Temporal Changes in Allocation and Partitioning of New Carbon as ^{11}C Elicited by Simulated Herbivory Suggest that Roots Shape Aboveground Responses in Arabidopsis[1][W][OA]

Abigail P. Ferrieri[2]*, Beverly Agtuca, Heidi M. Appel, Richard A. Ferrieri, and Jack C. Schultz

University of Missouri, Division of Plant Sciences, Bond Life Sciences Center, Columbia, Missouri 65211 (A.P.F., H.M.A., J.C.S.); State University of New York Environmental Science and Forestry, Syracuse, New York 13210 (B.A.); and Brookhaven National Laboratory, Biosciences Department, Upton, New York 11973 (R.A.F.)

Using the short-lived isotope ^{11}C ($t_{1/2}$ = 20.4 min) as $^{11}CO_2$, we captured temporal changes in whole-plant carbon movement and partitioning of recently fixed carbon into primary and secondary metabolites in a time course (2, 6, and 24 h) following simulated herbivory with the well-known defense elicitor methyl jasmonate (MeJA) to young leaves of Arabidopsis (*Arabidopsis thaliana*). Both $^{11}CO_2$ fixation and ^{11}C-photosynthate export from the labeled source leaf increased rapidly (2 h) following MeJA treatment relative to controls, with preferential allocation of radiolabeled resources belowground. At the same time, ^{11}C-photosynthate remaining in the aboveground sink tissues showed preferential allocation to MeJA-treated, young leaves, where it was incorporated into ^{11}C-cinnamic acid. By 24 h, resource allocation toward roots returned to control levels, while allocation to the young leaves increased. This corresponded to an increase in invertase activity and the accumulation of phenolic compounds, particularly anthocyanins, in young leaves. Induction of phenolics was suppressed in sucrose transporter mutant plants (*suc2-1*), indicating that this phenomenon may be controlled, in part, by phloem loading at source leaves. However, when plant roots were chilled to 5°C to disrupt carbon flow between above- and belowground tissues, source leaves failed to allocate resources belowground or toward damaged leaves following wounding and MeJA treatment to young leaves, suggesting that roots may play an integral role in controlling how plants respond defensively aboveground.

Plant responses induced by herbivorous insects depend on the coordination of molecular, biochemical, and physiological events (Mithöfer and Boland, 2012). Plant tissues are not attacked uniformly nor are they uniformly responsive to insect herbivores, in part because they are modular organisms made up of source and sink tissues. While sink tissues (e.g. young leaves, flowers, and roots) are usually the major determinants of carbon partitioning in plants, defense induction can also reconfigure primary metabolism to provide additional substrates and energy required to produce secondary metabolites (Schwachtje and Baldwin, 2008). This results in complex patterns of defense that may vary spatially and temporally (Karban and Baldwin, 1997).

The idea that there are significant tradeoffs among growth, reproduction, and defense function in plants dates back to Darwin and has shaped much of modern plant and chemical ecology. Developing fruits, growing tissues, and storage organs are all significant sinks that compete for a common pool of carbon resources in plants. In past studies, for example, the number and size of competing sinks have been shown to strongly influence the success of galling aphids on cottonwood trees (i.e. their ability to enhance the sink strength at the feeding site and out compete other plant sinks for resources; Larson and Whitham, 1997; Wool, 2004). While investigators have acknowledged different responses in defense chemistry and downstream effects on herbivores as plants enter their reproductive stage (Bazzaz et al., 1987; Zangerl and Bazzaz, 1992; Baldwin, 1998; Barton and Koricheva, 2010), there are no clear generalizations regarding the relationship between other naturally occurring competing sinks (young leaves and roots) and plant defense responses. Moreover, recent attempts to develop a conceptual model of changes made to plant resource allocation

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[2] Present address: Max Planck Institute for Chemical Ecology, Department of Molecular Ecology, Hans-Knöll-Straße 8, D–07745 Jena, Germany.

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

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Abigail P. Ferrieri (aferrieri@ice.mpg.de).

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following herbivore attack that takes into account vascular architecture, tissue development, variation over time, and local environmental variation have been impeded by differences in temporal and spatial variation in responses and by the dependency of induction of local defense responses on other plant modules (Hanhimäki and Senn, 1992; Orians and Jones, 2001).

Recent studies utilized the short-lived positron-emitting radioisotope, ^{11}C ($t_{1/2}$ = 20.4 min) as $^{11}CO_2$ to study changes made to carbon allocation during plant defense induction (Babst et al., 2005, 2008; Thorpe et al., 2007; Gómez et al., 2010) and to measure changes in their metabolic partitioning into key secondary metabolites (Ferrieri et al., 2005; Hanik et al., 2010). Previous studies using this short-lived isotope have narrowed their focus to short-term dynamics in resource allocation occurring immediately (within hours) following herbivory. These experiments suggest that plants respond to leaf damage by rapidly transporting recently fixed carbon from both damaged and undamaged tissues to roots, a response thought to be employed by plants to tolerate herbivory by bunkering resources away from sites of damage and into storage belowground for later regrowth (Babst et al., 2005, 2008; Schwachtje et al., 2006; Newingham et al., 2007; Frost and Hunter, 2008; Kaplan et al., 2008; Gómez et al., 2010, 2012; Orians et al., 2011).

In contrast, previous studies in *Populus* spp. show that the ability of young, developing leaves to respond defensively relies on their capacity to induce their sink strength for carbon-based resources by increasing the activity of Suc metabolizing enzymes (invertases) and importing carbon toward damaged sites from orthostichous (directly connected) source leaves (Arnold and Schultz, 2002; Arnold et al., 2004). This response was observed at 24 and 48 h following insect damage using stable isotope labeling (^{13}C) techniques. Our previous work in the model plant Arabidopsis (*Arabidopsis thaliana*) also demonstrates the movement of a radioactive Glc surrogate, [^{18}F]fluoro-2-deoxy-D-Glc, toward wounded leaves where it was incorporated into phenolic glycosides 4 h after simulated herbivory (Ferrieri et al., 2012).

Given these apparent contradictions in the literature, these studies aimed to examine the movement of recently fixed carbon within Arabidopsis. Using $^{11}CO_2$, we captured temporal changes in whole-plant carbon movement and partitioning of recently fixed carbon into primary and secondary metabolites in a time course (2, 6, and 24 h) following simulated herbivory with the well-known defense elicitor methyl jasmonate (MeJA). In Arabidopsis, MeJA is essential for the induction of biologically effective defense responses and is responsible for the transcriptional activation of pathways leading to the production of several classes of secondary metabolites, including glucosinolates, phenylpropanoids, anthocyanins, and isoprenoids (Gundlach et al., 1992; McConn et al., 1997; Pauwels et al., 2009).

We hypothesized that the movement of ^{11}C-photosynthate away from damaged sites (to roots) is an immediate response to defense elicitation and is followed by movement toward damaged sites at later time points. If transported carbohydrates serve as building blocks for the production of secondary metabolites in developing sink leaves, we predicted that transport from source to sink would be required as a prerequisite to induction in these young leaves. To determine whether roots are a dominant sink for resources in Arabidopsis and compete with secondary metabolites for recently fixed carbon, we performed root chilling treatments to disrupt carbon flow between above- and belowground tissues. We predicted that this treatment would suppress the flow of carbohydrates to and from roots, thus removing them as a competing sink and increasing carbon precursors available for primary (sugar) and secondary (phenolic acid) metabolisms in young, developing sink leaves. The short-lived nature of ^{11}C provides a unique opportunity to determine how newly acquired carbon resources are distributed throughout the plant and partitioned into key metabolite pools during times of stress.

RESULTS

Temporal Changes in Fixation and Export of $^{11}CO_2$

To capture temporal changes in Arabidopsis carbon dynamics in response to simulated herbivory, an individual source leave (load leaf [LL]) was pulse labeled with $^{11}CO_2$, and ^{11}C-uptake and export by this leaf were measured at 2, 6, and 24 h following the combination of wounding and MeJA application (referred to as wound/MeJA treatment throughout remaining text) to two young orthostichous leaves (YO).

Diurnal fluctuations in $^{11}CO_2$ fixation and ^{11}C-photosynthate export from the LL were not observed during our time course; there were no significant differences between control plants labeled at 2 h (AM labeling) and 6 h (PM labeling; fixation, P = 0.2126; export, P = 0.3009). However, 2 h following wound/MeJA application to YO leaves, both fixation and export were significantly elevated (Fig. 1, A and B; P = 0.015 and P = 0.000072, respectively). ^{11}C-photosynthate export was elevated at 6 h post treatment as well, despite no change in fixation (Fig. 1B; P = 0.014). By 24 h, both fixation and export had returned to baseline levels.

Whole-Plant Allocation of ^{11}C-Photosynthate in Response to Wound/MeJA

The increases in $^{11}CO_2$ fixation and export observed 2 h following wound/MeJA treatment co-occurred with preferential allocation of radiolabeled resources belowground relative to control plants (see Supplemental Fig. S1; P = 0.0235). Of the ^{11}C-photosynthate remaining

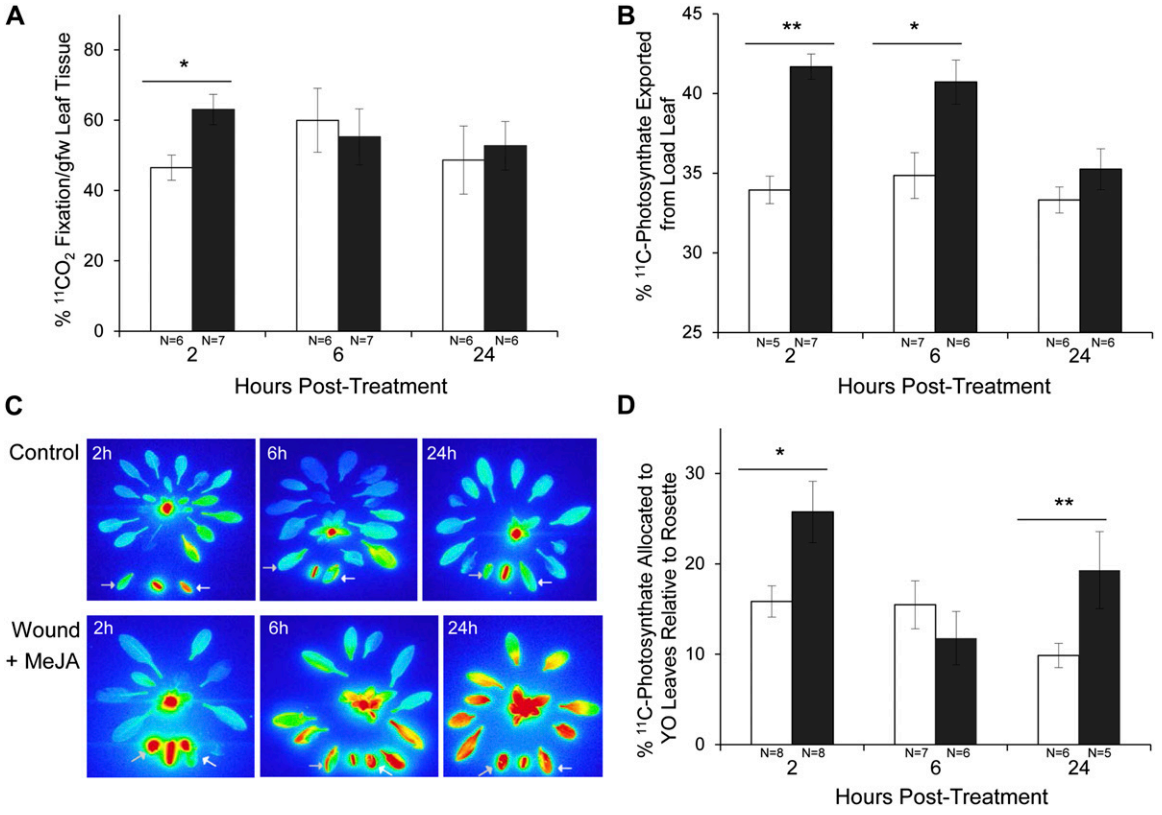

Figure 1. Temporal changes in $^{11}CO_2$ fixation and export by the LL and subsequent distribution of ^{11}C-photosynthates among leaves following combined wound/MeJA treatment to two young leaves. Changes made by wound/MeJA treatment (black bars) are compared with representative control groups (white bars). A, Percentage of $^{11}CO_2$ fixed by the LL normalized to gram fresh weight (gfw) of leaf tissue enclosed within the leaf cell. B, Percentage of ^{11}C-photosynthate exported by the LL following treatment. $^{**}P < 0.01$ and $^{*}P < 0.05$ show significance of the effect of treatment (A and B). C, Positron autoradiographs of aboveground tissues. Blue coloration indicates low radioactivity with increasing radioactivity for yellow and then red. White arrows indicate two young leaves targeted for treatment during experiments. Leaves and the remaining LL petiole (located between two targeted young leaves) were cut away from the rosette to avoid overlap. D, Effect of wound/MeJA on fine-scale distribution of ^{11}C-photosynthates in aboveground tissues. Percentage values were calculated as radioactivity in treated YO leaves relative to total rosette activity, not including radioactivity in the LL petiole ($^{*}P = 0.0257$; $^{**}P = 0.0122$). All bars are mean ± SE.

in aboveground sink tissues, we found preferential allocation to the treated YO leaves at 2 h post treatment (Fig. 1, C and D; $P = 0.0257$). This response returned to control levels by 6 h (Fig. 1D; $P = 0.6573$). By 24 h post treatment, resource allocation to roots was comparable to controls (Supplemental Fig. S1), but allocation to the YO leaves increased (Fig. 1D; $P = 0.0122$).

Partitioning of ^{11}C into Soluble Sugars

We observed marginally significant changes in the partitioning of recently fixed $^{11}CO_2$ into soluble sugars, including ^{11}C-Glc and ^{11}C-Fru across the time course (Supplemental Figs. S2 and S3). For example, in the LL, ^{11}C-Glc and ^{11}C-Fru decreased from 2 to 6 h in both treatments (Supplemental Fig. S2, C and D; ^{11}C-Glc, $P = 0.051$; ^{11}C-Fru, $P = 0.023$) but by 24 h had returned to the 2 h levels. There was no significant effect of wound/MeJA treatment on ^{11}C-sugars in the LL. In YO leaves, ^{11}C-Glc and ^{11}C-Fru also decreased marginally 24 h following wound/MeJA treatment (Supplemental Fig. S3, C and D; ^{11}C-Glc, $P = 0.085$; ^{11}C-Fru, $P = 0.078$). Wound/MeJA elicited no change to total ^{11}C-sugars in the LL or YO leaves.

Partitioning of ^{11}C into Organic Acids

In general, YO leaves allocated more ^{11}C-photosynthate to the production of ^{11}C-organic acids, including ^{11}C-phenolic acids and ^{11}C-cinnamic acid, compared with the older LL (Supplemental Fig. S4). Wound/MeJA treatment to two YO leaves induced total ^{11}C-phenolic acids (Supplemental Fig. S4A; $P = 0.0495$) and ^{11}C-cinnamic acid (Supplemental Fig. S5A; $P = 0.0413$) in the LL 2 h following treatment. No changes were detected in YO leaves following wound/MeJA treatments for any time point (Supplemental Figs. S4B and S5B).

Total ^{12}C-Phenolics

The same time course experiment was repeated at a larger scale, in the absence of $^{11}CO_2$ labeling, to assess changes in the accumulation ^{12}C-phenolics as well as cell wall (CWI) and soluble (SOL) invertase activity. The accumulation of ^{12}C-phenolics was elevated in young leaves as well as in their respective source leaf 24 h following wound/MeJA treatment to young leaves (Supplemental Fig. S6, A and B). When concentrations of various phenolic classes were assayed separately, we specifically found anthocyanins to be induced in all tissue types 24 h following wound/MeJA treatment (Supplemental Fig. S6, D–F). Flavonoid concentrations in leaves were not affected by wound/MeJA treatment (Supplemental Fig. S6, G and H), but were significantly suppressed in roots at 2 and 6 h following treatment aboveground (Supplemental Fig. S6I). By 24 h posttreatment, this pattern was reversed; flavonoids were significantly elevated in roots relative to respective control plants.

Source Constraint on Phenolic Induction

To test whether the flow of phloem-borne signals or resources traveling from sources to sink leaves facilitates changes made to phenolic chemistry in young, sink leaves, responses were measured in plants where export of carbohydrates from source leaves was disrupted. This was accomplished through the use of a Suc transporter insertion mutant (*suc2-1*, At1G22710), which is compromised in its ability to load carbohydrates in the phloem of source leaves (Gottwald et al., 2000).

Compared with wild-type plants, *suc2-1* mutants contained similar levels of total phenolic compounds in young leaves (Fig. 2). In wild-type plants, wound/MeJA elicitation to two young leaves induced the accumulation of total phenolics locally (Fig. 2A; $P = 0.013$) as well as systemically (Fig. 2B; $P = 0.002$). When individual classes of phenolics were analyzed separately, we found that anthocyanins were specifically elevated following treatment (Supplemental Fig. S7). The application of MeJA to *suc2-1* plants induced total phenolic compounds locally (Fig. 2A; $P = 0.042$), but not to the same extent as wild-type plants (8% increase in *suc2-1* plants compared with 17% increase in the wild type). In contrast to wild-type plants, the concentration of total phenolics did not increase in young, systemic tissues of *suc2-1* plants (Fig. 2B; $P = 0.283$). Within these leaves, anthocyanin levels were also no different than controls following wound/MeJA treatment of *suc2-1* plants (Supplemental Fig. S7B; $P = 0.967$).

Invertase Activity

Additional experiments were conducted to quantify changes made to the activity of CWI and SOL invertases in leaves and roots to provide insight into the mechanism underlying changes in carbon dynamics

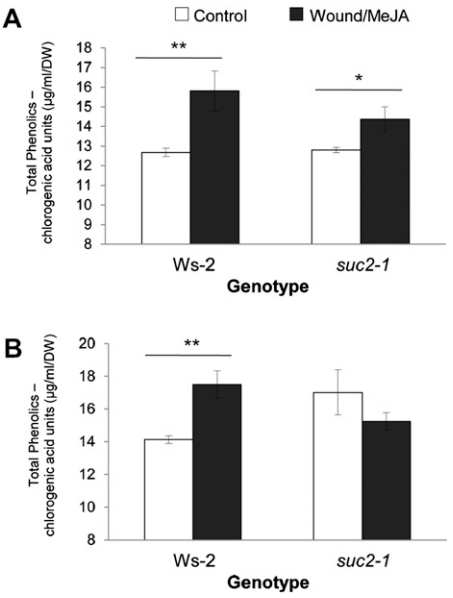

Figure 2. Effect of wound/MeJA treatment on Folin-reactive phenolic compounds in two young, treated leaves and systemic leaves of *suc2-1* and wild-type (Wassilewskija-2 [Ws-2]) plants. A, Two young leaves targeted for wound/MeJA treatments. B, Young cohort of undamaged leaves. Leaves were harvested 48 h after wound/MeJA treatment for phenolic measurements. All bars are mean ± SE. $^{**}P < 0.0001$ and $^{*}P < 0.05$ show significant treatment effect relative to controls. $n = 8$/treatment group. DW, Dry weight.

observed in our labeling studies. In Arabidopsis, invertases are not only the main route of Suc metabolism but are essential for normal plant growth and development (Barratt et al., 2009). Invertases also play a crucial role in regulating the supply of photosynthate to naturally occurring sink tissues (Tang et al., 1999; Weschke et al., 2003; Heyer et al., 2004; Roitsch and González, 2004) and are known to be up-regulated during gall formation (Rehill and Schultz, 2003; Siemens et al., 2011) and after wounding and insect attack (Zhang et al., 1996; Ohyama et al., 1998; Rosenkranz et al., 2001; Arnold and Schultz, 2002).

In our study, rapid sequestration of ^{11}C-photosynthates belowground following wound/MeJA treatment aboveground corresponded to the induction of CWI and SOL in roots (Supplemental Fig. S8, C and F). Aboveground, young damaged leaves marginally induced CWI activity by 6 h after wound/MeJA treatment (Supplemental Fig. S8A; $P = 0.07$). By 24 h, both CWI and SOL increased in all tissue types following wound/MeJA treatment (Supplemental Fig. S8).

Effect of Root Chilling on Resource Allocation

Root chilling did not alter $^{11}CO_2$ fixation (Fig. 3A; $P = 0.4815$) or ^{11}C-photosynthate export (Fig. 3B; $P = 0.2027$) from the LL. However, export was suppressed 2 h after wound/MeJA treatment to young leaves (Fig. 3B; $P = 0.0012$). This response occurred without changes in

fixation by the LL (Fig. 3A; $P = 0.1475$). In general, root chilling treatment decreased ^{11}C-photosynthate allocated to YO leaves (Fig. 3, C and D; 21°C controls versus 5°C controls $P = 0.0358$). Unlike plants with 21°C roots, plants with 5°C roots did not allocate more ^{11}C-photosynthates to YO leaves 2 h after wound/MeJA treatment (Fig. 3, C and D; $P = 0.4567$). Interestingly, plants with chilled roots also failed to allocate more ^{11}C-photosynthate to roots following wound/MeJA treatment aboveground (Fig. 4; significant effect of wound/MeJA treatment at 21°C $P = 0.0235$; nonsignificant treatment effect at 5°C $P = 0.5712$).

Root chilling alone induced CWI in YO leaves and roots (Fig. 5, A and C; $P < 0.05$). Under ambient root conditions, wound/MeJA treatment aboveground rapidly induced CWI and SOL activity in roots (Fig. 5, C and F; $P < 0.0001$). However, in plants with roots at 5°C, wound/MeJA treatment did not alter SOL (Fig. 5F) and significantly suppressed CWI activity (Fig. 5C; $P < 0.0001$) belowground. This pattern was also evident aboveground; CWI normally increased in YO leaves following wound/MeJA (Fig. 5A; $P = 0.0075$). However, when roots were chilled, invertases were either no different than controls (Fig. 5A) or decreased in these leaves (Fig. 5D; $P = 0.0019$).

Effect of Root Chilling on Metabolic Partitioning of ^{11}C into Soluble Sugars

Root chilling treatment alone significantly decreased the partitioning of ^{11}C into soluble sugars in the LL

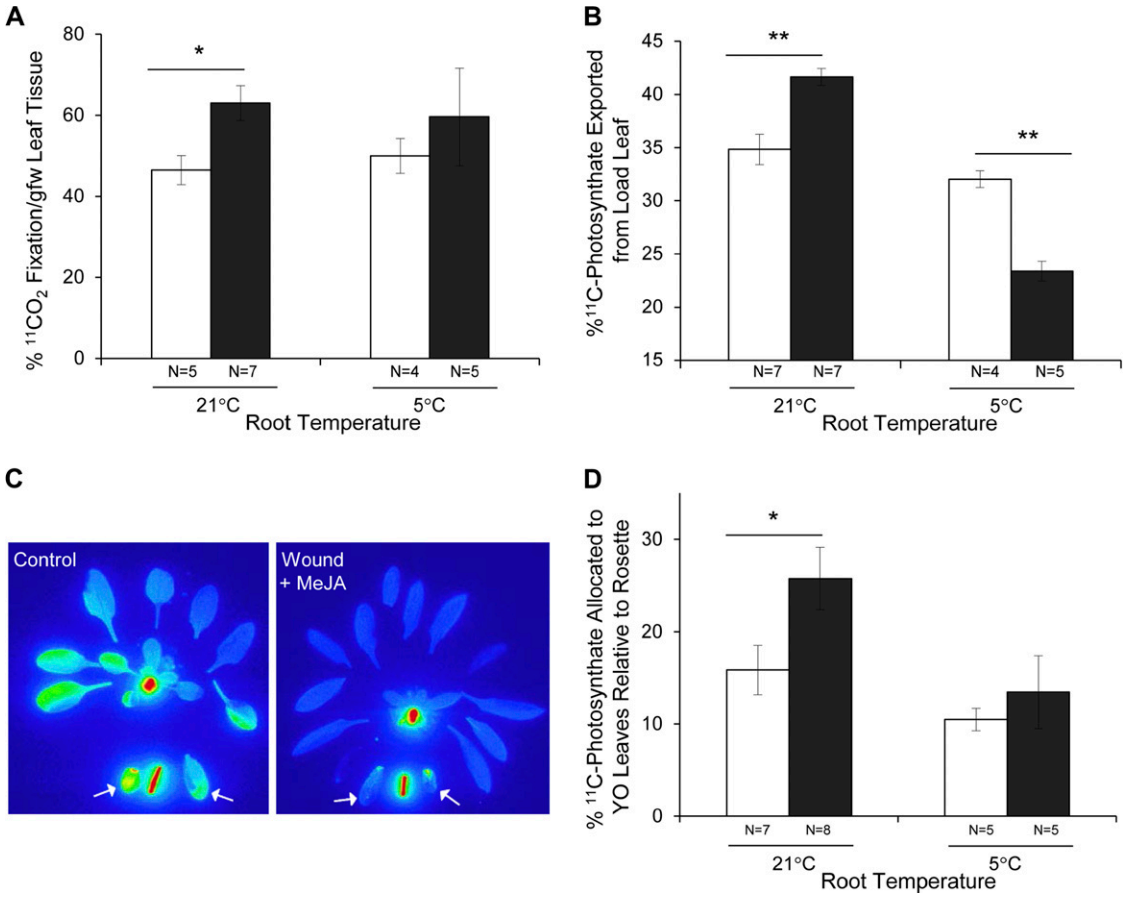

Figure 3. Combined effect of root chilling and wound/MeJA treatment to $^{11}CO_2$ fixation, export, and distribution of ^{11}C-photosynthates among leaves. Changes made by wound/MeJA treatment (black bars) are compared with representative control groups (white bars). A, Effect of root chilling treatment on percentage of $^{11}CO_2$ fixed by the LL normalized to gram fresh weight (gfw) of leaf tissue enclosed within the leaf cell. $*P < 0.05$ shows significance of the effect of treatment. B, Effect of root chilling treatment on percentage of ^{11}C-photosynthate exported by the LL following treatment. $**P < 0.01$ shows significance of the effect of treatment. C, Positron autoradiographs showing the combined effect of root chilling and wound/MeJA treatment on the distribution of ^{11}C-photosynthates among aboveground tissues. Control (left) depicts the effect of root chill treatment only. Blue coloration indicates low radioactivity with increasing radioactivity for yellow and then red. White arrows indicate two young leaves targeted for treatment during experiments. Leaves and the remaining LL petiole (located between two targeted young leaves) were cut away from the rosette to avoid overlap. D, Results of ImageGauge analyses of autoradiographs. Percentage values were calculated as radioactivity in YO leaves relative to total rosette activity, not including radioactivity in the LL petiole ($*P = 0.0257$). All bars are mean ± SE.

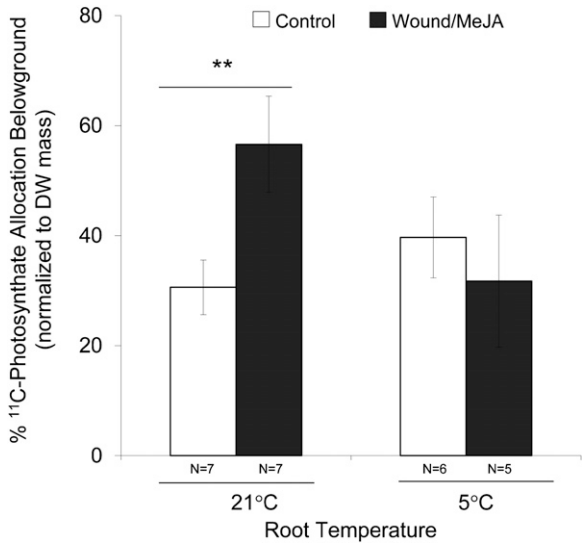

Figure 4. Combined effect of root chilling and wound/MeJA treatment on the percentage of ^{11}C-photosynthates allocated to Arabidopsis roots. All bars are mean ± SE. Asterisks show significant effect of wound/MeJA treatment ($P = 0.0235$). DW, Dry weight.

(Supplemental Fig. S9A; $P = 0.02$); the accumulation of ^{11}C-Suc and ^{11}C-Fru was significantly suppressed (Supplemental Fig. S9, B and D; ^{11}C-Suc, $P = 0.0331$; ^{11}C-Fru, $P = 0.0253$). In contrast, chilling had no effect on total or individually assayed ^{11}C-sugars in YO leaves (Supplemental Fig. S10); total ^{11}C-sugars increased slightly following this treatment, but means were no different from 21°C controls (Supplemental Fig. S10A; $P = 0.161$). Wound/MeJA had no effect on total or individually assayed ^{11}C-sugars in the LL or YO leaves of plants with chilled roots (Supplemental Figs. S9 and S10).

Effect of Root Chilling on Metabolic Partitioning of ^{11}C into Organic Acids

Root chilling marginally suppressed the production of total ^{11}C-phenolic acids (Supplemental Fig. S11) and ^{11}C-cinnamic acid in the LL (Fig. 6A; $P = 0.0658$) and YO leaves (Fig. 6B; $P = 0.009$). Interestingly, the production of these compounds in the LL was suppressed even more following wound/MeJA treatment (Fig. 6A; Supplemental Fig. S11A).

DISCUSSION

Our results demonstrate that the combination of wounding and MeJA applied as a treatment to young leaves induces rapid temporal changes in the physiological and metabolic pathways in Arabidopsis as reflected by changes in allocation and metabolic partitioning of newly acquired ^{11}C resources. Despite the advantages of this short-lived isotope, few studies, to our knowledge, have examined both temporal changes and the role of roots in shaping whole-plant ^{11}C distribution and its use in primary and secondary metabolites following simulated herbivory.

Newly Acquired ^{11}C Is Exported to Treated Young Leaves: Evidence for the Induced Sink Strength Model of Defense

Within 2 h of wound/MeJA treatment to two young leaves, we observed elevations in both $^{11}CO_2$ fixed and ^{11}C-photosynthate exported from the untreated LL.

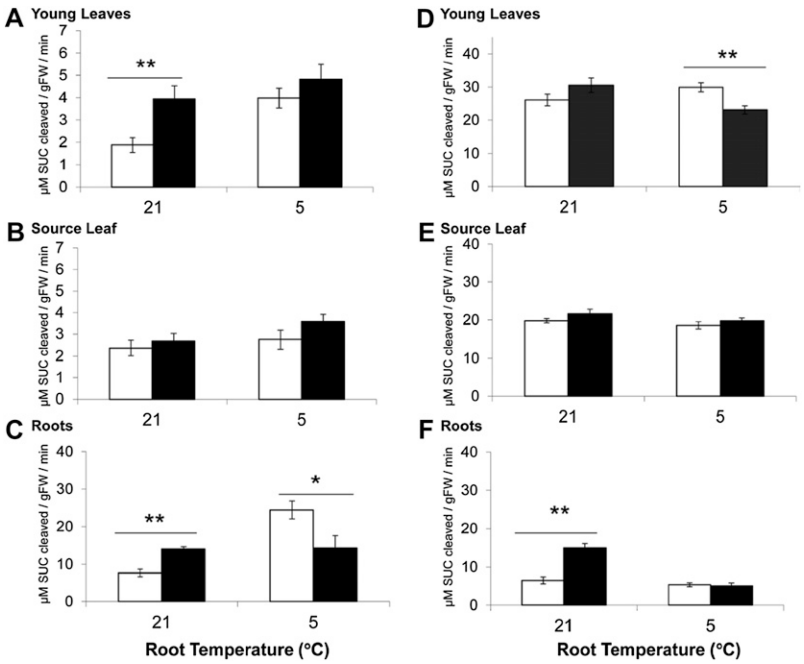

Figure 5. Combined effect of root chilling and wound/MeJA treatment on invertase activities in leaves and roots. Changes made by wound/MeJA treatment (black bars) are compared with representative control groups (white bars). CWI (A–C) and SOL (D–F) invertase activities in two young leaves (A and D), their respective source leaf (B and E), and root tissue (C and F) were measured at 2 h following wound/MeJA treatment. All bars are mean ± SE. $^{**}P < 0.0001$ and $^{*}P < 0.05$ show significant effect of wound/MeJA treatment. $n = 10$ to 12/treatment group. FW, Fresh weight.

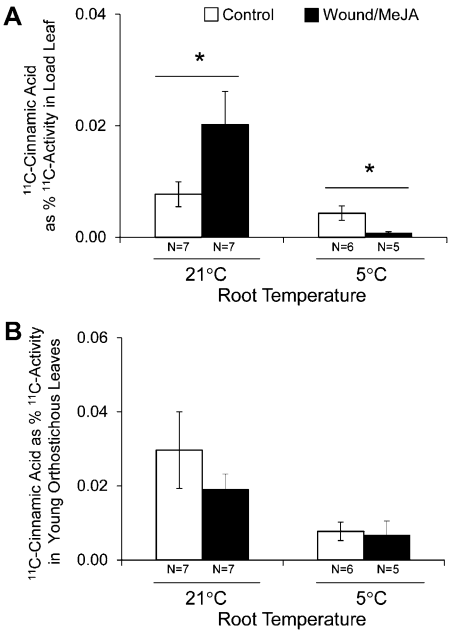

Figure 6. Combined effect of root chilling and wound/MeJA treatment on partitioning of recently fixed $^{11}CO_2$ into ^{11}C-cinnamic acid in the LL (A) and two YO (B) leaves. Percentage values were calculated as amount radioactivity present in all ^{11}C-phenolics relative to total radioactivity in these leaves. $*P < 0.05$ shows significant effect of wound/MeJA treatment. All bars are mean ± SE.

These findings are in agreement with studies observing increases in photosynthetic activity in unattacked leaves following damage by defoliating herbivores (Nowak and Caldwell, 1984; Welter, 1989; Schwachtje and Baldwin, 2008), a phenomenon thought to reflect a strategy to partially compensate for defoliation during herbivore attack (Detling and Painter, 1983; Nowak and Caldwell, 1984). Jasmonates are well known to decrease the photosynthetic apparatus locally (Creelman and Mullet, 1997; Beltrano et al., 1998; Hristova and Popova, 2002; Izaguirre et al., 2003; Schwachtje and Baldwin, 2008; Gómez et al., 2010). Thus, it is possible that other factors, such as local suppression of photosynthesis and the induction of invertases at the wound sites, create a strong sink for resources in these young leaves and subsequently induce carbon fixation and export by source leaves. This would be consistent with the current hypothesis that young leaves induced their sink strength for resources following wound/MeJA treatment (Arnold et al., 2004).

The increase in ^{11}C-photosynthate exported from the LL observed in our study is also consistent with other physiological studies using ^{11}C as a tracer to examine changes in carbon dynamics following jasmonate treatments (Babst et al., 2005; Gómez et al., 2010; Orians et al., 2011). These studies hypothesize that this response acts primarily as a preventive strategy employed by plants to safeguard valuable resources in distant tissues, away from herbivores. However, we suspect that increased ^{11}C-photosynthate export also reflects changes in source-sink relationships followed by large-scale metabolic reprogramming associated with an increased demand for energy and carbon-based resources necessary for the production of defensive compounds. Although separating where and for what purposes additional carbon and energy are used is difficult, this alternative hypothesis is consistent with previous work in *Populus* spp., where increasing carbon export in unattacked source leaves had a positive effect on the production of secondary defense compounds in young sink leaves (Arnold and Schultz, 2002; Arnold et al., 2004).

Of the tracer remaining in aboveground sink tissues, autoradiography showed preferential allocation of ^{11}C-photosynthate toward YO leaves rapidly (2 h) following wound/MeJA treatment. The accumulation of ^{11}C-photosynthate in damaged YO leaves corresponded with the production of ^{11}C-organic acids, including total ^{11}C-phenolics and ^{11}C-cinnamic acid, as well as the accumulation ^{12}C-phenolics (anthocyanins) by 24 h. This is consistent with previous studies observing the induction of gene transcripts and enzyme activity associated with phenylpropanoid biosynthesis (Richard et al., 2000) and the accumulation of phenolic metabolites following treatment with jasmonates and insect herbivores (Arnold et al., 2004; Hanik et al., 2010). This pattern is also consistent with previous studies demonstrating that the ability of developing leaves to respond defensively to MeJA, wounding, or insect treatment relies on their capacity to induce their sink strength for carbon-based resources and import carbon from orthostichous source leaves (Arnold and Schultz, 2002; Arnold et al., 2004).

Radioactivity present in monosaccharide sugars ^{11}C-Glc and ^{11}C-Fru in the LL fluctuated dramatically across the time course examined; we observed a significant decrease in these sugars from 2 to 6 h, followed by a return to baseline levels by 24 h. In our study, the 2 and 24 h time points fell at the beginning of the photoperiod (early morning), while 6 h plants were harvested in late afternoon. Thus, it is possible that the normal fluctuations in primary metabolite pools helped to accelerate the responses we observed at 2 and 24 h in young leaves. According to a recent study in *Nicotiana attenuata*, metabolites often have pronounced diurnal rhythms to their production, which may play an important role in shaping a plant's responses to herbivore attack (Kim et al., 2011). Given these findings, and the 8-h photoperiod in which the plants for our studies were grown, it is possible that induced responses to wound/MeJA were waning by our 6 h time point, which would provide support for an underlying mechanism of circadian clock-enhanced defense in Arabidopsis (Goodspeed et al., 2012).

To our surprise, the accumulation of radiolabel in treated YO leaves at 2 h did not appear to be primarily driven by CWI or SOL invertase activity; in our time-course experiment, invertases were not significantly induced until 24 h post treatment. This result suggests that induced sink strength in young, treated leaves

may also be facilitated by the push of photosynthate from source leaves, rather than these leaves locally pulling for carbon. This idea is supported by the increase in ^{11}C-photosynthate export from the LL observed 2 h following wound/MeJA elicitation. We also found that in *suc2-1* mutant plants, compromised in their ability to load photosynthate at source leaves, reactive phenolics, including anthocyanins, were not induced as dramatically in young, treated leaves, compared with their wild-type counterparts. This result provides additional support for the hypothesis that phloem-borne signals and/or resources transported from source leaves to damaged sinks is required for the induction of secondary metabolites in young leaves.

Wound/MeJA Treatment Induces Rapid Allocation of ^{11}C-Photosynthate toward Roots

In our study, we found both allocation of newly acquired ^{11}C-resources to wounded, young leaves and sequestration to roots to occur rapidly and simultaneously. These findings contradict our original hypothesis regarding the timeline of carbon movement following damage but are nevertheless consistent with previous work showing that enhanced movement of carbon from labeled source leaves into damaged, young leaves is coincident with transport belowground (Frost and Hunter, 2008).

The rapid movement of resources belowground observed in our study is also in agreement with the growing body of literature demonstrating the transport of resources away from damaged sites and into roots and storage organs within hours following herbivory (Babst et al., 2005, 2008; Schwachtje et al., 2006; Newingham et al., 2007; Frost and Hunter, 2008; Kaplan et al., 2008; Gómez et al., 2010, 2012). The adaptive value of sequestering resources to roots has been explained in various ways, including storing reserves for later regrowth to tolerate herbivory (Schwachtje et al., 2006). However, concrete evidence linking this response to tolerance remains elusive, and to date, no study exists showing the incorporation of these bunkered resources into regrown tissues.

In our study, rapid transport of ^{11}C-photosynthate belowground corresponded to the induction of CWI and SOL invertases in roots following wound/MeJA treatment aboveground. These results suggest that carbon allocation to roots may be driven by a combination of mechanisms, including invertase-induced sink strength in the roots and increases in fixation and loading of ^{11}C in the source leaves. By 24 h post-treatment, resource allocation to roots diminished, while allocation to the young, treated leaves remained elevated relative to controls. This pattern may reflect increases in invertase at later time points in the young leaves and/or changes made to whole-plant allocation patterns; overall, we found more radiolabel accumulating in all leaves by 24 h after treatment (see autoradiographs). It is also possible that ^{11}C-phenolics accumulating in the LL are being transported in their intact form toward YO leaves, which may explain the increase in radiolabel in YO leaves following wound/MeJA treatment.

Root Chilling Abolishes Wound/MeJA-Induced Carbon Dynamics in Arabidopsis

To determine whether roots are a dominant sink for resources in Arabidopsis and compete with aboveground tissues for carbon resources, we used root chilling treatments to disrupt carbon flow between above- and belowground tissues. In contrast with our expectations, root chilling did not decrease root invertase activity or disrupt constitutive whole-plant allocation patterns of ^{11}C-photosynthate. In fact, root chilling significantly induced invertase activity in roots. Despite the fact that we observed no changes in newly acquired carbon allocation patterns following root chilling, it is still possible that this treatment was capable of influencing turnover in ^{12}C-sugars pools in roots (e.g. by increasing root exudation) to drive the observed changes in invertases.

Compared with plants with roots at ambient temperature, plants with chilled roots failed to allocate newly acquired carbon resources belowground or transport carbon toward wounded YO leaves following wound/MeJA. The increase in $^{11}CO_2$ fixed and in ^{11}C-photosynthate exported by the LL following wound/MeJA treatment was also eliminated when roots were chilled, suggesting that roots may play an integral role in controlling how plants respond defensively aboveground in Arabidopsis. Placement of plants under low temperature conditions is known to trigger the biosynthesis of the plant hormone abscisic acid, which not only activates genes induced by drought, salt, and cold stress, but is also known to down-regulate genes related to defense (Anderson et al., 2004). In Arabidopsis, Suc synthesis genes, including those encoding Suc phosphate synthase, are induced by low temperature (Usadel et al., 2008), while transcript levels of several members of the invertase family, as well as overall levels of invertase activity, are suppressed. In contrast, other plant species (e.g. wheat [*Triticum aestivum*], potato [*Solanum tuberosum*], and tomato [*Solanum lycopersicum*]) exhibit the up-regulation of invertase following decreases in temperature (Artuso et al., 2000; Vargas et al., 2007; Janská et al., 2010). Together, these findings underline a key role for carbohydrate metabolism under low temperature conditions (Kaplan et al., 2007; Guy et al., 2008). In our study, while ^{11}C-photosynthate appeared to accumulate in excess in the LL following root chilling treatment, this accumulation did not correspond to increases in any of the primary or secondary metabolites measured. This finding suggests that newly acquired carbon may be partitioned to other functions such as long-term storage pools (starch) or carbon skeletons for compounds such as amino acids.

Resolving the Bunkering Conundrum

This work reveals that many induced plant defense responses are rapid, complex, and transient in nature. Our results indicate that Arabidopsis simultaneously transports newly acquired carbon belowground even as it increases transport toward damaged sites. These responses likely reflect mechanisms to tolerate herbivory and to up-regulate secondary defense chemistry at sites of attack, both of which may be driven in part by increased fixation and export by source leaves and systemic induction of invertases. Whole-plant tracer dynamics corresponded to the local induction of phenolic compounds in the plant's defense chemistry production. However, when root chilling treatments were combined with wound/MeJA elicitation, aboveground tissues were unresponsive, suggesting that roots play a crucial role in shaping how plants respond defensively aboveground through an integrated communication network.

Growing root apices are well known to sense changes in their local abiotic and biotic environments and respond to them accordingly with changes to growth (Monshausen and Gilroy, 2009). Therefore it is possible that roots may direct the signaling that causes a rapid shuttling of resources to roots following herbivory and subsequent return to leaves to cue systemic defenses aboveground at later time points. Current evidence in maize (*Zea mays*) and tobacco (*Nicotiana tabacum*), as well as studies using Arabidopsis, suggest that roots are capable of synthesizing many secondary metabolites, serve as dynamic storage organs, and may also relay information regarding changes to their abiotic and biotic environment in a shoot-root-shoot loop in plant defense systems (Erb et al., 2009). Thus, future studies using short-lived isotopes with Arabidopsis may elucidate shoot-root and root-shoot signals that underlie carbon resource allocation and metabolic partitioning to plant defense chemistry to provide a better understanding of source-sink interactions in relation to plant defenses and their importance for pest resistance in other plant species.

MATERIALS AND METHODS

Plant Material

For $^{11}CO_2$ labeling studies, Arabidopsis (*Arabidopsis thaliana*; Columba-0) seeds were germinated on Rockwool cylinders soaked in Hoagland solution (Hoagland modified basal salt mixture; Phyto Technology Laboratories) at 21°C and 62% relative humidity with an 8/16-h (180 μmol m^{-2} s^{-1}) photoperiod. Two-week-old seedlings were transplanted into individual pots containing a 4:1 mixture of sand:zeolite (Sakrete multipurpose sand, Sakrete of North America; Zeopro zeolite mix, Zeoponix). Zeolite was soaked in Hoagland solution to wet, then mixed by hand with sand. For large-scale experiments measuring ^{12}C-phenolics and invertase, Arabidopsis plants were germinated on agar plates containing Murashige and Skoog media and transplanted at 2 weeks of age to pots containing a 4:1 mixture of sand:zeolite supplemented with 1.8 kg of Osmocote slow-release fertilizer (The Scotts Company) per cubic meter of soil. Plants were maintained under the same growth conditions described above. In all experiments, plants were approximately 4 weeks postgermination and contained 21 ± 2 leaves.

Treatments and Experimental Design

MeJA is a well-known plant defense elicitor that up-regulates the production of defensive compounds against herbivores and is often used to mimic the effect of herbivory (Gundlach et al., 1992; McConn et al., 1997; Baldwin and Hamilton, 2000). Young leaves are frequently preferred by herbivores as a result of their higher nutrient content and reduced toughness (McKey, 1974; Coley et al., 2006). Thus, we simulated herbivore damage on two young leaves (Supplemental Fig. S12, shown in purple) sharing direct vascular connections (orthostichous) to a mature source leaf (approximately ninth leaf; yellow in Supplemental Fig. S12) by damaging leaves perpendicular to the midvein with a pattern wheel and applying 20 μL of an aqueous dilution of MeJA (115 μM; Sigma-Aldrich) to each leaf. Leaves were assigned numbers by counting down from the apex, with leaf 1 designated as the youngest leaf that was at least 5 mm in length.

To capture temporal changes in resource allocation in response to simulated herbivory, the LL was pulse labeled with $^{11}CO_2$ at 1, 5, and 23 h following wound and MeJA treatment. Plants were allowed to incubate with the tracer for 1 h, during which ^{11}C-uptake and export by the LL were measured. Partitioning of ^{11}C-photosynthate into primary and secondary metabolites was measured in the LL and two YO leaves immediately following this incubation period.

During the labeling procedure, the emission of radioactivity by the ^{11}C-labeled plants required special containment, limiting analysis to a single plant per time point. Labeling experiments were repeated to average approximately six plants per time point (2, 6, and 24 h). Control plants were left untreated but were placed in the tracer administration box during the time course. The time-course experiment was repeated in the absence of $^{11}CO_2$ labeling to assess changes in CWI and SOL invertase activity as well the accumulation ^{12}C-phenolics.

suc2-1 Study

Arabidopsis loads phloem from source leaves into the companion cell/sieve element (CC/SE) complex apoplastically (i.e. CC/SE complexes are isolated from the symplast of the surrounding cells by a shortage of plasmodesmatal connections; Haritatos et al., 2000). As a result, photosynthate and presumably other phloem-transported metabolites must first be exported from mesophyll cells into the apoplast and then loaded into the CC/SE complex by an energy-dependent transport system (DeWitt and Sussman, 1995). In Arabidopsis, this process is primarily driven by Suc transporters (Stadler and Sauer, 1996). Source leaves of the Arabidopsis T-DNA insertion line in AtSUC2 (*suc2-1*), a phloem-specific Suc transporter gene (Truernit and Sauer, 1995; Stadler and Sauer, 1996), contain excess starch and fail to transport radiolabeled sugar efficiently to roots and inflorescences (Gottwald et al., 2000). This mutant not only provides strong evidence in support of apoplastic loading as the primary method for initiating long-distance transport in Arabidopsis, but offers a unique opportunity to test whether phloem loading at the source and subsequent transport to sink tissues is required for the induction of secondary chemistry.

Mutant seeds (The Arabidopsis Information Resource) were grown side by side with wild-type (Wassilewskija-2) and heterozygous siblings for controlled comparisons. Seeds were surface sterilized and germinated on agar plants containing Murashige and Skoog media. Homozygote seedlings were screened for experiments by their characteristic phenotype following germination on media without Suc; mutants are visibly smaller than the wild type, with very short primary roots and translucent cotyledons (Gottwald et al., 2000). Approximately 2 weeks post germination, homozygous plants were rescued on fresh media containing 1% supplemental Suc. Plants that were successfully rescued were transplanted to pots containing Metro-Mix 200 soil (Sun Gro Horticulture) supplemented with 1.8 kg of Osmocote slow-release fertilizer (The Scotts Company) per cubic meter of soil. Homozygous and wild-type plants were approximately 6 weeks post germination at the time of the experiment. Homozygous plants were generally slower to development than their wild-type siblings and so were smaller at the time of the experiment (contained approximately four less leaves).

We used a factorial design comprised of 32 plants randomly assigned to one of the following treatments: (1) control, wild type; (2) wound/MeJA, wild type; (3) control, *suc2-1*; and (4) wound/MeJA, *suc2-1*. As in the labeling studies, MeJA treatments were applied to sink leaves 1 and 4. Damaged sink leaves and leaves from the apex of the rosette were harvested in liquid nitrogen 48 h later for phenolic measurements.

Root Chilling Treatment

In Arabidopsis, roots are normally a significant sink, drawing resources to a greater extent than aboveground tissues (Ferrieri et al., 2012). To determine whether roots effectively compete with defense-induced primary and secondary metabolites for carbon resources, root chilling treatments were used to disrupt carbon flow between above- and belowground tissues. The inhibition of phloem transport by chilling roots has been particularly well investigated (Geiger, 1969; Minchin et al., 1983, 1994; Poiré et al., 2010; Thorpe et al., 2010) and can be accomplished with minor drops in temperature (Minchin and Thorpe, 1983). In our experiments, individual plants were seated inside an epoxy-sealed pot affixed in an ice water bath (0°C; Supplemental Fig. S13A). Plants incubated for 1 h (the time needed for soil to reach 5°C; Supplemental Fig. S13B) before wound/MeJA treatments occurred and remained in the ice bath during the 1-h incubation following treatment as well as for the 1-h incubation with the tracer. Control plants were left untreated but were placed in the root chilling station for the same duration as wound/MeJA-treated plants. The experiment was repeated at a larger scale (root chilling array; Supplemental Fig. S13C) to assess changes in CWI and SOL invertase activities as well as total ^{12}C-phenolics in two young developing leaves, their source leaf, and roots.

Radiotracer Production

^{11}C, a short-lived radioisotope ($t_{1/2}$ = 20.4 min) was used to examine the uptake, transport, and allocation of ^{11}C-photosynthates. ^{11}C was made by irradiating a nitrogen gas (N_2) target with 17-MeV protons from the TR-19 cyclotron (Ebco Industries) at Brookhaven National Laboratory to induce the $^{14}N(p,\alpha)^{11}C$ nuclear transformation (Ferrieri and Wolf, 1983).

Radiotracer Administration

Carbon dioxide labeled with ^{11}C was captured on a molecular sieve (4 Å), desorbed, and quickly released into an air stream at 200 mL min^{-1} as a discrete pulse to the targeted LL (leaf 9) fixed inside a 5 × 10-cm airtight cell maintained at 21°C and fitted with red/blue light-emitting diodes (290 μmol m^{-2} s^{-1}) to ensure a steady level of fixation (Supplemental Fig. S14A). The LL was pulse fed $^{11}CO_2$ for 1 min, then chased with normal air for the duration of the 1-h incubation with the tracer. A PIN diode radiation detector (Carroll Ramsey Associates) affixed to the bottom of the leaf cell enabled continuous measurement of radioactivity levels within the cell.

Measuring Whole-Plant Carbon Dynamics

$^{11}CO_2$ fixation and leaf export of ^{11}C-photosyntate were measured for each plant and compared with controls. The difference between the amount of radioactivity registered in the pulse height and residual radioactivity after the pulse had passed through the cell was a reflection of leaf tracer fixation (Supplemental Fig. S14B). Export of the radiotracer from the LL was calculated as the percentage of activity that left the labeled leaf after 1 h (Supplemental Fig. S14B). All calculations were performed using activity decay-corrected values to take into account the decay of the radioisotope over the time course of the experiment. Leaves and roots were placed into separate scintillation vials and counted using a γ counter (Picker). All radioactivity measurements were decay corrected to a standard zero time of each study to quantify allocation of ^{11}C-photosynthate above- and belowground.

Positron Autoradiography

To determine the distribution of ^{11}C-photosynthates throughout aboveground tissues, we used phosphor plate imaging of positron emissions (Fuji 2500 imager). One hour after pulse labeling of the targeted LL, treated and untreated rosette leaves (not including the LL) were excised at the soil line and exposed for 15 min on a phosphor imaging plate (Fuji BAS2500). The remaining LL petiole, YO leaves (wound/MeJA or corresponding control leaves of same leaf number) and untreated rosette leaves were cut away from the central stem of the rosette to minimize leaf overlap and aid in the image analysis. In each image, background radiation was subtracted from the number of photostimulated luminescence units determined in a defined region of interest using Science Laboratory 99 Image Gauge software (Fuji Photo Film). Radioactivity present specifically in two targeted YO leaves was related to total radioactivity present in the rest of the rosette, excluding the remaining LL petiole; this tissue was commonly oversaturated in autoradiographs and was therefore eliminated from the image analyses.

Tissue Extraction and Analysis of Radioactive Soluble Sugars

One hour after tracer administration, the portion of LL tissue enclosed in the cell (exposed to $^{11}CO_2$) was cut away from the remaining leaf, weighed, and flash frozen in a microtube immersed in liquid nitrogen. Frozen tissue was ground to a fine powder using a pellet pestle tissue grinder (Kontes disposable pellet pestles; 1.5 mL) and extracted in 1 mL of acidified methanol (1% [v/v] HCl in methanol) for 10 min at 90°C. The extract was separated by pipette and filtered through a 0.22-μM filter (Millipore). Immediately following autoradiography of aboveground tissues, two treated YO leaves (or equivalent controls) were weighed together and subjected to the same extraction procedure. ^{11}C-soluble sugars were separated from final extracts by thin-layer chromatography by spotting 2 μL onto silica NH_2 HP TLC plates (Sorbent Technologies; B. Babst, personal communication). Plates were developed in 4:1 acetonitrile:water, air-dried, and exposed for 30 min on a phosphor imaging plate (Fuji BAS2500). The incorporation of ^{11}C into soluble sugars was quantified using ImageGauge software and related to total extract radioactivity determined by γ counting (Picker). Individual soluble sugars (Suc, Glc, Fru, raffinose, and maltose) were identified by comparing retention factor values of sugars from sample extracts to those of authentic ^{12}C-sugar standards. ^{12}C-sugars were visualized colorimetrically by dipping plates into a derivatization reagent of 5% 1-naphthol in concentrated H_2SO_4.

Analysis of ^{11}C-Organic Acids

Following ^{11}C-soluble sugar analysis, remaining acidified methanol extracts were hydrolyzed by adding 2 μL KOH (6 N) and heating for 10 min at 90°C. Extracts were transferred by glass pipette to NH_2 sep-pak cartridges (Waters) and washed with 2 mL each of water, isopropyl alcohol:ethyl acetate (1:1 [v/v]), ether, and acetonitrile. Elute from each wash was collected and counted using a γ counter (Picker). Phenolic acids were eluted in 2 mL 2% formic acid in methanol, heated to dryness, and reconstituted in 50 μL methanol.

The extent to which ^{11}C-photosynthates were incorporated into Arabidopsis organic acids was determined in 5 μL of this final methanol extract using two-dimensional thin-layer chromatography (Chapple et al., 1992). Silica NH_2 HP TLC plates (Sorbent Technologies) were run first in petroleum ether:ethyl acetate:methanol:acetic acid (10:10:1:0.2) and then in toluene: glacial acetic acid (2:1) saturated with water. Plates were air-dried and immediately exposed for a minimum of 3 h on phosphor plates. The percentage of radioactivity present in ^{11}C-organic acid compounds in each extract was quantified using ImageGauge software and related to total extract radioactivity.

^{11}C-labeled organic acids (total phenolics and cinnamic acid) were identified by comparing retention factor values to those of phenolics commonly found in Arabidopsis. ^{12}C-phenolic standards were visualized under longwave UV light (365 nm) by their characteristic fluorescence under shortwave UV light (254 nm) by their characteristic absorbance (Chapple et al., 1992).

^{12}C-Phenolics Extraction and Quantification

Individual leaves and roots were freeze-dried (4 to 6 mg dry weight) and ground in a Talboys high-throughput homogenizer (Troemner). Phenolics were extracted overnight in 200 μL of 1% (v/v) HCl in methanol at 4°C. An additional extraction with 250 μL distilled water and 500 μL of chloroform was used to remove chlorophyll from all samples (Choi et al., 2009). Samples were vortexed and centrifuged for 3 min at 3000*g*. Relative anthocyanin levels in the aqueous phase were determined spectrophotometrically by measuring A_{530}. Total flavonoid compounds were also estimated in the same extracts at absorbance 320 nm (Fukumoto and Mazza, 2000; Shao et al., 2008). The concentration of total reactive phenolics present in leaf extracts was determined using the Folin-Denis assay. Standard curves were developed using chlorogenic and gallic acids as well as standards purified from each treatment group (Appel et al., 2001).

Invertase Activity Assay

The activity of CWI and SOL invertases were measured in leaves and roots according to previously described methods (Arnold and Schultz, 2002).

Briefly, proteins were extracted from fresh frozen and ground tissue in 6 volumes (volume g fresh weight^{-1}) of MES buffer (pH 7.0) containing 5 mM EDTA, 5% (w/v) polyvinylpolypyrrolidone, Ser proteinase inhibitor DTT (20 mM), and benzamidine (2.5 mM). Samples were sonicated on ice for 30 min and SOL fractions were transferred to new tubes. Remaining pellets (containing CWI) were washed three times with deionized water. CWI and SOL fractions were suspended in assay buffer (0.05 M acetic acid, 0.05 M MES, 0.01 M Trizma, and 3 mM sodium azide, pH 4.5) containing 200 mM Suc. Suc cleaved by CWI and SOL fractions was assayed by the generation of Glc monomers during a 15-min incubation period (pH 4.5, 37°C) and quantified (μM Suc cleaved g fresh weight^{-1} min^{-1}) colorimetrically by their A_{562}.

Statistical Analyses

One-way and two-way ANOVAs were performed, followed by Tukey honestly significant difference post-hoc comparisons. Normality and equality of variance were verified using Kolmogorov-Smirnov and Levene's tests, respectively. All data were analyzed using SAS statistical package version 9.3 (SAS Institute).

Supplemental Data

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

Supplemental Figure S1. Percentage of total ^{11}C-photosynthates allocated to Arabidopsis roots.

Supplemental Figure S2. ^{11}C-soluble sugar profiles in the load leaf.

Supplemental Figure S3. ^{11}C-soluble sugar profiles in two young treated leaves.

Supplemental Figure S4. Partitioning of recently fixed $^{11}CO_2$ into total phenolic acids.

Supplemental Figure S5. Partitioning of recently fixed $^{11}CO_2$ into cinnamic acid.

Supplemental Figure S6. Effect of wound/MeJA treatment on Folin-Denis reactive phenolic compounds.

Supplemental Figure S7. Effect of wound/MeJA treatment on anthocyanin accumulation in two young treated leaves and systemic leaves of *suc2-1* and wild-type (Ws-2) plants.

Supplemental Figure S8. Effect of wound/MeJA treatment on invertase activities in leaves and roots.

Supplemental Figure S9. Combined effect of root chilling and wound/MeJA treatments on ^{11}C-soluble sugars in the load leaf.

Supplemental Figure S10. Combined effect of root chilling and wound/MeJA treatments on ^{11}C-soluble sugars in two young treated leaves.

Supplemental Figure S11. Combined effect of root chilling and wound/MeJA treatments on the partitioning of recently fixed $^{11}CO_2$ into total phenolic acids.

Supplemental Figure S12. Arabidopsis plant illustrating the load leaf and YO, treated leaves.

Supplemental Figure S13. Experimental setup for root chilling studies.

Supplemental Figure S14. Experimental setup for pulse labeling with $^{11}CO_2$.

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