Dynamic Precision Phenotyping Reveals Mechanism of Crop Tolerance to Root Herbivory[OPEN]

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The western corn rootworm (WCR; Diabrotica virgifera virgifera LeConte) is a major pest of maize (Zea mays) that is well adapted to most crop management strategies. Breeding for tolerance is a promising alternative to combat WCR but is currently constrained by a lack of physiological understanding and phenotyping tools. We developed dynamic precision phenotyping approaches using 13C with positron emission tomography, root autoradiography, and radiometabolite flux analysis to understand maize tolerance to WCR. Our results reveal that WCR attack induces specific patterns of lateral root growth that are associated with a shift in auxin biosynthesis from indole-3-pyruvic acid to indole-3-acetonitrile. WCR attack also increases transport of newly synthesized amino acids to the roots, including the accumulation of Gln. Finally, the regrowth zones of WCR-attacked roots show an increase in Gln biosynthesis from indole-3-pyruvic acid to indole-3-acetonitrile. WCR attack also increases transport of newly synthesized amino acids to the roots, including the accumulation of Gln. Finally, the regrowth zones of WCR-attacked roots show an increase in Gln turnover, which strongly correlates with the induction of indole-3-acetonitrile-dependent auxin biosynthesis. In summary, our findings identify local changes in the auxin biosynthesis flux network as a promising marker for induced WCR tolerance.

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rootworm with resistance to crop rotation can be found in parts of Illinois, Indiana, and parts of bordering states (Gray et al., 2009; Levine et al., 2002).

More recently, D. v. virgifera resistance to deployed genetically modified organisms has been reported. First introduced into the market to target this pest back in 2003, genetically altered Bt-maize expressing one or more proteins from the soil bacteria Bacillus thuringiensis provided enhanced plant defenses to larval feeding. When a vulnerable insect ate the Bt-containing plant, the protein became activated in its gut, forming a toxin that paralyzed the digestive system and caused it to stop feeding. Unfortunately, resistance began to show within three generations of selection (Meihls et al., 2008).

An alternative strategy to reduce the negative impact of D. v. virgifera attack without triggering counter adaptations in the pest is plant tolerance, which relies on a plant’s capacity to maintain growth and yield even in the presence of substantial damage. While D. v. virgifera-tolerant maize germplasms exhibiting slight to moderate tolerances to D. v. virgifera have been reported (Flint-Garcia et al., 2009), more effective lines are needed. Unfortunately, we know very little about the underlying mechanisms for crop tolerance. Over the years, one resounding message has been that the physiological processes affected by herbivory should be better characterized before breeding tools can be leveraged in a rational way to generate improved varieties that maintain high yields under herbivore pressure (Riedell, 1990). Rational decision making in the breeding selection process requires rigorous phenotyping; however, present phenotyping tools tell us little about the plasticity of root systems, especially when it comes to understanding mechanisms for crop tolerance to attack belowground. It was recently suggested that the timing for allocation of newly fixed carbon resources as soluble sugars between leaves, stalks, and root systems, and their coordination with mobilization of other resources including amino acids, may play significant roles in determining the ability of maize plants to survive an attack by D. v. virgifera (Orians et al., 2011; Robert et al., 2014).

In this work, our systematic evaluation of the physiological, metabolic, and genetic basis for root regrowth as a tolerance trait sheds new light on the regulation of the growth hormone auxin (indole-3-acetic acid [IAA]) and its role in this process. Radioactive decay of $^{11}$C ($\beta^+$ emitter, $t_{1/2} = 20.4$ min), dynamic whole-plant positron emission tomography, root autoradiography, and radiometabolite flux analyses allowed us to map the transport, allocation and metabolism of carbon and nitrogen resources against genetic and radiolabeled biochemical markers including $[^{11}$C]IAA, $[^{11}$C]indole, $[^{11}$C]indole-3-acetonitrile ($[^{11}$C]IAN), $[^{11}$C]indole-3-acetamide ($[^{11}$C]IAM), and $[^{11}$C]Gln (Supplemental Fig. S2). Taken together, these tools enabled us for the first time, to our knowledge, to rigorously map out the auxin biosynthesis flux network at regional tissue levels and in turn provide new insights on auxin regulation and its coordination with the availability of a key amino acid, L-Gln. The developed phenotyping tools can now be employed for the rapid identification and selection of D. v. virgifera-tolerant maize germplasm.

RESULTS

Root Herbivore Attack Induces Asymmetric Formation of Lateral Root Primordia

In the field, D. v. virgifera-tolerant maize plants often display a pronounced increase in lateral root growth (Fig. 1A). Initial imaging studies set out to determine whether this trait can be characterized in a laboratory setting using 3-week-old maize seedlings at their V2 stage. Seedlings at this stage of development have mature nodal or crown roots showing developing lateral root primordia (LRP) and lateral root structures (Fig. 1B). The high degree of LRP spatial symmetry (Fig. 1B) found in healthy nodal roots is lost upon larva feeding (Fig. 1C). After leaf administration of $^{13}$CO$_2$, autoradiographic imaging of the gross $[^{11}$C]photosynthate distribution reveals that in healthy undamaged roots, the lateral root meristems and LRP have a high accumulation of $^{11}$C radioactivity, indicating that these sites are strong sinks for resources (Fig. 1D). Furthermore, the spatial patterning of radioactivity in the healthy root image was highly symmetrical. In contrast to this, roots mildly damaged by herbivore feeding show a nonsymmetrical patterning of $[^{11}$C]photosynthate in the LRP (Fig. 1E). These LRP sites of high sink strength (and presumably high metabolic activity) coincide with high specific binding of $[^{11}$C]IAA both in healthy roots (Fig. 1F) and in mildly damaged roots (Fig. 1G) suggesting a strong correlation between auxin signaling, cellular metabolism, and compensatory growth as a form of herbivore tolerance.

Compensatory Growth Is Associated with Distinct Shifts in Auxin Biosynthesis and Transport

Root growth is in part determined by the hormone auxin. We therefore conducted a series of experiments to understand whether the observed regrowth phenotype is accompanied by changes in auxin biosynthesis and its distribution. Endogenous auxin (IAA) concentrations in nodal roots were seen to increase significantly (by 45%) as a function of root herbivore attack reaching levels of 0.051 ± 0.012 ng mg FW$^{-1}$ (Supplemental Fig. S3). Even so, this result says little about auxin biosynthesis and its regulation. IAA can be traced back to chorismic acid, which is biosynthesized via the shikimate pathway within the cell chloroplast (Fig. 2A). Chorismic acid undergoes rapid amination to anthranilic acid via the action of L-Gln. Anthranilic acid is then transformed through several steps into different indole compounds and eventually into key aromatic amino acids including L-Trp, Phe, and Tyr that are essential for primary and secondary metabolism in plants (Maeda and Dudareva, 2012).

Two major pathways for IAA biosynthesis have been postulated in higher plants (Fig. 2A; McSteen, 2010; Normanly, 2010; Mashiguchi et al., 2011; Won et al., 2013).
2011; Ljung, 2013; Wang et al., 2015): a Trp-dependent route including (1) the indole-3-acetaldoxime (IAOx) pathway, (2) the IAM pathway, (3) the indole-3-pyruvic acid (IPyA) pathway, and (4) the tryptamine (TAM) pathway, as well as a Trp-independent route branching either from indole-3-glycerol phosphate or indole has also been suggested (Wright et al., 1991). Because the scientific community has lacked a strong set of biochemical markers enabling rigorous mapping of the IAA biosynthesis flux network, little is known about the importance of individual pathways as a function of environmental stress. We applied several [11C]indole-containing tracers to rigorously map key branch points of this complex network (Reid et al., 2011; Lee et al., 2015a, 2015b). Using [11C]indole, we mapped the Trp-dependent IAA biosynthesis pathways occurring within the upper nodal root tissues 1 h after incubation with tracer for two experimental conditions including (1) unstressed (control) conditions (Fig. 2B) and (2) biotic stress conditions elicited by WCR larvae feeding (Fig. 2C). We observed the following: (1) IAA biosynthesis in unstressed control roots is dominated by the IPyA pathway with no detectable contributions from the IAM pathway; and (2) IAA biosynthesis in herbivore stressed roots is dominated by the IAOx pathway (leading through IAN), and the IAM pathway is now active. These results prompted our further investigation into the kinetics of some of these pathways. Using [11C]IAN, we mapped the temporal profile for the disappearance of this substrate in upper nodal roots with and without WCR attack (Fig. 3A). The kinetic loss of substrate is first order, and the rate of metabolism increases significantly upon treatment. We also mapped the growth of the [11C]IAA product derived from [11C]IAN metabolism (Fig. 3B) and found that the rate of product formation increases significantly with treatment but that the rate of free [11C]IAA utilization (either through conjugation, metabolism, or protein binding) increases significantly as well. Finally, using the [11C]IAM tracer (Fig. 3C), we verified that the IAM pathway is not active in unstressed control roots but becomes active with treatment exhibiting first-order kinetics for substrate metabolism.

Next, we spatially mapped the amount of [11C]IAA that was formed from [11C]IAN after 1 h incubation with tracer (Fig. 4A) across three regions of the nodal root length including the upper proximal portion where lateral roots are visibly growing (zone 1), the midroot section extending down to the elongation zone (zone 2), and the root tip (zone 3). Overall, our findings show a significantly higher rate of metabolic turnover of [11C]IAN in the upper proximal zone than in lower zones, as well as a significant treatment effect in this zone.

Because enzymes catalyzing the conversion of IAOx to IAM are not known, we decided to target the nitrilase (NIT) genes that have been characterized in maize and may play an important role in the conversion of IAM to IAA. NIT1 and NIT2 gene expression patterns were quantified by reverse transcriptase real-time polymerase chain reactions and mapped across the same three root zones as described above (Fig. 4B). NIT1 gene expression...
does not show a statistical difference across the three root zones, nor does root herbivore treatment impose any effect on this gene’s expression level. NIT2 also doesn’t exhibit spatial patterning in gene expression across the root zones for the unstressed controls. However, WCR attack significantly lowers NIT2 expression in zones 2 and 3 relative to controls while possibly slightly increasing expression in zone 1, though this was not considered statistically significant ($P = 0.062$).

Finally, redistribution of [1^1C]IAA via physical transport was measured using dynamic positron emission tomography (PET) imaging (Fig. 5A). Using subcortical microliter injections of tracer in the upper root zone 1, PET imaging enabled us to visualize the movement of [1^1C]IAA over time, as well as enabled us to quantify its transport speed (Fig. 5B). By measuring the time of arrival of tracer across two distinct ROIs, an auxin transport speed of $17.0 \pm 5.7 \text{ mm h}^{-1}$ is observed for the unstressed control state. WCR attack significantly lowers transport by a factor of 2.6. Taken together, these results show that WCR attack leads to a pronounced reorganization of auxin biosynthesis and transport.

### Root Herbivore Attack Increases Amino Acid Transport to the Roots

The regulation of key hormones like auxin that are important for controlling root growth requires a steady
supply of essential carbon and nitrogen resources. To understand how WCR influences resource allocation, we used $^{11}$CO$_2$ administered to source leaves in combination with dynamic PET imaging to trace the physiological and biochemical fate of “new carbon” (as $^{11}$C) at the whole-plant level. We observed that allocation of gross $^{11}$C photosynthates belowground decreases significantly with WCR attack from 38.87% ± 4.23% of fixed $^{11}$CO$_2$ to 25.91% ± 6.64% (Fig. 6A). By contrast, transport speeds of gross $^{11}$C photosynthates more than doubled with herbivore treatment from 1.67 ± 0.24 to 3.43 ± 0.71 mm min$^{-1}$ (Fig. 6B), suggesting that the turnover of photosynthates increases under WCR attack.

Figure 3. A, Logarithmic plot of $[^{11}$C$]$IAN levels after sub cortical injection of tracer into the upper proximal zone of a nodal root ($n = 5$, control; $n = 7$, root herbivory). Results reflect first-order kinetics for $[^{11}$C$]$IAN metabolism and a strong root herbivore treatment effect with increased rate of substrate metabolism. B, Relative percent of $[^{11}$C$]$IAA product derived from $[^{11}$C$]$IAN metabolism shown in A. Herbivory increases the rate of $[^{11}$C$]$IAA formation, as well as increases loss of free $[^{11}$C$]$IAA from metabolism, conjugation, and/or receptor binding. C, Logarithmic plot of $[^{11}$C$]$IAM levels after sub cortical injection of tracer into the upper proximal zone of a nodal root ($n = 4$, control; $n = 4$, root herbivory). Results show that $[^{11}$C$]$IAM is not metabolized in unstressed control roots but is actively metabolized in first-order kinetics after root herbivore treatment.

Figure 4. A, Spatial mapping of $[^{11}$C$]$IAN biosynthesis from $[^{11}$C$]$IAN across three nodal root zones, including the upper proximal region (zone 1), the midroot region including the elongation zone (zone 2), and the root tip (zone 3; $n = 5$, control; $n = 6$, root herbivory). Higher levels of $[^{11}$C$]$IAN metabolism are indicated in zone 1. Root herbivore treatment significantly increased tracer metabolism in this zone but had no effect in zones 2 and 3. B, Ln-fold change in NIT1 and NIT2 gene expression measured across the same three root zones as a function of root herbivore treatment. No change in NIT1 gene expression was noted across any of the zones or as a function of treatment ($n = 3$). On the other hand, NIT2 gene expression was significantly down-regulated with herbivore treatment in zones 2 and 3 but exhibited a slight up-regulation of expression in zone 1 ($n = 4$), though not statistically significant ($P = 0.062$). Where appropriate, statistical significance was denoted as *$P$ ≤ 0.05; **$P$ ≤ 0.01; ***$P$ ≤ 0.001.
Absolute amounts of $^{11}$C-soluble sugars were measured both in the load (source) leaf at 10 and 90 min postadministration of $^{11}$CO$_2$ as well as in the roots at 90 min, for control plants and plants subjected to root herbivore treatment (Fig. 7A). Gross export of $^{11}$C-labeled sugars was calculated from the difference between the load leaf levels of $^{11}$C-labeled sugars measured at the 10 and 90 min time points (SEs propagated). Three general observations are worth noting on the effects of root herbivore treatment on whole-plant sugar status: (1) it increases the flux of new carbon into source-leaf-soluble sugar pools; (2) it increases the export of these sugar resources from those leaves (though not statistically significant); and (3) it decreases their transport belowground to sites of attack. Specifically, gross sugar export from the source load leaf increases from 10.64% ± 2.04% to 13.85% ± 2.66% (based on total $^{11}$CO$_2$ fixed) as a function of treatment, but roots receive less of these sugars, showing a significant decrease from 7.49% ± 0.91% to 4.60% ± 0.32%. The relative profile of the individual sugars is also revealing. Here, we captured profile information for both the load leaf and the roots at 90 min (Fig. 7B). At the source leaf supply side, root herbivore treatment significantly increases the metabolic partitioning of new carbon into $[^{11}]$C-maltose and $[^{11}]$C-Suc, relative to controls, while generating a slightly higher proportion of $^{11}$C-Suc in the roots.

Similarly, absolute amounts of $^{11}$C-labeled amino acids were measured in the load (source) leaf at 10 and 90 min postadministration of $^{11}$CO$_2$, as well as in the roots at 90 min, for control plants and plants subjected root herbivore treatment (Fig. 8A). Like before, the gross export of $^{11}$C-labeled amino acids was calculated from the difference between the levels of $^{11}$C-labeled amino acids measured at the 10 and 90 min time points. Three general observations are worth noting on the effects of WCR attack on whole-plant amino acid status: (1) it significantly increases the flux of new carbon into source leaf amino acid pools; (2) it significantly increases the export of these resources from those source leaves; and (3) it significantly increases their transport belowground to sites of attack. Specifically, gross amino acid export from the source load leaf
increases from 1.41% ± 0.56% to 4.21% ± 0.89% as a function of treatment. Roots receive more of these amino acids where allocations significantly increase from 0.47% ± 0.09% to 1.74% ± 0.28%. Furthermore, studies using the L-[5-11C]Gln tracer, applied to an abraded source leaf tip, show that root herbivore treatment doubles the phloem transport speed of this substrate (Supplemental Fig. S4). Like before, we captured amino acid profile information for the load leaf (Fig. 8B) and the roots (Fig. 8C) at 90 min. On the source leaf supply side, root herbivore treatment significantly increases the metabolic partitioning of new carbon into [11C]Gln, [11C]Glu, and [11C]Asn relative to controls. Roots also show a significantly higher proportion of [11C]Gln with treatment but lower proportions of [11C]Ala and other [11C] amino acids. These results show that WCR attack triggers strong and opposing changes in carbohydrate and amino acid allocation to the roots, which reflects the reorganization of root metabolism and growth upon herbivore infestation.

Changes in Gln and Auxin Metabolism Are Strongly Correlated

Because Gln is important to maintaining auxin homeostasis (Ljung, 2013) through its involvement in the shikimate pathway (Maeda and Dudareva, 2012) providing indole-like substrates in support of auxin biosynthesis, we further investigated the dynamics of its metabolism in roots. Using the L-[5-11C]Gln tracer, we mapped its metabolism to [11C]Glu as a function of root tissue type (upper nodal root versus lower root tip) and root herbivore treatment (Fig. 9A). Results show that Gln metabolism is significantly faster in upper root tissues than in tips. Furthermore, WCR attack increases metabolic turnover of L-[5-11C]Gln, for both of these tissue types. Lastly, by mapping L-[5-11C]Gln metabolism in upper root tissues (Fig. 9B) against [11C]IAA biosynthesis (derived from the [11C]IAN precursor) we see a strong correlation between the two processes and their response to WCR attack, suggesting that Gln accumulation and IAA signaling are tightly coupled in maize roots.

DISCUSSION

Influence of Biotic Stress on Auxin Regulation

In an environment where higher plants are continuously subjected to a broad array of biotic and abiotic stimuli, their adaptability to such conditions requires a high degree of plasticity across scales of the whole plant. Plasticity, best reflected by rapid changes in a plant’s physiology and/or central metabolism, can give rise to significant changes in growth and morphology, as well as in changes secondary metabolism. Often, active defensive strategies of a plant against would-be “attackers” involves changes to secondary metabolism, producing toxins that can repel, deter, or kill their attacker (Howe and Jander, 2008). Defense can also involve tolerance mechanisms that allow the plant to regrow or reproduce after significant damage and/or tissue loss (Strauss and Agrawal, 1999). Underlying mechanisms for triggering such responses are traced to long-range signaling involving specialized hormones that help coordinate allocation of resources to targeted distal tissues and organs, as well as the metabolic partitioning of these resources (Thorpe et al., 2007).

Hormones like auxin are known classically for their roles in regulating plant development including axis formation and patterning during postembryogenesis,
vascular elongation, leaf expansion, inflorescence, fruit development, tropism, and apical dominance (Woodward and Bartel, 2005; Kazan and Manners, 2009). Most particularly, auxin can stimulate “reshaping” of root system architecture by triggering branch root patterning and root hair formation (McSteen, 2010).

The supply of IAA to targeted tissues and organs of the plant is thought to rely on a fine balance between its biosynthesis and its physical transport both in and out of cells, as well as across tissues. Through a combination of efflux carrier proteins, including the PIN family and ABCB family (Zázímalová et al., 2010) and influx carrier proteins, including the AUX1/LAX family (Swarup and Père, 2012), polar auxin transport can be established at a cellular level, providing the plant with a mechanism for effectively transporting this polar substrate across longer distances. Often, gradients in auxin concentration are found in targeted tissues that are thought to provide developmental cues for the plant (Blilou et al., 2005; Benková et al., 2009). For example, auxin is typically found at its highest concentration at the root tip (Ágútca et al., 2013).

As described earlier (Fig. 2A), several pathways can play a role in producing auxin. Of these, we were primarily interested in the IAOx and IAM pathways. IAN is a putative IAA precursor that is synthesized from IAOx. NITs are important in converting IAN-to-IAA. ZmNIT1 and ZmNIT2, which are encoded in the maize genome and are expressed in developing kernels and seedlings (Park et al., 2003), have been identified. Prior research using a ZmNIT2 knockout maize mutant showed significantly lower root IAA concentration than wild type, and it exhibited a strong root phenotype, suggesting that a pathway through IAN is important (Kriechbaumer et al., 2007). Indeed, our metabolic flux assays show that this pathway is the second most dominant pathway, next to the IPyA pathway, contributing to auxin in unstressed maize roots, and is the most dominant pathway when roots come under herbivore attack. Furthermore, the regional specificity exhibited by this pathway, as evidenced by higher NIT2 gene expression and faster [11C]IAN kinetics in the upper root zone where lateral root growth is evident, directly links this biochemical response to regrowth and crop tolerance. We note that past studies that spatially mapped both the IAA biosynthesis rate (Ljung et al., 2005) and the expression level of key genes linked with auxin biosynthesis (Birnbaum et al., 2003) across similar root zones in Arabidopsis (Arabidopsis thaliana) found higher rates of auxin biosynthesis and higher levels of NIT expression in the upper zone where lateral root growth occurs. Hence, the significant down-regulation that we see in NIT2 expression in the lower maize root zones where no lateral root growth occurs suggests that the IAOx pathway may be uniquely tied to a plant stress response mechanism that aligns with the development of lateral roots.

The IAM pathway, though considered an important pathway in bacteria, manifests in only very minute amounts of IAM in certain plants (Novák et al., 2012). The IAM hydrolase (AtAM1) enzyme, responsible for converting IAM-to-IAA, has also been observed in some plant species (Pollmann et al., 2006; Nemoto et al., 2009). Even so, little is known about the importance of
the IAM pathway across the plant kingdom, or of its connectivity with other pathway intermediates such as IAN in making IAA (see Fig. 2A). Through two different tracer experiments, our results clearly show that the IAM pathway is not active in unstressed plants, but activates under biotic stress. Even so, this pathway only contributes a minor portion of substrate to the overall auxin biosynthesis flux network. Furthermore, the first-order kinetics for metabolism of both $[^{11}\text{C}]$IAN and $[^{11}\text{C}]$IAM substrates clearly indicates that pathway interconnectivity does not exist for this system. As noted in Figure 2, there has been some speculation on the interconnectivity of these pathways based on identification of key enzymes. However, we note that these precursors can also play important roles in plant secondary defense chemistry, leading to defense compounds such as indole glucosinolates and camalexin, and perhaps the existence of these enzymes in plants is to serve this purpose and not auxin biosynthesis (Ljung, 2013).

**Influence of Biotic Stress on Central Metabolism and Resource Allocation**

While resistance mechanisms have been extensively studied over the years, much less is known about the mechanistic basis of tolerance (Stowe et al., 2000). Resource reallocation following real or simulated leaf attack aboveground has been well documented across numerous plant species, including tomato, tobacco, maize, barley, and poplar, where the response has been increased export of leaf photosynthates to the stem and roots (Babst et al., 2005, 2008; Gómez et al., 2010; Hanik et al., 2010a, 2010b; Henkes et al., 2008; Schwachtje et al., 2006). Similarly, nitrogen reallocation from leaves-to-roots was found to increase in tomato plants that were subjected to simulated herbivory aboveground (Gómez et al., 2010). More recently, our work using real root herbivore treatments showed decreased allocation of gross $[^{11}\text{C}]$photosynthates from source leaves to nodal roots in maize, as well as decreased root meristematic activity (Hanik et al., 2010a) and abiotic stresses (Pankievicz et al., 2015) can drastically and rapidly alter new carbon fluxes into key metabolite pools that make up plant photosynthates. Most particularly, increased partitioning of new carbon into $^{12}$C-labeled amino acid pools was noted, which coincided with compensatory reduction of the $^{12}$C-labeled sugar pools. In this work, we also observe a 2.6-fold increase in new carbon partitioning into source leaf $^{12}$C-labeled amino acid pools as a function of root herbivory. Unlike before, there was no compensatory decrease in the $^{12}$C-labeled sugar pool, but rather a slight increase

![Figure 9.](https://example.com/figure9.png)
following WCR attack. This may be due to the fact that 11C CO2 fixation (i.e. input of new carbon) actually increases when roots come under attack (Robert et al., 2014)—a feature that was not evident in prior above-ground herbivore studies. Upon closer inspection of our data, we begin to see an interesting story unfold where an increase in metabolic partitioning of new carbon into source leaf amino acids (specifically into highly transportable amino acids like Glu) enables the plant to quickly translocate more nitrogen resources to the roots when they come under attack. This statement is substantiated by key observations, including: (1) increases in allocation of 11C-labeled amino acids to roots, (2) increases in relative amounts of 11C-Gln in the root amino acid pool, and (3) increases in the rate of transport of the l-[5-11C]Gln tracer. We suspect this strong physiological response is being driven by the increase in 11C-Gln metabolism we see in roots when they come under attack. In concert with this, there is significant inhibition in the flow of sugars to roots. Because the profile of 11C-labeled sugars in these sink tissues reflect higher levels of 11C-Suc, we suspect that this strong physiological response is driven by decreased sink demand for sugars. Indeed, our prior studies revealed a significant reduction in root invertase enzyme activity with root herbivore treatment (Robert et al., 2014).

CONCLUSION

In summary, this work has revealed several new insights about root regrowth in crop tolerance to root herbivore attack as it relates to auxin biosynthesis and its regulation, including the following: (1) auxin biosynthesis is tightly regulated at the local root tissue level with minimal hormone translocation away from site of biosynthesis; (2) auxin biosynthesis is highly pathway specific, with the IAOx pathway through IAN dominating under stress; and (3) auxin biosynthesis is tightly coordinated, with Glu mobilization to roots, and with increased Glu metabolism at sites of regrowth. Given the redundant nature through which auxin is biosynthesized in higher plants, it is unlikely that up-regulating a specific pathway using genetic engineering will satisfy the local tissue demands for this hormone. Other pathways will likely just down-regulate, enabling the auxin flux network to maintain status quo based on the supply of substrates needed to support the overall process. A more viable approach is to target increasing the supply of these essential substrates. Our observations suggest that improving upon whole plant Glu utilization through increased Glu biosynthesis and/or transport would be a good place to start for crop breeding programs or for engineering new genetically modified organisms.

MATERIALS AND METHODS

Materials

All chemicals used in these studies were obtained from Sigma-Aldrich and used without any further purification.

Plant Growth and Treatment

Corn (Zea mays) kernels of the B73 line (U.S. Department of Agriculture Agricultural Research Service-Germplasm Resource Network) were germinated in darkness for 48 h on wetted KimWipe tissues placed in 7 × 7 cm petri dishes. The dishes were wrapped with aluminum foil and kept at room temperature. After 48 h, the petri dishes were unwrapped and placed under Agro lights giving 100 μmol m−2 s−1 of light intensity during a 16 h photoperiod. Once primary roots of germinating seedlings reached 1 to 2 cm in length, they were transplanted into 600 mL glass growth cells filled with Hoagland’s fortified agar gel.

Agar gels were prepared out of 3 L of deionized water, 4.9 g Hoagland modified basal salt mixture (PhytoTechnology Laboratories), and 1.66 g MES hydroxide. The pH of the solution was adjusted to 5.9 by adding 1 N sodium hydroxide solution. While stirring, 8.4 g Gelzan CM (Sigma-Aldrich) were added. The solution was autoclaved (Harvey SterileMax; Thermo Fisher Scientific) for 15 min at 121°C and mixed at high speed to enable aeration of the viscous solution before it had a chance to set as a gel. Sterilized plastic inserts were placed within each cell before the gel set. This allowed us to position the germinated kernel into a notch in the top portion of the cell. Following this, the tops of the cells were sealed with Parafilm and completely wrapped with aluminum foil to block out light. A small hole at the top of the cell enabled the developing plant to grow through. The cells were cultivated in growth chambers (Convirion) at 23°C using 320 μmol m−2 s−1 of light intensity on a 12-h photoperiod. Three-week-old plants that measured ~35 to 40 cm in height were used for studies.

For root herbivore studies involving attack by Diabrotica virgifera virgifera, 3-week-old plants were subjected to feeding by twelve D. v. virgifera larvae at their second-instar stage. Larvae were allowed to free-feed for 4 d prior to screening plant physiological and biochemical responses using 11C.

11CO2 Production and Administration

11CO2 was produced via the 18N(p, n)11C nuclear transformation from a 20 mL target filled with high-purity nitrogen gas containing 1.13 × 107 mg m−3 oxygen (400 mL O2 STP) using 18 MeV protons from the TR-19 (Ebbe Industries) cyclotron at Brookhaven National Laboratory and captured on a molecular sieve (4 Å). The 11CO2 that was trapped on the molecular sieve was desorbed and quickly released into an air stream at 200 mL/min as a discrete pulse for labeling a leaf affixed within a 5 × 10 cm lighted (320 μmol m−2 s−1) leaf cell at 21°C to ensure a steady level of fixation. The load leaf affixed within the cell was pulse-fed 11CO2 for 1 min, then chased with normal air for the duration of exposure. A PIN diode radiation detector (Carroll Ramsey Associates) affixed to the bottom of the cell enabled continuous measurement of radioactivity levels within the cell during the initial pulse and in the minutes right after the pulse giving information on 11CO2 fixation (Ferrieri et al., 2005).

Radiosyntheses of Complex 11C-Labeled Substrates

The radiosynthesis of 11C-IAA and its biosynthetic precursors 11C-IAN and 11C-IAM, using nucleophilic 11C-cyanations on gramine, was previously reported by us as a way to examine hormone signaling pathways in plants noninvasively (Reid et al., 2011). In the past year, we further refined this original synthesis method to include a two-step, one-pot process with integrated solid-phase extractions that enabled reliable and remotely controllable production of these radioisotopes in less than 1 h yielding 0.22 to 0.46 GBq of product at specific activities of 47.4 ± 12.5 Bq μmol−1 and >98% radiochemical purity (Reid et al., 2011; Lee et al., 2015a). The improvements to radiochemistry allowed for final product formulation in extremely small volumes (0.15–0.3 mL of deionized water at pH 6–7) that were suitable for in vivo PET plant imaging and/or metabolic flux assays.

The radiosynthesis of 11C-indole was recently reported by us (Lee et al., 2015b), which leveraged the nucleophilic 11C-cyanation reaction on 2-nitrobenzyl bromide generating a highly reactive 2-(2-nitrophenyl)-l-[11C]acetanilide intermedium that was subjected to nickel-catalyzed reductive cyclization to yield the desired product in less than 1 h at >98% radiochemical purity and with a specific activity of 176 ± 24.8 GBq μmol−1.

A previously reported 11C-cyanamide end-capping labeling method (Qu et al., 2012) was recently refined by us (Gleede et al., 2013) to create a milder, more reliable, two-step method for radiosynthesizing 185 to 370 MBq batches of l-[5-11C]Gln in less than 50 min at >90% radiochemical purity and possessing a L/D enantiomeric ratio of 94 ± 2.5/2.2 ± 1.7, and specific activity of 7.0 ± 1.5 GBq μmol−1.
Complex organic radiotracers were introduced into targeted root tissues using subcortical injections (< 1 µL). We compared this method for tracer administration to that of grinding fresh tissue with tracer and obtained similar results on radiometabolite profiles.

Plant Imaging

We used microPET imaging (Concorde MicroSystems) to carry out 90 min dynamic scanning on roots. The system was used in its default configuration as described previously for all dynamic imaging experiments (Knoesel et al., 2003; Alexoff et al., 2003). Images reconstructed by filtered back projection had a volumetric resolution of ~5 µL at the center of the field of view increasing to ~30 µL at a radius of 4 cm (Knoesel et al., 2003). Data corrections for random coincidences and dead-time losses were provided by the manufacturer. Scattered coincidences were subtracted using a tail-fitting method described previously (Alexoff et al., 2003). ASIPro VM software (BSI Research Systems) was used to create regions of interest (ROI) on reconstructed images giving information on radioactivity time-of-arrival between those ROIs. Distances between ROIs were also measured in pixels using the same software and related to mm spatial scales for calculating velocities (mm min⁻¹). After the microPET imaging, plants were removed from their growth cells and selected tissues (roots and leaves) were imaged using autoradiography (Typhoon 7000; GE Healthcare). Image data were quantified using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB).

[^1]CSugar and [^1]CAmino Acid Analyses

Targeted tissues were extracted in 4× (w/v) methanol, briefly vortexed (VWR analog vortex mixer; Sigma-Aldrich), and then sonicated (Branson Bransonic 32; Sigma-Aldrich) in an iced water bath for 10 min with intermittent vortexing to ensure complete mixing. The tubes were centrifuged (Eppendorf Centrifuge 5424) for 2 min at 15,000 rpm, and the supernatant was filtered through 0.2 µm Acrodiscs (Gelman Sciences). The pellet contained all insoluble components that comprised mostly cell wall polymers and starch. The filtrate contained small soluble compounds, including soluble sugars and amino acids. Sugars were separated and analyzed by thin-layer chromatography (TLC; Babst et al., 2013). Glass backed NH₂-silica HPTLC plates (200 µm, w/UV254) were used for the sugar separation (Sorbent Technologies). Plates were pre-spotted with sugar standards of maltose, Suc, Fru, Glc, and Xyl. Aliquots of radioactive tissue extract were applied to TLC plates using a semiautomated Linomat 5 sample applicator (Camag Scientific) for high precision of spot size and sample volume. TLC plates were developed using a mobile phase consisting of 75:25 acetonitrile:water (v/v). Developed plates were imaged using autoradiography to determine the fraction of each radiolabeled sugar. The plates were then heat treated (200°C for 10 min) to initiate chemical reaction of individual sugars with the amino functionalized silica support that gave fluorescence under long-wavelength (365 nm) UV light providing coregistration of the [¹³C]sugar standards with individual [¹³C]-labeled components. ImageQuant TL software 7.0 (GE Healthcare Bio-Sciences AB) was used to analyze the radiographic image data to determine the amount of [¹³C] in each sugar.

An additional 130 µL of tissue extract was also delivered into 2 mL brown-glass vials (Fisher Scientific) where equal volumes of O-phthalaldehyde amino acid derivatizing reagent containing 0.1% (v/v) mercaptoethanol (Sigma Life Science) were delivered. Mixtures were vortexed and then allowed to react at ambient temperature for 3 min. Fifty microliters of the derivatized extracts were injected onto a reversed-phase analytical HPLC column (Phenomenex; Ultratex C18, 10 µm particle size, 250 mm × 4.6 mm inner diameter) using a precolumn gradient mixer (Sonnetek) and a mobile phase composed of A (2.5% aqueous acetic acid adjusted to pH 3.8 using 1 M KOH) and B (80:20 acetonitrile:water). At injection, the mobile phase (1.0 mL min⁻¹) was programmed to adjust from 80% A; 20% B, to 50% A; and 50% B over 25 min. Two radio-TLC methods were also deployed to enable rapid assay of pathway-specific auxin biosynthesis kinetic fluxes using [¹³C]JAN and [¹³C]JAM radiotracers. In the first method, the silica TLC plate was developed using 1:5.1, hexamethylene acetate (0.1% formic acid [v/v]) as the solvent, which allowed rapid separation of [¹³C]JAN and [¹³C]JAM and leaving [¹³C]JAM at the origin. In the second method, the silica TLC plates were pretreated using NH₄OH (29%) and developed using pure ethyl acetate solvent to enable a different Rₑ elution profile of the three components with [¹³C]JAN carried with the solvent front. Activity distributions on the plates were made visible by autoradiography and quantified using ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences AB).

Endogenous Auxin Hormone Analysis

Root tissues were placed in Eppendorf tubes and ground to a fine powder at liquid nitrogen temperature using a Retsch ball mill grinder (Verder Scientific). Five hundred microliters of 0.05 M sodium phosphate buffer, pH 7.0, containing 0.02% (w/v) sodium dihydrodithiocarbamate as antioxidant and 250 µg of anthrancic acid (Sigma-Aldrich) as an internal standard were added to the Eppendorf tube, and the sample was extracted under continuous shaking for 1 h at 4°C. After extraction, the pH was adjusted to approximately 2.6 with 1 M HCl, and the sample was slurried with 35 mg of Amberlite XAD-7HP (weakly polar polymeric resin, 20–60 mesh; Sigma-Aldrich) for 30 min. After removal of the buffer, the XAD-7 was washed twice using 500 µL of 1% (v/v) acetic acid before being slurried twice more using 500 µL of dichloromethane for 30 min. The combined dichloromethane fractions were reduced to dryness in a vacuum centrifuge (LabConco) and then trimethylsilylated with 25 µL of acetamide and 25 µL of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% (v/v) trimethylchlorosilane (Sigma-Aldrich) at 70°C for 15 min. After evaporation to dryness (LabConco), samples were dissolved in hexane. One micro liter aliquots were split injected (100:1 [v/v]) onto a Hewlett-Packard 5890A capillary gas chromatograph equipped with a 30 m HP-5 column (0.25 mm diameter, 0.25 µm film thickness; Agilent Technologies) and a nitrogen-phosphorous detector.

Quantitative RT-PCR Analysis

NIT1 and NIT2 gene expression patterns along three root zones (including upper proximal, midroot, and root tip) were quantified by performing a quantitative reverse transcriptase real-time polymerase chain reactions as previously described (Ezbr et al., 2010). The gene-specific primers were used for NIT1 and NIT2 as previously described (Park et al., 2003). The Ct values were corrected for the housekeeping genes Actin and GapC and normalized to controls.

Statistical Analysis

Data were subjected to the Student’s t test for unpaired samples assuming an unequal variance. Statistical significance levels were assigned to the following rating scale: *p < 0.05; **p < 0.01; ***p < 0.001.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. A, Adult beetle of D. v. virgifera, LeConte; B, mature larva of D. v. virgifera.

Supplemental Figure S2. Complex organic [¹³C]-labeled radiotracers used in this study.

Supplemental Figure S3. Endogeneous nodal root IAA concentrations presented as mg ng FW⁻¹ and plotted as a function of root herbivore treatment.

Supplemental Figure S4. Transport speed (mm min⁻¹) of L-[5-¹³C]Gln plotted as a function of root herbivore treatment.
Meinke LJ, Siegfried BD, Wright RJ, Chandler LD (1998) Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. J Econ Entomol 91: 678–685
Qu et al.


