

# A *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) reduces growth and increases secondary metabolite biosynthesis in herbivore-attacked plants

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## Summary

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## Introduction

The induction of plant defense following herbivory is characterized by a complex suite of chemical changes, including the reconfiguration of primary and secondary metabolism (Zangerl *et al.*, 2002; Schwachtje & Baldwin, 2008). The suppression of the photosynthetic apparatus that typically occurs following insect attack prevents the addition of photosynthate to the existing carbon pool (Creelman & Mullet, 1997; Beltrano *et al.*, 1998; Hristova & Popova, 2002; Izaguirre *et al.*, 2007; Schwachtje & Baldwin, 2008; Gómez *et al.*, 2010). As a consequence, metabolic reconfiguration may incur a cost in terms of biosynthesis, storage, and fitness impacts as plants must rely on other means to accrue the building blocks to support secondary metabolism responsible for plant defenses, and maintaining optimal growth (Chapin *et al.*, 1990). Plants meet this increase in demand by maintaining a degree of flexibility in resource allocation. This may act to

- Plant invertases are sucrolytic enzymes that are essential for the regulation of carbohydrate metabolism and source–sink relationships. While their activity has been well documented during abiotic and biotic stresses, the role of proteinaceous invertase inhibitors in regulating these changes is unknown.
- Here, we identify a putative *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) which is strongly up-regulated in a jasmonate (JA)-dependent manner following simulated attack by the specialist herbivore *Manduca sexta*. To understand the role of NaCWII in *planta*, we silenced its expression by RNA interference and measured changes in primary and secondary metabolism and plant growth following simulated herbivory.
- NaCWII-silenced plants displayed a stronger depletion of carbohydrates and a reduced capacity to increase secondary metabolite pools relative to their empty vector control counterparts. This coincided with the attenuation of herbivore-induced CWI inhibition and growth suppression characteristic of wild-type plants.
- Together our findings suggest that NaCWII may act as a regulatory switch located downstream of JA accumulation which fine-tunes the plant's balance between growth and defense metabolism under herbivore attack. Although carbohydrates are not typically viewed as key factors in plant growth and defense, our study shows that interfering with their catabolism strongly influences plant responses to herbivory.

prevent further loss of photosynthetic tissues and desiccation from wounds, rebuild damaged cell walls, and allocate resources to tissues that are inaccessible to feeding herbivores, or those that have been damaged locally to support local defense induction (Schultz *et al.*, 2013).

Cell wall invertases (CWIs) are ionically cell wall-bound enzymes that facilitate phloem unloading at sink tissues by cleaving sucrose into fructose and glucose (Sturm & Tang, 1999). In addition to their pivotal role in plant development (Roitsch & González, 2004), a growing body of literature suggests that invertases are a central component of plant defense responses to biotic stresses, given their ability to alter whole-plant carbon allocation patterns by turning damaged tissues into metabolic sinks for resources (Tauzin & Giardina, 2014). The activity of invertases is well known to be affected by a range of environmental stimuli, including mechanical damage, herbivore grazing, pathogen infection, galling, and plant hormones (Roitsch & González, 2004; Sonnewald *et al.*, 2012). Many studies highlight the link between plant defense induction and invertase activity (Bolton, 2009;

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Schultz *et al.*, 2013; Proels & Hückelhoven, 2014; Tazuin & Giardina, 2014). For example, enzyme activity and levels of acid invertase mRNAs have been found to increase in wounded leaves of tomato (*Lycopersicon esculentum* and *Lycopersicon peruvianum*) (Ohyama *et al.*, 1998), carrot (*Daucus carota*; Sturm & Chrispeels, 1990), *Chenopodium rubrum* (Ehneß & Roitsch, 1997), pea (*Pisum sativum*; Zhang *et al.*, 1996), and sugar beet (*Beta vulgaris*; Rosenkranz *et al.*, 2001), suggesting that inducible CWI activities are significant components of plant wound responses. Studies in *Populus* demonstrate that the ability of young, developing leaves to respond defensively to jasmonate (JA) treatments relies on their capacity to enhance sink strength for carbon-based resources by increasing the activity of CWI and exporting carbon toward damaged sites (Arnold & Schultz, 2002; Arnold *et al.*, 2004; Appel *et al.*, 2012). Similar findings have been reported in *Arabidopsis thaliana*, where the transport of a radioactive glucose surrogate, [<sup>18</sup>F]fluoro-2-deoxy-D-glucose (Ferrieri *et al.*, 2012), and <sup>11</sup>C-photosynthate (Ferrieri *et al.*, 2013) to leaves elicited with methyl jasmonate was correlated with changes in CWI activity.

Cell wall invertase activity is tightly regulated by both transcriptional and translational mechanisms during plant growth and development, as well as by changes in endogenous hexose pools (Huang *et al.*, 2007). Studies on the role of invertases have primarily focused on the transcriptional level; however, recent evidence suggests that their activity may also be highly dependent on post-translational regulation by a small group of inhibitory proteins with molecular masses between 15 and 23 kDa (Krausgrill *et al.*, 1998; Rausch & Greiner, 2004; Essmann *et al.*, 2008; Ruan *et al.*, 2009). While proteinaceous invertase inhibitors have been identified in many plant species (Pressey, 1994; Weil *et al.*, 1994; Weber *et al.*, 1996; Greiner *et al.*, 1998; Krausgrill *et al.*, 1998; Bate *et al.*, 2004; Link *et al.*, 2004; Reca *et al.*, 2008), their physiological roles remain to be elucidated (Rausch & Greiner, 2004; Ruan *et al.*, 2009). Jin *et al.* (2009) found that silencing a tomato invertase inhibitor (INVINH1) specifically increased the activity of CWI and sugar concentrations in developing fruits. The elevation of CWI also resulted in an attenuation of abscisic acid (ABA)-induced leaf senescence and an increase in seed size, suggesting a key role for INVINH1 in regulating leaf longevity and seed development. More recently, Bonfig *et al.* (2010) found that the expression and activity of invertase inhibitors were suppressed in *A. thaliana* during *Pseudomonas syringae* infection, which released CWI from post-translational inhibition (Bonfig *et al.*, 2010). In this study, infiltration of *A. thaliana* leaves with a chemical invertase inhibitor, acarbose, increased plant susceptibility to this pathogen, suggesting a role for proteinaceous invertase inhibitors in post-translational regulation of CWI activity during pathogen infection.

Despite these advances, the involvement of invertase inhibitors in regulating plant growth and metabolism during plant responses to insect herbivores remains unknown. Here, we explore the function of INVINH1 in the interaction between *Nicotiana attenuata* and the specialist herbivore *Manduca sexta* by cloning its respective homolog in *N. attenuata* (NaCWII) and

reducing transcript levels by RNA interference. Changes in plant growth and metabolism following simulated herbivore attack are examined to elucidate potential regulatory and defensive roles for NaCWII in *N. attenuata*.

## Materials and Methods

### Plant growth conditions

Seeds of the 31<sup>st</sup> generation of an inbred line of *Nicotiana attenuata* Torr. Ex Watts originally collected from Utah, USA, were used for experiments. Seeds were first surface-sterilized and incubated with 1 : 50 (v/v) diluted liquid smoke (House of Herbs, Passaic, NY, USA) and 0.1 M gibberellic acid (GA<sub>3</sub>), and germinated on plates containing Gamborg's B5 medium as described in Krügel *et al.* (2002). Ten days after germination, seedlings were transferred to Teku pots (Poppelmann GmbH & Co. KG, Lohne, Germany) for an additional 10–12 d, followed by a final transplanting to 1-l pots filled with washed sand. Plants were grown under glasshouse conditions at 45–55% relative humidity, 24–26°C, and 16 h : 8 h, light : dark (Krügel *et al.*, 2002).

### Identification of a CWII homolog in *N. attenuata* and plant transformation

The amino acid sequence of an invertase inhibitor in tomato INVINH1 (*Solanum lycopersicum*) (Jin *et al.*, 2009) was used to identify homologs in *N. attenuata*. Accession numbers of tobacco and tomato can be found in the GenBank/EMBL database under: NT VINVINH, AY594170; NT CWINVINH, Y12805; LE INVINH1, AJ010943. Specific primers (forward 5'-ACAT GCAAGAACACACCAAATTACCAA and reverse 5'-TCCC CTGTTCACTCCGTTGTCC) were designed to PCR amplify the respective homolog in *N. attenuata* (NaCWII). A PCR product was obtained using *N. attenuata* leaf cDNA as template and a 293-bp fragment (Supporting Information, Fig. S1) was cloned into the pRESC8 transformation vector in an inverted repeat orientation driven by the cauliflower mosaic virus (CaMV) 35S promoter (Gase *et al.*, 2011) for gene silencing. This vector was transformed into wild-type *N. attenuata* plants using *Agrobacterium tumefaciens*-mediated transformation methods described by (Krügel *et al.*, 2002). Independent transgenic lines were selected for homozygosity by hygromycin resistance (Gase *et al.*, 2011). Southern blotting was performed following digestion of 5 µg of genomic DNA with *Eco*RV and *Bgl*II (New England Biolabs; <http://www.neb.com>). Labeling was performed with the GE Healthcare (<http://www.gehealthcare.com>) Ready-prime DNA labeling system and ProbeQuant g-50 microcolumns according to the manufacturer's instructions. Quality, concentration and digestion were checked by agarose 1% gel electrophoresis and gDNA was blotted onto a nylon membrane (GeneScreenPlus; PerkinElmer; <http://www.perkinelmer.com>) according to the manufacturer's procedure. Phospho-imaging plates where exposed overnight and scanned with a Fluorescent Image Analyzer 3000 (FLA-3000; FujiFilm Europe GmbH, Düsseldorf, Germany; <http://www.fujifilm.eu/>). Flow cytometric

analysis (Bubner *et al.*, 2006) confirmed that selected lines were diploid.

#### Regulation of a *N. attenuata* CWII by wounding and *M. sexta* simulated herbivory

Rosettes of empty vector (EV) and two independently transformed NaCWII-silenced lines (inverted repeat cell wall invertase inhibitor, irCWII-1 and irCWII-2) were treated by rolling a serrated fabric pattern wheel three times on each side of the midvein of three middle-aged leaves per plant, and by immediately applying 20 µl of water (W + W) or *M. sexta* oral secretions (W + OS) diluted 1 : 5 (v/v) in distilled water to the fresh puncture wounds of each leaf. The application of W + OS was chosen as it mimics herbivory-specific changes in *N. attenuata* without removing extensive amounts of leaf tissue (Halitschke *et al.*, 2003) and to precisely control the kinetics of the elicitation process. Control leaves remained untreated. Leaves were harvested 1, 3 and 6 h after treatments and immediately frozen in liquid nitrogen for subsequent analysis.

For qPCR analysis, total RNA was extracted by the TRIZOL method, followed by DNase-I treatment (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. Five micrograms of total RNA was reverse-transcribed using oligo (dT)18 and the SuperScript-II Reverse Transcriptase kit (Invitrogen). Resulting cDNA was used for quantitative analysis with SYBR Green I following the manufacturer's protocol, and the  $\Delta C_t$  method was used for transcript evaluation. ACTIN was used as an endogenous reference. Data were analyzed using three-way ANOVAs to test for the effect of plant genotype, herbivory and time-point on relative transcript abundance of NaCWII ( $n=3$ ). The data presented are supported by two independent experiments with similar results.

#### Regulation of cell wall and soluble invertase activity by NaCWII after simulated herbivory

To investigate whether *M. sexta* attack influences invertase activity in *N. attenuata*, we measured cell wall and soluble invertase activities in leaf extracts of EV, irCWII-1 and irCWII-2 plants following repeated W + OS treatments. Every 48 h, two leaves per plant were treated with 20 µl per leaf of 1 : 5 diluted *M. sexta* oral secretions over a total of 6 d, resulting in six treated leaves per plant ( $n=5$ ). Leaves were harvested 6 d after the first treatment at midday. Cell wall and soluble invertase activities were determined according to Ferrieri *et al.* (2013). Results were analyzed using two-way ANOVA to test for the effect of plant genotype and simulated herbivory on invertase activity ( $\mu\text{mol glucose produced g}^{-1} \text{ FW min}^{-1}$ ).

#### Role of NaCWII in plant growth following simulated herbivory

Treatments were applied to three leaves per plant ( $n=35$ ) every other day for 6 d beginning in the early elongation stage (refer to Fig. 5(a) for experimental plan). Growth parameters, including

rosette diameter, branch height and elongation rate, were monitored at 24 and 48 h following the first treatment, as well as three time-points surrounding the last treatment on day 5 (at the time of the final W + OS treatment day, and 12- and 24-h following this final treatment). Three-way ANOVAs were used to evaluate the effect of genotype, herbivory, and time-point on short-term growth responses following one treatment. *Post hoc* comparisons were carried out within the 24- and 48-h time-points to assess herbivory treatment effects. Long-term growth measurements were evaluated using two-way repeated measures ANOVAs and pairwise multiple comparisons procedures (Holm–Sidak *post hoc* tests) to test for genotypic effects.

#### Primary and secondary metabolite profiles of NaCWII-silenced plants

An additional time course experiment was performed to assess the impact of silencing NaCWII on the herbivore-induced reconfiguration of primary and secondary metabolism. Herbivory was simulated every other day for 8 d by applying 20 µl per leaf of 1 : 5 diluted *M. sexta* oral secretions (W+OS) on two wounded leaves per plant (four treatments; eight damaged leaves per plant in total). Leaves were harvested at 02:00, 06:00, 14:00 and 20:00 h following the last day of treatment. Soluble sugars (glucose, fructose, and sucrose) and starch were extracted from 100 mg of frozen leaf material using an 80% (v/v) ethanol extraction procedure according to Machado *et al.* (2013). Sucrose, glucose, and fructose were quantified enzymatically in supernatant pooled during the extraction steps as previously described (Velterop & Vos, 2001), and remaining pellets were used for starch determination (Smith & Zeeman, 2006). To investigate whether silencing NaCWII influences the abundance of sugars and starch following simulated herbivory, we analyzed the effect of plant genotype, herbivory treatment and time-point on the amount of a given metabolite (glucose + fructose, sucrose and starch) using a three-way ANOVA followed by Tukey's *post hoc* comparisons ( $n=4$ ).

Secondary metabolites were extracted from 100 mg of frozen leaf material using a 40% methanol extraction procedure (Gaqerel *et al.*, 2010). Separation of leaf extracts was performed using an RSLC system (Dionex Corp., Sunnyvale, CA, USA) according to Kim *et al.* (2011). Briefly, 4 µl of extract was injected onto a C18 column (Acclaim; 2.2 µm particle size and 150 mm × 2.1 mm inner diameter; Dionex Corp.). The mobile phase consisted of solvent A (0.1% (v/v) acetonitrile and 0.05% (v/v) formic acid in deionized water) and solvent B (0.05% (v/v) formic acid in acetonitrile) with the following elution profile: 0–0.5 min, 10% B in A; 0.5–6.5 min, linear gradient 10–80% B in A; 6.5–10 min, 80% B in A, followed by re-equilibration at 10% B for 3 min with a flow rate of 300 µl min<sup>-1</sup>. A time-of-flight mass spectrometer equipped with an electrospray ionization source (Bruker Daltonic, Bremen, Germany) was used to determine the molecular mass of ionized molecular fragments and the amounts of the eluted analytes. The capillary voltage was 4500 V, and the dry gas (200°C) flow rate was 8 ml min<sup>-1</sup>. The detected ion range was from *m/z* 200 to 1400 at a repetition rate of 1 Hz.

The mass calibration was achieved using a sodium formate solution (10 mM sodium hydroxide and 0.2% formic acid in isopropanol/water 1:1, v/v). Data were analyzed using three-way ANOVAs to test for the effect of plant genotype, herbivory, and time-point on metabolite concentration ( $n=4$ ).

### Phytohormone measurements

To investigate whether functional NaCWII is required for the herbivore-induced changes in phytohormones, we quantified concentrations of jasmonic acid (JA), (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile), ABA, and salicylic acid (SA) 1 h following W+OS simulated herbivory on EV, irCWII-1 and irCWII-2 plants ( $n=5$ ). Phytohormone extraction and quantification were performed according to Machado *et al.*, 2013;. For ethylene measurements, three leaves of EV and irCWII-1 plants ( $n=3$ ) were W+OS elicited or left as untreated control ( $n=3$ ). Immediately following this treatment, leaves were transferred to 100-ml cuvettes and ethylene was allowed to accumulate in the headspace for 4 h. Stop-flow measurements were taken continuously and noninvasively in real time using a laser acoustic spectrometer (Invivo GmbH, Sankt Augustin, Germany) as previously described (von Dahl *et al.*, 2007).

### The effect of silencing NaCWII on *M. sexta* performance

To determine whether the *N. attenuata* cell wall invertase inhibitor NaCWII affects the activity of *M. sexta* midgut invertases, insect nutritional indices were assessed for larvae feeding on EV, irCWII-1 and irCWII-2 tissue (Waldbauer, 1968) and midgut tissues were harvested for invertase activity assays. *Manduca sexta* larvae were reared to third instar on irCWII-1, irCWII-2 or EV plants ( $n=50$  per genotype) under normal glasshouse conditions. A total of 75 larvae were used for mid-gut invertase activity measurements ( $n=25$  per genotype). To extract larval mid-gut invertase, larvae were starved for 4 h and placed on ice to facilitate mid-gut removal. The third and fifth segments from the head were cut and the mid-gut was removed using a fine forceps. Mid-guts were washed in distilled water, dried, weighed, and placed in Eppendorf tubes containing 100  $\mu$ l of 50% (v/v)

MeOH. Samples were centrifuged for 20 min at 4°C and 18 000 g and supernatants were used to quantify soluble invertase activity levels according to Ferrieri *et al.* (2013). The effect of plant genotype on *M. sexta* mid-gut invertase activity was analyzed using one-way ANOVA.

Remaining larvae were transferred to individual Petri dishes (3.5  $\times$  4.5 cm; DG-Distler-Gastro GmbH, Erfurt, Germany) placed in a climate chamber (70  $\pm$  5% RH, 28  $\pm$  2°C and 16 h : 8 h, light : dark) for 24 h. Larvae were weighed before and after this starvation period. During the following 2 d, larvae ( $n=22$ –23) were fed *ad libitum* on excised, W+OS-treated leaves of irCWII-1, irCWII-2, or EV plants. Initial leaf fresh masses were recorded at each feeding time. At the end of the experiment, remaining plant tissue, larvae, and frass were dried and weighed. The following nutritional indices were calculated (Waldbauer, 1968): Consumption index (CI) measures the amount of leaf mass consumed; approximate digestibility (AD) approximates digestibility; the efficiency of digestion of ingested food (ECI); the efficiency with which digested food is converted to body mass (ECD) (Table 1). The effect of plant genotype on nutritional indices (CI, ECI, AD and ECD) was analyzed separately using one-way ANOVA generalized linear models (GLMs) under a normal distribution in R (R Development Core Team, 2012).

Larval performance was also assessed under glasshouse conditions by placing freshly hatched *M. sexta* neonates directly on fully developed leaves of rosette-stage plants (1 neo-nate per plant;  $n=25$  per genotype) and allowing them to feed freely for 10 d. Larvae were weighed after 6, 8 and 10 d of feeding using a microbalance (Sartorius TE214S; Data Weighing Systems Inc., Elk Grove, IL, USA). Data were analyzed using one-way ANOVA to test for the effect of plant genotype on *M. sexta* mass gain.

### Statistical analyses

Unless otherwise stated, data were analyzed by ANOVA using SIGMA PLOT 12.0 (Systat Software Inc., San Jose, CA, USA). Normality and equality of variance were verified using Shapiro–Wilk and Levene's tests, respectively.

**Table 1** Silencing *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) increases leaf digestibility to *Manduca sexta* larvae

Nutritional index	Formula	EV	irCWII-1	irCWII-2	F value	P value
Consumption index (CI)	[C/(G $\times$ T)]	2.13 $\pm$ 0.07a	2.29 $\pm$ 0.06a	2.24 $\pm$ 0.06a	[2,67] 1.328	0.271
Approximate digestibility (AD)	[(C-F)/C] $\times$ 100	74 $\pm$ 0.9a	79 $\pm$ 0.9b	77 $\pm$ 1.2b	[1,68] 9.897	0.002**
Efficiency of conversion of ingested food (ECI)	(G/C) $\times$ 100	24 $\pm$ 0.8a	22 $\pm$ 0.6a	23 $\pm$ 0.6a	[2,67] 1.633	0.203
Efficiency of conversion of digested food (ECD)	[G/(C-F)] $\times$ 100	33 $\pm$ 1.4a	28 $\pm$ 0.9b	30 $\pm$ 1.1b	[1,68] 5.821	0.01**
Frass		0.274 $\pm$ 0.01a	0.276 $\pm$ 0.01a	0.278 $\pm$ 0.02a	[2,66] 0.092	0.912
Larval mass		1246 $\pm$ 59.2a	1268 $\pm$ 61a	1313 $\pm$ 81a	[2,66] 2.035	0.138

Nutritional indices were calculated for *M. sexta* feeding on excised, W+OS (1:5 water-diluted *M. sexta* oral secretions)-elicited leaves of empty vector (EV) and NaCWII-silenced (irCWII-1 and irCWII-2) plants according to Waldbauer (1968) ( $n=22$ –23). Mean consumption index (g FW plant tissue g $^{-1}$  FW larvae d $^{-1}$ ), approximate digestibility (%), efficiency of conversion of ingested food (%), efficiency of conversion of digested food (%), 2-d frass production (g), and mass gain of larvae (mg) are shown  $\pm$  SE. C, ingested food (g); G, insect mass gain; T, duration of feeding period (d); F, frass produced (mg). Results of one-way ANOVA with pairwise GLM comparisons under normal distribution are shown (\*\*,  $P \leq 0.01$ ). Different letters (shown in bold) indicate significant differences among plant genotypes ( $P < 0.05$ ).

## Results

### Cloning of NaCWII, a cDNA encoding a putative invertase inhibitor in *N. attenuata*

As the first step toward elucidating the role of cell wall invertase inhibitors in *N. attenuata*, we identified a putative *N. attenuata* invertase inhibitor gene sequence that was shown previously to be up-regulated after simulated *M. sexta* attack by mining our recently established Agilent microarray platform (GEO microarray repository, GPL13527) (Onkokesung *et al.*, 2012). BLAST of the known tomato and tobacco CWIIs against 454 transcriptome sequencing data revealed a single CWII homolog in *N. attenuata*. At present, we cannot exclude the possibility that other CWIIs may be present in the *N. attenuata* genome which we may not have picked up from the transcriptome, for instance because of their low or highly tissue-specific expression. Full-length cDNA was cloned from *N. attenuata* leaves and named NaCWII (Fig. S1). Sequence alignment of NaCWII showed high overall homology with known invertase inhibitors from tobacco (cell wall invertase inhibitor, NtCWINVIN; vacuolar invertase inhibitor, NtVINVINH) and tomato (invertase inhibitor 1, LeINVINH1), and the presence of the conserved four Cys residues (Fig. 1), which are a hallmark of all known plant invertase inhibitors (Rausch & Greiner, 2004). Real-time qPCR was used to confirm silencing efficiency and to select two independently transformed lines (A-12-439-5 and A-12-432-3), which were used in further experiments and named irCWII-1 and irCWII-2, respectively (Fig. S2). For all experiments, wild-type plants transformed with an empty vector construct (EV; line A-03-9-1) were used as controls.

### NaCWII is up-regulated by simulated herbivory in a JA-dependent manner

A time course experiment was conducted to establish the expression dynamics of NaCWII following simulated

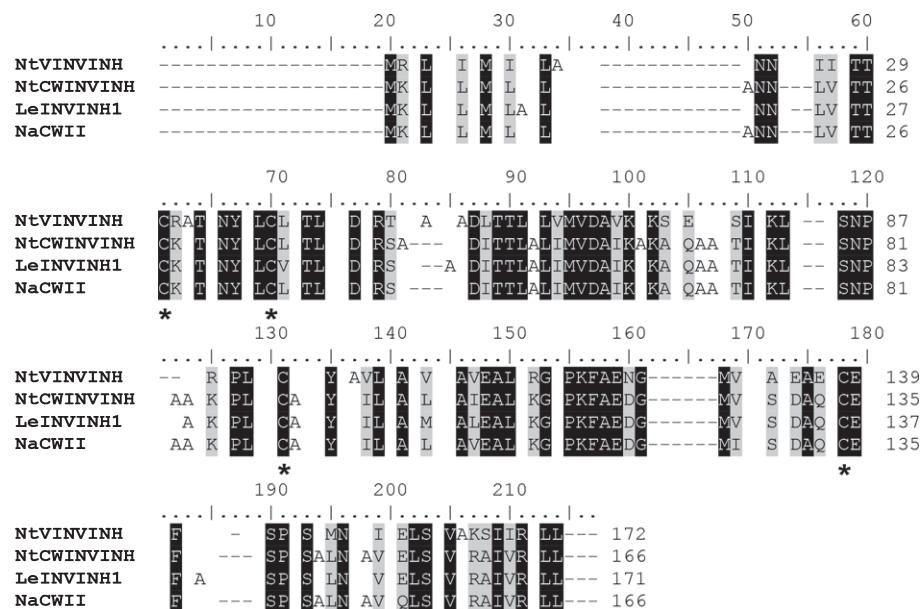
herbivory. The expression of NaCWII was concurrently assessed in a set of previously characterized JA biosynthesis-impaired plants (inverted repeat allene oxide cyclase (irAOC) line; Kallenbach *et al.*, 2012) to evaluate the contribution of JA signaling to herbivore-induced changes in gene expression. We found a strong, OS-specific up-regulation of NaCWII (16-fold increase relative to control) 6 h following simulated herbivory on EV plants (Fig. 2). By contrast, no significant changes in NaCWII expression were observed in JA-deficient irAOC plants (Fig. 3). In comparison to EV plants (Fig. 2a), the OS-specific expression of NaCWII at 6 h was reduced by c. 83% and 87% in leaves of irCWII-1 and irCWII-2 plants, respectively (Fig. 2b,c).

### Simulated herbivory decreases plant cell wall invertase activity in a NaCWII-dependent manner

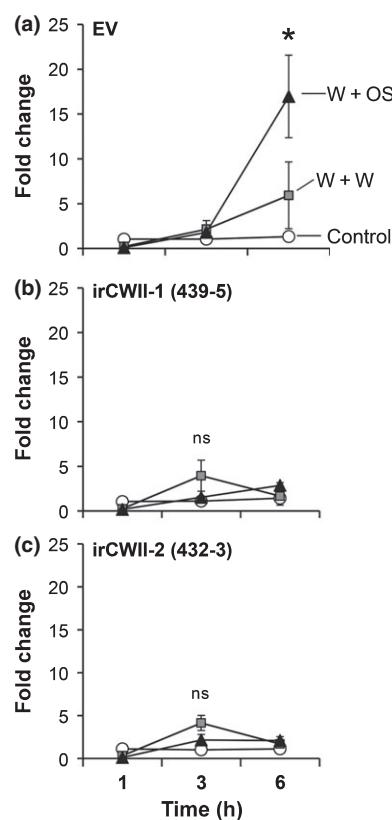
To investigate whether NaCWII regulates the activity of plant invertase, we quantified cell wall and soluble invertase activities in control and W + OS-treated EV and irCWII plants. Consistent with an increase of cell wall invertase activity, we found that multiple W + OS treatments significantly suppressed cell wall invertase activity in EV, but not in irCWII plants (Fig. 4a; two-way ANOVA with *post hoc* Tukey's tests;  $P = 0.01$ ). Soluble invertase activity was constitutively higher in irCWII lines compared with EV (Fig. 4b; two-way ANOVA with *post hoc* Tukey's tests;  $P < 0.05$ ).

### Silencing NaCWII attenuates herbivore-induced growth suppression aboveground

To determine whether NaCWII regulates the capacity of *N. attenuata* plants to maintain normal growth under defense elicitation, we simulated *M. sexta* attack using procedures described in Machado *et al.* (2013) (Fig. 5a). Silencing NaCWII had no effect on constitutive rosette growth during early



**Fig. 1** *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) shares high homology with known plant invertase inhibitors. Alignment of NaCWII with amino acid sequences of tobacco (cell wall invertase inhibitor, NtCWINVINH; vacuolar invertase inhibitor, NtVINVINH) and tomato (invertase inhibitor 1, LeINVINH1) invertase inhibitors is shown. Asterisks denote four conserved Cys residues that are characteristic of plant invertase inhibitors. Gray and black vertical shading represents regions exhibiting medium and high degrees of amino acid identities, respectively.

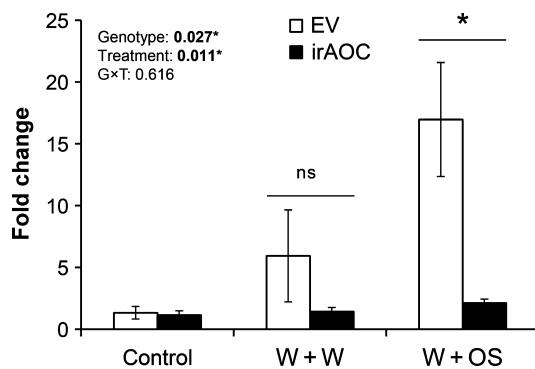


**Fig. 2** Silencing *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) suppresses herbivore-induced up-regulation of NaCWII. Relative transcript abundance of NaCWII in empty vector (EV) plants (a) and two independently transformed NaCWII-silenced lines (inverted repeat cell wall invertase inhibitor, irCWII), irCWII-1 (b) and irCWII-2 (c), is shown. mRNA was quantified by RT-qPCR in unelicited *N. attenuata* leaves, and at three time-points (1, 3, and 6 h) following wounding and treatment with 20  $\mu$ l of water (W + W) or 1 : 5 water-diluted *Manduca sexta* oral secretions (W + OS). NaCWII mRNA levels are expressed as the ratio of abundance relative to ACTIN. Mean  $\pm$  SE ( $n = 3$ ) are shown. The asterisk indicates a significant W + OS effect ( $P < 0.05$ ). ns, not significant.

development (Fig. S3). In EV plants, we observed a reduction in the growth of elongating stems (Fig. 5b) and rosettes (Fig. 5c) following one W + OS elicitation. This reduction was absent in irCWII plants (Fig. 5d–g). Following multiple elicitations (three rounds of damage across 6 d), a reduction in stem height was observed across all genotypes. This reduction was more pronounced in EV plants than in irCWII-1 and irCWII-2 plants (Fig. 5h).

#### Silencing NaCWII increases carbohydrate depletion

To examine whether NaCWII affects the accumulation of plant primary and secondary metabolites following simulated herbivory, we measured foliar concentrations of soluble sugars and starch in EV and irCWII-1 plants in a time course following repeated damage. As we expected based on previously published studies (Machado *et al.*, 2013), soluble sugars (Fig. 6a,b) and starch (Fig. 6c) were significantly depleted in leaves in response to repeated W + OS treatments. This depletion was driven primarily by a reduction in glucose and



**Fig. 3** *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) is induced by simulated herbivory in a jasmonate (JA)-dependent manner. Analysis of mRNA was quantified by RT-qPCR in unelicited *N. attenuata* leaves, and 6 h following wounding and treatment with 20  $\mu$ l of water (W + W) or 1 : 5 water-diluted *Manduca sexta* oral secretions (W + OS). NaCWII mRNA levels are expressed as the ratio of abundance relative to ACTIN. Bars are mean  $\pm$  SE ( $n = 3$ ). Open bars are empty vector (EV) and closed bars are inverted repeat allene oxide cyclase line (irAOC) genotypes. Significant difference between genotypes under W + OS treatment: \*,  $P < 0.05$ . ns, not significant.

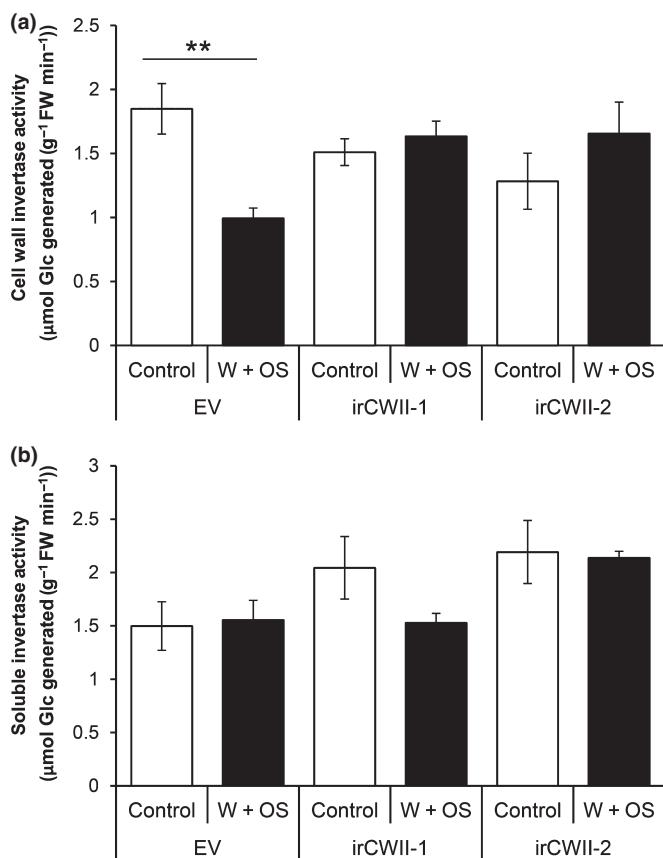
fructose (Fig. 6a), but not sucrose (Fig. 6b). Relative to EV plants, irCWII-1 plants displayed a greater depletion in glucose and fructose, which occurred during the early morning time-point (06:00 h; Tukey's *post hoc* tests;  $P < 0.05$ ) and at midday (14:00 h;  $P < 0.001$ ) (Fig. 6a). Silencing NaCWII also increased starch depletion following W + OS at the 14:00 and 22:00 h time-points (Fig. 6c;  $P < 0.01$ ). By contrast, sucrose concentrations were higher in irCWII-1 plants relative to EV at 22:00 h (Fig. 6b;  $P < 0.01$ ). NaCWII-silenced plants displayed constitutively higher concentrations of glucose and fructose (Fig. S4a) and sucrose (Fig. S4b) relative to EV controls, which was most pronounced during the day (14:00 h; Fig. S4).

#### NaCWII-silenced plants are attenuated in herbivore-induced secondary metabolites

Similar to previous studies, we found that foliar concentrations of nicotine, caffeoylputrescine, and dicafeoylspermidine were significantly elevated upon repeated W + OS treatments (Fig. 7a–c). Relative to EV controls, irCWII-1 plants were attenuated in the production of dicafeoylspermidine following W + OS (Fig. 7c; significant effect of genotype under W + OS;  $P < 0.05$ ). Silencing NaCWII had no effect on herbivore-induced 17-hydroxygeranylinalool diterpenoid glycoside (HGL-DTG) concentrations (Fig. S5a). Foliar rutin concentration was reduced following W + OS, an effect that was more pronounced in EV plants (Fig. S5b).

#### Silencing NaCWII does not affect herbivore-induced changes in phytohormones

To examine possible systemic signals that trigger the herbivore-induced changes in *N. attenuata* metabolism and growth, we evaluated the induction of defense-related phytohormones in



**Fig. 4** Simulated herbivory decreases cell wall invertase activity in a *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII)-dependent manner. Cell wall (a) and soluble (b) invertase activities in empty vector (EV) and two NaCWII-silenced lines (inverted repeat cell wall invertase inhibitor, irCWII-1 and irCWII-2) are shown. Two *N. attenuata* rosette leaves were treated every 48 h for 6 d with 1 : 5 water-diluted *Manduca sexta* oral secretions (W + OS; closed bars) from *M. sexta* larvae or remained untreated as a control (open bars). Bars represent mean  $\pm$  SE ( $n = 5$ ). Significant difference between treatments within each genotype: \*\*,  $P = 0.01$ .

irCWII and EV plants in response to *M. sexta* attack. We found that irCWII plants retained an intact JA burst following simulated herbivory (Fig. S6), and accumulated other phytohormones such as ABA, SA and ethylene to the same extent as EV plants (Figs S7, S8).

#### NaCWII regulates *N. attenuata*, not *M. sexta*, invertase activity

We took a two-pronged approach to assess whether NaCWII influences the growth and performance of *M. sexta* larvae. First, we assessed the utilization of leaf material by *M. sexta* larvae using Waldbauer (Waldbauer, 1968) and glasshouse performance assays. Second, we measured the activity of mid-gut invertases of larvae fed on EV or irCWII tissue. Results from the Waldbauer assay showed that *M. sexta* both consumed (CI) and effectively converted ingested material (ECI) to the same degree on EV and irCWII plants (Table 1). Larvae feeding on elicited irCWII leaves displayed a greater ability to digest this leaf material (AD;

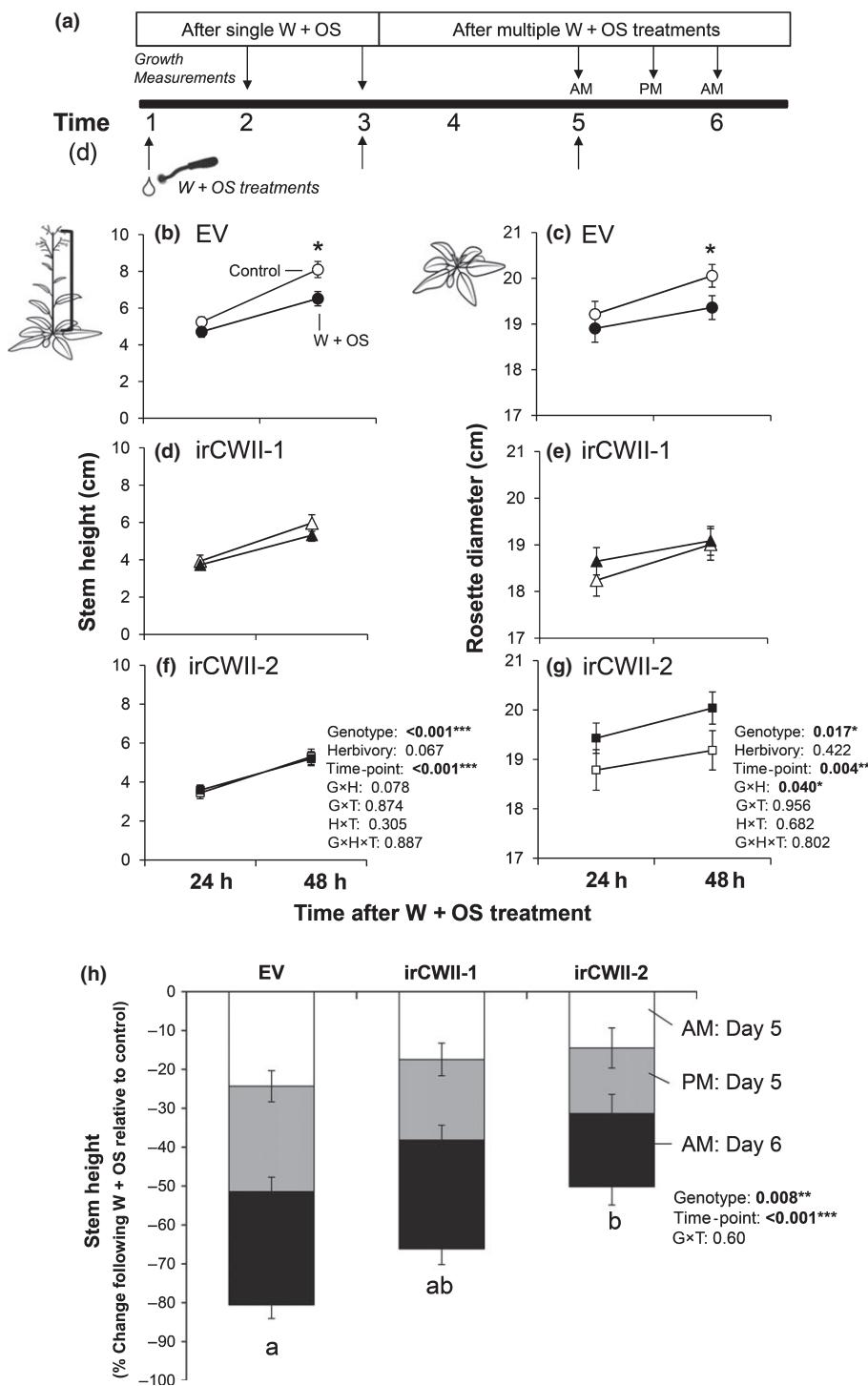
$P = 0.002$ ); however, the conversion of digested material to larval biomass occurred at a reduced capacity compared with larvae fed on elicited EV leaves (ECD;  $P = 0.01$ ). All larvae produced equivalent amounts of frass and attained similar masses by the end of the experiment (Table 1). Consistent with these findings, *M. sexta* larval mass gain was not influenced by plant genotype in the glasshouse performance assay (two-way ANOVA with *post hoc* Tukey's comparisons;  $P = 0.199$ ; Fig. S9). We found no difference in the activities of insoluble (Fig. S10a) or soluble (Fig. S10b) invertases extracted from the mid-gut of larvae fed EV or irCWII tissue (one-way ANOVA;  $P = 0.713$ ).

#### Discussion

We identified NaCWII, a homolog of INVINH1 which is known to specifically inhibit CWI activity in tomato (Jin *et al.*, 2009), and used a reverse genetics approach to silence its expression in *N. attenuata* by RNA interference in order to understand the role of this putative invertase inhibitor in herbivore-induced plant responses. Our analysis of invertase activity, downstream changes in primary and secondary metabolites, and subsequent changes in plant growth of EV control and NaCWII-silenced lines provides new insights into the post-translational regulation of CWI during plant defense and suggests a role for NaCWII in regulating the balance between growth and defense in herbivore-attacked *N. attenuata* plants.

In wild-type *N. attenuata*, we found that the expression of NaCWII increased dramatically following simulated herbivore attack. This corresponded to the suppression of CWI activity, but not of soluble invertases. The specificity of NaCWII for regulating CWI activity is consistent with other studies demonstrating that the overexpression of an invertase inhibitor in *A. thaliana* (INVINH1) specifically reduces CWI activity, while silencing its expression in tomato significantly increased the activity of CWI, without altering activities of cytoplasmic and vacuolar invertases (Jin *et al.*, 2009). The suppression of CWI activity following simulated herbivory observed in our study may serve to 'protect' sucrose from cleavage to buffer against the characteristic decrease in photosynthetic rate that occurs in local tissues following herbivory (Zangerl *et al.*, 2002). Alternatively, the response may reflect the global inhibition of protein synthesis, which is thought to anticipate the need to redirect resources to defensive functions (Schwachtje & Baldwin, 2008).

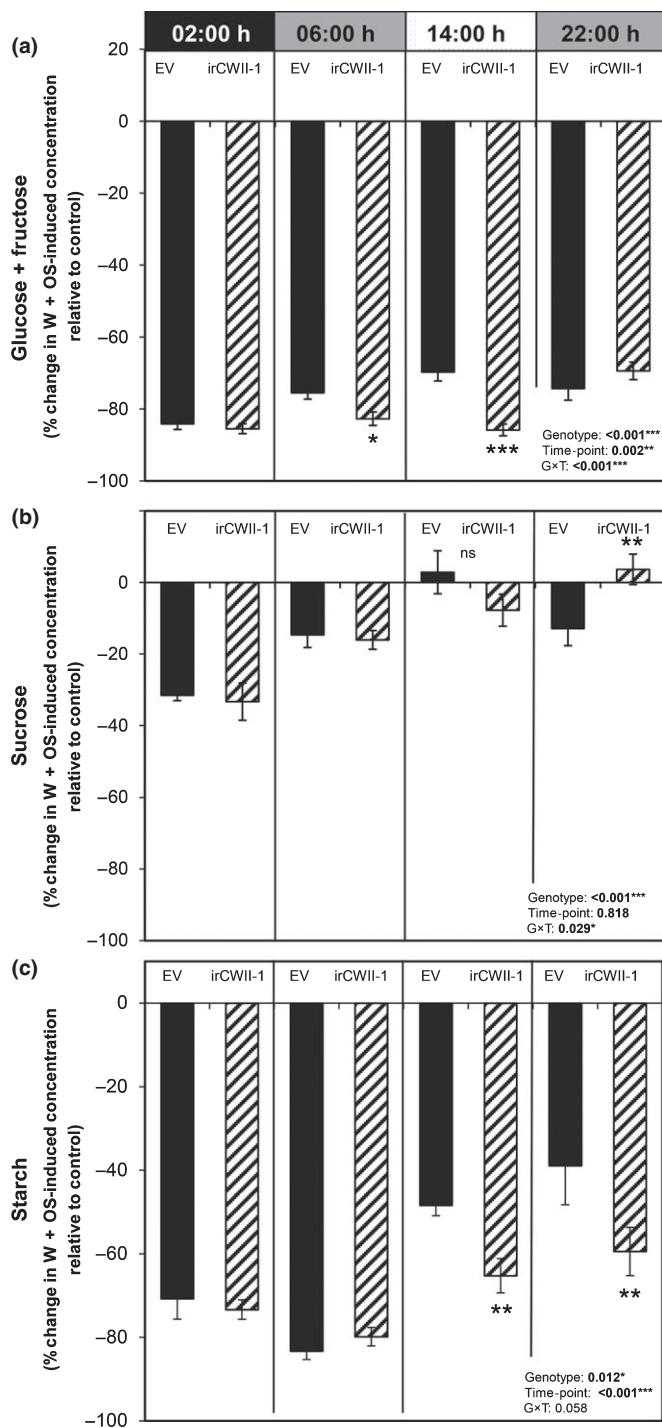
As expected based on our previous work (Machado *et al.*, 2013), the concentrations of soluble sugars and starch were significantly depleted in leaves of EV plants following W + OS events – a phenomenon assumed to reflect the increase in localized sink metabolism that is necessary to satisfy the increased demand for energy and building blocks to support secondary metabolism during plant defense responses. However, in contrast to wild-type conditions, silencing NaCWII led to a greater depletion in the plant's soluble carbohydrate pools following simulated herbivory. This co-occurred with the attenuation of OS-induced secondary metabolism, in particular a decrease in phenolamide accumulation. In contrast, irCWII plants retained their ability to sustain normal growth



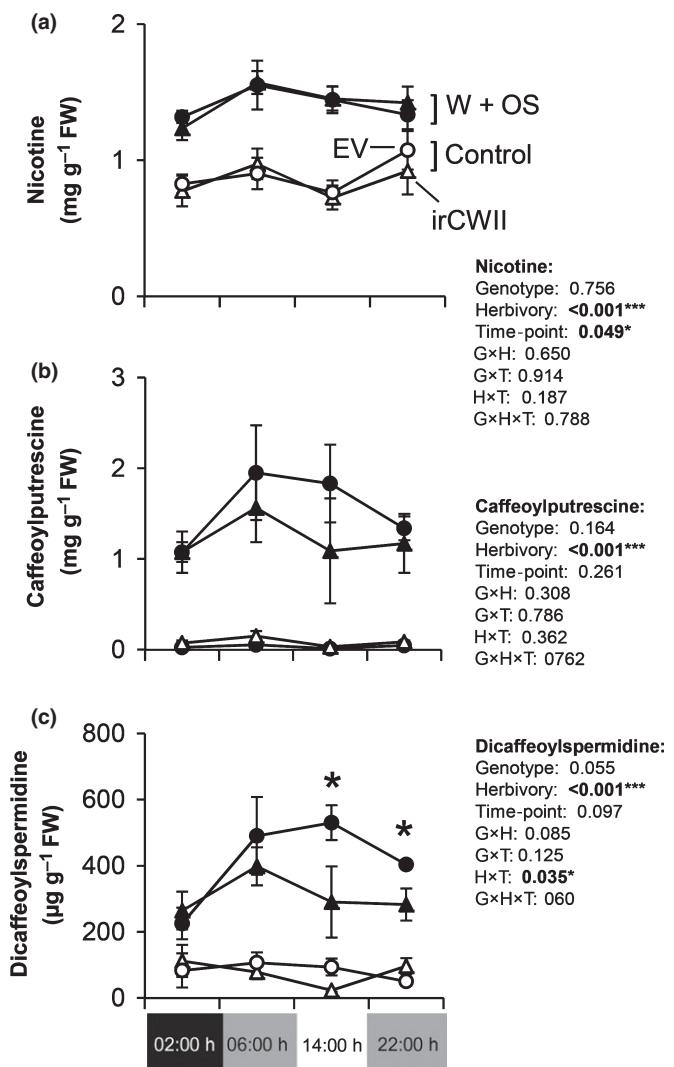
**Fig. 5** Silencing *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) attenuates herbivore-induced growth suppression. (a) Experimental plan to assess the role of NaCWII in *N. attenuata* growth following single and multiple 1 : 5 water-diluted *Manduca sexta* oral secretion (W + OS) treatments. All W + OS treatments were performed at 08:00 h. (b–g) Short-term growth suppression aboveground following W + OS is NaCWII-dependent (control, open points; W + OS, closed points). Stem height (b, d, f) and rosette diameter (c, e, g) were measured at 24 and 48 h following one W + OS treatment. Results of three-way ANOVAs are shown (\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ). Asterisks indicate a significant difference between empty vector (EV) and NaCWII-silenced (inverted repeat cell wall invertase inhibitor, irCWII) genotypes within a given time-point ( $P < 0.05$ ). (h) Silencing NaCWII affects the magnitude of W + OS-induced growth suppression following multiple W + OS treatments. Stem heights of EV and two irCWII lines were measured at three time-points following W + OS elicitation (AM: Day 5, morning of the final W + OS treatment day, white; PM: Day 5, 12 h following the final treatment, gray; AM: Day 6, 24 h following final W + OS treatment, black). Data are shown as per cent decrease in W + OS-treated plants relative to control groups. Mean  $\pm$  SE are shown in all graphs ( $n = 35$ ). Letters indicate significant differences between final stem heights ( $P < 0.05$ ).

aboveground. Phenolamide accumulation across tissue types of wild-type *N. attenuata* is known to be highly tuned to changes in plant ontogeny (Kaur *et al.*, 2010; Onkokesung *et al.*, 2012; Gaquerel *et al.*, 2014). Dicaffeoylspermidine, for example, has been shown to accumulate to a greater extent during early elongating stages in *N. attenuata*, where its accumulation peaks in the young rosette leaves and the basal stem following W + OS elicitation (Onkokesung *et al.*, 2012). Similar tissue- and age-specific patterns have been noted for phenolamides in

other plant species (Martin-Tanguy, 1985; Facchini *et al.*, 2002; Edreva *et al.*, 2007; Grienberger *et al.*, 2009; Luo *et al.*, 2009). In our study, growth parameters were monitored during the early elongation stage. Thus, it is possible that the exaggeration of OS-induced carbohydrate depletion and attenuation of OS-induced growth depression in irCWII plants suggests that NaCWII may function to regulate the balance between stem elongation and phenolamide production during this time of rapid growth.



**Fig. 6** Silencing *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) increases herbivore-induced leaf carbohydrate depletion. Combined glucose and fructose (a), sucrose (b) and starch (c) concentrations were measured in a time course following four rounds of simulated herbivory (1 : 5 water-diluted *Manduca sexta* oral secretions; W + OS). Bars represent mean concentration in W + OS tissue relative to mean control  $\pm$  SE ( $n = 4$ ). Asterisks indicate significant differences between empty vector (EV; black bars) and NaCWII-silenced *N. attenuata* plants (inverted repeat cell wall invertase inhibitor, irCWII-1; hatched bars) within each time-point. \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P < 0.05$ ; ns, not significant.



**Fig. 7** Silencing *Nicotiana attenuata* cell wall invertase inhibitor (NaCWII) attenuates the production of herbivore-induced leaf secondary metabolites. Nicotine (a), caffeoylputrescine (b), and dicaffeoylspermidine (c) were quantified in local *N. attenuata* leaves at four time-points following repeated simulated herbivory. Open points, untreated controls; closed points, simulated herbivory (1 : 5 water-diluted *Manduca sexta* oral secretions (W + OS)). Results of three-way ANOVAs are shown (\*\*\*,  $P < 0.001$ ; \*,  $P < 0.05$ ). All points represent mean  $\pm$  SE ( $n = 4$ ). Asterisks indicate significant differences between empty vector (EV) (circles) and irCWII-1 (triangles) within herbivory treatment and time-point ( $P < 0.05$ ).

In our study, we found that the attenuation of OS-induced phenolamides in irCWII plants corresponded with an increase in the digestibility of tissues to feeding *M. sexta* larvae. While this result is consistent with previous work linking a decrease in phenolamides to increased herbivore performance (Kaur *et al.*, 2010), a clear defensive role for NaCWII mediated through changes in plant secondary chemistry remains elusive, as *M. sexta* feeding on irCWII plants did not outperform larvae feeding on EV plants (Fig. S9). Changes in phenolamide accumulation elicited by

*M. sexta* feeding on *N. attenuata* are known to intersect and therefore shape other connected metabolic pathways (Giquerel *et al.*, 2014), which may influence other aspects of herbivore feeding behavior and performance. Despite recent evidence suggesting that an excess of soluble sugars may be detrimental to *M. sexta* growth (Machado *et al.*, 2015), we found that the higher constitutive carbohydrate accumulation observed in irCWII lines relative to EV (Fig. S4) was unlikely to be a major determinant of *M. sexta* resistance (Fig. S9). Nevertheless, it is possible that the transgene may impact plant fitness in other ways, for example through changes in development or chemical composition of belowground biomass, which may affect a plant's ability to tolerate herbivory (Machado *et al.*, 2013); such aspects might be addressed in future investigations.

Given the essential role that CWI plays in carbon allocation processes, it is fitting to ask why an inhibitory protein such as NaCWII might exist to limit CWI activity in plants after herbivore attack. Based on our current findings, the regulation of CWI by its inhibitor NaCWII appears to function to optimize the allocation of carbon among primary and secondary metabolism, and plant growth. Having additional regulatory switches downstream of JA signaling may act as a strategy to ensure efficient utilization of limiting resources for growth and defense. This hypothesis is supported by our observation that growth and secondary metabolisms become deregulated in NaCWII-silenced plants. The limitation of CWI activity by its inhibitor following herbivore attack may allow plants to dictate the timing of defense induction, thereby guaranteeing optimal carbon allocation to growth and defense.

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## Supporting Information

Additional supporting information may be found in the online version of this article.

**Fig. S1** The cDNA sequence of the putative cell wall invertase inhibitor in *N. attenuata*.

**Fig. S2** NaCWII expression in independent transgenic lines.

**Fig. S3** Silencing NaCWII does not influence early rosette growth.

**Fig. S4** Silencing NaCWII increases constitutive carbohydrate accumulation in *N. attenuata* leaves during the day.

**Fig. S5** Silencing NaCWII does not alter the accumulation of HGL-DGTs, but attenuates rutin production following simulated herbivory.

**Fig. S6** Silencing NaCWII does not affect the herbivore-induced accumulation of JA and JA-Ile.

**Fig. S7** Silencing NaCWII does not affect the herbivore-induced accumulation of abscisic acid or salicylic acid.

**Fig. S8** Silencing NaCWII does not affect the herbivore-induced accumulation of ethylene.

**Fig. S9** Silencing NaCWII does not affect *M. sexta* growth.

**Fig. S10** Silencing the *N. attenuata* invertase inhibitor does not affect the activity of invertases present in the mid-gut of *M. sexta* larvae.

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