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Metabolomic profiling from leaves and roots of tomato (*Solanum lycopersicum* L.) plants grown under nitrogen, phosphorus or potassium-deficient condition

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ABSTRACT

Specific metabolic network responses to mineral deficiencies are not well-defined. Here, we conducted a detailed broad-scale identification of metabolic responses of tomato leaves and roots to N, P or K deficiency. Tomato plants were grown hydroponically under optimal (5 mM N, 0.5 mM P, or 5 mM K) and deficient (0.5 mM N, 0.05 mM P, or 0.5 mM K) conditions and metabolites were measured by LC–MS and GC–MS. Based on these results, deficiency of any of these three minerals affected energy production and amino acid metabolism. N deficiency generally led to decreased amino acids and organic acids, and increased soluble sugars. P deficiency resulted in increased amino acids and organic acids in roots, and decreased soluble sugars. K deficiency caused accumulation of soluble sugars and amino acids in roots, and decreased organic acids and amino acids in leaves. Notable metabolic pathway alterations included; (1) increased levels of α-*keto*glutarate and raffinose family oligosaccharides in N, P or K-deficient tomato roots, and (2) increased putrescine in K-deficient roots. These findings provide new knowledge of metabolic changes in response to mineral deficiencies.

1. Introduction

Nitrogen (N), phosphorus (P) and potassium (K) are the mineral nutrients required by plants in the greatest quantities. These elements frequently limit plant growth. Plants use nitrate (NO_3^-) or ammonium (NH_4^+) as primary N source and primarily in the form of $H_2PO_4^-$ and K^+ as P and K sources, and acquire them through each mineral-specific transport from the rhizosphere solution. Nitrogen metabolism includes uptake, reduction and assimilation, amino acid metabolism and transport, and translocation and remobilization of N [1–5]. Inorganic phosphate is a component of many cellular molecules, plays essential roles in structural maintenance, and also has roles in primary and secondary metabolism in plants. P-deficient plants modify metabolic processes to increase root surface area for the acquisition of external P [6]. Furthermore, P-deficient plants enhance P utilization through P mobilization from different subcellular compartments and organs, and through metabolic modification of P-containing molecules, nucleic acids, phospholipids and phosphorylated metabolites [7–9]. Although K is not assimilated into organic compounds, it influences primary and secondary metabolites with mechanical stability, and is important for pathogen/pest resistance in crops [10]. Molecular approaches to understanding K nutrition have focused on the function and regulation of transporters at the cellular level [11,12]. However, biochemical and molecular knowledge regarding the regulation of K and metabolites is not well characterized [13].

Primary and secondary metabolites are the intermediate or ultimate products of complex networks of biochemical pathways

Abbreviations: GC–MS, gas chromatography–mass spectrometry; LC–MS, liquid chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–mass spectrometry–mass spectrometry.

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involved in plant metabolism [14]. Because of their various roles in plant growth and function, in addition to biochemical roles, N, P, or K deficiencies can indirectly or directly affect plant metabolic pathways and thus affect the quantities of metabolites. Complex interactions between networks of metabolic pathways exist in many aspects of N, P and K metabolism [15,16]. For example, through amino acid metabolism, C and N metabolism are intimately connected. A more detailed understanding of which metabolites change in abundance in response to N, P, or K deficiency, and how these changes are similar or different in root and leaf tissues, will increase our understanding of plant nutrient responses, interactions between metabolic networks, and basic plant metabolism.

Recent advances in analytical technologies such as liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (MS) have increased our understanding of biochemical and physiological aspects by allowing analysis of thousands of metabolites from a single sample. Metabolite profiling is extensively used for diagnostics, and for mechanistic insight into plant responses and adaptations to a wide range of stresses including, but not limited to, nutrient deficiency [17–21], mineral toxicity [22], temperature and oxidative stress [23] and osmotic stress [24]. Metabolite-metabolite correlations can be evaluated using a data matrix generated by metabolomic techniques, allowing a variety of regulatory mechanisms to be widely explained. Furthermore, metabolite profiles provide a much broader view of systematic adjustment in metabolic processes compared to the conventional biochemical approaches, and also abundant opportunities to reveal new insights on metabolism.

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops, and it has continuously faced to unfavorable mineral conditions, especially early growth stage, because of a year-round intensive cultivation. Despite its importance, little information is available about metabolite changes in response to mineral deficiency. Because homeostasis of N, P and K is regulated at the whole-plant level, we studied biochemical metabolism in both leaves and roots to understand whole-plant adaptation to low mineral environments. In this study, commercial tomato seedlings were grown hydroponically on liquid culture under different N, P or K regimes. The levels of a wide range of metabolites (171 for leaf and 227 for root) were significantly changed in leaf and root materials. The results of this study provide new insights how N, P or K deficiency acts on primary and secondary metabolism.

2. Materials and methods

2.1. Plant materials and growth conditions

Tomato seeds (*Solamum lycopersicum* cv. Seonmyoung) were germinated on perlite supplied with deionized water for 2 weeks. Twelve uniformly sized seedlings were transplanted into holes in lids of aerated 20 L hydroponic containers containing 1/3 strength Hoagland solution, and grown for another 2 weeks prior to initiation of treatments. The composition of mineral solution (NPK sufficient) was: 5 mM $Ca(NO_3)_2{\cdot}4H_2O$, 5 mM KNO_3, 2 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM KH_2PO_4, 1.5 mM Fe-EDTA, 1 mM NH_4NO_3, and 1 mL micronutrients mixture (H_3BO_3, 2.86 g L^{-1}; $MnCl_2{\cdot}4H_2O$, 1.81 g L^{-1}; $ZnSO_4{\cdot}7H_2O$, 0.22 g L^{-1}; $CuSO_4{\cdot}5H_2O$, 0.051 g L^{-1}; $H_2MoO{\cdot}4H_2O$, 0.09 g L^{-1}). In order to induce mineral deficiency of tomato plants, each N, P or K-deficient medium was adjusted to 1/10 strength of mineral sufficient medium as follows; 0.5 mM $Ca(NO_3)_2{\cdot}4H_2O$ and 0.5 mM KNO_3 for N deficiency, 0.05 mM KH_2PO_4 for P deficiency, and 0.5 mM KNO_3 for K deficiency, respectively. The shortage of Ca, K and NO_3 resulted from N, P or K deficiency was equivalently compensated with $CaCl_2$, KCl and NH_4NO_3, respectively. Plants were grown at 30 ± 3 °C during the day and of 20 ± °C during the night. Mid-day photosynthetic pho-

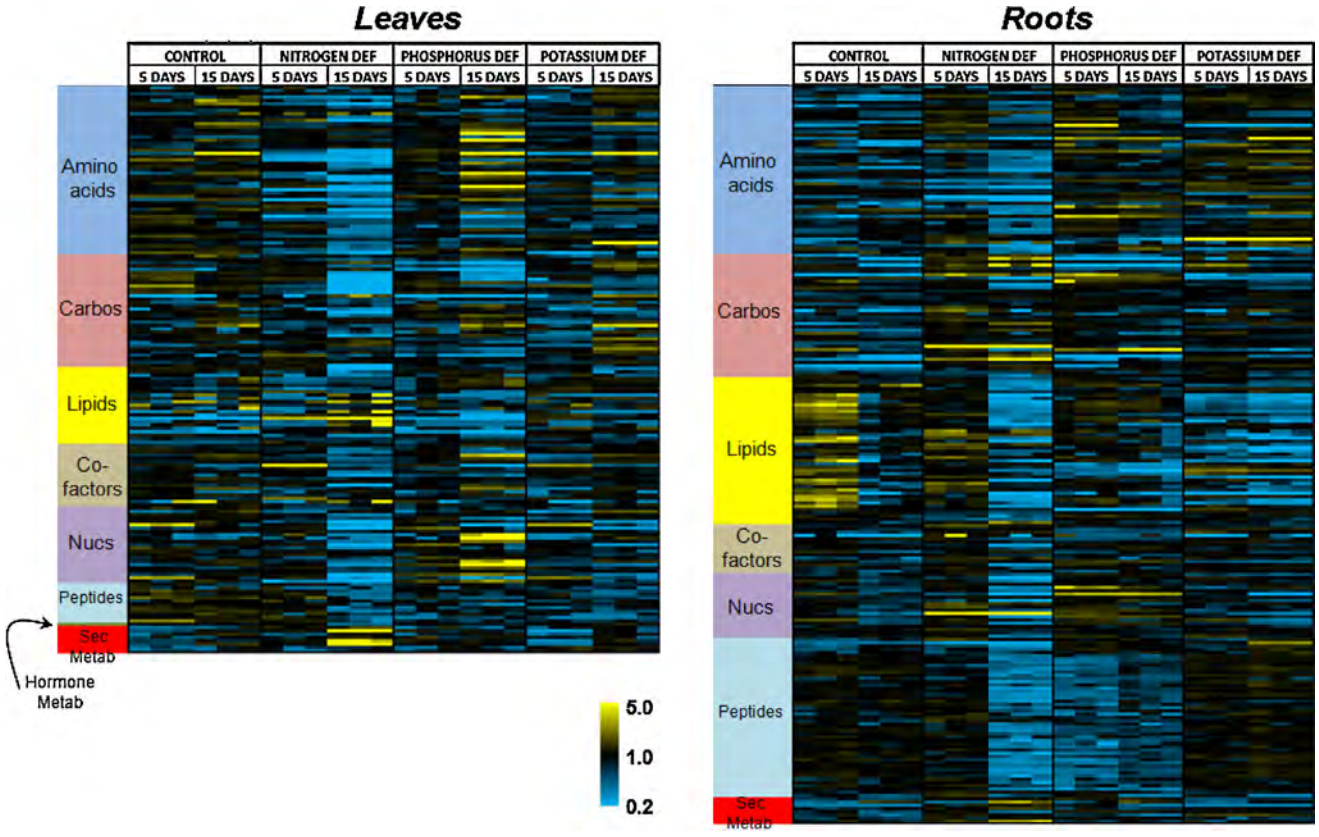


Fig. 1. Heat maps of untargeted compounds obtained from 171 in leaves (left) and 227 in roots (right) of N, P or K-deficient tomato plants.

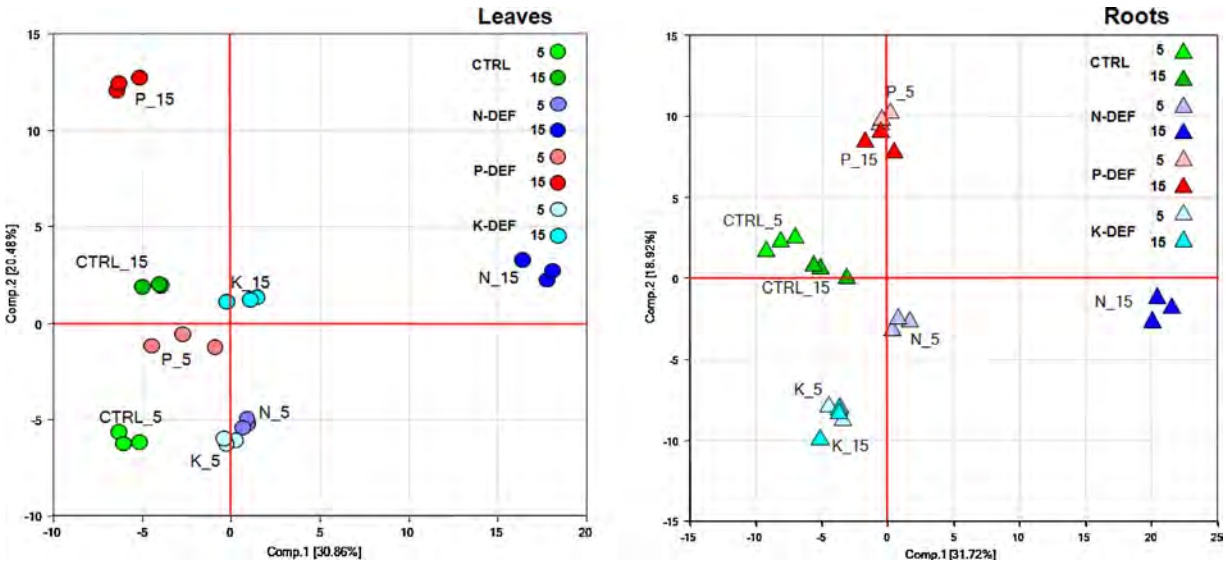


Fig. 2. Sample scores for the first (PC1) and second (PC2) principal components provided by PCA analysis for identified metabolites in leaf and root samples obtained from N, P and K-deficient tomato plants. Each group is represented by 3 samples.

ton flux density was 800–1200 μmol m^{-2} s^{-1}. The nutrient solution was replaced every 3 days. Three plants having a similar growth from each treatment were harvested between 10:00 and 12:00 to minimize diurnal effects on metabolite levels at 5, and 15 days from four mineral conditions (NPK sufficiency and N, P or K deficiency). Upper fully-expanded leaves and whole roots were rinsed briefly in deionized water and immediately frozen in liquid nitrogen, and stored at −80 °C until biochemical analysis.

2.2. Metabolomic profiling

The GC–MS method for the analysis of leaf metabolites was previously developed [19]. Sample preparation used the automated MicroLab STAR® system (Hamilton Company). For quality controls, recovery standards were added prior to the first step of the extraction process. A proprietary series of organic and aqueous extractions removed the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by LC and one for analysis by GC. The organic solvent was removed using a TurboVap® (Zymark). Each sample was then frozen and dried under vacuum. Samples were then prepared for either LC/MS or GC/MS. The LC/MS was based on a Waters ACQUITY UPLC (Waters Corporation) and a LTQ mass spectrometer (Thermo Fisher Scientific Inc.), which consisted of an electro spray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The sample extract was split into two aliquots, dried, then reconstituted in acidic or basic LC-compatible solvents, both of which contained 11 or more injection standards at known concentrations. One aliquot was analyzed using acidic positive ion optimized conditions and a second aliquot using basic negative ion optimized conditions in two independent injections into separate dedicated columns. Extracts reconstituted in acidic conditions were gradient eluted using water and methanol, both containing 0.1% Formic acid. The basic extracts also used water and methanol, both containing 6.5 mM ammonium bicarbonate. The MS analysis alternated between MS and data-dependent MS^2 scans using dynamic exclusion. The samples destined for GC/MS analysis were re-dried under vacuum desiccation for at least 24 h, then were derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA). The GC column was 5% phenyl and the temperature was ramped from 40 °C to 300 °C over a 16 min period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS using electron impact ionization. The instrument was tuned and calibrated daily for mass resolution and mass accuracy. The output from the raw data files was automatically extracted as discussed below. The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which had a linear ion-trap (LIT) front end and a Fourier transform ion cyclotron resonance (FT-ICR) MS back-end. An accurate mass measurement was performed for ions with counts greater than 2 million. The typical mass error was less than 5 ppm.

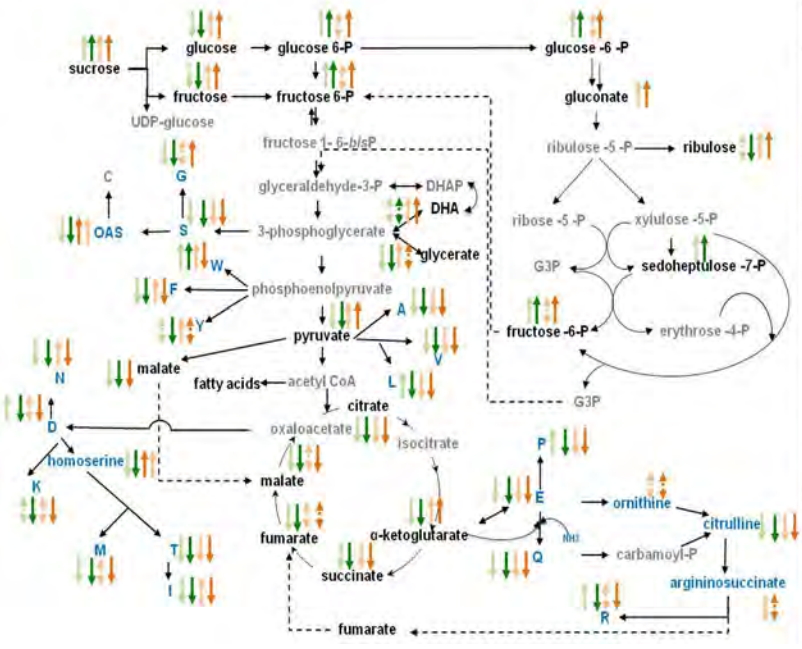
2.3. Compound identification and normalization

Compounds were identified by comparison to purified standards or recurrent unknown entities. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. More than 1000 commercially available purified standard compounds were in the Laboratory Information Management System (LIMS) for distribution to both the LC and GC platforms for determination of their analytical characteristics. The combination of chromatographic properties and mass spectra indicated a match to a specific compound or an isobaric entity. Additional entities were identified by recurrence (both chromatographic and mass spectral). A data normalization step was performed to correct variation from instrument inter-day tuning differences [25]. Essentially, each compound was corrected in run-day blocks by registering the medians to equal one (1.00) and normalizing each data point proportionately.

2.4. Statistical analysis

To visualize the entire data set, we generated heat maps to show fold change for each compound identified by GC–MS and LC–MS analyses of the tissue samples (see Supplementary datasets I and II). Fold change for each metabolite was calculated as the mean relative quantity of that compound in each treatment divided by the quantity in the control treatment (NPK optimal). Statistical analysis of the data was performed using the program R (http://cran.r-project.org). A p-value was obtained by Welch's t test, and if $p \leq 0.05$, the metabolite was considered significantly increased or decreased in abundance. We used the FDR to correct for Welch's two sample t test comparisons for the hundreds of compounds detected. The

(A)



(B)

Pathway	Metabolite	N deficiency/NPK suffficiency			
		Leaves		Roots	
		Day 5	Day 15	Day 5	Day 15
Amino acid	glycine	0.43	0.25	1.16	0.67
	o-acetylserine	0.38	0.06	2.14	3.07
	serine	0.73	0.58	0.82	0.49
	phenylalanine	0.86	0.42	1.26	0.66
	tryptophan	1.28	1.6	1.32	0.62
	tyrosine	0.99	0.62	6	1.12
	alanine	0.59	0.26	0.6	0.58
	asparagine	0.81	0.02	1.36	0.29
	aspartate	0.19	0.21	1.05	3.77
	homoserine	0.1	0.07	1.59	3.28
	lysine	0.99	0.39	1.17	0.36
	methionine	0.7	0.37	1.1	0.47
	threonine	0.42	0.16	0.85	0.27
	arginine	1.69	0.41	1	0.76
	argininosuccinate			0.44	0.56
	citrulline	0.24	0.11	0.38	0.17
	glutamate	0.43	0.69	0.73	0.72
	glutamine	0.15	0.12	0.32	0.12
	histidine			0.41	0.26
	ornithine			0.66	0.66
	proline	1.58	0.5	0.52	0.2
	isoleucine	0.76	0.42	1.29	0.36
	leucine	1.33	0.44	1.56	0.36
	valine	0.83	0.45	1.3	0.46
Glycolysis and carbohydrate metabolism	1,3-dihydroxyacetone	0.95	0.56	4.43	2.1
	fructose-6-phosphate	1.5	1.94	1.38	21.46
	glucose	0.9	0.64	1.71	1.58
	glucose-6-phosphate (G6P)	2.26	1.94	1.35	23.2
	glycerate	0.66	0.21	1.17	0.96
	pyruvate	0.61	0.17	1.5	1.39
	sedoheptulose-7-phosphate	14.03	4.47		
	ribulose			1.89	1.55
	fructose	0.83	0.81	2.09	2.37
	sucrose	1.66	1.38	5.17	11.76
	gluconate			1.81	1.92
TCA	alpha-ketoglutarate	0.89	0.06	22	14.19
	citrate	0.7	0.01	0.62	0.13
	fumarate	0.2	0.08	0.65	1.97
	malate	0.44	0.02	0.96	0.29
	succinate	0.37	0.12	0.59	0.58

Fig. 3. Differences in the metabolites in glycolysis/TCA cycle and amino acids biosynthesis in the leaves and roots. (A) Glycolysis/TCA cycle and amino acid biosynthesis pathway. The metabolites in black and blue indicate carbohydrates/organic acids and amino acids, respectively. The metabolites in gray indicate that they are below detection level (not detected). The arrow in light and dark green indicates Day 5 and 15 in the leaves, respectively, and the arrows in light and dark red indicate Day 5 and 15 in the roots. Upward-pointing arrows show increased amounts under N deficiency compared of the control (optimal N, see Section 2), downward-pointing arrows show decreased amount under N deficiency, and the bidirectional arrows mean unchanged. (B) Heat map showing the ratio of the metabolite levels between N deficiency and control and their statistical significance of the differences. Cells shaded with green indicate significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of <1.00. Cells shaded with indicate narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of <1.00. Cells shaded with red indicate significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of ≥1.00. Cells shaded with light red indicate narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of ≥1.00. Cells shaded with non-colored text and cell mean values are not significantly different for that comparison. The score in each cell indicates the fold changes between N deficiency and NPK sufficiency.

FDR for a given set of metabolites was estimated by the q value [26]. Box plots were generated for compounds with a significant increase or decrease using both t test and FDR, with $p < 0.05$ and $q < 0.10$ as significance values.

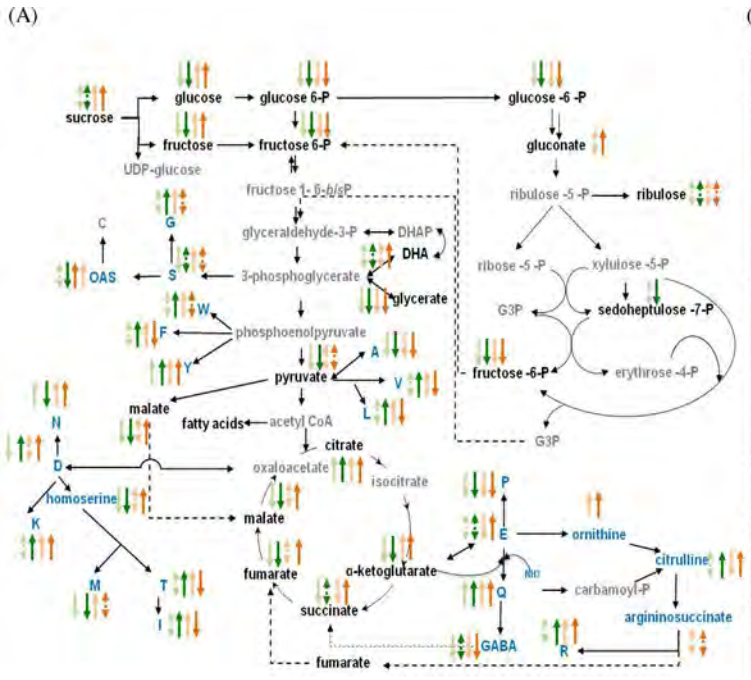
3. Results

3.1. LC–MS and GC–MS determination of metabolite levels

Since N, P, or K deficiency can affect plant metabolic pathways and thus affect the quantities of metabolites we performed metabolomics profiling of leaves and roots of tomato seedlings at two time points. Relative levels of differentially abundant metabolites in tomato leaves and roots were measured by LC–MS and GC–MS. In this study we identified 171 metabolites from leaves and 227 metabolites from roots, and they were expressed by heat map (Fig. 1). Plots of the first and second principal component analysis (PCA) scores revealed the differences in metabolic profiles: the results formed distinct clusters that clearly corresponded to the differences in N, P or K-deficiency and time points in both leaves and roots (Fig. 2). First is differential response to mineral conditions. The first principal component accounting for 30.86 in leaves and 31.72% in roots, respectively, indicated a strong differential response in between mineral conditions at the metabolic level. Second is differential response to time points. The second principal component accounting for 20.48 in leaves and 18.92% in roots, respectively, indicated a strong differential response in between time points at the metabolic level. PCA analysis showed that the effects of mineral conditions and time points clearly contributed most to the total variance within the data set. To understand the biological implications of these metabolic changes, the compounds were categorized into specific biological pathways (see Supplementary data set I and II).

3.2. Response of primary metabolites to N deficiency

Most of primary metabolites changed markedly in abundance in leaves and roots during nitrogen deficiency (Fig. 3A and B). Overall, most amino and organic acids of the tricarboxylic acid (TCA) cycle declined in both tissues (green-colored cells of the heat-map) markedly at day 5 and 15 of nitrogen deficiency. In contrast, the change in carbohydrates was tissue-specific, in that they decreased in leaves by 25–50%, but increased several-fold in roots. In the N-deficient leaves, changes in abundance were strongly dependent on the tissue and the carbohydrate species (Fig. 3A). Fru-6-P, Glc-6-P, sedoheptulose-7-P and Suc accumulated in leaves, and sedoheptulose-7-P had a high increase. Conversely, N-deficient roots displayed a clear trend of increasing carbohydrates and glycolysis intermediates, where 1,3-dihydroxyacetone (DHA), Fru-6-P, Glc, Glc-6-P, glycerate, pyruvate, ribulose, Fru and Suc significantly increased during deficiency. A surprising event was the significant increase in major carbohydrates, Fru-6-P (21.5 fold increase), Glc-6-P (23.2 fold increase) and Suc (11.8 fold increase). The levels of TCA cycle-involved organic acids, citrate, fumarate, malate and succinate, were decreased at day 5, and recovered at day 15. A dramatic and rapid increase was observed in the level of the α-*keto*glutarate in roots, which increased 22- and 14-fold compared to NPK sufficiency at day 5 and 15, respectively. In addition, a gluconate involved in ribulose biosynthesis was largely increased under N deficiency. The majority of amino acids in



Pathway	Metabolite	P deficiency/NPK sufficiency			
		Leaves		Roots	
		Day 5	Day 15	Day 5	Day 15
Amino acid	glycine	1.14	2.27	2.19	1.06
	O-acetylserine	0.96	0.29	1.4	1.77
	serine	0.76	1.01	1.17	0.96
	phenylalanine	1.02	1.38	1.3	0.88
	tryptophan	1.14	2.0	2.14	0.94
	tyrosine	1.2	6.9	15.33	1.19
	alanine	0.85	0.71	0.65	0.7
	asparagine	2.61	0.75	6.26	3.34
	aspartate	0.82	1.27	0.96	4.62
	homoserine	0.56	0.31	1.32	2.48
	lysine	0.86	3.12	4.64	1.77
	methionine	0.69	0.71	1.37	0.91
	threonine	0.91	1.67	1.39	0.73
	arginine	1.18	9.26	3.92	2.76
	argininosuccinate			0.56	0.71
	citrulline	1.09	1.32	0.9	1.4
	gamma-aminobutyrate (GABA)	0.74	1	0.72	0.52
	glutamate	0.83	1.16	1.1	1.28
	glutamine	2.03	1.44	2.06	1.5
	histidine			1.24	2.42
	ornithine			2.9	3.66
	proline	0.8	0.55	0.71	0.45
	isoleucine	1.08	2.11	2.03	0.72
	leucine	1	1.76	2.37	0.74
	valine	0.97	1.3	1.87	0.79
Glycolysis and carbohydrate metabolism	1,3-dihydroxyacetone	0.95	0.88	1.44	0.68
	fructose-6-phosphate	0.59	0.1	0.05	0.69
	glucose	0.59	0.52	1.3	1.1
	glucose-6-phosphate (G6P)	0.64	0.05	0.07	0.36
	glycerate	0.58	0.24	0.4	0.75
	pyruvate	0.56	0.3	1	0.99
	sedoheptulose-7-phosphate	1	0.08	0.96	1.29
	ribulose	0.69	1.03	1.01	0.92
	fructose	0.55	0.47	1.57	1.25
	sucrose	1.14	1.1	1.71	7.12
TCA	alpha-ketoglutarate	0.55	0.23	27.34	10.44
	citrate	1.17	1.93	1.8	1.21
	fumarate	0.47	0.45	1.56	1.55
	malate	0.63	0.63	1.05	1.07
	succinate	0.79	1.12	0.69	1.52

Fig. 4. Differences in the metabolites in glycolysis/TCA cycle and amino acids biosynthesis in the leaves and roots. Tomato plants were grown in a Hoagland nutrient solution with 0.5 mM KH_2PO_4 (optimal P) or 0.05 mM KH_2PO_4 (low P), harvested at day 5 and 15 after the treatment, and separated into leaves and roots. (A) Glycolysis/TCA cycle and amino acid biosynthesis pathway. The metabolites in black and blue indicate carbohydrates/organic acids and amino acids, respectively. The metabolites in gray indicate that they are below detection level (not detected). The arrow in light and dark green indicates Day 5 and 15 in the leaves, respectively, and the arrows in light and dark red indicate Day 5 and 15 in the roots. Upward-pointing arrows show increased amounts under P deficiency compared of the control (optimal P, see Section 2), downward-pointing arrows show decreased amount under N deficiency, and the bidirectional arrows mean unchanged. (B) Heat map showing the ratio of the metabolite levels between N deficiency and control and their statistical significance of the differences. Cells shaded with green indicate significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of <1.00. Cells shaded with indicate narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of <1.00. Cells shaded with red indicate significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of ≥1.00. Cells shaded with light red indicate narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of ≥1.00. Cells shaded with non-colored text and cell mean values are not significantly different for that comparison. The score in each cell indicates the fold changes between N deficiency and NPK sufficiency.

leaves decreased progressively during N deficiency. The changes in Arg and Pro (α-*keto*glutarate derived), and Leu (pyruvate-derived) were somewhat different, with an initial increase in levels followed by a decline. The level of Trp (PEP-derived) remained remarkably high throughout the experiment. Variation in the levels in roots was remarkable, and Phe, Trp and Tyr (PEP-derived), Asn and Ile (OAA-derived), and Leu and Val (pyruvate-derived) was largely increased at day 5 and decreased at day 15. By contrast, Asp and homo-serine (OAA-derived) remained unchanged at the first time point, then increased. In addition, the level of *o*-acetyl-serine (phosphoglycerat-derived) was remarkably high throughout the experiment.

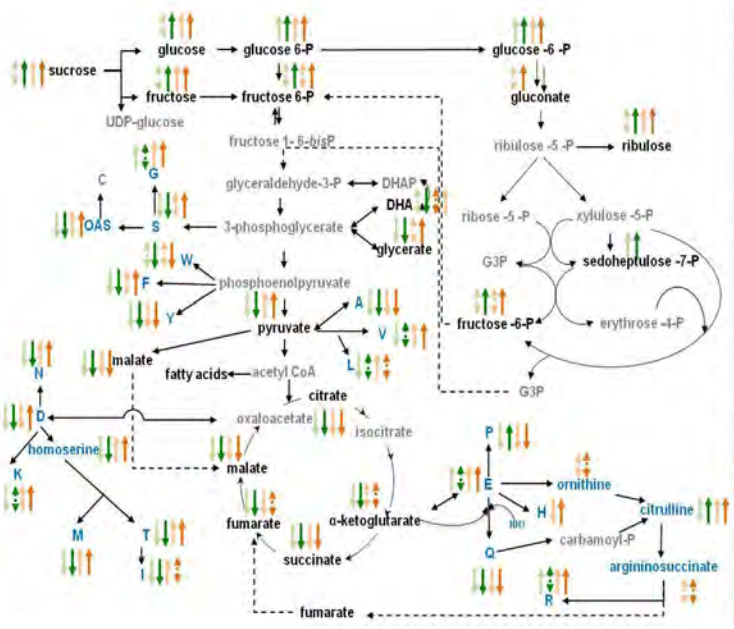
3.3. Response of primary metabolites to P deficiency

The levels of carbohydrates and glycolysis intermediates in leaves were extremely decreased by P deficiency (Fig. 4A and B), and most of them (except DHA and Suc, which remained unchanged during the experiment period) were at ~50% of NPK sufficiency at day 5, and less than 25% of NPK sufficiency at day 15. The levels of Glc, sedoheptulose-7-P, Fru and Suc in roots showed a clear increase in response to P deficiency, while Fru-6-P, Glc-6-P and glycerate remained at low levels. The response of organic acids to P deficiency was largely tissue-specific, where they decreased in leaves and increased in roots. The levels of α-*keto*glutrate, fumarate and malate in leaves decreased sharply over the time-course, while citrate accumulated. However, the levels of organic acids in roots largely increased during the experiment. As observed with N deficiency, a surprising result was the level of α-*keto*glutrate, which increased more than 10-fold during the experiment. A large change in the amino acids occurred in both leaves and roots under P deficiency. In leaves, Tyr, Asn and Gln increased at day 5, while most of the amino acids had increased at day 15. An increasing ratio of Tyr and Arg was distinct. The change in amino acids in roots was extremely different than in leaves, with an initial (day 5) dramatic increase followed by a progressive decline for some. The levels of GABA and Pro in both tissues decreased remarkably during the experiment, while *o*-acetyl-serine (phosphoglycerate-derived), Asn and Lys (OAA-derived), and Arg, Gln and ornithine (α-*keto*glutrate-derived) increased several fold. The most remarkable change was Tyr level in both tissues, which increased 1.2-fold at day 5 to 6.9-fold at day 15 in leaves, and increased 15.3-fold at day 5 in roots whereas shown no significant difference from NPK sufficiency at day 15. A

3.4. Response of primary metabolites to K deficiency

There was a substantial effect of potassium deficiency on the levels of many primary metabolites (Fig. 5 and B) in both tissues and time points. The profile in leaves was marked by decreased levels of most carbohydrates, amino and organic acids, and increased levels of only a few metabolites. Increased metabolites included the carbohydrates, Glc-6-P, Fru-6-P, Glc, ribulose, Fru, Suc and sedoheptulose-7-P, and the amino acids, Arg, Gln, citrulline and Pro, during the experimentThe time-course of metabolite changes under K deficiency in roots was different than in leaves, except for organic acids, which remained relatively low. Most amino acids increased in roots, and only a few amino acids such as Tyr, Ala, Asp, Glu, His and Pro decreased. The levels of Asp and His were lower at day 15 compared to at day 5. The carbohydrates and glycolysis

(A)



(B)

Pathway	Metabolite	K deficiency/NPK sufficiency			
		Leaves		Roots	
		Day 5	Day 15	Day 5	Day 15
Amino acid	glycine	0.59	1.45	1.21	1.24
	O-acetylserine	0.59	0.42	3.43	3.36
	serine	0.67	0.87	1.27	1.47
	phenylalanine	0.74	0.74	1.14	1.17
	tryptophan	1.07	0.71	0.98	1.16
	tyrosine	0.81	0.81	0.63	0.19
	alanine	0.73	0.77	0.92	0.73
	asparagine	0.53	0.75	2.11	4.71
	aspartate	0.66	0.61	0.6	4.34
	homoserine	0.75	0.54	2.15	5.85
	lysine	0.76	1.1	1.58	2.21
	methionine	0.7	0.85	1.28	1.12
	threonine	0.71	0.63	1.37	1.11
	arginine	1.56	1.03	2.92	2.21
	argininosuccinate			1.17	0.72
	citrulline	0.82	1.23	1.56	2.65
	GABA			1.53	1.24
	glutamate	0.72	0.41	0.45	0.58
	glutamine	1.49	1.05	1.84	2.47
	histidine			0.75	1.56
	ornithine			0.81	1.39
	proline	0.74	1.35	0.6	0.72
	isoleucine	0.82	0.89	1.17	1.06
	leucine	0.73	1.02	1.34	1.01
	valine	0.81	1	1.44	1.36
Glycolysis and carbohydrate metabolism	1,3-dihydroxyacetone	0.82	0.43	4.16	1.36
	fructose-6-phosphate	1.22	1.92	1.03	3.11
	glucose	0.85	2.97	1.43	1.36
	glucose-6-phosphate (G6P)	1.79	2.2	2.24	4.12
	glycerate	0.45	0.74	1.07	1.25
	pyruvate	0.69	0.85	1.4	1.24
	sedoheptulose-7-phosphate	12.13	4.82		
	ribulose	1.08	1.5	1.7	1.23
	fructose	0.79	2.92	1.65	1.58
	sucrose	1.01	1.72	5.6	11
TCA	alpha-ketoglutarate	0.45	0.48	2.16	2.23
	citrate	0.52	0.55	0.3	0.27
	fumarate	0.7	0.49	0.5	0.68
	malate	0.59	0.55	0.73	0.86
	succinate	0.52	0.46	0.58	0.77

Fig. 5. Differences in the metabolites in glycolysis/TCA cycle and amino acids biosynthesis in the leaves and roots. Tomato plants were grown in a Hoagland nutrient solution with 5 mM KNO_3 (optimal K) or 0.5 mM KNO_3 (low K), harvested at day 5 and 15 after the treatment, and separated into leaves and roots. (A) Glycolysis/TCA cycle and amino acid biosynthesis pathway. The metabolites in black and blue indicate carbohydrates/organic acids and amino acids, respectively. The metabolites in gray indicate that they are below detection level (not detected). The arrow in light and dark green indicates Day 5 and 15 in the leaves, respectively, and the arrows in light and dark red indicate Day 5 and 15 in the roots. Upward-pointing arrows show increased amounts under K deficiency compared of the control (optimal K, see Section 2), downward-pointing arrows show decreased amount under N deficiency, and the bidirectional arrows mean unchanged. (B) Heat map showing the ratio of the metabolite levels between N deficiency and control and their statistical significance of the differences. Cells shaded with green indicate significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of <1.00. Cells shaded with indicate narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of <1.00. Cells shaded with red indicate significant difference ($p \leq 0.05$) between the groups shown, metabolite ratio of ≥1.00. Cells shaded with light red indicate narrowly missed statistical cutoff for significance $0.05 < p < 0.10$, metabolite ratio of ≥1.00. Cells shaded with non-colored text and cell mean values are not significantly different for that comparison. The score in each cell indicates the fold changes between N deficiency and NPK sufficiency.

intermediates increased or were not significant compared to NPK sufficiency, and, in addition, the levels of Fru-6-P, Glc-6-P and Suc became progressively higher with time, while DHA and ribulose decreased.

3.5. N, P or K deficiency-induced alternative carbohydrate biosynthesis

The dramatic elevation of raffinose (a trisaccharide composed of galactose, glucose and fructose) biosynthesis metabolites was observed in N, P or K-deficient roots (Fig. 6). The level of raffinose in N, P or K-deficiency was 2.9, 3.9 and 3.6 fold as high, respectively, at day 15. Additionally, Glc-6-P and galactinol, intermediates for raffinose biosynthesis, was greatly increased in P deficiency (23.2 and 29.5 fold) at day 5 whereas N or K-deficiency was not observed in the constant trend of fluctuation.

3.6. Polyamine metabolism in response to N, P or K deficiency

One of the most significant metabolic changes in K-deficient roots was the higher levels of putrescine (13.9 and 5.8 fold increase at day 5 and 15) and spermidine (1.3 and 2.4 fold increase) (Fig. 7). N deficiency also led to significant increase in the levels of putrecine (4.2 fold) and spermidine (2.8 fold) at day 5 but not day 15. Furthermore, in K deficiency, Arg, the precursor of putrescine and *N*-acetyl putrescine, was accumulated at higher amounts (2.2–2.9 fold increase). However, the level of ornithine, another precursor of the diamine putrescine, remained unchanged during the experiment. In addition, levels of γ-aminobutyric acid (GABA) and β-alanine, compounds closely associated with polyamine metabolism, also increased from 1.2 to 1.8 fold.

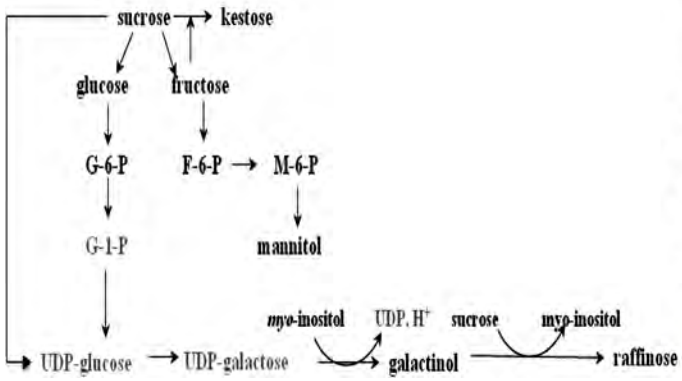
4. Discussion

Our objective in this study was to obtain an insight into plant responses to N, P or K deficiency by discovering new information about changes in metabolite abundance in tomato plants. Because homeostasis of N, P and K is regulated at the whole-plant level, we sampled both leaves and roots. We used LC–MS and GC–MS techniques to investigate abundance and identities of metabolites, which gave clues to changes in key biochemical pathways under these mineral deficiencies.

4.1. Effect of N deficiency on metabolic responses

N metabolism in plants is controlled by a complex network that includes nitrate, sugars, organic acids, amino acids, hormones and other chemicals [27]. The uptake and assimilation of N interacts to affect metabolites further downstream [28]. It is well known that nitrate assimilation is tightly regulated by nitrate reductase, which is subject to a hierarchy of transcriptional, translational and post-translational regulation [29,30]. Amino acids significantly decrease when N is limiting [20,27,31–34]. We saw similar results in the present study (Fig. 3), in which amino acids substantially decreased in the leaves. Surprisingly, however, the levels of several amino acids that are derived from 3-PG, PEP and oxaloacetate pathways significantly increased in roots, e.g., 6.0 fold increase for Tyr. This implies that there is feedback from shoots to roots, and suggests that accumulation of DHA, glycerate and pyruvate resulted

(A)



(B)

Carbohydrates	N def./NPK suf.		P def./NPK suf.		K def./NPK suf.	
	Day 5	Day 15	Day 5	Day 15	Day 5	Day 15
sucrose	5.37	7.12	11.78	5.6	1.71	11
Isobar: 1-kestose, levan	1.92	2.18	2.86	2.51	1.81	2.11
glucose	1.73	1.1	1.58	1.43	1.3	1.36
glucose-6-phosphate (G6P)	1.35	0.36	23.2	2.24	0.07	4.12
fructose	2.09	1.25	2.37	1.65	1.57	1.58
fructose-6-phosphate	1.38	0.69	21.46	1.03	0.09	3.11
galactinol	8.96	1.07	29.49	1.16	1.01	1.27
raffinose	6.68	2.85	22	3.91	0.87	3.61
mannose-6-phosphate	1.27	0.87	13.3	1.4	0.18	2.88
mannitol	1.36	0.98	1.74	1.1	1.01	1.25

Fig. 6. Elevation of raffinose biosynthesis pathway in tomato roots in response to N, P or K deficiency. Tomato plants were grown in a Hoagland nutrient solution with 0.5 mM $Ca(NO_3)_2{\cdot}4H_2O$ and 0.5 mM KNO_3 (low N), 0.05 mM KH_2PO_4 (low P), or 0.5 mM KNO_3 (low K), harvested at day 5 and 15 after the treatment (See the Section 2 for the detail).

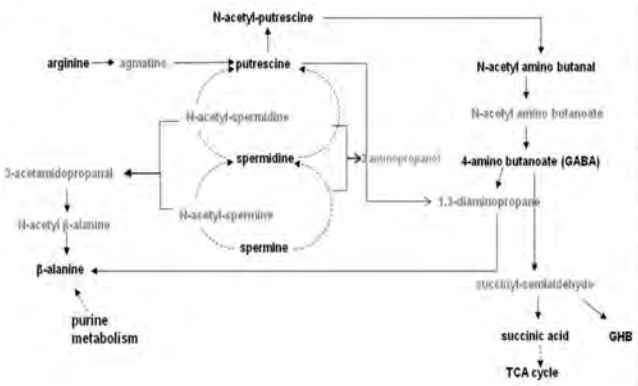
in altered fluxes of amino acid biosynthesis. Further experiments are needed to understand the high accumulation of amino acids. Starch accumulates under N-limited conditions [35,36], whereas the major soluble sugars response is less clear, with reports that they decrease [37] or increase [20,38,39]. The concentration of a wide range of soluble sugars changed markedly in response to N deficiency, and the changes were different between root and shoot tissues. A large increase in Glc-6-P and sedoheptulose-7-P in the leaves suggests that Glc-6-P is predominantly used in the pentose-P pathway when the downstream glycolysis pathway is restricted. Furthermore, soluble sugars and the intermediates of glycolysis accumulated in roots, with considerable levels of Fru-6-P, Glc-6-P and Suc. The TCA-cycle organic acids citrate, succinate, fumarate and malate were significantly reduced under N deficiency, which is in agreement with previous studies [20,37]. In the present study, results suggest that N deficiency resulted in decomposition or restricted synthesis of Glu and Gln, and subsequently a large accumulation of α-*keto*glutarate (14.19–22.0 fold increase) and major soluble sugars. The importance of α-*keto*glutarate and Glu as key regulators of C/N interactions and amino acid biosynthesis has been demonstrated in previous studies [40,41]. Most strikingly in our study, the levels of α-*keto*glutarate decreased significantly in leaves and largely increased in roots. Therefore, the changes in the levels of α-*keto*glutarate led to a decrease in amino acids in leaves (with the exception of P deficiency) and an increase in roots (with the exception of N deficiency). There are contrasting studies; increased levels of many amino acids in *Arabidopsis* shoots [20,42–44] and decreased levels in tomato [20] under low N condition; accumulation of amino acids, especially Gln and Asn, under low P conditions [45], and slight increases in amino acids in K-deficient *Arabidopsis* roots [13]. In contrast to previous studies [13,15,17,45,46] the level of α-*keto*glutarate in our work increased only in roots but not in leaves. On the basis of these differences, further experiments are required to establish a direct link of N, P or K deficiency to the increased accumulation of α-*keto*glutarate in plants. In addition, it is well documented that nitrogen deficiency induces phloem loading and partitioning of sugar, particularly sucrose, from source to sink tissues. Sugar signal transduction triggers nutrient-responsive gene expression to produce C- and N-containing compounds such as amino acids and organic acids. Morphological responses include enlarging the root surface area [47]. The present study suggests that the reduced levels of amino acids under N deficiency are derived from both limitation of the N available for N-containing compounds, and from accumulation of several intermediates such as soluble sugars, pyruvate, *o*-acetylserine, homoserine, and α-*keto*glutarate.

4.2. *Effect of P deficiency on metabolic responses*

Metabolic responses to P status were well-coordinated, as soluble sugars and organic acids decreased and amino acids increased in leaves of P-deficient plants. In roots, three major metabolite pools such as soluble sugars, organic acids and amino acids had an increasing pattern. P deficiency leads to increased transcripts of genes for enzymes involved in protein degradation and, simultaneously, the suppression of genes for protein synthesis [21,48,49]. The increase in total free amino acids in both tissues under P deficiency (Fig. 4) is in agreement with studies that showed increased protein degradation, increased total free amino acids, repressed protein synthesis [45], and accumulation of most amino acids [15]. Gln and Asn were elevated in both leaves and roots of severely P-deficient tomato plants, although Asn tended to decrease with extended treatment. Gln and Asn require a carbon skeleton derived from the TCA cycle, such as α-*keto*glutarate and oxaloacetate, and subsequently lead to further consumption of organic acids and restriction in energy production under P deficiency. Increases in major amino acids, including Gln and Asn, also occur in other nutrient stresses, including N and sulfate deficiency [17,50]. Most strikingly, P deficiency resulted in significant increases in Tyr (PEP derived) and Arg (α-*keto*glutarate derived) in both leaf and root tissues. Tyrosine functions as a receiver of phosphate groups that are transferred by protein kinases to activate target proteins, and thus the accumulation of Tyr and aromatic amino acids, Phe and Trp might be from degradation of proteins [45]. Additionally, the large accumulation of Arg during P deficiency is consistent with previous reports [21,51] that ammonia accumulates during P deficiency and that the Arg biosynthetic pathway is an early response to detoxify ammonia from P-deficient tissue. However, the reason for the differences in the levels of amino acids is not clear, and it is merely presumed that enzymes using these amino acids as substrates might be activated under P deficiency.

Soluble sugars decreased in P-deficient leaves over 15 days. In roots, P deficiency greatly inhibited the phosphorylation of hexoses, such as glucose and fructose (Fig. 4). The levels of small phosphorylated metabolites, including Glc-6-P and Fru-6-P, largely decreased in both leaves and roots, consistent with previous reports [45,52]. Under P deficiency, organic acids had a tissue-dependent response with a significant decrease in leaves (except citrate), whereas roots had a substantial accumulation of most organic acids (Fig. 4). Further, the most striking organic acid change was the great accumulation of α-*keto*glutarate in roots. The reports of organic acid metabolism in response to P deficiency are controversial. The concentration of pyruvate and succinate largely increased

(A)



(B)

Metabolite name	N def./NPK suf.		P def./NPK suf.		K def./NPK suf.	
	Day 5	Day 15	Day 5	Day 15	Day 5	Day 15
arginine	1	0.76	3.92	2.76	2.92	2.21
putrescine	4.22	1.01	1.87	0.33	13.87	5.84
spermidine	2.75	2.21	0.97	0.81	1.25	2.4
N-acetylputrescine	1.54	3.44	0.85	1.72	2.23	3.84
gamma-aminobutyrate (GABA)	0.81	0.71	0.72	0.82	1.53	1.24
beta-alanine	1.21	0.44	1.57	0.78	1.75	0.86

Fig. 7. Polyamine metabolism in tomato roots in response to N, P or K deficiency. Tomato plants were grown in a Hoagland nutrient solution with 0.5 mM $Ca(NO_3)_2{\cdot}4H_2O$ and 0.5 mM KNO_3 (low N), 0.05 mM KH_2PO_4 (low P), or 0.5 mM KNO_3 (low K), harvested at day 5 and 15 after the treatment (See the Section 2 for the detail).

under P deficiency [15]. By contrast, the reduced levels of several organic acids of the TCA cycle including α-*keto*glutarate, succinate, fumarate and malate were observed in roots of *Arabidopsis* [49], common bean [21], and barley [45] under P deficiency. It is suggested from our data that the root-specific increase in organic acids is closely connected with carbohydrate metabolism, as indicated by large accumulation of Glc, Fru and Suc without a change in sucrose in leaves. It is well known that organic acids are secreted as a P adaptive mechanism, and this increases rhizosphere P availability [53–56].

4.3. Effect of K deficiency on metabolic responses

Because K is closely involved in the biosynthesis, conversion of, and allocation of metabolites, it is helpful to gain insight into the most crucial indicators of metabolic alterations in K-deficient plants. The metabolite profile of K-deficient tomato plants (Fig. 5) was characterized by a strong increase in the concentration of Fru-6-P, Glc-6-P and Suc which was especially notable in roots, and a large decrease in organic acids in leaves and an increase in roots. Our metabolite analysis included more types of metabolites, tissues and time points than previous reports. Some of our metabolite changes have been observed in K-deficient crops; e.g. strong increase in soluble sugars in leaves of *Arabidopsis* [13], bean [57], cotton [58,59], soybean [60] and wheat [61] and in roots of alfalfa [62], *Arabidopsis* [13] and sugar beet [63]. Interestingly, it was also observed that sedoheptulose-7-P strongly increased in leaves of K-deficient tomato plants, similar to N deficiency, which suggests that high levels of intermediates of the pentose-P pathway are largely associated with a restricted glycolytic pathway, potentially to minimize the damage of excessive accumulation of soluble sugars. K deficiency strictly restricts the distribution of photosynthetically fixed carbon (triose-P) to free amino acids [64], and temporary enhancement of oxidative pentose-P pathway to supply the intermediates for glycolysis [65]. Thus, it is required that the effect of K deficiency on major carbohydrate metabolism, such as triose-P and pentose-P, should be studied in more detail. The responses of amino acids to K deficiency revealed tissue-specific patterns (i.e. decrease in leaves and increase in roots). Most amino acids accumulate in K-deficient plants of many species [13,15,66–68] although acidic amino acids such as Glu and Asp decrease. The difference in amino acids between leaves and roots in our data may represent restricted transport of nitrate toward the leaves, resulting from predominant assimilation in roots, as suggested by reduced activities of nitrogen reductase (NR), glutamine synthetase (GS) and glutamate synthase (GOGAT) [13] or significantly increased protein degradation in roots. K deficiency results in reversible changes in several enzyme activities involved in sugar metabolism, glycolysis, TCA cycle and nitrogen assimilation [16]. However, the reason for the differences in amino acid metabolism between leaf and root tissue is unknown, and requires further study.

4.4. Effect of N, P or K deficiency on alternative carbohydrate biosynthesis

The biosynthesis of raffinose family oligosaccharides (RFOs) is initiated by the formation from UDP-galactose and myo-inositol by galactinol synthase, and sequential addition of sucrose leads to the formation of raffinose and RFOs. The RFOs accumulate in plants experiencing environmental stresses such as cold, heat, drought or salinity [23,69–75]. In our study, we found that mineral deficiency, especially N and K, increased RFO biosynthetic cascades (Fig. 6), which could be an alternative route for the consumption of sucrose and hexoses. This metabolic alteration appears to be intimately connected with C/N metabolism concerning the restricted glycolytic pathway in response to unfavorable mineral conditions.

4.5. Effect of N, P or K deficiency on polyamine metabolism

Cellular K acts as a counter-ion to negatively charged ions and compounds, and cellular K concentration is closely related to the concentration of organic acids. K deficiency led to a significant decrease in organic acids in both leaves and roots over the experimental period. Considering the equilibrium relationship, decreased in organic acids in both leaves and roots is presumed to contribute to maintaining charge balance. It is well established that organic acids in the TCA cycle are decreased by K deficiency [13,15]. Polyamines, especially putrescine, are involved in K deficiency [76,77] as well as development, senescence and various stress responses [78,79]. Putrescine is the simplest polyamine, and had the most considerable increase in tomato roots under K-deficiency, 13.84-fold at day 5 and 5.84-fold at day 15 (Fig. 7). It was suggested by our study that polyamines are a strong alternative to K for adjusting charge balance, as they were closely correlated with negatively charged compounds such as amino acids and organic acids.

5. Conclusions

The data presented here suggest that mineral deficiencies, especially N, P and K, have wide-ranging effects on plant metabolism as indicated by considerable changes, mineral and time-dependent increase or decrease, in primary and secondary metabolites. The

results of our study corroborate and extend previous studies in various plant species. We found that certain metabolic responses greatly differed in each mineral deficiency. From an overall point of view, mineral deficiency led to a decrease in metabolites in leaves and an increase in roots, and an increase in soluble carbohydrates and a decrease in amino acids and organic acids. Our present data provide new insights to understand and interpret the interaction between mineral deficiency and accumulation of metabolites. Depletion of minerals leads to alternative biochemical pathways leading to the production of mineral-deficient-induced primary to secondary metabolites. Most strikingly, we identified several changing metabolites from soluble sugars, amino acids, organic acids, nucleic acids and secondary metabolite in response to each mineral deficiency. This work provides a starting point to further elucidate the complex regulation involved in the metabolic networks affected by mineral stress by identifying specific metabolites and pathways that are uniquely affected by N, P, or K deficiency.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.09.027.

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