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Research

RuBPCase activase (RCA) mediates growth–defense trade-offs: silencing RCA redirects jasmonic acid (JA) flux from JA-isoleucine to methyl jasmonate (MeJA) to attenuate induced defense responses in *Nicotiana attenuata*

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Summary

- RuBPCase activase (RCA), an abundant photosynthetic protein, is strongly down-regulated in response to *Manduca sexta*'s oral secretion (OS) in *Nicotiana attenuata*. RCA-silenced plants are impaired not only in photosynthetic capacity and growth, but also in jasmonic acid-isoleucine (JA-Ile) signaling, and herbivore resistance mediated by JA-Ile-dependent defense traits. These responses are consistent with a resource-based growth–defense trade-off.
- As JA + Ile supplementation of OS restored wild-type (WT) levels of JA-Ile, defenses and resistance to *M. sexta*, but OS supplemented individually with JA or Ile did not, the JA-Ile deficiency of RCA-silenced plants could not be attributed to lower JA or Ile pools or JAR4/6 conjugating activity. Similar levels of JA-Ile derivatives after OS elicitation indicated unaltered JA-Ile turnover, and lower levels of other JA conjugates ruled out competition from other conjugation reactions. RCA-silenced plants accumulated more methyl jasmonate (MeJA) after OS elicitation, which corresponded to increased jasmonate methyltransferase (JMT) activity.
- RCA silencing phenocopies JMT overexpression, wherein elevated JMT activity redirects OS-elicited JA flux towards inactive MeJA, creating a JA sink which depletes JA-Ile and its associated defense responses.
- Hence, RCA plays an additional non-photosynthetic role in attenuating JA-mediated defenses and their associated costs, potentially allowing plants to anticipate resource-based constraints on growth before they actually occur.

Introduction

Plants are attacked by a variety of herbivores and, in response, can activate defenses which affect directly or indirectly the attacking herbivores (Kessler & Baldwin, 2001; Steppuhn *et al.*, 2004; Zavala *et al.*, 2004). Plants are thought to deploy two alternative strategies against herbivores: (1) resistance; and (2) tolerance. These strategies have been well studied and have been explained by different theories. Among these, the optimal defense (OD) theory (Mckey, 1974, 1979; Rhoades, 1979) enjoys the most empirical support. This theory proposes that the distribution of defenses within a plant reflects the fitness value of the tissue for the plant, with higher value tissues being better defended than less valuable tissues. Moreover, this theory assumes that defenses are costly and that a trade-off exists between defense and growth, which, in turn, explains the prevalence of inducible defenses (Coley *et al.*, 1985; Heil & Baldwin, 2002). Hence, during herbivore attack, plants re-adjust their resource investment strategies to re-optimize their allocation of resources to resistance and tolerance mechanisms, growth and reproduction (Herms & Mattson, 1992). Under these circumstances, a rapid reallocation of resources to tolerance rather than the defense response could maximally reduce the negative fitness consequences of herbivore attack (Schwachtje & Baldwin, 2008). However, very little is known about the molecular mechanisms used by plants to optimize their resource allocation after herbivore attack. For example, whilst tolerating herbivory, plants allocate newly assimilated carbon (C) to their roots to be used for post-herbivory re-growth, rather than transporting it to the young leaves (Schwachtje *et al.*, 2006).

When *Nicotiana attenuata* is attacked by its specialist lepidopteran herbivore, *Manduca sexta*, fatty acid amino acid conjugates (FACs), present in larval oral secretions (OSs), activate early defense responses by activating the jasmonic acid (JA) signaling network (Halitschke *et al.*, 2001). JA, a linolenic acid-derived compound, is rapidly and transiently accumulated after herbivory (Creelman *et al.*, 1992; Farmer & Ryan, 1992; Baldwin *et al.*, 1994). JA biosynthesis begins in chloroplasts after lipase activation, which releases fatty acids from the membrane lipids. Free linolenic acid is converted to 13S-hydroperoxyoctadecatrienoic acid (HPOT) by a specific lipoxygenase, which is

subsequently converted to 12-oxo-phytodienoic acid (OPDA) by allene oxide synthase (AOS) and allene oxide cyclase (AOC). OPDA is transported to the peroxisome and, after reduction and three cycles of β-oxidation by the acyl CoA oxidase 1 enzymes, multifunctional protein and L-3-ketoacyl CoA-thiolase, is transformed to JA (Schaller & Stintzi, 2009). JA is then exported to the cytosol through the peroxisomal membrane by membrane proteins (Arai *et al.*, 2008), where it is further metabolized. The accumulation of JA is regulated not only by JA biosynthetic genes and their associated transcription factors, but also by the availability of fatty acid precursors (Howe & Schilmiller, 2002; Chung *et al.*, 2008; Paschold *et al.*, 2008; Skibbe *et al.*, 2008; Kallenbach *et al.*, 2010). A portion of JA is conjugated to different amino acids, with the isoleucine conjugate (JA-Ile) associating with Coronatine insensitive 1 (COI1) to promote the degradation of Jasmonate ZIM domain (JAZ) repressors by the 26S proteasome (Thines *et al.*, 2007). The degradation of the JAZ repressor releases the MYC 2 transcription factor from repression and activates JA-responsive genes involved in plant defense (Fonseca *et al.*, 2009; Memelink, 2009). JA-Ile is the active molecule triggering downstream defense responses, and therefore the amount of JA-Ile is directly correlated with the magnitude of a plant's defense response. In *N. attenuata*, silencing the expression of JAR4/6, the enzyme conjugating JA and Ile, attenuates JA-Ile production and resistance against attack from *M. sexta* larvae (Wang *et al.*, 2007).

A comparative proteomic–transcriptomic study revealed that, although defense-related genes are up-regulated after herbivory, photosynthesis-related genes are down-regulated (Giri *et al.*, 2006; Bilgin *et al.*, 2010). Remarkably, herbivore attack causes a greater reduction in a plant's photosynthetic capacity than would be predicted on the basis of the canopy area removed by the herbivore (Zangerl *et al.*, 2002). RuBPCase activase (RCA), an abundant photosynthetic protein, is strongly down-regulated after herbivore attack or simulated herbivory (Giri *et al.*, 2006). RCA modulates the activity of RuBPCase, the major photosynthetic protein involved in C fixation, by removing inhibitory sugar phosphates from the active site of the enzyme (Portis, 2003). RCA's role in photosynthesis and growth has been well studied, and RCA-deficient plants show reduced photosynthetic rates and growth, and accumulate less biomass (He *et al.*, 1997; Ilyin *et al.*, 2005). Reduced growth limits the food available for herbivores; therefore, decreasing growth could be a part of a plant's defense strategy (Hermsmeier *et al.*, 2001; Hahlbrock *et al.*, 2003). In addition, a decrease in C supply could alter the expression of genes of enzymes involved in C utilization and storage (Koch, 1996).

Previously, we have observed that, in addition to impaired photosynthetic capacity and growth, RCA-silenced *N. attenuata* plants are impaired in JA-Ile signaling, herbivore resistance and many defense traits that mediate resistance (Mitra & Baldwin, 2008) (Fig. 1). The reduction in photosynthesis and growth associated with RCA silencing is congruent with RCA's biochemical function, as revealed by work with Arabidopsis, tobacco and rice (He *et al.*, 1997; Ilyin *et al.*, 2005). However, the decrease in JA-Ile accumulation after RCA silencing was novel. Previous

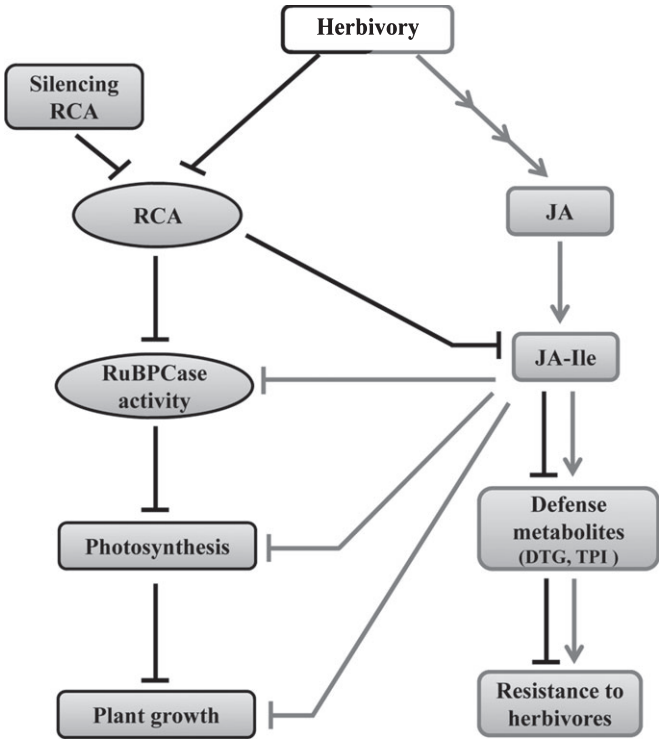

Fig. 1 An overview of the consequences of herbivory and RuBPCase activase (RCA) silencing in *Nicotiana attenuata* plants. In *N. attenuata*, attack from *Manduca sexta* larvae results in a jasmonic acid-isoleucine (JA-Ile) burst, which increases a suite of JA-induced defense compounds (diterpene glycosides (DTGs) and trypsin protease inhibitors (TPIs)) and herbivore resistance, and reduces the levels of major photosynthetic proteins RuBPCase and RCA, the plant's photosynthetic rate and growth. RCA-silenced plants are impaired in their RuBPCase activity, photosynthetic capacity, growth, JA-Ile signaling, JA-induced defense compounds (DTGs and TPIs) and, consequently, herbivore resistance. Black lines depict the consequences of RCA silencing and gray arrows show the interaction between the herbivory-induced JA-mediated defense pathway and photosynthesis and growth. The research presented here reveals how RCA down-regulation also down-regulates JA signaling and its associated defenses.

experimentation had ruled out limitations in the Ile pool at the wound site or activity of the conjugating enzyme as being responsible for the attenuated JA-Ile levels of RCA-silenced plants (Mitra & Baldwin, 2008). As a member of the AAA+ (for ATPases associated with a variety of cellular activities) protein family, RCA may also be involved in other cellular processes (Ogura & Wilkinson, 2001). In different plant systems, the regulation of RCA in response to UV-B light, ozone, drought and heat stress (Pelloux *et al.*, 2001; Liu *et al.*, 2002; Bota *et al.*, 2004; Demirevska-Kepova *et al.*, 2005) suggests that RCA is involved in diverse stress-related functions. Recently, RCA in Arabidopsis was found to be down-regulated at both transcript and protein levels in a COI1-dependent manner, after elicitation with JA (Shan *et al.*, 2011). The increased susceptibility of RCA-deficient *N. attenuata* plants to herbivore attack (Mitra & Baldwin, 2008) suggests an additional, defense-related role for RCA other than in RuBPCase activation.

Previously, we have characterized two independently transformed RCA-silenced lines (line 1 and line 2) with similar

degrees of reduction in photosynthetic rate, JA-Ile levels and resistance against *M. sexta* larvae (Mitra & Baldwin, 2008). Here, we used a single RCA-silenced line (line 2) to elucidate the mechanisms responsible for its attenuated JA-Ile accumulation and herbivore resistance. We examined its JA metabolism and JA signaling after simulated herbivory with JA or Ile or both (JA + Ile) supplementation. As adenylation of JA initiates its conjugation to amino acids, and adenylation is an energy-demanding process (Staswick *et al.*, 2002), a decrease in photosynthetic capacity may reduce the ATP supply required for JA adenylation. By extending the dark period in wild-type (WT) plants, we examined the effect of reduced net C gain on JA adenylation and, consequently, on JA-Ile accumulation. Lastly, we examined the growth of RCA-silenced plants after simulated herbivory and methyl jasmonate (MeJA) treatment.

Materials and Methods

Plant material and growth conditions

A previously characterized RCA-silenced homozygous line was used in all experiments (Mitra & Baldwin, 2008). The plants of the 31st inbred generation of *N. attenuata* (originally collected from Utah, USA, and the same accession was used to create the transformed plants) were used as WT plants. Previously, we have shown that the growth and herbivory-induced responses of empty vector transformed plants (which can be used as transgenic controls) are similar to those of WT plants (Mitra & Baldwin, 2008; Schwachtje *et al.*, 2008); therefore, we used WT plants as controls in all experiments. Seeds were smoke germinated on Gamborg's B5 medium (Kruegel *et al.*, 2002). Plants were grown in 1-l pots containing a peat-based substrate (Klasmann Tonsubstrat, Geeste-Groß Hesepe, Germany) in the glasshouse of the Max Planck Institute for Chemical Ecology (Jena, Germany) at 24–26°C, 16 h light (supplemental lighting by Philips Sun-T Agro 400 W and 600 W sodium lights) and 55% humidity. Four- to 5-wk-old rosette plants were used for all experiments.

Supplementation experiments

Fully expanded (+ 1) rosette leaves were punctured with a pattern wheel and the wounds were immediately treated with 20 µl of OS that contained 0.625 µmol JA or 0.625 µmol [$^{13}C_6$]Ile or both 0.625 µmol [$^{13}C_6$]JA and 0.625 µmol Ile (dissolved in 30% (v/v) ethanol–water); control plants were wounded and treated with similarly diluted OS (diluted in 30% (v/v) ethanol–water). These concentrations of JA and Ile have been shown in previous research to restore the deficiencies of either JA or Ile required to activate JA-Ile signaling at WT levels in either JA- or Ile-deficient plants (Kang *et al.*, 2006; Paschold *et al.*, 2007). The levels of JA and JA-Ile in WT *N. attenuata* plants reached their maxima after 60 min of wound + oral secretion (W + OS) elicitation and returned to basal levels 180 min after W + OS elicitation. Therefore, leaf tissue was harvested 45, 60 and 180 min after the treatments.

Analysis of JA and JA conjugates

Approximately 200 mg of harvested leaf tissue from each genotype were extracted and analyzed for JA and JA-Ile levels by an LC/MS/MS system configured with an electro-spray ionization source (1200L Varian, Palo Alto, CA, USA), as described previously (Wang *et al.*, 2007). Negative or positive ionization mode was used depending on the jasmonate structure, as described in Stitz *et al.* (2011b) [$^{13}C_6$]JA-Ile was used as an internal standard for the relative quantification of hydroxylated (OH)-JA, OH-JA-Ile, carboxylated (COOH)-JA-Ile, JA-valine (JA-Val) and JA-glucose (JA-Glc).

Extended night experiment

In tobacco, photosynthetic C assimilates, the sugars, play an important role in the regulation of nitrogen (N) metabolism. Under a short-day condition, tobacco plants become C and N limited compared with plants grown under long days, which fix more C and accumulate more N (Matt *et al.*, 1998). As RCA-silenced plants are probably C limited, we evaluated whether the C limitations could be responsible for shortages in the levels of N-containing Ile and, consequently, the impaired JA-Ile accumulations observed in RCA-silenced plants. In addition, the C limitation may limit the ATP available for JA adenylation (Staswick, 2002). To test these hypotheses, we grew rosette-stage WT plants, normally grown under 16 h : 8 h (light : dark) regimes, under three different light : dark periods, namely 16 h : 8 h, 12 h : 12 h and 8 h : 16 h for 1 d, and the levels of starch were determined as a measure of net C gain as described previously (Smith & Zeeman, 2006; Machado *et al.*, 2013). We considered 16 h : 8 h, 12 h : 12 h and 8 h : 16 h light regimes as providing normal, moderate and severely depleted C regimes, respectively. Fully expanded (+ 1) rosette leaves were wounded with a pattern wheel and the wounds were treated with *M. sexta* OS (1 : 1 diluted with water). Tissues were harvested after 1 h of elicitation, which corresponded to the end of the light period (at 22:00, 18:00 and 14:00 h) or the end of the dark period (06:00 h). Harvested tissues were analyzed for starch, JA, JA-Ile and MeJA contents.

Manduca sexta larval performance

Rosette (+ 1) leaves were wounded and treated with OS or OS containing JA or Ile or JA + Ile. To evaluate the effects of supplementation with JA, Ile and JA + Ile on *M. sexta* larval mass, freshly hatched larvae were placed on the treated leaves of 15 replicate plants of each genotype, 24 h after elicitation, and the larval mass was recorded after 12 d of feeding on these elicited plants.

In vivo and *in vitro* enzyme assays

Fully expanded (+ 1) rosette leaves were punctured with a pattern wheel and immediately treated with 20 µl OS containing 0.625 µmol JA (dissolved in 30% (v/v) ethanol–water) or lanolin containing 150 µg MeJA; control plants were wounded and treated with similarly diluted OS (diluted in 30% (v/v)

ethanol–water) or pure lanolin, respectively. Tissues were harvested 60 min after elicitation and the levels of MeJA and JA were quantified.

For the *in vitro* enzyme assays, fully expanded (+1) rosette leaves were punctured with a pattern wheel and the puncture wounds were immediately treated with 20 µl OS (1 : 1 diluted with water). Leaf tissue was harvested 60 min after elicitation. Untreated samples served as controls. Total protein was extracted from 200 mg of leaf tissue in a buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM EDTA and 10 mM β-mercaptoethanol. Protein concentration was determined by the 2D quant kit (Amersham) with BSA as a standard.

Jasmonate methyltransferase (JMT) activity was determined by measuring the production of MeJA from 1 mM JA and 1 mM *S*-adenosylmethionine (SAM). The 50 µl assay buffer contained 50 mM Tris-HCl (pH 7.5), 100 mM KCl and 10 mM β-mercaptoethanol. The reaction mixture was incubated at 20°C for 30 min, and MeJA was extracted with 100 µl of ethyl acetate (Seo *et al.*, 2001). The amounts of MeJA produced were determined by LC/MS/MS as described previously (Stitz *et al.*, 2011b). *In vitro* MeJA esterase (JME) activity was estimated by measuring the amount of JA released from de-esterified MeJA as described previously (Wu *et al.*, 2008).

Plant growth

Growth performance was estimated by repeated measures of stalk lengths during the rosette-to-flowering transition. Fully expanded rosette leaves (+1) of WT and RCA-silenced plants were wounded with a pattern wheel and treated with *M. sexta* OS (diluted with water 1 : 1) or lanolin containing 150 µg MeJA. Stalk lengths were determined 7 d after treatment. Untreated plants served as controls.

Statistical analysis

Data were analyzed with Stat View (Abacus Concepts Inc., Berkeley, CA, USA) in all the experiments, data were subjected to one-way ANOVA and statistical significance was determined using Fisher's least-significant difference (LSD) *post-hoc* test.

Results

Consequences of herbivory and RCA silencing in *N. attenuata* plants

Previously, we have described the consequences of herbivory and RCA silencing on *N. attenuata* plants. Here, we summarize the previous results (Fig. 1) to facilitate the understanding of the relationship between 'responses to herbivory' and 'RCA silencing' in *N. attenuata* plants. When *N. attenuata* is attacked by its native herbivore *M. sexta*, JA and JA-Ile levels accumulate rapidly and transiently. JA-Ile is the main signaling molecule which increases a suite of JA-dependent defense compounds (namely nicotine, diterpene glycosides (DTGs) and trypsin protease inhibitors (TPIs)) and herbivore resistance. At the same time, the levels of the major photosynthetic proteins RuBPCase and RCA, and the plant's photosynthetic capacity and growth, are reduced. RCA-silenced plants, however, show reduced RCA activity, photosynthetic rate, growth, JA-Ile signaling, JA-induced defense compounds (DTGs and TPI) and, consequently, herbivore resistance (Fig. 1). Therefore, RCA silencing affects not only a plant's photosynthetic rate and growth, but also impairs its defense responses. These results suggest that RCA plays a direct role in the optimization of growth and defense in *N. attenuata*.

Impaired JA-Ile signaling in RCA-silenced plants does not result from lower JA pools or reduced JA adenylation or conjugation activity at the wound site

Previously, we have reported that the attenuated JA-Ile levels observed in RCA-silenced plants do not result from decreased Ile pools at the wound site or lower transcript levels of JAR4/6 (Mitra & Baldwin, 2008). However, compartmentalization of wound-induced JA might restrict the conjugation of JA with Ile, and influence the accumulation of JA-Ile after W + OS elicitation. To evaluate whether deficiencies in the availability of JA for conjugation were responsible, we measured JA-Ile accumulation in RCA-silenced plants treated with JA-supplemented OS (W + OS + JA). W + OS + JA treatment increased the basal levels, but did not restore JA-Ile to the levels found in OS-elicited WT plants (ANOVA; $F_{3,12} = 17.56$; $P = 0.005$) (Fig. 2a). From these results, we infer that lower JA pools at the wound site are not responsible for the attenuated JA-Ile burst in RCA-silenced plants.

Adenylation of JA initiates its conjugation to amino acids (Staswick *et al.*, 2002), and adenylation is known to be an energy-demanding process; therefore, to test whether decreases in JA-Ile levels in RCA-silenced plants are caused by impaired JA adenylation, we measured JA-Ile accumulation in WT and RCA-silenced plants treated with W + OS + JA + [$^{13}C_6$]Ile. If RCA-silenced plants showed attenuated conjugating enzyme activity or JA adenylation, they would not have been able to make JA-Ile at WT levels even after JA and [$^{13}C_6$]Ile supplementation. However, this treatment restored JA-Ile levels of RCA-silenced plants to those of WT plants (ANOVA; $F_{3,12} = 6.98$; $P = 0.19$) (Fig. 2b), demonstrating that neither the activity of the conjugating enzyme nor the efficiency of JA adenylation is altered after RCA silencing.

In addition, to examine the influence of net C gain on JA and JA-Ile accumulation, WT *N. attenuata* plants were grown under three different light regimes for 1 d and their starch, JA and JA-Ile levels were measured. The levels of starch accumulated at the end of the light period reflect the net C gain. We found that the extended night depleted the net C gain by 24% and 41% in 12 h : 12 h and 8 h : 16 h (light : dark) regimes, respectively, compared with the norm, that is, 16 h : 8 h (light : dark) regime (ANOVA; $F_{2,6} = 4.5$; $P_{12\,h:12\,h} = 0.09$; $P_{8\,h:16\,h} = 0.02$; Supporting Information Fig. S1a). JA accumulation was significantly decreased (ANOVA; $F_{5,12} = 6.7$; $P = 0.003$) in all samples collected at the end of the dark period compared with the samples collected at the end of the light period; however, JA-Ile accumulation was only lower in samples collected at the end of the 16 h

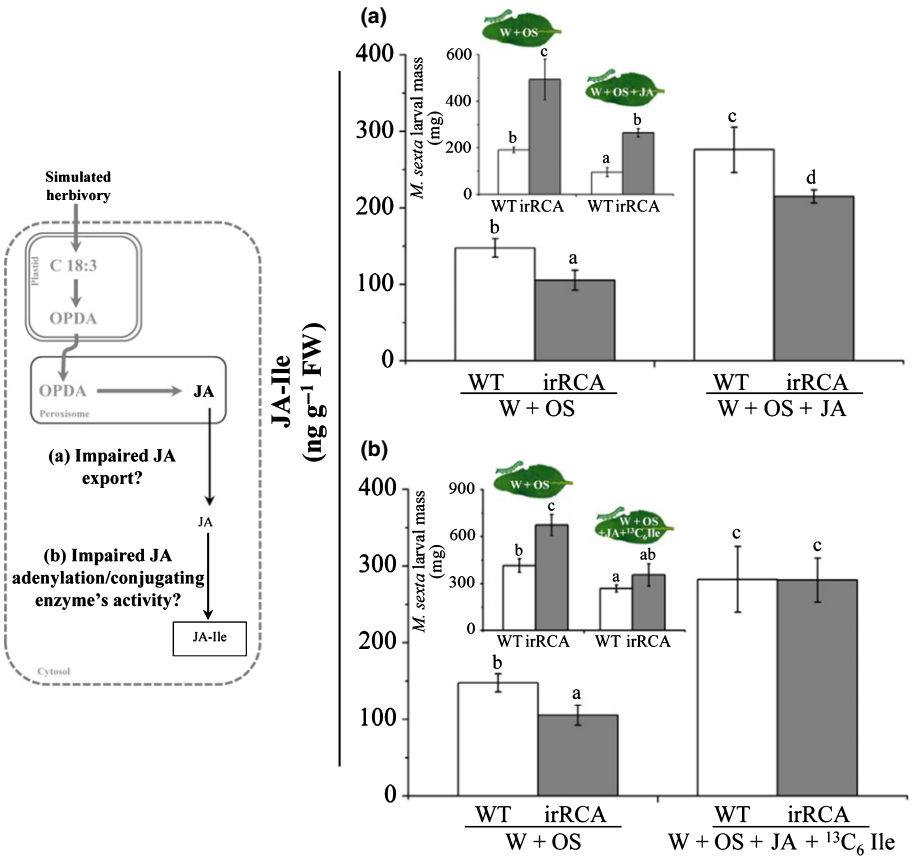

Fig. 2 Attenuated jasmonic acid (JA) pools or jasmonic acid-isoleucine (JA-Ile) conjugating activity at the wound site does not account for the attenuated JA-Ile burst and herbivore resistance in RuBPCase activase (RCA)-silenced *Nicotiana attenuata* plants. Left panel depicts JA and JA-Ile biosynthesis, which takes place in three different cellular compartments: 12-oxo-phytodienoic acid (OPDA) is synthesized in chloroplasts; OPDA is converted to JA in peroxisomes and subsequently conjugated with Ile in the cytosol. The thickness of the arrow and the font size indicate relative metabolite accumulations. Right panels show results. Oral secretion (OS)-elicited RCA-silenced plants accumulated (a) significantly less JA-Ile compared with wild-type (WT) plants with or without JA supplementation to the OS, but, when the OS was supplemented with both JA and [$^{13}C_6$]Ile, the elicited JA-Ile levels of RCA-silenced plants were fully restored to WT levels, as was herbivore resistance (b). Values are means (± SE) of four replicate plants from each genotype and treatment. *Manduca sexta* larvae reared on W + OS- or W + OS + JA-elicited RCA-silenced plants gained significantly more body mass than did larvae on similarly elicited WT plants (a, inset). However, resistance was fully restored when larvae were reared on W + OS + JA + [$^{13}C_6$]Ile-elicited RCA-silenced plants. These larvae attained a similar body mass to those on WT plants (b, inset). Values are means of 15 (± SE) replicate larvae per genotype and treatment. Different letters indicate significant differences at $P \leq 0.05$ by one-way ANOVA. irRCA, inverted repeat RCA; W + OS, wound + *M. sexta* oral secretion.

dark period (ANOVA; $F_{5,12} = 3.18$; $P = 0.02$; Fig. S1b). JA and JA-Ile levels in the samples collected at the end of the 12 h and 8 h dark periods were similar (ANOVA; $F_{5,12} = 6.7$; $P_{JA} > 0.05$; ANOVA; $F_{5,12} = 3.18$; $P_{JA\text{-}Ile} > 0.05$), but significantly lower for JA-Ile levels in the samples collected at the end of the 16 h dark period, compared with the samples collected at the end of the 8 h dark period (ANOVA; $F_{5,12} = 6.7$; $P = 0.008$; ANOVA; $F_{5,12} = 3.18$; $P_{JA\text{-}Ile} = 0.005$; Fig. S1b). From these results, we infer that a severe decrease in net C gain reduces JA-Ile bursts, but not JA bursts, after OS elicitation.

Resistance to *M. sexta* attack in RCA-silenced plants is restored by treatment with both JA and Ile, but not JA or Ile alone

To examine the impact of JA, Ile or JA + Ile supplementation on the performance of the herbivore, we compared the growth of *M. sexta* larvae fed on WT and RCA-silenced plants treated with W + OS, W + OS + JA, W + OS + [$^{13}C_6$]Ile or W + OS + JA + [$^{13}C_6$]Ile. We found that the larvae fed on W + OS- (ANOVA; $F_{1,28} = 11.97$; $P = 0.002$), W + OS + JA- (ANOVA; $F_{3,56} = 12.92$; $P = 0.01$) (Fig. 2a, inset) and W + OS + [$^{13}C_6$]Ile- (ANOVA; $F_{3,56} = 13.15$; $P < 0.0001$; Fig. S2) treated RCA-silenced plants gained significantly more mass than did larvae on comparably treated WT plants. Larvae fed on W + OS + JA + [$^{13}C_6$]Ile-treated plants did not differ in mass gain between WT and RCA-silenced plants (ANOVA; $F_{3,56} = 9.8$; $P = 0.24$) (Fig. 2b, inset). These results demonstrate that JA-Ile accumulation determines larval performance, from which we infer that the attenuated JA-Ile levels of RCA-silenced plants are responsible for the impaired herbivore resistance of these plants.

JA-Ile turnover is unaltered after RCA silencing

To evaluate whether increased conversion of JA-Ile to its inactive derivatives, namely OH- and COOH-JA-Ile, could explain the

lower levels of JA-Ile in RCA-silenced plants, we measured the levels of OH-JA-Ile and COOH-JA-Ile after W + OS elicitation in WT and RCA-silenced plants. In addition, WT and RCA-silenced plants were treated with W + OS + JA to increase the levels of JA-Ile derivatives, and their accumulations were measured over a 3-h period. W + OS- and W + OS + JA-treated WT and RCA-silenced plants showed similar levels of OH-JA-Ile after 45 and 60 min of induction (ANOVA$_{(W + OS)}$; $F_{7,24} = 21.29$; $P > 0.05$; ANOVA$_{(W + OS + JA)}$; $F_{7,24} = 272.64$; $P > 0.05$; Fig. 3a). However, after 180 min, RCA-silenced plants showed a significant decrease in OH-JA-Ile levels compared with WT plants (Fig. 3a) in both W + OS- (ANOVA$_{(W + OS)}$; $F_{7,24} = 21.29$; $P = 0.01$) and W + OS + JA- (ANOVA$_{(W + OS + JA)}$; $F_{7,24} = 272.64$; $P = 0.02$) treated plants. The levels of COOH-JA-Ile in W + OS-treated RCA-silenced plants were similar to those of WT plants throughout the time course (ANOVA$_{(W + OS)}$; $F_{7,24} = 25.59$; $P > 0.05$; Fig. 3a). However, W + OS + JA-treated RCA-silenced plants showed a significant decrease in COOH-JA-Ile levels only 180 min after elicitation (ANOVA$_{(W + OS + JA)}$; $F_{7,24} = 39.01$; $P = 0.002$). From these results, we infer that the JA-Ile turnover remains unaltered after RCA silencing.

JA flux is not redirected from Ile to other known JA derivatives in RCA-silenced plants

JA metabolism is controlled by multiple competing enzymes and the lower JA-Ile levels could result from increased flux to other conjugates. To evaluate this possibility, we measured the accumulation of all other major JA conjugates known to occur in *N. attenuata*, namely OH-JA, JA-Val and JA-Glc, after W + OS and W + OS + JA elicitations. The levels of OH-JA (ANOVA; $F_{7,24} = 14.13$; $P_{OH\text{-}JA} = 0.0009$) and JA-Val (ANOVA; $F_{7,24} = 55.5$; $P_{JA\text{-}Val} = 0.05$) were significantly lower in RCA-silenced plants (compared with WT plants) after 180 and 60 min of W + OS elicitation (Fig. 3b). The levels of JA-Glc were similar in W + OS-treated WT and RCA-silenced plants (ANOVA; $F_{7,24} = 7.58$; $P_{JA\text{-}Glc} = 0.055$). JA supplementation substantially increased the levels of OH-JA (23-fold), JA-Val (25-fold) and JA-Glc (125-fold) compared with the OS treatment. However, the accumulations of OH-JA (ANOVA; $F_{7,24} = 79.35$; $P_{OH\text{-}JA} > 0.05$), JA-Val (ANOVA; $F_{7,24} = 36.85$; $P_{JA\text{-}Val} > 0.05$) and JA-Glc (ANOVA; $F_{7,24} = 67.12$; $P_{JA\text{-}Glc} > 0.05$) were similar in WT and RCA-silenced plants (Fig. 3b).

RCA-silenced plants show elevated MeJA levels and JA methylation activity

A proportion of herbivory-elicited JA can be esterified to its volatile form MeJA, a reaction mediated by JMT (Seo *et al.*, 2001). However, in WT *N. attenuata*, the amounts of MeJA produced are very low (von Dahl & Baldwin, 2004). To determine whether JA methylation activity contributes to the decreased JA-Ile level in RCA-silenced plants, we measured the JA methylation (*in vivo* and *in vitro*) ability of both RCA-silenced and WT plants. *In vivo*

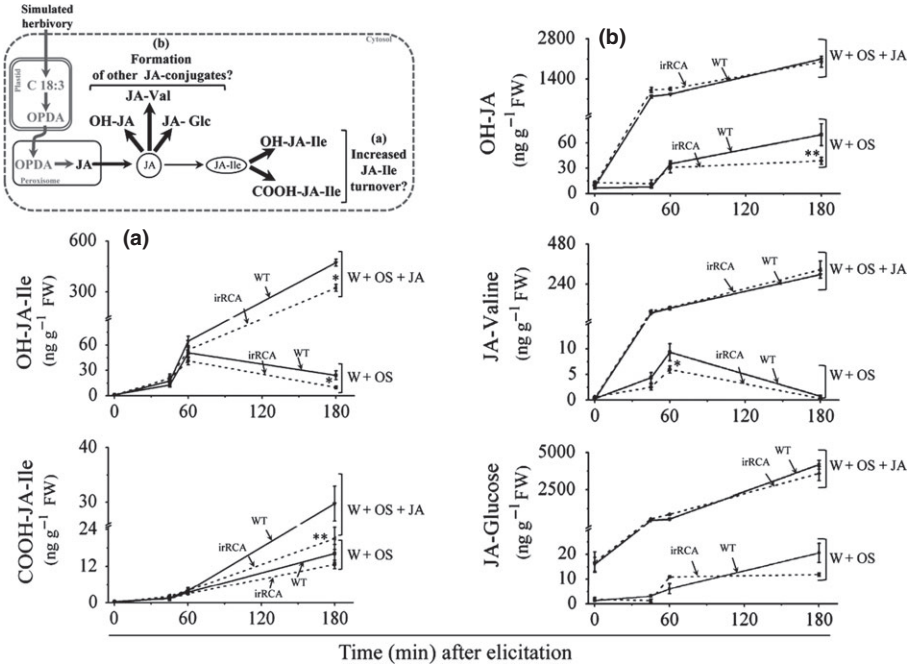

Fig. 3 Attenuated jasmonic acid-isoleucine (JA-Ile) bursts in RuBPCase activase (RCA)-silenced plants is not caused by either increased JA-Ile turnover or competition from other known conjugation reactions for JA. The accumulation of (a) hydroxy (OH)-JA-Ile and carboxy (COOH)-JA-Ile in RCA-silenced *Nicotiana attenuata* plants was significantly decreased after W + OS elicitation. After W + OS + JA elicitation, the level of OH-JA-Ile in RCA-silenced plants was similar to that of wild-type (WT) plants, but the level of COOH-JA-Ile was significantly lower than that of WT plants. The accumulation of (b) OH-JA, JA-valine (JA-Val) and JA-glucose (JA-Glc) was significantly lower in RCA-silenced plants than in WT plants after W + OS elicitation; however, after W + OS + JA elicitation, the levels of OH-JA, JA-Val and JA-Glc accumulation were similar to those of WT plants. Values are means (± SE) of four replicate plants from each genotype and treatment. Significant differences by one-way ANOVA: *, $P \leq 0.05$; **, $P \leq 0.005$. OPDA, 12-oxo-phytodienoic acid; W + OS, wound + *Manduca sexta* oral secretion.

methylation ability was determined by measuring the formation of MeJA in RCA-silenced plants after treating with W + OS or W + OS + JA. We found that RCA-silenced plants synthesized 40% more MeJA than did WT plants (ANOVA; $F_{3,12} = 37.84$; $P_{W+OS} = 0.03$; $P_{W+OS+JA} = 0.007$) (Fig. 4a). *In vitro* JA methylation activity was determined using the total protein extracts of untreated and W + OS-treated leaves. We found that the methylation activity was similar in untreated WT and RCA-silenced plants but, after W + OS elicitation, the methylation activity increased by 36% in RCA-silenced plants compared with WT plants (ANOVA; $F_{3,15} = 1.65$; $P_{Untreated} = 0.59$; $P_{W+OS} = 0.049$) (Fig. 4b).

It is known that JA and MeJA are interconvertible and that MeJA is hydrolyzed to JA by JME (Stuhlfelder *et al.*, 2002, 2004). Therefore, a decrease in MeJA demethylation activity might also contribute to the increased MeJA levels in RCA-silenced plants. *In vivo* demethylation ability was determined by measuring the formation of JA in RCA-silenced plants after treating with lanolin containing MeJA or pure lanolin. JA was not detected in lanolin-treated control samples, and no significant difference was found in the levels of the cleavage product of MeJA in WT and RCA-silenced plants (ANOVA; $F_{1,6} = 0.52$; $P = 0.49$) (Fig. S3a). Similarly, in an *in vitro* assay, protein extracts of WT and RCA-silenced leaves hydrolyzed similar amounts of MeJA to form JA (ANOVA; $F_{3,16} = 2.98$; $P_{Untreated} = 0.07$; $P_{W+OS} = 0.1$) (Fig. S3b). From these results, we infer that the increase in MeJA levels in RCA-silenced plants could be attributed to an increase in JA methylation activity and not to a decrease in demethylation activity.

RCA-silenced plants phenocopy the JA metabolism and signaling behavior of JMT-overexpressing plants

The ectopic expression of *Arabidopsis thaliana* (*At*)-JMT in *N. attenuata* reduced the accumulation of JA-Ile by 95% and that of the other amino acid-JA derivatives by 30% (Table 1). The resulting increase in the JA methylation activity (93%) redirected the flux of JA to MeJA and compromised the plant's defenses (Stitz *et al.*, 2011a,b). As such, ectopic overexpression of JMT creates a JA sink, diverting JA to inactive MeJA without influencing the JA pathway before the formation of JA, and hence phenocopies RCA-silenced plants (Fig. 5). JA-Ile and other amino acid-JA derivatives decreased by 28% and 36%, respectively, and the JA methylation activity and MeJA accumulation increased by 40% and 36%, respectively. Interestingly, the decrease in JA-Ile corresponded to a stoichiometric increase in MeJA levels (Fig. S4).

Table 1 Jasmonic acid (JA) metabolism and signaling in RuBPCase activase (RCA)-silenced and jasmonate methyltransferase (JMT)-overexpressing *Nicotiana attenuata* plants are similarly regulated after simulated herbivory

	Regulation after simulated herbivory (with respect to WT plants)	
Traits	Overexpressed JMT (%)	Silenced RCA (%)
JA	27 ↓	No change
JA-Ile	95 ↓	28 ↓
JA-Val	31 ↓	36 ↓
MeJA	96 ↑	40 ↑
JME activity	30 ↓	No change
JMT activity	93 ↑	36 ↑
Manduca sexta larval mass	††61 ↑	61 ↑

Table 1 shows the percentage increase or decrease in the accumulation of JA, jasmonic acid-isoleucine (JA-Ile) and methyl jasmonate (MeJA), the activity of methyltransferase (JMT) and methylesterase (JME) and the mass gain of *Manduca sexta* larvae in JMT-overexpressing and RCA-silenced plants relative to wild-type (WT) plants. Up arrows (↑) and down arrows (↓) signify increases and decreases, respectively. The values of JMT-overexpressing plants are from Stitz *et al.* (2011a,b) and, in this study, *M. sexta* larvae were fed *untreated* JMT-overexpressing plants and hence their larval mass gain cannot be compared directly with the larval mass gain of larvae fed on *oral secretion (OS)-elicited* RCA-silenced plants (††).

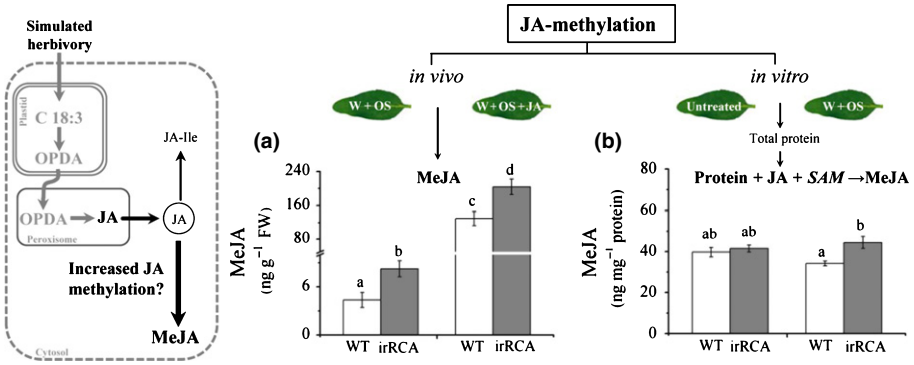

Fig. 4 RuBPCase activase (RCA)-silenced *Nicotiana attenuata* plants accumulate more methyl jasmonate (MeJA) than do wild-type (WT) plants, which correlates with the increased methylation activity of free JA. The accumulation of (a) MeJA (*in vivo*) increased after W + OS and W + OS + JA elicitation, and basal levels were higher in RCA-silenced plants. To measure the JA methylation activity *in vitro*, total protein was extracted from W + OS-treated rosette leaves (+ 1) of RCA-silenced and WT plants. Untreated plants served as controls. Methyltransferase activity was determined by measuring the production of MeJA from JA and *S*-adenosylmethionine (SAM). (b) Protein extracts of RCA-silenced plants produced significantly more MeJA when supplemented with JA and SAM. Values are means (± SE) of four to five replicate plants from each genotype and treatment. Different letters indicate a significant difference at $P \leq 0.05$ by one-way ANOVA. OPDA, 12-oxo-phytodienoic acid; irRCA, inverted repeat RCA; W + OS, wound + *Manduca sexta* oral secretion.

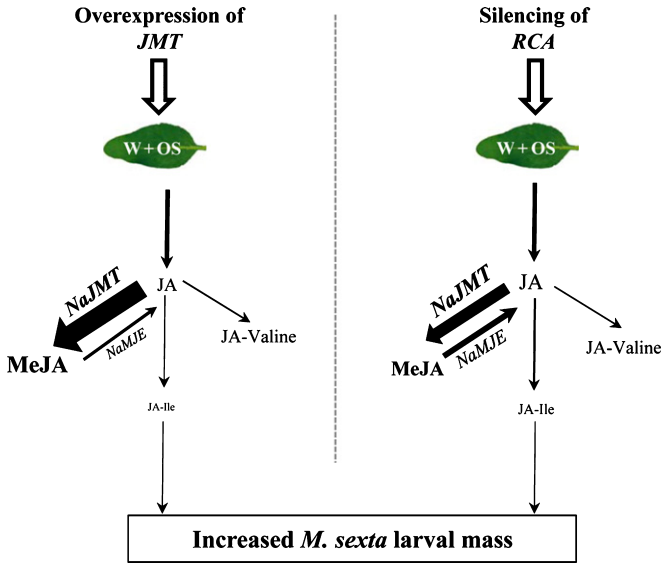

Fig. 5 RuBPCase activase (RCA) silencing phenocopies jasmonate methyltransferase (JMT) overexpression in *Nicotiana attenuata* plants. A model of jasmonic acid (JA) metabolism and signaling in oral secretion (OS)-induced RCA-silenced plants, in which the elevated methyltransferase activity redirects OS-elicited JA flux to the inactive methyl jasmonate (MeJA) rather than to the active signaling molecule jasmonic acid-isoleucine (JA-Ile) or to other less active JA derivatives, thereby compromising the elicited defense responses. The font size and arrow thickness are proportional to the intensity of metabolite flux, enzyme activity and *Manduca sexta* larval mass. W + OS, wound + *M. sexta* oral secretion.

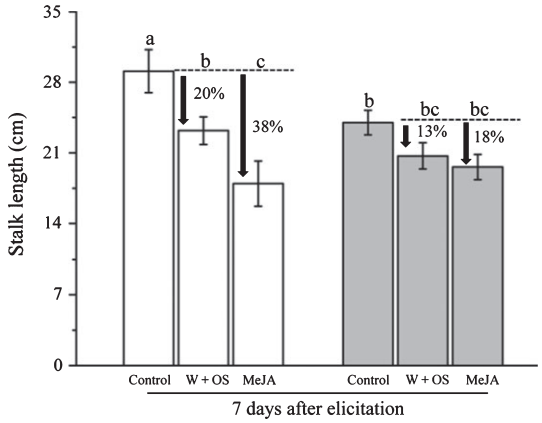

Fig. 6 Growth of RuBPCase activase (RCA)-silenced *Nicotiana attenuata* plants is less affected by simulated herbivory and methyl jasmonate (MeJA) treatment relative to wild-type (WT) plants. Fully expanded (+ 1) leaves of WT and RCA-silenced plants were wounded (W) with a pattern wheel and treated with *Manduca sexta* oral secretion (OS) or MeJA, and the stalk length was recorded 7 d after elicitation. Untreated plants served as controls. W + OS or MeJA treatments significantly delayed stalk elongation in WT plants, but the effects in RCA-silenced plants were not significantly different from those in the respective controls. Values are means (± SE) of five replicate plants from each genotype and treatment. Different letters indicate a significant difference at $P \leq 0.05$ by one-way ANOVA. WT, white bars; inverted repeat RCA (irRCA), gray bars

RCA silencing attenuates the OS- and MeJA-elicited growth reductions

OS elicitation and MeJA treatment of WT plants are both known to decrease *N. attenuata* growth, particularly under competitive growth in both the field and glasshouse (Baldwin, 1998; Halitschke *et al.*, 2001; Zavala *et al.*, 2004). To evaluate the growth performance of RCA-silenced plants after OS and MeJA treatments, we recorded the stalk length of W + OS- and MeJA-treated RCA-silenced plants and compared the results with those of similarly treated WT plants. Plants were grown in individual pots to provide a conservative measure of growth effects. After treating with OS and MeJA, the growth of WT plants was reduced by 20% (ANOVA; $F_{5,24} = 5.7$, $P_{W+OS} = 0.01$) and 38% (ANOVA; $F_{5,24} = 5.7$, $P_{MeJA} < 0.0001$), respectively, whereas the growth of RCA-silenced plants was only reduced by 13% (ANOVA; $F_{5,24} = 5.7$, $P_{W+OS} = 0.17$) and 18% (ANOVA; $F_{5,24} = 5.7$, $P_{MeJA} = 0.07$), respectively (Fig. 6); these reductions were significantly different from each other in the WT, whereas, in RCA, they were not. From these results, we infer that the attenuated induced defenses of RCA-silenced plants may contribute to the sustained growth of these plants after elicitation by herbivores.

Discussion

As the herbivory-elicited accumulation of JA in RCA-silenced plants was indistinguishable from that of WT plants, the attenuated JA-Ile signaling of RCA-silenced plants could result from: (1) a reduced JA pool at the wound site; (2) impaired JA adenylation; (3) impaired JA-Ile conjugating enzyme activity; or (4) altered JA metabolism. The results from the JA and Ile supplementation experiments ruled out the first hypothesis and demonstrated that a reduced JA pool at the wound site could not account for the impaired JA-Ile accumulation. Moreover, the results from supplementation experiments with JA + [$^{13}C_6$]Ile and the extended night experiments allowed us to rule out deficiencies in JA adenylation or conjugating enzyme activity. Attack-elicited JA and JA-Ile are metabolized to their OH or COOH form, or JA is conjugated with other molecules (Miersch *et al.*, 2008; Wang *et al.*, 2008; Koo *et al.*, 2011). Conversion of JA or JA-Ile to its OH or COOH derivative deactivates JA signaling (Miersch *et al.*, 2008; Koo *et al.*, 2011), and the conjugation of JA with molecules other than Ile also disables defense signaling (Wang *et al.*, 2008). Therefore, RCA-silenced plants could have an increased JA-Ile turnover or JA could be conjugated to amino acids other than Ile. However, the levels of JA-Ile and of the major JA derivatives in RCA-silenced plants suggested that JA-Ile accumulation is not influenced by increased JA-Ile turnover and is not outcompeted by conjugation reactions involving other amino acids or glucose.

JA can also be methylated to MeJA, a semi-volatile organic compound involved in plant defense and many other developmental pathways (Creelman & Mullet, 1997; Wasternack & Hause, 2002, 2013). In Arabidopsis, the methylation of JA is catalyzed by the enzyme JMT, an enzyme whose corresponding transcripts are up-regulated in response to wounding or JA application (Seo *et al.*, 2001). Ectopic expression of *A. thaliana JMT* (*AtJMT*) in *N. attenuata* creates a metabolic sink in the JA pathway which redirects the flux of JA towards MeJA and strongly

reduces the accumulation of herbivory-induced JA and JA-Ile, and the JA-associated defense responses. RCA-silenced plants, like the JMT-overexpressing *N. attenuata* plants ($_{ov}$*AtJMT*), show high MeJA accumulations and JMT activity both *in vivo* and *in vitro*. $_{ov}$*AtJMT* plants show JMT activity that is elevated by 93%, which corresponds to a 96% increase in MeJA levels and 27% and 95% decreases in JA and JA-Ile levels, respectively. Similarly, RCA-silenced plants show a 36% increase in JMT activity, corresponding to a 40% increase in MeJA level and a 28% decrease in JA-Ile levels, all without any changes in JA levels. RCA-silenced plants therefore phenocopy $_{ov}$*AtJMT* plants in all aspects, with the exception of their unaltered JA levels. In this regard, RCA-silenced plants are more similar to Arabidopsis *JMT*-overexpressing plants in which the MeJA level is increased without altering the normal JA burst (Seo *et al.*, 2001). The difference may simply reflect differences in the strength of the JA sink. In both RCA-silenced *N. attenuata* plants and JMT-overexpressing Arabidopsis, the JA sink strengths are substantially less (*c.* 50%) than that of the JMT-overexpressing *N. attenuata* plants. The involvement of the abundant and important photosynthetic protein, RCA, in regulating JA signaling is novel (Fig. S4), and will require substantially more research to understand its underlying mechanisms. Being a molecular chaperone, RCA may directly interact with the JMT protein and negatively regulate its function, something which will be possible to test once the putative *NaJMT* has been identified and characterized in *N. attenuata.*

At a functional level, it is not clear why RCA would have evolved to play an additional role in negatively regulating JA signaling. Phytohormones and the signaling cascades they activate are known to be tightly regulated by catalytic reactions that control the pools of active hormone signals (Qin *et al.*, 2005; Varbanova *et al.*, 2007; Tieman *et al.*, 2010). For example, JA is inactivated when converted to 12-OH-JA, as clearly seen in the termination of the expression of a subset of genes involved in JA signaling. Similarly, the catabolism of JA-Ile into OH-JA-Ile or COOH-JA-Ile down-regulates JA signaling (Miersch *et al.*, 2008) and, recently, the hydrolysis of JA-Ile by jasmonyl-L-isoleucine hydrolase 1 (JIH1) has been shown to attenuate the JA-Ile burst and allow *N. attenuata* plants to tailor their defense responses (Woldemariam *et al.*, 2012). RCA should now be added to the many layers by which plants can tailor their JA-Ile signaling.

Jasmonate-induced defenses impose significant costs on a plant's growth (Redman *et al.*, 2001); therefore, a trade-off occurs between growth and defense (Coley *et al.*, 1985; Heil & Baldwin, 2002). Hormonal cross-talk has been proposed as a mechanism for the resource-based trade-offs between growth and defense (Yang *et al.*, 2012; Machado *et al.*, 2013) and, recently, the JA-dependent reduction in photosynthetic rate has been proposed to be responsible for a plant's decreased growth (Nabity *et al.*, 2013). Thus, on the one hand, JA signaling reduces photosynthetic capacity and, on the other, it depletes C resources by incorporating them in defense metabolites. Moreover, the results of our extended night experiments demonstrate that JA-Ile production is also resource dependent. In addition, regulatory elements other than JA may influence a plant's root storage regime and re-growth capacity (Machado *et al.*, 2013). The C-limited RCA-silenced plants were impaired in their JA-Ile bursts and JA-Ile-induced defense metabolites, suggesting that RCA could be one of the factors suggested by Machado *et al.* (2013). When herbivore attack elicits a JA burst, *N. attenuata* plants could anticipate the upcoming resource constraint resulting from reduced C assimilation through the signaling mediated by the down-regulation of RCA. Therefore, the C-limited RCA-silenced plants reorganize their resource investment strategy and redirect the attack-elicited JA flux from JA-Ile towards MeJA, thereby attenuating the expensive JA-Ile-mediated defense responses.

Resistance is thought to be costly in terms of a plant's growth and fitness; therefore a trade-off is assumed between growth and defense (Coley *et al.*, 1985; Heil & Baldwin, 2002). Many plants employ both resistance and tolerance strategies to cope with their herbivore communities (Leimu & Koricheva, 2006; Carmona & Fornoni, 2013). In response to herbivory, many plant species allocate C to the roots (Dyer *et al.*, 1991; Holland *et al.*, 1996; Schwachtje *et al.*, 2006). However, the reallocated C is not used for root growth; instead, it is used for post-herbivory regrowth of the shoot (Schwachtje *et al.*, 2006). This is an example of an anticipatory response which occurs before the resource supply limits the activation of defense to optimize the capacity for sustained growth (Smith & Stitt, 2007). We observed that RCA-silenced plants tolerated simulated herbivory and MeJA treatments better than similarly treated WT plants. Thus, by making more MeJA, RCA-silenced plants may avoid the production of JA-Ile and the associated expensive defense responses, conserving C resources for post-herbivory growth. This suggests that, normally, RCA is down-regulated after herbivory to redirect the JA flux towards MeJA and away from JA-Ile to facilitate the transition from growth to defense and, subsequently, from resistance to tolerance. We therefore propose that RCA plays a direct role in attenuating JA-induced defense responses, which, in turn, allows *N. attenuata* plants to anticipate the forthcoming resource constraints.

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Supporting Information

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

Fig. S1 Extended night series depletes starch levels in wild-type (WT) *Nicotiana attenuata* plants, and only a severe decrease in net carbon gain attenuates the oral secretion (OS)-elicited jasmonic acid-isoleucine (JA-Ile) burst in WT plants.

Fig. S2 Impaired herbivore resistance in RuBPCase activase (RCA)-silenced plants could not be attributed to the lower isoleucine (Ile) pools at the wound site.

Fig. S3 Increased methyl jasmonate (MeJA) level in RuBPCase activase (RCA)-silenced plants does not result from impaired demethylation activity.

Fig. S4 An overview of the consequences of herbivory and RuBPCase activase (RCA)'s novel role in the attenuation of *Nicotiana attenuata*'s defense responses.

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