was used for all experiments described in this study. Importation and release of transgenic plants were carried out under Animal and Plant Health Inspection Service (APHIS) import permit numbers 07-341-101n (EV) and 11-350-102m (irTOC1) and release permit number 16-013-102r. Field growth conditions were described by McGale and colleagues (McGale, Diezel, Schuman, & Baldwin, 2018). Briefly, seedlings were germinated on Gamborg's B5 media under illumination from fluorescent lights (GE Plant & Aquarium 40 W and GE Warm White 18 W) at ambient temperatures at the field station. One week after germination, seedlings were grafted (see below, *Micrografting*, and Fragoso and colleagues (Fragoso, Goddard, Baldwin, & Kim, 2011). One to two weeks after grafting, seedlings with four visible leaves were transferred into previously hydrated 50-mm peat pellets (Jiffy 703, www.jiffypot.com) treated with Borax to provide boron, an essential micronutrient (1:100 dilution of a 1.1 g L-1 stock solution) and adapted over two weeks to the field conditions of high light intensity and low relative humidity by keeping seedlings first in shaded, closed translucent plastic 34-quart boxes (Sterilite), then opening the boxes, and subsequently transferring open boxes to partial sunlight in mesh tents (Tatonka). Adapted size-matched seedlings were transplanted into an irrigated field plot at the Lytle Ranch Preserve, Santa Clara, Utah, in April 2016.

Simulated herbivory treatments

M. sexta simulated feeding was performed by applying 20µL of eliciting solution of 1:5 (v/v) water-diluted oral secretions (W+OS) immediately to fresh leaf wounds generated by three lines of pattern wheel punctures on either side of a lea and gently dispersed across the leaf surface with a gloved finger.

Micrografting

Seven-day-old seedlings were micro-grafted as described by Fragoso and colleagues (Fragoso *et al.*, 2011). EV (E) and irTOC1 (T) genotypes were used as scions or rootstocks yielding EE, TT, and ET grafts - where the first letter refers to the scion genotype while the second letter, to the genotype of the rootstock. The average grafting success was 80% under glasshouse conditions ($p > 0.05$ between genotypes, ANOVA followed by Tukey HSD post-hoc) and 59% under field conditions ($p < 0.05$ among genotypes: 68% EE, 49% ET, and 54% TT, ANOVA followed by Tukey HSD post-hoc).

Transcript abundance

150 mg of leaf or 300 mg of root tissue were harvested, and RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified using a NanoDrop (Thermo Scientific, Wilmington, USA) and cDNA was synthesized from 500 ng of total RNA using RevertAid H Minus reverse transcriptase (Fermentas) and oligo (dT) primer (Fermentas). Reverse transcription quantitative PCR (RT-qPCR) was performed in a Mx3005P PCR cycler (Stratagene) using SYBR GREEN1 kit (Eurogentec). The *N. attenuata Translation initiation factor 5a-2 (IF5a-2)* gene and the *N. attenuata elongation factor 1a (EF1a)* were used as a standard housekeeping gene for normalization. The sequences of primers used for RT-qPCR are provided in Supplemental Table S1). All RT-qPCRqRT-PCR data were normalized using the delta-Ct method.

^{15}N-labeling treatment

Plants were pulse labelled with 5.1 mg of ^{15}N-labelled nitrogen administered as $K^{15}NO_3$ three days prior to W+OS elicitation. Incorporation of ^{15}N into nitrogen-containing metabolites was determined as described by Ullmann-Zeunert and colleagues (Ullmann-Zeunert *et al.*, 2012, 2013). Briefly, secondary metabolites were extracted in pre-cooled acidified methanol and analysis was performed by an electrospray ionization-time of flight mass spectrometer (Bruker Daltonic) as described previously by Gaquerel and colleagues (Gaquerel, Heiling, Schoettner, Zurek, & Baldwin, 2010). Average mass spectra were extracted and ^{15}N-incorporation analysis was performed using the Excel spreadsheet ProSIPQuant (Taubert *et al.*, 2011), modified for small metabolites based on compound sum formulae.

1-methylcyclopropene treatment

Plants were pre-exposed overnight (22:00 until 06:00) to 1-methylcyclopropene (1-MCP) before W+OS treatments were performed as described above. 10 plants along with the activated solution of 1-MCP were placed within growth chambers fitted with Plexiglas lids and a 14.5 cm fan for air circulation. Following Kahl and colleagues (Kahl *et al.*, 2000), 500 mg of Ethylblock (0.43% 1-MCP [van der Sprong, Postbus, Netherlands]) was dissolved in a vial containing 50 mL of alkaline solution (0.75% KOH + NaOH in a 1:1 ratio) to release the active substance, 1-MCP and immediately placed within the chamber. To control for any potential stress responses

associated with confinement in chambers and exposure to 1-MCP, control plants were kept in identical chambers supplied with an equivalent amount of alkaline solution.

Phytohormone and secondary metabolite extraction and quantification

Phytohormone analysis of leaf material was performed on a UPLC-MS/MS (EvoQ Elite Triple quad-MS; 296 BRUKER DALTONIK GmbH, Bremen, Germany) following extraction in pre-cooled acidified methanol and column purification as described by Schäfer and colleagues (Schäfer *et al.*, 2016). Secondary metabolites were extracted similarly in pre-cooled acidified methanol and analysis was performed by an electrospray ionization-time of flight mass spectrometer (Bruker Daltonic) as described previously by Li and colleagues (Li *et al.*, 2018, 2017).

Statistical analyses

All data were analyzed using R version 3.4.2 (R Core Team, 2018) and RStudio version 1.0.153(Rstudio Team, 2016). Datasets were fit to LM, GLS, or GLM models after outlier removal and homoscedasticity and normality tests had been applied before model reduction. Pairwise post hoc comparisons were made using the R package *EMMeans* (Lenth, Singmann, Love, Buerkner, & Herve, 2018) using Tukey HSD tests after significant results in a two-way ANOVA.

4.3. Results

4.3.1. *TOC1* silencing in *Nicotiana attenuata* leads to a shift in JA signaling, and defense-related transcripts after simulated herbivory

ZTL and TOC1 interact directly within the circadian clock (Más *et al.*, 2003; Yon *et al.*, 2012), and silencing ZTL has been shown to decrease nicotine but not phenolamide levels in *N. attenuata* in a JA-independent manner after elicitation with oral secretions of the generalist herbivore *Spodoptera littoralis* (Li *et al.*, 2018). We therefore investigated whether these phenotypes were similar under simulated herbivory after elicitation with oral secretions of a nicotine-tolerant specialist herbivore, *Manduca sexta.* We found that transcript abundance of JA biosynthetic genes was not strongly affected by either *ZTL*- or *TOC1*-silencing (Fig. **1a-d**), although JA and JA-Ile levels were strongly reduced in *ZTL*-silenced plants only (Fig. **1e,f**). Although the peak of JA-Ile induction was shifted in irTOC1 plants, we did not observe a significant change in transcript levels of *Theorine Deaminase,* whose expression is strongly

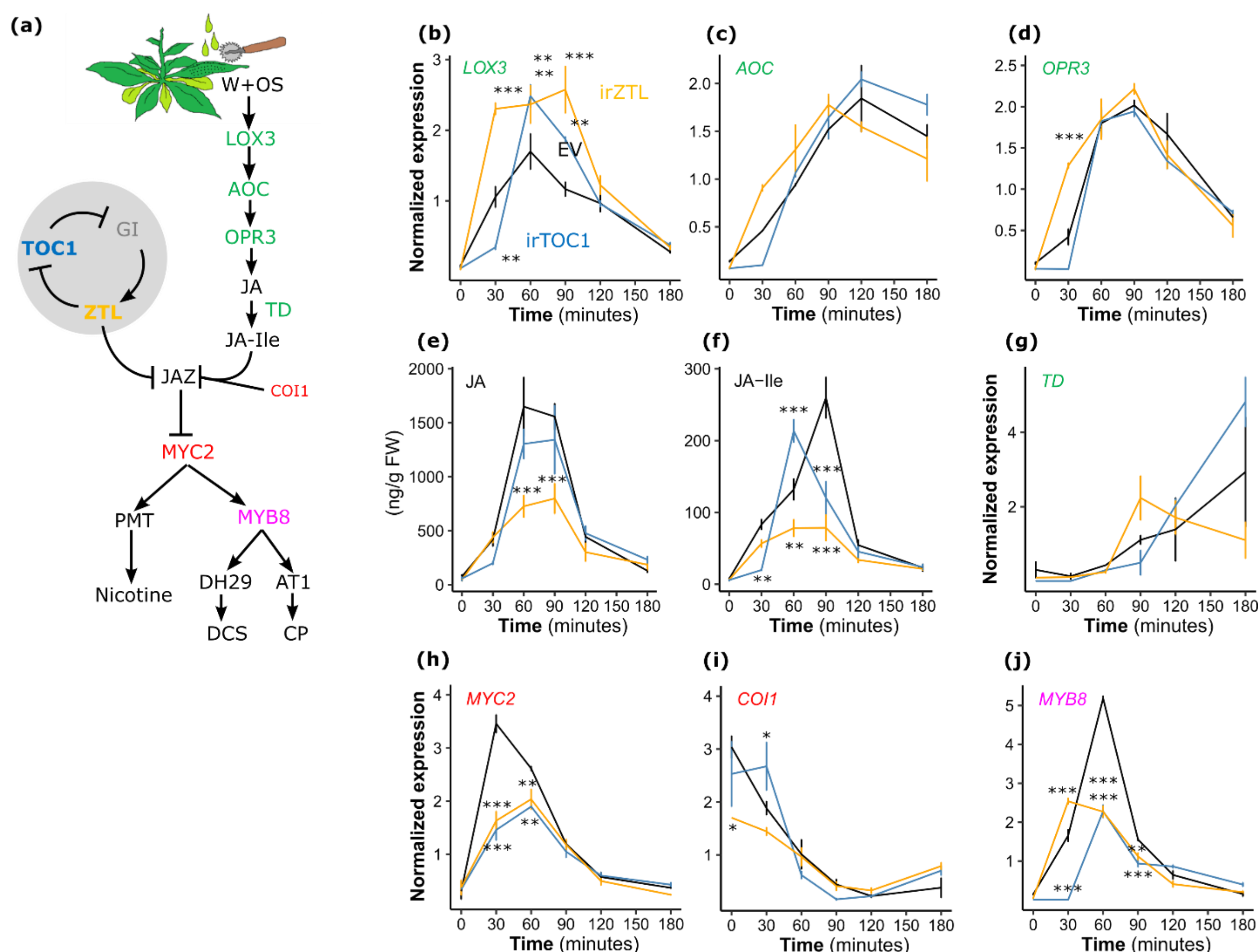

Figure 1: *TOC1* silencing in *Nicotiana attenuata* leads to a shift in JA-Ile peak accumulation, delays in JA signaling, and reduced peaks of defense-related transcripts after elicitation with *Manduca sexta* oral secretion. (a) Key biosynthetic and regulatory steps in JA signaling and the differences in transcript accumulation kinetics between TOC1-silenced and EV plants elicited by *M. sexta* oral secretions. (b-d) W+OS-induced transcripts related to jasmonate biosynthesis are somewhat attenuated in irTOC1 and irZTL lines, but (e-g) JA and JA-Ile levels, and transcript levels of *TD,* which mediates the conjugation of isoleucine to JA, are less affected in irTOC1 than in irZTL relative to EV plants. (h-j) Downstream of JA biosynthesis, irTOC1 plants show attenuation of defense signaling similar to irZTL plants relative to EV. N=3, error bars represent mean + SEM for individual compounds. Stars represent significant differences in estimated marginal means relative to EV, Tukey-adjusted pairwise contrasts. JA: Jasmonic acid; JA-Ile: Jasmonyl isoleucine; *GI: GIGANTEA; TOC1: TIMING OF CAB EXPRESSION 1; ZTL: ZEITLUPE; LOX3: LIPOXYGENASE3; AOC: ALLENE OXIDE CYCLASE; OPR3: 12-OXOPHYTODIENOATE REDUCTASE3; TD: THEORINE DEAMINASE; COI1: CORONATINE-INSENSITIVE1*

induced by herbivory and contributes to the conversion of threonine into isoleucine, a necessary step in the production of bioactive JA-Ile. (Fig. **1g**, see Kang, Wang, Giri & Baldwin 2006). The peak of transcript abundance following simulated herbivory significantly decreased in both irTOC1 and irZTL plants for MYC2 and MYB8, two important coordinators of defense responses following herbivory (Fig. **1h,j**), while basal transcript abundance of COI1 decreased in *ZTL*-silenced plants only (Fig. **1i**).

4.3.2. Silencing *TOC1* leads to increases in nicotine accumulation and decreases in accumulation of both inducible and constitutive phenolamides after simulated herbivory

Given the lower elicitation of MYC2 and MYB8 in both irTOC1 and irZTL plants, we examined the levels of three of *N. attenuata's* most abundant secondary metabolites, the alkaloid nicotine and the two phenolamides caffeoylputrescine (CP) and dicaffeoyl spermidine (DCS). Nicotine, the signature compound of *Nicotiana* spp., functions as both a constitutive and elicited defense, but is not induced by oral secretions of the specialist herbivore *M. sexta* (Ian T. Baldwin *et al.*, 1997; Halitschke *et al.*, 2001; McCloud & Baldwin, 1997). Interestingly, we observed a nearly opposite phenotype in irTOC1 as compared to irZTL plants: while levels of both the constitutive and induced phenolamides DCS and CP were lower, nicotine levels were significantly higher in irTOC1 plants 72 hours after simulated herbivory (Fig. **2b-f**). Similar to the results obtained by Li and colleagues, irZTL plants displayed decreased levels of nicotine after simulated herbivory and similar levels of CP relative to EV plants, although levels of DCS were slightly attenuated (Fig. **2b-f**).

4.3.3. Allocation of nitrogen is shifted towards nicotine in *TOC1*-silenced plants

Nicotine, CP, and DCS are nitrogen-rich compounds are found in high abundance both constitutively and after herbivore induction and share a nitrogen-rich precursor, putrescine (Fig. **2a**). We examined the allocation pattern of nitrogen between these three compounds using a ^{15}N-pulse labeling approach described previously in *N. attenuata* (Ullmann-Zeunert *et al.*, 2012, 2013). Briefly, plants in hydroponic cultures were treated with ^{15}N-labeled KNO_3 three days before simulated herbivory, and both control and elicited plants were sampled at various time intervals over the next four days. Interestingly, while ^{15}N-incorporation patterns (measured as % of atomic incorporation of ^{15}N) under control conditions were temporally shifted for nicotine and appeared to be lower at most time points for CP and DCS, after elicitation these incorporation patterns became more similar between EV and irTOC1 plants (Fig. **3b,c,d**). The variation in incorporation rates implied a potential shift in the allocation of nitrogen pools between these compounds, which we measured as ratios of nicotine to either CP or DCS. We observed that under control conditions, nitrogen allocation favored nicotine in irTOC1 plants, while incorporation rates were nearly 1:1 between nicotine and both CP and DCS in EV plants (Fig. **3e,f**). Both EV and irTOC1 allocation patterns shifted towards phenolamide pools and became statistically indistinguishable after elicitation (Fig. **3e,f**).

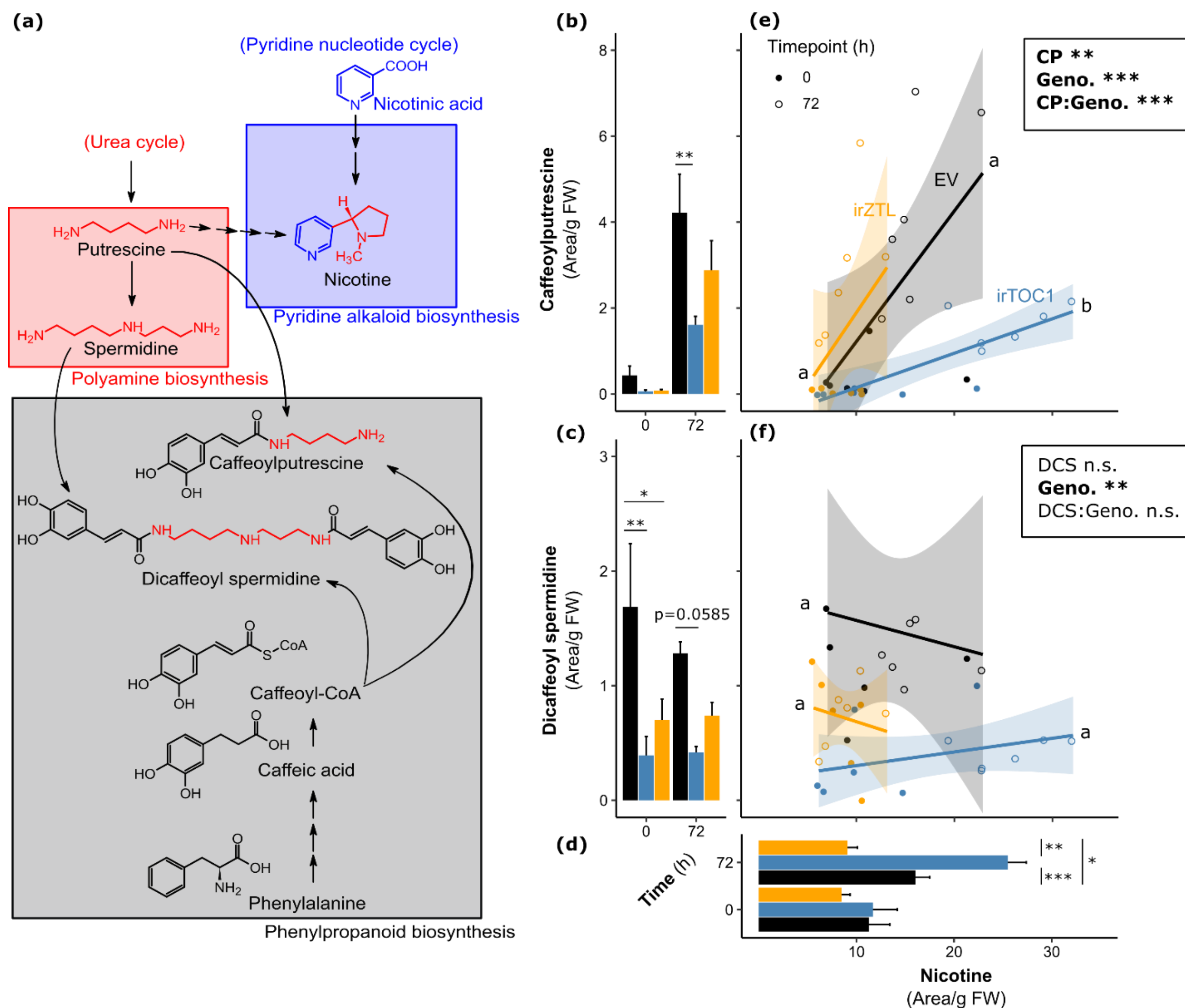

Figure 2: Silencing *TOC1* changes the ratio between nicotine and phenolamides. (a) Schematic representation of the biosynthetic pathways linking nicotine and caffeoyl-based amines. The relationship between the herbivore-induced phenolamide caffeoylputrescine (b) or the constitutive di-caffeoyl spermidine (c) and nicotine (d) are shown to demonstrate the divergent relationship in irTOC1 plants (blue bars) as compared to EV (black bars) or irZTL plants (yellow bars). While irZTL shows reductions in all compounds compared to EV, irTOC1 produces relatively more nicotine and less of the two phenolamides, leading to significant changes in the trait correlation between these compounds for irTOC1 plants (e, f). Letters represent significant differences in estimated marginal means of linear trends, Tukey-adjusted pairwise contrasts. Error bars represent mean + SEM for individual compounds for panels (b), (c) and (d). Shading represents 95% confidence level intervals along correlation axes for panels (e) and (f). CoA: Coenzyme A.

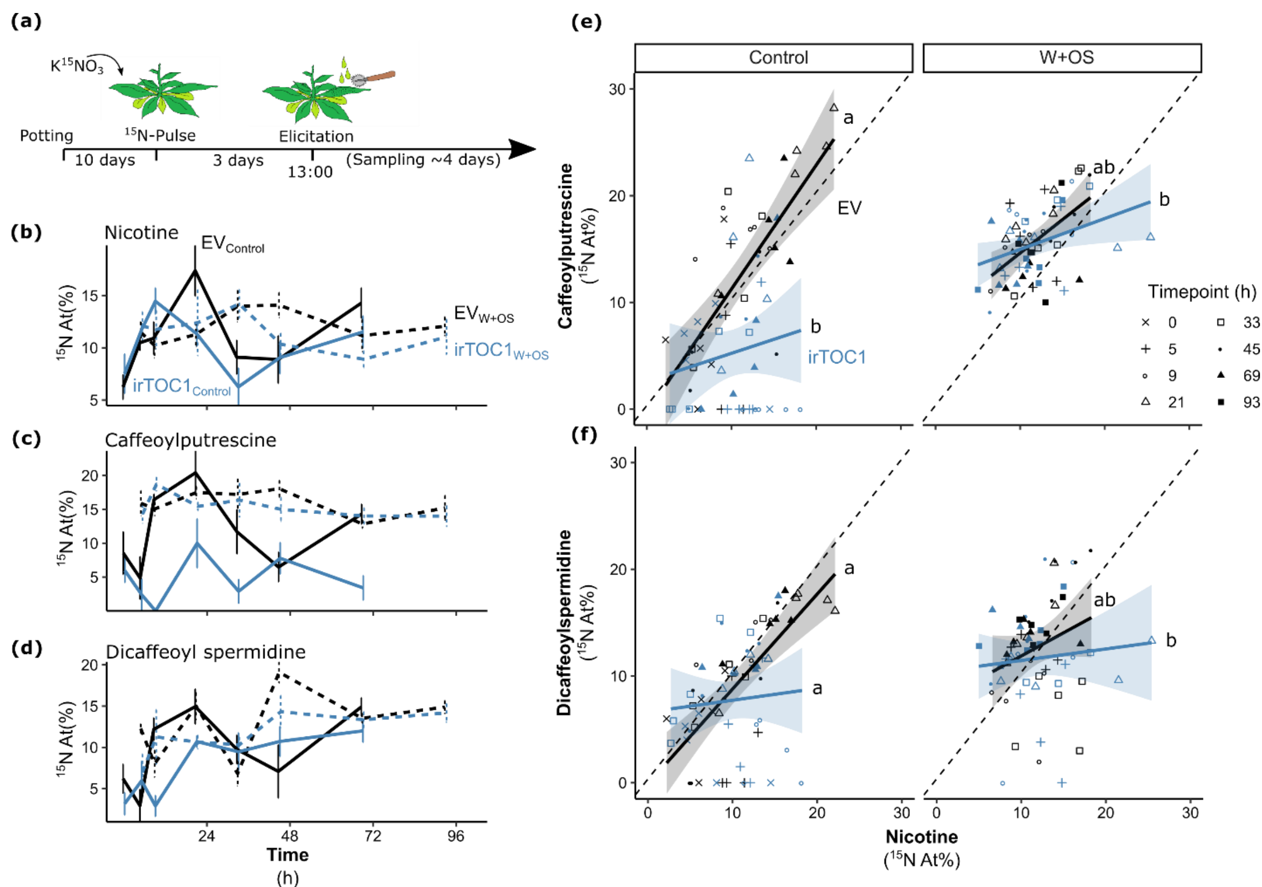

Figure 3: Silencing *TOC1* in *N. attenuata* causes a shift in nitrogen allocation patterns away from phenolamides and towards nicotine under control conditions. (a) Experimental design of 15N-labeling experiments: plants were irrigated with ^{15}N-labeled KNO_3 3 days before start of the experiment, after which plants were treated by controlled wounding and *Manduca sexta* oral secretions and sampled for 93 hours to determine shifts in ^{15}N allocation between nicotine and the phenolamides caffeoylputrescine (CP) and di-caffeoyl spermidine (DCS). Patterns for all three compounds (b,c,d) suggested differences between EV and irTOC1 control plants, but more similar patterns after elicitation. We compared allocation ratios between nicotine and either CP (e) or DCS (f) and found significant differences in allocation patterns under control conditions that were largely abolished under elicited conditions. Letters represent significant differences in estimated marginal means of linear trends, Tukey-adjusted pairwise contrasts. Shading represents 95% confidence level intervals along correlation axes.

4.3.4. Blocking ethylene signaling by 1-methylcyclopropene treatment abolishes differences between EV and *TOC1*-silenced nicotine accumulation

It is known that the JA-induced increase in nicotine accumulation in *N. attenuata* is suppressed by oral secretions of *M. sexta* (Von Dahl *et al.*, 2007; Winz & Baldwin, 2001), and the observed increase in nicotine accumulation in irTOC1 plants after simulated herbivory mirrors the release from suppression in ethylene-deficient plants. However, the herbivory-elicited ethylene burst is not affected in *TOC1*-silenced plants (Fig. S1), and the transcript abundance of various components of the ethylene signaling pathway are not significantly affected by *TOC1* silencing (Fig. S2). To explore whether increased levels of nicotine in irTOC1 plants were related to ethylene sensitivity, we treated plants with 1-methylcyclopropene (1-MCP), an ethylene agonist that binds to the ethylene receptor ETR1 and prevents inactivation of downstream ethylene-sensitive transcription factors (Fig. **4b**; see Wang, Li & Ecker 2002; Blankenship & Dole 2003; Watkins 2006; Ju & Chang 2015). Differences in nicotine accumulation after elicitation between EV and irTOC1 plants were abolished by 1-MCP treatment (Fig. **1c**), while accumulation of CP increased proportionally after 1-MCP treatment, remaining lower in irTOC1 plants (Fig. **1e**). Given that nicotine is primarily synthesized in the roots, we also observed a decrease in the variation of nicotine levels between EV and irTOC1 roots treated with 1-MCP (Fig. **4d**). Consistent with previous findings, both CP and DCS were largely absent and unaffected in the roots (Fig. **4f,h**), and DCS levels increased slightly in EV plants after 1-MCP treatment and elicitation, remaining significantly higher than irTOC1 levels (Fig. **4g**).

4.3.5. *TOC1* in roots limits nicotine accumulation, but changes to reproductive allocation depend on *TOC1* in shoots

Given that nicotine is synthesized in the roots, and that changes in the allocation of nitrogen between metabolically expensive compounds might affect the plant's ability to defend itself against native herbivores, we examined the effect of silencing *TOC1* under field conditions, and in roots only or in whole plants using a micrografting approach (Fig. **5a**). We found that differences in nicotine accumulation of the heterograft (EV shoots and irTOC1 roots) mirrored the levels of nicotine after whole-plant *TOC1* silencing (Fig. **5b**), while levels of CP were statistically indistinguishable from EV levels, their tendency was to be greater in the heterografts (Fig. **5c**). Curiously, DCS levels appeared to vary in root *TOC1*-silenced heterografts more than under whole-plant *TOC1*-silencing (Fig. **5d**). Allometric analysis of ratios of biomass to seed capsule production, taken as a measure of efficient conversion of vegetative resources into fitness correlates, showed that whole-plant *TOC1* silencing had much stronger effects on allometric trajectories, while root *TOC1*-silenced heterografts were indistinguishable from EV plants under control field conditions, and displayed an intermediate phenotype under simulated herbivory.

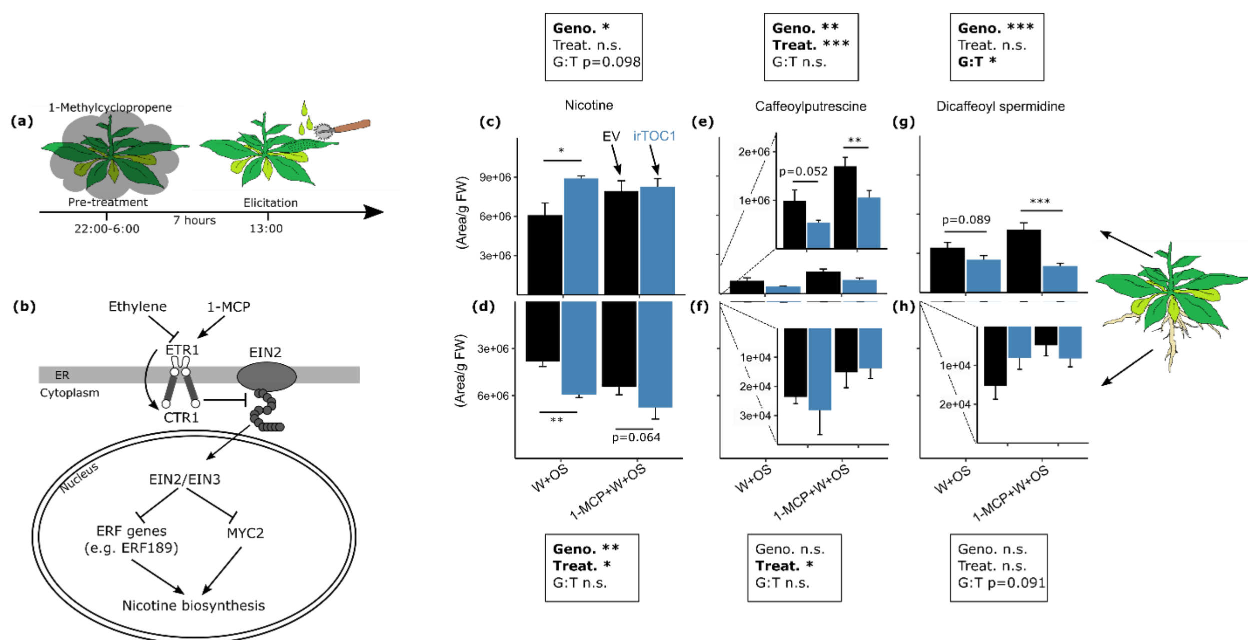

Figure 4: Shifts in nicotine levels after wounding and 1-methylcyclopropene are consistent with an abrogated ethylene signaling/feedback loop in irTOC1 plants. (a) Treatment and sampling scheme for 1-methylcyclopropene (MCP) experiment. (b) 1-methylcyclopropene acts as an ethylene agonist, preventing ethylene from engaging in downstream signaling that modulates biosynthesis of nicotine in *Nicotiana attenuata*. 1-methylcyclopropene-treatment followed by elicitation (W+OS +MCP) abolishes differences between irTOC1 and EV plants in nicotine accumulation in leaves (c) and roots (d) consistent with an abrogation of the ethylene-dependent dampening of nicotine production following elicitation with *Manduca sexta* oral secretions. 1-methylcyclopropene treatment leads to increased levels of an inducible phenolamide in both irTOC1 and EV leaves (e), but does not cause an increase in levels of a constitutive phenolamide in irTOC1 leaves (g). Root levels of both caffeoylputrescine (f) and dicaffeoyl spermidine (h) are generally very low and unaffected by abbrogated ethylene signaling. N=4-5, error bars represent mean + SEM for individual compounds. Stars represent significant differences in estimated marginal means, Tukey-adjusted pairwise contrasts.

4.4. Discussion

Selective allocation by plants, both of resources to defense versus growth and reproduction, and of defense traits among tissues, is a common theme in theories of plant defense (Herms & Mattson, 1992; Doyle McKey, 1974; Meldau *et al.*, 2012). Although plant defense theories generally do not consider the temporal dimension of selective allocation, two frameworks, apparency theory and optimal defense theory, incorporate different probabilities of herbivore attack based on plant phenotypes. In particular, optimal defense theory predicts that tissue defense should be correlated with likelihood of attack combined with tissue value, and this framework could incorporate diurnal changes in likelihood of attack, in addition to the traditional view of changing likelihood over ontogeny (Meldau & Baldwin, 2013). Previous work in *N. attenuata* is consistent with the predictions of optimal defense theory: inducible defenses in *N. attenuata* are regulated by levels of cytokinins, a class of developmental growth hormones (Brütting *et al.*, 2017), and developmental transitions such as flowering alter the ethylene/JA-mediated defense signaling at the heart of inducible plant defenses (Diezel *et al.*, 2011). However, what role the circadian clock plays in optimizing defenses across development has not been previously investigated.

Our results provide new mechanistic insight into the circadian clock's role in optimizing the allocation of resources in *N. attenuata's* defense chemistry, building on the evidence outlined in Li *et al.* (2018) that the clock component ZTL interacts with jasmonate signaling to promote synthesis of the abundant, nitrogen-rich defense compound nicotine. Here, we find that *TOC1* interacts with ethylene signaling to dampen nicotine accumulation, and that *TOC1* additionally mediates the allocation of nitrogen between nicotine and another group of abundant nitrogen-containing defense compounds, the phenolamides. Testing the functional consequences of these allocation differences under field conditions demonstrates that these trade-offs in nitrogen allocation do not explain the large effect of *TOC1* function on plant fitness, which furthermore seems to be specific to the function of *TOC1* in shoots, while nicotine is synthesized in the root. Given that the circadian clock has been shown to be mechanistically distinct (Endo, Shimizu, Nohales, Araki, & Kay, 2014) and vary in functional outputs across tissue types (Bordage, Sullivan, Laird, Millar, & Nimmo, 2016), there should be a greater effort to explore the functional consequences of individual circadian clock components across tissues for defense responses.

Another theory of plant defense that has been applied in understanding resource allocation, the carbon/nutrient balance theory, predicts that resources such as carbon or micronutrients such as nitrogen, phosphorus, or potassium may be rate-limiting for growth or defense compounds (Bryant *et al.*, 1983; Massad *et al.*, 2012). Nitrogen pools in particular provide a valuable testing ground for questions of resource allocation, as they can be limited and subject to changing needs and intense intraspecific competition in *N. attenuata* populations, and many of *N. attenuata's* highly abundant defense metabolites are nitrogen-rich, including nicotine (Ian T Baldwin *et al.*,

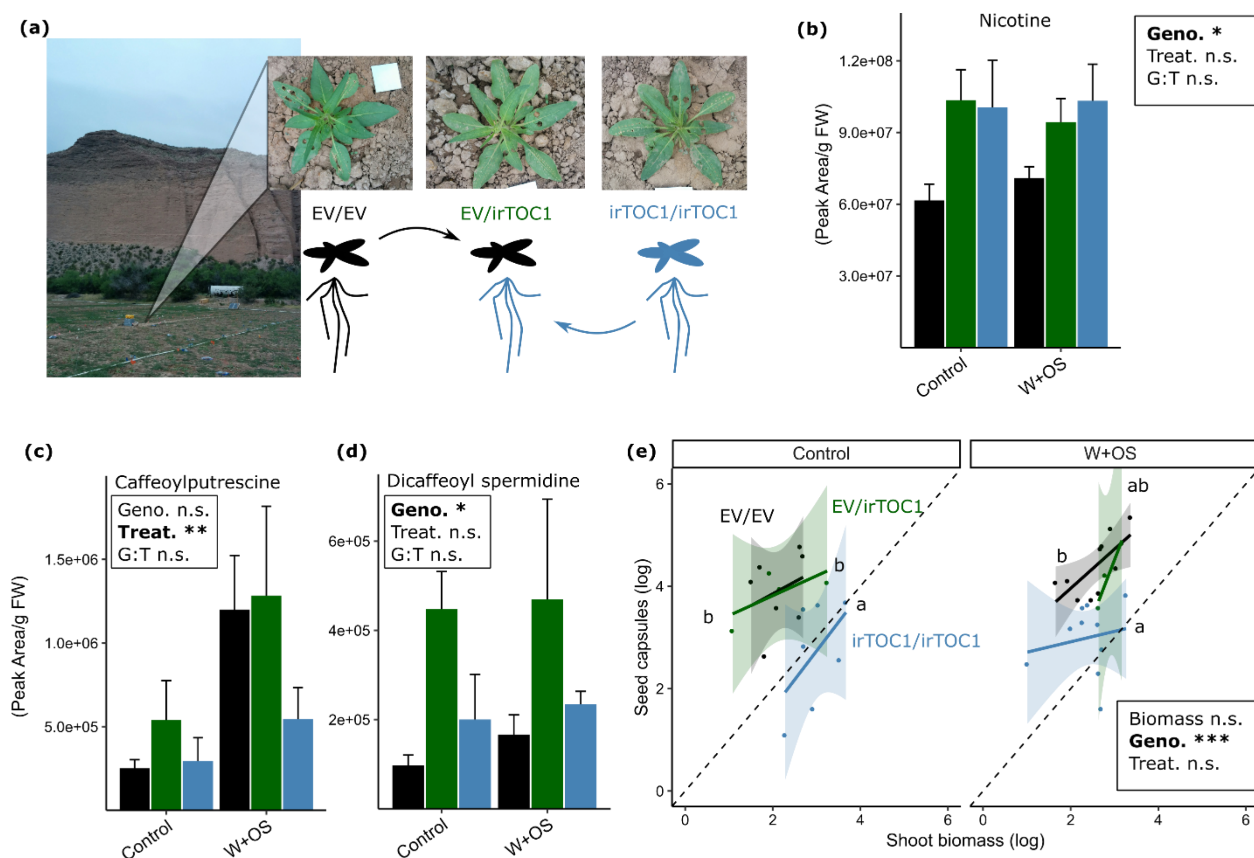

Figure 5: Silencing TOC1 in the shoot under field conditions in *N. attenuata* shoots causes severe fitness, while root *TOC1* silencing is sufficient to explain differential nicotine accumulation compared to EV. (a) Representation of field plot at Lytle Preserve, UT, USA, as well as representative photos of each genotype at treatment and sampling. Production of secondary metabolites nicotine (b) caffeoylputrescine (c) and dicaffeoylspermidine (d) 72h after elicitation with *M. sexta* oral secretions. (e) Final biomass was severely affected in irTOC1/irTOC1 lines, but not in EV-shoot lines, leading to a differential allometric trajectory for irTOC1/irTOC1 plants. N=4-6, error bars represent mean + SEM for individual compounds for (b), (c), and (d). Shading represents 95% confidence level intervals along correlation axes for (e).

1998; Ullmann-Zeunert *et al.*, 2013; van Dam & Baldwin, 2001). Resource allocation can be manipulated directly by the circadian clock, as has been shown for carbon metabolism (Kölling, Thalmann, Müller, Jenny, & Zeeman, 2015; L. M. Müller, Von Korff, & Davis, 2014; Sanchez-Villarreal *et al.*, 2013). Our results suggest that TOC1 modulates nitrogen allocation indirectly as an output of defense signaling, given that levels of chlorogenic acid (CGA) are also attenuated in irTOC1 plants, and CGA is a polyphenolic compound also regulated by *MYB8* much like CP and DCS (Fig. S3). The contrast to the irZTL phenotype in nitrogen-rich defense metabolite accumulation as well as TOC1's apparent role in ethylene-mediated defense signaling indicates that individual members of the circadian clock may have specific, if nested, metabolic roles in optimizing responses to stress, and that optimizing these responses is essential for maintaining plant fitness.

Previous data suggests that herbivory disrupts normal clock function and its outputs (Joo *et al.*, 2018). It is reasonable to expect that, whatever the adaptive value of circadian-regulated inducibiity of JA or concentration of defense metabolites, plant resources are preferentially if temporarily mobilized for defense after elicitation occurs. Our result support this hypothesis, given that variation in nitrogen allocation under control conditions between EV and irTOC1 plants disappears after a simulated elicitation event (Fig. **3**). Interestingly, this effect cannot be observed when looking at secondary metabolite accumulation under elicited conditions (Fig. **2**), highlighting an important difference between investigating variation in abundance and allocation.

Ethylene emissions are rhythmic and circadian in *Arabidopsis,* and ethylene signaling is known to be regulated by the circadian clock (Song *et al.*, 2018; Thain *et al.*, 2004). Previous work had ruled out the possibility that ethylene feedback mechanisms are circadian related, as ethylene-insensitive mutants overproduce ethylene but do not display altered rhythmicity in ethylene production (Thain *et al.*, 2004). Despite this, our results provide evidence that for a well-known ethylene response, the herbivore-elicited dampening of nicotine accumulation, the circadian clock component *TOC1* seems to play a key role in signal transduction. The fact that the induction of high levels of ethylene after elicitation with *M. sexta* oral secretions is not diminished by *TOC1* silencing and are, rather, slightly but not significantly increased also supports the hypothesis that *TOC1* abrogation may lead to partially ethylene insensitive phenotypes, at least in some contexts like herbivore-mediated nicotine production.

If *TOC1*-silenced plants are ethylene insensitive, the generation of crosses with some available lines affecting ethylene production (irACO) or affecting ethylene perception but with extreme pleiotropic effects (asETR1) would not be helpful in elucidating *TOC1's* role in herbivore response. Given that *ACC SYNTHASE 8 (ACS8)*, one of a family of 12 enzymes in *A. thaliana* producing the ethylene precursor ACC*,* displays a circadian rhythmic phenotype in transcript accumulation, it may be worthwhile to examine the nicotine accumulation of elicited plants silenced in *ACS8* by transient virus-induced gene silencing, and to investigate the role of TOC1 in repressing *ACS8*.

The results of our field experiment demonstrate that the shifted ratio between nicotine and phenolamides in *TOC1*-silenced plants persists, and moreover that this shift may have consequences for fitness outcomes under field conditions. Micrografting of *TOC1*-silenced roots to EV shoots further demonstrates that *TOC1's* role in mediating herbivore-elicited phenolamide production may be shoot-specific, while *TOC1's* role in nicotine biosynthesis, and thus its ethylene insensitivity, may be root-specific. Given the fitness metrics of root-only *TOC1* silenced heterografts remained similar to EV plants under field conditions, the fitness consequences of whole-plant *TOC1* silencing may have less to do with a differential allocation of nitrogen generally, but rather a specific shift towards overproduction of a single defense such as nicotine at the expense of a more diverse defensive metabolite profile. Future work should dissect whether the fitness disadvantages of irTOC1 or other clock mutants under field conditions are due to allocation costs or to the opportunity costs of mis-timing defenses.

The results of the ^{15}N-labeling experiment (Fig. **3e**), taken together with previous work by Joo and colleagues (Joo *et al.*, 2018) suggest that the circadian machinery plays a greater role in the metabolic state of the plant preceding and immediately at the beginning of herbivory events, and that subsequent metabolic reorganization decouples the clock from its normal function under control conditions. The use of inducible promoters (see e.g. Schäfer *et al.*, 2013) may help to dissect the role of the clock between these preparatory or anticipatory stages of herbivory and the role of circadian clock components such as TOC1 and particularly ZTL on the remobilization of defense responses during the course of an herbivory event.

Acknowledgements

We thank the glasshouse department at the Max Planck Institute for Chemical Ecology and the field team in 2015 and 2016 for support; Brigham Young University for the use of the Lytle Ranch Preserve field station in Utah, USA, and APHIS for constructive regulatory oversight; the technical staff at the department of Molecular Ecology for providing seeds; L. Cortes-Llorca for helpful discussions; and both 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 H.V. and E.M.

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Supplemental files

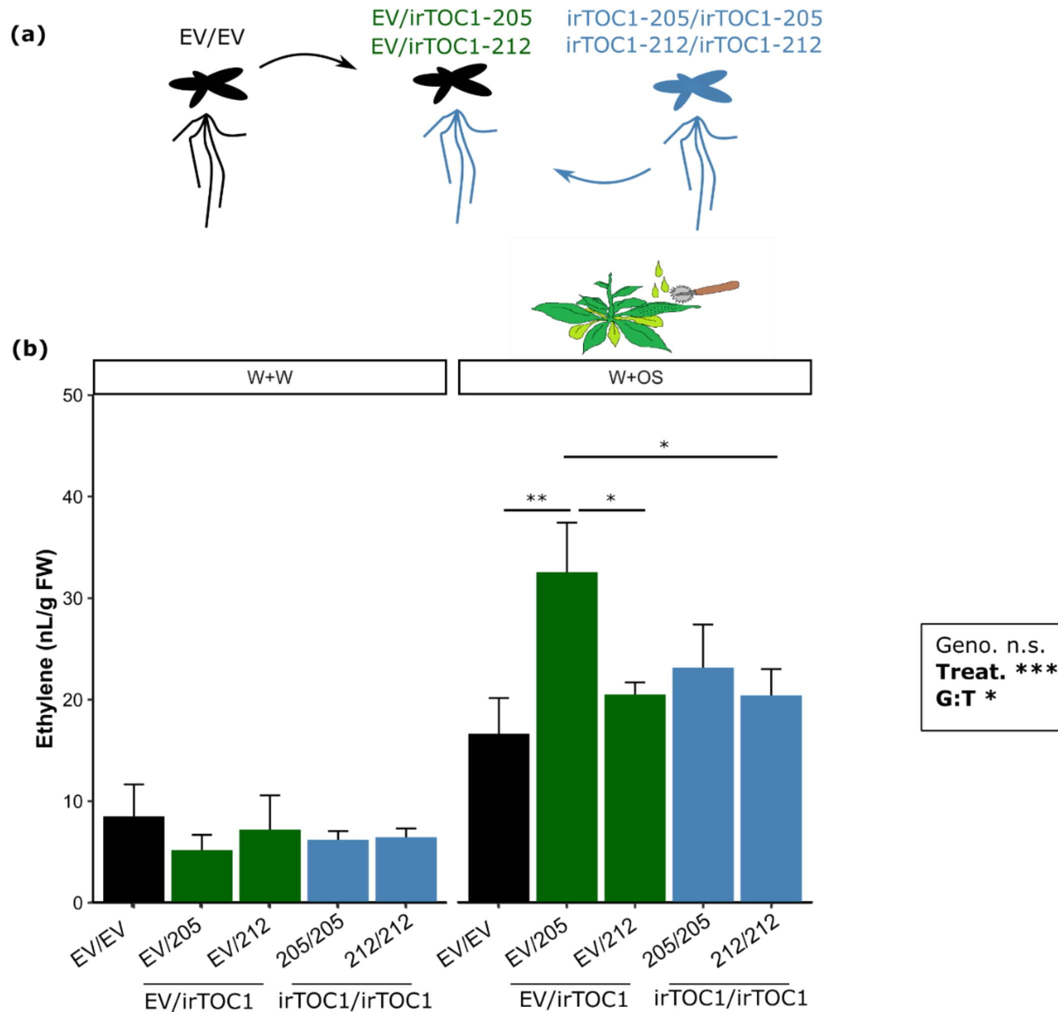

Figure S1: Ethylene burst after elicitation with *Manduca sexta* oral secretions is not affected by whole-plant nor root-only *TOC1* silencing. (a) Schematic of two independent silenced lines used for grafting and ethylene analysis in the glasshouse. (b) Ethylene levels 5h after elicitation are not considerably different between EV and *TOC1*-silenced lines. N=6, error bars represent mean + SEM for individual compounds. Stars represent significant differences in estimated marginal means, Tukey-adjusted pairwise contrasts.

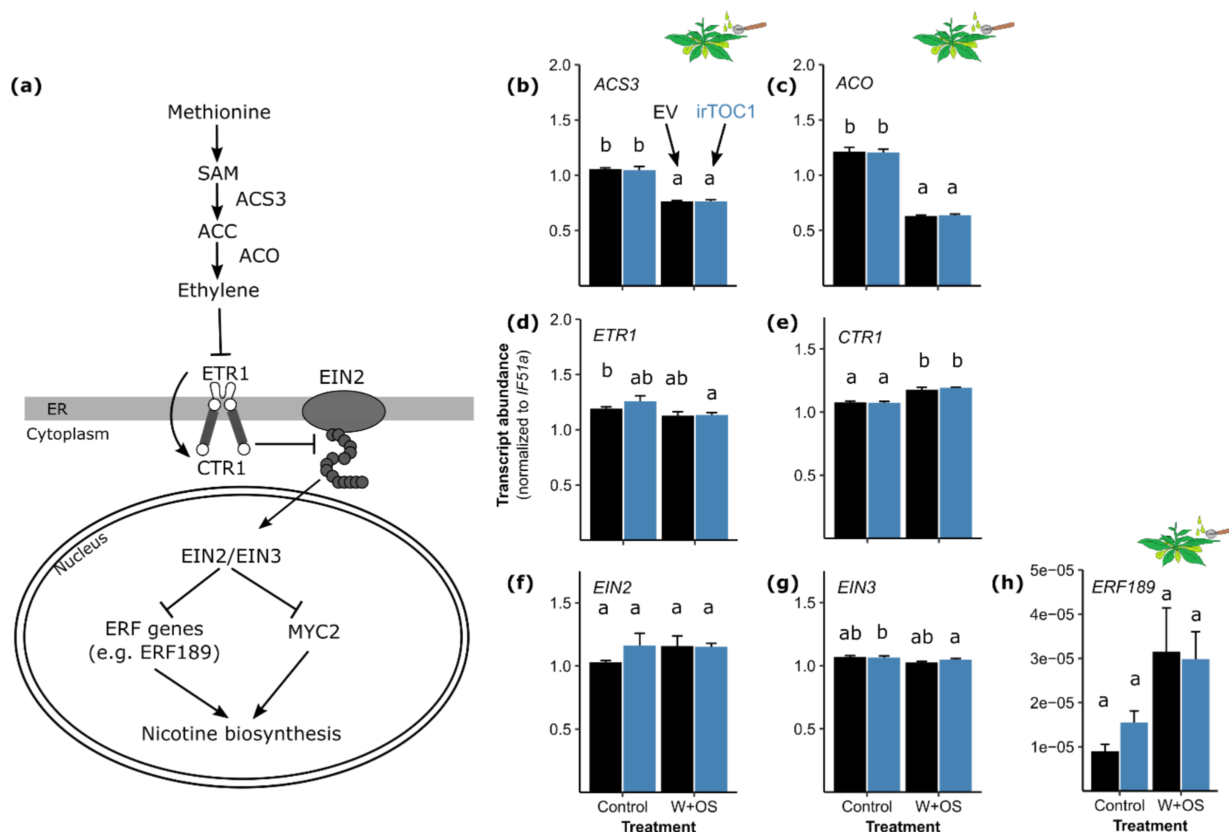

Figure S2: Effect of*TOC1* silencing on transcript abundance of various ethylene receptors and downstream targets 1 hour after simulated herbivory. (a) Schematic of ethylene biosynthesis and signaling. Ethylene biosynthesis (b,c), perception (d,e) and downstream signaling (f-g) are significantly affected by W+OS treatment, but not by TOC1 silencing 1h after elicitation. (h) Nicotine biosynthesis-targeting transcription factors, such as ERF189, are not abundant and unaffected by treatment or genotype in leaf tissues. N=6, error bars represent mean + SEM for individual compounds. Stars represent significant differences in estimated marginal means, Tukey-adjusted pairwise contrasts. SAM: S-adenosyl-l-methionine; ACC: 1-Aminocyclopropane-1-carboxylic acid; *ACS3: ACC SYNTHASE3; ACO: ACC OXIDASE; ETR1: ETHYLENE RESPONSE1; CTR1: CONSTITUTIVE RESPONSE1; EIN2: ETHYLENE INSENSITIVE2; EIN3: ETHYLENE INSENSITIVE3; ERF189: ETHYLENE RESPONSE FACTOR189*

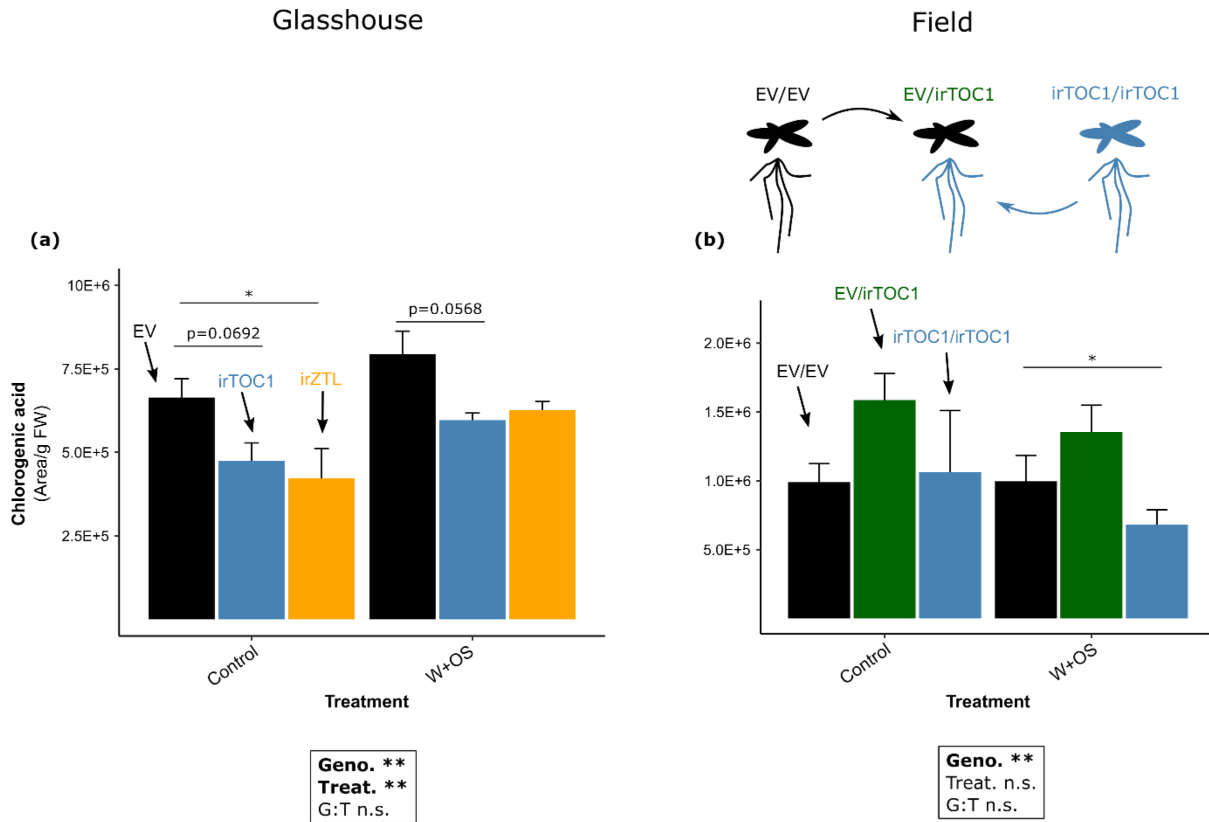

Figure S3: Chlorogenic acid accumulation is attenuated in irTOC1 plants 72h after simulated herbivory. (a) Levels of chlorogenic acid in EV, irTOC1, and irZTL plants under glasshouse conditions. (b) Levels of chlorogenic acid under field conditions in whole-plant and root-only *TOC1*-silenced plants. N=6 for (a), 4-6 for (b); error bars represent mean + SEM for individual compounds. Stars represent significant differences in estimated marginal means, Tukey-adjusted pairwise contrasts.

Discussion

In this dissertation, I investigated the role of the circadian clock component *TOC1* in mediating fitness outcomes for *N. attenuata* under both biotic and abiotic stress conditions. In the following discussion, I will elaborate further on the significance of these findings in the context of elucidating the function of the plant circadian clock in nature, for which much work remains to be done. I will also discuss coming technologies for expanding the specificity of our functional knowledge about the clock, and how emerging technologies will enable parallel investigations in non-model systems. I discuss the potential to elucidate the role of circadian and photoperiodic alleles in domesticated crops as a case study, and how these investigations can elucidate plant evolutionary history with potential applications for plant breeding and cultivar development in agriculture.

Adaptability conferred by the clock over developmental-scale responses to stress

Every hypothesis exploring biological phenomena must be explored at one of four separate but complementary levels of analysis: evolutionary origins, exploring how organisms have evolved from ancestral forms; functional consequences, querying the fitness or adaptive value of particular phenomena; ontogenetic processes, exploring responses throughout age and development; and finally mechanistic questions, exploring the physiological, chemical and molecular processes by which biological phenomena happen at their most proximal level (Schuman & Baldwin, 2016; Sherman, 1988; Tinbergen, 1963). The majority of circadian clock literature to date has focused largely on mechanistic questions: what are the components of the circadian clock? Which components interact directly with which others, and what are their regulators and downstream targets? Specifically, how do shifts in circadian rhythms gate stomatal aperture closure sensitivity to abscisic acid, or secondary metabolite production to herbivore attack? These investigations are highly important for elucidating which mechanisms can be manipulated to best understand the clock's role in maintaining plant fitness, but an intimate understanding of physiological mechanism cannot replace functional elucidation of whether that mechanism is adaptive, i.e. contributes to the fitness of the organism. Aside from physiological mechanistic studies, some ontogenetic mechanistic studies on the role of the components of the clock regulating photoperiodic flowering have been undertaken, including examining their role in stress responses (Galbiati *et al.*, 2016; Izawa, 2012; Matsuzaki, Kawahara, & Izawa, 2015; Riboni, Galbiati, Tonelli, & Conti, 2013). However, the role of central circadian clock components in modifying or optimizing plant fitness in response to stress have not been explored. **Chapter 1** provides a review linking diurnal and circadian responses to ontogenetic responses, using plant volatiles as case study. Importantly, because many studies investigating circadian stress responses have been designed to answer mechanistic questions, they cannot ask or answer functional questions or investigate the fitness value of the circadian clock's gating of stress responses.

Compared to the focus on mechanistic questions in the circadian literature, there have been fewer studies of the functional role of circadian rhythms. To truly understand how plants adapt to changes in climatic conditions, we must ask functional questions of plant systems in order to better understand how mechanisms of stress response work to maximize fitness. Many functional questions remain under-investigated, likely because they must be pursued under field conditions and thus require a more extensive and fundamental understanding of plant natural history, which

can only be arrived at by experience observing plant-environment interactions in the field. Why do plants maintain such well-conserved circadian mechanisms, despite the presence of diurnal zeitgebers such as light and temperature that sometimes entrain mutants or knockouts targeting key circadian clock regulators to daily rhythms? Do plants with functioning circadian clocks produce more offspring or optimize their fitness in other ways relative to arrhythmic or clock-shifted plants? Finally, what is the function of the clock in mediating responses to either predictable or stochastic stress events? Few formal hypotheses and tests have been framed to address questions such as these in the circadian literature.

Circadian adaptation to drought stress

Drought provides an interesting test of the functional consequences of plant circadian clocks given the variability of responses to low water availability. Plant drought resistance is sometimes invoked in the mechanistic literature as a response without much discussion of the mechanisms by which drought resistance can be manifested to maintain plant fitness. Drought resistance can be categorized into three classes of mechanisms: **drought tolerance**, wherein plants produce osmoprotectants and physical barriers to dehydration such as thick cuticles; **drought avoidance**, involving a decrease in transpiration, limiting aerial vegetative growth, and increased root growth to avoid dehydration and maximize water uptake; and **drought escape**, wherein plants speed up developmental transitions and reproduction in order to ensure the production of the next generation before drought conditions become lethal (Chapin, Autumn, & Pugnaire, 1993; Kooyers, 2015).

As discussed in **Chapter 2,** plants may respond using different strategies depending on the severity and length of water stress conditions, and thus carefully applying stress to test particular responses is paramount to interpreting the results of studies exploring the molecular mechanisms underpinning drought resistance. As an example, rice plants treated with a temporary drought event delayed flowering predictably, with more days of low water availability directly correlating to a greater delay in heading date (Galbiati *et al.*, 2016). In another study, rice plants subjected to longer periods of consistently low water availability were found to flower early (Du *et al.*, 2018). In the first experiment, plant ontogeny approximates more closely a drought avoidance response, where decreased transpiration, lower vegetative growth rates, and increased root investment lead to delayed developmental transitions after the drought event has passed. Because of these differences in how stress was applied, it is possible that wild-type plants in each study experienced different levels of drought stress, leading to different physiological responses to growth and ultimately to floral transition. When one considers furthermore the variable water use and developmental phenotypes of genotypes used in each experiment, predicting the functional consequences of circadian and photoperiodic systems becomes difficult.

In **Chapter 3**, I explore some of the missing gaps in understanding the functional role of the core circadian clock component, *TOC1*. The literature linking *TOC1* to drought responses has been elucidated in *Arabidopsis thaliana,* demonstrating the direct role of *TOC1* in gating ABA responsiveness and further demonstrating the feedback loop that exists between *TOC1* and ABA signaling via the MYB transcription factor MYB96 (H. G. Lee *et al.*, 2016; K. Lee *et al.*, 2015; Legnaioli *et al.*, 2009; Seo *et al.*, 2011, 2009; Seo & Park, 2010). The central questions underlying this study were the following: does silencing *TOC1* in *N. attenuata* increase water-use efficiency and decrease water loss under drought, as it does in *A. thaliana*? If so, what are the functional consequences of this increase in water use efficiency? And finally, given *TOC1*'s

variable contribution to the root clock (James *et al.*, 2008), and the root's importance in drought responses (Giuliani *et al.*, 2005; Montero-Tavera, Ruiz-Medrano, & Xoconostle-Cázares, 2008; Rasheed, Bashir, Matsui, Tanaka, & Seki, 2016), does *TOC1* play a role in both roots and shoots in maintaining fitness during drought?

Investigating the first of these questions, regarding mechanisms of physiological response to drought, suggests that irTOC1 *N. attenuata* plants perform better than unsilenced plants under drought, as evidenced by losing less water from excised leaves and increasing water use efficiency. However, strong evidence suggests that irTOC1 plants fail to optimize their fitness under drought condition. In the glasshouse, where watering was carefully controlled to ensure that plant water status was the same between silenced and unsilenced plants, drought stressed irTOC1 plants failed to produce the same amount of seed capsules than their well-watered counterparts, unlike unsilenced plants, which produced the same number of capsules under both treatments. Under field conditions where plants experienced similar water deficit stress under drought conditions, irTOC1 plants did not manage to produce similar numbers of seed capsules to unsilenced plants, which significantly increased their seed capsule production under drought. These changes in seed capsule production, when compared to final biomass accumulation, suggest that irTOC1 plants fail to convert biomass efficiently into seed capsules under duress, a hallmark of the drought escape strategy that is most adaptive for small annual herbaceous plants that grow in temperate climates (Franks, 2011; Franks, Sim, & Weis, 2007; Kooyers, 2015). These results suggest that, despite similar findings to *A. thaliana* regarding increased performance, it would be unlikely for polymorphisms in a circadian clock component like *TOC1* to be adaptive under natural conditions.

Circadian adaptation of plant defense

In **Chapter 4,** I expand functional analyses of *TOC1* expression to plant defense against the specialist herbivore *Manduca sexta*. Plant defenses are known to be rhythmically regulated in *N. attenuata*, rather than circadian regulated, and this may be linked to the diurnal feeding phenotype of the specialist herbivore *M. sexta*, which causes some of the most severe fitness consequences for the plant among its many herbivores (Herden *et al.*, 2016; Joo *et al.*, 2018). There has been several theories posited to explain how and why plants produce secondary metabolites to defend themselves successfully against unrelenting attacks by heterotrophs, both animal and microbial (Schuman & Baldwin, 2016; Stamp, 2003). The hypotheses tested in **Chapter 4** are divided between two different theories of plant defense, operating at two different levels of analysis.

Queries of how plant defense is allocated within plants have led, among others, to **carbon:nutrient balance theory** (**CNB**) and **optimal defense theory** (**OD**). CNB provides mechanistic hypotheses of how plants allocate resources to their defense chemistry, and predicts that the allocation costs of defense are mediated by inputs of sunlight (carbon) and micronutrients such as nitrogen into the plant from the environment, and further that allocation costs to defenses increase as these inputs are limited, because allocation to constitutive defenses are fixed at a genotypic level (Stamp, 2003; Tuomi, Niemelä, Stuart Chapin, Bryant, & Sirén, 1988). One sub-hypothesis of OD predicts that plants should favor commitment to lower-cost, less effective defenses over higher-cost, more effective ones when there is a fitness-limiting trade-off between them (for example, when plants are stressed or at particular developmental stages).

In **Chapter 4,** I explore both mechanistic questions of allocation costs between *TOC1*-silenced and unsilenced plants as well as functional questions regarding how fitness is affected under herbivory-induced conditions. Thus, the first question of this study was: given previous evidence of the circadian clock component ZEITLUPE's direct role in regulating nicotine production in *N. attenuata* (Li *et al.*, 2018), is *TOC1* also involved in regulating defense chemistry? And if so, what are the fitness consequences, if any, of mis-regulation of defense allocation, particularly under natural conditions? Finally, are there differing consequences to silencing *TOC1* in roots only, given that the function of the clock in roots is expected to vary from its function in the shoot? Given the circadian clock's known role in maintaining efficient resource uptake in plants (Graf, Schlereth, Stitt, & Smith, 2010; Joo, Fragoso, Yon, Baldwin, & Kim, 2017; Kölling *et al.*, 2015; L. M. Müller *et al.*, 2014) and *TOC1's* role in mediating plant growth responses down to the cellular level (Fung-Uceda *et al.*, 2018; Soy *et al.*, 2016; Zhu *et al.*, 2016), we predicted that either *TOC1*-deficient plants would produce less induced defense, or else less high-cost defense. As predicted by CNB, *TOC1*-deficient *N. attenuata* plants produce large amounts of nicotine and yet fail to induce wild-type levels of an induced defense, caffeoylputrescine. Given that phenolamides are more effective against and specifically induced by *M. sexta* feeding (Gaquerel *et al.*, 2014; Kaur *et al.*, 2010), however, they may be valid candidates for high-cost defenses in this context, despite the fact that nicotine is produced in large quantities and is "non-refundable," metabolically speaking, for the plant (Ian T. Baldwin & Ohnmeiss, 1994).

We further queried whether the defense allocation differences in irTOC1 plants may have led to fitness consequences under field conditions. Nicotine in *N. attenuata* is primarily root-produced and then trafficked to the shoot (I. T. Baldwin, 1999; Fragoso, Rothe, Baldwin, & Kim, 2016), unlike phenolamides, which accumulate primarily in above-ground tissues (Kaur *et al.*, 2010), and that induction by *M. sexta* oral secretions shifts nicotine content towards plant roots (Ullmann-Zeunert *et al.*, 2013). Therefore, micrografting irTOC1 roots to unsilenced shoots allowed us to ask, first, whether *TOC1's* effect on nicotine accumulation could be isolated to the root, and thus whether the shift in defense chemistry allocation between nicotine and phenolamides had strong fitness consequences for plants under field conditions. As expected, plants with root-only *TOC1* silencing displayed increased nicotine accumulation in leaves, similar to whole-plant silencing, and unsilenced levels of phenolamides. Interestingly, despite the large increase in nicotine production, root-only *TOC1* silenced plants maintained fitness levels similar to unsilenced plants, while whole-plant silencing led to severe fitness disadvantages under *M. sexta* elicitation. Similar to **Chapter 3**, these results suggest that mechanistic differences in defense chemistry cannot be considered adaptive in a straightforward manner, but must rather be tested under natural conditions.

Moving forward with the elucidation of circadian clock function

Evolutionary evidence, such as the ubiquity of circadian clock mechanisms, provide a strong argument for the adaptive value of the circadian clock (Dakhiya, Hussien, Fridman, Kiflawi, & Green, 2017; Guenther *et al.*, 2012; Yerushalmi & Green, 2009; Yerushalmi, Yakir, & Green, 2011). That circadian mechanisms vary naturally and seem to be adaptive under different conditions (e.g. at different latitudes) is also known, and provides further evidence from a genetic and evolutionary perspective of the importance of circadian mechanisms for plant fitness (Anwer & Davis, 2013; Edwards *et al.*, 2005; Greenham *et al.*, 2017; Lopes, Kato, De Aguiar Andrade, Maia, & Yoshida, 1997; Michael *et al.*, 2003; Xie *et al.*, 2015). Likewise, there is a

wealth of mechanistic evidence that supports the performance advantages of a functioning circadian clock in a variety of contexts (Goodspeed, Chehab, *et al.*, 2013; Legnaioli *et al.*, 2009; L. M. Müller *et al.*, 2014; Nolte & Staiger, 2015; C. Zhang *et al.*, 2013).

Evidence for the actual fitness consequences of a mis-regulated circadian apparatus, on the other hand, has not been explored in-depth. Some work in *Oryza sativa* by Izawa and colleagues has investigated the consequences of manipulating circadian/photoperiodic mechanisms under field conditions (Izawa, 2012; Izawa *et al.*, 2011), and the work in **Chapters 3** and **4** helps to bridge some of the gaps of knowledge in regards to the functional significance of the clock in the face of biotic and abiotic challenges. Perhaps most interestingly, the disconnect between the functional importance of the root and shoot circadian clocks merits further exploration, and demonstrates the need for further elucidation of how clock components contribute to stress response mechanisms in different tissues. Further functional studies under natural conditions that examine the fitness consequences for how plants of different species maintain or optimize fitness under stressful conditions will be necessary to apply our mechanistic understanding of the clock in order to determine the full extent to which the circadian clock contributes to fitness.

Reverse genetics in the genome editing era: querying non-model systems

The analysis undertaken in **Chapter 4** also highlights a further need: to determine the specifics of how the circadian clock contributes to plant fitness in individual systems, given that specialized defense chemistry varies highly between species. With the advent of gene editing technology, it has become feasible to query individual plant-herbivore systems in detail and at the tissue level to determine the range of possibilities for how the circadian clock and plant defenses intersect to maximize fitness. Traditional systems of genetic manipulation have, until now, been restricted to a select number of model systems and essential crop species. Genetic manipulation was largely restricted to these few model systems not only because of the time-consuming nature of designing genetic manipulation tools, but also by the extreme cost of whole-genome sequencing. However, more efficient tools for genetic manipulation are now available, including the **Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9)** system, which allows bacteria and archaea to detect and silence viral DNA through the use of small RNA guide strands to allow for targeted base pair cleavage by Cas9 proteins (Gasiunas, Barrangou, Horvath, & Siksnys, 2012; Jinek *et al.*, 2012). Furthermore, rapid advances in sequencing technology and the large drop in per-base pair sequencing costs have made the prospect of not only sequencing reference genomes for non-model species, but also the whole-genome sequencing of individual variants a reality. These advances combined provide the potential for efficient genomic investigation, revolutionizing the potential for rapidly elucidating gene function in non-model systems (Cong *et al.*, 2013; Dominguez, Lim, & Qi, 2016; Knott & Doudna, 2018).

Genome editing has been successfully applied to a variety of biological systems, several biomedical applications (Heidenreich & Zhang, 2016; Waddington, Privolizzi, Karda, & O'Neill, 2016), but it is its potential for agricultural non-model systems that is of interest for the purposes of this discussion (Chen, Wang, Zhang, Zhang, & Gao, 2019). In barley, the advent of genome editing has allowed the rapid generation of knockouts for investigating gene function (Lawrenson & Harwood, 2019), and more generally for other cereal crops (Zong *et al.*, 2017). In tomatoes and other fruits, genome editing has allowed higher-throughput investigation of applications to pest resistance, fruit quality improvement (T. Wang, Zhang, & Zhu, 2019;

Yamamoto *et al.*, 2018). In animal systems, the use of genome editing for more rapid querying of circadian clock functioning is also underway (Börding, Abdo, Maier, Gabriel, & Kramer, 2019; Korge, Grudziecki, & Kramer, 2015).

With the elucidation of the plant circadian clock having been undertaken over the last decades, prior to the CRISPR revolution, there is less cause for the development of CRISPR/Cas9 systems for the piecing together of individual circadian clock components and their interactions. However, as has been investigated in this thesis, there is ample room for functional elucidation, which must remain the next step of circadian studies to enable the understanding and application of the circadian clock's adaptive value. The additional development of tissue-specific knock-out targeting in *A. thaliana* raises the prospect of rapid screening of circadian function at the tissue level across a variety of potential plant systems and, ideally, the investigation of these functions under natural conditions (Decaestecker *et al.*, 2019).

Plant domestication as a case study of circadian adaptation

Humans have been domesticating plants since the beginning of the Holocene 12,000 years before the present, following the end of the last major glaciation event (Larson *et al.*, 2014; Purugganan, 2019). From the very beginning, the study of domestication has been a specialized case study of adaptation and evolution, with the first chapter of Darwin's seminal work *On the Origin of Species* devoted to comparing the creation of new cultivars and varieties of both animals and plants by artificial selection, to the generation of new species by natural selection (C. Darwin, 1859). Indeed, despite the fixation with Darwin's finches in the public mind, Darwin himself was highly interested in understanding evolution and selection in an agricultural context, devoting to this topic the first half of his intended "big book" on plant and animal domestication, titled *The Variation of Animals and Plants under Domestication* (Darwin 1868; see Freeman 1977 p. 74). Both Charles Darwin and his son Francis Darwin explored circadian-regulated plant responses and movements, such as stomatal responsiveness and circumnutation, the circadian bowing and bending movements of growing plant meristems (Jackson 2009; Friedman & Diggle 2011; see e.g. F. Darwin 1898 and Ch. I and VI of C. Darwin and F. Darwin 1880; for a more modern review of circumnutation, see Stolarz 2009).

Crop species are a particularly interesting area for the study of the adaptive value of the circadian clock, given that many crops have been domesticated in one area, particularly at tropical latitudes, and then dispersed alongside human populations to more distant environments, where day length and many other environmental variables change (Cockram *et al.*, 2007). Mechanistic studies of the circadian clock in model crop systems, which have begun to elucidate both the variation in circadian rhythms across cultivars (Greenham *et al.*, 2017) as well as between closely-related domesticated and wild species (N. A. Müller *et al.*, 2015), point to the adaptive value of circadian or photoperiodic variations being selected for in cultivars. In cultivated barley (*Hordeum vulgare*), a polymorphism of the gene Photoperiod-H1, which regulates photoperiodic flowering, has been identified that accounts for variations in barley flowering time among domesticated landraces and wild barley (H. Jones *et al.*, 2008). Interestingly, this polymorphism has been linked to variation in phenotypic characteristics of barley landraces that are suggestive of variable adaptation to different climates (Glynis Jones *et al.*, 2012), and further analysis has shown that these landraces are structured into various haplotypes, including a haplotype

unresponsive to photoperiodic flowering that may have been a later introduction with higher performance in northern climates (G. Jones *et al.*, 2013).

The concurrent loss of photoperiodic-sensitive flowering traits and cultivation at less equatorial latitudes is a common theme across domesticated crop species (see e.g. Liu, Li & Xing 2018), and generates a compelling case for the functional study of circadian clock components under variable conditions. Given the mounting mechanistic data on how the clock functions and the evolutionary data implying its important role in plant fitness, it is now time to properly explore the functional consequences for fitness at the individual and tissue-specific level of the circadian clock. Previous evidence has shown that focusing on performance traits such as stomatal conductance, defense chemistry accumulation and herbivore mass gain, particularly without consideration of developmental or ontogenetic consequences of altering the circadian clock, can lead to predictions that field studies ultimately reveal to be erroneous. We must be careful not to conflate increased performance under stressful conditions at the physiological level, e.g. the water use efficiency of *toc1* mutants under drought stress (Legnaioli *et al.*, 2009) with an increase in fitness that can only be measured at a functional level, as in **Chapter 2** of this dissertation.

Further studies of how circadian and photoperiodic variation have been selected for both by natural and artificial selection will yield the next piece of the evolutionary puzzle of circadian clock function. These mechanistic and functional insights can then better inform the study of how to prepare agricultural systems for the challenges of the twenty-first century, particularly in the face of mounting evidence for great disruptions to food supply and the availability and quality of arable land in the face of climate change (X. Zhang & Cai, 2011). Policy shifts in the European Union correctly predict the need for more adaptive crop varieties as part of the response to these challenges, and it is necessary to better understand the true functional consequences of particular variations and modifications may have on plant yield and fitness to safeguard agricultural production in the future (*Report from the Commission to the European Parliament and the Council - on the implementation of the EU Strategy on adaptation to climate change*, 2018). Applying our knowledge of how the circadian clock optimizes plant phenotypic plasticity and adaptation will hopefully provide better breeding strategies for an uncertain future than selection for individual performance traits, which may be less adaptive in variable unpredictable environments.

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Summary

As sessile organisms that "escape" across temporal rather than spatial ranges, and which produce energy from sunlight, plants' lives are intimately tuned to the Earth's diurnal and circadian rhythms. Plant rhythmic responses have been observed since antiquity, and at least since Darwin's time, rhythmic mechanisms in plants have been measured. These findings presaged the discovery and description of a molecular circadian clock, an intrinsic and ancestral timekeeping mechanism in nearly all life forms. In recent years, the mechanisms by which the circadian clock influences and contextualizes plant responses ranging from flowering time to protection against biotic and abiotic stresses have been elucidated.

Our mechanistic understanding of the clock is highly sophisticated, and evolutionary studies point towards its adaptive function. However, functional analyses of plant circadian clocks must be undertaken to better understand how the clock contributes to plant fitness under ever-changing natural conditions. In this work, I have sought to expand our understanding of the functional role of the circadian clock. I present evidence of the central circadian clock component, *TIMING OF CAB EXPRESSION1* (*TOC1*)'s role in optimizing fitness outcomes for *Nicotiana attenuata* plants under water limitation and simulated herbivory stress under field and glasshouse conditions.

I present evidence that *TOC1*-silenced *N. attenuata* plants (irTOC1) demonstrate decreased water loss relative to empty vector plants (EV) during controlled drought events under glasshouse conditions. Despite this, irTOC1 plants show decreased fitness correlate production under drought conditions relative to well-watered conditions, unlike EV plants, which maintain similar levels of fitness correlates across treatments. Allometric measurements under field conditions demonstrate that irTOC1 plants fail to efficiently increase their conversion of biomass to reproductive correlates, unlike EV plants. Furthermore, by employing a micrografting approach, I demonstrate that these fitness outcomes are abolished when plants are *TOC1*-silenced in roots only, and that these outcomes may be mediated by a red and far-red light signaling response co-expression module in *N. attenuata* shoots.

Because many plant herbivory responses are also mediated by the circadian clock, I further explored the potential function of *TOC1* in mediating plant defense chemistry against the specialist herbivore *Manduca sexta*. I present evidence that irTOC1 plants display increased nicotine accumulation after elicitation and accumulate significantly less phenolamides than EV plants, leading to a different ratio between these two nitrogen-rich defenses. Further investigation using a nitrogen (^{15}N) pulse-labeling approach revealed allocation differences between nicotine and two highly abundant phenolamides, caffeoylputrescine and dicaffeoyl spermidine. irTOC1 plants allocate less nitrogen to phenolamides under control conditions, and that elicitation disrupts this shift, despite not compensating for the shifted levels of defense chemistry accumulation after elicitation.

In *Nicotiana* spp., nicotine induction is dampened by a *M. sexta*-elicited ethylene burst, and blocking ethylene receptors prior to *M. sexta* simulated herbivory increased nicotine production in EV plants to irTOC1 levels, while differences in phenolamide accumulation between EV and irTOC1 did not change. Given that phenolamides accumulate largely in leaves, while nicotine is synthesized in roots, I performed micrografting experiments under field conditions, where root-only *TOC1* silencing led to increased levels of nicotine and EV levels of phenolamides. As a final functional test, allometric measurements revealed that the higher levels of nicotine in irTOC1 roots was not sufficient to cause a reduction in conversion from biomass to reproductive correlates.

Taken together, the findings of this work point towards a need for increased functional studies of the role of individual circadian clock components in natural conditions in order to better understand their adaptive value. Mechanistic elucidation has yielded many of the key circadian regulators of plant stress responses, and these insights should be leveraged to generate targeted functional assays to further our understanding of how plants adapt to their changing environments.

Zusamenfassung

Als sessile Organismen, die eher über zeitliche als über räumliche Bereiche "entkommen" und aus Sonnenlicht Energie gewinnen, sind die Leben der Pflanzen eng auf den Tages- und Tagesrhythmus der Erde abgestimmt. Rhythmische Reaktionen von Pflanzen wurden seit der Antike beobachtet, und zumindest seit Darwins Zeit wurden rhythmische Mechanismen in Pflanzen gemessen. Diese Erkenntnisse waren Voraussetzung für die Entdeckung und Beschreibung einer molekularen zirkadianen Uhr, eines intrinsischen und überlieferten Zeitnehmungsmechanismus in nahezu allen Lebensformen. In den letzten Jahren wurden die Mechanismen aufgeklärt, mit denen die circadiane Uhr die Reaktionen der Pflanzen beeinflusst und kontextualisiert, die von der Blüte bis zum Schutz vor biotischem und abiotischem Stress reichen.

Unser mechanistisches Verständnis der Uhr ist hochentwickelt, und Evolutionsstudien weisen auf ihre adaptive Funktion hin. Es müssen jedoch Funktionsanalysen der zirkadianen Pflanzenuhren durchgeführt werden, um besser zu verstehen, wie die Uhr unter sich ständig ändernden natürlichen Bedingungen zur Fitness der Pflanzen beiträgt. In dieser Arbeit habe ich versucht, unser Verständnis der funktionellen Rolle der circadianen Uhr zu erweitern. Ich präsentiere Beweise für die zentrale circadiane Uhr, die Rolle von *TIMING OF CAB EXPRESSION1* (*TOC1*) bei der Optimierung der Fitnessergebnisse für *Nicotiana attenuata*-Pflanzen unter Wassereinschränkung und simuliertem Pflanzenstress unter Feld- und Gewächshausbedingungen.

Ich lege Beweise dafür vor, dass durch *TOC1* zum Schweigen gebrachte *N. attenuata*-Pflanzen (irTOC1) bei kontrollierten Dürreereignissen unter Gewächshausbedingungen einen verringerten Wasserverlust im Vergleich zu leeren Vektorpflanzen (EV) aufweisen. Trotzdem zeigen irTOC1-Pflanzen unter Dürrebedingungen eine verringerte Fitness-Korrelat-Produktion im Vergleich zu gut bewässerten Bedingungen, im Gegensatz zu EV-Pflanzen, die ähnliche Fitness-Korrelate für alle Behandlungen aufrechterhalten. Allometrische Messungen unter Feldbedingungen zeigen, dass irTOC1-Pflanzen im Gegensatz zu EV-Pflanzen ihre Umwandlung von Biomasse in reproduktive Korrelate nicht effizient steigern können. Darüber hinaus zeige ich mit einem Mikrotransplantationsansatz, dass diese Fitnessergebnisse aufgehoben werden, wenn Pflanzen nur in Wurzeln mit TOC1 zum Schweigen gebracht werden, und dass diese Ergebnisse möglicherweise durch ein Modul zur Koexpression von Rot- und Far-Rot-Lichtsignalen in *N. attenuata*-Blättern vermittelt werden.

Da viele pflanzenfressende Reaktionen auch durch die circadiane Uhr vermittelt werden, untersuchte ich die mögliche Funktion von *TOC1* bei der Vermittlung von Pflanzenschutzchemikalien gegen den spezialisierten Pflanzenfresser *Manduca sexta*. Ich lege Beweise dafür vor, dass irTOC1-Pflanzen nach dem Auslösen eine erhöhte Nikotinakkumulation aufweisen und signifikant weniger Phenolamide als EV-Pflanzen akkumulieren, was zu einem unterschiedlichen Verhältnis zwischen diesen beiden stickstoffreichen Abwehrmechanismen

führt. Weitere Untersuchungen unter Verwendung eines Stickstoff (^{15}N) - Pulsmarkierungsansatzes ergaben Zuordnungsunterschiede zwischen Nikotin und zwei sehr häufig vorkommenden Phenolamiden, Caffeoylputrescin und Dicaffeoylspermidin. irTOC1-Pflanzen weisen Phenolamiden unter Kontrollbedingungen weniger Stickstoff zu, und dieser Auslöser stört diese Verschiebung, obwohl er die verschobenen Niveaus der Anreicherung von Verteidigungschemikalien nach dem Auslösen nicht kompensiert.

In Nicotiana spp. Wird die Nikotininduktion durch einen durch *M. sexta* ausgelösten Ethylenstoß gedämpft, und die Blockierung von Ethylenrezeptoren vor *M. sexta* erhöhte die Nikotinproduktion in EV-Pflanzen auf irTOC1-Werte, während Unterschiede in der Phenolamid-Akkumulation zwischen EV und irTOC1 auftraten nicht ändern. Da sich Phenolamide weitgehend in Blättern anreichern, während Nikotin in Wurzeln synthetisiert wird, führte ich Mikrotransplantationsexperimente unter Feldbedingungen durch, bei denen die reine *TOC1*-Stummschaltung zu erhöhten Nikotin- und EV-Gehalten von Phenolamiden führte. Als letzten Funktionstest zeigten allometrische Messungen, dass die höheren Nikotingehalte in den irTOC1-Wurzeln nicht ausreichten, um die Umwandlung von Biomasse in reproduktive Korrelate zu verringern.

Zusammengenommen deuten die Ergebnisse dieser Arbeit auf die Notwendigkeit hin, die Rolle der einzelnen circadianen Uhrenkomponenten unter natürlichen Bedingungen genauer zu untersuchen, um ihren Anpassungswert besser zu verstehen. Die mechanistische Aufklärung hat zu vielen der wichtigsten zirkadianen Regulatoren von Pflanzenstressreaktionen geführt. Diese Erkenntnisse sollten genutzt werden, um gezielte funktionelle Assays zu generieren, mit denen wir besser verstehen, wie sich Pflanzen an veränderte Umgebungen anpassen.

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Zong, Y., Wang, Y., Li, C., Zhang, R., Chen, K., Ran, Y., Qiu, J.L., Wang, D., & Gao, C. (2017). Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. *Nature Biotechnology 35*(5):438-440. doi: 10.1038/nbt.3811

Züst, T., & Agrawal, A. A. (2017). Trade-Offs Between Plant Growth and Defense Against Insect Herbivory: An Emerging Mechanistic Synthesis. *Annual Review of Plant Biology*, *68*(1), 513–534. https://doi.org/10.1146/annurev-arplant-042916-040856

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, Henrique Valim

Bestätigung des Betreuers

Der Betreuer ist über die Einreichung der Dissertation informiert. Der Doktorand hat die Voraussetzungen für publikationsbasierte Dissertationen erfüllt.

Jena, Prof. Dr. Ian T. Baldwin

Curriculum Vitae

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Born 16th October 1991 in Araras, SP, Brazil
Nationality: Brazil/USA

EDUCATION

Doctoral thesis May 2015-Present
Friedrich Schiller University, Jena, Germany
Max Planck Research School for the Exploration of Ecological Interactions with Molecular and Chemical Techniques (IMPRS), Jena, Germany
Supervisors: Prof. Dr. Ian T. Baldwin, Prof. Dr. Nicole van Dam, Prof. Dr. Meredith C. Schuman

Fulbright Research Fellow September 2014-July 2015
Institute of International Education
Max Planck Institute of Chemical Ecology, Jena, Germany
Supervisors: Prof. Dr. Ian T. Baldwin, Prof. Dr. Meredith C. Schuman

Bachelor of Arts, Natural Sciences August 2010-May 2014
Fordham University, New York, NY, USA
Concentration in Molecular Biology
Minor in Bioinformatics
G.P.A. 3.650/4.000

- Departmental Honors
- *cum laude*
- *in cursu honorum*

IB Diploma August 2006-May 2010
Haines City High School
Haines City, FL, USA
Total: 29 points

RESEARCH EXPERIENCE

Doctoral Thesis September 2014-July 2015

Max Planck Institute of Chemical Ecology, Jena, Germany

Advisors: Prof. Dr. Ian T. Baldwin, Prof. Dr. Nicole van Dam, Prof. Dr. Meredith C. Schuman

- Dissertation: "Long-term effects of the circadian clock on plant fitness in the face of abiotic and biotic stress"

Fulbright Research Fellow September 2014-July 2015

Max Planck Institute of Chemical Ecology, Jena, Germany

- Investigation of the effect of the circadian clock gene *TOC1* on plant-plant competition and growth in root and shoot tissues through implementation of micrografting techniques
- Effect of competition and herbivory stress on plants deficient in the circadian clock gene *TOC1*

Research Assistant Summer 2014

Steven J. Franks and Acer VanWallendael, Fordham University, New York, NY

- Field study on the effects of Japanese barberry (Ranunculales: Berberidaceae) removal on deer tick (*Ixodes scapularis*, Acari: Ixodidae) abundances in Westchester, NY, USA
- Analysis of glyphosate tolerance in Japanese knotweed (*Reynoutria japonica*) in New York by shikimic acid spectrometric assay

Research Assistant Summer 2013-Summer 2014

Frank D. Hsu, Fordham University, New York, NY

- Combination of two visual cognition systems using Combinatorial Fusion
- Data-gathering from human participants, application of Combinatorial Fusion framework and statistical analysis, as well as examination of relevant literature

Research Assistant Summer 2013- Fall 2013

Xiaoxu Han, Fordham University, New York, NY

- Analysis of next-generation sequencing (NGS) data from a variety of different cancers, including prostate, kidney, and liver, using MATLAB and Cytoscape

Student Ambassador Summer 2013

International Conference on Cybersecurity 2013

International Conference on Cognitive Informatics and Cognitive Computing 2013

Fordham University, New York, NY

- Assisted with IT-related issues for speakers and general organization and execution of the conference

Research Assistant Summer 2012
Rui Martinho, Early Fly Development Lab
Instituto Gulbenkian de Ciˆencia, Oeiras, Portugal

- Three separate projects looking at splicing defects in *Drosophila melanogaster* early development

Research Assistant Summer 2011
Jason Morris, Fordham University, New York, NY

- Identification of the mutation *fried* as the gene *CG31320* in *Drosophila melanogaster*

TEACHING EXPERIENCE

Supervisor, May 2019-July 2019
Max Planck Institute of Chemical Ecology, Jena, Germany

- Co-supervision of 2 interns a project entitled: MAGIC – a forward genetics approach to dissect ecologically functional phenotypic variation in *Nicotiana attenuata*
- Teaching one-day courses: introduction to R, introduction to QTL mapping, data visualization in R

Supervisor, May 2018-July 2018
Max Planck Institute of Chemical Ecology, Jena, Germany

- Co-supervision of 12 interns for a large-scale foward genetics screen of a MAGIC-RIL population
- Teaching one-day courses: introduction to R, introduction to QTL mapping, data visualization in R

COURSES ATTENDED

1. Mar 2017 Grant Proposal Writing, speaker: Dr. Sabine Preusse
2. Sep 2016 Black Forest Summer School 2016: To See the (Black) Forest for the Trees. NGS Data for Phylogenetics
3. Dec 2015 Data Visualization Workshop, speaker: Dr. Rick Scavetta
4. Oct 2015 Scientific Writing, speaker: Brian Cusack/Science Craft
5. Nov 2014 Ecological interactions and evolutionary game theory, Christian Kost

LANGUAGE SKILLS

Portuguese: Native speaker
English: Fluent
German: Advanced speaking and reading, moderate writing
Spanish: Moderate speaking, reading and writing

Jena, Henrique Valim

List of publications

Scientific articles

Valim, H., Dalton, H., Joo, Y., McGale, E., Halitschke, R., Gaquerel, E., Baldwin, I.T* and Schuman, M.C.* (in preparation) *TOC1* in *Nicotiana attenuata* regulates efficient production of nitrogen-rich defense metabolites under herbivory stress. (*co-corresponding authors)

McGale, E.*, Valim, H.*, Halitschke, R., Baldwin, I.T. (submitted to *Plant, Cell & Environment*) Early developmental transitions influence drought resistance strategies and confound the quantification of genotypic diversity in drought responses. (*equal contribution)

McGale, E., Valim, H., Mittal, D., Morales Jiminez, J., Halitschke, R., Schuman, M.C., Baldwin, I.T. (in review) Determining the scale at which variation in WUE traits changes population yield. Submitted to *eLife.*

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. (*co-corresponding authors)

Schuman, M. C., Valim, H., Joo, Y. (2015). "Temporal dynamics of plant volatiles: mechanistic bases and functional consequences." In J. Blande, R. Glinwood (Eds.), *Deciphering Chemical Language of Plant Communication.* (pp. 3-34). Cham: Springer International Publishing. doi:10.1007/978-3-319-33498-1_1.

Valim, H., Clemens, M., Hsu, D.F. "Joint decision-making on two visual perception systems." *2014 IEEE Symposium on Computational Intelligence, Cognitive Algorithms, Mind, and Brain (CCMB),* Orlando, FL, 9-12 Dec. 2014, pp.92-99 .

Presentations

Valim, H. (2019) “Investigating developmental and circadian stress responses in *Nicotiana attenuata*.” Institute Symposium, Max Planck Institute for Chemical Ecology, Jena, DE

Valim, H. (2018) "*TOC1* function in shoots, but not roots, mediates *Nicotiana attenuata's* drought responses in nature," 17th IMPRS Symposium, International Max Planck Research School, Dornburg, DE

Valim, H. (2015) "How does variation in the core circadian clock mechanism alter lifestyle choices in *Nicotiana attenuata*?" iDiv Annual Conference, Leipzig, DE

Valim, H. (2014) "Joint decision-making on two visual perception systems," 2014 IEEE Symposium on Computational Intelligence, Cognitive Algorithms, Mind, and Brain, Orlando, FL, USA

Posters

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," Institute Symposium, MPI-CE, Jena, DE

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," SAB Meeting 2016, MPI-CE, Jena, DE

VanWallendael, A., Valim, H., Franks, S.J. (2015) "Evidence for evolution in glyphosate resistance, but not tolerance, based on history of exposure to the herbicide in Japanese Knotweed (*Reynoutria japonica*) in New York," 2015 Ecological Society of America Annual Meeting, Baltimore, MD, USA

Valim, H., Guilgur, L., Martinho, R.G. (2013) "Genetics of Drosophila zygotic transcription," Fordham University Spring Research Fair, Fordham University, New York, NY, USA

Seoane, K., Valim, H., Morris, J. (2012) "Idetification of CG31320 as the gene fried," Eastern Colleges Science Conference, William Paterson University, Wayne, NJ

Seoane, K., Valim, H., Morris, J. (2012) "Idetification of CG31320 as the gene fried," Drosophila Research Conference, Chicago, IL, USA

Jena, Henrique Valim