Integrated Metabolomics and Transcriptomics Reveal Enhanced Specialized Metabolism in Medicago truncatula Root Border Cells

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SUMMARY

Medicago truncatula border cells contain elevated levels of specialized metabolites which are important in plant-microbe signaling and defense.
Financial Source
The Samuel Roberts Noble Foundation

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ABSTRACT

Integrated metabolomics and transcriptomics of *Medicago truncatula* seedling border cells and root tips revealed substantial metabolic differences between these distinct and spatially segregated root regions. Large differential increases in oxylipin-pathway lipoxygenases and auxin-responsive transcript levels in border cells corresponded to differences in phytohormone and volatile levels compared to adjacent root tips. Morphological examinations of border cells revealed the presence of significant starch deposits which serve as critical energy and carbon reserves as documented through increased β-amylase transcript levels and associated starch hydrolysis metabolites. A substantial proportion of primary metabolism transcripts were decreased in border cells while many flavonoid- and triterpenoid-related metabolite and transcript levels were dramatically increased. The cumulative data provide compounding evidence that primary and secondary metabolism are differentially programmed in border cells relative to root tips. Metabolic resources normally destined for growth and development are redirected towards elevated accumulation of specialized metabolites in border cells, resulting in constitutively elevated defense and signaling compounds needed to protect the delicate root cap and signal motile rhizobia required for symbiotic nitrogen fixation. Elevated levels of 7,4′-dihydroxyflavone (DHF) were further increased in border cells of roots exposed to *Phymatotrichopsis omnivora*, and the value of DHF as an antimicrobial compound was demonstrated using in vitro growth inhibition assays. The cumulative and pathway-specific data provide key insights into the metabolic programming of border cells that strongly implicate a more prominent mechanistic role for border cells in plant-microbe signaling, defense and interactions than previously envisioned.
INTRODUCTION

The plant root tip includes the apical meristem and root cap initials which are progenitor cells to all new growth in the root. Root cell division and elongation originate in the apical meristem and proceed toward the mature root. Root cap growth begins in the root cap initials and continues to the root apex. Damage or destruction of the root tip can reduce growth or kill a plant. Fortunately, the root tip in most plants is not left unguarded or defenseless. The root cap of many species produces thousands of differentiated cells which separate from the root but remain appressed to it in a water-soluble polysaccharide matrix, or mucilage, until released by exposure to water. These cells are termed border cells because they form a boundary between the root and the rhizosphere, and are defined as the cells that disperse into suspension when root tips are placed in water (Hawes et al., 2000).

Border cells are more than by-products of root cap growth, and they provide a biotic boundary fundamental in rhizosphere modifications. For example, co-cultivation of oat border cells with micromolar levels of aluminum resulted in increased border cell and mucilage production in a dose-dependent manner (Miyasaka and Hawes, 2001). In addition, border cells serve key roles in plant defense and plant-symbiont interactions. They attract and immobilize nematodes (Hawes et al., 2000), orchestrate interactions with both mutualistic fungi (Kosuta et al., 2003; Nagahashi and Douds, 2004) and pathogenic fungi (Hawes et al., 2000; Gunawardena and Hawes, 2002; Woo et al., 2004; Gunawardena et al., 2005), and bind and repel bacteria (Hawes et al., 2000).

The production of border cells appears to be tightly regulated, but little is known about this process (Brigham et al., 1998; Hawes et al., 2000). Once a full complement of border cells is produced, root cap cell division ceases and border cells remain tightly appressed to the root tip until exposed to water (Hawes et al., 2000). When border cells are removed from the root, cell division in the root cap initials resumes within five minutes, remains high for two hours, and a complete complement of new border cells is produced within 24 hours, maintaining a species-specific number of border cells (Hawes et al., 2003). Border cells are a determinate cell type which serve several functions during their journey from meristem through columella and on to peripheral cells, i.e., gravity sensing and mucilage secretion, before arriving at the outer layer of the root. Border cells undergo an increase in metabolic activity after release from the root cap, resulting in the production of specific metabolites and the secretion of mucilage and proteins into the rhizosphere (Hawes et al., 2000; Wen et al., 2007)).

Two landmark publications that characterized root development in Arabidopsis were based on comparisons of anatomically distinct cell types over a developmental time-series using microarray gene expression data (Birnbaum et al., 2003; Brady et al., 2007). These studies provided a model for understanding root architecture and its relationship to root development in both space and time. A more recent study demonstrated the involvement of programmed cell death of Arabidopsis lateral root cap cells in the maintenance of root cap size (Fendrych et al., 2014). However, none of these studies addressed the biochemistry of border cells, the role of border cells in root physiology, or the signals produced by these specialized and spatially resolved cells because Arabidopsis roots do not produce
border cells but instead produce a few “border-like” cells in plants older than 5 days (Vicre et al., 2005; Driouich et al., 2007). In contrast, legume roots produce numerous border cells that are viable even after release from the root (Hawes et al., 1998).

Legume root biology is fundamentally important to agriculture, in part because legumes form symbiotic relationships with both mycorrhizal fungi and nodulating soil bacteria which are beneficial to plant growth and yield. Legumes also synthesize numerous natural products critical in plant defense, development and nutrition, including flavonoids, isoflavonoids, lignin and saponins (Dixon and Sumner, 2003). Information about the spatial localization and biosynthesis of these natural products in roots is sparse, and much of the present knowledge of root secondary metabolism comes from work done in the model legume Medicago truncatula (Achnine et al., 2005; Schliemann et al., 2008). In addition, genetic, genomic and biochemical resources are available for M. truncatula, including a genome sequence (Young et al., 2005; Young et al., 2011), high density microarray chipsets (Stacey et al., 2006) and a gene expression atlas for many organs, including specific root tissues (Benedito et al., 2008). These resources support M. truncatula as an ideal model to investigate the basal capacity of border cells and their ability to respond metabolically to environmental stimuli.

The present study integrated metabolic, transcriptional and morphological analyses of anatomically distinct M. truncatula seedling root tissues to better characterize the spatial distribution of metabolism in legume roots. Cumulative and pathway-specific data provided compounding evidence that border cells are metabolically differentiated relative to root tips. Border cells possess a pronounced enhancement in secondary metabolism which suggests a prominent biochemical role for these unique cells in defense, plant-microbe signaling and rhizosphere transformation. The high constitutive level of 7,4’-dihydroxyflavone and its subsequent increase in border cells exposed to Phymatotrichopsis omnivora is reported as an example of the role of border cells in root defense.

RESULTS

Microscopy
A polysaccharide matrix surrounds and adheres border cells to the tip of M. truncatula seedling roots (Figure 1A, Figure S1A ). Gentle agitation in or contact with water solubilizes the matrix and frees border cells from the root (Figure 1B, Figure S1B, C, Figure S2). M. truncatula border cells (used throughout to mean border cells with their associated mucilage) can be reproducibly harvested with over 95% viability as determined using fluorescein diacetate viability staining (Figure S1D-F) and cell counting. The number of M. truncatula seedling border cells was counted and determined to be approximately 1,700-2,000 per root, comparable to the numbers reported for alfalfa seedling roots (Woo et al., 2004). Many M. truncatula border cells have an elongated appearance and thick cell walls, similar to other species (Hamamoto et al., 2006), and large iodine-stained starch bodies were clearly visible in numerous detached border cells (Figure 1C, D, Figure S3A, B ). The relative amount of starch in border cells was lower than in most other root tip cell types, especially the columella cells.
(compare Figure S3 C, D with Figure 1C, D and Figure S3A, B) (Blancaflor et al., 1998; Barlow, 2003), but substantially higher than observed in the elongation and mature root zones (Figure S4).

**Gene expression analysis**

RNA was isolated from root tissues of 3-day-old pooled seedlings and used for the microarray gene expression comparisons of border cells to that of root tips lacking border cells and to whole roots. “Root tip” is used in the remainder of this text to describe the terminal 2-4 mm of the root minus border cells, “whole roots” refers to unaltered roots containing border cells and “border cells” were defined above. Three biological replicates were analyzed using the Affymetrix *M. truncatula* genome array as described by Benedito et al (2008), a selection threshold of 2 for transcript ratios and a Bonferroni-correction P value threshold of 8.15954E-07. The raw expression data were analyzed, and each transcript was assigned an absolute expression level and a “present” or “absent” call based on the signal-to-noise ratio. Approximately 50% of the plant probe sets from the *M. truncatula* GeneChip array produced “present” calls when hybridized with biotin-labeled cRNA from the three sample types, similar to previously reported hybridization percentages for *M. truncatula* (Holmes et al., 2008). Following normalization, 1,995 transcripts were identified as statistically increased and 4,519 as decreased in border cells when compared with whole seedling roots (Table SI). Changes at the transcript level between border cells and root tip samples were more pronounced with 5,140 transcripts higher and 7,084 transcripts lower in border cells when compared with root tips (Table SI). The full data set has been deposited in the Array Express database and is publicly available as accession E-MEXP-2883 and in the *Medicago truncatula* Gene Expression Atlas V3 (http://mtgea.noble.org/v3/).

MapMan software (Thimm et al., 2004); (Urbanczyk-Wochniak et al., 2006) was adopted for visualizing *M. truncatula* transcript data by generating species-specific mapping files for the Affymetrix *Medicago* chip (Uppalapati et al., 2009). Differentially expressed genes from the three different sample types were functionally classified using MapMan categories (Figure 2, Figure S5) and displayed on pathway diagrams. Less than 50% of differentially expressed transcripts could be assigned functional categories (Figure 3, Figure S6). The assigned transcript classes most strongly differentiating border cells from whole root and root tips were associated with RNA regulation and protein post-translational regulation (Figure 3, Figure S6). In these classes, more transcripts were decreased than increased in border cells. The total number of transcripts involved in nucleotide and DNA metabolism was also lower in border cells, consistent with a determinate cell type with a slowing rate of replication and cell division. Cell wall metabolism, lipid metabolism, stress, hormone metabolism and miscellaneous (UDP glycosyl transferases, peroxidases, oxidases, etc.) also accounted for substantial differences between border cells and whole roots and/or root tips (Figure 3, Figure S6). Transcripts in these categories were higher in border cells, as were the overall number of border cell transcripts related to secondary metabolism and transport, two categories linked to defense.
There were 396 transcripts observed only in border cells and not in other *M. truncatula* root tissues (Table SI). Most of these transcripts were detected at low levels, and 75% were novel transcripts categorized as “not annotated”. Several other transcripts were observed at reproducible levels in only a few tissue types besides border cells. For example, a pectin methyl esterase inhibitor was present in young roots prior to nodulation (equivalent to whole roots in this analysis) and in border cells but absent in all other tissues analyzed (Figure 4A).

Quantitative RT-PCR was performed to provide a more rigorous quantitative measure of gene expression for select genes. Expression levels for five genes representative of central steps in primary metabolism and eight genes related to major changes in secondary metabolism were validated by quantitative real-time PCR (Table I). Additionally, the expression level of a pectin methylesterase (PME), a known marker for root tip-border cell separation, and a PME inhibitor were also re-analyzed by qRT-PCR. Two genes important in hormone response and metabolism in different areas of the root were also analyzed. These genes, an auxin-responsive SAUR (small auxin up RNA) protein and a lipoxygenase, showed very large expression increases in border cells compared to root tips. In total, 17 genes were analyzed by qRT-PCR, and the results from the microarray analysis were confirmed by the qRT-PCR (Table I) in every case. These results are discussed in more detail in the Discussion section. A complete list of primers can be found in Table SII.

**Metabolomics**

This report focuses on the metabolic comparison of anatomically distinct root tips and border cells while noting that a few prior publications have reported cumulative metabolic profiles of whole *M. truncatula* roots (Achnine et al., 2005; Huhman et al., 2005; Schliemann et al., 2008; Zhang et al., 2014). Metabolomics analyses were performed using a series of GC-MS and LC-MS experiments. The GC-MS profiling identified distinct, reproducible tissue differences (Figure 5A) between border cells and root tips. The levels of most sugars were unchanged or lower in border cells (Table II), though fructose, glucose, galactose, sucrose and arabinose were all abundant in these cells. Sucrose was the only sugar significantly elevated in comparison to root tips. Fructose was the most abundant sugar observed in the metabolite analyses (Table SIII), and this was reflected in the high percentage of glycolytic transcripts (> than 70%) linked to fructose metabolism in border cells (Table SI). Fructose and glucose are produced from the degradation of sucrose, and glucose was observed at lower levels in border cells than root tips. However starch, a product of glucose, was abundant in both (Figure 1 C, D, Figure S3).

Several organic acids were found at higher levels in border cells, including four (Table II) intermediates in the TCA cycle. The level of citrate, an early TCA intermediary, was fivefold higher in border cells than in root tips. Fumarate, a precursor for the amino acids aspartic acid and asparagine, was also more abundant in border cells compared to root tips (Table II). The level of malic acid was approximately 2.5-fold higher when compared to root tips. Malate is an important precursor in the formation of pyruvate, and thus of the branched-chain amino acids and CoA (Figure 6).
Nineteen standard and two non-standard amino acids were identified in border cells (Table II, Table SIII). Of these, 10 were significantly higher in border cells while the level of 11 amino acids was statistically unchanged (± twofold, p<0.05) compared to root tips. The most abundant amino acids in border cells were among those most elevated in comparison to root tips. These included asparagine, one of the most abundant metabolites in border cells, and serine, homoserine, and glycine (Table I, Table SIII). Proline and threonine were also highly abundant in border cells, with levels that were elevated in comparison to root tips. The branched-chain amino acids isoleucine, valine and leucine, precursors for CoA biosynthesis, were also observed at high levels in border cells. Phenylalanine, the precursor for phenylpropanoids, was abundant in both root tips and border cells (Table I, Table SIII).

An ultra high-pressure liquid chromatography coupled to a hybrid quadrupole time-of-flight mass spectrometer (UHPLC-QTofMS) method was used to profile a range of saponins and flavonoids because of their importance to defense and signaling in legumes. Consistent with the Metabolomics Standard Initiative (Sumner et al., 2007), this profiling method provided a significant number of confident identifications for a number of differentially accumulated secondary metabolites by comparison to authentic standards and a number of tentative identifications through accurate mass matches to metabolites within public databases (Table III). Distinct and reproducible tissue differences in secondary metabolite levels were observed (Figure 5B). Almost twenty saponins and sapogenin aglycones were observed at increased levels in border cells. Five of these were observed at a 10-fold or greater excess in comparison to root tips. Root tips also contained many saponins; some of these were in excess of the levels observed in border cells or not detected in border cells (Table III).

Flavonoids are important in plant defense, symbiosis, development and pollination (Modolo et al., 2007). Two flavonoids observed at higher levels in border cells were apigenin and 7,4’-dihydroxyflavone (Table III). Naringenin chalcone 4’-O-glucoside, a glycosylated form of an apigenin precursor, was also identified in border cells at elevated levels. Table SIII contains a comprehensive list of primary and secondary metabolites observed in this study with the supporting chromatographic retention times and mass spectral m/z values.

M. truncatula seedlings were treated with water or a suspension of mycelia from the non-sporulating fungus Phymatotrichopsis omnivora (cotton root rot), a devastating root pathogen that attacks many plants, including legumes (Marek et al., 2009; Uppalapati et al., 2009; Uppalapati et al., 2010). Border cells and root tips without border cells were collected 24 and 48 hours after inoculation and analyzed by UHPLC-QTofMS. The flavone 7,4’-dihydroxyflavone (DHF) was found constitutively at higher levels in border cells, and was further increased ~ twofold in border cells after a 24 hour exposure to P. omnivora, while there was no change in root tips (Figure 7A). After a 48 hour exposure to P. omnivora the level of DHF had further increased in border cells but was unchanged in root tips. The level of DHF in control border cells dropped at the 48 hour time point (Figure 7A). DHF was tested for antifungal properties against P. omnivora and was effective at concentrations as low as 100 µM (Figure 7B). DHF showed greater growth inhibition than catechol, a
known potent inhibitor of *P. omnivora* growth (Greathouse and Rigler, 1940), and similar to or slightly better than the phytoalexin medicarpin (Naoumkina et al., 2007) (Table IV).

Phytohormones were analyzed in root tips without border cells and border cells using ultra high-pressure liquid chromatography coupled to a triple quadrupole tandem mass spectrometer (UHPLC-QqQMS/MS). Phytohormone analyses in root tips revealed a high concentration of jasmonic acid (JA), indole acetic acid (IAA) and salicylic acid (SA), along with a lesser concentration of abscisic acid (ABA) (Figure 8A). SA was also abundant in border cells while JA and ABA were less abundant, and IAA was not detected in border cells (Figure 8A).

The volatile compound hexanal was observed in border cells (350 + 100 pmol/50 roots) using solid-phase microextraction (SPME) GC-MS analysis, but not in roots without border cells or whole seedlings without border cells (Figure 8B). Volatiles from root tips were not measured because excising the tip would cause a wounding response.

**DISCUSSION**

Border cells are determinate cells capable of responding to many different stimuli encountered in the rhizosphere (Hawes et al., 2000) and are differentiated from other root cells. Differentiation of border cells begins with important developmental cues and heightened hormonal activity resulting in substantial differences in primary and secondary metabolism fueled through intracellular starch-based energy production (Figure 6). Discussions follow that provide detailed gene expression and metabolomics data supporting our conclusion that border cells are differentially programmed with enhanced secondary metabolism. We further demonstrate that the differential metabolic programming and composition of border cells facilitate a unique role in root growth, development, defense and plant-microbe signaling.

**Border cell production includes pectin methylesterase (PME), PME inhibitor (PMEI) and localized hormone activity**

Border cells are produced within two to three days after germination and originate from the root cap meristem initials (Brigham et al., 1998; Hawes et al., 2000). They transition through columella cells, peripheral cells and, ultimately, into border cells (Brigham et al., 1998; Hawes et al., 2000). Border cells are released from the root, in part by PME activity. This enzyme demethylates pectin and allows cell wall degradation which leads to separation of border cells from roots. Inhibition of this gene blocks the normal detachment of border cells (Wen et al., 1999). Expression levels for PME were low in border cells as determined by both microarray and qRT-PCR data (Table I, Table SI), and substantially higher in root tips (7-16 fold higher). The action of PME in roots has been well documented (Wen et al., 1999) and confirmation of PME expression in *M. truncatula* root tips and border cells provided reassurance of our experimental approaches.

Interestingly, a novel transcript for a gene annotated as a PME inhibitor (PMEI) was greatly enhanced in border cells (115 fold higher) (Figure 4A, Table I). PMEI activity has been characterized
in kiwi fruit and Arabidopsis (Balestrieri et al., 1990; Raiola et al., 2004), and in pepper leaves (An et al., 2008) where it also exhibited antifungal properties. However, this is the first time a putative PMEI has been identified in border cells. PMEI and PME form a complex in a 1:1 stoichiometric ratio (Di Matteo et al., 2005). Therefore, PMEI expression in border cells appears to be a negative regulator of PME activity and associated with border cell detachment. PMEI levels increase once PME activity is no longer needed.

All cell types in the root tip of Arabidopsis seedlings synthesize auxin (Ljung et al., 2005). The auxin is transported basipetally by polar transport from most root tip cells, and IAA levels in columella cells are lower than in the rest of the root tip. In contrast, the quiescent center maintains a high concentration of IAA (Ljung et al., 2005), and high auxin levels contribute to the mitotic activity in meristematic cells while the auxin gradient in other root tip cells is tightly connected to differentiation and development (Ljung et al., 2005). Border cells in Medicago progress from columella cells and are the most mature cells in the root tip; thus, the lack of detectable auxin in border cells and high levels of auxin in the root tip (Figure 8A) correlate with auxin-related root development. In border cells, elevated auxin-related transcripts are the predominant class of hormone transcripts (40% of BC/RT); many of these are negative regulators of auxin. For example, multiple auxin-responsive SAUR (small auxin up RNA) transcripts are elevated in border cells. These proteins are short-lived nuclear proteins hypothesized to be negative regulators of auxin synthesis and transport (Kant et al., 2009). Overall, 19 of 21 SAUR transcripts were higher in border cells, and the transcript level for one SAUR verified by qRT-PCR was especially elevated (> 900-fold) (Table I). Expression of this SAUR was not observed in other root tissues contained within the Medicago gene atlas (Figure 4B). SAURs are involved in auxin signal transduction (Davies, 2004) and in auxin-induced cell elongation (Knauss et al., 2003), and likely play a role in elongation of border cells. Transcript levels of several auxin response factors, transcription factors that regulate auxin-mediated responses, were lower in border cells (Table SI), further confirming a lack of auxin in these cells.

Jasmonic acid expression is high in root tips (Hayashi et al., 2008; Birnbaum et al., 2003) and levels of JA in this study were also higher in root tips than border cells (Figure 8A). LOX genes function early in the JA pathway, but the transcript levels for three LOX transcripts were 84- to 225-fold higher (microarray data) in border cells. The level of one LOX was further validated by qRT-PCR and confirmed as 126-fold higher in border cells (Table I). This LOX catalyzes an early step in the oxylipin pathway, and its product is the branch-point compound for either JA synthesis or the synthesis of hexanal (Figure S7B), a stress volatile produced in response to biotic and abiotic stimuli (Chehab et al., 2006). Hexanal was the only volatile detected by SPME analysis of border cells, and there was no discernible peak for hexanal in roots or seedlings without border cells (Figure 8B). Transcript levels for hydroperoxide lyase, the next enzyme in the biosynthetic pathway for hexanal, were fourfold higher in border cells than root tips (Table SI). Levels of allene oxide synthase and allene oxide cyclase transcripts, enzymes that catalyze subsequent reactions in the JA pathway, were either unchanged or higher in root tips (Table SI). Elevated levels of JA, hexanal (Figure 8) and transcripts for enzymes in the biosynthetic pathway of these compounds support an important role for
border cells in stress metabolism and in protection against pathogen and insect attack, processes in which oxylipins have an established role (Reymond and Farmer, 1998; Uppalapati et al., 2009).

SA is a key signaling molecule synthesized in response to both biotic and abiotic stress (Horvath et al., 2007). It is abundant in root tips and even more abundant in border cells (Figure 8A), yet there are few transcripts annotated as SA-related in either tissue (Table SI). Two pathways for SA synthesis are proposed in plants, one of which is through benzoic acid (BA) (Chen et al., 2009). Border cells and root tips contain BA (Table II, Table SIII), and only one additional hydroxyl group differentiates SA from BA. Benzoic acid 2 hydroxylase (BA2H) activity has been detected in tobacco and rice, and the tobacco protein has been partially purified, although a gene has not yet been isolated (Chen et al., 2009). Removal of border cells from root tips could be enough stress to cause an as yet unannotated Medicago BA2H to synthesize SA from BA to aid in root tip defense (Naoumkina et al., 2010).

Border cells utilize starch for energy and carbon

Border cells are detached root cells and unable to directly benefit from energy sources transported through the vascular system. However, border cells contain starch-filled plastids (Figure 1C, D), which are a common source of stored energy and carbon (Blancaflor et al., 1998; Barlow, 2003). Starch is synthesized from glucose made available when sucrose is degraded, and over seventy percent of sucrose transcripts in border cells and root tips are annotated as degradation-related (Table SI). Fifty-six percent of the starch-annotated transcripts in root tips are associated with synthesis, and root cap columella cells are packed with starch bodies, as determined through iodine staining (Figure S3C), supporting the importance of the root tip as a site for conversion of glucose to starch. Starch levels are lower in border cells than in their progenitor root cap cells, and eighty percent of observed border cell transcripts involved in starch metabolism were annotated as degradation genes (Table SI). In addition the transcript level of the starch-degrading enzyme β-amylase was validated by qRT-PCR as ~15-fold higher in border cells (Table I). Cumulative transcriptomic and microscopic data indicate that starch reserves accumulated during border cell production are utilized as an energy and carbon source.

Primary metabolism is reduced in border cells and redirected toward secondary metabolism

Seventy percent of transcripts involved in primary metabolism were lower in border cells compared to root tips (Table V). Exceptions to this trend are discussed in more detail below and include compounds that serve important roles in supplying primary metabolic precursors for the synthesis of important secondary metabolites.

Levels of many amino acids were much higher in border cells than root tips (Table II), while overall transcript levels for amino acid synthesis in border cells was decreased (Figure 3, Table SI), suggesting most amino acids were synthesized during early border cell development. Alternatively, border cell amino acids may have originated from protein degradation; this is less likely because the
percentage of protein degradation transcripts elevated in border cells and root tips was equivalent, with fewer total degradation transcripts in border cells. In addition, border cells are reported to actively synthesize proteins even after release from the root (Brigham et al., 1995; Wen et al., 2007). Thus, the origin of increased amino acids in border cells is unknown, but evidence supports their utilization as precursors for synthesis of proteins and metabolites.

Asparagine was the most abundant amino acid in border cells and was increased compared to root tips. Transcript levels for asparagine synthetase were also elevated three- to six-fold (Table I) although the transcript levels for most other amino acid syntheses were decreased in border cells. Asparagine is an endpoint amino acid that serves as a major nitrogen transport and storage compound in plant cells (Ta et al., 1984). Amino acids also serve as precursors for the rapid production of defense compounds. For example, phenylalanine is the entry point for lignin, flavonoid, and salicylate biosynthesis. Phenylalanine was present in border cells and root tips, and lignin transcripts plus multiple flavonoids and SA (compounds and/or transcripts) important in plant defense were identified in both (Tables I, III, Figure 6).

Transcript levels for the enzyme that reversibly converts fructose-6P to fructose-1,6-P were 28-fold higher in border cells as determined by qRT-PCR (Table I). Fructose is a product of sucrose degradation and the most abundant sugar measured. Fructose is a precursor metabolite in glycolysis, and the glycolysis of fructose yields ATP and NADH with an end product of pyruvate. Pyruvate can be metabolized in the TCA cycle to form acetyl-CoA, central to the process of shuttling carbon from primary to secondary metabolism.

The level of citrate was up fivefold (Table II) in border cells, and the correlated transcript level of citrate synthase, as measured by qRT-PCR, was slightly higher (Table I). Citrate is an important intermediary in the TCA cycle (Figure 6) and also serves as a substrate for the cytosolic production of acetyl-CoA, an essential precursor in the synthesis of secondary metabolites. As a side note, citrate is secreted in response to aluminum (Li et al., 2000), and the importance of border cells in protecting the root tip from aluminum has been documented (Miyasaka and Hawes, 2001). Prior literature and increased levels of citrate in border cells support their role as a major quantitative source for the secretion of citrate.

Coenzyme A is important in numerous metabolic processes, especially in providing carbon substrates for secondary metabolism. Precursors for CoA biosynthesis, including β-alanine and the branched-chain amino acids isoleucine and valine, were all found at increased levels in border cells (Table II). Beta-alanine is a precursor of pantothenate and CoA, and the transcript for β-ureidopropionase, an enzyme involved in β-alanine synthesis, was determined to be threefold higher in border cells by qRT-PCR (Table I). CoA is necessary for the production of acetyl-CoA, a central metabolite in the shuttling of carbon from primary to secondary metabolism, and synthesis of many natural products, including flavonoids and terpenoids.

Although flux was not measured, the integrated metabolite and transcript data strongly support that carbon and energy necessary for growth and development is redirected toward secondary metabolism in border cells.
Secondary metabolism is enhanced in border cells

Border cells are rich in secondary metabolites and contain numerous elevated transcripts for secondary metabolism (Figures 2, 3, Table V). This is highly evident in the differential MapMan visualizations where the majority of transcripts for waxes, phenylpropanoids, phenolics and flavonoids were distinctly higher in border cells (Figure 2). The elevation in secondary metabolism transcripts is in sharp contrast to the decrease of many primary metabolite transcripts, indicating energy and carbon from primary metabolism are channeled into border cell secondary metabolism.

Border cells are mature lignified cells (Hamamoto et al., 2006), and the transcript level for caffeoyl CoA O-methyltransferase (CCoMT), an important enzyme in phenylpropanoid based monolignol biosynthesis, was determined by qRT-PCR and found to be 24-fold higher in border cells (Table I). The relative increase in CCoMT supports continued lignification and secondary cell wall reinforcement in border cells after detachment from the root. Lignin provides strengthened cell walls for enhanced protection against mechanical damage and during encounters with plant pathogens (Vance et al., 1980).

Many triterpene saponins and flavonoids were identified in border cells, and the levels of many of these compounds were dramatically higher than in root tips. Saponins have reported allelopathic, antimicrobial and insecticidal properties important in plant protection (Shao et al., 2005) and are generally toxic to cold-blooded animals and insects (Tava and Odoardi, 1996; Waterman, 1996). Legumes have a rich variety of saponins (Huhman and Sumner, 2002; Dixon and Sumner, 2003; Huhman et al., 2005; Pollier et al., 2011), many of which are present in M. truncatula root tissue. Triterpene saponins were quantitatively the most abundant class of secondary metabolites identified in border cells, and the largest fold increases in border cell metabolites were observed for saponins.

Much of the biosynthetic pathway for triterpenoid saponins is unknown, but the first committed step is the cyclization of 2,3-oxidosqualene by β-amyrin synthase to form β-amyrin (Hayashi et al., 2001; Suzuki et al., 2002). This is the starting point for the synthesis of at least seven different sapogenins (aglycone form of saponins). The transcript levels for β-amyrin synthase were similar in border cells and root tips as measured by qRT-PCR (Table I), suggesting that early steps in saponin biosynthesis occur at approximately equivalent rates in root tissues. The transcript level of CYP93E, an isoform of the enzyme reported to catalyze the hydroxylation of β-amyrin and sophoradiol in soybean and licorice to form the first soyasapogenin (Shibuya et al., 2006; Seki et al., 2008), was sevenfold higher in border cells while the P450 that functions in the first committed step of the oleanate sapogenol pathway, CYP 716A12 (Carelli et al., 2011), is threefold higher in root tips (Table SI). Subsequent oxidation of the triterpene aglycone skeletons is believed to involve several currently unknown cytochrome P450s (CYPs). The transcript level of a proposed CYP family member associated with terpenoid biosynthesis, CYP71A8 (Naoumkina et al., 2010), was 91-fold higher in border cells, implying a tissue/cell specificity for certain steps in terpenoid biosynthesis. The transcript for another gene involved in sesquiterpenoid biosynthesis, (-)-germacrene synthase, was
validated by qRT-PCR and found in border cells at levels 167-fold higher than in root tips. Unfortunately, the product of this enzyme, (-)-germacrene D, was not observed in the GC-MS analysis due to its volatility. However, these data cooperatively support the elevated biosynthesis of terpenes in border cells and a role for border cell terpenoids in defense and rhizosphere modification.

Glycosylation typically influences the bioactivity of secondary metabolites as well as their cellular localization, stability and metabolism (Modolo et al., 2007). More specifically, glycosylation can increase biological activity of triterpenoid saponins in comparison to the aglycone, presumably due to the increased ability of the molecules to complex in fungal membranes, and the activity is dependent on the number of sugar molecules in the attached chain (Haridas et al., 2001; Osbourn, 2003). In border cells, multiple sapogenin aglycones and various conjugated forms of each sapogenin were identified (Ex: bayogenin, rha-hex-hex-bayogenin, hex-hex-hex-bayogenin), thereby increasing the saponin diversity (Table III) and potential defense compounds useful in responses to rhizosphere microbes and environmental stresses.

Flavonoids are associated with symbiosis, signaling, plant development and plant defense (Kape et al., 1991; Stafford, 1997; Shirley, 1998; Aoki et al., 2000; Forkmann and Martens, 2001); and there is a net increase in flavonoids in border cells (Table III). Phenylpropanoids are the precursors of flavonoids, isoflavonoids, anthocyanins and lignin, and are synthesized from the primary amino acid phenylalanine. An early step in the biosynthesis of these compounds is the conversion of phenylalanine to cinnamic acid by phenylalanine ammonia-lyase (PAL). Phenylalanine was abundant in border cells, and three isoforms of the PAL transcript were identified at increased levels (Table SI). This increase was confirmed for one isoform by qRT-PCR (Table I) as 30-fold higher in border cells. Isoflavone synthase (IFS) is the branch point of phenylpropanoids into isoflavone synthesis, and the transcript for an IFS-like protein was increased 13-fold in border cells. These data demonstrate elevated isoflavone synthesis in border cells.

Secreted flavonoids are important in the signaling processes between plants and other organisms in the rhizosphere. One example is nod gene induction in rhizobacteria (Peck et al., 2006) where apigenin and 7,4’-dihydroxyflavone (DHF) are among the most potent inducers of nod genes in Sinorhizobium meliloti during pre-symbiotic interactions with Medicago (Zhang et al., 2007). Multiple flavonoids involved in rhizobial signaling and symbiosis (Modolo et al., 2007) were substantially elevated in border cells. Specifically, apigenin was sixfold higher, DHF was increased by more than 12-fold, and naringenin-chalcone glucoside was twofold higher in border cells than root tips (Table III). Transcripts associated with the production of apigenin, naringenin-chalcone synthase and flavone synthase II were also measured by qRT-PCR at substantially increased levels in border cells (64-fold and ~sevenfold, respectively) (Table I). These data illustrate that border cells contain substantially greater quantitative amounts of important flavonoid signaling molecules than do other root tissues. Thus, border cells are equipped to recruit motile rhizobia to inoculate root hairs and initiate nodule development as the root continues its developmental processes.

Flavonoids also serves as defense compounds (Ferreyra et al., 2012) and the differentially accumulated specialized metabolites in border cells likely serve important defense roles as well as
signaling roles. Hence, the metabolic response of root tips and border cells were measured in response to exposure to *P. omnivora*, a devastating fungal root rot pathogen with limited treatment options and no known resistance in Medicago or any crop species (Uppalapati et al., 2010). The flavone DHF was strongly increased in border cells after exposure to *P. omnivora*. The constitutively high levels of DHF in border cells doubled after 24 hours of exposure to the fungus, and increased even further after 48 hours (Figure 7A). Yet the levels of DHF remained low at all tested time points in the root tip. To further demonstrate the defense roles of DHF and border cells in plant defense, DHF was tested for growth inhibition against *P. omnivora*. DHF showed strong fungal growth inhibition, and was as potent an antifungal agent as medicarpin and 20-fold better than catechol, one of the few reported chemical treatments for *P. omnivora* (Table IV). This novel bioprotection against *P. omnivora*, in conjunction with high levels of DHF in non-elicited border cells and a robust increase in DHF after exposure to cotton root rot, led us to conclude that border cells produce DHF and other secondary metabolites as phytoanticipins and/or phytoalexins to protect the critically important meristemmatic root tip.

**A systems model for metabolic programming and enhanced metabolic capacity of *M. truncatula* border cells**

Integrated transcriptome and metabolome data detailing differences observed between *M. truncatula* border cells and root tips are provided in Figure 6. This figure includes a model describing the enhanced secondary metabolic capacity of border cells. Border cells begin their life cycle as root cap initial cells, develop as columella and peripheral root cells, and then transition into border cells. Differentiated border cells are characterized by large expression differences when compared to root tips, and the most dramatic differences are in hormone-associated transcripts. Border cells contain SA, JA and ABA, but IAA is not detected in border cells. This contrasts with the root tip, which contains the highest concentration of IAA in roots. The volatile compound hexanal is produced by border cells and is absent in roots without border cells. Iodine staining revealed starch deposition in the root cap and border cells, and comparative microarray data revealed increases in border cell β-amylase. These data support starch reserves as a critical energy source and carbon reserve for detached border cells. Transcript data document an overall general decrease in primary metabolism with exceptions associated with branched-chain amino acid and β-alanine biosynthesis, which are associated with CoA biosynthesis and carbon shuttling into secondary metabolic pathways, i.e., flavonoid and triterpene biosynthesis. Flavonoid transcripts and related metabolites are substantially increased in border cells, and many triterpenoid transcripts and metabolites are also observed at elevated levels.

The cumulative pathway-specific data provide compounding evidence that primary and secondary metabolism are differentially regulated in border cells relative to root tips. Although flux was not measured, the integrated metabolite and transcript data strongly support that carbon and energy are reallocated from biosynthesis for growth and development toward enhanced secondary metabolism in border cells. Quantitative increases in specific secondary metabolites implicate an important role for border cells in defense and plant-microbe interactions, a hypothesis validated by the
antifungal effect of DHF against *P. omnivora*. Future work will concentrate on expanding our understanding of the molecular and metabolic basis for border cells in plant-microbe signaling and defense, and the specificity of plant-microbe interactions.

**Materials and Methods**

**Sample growth conditions and collection**

*M. truncatula* (A17) seeds were scarified by soaking in concentrated sulfuric acid for five minutes and then rinsed three times with chilled, distilled water. Scarified seeds were sterilized in bleach for 10 minutes, rinsed three times with distilled water and placed on sterile filter paper atop 1% water agar plates to germinate at 24° C in a dark growth chamber for three days. Twenty µl of a mycelial suspension of *P. omnivora* was pipetted along the germinated root two days after plating, and the plates returned to the dark. Border cells and root tips were collected 24 and 48 hours later. For the metabolite analyses, border cells were collected from 40 replicate seedlings, and the resulting root tips without border cells were excised and collected. Five whole roots consisting of the complete radicle were collected separately. All samples were frozen immediately in liquid N\textsubscript{2}.

**Histology**

Border cells from 10 seedlings were collected in water and incubated for five minutes with fluorescein diacetate (50 ng/µl) to detect live cells and propidium iodide (500 ng/µl) to stain dead cells. Dead and live cells were counted using a hemocytometer. The count was repeated at least three times. A Nikon Microphot-FX microscope was used for cell counts and starch body visualization.

Starch-stained plastids in border cells were confirmed by examining five or more seedlings on three separate occasions. Dilute iodine stain (1/4 strength) was added to visualize starch in seedling roots and detached border cells. Seedling roots were sectioned to a thickness of 70 µm and iodine-stained to image starch bodies in columella cells. DIC (differential interference contrast) microscopy was utilized to visualize starch bodies in detached border cells.

Roots and border cells stained with fluorescein diacetate or double-stained with fluorescein diacetate and propidium iodide were imaged with a Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Exton, Pennsylvania) using a 63x HCX Plan-Apo water immersion objective with a numerical aperture of 1.2. Fluorescein diacetate was detected by illuminating with the 488 nm line of the Argon laser and emission detected at 510 nm. Propidium iodide was detected after illumination with the 543 nm line of the Argon laser and emission detected at 617 nm.

Roots with appressed border cells and matrix were attached to a specimen holder frozen in liquid nitrogen and imaged on a Hitachi TM3000 Tabletop SEM (scanning electron microscope). Environmental SEM of border cells floating off seedling roots was performed using an FEI Quanta 600F eSEM at 6.5T, 5° C. The root was placed on a thin strip of agar with water droplets initially surrounding the root tip.
Ruthenium red at a concentration of 0.02% in distilled water was used to stain acidic pectins in mucilage and border cells released from seedling roots. The roots were placed in a drop of stain on a microscope slide and monitored for 20-30 minutes. Images were made using a Nikon Microphot-FX once sufficient color developed.

**Fungal growth and inhibition assays**

*Phymatotrichopsis omnivora* cultures were grown at 28°C on sterile plates of PDA (Potato Dextrose Agar 18 g/L, malt extract 1 g/L, yeast extract 1 g/L and peptone 1 g/L). Fungal inhibition was assayed on PDA plates for 5 days. Flavonoid molecules were pre-dissolved in DMSO to make 25 mM and 5 mM stock solutions, which were diluted to 1 mL with PDA medium to a final concentration of 0.5 mM and 0.1 mM. A two mm fungal plug of *P. omnivora* was incubated on the assay plates and fungal growth was recorded every 12 h starting at 48 h. The fungal growth for each tested molecule was scored on a qualitative scale from 0 to 5 with 0 as no growth and 5 as the most growth by comparing with PDA only medium and DMSO control (20 µL in 1 mL PDA). Catechol at concentrations of 5 mM and 10 mM was used as a positive control. Formononetin, which bears no antifungal activity, was used as the negative control. All experiments were replicated four times.

**Gene expression analysis**

Border cells for each replicate were collected by gently agitating roots directly in Qiagen buffer RLT from ~150-200 seedlings. Root tips were collected by agitating roots in water (detailed in Mass Spectrometry Analysis section), and the root tips (2-4 mm) minus border cells from 10 roots were excised and frozen immediately in liquid N₂. Five whole roots were excised from seedlings and frozen immediately in liquid N₂. Three biological replicates were performed for each tissue sample. Total RNA was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, California). RNA was quantified and evaluated for purity using a Nanodrop Spectrophotometer ND-100 (NanoDrop Technologies, Wilmington, Delaware) and Bioanalyzer 2100 (Agilent, Santa Clara, California). Four µg of total RNA was used for the expression analysis of each sample using the Affymetrix GeneChip® Medicago Genome Array (Affymetrix, Santa Clara, California). Probe labeling, chip hybridization and scanning were performed according to the manufacturer’s instructions for one-cycle labeling (Affymetrix). Data normalization between chips was conducted using RMA (Robust Multi-chip Average) ([Irizarry et al., 2003]). Presence/absence calls for each probe set were obtained using dCHIP ([Forkmann and Martens, 2001]). Gene selections based on Associative T-test ([Dozmorov and Centola, 2003]) were made using Matlab (MathWorks, Natick, Massachusetts). A selection threshold of 2 for transcript ratios and a Bonferroni-corrected P value threshold of 8.15954E-07 were used (where the threshold was derived from 0.05/N, and N is the number of probe sets on the chip). False discovery rate of all significant genes was monitored with Q-Values obtained by EDGE software ([Storey and Tibshirani, 2003]; [Leek et al., 2006]). Transcriptome data were pre-processed.
independently and integrated using MapMan software tools customized for *Medicago* (Urbanczyk-Wochniak and Sumner, 2007).

Genes of interest were selected for further confirmation by qRT-PCR following manufacturer’s protocols (Power SYBR Green, Life Technologies). Total RNA was isolated as above, and primer pairs were designed using Primer3 software and located in the same region of the gene as the microarray probes whenever possible. LinRegPCR was used to assess amplification efficiency, and the expression data were analyzed according to (Czechowski et al., 2004) for samples without a control. Ubiquitin and helicase genes were used for normalization, as these were stably expressed in the microarray experiments. The mean ratio of the two normalization genes was used to present the data in Table I. A list of the primer pairs used for qRT-PCR is included in Table SII. The melting curves of all primer pairs except those for β-amylase and citrate synthase showed amplification of a single product. The curve for β-amylase showed a minor secondary product and the curve for citrate synthase also showed multiple products, but both were of satisfactory quality for this purpose.

**Mass spectrometry analysis**

Border cells were collected by gently agitating roots in water for 30-60 sec, and an equal volume of methanol was added to stop enzyme activity during drying. For the *P. omnivorum* experiment, border cells were collected directly in 80% methanol, 20% water (v/v). The border cells were frozen in liquid N₂, dried, ground in an Eppendorf tube and extracted for two hours in 80% methanol containing 20 μg/ml umbelliferone as an internal standard. After border cells were removed, roots were rinsed in water and root tips were excised and frozen in liquid N₂. Whole roots containing border cells and root tips were separated from the seedling hypocotyl and frozen in liquid nitrogen. The secondary metabolite analyses consisted of three replicates for each tissue. Samples were lyophilized, ground and extracted with 80% methanol as above. Samples were centrifuged and the supernatant analyzed by UPLC-QToFMS Premier™ (Waters, Inc.). Separations were achieved using a Waters Acquity UPLC, 2.1 x 150 mm BEH C18 column, mobile phases of (A) 0.1% aqueous acetic acid and (B) acetonitrile, and a linear gradient of 95%:5% to 30%:70% eluents A:B in 30 min. The mass spectrometer was operated in negative electrospray ionization mode. Raffinose was used as the reference compound. Peak picking, alignment and quantification were performed using Waters MarkerLynx software. The *P. omnivorum* samples were examined using a Waters Acquity UPLC coupled with LECO’s fast acquisition speed Unique HT TOFMS operated in negative electrospray ionization mode, follow by ChromaTOF software deconvolution. All conditions for UPLC were as listed above except eluent A was 0.1% formic acid in water. Compounds were normalized relative to the internal standard, then to the total ion abundance. Metabolite identifications were achieved via comparison of retention time and accurate mass to those of authentic standards. Tentative identifications were performed by matching experimental accurate mass data to those in plant metabolite databases and public literature within a 5 ppm mass accuracy tolerance.

For GC-MS analyses, dried polar extracts were derivatized with methoxyamine hydrochloride in pyridine followed by TMS derivatization using *N*-methyl-*N*-trimethylsilylt trifluoroacetamide and
analyzed as previously reported (Broeckling et al., 2005). Four replicates were performed. Mass spectra deconvolution and metabolite identification were performed using AMDIS software (http://chemdata.-nist.gov/mass-spc/amdis/) and a custom in-house EI-MS metabolite library. Peak picking, alignment and quantification were achieved using MET-IDEA software (Broeckling et al., 2006) (http://bioinfo.noble.org/download). Normalization was performed as described above.

Phytohormone analyses were based upon (Pan et al., 2010), with some modifications. Briefly, border cells were collected from 50 seedlings and frozen in liquid nitrogen. Root tips without border cells were collected from the same seedlings, frozen in liquid nitrogen and ground to a fine powder. Ten mg of root tip tissue and all border cell tissue was extracted in 1 mL of Isopropanol : Water : HCl (2 : 1 : 0.002 ) for one hour at 4° C with 50 pmol of the internal standards d5-IAA (C/D/N Isotopes), d6-SA (Sigma), dh-JA (TCI), d6-ABA (Icon). 0.5 mL of dichloromethane was added to each sample, and samples were shaken for another 30 minutes at 4° C. Samples were centrifuged and two phases formed. One mL of the bottom layer was transferred to a 2 mL glass vial, and the solvent was dried under nitrogen. The residue was redissolved in 0.1 mL methanol and diluted to 1 mL with 1% acetic acid. The solution was applied to a conditioned Waters Hydrophilic/Lipophilic Balanced HLB column, and the column was rinsed with 1mL of 1% acetic acid. The rinse was discarded, and phytohormones were eluted from the column using 1.8 mL 80% methanol containing 1% acetic acid and collected in a 2 mL autosampler glass vial. Solvents were dried under nitrogen and redissolved in 50 uL of 50% methanol in 1% acetic acid. 10 uL was injected onto an Agilent 1290/6430 UHPLC/MS TripleQuad system. Separations were achieved using a Waters Acquity UPLC 2.1 x 150 mm, BEH C18 column with a mobile phase of (A) 0.1% aqueous formic acid and (B) acetonitrile, and a linear gradient of 5% to 46% acetonitrile in 25 minutes. Phytohormones were detected and quantified using multiple reaction monitoring (MRM) as described in (Pan et al., 2010). At least four replicates were performed for each tissue.

Volatile emitted from border cells, root tissues without border cells and seedlings without border cells were extracted using an SPME fiber and analyzed by GC-MS. Prior to the analysis, each sample was prepared in a 10 ml glass vial. Border cells were collected from 100 seedlings into approximately 0.5 ml water. The collected border cells were transferred into a 10 ml glass vial and the vial was tightly capped. For the analysis of root volatiles, the root portion of 50 seedlings without border cells was placed inside a 10 ml glass vial. The top part of the seedlings was covered with aluminum foil. The analysis of volatiles from whole seedling minus border cells was conducted on 50 seedlings in a tightly capped 10 ml glass vial. A divinylbenzene / carboxen / polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm, 2 cm) SPME fiber (Supelco, Bellefonte, Pennsylvania) was used to extract the headspace volatiles for 60 minutes at 30° C. SPME-absorbed volatiles were desorbed at 250° C for 90 seconds in a splitless GC injector. Separation of volatiles was achieved with an Agilent 6890/5973 GC-MS (Palo Alto, California) equipped with a 60 m length, 0.25 mm i.d., 0.25 μm film thickness, fused silica capillary column (DB-5, Agilent). Helium was used as the carrier gas with a flow rate of 1.0 ml min⁻¹. The column temperature was held at 40° C for two minutes and then programmed at 5° C min⁻¹ to 250° C and held for three minutes. MS conditions were as follows: ion
source, 200° C; electron energy, 70 eV; quadrupole temperature, 150° C; GC-MS interface zone, 280° C; scan range, 35-350 mass units. The SPME fiber was heated to 250° C for 20 minutes to remove carryover between extractions, and three replicates of each tissue were analyzed. Hexanal was identified by its Kovats’ retention index (RI) and by comparison to the mass spectra of an authentic standard. The amount of hexanal was quantified using a standard curve of hexanal as the external standard and calculated on a per-plant basis.

ACKNOWLEDGMENTS
The authors thank Vagner Benedito for assistance analyzing qRT-PCR data; Jin Nakashima, Terry Colberg and Elison Blancaflor for help with microscopy; and Zhentian Lei, Elison Blancaflor and Michael Udvardi for a careful reading of the manuscript. The Noble Foundation provided funding for this research. NSF equipment grant DBI 0400580 provided funds to purchase the confocal microscope. Instrumental support was provided by LECO Corporation. Metabolite identifications were partially enabled through resources provided by NSF Awards #1139489 and #1126719.

Supporting Information
Supporting information may be found in the online version of this article.

Figure S1. Border cells and root tips of M. truncatula. A, SEM of matrix and entangled border cells; B, eSEM of seedling root with detaching border cells; C, fluorescein diacetate confocal of root tip with detached border cells; D-F, Confocal image of detached border cells stained with D, fluorescein diacetate, E, propidium iodide, F, overlay of D and E; the blue arrows point to a non-viable cell. Bars for: A = 25µm, B-F = 50 µm.

Figure S2. A seedling root with detaching border cells in their associated mucilage stained with 0.02% ruthenium red. Size bar = 100 µm.

Figure S3. Starch in different root cell types. A-B, DIC of free border cells with the number of starch granules ranging from 0 to ~ 22; black arrows point to starch granules. C, Columella cells filled with starch granules in a median root tip section (red arrows). D, non-median section of root tip cells containing starch granules. Size bars = 25µm.

Figure S4. The root cap cells of an iodine-stained seedling root contain many starch bodies and stain darker than cells in the elongation zone of the root. Size bar = 50 µm.

Figure S5. Overview of border cells/whole roots transcript ratios in major metabolic pathways visualized by MapMan. Transcripts significantly up- and downregulated are indicated in blue and yellow, respectively. Scale bars display fold changes.

Figure S6. Overview of transcript profiling results. The graph represents the percent of transcripts assigned to each non-redundant functional category based on MapMan software. The smaller pie charts represent all transcripts, and the white sections represent the percentage of assigned transcripts. BC>WR – transcripts increased in border cells relative to whole roots. BC<WR – transcripts decreased in border cells compared to whole roots.

Figure S7. A. Phytohormone analyses of root tips, the remainder of the root after the root tip is removed, and whole roots. B. Early steps in the oxylipin pathway highlighting the connection between JA and hexanal biosynthesis.
Table SI. Detailed methods for the microarray analysis and a complete list of normalized expression values of Medicago Affymetrix Genechip transcripts of three different root tissues: border cells; root tip minus border cells; and whole roots.

Table SII. Primers used for qRT-PCR validation of microarray data.

Table SIII. Metabolomics Standards Initiative compliant metadata and complete list of metabolites from *M. truncatula* border cells, root tips minus border cells and whole roots.

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Figure Legends

Figure 1. Border cells and root tips of *M. truncatula*. A, SEM of seedling root with attached border cells and polysaccharide matrix; B, Confocal image of detached border cells stained with fluorescein diacetate; C, Border cell at root tip containing starch granules; blue arrow points to border cell. D, Free border cells with starch granules. Bars for: A, B = 50µm, C, D = 25µm.

Figure 2. Overview of border cells/root tips transcript ratios in major metabolic pathways visualized using MapMan. Transcripts significantly up- and downregulated are indicated in blue and yellow, respectively. Scale bars display fold changes.
Figure 3. Overview of transcript profiling results. The graph represents the percent of transcripts assigned to each non-redundant functional category based on MapMan software. The smaller pie charts represent all transcripts, and the white sections represent the percentage of assigned transcripts. BC Only – transcripts observed only in border cells. BC>RT – transcripts increased in border cells relative to root tips. BC<RT – transcripts decreased in border cells compared to root tips.

Figure 4. Relative expression levels of A, pectin methylesterase inhibitor (PMEI) and B, auxin responsive small auxin up RNA (SAUR) protein determined using the *M. truncatula* Gene Expression Atlas (Benedito et al., 2008).

Figure 5. Principal Component Analysis of A, primary and B, secondary metabolite profiling data from border cells (BC) and root tips (RT). PC1 for primary metabolites explains 57% of variance and PC2 explains 28% of variance using ~500 mass features. PC1 for secondary metabolites explains 79% of variance and PC2 explains 7% of variance using ~3100 mass features.

Figure 6. Systems model of major metabolic and transcriptional differences in *M. truncatula* border cells. Cumulative constitutive data provide evidence that border cells have enhanced metabolic capacity and content relative to root tips. Starch reserves in border cells are directed towards increased secondary metabolism as opposed to fueling continued cell growth and division. The enhanced secondary metabolism of border cells fortifies them as front-line defenders in plant-pathogen interactions and important ambassadors in mutualistic signaling.

Figure 7. A, Relative abundance of 7,4’-dihydroxyflavone in water control (con) and *P. omnivora* (*Po*) treated border cells (gray bars) and root tips (black bars) after 24 hours (24) and 48 hours (48) exposure; n = 3 or 4 and the error bars represent standard error. B, In-vitro assay of antifungal activity after five days of no-treatment control, DMSO only, and two concentrations of 7,4’-dihydroxyflavone against *P. omnivora*. Fungal growth was quantified on a scale of 0-5 with 0 meaning no growth and 5 no inhibition: control and DMSO = 5, 0.1mM = 1, 0.5 mM = 0.

Figure 8. A, Phytohormone content of border cells (black bars) and root tips (gray bars). B, headspace hexanal concentration of border cells, roots without border cells and seedlings without border cells. Volatiles were analyzed from border cells collected from 100 roots and compared on the basis of 50 roots. The error bars represent standard errors. ND = not detected.
### Table I. Summary of validated genes, expression levels and related metabolite levels (BC/RT).

| Class                      | Gene                          | Microarray BC/RT | qRT-PCR BC/RT | Metabolites BC/RT |
|----------------------------|-------------------------------|------------------|---------------|-------------------|
| Genes in Primary Metabolism|                               |                  |               |                   |
| Major Carbohydrate Metabolism | β-amylase TC94273              | 18x increase     | 15x increase  | Starch detected by microscopy |
| Glycolysis                 | PF6P1P TC101885                | 12x increase     | 28x increase  | Fructose derivatives 3-4x decrease |
| TCA Cycle                  | Citrate synthase-like BQ153338| 3x increase      | 2x increase   | Citrate 5x increase |
| Amino Acids                | Asparagine Synthetase TC100391| 6x increase      | 3x increase   | Asparagine cumulative 3x increase in border cells |
| Not assigned (β-alanine)   | β–ureido-propionase TC100938   | 7x increase      | 3x increase   | β-alanine 6x increase |
| Genes in Secondary Metabolism|                               |                  |               |                   |
| Phenylpropo-noids          | PAL TC101026                   | 16x increase     | 30x increase  | Phenylalanine abundant in border cells |
| Lignin and lignans         | CCoAOMT-like protein BM814917 | 18x increase     | 24x increase  | Lignin in border cells (from literature) |
| Flavonoids                 | Naringenin-chalcone synthase TC102405 | 50x increase | 64x increase | Apigenin 6x increase |
| Flavonoids                 | Flavone synthase II BM779623  | 8x increase      | 7x increase   | 7, 4’-DHF 13x increase |
| Isoflavonoids              | Isoflavone synthase-like TC106940 | 13x increase | 13x increase | not detected |
| Terpenoids                 | β-amyrin synthase AW689929     | 3x decrease      | 1.2x decrease | up to 32x increase in saponins |
| Terpenoids                 | CYP71A8 BE943181               | 21x increase     | 91x increase  | up to 32x increase in saponins |
| Terpenoids | (-)-germacrene D synthase TC94781 | 34x increase | 167x increase | Volatile terpenes not detected with this method |
|------------|----------------------------------|--------------|--------------|------------------------------------------------|
| **Additional Categories** | | | | |
| Jasmonate | Lipoxygenase TC106479 | 224x increase | 126x increase | less than in root tips |
| IAA | Auxin responsive SAUR protein BQ157435 | 38x increase | 884x increase | not detected in border cells |
| Cell Walls | PME TC103769 | 16x decrease | 7x decrease | (Wen et al, 1999) |
| Cell Wall | PME Inhibitor AC134522_38.4 | 45x increase | 115x increase | novel |
Table II Primary metabolites identified in border cells and root tips

| SUGARS                          | BC/RT | x fold | p value | ion    | retention |
|---------------------------------|-------|--------|---------|--------|-----------|
| Sucrose TMS                     |       | 3.68   | 0.0092  | 361.2  | 45.1907   |
| Fructose 5-TMS MEOX2            |       | 0.29   | 0.0003  | 217.2  | 32.0651   |
| Fructose 5-TMS MEOX1            |       | 0.27   | 0.0006  | 217.2  | 31.8745   |
| Xylose 4-TMS MEOX2              |       | 0.65   | 0.0219  | 217.1  | 27.1635   |
| Arabinose 4-TMS MEOX1           |       | 0.62   | 0.0015  | 217.1  | 27.6668   |
| Ribose 4-TMS MEOX               |       | 0.55   | 0.0089  | 308.2  | 27.3277   |
| Fructose-6-phosphate 6-TMS MEOX |       | 0.38   | 0.0149  | 315.1  | 39.9454   |
| Glucose-6-phosphate TMS MEOX1   |       | 0.21   | 0.0020  | 387.1  | 40.1773   |
| Galactose 5-TMS MEOX1           |       | 0.20   | 0.0001  | 319.2  | 32.2821   |
| Glucose 5-TMS MEOX2             |       | 0.20   | 0.0001  | 319.2  | 32.4089   |

| ORGANIC ACIDS                   |       |        |         |        |           |
| Succinic acid 2-TMS             |       | 5.78   | 0.0060  | 247.1  | 18.8065   |
| Citric Acid TMS                 |       | 5.39   | 0.0109  | 273.1  | 30.8361   |
| Shikimic acid 4-TMS             |       | 4.17   | 0.0014  | 204.1  | 30.5913   |
| Benzoic Acid TMS                |       | 3.96   | 0.0010  | 194.1  | 17.1936   |
| Hexanoic acid TMS               |       | 3.92   | 0.0002  | 173.1  | 12.1131   |
| Nicotinic Acid TMS              |       | 3.55   | 0.0032  | 180.1  | 18.487    |
| Propionic Acid 3-TMS            |       | 3.53   | 0.0029  | 189.1  | 19.1171   |
| Fumaric Acid 2-TMS              |       | 3.08   | 0.0056  | 245.1  | 19.7831   |
| Hexadecanoic Acid TMS           |       | 2.81   | 0.0045  | 313.3  | 35.5837   |
| Malic acid 3-TMS                |       | 2.53   | 0.0188  | 233.1  | 23.2597   |
| Butanoic acid 4-TMS             |       | 2.13   | 0.0362  | 174.1  | 28.4915   |
| Nonanoic Acid                   |       | 1.96   | 0.0251  | 215.1  | 20.1453   |
| Pyruvic acid TMS MEOX1          |       | 1.66   | 0.0113  | 174.1  | 11.3521   |

| AMINO ACIDS                     |       |        |         |        |           |
| Glutamine 3-TMS                 |       | 97.50  | 0.0226  | 246.1  | 29.9694   |
| Isoleucine TMS                  |       | 23.76  | 0.0018  | 188.2  | 15.1115   |
| Proline +CO₂ 2-TMS              |       | 9.66   | 0.0013  | 186.1  | 25.7291   |
| Proline 2-TMS                   |       | 7.48   | 0.0046  | 216.1  | 18.4395   |
| Proline TMS                     |       | 6.10   | 0.0002  | 172.1  | 15.1135   |
| Glycine 3-TMS                   |       | 7.49   | 0.0017  | 174.1  | 18.6267   |
| Glycine 2-TMS                   |       | 6.35   | 0.0008  | 204.1  | 13.399    |
| Homoserine 3-TMS                |       | 7.47   | 0.0018  | 218.2  | 22.317    |
| Serine 4-TMS                    |       | 6.65   | 0.0011  | 290.1  | 25.3873   |
| Serine 2-TMS                    |       | 4.36   | 0.0008  | 219.1  | 17.2432   |
| Serine 3-TMS                    |       | 4.06   | 0.0017  | 204.1  | 19.9493   |
| Threonine 3-TMS                 |       | 4.92   | 0.0044  | 218.1  | 20.6207   |
| Threonine 2-TMS                 |       | 3.01   | 0.0032  | 219.1  | 18.2625   |
| Asparagine +CO₂ 4-TMS           |       | 4.58   | 0.0003  | 232.1  | 33.5789   |
| AMINO ACIDS continued | BC/RT | p value | ion | time  |
|-----------------------|-------|---------|-----|-------|
| Asparagine 4-TMS      | 3.05  | 0.0161  | 188.1| 32.0088|
| Asparagine 2-TMS      | 2.84  | 0.0010  | 159.1| 26.077 |
| Asparagine 3-TMS      | 2.60  | 0.0118  | 231.2| 27.6817|
| Alanine +CO₂ 2-TMS    | 3.26  | 0.0020  | 160.1| 21.0853|
| Alanine 3-TMS         | 3.21  | 0.0007  | 188.2| 20.0924|
| Valine TMS ester      | 2.72  | 0.0001  | 156.1| 12.5797|
| Valine 2-TMS          | 2.36  | 0.0169  | 218.1| 16.0488|
| Tyrosine 2-TMS        | 1.70  | 0.0416  | 219.1| 33.4378|
| Lysine 3-TMS          | 0.51  | 0.0014  | 174.1| 31.7517|
| Glutamic acid 3-TMS   | 0.72  | 0.0062  | 246.1| 26.4894|
| Phenylalanine 2-TMS   | 0.67  | 0.0101  | 218.1| 26.8129|
| **CoA SYNTHESIS**     |       |         |     |       |
| beta-Alanine 3-TMS    | 5.53  | 0.0007  | 174.1| 21.8571|
| **MISCELLANEOUS**     |       |         |     |       |
| Urea 2-TMS            | 5.19  | 0.0022  | 171.1| 16.8071|

*italicized amino acids are non standard*
TMS= trimethylsilyl
MEOX=methyloxime
| PHENOLICS | ID | BC/RT | retention | m/z |
|-----------|----|-------|-----------|-----|
| 4-hydroxy-7-methoxy flavone | 1 | nd in rt | 0.0042 | 12.30 | 267.0685 |
| Epicatechin Pentose | 2 | 21.45 | 0.0005 | 8.88 | 421.2085 |
| Epicatechin Pentose | 2 | 15.30 | 0.0000 | 9.42 | 421.2073 |
| 7,4-dihydroxyflavone | 1 | 12.51 | 0.0004 | 7.42 | 253.0492 |
| Apigenin | 1 | 5.92 | 0.0002 | 9.96 | 269.0445 |
| Luteolin 7-glucoside | 2 | 1.96 | 0.0040 | 3.91 | 447.097 |
| Naringenin chalcone 4-O-glucoside | 2 | 1.92 | 0.0034 | 3.92 | 433.115 |
| 4-Methylumbelliferone | 1 | 1.68 | 0.0012 | 5.98 | 175.0391 |
| Kaempferol-3-O-rutinoside | 1 | 0.33 | 0.0031 | 6.05 | 593.1519 |
| Unknown phenolic | 2 | 0.30 | 0.0005 | 5.27 | 577.156 |
| Daidzin | 1 | 0.10 | 0.0028 | 4.56 | 415.1044 |

| SAPONINS OR SAPOGENINS | ID | BC/RT | retention | m/z |
|-------------------------|----|-------|-----------|-----|
| Hex-Hex-Hex-Bayogenin | 2 | nd in rt | 0.0002 | 13.88 | 973.5013 |
| Rha-Hex-Hex-Bayogenin | 2 | 31.90 | 0.0016 | 13.97 | 957.5092 |
| Rha-Hex-Hex-Bayogenin | 2 | 22.38 | 0.0072 | 8.46 | 1119.5665 |
| 3-Glc-Glc-28-Ara-Rha-Xyl Medicagenic Acid | 1 | 19.25 | 0.0017 | 13.12 | 1087.4988 |
| Rha-Hex-Hex-Bayogenin | 2 | 18.63 | 0.0001 | 13.38 | 957.5135 |
| 3-Glc-Malonyl-Medicagenic Acid | 2 | 12.50 | 0.0266 | 16.76 | 705.3861 |
| HexA-Hex-Soyasapogenol E | 2 | 6.32 | 0.0000 | 18.74 | 793.4370 |
| 3-Glc-Malonyl-Medicagenic Acid | 2 | 6.30 | 0.0006 | 17.92 | 705.3877 |
| 3-Rha-Gal-GlcA-Soyasapogenol B | 2 | 5.92 | 0.0004 | 17.02 | 941.5163 |
| Arab/Xyl-Gypsogenin | 3 | 5.81 | 0.0046 | 11.63 | 1221.5609 |
| 3-Glc-Malonyl-Medicagenic Acid | 2 | 5.31 | 0.0392 | 14.79 | 705.3874 |
| Hex-Hex-Hex-Hederagenin | 2 | 4.90 | 0.0014 | 14.74 | 957.5079 |
| Hex-Hex-Hex-Bayogenin | 2 | 4.67 | 0.0003 | 11.11 | 973.5040 |
| Hex-Gypsogenic acid | 2 | 4.56 | 0.0009 | 16.29 | 647.3811 |
| 3-Glc-28-Ara-Rha-Xyl Medicagenic Acid | 2 | 4.50 | 0.0291 | 13.68 | 1073.5138 |
| 3-Rha-Xyl-GlcA-Gypsogenic Acid | 2 | 4.33 | 0.0053 | 18.53 | 939.4972 |
| 3-Glc-Malonyl-Medicagenic Acid | 2 | 2.89 | 0.0287 | 17.41 | 705.3878 |
| 3-Glc-Medicagenic Acid | 1 | 2.10 | 0.0029 | 17.02 | 663.3768 |
| Echinocystic Acid | 2 | 1.66 | 0.0277 | 26.10 | 471.3476 |
| Rha-Hex-Hex-Bayogenin | 2 | 1.59 | 0.0142 | 15.54 | 957.5125 |
| Hex-Gypsogenic Acid | 2 | 1.33 | 0.0178 | 18.22 | 647.3803 |
| 3-Rha-Gal-GlcA-Soyasapogenol B | 2 | 1.18 | 0.0431 | 16.47 | 941.5138 |
| Hex-Soyasapogenol E | 2 | 0.45 | 0.0056 | 21.44 | 617.4059 |
| 3-Rha-Gal-GlcA-Soyasapogenol B | 2 | 0.13 | 0.0021 | 18.89 | 941.5139 |
| Hex-Hex-Rha-Bayogenin | 2 | 0.12 | 0.0032 | 12.00 | 957.5054 |
| Rha-Hex-Hex-Bayogenin | 2 | 0.07 | 0.0001 | 11.17 | 1119.5549 |
| 3-Glc-28-Glc-Medicagenic Acid | 1 | 0.02 | 0.0000 | 13.03 | 825.4293 |
| Hex-Hex-Bayogenin | 2 | 0.01 | 0.0000 | 12.41 | 973.5075 |
Table III continued

| Compound                        | RT (min) | nd in bc | m/z  | ppm  |
|---------------------------------|----------|----------|------|------|
| Hex-Rha-Hex-Hex-Hederagenin     | 12.85    | nd in bc | 1103.5693 |
| Rha-Hex-Hex-Hex-Bayogenin       | 12.00    | nd in bc | 1119.5616  |
| Hex-Hex-Hex-Bayogenin           | 11.64    | nd in bc | 973.5012    |
| Hex-Hex-Hex-Medicagenic Acid    | 12.70    | nd in bc | 987.4860    |
| Hex-Hex-Rha-Bayogenin           | 11.18    | nd in bc | 957.5043    |

1 = identification using authentic standards
2 = putative identification using accurate mass (database search ± 6ppm)  Echinocystic acid has been identified by Tava, et al 2011, [M-H]- 471
3= putative identification using tandem MS. The aglycone has the same m/z as Aglycone B in Pollier, et al 2011.
nd = not detected
minimum area of border cell peak is 1/100 of Internal Standard
Table IV. In-vitro antifungal activity of catechol and flavonoids against *P. omnivora*

| Compounds              | control | DMSO  | 5.0 mM | 10.0 mM |
|------------------------|---------|-------|--------|---------|
| catechol               | 5       | 5     | 4      | 4       |
|                        |         |       | 2      | 2       |
|                        |         |       | 0      | 0       |

| Compounds              | control | DMSO  | 0.1 mM | 0.5 mM |
|------------------------|---------|-------|--------|--------|
| formononetin           | 5       | 5     | 4      | 5      |
|                        |         |       | 5      | 5      |
|                        |         |       | 5      | 5      |
| narigenin              | 5       | 5     | 4      | 4      |
|                        |         |       | 4      | 4      |
|                        |         |       | 4      | 4      |
| isoliquirifigenin      | 5       | 5     | 4      | 4      |
|                        |         |       | 4      | 4      |
|                        |         |       | 4      | 4      |
| apigenin               | 5       | 5     | 4      | 4      |
|                        |         |       | 4      | 3      |
|                        |         |       | 4      | 4      |
| medicarpin             | 5       | 5     | 4      | 4      |
|                        |         |       | 2      | 1      |
|                        |         |       | 1      | 0      |
| 7,4’-dihydroxyflavone  | 5       | 5     | 5      | 5      |
|                        |         |       | 1      | 1      |
|                        |         |       | 0      | 0      |

Qualitative measurement of fungal infection – inhibition effect is scored by numbers with 5 standing for no inhibition and 0 for complete inhibition.
Table V Comparison of border cell and root tip transcripts increased/decreased in primary and secondary metabolism

| Category                                      | Number transcripts increased in BC_RT | Number transcripts decreased in BC_RT |
|-----------------------------------------------|---------------------------------------|---------------------------------------|
| Carbohydrate metabolism (major and minor)     | 32                                    | 55                                    |
| Glycolysis                                    | 7                                     | 23                                    |
| TCA/organic acid transformation               | 9                                     | 22                                    |
| Amino acid metabolism                         | 39                                    | 96                                    |
| Secondary metabolism                          | 142                                   | 82                                    |
| Flavonoids                                    | 56                                    | 20                                    |
| Terpenoids                                    | 24                                    | 19                                    |
Figure 1. Border cells and root tips of *M. truncatula*. A, SEM of seedling root with attached border cells and polysaccharide matrix; B, Confocal image of detached border cells stained with fluorescein diacetate; C, Border cell at root tip containing starch granules; blue arrow points to border cell. D, Free border cells with starch granules. Bars for: A, B = 50 µm, C, D = 25 µm.
Figure 2. Overview of border cells/root tips transcript ratios in major metabolic pathways visualized using MapMan. Transcripts significantly up- and downregulated are indicated in blue and yellow, respectively. Scale bars display fold changes.
Figure 3. Overview of transcript profiling results. The graph represents the percent of transcripts assigned to each non-redundant functional category based on MapMan software. The smaller pie charts represent all transcripts and the white sections represent the percentage of assigned transcripts. BC Only – transcripts observed only in border cells; BC>RT – transcripts increased in border cells relative to root tips; BC<RT – transcripts decreased in border cells compared to root tips.
Figure 4. Relative expression levels of A, pectin methylesterase inhibitor (PMEI) and B, auxin responsive small auxin up RNA (SAUR) protein determined using the *M. truncatula* Gene Expression Atlas (Benedito et al., 2008).
Figure 5. Principal Component Analysis of A, primary and B, secondary metabolite profiling data from border cells (BC) and root tips (RT). PC1 for primary metabolites explains 57% of variance and PC2 explains 28% of variance using ~500 mass features. PC1 for secondary metabolites explains 79% of variance and PC2 explains 7% of variance using ~3100 mass features.
Figure 6. Systems model of major metabolic and transcriptional differences in *M. truncatula* border cells. Cumulative constitutive data provide evidence that border cells have enhanced metabolic capacity and content relative to root tips. Starch reserves in border cells are directed towards increased secondary metabolism as opposed to fueling continued cell growth and division. The enhanced secondary metabolism of border cells fortifies them as front-line defenders in plant-pathogen interactions and important ambassadors in mutualistic signaling.
Figure 7. A, Relative abundance of 7,4'-dihydroxyflavone in water control (con) and \textit{P. omnivora} (Po) treated border cells (gray bars) and root tips (black bars) after 24 hours (24) and 48 hours (48) exposure; \( n = 3 \) or 4 and the error bars represent standard error. B, In-vitro assay of antifungal activity after five days of no-treatment control, DMSO only, and two concentrations of 7,4'-dihydroxyflavone against \textit{P. omnivora}. Fungal growth was quantified on a scale of 0-5 with 0 meaning no growth and 5 no inhibition: control and DMSO = 5, 0.1mM = 1, 0.5 mM = 0.
Figure 8. A, Phytohormone content of border cells (black bars) and root tips (gray bars). B, headspace hexanal concentration of border cells, roots without border cells and seedlings without border cells. Volatiles were analyzed from border cells collected from 100 roots and compared on the basis of 50 roots. The error bars represent standard errors. ND = not detected.