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探討 NPF 家族及 CLC 家族在葉片之硝酸鹽分佈及硝酸鹽的訊息傳遞

Investigation of NPFs and CLCs in Nitrate Signaling and Nitrate Distribution among Leaves

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To my parents and beloved ones

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中文摘要

氮源在植物的生長中扮演不可或缺的角色，而硝酸鹽是植物最常利用的形式。為了有效地利用硝酸鹽，植物發展出可以有效吸收、轉移和儲存硝酸鹽的各種機制。NPF 的成員是植物很重要的硝酸鹽轉運蛋白，然而在 53 個成員裡，除了少數成員的角色已被確立之外，仍有一大部分尚未被了解。為了進一步探討哪些基因參與在硝酸鹽在地上部的分布，首先利用公開資料庫（MPSS 以及 e-FP Browser）所提供的基因表達資料篩選出在葉片表達量較高的基因，並選定 19 個基因剔除變異株進行硝酸鹽含量測定。研究發現，*gtr2-1* 的基因剔除株中年輕葉的硝酸鹽含量比野生株高，在氮 15 標定硝酸鹽追蹤實驗中，氮 15 在年輕葉的含量也比野生株高，此外，在低硝酸鹽環境下，*GTR2* 的基因表現量在年輕葉片中高於老葉，且利用 GUS 報導基因發現 GTR2 主要表現在根、地上部與地下部連接處、以及年輕葉片，這些證據指出 GTR2 可能負責降低年輕葉片中的硝酸鹽。另一方面，為了探討細胞內部硝酸鹽含量是否會影響植物感應外在硝酸鹽濃度的變化，在初期硝酸鹽反應實驗中採用了硝酸鹽儲存缺失的基因剔除株 *clca/clcb*，目前的結果顯示內部硝酸鹽的含量可能在根部輕微影響初期硝酸鹽反應，但對地上部卻沒有影響。本研究針對 NPF 家族，提供硝酸鹽在葉片儲存及分布的系統化分析，並得知 GTR2 確實參與在年輕葉片硝酸鹽的分布，另外也藉由 *clca/clcb* 的基因剔除株了解細胞內部硝酸鹽的含量是否會影響初期硝酸鹽反應。

關鍵字：阿拉伯芥; 硝酸鹽; 轉運蛋白; 硝酸鹽分布; 初期硝酸鹽反應

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Abstract

Nitrogen is one of the most important nutrients for plants to grow, and nitrate is the most common form of nitrogen to be absorbed. To utilize nitrate efficiently, higher plants have developed various transporters, including *NPFs* being responsible for uptake, transport and storage. Of all the 53 genes in *NPF*, despite some of the members have been well characterized, a lot of them remain unclear. To find if any *NPF* genes participate in nitrate distribution among leaves, gene expression from public databases, MPSS and e-FP Browser, was analyzed, and nitrate content among leaves of mutants of 19 candidates was measured. The results showed that the nitrate content of young leaf in *gtr2-1* was higher than wild type, and more root-fed ^{15}N was transported to young leaf in *gtr2-1*. The gene expression of GTR2 was higher in young leaf under low nitrate concentration, and β-glucuronidase reporter analyses indicated that GTR2 were expressed in roots, root-shoot junction, and the young leaves. Taken together, these results suggest that GTR2 might be involved in repressing nitrate allocation to young leaves. In addition, to find out if internal nitrate level would affect primary nitrate response, a nitrate storage defective mutant, *clca/clcb*, was characterized, and the data showed that CLCa and CLCb might be partly involved in regulating primary nitrate response in roots but not in shoots. This study

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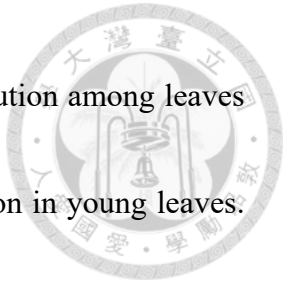

provides a systematic analysis of the roles of NPFs in nitrate distribution among leaves and found out that GTR2 has a negative impact on nitrate distribution in young leaves. Besides, the study of *clca/clcb* suggests that external nitrate plays a major role in inducing primary nitrate response.

Keyword: *Arabidopsis*; nitrate; transporter; nitrate distribution; primary nitrate response

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1. Introduction

1-1. Nitrate metabolism in plants

Aside from carbon (C), hydrogen (H), and oxygen (O), nitrogen (N) is also a principal constituent of nucleic acids, proteins, and other essential biomolecules, and nitrogen is a major limiting factor for plant growth and crop production; however, the atomic nitrogen (N) is too stable for plants to absorb, thus plants can only consume nitrogen in soluble forms, such as nitrate, ammonium, amino acid and peptides, and among them, nitrate is the most abundant form to be absorbed (Crawford, 1995). After being taken up by roots, nitrate could either be stored in the vacuole, or be reduced to nitrite by nitrate reductase (NR) in the cytosol then to ammonium by nitrite reductase (NiR) in the chloroplast in the shoot or the plastid in the root. Finally, with the cooperation of glutamine synthetase (GS) and glutamate synthase (GOGAT), ammonium would be converted to amino acids.

1-2. Nitrate transport systems in Arabidopsis thaliana.

Four gene families have been characterized for their diverse roles in plants, including *NPF* (formerly NRT1/PTR, Nitrate Transporter 1/Peptide Transporter), *NRT2*

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(Nitrate Transporter 2), *CLC* (Chloride Channel), and *SLAC1/SLAH* (Slow Anion Channel-associated1) (Dechorgnat et al., 2011; Wang et al., 2012; Leran et al., 2014).

As sessile organisms, plants have developed two nitrate uptake systems to adapt to the fluctuating nitrogen conditions, which are the high-affinity transport system (HATS) and the low-affinity transport system (LATS). The HATS operates when the external nitrate concentration is lower than 0.5 mM, while the LATS has Km over 2 mM. The transporters from NRT2 family have been known as HAT (Kotur et al., 2012), and those from NPF are mostly LAT (Crawford and Glass, 1998; Forde, 2000; Glass et al., 2001; Williams and Miller, 2001). Nonetheless, it is worth noticing that the first identified nitrate transporter, NPF6.3 (NRT1.1/CHL1), displays dual affinity in the high and low affinity ranges for nitrate, and apart from that, it is also a sensor, playing an essential role in plant development (Tsay et al., 1993; Wang et al., 1998; Liu et al., 1999; Ho et al., 2009).

1-3. NPF members have diverse functions in plants.

Up to now, NPF family in *Arabidopsis* have been identified to have transport ability with a huge range of substrates, including nitrate, nitrite, chloride, glucosinolates, amino

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acids, peptides, and several hormones (Corratge-Faillie and Lacombe, 2017).

Since the first nitrate transporter, NPF6.3 (NRT1.1/CHL1), was identified, numerous groups have dived into this field and explored the uncharted territory. For these two decades, 17 out of 53 NPF family members have been reported to be able to transport nitrate. For instance, NPF4.6 (NRT1.2) is expressed in roots and is responsible for low-affinity nitrate uptake (Huang et al., 1999). NPF7.3 (NRT1.5) is expressed in pericycle cells close to the xylem. In *npf7.3* knockout mutants, nitrate accumulates in roots, indicating that NPF7.3 participates in loading nitrate into xylem and regulates long-distance transportation (Lin et al., 2008). NPF7.2 (NRT1.8) is expressed in parenchyma cells within the vasculature, playing the role of removing nitrate from xylem sap, and therefore has a negative impact on the root-to-shoot nitrate transport (Li et al., 2010). On the other hand, NPF2.9 (NRT1.9) is expressed in the companion cells of root phloem. In *npf2.9* knockout mutant, the root-to-shoot nitrate transport and plant growth are enhanced, suggesting that NPF2.9 facilitates nitrate loading into the phloem and enhances downward nitrate flow (Wang and Tsay, 2011).

Except for the roots, NPF family also has functions in shoots. In shoots, NPF6.2 (NRT1.4) is expressed in the petiole, modulating nitrate storage in petiole (Chiu et al.,

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2004). NPF6.4 (NRT1.3) is expressed in parenchymal tissues and up-regulated by light, suggesting that NRT1.3 may participate in transporting nitrate to photosynthesizing cells (Lejay et al., 2008; Tong et al., 2016). NPF3.1 is expressed in smaller veins, playing the role as a nitrite/nitrate transporter in the chloroplast envelope (Sugiura et al., 2007; Pike et al., 2014). Oocytes expressing GTR1 or GTR2 exhibit nitrate induced current, and these two NPF members are both high-affinity H^+/glucosinolates transporters (Nour-Eldin et al., 2012; Andersen et al., 2013; Ishimaru et al., 2017). PTR family is reported to have dipeptide transport activity: PTR1 is proton-coupled dipeptide transporter with no nitrate transport activity; PTR2 also can transport histidine and dipeptides; as for PTR6, although it is homologous to PTR1, it does not have the transport activity for di- and tri-peptide, and PTR6 is expressed in the whole plant, with the highest expression in pollen and senescing leaves (Frommer et al., 1994; Chiang et al., 2004; Dietrich et al., 2004; Hammes et al., 2010; Weichert et al., 2012; Chiba et al., 2015; Qiu et al., 2017). NPF1.1 (NRT1.12) and NPF1.2 (NRT1.11) are expressed in the phloem of the leaf major vein of the mature leaves. In $^{15}NO_3^-$ labeling experiment, more ^{15}N was accumulated in mature leaves but less nitrate accumulation in young leaves in *npf1.1/npf1.2* double mutant, suggesting that NPF1.1 and NPF1.2 are responsible for redistributing nitrate from mature

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leaves to N-demanding leaves via phloem transport under N-sufficient condition (Hsu and Tsay, 2013). NPF2.13 (NRT1.7) also expressed in the phloem of the leaf minor vein in old leaves. Less ^{15}N was remobilized to young leaves in the knockout mutant in $^{15}NO_3^-$ tracing experiment, and more nitrate accumulated in old leaves in mutants. These data indicate that NPF2.13 is responsible for loading nitrate into phloem to transport nitrate out from old leaves to young leaves during nitrogen deficiency (Fan et al., 2009).

1-4. Nitrate serves as an important nutrient and a signal molecule.

In addition to being an important growth factor, nitrate also serves as a signal molecule. For instance, it could regulate root architecture, seed germination, flowering, hormones, and nitrogen/carbon balance (Forde, 2002; Miyawaki et al., 2004; Alboresi et al., 2005; Zheng, 2009; Lin and Tsay, 2017).

Moreover, nitrate itself can also regulate gene expression levels, such as nitrate transporters, nitrate assimilation enzymes, and even carbon assimilation enzymes (Gowri et al., 1992; Scheible et al., 1997; Lejay et al., 1999). At the absence of nitrate, these nitrate-regulated genes express at a low level, but when plants encounter nitrate, these genes would be induced within minutes, for instance, *NPF6.3* (*NRT1.1*), *NRT2.1*, *NR*, and

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NiR (Wang et al., 2003). This nitrate-induced rapid transcriptional response is called primary nitrate response (Redinbaugh and Campbell, 1991).

Nevertheless, before inducing these genes, nitrate has to be sensed by plants in the first place. Till now, we have already learned that NPF6.3 plays the role as transceptor (transporter-receptor), which can sense a wide range of nitrate concentration and trigger different levels of gene expression according to external nitrate concentration (Ho et al., 2009). NRT2.1 was also suggested to be a nitrate sensor or a signal transducer, repressing lateral root initiation (Little et al., 2005).

1-5. CLC family members are involved in nitrate compartmentalization in intracellular organelles

After entering cells via NPF or NRT2 families, nitrate would accumulate in the vacuoles, where the concentration of nitrate can go up to 50 mM, and the vacuolar nitrate contributes to the homeostasis of cytosolic nitrate (Martinoia et al., 2000; Cookson et al., 2005). In *Arabidopsis*, there are seven homologs have been identified, named AtCLCa to AtCLCg, and they are predominantly expressed in vascular tissues (Lv et al., 2009). Within a cell, CLCa, CLCb, CLCc and CLCg are localized in the vacuole, CLCd and

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CLCf are localized in the Golgi, and CLCe is localized in the chloroplast (Zifarelli and Pusch, 2010).

Among seven of them, CLCa is the most thoroughly studied plant CLC. CLCa, when mutated, leads to 50% of nitrate reduction in both shoots and roots (Geelen et al., 2000). CLCb had a stronger expression in young roots, cotyledons, leaves and flowers, showing strong selectivity of nitrate when expressed in oocytes, but the nitrate content of *clcb* was identical to wild type (von der Fecht-Bartenbach et al., 2010). CLCc also showed decreased nitrate content compared to wild type, but had a broader anion selectivity (Harada et al., 2004).

1-6. Nitrate transporters in Poplar

In contrast to most crop species preferring nitrate, the source of nitrogen taken up by trees mainly depends on the tree species and the environment. For example, *Populus tremuloides* had higher capacity for nitrate acquisition over ammonium, and the ability was mediated by both HATS and LATS (Min et al., 2000). In Grey poplar (*Populus tremula×Populus alba*), under saline conditions, uptake, assimilation, and accumulation of N were enhanced when supplied with nitrate instead of ammonium (Ehlting et al.,

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2007), while roots of *Populus simonii* uptake more ammonium than nitrate in salt-treated experiment (Zhang et al., 2014).

Most research on nitrate has only been done at the physiological level, and more reports are focused preferably on the influence of N sources on the biomass, xylem production, and drought stress adaptation (Faustino et al., 2015; Omena-Garcia et al., 2015). Today, we have know that in *Populus trichocarpa*, 79 *NRTs* have been identified from the genome, including 68 *PtNRT/PTR*, 6 *PtNRT2*, and 5 *PtNRT3* genes (Bai et al., 2013). Microarray data showed that most of the *PtNRT* genes were expressed in wood, bark, and leaves, fewer *PtNRT* genes expressed in roots, and very few *PtNRT* genes were found expressed in the developing xylem and the elongation zone (Bai et al., 2013).

1-7. Aim of this research

With all the efforts from numerous scientist for these two decades, we still lack a systematic research on which genes might participate in nitrate distribution among leaves, and whether internal nitrate concentration affects nitrate sensing. Here I focused on NPF family, using public databases to screen for my candidates based on their gene expression among leaves, and tried to find out within this family, which genes might be involved in

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nitrate distribution in leaves.

In addition, it has not been reported that if there is any sensor inside the cell and can detect the internal nitrate concentration. In this experiment, *clc* mutants, which has significant nitrate storage defect, were used to perform low- and high-affinity primary nitrate response, trying to understand if internal nitrate concentration would affect nitrate signaling.

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2. Material and method

2-1. Plant Material and Growth Condition

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type control, and 19 *npf* mutants used in nitrate content analysis were obtained from Arabidopsis Biological Resource Center (ABRC) or The European Arabidopsis Stock Centre (NASC).

AGI code	NPF names	Published names	Mutant line
At3g47960	AtNPF2.10	GTR1	*gtr1* (CS879742)
At1g12110	AtNPF6.3	NRT1.1(CHL1)	*chl1-5*
At3g21670	AtNPF6.4	NRT1.3	*sper3-3* (SALK_001553)
At1g69870	AtNPF2.13	NRT1.7	*nrt1.7-2* (SALK_053264)
At1g22570	AtNPF5.15		*npf5.15-1** (CS859376)
At2g26690	AtNPF6.2	NRT1.4	*nrt1.4-2* (WiscDsLox322H05)
At5g62680	AtNPF2.11	GTR2	*gtr2-1* (Garlic_20_B07)
At1g69850	AtNPF4.6	NRT1.2	*ait1-1* (SALK_146143)
At2g02040	AtNPF8.3	PTR2	*ptr2-1** (SALK_079073)
At1g62200	AtNPF8.5	PTR6	*ptr6-1** (GK-651C03)
At1g68570	AtNPF3.1		*npf3.1-1** (SALK_076121)
At3g53960	AtNPF5.7		*npf5.7-1** (SALK_068690C)
At3g54140	AtNPF8.1	PTR1	*ptr1-1* (SALK_131530)
At2g40460	AtNPF5.1		*npf5.1-1** (SALK_000464)
At1g22540	AtNPF5.10		*npf5.10-1** (SALK_141062)
At5g13400	AtNPF6.1		*npf6.1-1** (SALK 007230)
At5g14940	AtNPF5.8		*npf5.8-1** (SALK_039348)
At1g72140	AtNPF5.12		*npf5.12-1** (Garlic_168_G10)
At1g72130	AtNPF5.11		*npf5.11-1** (SALK_042211)

* The novel mutants, which have not been published, were named here with their NPF names or published name of the gene. Their gene structures were presented in Supplementary Figure 1 to 11, with the primers list in Supplementary Table 1.

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Plants were grown hydroponically containing different nitrate concentrations depending on experimental designs with 1 mM KH_2PO_4/K_2HPO_4 and basal nutrient (2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM $FeSO_4$-EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4 \cdot 2H_2O$, 1 μM $ZnCl_2$, 1 μM $CuSO_4 \cdot 5H_2O$, 0.2 μM $NaMoO_4 \cdot 2H_2O$), 0.5g/L MES at pH 5.5 (adjust with KOH) under long day condition (16h of light/8h of dark cycles; 95-110 μmole m^{-2} s^{-1} PPDF). After four days, seeds were germinated on rockwool, and the full nutrient medium was applied twice a week after germination. The light intensity was measured with a LI-250A light meter with a LI-190SA quantum sensor (LI-COR).

For primary nitrate response, seeds of Col-0 and mutants were surface-sterilized with 70% ethanol for 2 minutes then sterilization solution (0.5% SDS and 20% bleach) for 15 minutes. Approximately 240 seeds were sown on nylon netting supported by Magenta vessels (Sigma) in about 57 ml of nitrate-free liquid growth medium. The medium contains 12.5 mM ammonium succinate as the sole nitrogen source, 10 mM KH_2PO4/K_2HPO_4, and basal nutrient (2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM $FeSO_4$-EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4 \cdot 2H_2O$, 1 μM $ZnCl_2$, 1 μM $CuSO_4 \cdot 5H_2O$, 0.2 μM $NaMoO_4 \cdot 2H_2O$, 1g/L MES, and 0.5% sucrose) at pH 6.5 (adjust with KOH). The eight-day-old seedlings were transferred to about 57 ml of 12.5 mM ammonium succinate

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medium, which the pH was changed to 5.5 by adding HCl, for two treatments, 16 hours and 3 hours respectively. After the 3-hour treatment, the plants were transferred to mediums containing 25 mM or 200 μM KNO_3, pH 5.5, and then the seedlings were collected at 0, 15, 25, 35, 45, 60, and 120 minutes after transferred. Two vessels were pooled together for each experiment (approximately 60 seedlings).

Plants for measurement of primary root length were grown on plates. Seeds of wild type and mutants were surface-sterilized as described previously and sown on 0.2 mM KNO_3, 5 mM KNO_3 plates at pH 5.7 and 5 mM ammonium succinate plate at pH 6.5. After stratification at 4°C for 3 days, the plates were taken out and put under continuous light for 4 days, and then the homogeneous seedlings were shifted to new plates with the same nutrient and pH level for another 6 days. The photos were taken every day at noon, and the measurement of the length of primary root was using ImageJ software (Schneider et al., 2012).

2-2. Genomic DNA extraction

7-day-old seedlings was ground with 0.5 mL Urea extraction buffer (7 M Urea, 1% Sarcosyl, 50 μM Tris, pH 8.0, 35 mM NaCl, 20 mM EDTA, pH 8.0) and mixed with 0.4

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mL phenol/chloroform/IAA (25:24:1) mixture. Mixed well and centrifuged at 14000 rpm for 15 minutes, and then the supernatant was transferred to a new microtube, the equal volume of IPA and 0.1 volume of 3 M sodium acetate and precipitated at 4°C overnight. The genomic DNA was pelleted by centrifuged at 14000 rpm for another 15 minutes and dissolved in water.

2-3. Quantitative PCR analysis

The RNA of samples were extracted with TRIzol reagent (Invitrogen), and then cDNAs were synthesized using oligo (dT) primers and ImProm-II reverse transcriptase (Promega). Quantitative PCR was performed with 2x LightCycler 480 SYBR green I Master Mix (Roche). The initial denaturing step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, 59°C for 5 seconds, and 72°C for 11 seconds. After PCR cycles, the melting temperature of the PCR product was measured. The gene expression was analyzed with gene-specific primers and was normalized with *UBQ10*.

2-4. Nitrate Content Analysis by HPLC

The plant tissue of root or shoot were collected and frozen in liquid nitrogen

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immediately, and then samples were dried by lyophilization. To extract nitrate, samples were boiled in water (1000 μL/mg dry weight) for 30 minutes and then freeze-thawed once. After filtering through 0.2 μm polyvinylidene fluoride membrane (Pall Corporation), nitrate content of samples was determined by HPLC (Thayer and Huffaker, 1980) using a PARTISIL 10 SAX (strong anion exchanger) column (HICHROM) with 50 mM KH_2PO_4 buffer, pH 3.0, as the mobile phase.

2-5. $^{15}NO3^-$ Labeling Assay

Plants were grown hydroponically 17 days as mentioned above. After the light was on, plants were transferred to 2 mM KNO_3 hydroponic medium containing a 49% excess of ^{15}N for 5 minutes, washed twice with 0.1 mM $CaSO_4$. The leaves were collected by order from old to young along with the root in tin capsule, and then dried in 80°C oven for two days. ^{15}N abundance in individual leaf was analyzed as described elsewhere using a continuous-flow isotope ratio mass spectrometer coupled with a carbon nitrogen elemental analyzer (SERCON)(Fan et al., 2009).

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2-6. GUS staining

Transgenic plants were generated by Ya-Yun Wang, with a 2.7-kb genomic fragment from the promoter to partial second exon (Wang, 2011). Plants were grown on MS plates (CAISSON) under continuous light. Histochemical staining for GUS activity was performed on plants at vegetative stage. The whole plant was vacuum infiltrated for 45 minutes at room temperature in 0.5% formaldehyde, 0.05% Triton X-100, 50 mM sodium phosphate buffer at 7.0. After rinsed three times with 50 mM sodium phosphate buffer at 7.0, the plants were incubated in staining buffer at 37°C in dark overnight, which contains 2 mM X-Glu (Gold BioTechnology, Inc.), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocycide, 0.05% Triton X-100, and 50 mM sodium phosphate buffer at 7.0. The plants were rinsed three times with 50 mM sodium phosphate buffer at 7.0, and then be fixed overnight in 2% formaldehyde, 0.5% glutaraldehyde, and 100 mM sodium phosphate buffer at 7.0. Pigments were removed by immersing plants in 15%, 30%, 50%, 70% ethanol successively.

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3. Results

3-1. Investigation of NPF genes for nitrate distribution among leaves

3-1-1. Bioinformatics research to identify potential candidates for further study.

To find out which genes might involve in regulating nitrate distribution in leaves, firstly I used public databases to narrow down my targets. The main database used was AtGenExpress Consortium (*Arabidopsis* eFP Browser, http://bbc.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Data from the eFP Browser are presented pictographically in Supplemental Figure 12. From this information, the gene expression pattern in various tissues for each NPF family member could be found. Among 53 NPF family members, the level of gene expression in leaves can vary from 1 to 876, including young leaves and old leaves, while 3 of the family members, At1g72120 (NPF5.14), At1g72130 (NPF5.11), and At3g54450 (NPF5.4), failed to be detected by this method. To screen for candidate genes involved in nitrate distribution in leaves, a cutoff level of gene expression in leaves at 100 was chosen to distinguish those genes that have higher expression in leaves from others. Under this criterion, 18 NPF members were chosen to be the candidates and subjected to the following nitrate content analyses (Table 1).

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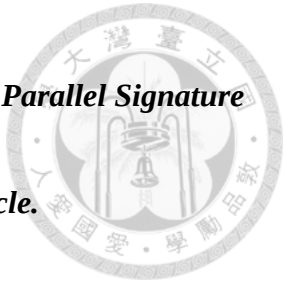

3-1-2. Three more NPF members were selected based on Massively Parallel Signature Sequencing (MPSS) and RT-PCR results presented in a review article.

To further confirm the selection and furthermore to identify genes that cannot be found in *Arabidopsis* eFP Browser, another public database, Massively Parallel Signature Sequencing (MPSS, https://mpss.danforthcenter.org/dbs/index.php?SITE=at_sRNA), and the data presented in a review article (Tsay et al., 2007) were examined.

From MPSS database, 28 out of 53 genes have signatures in leaves (Table 2). Among the 28 genes, even all of the genes showed signatures in leaf, however, some of the abundances of signature in leaf were too low compared to other tissues. Therefore, for the candidates chosen, the abundances of signature in leaf at least had to be 40% of that in the most abundant tissue. These 14 candidates genes were marked with light gray in Table 2.

On the other hand, in our previous study (Tsay et al., 2007), the tissue-specific expression pattern of the 53 genes was normalized with *UBQ10* and shown as circles with sizes proportional to the percentage in each tissue (Supplementary Figure 13). In comparison of shoots and roots, the same criterion was applied that the value in shoots had to be higher or at least 40% of that in roots to be chosen, and with this criterion, 16

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genes were choseon, marked with light gray in Supplementary Figure 13.

Taken together, as e-FP Browser showed detailed information of individual leaf, the genes selected based on e-FP Browser were all adopted, while the overlapping candidates selected from MPSS and RT-PCR results was also adopted in the following experiments. (Figure 1)

3-1-3. 19 candidates were chosen to measure nitrate content and biomass among leaves

According to the public databases mentioned above, we narrowed down to 21 *NPF* members; however, NPF1.1 and NPF1.2 together have been reported to participate in nitrate redistribution, so here they were excluded from the nitrate content experiment.

Mutants of the 19 candidates were randomly separated into batches and grown hydroponically supported with 2 mM KNO_3 (Supplementary Figure 14). Leaves were collected at 17^{th} day for all batches, except that in batch 8 were collected at 18^{th} day. In each batch, wild type was included as control. The medium of batch 3, 4 and 5 was refreshed every four days, while other batches' were refreshed twice a week, and this resulted in the smaller plants in batch 3, batch 4 and batch 5 (Supplementary Figure 15B). The biomass of these three batches was smaller than other batches and the seventh leaf

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was not able to be collected. As for batch 8, due to the difference of collecting day, the dry weight was higher in all the leaves, especially the big and young leaves (Supplementary Figure 15B). Nevertheless, the pattern of nitrate content was still comparable in all batches; the nitrate content was higher in old and big leaves (L1 to L4), and it was lower in young leaves (L5 to L7) (Supplementary Figure 15A).

To find out if the mutants of NPFs can consistently show the defects in nitrate distribution among leaves, the data were extracted from different batches and examined carefully. The results of all the mutants can be divided into three categories: no difference, slight difference, and consistent difference compared to wild type.

For the first category that had no difference between mutant and wild type, *npf3.1-1* showed no difference in both nitrate content and dry weight compared to wild type in all three batches, batch 1, batch 2, and batch 8 (Figure 2). Similarly, the nitrate content of *npf5.1-1* in both batches was similar and had no difference compared to wild type (Figure 3A and 3C). *npf5.7-1* mutant was examined in batch 9 and 10, and in both batches, the nitrate content and biomass were identical to those of wild type (Figure 4). Another mutant, *npf5.8-1*, also showed the same pattern that the nitrate content in batch 5 and batch 8 both showed no difference compared to wild type (Figure 5A and 5C). *npf5.10-1*

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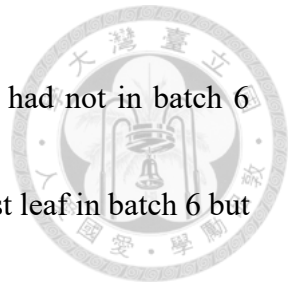

had smaller dry weight in the third leaf in batch 8 (Figure 6D) but had not in batch 6 (Figure 6B), and the mutant showed lower nitrate content in the oldest leaf in batch 6 but without statistical significance (Figure 6A). Overall, *npf5.10-1* had no difference compared to wild type (Figure 6). Data from two independent experiments showed that in nitrate content and biomass, *npf5.15-1* showed similar patterns as wild type did (Figure 7). In *gtr1* mutant, the patterns of nitrate content and dry weight in both batches, batch 9 and 10, were identical to wild type (Figure 8). In figure 9B, although the dry weight of *sper3-3* seemed to be bigger than wild type, there was no statistical significance in both nitrate content and dry weight compared to wild type (Figure 9). *ptr1-1* mutant was performed twice in batch 6 and batch 10. In batch 6, there was no difference between wild type and *ptr1-1* in both nitrate content and dry weight (Figure 10A and 10B), but in batch 10, the dry weight of *ptr1-1* was slightly higher than that of wild type, especially in fifth and seventh leaf, showing statistical difference (Figure 10D). Collectively, *ptr1-1* had no difference in nitrate content compared to wild type (Figure 10).In one batch of experiment, *ptr6-1* mutant showed similar pattern compared to wild type in both nitrate content and biomass (Figure 11).

As for mutants that had slight difference compared to wild type, *nrt1.4-2* had higher

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nitrate content in old leaves in batch 8 (Figure 12E), nitrate content in old leaves in batch 2 was slightly higher than wild type but had no statistical significance (Figure 12C), and in batch 1 it showed similar level compared to wild type (Figure 12A). For dry weight, there was no any differences compared to wild type (Figure 12B, 12D and 12F). In summary, *nrt1.4-2* overall had no consistent difference compared to wild type (Figure 12). As shown in Figure 13, *npf5.11-1* was performed three times in batch 6, 7, and 10, in which there were at least four different plants for mutants and wild type. However, the standard deviation of old leaves of nitrate content was extremely large that showed no statistical difference compared to wild type (Figure 13A, 13C and 13E), but the nitrate content slightly decreased in big leaves in *npf5.11-1* (Figure 13C and 13E), while there was no difference in dry weight (Figure 13B, 13D and 13F). *npf5.12-1* was examined in three batches, batch 5, 7, and 10. For *npf5.12-1*, nitrate content of the 6th leaf in batch 7 was higher than that of wild type (Figure 14C); the dry weight of 1st and 3rd leaf in batch 7 and 5th leaf in batch 10 was slightly higher than that of wild type (Figure 14D and 14F). Taken together, we concluded that *npf5.12-1* had no significant difference compared to wild type (Figure 14). As for *ait1-1*, the mutant had lower nitrate content in the oldest leaf in batch 1 (Figure 15A), and this was also observed in batch 4 (Figure 15C) and batch

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8 (Figure 15E) but no significant difference. In batch 8, mutant had lower dry weight in the oldest and the third leaf (Figure 15F), and this was also shown in batch 1 but without statistical significance (Figure 15B). Despite for the slight difference, *ait1-1* overall showed no dramatic difference compared to wild type (Figure 15). *npf6.1-1* showed higher nitrate content in the biggest leaf in batch 7 (Figure 16C), and this was also observed in batch 1 but without significance (Figure 16A). Also in batch 8, the mutant had higher dry weight in big and young leaves (Figure 16F), but this could not be seen in the other two batches. Taken together, *npf6.1-1* overall had no difference compared to wild type (Figure 16). For *ptr2-1*, the mutant showed higher nitrate content in the second leaf but lower nitrate content in young leaves in batch 6 (Figure 17A), while overall the mutant had smaller dry weight compared to wild type (Figure 17B and 17C).

In the third category, three mutants, *nrt1.7-2*, *chl1-5*, and *gtr2-1* showed the difference in independent batches consistently. In two independent batches, *nrt1.7-2* showed higher nitrate content (Figure 18A and 18C) and higher dry weight (Figure 18B and 18D) in most of the leaves compared to wild type. For *chl1-5*, in batch 4, it showed the minor difference of nitrate content in the first and third leaf, but there was no difference in biomass (Figure 19A and 19B). However, in batch 8 and 10, the mutant

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showed a significant decrease in both nitrate content and dry weight consistently. *chl1-5* only had about two-thirds of nitrate content in wild type (Figure 19C and 19E), and biomass was also smaller than wild type (Figure 19D and 19F), especially in batch 8 that the biomass was almost only half of it in wild type (Figure 19D). From three independent batches, it came to the conclusion that under this condition, *chl1-5* grew smaller and had lower nitrate content compared to wild type (Figure 19). Interestingly, *gtr2-1* showed lower nitrate content in old leaves but higher nitrate content in young leaves compared to wild type (Figure 20A and 20C), and although it did not show significance, this pattern could also be observed in batch 3 (Figure 20E). As for dry weight, only in batch 1 the third leaf of *gtr2-1* was smaller than it of wild type (Figure 20B), other than that there was no significant difference compared to wild type (Figure 20D and 20F).

In this study, nitrate content and biomass assay among leaves of most 19 *npf* mutants were performed at least twice in independent experiments, except that *ptr6-1* was only performed once. Three *npf* mutants, *nrt1.7-2*, *chl1-5*, and *gtr2-1* showed different nitrate distribution patterns and others had similar pattern compared to wild type. It was worth to note that *gtr2-1* showed low nitrate content of mutant in old leaves while high in young leaves compared to wild type (Figure 20), indicating that this gene might be involved in

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nitrate remobilization and distribution among leaves. Therefore, it was intriguing for me to go further and identify the function of this gene.

3-2. Functional analyses of Arabidopsis GTR2

3-2-1. GTR2 was mainly expressed in root, root-shoot junction and young leaves

In order to characterize the expression of *GTR2* at vegetative stage, GUS staining of P_{GTR2}-GUS transgenic lines was performed. Strong staining was presented mainly in the roots, root tips, and root-shoot junction (Figure 21A). The staining in leaves was relatively faint and could only be observed under a microscope. The GUS signal in shoot part was only shown in major veins in young leaves (Figure 21E to 21G), and was not shown in cotyledon, old leaf, and big leaf (Figure 21Bto 21D).

3-2-2. More ^{15}N was accumulated in young leaves of gtr2-1

As shown in Figure 20, when continuously grown with 2 mM KNO_3, *gtr2-1* mutant showed higher nitrate content in young leaves and lower in old leaves compared to wild type, indicating that *GTR2* might be involved in nitrate distribution among leaves. To understand how GTR2 participates in nitrate distribution, $^{15}NO_3^-$ allocation assay was

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performed by feeding $^{15}NO_3^-$ to the root for 5 minutes, and then the ^{15}N concentration in individual leaves was analyzed. Compared to wild type, the ^{15}N concentration was lower in old and big leaves (L1 to L4) of *gtr2*-1 mutant, despite no statistical significance (Figure 22). In contrast, ^{15}N concentration in the young leaf of *gtr2*-1 mutant showed 1.5 times higher than that of wild type (Figure 22). Both long-term nitrate accumulation and short-term ^{15}N distribution are changed in the mutant, so these results suggested that GTR2 might participate in regulating nitrate distribution among leaves, while nitrate is transported from root via xylem.

3-2-3. Expression of GTR2 in young leaves is increased at low nitrate condition

To find out the relative expression level of *GTR2* in different leaves under high and low nitrate concentrations, leaves of wild type plants were harvested at 17th day. RNA expression level was measured by RT-qPCR. As shown in Figure 23, *GTR2* expression level was higher under low nitrate concentration condition, while the expression level of high nitrate concentration was lower. Under low nitrate concentration, *GTR2* expression level in the youngest leaf was almost 4-fold higher than that in the oldest leaf. As for high nitrate concentration, *GTR2* expression level had no dramatic differences in all leaves.

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Taken together, the results indicated that *GTR2* had higher expression level under low nitrate concentration, participating in the young leaves.

3-2-4. Nitrate content in young leaves was higher under different nitrate concentrations in gtr2-1

Since the expression of GTR2 is higher at low nitrate, the influence of GTR2 on the nitrate content at low nitrate condition was examined in wild type and *gtr2-1* mutant grown with 0.2, 2, and 10 mM KNO_3 mediums at pH 5.5.

As shown in Figure 24A, 24C, and 24E, for all the three concentrations tested, 0.2 mM, 2 mM, and 10 mM, the nitrate content in young leaves of *gtr2-1* mutants were all higher than wild type. Different from what we are expected from the expression pattern, the nitrate content differences between wild type and mutant are more dramatic at higher nitrate concentrations like 2 mM and 10 mM KNO_3.

As for dry weight, under 0.2 mM KNO_3 condition, it was lower in old leaves in *gtr2-1* mutant (Figure 24B); under 2 mM and 10 mM KNO_3 conditions, the dry weight of young leaf was higher in *gtr2-1* mutant compared to wild type (Figure 24D and 24F). These data indicated that GTR2 might be involved in regulating nitrate distribution among leaves under low and high nitrate concentration conditions.

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3-2-5. Nitrate content dropped faster under starvation in gtr2-1

We are also interested to know if this gene would participate in regulating nitrate accumulation under starvation condition. Therefore, plants were shifted to nitrate-depleted medium after growing under 2 mM KNO_3 for 17 days, and samples were collected, before and after shifting, including 4 hours and 24 hours.

The nitrate content before shifting showed the same pattern observed before, as it was higher in young leaves and lower of it in old leaves in *gtr2-1* compared to wild type (Figure 25A). 4 hours after shifting, the nitrate content in both wild type and *gtr2-1* dropped. However, compared to wild type, nitrate content in *gtr2-1* declined faster. In old leaves, although the nitrate content was already lower in *gtr2-1* than in wild type before shifting, the nitrate content in wild type only dropped 22% to 24%, but in *gtr2-1*, it dropped 27% to 36%, and this phenomenon was more dramatic in young leaves. In young leaves, the nitrate content in the mutant was higher than in wild type before starvation, but 4 hours after starvation, the nitrate content dropped to the same level as wild type, and even lower than wild type in L7. 24 hours after starvation, the nitrate content of *gtr2-1* in old leaves was only half of that in wild type; while in mature leaves (L3 to L5), nitrate

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content was similar in wild type and mutant; and the nitrate was almost undetectable in young leaves (L6 and L7) in both wild type and mutant (Figure 25A).

For biomass, only in the oldest leaf and the fourth leaf, *gtr2-1* was slightly lower than wild type after 24 hours' starvation, but overall there was not so much difference. However, it brought our attention to that after 4 hours and 24 hours starvation, the growth of mature leaf (L3 to L5) and young leaf (L6 and L7) in wild type was faster than *gtr2-1*. In the mutant, the growth was about 0.1 to 0.2 mg in all leaves under starvation, but for wild type, the growth, especially in mature leaf, could up to almost 0.5 mg (Figure 25B). In summary, *gtr2-1* might grow slower than wild type under starvation, and nitrate depleted faster in *gtr2-1*.

3-2-6. Primary root length in gtr2-1 was longer under low nitrate condition

Although we selected GTR2 as one of our candidates based on its relatively high expression in leaves, the highest expression tissue of GTR2 was roots; therefore, we also examined root development to see if GTR2 played any roles in that. Seedlings were grown on plates containing 0.2 mM or 5 mM KNO_3, or 5 mM ammonium succinate. The primary root length was measured from the 4^{th} day after germination.

Under both high and low nitrate concentration plates, the primary root length of *gtr2-*

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1 was shorter at the beginning than wild type, while this difference was not observed under plates containing ammonia as nitrogen source. *gtr2-1* grew faster under low nitrate concentration plates, in which the primary root length was shorter in mutant compared to wild type at day 4, but started from day 7, the length of primary root was longer in the mutant (Figure 26A). On the other hand, *gtr2-1* in high nitrate concentration plates did not show longer root length at the end of this experiment but had similar length with wild type, though the difference at day 4 was shortened (Figure 26B). As for plants grown on ammonium plates, there was no difference between mutant and wild type in the period of this experiment (Figure 26C). In conclusion, the primary root grew longer in *gtr2-1* under low nitrate condition in this experiment, indicating that GTR2 might take part in primary root growth under low nitrate condition.

3-3. CLCa and CLCb are responsible for nitrate storage in vacuole and might have impact on nitrate sensing in roots

3-3-1. clca and clcb mutants showed a reduction in nitrate content among all leaves.

It has been reported that CLCa is responsible for nitrate storage in vacuole, and the nitrate content in *clca* in both shoots and roots is only 50% of that in wild type (Geelen

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et al., 2000), and CLCb was reported to localized in tonoplast as well and had strong selectivity for nitrate (von der Fecht-Bartenbach et al., 2010).

In Figure 27A, the nitrate content of *clca* and *clcb* was similar to previous research, in which the nitrate content was dropped dramatically in *clca* in all leaves, and the nitrate content of *clcb* was merely the same as wild type. Nevertheless, in mature leaves, the nitrate content of *clca/clcb* dropped even more than *clca* (Figure 27A). Despite the dramatic difference shown in nitrate content, the dry weight of wild type and mutants were identical (Figure 27B). To summarize, CLCa and CLCb do participate in nitrate storage in the vacuole, as the major and minor one, respectively, but the growth and biomass of mutants were not affected.

3-3-2. clca/clcb had no dramatic difference compared to wild type in terms of primary nitrate response under both high- and low-affinity nitrate conditions.

To investigate if internal nitrate content would affect nitrate sensing, primary nitrate response in wild type and *clca/clcb* under both low- and high-nitrate conditions, 200 μM and 25 mM, respectively, was performed. In most studies, primary nitrate response only focuses on roots, but since CLCa and CLCb are mainly expressed in shoots, both roots

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and shoots in this experiment were collected to examine the pattern of primary nitrate response. 9-day-old seedlings of wild type and *clca/clcb* were exposed to high- and low-affinity nitrate medium for 2 hours, and samples were collected at seven time points (0, 15, 25, 35, 45, 60, and 120 minute). Previous studies showed that under all nitrate concentrations tested, the expression of primary nitrate response genes peaked at 30 minutes in roots (Hu et al., 2009); however, there were not many studies on shoot primary nitrate response, so more time points were collected in case the peak in shoots were missed. For roots, *NPF6.3* (*CHL1*), *NRT2.1*, *NIA1*, and *NiR* were chosen as marker genes, which have been reported to be nitrate-inducible genes and can be upregulated in primary nitrate response (Wang et al., 2003); for shoots part, *NIA2*, *Glucose-6-phosphate-1-dehydrogenase* (*G6PDH3*), *Urophorphyrin III Methylase* (*UPM1*), *NiR*, and *PHOSPHOGLYCERATE MUTASE* were used as marker genes, which were induced rapidly by nitrate (Wang et al., 2003). Apart from quantifying relative expression of marker genes, nitrate content of wild type and *clca/clcb* was also measured to make a better correlation between internal nitrate content and gene induction level.

Since the plants for primary nitrate response were grown in nitrate-free ammonium medium, it was necessary for us to measure the gradually changed nitrate content in both

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shoots and roots after nitrate induction. In roots, under both high- and low-affinity conditions, the nitrate content of *clca/clcb* was significantly lower than wild type after nitrate induction (Figure 28A and 29A). On the other hand, in shoots, the nitrate content in mutant and wild type had no difference under high-affinity nitrate condition (Figure 28B), and the significant difference between mutant and wild type only showed after two hours under low-affinity nitrate condition (Figure 29B).

As for relative gene expression, the peak in roots was at 25 to 35 minutes after induction under both high- and low-affinity conditions (Figure 30 and 32), but the peak in shoots had different patterns. As shown in Figure 31, under high-affinity nitrate condition, the marker gene, *PHOSPHOGLYCERATE MUTASE*, peaked at 35 minutes, or at 45 to 60 minutes, including *NIA2*, *NiR*, *G6PDH3* and *UPM1*, and decreased within two hours. Under low-affinity nitrate condition, only *PHOSPHOGLYCERATE MUTASE* showed a peak at 35 minutes, and all the other marker genes tested seemed to reach the maximum at 45 to 60 minutes but did not decrease (Figure 33).

Under high-affinity nitrate condition, in both roots and shoots, the relative expression normalized to the 0 time point of the wild type of all genes examined was identical in wild type and *clca/clcb* (Figure 30 and 31). However, the fold change of

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CHL1 and *NiR* in roots, as shown in Figure 29, was slightly higher in the double mutant, indicating that even the nitrate content differed from wild type, CLCa and CLCb might only have a minor effect in roots on primary nitrate response under high-affinity nitrate condition.

Under low-affinity nitrate condition, in shoots, the expression of *G6PDH3*, *UPM1*, and *PHOSPHOGLYCERATE MUTASE* was slightly decreased in double mutant at 35 minutes, and the expression of *NIA2* was slightly decreased in mutant at 120 minutes, but overall, there was no dramatic difference between wild type and *clca/clcb* double mutant under low-affinity condition in shoots (Figure 33). Interestingly, in roots under low-affinity nitrate condition, the relative expression and fold change of marker genes in *clca/clcb* double mutant showed different patterns. When normalized to the 0 time point of wild type, the relative expression of marker genes in *clca/clcb* double mutant was significantly lower than wild type, especially in the first hour (Figure 32). However, the fold change of marker genes in wild type and *clca/clcb* double mutant was higher and showed the difference after 45 minutes (Figure 32).

In summary, under both the high- and low-nitrate conditions, the nitrate content in wild type and *clca/clcb* double mutant was significantly different, especially in roots, and

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the expression of target genes showed more differences in roots as well.

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4. Discussion

4-1. Most of the mutants of NPF members showed an identical pattern of nitrate distribution among leaves compared to wild type in this experiment.

Members of *NPF* have diverse functions in plants, modulating nitrate uptake, transport, and even allocate. With the coordination of *NPF* members, plants can survive in nitrate fluctuating condition. Once the nitrate is up taken by roots, it might be stored in the vacuole and be retrieved when needed.

In order to investigate in nitrate distribution among leaves in NPF efficiently, public databases, e-FP browser and MPSS, and results presented in a review article were adopted. Among 53 *NPF* members, a criterion that gene has higher expression in leaves helped us to identify 19 candidates for further study. Nitrate content and biomass were measured for the 19 candidates, and the results were categorized into three groups, including genes that had no difference, slight difference, and the consistent difference compared to wild type.

In the first category, containing mutants of genes had an identical pattern in nitrate content and biomass as wild type, there are NPF3.1, NPF5.1, NPF5.7, NPF5.8, NPF5.10, NPF5.15, GTR1, NRT1.3, PTR1, and PTR6. *npf3.1-1* showed no difference in both

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nitrate content and dry weight (Figure 2), although studies showed that NPF3.1 behaves as a nitrite/nitrate transporter of the chloroplast envelope in smaller veins (Sugiura et al., 2007; Pike et al., 2014), but it might not be involved in regulating nitrate distribution in leaves. GTR1 is well known for its contribution to translocate JA and JA-Ile and expressed in and surrounding the leaf veins (Ishimaru et al., 2017). Although 10 mM nitrate also elicits currents in oocytes expressing GTR1 (Nour-Eldin et al., 2012), in this experiment, no difference in nitrate content and dry weight can be detected in *gtr1* (Figure 8). It is reported that the expression of NRT1.3 is up-regulated by light (Lejay et al., 2008) and may take part in supplying nitrate to photosynthesizing cells (Tong et al., 2016). In this experiment, the light intensity was only 95-110 μmole m^{-2} s^{-1}, which was only half of that in the previous study, and this might lead to the results that *sper3-3* showed identical nitrate content and dry weight compared to wild type (Figure 9). Proton-coupled transporter PTR1 is documented to transport aromatic dipeptide and JA-Ile, and nitrate cannot induce currents in oocytes expressing PTR1 (Dietrich et al., 2004; Hammes et al., 2010; Chiba et al., 2015; Qiu et al., 2017). Together with our results that no dramatic difference can be detected in nitrate content and biomass between wild type and *ptr1-1* (Figure 10), the conclusion came to that PTR1 does not participate in nitrate distribution

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among leaves. PTR6 is homologous to di- and tripeptide transporters, and *AtPTR6-GUS* plants showed ubiquitous expression in the whole plant, with the highest expression in pollen and senescing leaves (Weichert et al., 2012). PTR6 did not show di- and tripeptide transport activity in a previous study (Weichert et al., 2012) and did not participate in nitrate distribution in this study (Figure 11), so its function in *Arabidopsis* still need further study to confirm.

In the second category, there are six genes in this category. Their mutants showed slight difference only once or twice of the repeats compared to wild type, including NRT1.4, NPF5.11, NPF5.12, NRT1.2, NPF6.1, and PTR2. NRT1.4 was expressed in the petiole, being responsible for nitrate storage, and in the null mutant, the nitrate content in petiole was reduced half of the wild type level (Chiu et al., 2004). It showed higher nitrate content in old leaves once (Figure 12E), but overall had no difference compared to wild type (Figure 12), which is reasonable due to this gene is mainly expressed in petiole but not in leaf lamina. NRT1.2 has been known as a constitutive component of low-affinity nitrate uptake and is expressed in root hairs (Huang et al., 1999). Consistent with results that nitrate uptake activity was about 90% of wild type in knockdown *nrt1.2* grown in 25 mM KNO_3 (Huang et al., 1999), the nitrate content and dry weight among leaves had no

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dramatic difference in this experiment (Figure 15). Similar to PTR1, PTR2 also has the ability to transport histidine and dipeptide as reported (Frommer et al., 1994; Chiang et al., 2004); therefore, the retardation of biomass observed (Figure 17B) might due to the defect of peptide transportation.

In the third category, NRT1.7, NRT1.1, and GTR2 showed the consistent difference in comparison between mutants and wild type. NRT1.7 has been well studied for its function in remobilizing nitrate from old leaves to young leaves, and under the nitrate-depleted condition, the knockout mutant, *nrt1.7-2*, shows severe growth retardation (Fan et al., 2009). On the contrary, in this experiment, *nrt1.7-2* was grown under the nitrate-sufficient condition, which might lead to the results that the mutant had bigger biomass (Figure 18B and 18D). NRT1.1 is famous for being a dual-affinity transceporter, playing an essential role in plant development (Tsay et al., 1993; Liu et al., 1999; Ho et al., 2009). With the severe defect of nitrate uptake, the plants grew smaller and had less nitrate storaged in leaves (Figure 19). Among all the candidates, GTR2 showed the most interesting pattern that the nitrate content was higher in young leaves but lower in old leaves (Figure 20). GTR2, similar to GTR1, is high-affinity H^+/glucosinolates symporters (Andersen et al., 2013). Though GTR2 also has nitrate transport activity when expressing

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in oocytes, there is not many studies on how GTR2 would participate in regulating nitrate distribution in plants (Nour-Eldin et al., 2012). Nevertheless, the pattern of higher nitrate content in young leaves gave us a hint that this gene might have more functions than only transporting glucosinolate in plants.

To conclude, in this experiment, most of the mutants showed no dramatic difference compared to wild type, but this does not mean that these genes do not participate in nitrate distribution among leaves. The plants were grown hydroponically under the nitrate-sufficient condition for 17 days, and samples were collected before bolting; therefore if the genes participate in nitrate distribution in early stage or function under different nitrate conditions, such as starvation or low nitrate condition, their phenotype would not show up in this experiment. In addition, the genes have similar functions might have redundancy, like NPF1.1 and NPF1.2 (Hsu and Tsay, 2013), and single mutants were used in this experiment, and it might lead to the results that not so many genes showed differences compared to wild type.

4-2. GTR2 might play the role of regulating nitrate storage in young leaves

20 hours after spotting $^{15}NO3^-$ on old leave, less ^{15}N content was detected in young

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leaves and more was accumulated in old leaves in *nrt1.7*. NPF2.13 (NRT1.7) is expressed in the phloem of the leaf minor vein in old leaves, and is responsible for loading nitrate into the phloem and transporting nitrate out from old leaves to young leaves (Fan et al., 2009). In another $^{15}NO_3^-$ labeling experiment, 30 minutes after feeding $^{15}NO_3^-$ from roots, more ^{15}N was accumulated in mature leaves in *npf1.1/npf1.2* double mutant. NPF1.1 (NRT1.12) and NPF1.2 (NRT1.11) are expressed in the phloem of the leaf major vein of the mature leaves, suggesting that NPF1.1 and NPF1.2 are responsible for redistributing nitrate from mature leaves to growing leaves via phloem transport (Hsu and Tsay, 2013). Previously, our lab has found that GTR2 is expressed in the xylem pole pericycle (Wang, 2011). In this study, 5 minutes after $^{15}NO_3^-$ was feeding on roots, more ^{15}N was accumulated in young leaves in *gtr2-1* (Figure 22), suggesting that the observed difference of ^{15}N distribution was due to the reallocation of $^{15}NO_3^-$ transported to shoots via xylem. And this is different from the function of NPF2.13 (NRT1.7), which is transporting stored nitrate out from old leaves, but similar to the function of NPF1.1 (NRT1.11) and NPF1.2 (NRT1.12), responsible of redistributing xylem-borne nitrate.

With the evidence that the nitrate content was higher in young leaves in *gtr2-1* (Figure 20), the ^{15}N concentration was higher in young leaves in *gtr2-1* (Figure 22),

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according to the staining of GUS lines and RT-qPCR analyses showing that expression of GTR2 was higher in young leaves (Figure 21E to 21G and Figure 23), it is believed that GTR2 might play the role as a negative regulator in young leaves nitrate accumulation. As the GUS staining shown in major veins in young leaves (Figure 21E to 21G), GTR2 is speculated to remove nitrate from xylem so as to store nitrate in other tissues, such as petiole.

Under low nitrate condition, the expression of *GTR2* in the youngest leaf was the highest among leaves in wild type (Figure 23) and had no big difference among various leaves under high nitrate condition; therefore, it was assumed to have more impact on nitrate content in young leaves under low nitrate condition. However, the unexpected results showed that under all tested conditions, 0.2 mM, 2 mM, and 10 mM KNO_3, the nitrate content in young leaf was higher in *gtr2-1* compared to wild type, and it did not show more significant difference under low nitrate condition (Figure 24). One possibility is that the low expression of *GTR2* under high nitrate condition could already affect the nitrate distribution in young leaf.

4-3. CLCa and CLCb do not affect nitrate sensing in short-term primary nitrate

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response

CLCa and CLCb have been reported to locate in the vacuole, in which CLCa is responsible for up to 50% of nitrate storage, while *clcb* showed no defect in nitrate content compared to wild type (Geelen et al., 2000; von der Fecht-Bartenbach et al., 2010; Zifarelli and Pusch, 2010). Nonetheless, as shown in Figure 26, the *clca/clcb* double mutant in this experiment showed significant defect in nitrate content compared to wild type, *clca*, and *clcb* single mutant; therefore, the *clca/clcb* double mutant was used in following experiments to understand if internal nitrate content would affect nitrate sensing within cells.

To find out if internal nitrate content would affect nitrate sensing, the level of nitrate content was measured in plants under the condition for primary nitrate response. Two hours after the nitrate treatment, no matter under high-affinity or low-affinity condition, the nitrate content in roots and shoots in *clca/clcb* was significantly decreased (Figure 28 and 29), and it was most severe in roots under low affinity, in which the nitrate content was only one-third of the wild type level (Figure 29A). It was interesting that the nitrate content in wild type had two phases, in roots under high-affinity condition, showing different slope before and after 60 minutes (Figure 28A), and it was different before and

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after 45 minutes under low-affinity condition in roots (Figure 29A). The reason for two-phase accumulation pattern could be that the supply of nitrate was first accumulated and assimilated in the cytosol, and after the first phase, the plants would start to accumulate nitrate into the vacuole. Therefore, the major defect of *clc* mutant showed up in the second phase when the nitrate started to be accumulated in the vacuole. In shoots, the slope of nitrate content was linear under both high- and low-affinity condition within two hours (Figure 28B and 29B), and this might due to the fact that it took more time to transport nitrate to the shoots, so the nitrate transported to the shoots was still under assimilation instead of being stored, so the turning point of nitrate content was delayed and could not be observed within two hours.

As for the relative expression of marker genes in shoots, although there was no dramatic difference between wild type and double mutant under both high- and low-affinity, the marker genes all peaked between 35 to 60 minutes under high-affinity condition (Figure 31), but under low-affinity condition, only *PHOSPHOGLYCERATE MUTASE* peaked at 35 minutes, and the relative expression of other marker genes kept at high level and did not drop to initial level (Figure 33). The primary nitrate response in shoots has not been well documented, and this study provides a view of it that under high-

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affinity nitrate condition, the examined marker genes would show a peak like it in roots, and under low-affinity nitrate condition, the expression level of marker genes might keep at maximum level up to 2 hours.

In *clca/clcb*, the fold changes of *CHL1* were higher under both high- and low-affinity conditions (Figure 30 and 32), which means the nitrate treatment induced a higher degree of changes of *CHL1* expression in the double mutant. Nonetheless, when the relative expression of marker genes was normalized to the 0 time point of wild type, the relative expression levels in *clca/clcb* were overall lower compared to wild type, and this might suggest that the initial mRNA level before nitrate treatment was already different between wild type and double mutant.

As mentioned above, the nitrate was started to be accumulated in roots after 45 minutes under low-affinity condition (Figure 28A) and after 60 minutes under high-affinity condition (Figure 27A), which means the defect of nitrate storage in *clc* mutants would only show up after one hour; therefore, the internal nitrate level might not have impact on nitrate sensing in short-term primary nitrate response, and it would be interesting to investigate the pattern of *clc* mutant in long-term nitrate response, as the plants already show internal nitrate difference.

doi:10.6342/NTU201703166

5. Reference

Alboresi, A., Gestin, C., Leydecker, M.T., Bedu, M., Meyer, C., and Truong, H.N. (2005). Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant Cell Environ. **28,** 500-512.

Andersen, T.G., Nour-Eldin, H.H., Fuller, V.L., Olsen, C.E., Burow, M., and Halkier, B.A. (2013). Integration of biosynthesis and long-distance transport establish organ-specific glucosinolate profiles in vegetative Arabidopsis. Plant Cell **25,** 3133-3145.

Bai, H., Euring, D., Volmer, K., Janz, D., and Polle, A. (2013). The nitrate transporter (NRT) gene family in poplar. PLoS One **8,** e72126.

Chiang, C.S., Stacey, G., and Tsay, Y.F. (2004). Mechanisms and functional properties of two peptide transporters, AtPTR2 and fPTR2. J. Biol. Chem. **279,** 30150-30157.

Chiba, Y., Shimizu, T., Miyakawa, S., Kanno, Y., Koshiba, T., Kamiya, Y., and Seo, M. (2015). Identification of Arabidopsis thaliana NRT1/PTR FAMILY (NPF) proteins capable of transporting plant hormones. J. Plant Res. **128,** 679-686.

Chiu, C.C., Lin, C.S., Hsia, A.P., Su, R.C., Lin, H.L., and Tsay, Y.F. (2004). Mutation of a nitrate transporter, AtNRT1:4, results in a reduced petiole nitrate content and

doi:10.6342/NTU201703166

altered leaf development. Plant Cell Physiol. **45,** 1139-1148.

Cookson, S.J., Williams, L.E., and Miller, A.J. (2005). Light-dark changes in cytosolic nitrate pools depend on nitrate reductase activity in Arabidopsis leaf cells. Plant Physiol. **138,** 1097-1105.

Corratge-Faillie, C., and Lacombe, B. (2017). Substrate (un)specificity of Arabidopsis NRT1/PTR FAMILY (NPF) proteins. J. Exp. Bot.

Crawford, N.M. (1995). Nitrate - Nutrient and Signal for Plant-Growth. Plant Cell **7,** 859-868.

Crawford, N.M., and Glass, A.D.M. (1998). Molecular and physiological aspects of nitrate uptake in plants. Trends Plant Sci. **3,** 389-395.

Dechorgnat, J., Nguyen, C.T., Armengaud, P., Jossier, M., Diatloff, E., Filleur, S., and Daniel-Vedele, F. (2011). From the soil to the seeds: the long journey of nitrate in plants. J. Exp. Bot. **62,** 1349-1359.

Dietrich, D., Hammes, U., Thor, K., Suter-Grotemeyer, M., Fluckiger, R., Slusarenko, A.J., Ward, J.M., and Rentsch, D. (2004). AtPTR1, a plasma membrane peptide transporter expressed during seed germination and in vascular tissue of Arabidopsis. Plant J. **40,** 488-499.

doi:10.6342/NTU201703166

Ehlting, B., Dluzniewska, P., Dietrich, H., Selle, A., Teuber, M., Hansch, R., Nehls, U., Polle, A., Schnitzler, J.P., Rennenberg, H., and Gessler, A. (2007). Interaction of nitrogen nutrition and salinity in Grey poplar (Populus tremula x alba). Plant Cell Environ. **30,** 796-811.

Fan, S.C., Lin, C.S., Hsu, P.K., Lin, S.H., and Tsay, Y.F. (2009). The Arabidopsis nitrate transporter NRT1.7, expressed in phloem, is responsible for source-to-sink remobilization of nitrate. Plant Cell **21,** 2750-2761.

Faustino, L.I., Moretti, A.P., and Graciano, C. (2015). Fertilization with urea, ammonium and nitrate produce different effects on growth, hydraulic traits and drought tolerance in Pinus taeda seedlings. Tree Physiol. **35,** 1062-1074.

Forde, B.G. (2000). Nitrate transporters in plants: structure, function and regulation. Bba-Biomembranes **1465,** 219-235.

Forde, B.G. (2002). Local and long-range signaling pathways regulating plant responses to nitrate. Annu. Rev. Plant. Biol. **53,** 203-224.

Frommer, W.B., Hummel, S., and Rentsch, D. (1994). Cloning of an Arabidopsis histidine transporting protein related to nitrate and peptide transporters. FEBS Lett. **347,** 185-189.

doi:10.6342/NTU201703166

Geelen, D., Lurin, C., Bouchez, D., Frachisse, J.M., Lelievre, F., Courtial, B., Barbier-Brygoo, H., and Maurel, C. (2000). Disruption of putative anion channel gene AtCLC-a in Arabidopsis suggests a role in the regulation of nitrate content. Plant J. **21,** 259-267.

Glass, A.D.M., Britto, D.T., Kaiser, B.N., Kronzucker, H.J., Kumar, A., Okamoto, M., Rawat, S.R., Siddiqi, M.Y., Silim, S.M., Vidmar, J.J., and Zhuo, D. (2001). Nitrogen transport in plants, with an emphasis on the regulation of fluxes to match plant demand. J. Plant Nutr. Soil Sc. **164,** 199-207.

Gowri, G., Kenis, J.D., Ingemarsson, B., Redinbaugh, M.G., and Campbell, W.H. (1992). Nitrate Reductase Transcript Is Expressed in the Primary Response of Maize to Environmental Nitrate. Plant Mol. Biol. **18,** 55-64.

Hammes, U.Z., Meier, S., Dietrich, D., Ward, J.M., and Rentsch, D. (2010). Functional Properties of the Arabidopsis Peptide Transporters AtPTR1 and AtPTR5. J. Biol. Chem. **285,** 39710-39717.

Harada, H., Kuromori, T., Hirayama, T., Shinozaki, K., and Leigh, R.A. (2004). Quantitative trait loci analysis of nitrate storage in Arabidopsis leading to an investigation of the contribution of the anion channel gene, AtCLC-c, to variation

doi:10.6342/NTU201703166

in nitrate levels. J. Exp. Bot. **55,** 2005-2014.

Ho, C.H., Lin, S.H., Hu, H.C., and Tsay, Y.F. (2009). CHL1 Functions as a Nitrate Sensor in Plants. Cell **138,** 1184-1194.

Hsu, P.K., and Tsay, Y.F. (2013). Two phloem nitrate transporters, NRT1.11 and NRT1.12, are important for redistributing xylem-borne nitrate to enhance plant growth. Plant Physiol. **163,** 844-856.

Hu, H.C., Wang, Y.Y., and Tsay, Y.F. (2009). AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. Plant J. **57,** 264-278.

Huang, N.C., Liu, K.H., Lo, H.J., and Tsay, Y.F. (1999). Cloning and functional characterization of an Arabidopsis nitrate transporter gene that encodes a constitutive component of low-affinity uptake. Plant Cell **11,** 1381-1392.

Ishimaru, Y., Oikawa, T., Suzuki, T., Takeishi, S., Matsuura, H., Takahashi, K., Hamamoto, S., Uozumi, N., Shimizu, T., Seo, M., Ohta, H., and Ueda, M. (2017). GTR1 is a jasmonic acid and jasmonoyl-L-isoleucine transporter in Arabidopsis thaliana. Biosci. Biotech. Bioch. **81,** 249-255.

Kotur, Z., Mackenzie, N., Ramesh, S., Tyerman, S.D., Kaiser, B.N., and Glass, A.D.

doi:10.6342/NTU201703166

(2012). Nitrate transport capacity of the Arabidopsis thaliana NRT2 family members and their interactions with AtNAR2.1. New Phytol. **194,** 724-731.

Lejay, L., Wirth, J., Pervent, M., Cross, J.M.F., Tillard, P., and Gojon, A. (2008). Oxidative pentose phosphate pathway-dependent sugar sensing as a mechanism for regulation of root ion transporters by photosynthesis. Plant Physiol. **146,** 2036-2053.

Lejay, L., Tillard, P., Lepetit, M., Olive, F.D., Filleur, S., Daniel-Vedele, F., and Gojon, A. (1999). Molecular and functional regulation of two NO3- uptake systems by N- and C-status of Arabidopsis plants. Plant J. **18,** 509-519.

Leran, S., Varala, K., Boyer, J.C., Chiurazzi, M., Crawford, N., Daniel-Vedele, F., David, L., Dickstein, R., Fernandez, E., Forde, B., Gassmann, W., Geiger, D., Gojon, A., Gong, J.M., Halkier, B.A., Harris, J.M., Hedrich, R., Limami, A.M., Rentsch, D., Seo, M., Tsay, Y.F., Zhang, M.Y., Coruzzi, G., and Lacombe, B. (2014). A unified nomenclature of NITRATE TRANSPORTER 1/PEPTIDE TRANSPORTER family members in plants. Trends Plant Sci. **19,** 5-9.

Li, J.Y., Fu, Y.L., Pike, S.M., Bao, J., Tian, W., Zhang, Y., Chen, C.Z., Zhang, Y.,

doi:10.6342/NTU201703166

Li, H.M., Huang, J., Li, L.G., Schroeder, J.I., Gassmann, W., and Gong, J.M. (2010). The Arabidopsis nitrate transporter NRT1.8 functions in nitrate removal from the xylem sap and mediates cadmium tolerance. Plant Cell **22,** 1633-1646.

Lin, S.H., Kuo, H.F., Canivenc, G., Lin, C.S., Lepetit, M., Hsu, P.K., Tillard, P., Lin, H.L., Wang, Y.Y., Tsai, C.B., Gojon, A., and Tsay, Y.F. (2008). Mutation of the Arabidopsis NRT1.5 Nitrate Transporter Causes Defective Root-to-Shoot Nitrate Transport. Plant Cell **20,** 2514-2528.

Lin, Y.L., and Tsay, Y.F. (2017). Influence of differing nitrate and nitrogen availability on flowering control in Arabidopsis. J. Exp. Bot.

Little, D.Y., Rao, H.Y., Oliva, S., Daniel-Vedele, F., Krapp, A., and Malamy, J.E. (2005). The putative high-affinity nitrate transporter NRT2.1 represses lateral root initiation in response to nutritional cues. Proc. Natl. Acad. Sci. U.S.A. **102,** 13693-13698.

Liu, K.H., Huang, C.Y., and Tsay, Y.F. (1999). CHL1 is a dual-affinity nitrate transporter of Arabidopsis involved in multiple phases of nitrate uptake. Plant Cell **11,** 865-874.

Lv, Q.D., Tang, R.J., Liu, H., Gao, X.S., Li, Y.Z., Zheng, H.Q., and Zhang, H.X.

doi:10.6342/NTU201703166

(2009). Cloning and molecular analyses of the Arabidopsis thaliana chloride channel gene family. Plant Sci. **176,** 650-661.

Martinoia, E., Massonneau, A., and Frangne, N. (2000). Transport processes of solutes across the vacuolar membrane of higher plants. Plant Cell Physiol. **41,** 1175-1186.

Min, X.J., Siddiqi, M.Y., Guy, R.D., Glass, A.D.M., and Kronzucker, H.J. (2000). A comparative kinetic analysis of nitrate and ammonium influx in two early-successional tree species of temperate and boreal forest ecosystems. Plant Cell Environ. **23,** 321-328.

Miyawaki, K., Matsumoto-Kitano, M., and Kakimoto, T. (2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. Plant J. **37,** 128-138.

Nour-Eldin, H.H., Andersen, T.G., Burow, M., Madsen, S.R., Jorgensen, M.E., Olsen, C.E., Dreyer, I., Hedrich, R., Geiger, D., and Halkier, B.A. (2012). NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. Nature **488,** 531-534.

Omena-Garcia, R.P., Justino, G.C., de Araujo, V.B.F., de Souza, L.A.G., de Camargos, L.S., and Goncalves, J.F.D. (2015). Mineral Nitrogen Associated

doi:10.6342/NTU201703166

Changes in Growth and Xylem-N Compounds in Amazonian Legume Tree. J. Plant Nutr. **38,** 584-595.

Pike, S., Gao, F., Kim, M.J., Kim, S.H., Schachtman, D.P., and Gassmann, W. (2014). Members of the NPF3 Transporter Subfamily Encode Pathogen-Inducible Nitrate/Nitrite Transporters in Grapevine and Arabidopsis. Plant Cell Physiol. **55,** 162-170.

Qiu, D., Hu, R., Li, Y., and Zhang, M. (2017). Aromatic dipeptide Trp-Ala can be transported by Arabidopsis peptide transporters AtPTR1 and AtPTR5. Channels 1-5.

Redinbaugh, M.G., and Campbell, W.H. (1991). Higher-Plant Responses to Environmental Nitrate. Physiol. Plantarum **82,** 640-650.

Scheible, W.R., GonzalezFontes, A., Lauerer, M., MullerRober, B., Caboche, M., and Stitt, M. (1997). Nitrate acts as a signal to induce organic acid metabolism and repress starch metabolism in tobacco. Plant Cell **9,** 783-798.

Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods **9,** 671-675.

Sugiura, M., Georgescu, M.N., and Takahashi, M. (2007). A nitrite transporter

doi:10.6342/NTU201703166

associated with nitrite uptake by higher plant chloroplasts. Plant Cell Physiol. **48,** 1022-1035.

Tong, W., Imai, A., Tabata, R., Shigenobu, S., Yamaguchi, K., Yamada, M., Hasebe, M., Sawa, S., Motose, H., and Takahashi, T. (2016). Polyamine Resistance Is Increased by Mutations in a Nitrate Transporter Gene NRT1.3 (AtNPF6.4) in Arabidopsis thaliana. Front Plant Sci. **7,** 834.

Tsay, Y.F., Schroeder, J.I., Feldmann, K.A., and Crawford, N.M. (1993). The Herbicide Sensitivity Gene Chl1 of Arabidopsis Encodes a Nitrate-Inducible Nitrate Transporter. Cell **72,** 705-713.

Tsay, Y.F., Chiu, C.C., Tsai, C.B., Ho, C.H., and Hsu, P.K. (2007). Nitrate transporters and peptide transporters. FEBS Lett. **581,** 2290-2300.

von der Fecht-Bartenbach, J., Bogner, M., Dynowski, M., and Ludewig, U. (2010). CLC-b-Mediated NO3-/H+ Exchange Across the Tonoplast of Arabidopsis Vacuoles. Plant Cell Physiol. **51,** 960-968.

Wang, R., Okamoto, M., Xing, X., and Crawford, N.M. (2003). Microarray analysis of the nitrate response in Arabidopsis roots and shoots reveals over 1,000 rapidly responding genes and new linkages to glucose, trehalose-6-phosphate, iron, and

doi:10.6342/NTU201703166

sulfate metabolism. Plant Physiol. **132,** 556-567.

Wang, R.C., Liu, D., and Crawford, N.M. (1998). The Arabidopsis CHL1 protein plays a major role in high-affinity nitrate uptake. Proc. Natl. Acad. Sci. U.S.A. **95,** 15134-15139.

Wang, Y.Y., and Tsay, Y.F. (2011). Arabidopsis Nitrate Transporter NRT1.9 Is Important in Phloem Nitrate Transport. Plant Cell **23,** 1945-1957.

Wang, Y.Y., Hsu, P.K., and Tsay, Y.F. (2012). Uptake, allocation and signaling of nitrate. Trends Plant Sci. **17,** 458-467.

Weichert, A., Brinkmann, C., Komarova, N.Y., Dietrich, D., Thor, K., Meier, S., Suter Grotemeyer, M., and Rentsch, D. (2012). AtPTR4 and AtPTR6 are differentially expressed, tonoplast-localized members of the peptide transporter/nitrate transporter 1 (PTR/NRT1) family. Planta **235,** 311-323.

Williams, L.E., and Miller, A.J. (2001). Transporters responsible for the uptake and partitioning of nitrogenous solutes. Annu. Rev. Plant Phys. **52,** 659-+.

Zhang, C.X., Meng, S., Li, Y.M., and Zhao, Z. (2014). Net NH4+ and NO3- fluxes, and expression of NH4+ and NO3- transporter genes in roots of Populus simonii after acclimation to moderate salinity. Trees-Struct. Funct. **28,** 1813-1821.

doi:10.6342/NTU201703166

Zheng, Z.L. (2009). Carbon and nitrogen nutrient balance signaling in plants. Plant Signal Behav. **4,** 584-591.

Zifarelli, G., and Pusch, M. (2010). CLC transport proteins in plants. FEBS Lett. **584,** 2122-2127.

doi:10.6342/NTU201703166

Table 1. 18 *NPF* members selected according to the data from *Arabidopsis* e-FP Browser.

AGI code	NPF names	Published names	Mainly expressed	Expression level in leaf	Highest expression in leaf
At3g47960	AtNPF2.10	GTR1	Leaf	452~876	Old leaf
At1g52190	AtNPF1.2	NRT1.11	Petiole	218~764	Mature leaf
At1g12110	AtNPF6.3	NRT1.1 (CHL1)	Leaf	309~676	Old leaf
At3g21670	AtNPF6.4	NRT1.3	Sepal	153~596	Old leaf
At1g69870	AtNPF2.13	NRT1.7	Sepal	106~506	Old leaf
At1g22570	AtNPF5.15		Leaf	93~460	Old leaf
At2g26690	AtNPF6.2	NRT1.4	Petiole	158~408	Mature leaf
At5g62680	AtNPF2.11	GTR2	Root	115~359	Old leaf
At1g69850	AtNPF4.6	NRT1.2	Leaf	105~340	Old leaf
At2g02040	AtNPF8.3	PTR2	Leaf	157~250	Old leaf
At1g62200	AtNPF8.5	PTR6	Pollen	100~230	Old leaf
At1g68570	AtNPF3.1		Sepal	89~207	Old leaf
At3g16180	AtNPF1.1	NRT1.12	2nd internode	87~191	Mature leaf
At3g53960	AtNPF5.7		Leaf	48~173	Old leaf
At3g54140	AtNPF8.1	PTR1	Root	41~172	Old leaf
At2g40460	AtNPF5.1		Silique	38~120	Old leaf
At1g22540	AtNPF5.10		Root	62~101	Old leaf
At5g13400	AtNPF6.1		Flower	68~100	Old leaf

The gene express level in leaves was retrieved from Arabidopsis e-FP Browser. A cutoff of gene expression level in leaf at 100 was made; therefore, the listed 18 genes were selected.

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Table 2. MPSS of 28 *NPF* members showing expression in leaves.

	Callus	Inflorescence	Leaves	Root	Silique
At1g12110	678	38	246	962	26
At5g62680	14	186	189	535	239
At1g52190	0	192	119	5	194
At1g18880	6	18	66	592	42
At1g32450	0	108	40	2148	8
At1g72140	101	0	40	100	0
At2g02040	89	27	37	50	42
At1g22540	42	10	36	42	12
At3g53960	0	26	29	2	11
At1g72130	0	0	21	20	8
At1g62200	33	30	20	24	0
At2g26690	2	4	19	0	0
At5g14940	6	36	17	17	1
At2g40460	3	74	16	5	34
At5g13400	15	35	16	0	23
At1g22550	39	15	13	44	4
At3g45680	0	29	11	15	68
At4g21680	356	0	10	3	0
At5g01180	2	0	9	0	62
At5g46050	0	2	7	4	0
At1g22570	6	0	6	4	0
At1g68570	0	44	6	0	20
At2g02020	0	0	3	25	2
At3g01350	0	2	3	21	0
At3g45710	0	0	2	70	2
At1g27040	0	19	1	0	0
At1g59740	257	146	1	2	6
At1g72120	90	18	1	12	16

Genes marked with light grey were chosen based on the criterion that the abundances of signature in leaf had to be larger than 40% of that in the most abundant tissue.

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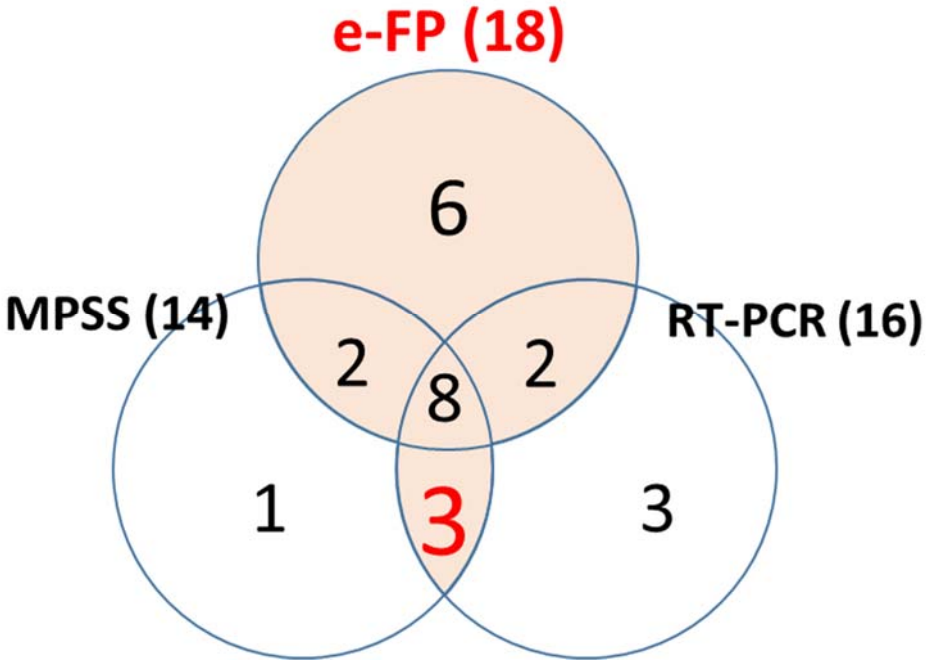

Figure 1. Venn diagram of selected *NPF* genes from three databases.

The numbers following the name of databases were the number of selected genes. All genes chosen based on e-FP Browser and genes in the intersection of MPSS and RT-PCR results were selected to conduct following experiment (marked in red).

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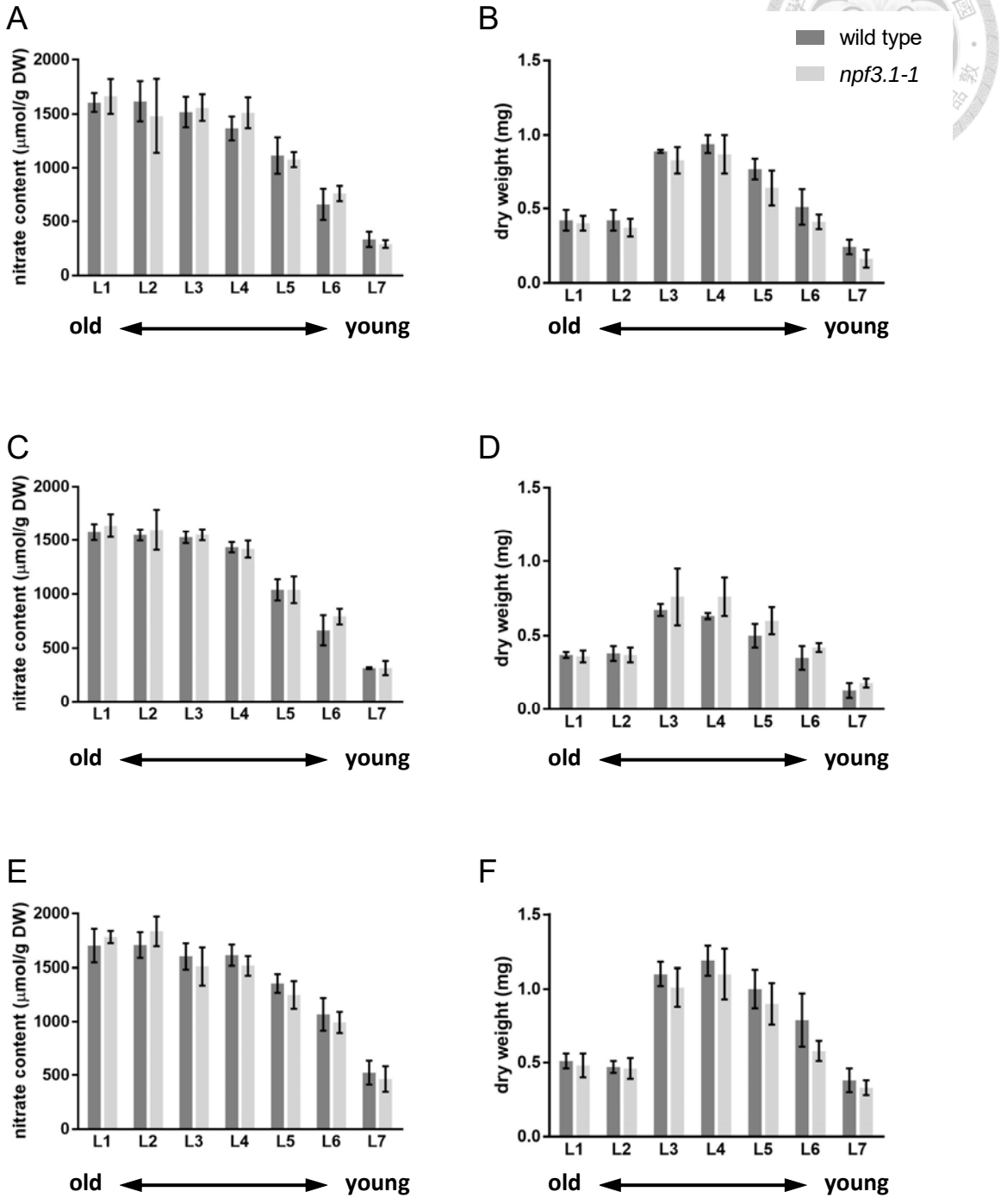

Figure 2. Nitrate content and biomass analysis of *npf3.1-1*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *npf3.1-1* mutant from three independent

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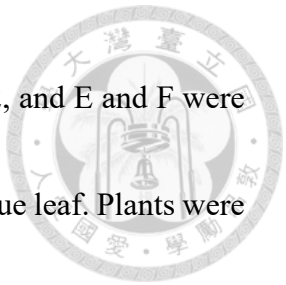

experiments. A and B were from batch 1, C and D were from batch 2, and E and F were from batch 8. L1 to L7 are true leaf numbers, where L1 is the oldest true leaf. Plants were grown hydroponically with 2 mM KNO_3. Each data represents the mean of 3 to 5 different plants and error bar is the standard deviation.

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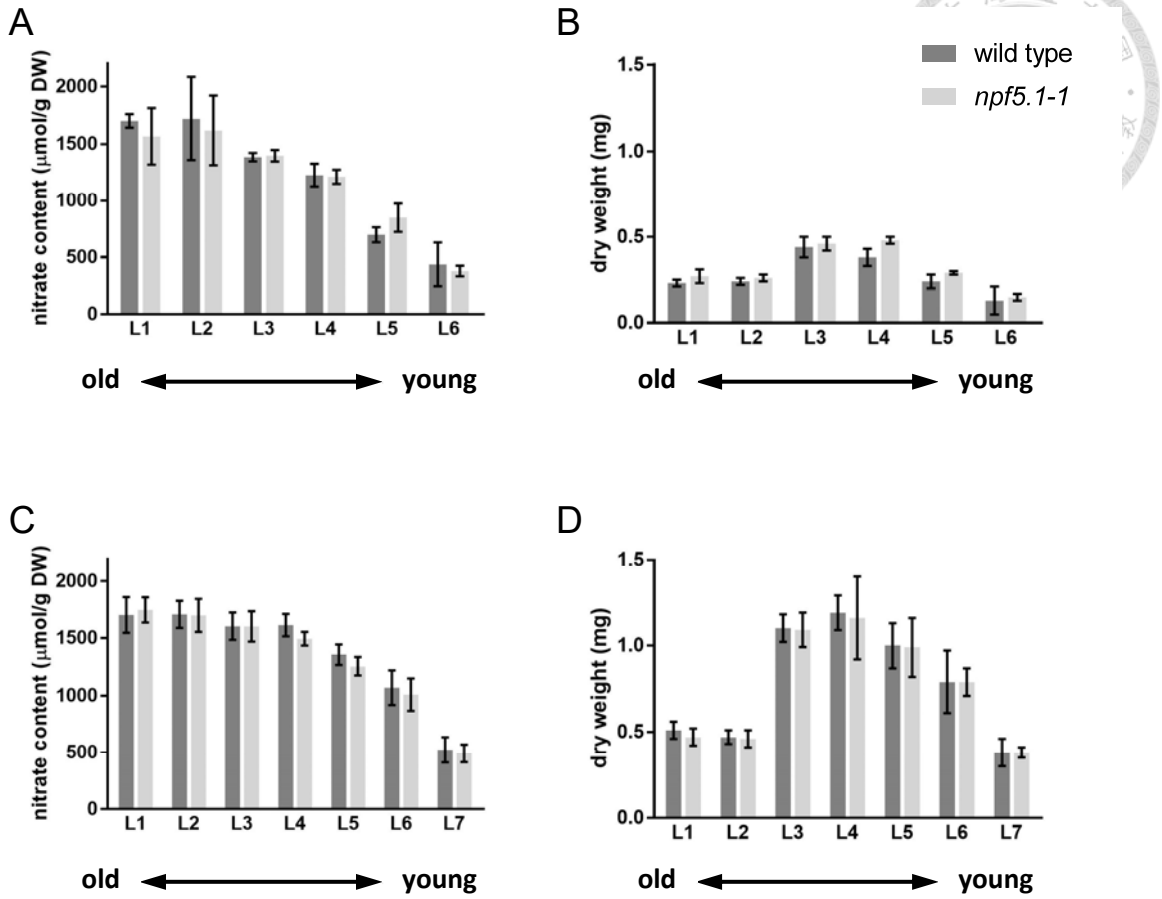

Figure 3. Nitrate content and biomass analysis of *npf5 .1-1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *npf5.1-1* mutant from two independent experiments. A and B were from batch 4, and C and D were from batch 8. Experiment details are described in Figure 1.

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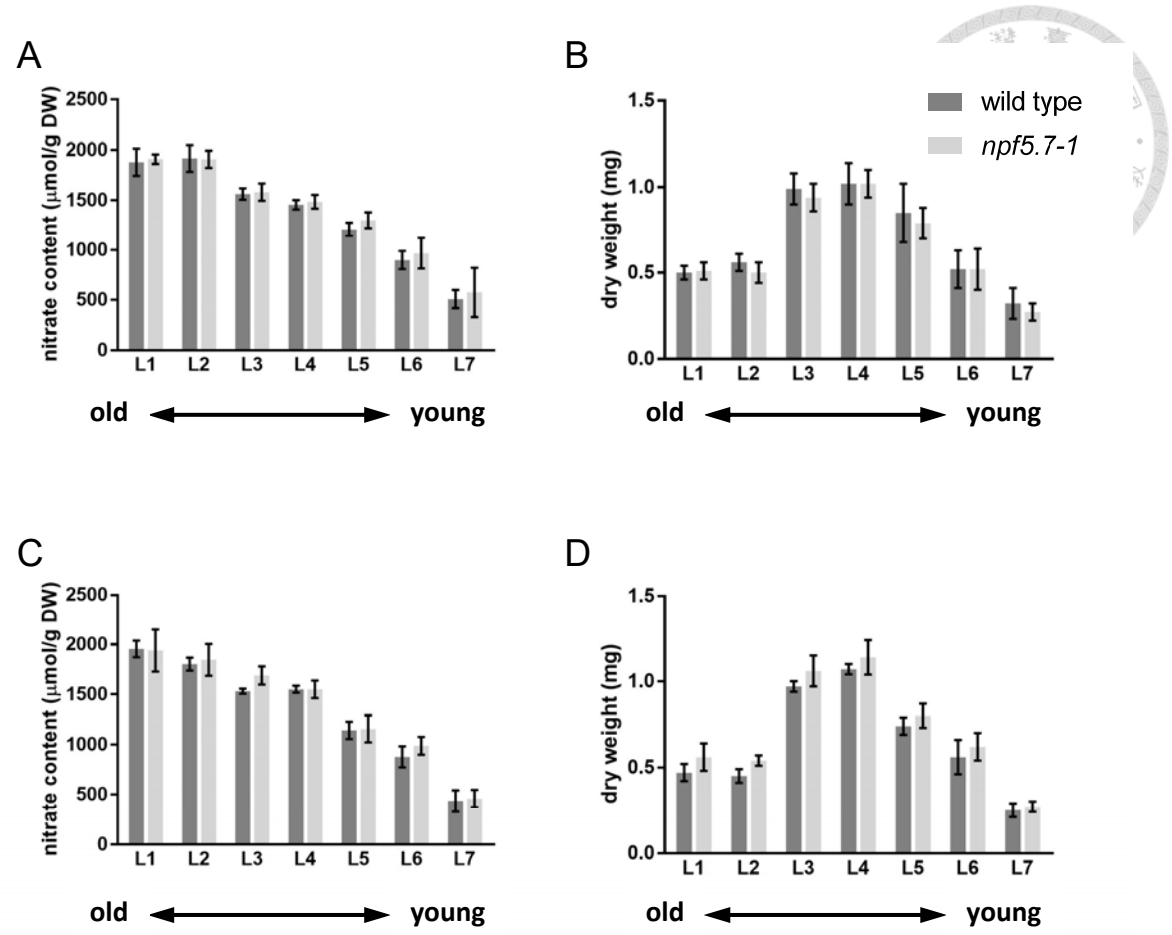

Figure 4. Nitrate content and biomass analysis of *npf5.7-1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *npf5.7-1* mutant from two independent experiments. A and B were from batch 9, and C and D were from batch 10. Experiment details are described in Figure 1.

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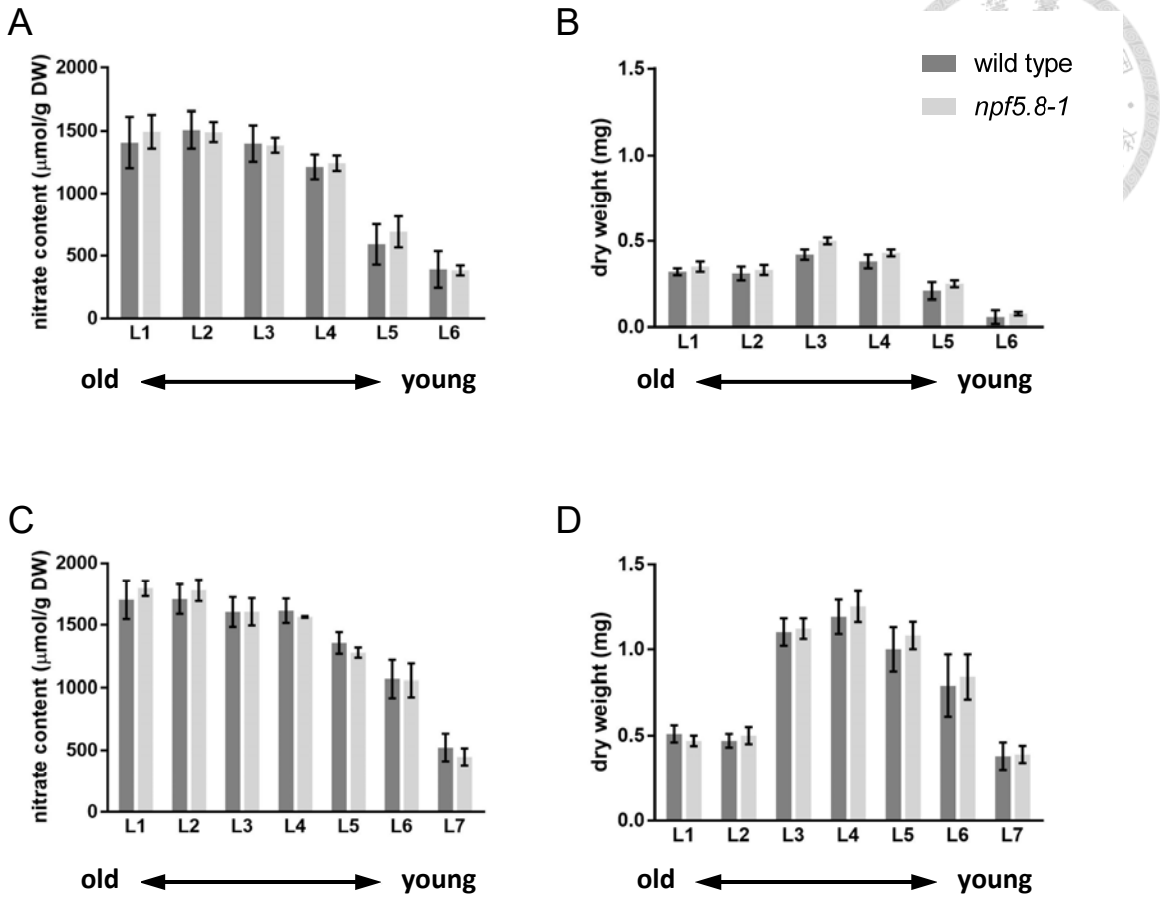

Figure 5. Nitrate content and biomass analysis of *npf5.8-1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *npf5.8-1* mutant from two independent experiments. A and B were from batch 5, and C and D were from batch 8. Experiment details are described in Figure 1.

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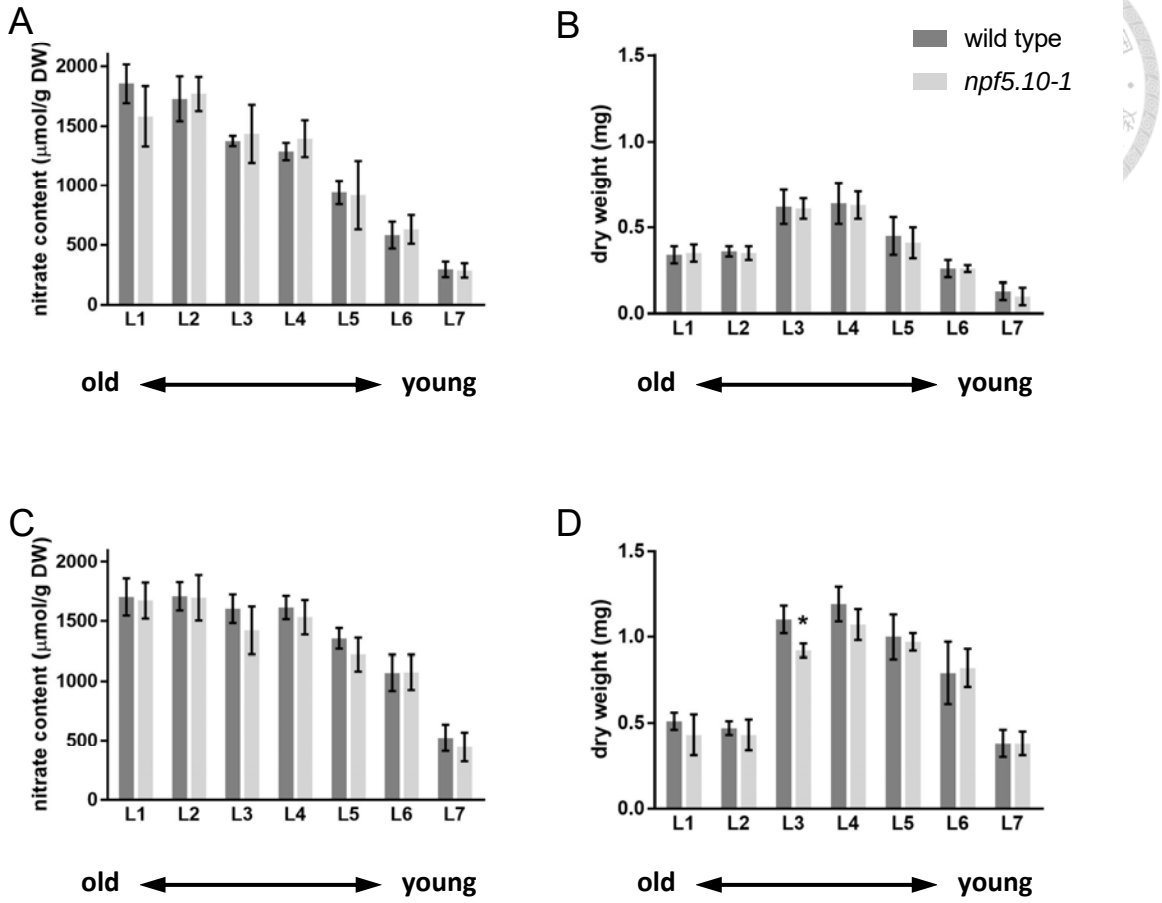

Figure 6. Nitrate content and biomass analysis of *npf5.10-1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *npf5.10-1* mutant from two independent experiments. A and B were from batch 6, and C and D were from batch 8. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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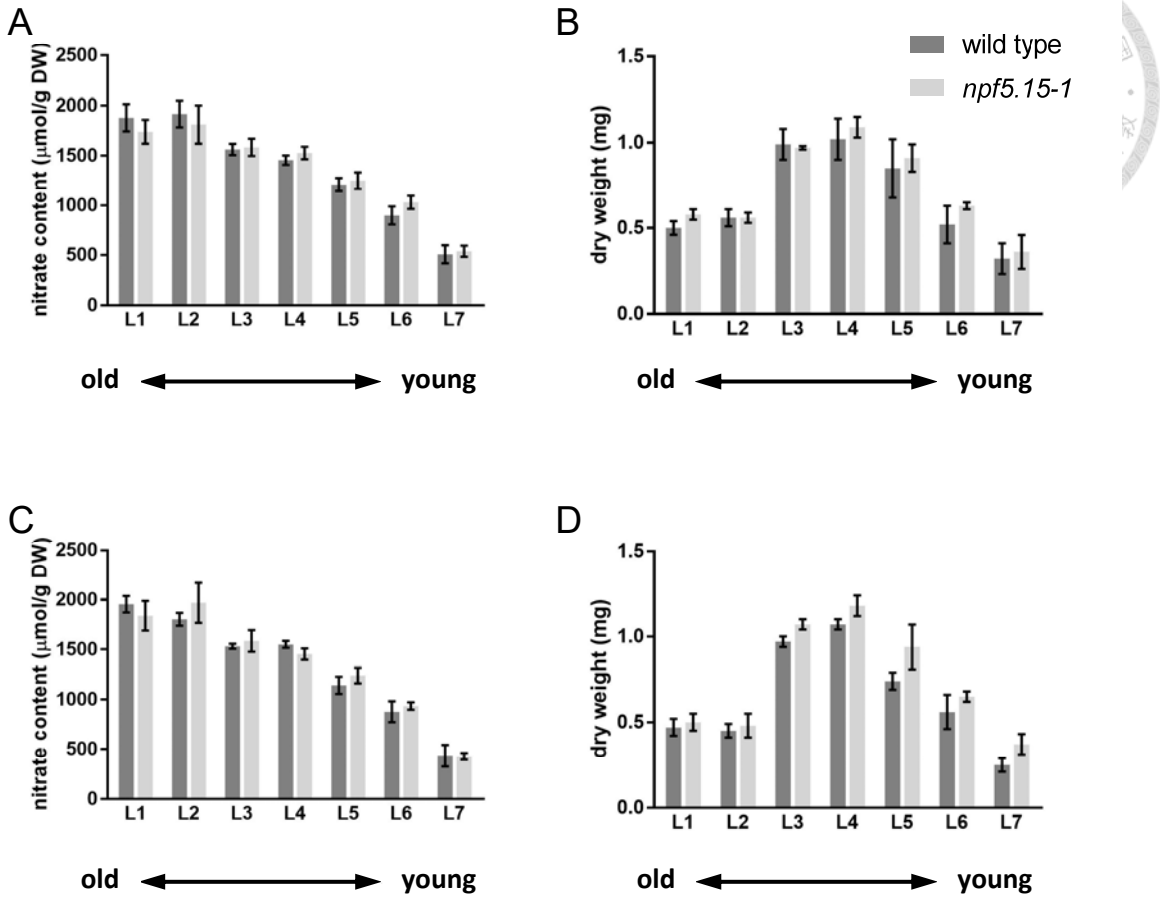

Figure 7. Nitrate content and biomass analysis of *npf5.15-1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *npf5.15-1* mutant from two independent experiments. A and B were from batch 9, and C and D were from batch 10. Experiment details are described in Figure 1.

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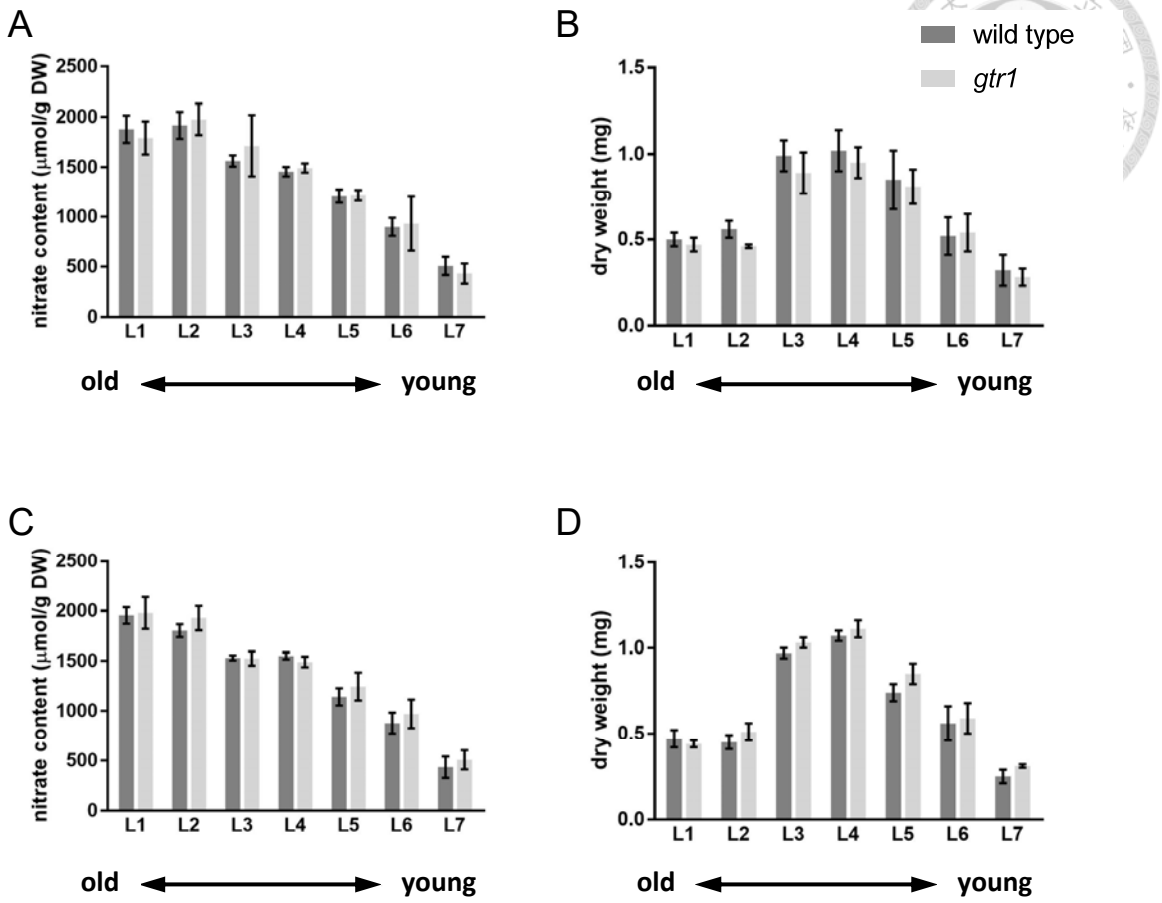

Figure 8. Nitrate content and biomass analysis of *gtr1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *gtr1* mutant from two independent experiments. A and B were from batch 9, and C and D were from batch 10. Experiment details are described in Figure 1.

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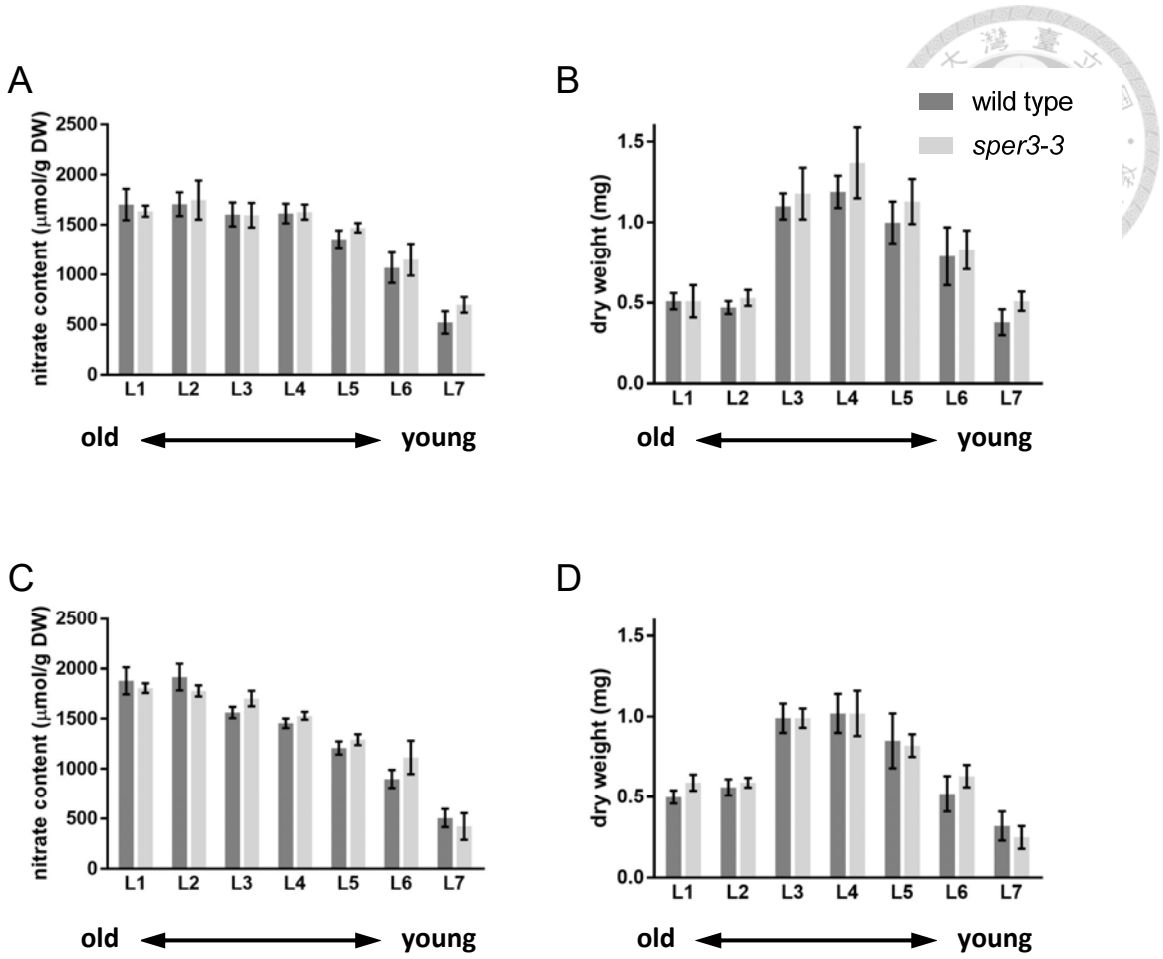

Figure 9. Nitrate content and biomass analysis of *sper3-3*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *sper3-3* mutant from two independent experiments. A and B were from batch 8, and C and D were from batch 9. Experiment details are described in Figure 1.

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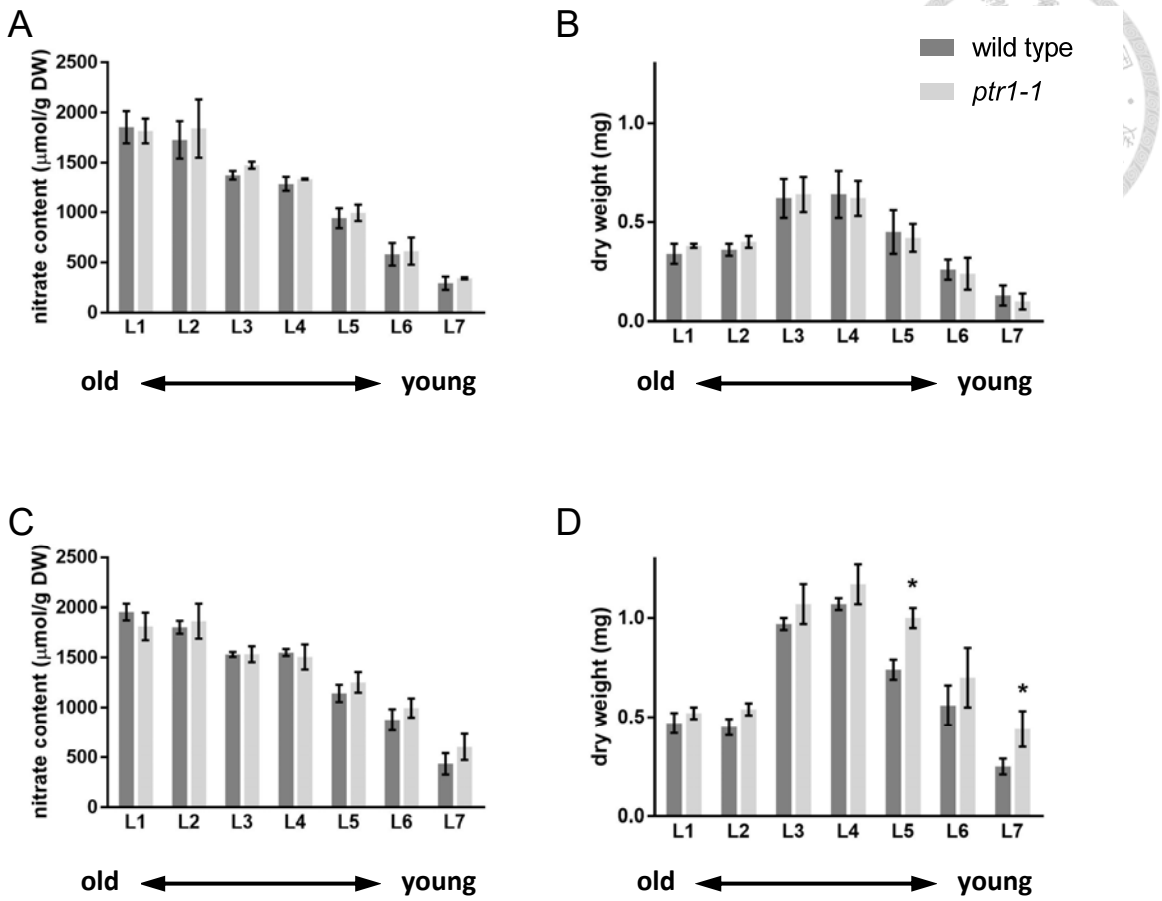

Figure 10. Nitrate content and biomass analysis of *ptr1-1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *ptr1-1* mutant from two independent experiments. A and B were from batch 6, and C and D were from batch 10. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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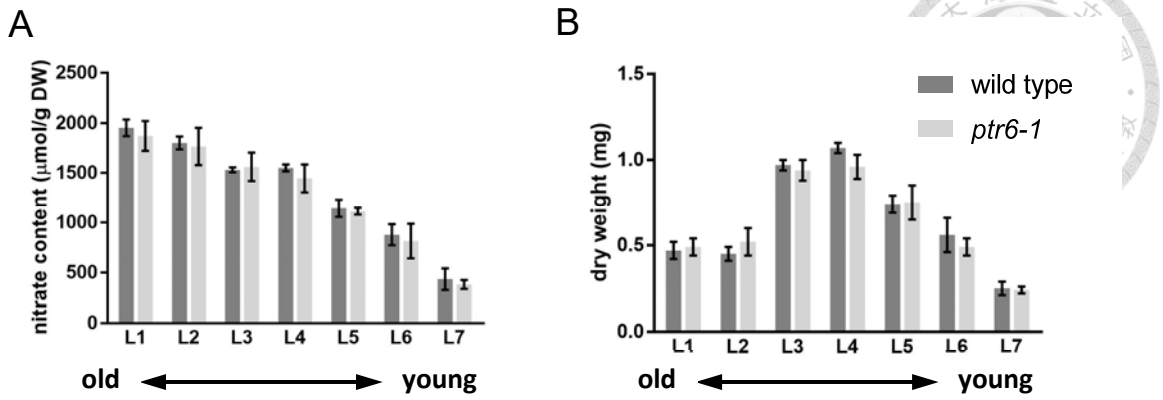

Figure 11. Nitrate content and biomass analysis of *ptr6-1*.

Left panel presented nitrate content (A) and right panel showed corresponding dry weight (B) of wild type and *sper3-3* mutant from batch 10. Experiment details are described in Figure 1.

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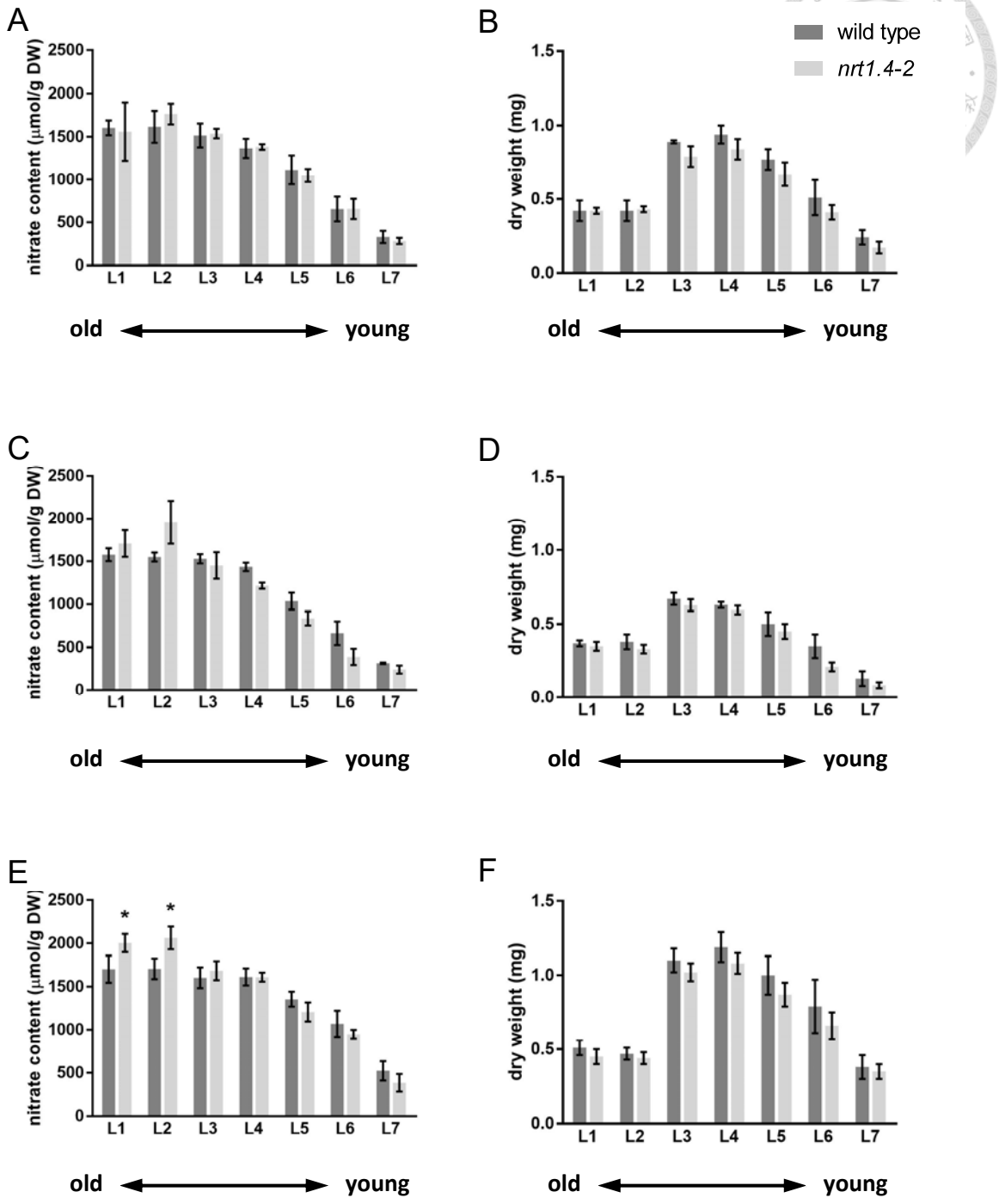

Figure 12. Nitrate content and biomass analysis of *nrt1.4-2*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *nrt1.4-2* mutant from three independent experiments. A and B were from batch 1, C and D were from batch 2, and E and F were

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from batch 8. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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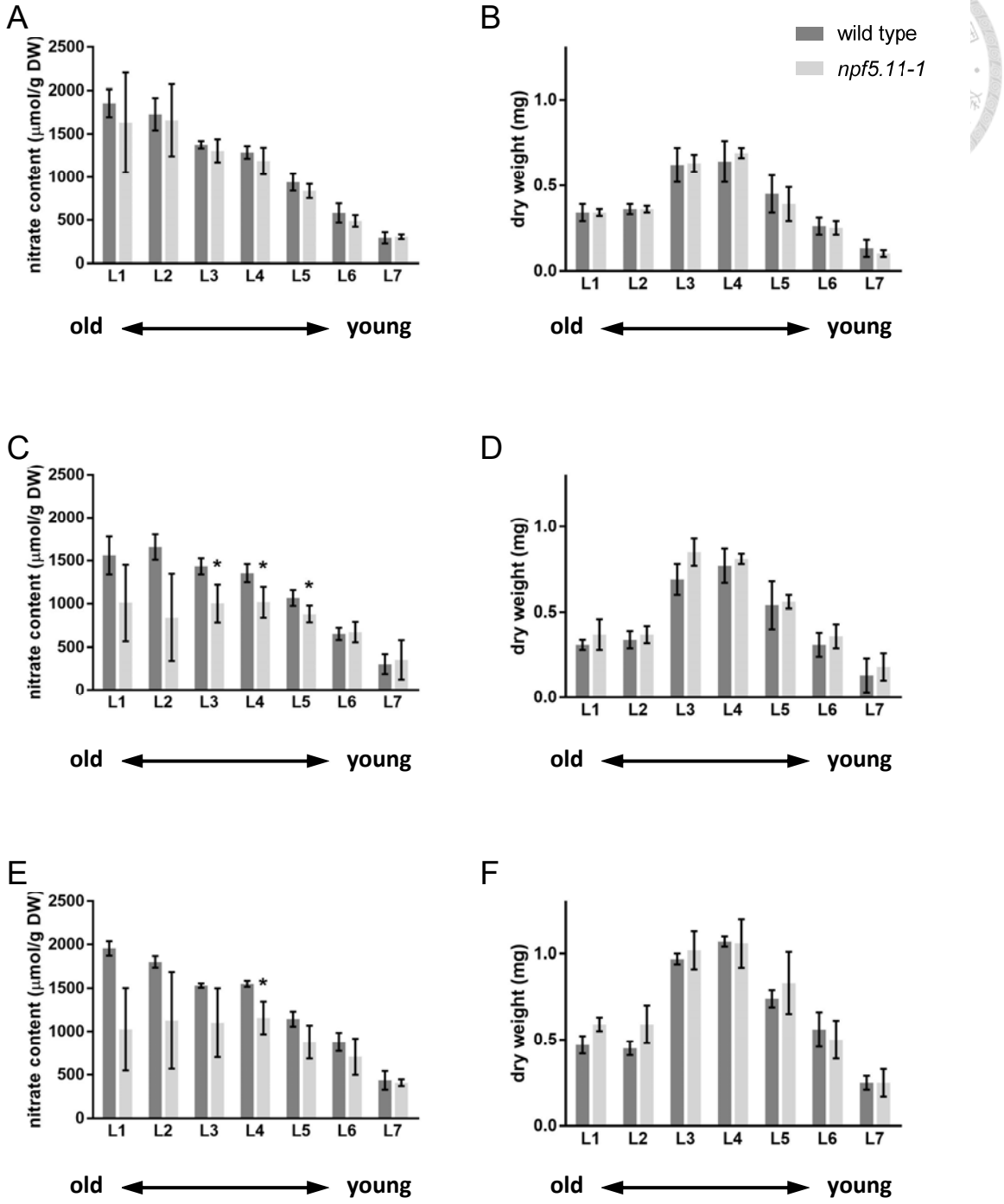

Figure 13. Nitrate content and biomass analysis of *npf5.11-1*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *npf5.11-1* mutant from three independent experiments. A and B were from batch 6, C and D were from batch 7, and E and F were

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from batch 10. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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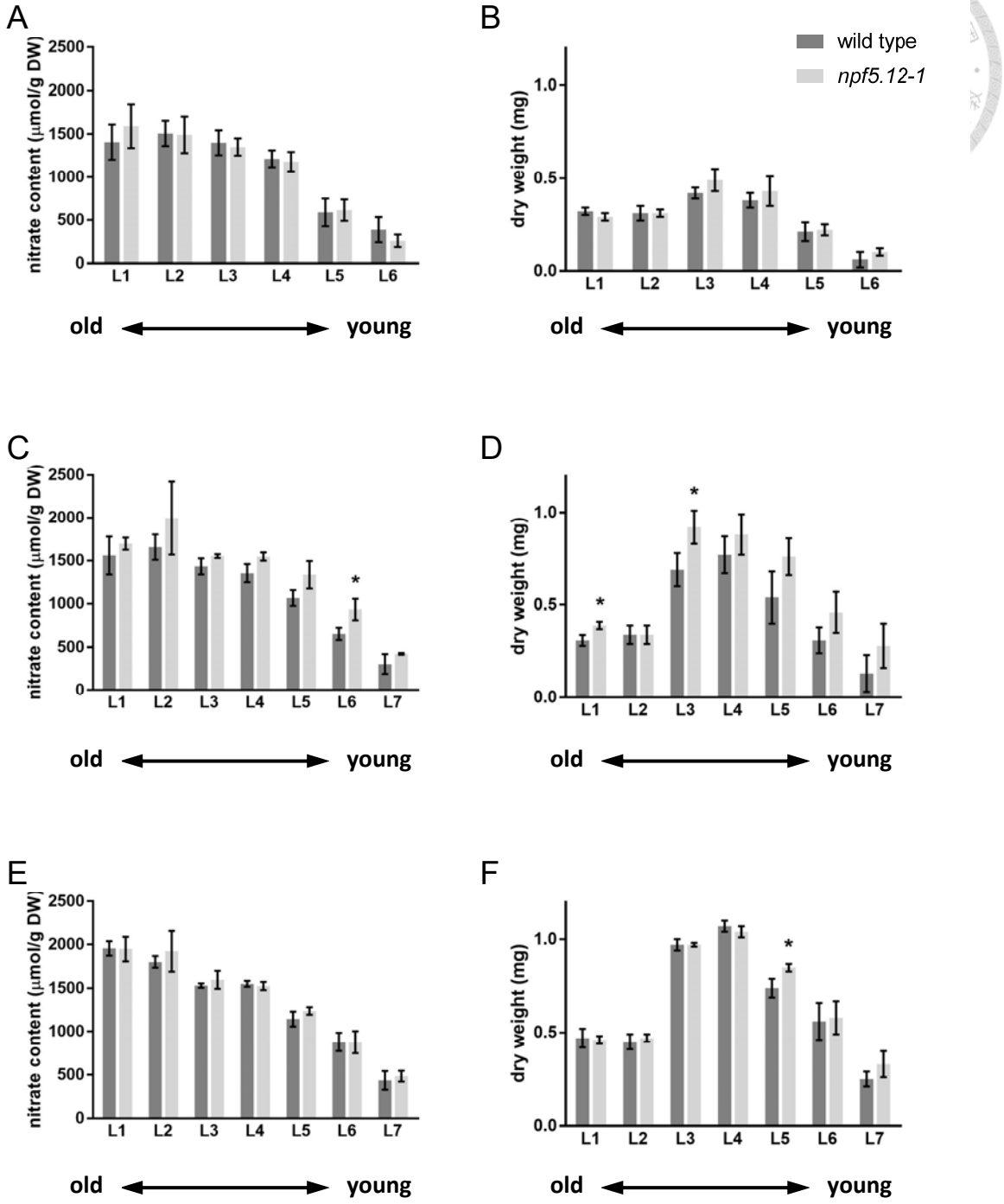

Figure 14. Nitrate content and biomass analysis of *npf5.12-1*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *npf5.12-1* mutant from three independent experiments. A and B were from batch 5, C and D were from batch 7, and E and F were

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from batch 10. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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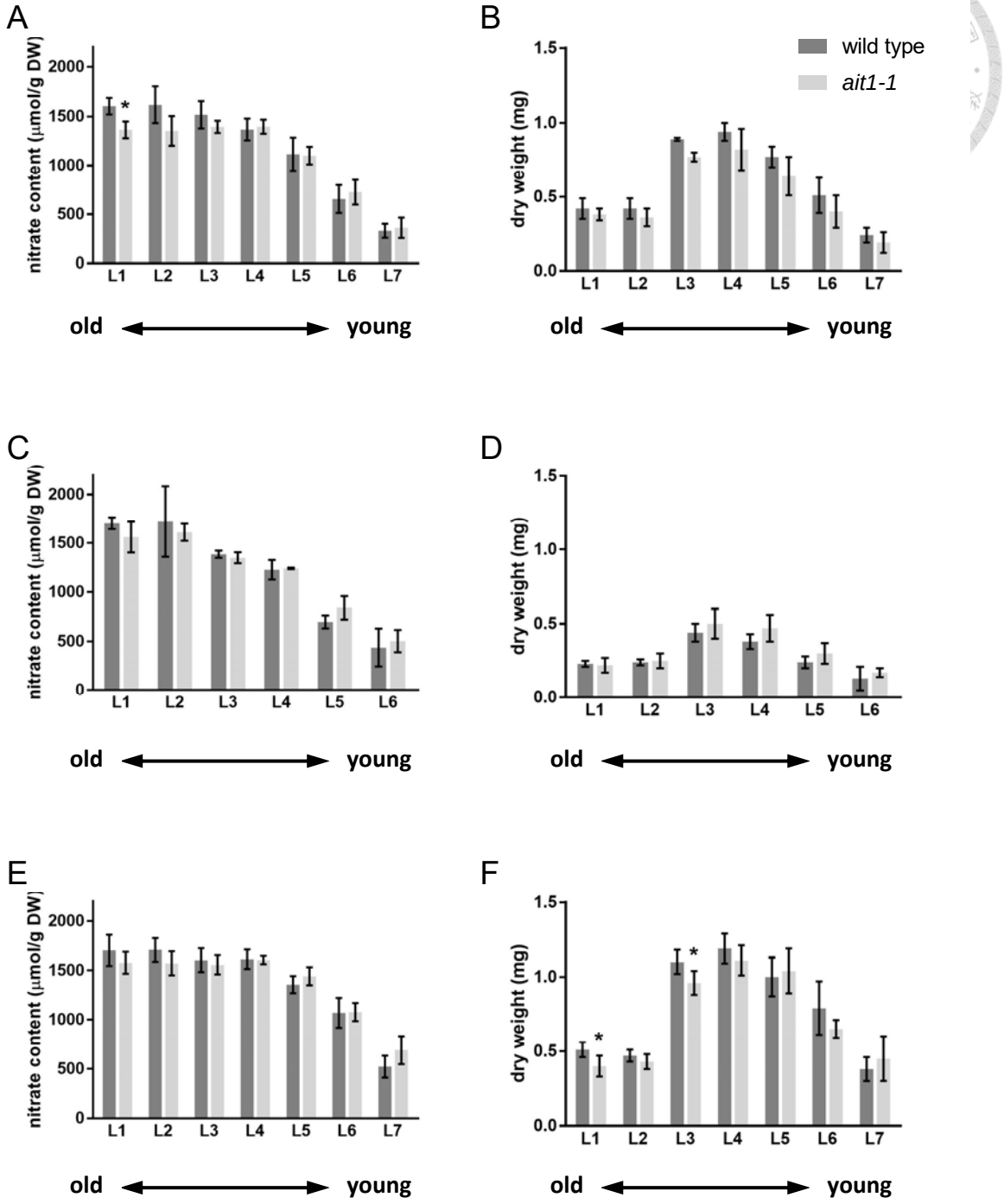

Figure 15. Nitrate content and biomass analysis of *ait1-1*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *ait1-1* mutant from three independent experiments. A and B were from batch 1, C and D were from batch 4, and E and F were

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from batch 8. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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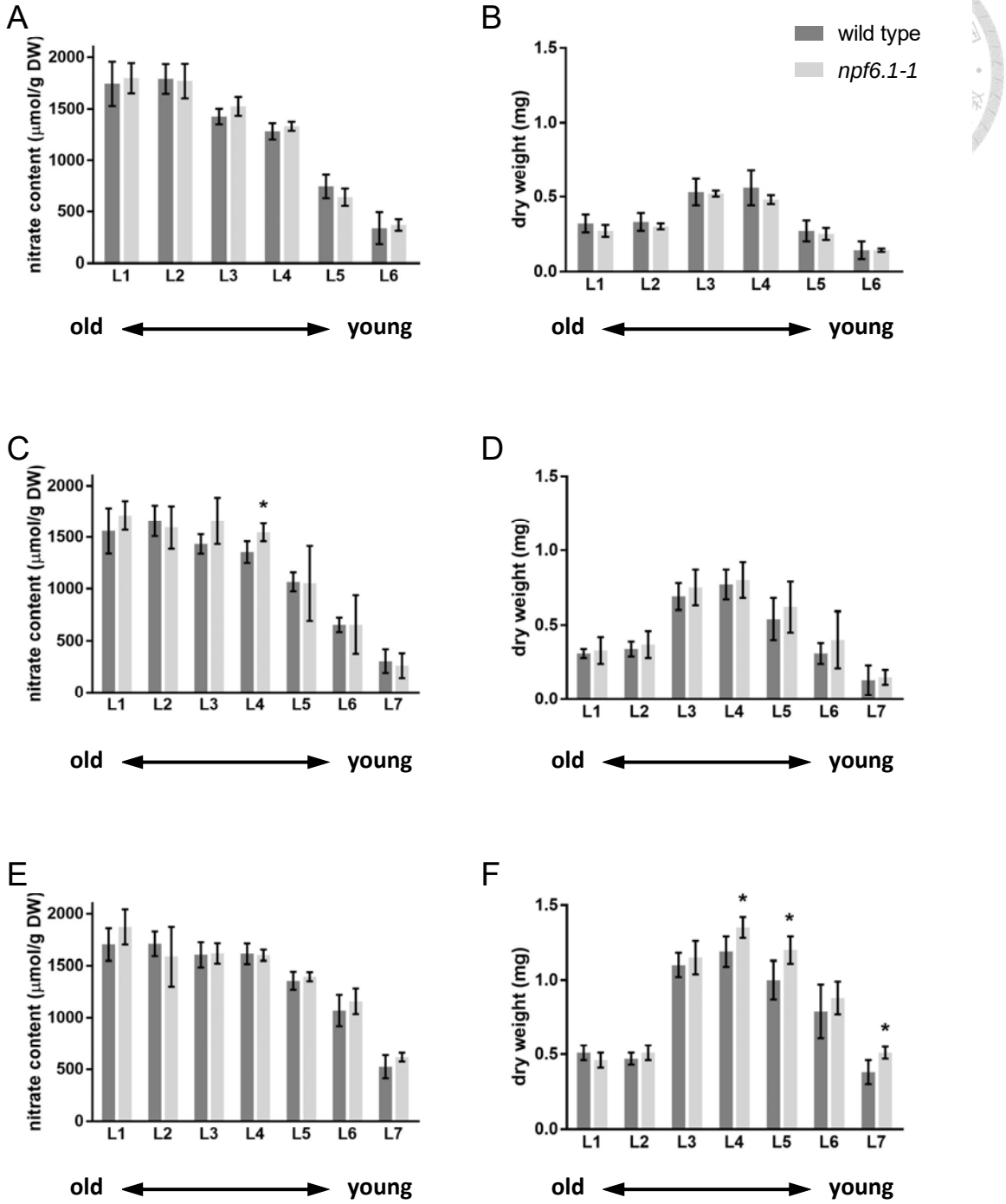

Figure 16. Nitrate content and biomass analysis of *npf6.1-1*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *npf6.1-1* mutant from three independent experiments. A and B were from batch 3, C and D were from batch 7, and E and F were

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from batch 8. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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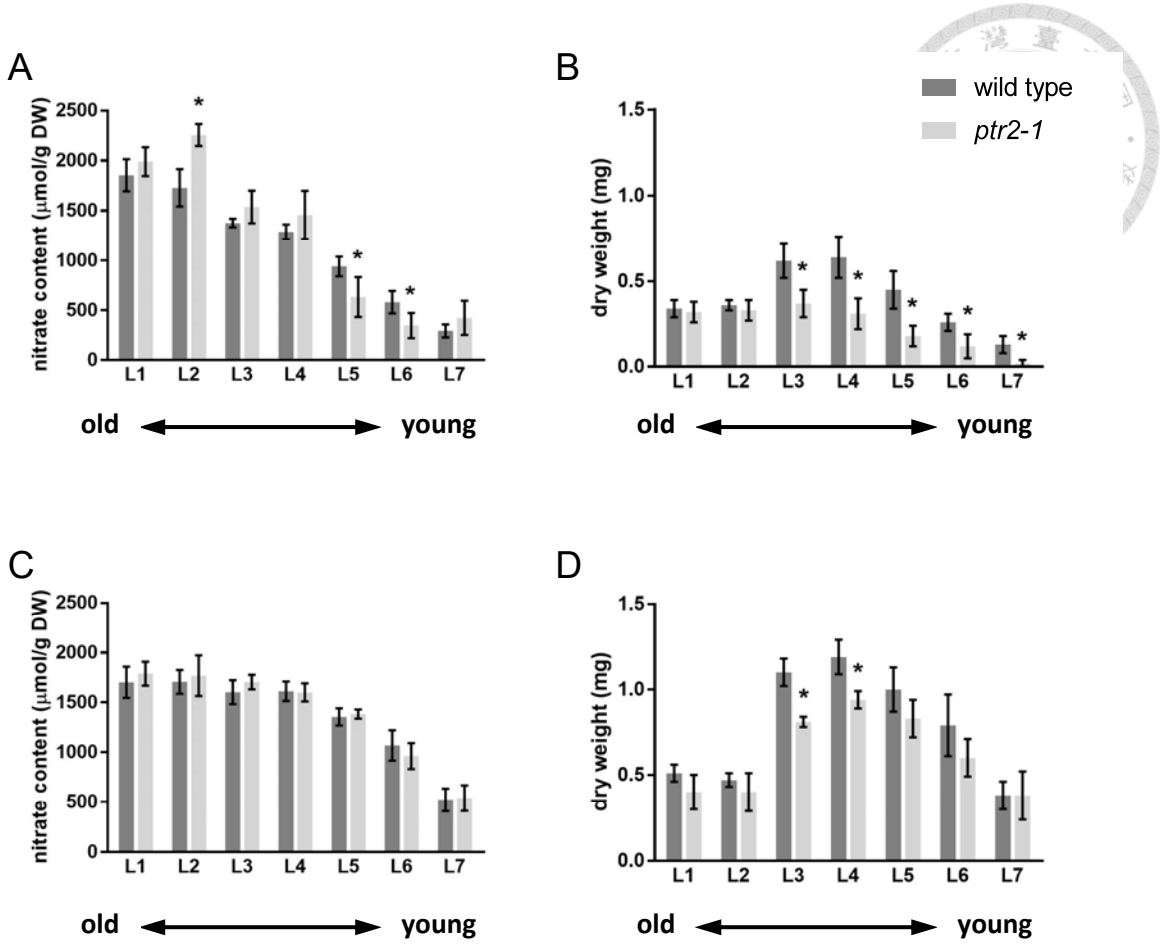

Figure 17. Nitrate content and biomass analysis of *ptr2-1*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *ptr2-1* mutant from two independent experiments. A and B were from batch 6, and C and D were from batch 8. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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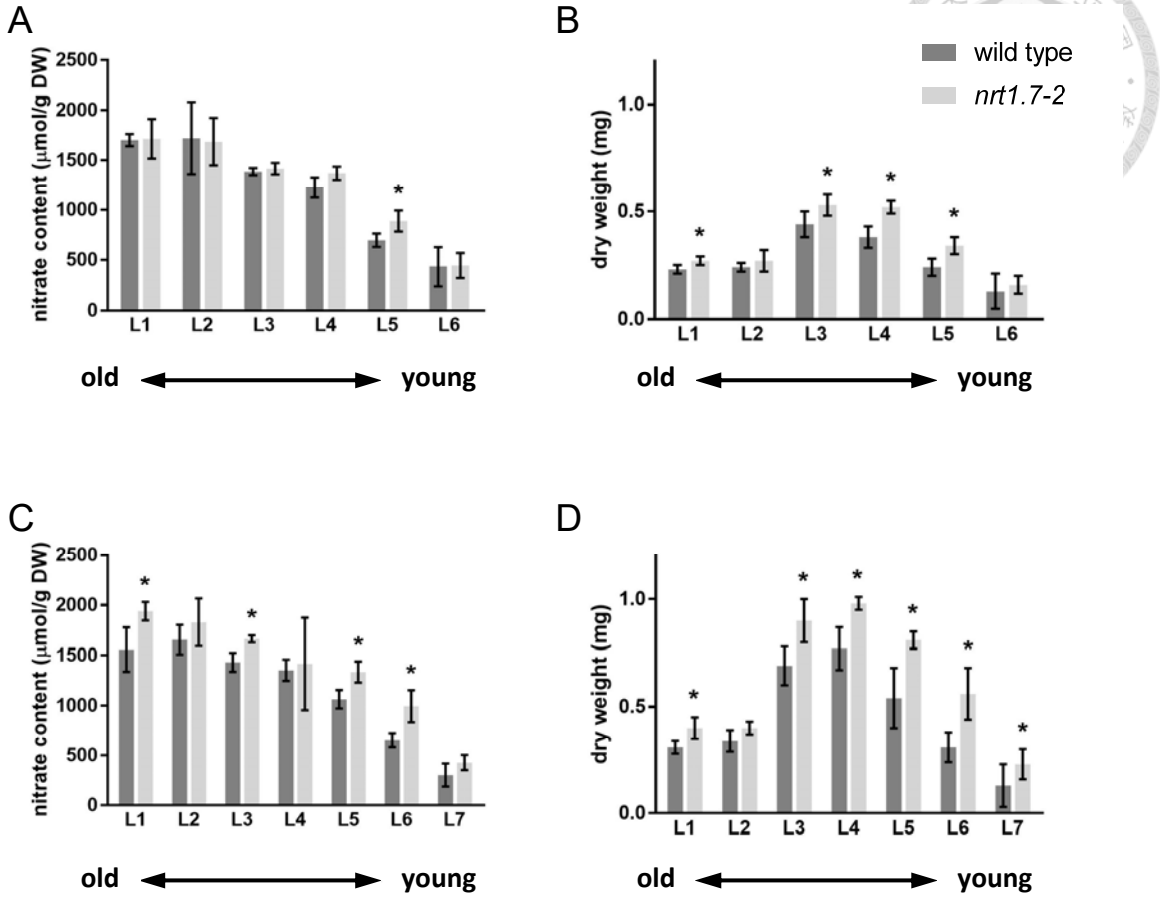

Figure 18. Nitrate content and biomass analysis of *nrt1.7-2*.

Left panels presented nitrate content (A and C) and right panels showed corresponding dry weight (B and D) of wild type and *nrt1.7-2* mutant from two independent experiments. A and B were from batch 4, and C and D were from batch 7. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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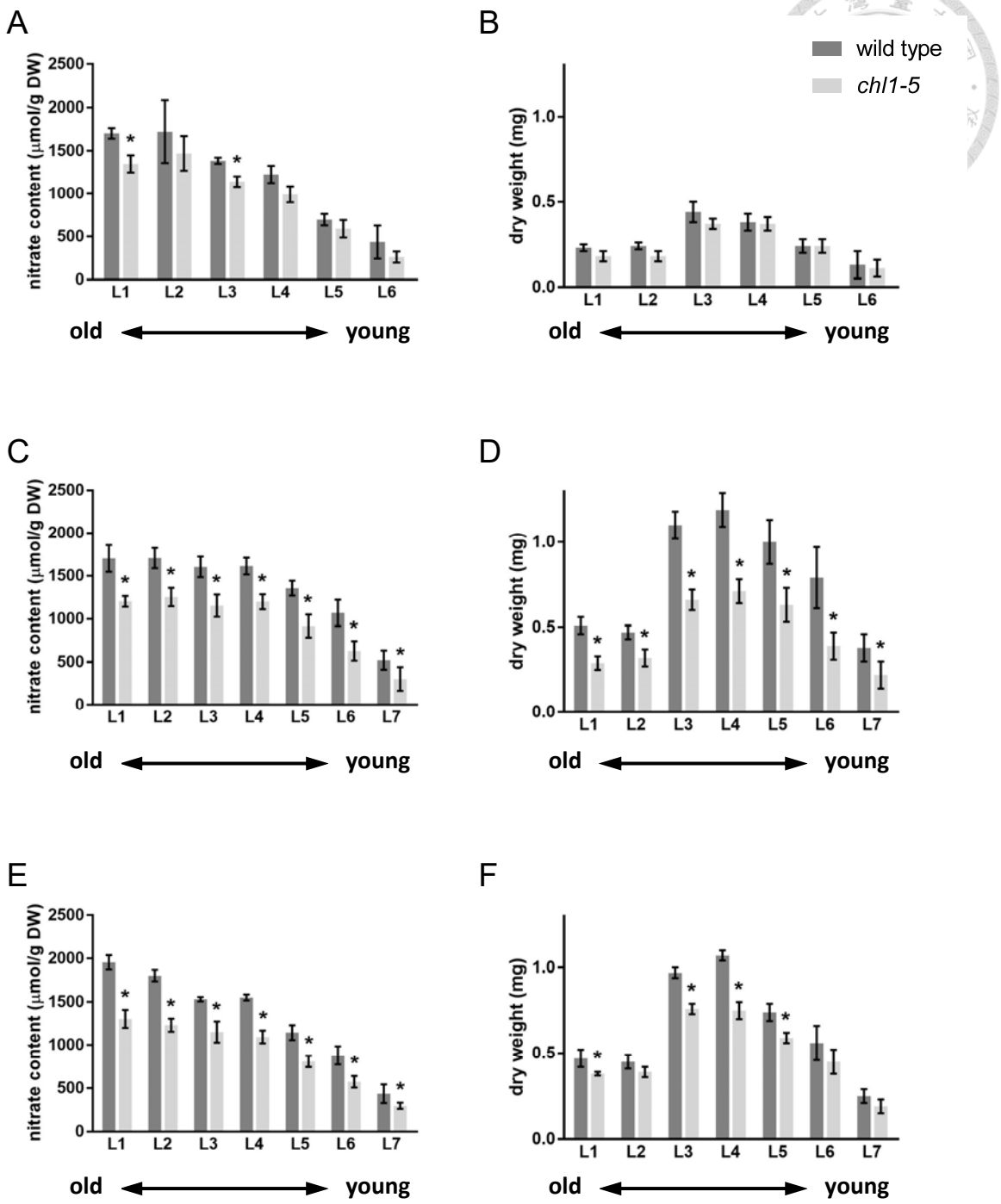

Figure 19. Nitrate content and biomass analysis of *chl1-5*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *chl1-5* mutant from three independent experiments. A and B were from batch 4, C and D were from batch 8, and E and F were

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from batch 10. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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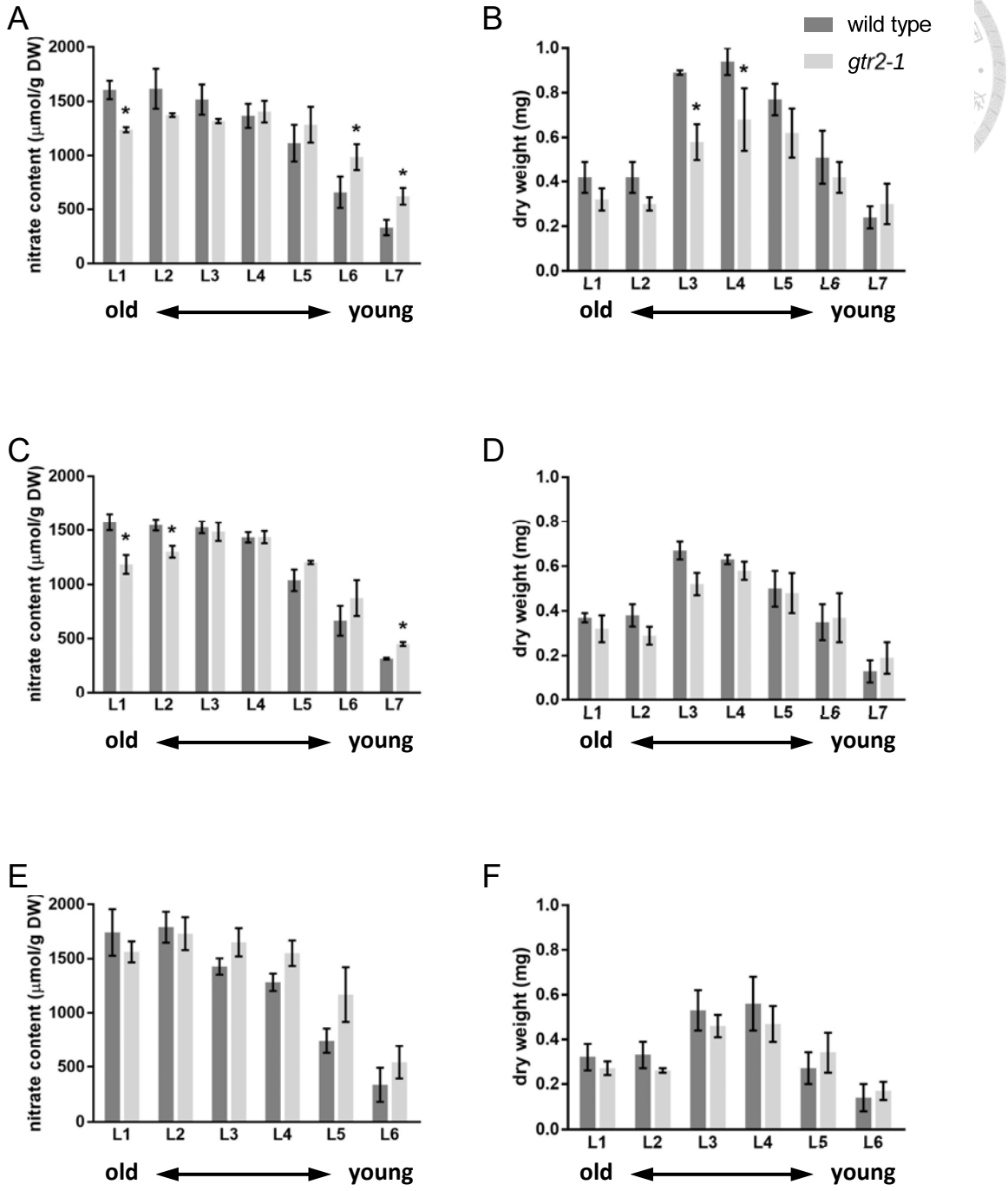

Figure 20. Nitrate content and biomass analysis of *gtr2-1*.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *gtr2-1* mutant from three independent experiments. A and B were from batch 1, C and D were from batch 2, and E and F were

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from batch 3. Experiment details are described in Figure 1. (*, $p<0.05$, compared to wild type based on Student's t test)

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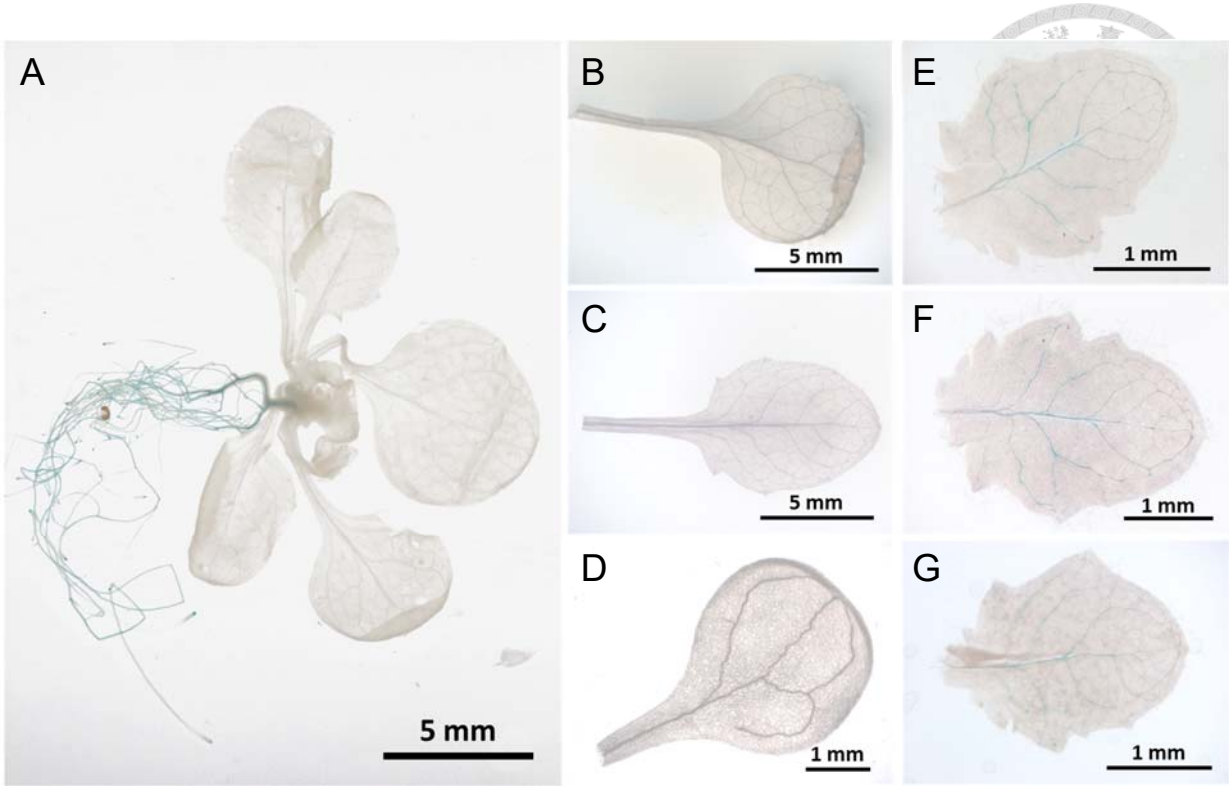

Figure 21. GTR2 was expressed in roots, root tips, root-shoot junction, and young leaves.

Plants were grown on MS plates under continuous light. The whole plant (A), old leaf (B), big leaf (C), cotyledon (D), young leaf (E to G) were presented. Scale bars are 5 mm (A to C), and 1 mm (D to G).

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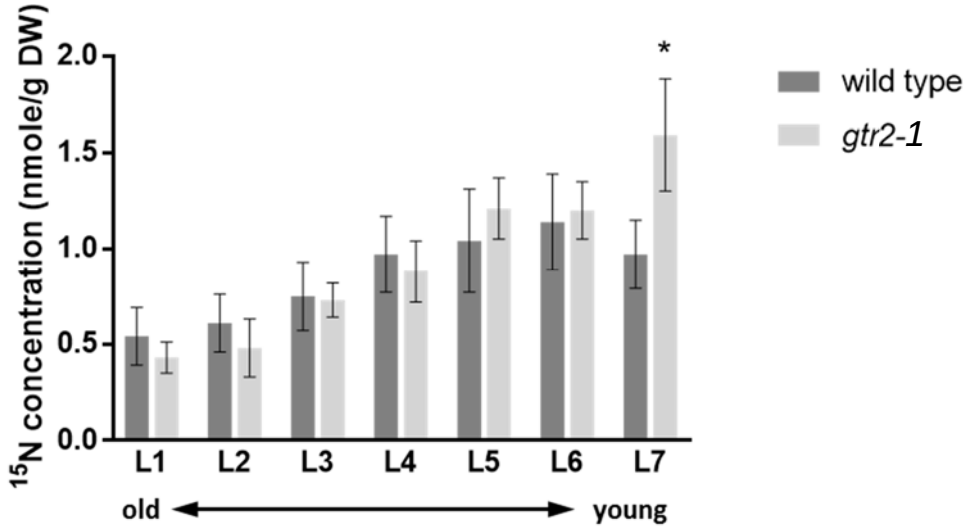

Figure 22. More ^{15}N was accumulated in young leaf in *gtr2-1*.

17-day-old plants were grown hydroponically and then transferred to 2 mM KNO_3 hydroponic medium containing a 49% excess of ^{15}N for 5 min, and then individual leaves were collected. L1 to L7 are true leaf numbers, where L1 is the oldest true leaf. Each data represents the mean of 4 different plants and error bar is the standard deviation, and the similar pattern was observed in two additional independent experiments. (*, $p<0.05$, compared to wild type based on Student's t test)

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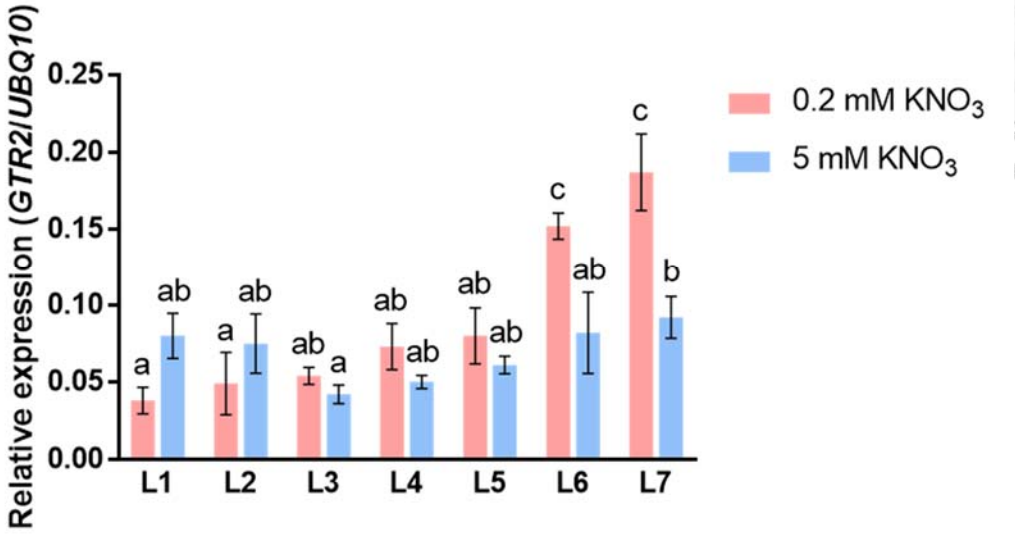

Figure 23. *GTR2* expression in leaves under different nitrate conditions.

Wild type plants were grown hydroponically with 0.2 mM or 5 mM KNO_3 for 17 days. L1 to L7 are true leaf numbers, where L1 is the oldest true leaf. *GTR2* expression was analyzed with gene-specific primers and was normalized to *UBQ10*. Each data represents the mean of 3 different plants and error bar is the standard deviation, and the similar pattern was observed in two additional experiments. The differences were compared using one-way ANOVA with Tukey HSD test at $p<0.05$.

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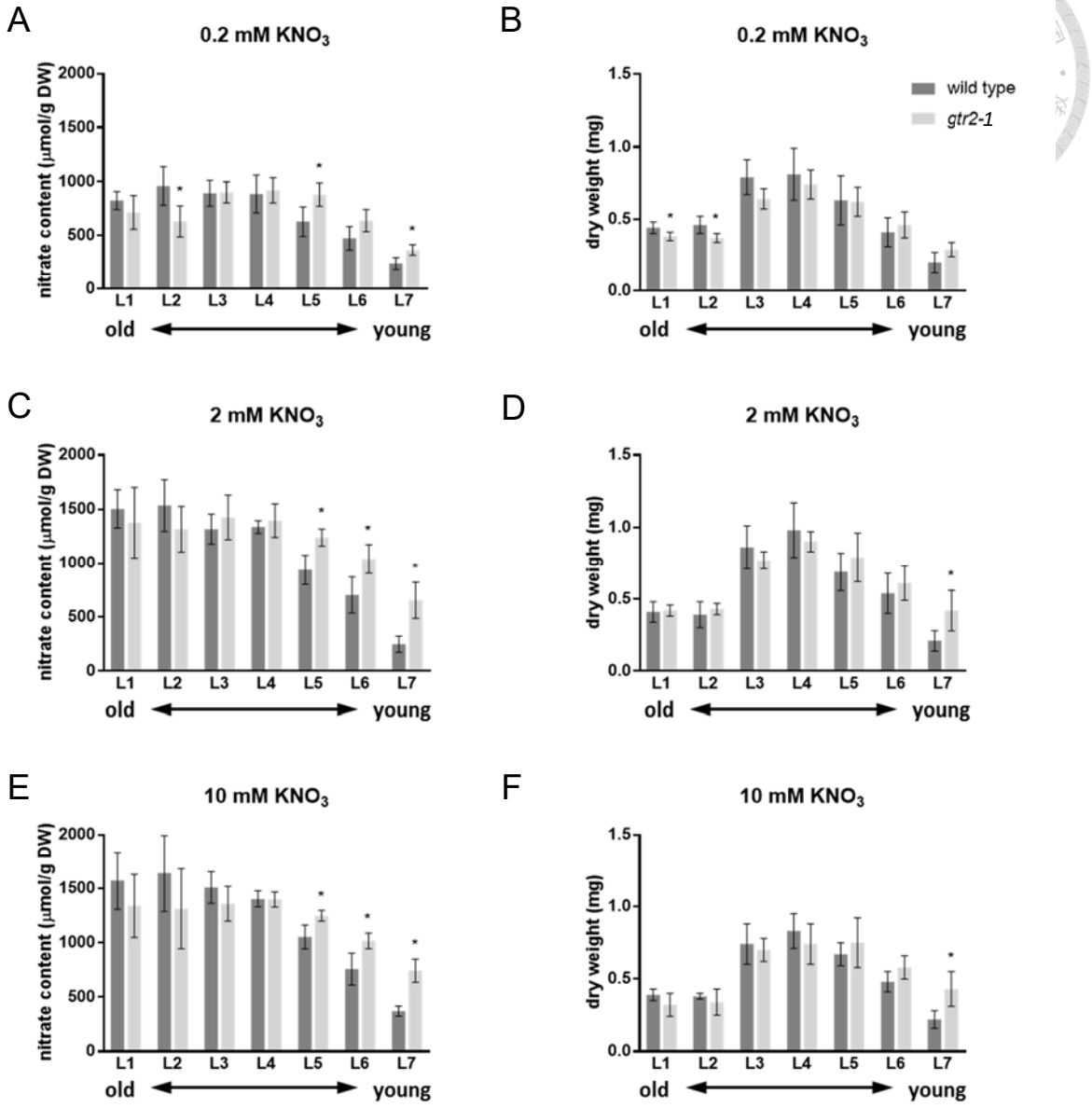

Figure 24. Nitrate content and biomass of wild type and *gtr2-1* under different nitrate concentrations.

Left panels presented nitrate content (A, C, and E) and right panels showed corresponding dry weight (B, D, and F) of wild type and *gtr2-1* mutant under different nitrate concentration conditions as indicated in one experiment. L1 to L7 are true leaf numbers,

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where L1 is the oldest true leaf. Plants were grown hydroponically for 17 days. Each data represents the mean of 7 different plants and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

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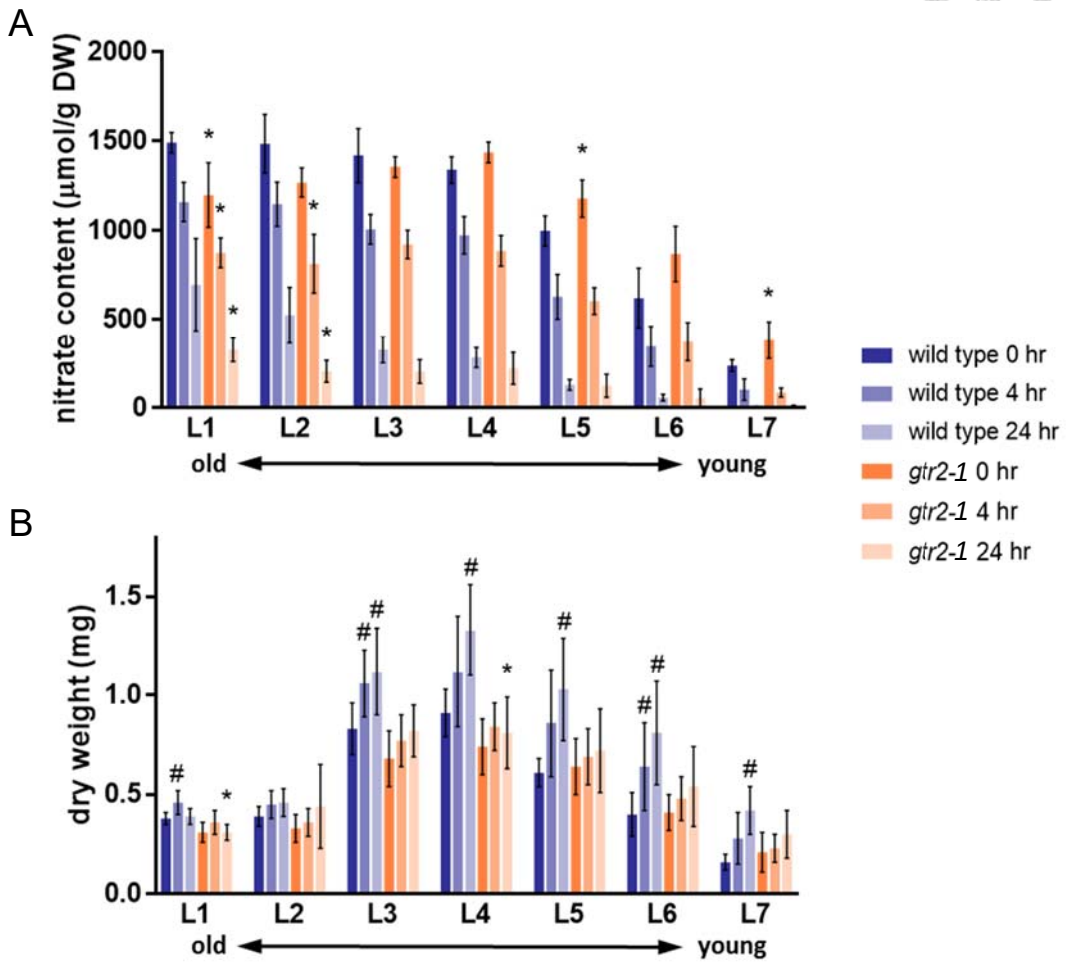

Figure 25. Nitrate content and biomass of wild type and *gtr2-1* under starvation.

Upper panel presented nitrate content (A) and lower panel showed corresponding dry weight (B) of wild type and *gtr2-1* mutant in one experiment. L1 to L7 are true leaf numbers, where L1 is the oldest true leaf. Plants were grown hydroponically with 2 mM KNO_3 for 17 days and then shifted to nitrate-depleted medium for another day. Samples were collected at 17th day before shifted to nitrate-depleted medium, and 4 and 24 hours after shifted. Each data represents the mean of 6 different plants and error bar is the standard deviation. (*, $p<0.05$, compared to corresponding time point of wild type based

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on Student's t test; #, $p<0.05$, biomass increased at 4 hour and 24 hour compared to that at 0 hour, respectively, based on Student's t test)

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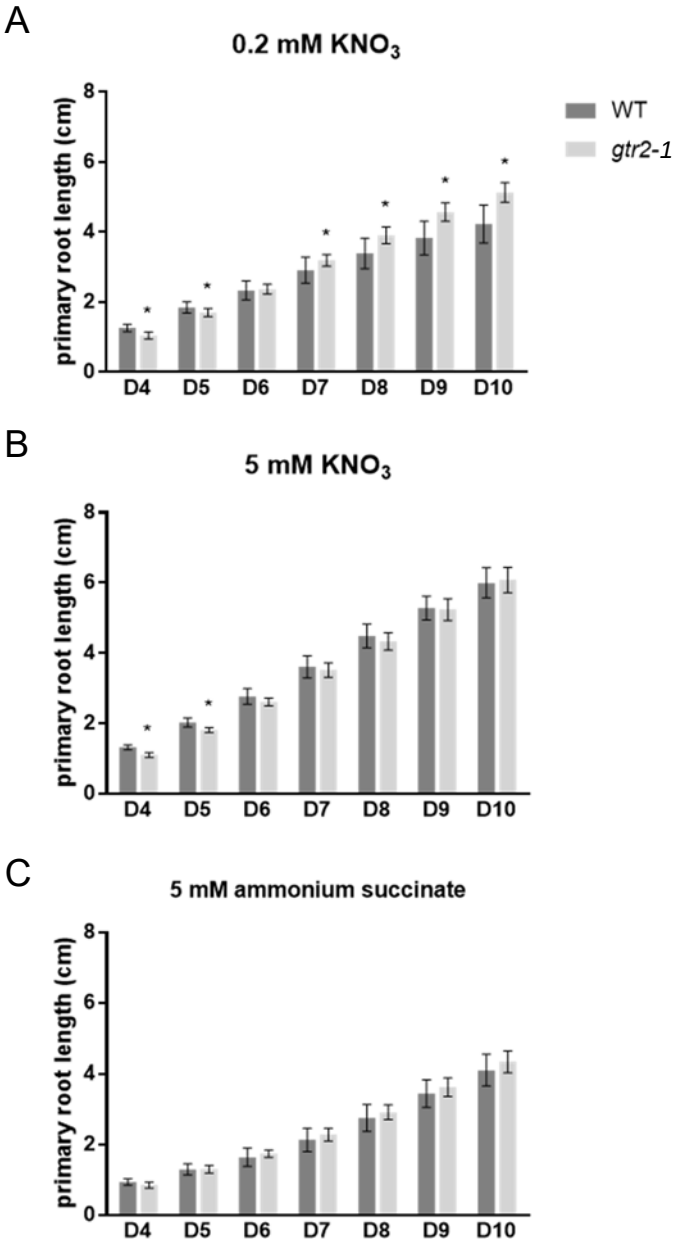

Figure 26. Primary root length of wild type and *gtr2-1* grown under different nitrogen sources.

Seedlings were grown under 0.2 mM KNO_3 (A), 5 mM KNO_3 (B), and 5 mM ammonium succinate (C) for 10 days. Each data represents the mean of 18 different plants and error

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bar is the standard deviation. The pattern was also observed in two additional independent experiments. (*, $p<0.05$, compared to wild type based on Student's t test)

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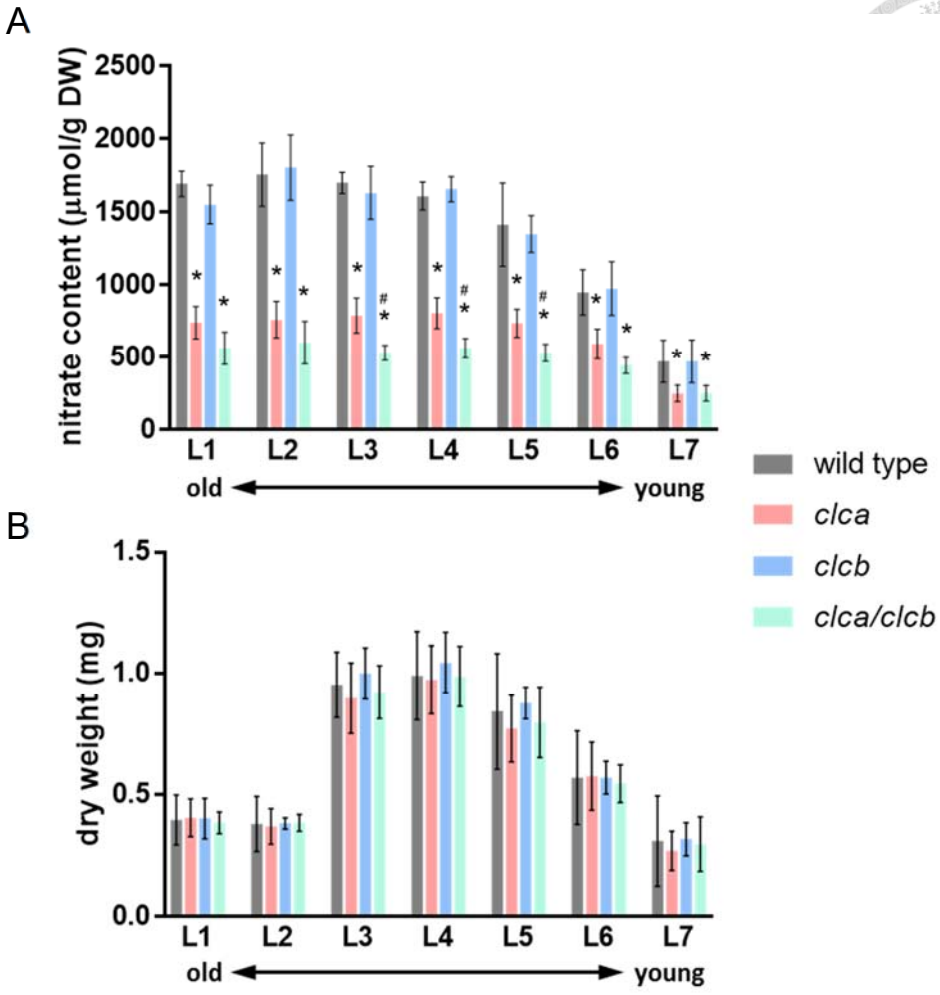

Figure 27. Nitrate content analysis of *clc* mutants grown with sufficient nitrate.

Upper panel presented nitrate content (A) and lower panel showed corresponding dry weight (B) of wild type and *clca*, *clcb*, *clca/clcb* mutant in one experiment. L1 to L7 are true leaf numbers, where L1 is the oldest true leaf. Plants were grown hydroponically with 2 mM KNO_3. Samples were collected at 17^{th} day. Each data represents the mean of 4 different plants and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test; #, $p<0.05$, comparison between *clca* and *clca*/*clcb* based on Student's t test)

doi:10.6342/NTU201703166

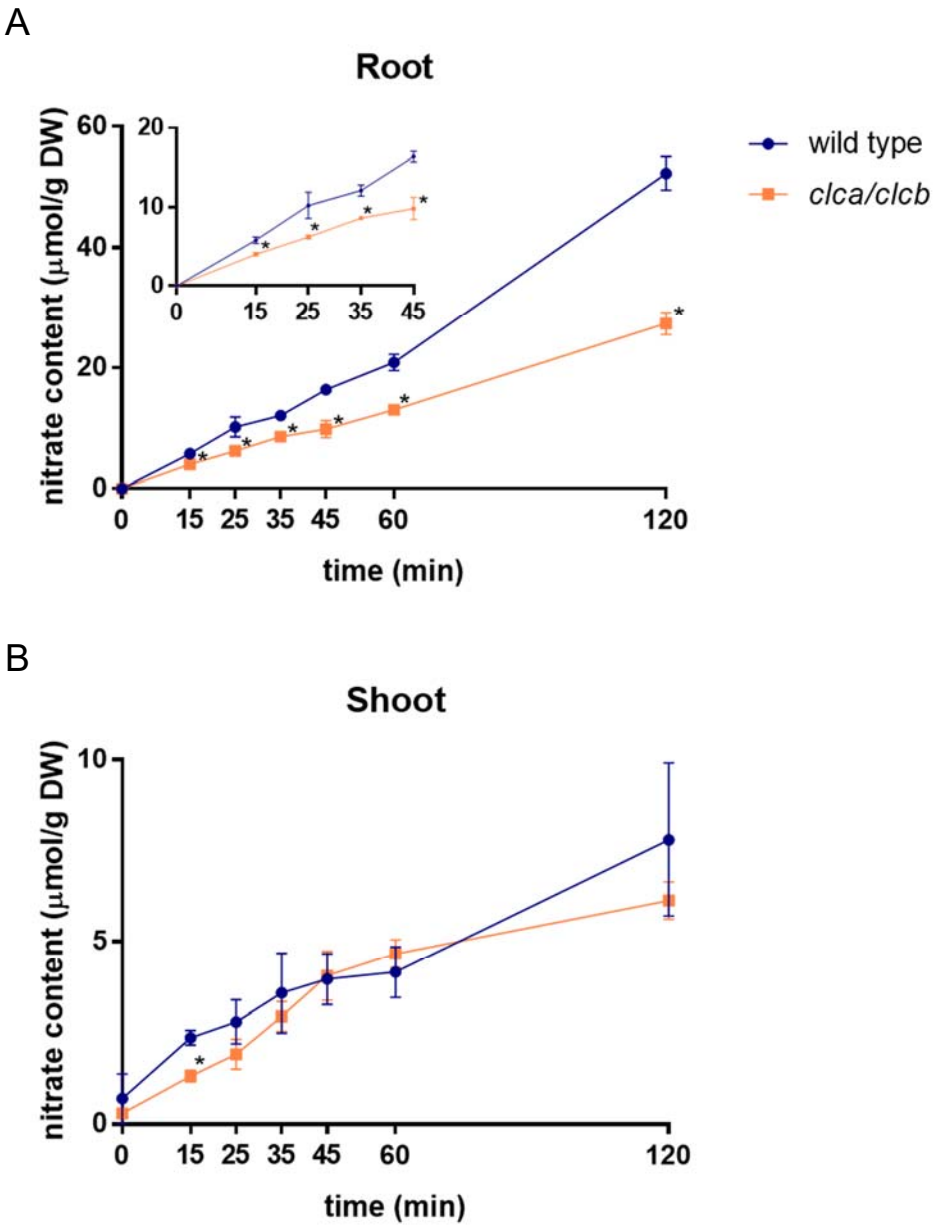

Figure 28. Nitrate content accumulation of *clca/clcb* mutant after high-affinity nitrate induction.

The nitrate content was analyzed in 9-day-old wild type and *clca/clcb* mutant by HPLC in one single experiment. Plants were grown in Magenta vessels in 12.5 mM ammonium

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succinate medium, pH 6.5, and shifted to pH 5.5 for two treatments, 16 hours and 3 hours respectively. After treated with 200 μM KNO_3 pH 5.5 medium, the medium was refreshed every 30 minutes and the samples of roots and shoots were collected at time as indicated after washed twice in 0.1 mM $CaSO_4$. Each data represents the mean of 4 biological replicates, each with about 60 plants, and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

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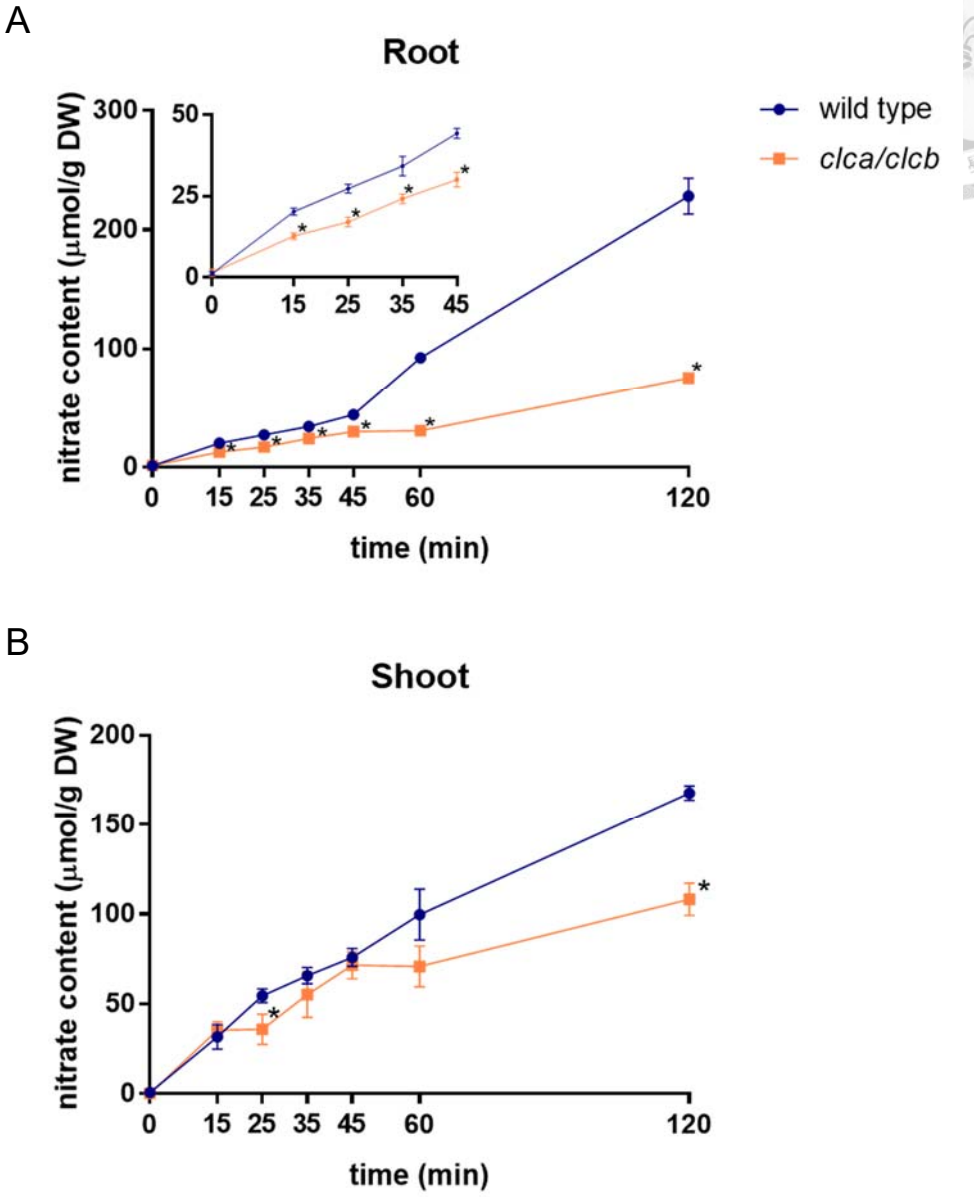

Figure 29. Nitrate content accumulation of *clca/clcb* mutant after low-affinity nitrate induction.

Experiment details are described in Figure 27 but with the treatment of 25 mM KNO_3, pH 5.5 medium. Each data represents the mean of 4 biological replicates, each with about

doi:10.6342/NTU201703166

60 plants, and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

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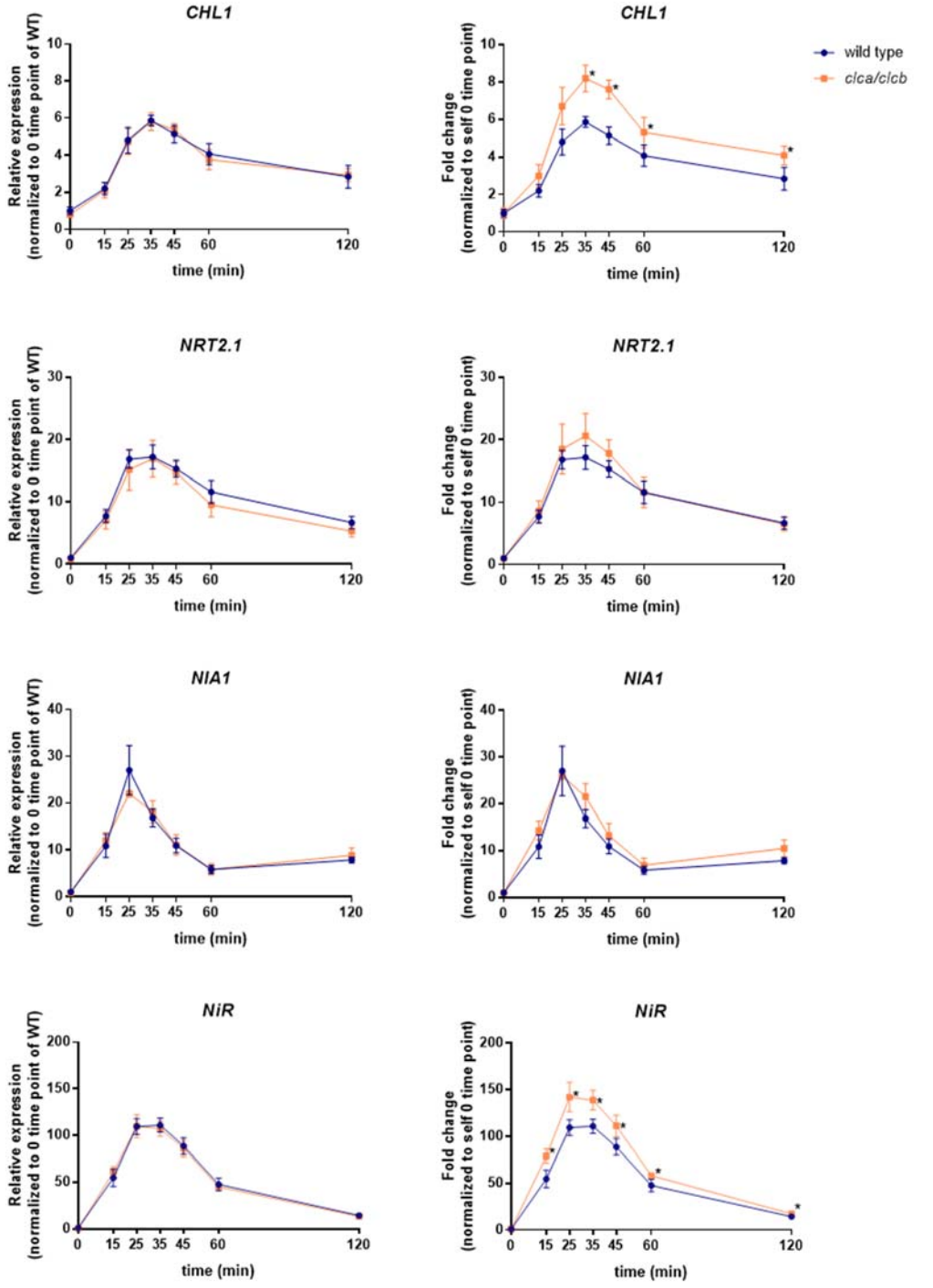

Figure 30. Root primary nitrate response of *clca/clcb* mutant after high-affinity nitrate induction.

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The expression of marker genes was analyzed in 9-day-old wild type and *clca/clcb* mutant by qRT-PCR in one single experiment. Plants were grown in Magenta vessels in 12.5 mM ammonium succinate medium, pH 6.5, and shifted to pH 5.5 for two treatments, 16 hours and 3 hours respectively. After treated with 200 μM KNO_3 pH 5.5 medium, the medium was refreshed every 30 minutes and the total RNA was collected from roots at the time as indicated. Expression of marker genes was analyzed with gene-specific primers and was normalized to *UBQ10*. Relative expression of marker genes was normalized to 0 time point of wild type (left panels) and the fold change was normalized to self 0 time point (right panels). Each data represents the mean of 4 biological replicates, each with about 60 plants, and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

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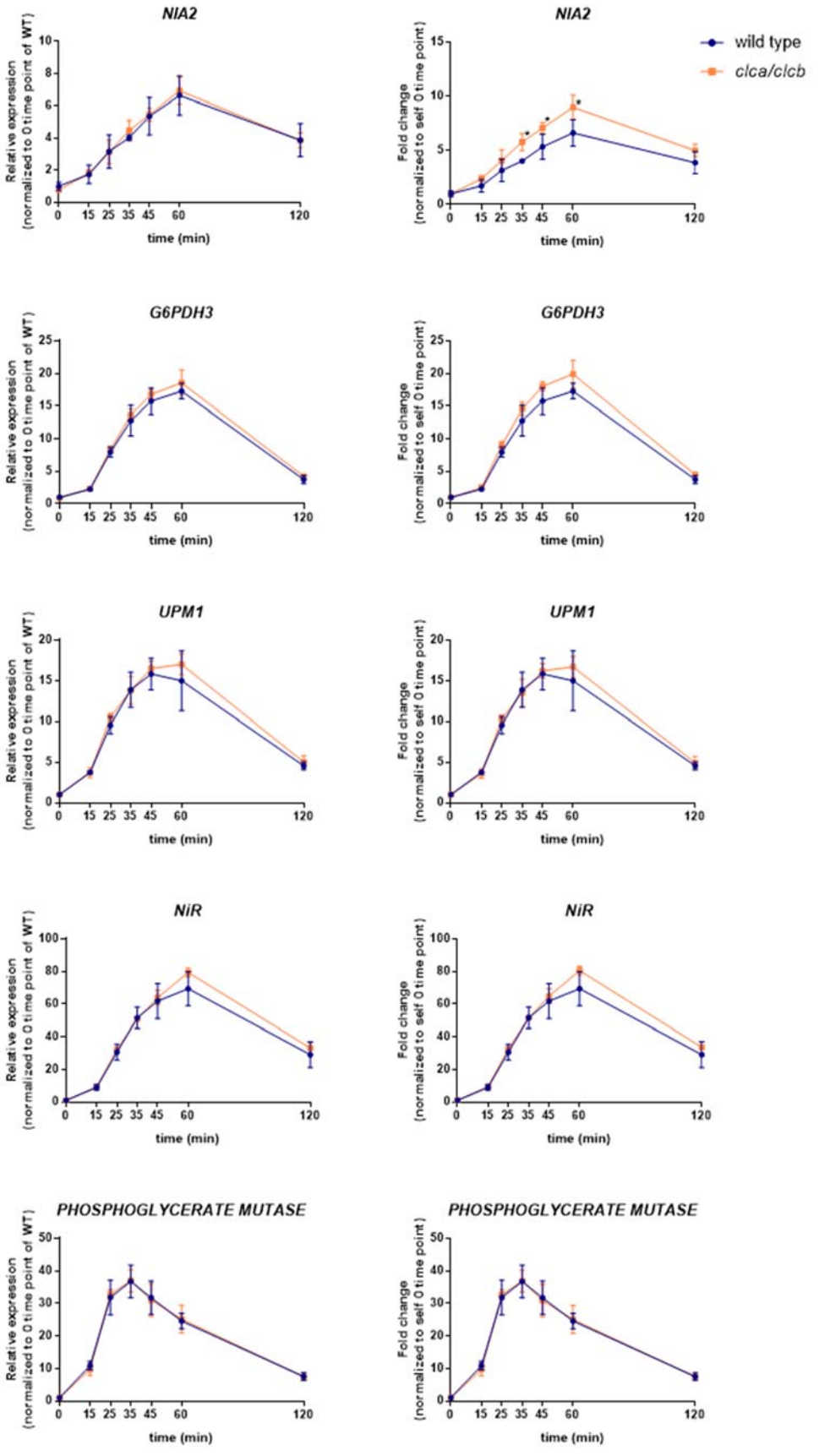


doi:10.6342/NTU201703166

Figure 31. Shoot primary nitrate response of *clca/clcb* mutant after high-affinity nitrate induction.

The experiment details are described in Figure 29. Each data represents the mean of 4 biological replicates, each with about 60 plants, and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

doi:10.6342/NTU201703166

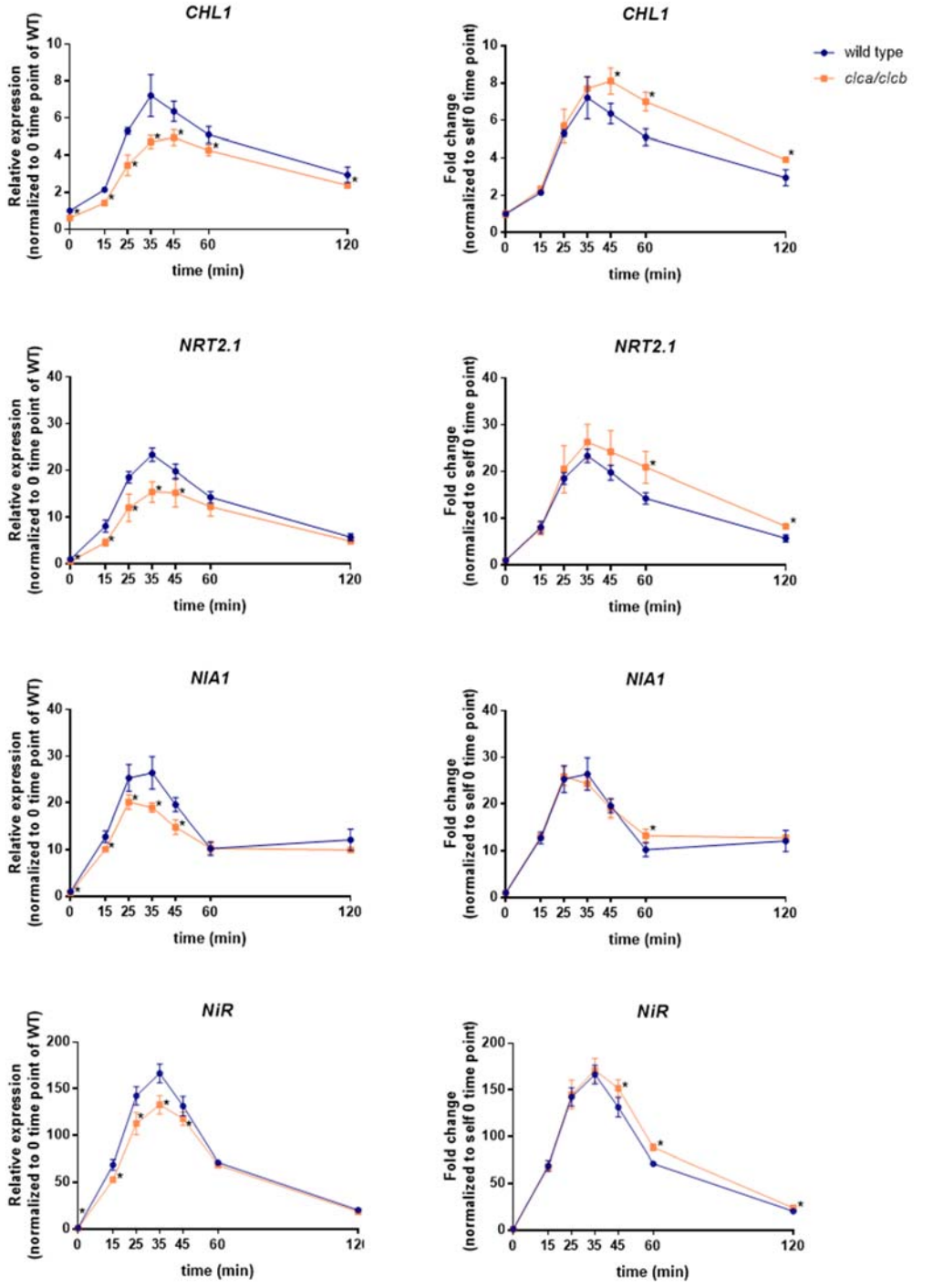
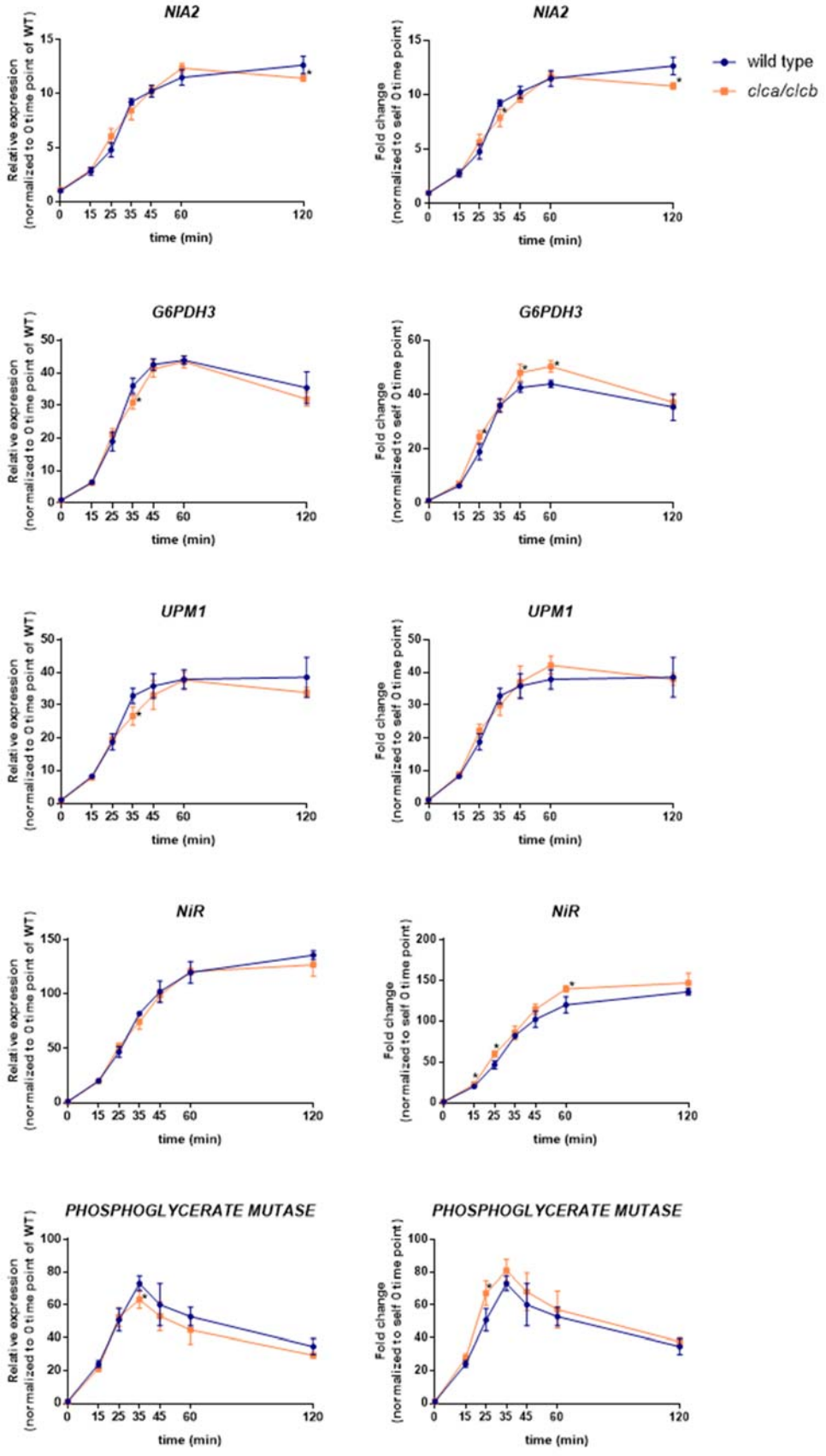


Figure 32. Root primary nitrate response of *clca/clcb* mutant after low-affinity nitrate induction.

doi:10.6342/NTU201703166

The experiment details are described in Figure 29 with 25 mM KNO_3 treatment instead of 200 μM KNO_3. Each data represents the mean of 4 biological replicates, each with about 60 plants, and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

doi:10.6342/NTU201703166



doi:10.6342/NTU201703166

Figure 33. Shoot primary nitrate response of *clca/clcb* mutant after low-affinity nitrate induction.

The experiment details are described in Figure 29 with 25 mM KNO_3 treatment instead of 200 μM KNO_3. Each data represents the mean of 4 biological replicates, each with about 60 plants, and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

doi:10.6342/NTU201703166

6. Appendix

Supplementary Table 1. Primers used in genotyping.

AGI code	NPF names	Primers (from 5' to 3')
At2g02040	AtNPF8.3	P4473-ATGGGTTCCATCGAAGAAGAAG P4474-GAAGCTTTCTTTTGCTTATACC P5264-ATGGGTTCCATCGAAGAAGAAGCA P5265-GCAGCAGAGAAGAAGTAAACCGCC LB-TGGTTCACGTAGTGGGCCATCG
At1g62200	AtNPF8.5	P4475-ATGGTGAATTCGAATGAAGAAG P4476-AAAGCCTTCTTCTTTGTGT P5266-TTCGAATGAAGAAGACGAAAGGAG P5267-CAAAGCCTTCTTCTTTGTGTGCTT LB-CCCATTTGGACGTGAATGTAGACAC
At1g68570	AtNPF3.1	P973-CCCTCCTGGCTAATGCCATTT P974-AAACACCTTTATAACCACTTCAAA P5253-ATGGAGGAGCAAAGCAAGAACAAG P5254-CCTTGCTATGATGAACCTGAACCGG LB-TGGTTCACGTAGTGGGCCATCG
At2g40460	AtNPF5.1	P855-TGTTCAAATGGAGGCCAAAGCCTT P856-TAGAATCCAAGCAACATAAT P5269-GGAGGCTGCAAAAGTTTACACAC P5270-CTAAGAGGAGATGTGTCTAAGGC LB-TGGTTCACGTAGTGGGCCATCG
At3g53960	AtNPF5.7	P4700-CGGCTAGCTTTGTCAACAATC P4701-GGACTTGGAATTCAGTTCTGTAAGATG P5251-ATGGAGCACAACAAGGTTGATACA P5252-CGTAAACTTGGACGTGTTATTCGTCC LB-TGGTTCACGTAGTGGGCCATCG
At5g14940	AtNPF5.8	P983-TAGCTTCCGACATATCATCTGCAAAC P1018-ATGGCTGGAGGAGAGAAAAG LB-TGGTTCACGTAGTGGGCCATCG

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At1g22540	AtNPF5.10	R010-ATCCGCTCCAAAAGCCTGAA P750-CACGAACGTCGCTGATTTTA P5271-GTCGATCTCCGGCGCTGTTGAT P5272-CTGGTGTCGAGCCTTTTAGAGACG LB-TGGTTCACGTAGTGGGCCATCG
At1g72130	AtNPF5.11	P751-GAAATTTCAGGCCGACAGTG P752-CAAAATTCCCGGGAGAAATA P5275-ATGGCTATCACCTACTCCTCCGC P5276-GACTTGGCGAACCATAAGTAGAAG LB-TGGTTCACGTAGTGGGCCATCG
At1g72140	AtNPF5.12	P510-GGACTTGGGCTGCTTACGTT P511-GGAGTTTGTCGCCGGTTTCT LB-TAGCATCTGAATTTCATAACCAATCTCGATACAC
At1g22570	AtNPF5.15	P4692-TACCCAAAGGGACATCATGAG P4693-TTGGTCTAGCCGACGATACAC P5255-AAGATACCAGAGGAAGAAGTTGC LB-AACGTCCGCAATGTGTTATTAAGTTGTC
At5g13400	AtNPF6.1	M021-GATAGCTCGGTACTTGTACC R095-AGCTTTCACAAGCAGTGAGT P5257-CCTCAAAATTGTACCCAATTG LB-TGGTTCACGTAGTGGGCCATCG

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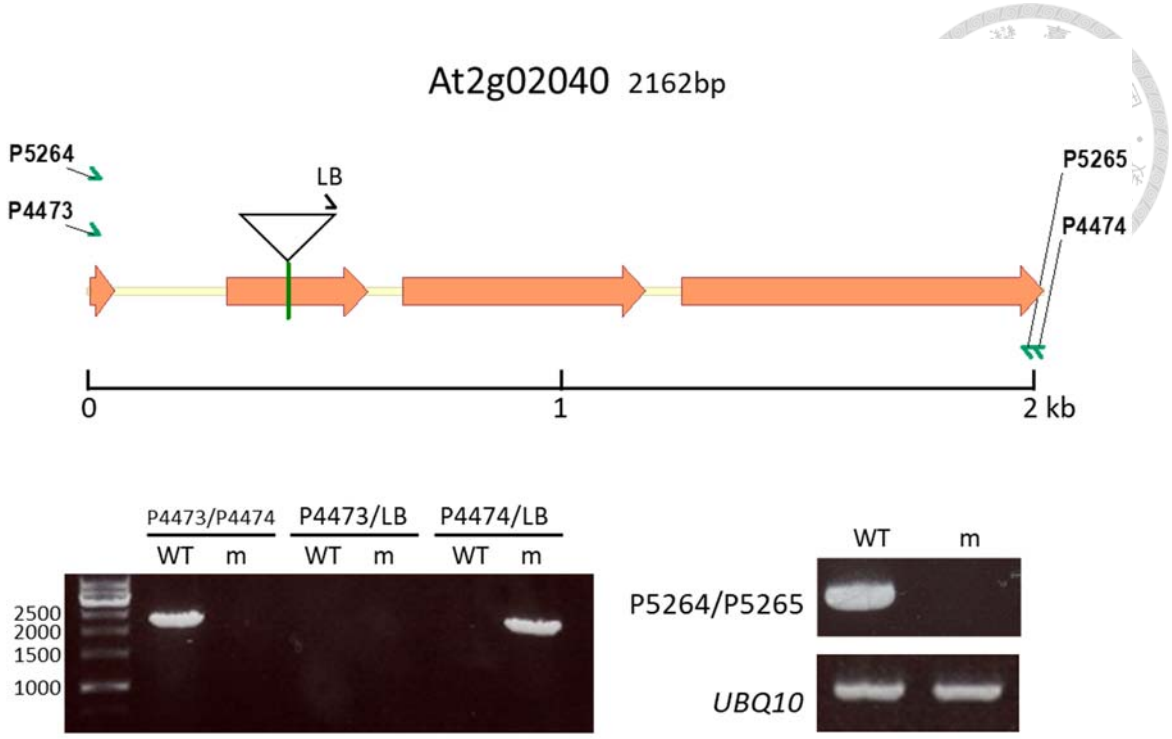
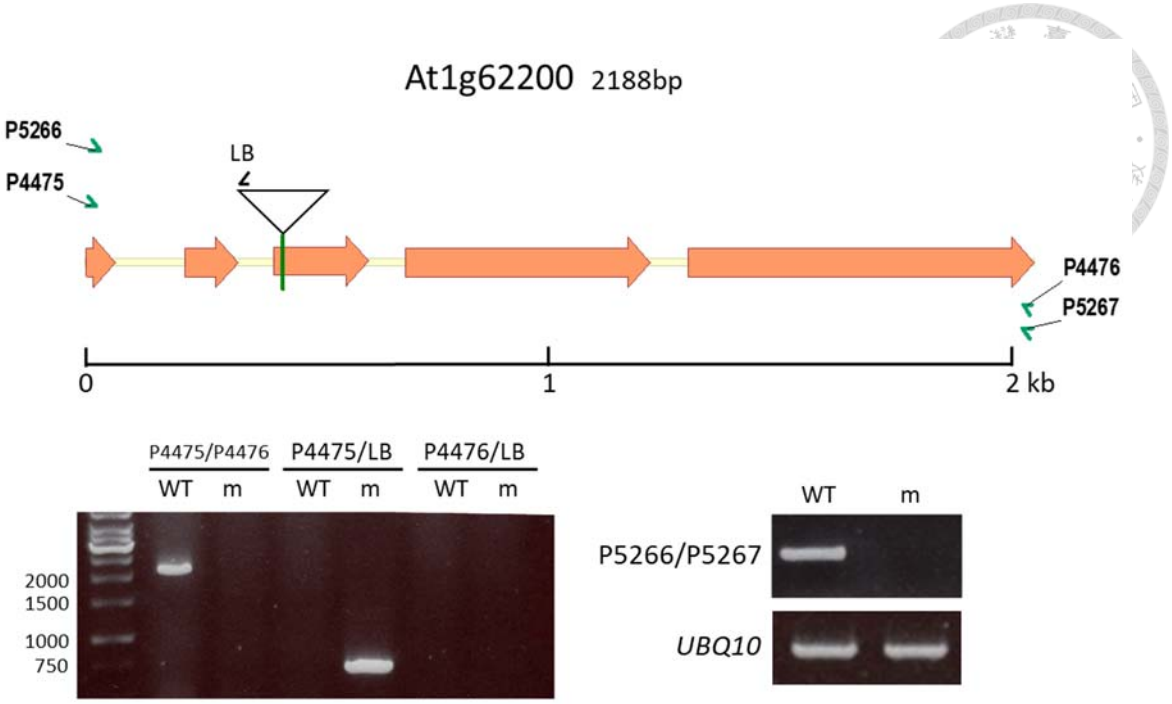


Supplementary Figure 1. T-DNA insertion site in *ptr2-1* mutant.

Gene structure was shown in upper panel, including exons (orange arrow), T-DNA insertion site (green line), forward primer, and reverse primer (green arrows). Genomic DNA PCR was performed with two out of three primers, gene specific primers and left border primer of T-DNA, to confirm the genotype of mutant (bottom left). RT-PCR analysis was performed with primers indicated, and *UBQ10* was used as control. Sequence of the primers were listed in Supplementary Table 1. WT, wild type; m, mutant; LB, left border primer of T-DNA.

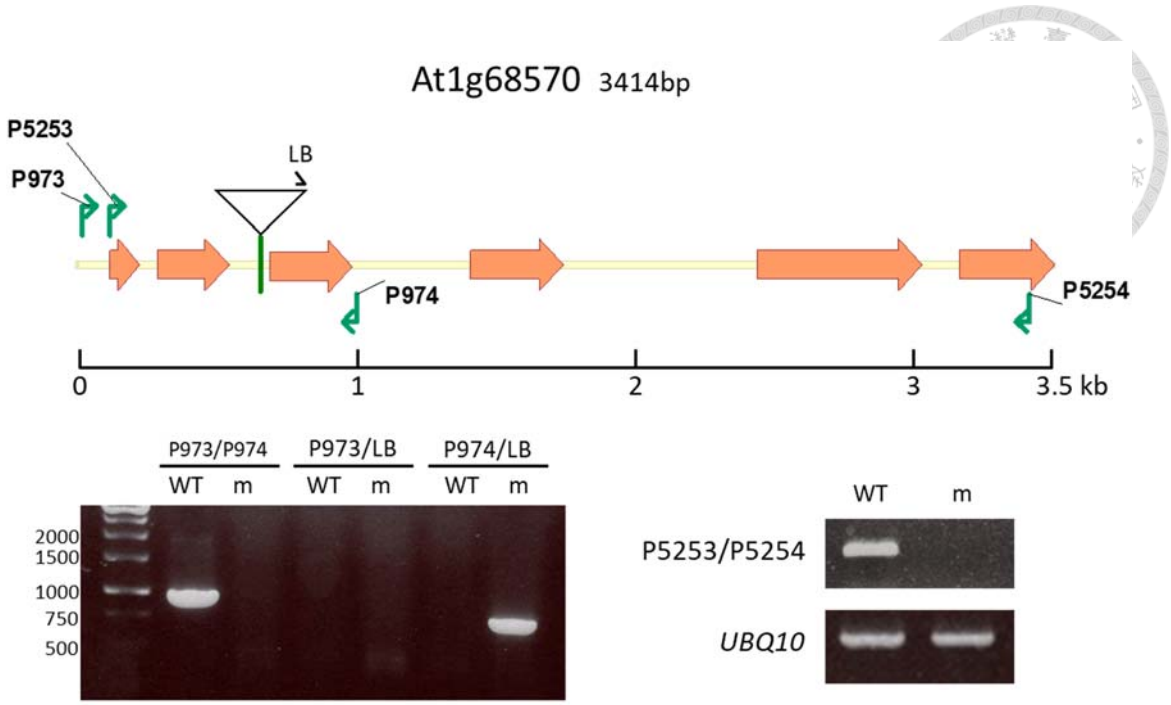
doi:10.6342/NTU201703166



Supplementary Figure 2. T-DNA insertion site in *ptr6-1* mutant.

Experiment details are described in Supplementary Figure 1.

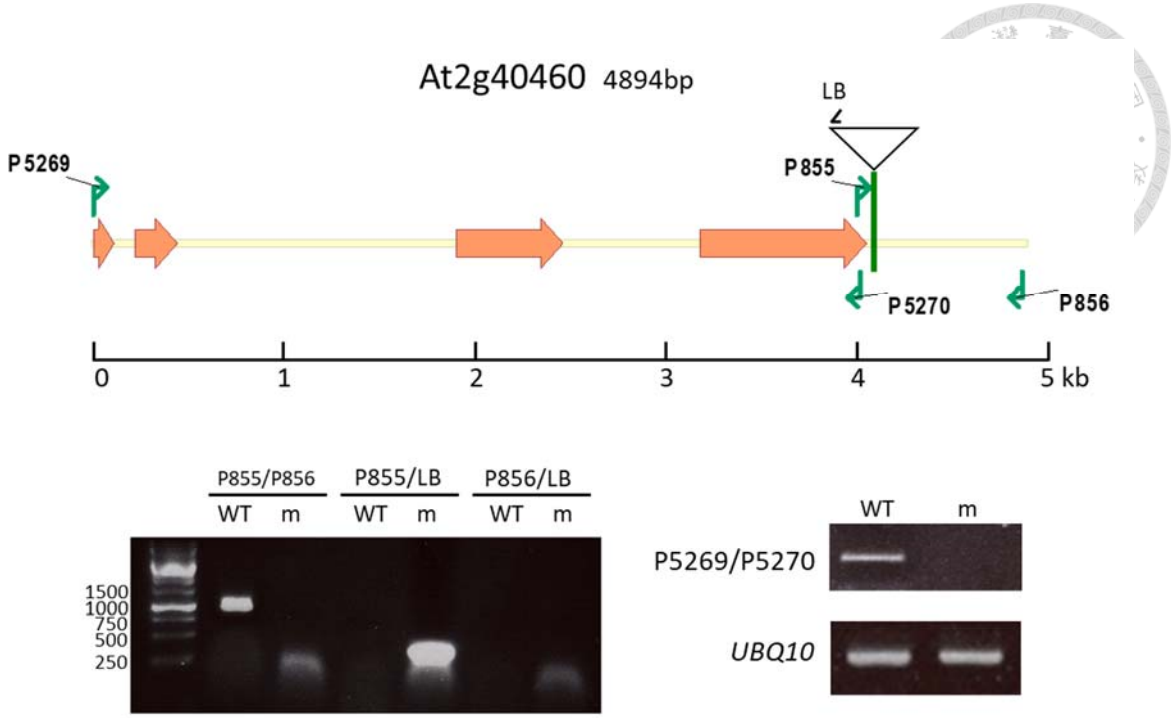
doi:10.6342/NTU201703166



Supplementary Figure 3. T-DNA insertion site in *npf3.1-1* mutant.

Experiment details are described in Supplementary Figure 1.

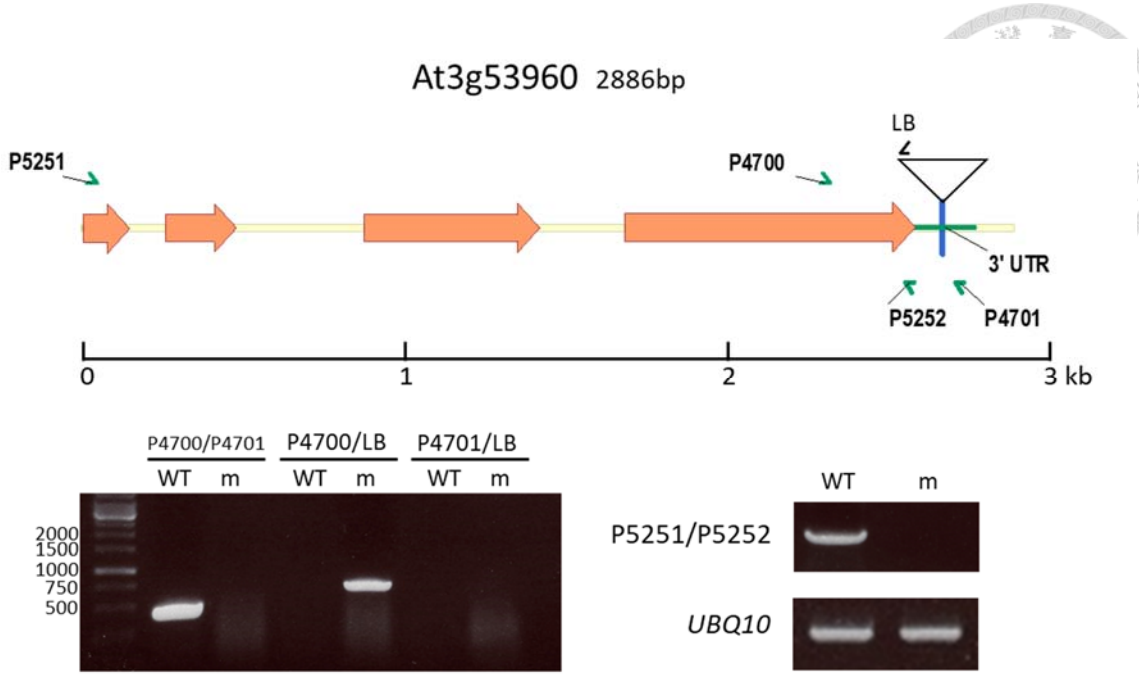
doi:10.6342/NTU201703166



Supplementary Figure 4. T-DNA insertion site in *npf5.1-1* mutant.

Experiment details are described in Supplementary Figure 1.

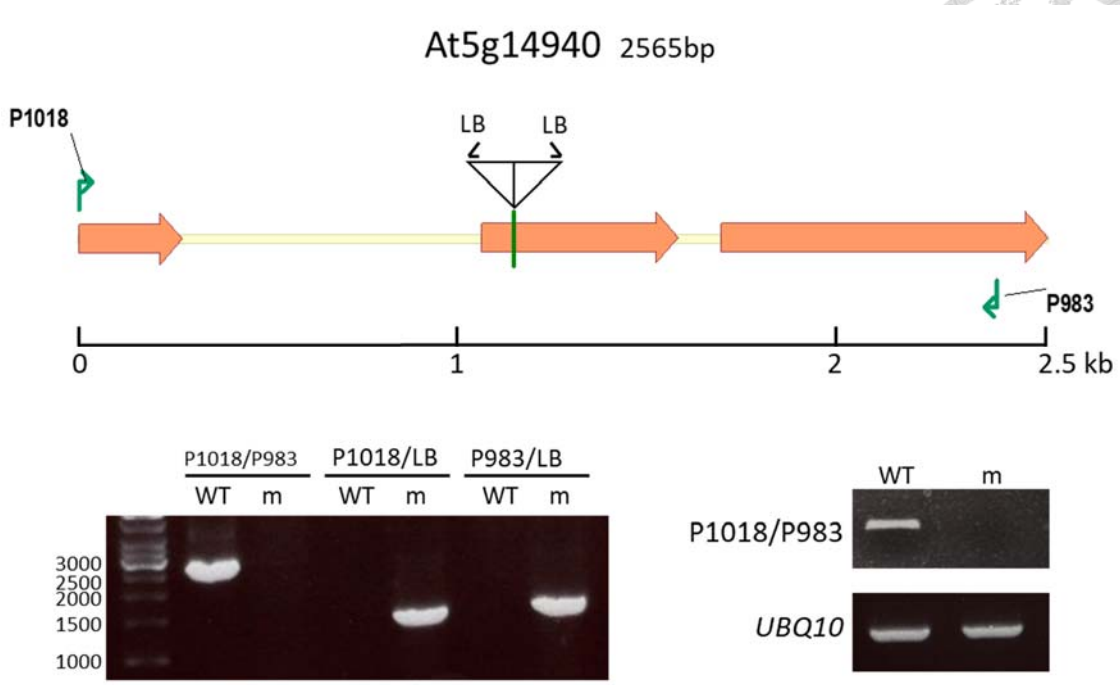
doi:10.6342/NTU201703166



Supplementary Figure 5. T-DNA insertion site in *npf5.7-1* mutant.

Experiment details are described in Supplementary Figure 1.

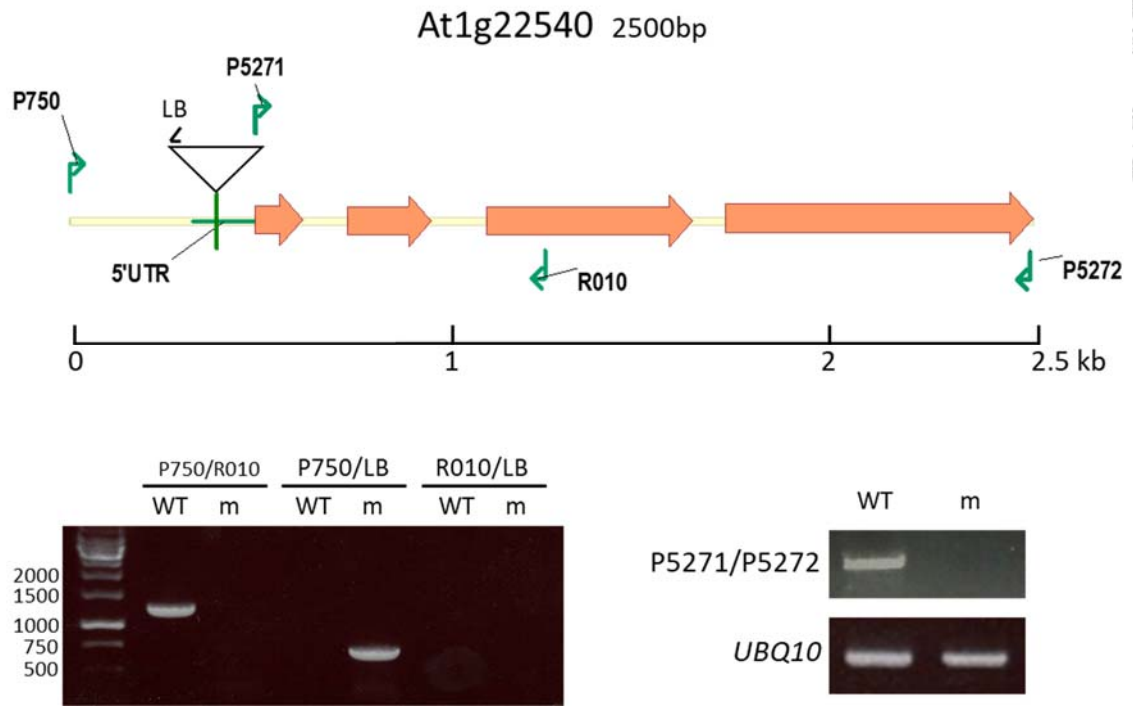
doi:10.6342/NTU201703166



Supplementary Figure 6. T-DNA insertion site in *npf5.8-1* mutant.

Experiment details are described in Supplementary Figure 1.

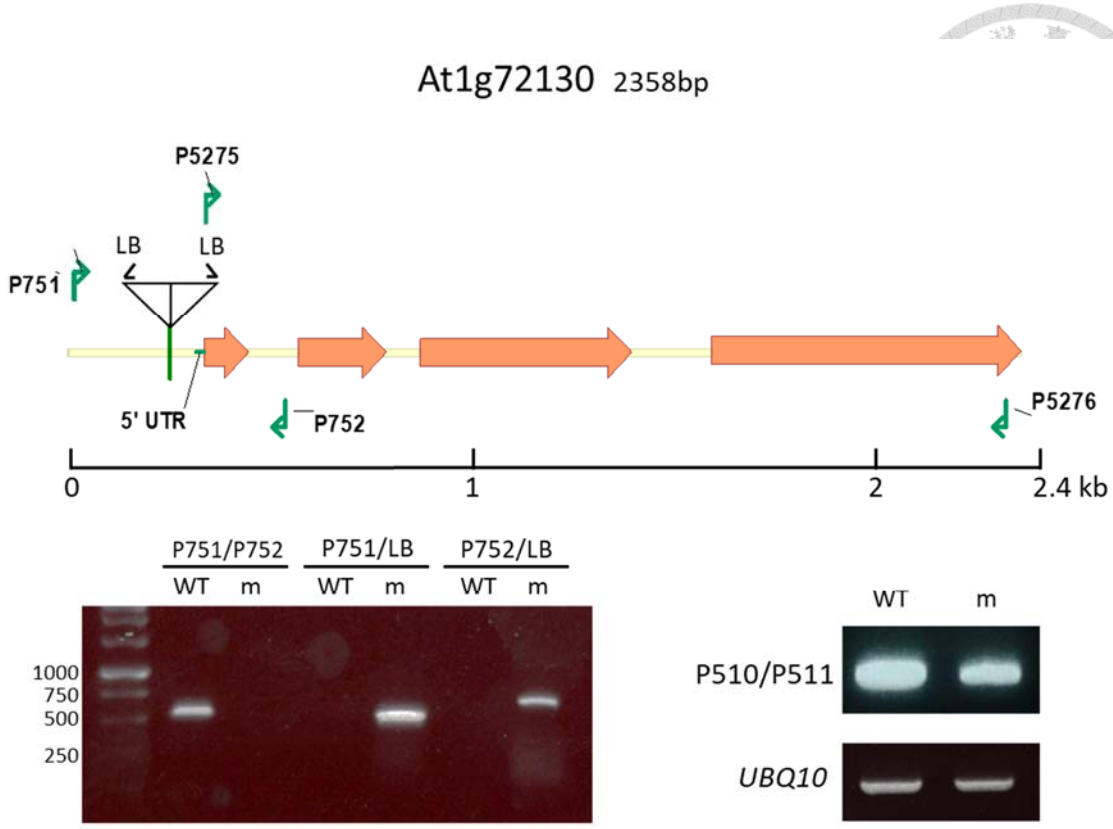
doi:10.6342/NTU201703166



Supplementary Figure 7. T-DNA insertion site in *npf5.10-1* mutant.

Experiment details are described in Supplementary Figure 1.

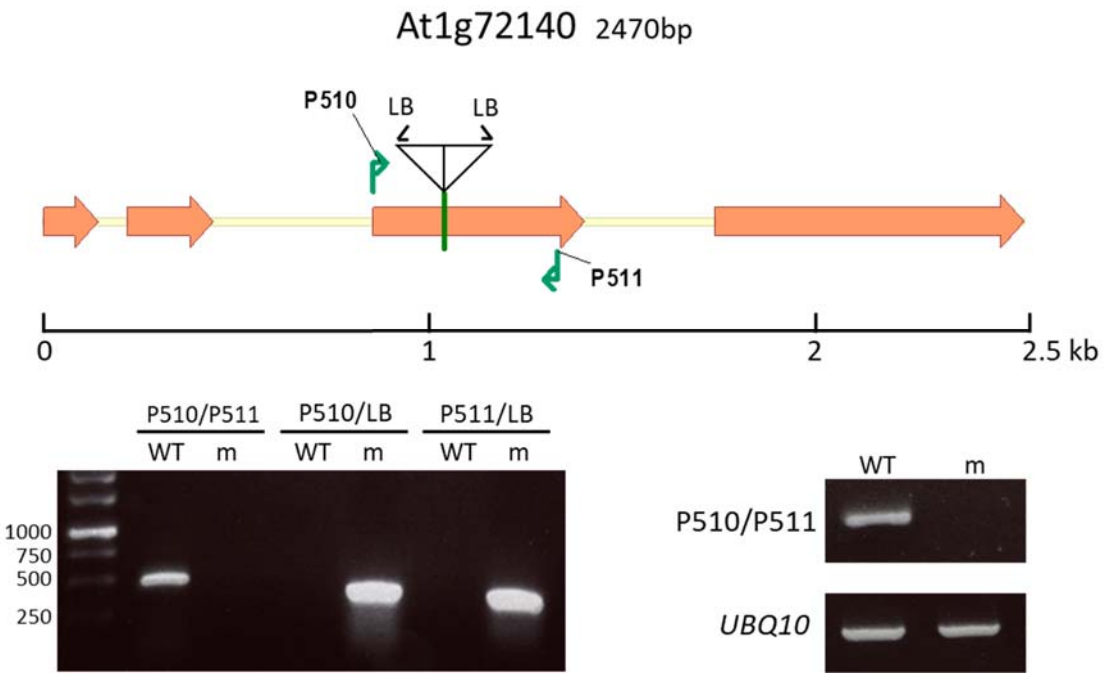
doi:10.6342/NTU201703166



Supplementary Figure 8. T-DNA insertion site in *npf5.11-1* mutant.

Experiment details are described in Supplementary Figure 1.

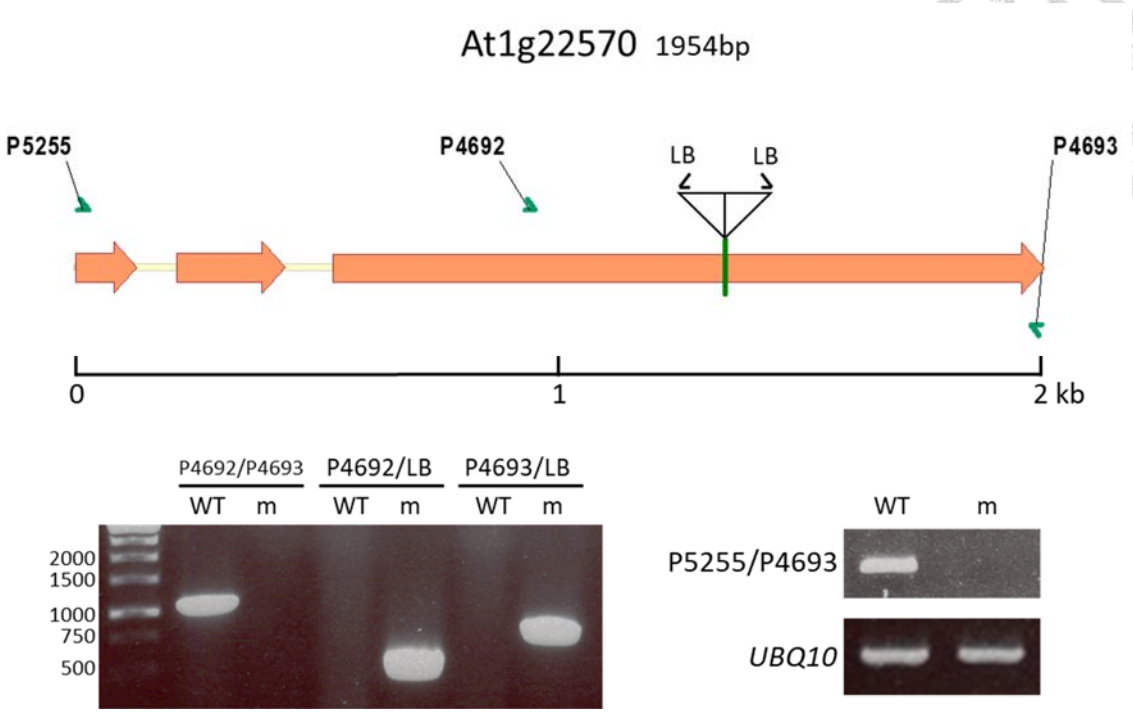
doi:10.6342/NTU201703166



Supplementary Figure 9. T-DNA insertion site in *npf5.12-1* mutant.

Experiment details are described in Supplementary Figure 1.

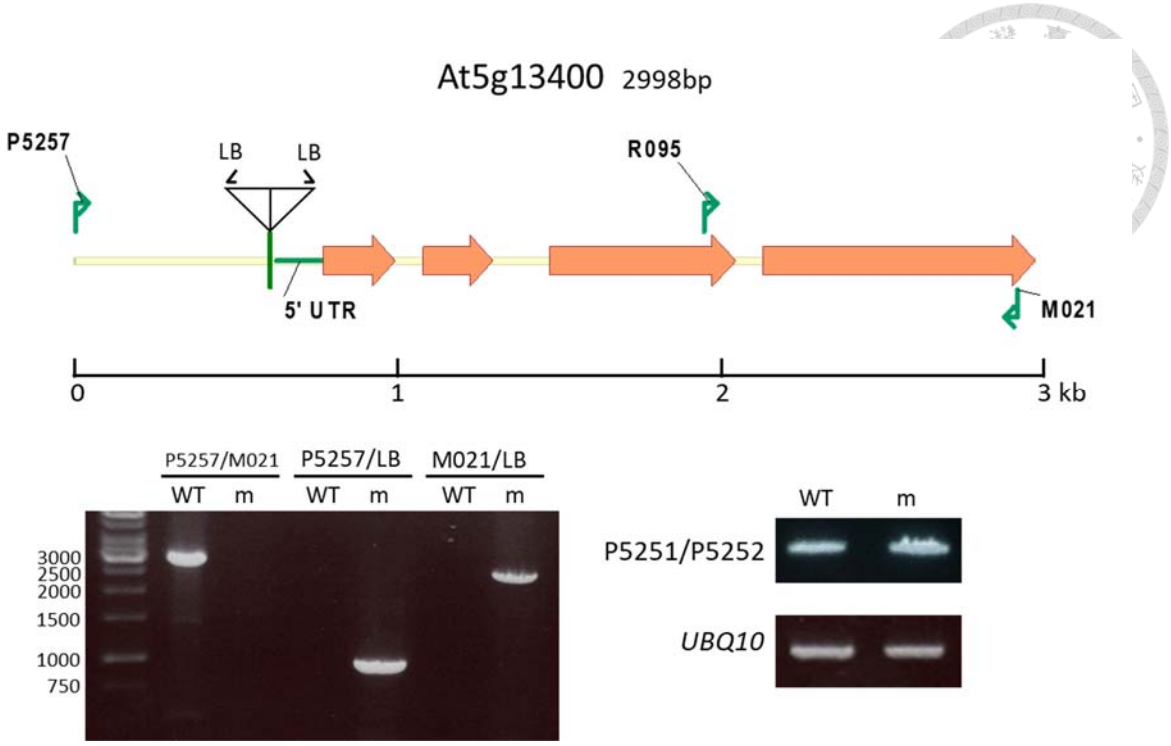
doi:10.6342/NTU201703166



Supplementary Figure 10. T-DNA insertion site in *npf5.15-1* mutant.

Experiment details are described in Supplementary Figure 1.

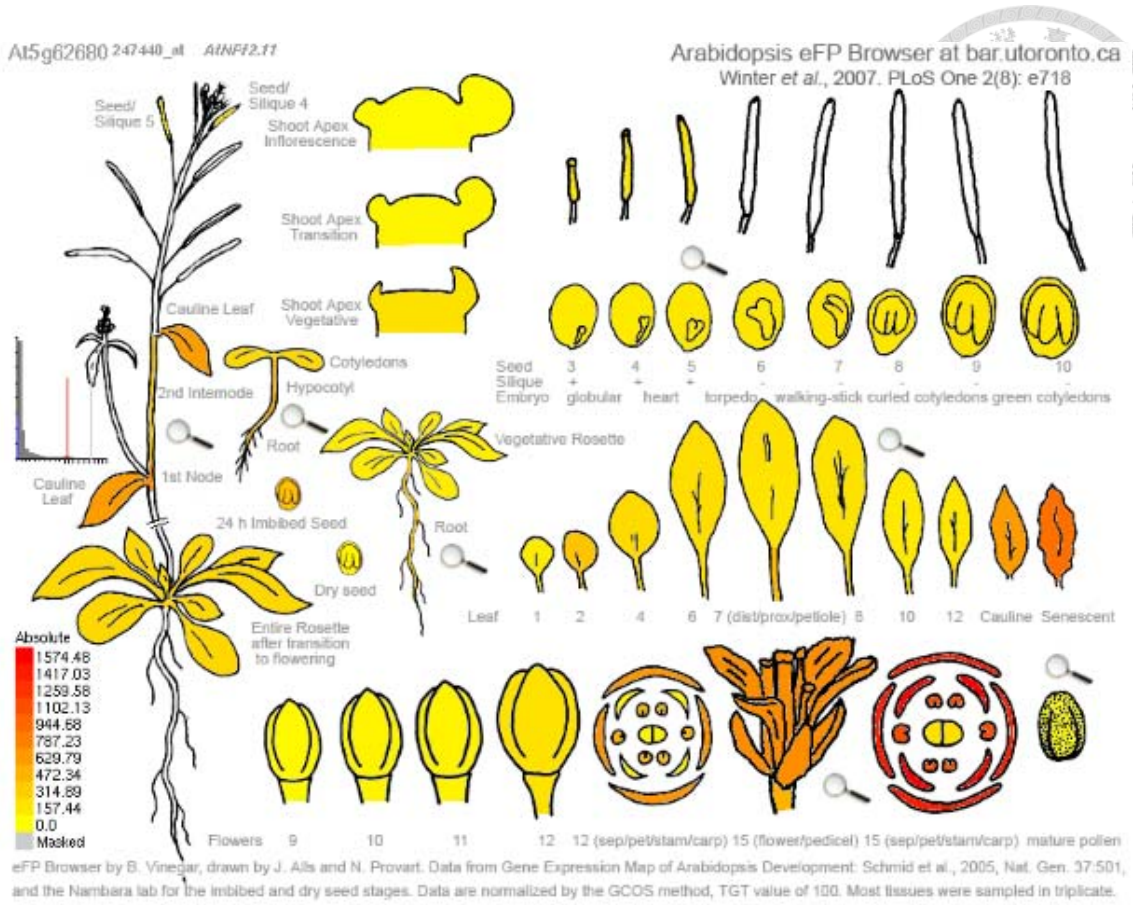
doi:10.6342/NTU201703166



Supplementary Figure 11. T-DNA insertion site in *npf6.1-1* mutant.

Experiment details are described in Supplementary Figure 1.

doi:10.6342/NTU201703166



Supplementary Figure 12. Pictographic data presented from the AtGenExpress Consortium (Arabidopsis e-FP Browser).

Tissue-specific gene expression level is shown in the graphic illustration, including root, leaf, silique, flower and other tissues. In this figure, GTR2 (NPF2.11/NRT1.10) is shown as an example. The old leaves are leaf number 1 and 2, the mature leaves are leaf number 4 to 8, and the young leaves are leaf number 10 and 12.

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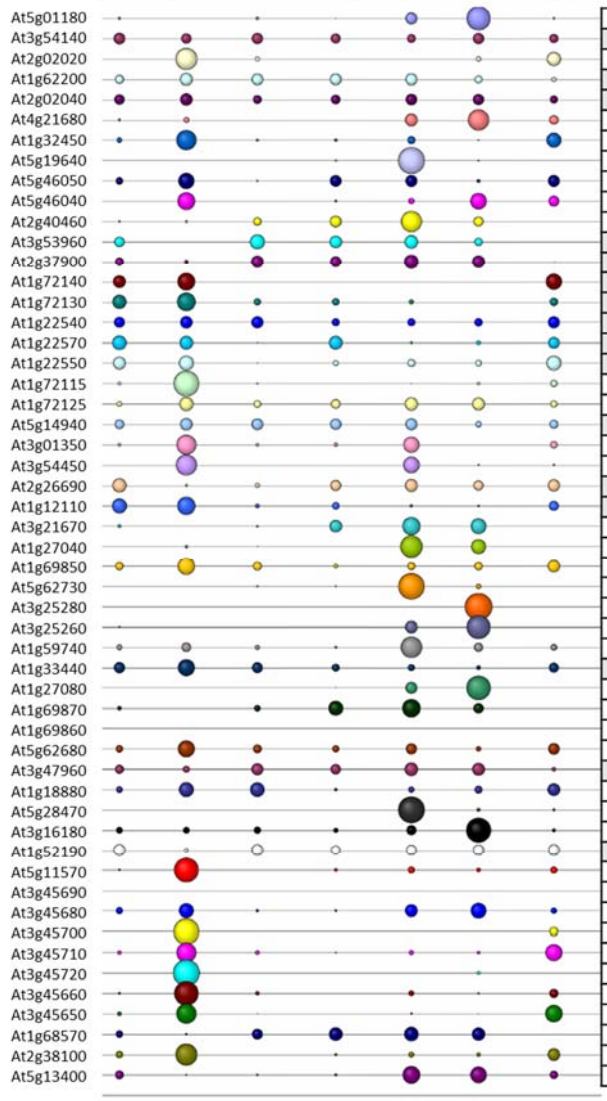

Shoot	Root	InfStem	CauLeaf	Flower	Silique	Seedling
1	0	1	0	20	77	1
18	14	17	13	10	17	11
0	66	3	0	0	4	27
11	22	17	18	20	8	3
14	21	10	12	18	17	8
1	4	0	0	22	61	12
4	55	1	1	8	0	30
0	0	0	0	99	0	0
7	35	0	18	19	2	19
0	42	0	1	5	37	16
1	1	9	17	59	13	0
15	0	30	21	26	9	0
10	2	20	17	28	22	0
22	43	0	0	0	0	35
27	47	7	8	3	0	9
15	21	19	8	8	9	20
28	25	0	24	1	2	18
22	30	0	4	8	6	29
2	90	1	0	0	1	7
4	26	7	12	24	22	7
12	21	18	17	19	5	10
1	54	1	2	34	0	7
0	61	0	0	38	1	1
26	1	3	15	23	13	19
30	45	3	7	1	1	13
2	0	1	20	45	32	0
0	2	0	0	68	30	0
9	41	9	3	8	9	21
0	0	0	0	96	4	0
0	0	0	0	0	100	0
0	0	0	0	22	78	0
4	11	3	1	64	11	6
17	39	15	8	7	3	13
0	0	0	0	19	81	0
3	0	6	30	47	14	0
0	0	0	0	0	0	0
7	38	10	6	16	4	18
11	6	19	14	25	22	2
6	30	29	1	5	9	21
0	0	0	0	97	2	1
4	4	4	2	10	75	1
19	2	17	12	16	17	18
1	82	0	2	6	3	6
0	0	0	0	0	0	0
7	31	1	1	22	34	5
0	90	0	0	0	0	10
2	52	3	0	3	1	39
0	98	0	0	0	2	0
1	82	2	0	4	0	10
3	56	0	0	1	0	40
7	1	16	25	27	24	0
7	65	0	1	4	2	20
10	1	1	1	42	36	9

Supplementary Figure 13. Tissue-specific expression pattern of 53 *NPF* members published in 2007 (Tsay et al., 2007).

The expression of genes was normalized with *UBQ10* and the sum of expression of all tissues for each gene was set as 100%. The expression level was shown as circles and the corresponding raw data was showed in the table. Genes marked with light grey were

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chosen based on the criterion that the value in the shoots at least had to be 40% in the most abundant tissue.

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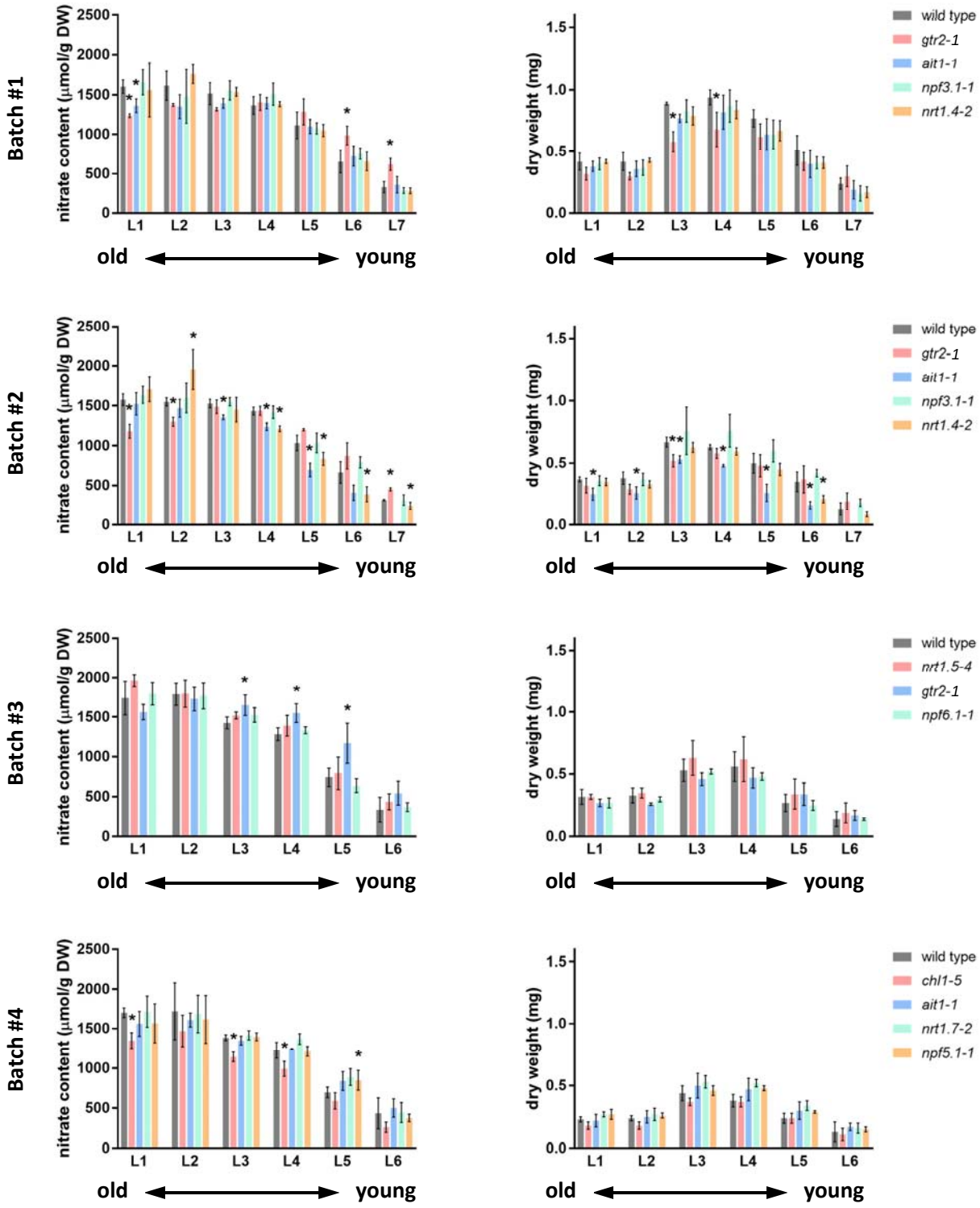

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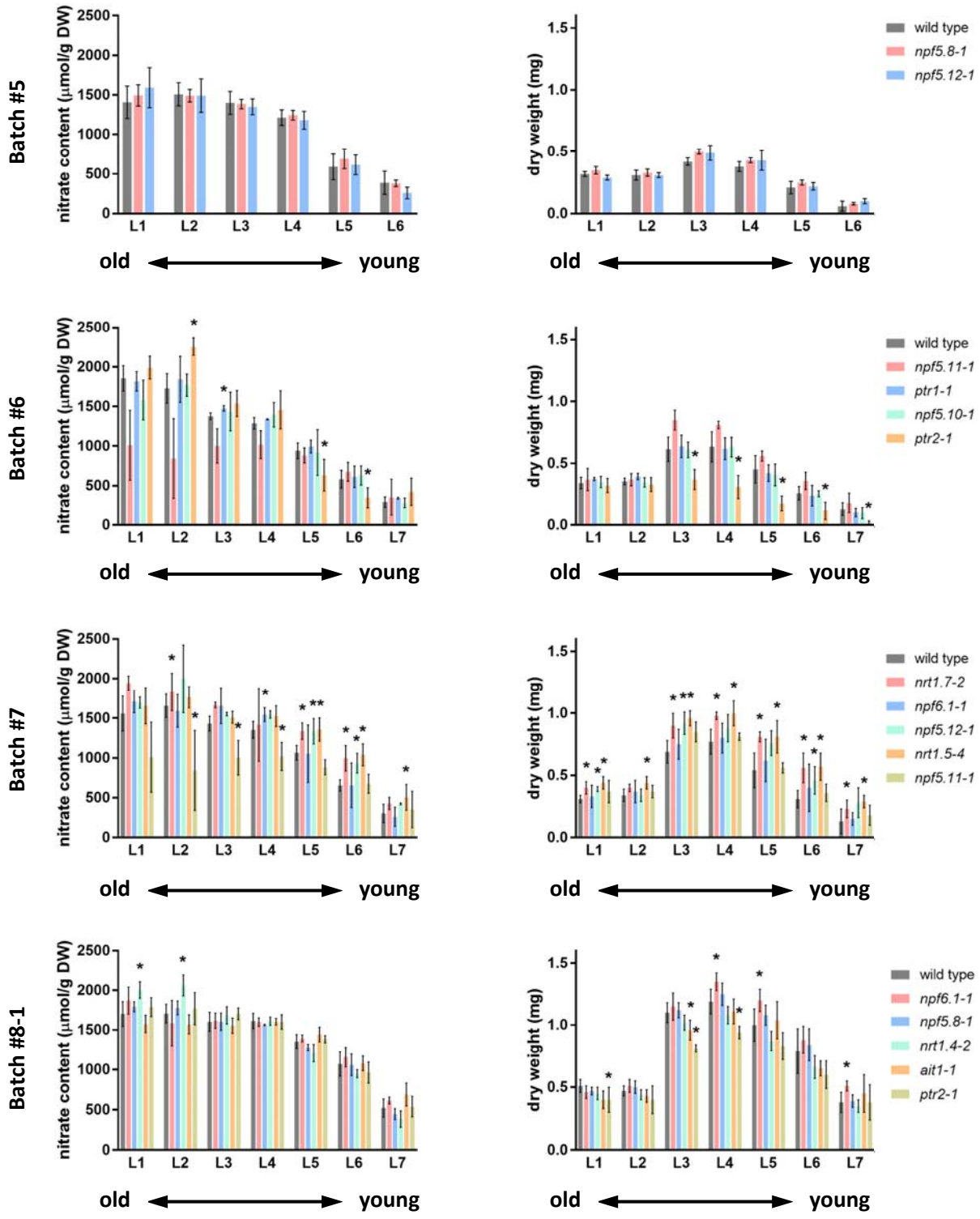

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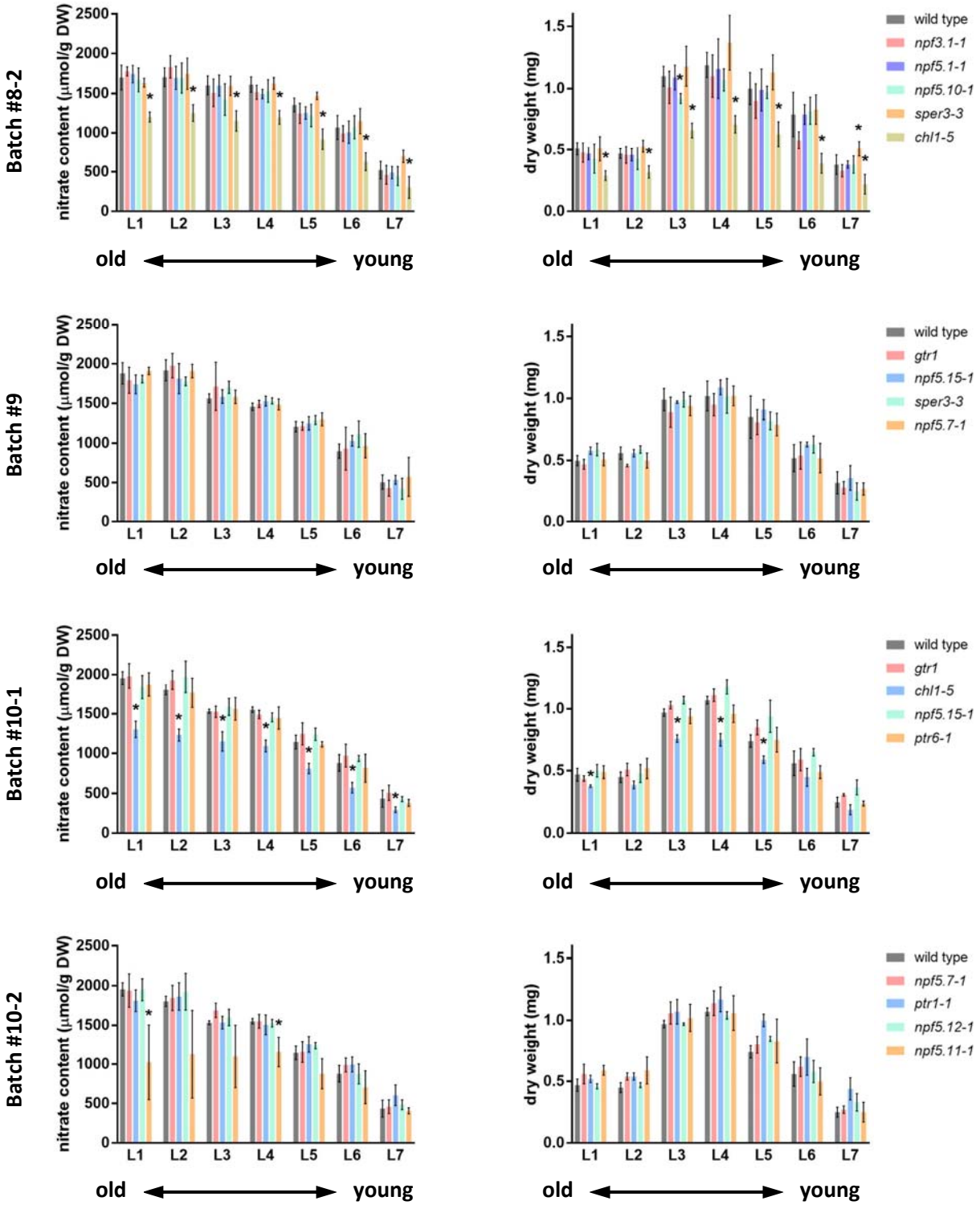

Supplementary Figure 14. Nitrate content and biomass analysis of *npf* mutants of ten batches.

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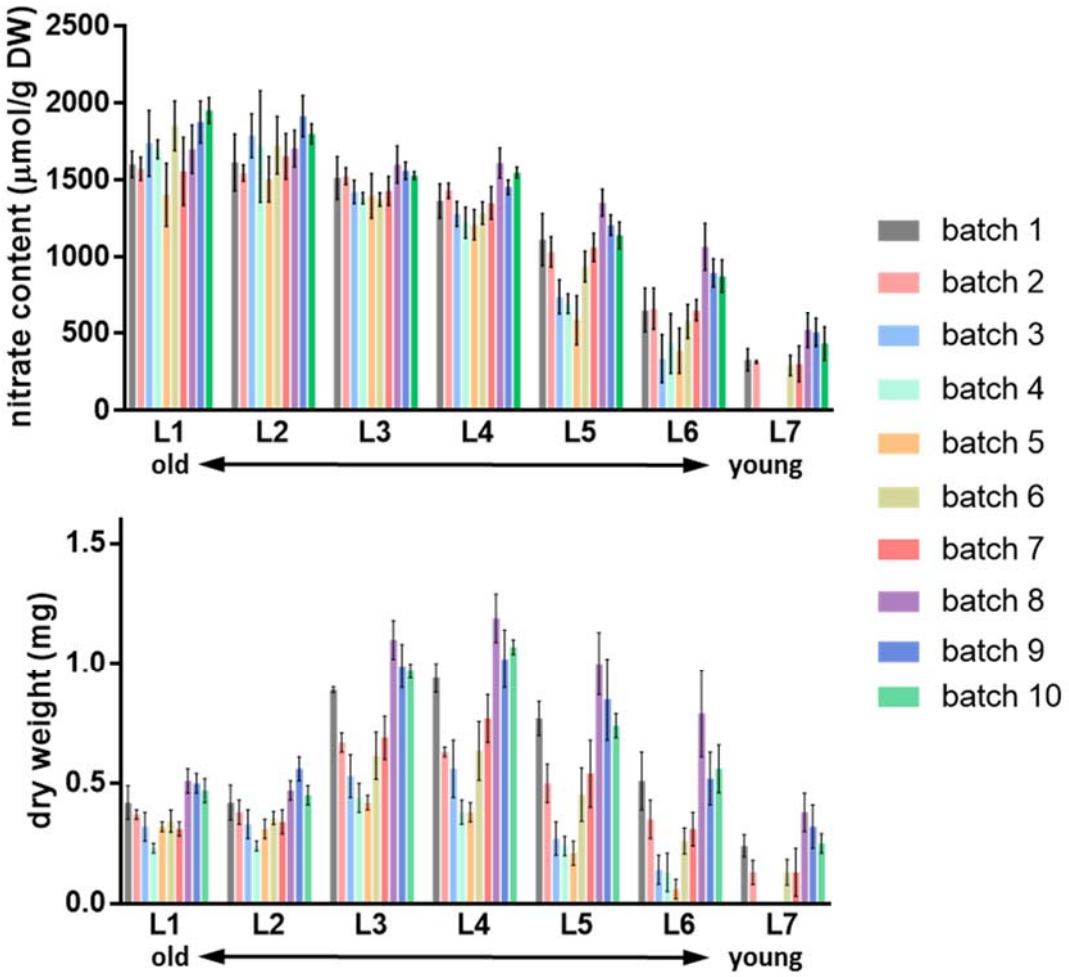
Left panels presented nitrate content and right panels showed the corresponding dry weight of wild type and *npf* mutants in ten batches. Plants were grown hydroponically with 2 mM KNO_3. Samples were collected at the 17^{th} day for batch 1-7 and at the 18^{th} day for batch 8. Each data represents the mean of 3 to 5 different plants and error bar is the standard deviation. (*, $p<0.05$, compared to wild type based on Student's t test)

doi:10.6342/NTU201703166



Supplementary Figure 15. Nitrate content and biomass analysis of wild type in ten batches.

Upper panel presented nitrate content (A) and lower panel showed corresponding dry weight (B) of wild type in ten batches. Plants were grown hydroponically with 2 mM KNO_3. Samples were collected at 17th day for all batches, except that samples in batch 8 was collected on 18th day. Each data represents the mean of 3 to 5 different plants and error bar is the standard deviation.

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