



# Natural variation in linalool metabolites: One genetic locus, many functions?<sup>oo</sup>

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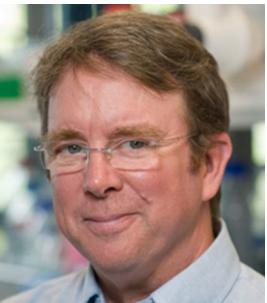
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## SUMMARY

The ubiquitous volatile linalool is metabolized in plants to nonvolatile derivatives. We studied *Nicotiana attenuata* plants which naturally vary in (S)-(+)-linalool contents, and lines engineered to

produce either (R)-(-)- or (S)-(+)-linalool. Only (S)-(+)-linalool production was associated with slower growth of a generalist herbivore, and a large fraction was present as nonvolatile derivatives. We found that variation in volatile linalool and its nonvolatile glycosides mapped to the same genetic locus which harbored the biosynthetic gene, *NaLIS*, but that free linalool varied more in environmental responses. This study reveals how (S)-(+)-linalool and conjugates differ in their regulation and possible functions in resistance.

Keywords: conjugated linalool, *Nicotiana attenuata*, *Spodoptera littoralis*

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## INTRODUCTION

Plant volatiles emitted into the headspace may convey important information from the plant to other organisms including neighboring plants and approaching animals (Baldwin, 2010; Loreto et al., 2014). Some volatiles can be metabolized to nonvolatile derivatives and stored in tissues. Linalool, a volatile monoterpenoid alcohol, mediates interactions between plants and pollinators, herbivores, carnivores, and microbes (Raguso, 2016). Linalool can be further metabolized to nonvolatile conjugates (Lücker et al., 2001; Aharoni et al., 2003; Yang et al., 2013). The formation of linalool oxides, glycosides and other derivatives has been reported from many organisms, but their function has rarely been investigated. In *Arabidopsis thaliana*, two P450 enzymes convert linalool to its oxygenated forms which can

be further metabolized (Ginglinger et al., 2013). These derivatives accumulate in floral tissues and can deter both floral visitors and attackers (Ginglinger et al., 2013; Boachon et al., 2015). Overexpressing the linalool/nerolidol synthase *FaNES1* in *Chrysanthemum morifolium* resulted in initial attraction of the herbivore *Frankliniella occidentalis* to volatile linalool, but later deterrence of the same herbivore by nonvolatile components (Yang et al., 2013).

Previously, we found that certain genotypes of the wild tobacco *Nicotiana attenuata* emit (S)-(+)-linalool from leaves upon herbivory, which increases predation by natural enemies of the specialist herbivore *Manduca sexta*, but has a subtler, context-dependent effect on the oviposition by adult *M. sexta* on the plant (Kessler and Baldwin, 2001; He et al., 2019). Here, we show that a very large fraction of the linalool produced by *N. attenuata* is metabolized to nonvolatile

glycosides. While the increased production of linalool does not affect the growth of specialist herbivore *M. sexta* larvae (He et al., 2019), here we found that it seems to slow the growth of the generalist herbivore *Spodoptera littoralis*. Given these apparently contrasting functions in resistance, we take a closer look at nonvolatile linalool metabolites in *N. attenuata* and investigate the regulation of free versus conjugated linalool, using an advanced intercross-recombinant inbred line (AI-RIL) population and plants engineered to ectopically express specific linalool enantiomers.

## RESULTS AND DISCUSSION

We observed that *N. attenuata* plants of an AI-RIL population in a field plot at the Walnut Creek Center for Education (Prescott National Forest, Arizona, USA) more frequently bore damage typical of Noctuidae larvae than other types of herbivores (Figure 1A, B). We previously used the same AI-RIL population to identify a locus controlling the emission of (S)-(+)-linalool from *N. attenuata*, which was associated with the removal of eggs and larvae of the specialist herbivore *M. sexta* from plants by naturally occurring predators (He et al., 2019). Ectopic expression of linalool synthase genes specific for either the endogenous (S)-(+)-, or the foreign (R)-(-)-enantiomer of linalool did not influence the growth of specialist *M. sexta* larvae (He et al., 2019). Here, we tested whether the ectopic enhancement of linalool production affects resistance to *S. littoralis* as a representative of the noctuid herbivores feeding on wild tobacco in the field in Arizona (Figure 1B). We used ectopic expression in both genetic backgrounds used to generate the AI-RILs: AZ collected from Arizona and UT collected from Utah (Baldwin et al., 1994; Glawe et al., 2003). In both backgrounds, the ectopically enhanced production of (S)-(+)-linalool reduced the growth of *S. littoralis* larvae while ectopic (R)-(-)-linalool production did not affect larval growth (Figures 1C, S1).

Using ultra-high performance liquid chromatography – tandem mass spectrometry, we detected several compounds which specifically accumulated in ectopic expression plants compared to the corresponding wild-type. Molecular ions and fragmentation patterns suggested that these compounds represent oxygenated monoterpenoid glycosides (Figure 1D; Table S1), including a putative linalool glucoside ( $C_{16}H_{28}O_6$ ;  $M = 316.19$ ), linalool malonyl-glucoside ( $C_{19}H_{30}O_9$ ;  $M = 402.19$ ), linalool pentosyl-hexoside ( $C_{21}H_{36}O_{10}$ ;  $M = 448.23$ ), linalool di-glucoside ( $C_{22}H_{38}O_{11}$ ;  $M = 478.24$ ), and the hydroxylated forms of the first three compounds. Several additional differential mass features were not clearly identified for lack of sufficient spectral information or suggested structures (Figure 1E; Table S2). The detailed information for annotation of these compounds is provided in supplementary material.

We used  $\beta$ -glucosidase treatment and chiral gas chromatography mass spectrometry to quantify linalool released from glycosylated conjugates in crude leaf tissue extracts of *N. attenuata* plants as done by Lücker et al. (2001). Glycosides of the

two linalool enantiomers accumulated specifically in AZ and UT plants with constitutive ectopic expression of either an (R)-(-)-linalool synthase gene from *Ocimum basilicum* (*ObLIS*; Iijima et al., 2004) or an (S)-(+)-linalool synthase gene from *Clarkia breweri* (*CbLIS*; Pichersky et al., 1995) (Figure 1F). Notably, AZ-*ObLIS* plants produced glycosides of both enantiomers, indicating that both native and ectopically expressed LIS enzymes were active and that endogenous metabolic enzymes could modify the foreign (R)-(-)-enantiomer. In contrast, in UT-*ObLIS* plants, the endogenous (S)-(+)-linalool was hardly detected while ectopic (R)-(-)-linalool was abundant (Figure 1F), as expected for this genotype which has a less active form of the *N. attenuata* LIS (He et al., 2019). We calculated the proportion of glycosylated/headspace linalool originating from the two enantiomers in the two AZ-*ObLIS* lines (Figure 1G). We found that a larger fraction of (S)-(+)-linalool was glycosylated in both lines, and thus a larger portion of (R)-(-)-linalool was emitted into the headspace. This partially explained our previous finding that plants expressing *ObLIS* emitted much more linalool than plants expressing *CbLIS* (He et al., 2019).

Subsequently, we asked to what extent there is natural variation in the endogenous (S)-(+)-linalool conjugates and how these vary with volatile linalool in *N. attenuata* accessions. We measured headspace linalool, and linalool released after glucosidase treatment, in the two natural accessions UT and AZ, respectively. We found that the sum of headspace and glycosylated linalool in AZ was about 54-fold that in UT plants (Figure 2A). However, in both accessions, linalool released after  $\beta$ -glucosidase treatment comprised more than 97% of the total linalool measured (Figure 2A). Next, we measured linalool released after glucosidase treatment of tissue from field-grown plants of the AI-RIL population developed from crossing UT and AZ (Figure 2B). We observed substantial variation, spanning over four orders of magnitude, among these RILs, which is considerably wider than that between the two parental lines, in amounts of linalool released by the  $\beta$ -glucosidase treatment. These differences were strongly correlated with previous measurements of headspace linalool (Figure 2B), consistent with the correlation observed in natural accessions of *N. attenuata*, although the RIL headspace data were collected from an independent experiment in which the plants were grown in a glasshouse (He et al., 2019). Subsequently, we mapped variation in the linalool glycosides as measured by  $\beta$ -glucosidase treatment to a quantitative trait locus (QTL) on chromosome VIII of *N. attenuata*. This QTL was mapped to a ~316 KB locus harboring four putative genes annotated to encode a pectin lyase, an unknown protein, *NaLIS*, and a Zinc finger protein, respectively (Figure 2C). *NaLIS* has been previously identified as the genetic factor controlling the variation of headspace linalool (He et al., 2019). Consistently, when we suppressed *NaLIS* expression using virus-induced gene silencing (VIGS), linalool glycoside contents as measured by  $\beta$ -glucosidase treatment were also reduced by about 80% (Figure 2D), even more than the ca. 53% reduction of headspace linalool previously reported (He et al., 2019).

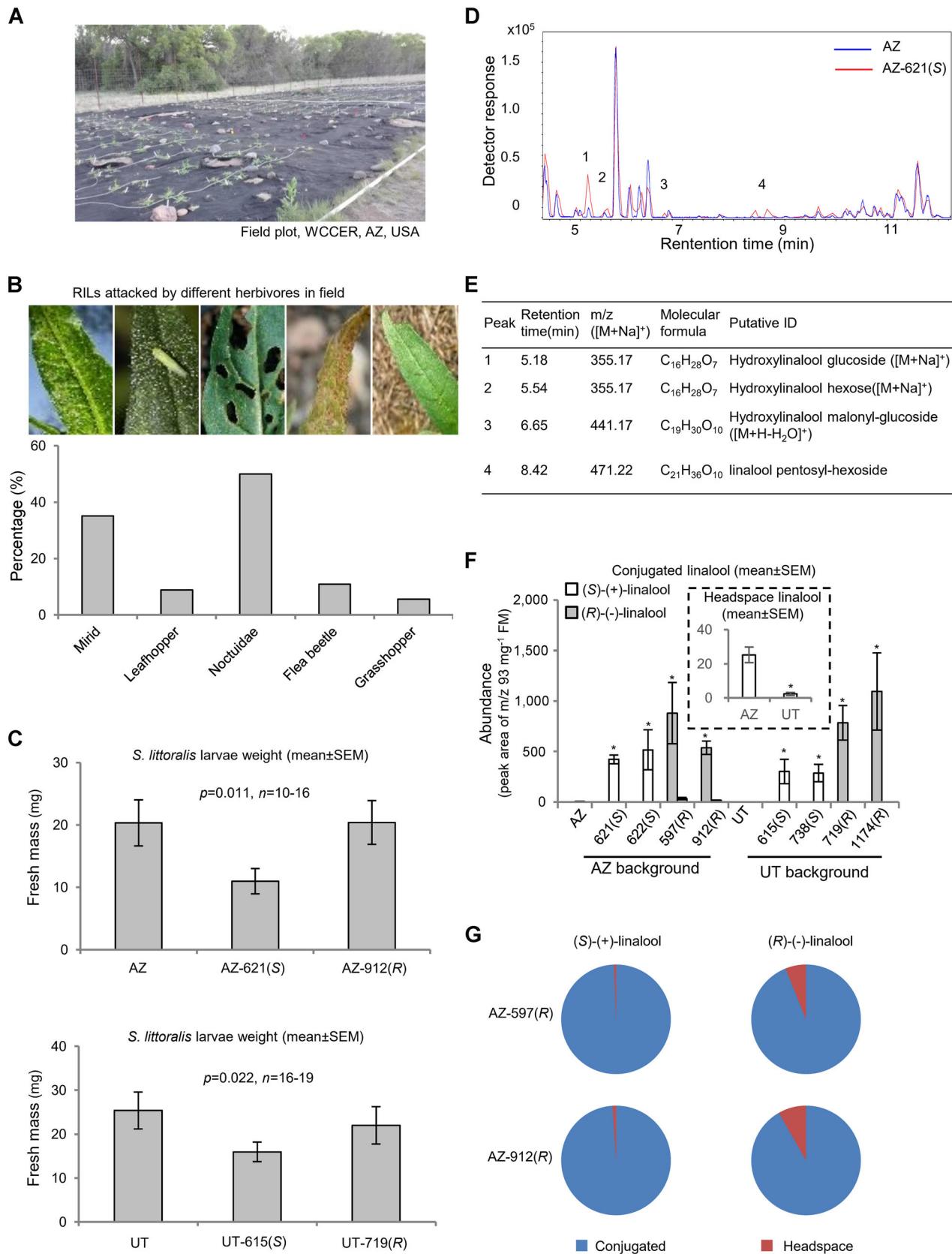


Figure 1. Continued

We further tested whether internal tissue stores of free linalool were co-regulated with linalool glycosides as measured by  $\beta$ -glucosidase treatment under different environmental conditions. Interestingly, as previously described for headspace linalool (Halitschke et al., 2000; He et al., 2019), the internal free linalool was much more concentrated during midday than in the early morning, but concentrations of linalool glycosides did not vary between these times of day (Figure 2E). Similarly, internal free linalool was suppressed by abscisic acid (ABA) treatment but linalool glycosides were not altered (Figure 2F). The regulation of free linalool under these conditions might occur directly through the diurnal transcriptional regulation of *NaLIS*, the upstream biosynthetic gene *NaGPPS1*, and ABA signaling (He et al., 2019). However, activity of the enzymes involved in glycosylation might buffer the responses in glycoside accumulation, or the relatively larger pools of glycosides may not be immediately responsive to changes in linalool biosynthesis.

In this study, we showed that ectopic expression of *CbLIS* and *ObLIS* not only boosted the specific enantiomers in the headspace, but also increased abundance of the corresponding linalool and hydroxylinalool glycosides (Figure 1E; Tables S1, S2). Linalool malonyl-glucoside, linalool pentosyl-hexoside and hydroxylinalool hexose were also detected in nonvolatile extracts from *Chrysanthemum morifolium* plants expressing the *FaLIS* gene, and hypothesized to deter thrips (Yang et al., 2013). Ectopic expression of *FaLIS* in *Arabidopsis* produced linalool, hydroxylated and glycosylated linalool derivatives, and retarded plant growth and development, while repelling aphids (Aharoni et al., 2003). Endogenous free linalool is hydroxylated or epoxidized by a set of cytochrome P450 oxygenases in flowers of *Arabidopsis* plants (Ginglinger et al., 2013; Boachon et al., 2015), and this oxidation is essential for defense against floral antagonists (Boachon et al., 2015). Recent work in *N. attenuata* has shown that further hydroxylations of both 17-hydroxygeranylinalool and 17-hydroxygeranylinalool diterpene glycosides are responsible for their autotoxicity in plants and their ability to reduce *M. sexta* growth after ingestion, respectively, by inhibiting sphingolipid biosynthesis (Li et al., 2021). Based on the similarities in structure and metabolic pathways between linalool and geranylinalool derivatives, a similar or shared mechanism might operate for

both. However, ectopic expression of *CbLIS* did not affect growth of *M. sexta* neonates feeding on *N. attenuata* plants, nor were there indications of plant autotoxicity (He et al., 2019). In contrast, we observed an effect on *S. littoralis* larvae (Figure 1), but not if experiments were started with larger larvae ( $>2.2$  mg) (Figure S1). We note that the function of volatile linalool as an indirect defense also primarily impacts early stage larvae of *M. sexta* via *Geocoris* bug attraction (Kessler and Baldwin, 2001; He et al., 2019). Taken together, linalool and its derivatives might be most hazardous to lepidopterans in the neonate stage. To determine how linalool derivatives function in plant direct defense against herbivores, their structure, bio-activities *in planta* and in herbivores, and the involvement of cytochrome P450 and glucosyltransferase should be investigated.

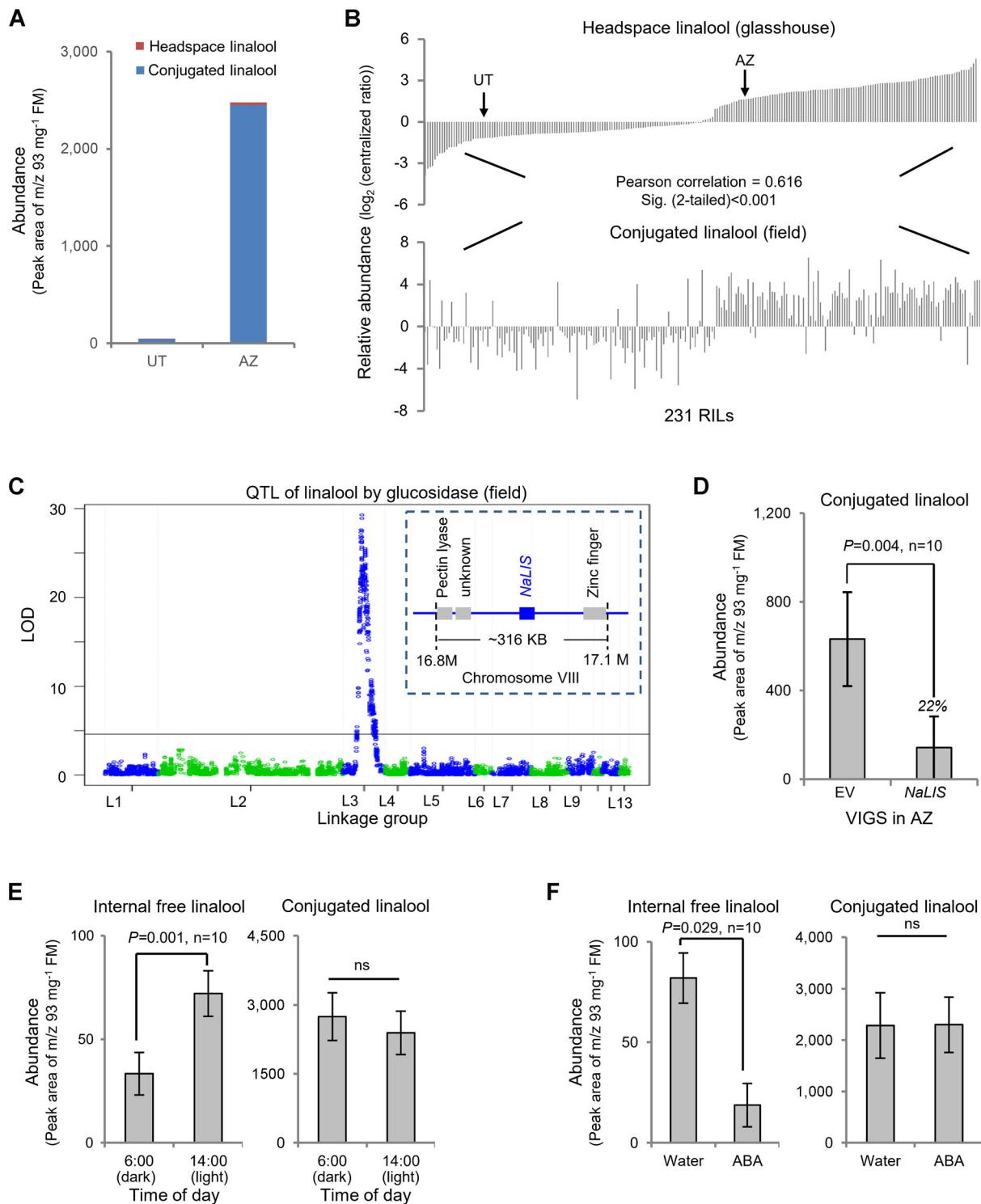
Linalool and linalool derivatives may serve *N. attenuata* plants as defensive compounds in nature. Some natural accessions of *N. attenuata* produce as much linalool as ectopic lines used in this study (He et al., 2019). Previously we found that variation in headspace linalool among *N. attenuata* accessions, including the UT and AZ accessions, is mapped to a single *NaLIS* gene responsible for the biosynthesis of (S)-(+)-linalool (He et al., 2019). In this study, we reveal that the same gene also provides the strongest statistical explanation for the variation in linalool glycoside accumulation (Figure 2C, D). No other QTL for linalool glycosides were identified in the AI-RIL population. It thus seems that production of free linalool by *NaLIS* as a substrate is the limiting step for the production of linalool glycosides. However, we found that the volatile and free forms of linalool were much more responsive to environmental factors whereas the nonvolatile conjugates accumulated in more stable tissue pools. Taken together with our recent work (He et al., 2019), this study indicates that linalool can serve both as a volatile signal and as part of nonvolatile resistance compounds, and that these two functions can be separately regulated in response to the environment, even though they are under the control of a common genetic locus.

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**Figure 1. Ectopic expression of an (S)-(+)-linalool but not an (R)-(-)-linalool synthase gene enhanced resistance in *Nicotiana attenuata* against the noctuid generalist *Spodoptera littoralis* while both increased plant tissue concentrations of conjugated linalool**

(A) The field plot at the Walnut Creek Center for Education and Research, Arizona, USA. (B) Noctuid-typical damage was the most frequently observed damage to a *N. attenuata* population grown in the field in the summer of 2017. Pictures of damages typical for the different herbivores are shown above the bar chart (photographs by D. Kessler). (C) Ectopic expression plants with increased (S)-(+)-linalool but not (R)-(-)-linalool conjugates hindered the growth of a noctuid generalist, *S. littoralis* larvae. Statistics: one-factor analysis of variance. (D) Representative liquid chromatography – mass spectrometry chromatograms of leaf extracts of wild-type and ectopic expression plants. Putative linalool conjugates (1–4) are indicated. (E) Compounds specifically accumulated in ectopic expression plants compared to wild-type plants were putatively identified based on the high-resolution mass spectra. Compound lists are shown in Tables S1 and S2. (F) Linalool enantiomers were quantified by chiral gas chromatography – mass spectrometry after  $\beta$ -glucosidase treatment of the leaf extracts. Ectopic expression plants accumulated higher levels of conjugated linalool enantiomers. The inserted figure shows the headspace linalool in the two background genotypes. \**t*-test,  $P < 0.01$ ,  $n = 3$ , compared with the corresponding wild-type or between the two wild-types. (G) (S)-(+)-linalool had a smaller conjugated ratio than (R)-(-)-linalool.



**Figure 2. Conjugated and headspace linalool are mapped to the same genetic locus but are differentially regulated over time of day and by exogenous abscisic acid (ABA) applications**

(A) Conjugated linalool varies between *Nicotiana attenuata* accessions from Utah (UT) and Arizona (AZ), and accounts for a larger portion of the total linalool pool than does headspace linalool in each accession. (B) Linalool released by  $\beta$ -glucosidase treatment is highly correlated with headspace linalool measurements within an intercross-recombinant inbred line (Al-RIL) population developed from a cross of UT and AZ. The data were collected from independent experiments in glasshouse (headspace) or field (glycosides). (C) Variation in glycosylated linalool was mapped to a quantitative trait locus in linkage group L3, which accounts for a ~316 KB genetic locus containing a previously identified linalool synthase gene (*NaLIS*) on chromosome VIII of the genome of *N. attenuata*. (D) Silencing *NaLIS* expression using virus-induced gene silencing led to reduction of linalool released from tissues by  $\beta$ -glucosidase treatment. (E, F) Concentrations of free linalool were lower in early morning and higher at midday, and were downregulated by ABA treatment, while conjugated linalool was not. ns, non-significant.

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## AUTHOR CONTRIBUTIONS

R.H., I.T.B., and M.C.S planned and supervised the project; J.H. performed the experiments; J.H., R.H., I.T.B., and M.C.S. designed the experiments; J.H. and R.H. analyzed the data; J.H. wrote the original draft, and all authors revised and finalized the manuscript. All authors read and approved of the article. Open Access funding enabled and organized by Projekt DEAL.

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## SUPPORTING INFORMATION

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**Figure S1.** Growth of *Spodoptera littoralis* larvae feeding on additional ectopic expression lines

These experiments are independent from those shown in Figure 1C and were conducted with larvae having a larger starting mass (20 caterpillars with overall weight of  $44 \pm 0.2$  mg).

**Table S1.** Putative linalool derivatives with differential accumulation in LIS expression lines

**Table S2.** Differential mass features without sufficient spectral information or suggested structures

**Material and Methods**