Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism

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Abstract

GC-MS-based metabolite profiling was used to analyse the response of *Medicago truncatula* cell cultures to elicitation with methyl jasmonate (MeJa), yeast elicitor (YE), or ultraviolet light (UV). Marked changes in the levels of primary metabolites, including several amino acids, organic acids, and carbohydrates, were observed following elicitation with MeJa. A similar, but attenuated response was observed following YE elicitation, whereas little response was observed following UV elicitation. MeJa induced the accumulation of the triterpene β-amyrin, a precursor to the triterpene saponins, and LC-MS analysis confirmed the accumulation of triterpene saponins in MeJa-elicited samples. In addition, YE induced a slight, but significant accumulation of shikimic acid, an early precursor to the phenylpropanoid pathway, which was also demonstrated to be YE-inducible by LC-MS analyses. Correlation analyses of metabolite relationships revealed perturbation of the glycine, serine, and threonine biosynthetic pathway, and suggested the induction of threonine aldolase activity, an enzyme as yet uncharacterized from plants. Members of the branched chain amino acid pathway accumulated in a concerted fashion, with the strongest correlation being that between leucine and isoleucine ($r^2=0.941$). While UV exposure itself had little effect on primary metabolites, the experimental procedure, as revealed by control treatments, induced changes in several metabolites which were similar to those following MeJa elicitation. Sucrose levels were lower in MJ- and YE-elicited samples compared with control samples, suggesting that a portion of the effects observed on the primary metabolic pool are a consequence of fundamental metabolic repartitioning of carbon resources rather than elicitor-specific induction. In addition, β-alanine levels were elevated in all elicited samples, which, when viewed in the context of other elicitation responses, suggests the altered metabolism of coenzyme A and its esters, which are essential in secondary metabolism.

Key words: Elicitation, *Medicago truncatula*, metabolite profiling, metabolomics, methyl jasmonate, primary metabolism, ultraviolet light.

Introduction

*Medicago truncatula* is a rapidly developing model for the study of legume biology, and is an excellent species for fundamental studies on the unique secondary metabolism of legumes (Dixon and Sumner, 2003). Two classes of secondary metabolites are of particular interest. Isoflavonoids are a subclass of the phenylpropanoids which have been attributed with health-promoting properties, and are nearly exclusive to leguminous plants. The biosynthetic pathway leading to the production of isoflavonoids can be elicited by the application of yeast cell wall extract (Kessmann et al., 1990) or pathogen attack and is relatively well understood, with several of the enzymes characterized (Liu et al., 2002). The phenylpropanoid pathway is also commonly induced following exposure to UV light, serving to limit photochemical damage through the UV absorbent character of phenylpropanoids (Mazza et al., 2000). Another important class of
secondary metabolites from *M. truncatula* are the triterpene saponins, which possess antiherbivore (Papadopoulos et al., 1999), allelopathic (Waller et al., 1993), anti-microbial (Osbourn, 2003), anti-insect (Tava and Odoardi, 1996), and anti-palatability activity, as well as anti-nutritional (Oleszek et al., 1999) effects. Saponins also exhibit anticholesterolemic, anti-cancer (Haridas et al., 2001), adjuvant (Behboudi et al., 1999), and haemolytic (Oh et al., 2000) activity. *M. truncatula* has a diverse saponin content (Huhman and Sumner, 2002) which is regulated by jasmonates, as exogenous application of methyl jasmonate to *M. truncatula* cell cultures results in the accumulation of saponins within 48 h of elicitation (Suzuki et al., 2002). Whereas the enzymes responsible for isoflavone biosynthesis are fairly well characterized, those involved in the biosynthesis of triterpene saponins are still relatively unknown.

Secondary metabolites are nearly universally derived from primary metabolic pathways. For example, flavonoids throughout the plant kingdom, and more specifically isoflavonoids of legumes, are derived initially through the phenylpropanoid pathway, originating from the protein amino acid phenylalanine (Kessmann et al., 1990). Likewise, the triterpene saponins are derived from the cyclization of 2,3-oxidosqualene, which also serves as the precursor to membrane phytosterols (Suzuki et al., 2002).

Only recently has the monitoring of metabolites grown into an ‘omics’ level field (Trehwey et al., 1999). Gas chromatography-mass spectrometry (GC-MS) has been applied to examine the effects of genetic and environmental manipulations (Roessner et al., 2001), to determine plasmid composition (Fiehn, 2003), for plant genotyping (Taylor et al., 2002) and, recently, for detecting silent phenotypes in transgenic potato (Weckwerth et al., 2004). GC-MS is currently the most developed of the available analytical tools, but other techniques are currently in use or being developed (Sumner et al., 2003). The growth of this technology offers the opportunity to view the effect of elicitation on metabolism at a larger scale than previously possible.

This study comprises a portion of an integrated functional genomics project studying the effects of elicitation with various biotic and abiotic elicitors on three biological levels of function: the transcriptome, the proteome, and the metabolome (VandenBosch and Stacey, 2003). This global approach should paint a more complete picture of the cellular response to elicitation than previously available. The approach to the metabolomics portion of this study attempts to cover a large portion of the metabolome, with primary metabolites monitored by gas chromatography-mass spectrometry (GC-MS), lower abundant intermediates by capillary electrophoresis, and secondary metabolites by liquid chromatography-mass spectrometry (LC-MS).

The results of GC-MS based metabolite analyses reveal the effect of elicitation on the accumulation of many primary metabolites and their interrelationships. In addition, correlation analyses revealed more universal metabolite relationships which are robust to elicitor-induced metabolic reprogramming. The results suggest both elicitor-specific changes in metabolite abundance and correlations as well as a more generic response in which metabolites demonstrate a similar trend regardless of the elicitor used.

### Materials and methods

#### Cell cultures and elicitation

A total of four separate experiments were performed. Three, one for each of the three elicitors, were highly detailed and included 21 sampling points over a 48 h period following elicitation (Table 1). The first time-course examined elicitation with methyl jasmonate (MeJa), the second with yeast elicitor (YE), and the third with UV-light (UV). Each of the three time-courses contained, in addition to the primary elicitor, 2–3 time points for each of the other elicitors, allowing evaluation of the potential effect of cell culture passage in monitoring the response. The fourth time-course contained fewer time points, but all three elicitors were examined simultaneously in order to allow a direct comparison of the cell culture responses to the elicitors at the same passage and to validate the responses previously demonstrated in the detailed time-courses using single elicitors.

Callus culture was initiated from *M. truncatula* roots, maintained on modified Schenk and Hilderbrandt (1971, SH) agar plates (see below) in the dark at 25 °C, and subcultured approximately every 4 weeks. Liquid subcultures were initiated in 40 ml modified SH medium in a 125 ml Erlenmeyer flask from 5.0 g callus and maintained in the dark at 22 °C with shaking at ~130 rpm. Liquid media were composed of sucrose (30.0 g l⁻¹), KNO₃ (2.525 g l⁻¹), MgSO₄ (370 mg l⁻¹), NH₄H₂PO₄ (290 mg l⁻¹), CaCl₂ (220 mg l⁻¹), myo-inositol (1.0 g l⁻¹), MnSO₄ (8.925 mg l⁻¹), H₂BO₃ (5 mg l⁻¹), ZnSO₄·7H₂O (1.0 mg l⁻¹), KI (1.0 mg l⁻¹), FeSO₄·7H₂O (15.0 mg l⁻¹) Na₂EDTA (20 mg l⁻¹), thiamine.HCl (5 mg l⁻¹), nicotinic acid (5 mg l⁻¹), pyridoxine.HCl (0.5 mg l⁻¹), Na₂MoO₄ (0.1 mg l⁻¹), CoCl₂·6H₂O (0.1 mg l⁻¹), CuSO₄·5H₂O (0.2 mg l⁻¹), potassium (0.11 mg l⁻¹), 2,4-D (0.45 mg l⁻¹), and PCPA (1.87 mg l⁻¹). Solid media (for UV elicitation) additionally contained 8 g Bacto™agar l⁻¹. Cultures were transferred to 250 ml flasks and subcultured approximately every 2 weeks until elicited.

Triplicate biological replicates were collected for both control and elicited samples at each time point, with each replicate collected from a separate culture flask. Thus, each elicitation time-course contained 126 culture flasks, in addition to 12–18 confirmatory samples of elicitations (with controls) other than the primary for that time-course.

For MeJa elicitation, 2.5 ml of a 50 mM solution of methyl jasmonate in ethanol was added to culture flasks to achieve a final concentration of 500 μM. Control flasks received 2.5 ml ethanol. MeJa elicitation was performed during the 9th passage. The YE time-course was conducted during the 11th passage by adding 2.5 ml of a concentration of 50 μg glucose equivalents ml⁻¹ (Schumacher et al., 1987). UV elicitation was performed during the 12th passage. Cultures were stained from cell culture media and spread onto 150 ml plates containing ~50 ml modified SH agar. Treatment plates were irradiated in a UV box for 5.5 min at 8000 J m⁻² while control plates received no exposure. Plates were then held on an illuminated shelf at 24 °C until harvested. At the time of harvest, the entire cell population was collected by vacuum filtration, washed with 50 ml 25% MS salts, divided into four 50 ml tubes, and flash-frozen in liquid N₂.

#### Metabolite analysis of cell culture tissue

One 50 ml tube containing frozen tissue was lyophilized for 48–72 h and harvested, noting that the tissue was maintained in its frozen state...
through evaporative cooling during the lyophilization process. Dried tissue was homogenized with a glass rod, and 6.0–6.05 mg of dried tissue was weighed into a 4.0 ml glass vial. The dried tissue was stored at –80 °C until extraction. Chloroform (1.5 ml) containing 25 μg ml⁻¹ docosanol (internal standard) was added to dried tissue. The sample was then vortexed and incubated for 45 min at 50 °C. After equilibrating to room temperature, 1.5 ml of HPLC-grade water containing 25 μg ml⁻¹ ribitol was added to the chloroform. The sample was then vortexed, and incubated for a second 45 min period. The biphasic solvent system was then centrifuged at 2900 g for 30 min and the resulting supernatant was evaporated under nitrogen to dryness. The residue was resuspended in 300 μl of 45% MeOH (isolavonoids) or 100 μl water (triterpine saponins) and the samples were analysed by LC-MS.

An Agilent 1100 series II LC system (Agilent Technologies, Palo Alto, CA) equipped with a photodiode array detector was coupled to a Bruker Esquire ion-trap mass spectrometer equipped with an electrospray-ionization source. UV spectra were obtained by scanning from 200 nm to 600 nm. A reverse-phase, C18, 5 μm, 4.6×250 mm column (JT Baker, Phillipsburg, NJ) was used for separations. The mobile phase consisted of eluent A (0.1% [v/v] CH₃COOH/water) and eluent B (acetonitrile), and separations achieved using a linear gradient of 5–90% B (v/v) over 70 min. The flow rate was 0.8 ml min⁻¹ and the temperature of the column was maintained at 28 °C. Both positive and negative ion mass spectra were acquired. Positive-ion ESI was performed using an ion source voltage of 4.0 kV and a capillary offset voltage of 86.0 V. Nebulization was aided with a coaxial nitrogen sheath gas provided at a pressure of 60 psi. Desolvation was aided using a counter current nitrogen flow set at a pressure of 12 psi and a capillary temperature of 300 °C. Mass spectra were recorded over the range 50–2200 m/z. The Bruker ion-trap mass spectrometer (ITMS) was operated under an ion current control (ICC) of approximately 10 000 with a maximum acquire time of 100 ms.

Data analysis

Relative metabolite abundances were calculated using a custom PERL script to extract peak areas of individual ions characteristic of each component. Metabolites were identified through spectral and retention time matching with authentic compounds prepared in an identical manner. Identities were further confirmed through spectral matching against the National Institutes of Standards and Technology (NIST) library. Peak areas were normalized by dividing each peak area value by the mean peak area for that compound, with each time-course treated independently. Correlation analyses were performed with a custom PERL script executing Pearson’s correlation formulas (Zar, 1999). Principal component analysis (PCA) was performed on normalized datasets with Pirouette™ (InfoMetrix, Woodinville, WA) software. Cumulative GC-MS metabolite profiling data is provided as supplementary materials (S1 and S2).

Results

Analytical and biological reproducibility

The instrumental variation attributed to multiple chromatographic analyses of the same sample was quantified to determine whether replicate GC-MS analyses of each extract were warranted. Duplicate GC-MS analyses were performed for each sample of the MeJa time-course, and all peaks from
the polar extracts consistently above the limit of detection, 249 in total, were analysed for peak area variation associated with replicate analyses. This variability was less than 2% for the majority of the metabolites and only 13% of the peaks varied by more than 5% (Fig. 1a). The most variable components were those of lowest abundance (Fig. 1b) and peaks with the highest total variation across the entire dataset also tended to possess the higher injection variation (Fig. 1c).

Based on these results, single GC-MS analyses were performed for nearly all subsequent samples, as the benefits of performing multiple analyses (slightly greater accuracy in peak areas) did not justify the additional resources (double the instrument time, file storage space, processing time, etc.), particularly for large datasets. However, biological triplicates were still utilized and triplicate instrumental analyses of individual biological replicates were performed on 4–6 samples throughout each time-course to provide an estimate of instrumental variability.

The analytical variation associated with the entire sampling, extraction, and analysis procedure was also quantified, as was the biological variance associated with different cell culture replicates. The analytical coefficient of variation (CV) was calculated using the internal standard peak area and ranged from 4.6% to 7.8% for polar extracts. For the calculation of biological variance, a list of approximately 120 components was compiled for comparative analyses of polar extracts from all elicited time-courses. This list was based on the consistent presence of these metabolites in all time-course data. The median biological CV (including elicitation responses and temporal trends) ranged from 27.4% to 33.3% over each time-course (mean biological variability values were approximately 10% to 15% higher than median values due to the influence of a few exceptionally variable peaks). Approximately 40% of quantified peaks have been identified (72 out of 169 for polar and non-polar metabolites). Peak area values based on individual representative ions for all metabolites used are presented as supplementary files and can be located at JXB online (S1 contains data on polar metabolites and S2 contains data on non-polar metabolites).

**MeJa elicitation**

The effect on primary metabolite pools was most dramatic following elicitation with MeJa. Increased levels of several amino acids, most notably valine, leucine, isoleucine, and threonine, were observed over the 48 h period (Fig. 2; Table 2). In addition, succinic and fumaric acid demonstrated similar trends. Phosphate accumulated to slightly higher levels in MeJa-treated compared with control cultures, as did the non-protein amino acids γ-aminobutyric acid (GABA) and β-alanine. Sucrose demonstrated the opposite trend, with decreased levels in elicited tissue relative to controls. The triterpene β-amyrin accumulated in MeJa-elicited samples, and was the only identified non-polar metabolite to demonstrate an elicitation response. Further, LC-MS analysis revealed the accumulation of triterpene saponins after 40 h (Fig. 3), suggesting that the accumulation of β-amyrin precedes increased saponin biosynthesis. The small but consistent accumulation of β-amyrin and triterpene saponins following MeJa elicitation, and of shikimic acid following YE elicitation discussed below, confirm that the *M. truncatula* cultures are responding in a similar manner to previously published reports (Suzuki et al., 2002).

Virtually all of the observed effects of elicitation were quantitative rather than qualitative. Two peaks, however, were only detected in extracts of MeJa-elicited tissue. The first was identified as jasmonic acid, presumably arising

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**Fig. 1.** Duplicate GC-MS analyses were performed on each sample of the polar extracts from the MeJa time-course. This dataset was analysed to evaluate the importance of multiple instrumental analyses of individual samples and guide the analytical approach for the remaining three time-courses. (A) The average difference between replicate peak areas for each of 249 peaks was calculated and tabulated as a percentage of the mean peak area. The majority of the peaks differed by less than 2% peak area, while less than 13% peaks differed by greater than 5%. (B) Deviation between analyses was found to be highly dependent on peak area, with the highest deviations demonstrated for the lowest abundance peaks. (C) Instrumental variability was also related to the total variation in peak area for the dataset. Peaks with high overall variability varied more between analyses, either due to low peak areas, compound instability, or other unknown factors. CV, Coefficient of variation; Diff CV, the difference between the CV for all samples of the first injection and the CV for all samples of the second injection.
from hydrolysis of exogenously applied MeJa (Swiatek et al., 2004). The second was an unidentified compound eluting approximately 5 min later. The spectral characteristics of the unknown compound are similar to those of derivatized jasmonic acid, with characteristic fragment ions with shifts of two m/z units in either direction. More specifically, jasmonic acid possesses fragment ions at m/z 280, 180, and 148, while the unknown possesses fragment ions at m/z 282, 178, and 146. The unknown peak is believed to be an intermediate in the enzymatic degradation or inactivation of jasmonic acid, as the second peak trails jasmonic acid in abundance by at least 18 h (Fig. 4). The putative molecular weight of the derivatized unidentified compound is 399, 88 m/z, greater than that of derivatized jasmonic acid. This difference can be explained by an additional hydroxylation (addition of 16) with subsequent TMS derivatization (addition of 72) and is consistent with hydroxylated jasmonic acid. Hydroxylated JA was recently identified as an intermediate in JA degradation in tobacco (Swiatek et al., 2004).

Correlation analyses and the related ‘connectivity’ of metabolites has recently been used to detect the metabolic consequences of sucrose synthase isoform II suppression, which fails to demonstrate a visible phenotype (Weckwerth et al., 2004). A similar approach using correlation analyses in a comparative fashion to reveal the effect of elicitors on metabolite relationships was used on this dataset. The dataset was divided into elicited and control samples, and metabolite-to-metabolite correlation analyses were performed on each dataset. The resulting correlation parameters were analysed for differences attributable to elicitation. This analysis utilized all time points for the estimation of correlation parameters, which is probably an oversimplification of the time-course nature of the data, but still valuable for comparative purposes. Several amino acids increased following elicitation while sucrose levels decreased. This trend is exemplified by the relationship between β-alanine and sucrose, which changed from absent in control samples ($r^2=0.028$) to negative ($r^2=0.796$, $r=−0.892$) following elicitation. In control extracts, threonine and pyroglutamic acid were very poorly correlated ($r^2=0.010$), but positively correlated following elicitation ($r^2=0.718$). An additional example of altered correlation parameters following elicitation was between the three
Metabolites showing a consistently (similar response in mixed-time course and respective individual elicitor time-courses) significant (P < 0.05 in mixed and individual elicitor time-courses) response to at least one elicitor (MeJa, UV, or YE). Values represent the fold change in peak area at the average of the 18, 24, and 36 h timepoints (values >1.0 represent increased levels in elicited samples, values <1.0 represent decreased levels due to elicitation; blank cells, statistically insignificant in one of both t-tests).

Table 2. Fold change in peak area following elicitation

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<tr>
<th>Metabolite</th>
<th>Retention time</th>
<th>MeJa Mixed MJ</th>
<th>YE Mixed YE</th>
<th>UV Mixed UV</th>
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Amino acids serine, glycine, and threonine. Glycine is biosynthetically linked to both serine and threonine by as few as one enzymatic step in micro-organisms. There was no correlation between glycine and threonine in unelicited samples, but a clear relationship was observed in elicited samples (Fig. 5).

Yeast elicitation

The effect of YE on primary metabolism was more subtle than that of MeJa, but several trends were observed, with some similar to those following MeJa elicitation while others were unique (Table 2). Phosphate levels increased following exposure to YE, whereas sucrose decreased. β-alanine levels were induced by YE, while other amino acids which showed MeJa responsiveness failed to show a clear response to YE. Shikimic acid, a precursor of the phenylpropanoid pathway, accumulated following YE elicitation, as did citric acid and glucose-6-phosphate. Several end-products of the phenylpropanoid pathway, of which shikimic acid is a precursor, also accumulated with maximal elicitation at either 10 h or over the 48 h period (Fig. 3).

Comparative correlation analysis of YE data yielded fewer changes in metabolite relationships than did the MeJa data. Valine and leucine were moderately correlated in control samples, and the strength of the correlation increased following treatment with YE from $r^2=0.445$ in controls to $r^2=0.860$. β-Alanine became negatively correlated with sucrose, with $r^2$ increasing from 0.051 to 0.465 following elicitation.

UV elicitation

UV elicitation had less of an effect on primary metabolism than either MeJa or YE. The procedure of transferring and spreading the original suspension cell cultures onto agar plates for UV exposure may have prevented observation of an elicitation response at the level of primary metabolism. In addition, the plates were maintained on an illuminated shelf exposed to a diurnal cycle following elicitation with strong UV exposure. The design of the experiment (see Materials and methods) may have induced changes in the cellular metabolic state which masked UV-elicitation effects at the level of primary metabolism. In fact, all UV samples, elicited and unelicited, looked similar to MeJa-elicited samples in many respects (see below).

Mixed elicitation

A fourth, mixed time-course served as a means of validating and correlating responses observed in each of the three more detailed individual time-courses. Although fewer time points were analysed, all elicitations were performed on cells from the same cell culture passage that were extracted and analysed in one large batch. Using the mixed time-course data, the elicitation responses were analysed using principal component analysis (Fig. 6a). Both UV control
and UV-elicited, and MeJa-elicited, samples segregated from the general cluster containing early time points from all samples and YE and MeJa controls. UV control and elicited samples were indistinguishable from each other in PCA space, and MeJa samples trended in the same direction as the UV samples. However, the two groups were clearly separated along the first principal component axis (Fig. 6a).

To identify the source of this similarity in elicitation response, the UV elicited and control samples were pooled, based on the lack of significant changes. The MeJa and YE controls were also pooled to provide a reliable estimate of control values. The similarity of the pooled UV samples to the MeJa-elicited samples (and to a lesser extent the YE-elicited samples) is reflected in the plots of individual compounds (Fig. 6b).

**Elicitor independent relationships**

While many of the metabolite correlations were altered by one or more elicitors, several relationships between metabolites were found to be robust to the effects of any of the elicitors, such that the correlation parameters between a metabolite pair were unaltered by elicitation procedures. To explore these elicitor-independent relationships thoroughly, correlation analysis was applied to a composite dataset compiled from all four elicited time-courses, with each metabolite normalized to its intra-time-course mean. The relationship between leucine and isoleucine was remarkably conserved through the entire dataset ($r^2=0.941$). Valine was also highly correlated with both leucine and isoleucine ($r^2=0.790$ and $r^2=0.822$, respectively), and leucine, isoleucine, and valine correlated moderately with threonine ($r^2=0.498$, 0.599, and 0.458, respectively). Alanine and pyroglutamic acid are correlated ($r^2=0.683$), despite the fact that there were no dramatic elicitor-induced changes in levels of either to buttress the $r^2$ value. The serine–threonine relationship previously discussed was considerably stronger ($r^2=0.652$) than either the glycine–serine ($r^2=0.353$) or glycine–threonine ($r^2=0.432$) correlations. Alanine was negatively correlated to fumarate with $r^2=0.467$, although a linear regression line is an overly simplistic model for this relationship.

**Discussion**

Biotic and abiotic elicitors are often applied in the examination of secondary metabolism and the responses of cultured plant cells to UV, MeJa or YE have been characterized at various levels of detail in several species. The effect of
elicitation on primary metabolite accumulation has largely been overlooked. However, at the transcript level, fungal elicitation altered expression of over 40 transcripts tested, including representatives from the phenylpropanoid, pentose phosphate, glycolytic, and fatty acid metabolic pathways (Batz et al., 1998), suggesting that the response to elicitation is much more than the simple induction of biosynthetic enzymes of secondary metabolism.

Primary metabolism provides critical substrates for secondary metabolic pathways. For example, the entry point into phenylpropanoid metabolism is phenylalanine. Further, many essential cofactors and ligands involved in primary metabolism are required for secondary metabolite biosynthesis, and these cofactors are also synthesized from primary metabolites. For example, Coenzyme A (CoA) is listed in over 300 metabolic reactions in the KEGG metabolism database (Kanehisa et al., 2004). CoA is an essential component in both primary and secondary metabolic reactions, and the regulation of enzymes utilizing CoA or its thioesters is often affected by the induction of secondary metabolism (Alex et al., 2000; Logemann et al., 2000). Batz et al. (1998) also demonstrated the induction of S-adenosyl-methionine synthase transcription following fungal elicitation, which serves as a methyl donor to furanocoumarins. In this study, dramatic changes in accumulation patterns for several metabolites and pathways of primary metabolism, both distant and proximal to secondary metabolic branch points, were observed and are discussed below.

**Carbon metabolism**

Exposure of *M. truncatula* cells to MeJa, YE, or UV resulted in decreased sucrose levels over 48 h, with the simultaneous accumulation of several amino acids and some organic acids. This pattern indicates altered carbohydrate metabolism following elicitation. A portion of the diverted carbon is shifted toward secondary metabolism, as revealed by increased triterpene saponin levels following MeJa elicitation and increased isoflavonoid accumulation following YE. Presumably, an additional portion of carbohydrate is consumed for production of energy to support secondary metabolite biosynthesis. In a similar fashion, elicitation of parsley cell cultures with *Phytophthora megasperma* extracts increased the rate of respiratory CO₂ evolution, particularly through glycolysis and the oxidative pentose phosphate pathways (Norman et al., 1994). The authors proposed that this response served to supply substrate for the synthesis of furanocoumarins. Although parsley and *M. truncatula* synthesize differing classes of secondary metabolites in response to elicitation, the elicitor-induced reallocation of carbon toward secondary metabolism appears similar. However, in addition to secondary metabolites, several primary metabolites, such as β-alanine, GABA, and succinic acid are observed to accumulate following MeJa elicitation. Accumulation of these metabolites cannot be explained by their ecological functions or common catabolic phenomena such as protein degradation.

Negative correlations between amino acids and sucrose have frequently been observed with the advent of global...
Fig. 6. A portion of the elicitation response seems to be common to MeJa and YE elicitation and exposure to light following UV elicitation. (a) Principal component analysis of time points 15 min, 18 h, 24 h, and 36 h samples from the mixed-elicitor time-course. The 15 min samples and YE and MeJa-unelicited samples from later time points are unseparated (unlabelled open diamonds), while the MeJa-elicited as well as the UV-elicited and control samples (filled diamonds) drift from the centre of the PCA plot in the same direction. However, MeJa-elicited and UV samples are clearly distinguishable along the first principal component axis. (b) The dynamics of several amino acids and sucrose nearly overlap for the pooled UV samples (open squares) and MeJa-elicited samples (closed squares), while the YE-elicited samples (open triangles) are generally more similar to pooled MeJa and YE control samples (closed diamonds).
metabolite profiling. To demonstrate the generality of the phenomenon, sucrose levels in potato tubers were altered in response to changes in light intensity, transgenic manipulation of sucrose transport from source leaves to sink tubers, or direct alteration of sucrose delivered to the tuber through a cut stolon (Roessner-Tunali et al., 2003). Although the method used to alter sucrose levels had some effect on the specific amino acid levels altered, the total amino acid content was consistently negatively correlated with sucrose levels ($r^2=0.70$) over 25 experimental conditions (Roessner-Tunali et al., 2003). In addition, repression of a constitutive sucrose transporter (SUT1) resulted in the increased expression of certain amino acid biosynthetic genes, including aspartate kinase, NADH-dependent glutamate synthase, and aspartate aminotransferase, suggesting that increased amino acid pools are not the result of increased protein degradation or decreased protein synthesis, but arise at least partially through increased biosynthesis. Likewise, the addition of sucrose to carrot cell suspension cultures resulted in a decrease in glutamate dehydrogenase activity and a resultant drop in glutamate concentration (Robinson et al., 1992).

**Non-protein amino acids and polyamines**

The strongest inverse carbohydrate-to-amino acid relationship observed was between sucrose and β-alanine. β-Alanine is a non-protein amino acid which can serve as an intermediate in coenzyme A synthesis through pantothenic acid. β-Alanine can be synthesized by different mechanisms, with the preferred biosynthetic route apparently being clade specific. The exact synthetic mechanism in plants is unknown, and a metabolic pathway based on *A. thaliana* sequence data cannot be reconstructed between sucrose and β-alanine. β-Ureidopropionase has been purified from maize seedlings and characterized (Walsh et al., 2001) and is thought to function primarily in the catabolism of pyrimidine bases. The products of the degradation of uracil and thymine include β-alanine and β-aminoisobutyric acid, affording the enzyme a simultaneous biosynthetic function. However, no β-aminoisobutyric acid was detected in this study, as might be expected if ureidopropionase were responsible for the observed accumulation of β-alanine. As the metabolic fate of β-aminoisobutyric acid is currently unknown, this cannot account for the absence of this metabolite through its conversion into an accumulating metabolite.

Recently, an additional biosynthetic route for the production of β-alanine through degradation of the polyamines spermidine and spermine was described in yeast (White et al., 2001). The enzymatic degradation of spermine results in the production of spermidine and 3-aminopropionaldehyde. The aldehyde is subsequently converted to β-alanine. In this study, the polyamine putrescine was observed at slightly increased levels following YE and MeJa elicitation, but neither spermine nor spermidine were detected. Polyamine synthesis is MeJa-inducible, apparently through the arginine decarboxylase pathway in tobacco (Biondi et al., 2001) and barley (Walters et al., 2002). This pathway proceeds through several steps to convert arginine through agmatine to putrescine, and genes encoding several enzymes of this pathway have been cloned (Piotrowski et al., 2003). However, over-expression of arginine decarboxylase, in an attempt to increase polyamine production in tobacco, resulted in either a 10–20-fold accumulation of agmatine without the accumulation of polyamines (Burtin and Michael, 1997), or a slight accumulation of polyamines which was correlated with a growth phenotype (Masgrau et al., 1997). In *A. thaliana*, MeJa induced a local induction of arginine decarboxylase, a transient accumulation of putrescine, no effect on spermidine, and a subtle decrease in spermine (Perez-Amador et al., 2002). The authors suggest that putrescine may be converted to GABA through 4-aminobutanol (Flores and Filner, 1985) or that degradation or conjugation of spermidine and/or spermine prevents the accumulation of the higher polyamines.

Increased polyamine biosynthesis without dramatic accumulation (Burtin and Michael, 1997; Masgrau et al., 1997) would simultaneously increase the availability of 3-aminopropionaldehyde, a possible precursor to β-alanine as well as 4-aminobutanaldehyde, an immediate precursor to GABA. Both β-alanine and GABA were found at higher concentrations in elicited compared with control cells following MeJa elicitation in this study. β-Alanine and the l-valine biosynthetic intermediate, 2-oxoisovalerate, are used in the formation of pantothentic acid (White et al., 2001) which is subsequently converted to Coenzyme A (Kupke et al., 2003). Valine, leucine, and isoleucine, which with their intermediates including 2-oxoisovalerate comprise the branched-chain amino acid pathway, accumulated following elicitation.

Assuming metabolite accumulation can be interpreted as a metabolic imbalance which appears as a consequence of increased flux through that metabolite, as was observed in this study for shikimic acid and β-aminic, then accumulation of branched chain amino acids, putrescine, GABA, and β-alanine, might collectively be interpreted as altered CoA biosynthesis (Fig. 7). This suggestion represents an hypothesis generated using metabolomics, rather than a conclusion based on experimental data, and will be pursued in future experiments.

CoA serves as a carrier for organic acids including acetic acid (utilized in fatty acid biosynthesis, glycolysis, citrate cycle, amino acid synthesis etc.), malonic, sinapic, and ferulic acid (intermediates in the phenylpropanoid pathway leading to lignin and flavonoids), and 3-hydroxyymethylglutaric acid (an intermediate in sterol and terpenoid biosynthesis). Thus, CoA is an essential cofactor, not only for primary metabolism, but also for the phenylpropanoid and triterpene saponin pathways which are up-regulated in *M. truncatula* by the elicitors used in this study. A tentative consensus (TC) sequence (TC78022)
with similarity to the *A. thaliana* pantothenate kinase (Kupke et al., 2003), which catalyses the initial step in the incorporation of pantothenate to CoA, was found in the TIGR *M. truncatula* database. This TC was most highly expressed in a library constructed from YE-treated cell cultures, further supporting the hypothesis that CoA biosynthesis may be inducible.

Branched-chain amino acids

A highly linear and precise correlation was observed between the levels of leucine and isoleucine which was robust to perturbation with elicitors. Valine, leucine, and isoleucine are all produced by the same biosynthetic pathway, the enzymes of which are plastid localized. Although the biosynthetic pathways are similar for each of these metabolites, the first enzymatic step uses different precursors for each branched-chain amino acid. Leucine is ultimately synthesized from acetyl-CoA through 2-oxoisovaleric acid, isoleucine from threonine through 2-oxobutyrate, and valine from pyruvate through 2-oxoisovalerate. The terminal step, converting 2-oxoacids to their corresponding amino acids, is accomplished by two separate enzymes in spinach chloroplasts (Hagelstein et al., 1997).

Fig. 7. Proposed metabolic model of the elicitation response based on experimental results. With exposure to YE, MeJa, or plating and exposure to UV light, sucrose levels (red box) decreased and concomitant increases in several other metabolites (yellow boxes) were observed. The subtle accumulation of putrescine following YE and MeJa elicitation was observed, as well as the accumulation of branched chain amino acids and \( \beta \)-alanine. Polyamine catabolism provides substrates for \( \beta \)-alanine and GABA synthesis, and \( \beta \)-alanine and 2-oxoisovaleric acid, an intermediate to valine, are incorporated into pantothenate which is converted subsequently to CoA. Altered CoA synthesis and accumulation is not yet experimentally validated, as no method exists to profile CoA organic acid esters broadly.

Glycine, serine, and threonine metabolism

Comparative correlation analyses revealed altered relationships between metabolites suggestive of the altered activity levels of particular enzymatic functions, some yet to be described from plants. For example, there was no correlation between glycine and threonine in unelicited samples,
but a clear relationship was evident in MeJa-elicited samples (Fig. 5). In yeast, glycine is biosynthetically linked to serine by serine hydroxymethyltransferase (EC 2.1.2.1) and to threonine by threonine aldolase (EC 4.1.2.5) (Woldman and Appling, 2002). In plants, serine hydroxymethyltransferase is well characterized (McClung et al., 2000); however, threonine aldolase is yet to be characterized from plants. The strength of the relationship between glycine and threonine increased following MeJa elicitation. The simplest explanation is that a threonine aldolase enzymatic function is present and inducible by MeJa in M. truncatula cell cultures, illustrating the value of correlation analyses and metabolomics for the discovery of potentially novel enzymatic functions.

Queries of the M. truncatula EST datasets utilizing the yeast amino acid sequence for threonine aldolase (TA) revealed a TC sequence (TC77640) with 35% amino acid identity (52% similarity) which was most highly expressed in libraries from nodulated root, irradiated seedlings, fungal-elicited cell cultures, and pathogen-infected whole tissues. In addition, this TC contains a lysine residue (Lys222) which is highly conserved in fungal (Lys199) threonine aldolase (Monschau et al., 1998) and is essential for pyridoxal 5′-phosphate binding and catalytic activity (Liu et al., 1997). TAs are very closely related to serine hydroxymethyltransferases (SHMT), which share structural and functional similarities (Contestabile et al., 2001). The amino acid sequence for MtTC77640 was aligned with various SHMT and TA sequences from yeast (Sc) E. coli (Ec), M. truncatula (Mt), and Arabidopsis thaliana (At). The putative MtTA sequence is similar to yeast and E. coli threonine aldolase sequences and distinct from the SHMT sequences (Fig. 8).

Alternatively, altered correlation parameters may be due to a less direct effect than changes in immediate biosynthetic connectivity. Utilizing the pathway reconstruction tool (PathComp) in the KEGG database (Kanehisa et al., 2004), enzyme functions were compiled to link glycine to threonine based on genome sequence data from Arabidopsis thaliana. This tool could identify no logical path of less than 15 steps between these two metabolites, suggesting the existence of either an unidentified mechanism of co-regulation or a novel biosynthetic pathway.

In summary, this report presents a detailed study of the response of Medicago truncatula primary metabolism to biotic and abiotic stimuli. Significant changes in the relative abundance of multiple metabolites were observed and are the result of genetic reprogramming of primary metabolism in response to stress. Of specific interest are decreased sucrose, increased branched-chain amino acids, and increased β-alanine levels, suggestive of a generic stress response. Further, these changes represent repartitioning of carbon from primary metabolism, specifically sucrose, into secondary metabolism such as the triterpene saponins and isoflavonoids. It has been speculated that elevated branched chain amino acids, putrescine, GABA, and β-alanine, collectively represent altered CoA biosynthesis, integral to the elicitation response and partially directed towards secondary metabolism. In addition, the data support the presence of a threonine aldolase in M. truncatula which has currently not been characterized in any plant. The evidence for both increased CoA metabolism and threonine aldolase activity is significant, but still speculative at this point. However, these are credible examples of an ‘omics’ approach successfully functioning as a discovery platform and producing new hypotheses for future investigations. It is perceived that these discovery hypotheses will continue to arise as transcriptome, proteome, and metabolome data from this project are integrated.

**Supplementary materials**

Two files containing peak area for all metabolites from all samples used in this study are available as supplemental materials at JXB online. S1 contains data for polar metabolite profiles and S2 contains data for non-polar metabolite profiles. The files are in a tab-delimited text format. Included for each analysis is the elicitor used, the time-course in which the sample was taken (Time_crs), the time after elicitation (Time (h)), treatment or control, the biological replicate number (Biol_rep), the injection replicate number (Inj_rep), the liquid extraction phase (P for polar; L for lipid or non-polar), and area data for each metabolite. Metabolite identifiers are encoded as follows: Metabolite-ID.Retention-time (Extracted-ion). All analyses in this
publication were performed on datasets which have been normalized as described above, S1 and S2 contain non-normalized data.

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