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Article

Temporal Changes in N Assimilation and Metabolite Composition of Nitrate-Affected Tomato Plants

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The role of inorganic nitrogen assimilation in the production of amino acids, organic acids and soluble sugars is one of the most important biochemical processes in plants, and, in order to achieve normally, nitrate uptake and assimilation is essential. For this reason, the characterization of nitrate assimilation and metabolite composition from leaves, roots and xylem sap of tomato (*Solanum lycopersicum*) was investigated under different nitrate levels in media. Tomato plants were grown hydroponically in liquid culture under five different nitrate regimes: deficient (0.25 and 0.75 mM NO_3^-), normal (2.5 mM NO_3^-) and excessive (5.0 and 10.0 mM NO_3^-). All samples, leaves, roots and xylem sap, were collected after 7 and 14 days after treatment. The levels of amino acids, soluble sugars and organic acids were significantly decreased by N-deficiency whereas, interestingly, they remained higher in xylem sap as compared with N-normal and -surplus. The N-excessive condition did not exert any significant changes in metabolites composition, and thus their levels were similar with N-normal. The gene expression and enzyme activity of nitrate reductase (NR), nitrite reductase (NIR) and glutamine synthetase (GS) were greatly influenced by nitrate. The data presented here suggest that metabolites, as a signal messenger, existed in xylem sap seem to play a crucial role to acquire nitrate, and, in addition, an increase in α-ketoglutarate pathway-derived amino acids under N-deficiency may help to better understand plant C/N metabolism.

Key words: Nitrate, Nitrate assimilation, Amino acids, Organic acids, Soluble sugars, Tomato

Introduction

Nitrogen (N) is one of the major environmental factors that control plant metabolism and closely related to crop production and quality. In particular, nitrate is well known to act as a signal manipulating the activity of many enzymes and transporters, such as nitrate reductase, phosphoenolpyruvate carboxylase, malate dehydrogenase, sucrose phosphate synthase and nitrate transporters involved in C and N balance in plants (Scheible et al., 1997). Thus, nitrate levels in a nutrient solution may influence the pathways that synthesize not only primary pathways (e.g. amino acids, carbohydrates and organic acids) but also secondary metabolites. Therefore, it is likely that the overall metabolic adaptation of a plant is affected by the N status, and in turn the C/N status. Although there have been many researches in relation to the effect of N application on the level of primary metabolites and nitrogen pools (Haynes and Goh, 1978; Darral and Wareing, 1981; Barneix and Causin, 1996), the effect of N status on temporal- and organ-based primary metabolites has been little known so far.

To understand changes in overall metabolism, a comprehensive and detailed analysis is required, and thus was first successfully applied to know metabolite profile (Roessner et al., 2000). Metabolite profiling has already proven to be a convenient and powerful tool, for example in characterizing the response to nutrient deprivation (Nikiforova et al., 2005). The response of key metabolic intermediates has been determined in many cases wherein nitrate assimilation has been modified by genetic and environmental perturbation (Muller et al., 2001; Masclaux-Daubresse et al., 2002). However, the study of metabolite profiling has not been attempted in order to characterize the responses to alter nitrogen nutrition and tissue-specific composition.

Tomato is one of the most popular vegetable crops

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grown commercially around the world, and its cultivation was ranked in scale the fifth among green houses-grown vegetable crops in South Korea. In this study, we used HPLC-based metabolite profiling in not only mature tomato leaves and roots to measure primary metabolites such as sugars, organic acids and amino acids, but also xylem exudates. The effect of N levels, which was subjected to the concentration of nitrate in the culture solution, on these metabolites was investigated. Additionally, to understand clearly N metabolism, we analyzed the gene expression and enzyme activity associated with the N assimilation.

Materials and Method

Plant materials, growth and treatment This study was conducted in a green house at NAAS, RDA in 2011. Seeds of tomato (*Lycopersicon esculentum* cv. Seonmyoung) were germinated in pearlite tray supplied with distilled-deionized water. Seedlings were transplanted into aerated containers with 1/3 strength of Hoagland nutrient solution. Four-weeks-old tomato plants were subjected to 12 holes-aerated 20L capacity containers with different nitrate levels as follows; 0.25, 0.75, 2.5 (Normal), 5.0 and 10.0 mM. Plants were constantly exposed for 2 weeks with average day temperature between 28±2℃ and night temperature between 18±2℃. Mid-day photosynthetic photon flux density was 900-1,000 μmol m^{-2} s^{-1}. The nutrient solution was replaced every 3 days. Plants were harvested between 10:00 and 14:00 at 7 and 14 days after treatment, immediately separated into leaves and roots, and used for further analysis. Xylem sap was obtained directly from intact tomato plants by cutting the stems with a sharp razor blade and collecting the sap (forced by the root pressure) from the cut stem ends.

N-assimilation enzyme activity NR and NIR activities were measured according to Merlo et al. (1995). For NR, frozen leaves (0.1 g) were homogenized with 1 ml of 50 mM HEPEs-KOH (pH 7.5) buffer, containing 0.5 mM EDTA, 5 mM $MgCl_2$, 14 mM ß-mercaptoethanol, 0.1% Triton×100 (v/v), 1% glycerol (v/v), 1% PVP (w/v), 50 mM leupeptin, and 0.5 mM PMSF. The homogenates were centrifuged at 5,000×g for 20 min and 100 μl of supernatants was supplemented with 400 μl of 50 mM HEPEs-KOH (pH 7.5) buffer containing 10 mM KNO_3, 2 mM EDTA, 0.2 mM NADH, and 10 μM FAD. For NIR, frozen leaves (0.1 g) were ground in a mortar and pestle homogenized with 1 ml of 100 mM Na-phosphate (pH 7.5) buffer, 1 mM L-cystein, and 1 mM EDTA. The homogenate was centrifuged at 10,000×g at 25 min for 4℃ and 100 μl of supernatants was supplemented with 2 ml Tris-HCl (pH 7.5) buffer containing 1 mM EDTA, 0.5 mM KNO_2, 200 mM sodium hydrosulfite in 200 mM sodium bicarbonate and 20 mM methyl viologen. The reaction was started after addition of 100 μl of the protein extracts. Incubation was carried out for 15 min at 30℃. The reaction was stopped by adding 50 μl of 0.5 M zinc acetate. Nitrite was revealed at 540 nm by adding 1 ml of 0.673 M sulphanilamide in 3 M HCl and 1 ml of 29.7 mM NED. Nitrate and nitrite reductase was expressed as μmol NO_2^- min^{-1} g^{-1} FW. For each measurement, one blank was prepared by adding zinc acetate prior to protein extract. GS activity was determined by using the hydroxylamine synthetase assay (Meister, 1985). Frozen leaves (0.1 g) were homogenized in 0.1 M Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol and 2 mM EDTA. After centrifugation at 20,000×g for 10 min at 4℃, the supernatant was used for enzyme assay as the crude extract. The standard reaction mixture (final volume, 1 ml) contained 100 mM imidazole-HCl (pH 7.2), 20 mM $MgCl_2$, 25 mM 2-mercaptoethanol, 50 mM sodium L-glutamate (pH 7.2), 125 mM hydroxylamine (pH 7.2), and 10 mM ATP. The reaction was started with the addition of the enzyme extract and after incubation for 15 min the reaction was stopped by the addition of 0.75 ml of ferric acid reagent (0.37 M $FeCl_3$, 0.2 M TCA and 0.67 M HCl). After centrifugation, the absorbance at 535 nm was read against a blank without enzyme. Assays without ATP and glutamate were used as controls. One unit of the enzyme activity was expressed as the amount that catalyzes the formation of 1 ml of γ-glutamylhydroxamate min^{-1}.

RNA isolation, cDNA preparation and RT-PCR Total RNA from fresh fully expanded leave samples was isolated by manufacturer's procedure (easy-spin Total RNA extraction Kit, iNtRON, Korea). The quality of total RNA without DNA contamination was tested by using 1% agarose-formaldehyde gel electrophoresis under standard protocols. Two micrograms of total RNA were used to synthesize cDNA by reverse

transcriptase (Maxmine RT premix, iNtRON, Korea). The cDNA samples were used as a template to quantify target gene expression level. The PCR analysis (i-MAX II, Maxime PCR premix, iNtRON, Korea) was carried out as follows: 95℃ for 15 mins, 30 cycles at 94℃ for 1 min; 55℃ for 1 min; and 72℃ for 1 min, then 72℃ for 10 min, and the PCR products of these genes were separated by 1.2% agarose gel electrophoresis. The PCR primers capable of amplifying segments of the nitrogen assimilation-related genes are: nitrate reductase (NR), F-GGTTCATCACTCCCGTACCACTT/R-TCTGCTTCACCATATTCTGCTCT; nitrite reductase (NIR), F-AGCTCGTTTGGCTGATGAGT/R-CTAGCAGGCATCCCATGAAT; glutamine synthetase-1 (GS-1), F-AAGTTGGACCTTCTGTTGGCATCT/R-GCCTTCTGTCCTCAAAGTATCCCT; glutamine synthetase-2 (GS-2), F-TGAGCCCATCCCAACAAACAA/R-AGATGCCAACAGAAGGTCCAA; and α-tubulin, F-TGAACAACTCATAAGTGGCAAAG/R-TCCAGCAGAAGTGACCCAAGAC.

Metabolites composition in leaves, roots and xylem sap of tomato plants Fresh leaves and roots (1g) were subjected to 10 ml of 0.5 N HCl (v/v) and heated at 80℃ for 10 min while shaking. The extracts (centrifuged at 12,000 rpm, 10 min, 4℃) and xylem sap were passed through 0.45 μm membrane filter and used for an analysis. Acid-soluble amino acids were determined by an Agilent 1100 HPLC (Agilent Technologies, Wilmington, DE, USA) procedure using a pre-column derivatization method. Known volumes of each sample and 16 amino acid standards were derivatized in a total volume of 100 μl using *o*-phthalaldehyde (OPA) for primary amino acids and 9-fluorenylmethyl chloroformate (FMOC) for secondary amino acids. Separations were performed on HPLC equipped with a 4.6×150 mm (5 μm) C18 column at 40℃. The mobile phase consisted of A (20 mM K-phosphate, pH 7.8) and B (ACN: MeOH:ddH2O=45:45:10, v/v/v). The column was preconditioned with 100% Eluent A for 10 min at a flow 1.5 ml min^{-1}. The injection volume for both standards and samples was 0.5 μl. Amino acids were eluted from the column by linearly increasing concentrations of Eluent B in the mobile phase. Eluent B was 0% between 0 and 1.9 min, 57% at 24 min and 100% at 26 min. The column was then washed with 100% Eluent A at 4 min before regenerating the column. Absorbance was detected with an UV detector (Agilent G1315A) at a wavelength of 338 nm. Quantification was based on standard curves obtained from 10 pmol to 1 nmol μl^{-1} of each standard amino acid. Acid-soluble sugars were determined using HPLC system (Dionex Ultimate 3000, USA) composed of an auto-sampler and detector (Shodex RI-101, Japan). Separation of sugars was carried out with a Waters sugar-pak (Temp. 75℃) under distilled-deionized water as a mobile phase. The injection volume of each sample was 20 μl and flow rate was maintained 0.5 ml min^{-1}. Quantification was based on standard curves obtained from 50 to 10,000 ppm of each standard of sugars. Organic acids were determined using HPLC system (Agilent 1100, USA). Separations were performed for 30 min with an Aminex 87H column (Temp., 40℃) under 0.01 N H_2SO_4 as a mobile phase. The injection volume of each sample was 20 μl and flow rate was maintained 0.5 ml min^{-1}. Absorbance was detected with an UV detector (Agilent G1315A) at a wavelength of 210 nm. Quantification was based on standard curves obtained from 0.1 mmol to 10 mmol μl^{-1} of each standard of organic acid.

Results

Changes in amino acids in the leaves, roots and xylem sap We examined changes in the amino acid profile of leaves, roots and xylem sap collected at 7 and 14 days under N-starvation and -surplus conditions (Table 1). The concentration of total soluble amino acids in leaves were 4,960 (0.25 mM N), 7,043 (2.5 mM N), and 11,277 (10 mM N) nmol g^{-1} fresh weight at 7 days, 3,745, 15,500, and 15,975 nmol g^{-1} at 14 days, respectively. Asparate (335, 2,183, and 2,311 nmol g^{-1} in 0.25 Mm, 2.5 mM, and 10 mM N, respectively, at 14 days) and glutamate (1,810, 5,169, and 5,885 nmol g^{-1} at 14 days) were the predominant amino acids found in the leaves irrespective of the N condition and time point. These two amino acids represented 47-70% of the total soluble amino acids. Most soluble amino acids in N-starved condition (0.25 mM N) were significantly reduced with an increase in the period of N deficiency, whereas their concentration in N-normal (2.5 mM N) and -surplus (10 mM N) increased obviously. In terms of the scientific context, amino acids are synthesized via the pathway of 3-phosphoglycerate, phosphoenol-

Table 1. Amino acid profile of different nitrate (NO_3)-fed tomato plants - leaves.

Amino acid	Day 7			Day 14		
	0.25mM	2.5mM	10mM	0.25mM	2.5mM	10mM
Aspartate	567±50	1,253±104	1,854±92	335±18	2,183±48	2,311±102
Glutamate	2,019±158	3,646±282	4,823±192	1,810±100	5,169±98	5,885±351
Asparagine	67±5	70±5	256±10	24±2	529±11	205±11
Glutamine	158±12	240±17	933±39	105±3	1,903±41	1,461±84
Arginine	48±4	61±1	134±6	20±1	97±4	68±3
Serine	409±34	180±14	400±15	306±17	1,185±27	1,539±97
Histidine	62±5	43±5	75±3	73±3	75±9	92±10
Glycine	52±3	56±4	336±13	170±8	1,016±18	1,178±81
Threonine	163±10	279±20	574±21	97±5	734±15	656±38
Alanine	287±24	421±34	721±29	103±6	1,177±29	1,314±85
Tyrosine	34±2	24±1	44±1	39±3	107±2	56±2
Valine	90±9	80±7	139±5	79±4	170±4	184±10
Tryptophan	40±3	20±2	30±3	109±7	93±2	58±6
Phenylalanine	133±9	63±4	107±3	82±5	225±6	173±9
Isoleucine	68±4	59±5	92±2	56±3	135±4	128±6
Leucine	60±3	58±3	73±1	53±4	76±1	64±3
Lysine	601±44	401±53	509±34	205±11	477±7	485±64
Proline	100±21	90±6	177±30	79±13	150±14	120±15
Total	4,960	7,043	11,277	3,745	15,500	15,975

Values are in nmol per gram fresh weight for leaves and roots and per mL for xylem sap. Values (mean) and standard deviation (SD) are reported from three replicates.

Table 1. continued - roots.

Amino acid	Day 7			Day 14		
	0.25mM	2.5mM	10mM	0.25mM	2.5mM	10mM
Aspartate	379±33	824±37	996±72	227±13	966±33	870±7
Glutamate	851±62	988±38	1,098±69	557±33	1,406±44	1,197±8
Asparagine	318±25	365±17	895±66	304±18	3,974±140	3,037±19
Glutamine	832±66	905±41	2,001±142	591±33	8,204±273	8,662±51
Arginine	202±15	264±11	403±28	143±8	945±30	703±3
Serine	260±21	438±20	526±39	196±10	786±27	908±2
Histidine	85±12	155±8	246±14	66±3	457±21	542±12
Glycine	52±4	66±3	78±5	56±1	131±4	143±5
Threonine	239±16	366±26	491±32	181±10	810±27	641±3
Alanine	126±7	131±5	187±12	85±5	213±6	167±3
Tyrosine	84±4	108±4	155±10	72±3	381±11	352±2
Valine	216±13	321±15	463±32	153±7	840±28	883±5
Tryptophan	82±6	63±1	72±5	47±3	110±4	152±2
Phenylalanine	62±3	100±4	141±9	46±2	342±10	320±2
Isoleucine	172±10	238±11	343±21	171±8	695±25	737±4
Leucine	272±17	351±15	468±30	180±9	933±38	69±7
Lysine	306±19	278±21	283±13	213±13	441±26	n.d.
Proline	171±9	208±14	181±19	91±13	313±14	206±9
Total	4,707	6,169	9,026	3,380	21,947	19,591

Table 1. continued − xylem sap.

Amino acid	Day 7			Day 14		
	0.25mM	2.5mM	10mM	0.25mM	2.5mM	10mM
Aspartate	9.2±0.2	5.1±0.1	6.8±0.3	4.1±0.4	3.7±0.3	7.4±0.2
Glutamate	19.5±0.9	18.5±0.2	22.1±0.3	7.3±0.9	8.6±0.3	13.4±0.5
Asparagine	33.8±1.7	20.1±0.6	8.4±0.2	26.4±1.5	2.6±0.1	14.9±0.6
Glutamine	207.3±9.4	54.3±1.8	32.5±1.0	203.0±10.3	9.5±0.4	30.4±1.2
Arginine	8.1±0.5	8.8±0.2	9.1±0.3	14.0±0.8	1.5±0.0	2.6±0.1
Serine	10.4±0.3	4.0±0.1	4.9±0.0	4.2±0.8	6.3±0.3	7.5±0.2
Histidine	7.7±0.4	4.4±0.0	4.3±0.1	9.6±0.7	n.d.	n.d.
Glycine	4.6±0.1	2.5±0.1	2.5±0.0	3.3±0.4	3.9±0.1	4.2±0.0
Threonine	9.0±0.4	7.8±0.3	7.4±0.2	7.1±0.4	2.3±0.2	3.9±0.1
Alanine	4.6±0.1	5.1±0.1	5.7±0.1	2.2±0.2	2.8±0.2	3.3±0.1
Tyrosine	3.2±0.2	3.4±0.1	2.4±0.1	3.4±0.2	n.d.	1.7±0.0
Valine	11.7±0.4	12.1±0.5	7.9±0.3	11.6±0.7	2.7±0.2	4.3±0.0
Tryptophan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylalanine	3.8±0.2	2.7±0.1	2.5±0.0	3.5±0.2	1.6±0.1	1.9±0.1
Isoleucine	12.6±0.6	10.9±0.3	6.8±0.2	12.7±0.8	2.2±0.2	4.3±0.2
Leucine	7.5±0.5	10.0±0.3	7.6±0.2	12.2±0.8	1.9±0.1	2.5±0.1
Lysine	n.d.	12.5±0.3	7.5±0.5	8.1±1.2	n.d.	n.d.
Proline	n.d.	3.5±0.4	4.5±0.2	n.d.	n.d.	n.d.
Total	353	186	143	333	50	102

pyruvate, pyruvate, α-ketoglutarate, and oxalate. According to our result, amino acids derived from α -ketoglutarate (glutamate, glutamine, arginine, histidine, and proline) and oxalate (aspartate, asparagines, lysine, methionine, threonine, and isoleucine) pathway are predominant, and both pathways revealed an inverse relationship in N-starved condition, but not in N-surplus. An increase in the period of treatment led to much more production of α-ketoglutarate-derived amino acids (from 43% to 50% of total amino acids), whereas the synthesis of oxalate-derived amino acids (from 30% to 19%) was significantly reduced. Interestingly, pyruvate-derived amino acids (alanine, leucine, and valine) were also decreased with an extension of N starvation. The concentration of total soluble amino acids in roots were 4,707 (0.25 mM N), 6,169 (2.5 mM N), and 9,026 (10 mM N) nmol g^{-1} fresh weight at 7 days, 3,380, 21,947, and 19,591 nmol g^{-1} at 14 days, respectively. In contrast with the leaves, asparagines (3,974 and 3,037 nmol g^{-1} in 2.5mM and 10mM N, respectively, at 14 days) and glutamine (8,204 and 8,662 nmol g^{-1}) were the predominant amino acids found in the roots in N-normal and -surplus conditions, but glutamate (557 nmol g^{-1}) and glutamine (591 nmol g^{-1}) in N-starved condition. These three amino acids represented 37-66% of the total soluble amino acids. Most soluble amino acids in N-starved condition (0.25mM N) were significantly reduced with an increase in the period of N deficiency, whereas their concentration in N-normal (2.5 mM N) and -surplus (10 mM N) increased obviously. Surprisingly, the concentration of total soluble amino acids in xylem sap was the highest in N-starvation (333 nmol mL^{-1}) at 14 days and followed by N-surplus (102 nmol mL^{-1}) and -normal (50 nmol mL^{-1}) conditions. Most amino acids were higher in N-starvation compared with other two N conditions, and the predominant amino acids revealed glutamate, asparagines, and glutamine. The percent contribution of three predominant amino acids of the xylem sap varied from 42 to 74%, and this was remarkable in N-starvation. The concentration of amino acids also represented a gradual decrease with an increase in the period of treatment irrespective of N conditions.

Changes in soluble sugars and organic acids in the leaves, roots and xylem sap Short-term changes in the concentration of selected soluble sugars and organic acids quantified from the leaves, roots and xylem sap at

Table 2. Metabolite profile of different nitrate (NO_3)-fed tomato plants.

Organ	Metabolite	Day 7			Day 14		
		0.25mM	2.5mM	10mM	0.25mM	2.5mM	10mM
Leaves	Sucrose	1.81±0.05	4.36±0.01	5.05±0.06	1.69±0.09	4.39±0.04	4.56±0.01
	Glucose	1.18±0.03	11.85±0.03	8.34±0.04	1.70±0.08	12.69±0.24	10.68±0.30
	Fructose	1.52±0.03	13.70±0.03	11.14±0.11	1.48±0.12	18.12±0.04	11.94±0.39
	Citrate	7.49±0.22	20.46±0.18	17.83±0.38	6.61±0.13	19.09±0.08	11.02±0.41
	Malate	1.31±0.08	21.09±0.51	15.43±0.87	2.99±0.33	32.83±1.00	24.64±0.15
	Succinate	n.d.	14.05±0.28	10.03±0.24	6.99±0.40	15.86±0.41	12.41±1.55
Roots	Sucrose	2.55±0.04	1.80±0.04	0.98±0.03	4.58±0.03	1.80±0.01	0.69±0.01
	Glucose	1.21±0.05	n.d.	0.28±0.02	2.49±0.02	0.53±0.07	n.d.
	Fructose	2.17±0.05	n.d.	n.d.	1.92±0.03	0.89±0.06	n.d.
	Citrate	1.27±0.09	0.35±0.01	0.34±0.04	0.26±0.02	1.63±0.01	0.32±0.02
	Malate	5.65±0.38	3.29±0.14	3.07±0.13	1.27±0.04	5.53±0.12	2.09±0.05
	Succinate	5.28±0.50	6.55±0.06	13.29±0.31	3.28±0.47	8.38±0.13	5.72±0.36
Xylem sap	Sucrose	75±1	29±2	23±2	55±1	18±2	14±1
	Glucose	14±3	n.d.	n.d.	20±3	n.d.	n.d.
	Fructose	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Citrate	91±5	16±2	n.d.	14±2	29±3	n.d.
	Malate	107±6	25±5	28±2	40±5	29±1	21±5
	Succinate	95±9	74±14	119±20	58±5	46±2	68±4

Values are in μmol per gram fresh weight for leaves and roots and nmol per mL for xylem sap. Values (mean) and standard deviation (SD) are reported from three replicates.

two time points were also noted (Table 2). The concentration of these metabolites in the leaves was 13.3 (0.25 mM N), 85.5 (2.5 mM N), and 67.8 μmol g^{-1} fresh weight (10 mM N) at 7 days after N treatment, and 21.5 (0.25 mM N), 103.0 (2.5 mM N), and 75.2 μ mol g^{-1} fresh weight (10 mM N) at 14 days, respectively. The predominant form of soluble sugar and organic acid was fructose (57.9 μmol g^{-1}) and malate (98.3 μmol g^{-1}), respectively. The concentration of these metabolites in the roots was 18.1 (0.25 mM N), 12.0 (2.5 mM N), and 18.0 μmol g^{-1} fresh weight (10 mM N) at 7 days after N treatment, and 13.8 (0.25 mM N), 18.8 (2.5 mM N), and 8.8 μmol g^{-1} fresh weight (10 mM N) at 14 days, respectively. On the contrary to the leaves, nitrogen-starved condition resulted in a significant increase in the levels of soluble sugars. The levels of metabolites in N-surplus condition didn't show a marked difference except for a great decrease in succinate at 14 days. The predominant form of soluble sugar and organic acid in the roots was sucrose (12.4 μmol g^{-1}) and succinate (42.5 μmol g^{-1}), respectively. Nitrogen deficiency led to significant increase in soluble sugars and organic acids existing in xylem sap of tomato plants. Sucrose concentration in N deficiency (0.25 mM N) represented 2 to 3 times higher than that in N normal and surplus whereas its concentration decreased slightly with time for all N conditions. Interestingly, glucose, which ranged from 14 to 20 μmol mL^{-1}, was detected in xylem sap of N-starved tomato plants. The fructose was not detected in the xylem sap irrespective of N levels. The xylem sap also contained huge amount of most abundant organic acids such as citrate, malate and succinate although their concentration were greatly dependent upon N condition. Likewise soluble sugars, N deficiency resulted in an accumulation of organic acids however their concentration decreased significantly with time.

Nitrogen-assimilated enzymes activities and genes expressions The activities of nitrogen-assimilating enzymes in fully-expanded tomato leaves at 14 days were strongly influenced by N conditions (Fig. 1). In N deficiency conditions (0.25 and 0.75 mM), nitrate reductase, nitrite reductase, and glutamine synthetase reveled only 6 to 7%, 32 to 76%, and 60 to 85% in comparison with N normal (100%), respectively. The

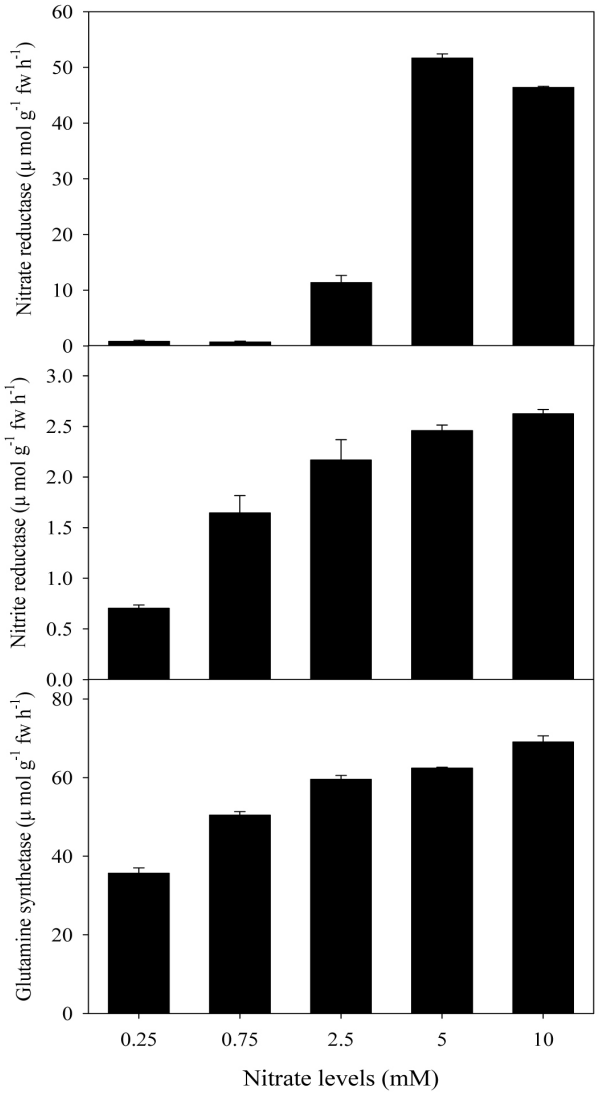

Fig. 1. Enzyme activities of nitrate reductase (NR), nitrite reductase (NIR), and glutamine synthetase (GS) from tomato leaves at 14 days after nitrate feeding (n=3).

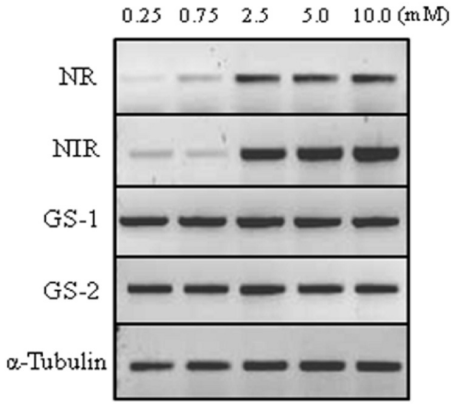

Fig. 2. mRNA levels for the nitrate assimilation-related genes in tomato leaves. Total RNA was extracted from tomato leaves at 14 days after nitrate feeding, converted to cDNA, and subjected to comparative RT-PCR quantification using 1.2 % agarose gel electrophoresis. NR (nitrate reductase), NIR (nitrite reductase), GS (glutamine synthetase).

N surplus conditions (5.0 and 10.0 mM) induced an activation of three enzymes and, especially, nirate reductase led to 4 to 4.5-fold higher compared with N normal. The expression intensities of three main N assimilation genes represented the same pattern with enzyme activities (Fig. 2). The nitrate reductase and nitrite reductase were marginally expressed in N deficiency conditions (0.25 and 0.75 mM) whereas similar expression for glutamine synthetase. The N surplus did not represent any obvious difference in comparison with N normal condition.

Discussion

In this study we have examined the metabolites composition of leaves, roots and xylem sap and N-assimilated enzymes activities and genes expressions from differently N-served tomato plants. The 2-oxoglutarate and glutamate are key regulators of amino acid biosynthesis (Masclaux-Daubresse et al., 2002), and these compounds increased in concert with a wide range of amino acids following transfer to nitrogen-saturated media. However, in the current study most abundant amino acids were identified as aspartate and glutamate in leaves and asparagine and glutamine in roots. Previous studies have shown that nitrogen regimes and sources influenced greatly on the concentration of free amino acids in the leaves and roots of plant species (Haynes and Goh, 1978; Darral and Wareing, 1981; Barneix et al., 1984). Nitrogen-deficient plants showed faster nitrate (Lee and Rudge, 1986; Rodgers and Barneix, 1989; Rufty et al., 1990) and ammonium (Morgan and Jackson, 1988a; Causin and Barneix, 1993) uptake rates than nitrogen-fed plants to sustain the levels of free amino acids in organs, and also ammonium uptake of plants were conversely enhanced against nitrate deficiency (Sung et al., 2011). Free amino acid concentration in both leaves and roots under nitrogen deficiency remained low while it showed similar level in nitrogen-normal and -surplus conditions (Table 1). Nitrogen deficiency also resulted in an accumulation of α-ketoglutarate-derived amino acids and, concurrently, obvious decrease in oxalate- and pyruvate-derived amino acids. Interestingly, it was found that most amino acids represent in nitrogen-normal and -excessive conditions were substantially increased with an extension of treatment period, and, therefore,

this suggested that absorbed nitrate was assimilated in shoots and roots at the same time, while slightly decreased for nitrogen-deficient condition. Under high N supply, the high concentration of amino acid in the leaf cytoplasm would allow the export of amino acids to sink such as growing leaves and roots, and the proteolysis rate would be maintained at a low level, however a shortage in the N supply would decrease the amino acid concentration and thus de-repress the nitrogen uptake mechanisms (Barneix and Causin, 1996). Amino acid concentration in xylem sap represented totally different tendency, which indicated 3 to 6 times high level at nitrogen deficiency, as compared with leaves and roots, and huge difference in amino acid concentration among treatments was derived from the concentration of glutamine and asparagines. Our finding is consistent with earlier reports that showed an increase in the proportion of glutamine and asparagines in the xylem sap (Krishnan et al., 2011; Ohtake et al., 1995). It has been reported that changes in the nitrogen metabolism can affect the relative composition and concentration of amino acids in the xylem sap. In our study it was observed to interact closely between shoots-xylem/phloem-roots by plant N status in that depressed nitrogen uptake led to a low amino acid production in both leaves and roots and high transport from shoots to roots whereas saturated- and/or excessive-uptake induced direct nitrogen assimilation and low transport rate. The contents of a wide range of soluble sugars and organic acids also changed markedly with respect to the level and period of nitrogen supply (Table 2), and, particularly, a change was remarkable in N-deficient condition. It is well known that the C/N metabolism is closely related and, therefore, the repressed N metabolism greatly affects photosynthetic activity and carbon assimilation. Nitrate regulates many genes assigned to sugar metabolism (Wang et al., 2003; Hirai et al., 2004), decreases sets of genes required for photosynthesis and export of photo-synthates (Hammond et al., 2005), and thus re-establishes the balance between photosynthesis and carbon use (Paul and Driscoll, 1997). Earlier studies have reported that nitrogen deficiency results in the accumulation of sugars and starch in leaves (Martin et al., 2002; Tranbarger et al., 2003), however, in our study nitrogen deficiency resulted in poor soluble sugar accumulation in tomato leaves, soluble sugars were quickly transported to the roots through the phloem/xylem routes, which indicated higher concentration of soluble sugars as compared with N-normal and -surplus conditions. This reduction of soluble sugar in leaves in N-deficiency is probably a direct consequence of reduced photosynthesis and sugar allocation because sugar plays a signal molecule to acquire mineral via controlling nutrient-responsive gene expression (Liu et al., 2005-2004 in References). Levels of citrate, malate, and succinate demonstrated a rapid and significant reduction following the imposition of nitrate stress and, furthermore, remained low for the experiment. These data are largely consistent with earlier study which represented a dramatic reduction in the levels of major organic acids of the tricarboxylic acid cycle (Urbanczyk-Wochniak and Fernie, 2005; Amtmann and Armengaud, 2009). However, the levels of citrate and malate in roots at 7 days revealed higher in N-deficiency than N-normal and -surplus, and, in addition, they were detected 4 times higher in xylem sap subjected to N-deficiency. Nitrogen-deficient plants secrete organic acid, malate, to enhance nitrate uptake by roots (Lopez-Bucio et al., 2000) and the transport of organic acids from shoots to roots was observed in this study. Nitrate reductase (NR) and nitrite reductase (NIR), the first enzymes involved in the metabolic route of nitrate assimilation, are regulated by nitrate (Oaks, 1994) and glutamine (Deng et al., 1991). In our study gene expression and enzyme activity of NR and NIR in N-deficient and -excessive tomato leaves were significantly reduced by N-deficiency. By contrast, N-surplus condition greatly induced NR activity which accounted 4-fold higher than N-normal although NIR activity was similar. Glutamine synthetase (GS) activity was also low by N-deficiency, however there was no significance in gene expression irrespective of nitrogen condition. Many researchers have identified and characterized a number of nitrate uptake and assimilation-related genes and enzymes with various plant species and the recycle of ammonium produced by degradation of proteins and amino acids in cells (Crawford, 1995; Crawford and Glass, 1998; Stitt, 1999; Forde, 2000), however they have focused on the mechanism occurring in roots. In conclusion, nitrogen deficiency led to a significant decrease in an accumulation of amino acids, soluble sugars, and organic acids in leaves and roots whereas they existed highly in xylem sap. Also, nitrate

assimilation-related genes and enzymes were greatly reduced. Therefore, N-deficiency induced the transport of primary metabolites to enhance nitrate uptake, and, especially, the modification of amino acid biosynthesis pathway, for example the α-ketoglutarate induction, is a challenging goal to understand better amino acid metabolism under nitrogen deficient condition.

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