Title: Genome-Wide Survey and Expression Analysis of NIN-Like Protein (NLP) Genes Reveals Its Potential Roles in the Response to Nutrition Deficiency in Tomato

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Abstract

Background: Tomato (*Solanum lycopersicum*) is one of the most important horticultural crops, with a marked preference of nitrate as inorganic nitrogen source. The molecular mechanisms of nitrate uptake and assimilation are poorly understood in tomato. NIN-Like Proteins (NLPs) are conserved, plant-specific transcription factors that play crucial roles in nitrate signaling.

Results: In this study, genome-wide analysis revealed six *NLP* members in tomato genome. They were clustered into three clades in a phylogenic tree. Comparative genomic analysis showed that *SlNLP* genes had collinear relationships to *NLP*s in Arabidopsis, canola, maize and rice, and that the expansion of the *SlNLP* family mainly resulted from segmental duplications in tomato genome. Tissue-specific expression analysis showed that the close homologues of *AtNLP6/7*, *SlNLP3*, was strongly expressed in roots during both seedling and flowering stages; *SlNLP4* and *SlNLP6* exhibited preferential expression in stems and leaves; and *SlNLP6* were expressed in high levels in fruits. Further, the nitrate uptake in tomato roots and expression patterns of *SlNLP* genes were measured under nitrogen/phosphate/potassium deficiency and nitrate resupply conditions. The transcript abundance of *SlNLP3* decreased to 70% under phosphate/potassium deficiency. Most of *SlNLPs* were up-regulated after nitrogen starvation. *SlNLP1* and *SlNLP5* were induced rapidly and temporally by nitrate.

Conclusions: These results provided significant insights into the potential diverse functions of *SlNLPs* to regulate nitrate uptake.

Background

Nitrogen (N), one of essential macro-nutrients for plants, serves as the component of amino acids, nucleotides, chlorophyll, hormones and co-enzymes. The growth and development of plants depends on proper nitrogen supply. And the availability of N in agricultural field affects crop yields significantly (Miller and Cramer, 2005). Plants absorb inorganic N from the soils mainly in two forms, nitrate (NO_3^-) and ammonium (NH_4^+). Under mild climatic conditions, nitrate is the main nitrogen source in dry land (Forde and Clarkson, 1999). The concentration of nitrate in the soils fluctuates between 10 μM to 100 mM (Crawford, 1995). To sustain vigorous growth, high-affinity and low-affinity transport systems have been evolved in plants to absorb nitrate efficiently from the environment. Nitrate is also one of important signaling molecules for lateral root development, flowering and synergistic absorption of the other nutrients (Vidal *et al.*, 2020).

For nitrate signaling, NIN-Like Proteins (NLPs) are identified as essential transcription factors (Konishi and Yanagisawa, 2013). It is reported that nutrient-Ca^{2+}-NLP regulatory pathway plays the central role in nitrate signaling and integrates transcription, transport, metabolism and systemic growth programs in plants (Castaings *et al.*, 2009; Marchive *et al.*, 2013; Liu *et al.*, 2017). In Arabidopsis, Nitrate tansporter 1.1 (NPF6.3/NRT1.1) has been identified as the nitrate sensor at the plasma membrane (Ho *et al.*, 2009). In the presence of nitrate, calcium-dependent protein kinases 10/30/32 (CPK10/30/32) mediate Ca^{2+}

signals by nitrate and phosphorylate NLP6/7 to ensure their location in the nucleus for transcriptional activation of the primary nitrate response genes (Liu *et al.*, 2017).

NIN protein was firstly identified in legume *Lotus japonicus*, with regulatory function on symbiotic root nodule formation (Schauser *et al.,*1999). Then, more members of NIN proteins and NLPs were found widely existing among other non-leguminous plants including Arabidopsis, rice, wheat, and maize, but not in animals (Schauser *et al.*, 2005; Kumar *et al.*, 2018; Wang *et al.*, 2018; Mu and Luo, 2019). Both NIN proteins and NLPs have RWP-RK domain for DNA binding; NLPs carry an additional PB1 domain for protein-protein interaction (Chardin *et al.*, 2014). Interactions between NLPs and other transcription factors such as nitrate regulatory gene 2 (NRG2) (Xu *et al.*, 2016), PCF (TCP)-domain family protein 20 (TCP20) (Guan *et al.*, 2017), and nitrate-inducible GARP-type transcriptional repressor 1 (NIGT1) (Meada *et al.*, 2018) have been reported. Beyond nitrate signaling, extra functions of NLPs in the N starvation response (Guan *et al.*, 2017), N and phosphate (P) interactions (Meada *et al.*, 2018), nitrate-promoted seed germination (Yan *et al.*, 2016), nitrate-dependent nodule symbiosis (Nishida *et al.*, 2018) and root cap cell release (Karve *et al.*, 2016) have been clarified.

As one of the most important crops, tomato (*Solanum lycopersicum*) shows a marked preference of nitrate as inorganic nitrogen source (Errebhi *et al.*, 1990).

In the present study, comparative bioinformatics analysis of the tomato *NLP* genes was performed. Further, the rate of root nitrate uptake and expressions of *SlNLP* genes under nutrition deficiency and nitrate resupply conditions were detected to evaluate their potential roles in nitrate uptake regulation in roots.

Results

Identification of *NLP* Genes in tomato

Table 1. Identification of NLP Genes in tomato

Gene Name	Gene ID	Protein Characteristics	Subcellular localization			
Length (aa)	Mw (Da)	pI	GRAVY			
SlNLP1	Solyc01g112190.3	841	93298.51	7.35	-0.524	Nucleus/cytosol
SlNLP2	Solyc04g082480.3	912	102467.99	5.58	-0.520	Nucleus/cytosol
SlNLP3	Solyc08g008410.3	1008	109783.94	5.70	-0.327	Nucleus/cytosol
SlNLP4	Solyc08g013900.3	961	106149.69	5.41	-0.347	Nucleus/cytosol
SlNLP5	Solyc08g082750.3	1611	180948.88	6.16	-0.473	Nucleus/cytosol
SlNLP6	Solyc11g045350.2	986	108349.29	5.30	-0.416	Nucleus/cytosol

Mw, molecular weight; pI, isoelectric point; GRAVY, grand average of hydropathicity.

A total of six *NLP* genes were identified from tomato genome for presence of conserved RWP-RK (hmm, PF02042) and PB1 domains (hmm, PF00564). The nomenclature used for *SlNLP* genes was based on their distribution on the chromosomes (Table 1). The numbers of amino acids coded by *SlNLP* genes ranging from 841 (*SlNLP1*) to 1611 (*SlNLP5*). The relative molecular weights (Mw) were between 93.30 kDa (SlNLP1) and 180.95 kDa (SlNLP5). All SlNLP proteins had a isoelectric point near neutral (5.30–7.35), and low hydrophilicity indicated by GRAVY values (−0.524 to −0.327). The subcellular localizations were predicted to be in the nucleus/cytosol for all six SlNLPs.

Conserved motifs and phylogenetic analysis of SlNLP proteins

Based on the previous study, Arabidopsis NLP proteins were divided into three clades (Schauser *et al.*, 2005). To analyze the evolutionary relationship of tomato NLP proteins, a Neighbor-Joining phylogenetic tree was constructed by comparing tomato NLP amino acid sequences with NLPs from four other plant species, including two dicotyledonous plants (Arabidopsis and canola) and two monocotyledonous plants (rice and maize) (Supplementary Table 1). The result (Fig. 1A) showed that Clade I contained 17 NLP members, including AtNLP1/2/3/4/5 and SlNLP1/2. Clade II contained 17 NLP members, including AtNLP6/7 and SlNLP3/5. Clade III contained 31 NLP members, including AtNLP8/9 and SlNLP4/6. Both dicotyledonous and monocotyledonous members existing in every clade indicated that gene expansion of the *NLP* gene family occurred before the ancestral divergence of monocotyledon and dicotyledon. The multiple sequence alignment (Fig. 1B and 1C) revealed all the NLP proteins share similar motif patterns, including the conserved RWP-RK domain and PB1 domain. Interestingly, SlNLP5 protein appeared to carry double RWP-RK domains and PB1 domains.

Chromosomal distribution and syntenic analysis of *SlNLP* genes

Six *SlNLP* genes were distributed unevenly in tomato genome (Fig. 2). *SlNLP3*, *SlNLP4* and *SlNLP5* were identified on chromosomes 8. The other three *SlNLP* genes, *SlNLP1*, *SlNLP2* and *SlNLP6* genes were identified on chromosomes 1, 4 and 11, respectively. Inter-chromosomal relationship of *SlNLP* genes showed two pairs of segmental duplications (*SlNLP1* and *SlNLP2*, *SlNLP3* and *SlNLP5*), indicating that tomato *NLP* genes were mainly generated by gene duplication during evolution.

Further, four comparative syntenic maps between tomato and Arabidopsis, canola, rice and maize, were constructed, to analyze the phylogenetic mechanisms of *SlNLPs* (Fig. 3). Tomato *SlNLP* genes showed 10 syntenic gene pairs with canola, 8 with Arabidopsis, 5 with maize and 3 with rice. Most background collinear blocks associated with *NLP* gene pairs identified between tomato and dicotyledon Arabidopsis/canola contained more genes than those between tomato and monocotyledon rice/maize (Supplementary Table 2). *SlNLP1*, *SlNLP2* and *SlNLP5* were found in the four comparative syntenic maps, suggesting that these orthologous pairs might already exist before evolutional divergence of monocotyledon and dicotyledon, and these three genes might have played fundamental roles in *NLP* gene family. The ratio of non-synonymous (Ka) to synonymous substitutions (Ks), presenting the

selection type acting on the coding sequences, were also calculated (Supplementary Table 2). Two *SlNLP* gene pairs, *SlNLP1* and *SlNLP2*, *SlNLP3* and *SlNLP5*, had Ka/Ks ratio of 1.01 and 1.46, respectively, indicating positive selection during evolution for functional divergence occurring after duplication. Most of the orthologous *NLP* gene pairs had a Ka/Ks ratio less than 1 (ranging from 0.10 to 0.96), suggesting purifying selective pressure during *NLP* gene family evolution and conserved functions of these genes. Three orthologous gene pairs, *SlNLP1* and *AtNLP5*, *SlNLP2* and *BnaNLP4-4*, *SlNLP1* and *ZmNLP1*, had a Ka/Ks ratio more than 1, indicating they have underwent positive selection pressure and might be evolved with some new functions to cope with their living environments.

Organ-dependent expression of *SlNLPs*

To obtain evidence of physiological function, tissue-specific transcript abundance of 6 *SlNLP* genes was analyzed by qRT-PCR at different developmental stages (Fig. 4). All of *SlNLP* genes had relatively low expression levels, 1/10000–4/100 of the level of internal control *SlEF1α* gene expression. *SlNLP1* had the lowest expression. Therefore, *SlNLP1* expression levels in roots or fruits were set to 1 for comparison of expression levels. At both the seedling and flowering stages, *SlNLP2* and *SlNLP3* were preferentially expressed in roots (Fig. 4A and 4B). *SlNLP2* and *SlNLP3* showed the highest transcript abundance in root at the seedling stage (Fig. 4A). When flowering, *SlNLP3* still showed the most abundance in roots, followed by *SlNLP3* and *SlNLP6* (Fig. 4B). At the flowering stage, the transcript abundance of *SlNLP4* and *SlNLP6* increased significantly in all the test tissues, with preferential expression in stems and leaves. And *SlNLP6* had highest transcript accumulation in leaves, stems and flowers. Particularly, significantly higher expression of *SlNLP6* was observed in fruits (Fig. 4C).

Expression of *SlNLPs* in response to nutrition deficiency

Nitrate absorption in tomato roots were found to be influenced by major mineral elements nutrition (nitrogen/phosphate/potassium) deficiency, indicated by $^{15}NO_3^-$ influx assay after different treatments (Fig. 5). The results showed that the root high-affinity nitrate uptake ability was enhanced under nitrogen starvation, but repressed under potassium/phosphate starvation (Fig. 5A). And the root low-affinity nitrate uptake ability was enhanced under potassium starvation, but repressed under nitrogen/phosphate starvation (Fig. 5B).

To obtain evidence of possible roles of SlNLPs in root nitrate absorption regulation during nutrition deficiency, the transcript abundance of *SlNLP* genes in roots was examined by qRT-PCR after starvation treatments (Fig. 6). The expression of *SlNLP1, SlNLP2, SlNLP4* and *SlNLP6* were up-regulated for 6.2, 3.1, 17 and 1.5 times, respectively, after nitrogen starvation. In response to phosphate starvation, *SlNLP3* showed expression decrease to 70% specifically. And the expression level of *SlNLP2*, *SlNLP3* and *SlNLP6* decreased to around 70% in response to potassium starvation.

Nitrate-dependent expression of *SlNLPs*

Both the root high-affinity and low-affinity nitrate uptake rates were enhanced after nitrate resupply to the nitrogen-starved plants, showed by results of $^{15}NO_3^-$ influx assay (Fig. 7). The nitrate-dependent expression of *SlNLP* genes in roots were examined at 0.5 h, 1 h and 2 h after nitrate was resupplied to the starved seedlings. The results (Fig. 8) showed that the transcript abundance of *SlNLP1* and *SlNLP5* increased rapidly and temporally in response to nitrate. The expression of *SlNLP1* and *SlNLP5* reached the maximum levels, 4.1 and 2.8 times respectively, 0.5 h after nitrate was supplied. The expression of *SlNLP2* and *SlNLP4* was repressed significantly after nitrate resupply for 1 h. By contrast, *SlNLP3* and *SlNLP6* did not show any response to nitrate in transcription level.

Discussion

In the present study, genome-wide analysis revealed six tomato *NLPs* (Table 1). The *Solanum lycopersicumNLP* family size is similar with *Arabidopsis thaliana* (9), *Oryza sativa* (5) and *Zea mays* (9), much smaller than *Brassica napus* (31). Phylogenetic analysis showed that every *NLP* family has members belongs to three groups (Fig. 1A). All of SlNLPs has conversed RWP-RK and PB1 domains. SlNLP5 is special for double RWP-RK and PB1 domains (Fig. 1B). The expansion of tomato *NLP* gene family was mainly generated by gene duplication in genome (Fig. 2). Orthologous gene pairs associated with *SlNLP1,SlNLP2* or *SlNLP5* were indicated existence before the ancestral divergence of dicotyledonous and monocotyledonous plants (Fig. 3). It is worth noting that Ka/Ks ratio of two paralogous *SlNLP* gene pairs (*SlNLP1* and *SlNLP2*, *SlNLP3* and *SlNLP5*) and three orthologous *NLP* gene pairs (*SlNLP1* and *AtNLP5*, *SlNLP2* and *BnaNLP4-4*, *SlNLP1* and *ZmNLP1*) were more than 1 (Supplementary Table 2), representing positive selection and fast evolutionary rates in these SlNLPs at the protein level. Therefore, it is implied that *NLPs* in tomato might evolve some new functions to meet their growth and development demands.

As one of fundamental regulatory elements at the transcriptional level, NLPs play important roles in nitrate uptake and assimilation regulation (Guan, 2017; Gaudinier *et al.*, 2018). Tissue-dependent expression pattern showed that all 6 *SlNLP* genes were expressed in all tested tissues including roots, stems, leaves, flowers and fruits (Fig. 4), which is similar with *NLPs* in Arabidopsis (Chardin *et al.*, 2014), maize (Ge *et al.*, 2018) and *Brassica napus* (Chardin *et al.*, 2014). *SlNLP3*, one of the close homologues of *AtNLP6/7* (Fig. 1A), the key component of nitrate signaling (Liu *et al.*, 2017), has the highest expression level in roots at both seedling and flowering stages. Besides *SlNLP3*, *SlNLP2* and *SlNLP6* were also expressed in high levels in roots, at different stages of development, implying their different functions in nitrate uptake regulation, rather than simple functional redundancy. Two *SlNLPs* from Clade III, *SlNLP4* and *SlNLP6*, showed preferentially expressed in aboveground tissues and were strongly up-regulated in their transcription abundance when flowering, suggesting that they might probably regulate nitrogen translocation and assimilation to support flower and fruit development. Different from *SlNLP4*, *SlNLP6* had higher transcript abundance both in roots and aboveground tissues. What is more, *SlNLP6* showed extremely higher expression level than all the other five *SlNLPs* in fruits. The close homologue of *SlNLP6*

is *AtNLP8* (Fig. 1A). AtNLP8 has been reported as a master regulator of nitrate-promoted seed germination (Yan *et al.*, 2016), which might provide some hints for functional research on *SlNLP6.*

Nitrate is more favorable inorganic nitrogen source form for tomato. The nitrate uptake in tomato roots must be under precise regulation with complex interactions between nitrogen and the other essential macro-nutrients phosphate and/or potassium availability (Vidal *et al.*, 2020). When environmental nitrogen source is depleted, the root low-affinity nitrate influx rate decreased, but high-affinity nitrate influx rate increased (Fig. 5). Similar results have been reported that higher nitrate influx was detected in tomatoes growing in nutrient solutions containing 5 mM nitrate than 0.1 mM (Abenavoli *et al.*, 2016). Both low-affinity and high-affinity nitrate uptake in roots increased after nitrate was resupplied to the nitrogen-starved tomato seedlings (Fig. 7). Distinct from nitrogen starvation, potassium deficiency led to enhanced low-affinity nitrate influx rate and deceased high-affinity nitrate rate in roots (Fig. 5), which is reasonable because some published data show that strong expression increase of the nitrate transporters *SlNRT1.2* and *SlNRT2.1* had been induced by potassium deprivation (Wang *et al.*, 2001). Slow-down of both low-affinity and high-affinity nitrate uptake rate were observed under phosphate deficiency (Fig. 5), which is consistent with the recent study in Arabidopsis (Wang *et al.*, 2020).

In Arabidopsis, *nlp7* mutants show features of a nitrogen-starved plant (Castaings *et al.*, 2009); *AtNLP7* overexpression increases plant biomass under both nitrogen-poor and -rich conditions (Yu *et al.*, 2016). Expression of rice *NLPs* (*OsNLP1*, *OsNLP4* and *OsNLP5*) was promoted by nitrogen deficiency as well as nitrate supply (Jagadhesan *et al.*, 2020). Overexpression of *OsNLP1* could enhance rice nitrogen use efficiency (Alfatih *et al.*, 2020). Here, the transcript abundance of *SlNLPs* in roots has been detected under various nutrition conditions (Fig. 6 and Fig. 8). Most of *SlNLPs* (*SlNLP1, SlNLP2,SlNLP4* and *SlNLP6*) showed up-regulated expression after nitrogen starvation for 2 days. When nitrate was resupplied, the temporal expression of *SlNLP2* and *SlNLP4* was repressed, but *SlNLP1* was still showed rapidly up-regulated. One of the two close homologues of *AtNLP6/7*, *SlNLP5*, was induced rapidly and temporally by nitrate. However, the other close homologue of *AtNLP6/7*, *SlNLP3*, which showed the highest expression level in roots during both seedling and flowering stages (Fig. 4), did not show any response to nitrate. It is noteworthy that AtNLP6/7 responds to nitrate signaling not in transcription level either (Liu *et al.*, 2017). Under phosphate deficiency or potassium deficiency, *SlNLP3* could be down-regulated in transcript abundance. After 2-days' phosphate starvation, *SlNLP3* was the only *SlNLP* gene to show altered expression level, 70% of control. *SlNLP3* also showed decreased transcript abundance to 70% after potassium starvation for 2 days, together with another two *SlNLP* genes, *SlNLP2* and *SlNLP6*. Therefore, it is interesting to figure out how *SlNLP3* participate in various nutrition deficiency signaling and/or nitrate signaling pathways.

Conclusions

In summary, this study provided genome-wide analysis of *NLP* genes in tomato. *NLP* genes are highly conserved among tomato, Arabidopsis, canola, maize and rice. Segmental duplication was the major driving force of *SlNLP* genes evolution. Some *SlNLP* genes had undergone positive selection during

evolution, probably leading to functional divergence in gene family. The expression patterns of *SlNLP* genes provided hints for their diverse physiological roles in tomato growth and development, especially in nitrate uptake regulation. Further functional analysis for each *SlNLP*, especially *SlNLP3* and *SlNLP6*, will be necessary to explore their regulatory functions. It is believed that a comprehensive understanding of the roles of *SlNLP* under fluctuating nutrition conditions is an essential step towards deciphering the molecular mechanism of nitrogen utilization and promoting nitrogen use efficiency in tomato.

Methods

Database search for NLP proteins

Raw Hidden Markov Model (HMM) data of the conserved RWP-RK (PF02042) and PB1 (PF00564) domain downloaded from Pfam (http://pfam.xfam.org) (Finn *et al.*, 2016) was used to search for their orthologs in the tomato genome (Solanum_lycopersicum.SL3.0), with e-value of less than 1e −10 in Phytozome (https://phytozome-next.jgi.doe.gov/info/Slycopersicum_ITAG2_4). Then, the results were confirmed by SMART (http://smart.embl.de/), NCBI Conserved Domains Database (CDD) (http://www.ncbi.nlm.nih.gov/cdd), and Plant Transcription Factor Database (TFDB) (http://planttfdb.cbi.pku.edu.cn/) database. The physicochemical properties of SlNLP proteins, including peptide length (aa), molecular weight (Mw), isoelectric point (pI) and grand average of hydrophilicity (GRAVY) were predicted using ExPASy ProtParam (http://web.expasy.org/protparam/) (Gasteiger *et al.*, 2005). Subcellular localizations of SlNLP proteins were predicted using CropPAL2020 (https://www.crop-pal.org) (Hooper *et al.*, 2020).

Multiple sequences alignment and phylogenetic analysis

Clustal W (version 2.1) was employed for the multiple sequences alignment and sequence identity matrix of the proteins (Larkin *et al.*, 2007). Then, the deduced amino acid sequences in RWP-RK and PB1 domains were adjusted manually using GeneDoc software. Phylogenetic tree was constructed with MEGAX program (http://www.megasoftware.net/) using the Neighbor-Joining method. Proportions of amino acid differences were computed using Poisson correction distance to estimate evolutionary distance. The pairwise deletion option was used to circumvent the gaps and missing data. The conserved protein motifs of SlNLP proteins were analyzed using MEME server v5.3.0 (http://meme-suite.org/tools/meme) (Bailey *et al.*, 2015). The parameters for the search were as follows: max motif number to find is 5 and min–max motif width to find is 2–40. The matched motifs with low quality were manually removed based on an e-value of less than 1e −15. Sequences of NLP proteins of tomato (*Solanum lycopersicum*), Arabidopsis (*Arabidopsis thaliana*), canola (*Brassica napus*), rice (*Oryza sativa*) and maize (*Zea mays*) were downloaded from Phytozome (https://phytozome.jgi.doe.gov/).

Chromosomal distribution and gene duplication

All *SlNLP* genes were mapped to chromosomes based on physical location information using Circos (Krzywinski *et al.*, 2009). Then, chromosome distribution was plotted with MapChart2.0

(https://mapchart.net/). The gene duplication events were analyzed using Multiple Collinearity Scan toolkit MCScanX. The syntenic analysis maps of orthologous *NLP* genes were constructed using the Dual Systeny Plotter software (https://github.com/CJ-Chen/TBtools) (Chen *et al.*, 2020). Non-synonymous (Ka) and synonymous (Ks) substitution of each duplicated *NLP* genes were calculated using KaKs_Calculator 2.0 (Wang *et al.*, 2010).

Plant materials and treatments

Tomato ecotype Micro-Tom was used in this study. The seeds were germinated and grown on vermiculite for 7 d before transferred to hydroponics. The hydroponic minimal medium comprised 2 mM KH_2PO_4, 2 mM $MgSO_4$, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnCl_2$, 0.5 μM $CuSO_4$, 0.5 μM Na_2MoO_4, and 20 μM Fe-EDTA. This was supplemented with 1.3 mM $Ca(NO_3)_2$, 1.5 mM KNO_3, 0.14 mM KH_2PO_4, and 1 mM $MgSO_4$ as normal condition. The pH of the solutions was maintained at approximately 5.8. Nutrient solutions were completely replaced weekly. Plants were grown at 28/22 ^oC with 16/8 h light/dark photoperiod. Plants grown in hydroponics for 4 weeks were used for nutrition deficiency treatments and nitrate treatment. For nitrogen starvation treatment (−N), hydroponic minimal medium with 1mM $CaCl_2$, 0.6 mM K_2SO_4, 0.25 mM KH_2PO_4, and 0.5 mM $MgSO_4$ were used for 2 days. For phosphate starvation treatment (−P), hydroponic minimal medium with 2 mM $Ca(NO_3)_2$, 0.35 mM KCl, 0.65 mM K_2SO_4 and 2 mM $MgSO_4$ were used for 2 days. For potassium starvation treatment (−K), hydroponic minimal medium with 2 mM $Ca(NO_3)_2$, 0.25 mM NaH_2PO_4 and 1.4 mM $MgSO_4$ were used for 2 days. For nitrate treatment, N-starved plants were resupplied with 5 mM nitrate medium (hydroponic minimal medium with KNO_3) for indicated time.

RNA extraction, cDNA synthesis, and qRT-PCR

Total RNA of different tissues was extracted using M5 SuperPure Total RNA Extraction Reagent (Mei5 Biotechnology Co. Ltd). Then, the DNA-free RNA was used for synthesis cDNA by using RevertAid First Strand cDNA Synthesis Kit (Cat. No. K1622, Thermo). The quantitative RT-PCR (qRT-PCR) was performed using SYBR Green PCR Master Mix (Life Technologies) in 7500 Real-Time PCR System (Applied Biosystems). The house-keeping tomato *EF1*a gene (*Solyc06g009970.3*) was used as an internal control. Primer Sequences used qRT-PCR were listed in Supplementary Table 3.

$^{15}NO_3^-$ Uptake Assay

$^{15}NO_3^-$ influx in roots was determined as previously described (Zou *et al.*, 2020). Tomato roots were washed in $CaSO_4$ for 1 min and then submerged in medium containing 1 mM or 0.1 mM $K^{15}NO_3$ for 5 min. ^{15}N concentration was measured using an isotope ratio mass spectrometer (IRMS; DELTA^{plus} XP).

Statistical analysis

Data were processed using the statistics program SPSS version 21. The statistical significance of differences in ^{15}N influx and gene expression was examined by student's t-test (*$p < 0.05$, **$p < 0.01$).

Declarations

Ethics approval and consent to participate

The experimental research on plants performed in this study complies with institutional, national and international guidelines.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

1. W. and M. L. designed the research plan and analyzed the data, M. L. performed the experiments, and X. Z. assisted in tomato hydroponics. The manuscript was written by Y. W.. Y. W. helped to revise the manuscript.

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Supplementary Information

Supplementary Table 1. *NLP* genes from tomato, Arabidopsis, canola, rice and maize.

Supplementary Table 2. One-to-one orthologous relationships between tomato and other four plant species.

Supplementary Table 3. Primers used in qRT-PCR.

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Figures

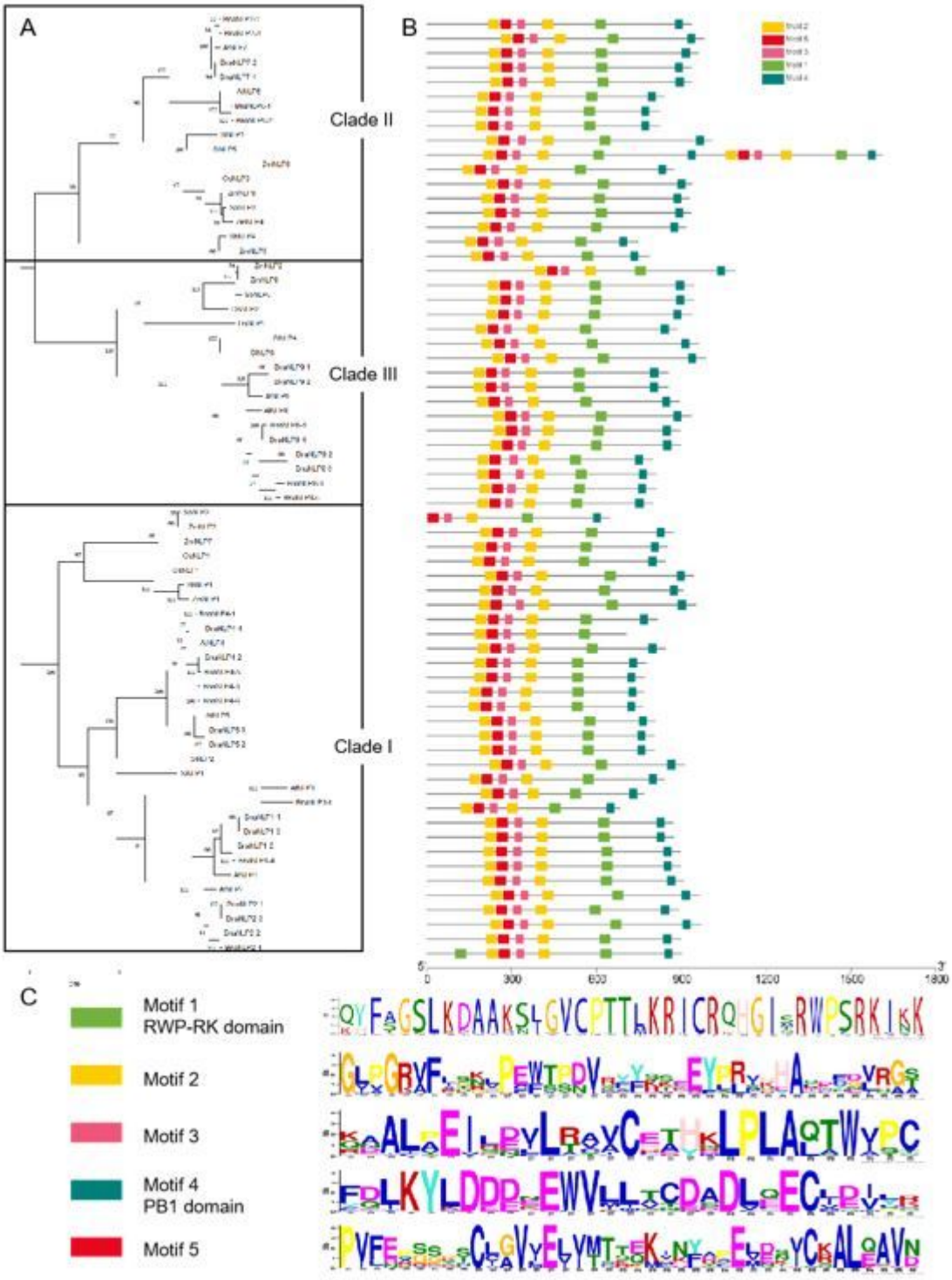

Figure 1

. Phylogenetic tree and conserved motifs of the NLP genes family. (A) A Neighbor-Joining phylogenetic tree of NLPs from tomato (Solanum lycopersicum), Arabidopsis (Arabidopsis thaliana), canola (Brassica napus), rice (Oryza sativa) and maize (Zea mays). All NLP proteins were assigned into three clades. (B) Motifs were identified by MEME. The motifs are displayed in different colors. The scale bar represents 300 amino acids. (C) Sequences of identified motifs including three unknown domains (yellow, pink and red), RWP-RK domain (light green) and PB1 domain (dark green).

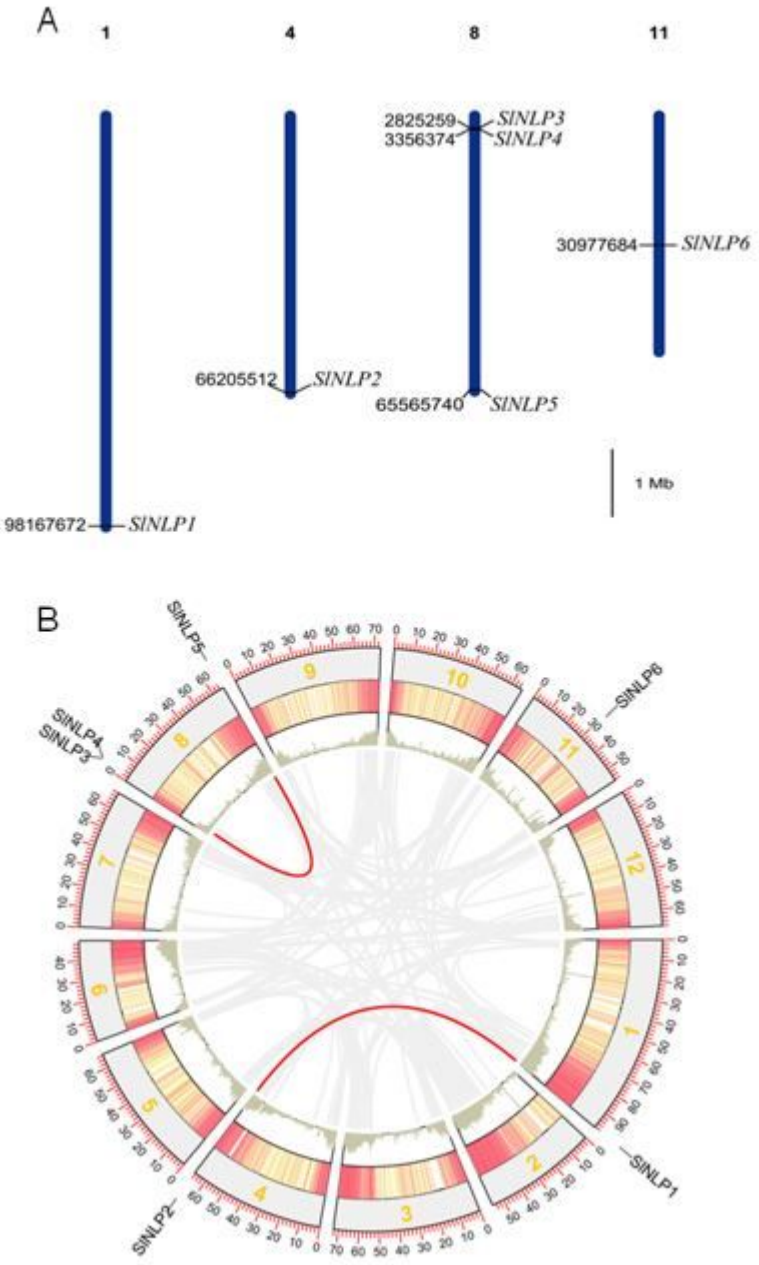

Figure 2

Chromosomal distribution and interchromosomal relationship of tomato NLP genes. (A) The distributions of SlNLP genes on tomato chromosomes 1, 4,8 and 11. The scale bar represents 1 Mb. (B) The inner-

species collinearity of SlNLPs. Gray lines indicate all syntenic blocks in tomato genome, and the red lines indicate the duplicated SlNLP gene pairs. The number in the grey box area is the chromosome number.

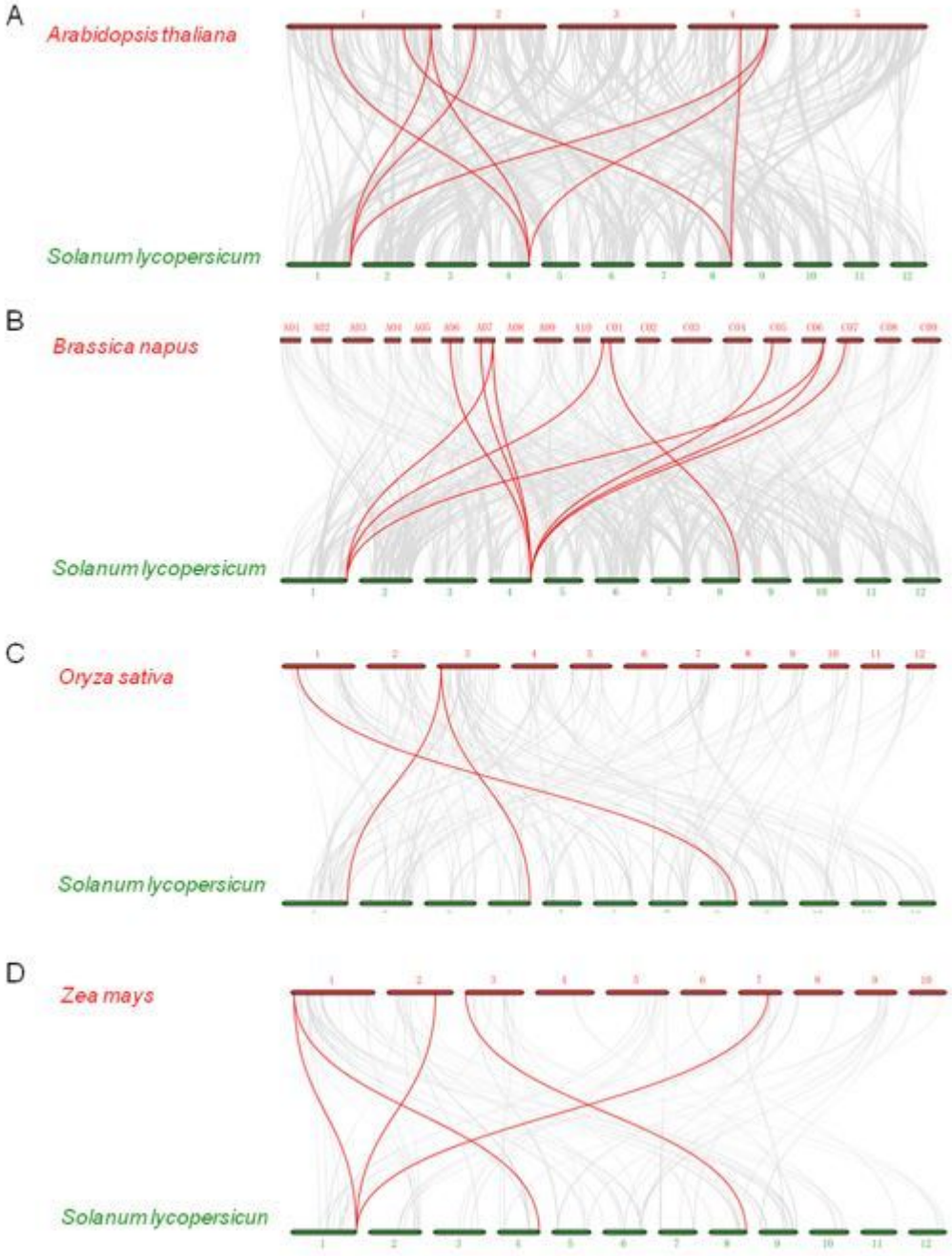

Figure 3

Syntenic NLP gene pairs between tomoto (Solanum lycopersicum) and four other plant species, including (A) Arabidopsis thaliana; (B) Brassica napus; (C) Oryza sativa; (D) Zea mays. Gray lines indicate all the collinear blocks in genome, while the red lines indicate the syntenic NLP gene pairs.

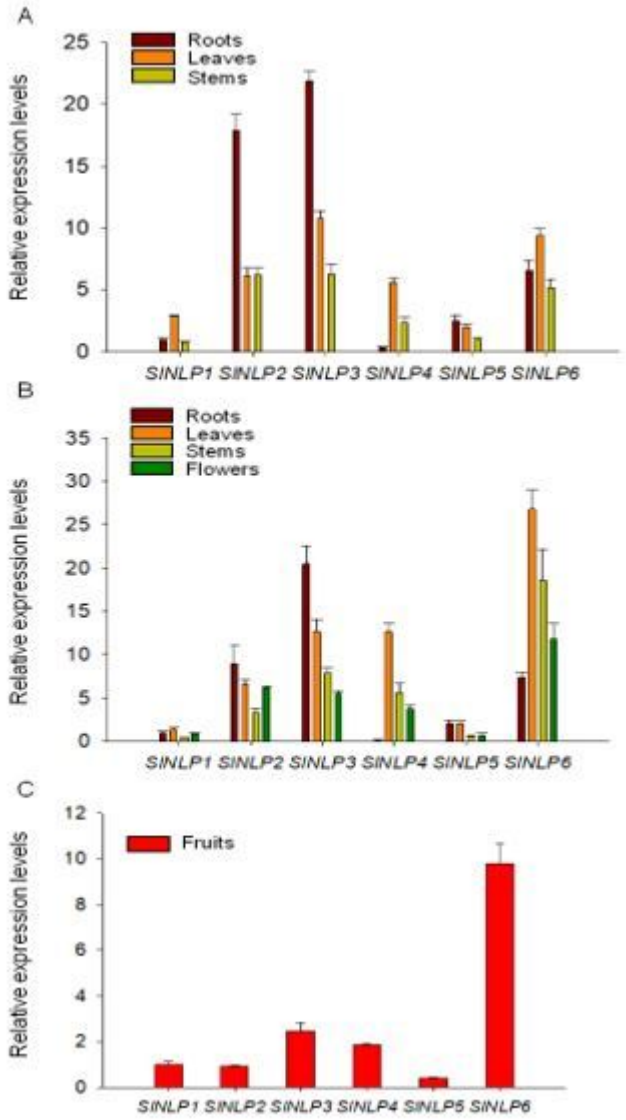

Figure 4

Tissue-specific expression of SlNLPs. (A) Relative expression levels of SlNLPs at the seedling stage; (B) Relative expression levels of SlNLPs at the flowering stage; (C) Relative expression levels of SlNLPs in red fruits. Gene expression levels were normalized to SlEF1a gene and the expression levels of SlNLP1 in roots and in fruits were set to 1 respectively. Four biological repeats were analyzed for each samples.

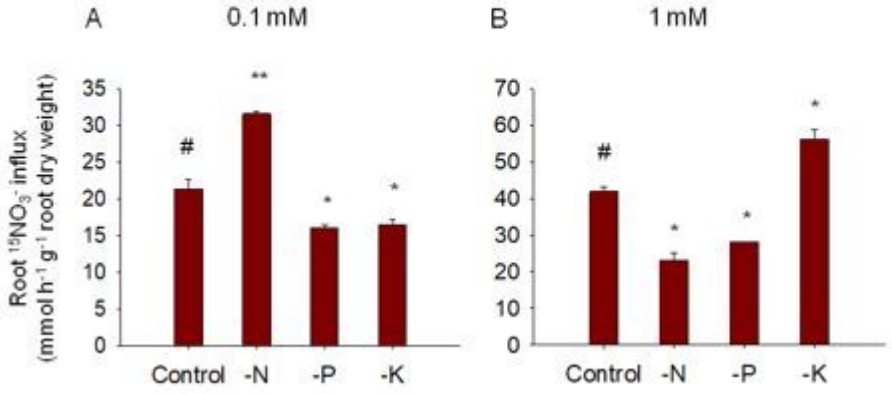

Figure 5

Root 15NO3– uptake assay under nutrition deficiency. Seedlings were treated with nitrogen starvation (−N), phosphate starvation (−P) or potassium starvation (−K) for 2 days. Then the root high-affinity and low-affinity 15NO3– uptake capacity were detected in 0.1 mM (A) or 1 mM (B) K15NO3 solution, respectively, for 5 min. Three biological repeats were analyzed for each sample. “#” represents the control, ** P < 0.01 and * P < 0.05.

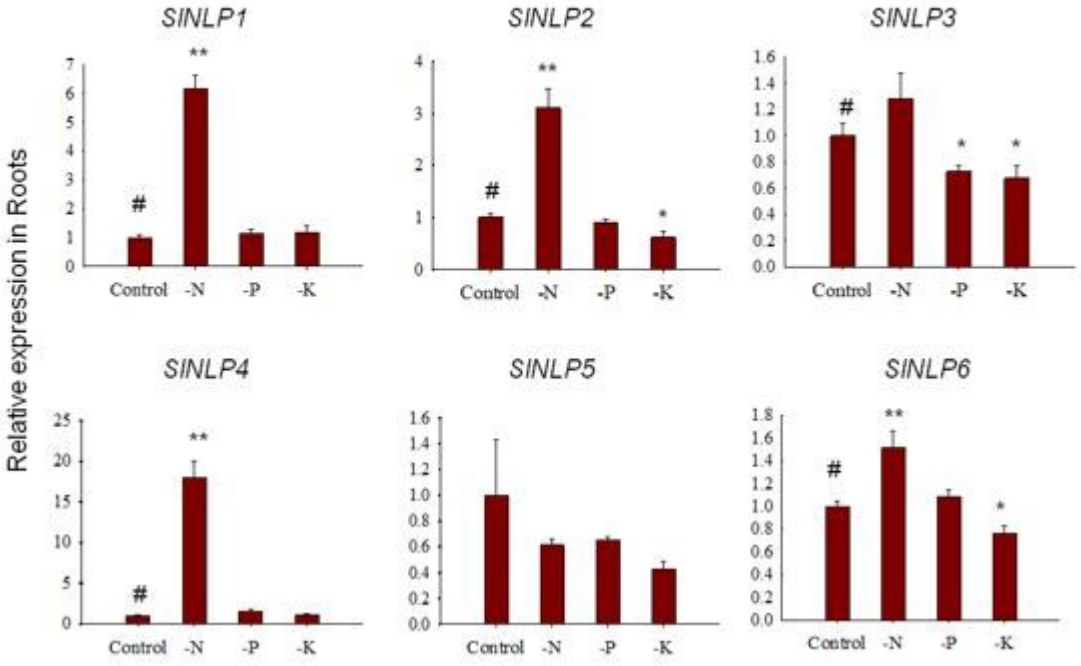

Figure 6

Expression of SlNLPs in response to nutrition deficiency. Seedlings were treated with nitrogen starvation (−N), phosphate starvation (−P) or potassium starvation (−K) for 2 days. Gene expression levels were

normalized to SlEF1a gene and the expression level in normal hydroponic medium was set to 1. Four biological repeats were analyzed for each sample. “#” represents the control, ** P < 0.01 and * P < 0.05.

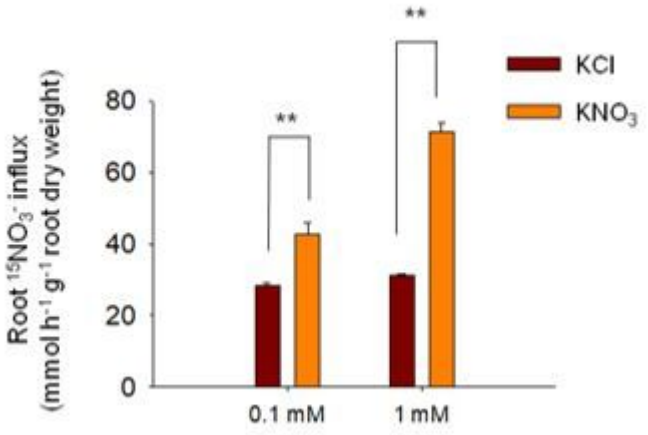

Figure 7

Root 15NO3– uptake assay after nitrate resupply. Seedlings were nitrogen-starved for 2 days and resupply with 5 mM KNO3 or 5 mM KCl for 2 hours. Then the root high-affinity and low-affinity 15NO3– uptake capacity were detected in 0.1 mM or 1 mM K15NO3 solution, respectively, for 5 min. Three biological repeats were analyzed for each sample. “#” represents the control, ** P < 0.01 and * P < 0.05.

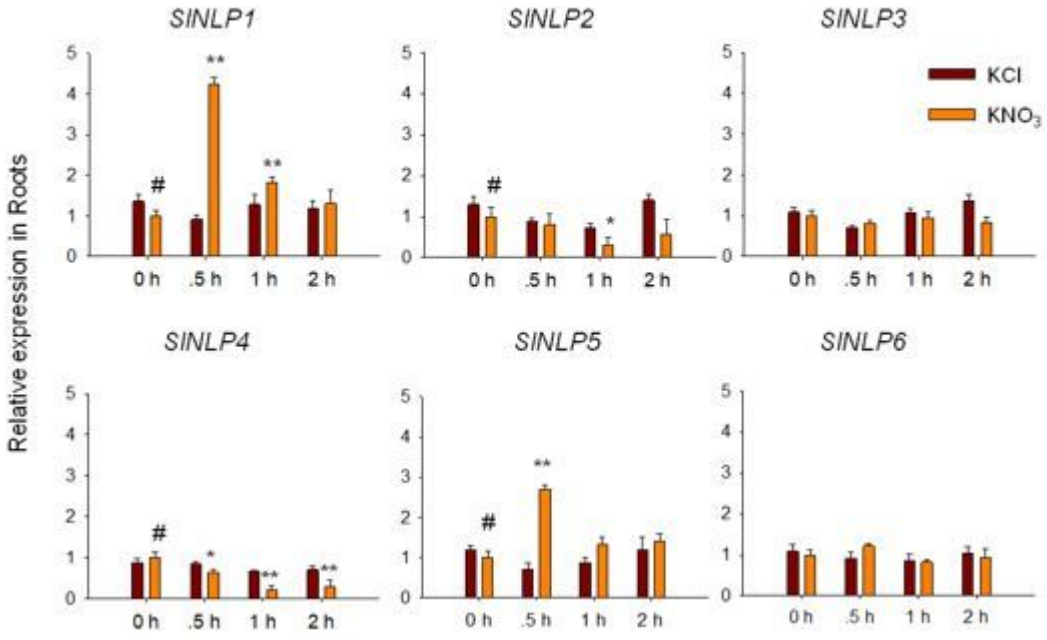

Figure 8

Nitrate-dependent expression of SlNLPs. Seedlings were nitrogen-starved for 2 days and resupply with 5 mM nitrate or 5 mM KCl as control for 2 hours. Total RNAs were extracted from roots at 0, 0.5, 1, 2 hours

after treatment and subjected to qRT-PCR analysis. Gene expression levels were normalized to SlEF1a gene and the expression level in samples at 0 h in 5 mM KCl medium was set to 1. Four biological repeats were analyzed for each sample. “#” represents the control, ** P < 0.01 and * P < 0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.NLPgenes.xlsx
- SupplementaryTable2.Onetooneorthologousrelationships.xls
- SupplementaryTable3.PrimersusedinqRTPCR.xlsx