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SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots

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Herbivore attack elicits costly defenses that are known to decrease plant fitness by using resources that are normally slated for growth and reproduction. Additionally, plants have evolved mechanisms for tolerating attack, which are not understood on a molecular level. Using ^{11}C-photosynthate labeling as well as sugar and enzyme measurements, we found rapid changes in sink–source relations in the annual *Nicotiana attenuata* after simulated herbivore attacks, which increased the allocation of sugars to roots. This herbivore-induced response is regulated by the β-subunit of an SnRK1 (SNF1-related kinase) protein kinase, GAL83, transcripts of which are rapidly down-regulated in source leaves after herbivore attack and, when silenced, increase assimilate transport to roots. This C diversion response is activated by herbivore-specific elicitors and is independent of jasmonate signaling, which regulates most of the plant's defense responses. Herbivore attack during early stages of development increases root reserves, which, in turn, delays senescence and prolongs flowering. That attacked GAL83-silenced plants use their enhanced root reserves to prolong reproduction demonstrates that SnRK1 alters resource allocation so that plants better tolerate herbivory. This tolerance mechanism complements the likely defensive value of diverting resources to a less vulnerable location within the plant.

carbon-11 | defense | plant–herbivore interactions | tolerance

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 with a variety of herbivores from different feeding guilds by 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

^{11}C 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 $^{11}CO_2$, 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 photoassimilate 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 $^{11}CO_2$ 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 ^{11}C to source leaves, we were able to measure C partitioning to roots and shoots of each unmanipulated plant (Figs. 1 *B* and *C* and 2). Source leaves were elicited and subsequently supplied for a second time with ^{11}C. 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. 2*A*).

Conflict of interest statement: No conflicts declared.

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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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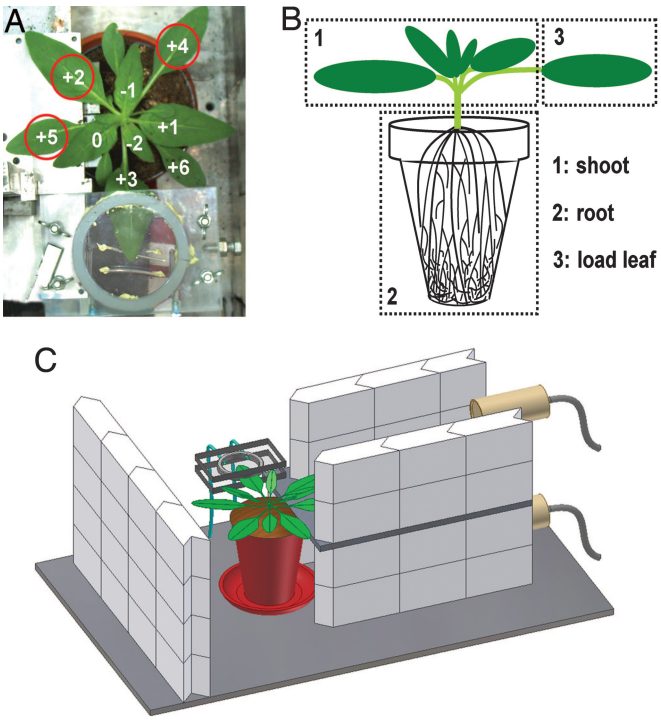

Fig. 1. Experimental setup. (*A*) Numbers denote the mature (source) leaves used for either $^{11}CO_2$ pulse feeding (+3) or elicitation (+2, +4, and +5); immature (sink) leaves are labeled with negative numbers. The sequence indicates the leaf age; the larger the number, the older the leaf. (*B*) Scheme of detection areas. The load leaf was separately measured to control $^{11}CO_2$ 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.

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. 2*A*). 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. 2*A*), 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. 3 *A* and *B*) or of SuSy (data not shown).

Only in roots did both treatments strongly increase soluble acid (vacuolar) invertase activity (Fig. 3*C*). 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 were strongly reduced (Fig. 3*B*), 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. 3*B*). 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. 3*A*). 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.

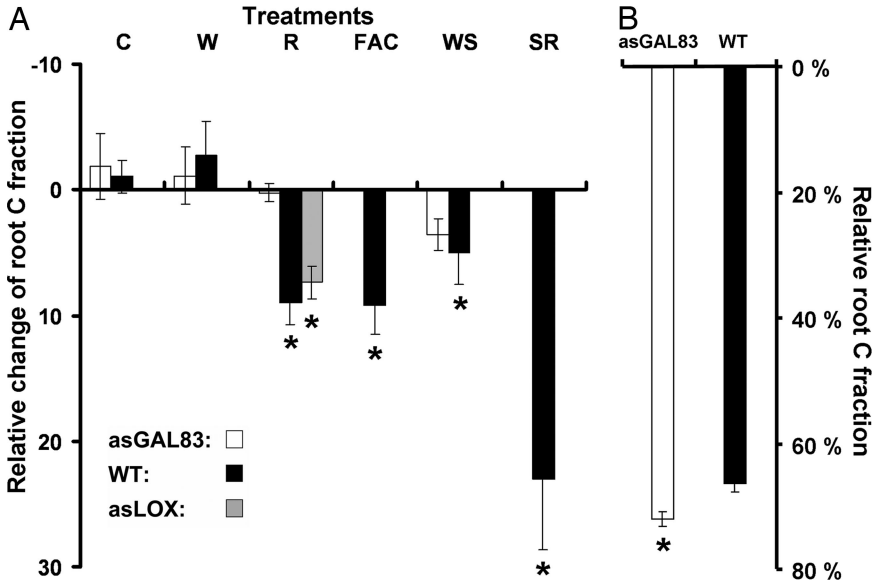

Fig. 2. C allocation in *N. attenuata*. (*A*) Relative change (mean ± SE, $n = 3–6$) of the root-partitioned C fraction of *as*GAL83, WT, and *as*LOX plants after 5 h in response to different types of induction (C, control; W, wounding; R, R elicitation; FAC, application of FACs; WS, wounding of sink leaves; SR, aboveground sink removal) as measured by $^{11}CO_2$ 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 as*GAL83 = 27) of assimilates partitioned to roots of unelicited plants (Mann–Whitney *U* test, $U < 462.5$, $P = 0.0134$).

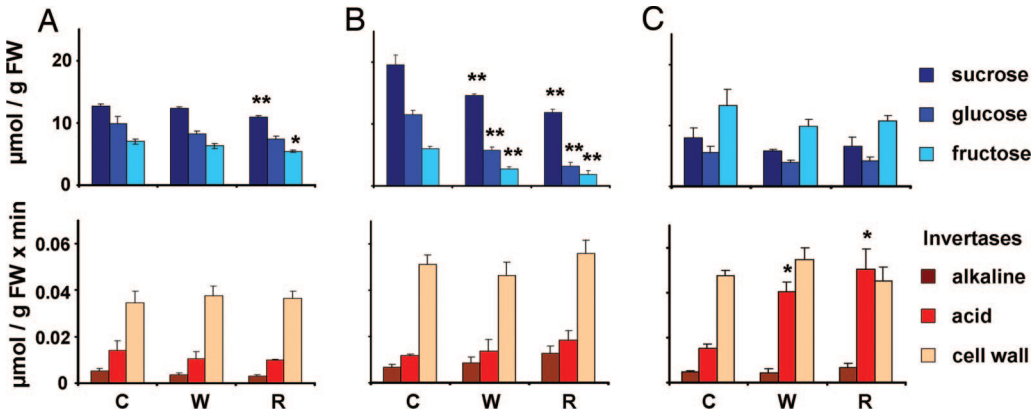

Fig. 3. Enzyme activities of alkaline invertase, soluble acid (vacuolar) invertase and cell wall invertase, and soluble sugar contents (sucrose, glucose, and fructose) of source leaves (*A*), sink leaves (*B*), and roots (*C*) 5 h after W and R treatment (see Fig. 1). (*A*) Mann–Whitney *U* test, sucrose, $U < 25$, $P = 0.009$; 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. *, $P < 0.05$; **, $P < 0.01$.

Apparently, additional sugar transporter activity is elicited in roots or source leaves of R-elicited plants compared with those of W-treated plants. The soluble sugar content in roots did not change after R treatment (Fig. 3*C*), and we infer that the extra supply of sugars was rapidly used for respiration, storage carbohydrate metabolism, or growth.

To determine whether the R/FAC-elicited response in C partitioning was mediated by JA signaling, we tested the response of *N. attenuata* plants (*as*LOX) that had been transformed with the endogenous lipoxygenase gene (NaLOX3); this gene supplies lipid hydroperoxide substrates for JA biosynthesis (12) and, when silenced by expression in an antisense (*as*) orientation, highly impairs a plant's defense responses (12). The elicited C partitioning response in these plants did not differ from that in WT plants (Fig. 2*A*), demonstrating that JA signaling is not required and that the rapid increase in C allocation to roots after herbivore elicitation is fully functional in defenseless plants with silenced JA signaling.

GAL83 Mediates C Allocation. To identify the genetic basis of the C partitioning response to *M. sexta* attack, we used a differential display PCR of control and *M. sexta*-attacked *N. attenuata* plants (27). We found that GAL83, a β-subunit of a heterotrimeric SnRK1 (SNF1-related kinase) kinase complex (28), was down-regulated in source leaves, whereas the catalytic α-subunit SNF1 was not (Fig. 4). That down-regulation is rapid (<1 h) (see *Supporting Text*, which is published as supporting information on the PNAS web site) and not elicited by methyl JA, a treatment that strongly elicits defenses (29), makes GAL83 an interesting candidate as a mediator of the resource allocation response.

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. *as*GAL83 potato plants, for example, show altered root and tuber development (34).

To study the role of GAL83 in the R/FAC-elicited C allocation response, we transformed *N. attenuata* plants to express GAL83 in an *as* orientation. From 20 independently transformed homozygous lines, 6 were 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 *as*GAL83 plants would mimic the C allocation pattern of elicited WT plants if GAL83 transcripts were continuously down-regulated. Two independently transformed single-insert homozygous *as*GAL83 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 $^{11}CO_2$ technique, we found that a single-insert homozygous line of *as*GAL83 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. 2*B*). R treatment of *as*GAL83 plants did not alter their constitutively increased allocation to roots (Fig. 2*A*). These results clearly demonstrate that the

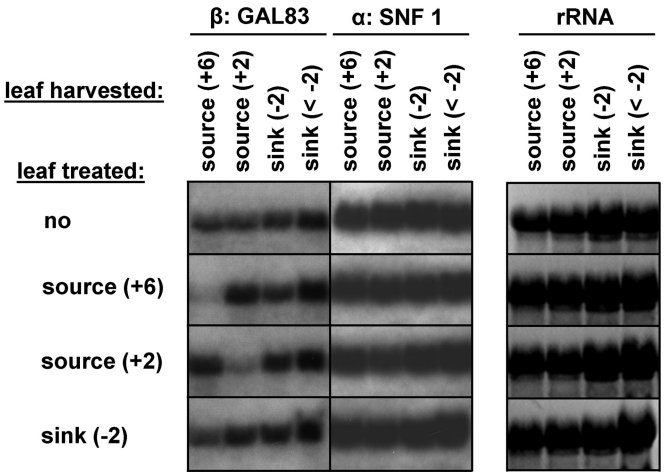

Fig. 4. Northern blot of GAL83 and SNF1 (α-subunit) mRNA expression in *N. attenuata* sink and source leaves (see Fig. 1*A*) 30 min after 4 h of repeated wounding and application of *M. sexta* R. Total RNA was pooled from 27 replicates for each treatment. GAL83 transcripts were down-regulated locally in treated source leaves and slightly down-regulated systemically after treatment of sink leaves. The transcript of the α-subunit, SNF1, was not regulated in leaves. rRNA was used as a loading control.

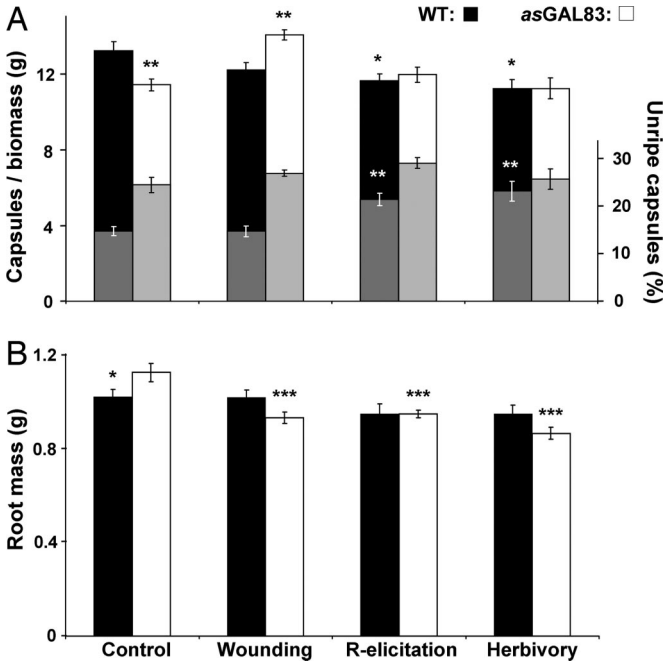

Fig. 5. Seed capsules per g of biomass and percentage of unripe capsules of the total capsules (*A*) and root (dry) masses (*B*) of WT and *as*GAL83 plants 54 days after elicitation. (*A*) Seed capsule number per g of biomass, mean ± SE (percentage of unripe capsules of total capsules is shown by gray bars). Asterisks over capsules/biomass bars (control and wounding) indicate significant differences between lines (Mann–Whitney *U* test, $P < 0.01$). Asterisks over capsules/biomass bars (R elicitation and herbivory) indicate significant differences between treatment and control plants (Mann–Whitney *U* test, $P < 0.01$). Asterisks over unripe capsules bars indicate differences compared with control (Mann–Whitney *U* test, $P < 0.01$). (*B*) Final root mass, mean ± SE. The *as*GAL83 controls do have a significantly larger root mass than WT controls (unpaired *t* test, $DF = 26$, $T = 2.071$, $P < 0.05$). All elicited *as*GAL83 root masses are significantly smaller than those of *as*GAL83 control plants (ANOVA, $F_{3,46} = 15.525$, $P < 0.0001$, post hoc $P < 0.001$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

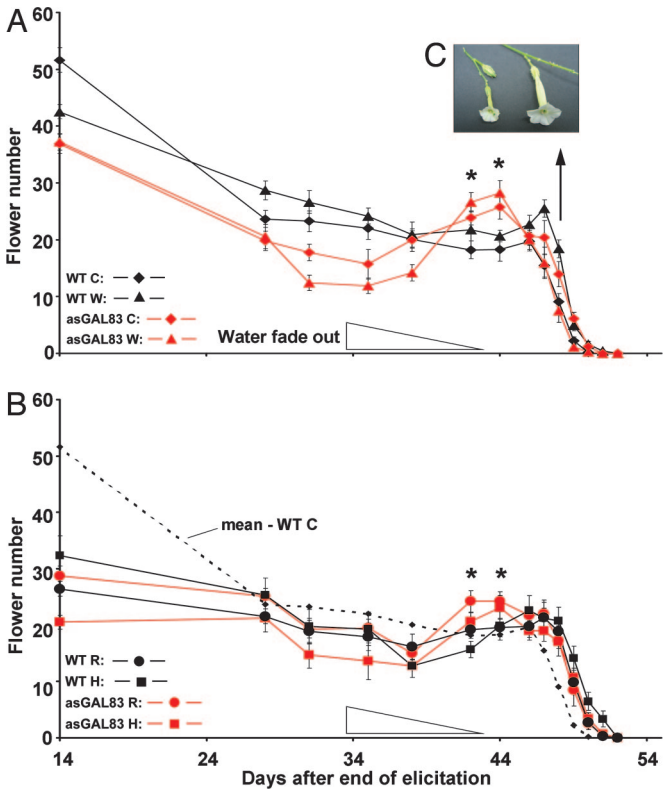

Fig. 6. Flower production of WT and *as*GAL83 plants after elicitation (mean ± SE). (*A*) Control and wounding treatment. (*B*) R elicitation and herbivory. (*A* and *B*) Fully opened flowers were measured. Watering of plants was gradually reduced over a 10-day period (see *Supporting Text*). Asterisks indicate that *as*GAL83 plants produced significantly more flowers than did WT (unpaired *t* test, $t > 3$, $P < 0.05$). (*C*) Late flowers of WT controls (left) are smaller than those of WT H-treated plants (right) on day 49 after elicitation; the same difference was observed for *as*GAL83 flowers.

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 *as*GAL83 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 *as*GAL83 plants (controls) produced significantly fewer capsules per gram of final biomass than did unelicited WT controls (Fig. 5*A*), and, accordingly, root mass at senescence of *as*GAL83 controls was significantly greater than that of WT controls (Fig. 5*B*). Interestingly, W-elicited *as*GAL83 plants produced significantly more capsules related to biomass than did W-treated WT plants, which did not regulate GAL83 (Fig. 5*A*). This compensatory response was associated with a 17% reduction in root mass in comparison with *as*GAL83 controls (Fig. 5*B*). Furthermore, root masses of *as*GAL83 plants were significantly reduced after all treatments (Fig. 5*B*). 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. 5*A*) 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 *as*GAL83 plants increased significantly more than in WT plants (Fig. 6 *A* 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, *as*GAL83 plants had significantly more unripe capsules relative to all capsules than did WT plants (24.51% ± 1.6% vs. 14.76% ± 0.9%; Fig. 5*A*), 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. 5*A*), which likely reflects the fitness costs of the elicited defense responses. In this species, R-elicited defensive trypsin proteinase 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. 6 *A* 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. 5*B*), 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-treated plants experienced no reduction of flower production or capsule number (absolute or related to biomass) compared with unwounded plants (Figs. 5*A*, 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: $t = -4.547$, $P < 0.0001$; *as*GAL83: $t = -2.657$, $P = 0.0144$), which was not observed in R- and H-treated plants (unpaired *t* test, WT R: $t = -1.402$, $P = 0.15$; WT H: $t = -0.655$, $P = 0.52$; *as*GAL83 R: $t = -1.742$, $P = 0.11$; *as*GAL83 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. The same elicitors (FACs) that activate the tuning of the induced defense responses after *M. sexta* attack activate this C storage in the roots, albeit by a different signal transduction cascade. By reconfiguring resource allocation, the plant gains a measure of tolerance to this voracious herbivore. That attacked GAL83-silenced plants use their enhanced root reserves to prolong reproduction demonstrates that SnRK1 supports a plant's tolerance to herbivory and that single genes can have large effects, contrary to the assumptions of previous quantitative genetic analyses (2, 42, 43).

The success of agriculture is based on breeding strategies that target the allocation of resources to harvestable parts (tubers, stems, fruit, etc.). The discovery that FACs trigger GAL83 extends to breeders the ability to alter the allocation of assimilates to different sink tissues (roots) by means of a simple environmental cue, which could represent a major biotechnological breakthrough.

Methods

For plant growth, treatment details, transformation, and RNA extraction and determination, see *Supporting Text*.

Plant Fitness Measurements. Stalk elongation, branch length, flowering, seed capsule production, and final root and shoot mass of all plants were measured as fitness determinants. To compare the lifetime reproductive performance among genotypes and treatments, we recorded for each plant: (*i*) stalk length starting on the day with measurable stalk growth (14 days after transplanting, when elicitation was finished) for 33 subsequent days, (*ii*) flower numbers (fully opened flowers) from day 14 after elicitation until end of flowering (52 days after elicitation), (*iii*) the number of ripe and unripe seed capsules 54 days after elicitation (the number of capsules per plant reflects the lifetime reproductive output in *N. attenuata* under natural or glasshouse conditions), and (*iv*) final root and shoot mass when capsule production had ended.

^{11}C Measurements. ^{11}C measurements were carried out at the Phytosphäre laboratory as described in ref. 19. $^{11}CO_2$ 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.

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