Independently Silencing Two Photosynthetic Proteins in *Nicotiana attenuata* Has Different Effects on Herbivore Resistance[1][W][OA]

Sirsha Mitra and Ian T. Baldwin*

Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, 07745 Jena, Germany

Insect attack frequently down-regulates photosynthetic proteins. To understand how this influences the plant-insect interaction, we transformed *Nicotiana attenuata* to independently silence ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) activase (*RCA*) and RuBPCase and selected lines whose photosynthetic capacity was similarly reduced. Decreases in plant growth mirrored the decreases in photosynthesis, but the effects on herbivore performance differed. Both generalist (*Spodoptera littoralis*) and specialist (*Manduca sexta*) larvae grew larger on *RCA*-silenced plants, which was consistent with decreased levels of trypsin protease inhibitors and diterpene glycosides and increased levels of RuBPCase, the larvae's main dietary protein. *RCA*-silenced plants were impaired in their attack-elicited jasmonate (JA)-isoleucine (Ile)/leucine levels, but *RuBPCase*-silenced plants were not, a deficiency that could not be restored by supplementation with Ile or attributed to lower transcript levels of *JAR4/6*, the key enzyme for JA-Ile conjugation. From these results, we infer that JA-Ile/leucine signaling and the herbivore resistance traits elicited by JA-Ile are influenced by adenylate charge, or more generally, carbon availability in *RCA*- but not *RuBPCase*-silenced plants. Growth of generalist larvae on *RuBPCase*-silenced plants did not differ from growth on empty vector controls, but the specialist larvae grew faster on *RuBPCase*-silenced plants, which suggests that the specialist can better tolerate the protein deficiency resulting from RuBPCase silencing than the generalist can. We conclude that the plant-herbivore interaction is more influenced by the particular mechanisms that reduce photosynthetic capacity after herbivore attack than by the magnitude of the decrease, which highlights the value of understanding defense mechanisms in evaluating growth-defense tradeoffs.

An herbivore-attacked plant is known to reduce its photosynthetic capacity while increasing the production and accumulation of defense-related compounds (Walling, 2000; Hermsmeier et al., 2001; Kessler and Baldwin, 2002; Hahlbrock et al., 2003). In response to attack, plants must grow rapidly to compete and simultaneously maintain the defenses necessary to survive in environments with herbivores (Herms and Mattson, 1992). The dramatic up-regulation of defense metabolites likely requires metabolic adjustments in plant growth and reproduction (Halitschke et al., 2003; Reymond et al., 2004; Ralph et al., 2006). However, reductions in plant growth can also be understood as part of a plant's defense strategy, as reduced growth limits the availability of food and nutrition for the feeding insect (Hermsmeier et al., 2001; Hahlbrock et al., 2003). Allocating resources for resistance mechanisms is believed to reduce the availability of resources for growth; hence, resistance is thought to be costly in terms of plant growth and fitness (Heil and Baldwin, 2002). However, very little is known about how plants optimize their resource allocation when attacked by herbivores. Plants may optimize growth by adjusting carbon demand with current or anticipated carbon supply (Smith and Stitt, 2007), and researchers are only just beginning to understand how plants reorganize carbon partitioning and assimilation in response to herbivore attack. For example, when attacked by *Manduca sexta* larvae, *Nicotiana attenuata* bunkers newly assimilated carbon in its roots rather than transporting the carbon to young leaves, thereby increasing the plant's ability to tolerate herbivore attack (Schwachtje et al., 2006).

The molecular interaction between the specialist herbivore *M. sexta* and its natural host *N. attenuata* has been well studied at the transcriptomic and proteomic levels (Schmidt et al., 2005; Giri et al., 2006). In a comparative proteomic-transcriptomic study, over 90 meaningful amino acid sequences were found to be differentially regulated by attack from this specialist herbivore. Proteins that increased were involved in primary metabolism, defense, and transcriptional and translational regulation; those that decreased were involved in photosynthesis (Giri et al., 2006). Here, we explore why plants commonly down-regulate their photosynthetic proteins in response to herbivore attack. In the *N. attenuata-M. sexta* system, we observed that herbivore attack and its simulation, which is readily accomplished by applying *M. sexta*

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* Corresponding author; e-mail baldwin@ice.mpg.de.

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regurgitate (oral secretions [OS]) to puncture wounds (W), strongly down-regulate ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCase) activase (*RCA*) and RuBPCase at the transcript and protein levels (Halitschke et al., 2001; Roda et al., 2004; Giri et al., 2006). RCA's biochemical function is to modulate the activity of RuBPCase, the major photosynthetic protein involved in carbon fixation, by removing the inhibitory sugar phosphates from the active site of the enzyme, whether or not the enzyme is carbamylated (Portis, 2003). The effect of *RCA* on photosynthesis and growth has been well studied in Arabidopsis (*Arabidopsis thaliana*) and tobacco (*Nicotiana tabacum*). In Arabidopsis, moderate reductions of *RCA* decrease growth and aboveground biomass and leaf area (Ilyin et al., 2005). While tobacco plants transformed with *RCA* expressed in an antisense orientation are slow growing, they eventually attain the same height and leaf number as wild-type plants (He et al., 1997).

As a member of the AAA^+ (for ATPases associated with a variety of cellular activities) protein family, RCA may also function in other cellular processes (Ogura and Wilkinson, 2001). For example, during sudden exposure to heat stress, *RCA* assumes the role of a chaperone in association with thylakoid-bound ribosomes, possibly protecting the thylakoid-associated protein synthesis machinery (Rokka et al., 2001). Moreover, the regulation of RCA and RuBPCase in response to UV-B light exposure, ozone, heat stress, and drought in different plant systems (Pelloux et al., 2001; Liu et al., 2002; Bota et al., 2004; Demirevska-Kepova et al., 2005) suggests the involvement of RCA in diverse stress-related functions. Hence, *RCA* may play other, more direct, roles in orchestrating the down-regulation of photosynthesis-related genes in response to herbivore stress, in addition to its ability to regulate photosynthetic capacity via RuBPCase activation.

RuBPCase accounts for 40% of total leaf proteins (Taiz and Zeiger, 1998) and is therefore the main dietary protein for herbivores (Felton, 2005). But down-regulating RuBPCase levels can have other profound effects on a plant's resistance to herbivores. The effect of RuBPCase silencing on photosynthesis, growth, and metabolite production has been well studied in tobacco. The decreased expression of RuBPCase strongly reduces photosynthesis rates and consequently a plant's carbon supply, which also has far-reaching effects on a plant's ability to assimilate nitrogen (N; Fichtner et al., 1993). Silencing RuBPCase activity in tobacco decreases nitrate reductase activity and levels of amino acids and increases nitrate accumulation; in addition, RuBPCase silencing also decreases levels of chlorogenic acid and nicotine, the major carbon- and N-rich secondary metabolites in tobacco leaves that function as chemical defenses against herbivores (Steppuhn et al., 2004). Hence, while total protein levels, and therefore a plant's nutritional value, will likely decrease when RuBPCase is silenced, the levels of chemical defenses are also likely to decrease, and what the net effect on herbivore performance will be is unclear. Plants are thought to prioritize resource allocation between growth and defense processes when attacked by herbivores (Herms and Mattson, 1992); however, the interplay between resistance and growth may involve anticipatory responses as well as resource depletion effects that result from carbon limitation (Smith and Stitt, 2007).

In our preliminary study, silencing the expression of *NaRCA* by virus-induced gene silencing decreased the plant's photosynthetic capacity and biomass (Giri et al., 2006), but how much of the growth phenotypes resulted from the *Agrobacterium tumefaciens* and virus inoculations that are part of the virus-induced gene silencing procedure is unknown. To better understand how *N. attenuata*'s resistance traits are influenced by the down-regulation of different photosynthetic proteins in plants whose photosynthetic capacity has been similarly decreased, we silenced the expression of the two major photosynthetic proteins, RCA and RuBPCase, in *N. attenuata* plants by "ir" and "as" constructs, respectively. Plants silenced with inverted-repeat RCA fragment are designated irRCA, and plants silenced with RuBPCase fragment in antisense orientation are designated asRUB. T_2 generations of homozygous transformed plants, each harboring a single insertion of the transgene, were screened for their photosynthetic rates to select lines with similarly suppressed photosynthetic capacity. After screening several irRCA and asRUB lines, we identified two irRCA lines and one asRUB line whose photosynthetic rates were similarly reduced compared with empty vector (EV) plants. These plants were evaluated for growth, levels of defense metabolites, jasmonate (JA) signaling, dietary proteins, and their resistance to generalist (*Spodoptera littoralis*) and specialist (*M. sexta*) herbivores and then compared with EV-transformed plants.

RESULTS

Silencing *RCA* and *RuBPCase* in *N. attenuata*

To generate transformants stably silenced in *NaRCA* and *NaRUB* expression, we employed an *Agrobacterium*-mediated transformation procedure (Krügel et al., 2002) using pRESC transformation vectors containing a 268-bp fragment of *NaRCA* in an inverted-repeat orientation or a 316-bp fragment of *NaRUB* in an antisense orientation. Previous experience in transforming plants with irRUB and asRCA constructs revealed that silencing RuBPCase with an inverted-repeat construct prevented the transformants from growing. Similarly, the silencing efficiency of RCA with antisense constructs was too weak to significantly reduce photosynthetic rates.

Using Southern hybridization, we identified two independently transformed *RCA* lines (irRCA lines 1 and 2) and one *RuBPCase* line (asRUB), each with a

single transgene insertion (Supplemental Fig. S1). These lines were bred to homozygosity and used for all further experiments. Homozygous *N. attenuata* plants of the same inbred generation harboring a single EV construct served as a control for all experiments.

The constitutive expression of *RCA* and *RuBPCase* in transformed plants was analyzed by quantitative reverse transcription (RT)-PCR. The accumulation of *RCA* transcripts in untreated leaves of *N. attenuata* plants was significantly lower (by 40%) in irRCA lines (ANOVA; $F_{3,8} = 4.13$; $P_{\text{irRCA1}} = 0.02$; $P_{\text{irRCA2}} = 0.01$), and the accumulation of *RuBPCase* transcripts of untreated leaves of asRUB plants was 50% that of untreated EV plants (ANOVA; $F_{3,8} = 6.39$; $P_{\text{asRUB}} = 0.03$; Fig. 1, A and B). The accumulation of *RCA* transcripts in asRUB plants (ANOVA; $F_{3,8} = 4.13$; $P_{\text{asRUB}} = 0.12$) and the accumulation of *RuBPCase* transcripts in irRCA plants (ANOVA; $F_{3,8} = 6.39$; $P_{\text{irRCA1}} = 0.17$; $P_{\text{irRCA2}} = 0.4$) were not significantly different from the accumulation in EV plants.

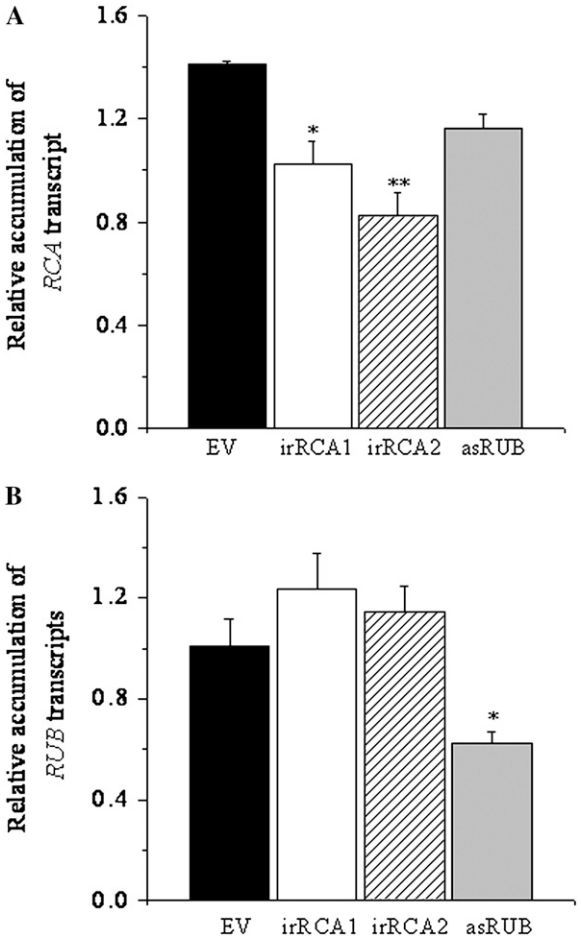

Figure 1. *RCA* and *RuBPCase* transcript levels in EV, irRCA, and asRUB plants. Transcript levels of *NaRCA* and *NaRUB* were analyzed in untreated EV, irRCA, and asRUB plants by quantitative RT-PCR and normalized to the levels of an unregulated gene, *actin*. A, irRCA plants accumulated significantly fewer *RCA* transcripts (40%– 42%) compared with EV-transformed plants; levels in *RuBPCase*-silenced plants also tended to be lower, but the differences were not statistically significant (ANOVA; $F_{3,8} = 4.13$; $P = 0.12$). B, *RuBPCase* transcript levels were significantly lower in asRUB plants than in EV plants. Values are means of three biological replicates. Asterisks indicate significant differences at $P < 0.05$ (*) and $P < 0.005$ (**).

Photosynthesis and Growth in Transformed Plants

The photosynthetic rates of irRCA and asRUB plants were lower than those of EV-transformed plants. Under ambient CO_2 concentration (400 μmol mol^{-1}) and different light regimes (0–2,000 μmol m^{-2} s^{-1}), irRCA and asRUB plants had only 50% of the photosynthetic capacity of EV plants (Supplemental Fig. S2), while under saturating light and different CO_2 concentrations (0–650 μmol mol^{-1}), they had 80% (Fig. 2A). EV-transformed plants showed efficient photosynthesis at ambient CO_2 as well as under limited CO_2 concentrations (200 μmol mol^{-1}); compared with EV-transformed plants, irRCA and asRUB plants had significantly lower photosynthetic rates even at higher (650 μmol mol^{-1}) CO_2 concentrations (ANOVA; $F_{3,16} = 5.77$; $P_{\text{irRCA1}} = 0.022$; $P_{\text{irRCA2}} = 0.007$; $P_{\text{asRUB}} < 0.001$). The photosynthesis rates of irRCA and asRUB plants are not significantly different from each other (ANOVA; $F_{3,16} = 5.77$; $P > 0.1$). At the whole plant level, silencing RCA reduced the photosynthesis rate by 50% to 55%, but silencing RuBPCase reduced it by only 25% (Fig. 2B).

Growth performance was estimated by repeated measures of stalk lengths when plants initiated reproductive growth. Stalk lengths of irRCA plants (Fig. 3) were significantly reduced (by 12%–20%; ANOVA; $F_{3,36} = 18.268$; $P_{\text{irRCA1}} = 0.0006$; $P_{\text{irRCA2}} < 0.0001$) after the expression of *RCA* was silenced. Stalk lengths of asRUB plants were also significantly shorter (by 60%) at the start of reproductive growth (ANOVA; $F_{3,36} = 18.396$; $P_{\text{asRUB}} = 0.0003$), but they eventually attained the heights of EV-transformed plants (Fig. 3). When irRCA plants were grown in competition with EV plants, their stalk lengths were about 40% shorter (paired *t* test; $n = 10$; $t = 5.81$; $P_{\text{irRCA1}} = 0.0003$; $t = 10.04$; $P_{\text{irRCA2}} < 0.0001$); even after 48 d of germination, the lengths of *RuBPCase*-silenced stalks did not catch up to those of EV-transformed plants and remained 8% shorter (paired *t* test; $n = 10$; $t = 4.29$; $P_{\text{asRUB}} = 0.002$; Supplemental Fig. S3).

Trypsin Proteinase Inhibitor Activity and Accumulation of Diterpene Glycosides and Nicotine in Transformed Plants

The OS-elicited trypsin proteinase inhibitor (TPI) activity was significantly (ANOVA; $F_{2,12} = 3.87$; $P_{\text{irRCA1}} = 0.04$; $P_{\text{irRCA2}} = 0.03$) lower in irRCA plants than it was in EV plants (Fig. 4A). In irRCA plants compared with EV plants, the levels of diterpene glycosides (DTGs) were also significantly lower both before (ANOVA;

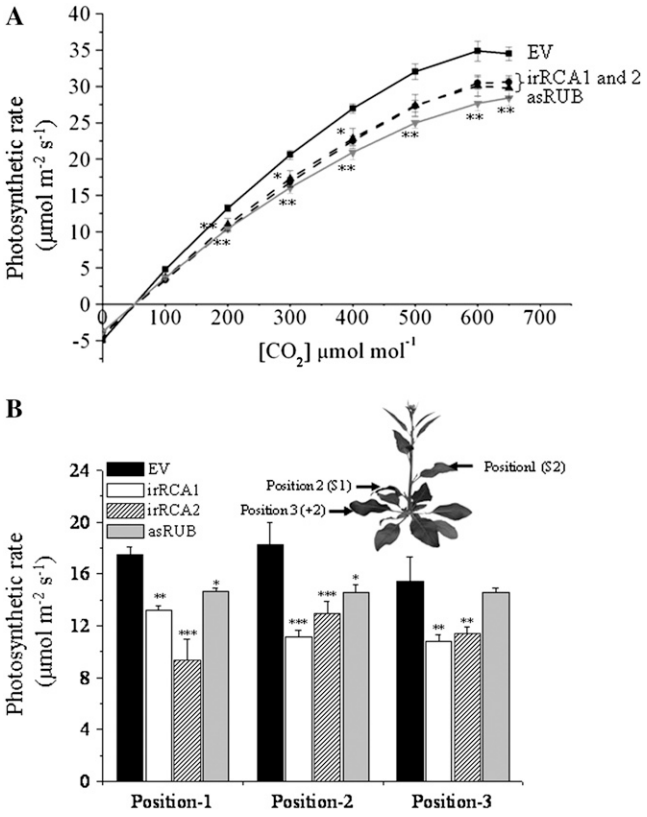

Figure 2. Silencing *RCA* and *RuBPCase* decreases photosynthetic rates in *N. attenuata*. A, CO_2 exchange (A/C_i) was measured at the irradiance of 1,200 μmol m^{-2} s^{-1} and at seven different CO_2 concentrations, namely 0, 100, 200, 400, 500, 600, and 650 μmol mol^{-1}, in EV-transformed irRCA (lines 1 and 2) and asRUB plants. The assimilation rates of irRCA and asRUB plants were 20% lower than those of EV plants at different CO_2 concentrations. The slope of the A/C_i curve revealed a 1.2- to 1.5-fold decrease in RuBPCase activity in transformed plants compared with EV-transformed plants. B, CO_2 exchange was measured at the light intensity of 1,200 μmol m^{-2} s^{-1} and optimum (400 μmol mol^{-1}) CO_2 concentration at three different leaf positions, position 1 (S2 leaf), position 2 (S1 leaf), and position 3 (+2 leaf) in EV, *RCA*-silenced, and *RuBPCase*-silenced plants. The assimilation rates of *RCA*- and *RuBPCase*-silenced plants were lower at all measured leaf positions compared with those of EV plants, with the exception that the decrease at position 3 of *RuBPCase*-silenced plants was not statistically significant. Values are means ± SE of five or four replicate plants from each genotype and each position. Asterisks indicate significant differences at $P < 0.005$ (*), $P < 0.005$ (**), and $P \leq 0.0001$ (***).

$F_{3,16} = 2.47$; $P_{irRCA1} = 0.04$; $P_{irRCA2} < 0.03$) and 4 d after (ANOVA; $F_{3,13} = 5.011$; $P_{irRCA1} = 0.05$; $P_{irRCA2} = 0.002$; Fig. 4B) OS elicitation. In contrast, the levels of both TPIs and DTGs before and after OS elicitation were similar in asRUB- and EV-transformed plants (TPI, ANOVA; $F_{1,8} = 0.56$; $P = 0.47$; DTG, $F_{1,7} = 1.82$; $P = 0.22$; Fig. 4, A and B). The accumulation of nicotine before and after OS elicitation in irRCA and asRUB plants was similar to that of EV-transformed plants (nicotine, ANOVA; $F_{7,29} = 5.39$; $P > 0.5$; Fig. 4C).

Accumulation of JA, JA-Ile, and *JAR4/6*

Since both TPIs (Zavala et al., 2004) and DTGs (Keinanen et al., 2001) are known to be jasmonate elicited, we measured JA after 0, 30, 45, 60, and 180 min of W + OS elicitation in all transformed plants. These time points are known to capture the OS-elicited dynamics of JA in *N. attenuata* plants (Kang et al., 2006). Our results showed that the highest accumulation of JA occurred at 45 min after OS elicitation. Since the JA-Ile burst is known to track the JA burst, we measured JA-Ile levels at this time to determine if the lower DTG and TPI levels could be attributed to deficiencies in JA-Ile signaling in *RCA*-silenced plants (Fig. 5, A and B). The kinetics of OS-elicited JA accumulation in *RCA*- and *RuBPCase*-silenced plants was not identical to that of EV-transformed plants; *RCA*- and *RuBPCase*-silenced plants showed increased levels of JA at 60 min, but maximum levels did not differ from those of EV-transformed plants (Fig. 5A). The accumulation of JA-Ile in irRCA plants, on the other hand, was significantly lower only at the 45-min harvest (ANOVA; $F_{3,16} = 5.143$; $P_{irRCA1} = 0.005$; $P_{irRCA2} = 0.05$) compared with that in EV-transformed plants. In contrast, the levels of JA-Ile in asRUB plants did not differ from the levels in EV-transformed plants (ANOVA; $F_{3,16} = 5.143$; $P_{asRUB} = 0.8$; Fig. 5B).

Decreased Ile pools at the wound site can influence the accumulation of JA-Ile after OS elicitation in Thr-deaminase-silenced plants, and this deficit can be readily restored by supplementing OS with Ile before it is added to puncture wounds (Kang et al., 2006). To determine whether the attenuated JA-Ile levels

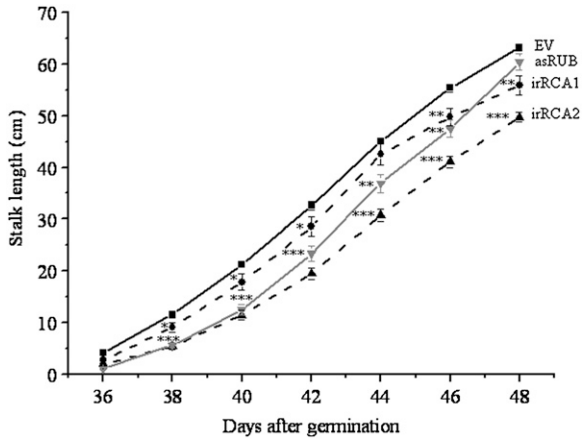

Figure 3. Growth of *RCA*- and *RuBPCase*-silenced plants is impaired under noncompetitive growth conditions. The stalk lengths of singly grown EV, irRCA, and asRUB plants were recorded for 7 d. The stalk lengths of irRCA plants (lines 1 and 2) were significantly less than those of EV plants until 48 d after germination; stalk lengths of asRUB plants were significantly less than those of EV plants until day 46 but eventually attained the same height as those of EV plants. Values are means ± SE of 10 replicate plants from each genotype. Asterisks indicate significant differences at $P < 0.005$ (*), $P < 0.005$ (**), and $P \leq 0.0001$ (***).

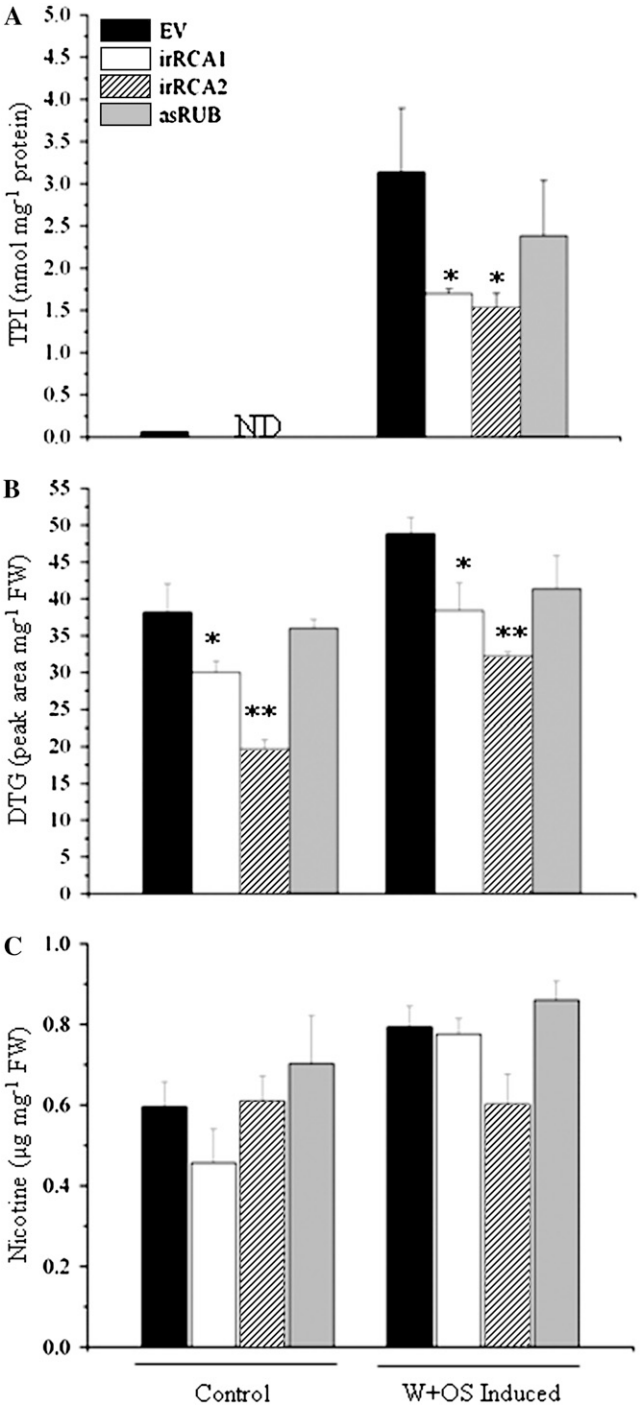

Figure 4. Direct defenses of *RCA*-silenced plants, but not *RuBPCase*-silenced plants, are lower than those of EV plants. Rosette-stage leaves (+1) of irRCA, asRUB, and EV plants were wounded with a pattern wheel and treated with 20 μL of OS of *M. sexta*. Tissues were harvested after 4 d of elicitation. A and B, Untreated leaves of each genotype served as a control. *RCA*-silenced plants accumulated fewer TPIs (A) and DTGs (B) than did EV-transformed plants. C, Nicotine levels in both lines did not differ from those in EV-transformed plants. Values are means ± SE of five replicate plants from each genotype. Asterisks indicate significant differences at $P < 0.005$ (*) and $P < 0.005$ (**). FW, Fresh weight.

observed in irRCA plants could be attributed to decreased Ile pools at the wound site, we measured JA-Ile accumulation in irRCA plants treated with W + OS + Ile. This treatment did not restore JA-Ile levels of *RCA*-silenced plants to those of EV plants (ANOVA; $F_{2,9} = 4.154$; $P_{irRCA1} = 0.04$; $P_{irRCA2} = 0.02$; Fig. 6A), and we conclude that reductions in Ile supply are not likely responsible for the attenuated JA-Ile burst.

When JA is conjugated to Ile, it is first adenylated by two redundant enzymes in *N. attenuata*, *NaJAR4* and *NaJAR6* (Wang et al., 2007). We measured transcript levels of these two genes at 60 min after OS elicitation, when transcripts are known to be strongly elicited

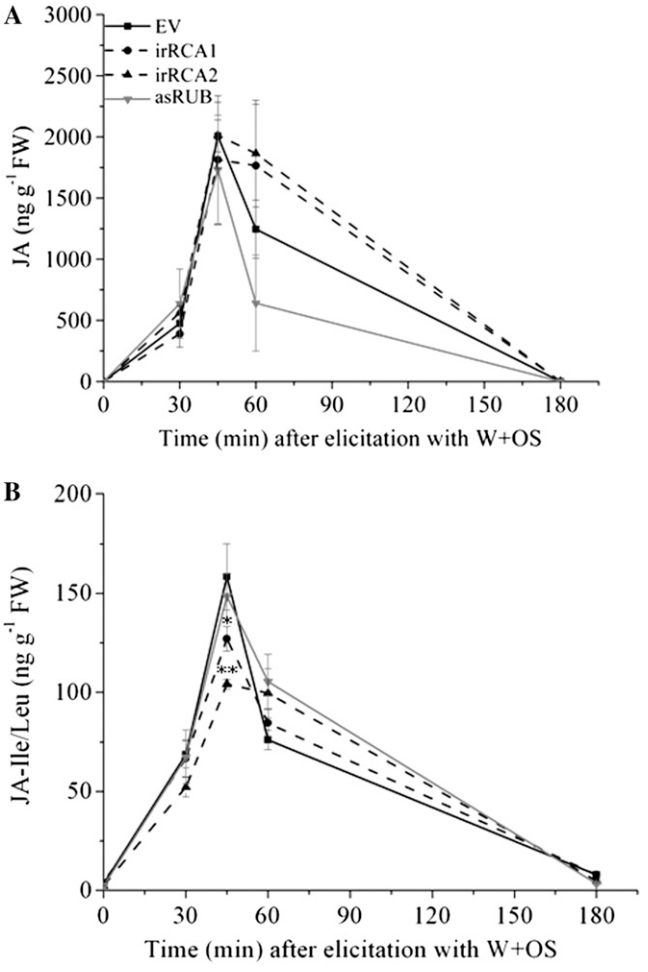

Figure 5. *RCA*-silenced plants, but not *RuBPCase*-silenced plants, are impaired in JA-Ile/Leu but not JA levels after OS elicitation. Rosette-stage leaves (+1) of irRCA, asRUB, and EV plants were wounded with a pattern wheel and immediately treated with 20 μL of OS of *M. sexta* larvae. Tissues were harvested at 0, 30, 45, 60, and 180 min after OS elicitation. A, *RCA*- and *RuBPCase*-silenced plants accumulated similar amounts of JA compared with EV-transformed plants. B, *RCA*-silenced plants accumulated less JA-Ile/Leu, but *RuBPCase*-silenced plants and EV plants accumulated similar amounts, at 45 min after elicitation. Values are means ± SE of five replicate plants from each genotype. Asterisks indicate significant differences at $P < 0.005$ (*) and $P < 0.005$ (**). FW, Fresh weight.

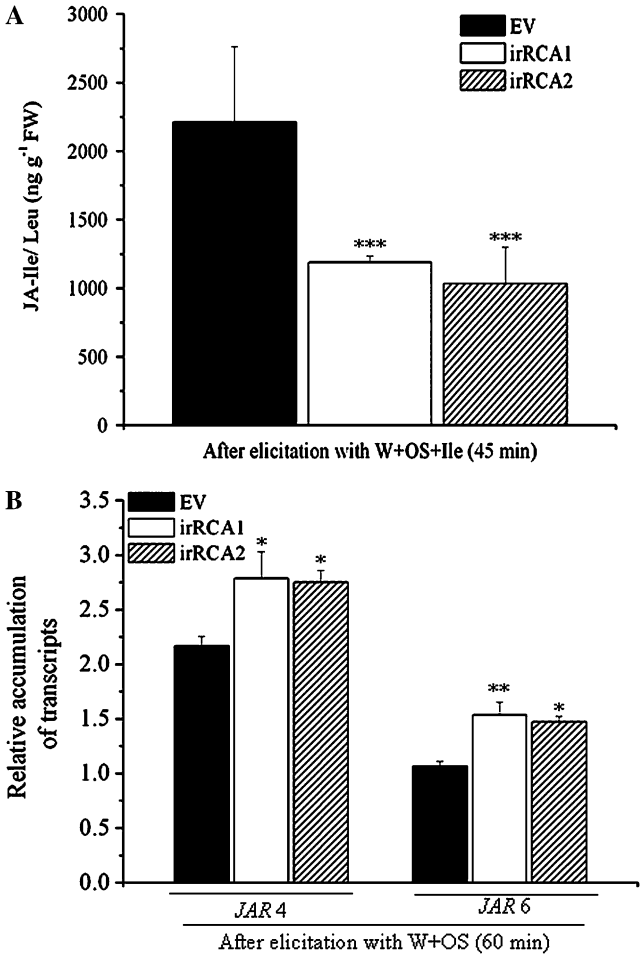

Figure 6. Lower JA-Ile/Leu levels in *RCA*-silenced plants are not due to Ile limitations at the wound site or to lower *JAR 4/6* transcript levels. Rosette-stage leaves (+1) of irRCA and EV plants were wounded with a pattern wheel, and the resulting puncture wounds were immediately treated with 20 μL of *M. sexta* OS supplemented with 625 μmol of Ile. Tissues were harvested after 45 min. A, *RCA*-silenced plants accumulated less JA-Ile/Leu compared with EV plants. B, Accumulation of *JAR4* and *JAR6* transcripts in *RCA*-silenced plants compared with EV-transformed plants at 60 min after OS elicitation. Values are means ± SE of three replicate plants from each genotype. Asterisks indicate significant differences at $P < 0.005$ (*), $P < 0.005$ (**), and $P \leq 0.0001$ (***). FW, Fresh weight.

(Kang et al., 2006). Transcript levels of *JAR4* and *JAR6* were significantly higher in irRCA plants than in EV plants (ANOVA; *JAR4*, $F_{2,6} = 4.56$; $P_{irRCA1} = 0.03$; $P_{irRCA2} = 0.04$; ANOVA; *JAR6*, $F_{2,6} = 10.65$; $P_{irRCA1} = 0.005$; $P_{irRCA2} = 0.01$; Fig. 6B). From these results, we infer that transcripts of the conjugating enzyme are not responsible for the JA-Ile phenotype of irRCA plants.

Performance of *S. littoralis* and *M. sexta* Larvae

To examine the effect of decreased photosynthesis on the performance of a native generalist and a native specialist herbivore, the growth of *S. littoralis* and *M. sexta* larvae fed on different transformed plants was evaluated (Fig. 7). *S. littoralis* larvae were reared on artificial diet for 6 d and subsequently transferred to the rosette-stage leaves of EV, irRCA, and asRUB plants, where they were allowed to feed for 12 d. *S. littoralis* larvae gained more mass only when fed on

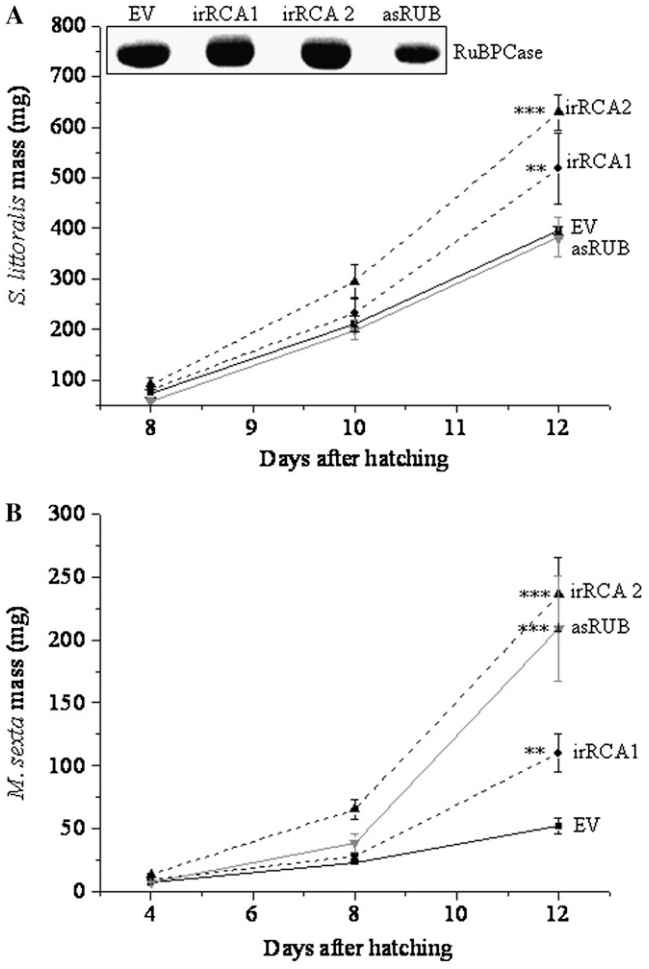

Figure 7. *S. littoralis* larvae performed better on irRCA than on EV plants but not as well on asRUB plants, and the improved larval performance corresponded to high levels of RuBPCase. *M. sexta* larvae, however, performed better on both irRCA and asRUB lines than on EV plants. A, Mass gain of *S. littoralis* larvae (means ± SE) on 12 replicate plants from EV, irRCA (lines 1 and 2), and asRUB genotypes. *S. littoralis* larvae reared on irRCA plants gained significantly more body mass than did larvae reared on asRUB and EV plants. One-dimensional gel electrophoresis of the large subunit of RuBPCase in irRCA and asRUB plants compared with EV plants is shown in the inset. The area of RuBPCase large subunit bands as quantified in EV, irRCA, and asRUB (lines 1 and 2) plants by PD-Quest software revealed an approximately 1- to 1.2-fold increase in the amount of RuBPCase in irRCA plants, and a 1.5-fold decrease in asRUB plants, compared with EV plants. B, *M. sexta* larval mass gain (means ± SE) of larvae on 15 replicate plants from EV, irRCA (lines 1 and 2), and asRUB genotypes. *M. sexta* larvae reared on both irRCA and asRUB plants gained significantly more body mass compared with larvae reared on EV plants. Asterisks indicate significant differences at $P < 0.005$ (**) and $P \leq 0.0001$ (***).

irRCA plants compared with larvae that fed on EV plants (ANOVA; $F_{3,36} = 6.25$; $P_{irRCA1} = 0.04$; $P_{irRCA2} = 0.001$; $P_{asRUB} = 0.86$; Fig. 7A). *M. sexta* neonates were placed on the rosette-stage leaves of EV, irRCA, and asRUB plants and allowed to feed for 12 d. Larvae that fed on irRCA (ANOVA; $F_{2,29} = 21.66$; $P_{irRCA1} = 0.04$; $P_{irRCA2} < 0.0001$) and asRUB (ANOVA; $F_{1,14} = 17.22$; $P = 0.001$) plants gained more body mass at the end of the experiment compared with larvae that fed on EV plants (Fig. 7B).

RuBPCase Levels

RuBPCase, which contributes up to 40% of plants' total protein, is the major dietary protein for insects (Johnson et al., 1996). He et al. (1997) have shown that silencing *RCA* increases RuBPCase content in cultivated tobacco. In order to determine if silencing RCA and RuBPCase influences the accumulation of large subunits of RuBPCase in *N. attenuata*, we performed a one-dimensional protein analysis of EV, irRCA, and asRUB plants (Fig. 7A, inset). Compared with the amounts of RuBPCase in EV-transformed plants, the amounts of RuBPCase increased 1- to 1.2-fold in irRCA plants but decreased 1.5-fold in asRUB plants.

DISCUSSION

Herbivore attack reduces a plant's photosynthetic capacity more than is expected, given the canopy area removed by the herbivore (Zangerl et al., 2002), a result that points to herbivore-induced reductions in *RCA* and *RuBPCase* as a potential explanation for the large decrease in the carbon assimilation rate that characterizes herbivore-attacked leaves. Silencing *RCA* and *RuBPCase* expression reduces photosynthetic capacity in a number of different species (Fichtner et al., 1993; Hammond et al., 1998; Ilyin et al., 2005; Jin et al., 2006), but these transformed lines have not been characterized in the context of the complex changes that are elicited by herbivore attack (Hermsmeier et al., 2001; Schmidt et al., 2005). We transformed *N. attenuata*, silencing its *RCA* and *RuBPCase* to determine whether the reductions in photosynthetic capacity that commonly result from herbivore attack translate into (1) reduced growth with and without intense intraspecific competition or (2) diminished resistance to herbivores.

Decreased expression of *NaRCA* and *NaRUB* was accompanied by a decrease in RuBPCase activity and a reduction in net photosynthetic rates at the whole plant level. At optimal CO_2 concentrations (400 μmol mol^{-1}) and different light intensities, photosynthetic rates decreased by 50%, but the magnitude of the reduction varied with leaf position, light, and CO_2 levels. Under different CO_2 concentrations and saturated light intensity, photosynthetic rates in *NaRCA* plants decreased by 20% compared with EV-transformed plants. Eckardt et al. (1997) demonstrated that *RCA* mutants of Arabidopsis with 30% to 40% reductions in *RCA* content were significantly diminished in their photosynthetic capacity and growth; this effect was even more pronounced under high light conditions (600 μmol m^{-2} s^{-1}). Our results are consistent with these observations and demonstrate that increases in light intensity restore the performance of transformed plants; however, transformed plants can recover up to 30% of their photosynthetic capacity when the CO_2 supply is increased. These results are consistent with the lower RuBPCase activity rates seen after silencing *RCA* and *RuBPCase*.

At the rosette stage, the photosynthesis rates of *RCA*- and *RuBPCase*-silenced plants were similarly reduced. The growth of reproductive stalks in *RCA*-silenced plants reflects differences in photosynthetic capacity throughout the plants' lives, but *RuBPCase*-silenced plants were able to eventually attain the stalk lengths of EV-transformed plants. We estimated whole plant photosynthetic capacity by averaging photosynthetic rates of leaves at three positions and found reductions of 50% in *RCA*-silenced plants but only 25% in *RuBPCase*-silenced plants, where older leaves had photosynthetic rates similar to EV plants; these results suggest that asRUB plants can adjust their photosynthetic capacity at later growth stages, enabling them to attain the same stalk heights of EV-transformed plants at a later growth phase. Aging of plant tissue is associated with the loss of soluble proteins, predominantly RuBPCase. The degradation and remobilization of the soluble proteins provide an important source of N and sulfur for the other parts of the developing plants (Friedrich and Huffaker, 1980). However, RuBPCase declines faster than RCA (He et al., 1997). Therefore, despite having less RuBPCase, asRUB plants have similar photosynthetic rates as EV plants. He et al. (1997) showed that the turnover of RuBPCase is repressed by 50% in RCA-deficient plants, which retained more RuBPCase in their older leaves compared with control plants. As a consequence, there is a large increase in the ratio of RuBPCase to soluble protein and a decrease in the ratio of RCA to RuBPCase in the older leaves of RCA-deficient plants (He et al., 1997). Therefore, RCA-silenced plants are not able to compensate for reduced photosynthetic rates. This suggests that *RCA*- and *RuBPCase*-silenced plants differ in their ability to use RuBPCase, not in their ontogeny. Regardless of how efficiently *RuBPCase* is silenced, asRUB plants can compensate for their photosynthetic rate and growth, which suggests that the amount of RuBPCase is more than sufficient to meet the requirements of plant growth, a result consistent with earlier observations of *RuBPCase*-silenced cultivated tobacco plants (Quick et al., 1991a, 1991b).

The performance of *S. littoralis* and *M. sexta* improved on *RCA*-silenced plants, possibly owing to decreases in the concentrations of defense metabolites, namely TPIs and DTGs, which are well known for their anti-herbivore activity (Zavala et al., 2004; Jassbi et al., 2008). Since both TPIs (Zavala et al., 2004) and

DTGs (Keinanen et al., 2001) are jasmonate elicited, we hypothesized that deficiencies in JA signaling could be responsible for the lower levels of these direct defenses. We found that although both *RCA*- and *RuBPCase*-silenced plants had similar OS-elicited JA bursts, only *RCA*-silenced plants had lower JA-Ile/Leu levels. JA-Ile is the main JA-amino acid conjugate required for direct defense signaling (Wang et al., 2007), and the decreased level of JA-Ile/Leu in *RCA*-silenced plants can account for the decreased level of TPIs and DTGs. Levels of another JA-induced defense, nicotine, were normal in *RCA*- and *RuBPCase*-silenced plants, which is consistent with the findings of Wang et al. (2007), who showed that decreases in JA-Ile levels negatively influenced the accumulation of TPIs but not the accumulation of nicotine. Moreover, a recent study showed that *N. attenuata* plants transiently silenced in the expression of their Gln synthase gene and clearly suffering from N-limited growth had higher levels of nicotine compared with EV plants but lower levels of another N-demanding inducible defense compound, TPI (Mitra et al., 2008). Therefore, in *N. attenuata*, the allocation of N appears to favor nicotine more than TPIs in N-limited conditions. The results from the Ile complementation experiments and transcription profiles of *JAR4/6* suggest that JA-Ile levels in RCA-silenced plants are influenced neither by the Ile pool nor by the accumulation of the conjugating enzyme. Adenylation of JA initiates its conjugation to amino acids and regulates the hormone activity (Staswick et al., 2002). Ile supplements increase the basal Ile pool of the plants, which causes 10-fold increases in the JA-Ile/Leu level. However, despite the increased Ile pool, JA-Ile/Leu accumulation was significantly less in *RCA*-silenced than EV plants. Adenylation is an energy-demanding process; therefore, decreases in photosynthetic rate may reduce the ATP supply required for the adenylation of JA. However, understanding how this process occurs requires additional experimentation.

High levels of dietary protein are known to negate the effects of TPI ingestion on the growth of larvae (Johnson et al., 1996). In this study, levels of RuBPCase, the main dietary protein in leaves, were about 1.2 times higher in irRCA plants than in EV-transformed plants and 1.5 times lower in asRUB plants than in EV-transformed plants. It is known that *RCA* silencing delays the turnover of RuBPCase and increases its proportional representation in the total protein pool of rice (*Oryza sativa*) and cultivated tobacco (He et al., 1997; Jin et al., 2006). Therefore, the increased level of RuBPCase in *RCA*-silenced plants likely increases the nutritional value of the plant to *S. littoralis* larvae, allowing them to gain more body mass compared with larvae that fed on *RuBPCase*- and EV-transformed plants. However, the performance of *M. sexta*, the specialist herbivore, was improved on both irRCA and asRUB plants. Mitra et al. (2008) showed that the performance of *M. sexta* larvae, a specialist herbivore, is not correlated with the level of RuBPCase. This suggests that the specialist herbivore can better tolerate decreases in protein intake than the generalist.

Plants gain resources via photosynthesis and allocate them to growth, reproduction, and defense. Zangerl and Berenbaum (1992) and Berenbaum (1995) wrestled with the question of how resources should be allocated to defense and other sinks, but they concluded that our understanding of the controls over the resource allocation process was not sufficiently mature for robust predictions. Investing resources in both reproduction and anti-herbivore defense is thought to be costly, particularly in the absence of damaging herbivores (Rausher et al., 1993; Saulnier and Reekie, 1995). Therefore, the resources gained and the allocation strategies used are predicted to vary according to the ecological situation and the plant species involved (Coley, 1998). Generally, growth is thought to be negatively related to defense (Coley, 1998). While these predictions are derived from considerations of evolved differences among plant taxa and therefore not directly applicable to the results of our study of transformation-related differences within a genotype, it is interesting that plants transformed to decrease growth rates were also less resistant to herbivores. These observations raise the question, how do *N. attenuata* plants allocate their carbon resources to different functions? According to the carbon-nutrient balance hypothesis (Coley, 1998), secondary metabolism is directed toward carbon-rich metabolites in N-limited plants and vice versa. Matt et al. (2002) demonstrated that decreasing photosynthetic capacity by down-regulating RuBPCase transcripts in cultivated tobacco strongly decreased the accumulation of carbon-rich secondary metabolites, namely chlorogenic acid and rutin. DTGs are also carbon-rich metabolites, and decreased photosynthetic capacity likely affects DTG accumulation. However, asRUB plants have similar rates of photosynthesis as irRCA plants at the rosette stage and yet have similar levels of DTGs compared with EV-transformed plants. From these results, we conclude that how the photosynthesis is decreased, namely by decreasing RuBPCase pools or its activation, is a more important determinant of defense allocation than the extent of the decrease.

These results highlight the value of understanding the mechanistic details of defense signaling and defense production when wrestling with the more abstract questions of growth-defense tradeoffs (Zangerl and Berenbaum, 1992; Berenbaum, 1995). Decreasing photosynthetic capacity by silencing RCA rather than by reducing RuBPCase pools appears to decrease plants' ability to elicit important herbivore resistance traits due to limitations in the production of JA-Ile. While more work is needed to place this inference on strong experimental footing, it suggests that carbon limitations on defense production can affect the elicitation of the defenses in addition to the production of the defenses. This is an unexpected result, given that defense signaling is thought to require relatively fewer resources than does defense production. This effect on

defense signaling is not a general result of carbon limitation and only occurs when plants are RCA silenced, not when their carbon limitations result from RuBPCase silencing. The performance of generalist and specialist larvae on silenced plants revealed that specialist larvae are more tolerant of changes in dietary protein pools than are generalist larvae, which may require higher nutritional diets to detoxify plant defenses (Green et al., 2001).

MATERIALS AND METHODS

Plant Growth Conditions

Seeds of a *Nicotiana attenuata* inbred line were smoke germinated on GB medium (Krügel et al., 2002). Ten days after germination, seedlings were planted in soil into Teku pots, and after an additional 12 d, they were transferred to 1 L (singly grown) or 2 L (competition-grown) pots with a peat-based substrate (Klasmann Tonsubstrat). Plants were grown in the glasshouse of the Max Planck Institute for Chemical Ecology at 24°C to 26°C (16 h of light; supplemental lighting by Philips Sun-T Agro [http://www.nam.lighting.philips.com/] 400- and 600-W sodium lights; 55% humidity). Four- to 5-week-old plants were used for all experiments except for growth and biomass measures.

Silencing *RCA* and *RuBPCase* in *N. attenuata*

Vector Construction and Agrobacterium-Mediated Transformation

As reported previously, a 268-bp inverted-repeat fragment (Voelckel and Baldwin, 2003) of the cDNA sequence of *NaRCA* (BU494545) was inserted into the pREC5 transformation vector and a 316-bp fragment of the cDNA sequence of *NaRUB* (AW191829; Hermsmeier et al., 2001) was inserted into the pREC2 transformation vector in the antisense orientation (Bubner et al., 2006). This vector, along with the *NaRCA* and *NaRUB* inserts, was transformed into *N. attenuata* wild-type plants using *Agrobacterium tumefaciens*-mediated transformation (Krügel et al., 2002). The presence of the hygromycin resistance gene (*hptII*) in the transformation vector allowed us to identify hygromycin-resistant transformants (Krügel et al., 2002). The number of insertions was determined by Southern hybridization of genomic DNA using a PCR fragment of the *hptII* gene as a probe. Single-insertion lines of irRCA and asRUB plants were identified and used in all subsequent experiments with the same wild-type generation transformed with an EV.

Southern-Blot Analysis and Quantitative RT-PCR

DNA was extracted from the fully expanded leaves of *N. attenuata* plants using the cetyltrimethylammonium bromide method (Rogers and Bendich, 1985) with slight modifications. Ten micrograms of the DNA samples was digested with different restriction enzymes, size fractionated on a 0.8% (w/v) agarose gel, and Southern blotted (Brown, 1997) onto a nylon membrane (GeneScreen Plus; Perkin-Elmer [http://las.perkinelmer.com/]). Fragments of *NaRCA* (forward primer, 5′-GAAGCTCCTTGAGTATGGTAACATG-3′; reverse primer, 5′-GGCAGCACTTGGAGATGCAAATG-3′), *NaRUB* (forward primer, 5′-CGGACGTTGTCGAATCCAATG-3′; reverse primer, 5′-CGAGACACTCTCATACCTTCC-3′), and *hptII* (forward primer, 5′-CGTCTGTCGAGAAGTTTCTG-3′; reverse primer, 3′-CCGGATCGGACGATTGCG-5′) were amplified by PCR and used as probes for Southern hybridization. The probes were labeled with the 32P Rediprime II DNA labeling system (Amersham Biosciences [http://www.amersham.com/]).

To analyze *NaRCA*, *NaRUB*, and *NaJAR4/6* expression, we extracted total RNA with the TRI reagent protocol (Sigma [http://www.sigmaaldrich.com/]). cDNA was synthesized from 2 μg of RNA using SuperScript II reverse transcriptase (Invitrogen [http://www.invitrogen.com/]). Quantitative RT-PCR was conducted using gene-specific primer pairs (for *NaRCA*, forward primer 5′-AGAACGCCAGGGTCCCTATT-3′ and reverse primer 5′-CGACCATCACGGATAAGAGGA-3′; for *NaRUB*, forward primer 5′-CGAGACACTCTCATACCTT-3′ and reverse primer 5′-GTAGTACCCTGGTGACTT-3′; for *NaJAR4*, forward primer 5′-ATGCCAGTCGGTCTAACTGAA-3′ and reverse primer 5′-TGCCATTGTGGAATCCTTTTAT-3′; for *NaJAR6*, forward primer 5′-TGGAGTAAACGTTAACCCGAAA-3′ and reverse primer 5′-AGAATTTGCTTGCTCAATGCCA-3′). The relative gene expression was calculated using an *actin* primer, which served as an endogenous control gene.

CO_2 Exchange and Plant Growth

In the glasshouse, 4-week-old plants were used for CO_2 exchange measurements. Net photosynthetic rates and intercellular CO_2 concentrations were measured on *RCA*-silenced, *RuBPCase*-silenced, and EV-transformed plants under saturating light (1,200 μmol m^{-2} s^{-1}) using the LI-COR 6400 Portable Photosynthesis System (Li-Cor Biosciences [http://www.licor.com/]). A light-responsive curve of photosynthesis was generated in at least five replicate plants, each at optimal CO_2 concentration (400 μmol mol^{-1}) and six different light intensities: 0, 200, 500, 1,200, 1,500, and 2,000 μmol m^{-2} s^{-1}. Net photosynthesis was also measured at seven different CO_2 concentrations, 0, 100, 200, 300, 400, 600, and 650 μmol mol^{-1}, in at least five replicate plants for each CO_2 concentration. Photosynthesis was also measured in the different leaf positions, S2 (position 1), S1 (position 2), and +2 (position3), at optimal (400 μmol mol^{-1}) CO_2 concentration and saturating light intensity (1,200 μmol m^{-2} s^{-1}). Whole plant photosynthesis refers to the average photosynthetic value from three different leaf positions. The slope of a regression of photosynthetic rate against C_i provides an in vivo measure of RuBPCase activity (Farquhar et al., 1980), and the slope of the A/C_i curve of EV plants is approximately 1.2 times greater than that of the irRCA plants, which clearly demonstrates that silencing *RCA* reduced RuBPCase activity. Reduced RuBPCase activity in turn decreased net photosynthesis. Stalk length was measured in 4-week-old plants for 7 consecutive days to evaluate growth.

Analysis of DTGs, Nicotine, and TPIs

Leaf tissue (approximately 100 mg) was sampled to analyze levels of DTGs, nicotine, and TPIs. The accumulation of DTGs and nicotine was analyzed by HPLC as described previously (Halitschke et al., 2003). TPI activity was analyzed by the radial diffusion assay described by Glawe et al. (2003).

Quantification of JA and JA-Ile

About 200 mg of harvested leaf tissue from each line was extracted and analyzed for JA and JA-Ile level by the 1200L liquid chromatography-mass spectrometry system (Varian [http://www.varianinc.com/]) as described (Wang et al., 2007).

Spodoptera littoralis and *Manduca sexta* Performance

Freshly hatched *S. littoralis* larvae were grown on artificial diet for 6 d and then placed on 12 replicate transformed plants of each genotype. After feeding for 2 d, larvae were weighed every 2nd d for 12 d. Freshly hatched *M. sexta* larvae were placed on 15 replicate transformed plants of each genotype. Larvae were weighed every 4th d for 12 d.

Statistical Analysis

Data were analyzed with StatView (Abacus Concepts [http://abacus-concepts.com/]). Experiments in the glasshouse were analyzed by ANOVA for singly grown plants and by paired *t* test for competition-grown plants.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers BU 494545 and AW 191829.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. T_2 generations of homozygous transformed plants harboring a single insertion of the transgene.

Supplemental Figure S2. Silencing *RCA* and *RuBPCase* decreases photosynthetic rates in *N. attenuata* (light curve).

Supplemental Figure S3. Growth of *RCA*- and *RuBPCase*-silenced plants is strongly impaired under competitive growth conditions.

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