Nicotiana attenuata sequences of herbivory. Have coevolved with adapted herbivores likely have elaborate mutant screens and other reverse genetic approaches with model responses require the tuning of primary metabolism, for which level, increases in photosynthetic rate, branching, and storage in genotype in environments with and without attackers, remains herbivore damage. Defense against, and would benefit from tolerance, which minimizes the fitness con-

Plants have evolved a variety of mechanisms for reducing the negative impact of herbivore attack on fitness; these mechanisms include direct and indirect defenses and tolerance (1). Defenses are costly, expending energy and resources that could otherwise be used to grow and generate offspring. Inducible defenses allow plants to invest resources into defense only when needed. Although defenses limit the extent of damage, even well defended plants lose large amounts of tissue when attacked by herbivores that have adapted to their defenses. Then, plants would benefit from tolerance, which minimizes the fitness consequences of tissue loss to herbivores (2–4). Defense against, and tolerance of, herbivory are not mutually exclusive; most plant–insect interactions likely combine both (5, 6). In contrast to the rapid advances in our understanding of defense mechanisms, little is known about the traits that allow plants to tolerate herbivore damage.

Tolerance, which is measured by comparing the fitness of a genotype in environments with and without attackers, remains uncharacterized at the molecular level (2, 7). At a physiological level, increases in photosynthetic rate, branching, and storage in belowground tissues are thought to be involved (8–10). These responses require the tuning of primary metabolism, for which mutant screens and other reverse genetic approaches with model plants have yet to yield molecular regulators. Host plants that have coevolved with adapted herbivores likely have elaborate defense and tolerance responses to minimize the fitness consequences of herbivory.

The postfire annual of the Great Basin Desert of the United States, Nicotiana attenuata Torr. ex Wats. (Solanaceae), copes dramatically up-regulating and tailoring the expression of a variety of defenses to particular attackers (11). For example, the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) has evolved resistance to nicotine (12), the plant’s major defense alkaloid. The plant recognizes attack from M. sexta larvae when fatty acid–amino acid conjugates (FACs) from larval oral secretions and regurgitants (Rs) are introduced into the wounds during feeding, which down-regulates nicotine production and up-regulates a suite of other direct and indirect defense responses, all requiring jasmonate (JA) signaling for their activation (13–15). Despite these defense responses, M. sexta larvae regularly defoliate N. attenuata plants in native North American populations and are responsible for most of the leaf damage in these populations (16, 17). Therefore, we predict that N. attenuata benefits from tolerance traits to complement its elaborate defense responses and that tolerance results from altered resource allocation (3) that is closely coordinated with herbivore attack.

Results and Discussion

11C Labeling Reveals C Partitioning to Roots. Because defense elicitation of N. attenuata occurs rapidly [transcriptional and metabolic responses start within minutes of attack (14, 18)], we measured C partitioning between shoot and root to estimate changes in resource allocation shortly after herbivore attack. We used 11CO2, a short-lived C isotope with a half-life of 20.4 min (<2% of initial activity after 2 h), which allows for in vivo tracking of photosynthate partitioning with several measurements per plant per day (19). Partitioning was measured both before and after elicitation in the same plant in real time. We supplied 11CO2 to source leaves of young rosette-stage WT plants. To elicit a strong and reproducible response to M. sexta attack, we wounded three source leaves (Fig. 1 A) with a fabric pattern wheel twice in 3 h and immediately applied R to the wounds, a treatment that elicits the same transcriptional and defensive responses as M. sexta feeding (20–22).

By providing 11C to source leaves, we were able to measure C partitioning to roots and shoots of each unmanipulated plant (Figs. 1B and C and 2). Source leaves were elicited and subsequently supplied for a second time with 11C. By calculating the relative change of root C fractions before (10 a.m.) and after (4 p.m.) treatments, we discovered a significant (10%) increase in C allocation to roots after treatment with R but not when puncture wounds were treated with distilled water (W) (Fig. 2A).

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: SnRK1, SNF1-related kinase; FAC, fatty acid–amino acid conjugate; R, regurgitant; JA, jasmonate; W, distilled water; SuSy, sucrose synthase; as, antisense.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY460336).

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www.pnas.org/cgi/doi/10.1073/pnas.0602316103
The effect of R was completely reproduced when FACs (N-linolenoyl-L-Gln and N-linolenoyl-L-Glu), which occur naturally in R and are known to elicit N. attenuata’s responses to M. sexta attack (13, 21), were added to puncture wounds (Fig. 2A). To better understand the magnitude of the R/FAC-elicited changes on C allocation to roots, we completely removed all aboveground sinks by removing the sink leaves and the stem of a 5-cm elongated plant while keeping source leaves intact. This treatment should have caused a dramatic alteration in sink–source balance between shoot and root, but it merely doubled the allocation of C to roots compared with the R/FAC treatment (Fig. 2A), demonstrating how strongly R elicitation influenced assimilate partitioning.

Furthermore, W and R treatments were accompanied by significant changes in sugar metabolism 5 h after elicitation. Sucrose transport by the phloem is understood to be a gradient-driven process whereby sucrose is actively loaded by transporters into source tissues and passively unloaded (symplasmically or apoplasmically) into sink tissues. Sink strength, which is partially regulated by sucrose-cleaving enzymes [invertases and sucrose synthase (SuSy)], helps drive the process (23–25). Neither W nor R treatments influenced the activity in leaves of any of the invertases measured (Fig. 3A and B) or of SuSy (data not shown).

Only in roots did both treatments strongly increase soluble acid (vacuolar) invertase activity (Fig. 3C). This increase in sugar-cleaving activity likely increases the sink strength of roots and facilitates root growth as recently shown by quantitative trait locus and mutant analysis of this invertase in Arabidopsis thaliana (26). Because a plant’s sink organs compete continuously with each other for photoassimilates, an increase in root sink strength will reduce the amount of photoassimilates transported to shoot sinks. Indeed, the amount of sugars measured in sink leaves of both W- and R-treated plants was strongly reduced (Fig. 3B), and R-treated plants had significantly lower sucrose contents in sink leaves than did W-treated plants (Mann–Whitney U test, P < 0.0143; n = 5; Fig. 3B). Significantly, sucrose and fructose levels were reduced in source leaves (which represent the major aboveground biomass of rosette-stage plants) in R-treated plants but not in W-treated plants (Fig. 3A). This finding indicates that roots of R-treated plants recruit sugars from source leaves much more efficiently than do roots of control- and W-treated plants.

Fig. 1. Experimental setup. (A) Numbers denote the mature (source) leaves used for either 11CO2 pulse feeding (+3) or elicitation (+2, +4, and +5); immature (sink) leaves are labeled with negative numbers. The sequence indicates the leaf age; the larger the number, the older the leaf. (B) Scheme of detection areas. The load leaf was separately measured to control 11CO2 pulses. (C) Scheme showing the positions of the shoot and root detectors as well as the lead and tungsten shielding (collimation) needed to separate the field of view of the different detectors.

Fig. 2. C allocation in N. attenuata. (A) Relative change (mean ± SE, n = 3–6) of the root-partitioned C fraction of asGAL83, WT, and asLOX plants after 5 h in response to different types of induction (C, control; W, wounding; R, R elicitation; FAC, application of FACs; WS, wounding of sink leaves; SR, aboveground sink removal) as measured by 11CO2 application. Asterisks indicate significant difference from WT C (for each comparison with WT C, Mann–Whitney U test, P < 0.05). (B) Fraction (mean ± SE, n WT = 45, n asGAL83 = 27) of assimilates partitioned to roots of unelicited plants (Mann–Whitney U test, U < 462.5, P = 0.0134).
4. SuSy activities (data not shown) were not changed after any treatment.

Collectively, SnRK1s are kinases that function as cellular fuel gauges and play central roles in cell energy metabolism, regulating several key enzymes in sugar metabolism (28, 30, 31). SnRK1s comprise three subunits: α, which is composed of SNF1; three possible β-subunits, SIP1, SIP2, and GAL83; and γ, which is composed of SNF4. Together, these subunits form the active complex. Homologues occur in all kingdoms and are well studied in yeast, where they activate glucose-repressed genes when the cells’ energy reserves run low (28, 30), and in mammals, where they are involved in regulating glucose uptake and gluconeogenesis and are also necessary for diabetes therapy (32). In yeast, GAL83 is thought to direct the kinase complex to the nucleus (33); however, its function in plants is not well understood. αGAL83 potato plants, for example, show altered root and tuber development (34).

Fig. 3. Enzyme activities of alkaline invertase, soluble acid (vacuolar) invertase and cell wall invertase, and soluble sugar contents (sucrose, glucose, and fructose) of source leaves (A), sink leaves (B), and roots (C) 5 h after W and R treatment (see Fig. 1). (A) Mann–Whitney U test, sucrose, U < 25, P = 0.009; fructose, U < 24, P = 0.0163; n = 5. (B) Mann–Whitney U test, U < 20, P < 0.01; n = 5. (C) Mann–Whitney U test, wounding, U < 9, P < 0.05; R elicitation, U < 9, P < 0.05; n = 4. SuSy activities (data not shown) were not changed after any treatment. *, P < 0.05; **, P < 0.01.

To study the role of GAL83 in the R/FAC-elicited C allocation response, we transformed N. attenuata plants to express GAL83 in an α orientation. From 20 independently transformed homozygous lines, we screened for transcriptional down-regulation of GAL83 in roots, where it is highly expressed in WT plants (Fig. 7, which is published as supporting information on the PNAS web site). We predicted that αGAL83 plants would mimic the C allocation pattern of elicited WT plants if GAL83 transcripts were continuously down-regulated. Two independently transformed single-insert homozygous αGAL83 lines were tested and found to have greater root:shoot dry mass ratios compared with WT plants, although total mass remained the same (Fig. 8, which is published as supporting information on the PNAS web site). With the 12CO2 technique, we found that a single-insert homozygous line of αGAL83 plants that accumulated only 22% of the GAL83 transcripts of WT plants (see Supporting Text) transported ~10% more C to the root than did WT plants (72.0% vs. 66.3%; Fig. 2B). R treatment of αGAL83 plants did not alter their constitutively increased allocation to roots (Fig. 2A). These results clearly demonstrate that the
are significantly smaller than those of control (Mann–Whitney test, P < 0.01). Asterisks over unripe capsules bars indicate differences compared with differences between treatment and control plants (Mann–Whitney test, P < 0.01). Asterisks over unripe capsules bars indicate differences compared with control (Mann–Whitney test, P < 0.01). (B) Final root mass, mean ± SE. The asGAL83 controls do have a significantly larger root mass than WT controls (unpaired t test, DF = 26, T = 2.071, P < 0.05). All elicited asGAL83 root masses are significantly smaller than those of asGAL83 control plants (ANOVA, F(3,46) = 15.525, P < 0.0001, post hoc P < 0.001). * P < 0.05; ** P < 0.01; *** P < 0.001.

GAL83 cofactor of the N. attenuata SnRK1 complex regulates the allocation of C within the plant in response to herbivore attack and is elicited by FACs of M. sexta R.

Root Resources Provide Tolerance. To determine whether M. sexta-attacked N. attenuata plants realize a fitness benefit from an increase in C allocation to roots, we conducted a long-term greenhouse experiment in which rosette-stage WT and asGAL83 plants were grown in 1-liter pots. For 6 days before stalk elongation commenced, we either (i) elicited plants with W or R twice per day (at 10 a.m. and 4 p.m.) with two source leaves treated simultaneously so that, each day, four different leaves were treated or (ii) allowed four M. sexta larvae to feed freely for 6 days on source leaves (a treatment that we call “H”). We monitored stalk height, flower number, and seed capsule production (as correlates of fitness through the male and female function, respectively) (29) for ~2 months until all plants had senesced and measured final root and shoot biomasses.

GAL83-silenced plants were smaller than WT plants after all treatments (Fig. 9, which is published as supporting information on the PNAS web site) because of increased assimilate allocation to roots and its associated opportunity costs for aboveground growth. Unelicited asGAL83 plants (controls) produced significantly fewer capsules per gram of final biomass than did unelicited WT controls (Fig. 5A), and, accordingly, root mass at senescence of asGAL83 controls was significantly greater than that of WT controls (Fig. 5B). Interestingly, W-elicited asGAL83 plants produced significantly more capsules related to biomass than did W-treated WT plants, which did not regulate GAL83 (Fig. 5A). This compensatory response was associated with a 17% reduction in root mass in comparison with asGAL83 controls (Fig. 5B).

Furthermore, root masses of asGAL83 plants were significantly reduced after all treatments (Fig. 5B). These results demonstrate that GAL83 regulates resource storage in the roots; these resources can be mobilized to support seed production, the principal fitness “currency” of this annual plant. Moreover, leaf damage during rosette-stage growth among all genotypes appears to allow a plant to use root resources more effectively during reproduction (Fig. 5A) by unknown mechanisms that deserve additional attention.

Watering was reduced over a 10-day period after plants had attained maximum stalk heights to simulate the normal soil-drying regime that these plants experience in their native habitat (see Supporting Text). During this period of decreased water availability, flower production in asGAL83 plants increased significantly more than in WT plants (Fig. 6A and B). In nature, soil desiccation appears to function as an (abiotic) signal that plants use to mobilize their remaining root storage for a final reproductive effort before completely senescing. At the final harvest of the experiment, which was conducted when flowering had ended, asGAL83 plants had significantly more unripe capsules relative to all capsules than did WT plants (24.51% ± 1.6% vs. 14.76% ± 0.9%; Fig. 5A), reflecting their larger final flowering effort, which in turn was likely fueled by their larger...
root reserves. Flowers mature into ripe capsules 10–12 days after pollination. Within-genotype comparisons showed that R and H treatments resulted in reduced growth and significantly fewer capsules in each genotype (Figs. 9 and 10, which are published as supporting information on the PNAS web site), as well as a significant reduction of capsules related to biomass (Fig. 5a), which likely reflects the fitness costs of the elicited defense responses. In this species, R-elicited defensive trypsin protease inhibitor production is known to decrease stalk height and reduce capsule production (35, 36). However, WT flowering was significantly prolonged (by 2–3 days) by herbivore elicitation, to the extent that the number of flowers produced in the last week was 1.67-fold greater than in unelicited controls (Fig. 6A and B). Consequently, R- and H-elicited WT plants produced significantly more unripe capsules than did wounded WT plants, which did not regulate GAL83 (unpaired t test, R elicitation: D = 26, t = 2.083, P < 0.05; herbivory: D = 22, t = 2.148, P < 0.05; data not shown), reflecting their increased use of reserves for the final flowering effort (Fig. 6). As a result, the proportion of unripe capsules of all capsules produced by WT plants significantly increased after R and H treatment (Fig. 5b), indicating a shift of resource investment into reproduction to a later stage of development. The delayed senescence of elicited plants correlated with their larger root reserves, which likely provided the resources required for the final reproductive effort.

In contrast to the plants in the R and H treatments, W-elicited plants experienced no reduction of flower production or capsule number (absolute or related to biomass) compared with unwounded plants (Figs. 5a, 6, and 10). Although wounding elicited some defenses, such as nicotine production, and resulted in the same amount of leaf damage experienced by R-treated plants, wounded plants were able to fully compensate for the associated costs. Wounding was accompanied by an increase in lateral branching (unpaired t test, WT: t = −4.547, P < 0.0001; asGAL83: t = −2.657, P = 0.0144), which was not observed in R- and H-elicited plants (unpaired t test, WT R: t = −1.402, P = 0.15; WT H: t = −0.655, P = 0.52; asGAL83 R: t = −1.742, P = 0.11; asGAL83 H: t = −1.465, P = 0.16), suggesting that a plant’s regrowth response to wounding is altered when the elicitors of insect herbivores are introduced into wounds.

Tolerance and Its Potential Application. All plants allocate resources among traits that function in growth, reproduction, and defense to optimize their chances of being represented in future generations. Tolerance may be the best strategy for a plant to extricate itself from cycles of defensive escalation with its adapted herbivores. When attacked by adapted herbivores, host plants are likely to combine defense and tolerance responses, yet how these responses are integrated has been unknown until now. When attacked by the nicotine-adapted Manduca larvae, N. attenuata tunes its repertoire of induced defenses for maximal effectiveness (37–41) but also begins to bunker recently fixed C in its roots. Because root tissue is safe from this folivore, C stored there may be a means of immediately removing it from harm’s way. Once allocated to the roots, the C can be used to sustain seed production at the end of the plant’s life, after the Manduca larvae have pupated. How GAL83, the β-subunit of the plant’s SnRK1, mediates this C hoarding behavior remains unknown.

11C Measurements. 11C measurements were carried out at the Phytosphère laboratory as described in ref. 19. 11CO2 was applied to the third fully developed source leaf, where it was rapidly incorporated in sucrose, the major form of C transport in the phloem. Plants were shielded with lead and tungsten to separately measure shoot and root activity with scintillation counters before and after treatments. For further details, see Supporting Text.

Enzyme Activity and Sugar Measurements. For measurements of soluble sugars (sucrose, glucose, and fructose) and enzyme activities [SuSy, soluble acid (vacuolar) invertase, cell wall invertase, and soluble alkaline (cytosolic) invertase], tissue samples were frozen in liquid nitrogen and homogenized. Sugars were measured after ethanol extraction according to ref. 44. Activity levels of SuSy and invertases were measured in desalted extracts according to refs. 45 and 46, respectively.

We thank M. Lim for plant transformation, R. Bahulikar for TaqMan assays, J. Schumacher for help with statistics, A. Steppuhn for comments, D. Schwachtje for help with Fig. 1, U. Schurr for financing the work of P.E.H.M. and S.J., and P. Geigenberger for financing the work of J.T.v.D. This work was supported by the Max-Planck-Gesellschaft.