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Research Article

Silencing Brassinosteroid Receptor *BRI1* Impairs Herbivory-elicited Accumulation of Jasmonic Acid-isoleucine and Diterpene Glycosides, but not Jasmonic Acid and Trypsin Proteinase Inhibitors in *Nicotiana attenuata*

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Abstract

The brassinosteroid (BR) receptor, BR insensitive 1 (BRI1), plays a critical role in plant development, but whether BRI1-mediated BR signaling is involved in plant defense responses to herbivores was largely unknown. Here, we examined the function of BRI1 in the resistance of *Nicotiana attenuata* (Solanaceae) to its specialist insect herbivore *Manduca sexta*. Jasmonic acid (JA) and JA-isoleucine conjugate (JA-Ile) are important hormones that mediate resistance to herbivores and we found that after wounding or simulated herbivory NaBRI1 had little effect on JA levels, but was important for the induction of JA-Ile. Further experiments revealed that decreased JAR (the enzyme for JA-Ile production) activity and availability of Ile in *NaBRI1*-silenced plants were likely responsible for the low JA-Ile levels. Consistently, *M. sexta* larvae gained more weight on *NaBRI1*-silenced plants than on the control plants. Quantification of insect feeding-induced secondary metabolites revealed that silencing *NaBRI1* resulted in decreased levels of carbon-rich defensive secondary metabolites (hydroxygeranyllinalool diterpene glycosides, chlorogenic acid, and rutin), but had little effect on the nitrogen-rich ones (nicotine and trypsin proteinase inhibitors). Thus, NaBRI1-mediated BR signaling is likely involved in plant defense responses to *M. sexta*, including maintaining JA-Ile levels and the accumulation of several carbon-rich defensive secondary metabolites.

Keywords: BRI1; herbivore; insect; jasmonic acid; *Nicotiana attenuata*; secondary metabolites.

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Introduction

To cope with various adverse environmental conditions, plants use complex signaling systems, including those mediated by phytohormones, to regulate proper responses. These phytohormones, which are involved in almost every aspect of a plant's life, include auxin, gibberellins, brassinosteroids (BRs), cytokinins, ethylene, abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), and strigolactones (Chow and McCourt 2006; Santner et al. 2009). Recent progress on the biosynthesis, transport, degradation, signal transduction, and physiological functions of these phytohormones have greatly advanced our understanding of the molecular mechanisms by which plants regulate their growth, development, and resistance to

environmental stresses. Beside their individual functions, it has become evident that complex synergistic and antagonistic interactions among these phytohormones fine-tune plant physiology (Chow and McCourt 2006; Grant and Jones 2009; Peng et al. 2011).

Among these hormones, jasmonic acid (JA) and its conjugate JA-isoleucine (JA-Ile) play key roles in mediating plant resistance to herbivores (Wasternack 2007; Howe and Jander 2008; Browse 2009; Wu and Baldwin 2009, 2010). Jasmonic acid is synthesized through an oxylipin pathway, which includes 13-lipoxygenase (13-LOX), allene-oxide synthase (AOS), allene-oxide cyclase (AOC), OPDA reductase (OPR), and acyl-coenzyme A oxidase (ACX) (Liechti and Farmer 2002; Wasternack 2007). Importantly, instead of JA, JA-Ile was found to elicit most of JA-induced responses (Staswick et al. 2002; Wang et al. 2008a). In *Arabidopsis*, jasmonate resistance 1 (JAR1) protein conjugates JA with isoleucine to form JA-Ile (Staswick et al. 2002). JA-Ile is perceived by the F-box protein coronatine insensitive protein 1 (COI1) complex (SCF^{COI1}) to promote the ubiquitination and degradation of Jasmonate-ZIM (JAZ) proteins, which suppress JA-induced responses; degradation of JAZ proteins releases the transcription factor MYC2 and thus activates MYC2-mediated transcriptional responses (Xie et al. 1998; Chini et al. 2007; Thines et al. 2007; Yan et al. 2009).

The ecological significance of JA and its signaling has been intensively studied in *Nicotiana attenuata-Manduca sexta* interactions. *N. attenuata* is a wild tobacco that inhabits the desert of Utah (USA) (Halitschke and Baldwin 2003; Paschold et al. 2007). Among the herbivores of *N. attenuata*, the specialist insect *M. sexta* causes heavy damages. *N. attenuata* plants have evolved sophisticated defense strategies against *M. sexta* feeding: *N. attenuata* plants perceive fatty-acid amino acid conjugates (FACs) in the oral secretions (OS) of *M. sexta*, which are introduced into the wounds during feeding (Halitschke et al. 2001; Wu et al. 2007) and rapidly activate the biosynthesis of JA and JA-Ile; these hormones in turn activates transcriptional reprogramming and the biosynthesis of defensive secondary metabolites such as nicotine, trypsin proteinase inhibitors (TPIs), hydroxygeranyllinalool diterpene glycosides (HG-DTGs), chlorogenic acid (CGA), and rutin (Halitschke and Baldwin 2003; Paschold et al. 2007). Silencing *LOX3, AOS* (JA biosynthesis) or *JAR4*/*6* (JA-Ile biosynthesis) in *N. attenuata* results in reduced JA or JA-Ile levels and in turn decreased anti-herbivore secondary metabolites and resistance to *M. sexta* (Halitschke and Baldwin 2003; Halitschke et al. 2004; Wang et al. 2007; Wang et al. 2008a; Wu et al. 2008). Silencing JA-Ile receptor *COI1* also highly impairs HG-DTGs and TPI levels, which makes *N. attenuata* plants very susceptible to *M. sexta* larvae (Paschold et al. 2007). All these data highlight the key roles of JA/JA-Ile, JA signaling, and JA-mediated anti-herbivore defense secondary metabolites in *N. attenuata* resistance to *M. sexta* (Howe and Jander 2008; Wu and Baldwin 2010).

Brassinosteroids are a class of steroid phytohormones that regulate many aspects of plant growth and development including seed germination, vascular differentiation, leaf expansion, apical dominance, and stem and root elongation (Clouse 2002a, 2002b; Halliday 2004; Karlova and de Vries 2006; Zhao and Li 2012). BR insensitive 1 (BRI1), a cell membrane-located leucine rich repeat-receptor kinase (LRR-RK), was identified as the BR receptor by genetic and biochemical approaches (Li and Chory 1997; Wang et al. 2001; Kinoshita et al. 2005). Another LRR-RLK, the somatic embryogenesis receptor kinase 3 (SERK3), also named BRI1-associated kinase 1 (BAK1), physically interacts with BRI1 and plays an essential role as an enhancer in BR signaling (Li et al. 2002; Nam and Li 2002). Upon perception of BR, BRI1 undergoes atuophosphorylation and also phosphorylates its substrates (such as BAK1) to activate downstream BR responses (Gendron and Wang 2007; Wang et al. 2008b; Kim and Wang 2010; Ye et al. 2011). Campos et al. (2009) reported that tomato BR biosynthesis-deficient mutant *dpy* has increased JA-inducible anti-herbivore secondary metabolite zingiberene and transcript levels of serine proteinase inhibitor *PI-I*, implying that BR signaling may influence plant defense responses against herbivores.

To study whether BRI1 is involved in the defense of *N. attenuata* against *M. sexta*, a virus-induced gene silencing (VIGS) approach was used to knock down *NaBRI1* transcript levels. We found that silencing *NaBRI1* compromised the availability of basal free Ile levels and likely JAR activity, which led to decreased herbivory-induced JA-Ile levels. *M. sexta* larvae gained more weight on *NaBRI1*-silenced plants than on control plants and quantification of anti-herbivore secondary metabolites revealed that *NaBRI1*-silenced plants had reduced levels of carbon-rich secondary metabolites, HG-DTGs, CGA, and rutin.

Results

Cloning of *NaBRI1* in *N. attenuata*

Based on *N. benthamiana NbBRI1* sequence (accession number: EF471738) (Holton et al. 2007), a 634 bp fragment of *NaBRI1* gene (accession number: EF623823) was cloned from the cDNA pool of *N. attenuata*. This partial *NaBRI1* sequence showed 98% identity to *NbBRI1* and *N. tabacum NtBRI1*. Using this cloned *NaBRI1* sequence and *NbBRI1* sequence as baits, we assembled a 2,697 bp fragment of *NaBRI1* opening reading frame (accession number: JX014434) from an *N. attenuata* transcriptome database prepared by 454 sequencing (Figure S1A). The deduced NaBRI1 protein sequence showed high homology to BRI1 proteins (98.8%, 98.7%, 91.1% and 73.5% homology to NbBRI1, NtBRI1, *Solanum lycopersicum* SlBRI1, and *Arabidopsis* AtBRI1 protein, respectively) (Figure S1B).

Simulated herbivory, but not wounding, specifically induces the accumulation of *NaBRI1* transcripts

To examine whether herbivory induces the accumulation of *NaBRI1* transcripts, *N. attenuata* rosette leaves were wounded with a pattern wheel and 20 μL of *M. sexta* OS were immediately applied to the wounds (W+OS); this treatment effectively induces herbivory-elicited responses in *N. attenuata* (Halitschke et al. 2001). Water (20 μL) was applied to wounds (W+W) as a comparison. The transcript levels of *NaBRI1* were measured using quantitative real-time polymerase chain reaction (qPCR) and gene-specific primers (**Figure 1**). *NaBRI1* transcripts increased 1.88- and 3.42-fold 1.0 h and 1.5 h after W+OS treatment and thereafter gradually decreased. In contrast, *NaBRI1* transcript levels did not change after W+W treatment (**Figure 1**). These data suggest that NaBRI1 is likely involved in the defense of *N. attenuata* against herbivores.

Silencing *NaBRI1* results in impaired herbivory-elicited accumulation of JA-Ile, but has little effect on JA

To study the function of NaBRI1 in *N. attenuata*, we knocked down *NaBRI1* transcript levels using a VIGS approach (Saedler and Baldwin 2004). A 359 bp fragment of *NaBRI1* cDNA was cloned into the pTV00 vector to form pTV-NaBRI1. Plants were inoculated with *Agrobacterium* carrying pTV-NaBRI1 to generate *NaBRI1*-silenced (hereafter, NaBRI1-VIGS) plants; plants inoculated with *Agrobacterium* carrying pTV00 empty vector were used as comparisons (hereafter, EV plants). To confirm the efficiency of gene silencing, *NaBRI1* transcript levels were quantified in EV and NaBRI1-VIGS plants using qPCR. In NaBRI1-VIGS plants, the transcript levels of NaBRI1 were at least 53% decreased (**Figure 2A**).

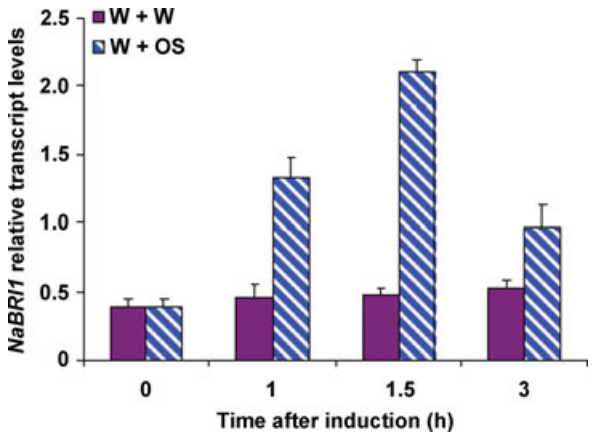

Figure 1. Wounding- and simulated herbivory-induced *NaBRI1* transcript levels.

Transcript levels of *NaBRI1* after wounding and simulated herbivory. Rosette leaves of empty vector (EV) and NaBRI1-virus-induced gene silencing (VIGS) plants were wounded with a pattern wheel and 20 μL 1 : 5 diluted oral secretion (OS) of *Manduca sexta* larvae were applied to the wounds (W+OS treatment) immediately. As a comparison, 20 μL of water were applied to the wounds (W+W). Relative expression levels of *NaBRI1* gene (mean ± *SE*) were quantified by quantitative real-time polymerase chain reaction (qPCR).

Compared with those of EV plants, the leaves of NaBRI1-VIGS plants were darker green and curly. Additionally, the NaBRI1-VIGS plants showed a dwarf phenotype similar to that of the tomato *curl3* (*cu3*) and *Arabidopsis bri1* mutants (Li and Chory 1997; Holton et al. 2007) (Figure S2). All experiments were performed on the rosette leaves of EV and NaBRI1-VIGS plants.

Next we examined whether NaBRI1 regulates wounding- and herbivory-induced JA and JA-Ile, two important phytohormones that mediate plant defense responses to herbivory. We quantified JA and JA-Ile levels in EV and NaBRI1-VIGS plants using a LC-MS/MS method. Before treatments, NaBRI1-VIGS and EV plants had similar JA levels and W+W and W+OS treatment did not induce different levels of JA in EV and NaBRI1-VIGS plants, except that 1.5 h after W+W NaBRI1-VIGS plants had about 42% less JA levels than those in EV plants (**Figure 2B**). In contrast, 1.0 h and 1.5 h after both W+W and W+OS treatment, NaBRI1-VIGS plants showed significantly reduced (34% and 28% decreased 1.0 and 1.5 h after W+OS, respectively; 29% and 61% decreased 1.0 h and 1.5 h after W+W, respectively) contents of JA-Ile, compared with those in EV plants (**Figure 2C**). These data suggest that NaBRI1 affects wounding- and herbivory-induced JA-Ile but has little effect on JA levels, and the decreased levels of wounding- and herbivory-induced JA-Ile was likely not due to the limitation of JA in NaBRI1-VIGS plants.

Both JAR activity and the availability of Ile likely limit herbivory-induced JA-Ile levels in *NaBRI1*-silenced plants

To study the possible reasons for the impaired JA-Ile levels in herbivore feeding-induced NaBRI1-VIGS plants, the transcripts of *JAR4* and *JAR6* (Wang et al. 2008a), which encode two important enzymes for conjugating JA with Ile to form JA-Ile, were measured by qPCR. When untreated, NaBRI1-VIGS plants had 25% lower *JAR4* transcripts than did EV plants. However, NaBRI1-VIGS plants accumulated 62% and 28% greater levels of *JAR4* transcripts, 1.0 h and 1.5 h after W+OS treatment, respectively; 1.0 h, 1.5 h, and 3.0 h after W+W treatment, NaBRI1-VIGS plants still exhibited approximately 69%, 92%, and 178% greater *JAR4* transcript levels than did EV plants, respectively (**Figure 3A**). For *JAR6*, NaBRI1-VIGS plants also had about 28% reduced basal *JAR6* transcripts. Although W+OS treatment did not induce any differences of

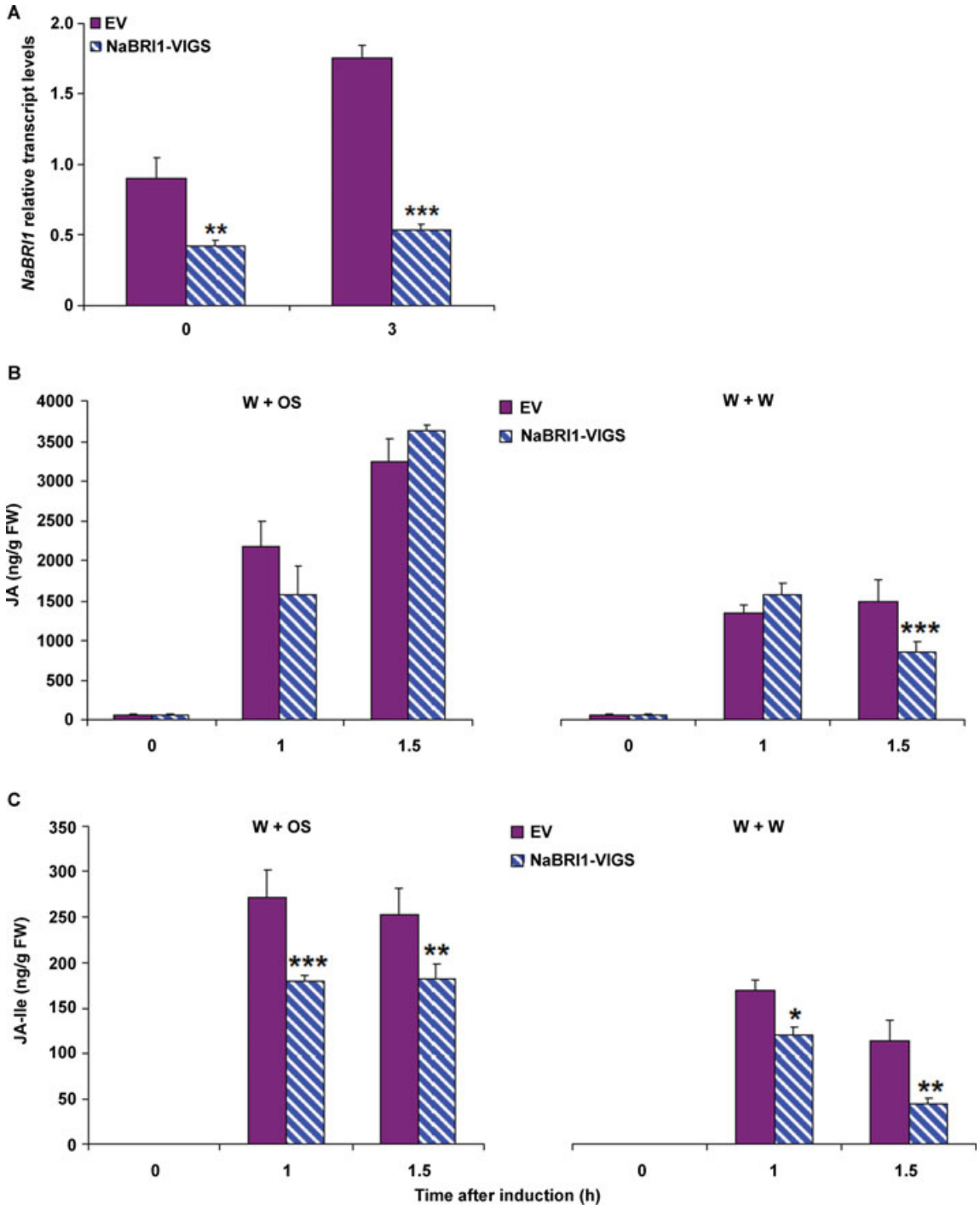

Figure 2. Jasmonic acid (JA) and JA-isoleucine conjugate (JA-Ile) levels in empty vector (EV) and NaBRI1-virus-induced gene silencing (VIGS) plants.

(A) Gene silencing of *NaBRI1*. *Nicotiana attenuata* plants were infiltrated with *Agrobacterium* carrying pTV00 and pTV-NaBRI1 to generate EV and NaBRI1-VIGS plants, respectively. Leaf materials were harvested at indicated times after W+OS treatment and the transcript levels of *NaBRI1* gene (mean ± *SE*) were measured. Stars indicate significant differences between EV and NaBAK1-VIGS plants.
(B) JA and **(C)** JA-Ile levels. Leaves of EV and NaBRI1-VIGS plants were wounded with a pattern wheel and 20 μL of water (W+W) or 1: 5 diluted *M. sexta* OS (W+OS) were applied to the wounds immediately. JA and JA-Ile levels (mean ± *SE*) were quantified on a LC-MS/MS. Stars indicate significant differences between EV and NaBAK1-VIGS plants (unpaired *t* test, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 5$).

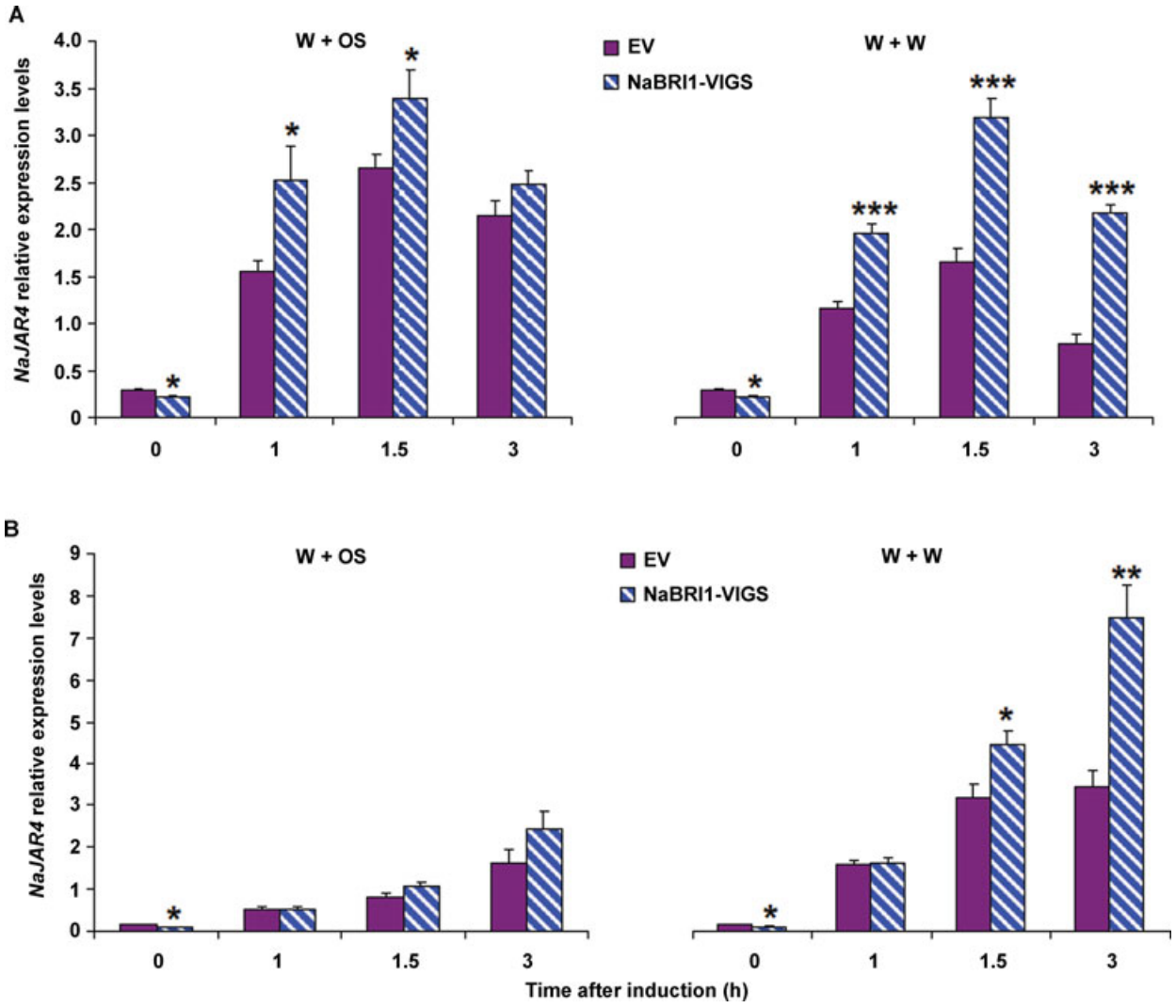

Figure 3. Herbivory- and wounding-induced *JAR* transcripts in empty vector (EV) and NaBRI1-virus-induced gene silencing (VIGS) plants.

Leaves of EV and NaBRI1-VIGS plants were wounded with a pattern wheel, and 20 μL of water (W+W) or 1 : 5 diluted *Manduca sexta* OS (W+OS) were applied to the wounds immediately. Transcript levels of *JAR4* **(A)** and *JAR6* **(B)** (mean ± *SE*) were measured in samples harvested at different times after the treatments. Stars indicate significant differences between EV and NaBAK1-VIGS plants (unpaired *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$).

JAR6 levels between EV and NaBRI1-VIGS plants, higher level of *JAR6* transcripts were detected in NaBRI1-VIGS plants 1.5 h and 3.0 h after W+W treatment (40% and 117% greater levels, respectively) (**Figure 3B**). These data indicate that the impaired JA-Ile levels were not due to reduced wounding- and herbivory-induced *JAR4* and *JAR6* transcripts.

To further examine whether JAR enzymatic activity and/or availability of substrate (Ile) limits the production of JA-Ile in NaBRI1-VIGS plants, we supplemented JA and/or Ile, that are needed for the formation of JA-Ile, to EV and NaBRI1-VIGS plant; namely, water (as control), Ile, or JA with Ile together, was added to wounds that were produced by W+W- and W+OS treatment (W+W+water, W+W+Ile, W+W+JA+Ile, W+OS+water, W+OS+Ile, W+OS+JA+Ile, respectively). When Ile was supplemented to W+W- and W+OS-treated plants, NaBRI1-VIGS plants showed 34% decreased JA-Ile compared with those in EV plants (**Figure 4**), suggesting that silencing BRI1 may have impaired the activity of JARs in *N. attenuata*. Consistently, when both JA and Ile were added to wounds generated by W+W and W+OS treatment

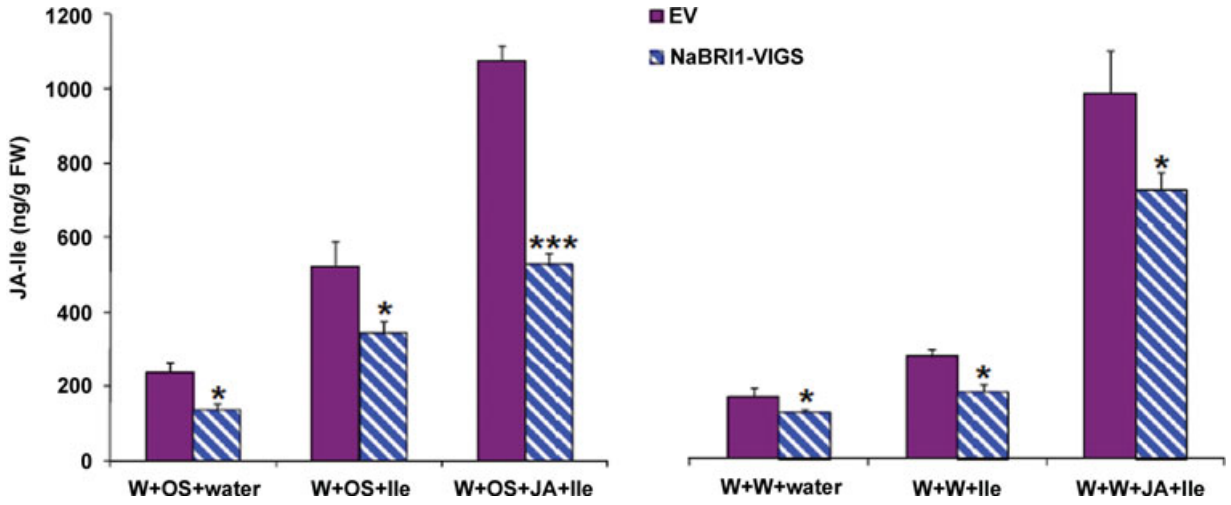

Figure 4. JAR activity in empty vector (EV) and NaBRI1-virus-induced gene silencing (VIGS) plants.

Leaves of EV and NaBRI1-VIGS plants were wounded with a pattern wheel and 20 μL *Manduca sexta* OS or water mixed with 0.625 μmol isoleucine (W+OS+Ile, W+water+Ile) or both JA and isoleucine (W+OS+JA+Ile, W+water+JA+Ile) were applied to the wounds immediately. Same volume of water was added into OS or water (W+OS+water, W+water+water) as controls. Mean JA-Ile levels (±*SE*) were quantified by LC-MS/MS before and 1 h after treatments. Stars indicate significant differences between EV and NaBAK1-VIGS plants (unpaired *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$).

(to exclude the possibility of limited endogenous JA or Ile), compared with those in EV, *NaBRI1*-silenced plants had 26% and 51% decreased JA-Ile contents, respectively (**Figure 4**). Thus, we concluded that the activity of JAR enzymes might be one of the limitations of herbivory-induced JA-Ile levels in NaBRI1-VIGS plants.

In addition, quantification of free isoleucine (Ile) levels showed NaBRI1-VIGS plants had 25% of the free Ile in EV plants, which further suggested that the availability of Ile was likely also a reason for the decreased JA-Ile levels in NaBRI1-VIGS plants (Figure S3). All together, these data suggest that impaired JAR activity and availability of Ile might be the limiting factors that caused the decreased accumulations of wounding- and herbivory-induced JA-Ile in NaBRI1-VIGS plants.

Silencing *NaBRI1* impairs *N. attenuata*'s resistance to insect herbivore *M. sexta*

To examine whether silencing *BRI1* alters the defense of *N. attenuata* against the specialist herbivore *M. sexta*, 30 newly hatched larvae were placed on the rosette leaves of EV and NaBRI1-VIGS plants (one neonate/plant). The larval masses were recorded on the 7, 9, 11, 13, and 15 d after infestation. *M. sexta* larvae gained higher masses after feeding for 13 d and 15 d (unpaired *t*-test $P = 0.0013$ and $P = 0.0089$, respectively) (**Figure 5**). These data suggest that silencing *NaBRI1* impairs *N. attenuata*'s resistance to *M. sexta*.

In *N. attenuata–M. sexta* interactions, carbon-rich anti-herbivore secondary metabolites HG-DTGs play important roles (Jassbi et al. 2008; Heiling et al. 2010). Therefore, HG-DTG levels were quantified 15 d after insect feeding. Insect feeding dramatically induced HG-DTG levels in EV plants.

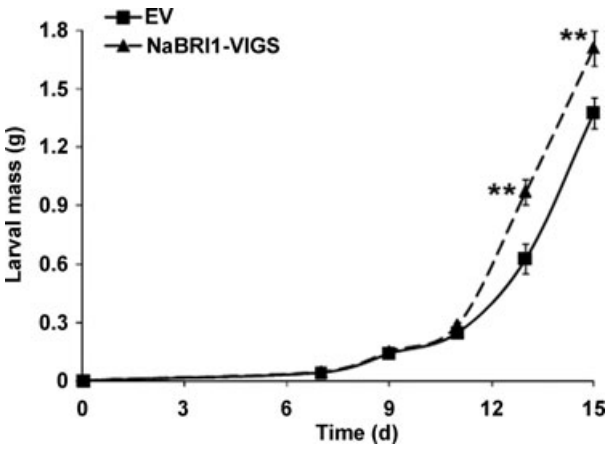

Figure 5. *Manduca sexta* growth Time (d) on empty vector (EV) and NaBAK1-virus-induced gene silencing (VIGS) plants.

One newly hatched *M. sexta* larva was placed on a rosette leaf of each EV and NaBRI1-VIGS plants and the masses of the larvae were recorded 7, 9, 11, 13, 15 d after feeding. Stars indicate significant differences between EV and NaBAK1-VIGS plants (unpaired *t* test, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 30$).

However, compared with EV plants, NaBRI1-VIGS plants had significantly reduced HG-DTG levels (67% decreased, **Figure 6A**). These data suggest NaBRI1 is required for herbivory-elicited HG-DTG accumulation. Chlorogenic acid (CGA) and rutin are both phenolic compounds whose levels increase after *M. sexta* attack, and CGA was found to be an anti-thrip phenylpropanoid (Leiss et al. 2009). In EV plants, *M. sexta* feeding enhanced CGA and rutin levels 1.9- and 1.7-fold, respectively; however, CGA levels did not significantly change after *M. sexta* feeding in NaBRI1-VIGS plants, and rutin levels even decreased to 3% of those in EV plants (**Figure 6B, C**). These data suggest that NaBRI1 is likely to be important for the accumulation of CGA and rutin induced by insect feeding.

Nitrogen-rich nicotine and trypsin proteinase inhibitors (TPIs) are also important anti-herbivore secondary metabolites (Steppuhn et al. 2004; Zavala et al. 2004). Quantification of nicotine contents revealed that insect feeding did not elevate nicotine levels in EV plants. Importantly, compared with EV plants, NaBRI1-VIGS plants had 1.6-fold greater and similar nicotine content before and 15 d after insect feeding, respectively (**Figure 6D**). Trypsin proteinase inhibitor activity levels were also highly elevated after insect feeding in EV and NaBRI1-VIGS plants. In contrast to the reduced herbivory-elicited HG-DTG, CGA, and rutin levels in NaBRI1-VIGS plants, NaBRI1-VIGS plants had 1.38-fold increased and similar TPI activity before and after insect feeding, respectively (**Figure 6E**). Thus, silencing *BRI1* resulted in similar herbivory-induced nicotine contents and TPI activity in *N. attenuata*.

Therefore, silencing *NaBRI1* resulted in decreased accumulation of certain herbivory-elicited carbon-rich defense secondary metabolites (HG-DTGs, CGA, and rutin) but had little effect on nitrogen-rich secondary metabolites, nicotine and TPI.

Discussion

Brassinosteroid biosynthesis mutant *dpy* has enhanced JA-inducible anti-herbivore secondary metabolite zingiberene and increased transcript levels of serine proteinase inhibitor, suggesting that BR negatively interacts with JA-mediated plant defense responses to insects (Campos et al. 2009). Here we silenced the BR receptor, *NaBRI1*, in *N. attenuata* to examine whether NaBRI1-mediated BR signaling is involved in plant-herbivore interactions. Compared with EV plants, *NaBRI1*-silenced plants exhibited reduced wounding- and herbivory-induced JA-Ile levels and insect feeding-elicited HG-DTG contents, although constitutive nicotine levels and TPI activity were significantly greater in *NaBRI1*-silenced plants. However, herbivory-induced JA levels, nicotine contents, and TPI activity were not strongly altered in *NaBRI1*-silencing plants.

BR signaling has little effect on JA accumulation but is important for JA-Ile biosynthesis

Although *NaBRI1*-silenced plants were impaired in growth and leaf development, wounding- and simulated herbivory-induced accumulation of JA was almost not affected, while JA-Ile levels were reduced. Analysis of *JAR4* and *JAR6* transcripts indicated that *NaBRI1*-silenced plants had even elevated expression of *JAR4* and *JAR6*. We speculated that this might be due to a feedback regulation of JA-Ile: the decreased contents of JA-Ile somehow activated the transcription of its biosynthetic genes.

Supplying substrates (JA and Ile) for JA-Ile biosynthesis to W+W– and W+OS-treated plants suggested that *NaBRI1*-silenced plants might have had attenuated JAR enzymatic activity. However, we cannot rule out the possibility that *NaBRI1*-silenced plants had limited adenosine triphosphate (ATP), which is required for conjugation of JA and Ile to form JA-Ile (Staswick et al. 2002), due to an energy shortage resulting from the possibly impaired photosynthetic capacity and globally changed metabolism and development. Further quantification of free Ile contents showed that *NaBRI1*-silenced plants had significantly less free Ile levels than did EV plants, suggesting that availability of Ile might also have limited the herbivory-induced JA-Ile levels in *NaBRI1*-silenced plants. It is also likely that BR signaling is implicated in plant primary metabolism, such as the biosynthesis of Ile.

NaBRI1 and NaBAK1 influence simulated herbivory-induced JA-Ile levels in different manners

Previously we found that NaBAK1 is important for wounding- and herbivory-induced JA and JA-Ile levels (Yang et al. 2011a). However, NaBRI1 is required for herbivory-induced accumulation of JA-Ile, but not JA. Furthermore, impaired JA-Ile levels in *NaBAK1*-silenced plants are mainly due to reduced JA accumulation, but not decreased JAR activity. In contrast, *NaBRI1*-silenced plants have reduced Ile availability and likely JAR activity. Clearly NaBAK1 and NaBRI1 influence different steps of JA-Ile biosynthesis, and NaBAK1 affects JA biosynthesis independently from NaBRI1-mediated BR signaling (Yang et al. 2011b). Other evidence has also suggested that BAK1 is not only involved in BR signaling, but also has other functions, for example, Du et al. (2012) reported that three *Arabidopsis* SERKs (AtSERK1, AtBAK1/SERK3, and AtBKK1/SERK4) control root development mainly in a BR-independent manner.

Arabidopsis bak1-3 mutants are susceptible to fungal infections, and Kemmerling et al. (2007) found that applying BR to *bak1-3* (to enhance BR signaling-mediated responses) restores the rosette leaf growth to WT levels, but does not complement the immunity to fungal infections; moreover, a group of BR signaling and biosynthesis mutants are not more susceptible to fungal infection than WT plants, indicating

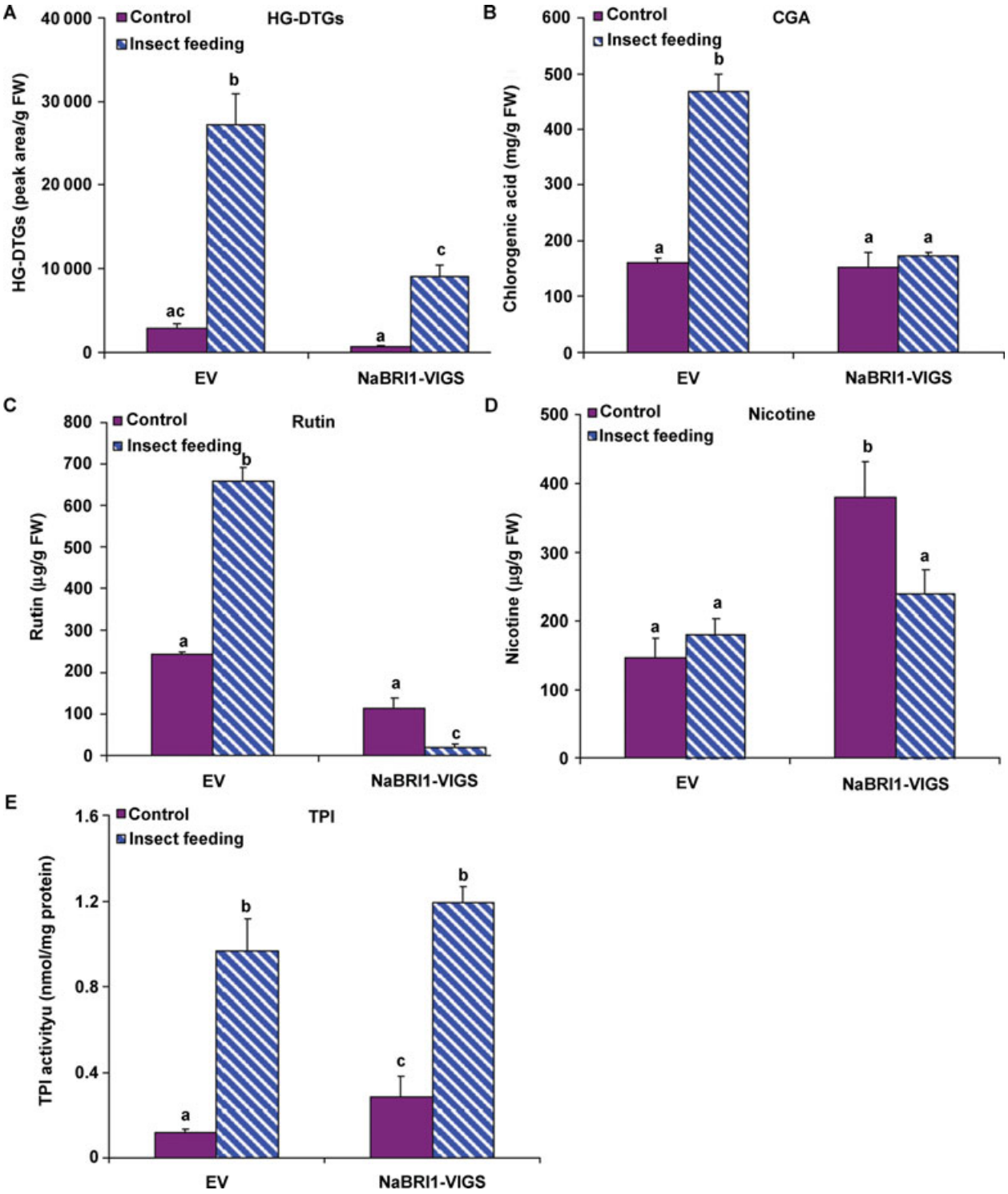

Figure 6. Herbivory-induced contents of herbivore defense-related secondary metabolites.

Leaves of empty vector (EV) and NaBRI1-virus-induced gene silencing (VIGS) plants were harvested 15 d after *Manduca sexta* feeding, non-treated ones served as controls. The contents (mean ± *SE*) of hydroxygeranyllinalool diterpene glycosides (HG-DTGs) **(A),** chlorogenic acid (CGA) **(B)**, and rutin **(C)** and nicotine **(D)**, and the activity (mean ± *SE*) of trypsin proteinase inhibitors (TPIs) **(E)** were determined. Different letters indicate significant differences (two-way ANOVA, Fisher's protected least significant difference (PLSD) test, $P < 0.05$; $n = 5$).

that BAK1 controls pathogen-induced programmed cell death (PCD) in a BR independent manner. Our finding that NaBAK1 affects herbivory-elicited accumulation of JA independent of NaBRI1-mediated BR signaling in plant-herbivore interactions also suggests that BRI1 and BAK1 have different functions in not only plant-pathogen interactions but also in plant-herbivore interactions.

NaBRI1-mediated BR signaling and the accumulation of defense-related metabolites

Investigation of gas-exchange, chlorophyll fluorescence characteristics, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity, and carbohydrate metabolism in cucumber, which were sprayed with 24-epibrassinolide (EBR), indicated increases in the capacity of CO_2 assimilation in the Calvin cycle, which was mainly attributed to an increase in the initial activity of Rubisco (Yu et al. 2004). Moreover, Xia et al. (2009) further showed supplementation of EBR to cucumber results in promotion of photosynthesis and plant growth by positively regulating synthesis and activation of a variety of photosynthetic enzymes including Rubisco. Therefore, BR application can promote plant growth through enhancing photosynthesis capacity and carbon assimilation. We hypothesize that impaired BRI1-mediated BR signaling in *NaBRI1*-VIGS plants might attenuate plant photosynthesis, and thus the carbon assimilation, which in turn affect the levels of carbon-based primary and secondary metabolites.

In cultivated tobacco, decreased photosynthetic capacity resulted in strongly reduced carbon-rich secondary metabolites, such as chlorogenic acid (CGA) and rutin (Matt et al. 2002). Similarly, silencing *ribulose-1,5-bisphosphate carboxylase/oxygenase activase* (*RCA*) in *N. attenuata* decreases simulated herbivory (W+OS)-induced accumulation of HG-DTGs, TPIs, and JA-Ile, but not JA, suggesting that photosynthesis capacity and carbon availability play important roles in plant defense responses to insect herbivory (Mitra and Baldwin 2008). Compared with EV, *NaBRI1*-silenced plants have reduced HG-DTG, CGA, and rutin levels after insect feeding. Furthermore, W+OS treatment elicited reduced JA-Ile levels in *NaBRI1*-silenced than in EV plants. These herbivory-induced defense responses in *NaBRI1*-silenced plants are highly reminiscent of those in *RCA*-silenced plants. Whether *NaBRI1*-silenced plants, like *RCA*-silenced plants, have reduced photosynthetic capacity and/or limited carbon assimilation should be investigated in the future.

Unlike carbon-rich metabolites, insect feeding-elicited nicotine contents and TPI activity were not different between *NaBRI1*-silenced and EV plants. Silencing *NaBRI1* might mainly affect insect feeding-induced carbon-rich secondary metabolites, but not nitrogen-rich ones. In contrast, *RCA*-silenced plants have decreased TPI levels after W+OS treatment (Mitra and Baldwin 2008). It is possible that BR signaling is not only implicated in photosynthesis but other processes to influence plant resistance to herbivores.

Importantly, it is necessary to further confirm the function of NaBRI1 in plant-herbivore interactions in other experimental settings (for example, using the stable RNA interference lines) in the future, as VIGS approach has an overall effect on *N. attenuata* plant growth and development, and might disturb phytohormone homeostasis.

In summary, our results suggest that BR signaling is likely involved in *N. attenuata-M. sexta* interactions, and NaBRI1 is required for wounding- and herbivory-induced accumulation of JA-Ile but has little effect on JA. NaBRI1 is important for the induction of HG-DTGs, CGA, and rutin, but not two nitrogen-based defensive metabolites, namely nicotine and TPI. Although BAK1 and BRI1 function synergistically in BR perception, they likely affect different aspects of plant-herbivore interactions.

Materials and Methods

Plant growth and sample treatments

Nicotiana attenuata (Solanaceae) seeds were from a line maintained in our laboratory that was originally collected from Utah (USA) and had been inbred for 30 generations in the greenhouse. Seed germination and growth condition followed Krügel et al. (2002). Each seedling was transfer to a 1-L pot and was maintained at 20–22 °C and under 16 h of light. A virus-induced gene silencing (VIGS) approach was used to silence the transcripts of *NaBRI1* following a VIGS procedure optimized for *N. attenuata* (Saedler and Baldwin 2004).

Rosette leaves were wounded with a pattern wheel and 20 μL of the oral secretions (OS) of *M. sexta* larvae (W+OS) was applied to the wounds immediately to mimic the insect attack. As a comparison, 20 μL of water (W+W) were applied. For supplying substrates of JA-Ile biosynthesis, a procedure described in Paschold et al. (2008) was adopted. Samples were harvested and frozen in liquid nitrogen immediately. Samples were kept in – 80 °C until analysis.

Cloning of *NaBRI1* gene, sequence analysis, and VIGS construct preparation

According to the sequences of *BRI1* from *N. benthamiana* and *N. tabacum* (accession numbers: EF471738 and EF623823), a 634 bp fragment of *NaBRI1* was amplified from an *N. attenuata* cDNA pool using primers *NaBRI1*-For and *NaBRI1*-Rev (Table S1). This fragment was cloned into the pJET vector (Fermentas, St. Leon-Rot, Germany) and sequenced. Using the fragments of *NaBRI1*, *NtBRI1*, and *NbBRI1* as baits, a 2697 bp fragment of *NaBRI1* gene (accession number: JX014434)

was assembled from an *N. attenuata* transcriptome database prepared by 454 sequencing. Cluster W algorithm (MegAlign, DNASTAR Lasergene 8) was used for sequence alignment.

A 369 bp fragment of *NaBRI1* gene was amplified with primers *NaBRI1*-VIGS-HindIII and *NaBRI1*-VIGS-BamHI (Table S1). This fragment was cloned into the vector pTV00 with HindIII and BamHI to form the pTV-NaBRI1-VIGS construct. After sequencing, pTV-NaBRI1-VIGS construct and pTV00 (empty vector) were transformed into *Agrobacterium* for VIGS.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from ground samples using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Five replicated biological samples were used for qPCR analysis. Total RNA samples (0.5 μg) were reverse transcribed to cDNA using oligo(dT) and the Superscript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed on a Stratagene MX3005P system (Agilent Technologies, Santa Clara, CA, USA) using qPCR Core kits (Eurogentec, Seraing, Belgium). The expression of *N. attenuata actin2* gene was used as the internal standard for normalizing cDNA concentrations. The primer sequences used for qPCR are listed in Table S2.

Quantification of phytohormones (JA and JA-Ile)

Jasmonic acid and JA-Ile concentrations were measured in five biological replicates following Wu et al. (2007). One milliliter ethyl acetate spiked with isotope labeled phytohormones (D_2-JA, 200ng; C_6-JA-Ile, 40 ng), which served as the internal standards for JA and JA-Ile, respectively, was added into ground leaf samples (100–200 mg). Samples were homogenized on a FastPrep homogenizer (Thermo Electron, Waltham, MA, USA) and centrifuged at 13,000 *g* for 20 min at 4 °C. The supernatants were transferred to fresh Eppendorf tubes and dried out in a vacuum centrifuge system (Eppendorf, Hamburg, Germany); subsequently, 0.5 mL of 70% methanol (v/v) was added to resuspend the residues. Phytohormone contents were quantified on a LC-MS/MS system following Wu et al. (2007) (Varian, Palo Alto, CA, USA).

Quantification of isoleucine

The isolation of free Ile followed Wünsche et al. (2011). Ground fine leaf powder was aliquoted to around 100 mg. Three hundred microliters of extraction buffer (0.1 M Tris-HCl, pH 7.6, 2 g/L phenylthiourea, 5 g/L diethyldithiocarbamate, and 50 g/L polyvinylpolypyrrolidone) were added and mixed by vortexing for 2 min. Insoluble particles were separated by centrifugation at 16,000 *g* for 10 min; 200 μL of the supernatants were mixed with 300 μL of 0.5 M sodium borate (pH 11). After 15 min of incubation at room temperature, samples were centrifuged again to remove particles. A standard curve was created by injecting 1, 2, 5 and 10 μL of an amino acid standard solution (Sigma-Aldrich, Steinheim, Germany). The autosampler was programmed to inject 30 μL of OPA-2ME (potassium borate buffer, pH 11, containing 10 μL/mL 2-mercaptoethanol and 11.4 mg/mL o-phthaldialdehyde) into each sample to derivatize amino acids. Samples were mixed twice and incubated for 3 min and injected into high performance liquid chromatography (HPLC). The HPLC running conditions is described in de Kraker et al. (2007).

Insect growth assay

The specialist herbivore *M. sexta* was obtained from a colony maintained in our laboratory. One newly hatched *M. sexta* neonate was placed on a rosette leaf of each plant. Larvae fed on the plants for 15 d, and masses of larvae were recorded on 7, 9, 11, 13, and 15 d after starting of insect feeding experiments.

Measuring plant secondary metabolites

Quantification of trypsin proteinase inhibitor (TPI) activity followed a radial diffusion assay described in van Dam et al. (2001). Analysis of hydroxygeranyllinalool diterpene glycosides (HG-DTGs), chlorogenic acid (CGA), rutin, and nicotine by HPLC is described in Keinanen et al. (2001).

Statistics analysis

Data were analyzed by unpaired *t* test or analysis of variance (ANOVA) using StatView, version 5.0 (SAS, Cary, NC, USA).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of *BRI1* genes and BRI1 proteins.
(A) Nucleotide sequence alignment of *Nicotiana attenuata NaBRI1* gene (partial) and other *BRI1* genes.
(B) Amino acid sequence alignment of the deduced NaBRI1 and other BRI1 proteins. National Center for Biotechnology Information (NCBI)(GenBank) accession numbers for *BRI1* genes and BRI1 proteins included in this analysis are: *N. attenuata NaBRI1* (JX014434), *Nicotiana tabacum NtBRI1* (EF623823), *Nicotiana benthamiana NbBRI1* (EF471738), *Solanum lycopersicum SlBRI1* (HQ699335), *Solanum tuberosum StBRI1* (EF471737), *Solanum pimpinellifolium SpBRI1* (EF471736); NaBRI1 (AFN44233), NtBRI1 (ABR18799), NbBRI1 (ABO27628), SlBRI1 (ADZ47880), StBRI1 (ABO27627), SpBRI1 (ABO27626), AtBRI1 (AAC49810). Nucleotides and amino acids in black background are different from the consensus sequence.

Figure S2. Morphology of empty vector (EV) and NaBRI1-virus-induced gene silencing (VIGS) plants.
Empty vector and NaBRI1-VIGS plants at the middle elongated stage. Note: NaBRI1-VIGS plants have dark green and curly leaves and show a dwarf phenotype. Plants silenced in *PDS* (*phytoene desaturase*) (PDS-VIGS) were used to monitor the degree of VIGS visually, since these plants have a photobleaching phenotype.

Figure S3. NaBRI1-virus-induced gene silencing (VIGS) plants have reduced free isoleucine contents.
Rosette leaves of empty vector (EV) and NaBRI1-VIGS plants were harvested and the free isoleucine contents were quantified by high performance liquid chromatography (HPLC). Stars indicate significant differences between EV and NaBAK1-VIGS plants (unpaired *t*-test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $n = 5$).

Table S1. Primers used for cloning of NaBRI1 and preparation of pTV-NaBRI1 Construct.

Table S2. Primer sequences used for quantitative real-time polymerase chain reaction (PCR) (SYBR Green analysis).