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Metabolomics Approach To Understand Mechanisms of β-*N*-Oxalyl-L-α,β-diaminopropionic Acid (β-ODAP) Biosynthesis in Grass Pea (*Lathyrus sativus* L.)

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Supporting Information

ABSTRACT: A study was performed to identify metabolic processes associated with β-ODAP synthesis in grass pea using a metabolomics approach. GC−MS metabolomics was performed on seedlings at 2, 6, and 25 days after sowing. A total of 141 metabolites were detected among the three time points representing much of grass pea primary metabolism, including amino acids, carbohydrates, purines, and others. Principal component analysis revealed unique metabolite profiles of grass pea tissues among the three time points. Fold change, hierarchical clustering, and orthogonal projections to latent structures-discriminant analyses, and biochemical pathway ontologies were used to characterize covariance of metabolites with β-ODAP content. The data indicates that alanine and nitrogen metabolism, cysteine and sulfur metabolism, and purine, pyrimidine, and pyridine metabolism were associated with β-ODAP metabolism. Our results reveal the metabolite profiles in grass pea development and provide insights into mechanisms of β-ODAP accumulation and degradation.

KEYWORDS: *β-ODAP, grass pea, metabolomics, nitrogen, sulfur, uracil*

INTRODUCTION

Grass pea (*Lathyrus sativus* L.) is pulse crop grown for food, animal feed, and forage in arid and semiarid regions of Asia and Africa, and it is important to human nutrition as a source of dietary protein. Grass pea also provides food security since it resists many abiotic stresses and plant pathogens.[1,2] However, incorporating grass pea as a major component in the diet is a challenge because the seed accumulates β-*N*-oxalyl-L-α,β-diaminopropionic acid (β-ODAP), a neurotoxic amine that can cause human lathyrism.[3−6] Further, while grass pea seed protein content is high, the protein has relatively low levels of sulfur-containing amino acids.[7−9]

There is support that β-ODAP neurotoxicity levels are affected by coconsumption of sulfur-containing amino acids such as methionine and cysteine. For example, Getahun et al.[5,6] demonstrated that consumption of grass pea in combination with vegetables rich in sulfur-containing amino acids lowered the neurotoxicity of β-ODAP. In plant studies, depleting exogenous methionine and cysteine in the plant growth medium can increase the neurotoxicity of β-ODAP to isolated neurons.[8] Thus, understanding the coregulation of grass pea toxicity with nitrogen and sulfur metabolism may be an effective method to reduce seed toxicity and improve the sulfur content for better human nutrition.[2,9]

The genetic and metabolic regulation of β-ODAP synthesis has been partially described and involves nitrogen and sulfur metabolism.[2] The initial steps involve the amino acid serine (the major precursor), which is transformed into *O*-acetylserine and isoxazolin-5-one, both of which are metabolized into β-(isoxazolin-5-on-2-yl)alanine (BIA) via the enzyme β-cyanoalanine synthase (β-CAS). BIA is then transformed into β-ODAP. β-CAS may be a major regulator of β-ODAP synthesis because it is also involved in retaining cysteine molecules within the β-ODAP pathway. Together, the collective structures of these precursor metabolites and pathway enzymes support that β-ODAP synthesis is coregulated with primary metabolism of nitrogen and sulfur.[2]

Metabolomics can be utilized as an effective approach to elucidate the metabolic coregulation of β-ODAP in grass pea. Seed β-ODAP content is highly variable, and it is influenced by genotype and environmental factors.[10−14] Recent studies have also utilized metabolomics to understand variation of physiology among varieties within the *Lathyrus* species.[15] However, the main focus of grass pea metabolomics has been to profile the chemical composition of various extractions, or understand grass pea antioxidant properties.[15−18] Another method to understand β-ODAP coregulation is to measure changes during the early stages of plant development and growth. β-ODAP content is very high in young shoots, and

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Table 1. Subset of Metabolites That Varied among Time Points[a]

Sub Pathway	chemicals	6d/2d		25d/6d		25d/2d	
		P-value	fold change	P-value	fold change	P-value	fold change
Alanine, aspartate and glutamate metabolism	L-Alanine	<0.001	1.87	ns		0.037	1.34
	L-Aspartic acid	ns		0.041	-0.540	0.041	-0.33
	Gamma-Aminobutyric acid	0.002	1.94	ns		0.018	1.8
	Oxalacetic acid/oxalic acid?	<0.001	2.41	0.032	8.270	0.032	22.71
	aspartic acid	ns		0.041	-0.540	0.041	-0.33
	Succinic acid	ns		0.039	1.440	ns	
Arginine and proline metabolism	L-Aspartic acid	ns		0.041	-0.540	0.041	-0.33
	N-Acetyl-L-alanine	ns		0.036	-21.230	0.007	-21.4
	Gamma-Aminobutyric acid	0.002	1.94	ns		0.018	1.8
	L-Proline	0.009	1.07	0.002	-2.570	0.004	-1.5
	Putrescine	ns		0.020	-22.920	0.002	-23.28
Beta-Alanine metabolism	L-Aspartic acid	ns		0.041	-0.540	0.041	-0.33
	N-Acetyl-L-alanine	ns		0.036	-21.230	0.007	-21.4
	Gamma-Aminobutyric acid	0.002	1.94	ns		0.018	1.8
	Beta-Alanine	0.001	2.92	0.004	-20.090	0.017	-20.3
	1,3-Diaminopropane	<0.001	0.55	ns		ns	
	Pantothenic acid	<0.001	0.55	<0.001	17.170	<0.001	17.72
Biosynthesis of unsaturated fatty acids	Alpha-Linolenic acid	<0.001	3.68	ns		ns	
	Palmitic acid	0.004	0.64	ns		0.013	0.64
Butanoate metabolism	Gamma-Aminobutyric acid	0.002	1.94	ns		0.018	1.8
	Succinic acid	ns		0.039	1.440	ns	
Carbon fixation in photosynthetic organisms	L-Alanine	<0.001	1.87	ns		0.037	1.34
	L-Aspartic acid	ns		0.041	-0.540	ns	
	Fructose-6-phosphate	0.004	1.12	<0.001	-3.150	0.019	-2.03
	Oxalacetic acid	<0.001	2.41	0.032	8.270	0.032	22.71
Citrate cycle (TCA cycle)	Oxalacetic acid	<0.001	2.41	0.032	8.270	0.032	22.71
	Succinic acid	ns		0.039	1.440	ns	
Cyanoamino acid metabolism	L-Aspartic acid	ns		0.041	-0.540	0.041	-0.33
	Glycine	<0.001	2.36	ns		0.021	2.07
	L-Serine	0.009	-2.24	ns		ns	
Cysteine and methionine metabolism	L-Aspartic acid	ns		0.041	-0.540	0.041	-0.33
	O-Acetylserine	ns		ns		0.025	-18.4
	L-cysteine	0.001	-19.88	ns		0.001	-19.85
	5'-methylthioadenosine	0.027	-16.68	ns		0.027	-16.65
	L-Methionine	0.017	2.4	0.008	-24.470	0.046	-22.07
	L-Serine	0.009	-2.24	ns		ns	
Galactose metabolism	Glycerol	0.010	0.62	ns		ns	
	Sorbitol	0.001	-21.85	ns		0.001	-21.81
Glucosinolate biosynthesis	ascorbate	ns		ns		ns	
	L-Isoleucine	<0.001	2.04	<0.001	-1.060	0.019	0.98
	L-Leucine	<0.001	0.55	ns		ns	
	L-Methionine	0.017	2.4	0.008	-24.470	0.046	-22.07
	L-Tryptophan	0.005	1.23	<0.001	-2.140	ns	
	L-Valine	<0.001	1.95	<0.001	-1.020	0.037	0.93
Glutathione metabolism	ascorbate	ns		ns		ns	
	L-cysteine	0.001	-19.88	ns		0.001	-19.85
	Glycine	<0.001	2.36	ns		0.021	2.07
	Putrescine	ns		0.020	-22.920	0.002	-23.28
Glycerolipid metabolism	Glycerol	0.010	0.62	ns		ns	
Glycerophospholipid metabolism	Ethanolamine	ns		ns		0.027	0.69
	Taxifolin	<0.001	0.55	ns		ns	
Glycine, serine and threonine metabolism	L-Aspartic acid	ns		0.041	-0.540	0.041	-0.33
	Glycine	<0.001	2.36	ns		0.021	2.07
	L-Serine	0.009	-2.24	ns		ns	
	L-Tryptophan	0.005	1.23	<0.001	-2.140	ns	
Glycolysis or Gluconeogenesis	L-Lactic acid	0.015	1.28	ns		0.029	1.23
	Oxalacetic acid	<0.001	2.41	0.032	8.270	0.032	22.71
Glyoxylate and dicarboxylate metabolism	Glycolic acid	<0.001	1.8	ns		0.022	2.44
	Oxalacetic acid	<0.001	2.41	0.032	8.270	0.032	22.71
	Succinic acid	ns		0.039	1.440	ns	

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Table 1. continued

Sub Pathway	chemicals	6d/2d		25d/6d		25d/2d	
		P-value	fold change	P-value	fold change	P-value	fold change
Inositol phosphate metabolism	Glucose-6-phosphate	0.013	2.04	0.008	-5.440	ns	
	D-Glucuronic acid	<0.001	0.55	ns		ns	
Lysine biosynthesis	L-Aspartic acid	ns		0.041	-0.540	0.041	-0.33
	L-Lysine	ns		<0.001	-3.090	0.004	-2.33
Nitrogen metabolism	Glycine	<0.001	2.36	ns		0.021	2.07
Pantothenate and CoA biosynthesis	Beta-Alanine	0.001	2.92	0.004	-20.090	0.017	-20.3
	Pantothenic acid	<0.001	0.55	<0.001	17.170	<0.001	17.72
	L-Valine	<0.001	1.95	<0.001	-1.020	0.037	0.93
Pentose and glucuronate interconversions	D-Xylose	0.002	3.94	0.002	-4.510	ns	
Phenylalanine metabolism	L-Phenylalanine	ns		ns		ns	
	Phenylethylamine	ns		ns		0.046	16.71
	Phenylpyruvic acid/phenylpyruvate?	0.016	-15.14	ns		0.016	-15.11
Phenylpropanoid biosynthesis	Sinapyl alcohol	<0.001	0.55	ns		ns	
Purine metabolism	L-Glutamine	0.026	-15.22	ns		ns	
	Guanine	<0.001	0.55	ns		ns	
	Sulfate/sulfuric acid	<0.001	1.12	0.003	0.750	<0.001	1.87
Pyrimidine metabolism	Beta-Alanine	0.001	2.92	0.004	-20.090	0.017	-20.3
	3-Aminoisobutyric acid	0.004	0.74	<0.001	-21.020	<0.001	-20.28
	L-Glutamine	ns		ns		ns	
	Uracil	0.017	2.27	ns		0.003	2.37
Pyruvate metabolism	L-Lactic acid	0.015	1.28	ns		0.029	1.23
	Oxalacetic acid	<0.001	2.41	0.032	8.270	0.032	22.71
Sulfur metabolism	O-Acetylserine	ns		ns		0.025	-18.4
	L-Cysteine	0.001	-19.88	ns		0.001	-19.85
	O-Succinylhomoserine	ns		0.028	-21.710	ns	
	L-Serine	0.009	-2.24	ns		ns	
	sulfuric acid	<0.001	1.12	0.003	0.750	<0.001	1.87
Tyrosine and tryptophan metabolism	Shikimic acid	ns		<0.001	23.360	<0.001	5.58
	Homogentisic acid	<0.001	0.55	ns		ns	
	2-ketoadipate	<0.001	0.55	ns		ns	
	succinic acid	ns		0.039	1.440	ns	
	L-Tryptophan	0.005	1.23	<0.001	-2.140	ns	
Thiamine metabolism	L-Cysteine	0.001	-19.88	ns		0.001	-19.85
	4-Methyl-5-thiazolethanol	<0.001	0.55	ns		ns	
Valine, leucine and isoleucine metabolism (Branched Chain Amino Acids)	alpha-ketoisocaproic acid	0.002	5.14	0.002	-3.250	ns	
	L-Isoleucine	<0.001	2.04	<0.001	-1.060	0.019	0.98
	L-Leucine	<0.001	0.55	ns		ns	
	L-Valine	<0.001	1.95	<0.001	-1.020	0.037	0.93

[a]Metabolites showing a significant increase or decrease between time points are highlighted in red and blue font, respectively.

then levels are reduced during early vegetative phase and later accumulate in the seeds.[14]

The biosynthetic pathway of β-ODAP is not fully described, and metabolomics can facilitate the understanding of this pathway, specifically for the involvement of 2,3-L-diaminopropanoic acid and synthesis toward isoxazolin-5-one.[19] Further, methods have been developed to evaluate the effect of exogenous applications of metabolites for effects on β-ODAP concentration. For example, an exogenous, foliar application of abscisic acid was found to increase the content of both abscisic acid and β-ODAP, indicating that this method is sufficient to study uptake of nutrients and the effects on β-ODAP levels.[20]

Here, metabolomics was performed on grass pea during a range of growth and development periods 2 days after sowing (DAS), and young shoots at 6 and 25 DAS to understand metabolite covariation with β-ODAP. Gas chromatography–mass spectrometry metabolomics was performed to measure primary metabolic variation during germination. We also validated metabolite findings related to nitrogen and sulfur metabolism in exogenous application assays. To our knowledge, this is the first report to integrate metabolomics with variation in β-ODAP content during different vegetative stages (2, 6, and 25 DAS) to elucidate factors that affect β-ODAP content in grass pea.

MATERIALS AND METHODS

Plant Growth Experiment for Metabolomics. Seeds of *L. sativus* cv. LZ(2) were sown at the beginning of March 2016 at an experimental farm of Northwest A&F University, China. Seeds of 2 DAS and young shoots of 6 DAS and 25 DAS (roots were excised) were harvested as independent samples, transferred to a 2 mL microcentrifuge tube, immediately flash frozen, and stored at −80 °C. Each time point had n = 6 plant replicates.

Metabolite Extraction and Derivatization. Metabolites were extracted by adding 400 μL of a water/methanol solvent (1:3, v/v), and 20 μL of adonitol (2 mg/mL) was added as an internal standard. The samples were homogenized in a ball mill for 4 min at 40 Hz, sonicated in an ice bath for 5 min, and centrifuged for 15 min at 12000g, 4 °C, and 350 μL of supernatant was transferred into a new 2 mL glass vial. The extracts were evaporated in a vacuum concentrator without heating and subjected to a two-step derivatization process. First, extracts were methoximated by adding 60 μL of methoxyamine HCl (20 mg/mL in pyridine) and incubated for 30 min at 80 °C. Next,

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80 μL of *N*,*O*-bis(trimethylsilyl) trifluoroacetamide with 1% TMCS-(BSTFA) was added, and samples were incubated at 70 °C for 2 h.

GC−TOF-MS Metabolic Detection and Data Processing. GC−TOF-MS analysis was performed using an Agilent 7890 gas chromatography system coupled with a Pegasus HT time-of-flight mass spectrometer. The system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30 m × 250 μm inner diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). A total of 1 μL of derivatized extract was injected in splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 3 mL min^{-1}, and the gas flow rate through the column was 1 mL min^{-1}. The initial temperature was kept at 50 °C for 1 min, then raised to 290 °C at a rate of 10 °C min^{-1}, and kept for 13 min at 290 °C. The injection, transfer line, and ion source temperatures were 280, 270, and 220 °C, respectively. The energy was −70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 50−500 at a rate of 20 spectra per second after a solvent delay of 366 s.

Peaks were detected using Chroma TOF4.3X software (LECO Corporation, St. Joseph, MI, USA) for raw peak exacting, data baseline filtering, calibration of the baseline, peak alignment, deconvolution, and quantitation via peak area under the chromatographic curve. Metabolite annotation was performed using retention index and mass spectral matching to the LECO-Fiehn Rtx5 database, with an index tolerance of 5000.[21] Metabolite detected more than once on the GC−MS platforms due to chromatographically separated isomers or partial derivatizations are indicated by numbers next to their annotations.

Plant Growth Experiment of Foliar Amino Acid Sprays and Association to β-ODAP. Two additional experiments were performed to measure effects of exogenous applications of nitrogen containing compounds on β-ODAP accumulation. In the first experiment, grass pea seeds were sown into vermiculite and germinated over a period of 10 d. Hoagland solution was applied (without nitrogen) every 2 days. After 10 d, seedlings were sprayed at dusk each day with 15 mL of an amino acid solution containing 15 mM of one of the following: glutamate, glutamine, asparate, asparagine, or alanine. After 7 days, leaves were excised and β-ODAP was measured as previously described.[14]

In the second experiment, different combinations of organic nitrogenous compounds were applied to grass pea seedlings that included 2 mM urea, and 1 mM of the following: guanine, xanthine, uric acid, allanotin, allantoate, uracil, serine, and oxalic acid. Specific combinations of the organic nitrogen applications are described in Results and Discussion. After 6 DAS, cotyledons were excised and seedlings were transferred so that roots were bathed in a nitrogen solution at 25 °C for 48 h, and then β-ODAP was measured as previously described.[14] The organic nitrogen solutions were changed every 12 h to avoid bacterial growth.

Data Analysis and Statistics. The metabolite data matrix was analyzed using principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) using SIMCA v14.1 software (Umetrics AB, Umea, Sweden). Data was mean-centered and scaled to unit variance prior to multivariate analyses. Exogenous application assays were evaluated using SPSS 16.0 software via one way ANOVA with a Duncan post hoc, comparing each treatment to the control, and utilized a p value threshold of 0.05. Student's t test was performed using SPSS 16.0 software to compare metabolite abundances between adjacent time points with a p threshold of 0.05. Fold changes were calculated as the $\log_2$ fold change between adjacent time points (6 DAS/2 DAS; 25 DAS/6 DAS). Heat maps and hierarchical clustering were performed using OmicsShare software and MeV (MultiExperiment Viewer). The metabolite data was z transformed (mean-centered and divided by the standard deviation of the metabolite) and then subjected to distance measurement with Euclidean correlation and an average clustering algorithm method.

RESULTS AND DISCUSSION

Metabolomics Analysis of Grass Pea Shows Chemical Variation. A previous study has shown that grass pea β-ODAP content in 6 DAS seedlings is approximately 1−3-fold higher than in dry seeds.[14] Here, studies were performed to identify metabolites that covary with the change in β-ODAP content over time. Grass pea tissue was sampled from a field study at 2 DAS, 6 DAS, and 25 DAS from six biological replicates per time point.

GC−MS profiling resulted in the identification and quantitation of 231 metabolites over the three time points. Metabolite data was interpreted based on their known biochemical pathways and chemical ontology and included organic acids (58 metabolites), carbohydrates (56), amino acids (43), amines (19), alcohol (14), nucleotides (10), lipids (8), secondary metabolites (5), and pyridines (4) (Table S1). A total of 55 metabolites were classified according to known biochemical pathways (Table 1). Principal component analysis (PCA) was performed to evaluate metabolite variation over the three time points. The principal component (PC) scores plot separated the three time points along PC1 (49.9% of the variation), and PC2 further separated 6 DAS from both 2 DAS and 25 DAS (Figure 1). This variation along PC2 supports that many metabolites are covarying with the overall increase in β-ODAP that occurs at 6 DAS.

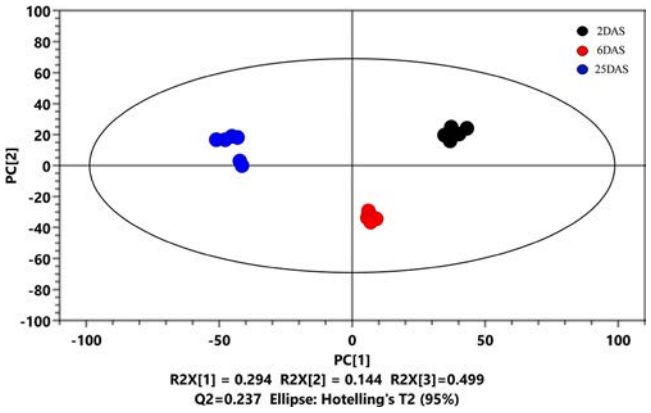

Figure 1. Principal component analysis (PCA) of global metabolite profiles in *L. sativus* at different developmental stages. PCA was performed on 670 metabolites for n = 6 replicate samples across the three time points. The data indicate metabolite variation at each time point. DAS = days after sowing.

Next, an orthogonal projection to latent structures-discriminant analysis (OPLS-DA) was performed to identify metabolites that are strongly associated with variation among the three time points. The OPLS-DA scores model explained 99.8% of the variation (R^2Y) for 2/6 DAS and 99.4% for of R^2Y for 6/25 DAS, and the model included a 7-fold cross validation (Q^2 = 97.1%) for 2/6 DAS and Q^2 = 93.0% for 6/25 DAS, indicating that the model is not overfit (Figure S1). The OPLS-DA biplot (scores and loadings, Figure 2) indicates separation of metabolite profiles over time. A variable importance projection (VIP) was used to characterize the metabolites that were the major contributors to the model (Table S1).

Metabolites were evaluated for VIP score (threshold of VIP > 1 for either OPLS model) data and Student's t test (p < 0.05) to identify metabolites that varied over time for a final data set of 141 metabolites. Hierarchical clustering (HCA) was

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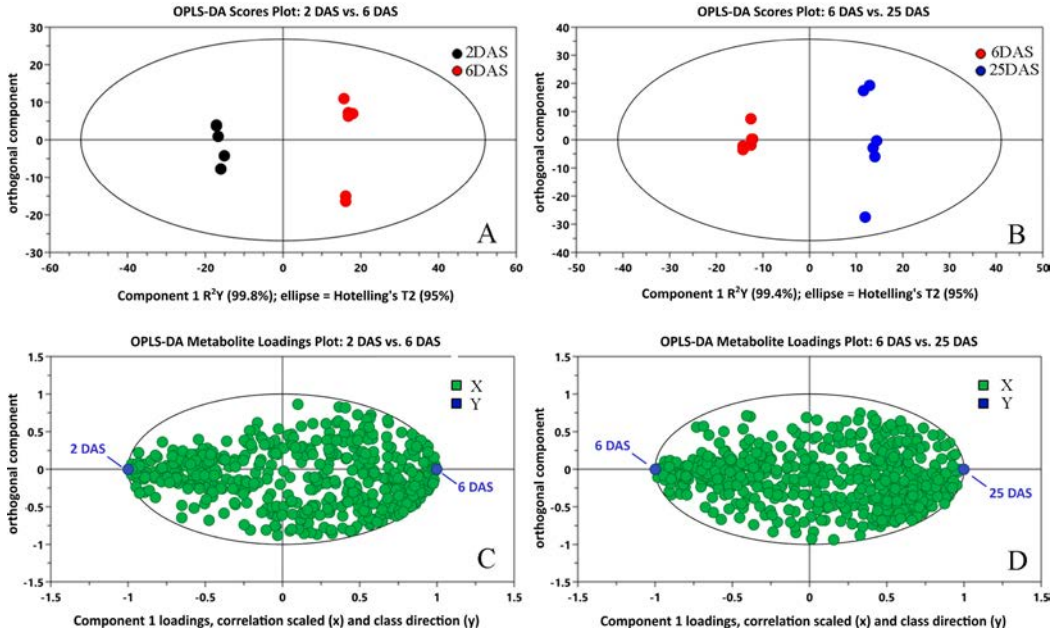

Figure 2. OPLS-DA analysis for change in metabolite abundances between time points. OPLS discriminant analysis was conducted twice to compare profiles between 2 DAS and 6 DAS and 6 DAS and 25 DAS. OPLS-DA scores (A and B) and loadings (C and D) (correlation scaled) for each model are displayed as top and bottom, respectively.

performed on the 141 metabolites to understand how metabolites varied over time (Figure S2), and it was found that 31 metabolites exhibited clear trends relevant to this study. The heat map reveals that the three time points had unique profiles (Figure 3). A metabolite cluster for 2 DAS revealed the samples to have higher abundance of several amines/amino acids (e.g., serine, glycocyamine, cysteine), a purine (5-methylthioadenosine), and an organic acid (phenylpyruvate) compared to 6 DAS and 25 DAS. The cluster of high-abundant metabolites for 6 DAS was solely composed of amines and amino acids (e.g., alanine, glycine, methionine, tryptophan, proline, *o*-acetylserine). The samples at 25 DAS were higher in some amines/amino acids (leucine, methionine sulfoxide, acetylleucine), organic acids (oxalic acid, oxalacetic acid), and sulfuric acid. Taken together, the heat map supports metabolite variation among the three time points that demonstrates a major role of nitrogen metabolism (i.e., variation in amines and amino acids) differing during early stages of grass pea development.

Many of the detected metabolites have relationships within the context of known biochemical pathways. The differences in metabolism among 2 DAS, 6 DAS, and 25 DAS samples were interpreted based on such pathways (Figure 4). The schematic supports that covariation of metabolites may be attributed to coregulation within biosynthetic pathways, such as carbohydrate metabolism (e.g., glycolysis via glucose/fructose 6P), links between carbohydrates and amino acids (e.g., pyruvate and alanine), and links between purines and amino acids (e.g., guanine, xanthine, glycine).

Carbohydrate and Alanine Metabolism Varied among the Three Time Points. Carbohydrates are known to be involved in glycolysis/gluconeogenesis, the citrate cycle and pentose phosphate pathway (PPP/HMS).[22] This study detected 56 carbohydrates (Table S1), and several varied over time (Figure 4). For example, glucose-6-phosphate and fructose-6-phosphate acid increased between 2 and 6 DAS ($\log_2$ fold change, FC = 2.04 and 1.12, respectively) and decreased between 6 and 25 DAS (−5.44 and −3.15). In contrast, several

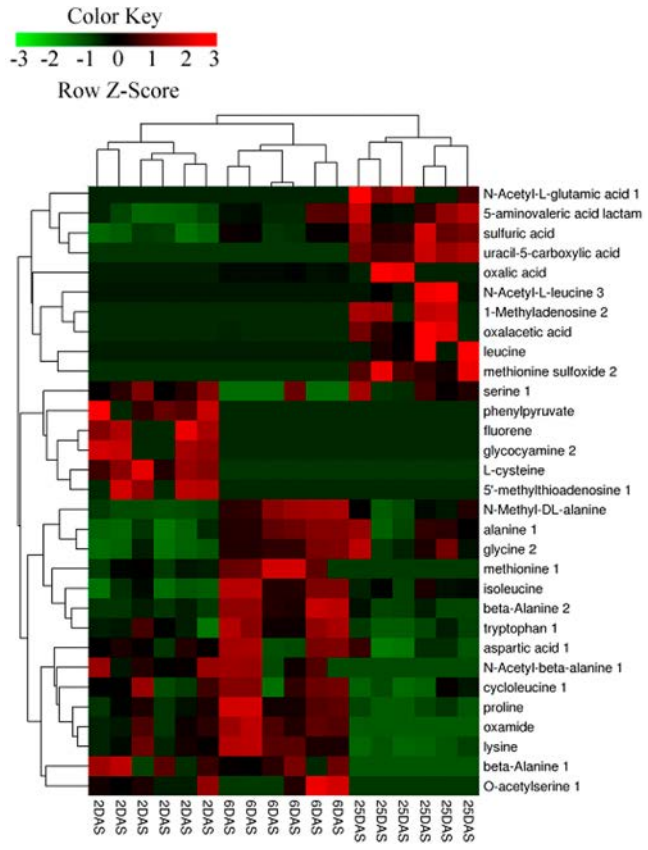

Figure 3. Heat map of 31 selected metabolites. Hierarchical clustering was performed on the normalized relative abundance (indicated by color) for 31 metabolites at each time point for $n = 6$ samples per time point. DAS = days after sowing.

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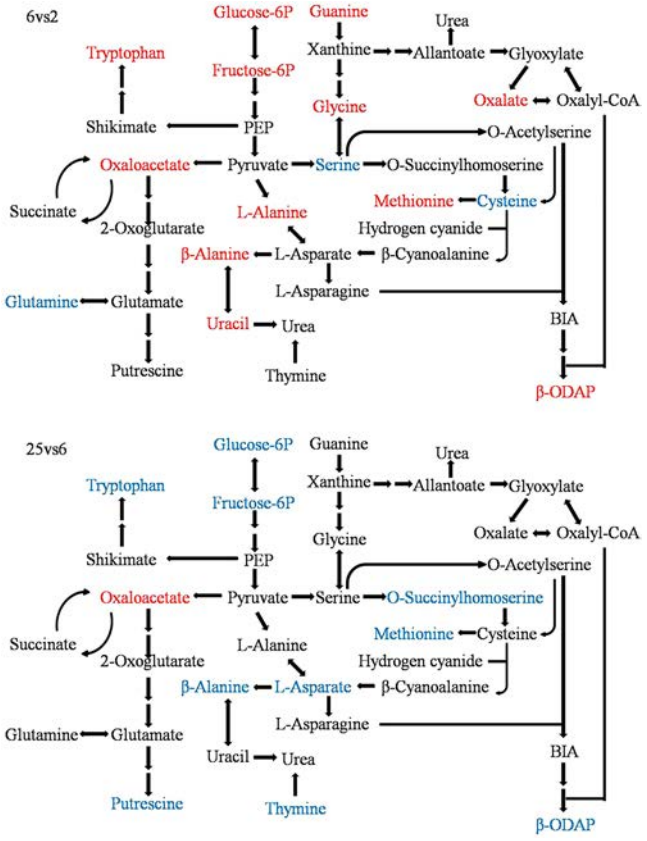

Figure 4. Schematic showing abundance of prominent metabolites from nitrogen, sulfur, purine, and pyrimidine pathways in seed of grass pea at different developmental time. Metabolites in red showed significant ($P < 0.05$) increase in 6 DAS over 2 DAS, metabolites in blue showed significant ($P < 0.05$) decrease in 6 DAS over 2 DAS, while metabolites in black are not changed or analyzed in this study.

metabolites steadily increased or decreased over time such as fructose-2,6-biphosphate (FC = −1.31 [6/2 DAS] and −1.85 [25/6 DAS]) and oxalacetic acid (FC = 14.44 [6/2 DAS] and 8.27 [25/6 DAS]). The trends observed for glucose and fructose-6 phosphate support that carbohydrate metabolism is involved in β-ODAP synthesis, especially in the generation of amino acid precursors and amino acids such as alanine and serine.

Importantly, seven metabolites of the pentose phosphate pathway (PPP) were detected and showed variation over time. Among them, gluconic lactone varied (FC = −18.09 [6/2 DAS] and 20.49 [25/6 DAS]) and glucuronic acid (FC = 0.55 [6/2 DAS] and 19.05 [25/6 DAS]). In PPP metabolism, the transformation of gluconic lactone and glucuronic acid is normally balanced within the cell. The relationship observed in this study (an imbalance between the two metabolites) indicates that the PPP may generate more downstream chemicals including ribulose-5-phosphate.

Given that ribulose-5-phosphate is necessary for nucleic acid biosynthesis,[22] we speculate that β-ODAP metabolism is related to nucleic acid metabolism, especially purine and pyrimidine. A total of 10 metabolites involved with purine and pyrimidine metabolism covaried with β-ODAP including 1-methyladenosine, guanine, glycine, sulfuric acid, beta-alanine, 3-aminoisobutyric acid, methylmalonic acid, thymine, uracil, and malonic acid. FC for these metabolites ranged between 0.55 and 2.92 6/2 DAS and 0.75−22.21 25/6 DAS. Interestingly, guanine and uracil increased 6/2 DAS, but did not decrease at 25/6 DAS.

Alanine is a known precursor for β-ODAP synthesis.[2] L-Alanine moderately varied among the time points in this study (FC = 1.87 [6/2 DAS] and −0.53 [25/6 DAS]). β-Alanine exhibited more change (FC = 2.92 [6/2 DAS] and −20.09 [25/6 DAS]), and also *N*-acetyl-L-alanine varied (FC = −0.16 [6/2 DAS] and −21.23 [25/6 DAS]). This supports that alanine metabolism is associated with β-ODAP metabolism, and a role for β-alanine and *N*-acetyl-L-alanine.

Serine, Cysteine, Methionine, and Pyridine Metabolism Varied among the Three Time Points. Recently, Xu et al.[2] reported that β-ODAP metabolism in *L. sativus* is an integration of nitrogen and sulfur metabolic pathways that is mediated through β-cyanoalanine synthase (CAS). In fact, β-ODAP is derived from heterocyclic BIA, which reaction is catalyzed by CAS,[23,24] and the formation of both cysteine and BIA competes for the same substrate (i.e., *O*-acetylserine). Therefore, the biosynthesis of β-ODAP is connected to the sulfur amino acid biosynthetic pathway,[23,24] and isoforms of the β-substituted alanine synthase (BSAS) family of enzymes play critical roles. These enzymes function as dimeric pyridoxal phosphate (PLP) dependent members, and the PLP-attachment site in GmCAS and LsCAS has been identified as Lys95.[25] Individual mutants at this site (LsCAS K95A, K95E, and K95R) would result in almost complete loss of CAS activity,[2] which might be caused via the pyridine ring rotating 15° toward the protein interior.[25] In fact, pyridine is one of the three central functional groups for PLP catalysis.[26]

In the context of this pathway, serine, cysteine, and methionine amino acids varied among the time points. Serine was inversely proportional to β-ODAP accumulation (FC = −2.24 [6/2 DAS] and 1.96 [25/6 DAS]), as well as cysteine (FC = −19.88 [6/2 DAS] and 0.03 [25/6 DAS]). Further, it is thought that cysteine transformation to methionine is a critical step for sulfur homeostasis in the plant. Methionine exhibited an opposite trend (FC = 2.40 [6/2 DAS] and −24.47 [25/6 DAS]). It is possible that the decrease in cysteine at 6 DAS may be due to decreased available serine. In support of this, the intermediate *O*-succinylhomoserine descreased 25/6 DAS (FC = −21.71). Taken together, these data support that the upstream serine pools may influence the content of β-ODAP content through cysteine.

In addition, our study detected several pyridine metabolites that covaried with β-ODAP. Specifically, 2,3-dihydroxypyridine and three isoforms of hydroxypyridine (2-, 3-, and 4-) increased 6/2 DAS (FC ranged between 1.65 and 21.01). The trend in pyridine is consistent with the high CAS activities necessary for β-ODAP biosynthesis.

Exogenous Application of Metabolites Validates Roles of Amino Acids and Purines in β-ODAP Synthesis. The metabolomics analysis indicated covariation of alanine with changes in β-ODAP content over time, and therefore an independent experiment was conducted to understand the effects of exogenously applied metabolites on β-ODAP concentration. The foliage of 10-DAS seedlings was sprayed daily with an amine/amino acid solution for a period of 7 days, after which leaves were excised and measured for β-ODAP. All amine/amino acid foliar treatments reduced seedling β-ODAP concentration (Table 2, ANOVA with Duncan post hoc $p < 0.05$). This is consistent with previous reports that availability of exogenous nitrogen is a regulator of β-ODAP accumulation,[12,14] whereby reduced exogenous nitrogen results in high

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Table 2. Effects of Different Nitrogen Sources on β-ODAP Content in *L. sativus*

treatment	content of β-ODAP (mg/g)
−N (control)	0.353 ± 0.013 a
urea	0.296 ± 0.004 b
glutamate	0.241 ± 0.010 c
glutamine	0.304 ± 0.003 b
asparate	0.303 ± 0.006 b
asparagine	0.297 ± 0.013 b
alanine	0.259 ± 0.008 c

concentrations of β-ODAP in young seedlings, and high nitrogen reduces β-ODAP. It can therefore be proposed that glutamate may regulate the nitrogen source to affect β-ODAP content further, while upstream alanine content regulates the concentrations of substrate for β-ODAP biosynthesis directly.

A second foliar spray experiment was conducted to investigate the effects of exogenous purines on β-ODAP concentration. In this experiment, urea was chosen as a control to include a terminal form of nitrogen for plant use. Grass pea exposed to exogenous guanine and guanine derivatives (xanthine, uric acid, allantoin, allantoate) in various combinations showed a minor influence on β-ODAP concentration (Table 3, ANOVA Duncan post hoc $p < 0.05$). Further, it is

Table 3. Effects of Pyrimidine, Purine, and Its Derivatives on β-ODAP Content in *L. sativus*

treatment	content of β-ODAP (mg/g)
urea (control)	0.294 ± 0.040 c
guanine	0.199 ± 0.098 d
xanthine	0.227 ± 0.130 cd
uric acid	0.289 ± 0.056 c
allantoin	0.273 ± 0.070 c
allantoate	0.310 ± 0.064 c
uracil	0.427 ± 0.040 b
serine	0.341 + 0.056 c
guanine + uracil	0.512 ± 0.068 a
guanine + serine	0.372 ± 0.054 bc
uracil + serine	0.326 + 0.041 c
guanine + uracil + serine	0.430 ± 0.072 b
uric acid + uracil + serine	0.454 ± 0.035 b
allantoin + uracil + serine	0.436 ± 0.040 b
allantoate + uracil + serine	0.505 ± 0.020 a
oxalic acid	0.211 ± 0.107 d
uracil + serine + oxalic acid	0.307 ± 0.073 c

important to note that β-ODAP content is not sensitive to allopurinol (1 mM), an inhibitor of xanthine oxidase, although the growing of seedlings was slightly reduced (data not shown). This indicates that xanthine oxidase has little effect on β-ODAP and therefore purine degradation may have a minor role. However, exogenous uracil and thymine increased β-ODAP compared with the urea control (ANOVA Duncan post hoc $p < 0.05$, Table 3). In addition, the uracil/guanine or uracil/guanine derivative treatments consistently revealed higher β-ODAP content when compared to the single metabolite applications. Therefore, these data support that β-ODAP biosynthesis is more sensitive to pyrimidine than purine content.

The study described herein performed metabolomic profiling of grass pea at three distinct germination stages to understand metabolite covariation with β-ODAP. The data supports that β-ODAP synthesis is coregulated with primary metabolism (Figure 4). Further, exogenous applications of different metabolites validated this influence of primary metabolism. Primary metabolism is tightly regulated during the initial stages of germination and seedling development, and therefore β-ODAP may be a critical component within these networks for grass pea. These data provide new insight into the biosynthesis of β-ODAP, but additional metabolomic profiling of tissue samples collected at different developmental stages (late vegetative stage, reproductive stage, and mature seeds) is required to fully understand the dynamics of β-ODAP synthesis.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04037.

Overfitting analysis of the OPLS-DA model, HCA map for metabolites, and all checked metabolites that varied among time points (PDF)

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Notes

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■ ABBREVIATIONS USED

β-CAS, β-cyanoalanine synthase; β-ODAP, β-*N*-oxalyl-L-α,β-diaminopropionic acid; BIA, β-(isoxazolin-5-on-2-yl)alanine; BSAS, β-substitute alanine synthase; DAS, days after sowing; HCA, hierarchical clustering; OPLS-DA, orthogonal projections to latent structures-discriminant analysis; PCA, principal component analysis; PPP/HMS, pentose phosphate pathway; VIP, variable importance projection

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