博士論文

The differential roles of NLPs in regulating nitrate-dependent growth in rice

（イネの異なる NLP の硝酸依存的な生育における役割）

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Contents

Abbreviations

N   nitrogen

NUE   nitrogen use efficiency

NRT   nitrate transporter

AMT   ammonium transporter

NIN   nodule inception protein

NLP   nodule inception protein-like protein

NR   nitrate reductase

NiR   nitrite reductase

NRE   nitrate-responsive *cis*-element

Na   sodium

P   phosphorus

B   boron

Mn   manganese

Ge   germanium

Cd   cadmium

Mo   molybdenum

Mg   magnesium

K   potassium

S   sulphur

Ni   nickel

Ca   calcium

Cu   copper

Co   cobalt

Introduction

Nitrogen is an essential macronutrient for plants and the limiting factor for growth and development when the supply is not sufficient. Nitrogen is an integral constituent of a number of biomolecules including proteins, nucleic acids, chlorophyll, co-enzymes, phytohormones and secondary metabolites (Crawford, 1995b). In addition to being an essential element, nitrogen itself also serves as a signaling molecule. For instance, it is known to regulate root architecture, stimulate shoot growth, delay flowering, regulate abscisic acid-independent stomata opening, and relieve seed dormancy (Walch-Liu et al., 2005). Furthermore, nitrate signaling and regulation is considered to be at the center of communications between plant intrinsic programs and the environment (Guan, 2017).

Nitrate and ammonium are the major forms of inorganic N in soil taken up by the root of higher plants. Nitrogen concentration in soil varies largely and the ratio of nitrate and ammonium is under a strong influence of soil redox status and microorganisms. Plants have evolved multiple strategies to cope with wide variation in the types and concentrations of soil nitrogen (Gaudin et al., 2011, Kant et al., 2011a). As most soil on the earth are aerobic, nitrate is a primary N source (Crawford and Forde, 2002). Rice are often grown under anaerobic conditions and under anaerobic conditions, ammonium becomes dominant form of inorganic nitrogen.

Rice is among the major crops in the world. Large amount of chemical nitrogen fertilizers is used globally to increase rice yield every year. But only 30% of the applied N is taken up by rice (Yi et al., 2018). The remaining 70% N is lost either through leaching out of the soil or in the gaseous forms into the atmosphere, both of which cause environmental pollution. In order to understand nitrogen signaling mechanisms and improve N use efficiency (NUE) of crop plants, nitrogen pathway including nitrogen transport, nitrogen assimilation and interaction with other signaling have been widely studied in recent years. The functions of some genes involved in nitrate signaling and response pathways have been characterized in details.

In the introduction, I will summarize the current knowledge of nitrate signaling and regulation network, the biological functions of nitrate related genes in rice, the role of NLPs in some model plants and present situation of ecological agriculture.

1. The importance of Nitrogen

After carbon, nitrogen (N) is the element required in the largest amount by plants: about 1-5% of total plant dry matter consists of nitrogen. Nitrogen is the mineral nutrient needed in greatest abundance by plants, which is necessary for growth and development (Crawford, 1995a).

1.1 The functions of nitrogen in plant

In plant, nitrogen is a primary constituent of the nucleotides, chlorophyll, proteins, co-enzymes phytohormones and secondary metabolites that are essential for life. Nitrogen is involved in many adaptive responses of plants, such as root nodule symbiosis, localized proliferation of roots, flowering or stomatal movements.

Nitrate itself also functions as a signal, and affects plant growth and development and disease resistance. For example, in *Arabidopsis thaliana*, nitrate acts as a signal at the shoot apical meristem and activates AtNLP6/7 to regulate flowering time via controlling the expression of SOC1 (SUPRESSOR OF OVEREXPRESSION OF CONSTANS 1) (Olas et al., 2019). On the other side, activation of nitrate signaling molecules may crosstalk with other signaling molecule (Ca^{2+}) and phytohormone (Singh et al., 2019). Totally, the functions of nitrogen are of great significance and also quite complicated.

1.2 The phenotypes of plants in different nitrogen conditions

Higher plants have evolved several mechanisms to adapt to changes in nitrogen supply and to use a variety of forms of nitrogen (Crawford, 1995a, Drechsel et al., 1990). For example, the localized proliferation of lateral roots occurs in nitrate-rich patches (Robinson, 1994). In addition to short-term fluctuations in the nutrient supply to the root, long-distance nitrogen signals arising in the root can provide the shoot with an early warning of fluctuations in external nitrogen concentrations or forms. And signals in the reverse direction are needed to ensure that root physiology and development are integrated with the nitrogen nutrient status of the whole plant (Forde, 2002b). For example, the rapid decline in leaf expansion rates that follows withdrawal of nitrate from the roots (McDonald and Davies, 1996). The recycling of amino acids between shoots and roots could provide a mechanism for communicating changes in the N status of the shoot (Cooper and Clarkson, 1989, Imsande and Touraine, 1994).

In *A. thaliana*, the accumulation of nitrate in shoot negatively affects the outgrowth of the laterals by inhibiting auxin biosynthesis or its transport to the root. But high sucrose concentration can overcome the inhibitor effect of nitrate in accordance with the plant's N/C balance (Zhang et al., 1999, Forde, 2002a). In legumes, nitrate is also influencing the establishment of beneficial plant-microbe association. High levels of nitrate inhibit the production of flavonoids, rhizobial infection, nodule initiation, nodule growth and nitrogen

fixation activity, while nodule senescence or disintegration are accelerated (Streeter, 1988, Kanayama and Yamamoto, 1990, Coronado et al., 1995, Matamoros et al., 1999, Nishida and Suzaki, 2018). In rice, excessive nitrogen inhibits root growth and promotes shoot growth, which means that the ratio of shoot to root increases (Ericsson, 1995, Fichtner and Schulze, 1992). The elongation of seminal and lateral roots of rice seedlings are markedly inhibited by high exogenous ammonium concentrations but not high exogenous nitrate levels (Hirano et al., 2008). The accumulation of ammonium significantly enhanced glutamine synthetase (GS) activity in roots, inducing abundant accumulation of glutamine (Gln) in shoots (Hirano et al., 2008). At millimolar concentrations, ammonium causes toxicity that is associated with ionic imbalances, disturbance of pH gradients across plant membranes or oxidative stress (Britto and Kronzucker, 2002, Li et al., 2014, Liu and von Wiren, 2017). Furthermore, many perennial plants can store large quantities of nitrogen rather than use them directly for growth. The resources may be stored to compete with their neighbours (Heilmeier et al., 1986) or bridge temporal gaps that exist between resource availability and resource demand (Bloom et al., 1985, Chapin et al., 1990).

1.3 Nitrogen use efficiency in agricultural systems

The concept of nitrogen use efficiency (NUE) itself is complex and indistinct. A number of reviews have summarized broader aspects of NUE, including N uptake efficiency (NUpE), N utilization efficiency (NUtE), N physiological use efficiency (NpUE), N transport efficiency (NTE), N remobilization efficiency (NRE), apparent N recovery rate (Broxmeyer et al.,1989) and agronomy efficiency of fertilizer N (AE) (Fageria and Baligar, 2005, Garnett et al., 2009, Glass et al., 1992, Good et al., 2004, Hirel et al., 2007, Masclaux-Daubresse et al., 2010, Robertson and Vitousek, 2009). Generally, among these sub-definitions, two plant physiological components, NUpE and NUtE contribute to plant NUE (Xu et al., 2012b).

Most non-legume plants need 20-50g nitrogen to produce 1 kg of dry biomass. In most agricultural cropping systems, the limited nitrogen in soil is the main reason responsible for low crop yields (Robertson and Vitousek, 2009). In nature, the biological conversion of N_2 in the air to plant-available ammonium by symbiotic bacteria is the major source of N input. The global annual N inputs through this way in agricultural systems total around 50-70 Tg (Herridge et al., 2008). But the amount doesn't reach the standard requirement in agricultural systems. During the past six decades, the application of chemical nitrogen fertilizers has increased global food production greatly (Good et al., 2004, Ju et al., 2009). For most crops, N fertilizers is the main effector for annual yield. Nevertheless, the benefits of N fertilizers added to cropping systems come with noticeable energy and environmental costs. Excess N compounds released from cropping systems threaten the quality of air, water

and soil (Guo et al., 2010, Robertson and Vitousek, 2009). N pollution has become the most serious problem in agriculture systems. Improving NUE is a crucial challenge faced with in the whole world.

2. Nitrogen signal

Plants display a high degree of physiological and development plasticity in response to changing nutritional conditions requiring the operation of both short- and long-range signaling pathways. In general, excessive nitrogen inhibits root growth and promotes shoot growth. And in hydroponic experiments, rice plants have shorter roots in ammonium condition compared with in nitrate condition. Some nutritional root responses are widely reported. For example, the localized proliferation of lateral roots that occurs in nitrate-rich soil patches (Robinson, 1994), and the rapid decline in leaf expansion rates induced by withdrawal of nitrate from roots (McDonald and Davies, 1996).

In the micro molar concentration range, ammonium promotes plant growth, while at high external supplies it often causes toxicity (Britto and Kronzucker, 2002). Ammonium toxicity inhibits root and shoot growth that is associated with leaf chlorosis and related to ionic imbalances, disturbance of pH gradients across plant membranes and oxidative stress (Gerendas et al., 1997, Britto and Kronzucker, 2002, Bittsanszky et al., 2015, Esteban et al., 2016). While the presence of ammonium inhibits the elongation of primary and lateral roots, localized ammonium supply to N-deficient plants strongly stimulates lateral root branching (Drew, 1975, Li et al., 2010b, Araya et al., 2016). If only a part of the root is exposed to elevated ammonium, plant will induce ammonium uptake and ammonium detoxification in different root parts simultaneously (Giehl and von Wiren, 2014). In addition, a number of studies have revealed that ammonium triggers multiple physiological and morphological responses independent of ammonium assimilation (Patterson et al., 2010, Li et al., 2010b, Lima et al., 2010). So ammonium itself can act as a signaling molecule.

In soil, nitrate and ammonium concentrations range from lower than 100 μM to higher than 10 mM (Miller et al., 2007b). In aerobic soils, the major form of inorganic N is nitrate while in flooded wetland or acidic soils, the major form is ammonium (Xu et al., 2012a). In fact, the growth and yield of most plant species are superior under mixed nitrate – ammonium with growth on either N source on its own. (Kirk and Kronzucker, 2005a). And ammonium uptake occurs before nitrate uptake (Sasakawa and Yamamoto, 1978). When nitrate and ammonium were provided together at the same total N concentration as in the single N species experiments, the uptake and assimilation of nitrate were repressed, but those of ammonium were stimulated (Kirk and Kronzucker, 2005a). Because very little free NH_4^+ is translocated to the shoot in rice (Kronzucker et al., 1998, Britto and Kronzucker, 2004), this indicates that nitrate can enhance ammonium assimilation in some way.

Totally, plants have evolved a very complex nitrogen signal pathway to adapt to the levels and types of N species received. The transport and assimilation of nitrate and ammonium are introduced in this part separately.

2.1 Nitrogen transport system in plant

In order to satisfy total demand for nitrogen, plants have evolved an active and multiphasic nitrate transport system. Nitrate uptake is an energy-consuming process which is driven by electrogenic proton co-transport. During nitrate uptake, the plasma membrane endures a speedy and transient depolarization, becoming up to 60 mV more positive inside the cell within 1 to 2 min (Ullrich and Novacky, 1981, Ullrich and Novacky, 1990, Ullricheberius et al., 1981). The initial uptake of nitrate occurs across the plasma membrane of epidermal and cortical cells of the root. Then nitrate is transported into the vacuole and the cells in vascular system and leaf (Miller and Smith, 1992). Both nitrate uptake and ammonium uptake increase during the day with a maximum at the end of the light period after which the uptake decrease (Gazzarrini et al., 1999). In addition, nitrate uptake is decreased by oxygen (Sasakawa and Yamamoto, 1978).

Plants have distinct transport systems with different affinities for nitrate. The high-affinity transport system is responsible for nitrate uptake at low concentration (below 1 mM). And the low-affinity transport system is accountable for inverse situation (above 1 mM) (Crawford, 1995a). In order to understand nitrate responses in plant, identifying and characterizing the function of the different players involved in nitrate signaling uptake, compartmentation, translocation, and remobilization and their functional relationships have been reported widely (Dechorgnat et al., 2011). And long-distance transport and root storage pools are thought to provide negative feedback signals regulating root uptake (Tang et al., 2012).

2.1.1 The family of nitrate transporter 1 (NRT1)

In *A. thaliana* genome, there are 53 members belonging to the NRT1 family (low-affinity transport system) (Miller et al., 2007a). CHL1 (AtNRT1.1) is the first reported nitrate transporter in higher plants, which has been shown to be a dual-affinity nitrate transporter and a sensor of external nitrate supply concentration (Liu et al., 1999, Wang et al., 1998, Liu and Tsay, 2003, Ho et al., 2009, Gojon et al., 2011) and an auxin transporter at nitrate-sufficient condition (Krouk et al., 2010b).CIPK23 can phosphorylate the Thr101 in AtNRT1.1 to elevate its nitrate affinity under low nitrate concentrations (Ho et al., 2009). *AtNRT1.1* is expressed in the epidermis in the root tip, but in the cortex and external half of the endodermis in other regions. In contrast, AtNRT1.2 is a constitutively expressed low-affinity nitrate transporter and mRNA is found only in epidermis (Tsay et al., 2007). *AtNRT1.3* is preferentially expressed in parenchymal tissues. (Tong et al., 2016). The induction of *AtNRT1.3* expression by nitrate or light is detected only in shoots. *AtNRT1.3* may play a role in the final step of nitrate supply to

photosynthesizing cells (Tong et al., 2016, Okamoto et al., 2003). AtNRT1.4 is required for petiole nitrate storage. *AtNRT1.4* is expressed in the leaf petiole and may be responsible for nitrate homeostasis in leaves (Chiu et al., 2004). AtNRT1.5 is a pH-dependent bidirectional nitrate transporter, deriving root-to-shoot nitrate transport. *AtNRT1.5* is expressed in root pericycle cells close to xylem and acts in root xylem loading of nitrate (Lin et al., 2008). AtNRT1.5 is also involved in modulating the response to K^+ deprivation affecting root development and K^+ transport (Cui et al., 2019). AtNRT1.6, only detectable in reproductive tissue, is involved in delivering nitrate from maternal tissue to the developing embryo (Almagro et al., 2008). AtNRT1.7 is responsible for phloem loading of nitrate in the source leaf to allow nitrate transport out of older leaves and into younger leaves (Fan et al., 2009). AtNRT1.8 is expressed in xylem parenchyma cells within the vasculature (Li et al., 2010a), while *AtNRT1.9* is expressed in companion cells of the root phloem. Both are involved in regulating root-to-shoot nitrate translocation (Wang and Tsay, 2011). In addition, *AtNRT1.8* is the only nitrate transporter gene that is strongly up-regulated by cadmium stress in roots (Li et al., 2010a). AtNRT1.11 and AtNRT1.12 are plasma membrane transporters expressed in the companion cells of the major vein, transferring root-derived nitrate into phloem (Hsu and Tsay, 2013).

Rice has almost 80 genes in the *NRT1* family. OsNRT1, a member of a growing transporter family called PTR, is a homolog of AtNRT1 and identified as a low-affinity nitrate transporter in rice (Lin et al., 2000). OsNRT1 is constitutively expressed in the most external layer of the root, epidermis and root hair (Lin et al., 2000). *OsNRT1.1a* has differential functions with *AtNRT1.1* and up-regulates the expression of N utilization-related genes not only for nitrate but also for ammonium, as well as flowering-related genes (Wang et al., 2018). In contrast, *OsNRT1.1B* is the functional homolog of *AtNRT1.1*, functioning in nitrate uptake, transport, and signaling (Hu et al., 2015).

2.1.2 The family of nitrate transporter 2 (NRT2)

In the *A. thaliana* genome, there are at least 7 members belonging to the NRT2 family (high-affinity transport system) (Tsay et al., 2007). AtNRT2.1 and AtNRT2.2 have been characterized as key root transporters, responsible for 80% of HATS (Filleur et al., 2001). In addition, AtNRT2.1 transport activity requires a second accessory protein, a nitrate assimilation related protein AtNAR2.1 (also known as AtNRT3.1) (Kotur et al., 2012, Okamoto et al., 2006, Orsel et al., 2006). Five other members (AtNRT2.3 to AtNRT2.7) and AtNRT1.1 contribute to the remaining 20%. *AtNRT2.4*, induced both in shoot and root by N starvation, is expressed in the epidermis of lateral roots and in or close to the shoot phloem (Kiba et al., 2012). Interestingly, *ATNRT2.7*, expressed highly in reproductive organs and dry seeds, plays a specific role in nitrate accumulation in seeds (Chopin et al., 2007).

Since the nitrate concentration in the rhizosphere of paddy field is less than 10 μm (Kirk and Kronzucker, 2005b), OsNRT2 family play a major role in nitrate transport in rice. Five *OsNRT2* genes have been identified in rice. *OsNRT2.1* and *OsNRT2.2* have high similarity to the *NRT2* genes of other monocotyledons, while *OsNRT2.3* and *OsNRT2.4* are more closely related to *AtNRT2* genes (Tang et al., 2012, Cai et al., 2008). *OsNRT2.1*, a root-specific key nitrate transporter gene, is most strongly induced in coleoptiles following nitrate supply initiation, while other *OsNRT2s* show modest induction (Takayanagi et al., 2011). Recently, it has been reported that overexpression of *OsNRT2.1* can increases yield and manganese accumulation under alternating wet and dry conditions (Luo et al., 2018). *OsNRT2.3a* is expressed mainly in xylem parenchyma cells of the stele of nitrate-supplied roots. Knockdown of *OsNRT2.3a* does not affect nitrate uptake and the expression of some root nitrate transporter genes (*OsNRT2.3b*, *OsNRT2.4* and *OsNAR2.1*). OsNRT2.3a is not directly involved in nitrate uptake by roots and plays a role in long-distance nitrate transport from roots to shoots at low nitrate conditions (Tang et al., 2012). In contrast, *OsNRT2.3b* is expressed abundantly in the phloem, with no effect of the N form and concentration on the amount of transcript and has a regulatory motif on the cytosolic side that acts to switch nitrate transport activity on or off by a pH-sensing mechanism (Feng et al., 2011a). Furthermore, high *OsNRT2.3b* expression in rice enhances the N, Fe, and P uptake of the plant (Fan et al., 2016, Feng et al., 2017). OsNAR2.1 can interact with OsNRT2.1, OsNRT2.2, and OsNRT2.3a and affect the activities of high- and low- affinity transporters (Yan et al., 2011). *OsNAR2.1*, *OsNRT2.1*, *OsNRT2.2*, and *OsNRT2.3a* are up-regulated by nitrate and suppressed by ammonium and high root temperature (37 °C). Root transcripts of *OsNRT2.3b* and *OsNRT2.4* are not affected by temperature. Expression of *OsNRT2.4* responds to changes in auxin supply unlike all the other *OsNRT2* genes (Feng et al., 2011b). OsNRT2.4 is supposed to work as a dual-affinity nitrate transporter. OsNRT2.4 does not have a nitrate efflux or an IAA influx activity. In rice roots, OsNRT2.4 is expressed mainly in the base of lateral root primordia. Knockout of *OsNRT2.4* decreases lateral root number and length, but does not affect rice growth and N uptake under conditions without N or with only ammonium supply (Wei et al., 2018).

2.1.3 Ammonium transporters

The ammonium transporters/methylamine permease/Rhesus (AMT/MEP/Rh) family mediate high-affinity ammonium transport. In *A. thaliana*, AtAMT2;1 is the only member of the MEP subfamily, while five homologs, AtAMT1;1 to AtAMT1;5, constitute the AMT clade (Ludewig et al., 2001). Transport activity of AtAMT1;1 is regulated via phosphorylation of a highly conserved threonine residue in its C-terminal domain (Loque et al., 2007). Trans-inactivation by C-terminal phosphorylation functions also extends to AtAMT1;2 (Neuhauser et al.,

2007) and AtAMT1;3 (Yuan et al., 2013). In the upstream, CIPK23 (Calcineurin B-like Interacting Protein Kinase23) can interact with AtAMT1;1 and AtAMT1;2 to inhibit ammonium transport (Straub et al., 2017). HY5 (Long Hypocotyles 5), a key transcription factor in *A. thaliana*, negatively regulates the *AtAMT1;2* under all nitrogen and light conditions (Huang et al., 2015). The prominent role of AtAMT1;3 is to promote ammonium-dependent higher-order root branching (Lima et al., 2010). Under nitrogen-deprived or ammonium-sufficient conditions, AtAMT1;3 oligomers show a relatively long-lived residence on the plasma membrane surface of root cells, while AtAMT1;3 proteins cluster disappear immediately after ammonium addition (Wang et al., 2013a). It is supposed that AtAMT1;1 and AtAMT1;3 serve as core components in different signaling pathways. AtAMT1;2 is also involved in uptake of ammonium from the apoplast for radial transport of ammonium. AtAMT1;4 is specifically expressed in pollen and mediates uptake of ammonium into the pollen grains (Yuan et al., 2009). AtAMT2;1 functions mainly in root-to-shoot translocation of ammonium. Under ammonium supply, the transcript levels of AtAMT2;1 increases and its promoter activity shifts preferentially to the pericycle (Giehl et al., 2017). Rice OsAMT family contains at least 10 *OsAMT*-like genes, three each for *OsAMT1*, *OsAMT2* and *OsAMT3*, respectively, and one for *OsAMT4* (Suenaga et al., 2003, Hoque et al., 2006). *OsAMT1;1* is known to be a prominent member showing a constitutive expression pattern in shoots and roots and its expression is ammonium responsive (Sonoda et al., 2003a). OsAMT1.1 is located in the plasma membrane and is mainly expressed in the root epidermis, stele and mesophyll cells. The *osamt1.1* mutant showed an increase in the potassium absorption rate under high ammonium condition and a decrease under low ammonium condition (Li et al., 2016). Transgenic rice lines overexpressing *OsAMT1;1* had the same root structure as the wild type but had 2-fold greater NH_4^+ permeability than the WT (Ranathunge et al., 2014). Taken together, OsAMT1.1 significantly contributes to the NH_4^+ uptake under both low and high NH_4^+ conditions and plays an important role in N-K homeostasis in rice. The expression of OsAMT1;2 is root-specific and ammonium-inducible while that of OsAMT1;3 is root-specific and nitrogen- suppressible (Sonoda et al., 2003b). And AMT3;1 is supposed to be the prime plant transporter involved in the mycorrhizal ammonium transfer in five model grasses (Koegel et al., 2017). The expressions of some ammonium transporters are under the control of a key transcription factor IDD10 (INDETERMINATE DOMAIN 10) (Xuan et al., 2019).

2.1.4 Nitrite transporter

The transfer of nitrite is a process requiring photochemical energy (Anderson and Done, 1978). Nitrite must be transferred immediately to the illuminated chloroplast stroma to prevent accumulation of toxic nitrite in the cytosol (Hoff et al., 1994). Since 2000, Rexach *et al.* found several nitrite transporters in *Chlamydomonas*

including NAR1 (Rexach et al., 2000). At the present time, there are a few report about nitrite transporter in higher plants. In higher plants, a NiTR gene is first reported in cucumber (Sugiura et al., 2007). In *A. thaliana*, a nitrite transporter named as CsNitr1-L, which is located in the inner envelop membrane of chloroplasts, is identified. CsNitr1-L, a member of the proton-dependent oligopeptide transporter (Potter and Talley) family, is an efflux-type nitrite transporter, enhancing the efflux of nitrite from the cell (Sugiura et al., 2007). Up to now, no definite nitrite transporter is known in rice. But a natural strain of *japonica* rice can accumulate a large amount of leaf nitrite and overexpression of *CsNitr1-L* in this strain reduced leaf nitrite concentration to one-third that in the untransformed rice (Sustiprijatno et al. 2006). In the genome of rice, there three genes (*AK070558*, *AK120596* and *AK110441*) encoding CsNir1-L homologs with putative nitrite or peptide transporters. To utilize nitrite efficiently, rice might have developed various nitrite transporters with different affinities.

2.2 Nitrogen assimilation mechanism

Plants must reduce nitrate to carbon-binding organic forms such as amino acids. Such activities are energy-consuming reactions depend on photosynthesis (Oaks et al., 1972, Yoneyama et al., 1977). In many plants, both shoots and roots are capable of nitrate reduction, and roots may reduce between 5% and 95% of nitrate taken up depending on various factors like level of nitrate supply, plant species and plant age (Andrews, 1986). In general, when the external nitrate supply is low, a high proportion of nitrate is reduced in the roots (Scheurwater et al., 2002). In natural conditions, the nitrate acquired by roots is transported to shoots before being assimilated usually and nitrate assimilation occurs mostly in leaf mesophyll cells (Smirnoff and Stewart, 1985).

Overall, the mechanisms of nitrate-to-glutamate transformation involve the sequential reduction and assimilation of nitrate to nitrite, ammonium, glutamine, and then glutamate. In the past 10 years, detailed molecular mechanisms of nitrogen assimilation have been established. In short, after absorption, nitrate is transferred from root epidermal cells to the cytosol of leaf cells via vascular tissues. Nitrate is stored in the vacuole or directly reduced to nitrite by nitrate reductase (NR). Then nitrite enters the chloroplast or plastid in root and reduced to ammonium by nitrite reductase (NiR) (Crawford, 1995b, Yoneyama and Suzuki, 2019).

2.2.1 Nitrate reductase

Nitrate reductases (NRs), cytosolic enzymes, catalyze the reduction of nitrate to ammonium. These enzymes function as homodimers, each with three co-factors (the Mo center, the Fe-heme of the cytochrome b5 domain and a C-terminal domain associated with a flavin adenine nucleotide (FAD) cofactor) covalently bound to specific domains (Hille, 1996). The Mo containing domain is responsible for the dimerization of NR and FAD transfers

electrons from NADH/NADPH to nitrate (Fischer et al., 2005, Campbell, 2001). NR protein has a half-life of only a few hours and is absent in plants not receiving nitrate (Patterson et al., 2010)

Most plant species have two nitrate reductase (*NIA*) genes which are expressed in shoots and roots (Crawford and Arst, 1993). In *A. thaliana*, expression of *NIA1* and *NIA2* is induced by nitrate and ammonium (Konishi and Yanagisawa, 2011) while NR protein levels are only higher in nitrate-treated leaves and roots (Kim et al., 2018). Additionally, the concentration of NR is increased by light, sucrose and cytokinin, whereas glutamine represses NR (Krapp et al., 1998). The methylation of *NIA2* is inhibited by ammonium, whereas that of *NIA1* is not affected by ammonium treatment (Kim et al., 2016). Furthermore, the expression of *NIA2* is positively controlled by HY5 and HY5 homolog (Jonassen et al., 2009), but NR activity is negatively regulated by sumoylation via the E3 ligase AtSIZ1 and a ring finger type E3 ubiquitin ligase (COP1). The function and stability of NR proteins might be post-translationally modulated by ubiquitination (Kim et al., 2018). It is also reported that NIA1 and NIA2 determine NO production in plants and are critical to ABA-induced stomatal closure by altering genes of core ABA signaling components (Zhao et al., 2016).

2.2.2 Nitrite reductase

Nitrite reduction mainly occurs in leaves. Nitrite reductase (NiR) localizes in the chloroplasts in leaves, in the pro-plastids of roots and other non-green tissues. A ferredoxin-binding domain, an iron-sulphur cluster and a siroheme co-factor bind to NiR. The electrons from reduced ferredoxin are passed to nitrite (Bowsher et al., 2007).

Nitrite reductase is encoded by a single gene (*NIR*) in higher plants (Rastogi et al., 1997, Kant et al., 2011b). To avoid the accumulation of nitrite which is toxic to plants cells, both NR and NiR activities are regulated by several mechanisms at different levels, such as enzyme synthesis, degradation and reversible inactivation. The expression of *NIR* is induced by nitrate (Estuardo et al., 2008).

Except the simple linear pathway, nitrite is also the a substrate for different molybdoenzymes in NO production (Wang et al., 2015, Cantu-Medellin and Kelley, 2013, Li et al., 2009, Sparacino-Watkins et al., 2014). NO has been demonstrated to be a key signaling molecule in several plant processes including such as whole plant development and different plant stress responses (Wendehenne and Hancock, 2011, Mur et al., 2012, Farnese et al., 2016, Sanz-Luque et al., 2015).

2.2.3 Ammonium reduction

It has been proposed that a plant's tolerance to ammonium is related to its capacity for ammonium assimilation (Cruz et al., 2006). Ammonium derived from nitrate reduction or directly taken up by the roots is rapidly

assimilated to glutamine amide via the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle and then transported in an organic form to the shoot (Xu et al., 2012a). In this pathway, the amino acid glutamate acts as the acceptor for ammonium, forming the amide glutamine (Tobin and Yamaya, 2001). Glutamine synthetase (GS) catalyzes the ATP-dependent incorporation of ammonium to glutamate, then glutamate synthase (GOGAT) catalyzes the conversion of L-glutamine and 2-oxoglutarate into two molecules of L-glutamate (Saez et al., 2000).

GS exists in multiple enzyme forms located in the cytosol or in plastids with multiple metabolic functions (Ferrario-Mery et al., 2001, Bernard et al., 2008). GS is categorized into two groups: the cytosol-localized GS1 group and the GS2 group localized mainly in the chloroplasts (Swarbreck et al., 2011). It has been reported that GS2 could assimilate the ammonium derived from photorespiration (Wallsgrove et al., 1987) while GS1 isozymes assimilate non-photorespiratory ammonium (Tobin and Yamaya, 2001). In rice, *OsGS1;1*, *OsGS1;2* and *OsGS1;3* are identified. *OsGS1;1* is expressed in all organs tested with higher expression in leaf blades, while *OsGS1;2*, and *OsGS1;3* are expressed mainly in roots and spikelets at the early stage of ripening , respectively (Tabuchi et al., 2005, Tabuchi et al., 2007). *OsGS1;1* is important in the processes of nitrogen remobilization though the phloem from source organs and grain filling (Tabuchi et al., 2005). *OsGS1;2* is important in the primary assimilation of ammonium ions taken up by roots, with *OsGS1;1* in the roots unable to compensate for *OsGS1;2* functions (Funayama et al., 2013). *OsGS1:3* is probably important in grain ripening and/or germination (Yamaya and Kusano, 2014).

GOGAT are complex iron-sulfur flavoproteins containing functional domains involved in the control and coordination of their catalytic activities in annual plants (Suzuki and Knaff, 2005). In plants, there are two forms of glutamate synthases that differ in their electron donors, NADH-GOGAT (EC 1.4.1.14) and Fd-GOGAT (EC 1.4.7.1) (Garcia-Gutierrez et al., 2018). In addition, two NADH-GOGAT genes have been identified in rice (*OsNADH-GOGAT1* and *OsNADH-GOGAT2*). *OsNADH-GOGAT1* is important in the development of active tiller number and hence panicle number of rice while *OsNADH-GOGAT2* is apparently important in controlling spikelet number per panicle (Tamura et al., 2011). *OsNADH-GOGAT1* is mainly expressed in surface cells of rice roots in an ammonium-dependent manner, like the case of *OsGS1.2* (Ishiyama et al., 2004a, Hamada et al., 2011). Overall, OsGS1;2 and NADH-GOGAT1 are important in the primary assimilation of ammonium taken up by rice roots (Yamaya and Kusano, 2014).

3. RWP-RKs Proteins

In the genome of *Lotus japonicus* and pea, *NIN* gene is identified as a core symbiotic gene required for

establishing symbiosis between legumes and *Rhizobium*. The most conserved region in NIN protein is a 60-amino acid –sequencing named as RWP-RK domain (Schauser et al., 1999). In non- leguminous plants like *A.thaliana* and rice, homologous sequences are also identified (Schauser et al., 2005). These RWP-RKs have been classified in two sub-families, NLPs (NIN-like proteins) and RKDs (RWP-RK domain proteins). NLPs regulate tissue-specific expression of genes involved in nitrogen use efficiency (NUE) and RKDs regulate expression of genes involved in gametogenesis/embryogenesis (Kumar et al., 2018).

The secondary structure of RWP-RK domain is α-helical basic region followed by a helix-turn-helix and a helical leucine zipper, spaced by loops (Schauser et al., 1999). RWP-RK domain is plant specific and has the function of DNA binding and protein dimerization (Riechmann et al., 2000). RWP-RK proteins acting as transcription factors have been widely reported (Waki et al., 2011, Suzuki et al., 2013, Konishi and Yanagisawa, 2013a). In addition to the RWP-RK domain, NLPs contain a PB1 domain at the C-terminus predicted to be a protein-protein interaction domain (Sumimoto et al., 2007) and a GAF-like domain that is not yet known (Grefen et al., 2008, Chardin et al., 2014).

3.1 The NLP family in *Arabidopsis thaliana*

In total, the *A. thaliana* genome encodes 14 proteins carrying a RWP-PK domain and 9 proteins belong to NLPs (AtNLP1-AtNLP9). The closet relative is AtNLP1 (Schauser et al., 2005). *AtNLPs* are expressed in almost all organs. *AtNLP8* and *AtNLP9* are mainly expressed in senescent leaves and seeds. *AtNLP8* expression is modified in response to many treatments while AtNLP9 only responses to specific stimuli like N treatments. And *AtNLP4* responses to heat stress (Chardin et al., 2014). AtNLPs can bind to the nitrate-responsive *cis*-element (NRE) in promoters of nitrate-responsive genes like *NIR1* and *NIA1* with transcriptional activator roles (Konishi and Yanagisawa, 2013b). It has been proved that the DNA-binding activity of the RWP-RK domain is unrelated to nitrate signaling. It is the N-terminal region is responsible for sensing nitrate signal and the activation of AtNLPs (Konishi and Yanagisawa, 2013b). AtNLPs activity is responsible for the expression of most nitrate-inducible genes, not only the nitrate assimilation process but also other nitrate-responsive processes (Konishi and Yanagisawa, 2013b). The post-translation activation of AtNLPs is a key step in mediating nitrate signaling to physiological and developmental events. The Ca^{2+}-sensor protein kinases (CPKs) are identified as master regulators in primary nitrate signaling and phosphorylate a conserved Ser205 in AtNLPs to convert AtNLPs from an inactive form to an active form (Liu et al., 2017b). And this induction is nitrate specific, not ammonium or glutamine (Yanagisawa, 2014b).

AtNLP7 is reported to regulate nitrate responses and works as a regulator of primary nitrate response

(Castaings et al., 2009b, Wang et al., 2009, Marchive et al., 2013b). The *atnlp7* mutants are impaired in transduction of nitrogen (Castaings et al., 2009a). And overexpression AtNLP7 improves plant biomass under both nitrogen-poor and –rich conditions with better-developed root system through enhancing photosynthesis rate and carbon assimilation (Yu et al., 2016). It is also reported that AtNLP7 controls of root cap cell release via the regulation of cell wall degrading enzymes, independent of columella cell identity and gravity sensing (Rucha et al., 2018). More interestingly, the localization of AtNLP7 is regulated by nitrate via a nuclear retention mechanism. AtNLP7 is mostly localized in the nucleus in the presence of nitrate while it is detected only in the cytoplasm in the absence of nitrate. Resupplying nitrate after N-starvation treatment AtNLP7 relocates into the nucleus within minutes (Marchive et al., 2013b). Furthermore, another transcription factor TCP20 (teosinte branched1/cycloidea/proliferating cell factora1-20) can interact with AtNLP6&7 via a bHLH-like domain. Under N starvation, TCP20-NLP6&7 heterodimers accumulate in the nuclei which regulate nitrate related genes and the mitotic cyclin gene *CYCB1;1* to counteract N-starvation stress (Guan et al., 2017b). In addition, the TCP20-NLP6&7 regulatory nexus link nitrate signaling and phytohormones, indicating signal of hormones are partly controlled by nitrate signaling (Guan, 2017).

AtNLP8 is essential for nitrate-promoted seed germination but nitrate doesn't regulate the mRNA level of AtNLP8 during seed germination. In details, AtNLP8 directly binds to the promoter region of *CYP707A2* (encoding an abscisic acid catabolic enzyme) to decrease ABA level and promote seed germination. Furthermore, AtNLP8 localizes to nuclei regardless of nitrate application not like the case of AtNLP7 (Yan et al., 2016b).

3.2 The NLP family in rice

The rice genome contains 6 OsNLPs and 4 annotated OsPKDs. Apart from the former two classes, a new class seems to have evolved in rice. But for now, nothing is known about the new class (Schauser et al., 2005). *OsNLPs* are expressed in almost all organs. *OsNLP1* is the closest rice homolog of *NIN* (Yokota et al., 2010). *OsNLP1* and *OsNLP3* are preferentially expressed in source organs. Expression levels of *OsNLP6* are very low in all organs (Chardin et al., 2014). *OsNLP3* is induced after germination and repressed after heat and submergence. *OsNLP4* is repressed by several abiotic stresses and induced by low P availability. *OsNLP6* expression is modified by N treatments (Chardin et al., 2014). For now, there is no report about the explicit pathway of NLP family in rice. OsNLPs is just predicated as central transcription factors of nitrate signaling, similar to AtNLPs in *A. thaliana,* but no experimental evidence has been reported. Recently, Hu et al reported that nitrate sensor OsNRT1.1b perceives nitrate signal and interacts with a phosphate signaling repressor

OsSPX4, ensuring N : P balance in rice. OsNLP3 is right under the control OsSPX4 (Hu et al., 2019).

3.3 The NLP family in other plants

In maize (*Zea mays* L.), nine *ZmNLP* genes are identified which have collinear relationships to the corresponding *OsNLPs* in rice (Wang et al., 2013b). The mRNA expression of *ZmNLP2.1, ZmNLP2.2, ZmNLP3.1, ZmNLP3.2, ZmNLP3.3* and *ZmNLP3.4* is induced by nitrate in maize roots, while *ZmNLP1.1 and ZmNLP1.2* expression is repressed. By contrast, *ZmNLP1.3* doesn't show any response to nitrate. The ZmNLP proteins are predicted to localize in the nucleus except ZmNLP3.4, which localize in the chloroplast/peroxisome (Wang et al., 2013b). One interesting thing is that there are two PB1 domains in ZmNLP1.2 and ZmNLP1.3 (Wang et al., 2013b). Overexpressing *ZmNLP3.1* in *A. thaliana atnlp7* mutant can restore the N-deficient phenotypes of *atnlp7* mutant (Wang et al., 2013b). In the downstream, the NRE domain is over-represented in the promoters of genes encoding nitrate transporters and ammonium transporters while the NRE is only reported in *NIR1* and *NIA1* in *A. thaliana* (Liseron-Monfils et al., 2013).

In *Lotus japonicus*, four *LjNLP* genes have been identified. Like all known NLPs, all LjNLP proteins have similar structure (Schauser et al., 2005, Yokota and Hayashi, 2011). LjNLP1 is closer to NIN than LjNLP2-4 in the phylogenic tree. NIN and LjNLP1 show similar ability for transcription activation but different nitrate-responsiveness *in vitro*. It is possible that NIN and LjNLPs regulate the expression of common genes in different cells in *L. japonicus* (Suzuki et al., 2013).

Purpose of this study

Till now, the clear functions of NLPs are only known in *A. thaliana*. In plants, homologous proteins might have evolved different molecular mechanisms between dicotyledons and monocotyledons. Rice is not only a model plant but also the most important crop in the world. However, the functions of OsNLP family are not well described. I initiated my Ph.D. study by analyzing *osnlp1*, *osnlp4* and *osnlp1/4* mutants, in order to understand the mechanisms of OsNLP family in rice growth under nitrate condition and provide a new sight on improving nitrogen use efficiency in paddy field.

Chapter 1. *OsNLP4* is a key gene regulating growth under nitrate condition in rice

Abstract

Nitrogen is a key nutrient in a number of physiological processes in plant. A number of nitrogen transporters and nitrate assimilation enzymes have been identified and functionally characterized. However, little is known about the nitrate sensor system and regulatory mechanisms of these nitrate related genes. In recent years, NIN-like proteins (NLPs) have been described as key regulators of nitrogen responses in *A. thaliana*. But the functions of OsNLPs in rice are still elusive.

I examined mRNA expressions of *OsNLPs* under four different nitrogen conditions (No nitrogen, Nitrate, Ammonium and Normal) and found that *OsNLP4* mRNA accumulation was increased significantly under nitrogen free condition, but not under other conditions. In a Tos-17 insertion line of *OsNLP4*, *OsNLP4* mRNA accumulation was reduced. The growth of the insertion line was reduced when nitrate was used as a sole source of nitrogen but not when ammonium was supplied, suggesting that *OsNLP4* is essential for growth under nitrate supply. These were reproduced in two CRISPR/Cas9 line of *OsNLP4*. In the *osnlp4* lines, mRNA accumulations of *NIA1* and *NIR1*, nitrate uptake rate, total N amount and NR activity were reduced, establishing that *OsNLP4* is responsible for these phenotypes. OsNLP4 was localized to nuclei and unlike AtNLP7, did not show nitrate-promoted nucleocytosolic shuttling, suggesting that OsNLP4 regulates nitrate responsive genes in a different mechanism from AtNLP7 of *A. thaliana*.

Results

OsNLP4 is essential for nitrate-dependent rice growth

To investigate the function of the rice NLP family, I obtained an _OsNLP4_ Tos-17 insertion line (_osnlp4-1_) which has a Tos-17 insertion in the first exon in the Nipponbare background (Figure 1-1a). A homozygous mutant line in terms of Tos-17 insertion in _OsNLP4_ were established and they were grown under the 2 mM KNO_3 (nitrate), 2 mM NH_4Cl (ammonium), 1 mM KNO_3 + 1 mM NH_4Cl (normal) and 2 mM KCl (nitrogen free) conditions for two weeks. When grown on the modified Kimura B solution in which nitrate was used as sole inorganic N source, the mutant line showed growth defects compared with that of wild type (WT) (Figure 1-1b, c). The _osnlp4-1_ plants grown hydroponically on 2 mM nitrate showed shorter shoot length and root length, almost 70% of wild type (Figure 1-1b). In addition, the shoot dry weight and root dry weight were reduced in the _osnlp4-1_ by 58% and 53% respectively (Figure 1-1b). Such reduction in growth was not observed when plants were grown on other three conditions (Figure 1-1b). These results suggest that _OsNLP4_ plays an important role in nitrate dependent growth in rice.

To further verify if the phenotype of _osnlp4-1_ is due to the disruption of the _OsNLP4_ gene, I constructed two _OsNLP4_ CRISPR/Cas9 lines, _osnlp4-2 and osnlp4-3_. The _osnlp4-2 and osnlp4-3_ have two bases deletion and one base insertion in the exon of _OsNLP4_, respectively (Figure 1-2a). The _osnlp4-2 and osnlp4-3_ reduced shoot length, root length, shoot dry weight and root dry weight only under nitrate condition compared with WT (Figure 1-2b). The fact that all the three independently obtained _osnlp4_ mutants exhibited impaired growth under nitrate condition, but not under ammonium supply, established that mutations in _OsNLP4_ are responsible for these phenotypes.

OsNLP4 mRNA accumulation is increased significantly under nitrogen free condition

There are six members encompassed RWP-RK domain and PBI domain in rice NLP family. OsNLPs can be classified into three clades (OsNLP1 and OsNLP4, OsNLP2 and OsNLP5, OsNLP3 and OsNLP6) (Figure 1-3a). RWP-RK domain is the most conserved region in OsNLPs. Database searches reveal that, in rice the RWP-RK domain sequences have diversified significantly more than those in _A. thaliana_ (Figure 1-4). OsNLP6 has a less conserved RWP-RK domain and is separated from most of other OsNLPs in the phylogenetic tree (Figure 1-3a).

Here I determined mRNA accumulation of _OsNLP_ genes in rice. I performed quantitative real-time PCR (qRT-PCR) using RNA extracted from shoots and roots of 15-day wild type plants grown under 2 mM KNO_3

(Nitrate), 2 mM NH₄Cl (Ammonium), 1 mM KNO₃ + 1 mM NH₄Cl (Normal) or 2 mM KCl (Nitrogen Free). Interestingly, the mRNA accumulation of *OsNLPs* was induced by nitrogen starvation except *OsNLP3* and *OsNLP6* (clade3). Especially, *OsNLP1* and *OsNLP4* (clade1) had significantly higher mRNA expression levels in both shoot and root (Figure 1-3b). But the *osnlp4* mutant lines did not show any different physiological phenotype with wild type under nitrogen free condition (Figure 1-5).*OsNLP5* was induced in shoot extensively while *OsNLP2* was induced only in root. *OsNLP6* was the only decreased genes in nitrogen free condition compared with other conditions. And in shoot, *OsNLP6* was up-regulated by ammonium not nitrate (Figure 1-3b).

Furthermore, the time-course experiment using plants with one-day nitrogen starvation treatment showed that *OsNLP1, OsNLP2, OsNLP4* and *OsNLP5* reacted to nitrate signal relatively quickly (Figure 1-6). In the shoot case, the mRNA expression levels of *OsNLP4* and *OsNLP5* were up-regulated extensively after plants were exposed to nitrate for 2 hours. In the root case, the mRNA expression levels of *OsNLP1* and *OsNLP2* were increased radically at 2 h while *OsNLP4* at around 4 h. *OsNLP6* was the single gene that had higher expression level at in nitrate condition compared with nitrogen free condition (Figure 1-6).

Taken together, our results suggested that the accumulation of *OsNLP4* transcript in rice is induced by nitrogen starvation.

Suppression of *OsNLP4* broadly down-regulates the expression of genes involved in nitrate assimilation

Our previous RNA-seq data showed there were 580 genes up-regulated and 622 genes down-regulated for the *osnlp4-1* compared with wild type. Most genes are related to catalytic activity, transporter activity and binding.

Expression of some selective genes related to nitrate assimilation and nitrate transport was analyzed by qRT-PCR to examine their mRNA expression levels. Result indicated that in roots and shoots nitrate reductase gene *NIA1* which had a relatively higher expression level in the wild type were declined in the *osnlp4* mutants. But the nitrite reductase gene *NIR1* was not affected (Figure 1-7). On the other hand, some genes related to nitrate transport (*NRT2.1*, high-affinity nitrate transporter; *NRT1.5a*, root-to-shoot transporter) had similar expression levels between wild type and the *osnlp4* mutants (Figure 1-7). However, in shoot, the accumulations of *NIA2* and *NRT2.1* transcripts were increased in the *osnlp4* mutants while *NIA1* was reduced (Figure 1-7). These results indicate that mRNA accumulation of genes related to nitrate assimilation but not nitrate transport are affected in the *osnlp4* mutants.

Overall, OsNLP4 plays a vital role in nitrate regulation of gene expression including genes involved in nitrate metabolism.

The ion concentrations in *osnlp4* mutants is changed in a large range

First, I detected the total nitrate concentration in plants grown under nitrate condition. In shoot part, the nitrate concentration reduced to around 20% of WT in *osnlp4* mutants (Figure 1-8a). But no difference existed in root part between WT and *osnlp4* mutants (Figure 1-8a).

Except N, other elements were further analyzed by ICP-MS. The concentrations of a number of elements were changed obviously in *osnlp4* mutants. In shoot, Na, P, B, Mn, Ge and Cd were higher in *osnlp4* mutants while Mo, Mg, K, Sand Ni were lower. In root, most elements were reduced in *osnlp4* mutants. Only the contents of Ca, Cu and Co were higher than WT (Figure 1-8b). These results suggest that low concentration of N in plants can cause the content change of a range of elements. Close connections might exist in different elements uptake or utilization.

OsNLP4 is involved in nitrate uptake and assimilation

To examine physiological defects of *osnlp4* mutants, I determined the nitrate absorption rate of wild type and the *osnlp4* mutants under continuous nitrate and one-day nitrate starvation conditions. It was found that the *osnlp4* mutants absorbed nitrate more slowly compared with wild type in both conditions (Figure 1-9a, b). In particular, after one-day nitrate starvation treatment, wild type showed high nitrate absorption rate at almost 1.5 NO_3^- (μmol)/root fresh weight(g)/h (Figure 1-9a) while the *osnlp4* mutants displayed less than half of that of wild type (Figure 1-9b).

In the aforementioned ICP-MS result, the concentrations of molybdenum and iron were reduced in the *osnlp4* mutants (Figure 1-8b). Molybdenum and iron have been reported to be essential for the activity of nitrate reductase through covalently bounding to specific domains of the enzyme (Liu et al., 2017c, Ford et al., 2016). I checked the mRNA expression levels of *MOT*, *IRT1* and *IRT2* via qRT-PCR. It is normal to see these three genes were mainly expressed in root. The expressions of *IRT1* and *IRT2* were strongly impaired in the *osnlp4* mutants root part. However, these genes were up-regulated in shoot (Figure 1-9c). It is possible that NR activity is also affected by OsNLP4. Furthermore, I compared the nitrate reductase (NR) activity of the *osnlp4* mutants with WT grown under nitrate condition and discovered that NR activity was lower in the *osnlp4* mutants, in root almost only 30% of WT (Figure 1-9d), establishing that OsNLP4 is required for normal NR activity.

I next analyzed shoot nitrogen content to verify that OsNLP4 is important for rice to assimilate nitrate by a CN coder. The C/N ratio (carbon-to-nitrogen ratio) in shoot was nearly two-fold higher in the *osnlp4* mutants under nitrate condition which means the nitrogen limitation existed in the *osnlp4* mutant lines (Figure 1-9e).

These results collectively suggest that OsNLP4 is indispensable for nitrate transduction pathway, including nitrate uptake and nitrate assimilation, which subsequently leads to changes in nitrate use efficiency.

OsNLP4 is localized in the nucleus

In *A. thaliana*, AtNLP6 and AtNLP7 are retained in the nucleus in the presence of nitrate, but AtNLP8 are not (Yan et al., 2016a, Yu et al., 2016, Guan et al., 2017a). To test whether the subcellular localization of OsNLP4 is under the control of nitrate, 35S::*OsNLP4-GFP* was transiently transformed into rice protoplasts. After 18h incubation with or without nitrate, GFP signal was observed using confocal microscopy to examine subcellular localization of GFP signal. OsNLP4-GFP signal was detected mainly in the nucleus with minor signals in the cytosol. These patterns of subcellular localization did not seem affected by the nitrate condition (Figure 1-10). These data indicate that OsNLP4 is localized to nucleus irrespective of nitrate conditions. This is in contrast to the case of AtNLP7, implying that OsNLP4 in rice is regulated by a different mechanism.

The *osnlp4-1* line has low rice yield in paddy field

Rice is the main cereal crop in the whole world and nitrogen fertilizers are frequently used with the aim to achieve high yields. In order to check agronomic traits of our *OsNLP4* Tos-17 insertion line, I planted WT (Nipponbare) and the *osnlp4-1* in fertilization (+N) and non-fertilization area in Tohoku paddy field (Sendai, Japan) in 2017. In fertilization area, although there was no significant difference of tiller number between WT and the *osnlp4-1*, the *osnlp4-1* line showed decreased productive panicle weight and straw weight (Figure 1-11). However, significant difference on grain yield was also observed between WT and the *osnlp4-1* line in non-fertilization area (Figure 1-11). It is possible that residual nitrogen existed in non-fertilization area. So I detected the soil composition via Tokachi Federation of Agricultural Cooperatives. In non-fertilization area, the nitrate concentration was lower than that of fertilization area while the ammonium concentration was similar between two areas (Table 1-1).

Totally, the study of *OsNLP4* has potential effects on the increase of rice yield.

Discussion

Rice accumulates *OsNLP4* at RNA level under no nitrogen condition

Most of the genes involved in nitrate signaling are induced by nitrate itself, although its mode of action is poorly understood (Hoff et al., 1994, Krouk et al., 2010a, Gutierrez, 2012, Krapp et al., 2014, Konishi and Yanagisawa, 2014). Recently, NLPs had proved to work as key transcription factors responsible for nitrate-inducible expression of a number of genes including nitrate transporter, nitrate reductase, nitrite transporter and nitrite reductase in response to nitrate in *A. thaliana* (Yu et al., 2016, Castaings et al., 2009a, Yanagisawa, 2014a). AtNLPs can directly bind to the NRE domain within some nitrate-induced genes probably through post-translational activation (Konishi and Yanagisawa, 2013a). This study identified *OsNLP4* as an important gene for regulating growth under nitrate condition in rice. The *osnlp4* mutants displayed prominent nutrient deficiency symptom when nitrate was used as sole nitrogen source (Figure 1-1b, c & Figure 1-2b). Notably, qRT-PCR results showed that mRNA expression level of *OsNLP4* was increased significantly under no nitrogen condition (Figure 1-3b). But the *osnlp4* mutant lines did not show any different physiological phenotype with wild type (Figure 1-5). In *A. thaliana*, no *AtNLP* was induced by N source or N starvation (Konishi and Yanagisawa, 2013a), but *AtNLP8* and *AtNLP9* were highly induced in imbibed seeds (Yan et al., 2016a). One possibility is that OsNLP4 protein is inactive when no nitrate is supplied. I speculate that rice accumulates a high level of OsNLP4 protein in order to react to nitrate quickly, converting the inactive form of OsNLP4 into active form when nitrate signaling is sensed.

Among OsNLP family, in addition to OsNLP4, it seemed like that rice also accumulate OsNLP1 and OsNLP2 especially in shoot and root under nitrate starvation while OsNLP6 was the only repressed one (Figure 1-3b & Figure 1-6). OsNLP6 might be related to ammonium uptake, transport or assimilation. OsNLP6 has a conserved N-terminal part of the RWP-RK domain, but lacks the downstream half (Schauser et al., 2005). This protein might have lost putative ancestral DNA-binding function and have acquired a divergent function. Our results support this hypothesis that NLPs for a specific functional role is different among plant species.

OsNLP4 triggers a number of nitrate specific responses

Except nitrate related genes (Figure 1-7), iron transporter genes *IRT1* and *IRT2* were also strongly impaired in the *osnlp4* mutants root part (Figure 1-9c). And our ICP-MS result showed that the concentrations of iron and molybdenum were lower in *osnlp4* mutants (Figure 1-8b). Nitrate reductase requires iron and molybdenum to consist co-factors to catalyze the reduction of nitrate-to-nitrite in cytosol. And the reduction needs NAD(P)H as

energy (Hoff et al., 1994). As expected, the NR activity was impaired in the *osnlp4* mutants (Figure 1-9d). Reduced NR activity further leaded to low total nitrogen content and negatively affected growth in *osnlp4* mutants under nitrate-sufficient condition (Figure 1-8a, Figure 1-9e & Figure 1-1b, c). On the other hand, no obvious difference was detected of *NRT2.1* and *NRT1.5a* mRNA levels between WT and the *osnlp4* mutants in roots, although *osnlp4* mutants took up nitrate more slowly than WT (Figure 1-9a, b). In shoots, the mRNA expression levels of *NIA2* and *NRT2.1* were even higher than WT in the *osnlp4* mutants (Figure 1-7). As reported, both nitrate assimilation and nitrate transport were impaired in *atnlp7* mutants (Castaings et al., 2009a). On the basis of our results, I propose that OsNLP4 mainly regulates the expression of nitrate assimilation genes but not nitrate transporter genes in root, acting as a nitrate sensor to trigger secondary responses. It is possible that OsNLP4 displays different requirements for NRE-like motif recognition and has different functions in shoots and roots. For now, I do not have evidence to prove OsNLP4 can bind to NRE domain directly. I just tried co-transfected rice protoplasts with effector construct and reporter construct to identify OsNLP4 can bind to the *OsNIA1* promoter or not and analyze the transactivation activity of OsNLP4. But the result was negative (Figure 1-12) probably because LUC and GUS signals are usually used in this kind of experiment. And in *A. thaliana*, not all genes regulated by AtNLPs have the NRE region in their promoters. There might be other regions NLPs can bind to.

OsNLP4 is localized in the nucleus

The localization or retention of AtNLP7 in nuclei is controlled by nitrate signaling and the phosphorylation of a conserved serine residue is essential for the nitrate-simulated rapid nuclear translocation (Marchive et al., 2013a, Liu et al., 2017a). However, OsNLP4-GFP was localized to the nucleus independent of nitrate and did not show nitrate-promoted nucleocytosolic shuttling mechanism (Figure 1-10). It is known that except nitrate multiple signals coordinately interact with each other through NLPs in plant to modulate downstream events, such as Ca^{2+} signaling, phytohormones and drought (Guan, 2017, Almeida et al., 2017, Liu et al., 2017a). It is possible that multiple mechanisms that activate NLPs functions exist in different plant species.

A schematic model of proposed OsNLP4 action in rice

I propose a working model to illustrate how OsNLP4 might react to nitrate signaling to regulate the nitrate assimilation pathway in rice. Nitrate is perceived as signal by some unknown sensors, then transmitted via a signal transduction pathway towards OsNLP4. OsNLP4 controls the expression of a wide range of genes related

to nitrate assimilation that have an impact on NR activity and other developmental processes (Figure 1-13). In addition, the expression of nitrate-assimilation related genes is also regulated by various environmental factors. The total nitrate concentration was reduced in *osnlp4* mutants (Figure 1-8a). And it is normal to see the concentrations of a number of elements were changed in *osnlp4* mutants (Figure 1-8b). For example, the high concentration of P may be related to nitrate transporter OsNRT2.3 because overexpression of *OsNRT2.3* improves rice P uptake and translocation (Feng et al., 2017). Probably, crosstalk may occur between OsNLPs and other transcription factors to coordinate the nitrate assimilation pathway.

Recently, it has been reported that in *A. thaliana* NLPs also take a role in developmentally programmed processes independent of nitrate. AtNLP7 can regulate root cap cell release likely through regulation of *CELLULASE5* (Karve and Iyer-Pascuzzi, 2018). So further studies are clearly required to understand how OsNLP4 reacts to nitrate signaling with changing from inactive form to active one and the functions of other OsNLPs in rice. NLPs might take a part in abundant developmental and metabolic processes as a transcription factor in plant. Identification and characterization of NLP family probably provide clues for understanding nitrogen or other metabolic pathway.

OsNLP4 have potential effect on sustainable agriculture

Moreover, nitrogen use efficiency (NUE) is an important indicator for the development of sustainable agriculture (Xu et al., 2012a). In order to increase grain yield, farmers use high amount of chemical fertilizers, especially nitrogen fertilizers, but with considerable negative impacts on the environment (Han et al., 2015). Our paddy field data indicated that the *osnlp4* impaired grain yield (Figure 1-11). The elevated gene expression in *OsNLP4* might enhance NUE in rice paddy field. Improving the NUE of rice will require a better understanding of nitrogen uptake and assimilation in different tissues and stages of development. Comprehension of the function of *OsNLP4* involved in nitrate signaling may increase crop growth and productivity under nitrogen constraints.

Materials and Methods

Plant materials and growth conditions

Oryza sativa L. cv. Nipponbare was used as the wild type. The *OsNLP4* Tos-17 insertion line (*osnlp4-1*), which has an insertion in first exon, was obtained from the collection of Tos-17 mutant panel by National Institute of Agro Biological Resources, Tsukuba, Japan. Homozygous Tos-17 line was identified using *OsNLP4* gene-specific primers and a Tos-17 left-border primer. To generate *OsNLP4* CRISPR/Cas9 lines (*osnlp4-2* and

osnlp4-3), sgRNAs designed on CRISPR-P (http://crispr.hzau.edu.cn/CRISPR2/) were annealed into pU6gRNA vector (Shan et al., 2013) and subsequently cloned into the modified binary vector pZDgRNA_Cas9 ver.2_HPT (Endo et al., 2015). All constructs were sequence verified and transformed into Nipponbar*e* using the *Agrobacterium*-mediated rice transformation method. The *osnlp4-2* line has a [867]GA deletion in first exon and the *osnlp4-3* line has a [1959]A insertion in third exon. The primers used are listed in the Table 1-2.

Wild type and the *osnlp4* lines were surface sterilized with 10% (v/v) bleach for 30 min and then rinsed thoroughly with deionized water. The sterilized seeds were germinated on plastic plate containing moist filter paper for 5 days. Uniform seedlings were selected and then transferred to a tank containing 3 L modified KimuraB solutions (Table 1-3) (Uraguchi et al., 2009) supplemented with one of the following as the sole N source: 2 mM KNO_3; 2 mM NH_4Cl; 1 mM KNO_3 + 1 mM NH_4Cl and 2 mM KCl. Only for ICP-MS analysis, added additional micronutrients in KimuraB solution. All the plants were grown in a chamber with a 16-h-light/ 8-h-dark photoperiod, and the temperature was controlled at 30 °C. The solution was refreshed every 7 days. Samples were harvested at 15 days for phenotypic analysis, RNA isolation and protein extraction. For nitrate uptake speed analysis and time-course experiment, 14-day old plants grown on 2 mM KNO_3 were transferred to 2 mM KCl and removed plants to 2 mM KNO_3 again after one day. For the preparation of rice protoplasts, Nipponbare were grown on 1/2MS medium at 30 °C for 10 days under continuous light.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from shoots and roots using NucleoSpin RNA Kit (MACHEREY-NAGEL). 500 ng total RNA was reverse-transcribed using PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa). Quantitative real-time PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus, TaKaRa) and monitored with the Thermal Cycler Dice Real Time System II (TaKaRa). The relative gene expression was normalized to the expression of *OsACT*. Triplicate biological replicates were analyzed. The primers used for qRT-PCR are listed in Supplementary Table 1-3.

Element concentration detection

For the nitrate concentration detection, the roots and shoots were separated, weighed and ground in a chilled mortar by liquid nitrogen. Then putted samples in 15 ml tubes and added 1 ml preheated water at 80 °C per 100 mg fresh weight to denature nitrate reductase quickly. The whole samples should be submerged in water. The tubes were incubated at 100 °C for 30 min with the lid closed and shaken by hand or vortexed every 5 min for

complete extraction. Finally, centrifuged the cooled tubes at 13,000 rpm for 10 min at room temperature and used the supernatants for nitrate determination by capillary electrophoresis (CE) (Agilent Technologies) after filtering with 0.22 µm filter membrane.

Other elements were detected by inductively coupled plasma mass spectrometry (ICP-MS) Agilent Technologies 7800). The 15-day shoot or root samples were dried in a 70 °C oven for 3 days then removed into glass tubes for nitrolysis. First added 2 ml HNO_3 into tubes heated by metal baths at 120 °C for 3 h. Then added additional 3 ml HNO_3 into tubes incubated at 100 °C for overnight. In the following day, added 1-2 ml HNO_3 at 120 °C until samples became buff powder totally. Finally, added 1 ml H_2O_2 into tubes and powder samples turned to white. Each powder sample was dissolved by 5 ml ICP-MS buffer (0.08 HNO_3 + 2 ppb In) completely and vortexed. The solution was diluted by 40 times (400 µl solution sample + 2.8 ml buffer) and analyzed by ICP-MS. The element concentrations of mutants were showed as Z-score finally. It is calculated by subtracting the population mean from an individual raw score and then dividing the difference by the population standard deviation (Z-socre$= \frac{\text{Sample value} - \text{WT mean}}{\text{WT S.D}}$).

Nitrate uptake rate assay

Three 15-day-old seedlings were used for a measurement of nitrate uptake. Three plants were transferred to a 50 ml tube containing 50 ml KimuraB solution with 2 mM KNO_3 and their roots were rinsed thoroughly with distilled H_2O. After 3 h and 6 h, sucked 500 µl solution from the tube to detect the concentrations of remaining nitrate using High Performance Capillary Electrophoresis System (Agilent Technologies). Then measured the root fresh weights and calculated the nitrate uptake speeds (NO_3^- (µmol)/root fresh weight (g)/h). Three biological replicates of WT and *osnlp4* mutant lines were analyzed for each treatment.

Measurement of C/N ratio

The total nitrogen content was investigated using CN coder Vario MAX cube (Elementar). The 15-day shoot samples were dried in a 70 °C oven for 3 days. Then removed samples into assorted metal containers. The machine burned samples and detected total contents of carbon and nitrogen automatically.

Analysis of NR activity

The nitrate reductase activity was measured by the modified method described by Maeda et al. and Pinto et al.

(Pinto et al., 2014). The roots and shoots (1.0 g fresh weight) were separated and ground in a chilled mortar containing 4 ml extraction buffer (50 mM HEPES-KOH pH 8.0, 5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM DTT, 0.5 mM PMSF, 5% (w/v) PVPP). The resulting homogenate was then centrifuged at 13,000 rpm for 30 min at 4 °C. Samples of the supernatant were used for the determination of protein content (NanoDrop ND-1000) and the assays of *in vitro* NR activity (300 µl). The reaction mixture (450 µl) of the latter consisted of 50 mM HEPES-KOH pH 8.0, 1 mM DTT, 2 mM KNO_3. After incubation at 30 °C for 1 min, the reaction was started by adding 150 µl 0.6 mM NADH. After incubation at 30 °C for 1h, the reaction was ended by the addition of 100 µl cold water. After 1h the NO_2^- produced was colorimetrically measured at 543 nm (SHIMADZU UV-1850) after the addition of 1% (w/v) sulphanilamide dissolved in 2 M HCl and 0.02% (w/v) N-1-naphthylamine. NR activity was expressed as µg NO_2^- mg^{-1} protein h^{-1}. The 0, 0.1mM, 0.2 mM and 0.5 mM nitrite solution were used to build a standard curve.

Protoplast transient assay

To understand the subcellular localization of OsNLP4, p35S-OsNLP4-GFP and p35S-GFP modified from pMDC83 vector (Curtis and Grossniklaus, 2003) were transformed into rice protoplasts. The effector plasmid was constructed by inserting the *OsNLP4* code region into pMDC32 vector. The reporter plasmid that contained the *GFP* reporter gene downstream of the *NIA1* promoter was constructed using the 2 kb DNA fragment for the *NIA1* promoter. The promoter was inserted in ahead of 35Smini promoter via Slice system. PCR primers are listed in Supplementary Table 1-1 and all constructs were verified by DNA sequencing.

The rice protoplast preparation and transfection followed preciously described procedures with some modifications (Bart et al., 2006). Rice protoplasts were isolated from stem of rice seedlings after sowing for 10 days. Briefly, 100 µl of protoplast suspension was transfected or co- transfected with 10 µg DNA for various constructs using PEG-mediated method. After transfection, cells were cultured with 0.25 ml inoculation solution containing 2 mM KCl or KNO_3 for 18 h at 25 °C. Cells were collected, then GFP signal and mCherry signal in the cells was analyzed using a confocal laser scanning microscope (OLYMPUS FV10-MCPSU) at λ_{500nm}～λ_{525nm} and λ_{600nm}～λ_{625nm}，respectively. PI channel (λ_{600nm}～λ_{660nm}) was used to show the auto fluorescence of chloroplasts. But PI channel and mCherry channel cannot be used at the same time because the wave lengths of the two channels are in adjacent wave range. In the experiment to analyze the transactivation activity of OsNLP4, the cell area and optical density were calculated by ImageJ.
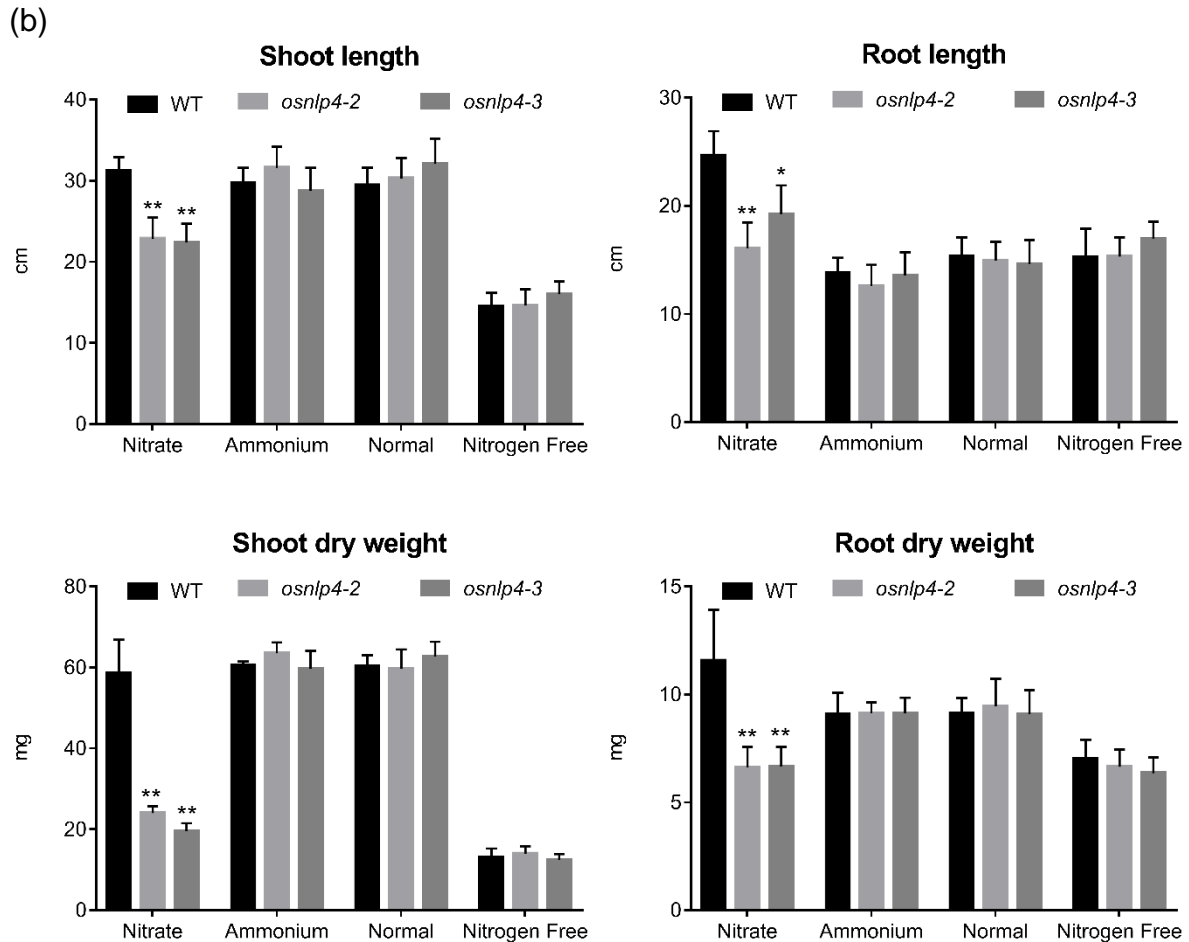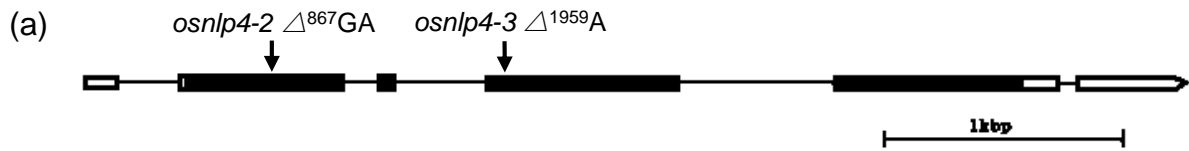
Rice plant sampling and analysis

To measure the rice grain yield, WT (Nipponbare) and the *osnlp4-1* line were planted in fertilization area (+N) and non-fertilization area of Tohoku paddy field (Sendai, Japan) in May 25, 2017. Tree frames (4 lines × 4 lines) for each genotype were harvested in each area in October 26, 2017. The panicle weight, straw weight and tiller number were measured from 20 lines sampled from the three harvested frames.

    The soil samples were taken from four corners of two areas. After drying, soil was grinded to powder and sent to Tokachi Federation of Agricultural Cooperatives (https://www.nokyoren.or.jp/) for composition analysis.
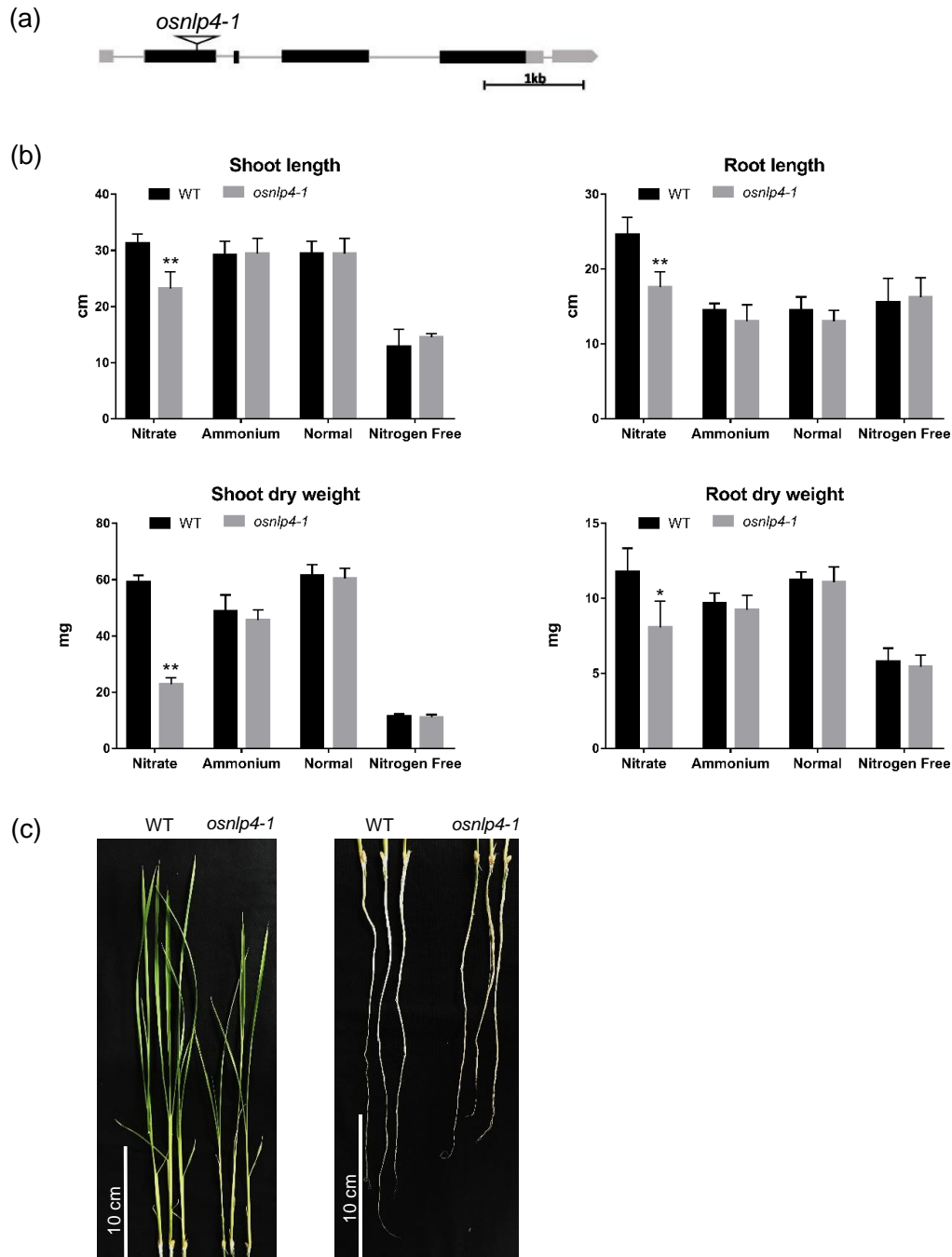
Figure 1-1. Morphological and physiological phenotype of *OsNLP4* Tos-17 insertion line. (a) Location of Tos-17 insertion in the *OsNLP4*. Lines represent introns, whereas grey and black boxes represent UTR regions and exons, respectively. (b) The shoot length, root length, shoot dry weight and root dry weight of the *osnlp4-1* were compared to wild type. Plants were grown in hydroponic culture for 15 days on modified KimuraB solution containing 2 mM KNO_3 (nitrate), 2 mM NH_4Cl (ammonium), 1 mM KNO_3 + 1 mM NH_4Cl (normal) and 2 mM KCl (nitrogen free) with of a density of　8 seeds/L. Error bars represent the standard deviation for 6 plants. Asterisks indicate significant differences between WT and the *osnlp4-1* line (t-test, *$p<0.05$, **$p<0.01$). (c) Photos of WT and the *osnlp4-1* in nitrate condition. Scale bars, 10cm.

(a)

osnlp4-2 $\triangle^{867}$GA   *osnlp4-3* $\triangle^{1959}$A

1kbp

(b)

Shoot length

cm

WT   *osnlp4-2*   *osnlp4-3*

Nitrate   Ammonium   Normal   Nitrogen Free

Root length

cm

WT   *osnlp4-2*   *osnlp4-3*

Nitrate   Ammonium   Normal   Nitrogen Free

Shoot dry weight

mg

WT   *osnlp4-2*   *osnlp4-3*

Nitrate   Ammonium   Normal   Nitrogen Free

Root dry weight

mg

WT   *osnlp4-2*   *osnlp4-3*

Nitrate   Ammonium   Normal   Nitrogen Free

Figure 1-2. Morphological and physiological phenotype of *OsNLP4* CRISPR/Cas9 lines. (a) The structure of *OsNLP4* CRISPR/Cas9 lines. The *osnlp4-2* has 2 bp deletion in the first exon, while *osnlp4-3* has 1 bp insertion in the third exon. (b) The shoot dry weight, root dry weight, shoot length and root length of *osnlp4-2* and *osnlp4-3* compared to wild type for plants grown in different nitrogen conditions. Error bars represent the standard deviation for 6 plants. Differences are statistically significant (Dunnett's test, *p<0.05, **p<0.01).
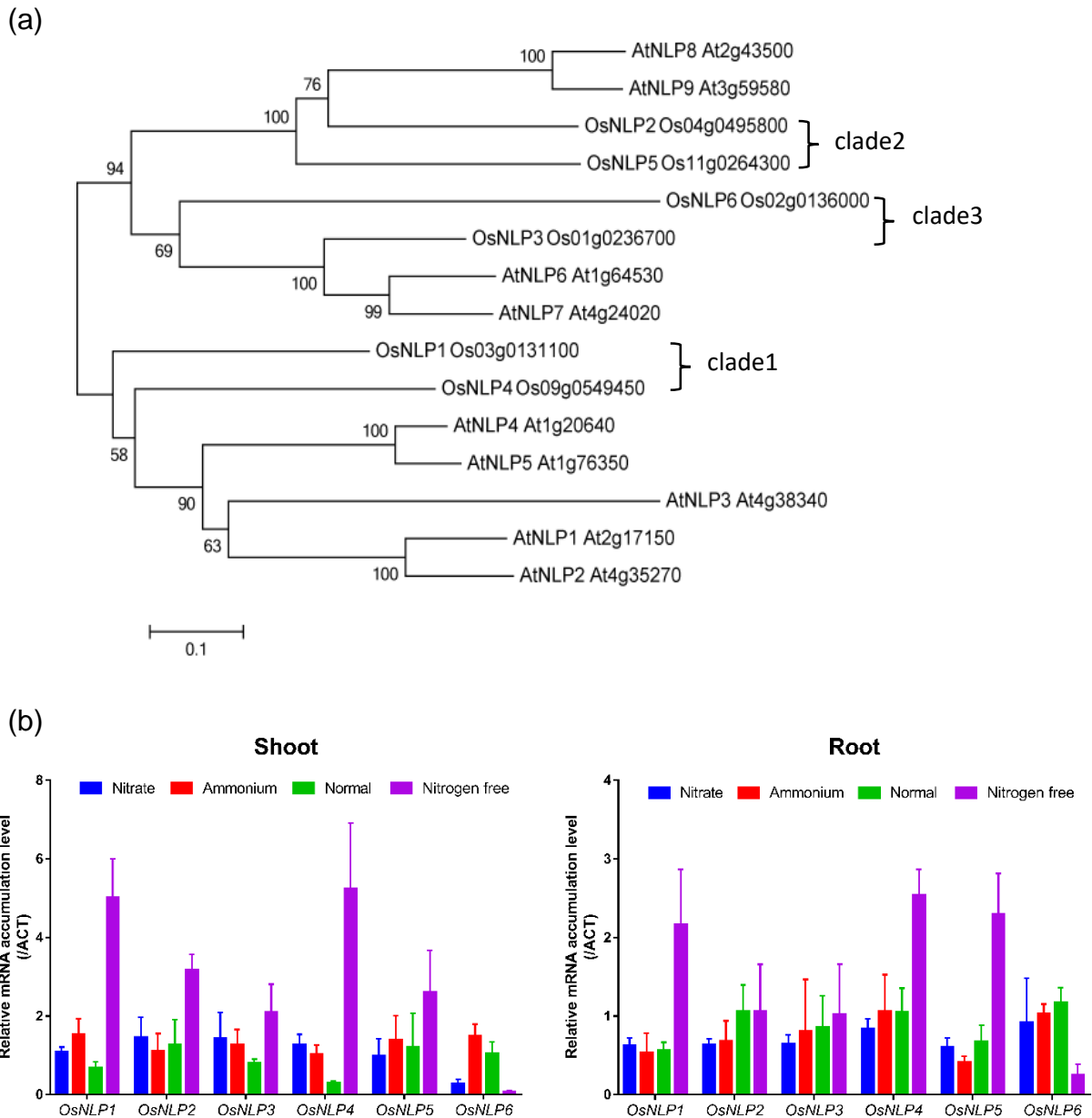
Figure 1-3. The mRNA expression levels of *OsNLPs* in wild type under a range of nitrogen conditions.

(a) Phylogenetic tree presented evolutionary relationships between rice and *A. thaliana*. The scale bar represents the evolutionary distance. Six OsNLP family members were classified into three clades. *OsNLPs* sequences and *AtNLPs* sequences were obtained from RAP-DB (http://rapdb.dna.affrc.go.jp/) and TAIR (https://www.arabidopsis.org/) relatively. Phylogenetic tree computed by the MEGA5 software. (b) Relative expression of *OsNLP*s was analyzed by qRT-PCR and normalized to the expression of *ACT*. Each gene had three biological replicates and the assays were repeated twice.
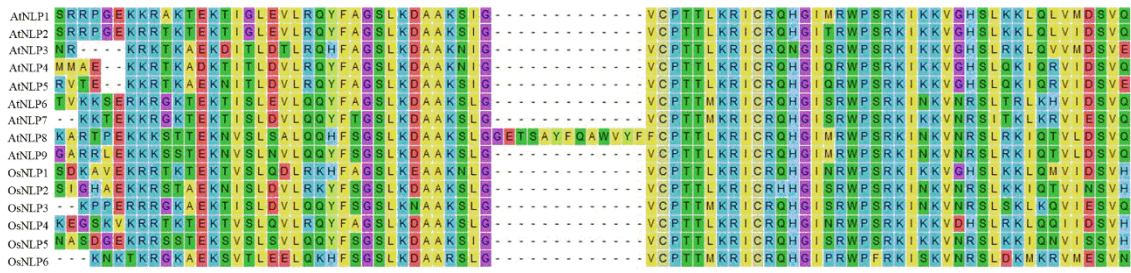
Figure 1-4. RWP-RK domain of AtNLPs and OsNLPs. Amino acid sequence from UniProt (http://www.uniprot.org/).
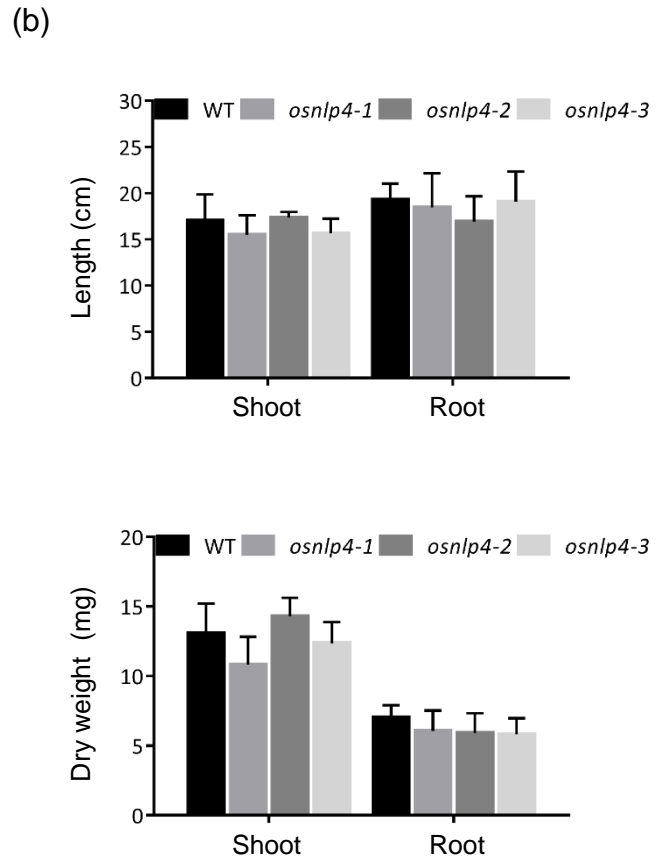
Figure 1-5. Physiological phenotype of WT and the osnlp4 mutant lines under nitrogen free condition.

(a) Plants were grown in hydroponic culture without nitrogen for 15 days. Scale bars, 10cm. (b) The shoot length, root length, shoot dry weight and root dry weight of the osnlp4 mutants were compared to wild type. Error bars represent the standard deviation for 6 plants.
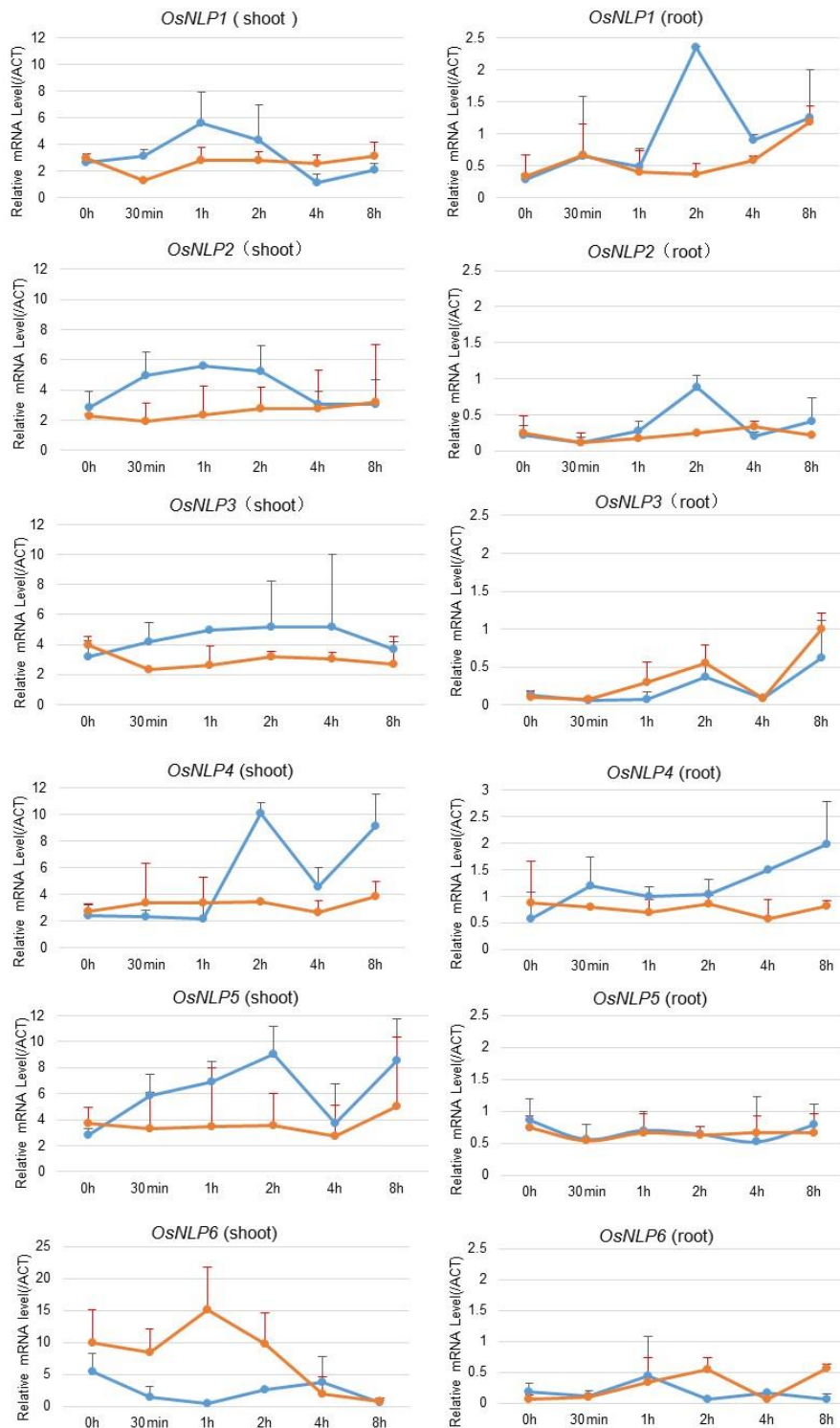
Figure 1-6. The reactions of *OsNLPs* to nitrate signal in short time. Plants (WT) were grown in hydroponic culture with nitrate for 14 days. Half of plants were transferred to 2 mM KCl and remaining plants were still in nitrate condition. After one day, removed all plants to refresh hydroponic culture with nitrate and sampled at 0 h, 30 min, 1 h, 2 h, 4 h and 8 h. The mRNA accumulations were normalized by that of *ACT*. Error bars, s.d, n=3.
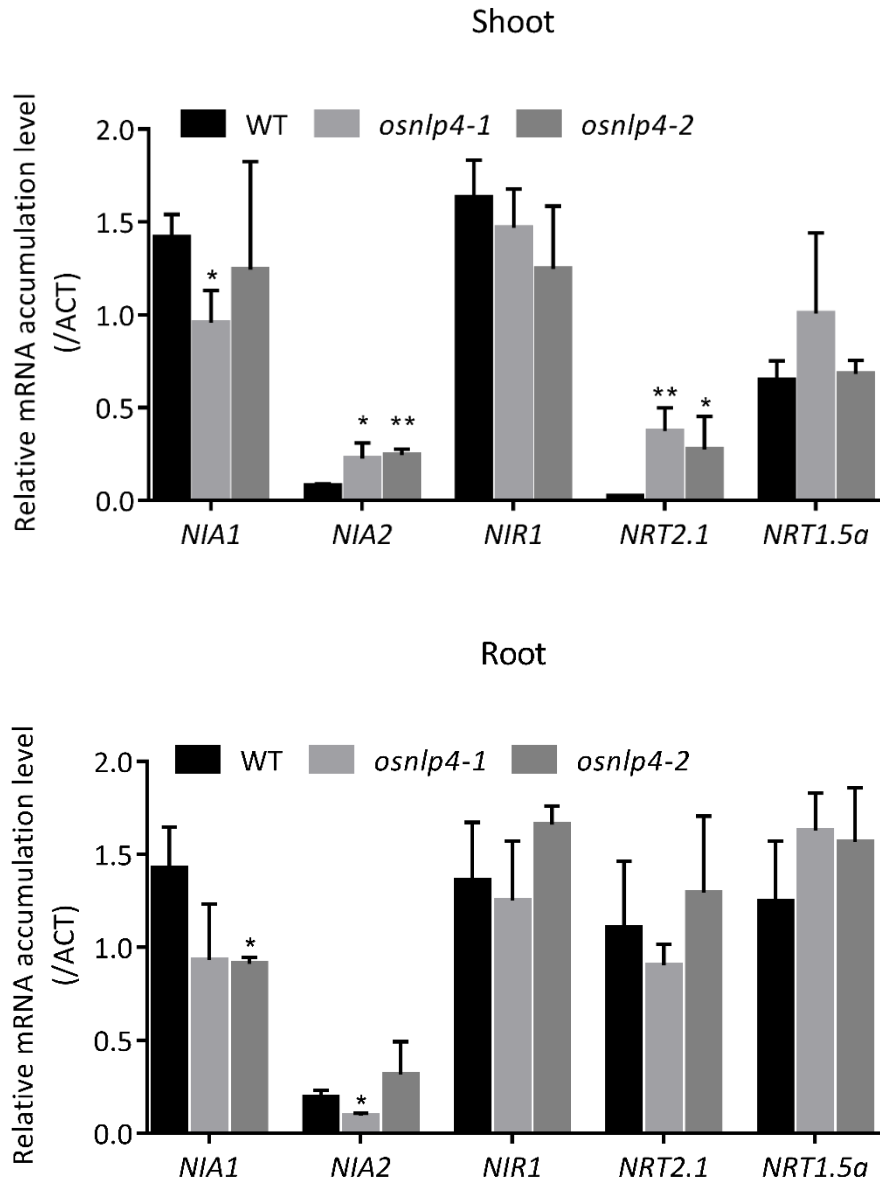
Figure 1-7. The mRNA expression levels of nitrate reductase genes and nitrate transporter genes in the osnlp4 mutants under nitrate condition. The steady-state transcript amounts for the nitrate reductase genes NIA1 and NIA2, nitrite reductase gene NIR1, high-affinity nitrate transporter genes NRT2.1 and root-to-shoot nitrate transporter gene NRT1.5a were determined by qRT-PCR. The results given are a percentage of the level of mRNA for the ACT gene. Error bars, s.d, n=3 biological replicates, Asterisks indicate significant differences between WT and the osnlp4-1 line or the osnlp4-2 line (Dunnett's test, $*p<0.05$, $**p<0.01$).
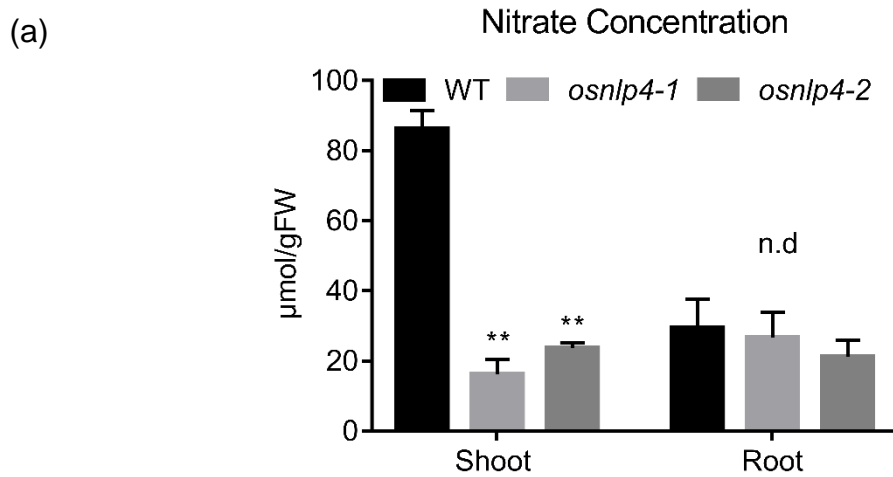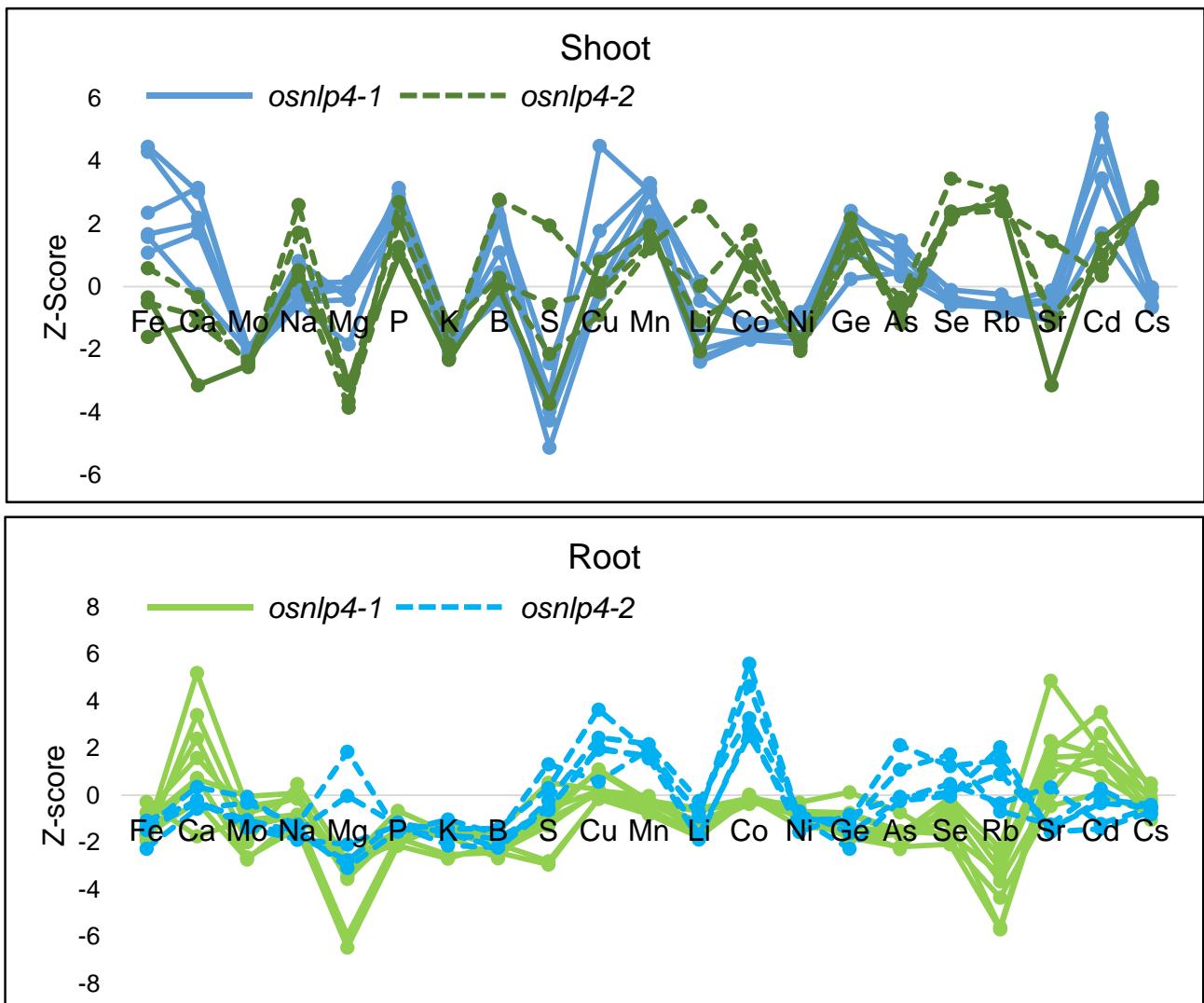
Figure 1-8. The element concentrations in *osnlp4* mutants under nitrate condition. (a) The total nitrate concentration of 15-day plants grown under nitrate condition was detected by HPCE. Error bars, s.d, n=3 (Dunnett's test, **$p<0.01$). (b) Other elements except N were detected by ICP-MS. n=4 or 6.
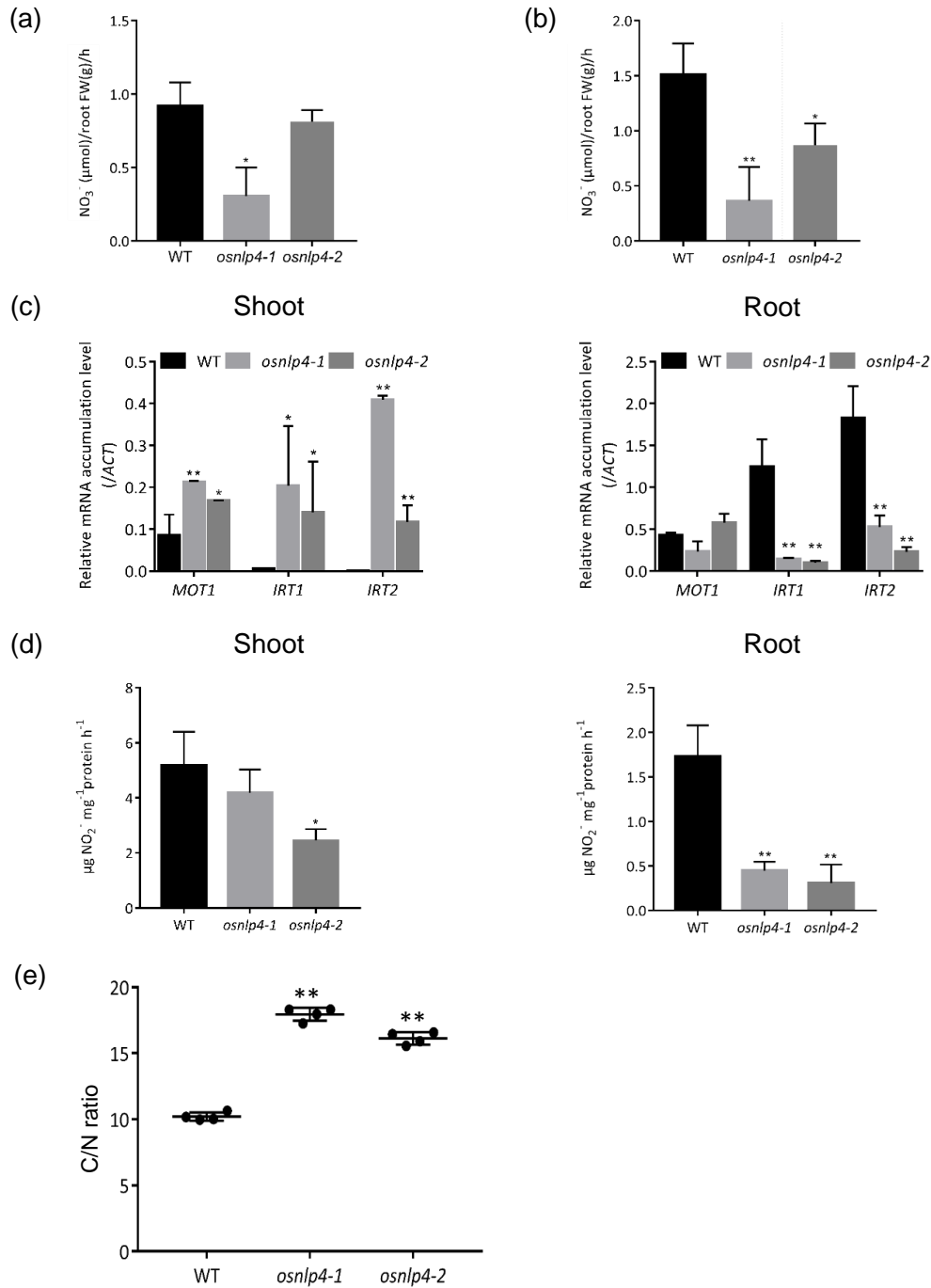
Figure 1-9. The *osnlp4* mutants impair nitrate uptake rate and nitrate assimilation. (a) Nitrate absorption rate of WT and *osnlp4* mutants under continuous nitrate condition. (b) The 14-day old plants grown on 2 mM KNO$_3$ were transferred to 2 mM KCl. After one day, removed plants to 2 mM KNO$_3$ again and detected nitrate absorption rate. (c) The mRNA expression levels of molybdenum transporter gene *MOT1* and iron transporter genes *IRT1*, *IRT2* were determined by qRT-PCR. The mRNA accumulations were normalized by that of *ACT*. (d) Nitrate reductase activity. (e) The carbon to nitrogen ratio. Error bars, s.d, n=3 or 4 (Dunnett's test, *$p<0.05$, **$p<0.01$).
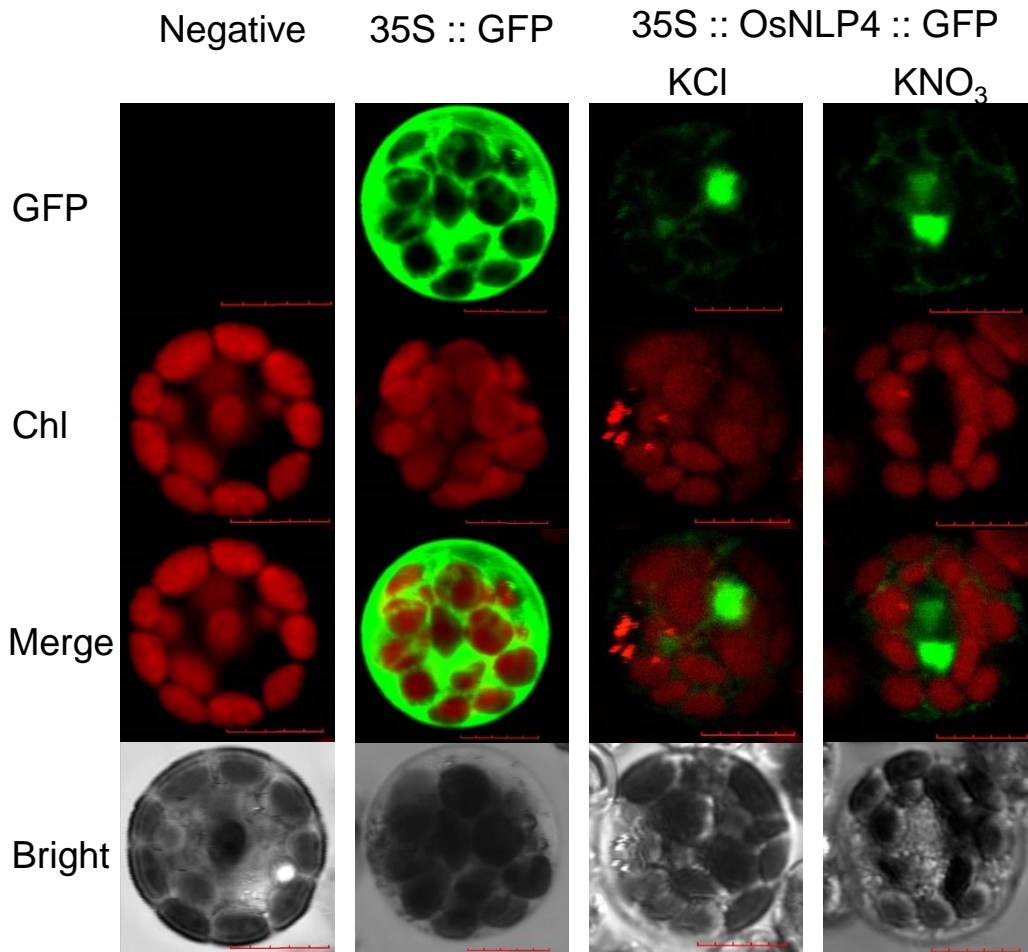
Figure 1-10. Subcellular localization of OsNLP4-GFP in the KCl- or KNO$_3$- treated rice protoplasts. The pMDC83 vector was used as a negative control and 35S :: GFP was used as a positive control of GFP signal. After PEG-mediated transformation, added KCl or KNO$_3$ to the protoplast incubation medium at 2 mM final concentration. Chl : chloroplasts autofluorescence. A bar indicates 10 μm. Images are representative of 10 protoplasts.
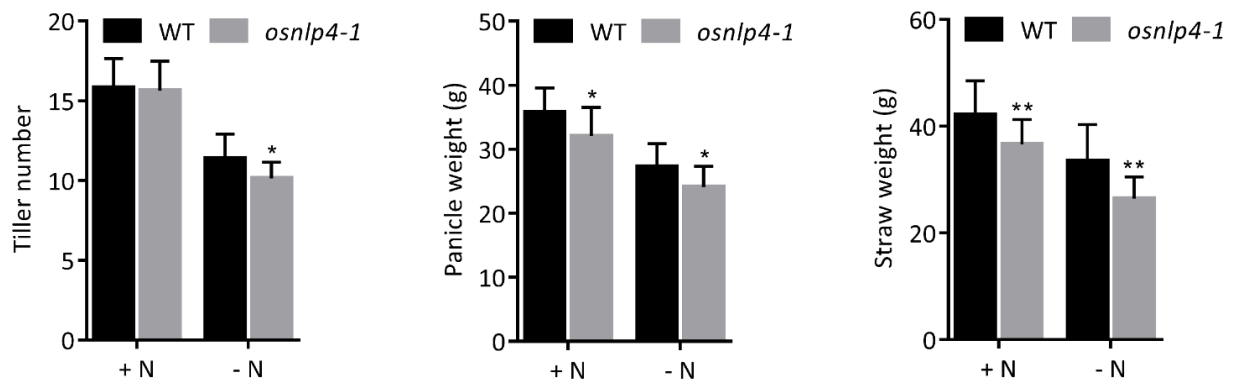
Figure 1-11. Agronomic traits of the *osnlp4* Tos-17 insertion line in paddy field (2017). Tiller number, panicle weight and straw weight per plant were determined for the *osnlp4-1* in comparison to WT in a field trial conducted in Sendai, Japan in 2017 (+ N, Fertilizer paddy field; -N, No-fertilizer paddy field). Error bars represent s.d, n=20 (t-test, *p<0.05, **p<0.01).

Table 1-1. Soil diagnosis in Tohoku paddy field

Area	Ammonium (mg/100g)	Nitrate (mg/100g)
- N	1.17	0.15
	1.67	0.15
	1.88	0.15
	1.75	0.18
+ N	2.15	0.51
	1.54	0.17
	1.86	0.62
	1.79	0.27

(a)
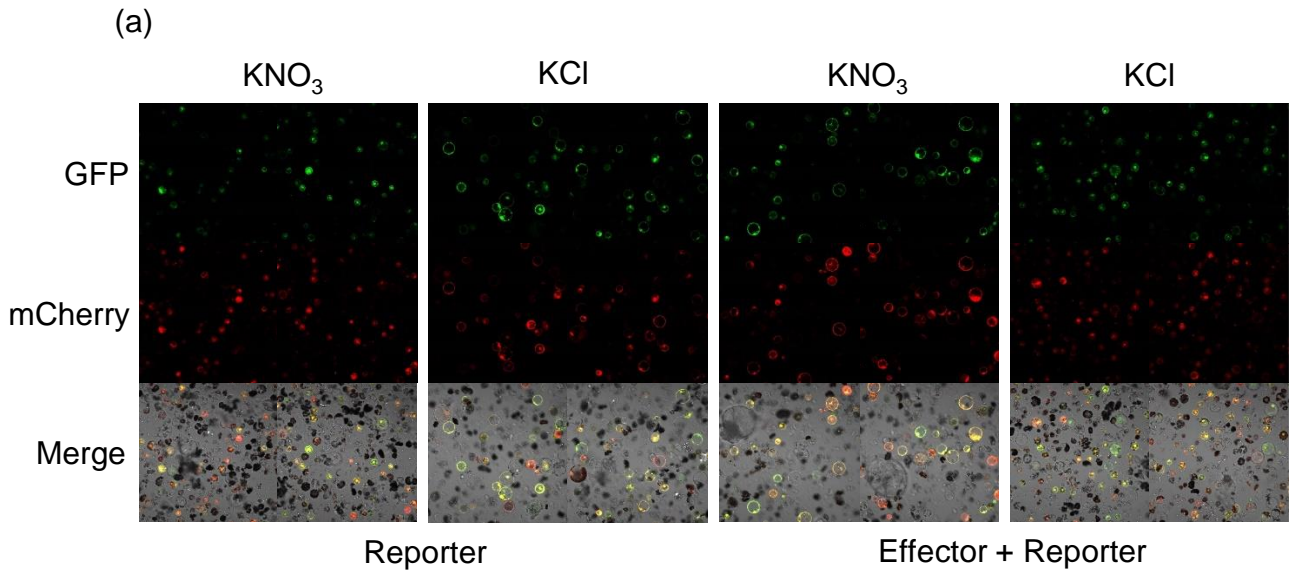
Effector construct   2*35S::*OsNLP4*
Reporter constructs   prom*OsNIA1*::35Smini::GFP
                          35S::mCherry

(b)
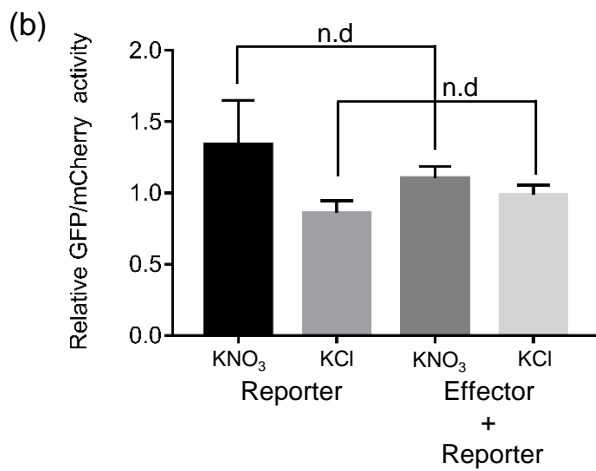
Figure 1-12. The transactivation activity of OsNLP4. (a) The photos of rice protoplasts co-transfected effector construct and reporter constructs and treated with 2 mM KNO_3 or KCl. (b) Protoplast transient assays using photos shown in (a) by ImageJ. An internal control plasmid (35S::mCherry) was used to normalize the GFP reporter activity levels. Error bars, s.d, n=5 (t-test).
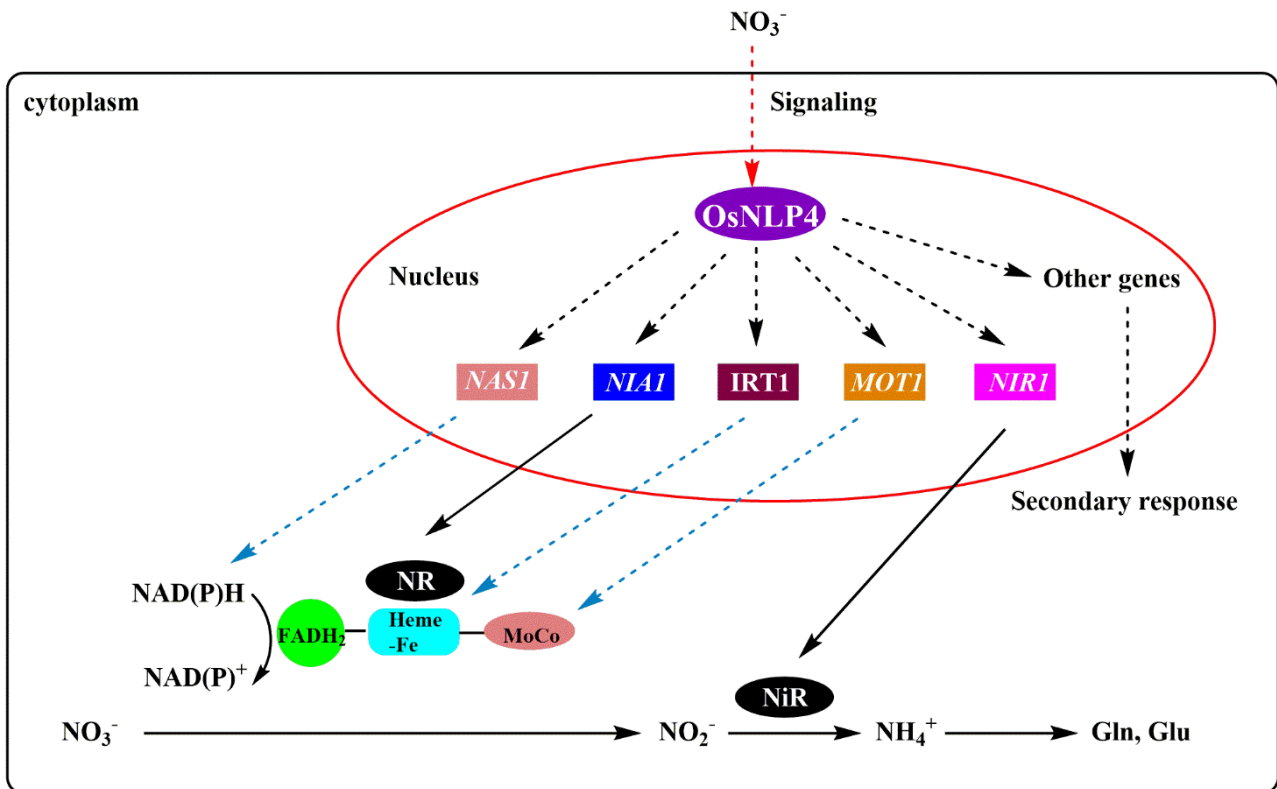
Figure 1-13. A schematic model of proposed OsNLP4-regulated nitrate assimilation in response to nitrate provision in rice. Nitrate is perceived as signal and transmitted via unknown pathway towards transcription factor OsNLP4. Then OsNLP4 promotes the expression of nitrate assimilation-related genes and other unknown regulatory genes. High active nitrate reductase catalyzes nitrate reduction to trigger nitrate specific growth responses.

Table 1-2. Primer pairs used for constructing *osnlp4* mutant lines and plasmids.

Mutant Line	Primer Name	Sequence (5'-3')
osnlp4-1	oAN_208	TCCAACTGCTTGACAGATGC
	oAN_136_Tos_left	ATTGTTAGGTTGCAAGTTAGTTAAGA
osnlp4-2	*OsNLP4*_CRISPR_Guide20_F	GTTGAGCCATTCACTCTCGATCAG
	*OsNLP4*_CRISPR_Guide20_R	AAACCTGATCGAGAGTGAATGGCT
osnlp4-3	*OsNLP4*_CRISPR_Guide4_F	GTTGGTGACCCAGGTCTGAGCTAA
	*OsNLP4*_CRISPR_Guide4_R	AAACTTAGCTCAGACCTGGGTCAC
Vectors	**Primer Name**	**Sequence (5'-3')**
35S::*OsNLP4*::*GFP*	*OsNLP4*-gateway-F	CAACATGGAAGAGGGAGACCCCCAGC
	OsNLP4-gateway-R	TGAGAAACCAGTGTGACCAA
P$_{NIA1}$::35Smini::*GFP*	35Smini-F	ATCTCCACTGACGTAAGGGA
	35Smini-R	TCCTCTCCAAATGAAATGAAC
	P$_{NIA1}$-35Smini-Slice--F	GCTAGAACCGAGCACTAAACATCTCCACTGACGT
	35Smini-Slice-R	GAAAGCTGGGTCTAGATATCCTCTCCAAATGAAATGAAC
	P$_{NIA1}$-Slice-F	CAATTCAGTCGACTGGATCCGTGTTTCGTTCTTAATTTCAG
	P$_{NIA1}$-35Smini-Slice-F	TCCCTTACGTCAGTGGAGATGTTTAGTGCTCGGTTCTAGC

Table 1-3. Composition of KimuraB hydroponics solution.

Composition	Concentration
MES (KOH, pH5.7)	2 mM
$Na_2HPO_4.12H_2O$	0.17 mM
K_2SO_4	0.27 mM
$CaSO_4.2H_2O$	0.08 mM
$CaCl_2.2H_2O$	0.19 mM
$MgSO_4.7H_2O$	0.47 mM
Fe-citrate	0.09 mM
$CuSO_4.5H_2O$	0.16 μM
$ZnSO_4.7H_2O$	0.15 μM
$Na_2MoO_4.2H_2O$	0.1 μM
$MnSO_4.5H_2O$	4.6 μM
H_3BO_3	18 μM
$KNO_3/NH_4Cl/KNO_2$/Glutamine	2 mM
LiCl	0.073 μM
RbCl	0.012 μM
CsCl	0.29 μM
$Sr(NO_3)_2$	0.36 μM
$CdCl_2.5H_2O$	0.39 μM
$NaAsO_2$	0.22 μM
Na_2SeO_4	0.33 μM
$CoCl_2.6H_2O$	0.41 μM
$NiCl_2.6H_2O$	0.41 μM
GeO_2	0.18 μM

Table 1-4. Primer pairs used for qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
OsNLP1	TCCGTCTTCAGCCTAGTTGG	AGCAGGTCTGCATCACAGGT
OsNLP2	CTTGCCAACTGGACACACAG	TGCATGTCCTATGGAACTAGGC
OsNLP3	GCACTGAGATATTGGACCATCC	AGGAAGCTTGTGCTCTTCACA
OsNLP4	TCCTTCGGCAGTACTTTGCT	CAGCGGTTTATGCCATGCT
OsNLP5	ATCTGGGCTCCAATCGAAGC	ACACTCTTCCTCAGCACCCA
OsNLP6	GAGGAGGAGGTTGAAGTCGC	CCATCGATTCGGGTCGCATA
OsNIA1	ACTGGTGCTGGTGCTTCTGG	CGGCTGGGTGTTGAGGGACT
OsNIA2	CATGCAGCATTTCGTTTCT	CCAATTCTTTCATCGTGTTCT
OsNIR1	GTGACAGTACAAGCGTGCCA	TTGCTTGCTCCGGTGATCTC
OsNRT2.1	GCGACCGAGACCAGCAATAC	TTCATCACCGTTTGCAACAAG
OsNRT1.5a	AGACCTGCGCCATATTCCAG	CCTGATCAGGTACAGCCGC
OsMOT1	CAAGCAGGAGTCGTTCGTCA	CTCAGGCGTAGCAACAGGTA
OsIRT1	AGGTCGGTGCTCGTCTTCT	TGTCCCTGTACACCCTGGTC
OsIRT2	TGCATGATGTATAGGTGAAGGTG	CGGCAGAAGCTGGTCTTTATTA

Chapter 2. The differential role of *OsNLP1* in regulating growth under nitrate conditions in rice

Abstract

Among six members of *OsNLP* family in rice, except *OsNLP4*, the mRNA expression level of *OsNLP1* was also induced significantly only under nitrogen free condition. I examined the growth of a Tos-17 insertion line of *OsNLP1* and a double mutant line (*osnlp1/4*) under different nitrogen conditions. The root growth of the *osnlp1* was reduced when nitrate was used as a sole source of nitrogen. But the shoot part did not show any obvious phenotype compared with WT. The *osnlp1/4* line showed severer growth defects compared with the *osnlp4* single mutant. Total nitrate concentration and NR activity were decreased in the *osnlp1/4* but not in the *osnlp1*. In the *osnlp4*, mRNA accumulation of genes related to nitrate assimilation were reduced, while those of nitrate transport genes were reduced in the *osnlp1* mutant line. More interestingly, translational fusions with GFP showed that OsNLP1 appeared to be localized to nucleus in a nitrate-dependent manner activated by nitrate-dependent nuclear retention. Additionally, no protein interaction of OsNLP1 and OsNLP4 was detected. On the other side, the phenotypes of *osnlps* mutants were not nitrate-specific. Nitrite and glutamine, the products of nitrate in assimilation pathway, cannot recover the growth defects of mutants totally. Our results suggest that compared with OsNLP4, OsNLP1 plays a redundant or different role in nitrate response pathway.

Result

***OsNLP1* is essential for nitrate-dependent rice root growth**

In order to compare the functions of *OsNLP1* and *OsNLP4*, I obtained an *OsNLP1* Tos-17 insertion line (*osnlp1*) which has a Tos-17 insertion in the fourth exon in Nipponbare background (Figure 2-1a). And I screened a homozygous line of double mutant (*osnlp1/4*) by hybridizing the *osnlp1* line and the *osnlp4-1* line. The WT, *osnlp1* single mutant, *osnlp4* single mutant and *osnlp1/4* double mutant were grown under the 2 mM KNO$_3$ (nitrate), 2 mM NH$_4$Cl (ammonium) and 2 mM KCl (nitrogen free) conditions for two weeks. In ammonium and nitrogen free conditions, no differences were observed in all mutants compared with WT (Figure 2-1b, c), although the mRNA level of *OsNLP1* was increased in nitrogen free condition (Figure 1-3b). However, under nitrate condition, the *osnlp1* showed shorter root length and reduced root dry weight as the *osnlp4* (Figure 2-2a, b). But the shoot part of the *osnlp1* did not show any obvious phenotypes, unlike the case of the *osnlp4* (Figure 2-2b). Interestingly, the *osnlp1/4* line showed severer growth defects compared with the *osnlp4* single mutant in shoot length (Figure 2-2b). Although *osnlp1* and *osnlp4* had shorter roots compared with that of WT, no difference existed between the two single mutant lines (Figure 2-2b).

To confirm this kind of phenotypes, I also tried low (0.2 mM) and high (5 mM) nitrate conditions using 3-week plants. Similar to the normal nitrate condition, the growth of both shoots and roots of *osnlp4* and *osnlp1/4* were impaired (Figure 2-3a, b). In the case of *osnlp1*, only the root part was weakened especially in higher nitrate condition (Figure 2-3a, b). The phenotypes of all mutants under low and high nitrate conditions were consistent with normal nitrate condition.

Taken together, different with *OsNLP4*, *OsNLP1* is only essential for nitrate-dependent rice root growth. The root part phenotypes of *osnlp1* are induced by nitrate independent of concentration.

Suppression of *OsNLP1* down-regulates nitrate transporter genes *NRT1.5a* and *NRT2.1* in root

Expression of some selective genes related to nitrate assimilation and nitrate transport was analyzed by qRT-PCR to examine their mRNA expression levels in the *osnlp1* and the *osnlp1/4*. Result indicated that nitrate reductase gene *NIA1*, which declined in the *osnlp4* both shoot and root, had a relatively lower expression level only in the *osnlp1* root not shoot (Figure 2-4). The mRNA level of nitrite reductase gene *NIR1* was similar with WT in all three mutants (Figure 2-4). More interestingly, nitrate transport genes *NRT2.1* and *NRT1.5a*, which were not impaired in the *osnlp4*, were appreciably reduced in the *osnlp1* root (Figure 2-4). In the *osnlp1* shoot, *NRT1.5a* was also down-regulated while *NRT2.1* was up-regulated even higher than the *osnlp4* (Figure 2-4). In

the way of the double mutant *osnlp1/4*, it was reasonable to see both nitrate assimilation genes and nitrate transporter genes were impacted (Figure 2-4).

Overall, *OsNLP1* plays a vital role in the regulation of nitrate transporter genes expression outstandingly in root part whereas OsNLP4 controls the expression of nitrate assimilation genes.

The element concentrations in *osnlp1* mutants is changed in a large range

As usual, I investigated the total nitrate concentration in plants grown under nitrate condition. In shoot part, the nitrate concentration of the *osnlp1* was alike WT while the *osnlp1/4* was similar to the *osnlp4* (Figure 2-5a). In root part, the nitrate concentration of *osnlp1* and *osnlp1/4* seemed a little lower than WT and *osnlp4.* But significance test showed no difference (Figure 2-5a).

The concentrations of other elements were also analyzed in the *osnlp1* by ICP-MS. The concentration of Na was higher in shoot and lower in root. The situation of Mg was reverse. In addition, Co concentration was quite higher in both shoot and root. But Li and Ni were reduced in the whole plant (Figure 2-5b). These results suggest that *osnlp1* does not affect nitrate concentration in rice. Possibly, OsNLP1 works as a transcription factor taking a role in other elements uptake or translocation.

OsNLP1 does not affect nitrate assimilation

The ICP-MS result showed the concentration of iron was slightly lower in the *osnlp1* mutant (Figure 2-5b). I checked the NR activity as before. The NR activity of the *osnlp1* had a slight reduction, but not as significant as the *osnlp4* and *osnlp1/4* both in shoot and root (Figure 2-6a). In addition, I analyzed C/N ratio of all mutants in nitrate and ammonium conditions. In nitrate condition, there was no difference between WT and the *osnlp1* while the *osnlp1/4* had higher C/N ratio in shoot than the *osnlp4.* And the *osnlp1/4* had higher C/N ratio in both shoot and root as the way of the *osnlp4* (Figure 2-6b). In ammonium condition, all mutants seemed similar to WT (Figure 2-6c).

In fact, I also examined nitrate uptake speed and nitrite reductase (NiR) activities in mutants. The unit of nitrate absorption rate is NO_3^- (µmol)/ root fresh weight (g)/h. Because the *osnlp1* root was shorter than WT root, the calculated result showed the *osnlp1* took nitrate faster than WT even the amount of absorbed nitrate was same between the two lines (Figure 2-7a & Figure 2-5a). *In vitro* test, the NiR activity was quit low and there was no significant difference between WT and all mutants (Figure 2-7b).

These results suggest that the mutation in *osnlp1* does not impair the nitrate assimilation pathway in rice.

OsNLP1 has nitrate-promoted nucleocytosolic shuttling mechanism

In chapter one, OsNLP4 was always found primarily in the nucleus. In this chapter, I tested the effect of nitrate signal on the subcellular localization of the OsNLP1 protein. Cellular localization was examined under two conditions: 2 mM KNO_3 and 2 mM KCl. Under nitrate starvation, signal for OsNLP1 was found in the nucleus with some addition signal outside the nucleus. Under nitrate supply condition, OsNLP1 was located in the nucleus (Figure 2-8). These data indicate that OsNLP1 is retained in the nucleus in the presence of nitrate, like the case of AtNLP7.

In *A. thaliana*, AtNLP6 can interact with AtNLP7 and take function as heterodimers (Guan et al., 2017b). The bimolecular fluorescence complementation (BiFC) constructs of OsNLP4-nYFP and OsNLP1-cYFP were transformed and tested in rice protoplasts. But no physical interaction between OsNLP1 and OsNLP4 was observed in my conditions (Figure 2-9).

Nitrite and glutamine can't recover the growth of mutants totally

In *A. thaliana*, the growth defects of *nlp* mutants are nitrate specific. The shoot development and root system architecture of *nlp* mutants in other nitrogen sources, such as ammonium and glutamine, are similar to WT (Liu et al., 2017b). Nitrite and glutamine are productions of nitrate in the assimilation pathway. To test nitrite or glutamine can recover the *osnlps* nitrate-starved features or not, I checked phenotypes of all mutants and WT grown hydroponically under 2 mM nitrite or 2 glutamine conditions for 15 days first. In nitrite condition, rice had very short roots and the *osnlps* mutants showed similar development defects with in nitrate condition (Figure 2-10a). Compared with WT, the *osnlp1* had shorter root while both shoot and root of the *osnlp1* and *osnlp1/4* were limited (Figure 2-10a). But in natural condition, some bacteria can produce nitrate from nitrite. And nitrate can be detected in KimuraB solution after 7 days. So it is difficult to confirm the phenotypes of mutants were caused by nitrite in hydroponic cultivation. Then I tried the agar medium to make sure it was the real nitrite condition. But the space in plate is limited, the shoots reach the top and the roots curl at the bottom just after one week (Figure 2-10b). So I checked the phenotypes of 7-day plants this time. And nitrate specific phenotypes of all mutants were repeatable in ager medium (Figure 2-12). In agar medium containing nitrite as the sole N source, the root parts of all mutants still showed obvious phenotypes (Figure 2-10b, c). The *osnlp1/4* had a severe phenotype than *osnlp4* (Figure 2-10c). Overall, the *osnlps* mutants showed similar development defects as them in nitrate condition. However, the C/N ratios of the *osnlp4* and *osnlp1/4* were reduced to WT level (Figure 2-10d).

In glutamine hydroponic culture, KimuraB solution was easily polluted because various bacteria feed on glutamine. The roots of plants were dirty and slimy, although the *osnlp4* showed visible shorter shoot compared with WT and the *osnlp1* (Figure 2-11a). So it was impossible to observe the root phenotype and plants might be in unhealthy condition in hydroponic culture. Then I used 7-day plants in agar medium containing glutamine. The root of the *osnlp1* was still shorter than WT. And the *osnlp1* and the *osnlp1/4* still had shorter shoot and root than WT, though not so significant as in nitrate or nitrite conditions (Figure 2-11b, c). The C/N ratios of all mutants were also similar to WT in glutamine condition (Figure 2-11d).

In total, nitrate and glutamine can't recover the growth defects of the *osnlps* mutants. Especially in nitrite condition, the root part of mutants showed more significant phenotypes while the shoots had a less severe phenotype than in the nitrate condition.

The *osnlp1* line doesn't has obvious agronomic trait

In order to check agronomic traits of our *OsNLP1* Tos-17 insertion line, I planted WT, *osnlp1*, *osnlp4* and *osnlp1/4* in fertilization (+N) and non-fertilization area in Tohoku paddy field (Sendai, Japan) in 2018. In fertilization area, there was no significant difference between WT and the *osnlp1* in tiller number, straw weight and panicle weight. Only the *osnlp4* and *osnlp1/4* showed decreased straw weight (Figure 2-13). Honestly, this result was not consistent with last year (Figure 1-11). One possible reason is the range between maximum and minimum were very large, which means individual difference was considerable. In short, knockoff the *OsNLP1* doesn't affect rice yield in paddy field.

Discussion

***OsNLP1* takes a role in root elongation or shoot-root allocation**

The phenotypes of the *osnlp1* line were different from those of the *osnlp4* line in nitrate condition. The *osnlp4* had shorter shoots and roots compared with WT while in *osnlp1* only root elongation was inhibited (Figure 2-2a, b). And the inhibition of root elongation was unrelated to nitrate concentrations (Figure 2-3). In addition, nitrite and glutamine cannot recover this inhibition (Figure 2-10 & Figure 2-11). The double mutant line *osnlp1/4* even showed shorter root length and lower root dry weight than the *osnlp1* single mutant. However, the shoot part of the *osnlp1* was always similar to WT under all nitrogen supply conditions (Figure 2-1b, Figure 2-2, Figure 2-10 & Figure 2-11). This kind of phenotypes proves *OsNLP1* gene functions in root elongation or shoot-root allocation, unlike the case of *OsNLP4* that regulates growth of both shoot and root. The roles of *OsNLP1* and

OsNLP4 might be separated, though the two genes are classified in clade 1 (Figure 1-3a). Sometimes, the most homologous proteins are not functional orthologous. Root elongation is determined by two linked cell biological processes, namely cell division and cell expansion (Beemster and Baskin, 1998). OsNLP1 probably controls the expression of related genes in the processes as a transcription factor. I have send *osnlp1* RNA samples for RNA sequencing and it is possible to identify which genes were impaired in *osnlp1* root.

It has also been shown that growth rate of roots relative to shoots is negatively correlated with total nitrogen content in plants (Agren and Ingestad, 1987, Levin et al., 1989). Root:shoot (R:S) biomass partitioning is one of the keys to the plants' ability to compensate for limiting resources. The R:S ratio of the *osnlp1* was lower than WT but the nitrate content was not higher in *osnlp1* (Figure 2-5a). Probably, *OsNLP1* can control shoot-root allocation through other pathways. Because the R:S ratio is not only related to nitrogen availability but also stomatal conductance, photosynthesis and leaf relative water content (Nada and Abogadallah, 2016, Maskova and Herben, 2018).

Only a part of nitrate transporter genes is impaired in the *osnlp1* line

My qRT-PCR result indicated that the mRNA expression of nitrate transporter genes *NRT1.5a* and *NRT2.1* were strongly reduced in the roots of *osnlp1* and *osnlp1/4* but not *osnlp4.* And the nitrate assimilation gene *NIA1* was only reduced in *osnlp1* root (Figure 2-4). However, the total nitrate concentration, nitrate uptake ratio, NR activity, NiR activity and C/N ratio were not affected in the *osnlp1* line compared with WT in nitrate condition (Figure 2-5a, Figure 2-6a, b & Figure 2-7b). There are so many nitrate transporters acting in root nitrate uptake or nitrate translocation. Based on my results, OsNLP1 doesn't regulate nitrate assimilation pathway and maybe only a part of nitrate transporter genes are under the regulation of OsNLP1. In the rice NLP family, *OsNLP4* seems a central gene in regulation nitrate assimilation and growth in nitrate condition, while *OsNLP1* might works as a redundant gene in regulating nitrate transport.

The OsNLP1 protein can receive the nitrate signaling

The subcellular localization of OsNLP1 protein is nitrate-dependent, similar to the situation of AtNLP7/6. OsNLP1-GFP signal was detected in the nucleus, but also in the cytosol or other organelles without nitrate application. After nitrate supply, OsNLP1 protein assembled in the nucleus (Figure 2-8). It is proved that OsNLP1 can sense the external nitrate signaling and has the nitrate-dependent nucleocytosolic shuttling mechanism. In *A.thaliana*, nitrate is sensed by the AtNRT1.1 that modulates the primary nitrate response (Liu and Tsay, 2003,

Ho et al., 2009). Then the N-terminal region of AtNLP7 receives nitrate signaling through the phosphorylation of a Ser205 and this process induces the changes in AtNLP7 form and localization (Liu et al., 2017b). One hypothesis is that the same mechanism exists in OsNLP1 protein. But the OsNLP1 protein cannot sense the nitrate signaling directly. OsNRT1.1b has been demonstrated to mediate nitrate signal transduction (Hu et al., 2015). But the kinase in the transduction process is still unknown. And if OsNLP1 protein is in an inactive form when no nitrate is supplied, more in-depth researches are needed to explain the reason rice accumulates the mRNA of *OsNLP1* under nitrogen free condition (Figure 1-3b) and the *osnlp1* still showed similar phenotypes to nitrate condition under nitrite or glutamine conditions (Figure 2-10 & Figure 2-11).

Recently, a phosphate signaling repressor SPX4 is hypothesized to control the localization of NLPs in rice (Hu et al., 2019).In rice, the proper N:P balance is essential for healthy growth. Like N, P also functions as a signaling molecule to regulate gene expression and activate nutritional responses (Gusewell, 2004, Chaturvedi et al., 2014, Luo et al., 2016). Notably, high N concentration greatly promotes P uptake (Gusewell, 2005). The nitrate-phosphate signaling crosstalk may contribute to the localization or function of OsNLP1.

On the other side, in *A. thaliana*, AtNLP6 can bind to AtNLP7 as a heterodimer (Guan et al., 2017a) but AtNLP8 works solely (Yan et al., 2016a). The subcellular localization of OsNLP1 is different with OsNLP4 responding to nitrate signal (Figure 1-10 & Figure 2-8). And the physical interaction between two proteins was not observed (Figure 2-9). It proves that OsNLP1 and OsNLP4 functions separately.

Nitrite and glutamine cannot recover the phenotypes of *osnlps* mutants totally

In *A. thaliana*, the root system architecture defects of *icpk* mutant (in which all AtNLPs are inactive) are nitrate specific (Liu et al., 2017a). However, the situation in rice is dissimilar. The *osnlp4* and *osnlp1/4* still showed growth defects in both shoot and root and the root elongation was inhibited in the *osnlp1* under nitrite and glutamine conditions. But the C/N ratios of all mutants were reduced to the level of WT (Figure 2-10d & Figure 2-11d).

In the shoot part, the *osnlp1* line was always similar to WT under a range of nitrogen conditions (Figure 2-14a). Even in the paddy field, knockout of *OsNLP1* didn't affect biomass and rice yield (Figure 2-13), though root architecture and maintaining activity are important for grain N content and NUE (Bogard et al., 2010). Probably, the *OsNLP1* is only a regulatory target for improving NUE during early vegetative growth not at senescence. But in the case of *osnlp4* and *osnlp1/4*, the mutants showed shortest shoot length in nitrate condition. Nitrite, ammonium and glutamine can promote the shoot growth but didn't recuperate totally to the

height of WT (Figure 2-14a). Moreover, the inhibition of shoot growth was severer than that in root in the *osnlp4* (Figure 2-2b). Overall, the OsNLP4 is essential for rice growth at the whole plant level with main role in shoot. In the *osnlp4*, the reduction from nitrate to nitrite is the key inhibited step. The following reactions and GS/GOCAT cycle are also impaired at some extend via crosstalk with phytohormones or nutrient balance *et al* (Figure 2-14b). Because it is generally assumed that auxin is transported basipetally and mediates N signals from shoot to root and cytokinins may functions as both a local and long-distance signal of N status in plants in both directions between root and shoot (Kiba et al., 2011, Krouk et al., 2010b, Guan, 2017).

In the root part, the *osnlp1* line showed obvious phenotypes in nitrate, nitrite and glutamine conditions (Figure 2-2b, Figure 2-10 & Figure 2-11). Unlike OsNLP4, OsNLP1 might functions only in root responding to local nitrate signaling. Although OsNLPs are reported to express in almost all organs, OsNLP1 may be expressed in special cell layers of roots, such as epidermis, cortex and endodermis et al, to regulate the expression of specific nitrate transporter genes like *NRT1.5a* and *NRT2.1* (Figure 2-4). Under nitrite condition, the root elongation of all *osnlps* mutants was intensely inhibited. Even WT also had quit short root (Figure 2-10a, b, c). It might be related to nitric oxide (NO), a product of nitrite, which can acts as a signal to regulate root formation and primary root biomass accumulation in plants. The GS/GOCAT cycle are also a bit impaired in the *osnlp1* that still had slightly shorter root than WT under glutamine condition (Figure 2-11c). What happened in GS/GOCAT cycle is elusive. The GS activity and GOCAT activity are not regulated in similar manner (Ishiyama et al., 2004b, Martin et al., 2006, Hauck, 2010). In addition, glutamine or glutamine-derived signals provide a link between plant N-assimilatory pathway and circadian rhythms (Gutierrez et al., 2008).

In hypothesis, the *OsNLPs* are not only response to nitrate signal but also others. And OsNLP1 and OsNLP4 take different roles in regulating rice growth in nitrate condition. The former only functions in root via regulation of some nitrate or nitrite transporter genes and root elongation related genes, while the latter acts mainly in shoot by nitrate assimilation pathway controlling the growth at the whole plant level. Trying to understand the clear mechanisms of OsNLPs is likely challenging.

Materials and Methods

Plant materials and growth conditions

The *OsNLP1* Tos-17 insertion line (*osnlp1*), which has an insertion in fourth exon, was obtained from the collection of Tos-17 mutant panel by National Institute of Agro Biological Resources, Tsukuba, Japan. Homozygous Tos-17 line was identified using an *OsNLP1* gene-specific primer (5'-

TCCAACTGCTTGACAGATGC-3') and a Tos-17 left-border primer (5'-ATTGTTAGGTTGCAAGTTAGTTAAGA-3'). The double mutant line of *OsNLP1* and *OsNLP4* (*osnlp1/4*) was constructed by hybridizing the *osnlp1* and the *onslp4-1* introduced in Chapter one.

Hydroponic cultivation had been introduced in Chapter one in details. In the case of agar medium, 3 g ager purified powder (nacalai tesque) was added in every 300 ml KimuraB without N. Then add filtrated nitrate, nitrite, ammonium or glutamine into KimuraB solution at final concentration of 2 mM after sterilization and mixed fully. And put 50 ml medium in each plate. Total six sterilized seeds were placed in each plate at the same height. Plates were put in a chamber for one week. The growth conditions was same with Chapter One.

Analysis of NiR activity

The roots and shoots (1.0 g fresh weight) were separated and ground in a chilled mortar containing 4 ml extraction buffer (50 mM HEPES-KOH pH 8.0, 5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM DTT, 0.5 mM PMSF, 5% (w/v) PVPP). The resulting homogenate was then centrifuged at 8,000 rpm for 10 min at 4 °C. Samples of the supernatant were used for the determination of protein content (NanoDrop ND-1000) and the assays of *in vitro* NiR activity (100 μl). The reaction mixture (800 μl) of the latter consisted of 50 mM Tris-HCl (pH =7.5), 1 mM methyl viologen, 0.5 mM KNO_2 and 50 mg/ml sodium dithionite (dissolved in 100 mM $NaHCO_3$). After incubation at 25 °C for 3 h, the reaction was ended by the addition of 100 μl cold water. Took 100 μl mixture to measure concentrations of remaining NO_2^- at 543 nm (SHIMADZU UV-1850) after the addition of 400 μl 1% (w/v) sulphanilamide (dissolved in 2 M HCl), 300 μl 0.02% (w/v) N-1-naphthylamine and 3.2ml H_2O. NiR activity was expressed as μg NO_2^- mg^{-1} protein h^{-1}. The 0, 0.1mM, 0.2 mM and 0.5 mM nitrite solution were used to build a standard curve.

Bimolecular florescence complementation

The p35S-OsNLP1-GFP plasmid was transfected into rice protoplasts to observe the subcellular localization of OsNLP1. For OsNLP1-cYFP and OsNLP4-nYFP constructs, the full length of *OsNLP1* and *OsNLP4* were cloned into pGW-cYFP (YFP C-terminal portion) and pGW-cYFP (YFP N-terminal portion) vector, respectively (Hino et al., 2011). The p35S-mCherry was used as a control to show successful transient expression. All the constructs were transformed into the rice protoplast (introduced in Chapter One) in BiFC experiment. After transfection, cells were cultured with 0.25 ml inoculation solution containing 2 mM KCl or KNO_3 for 18 h at 25 °C. Then YFP signal and mCherry signal in the cells were analyzed using a confocal laser scanning microscope (OLYMPUS

FV10-MCPSU) at $\lambda_{525nm} \sim \lambda_{550nm}$ and $\lambda_{600nm} \sim \lambda_{625nm}$, respectively. The primers used to construct plasmids were listed in Table 2-1

Rice plant sampling and analysis

WT (Nipponbare), *osnlp1* line, *osnlp4* line and *osnlp1/4* line were planted in fertilization area (+N) and non-fertilization area of Tohoku paddy field (Sendai, Japan) in May, 2018. Tree frames (4 lines × 5 lines) for each genotype were harvested in each area in October, 2018. The panicle weight, straw weight and tiller number were measured from 30 plants sampled from the three harvested frames.

The protocols of qRT-PCR, ICP-MS, nitrate concentration, nitrate uptake rate, C/N ratio and NR activity have been described in Chapter One. In this chapter, the *osnlp1*, *osnlp4* and *osnlp1/4* were used as materials to do these experiments.
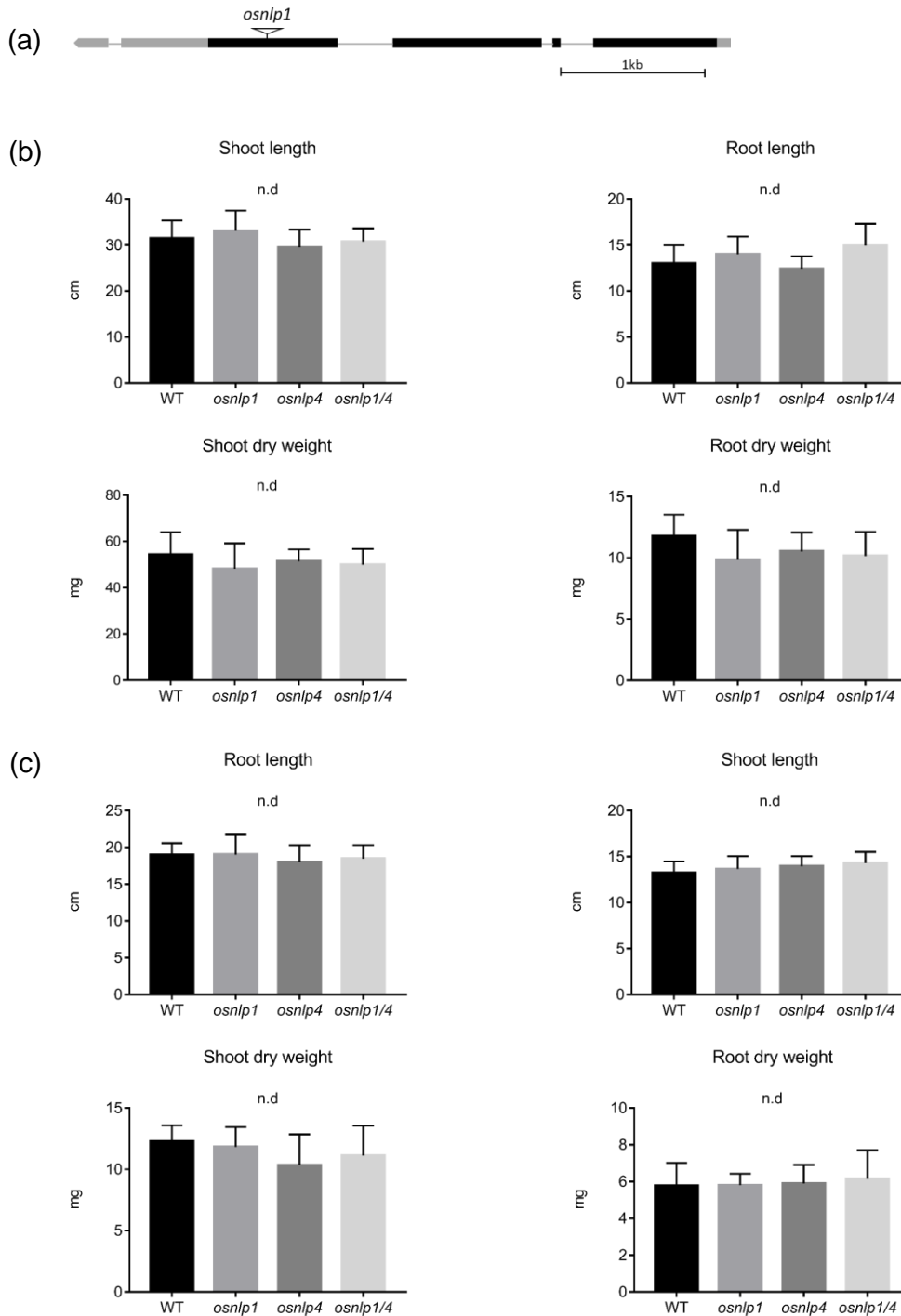
Figure 2-1 Morphological and physiological phenotype of the *osnlp1* line and the double mutant *osnlp1/4* line under ammonium or nitrogen free condition. (a) Location of Tos-17 insertion in the *OsNLP1*. Lines represent introns, whereas grey and black boxes represent UTR regions and exons, respectively. The shoot length, root length, shoot dry weight and root dry weight of *osnlp1* compared to wild type and *osnlp4* for plants grown in hydroponic culture for 15 days on 2mM ammonium (b) or no nitrogen (c) KimuraB solution. Error bars represent the standard deviation for 6 plants. n.d means no difference (REGWQ test).
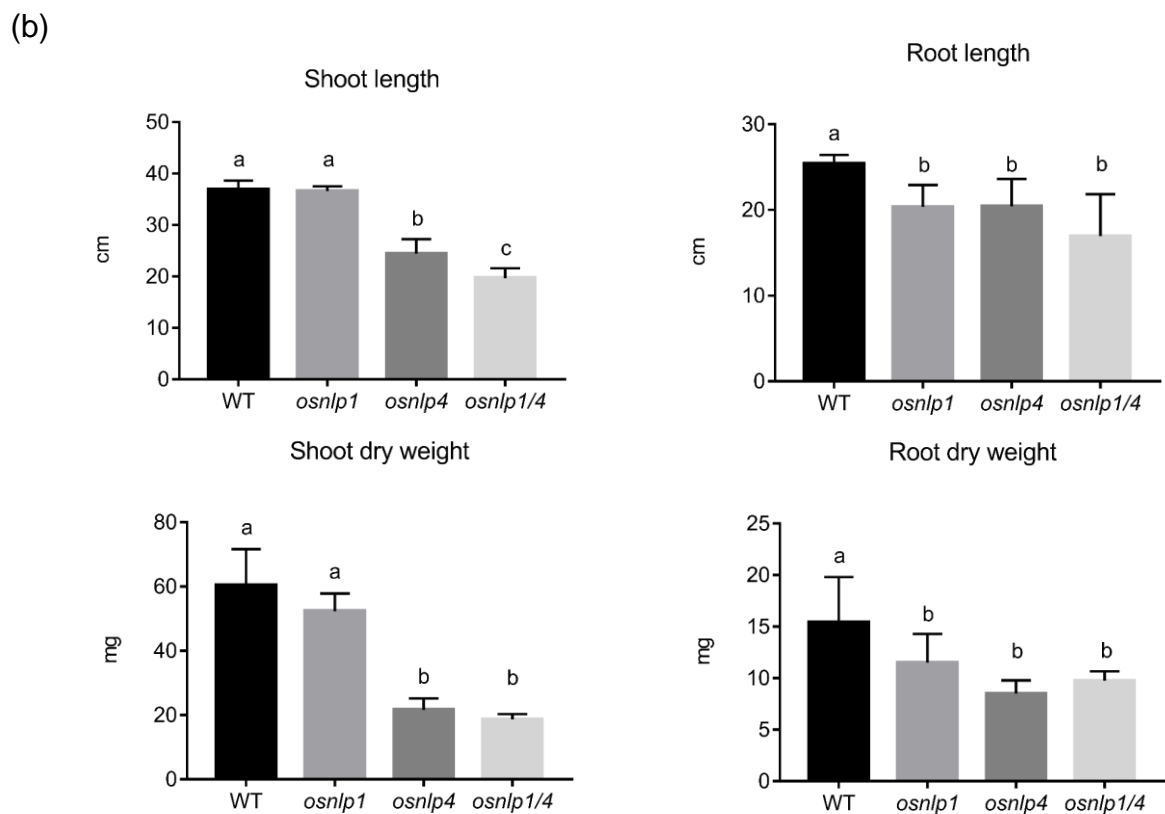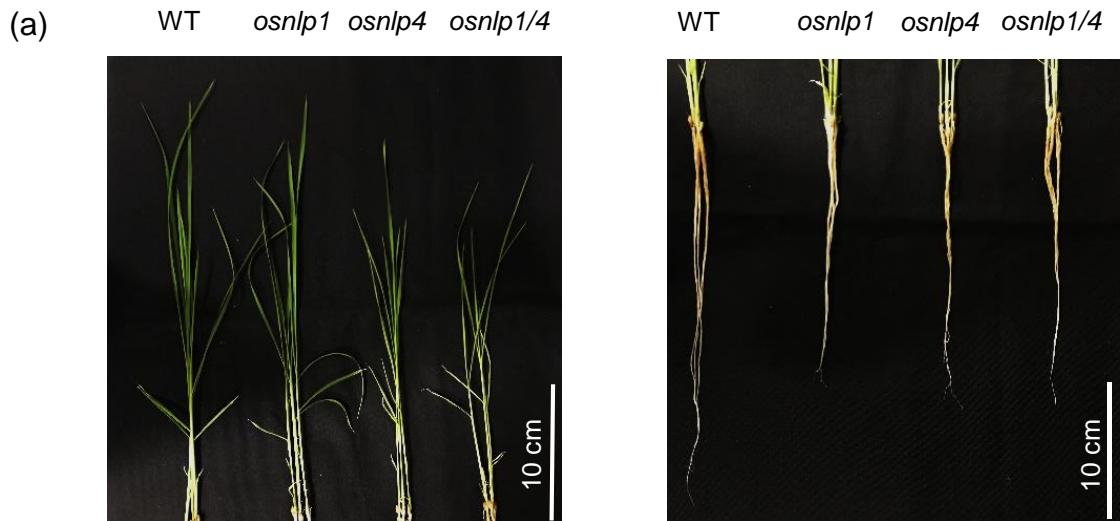
Figure 2-2 Morphological and physiological phenotype of the *osnlp1* line and the double mutant *osnlp1/4* line under nitrate condition. (a) Plants grown in hydroponic culture for 15 days on 2 mM nitrate (KNO_3) modified KimuraB solution. Scale bars, 10cm. (b) The shoot length, root length, shoot dry weight and root dry weight of *osnlp1, osnlp4* and *osnlp1/4* compared to wild type for plants grown as in (a). Error bars represent the standard deviation for 6 plants (REGWQ test, *$p<0.05$).
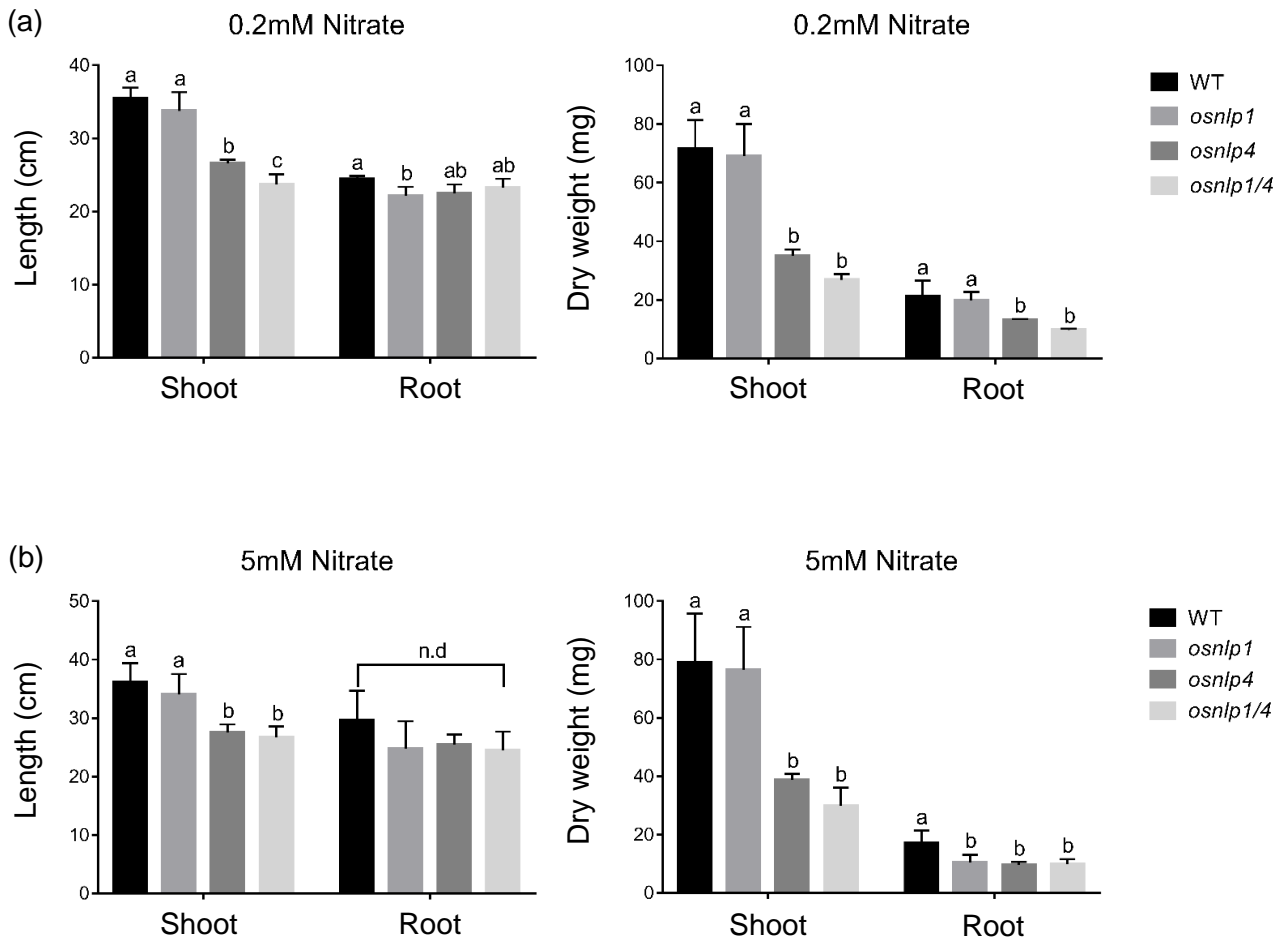
Figure 2-3. Morphological and physiological phenotype of mutants under low and high nitrate condition. The shoot length, root length, shoot dry weight and root dry weight of *osnlp1, osnlp4 and osnlp1/4* compared to wild type grown in hydroponic culture for 21 days on 0.2 mM nitrate (a) or 5 mM nitrate (b) KimuraB solution with of a density of 8 seeds/L. Error bars represent the standard deviation for 6 plants. n.d means no difference (REGWQ test, *$p<0.05$).
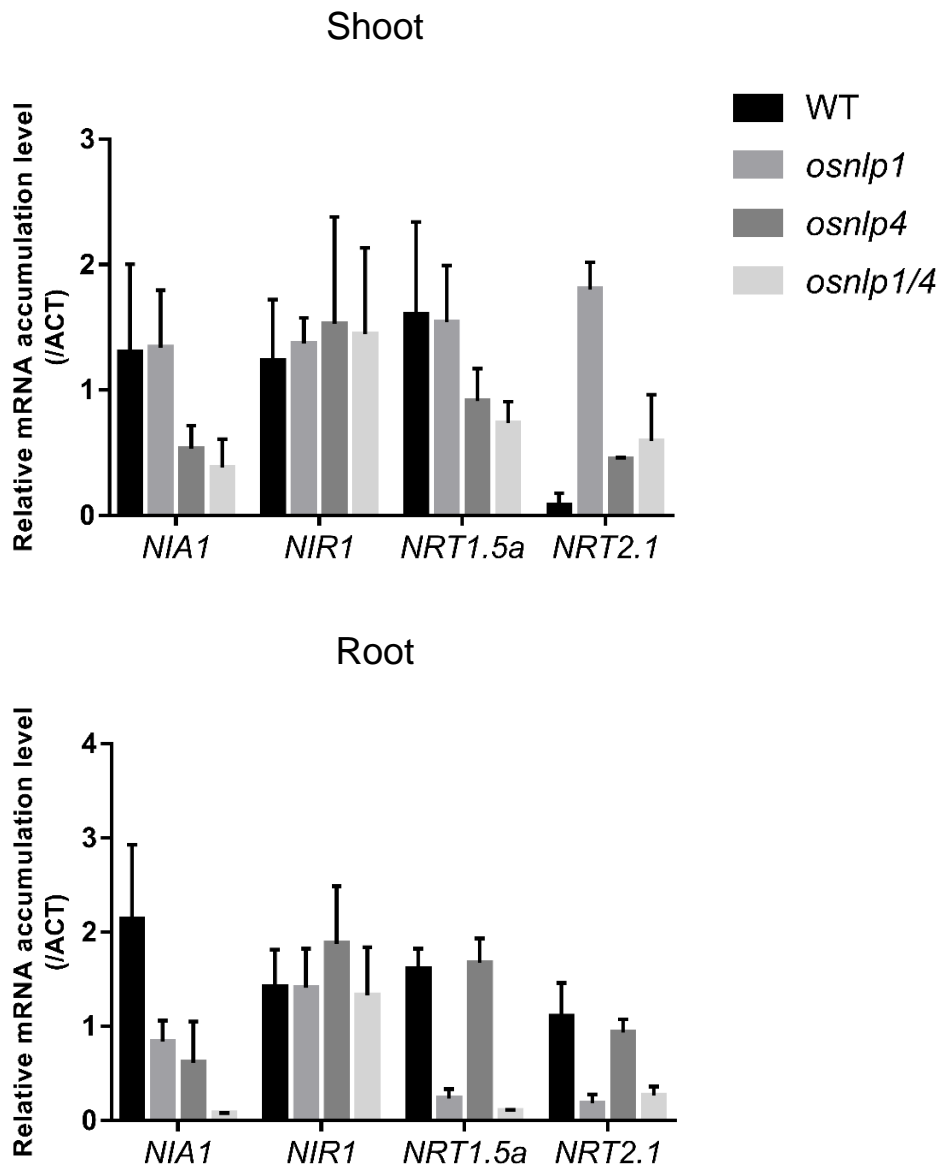
Figure 2-4. The mRNA expression levels of nitrate reductase genes and nitrate transporter genes in the all mutants under nitrate condition. The steady-state transcript amounts for the nitrate reductase genes *NIA1*, nitrite reductase gene *NIR1*, high-affinity nitrate transporter genes *NRT2.1* and root-to-shoot nitrate transporter gene *NRT1.5a* were determined by qRT-PCR. The results given are a percentage of the level of mRNA for the *ACT* gene. Error bars, s.d, n=3 biological replicates.

(a)

Nitrate Concentration

(b)
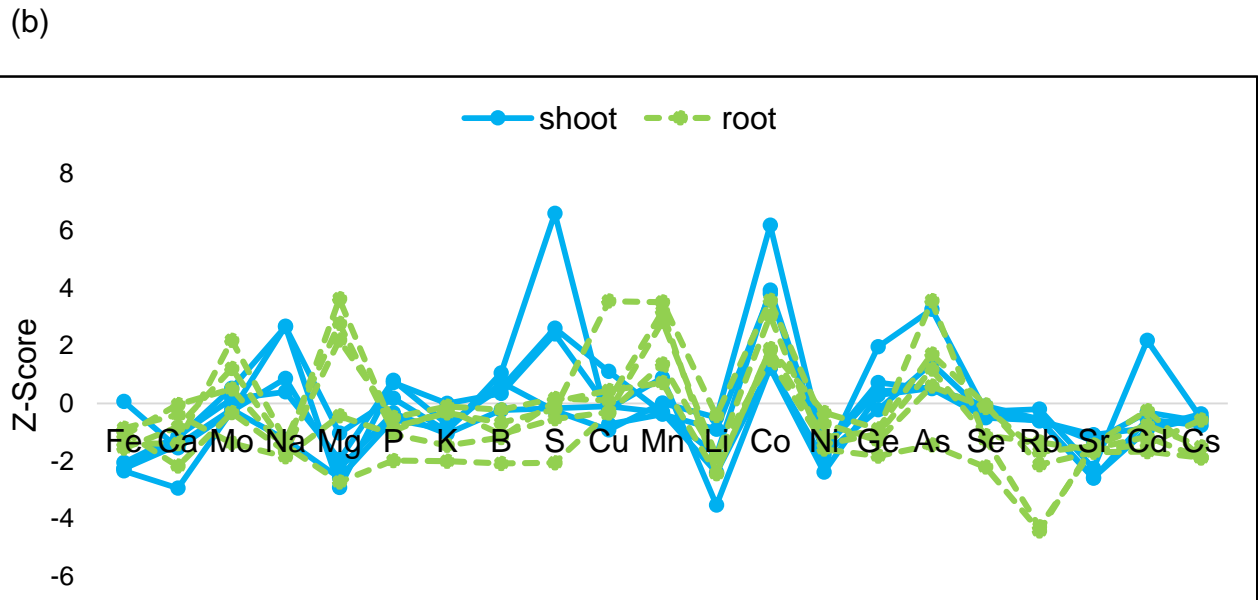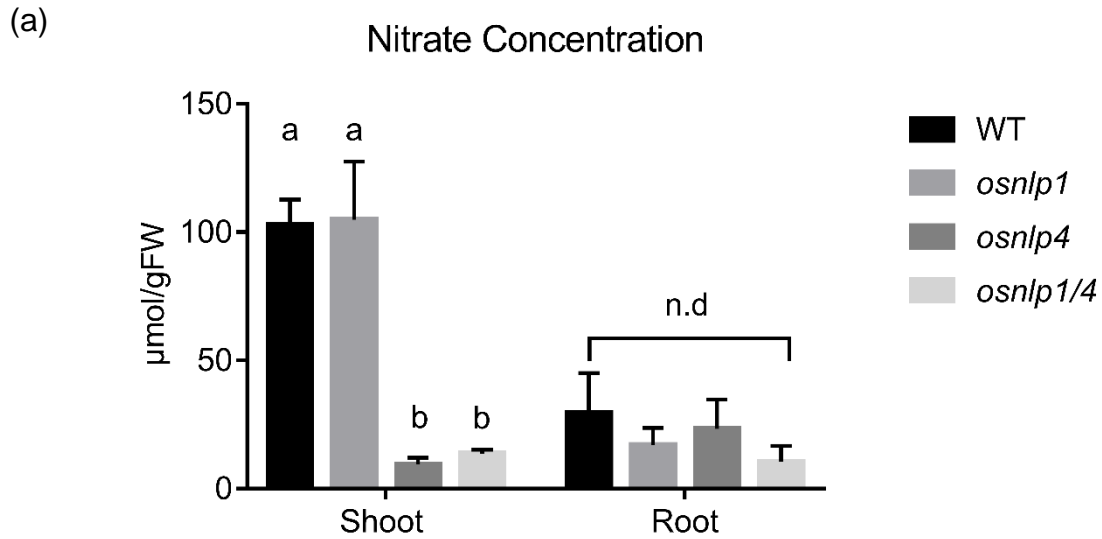
Figure 2-5. The element concentrations in *osnlp1* mutants under nitrate condition. (a) The total nitrate concentration of 15-day plants grown under nitrate condition was detected by HPCE. Error bars, s.d, n=3 (REGWQ test, *p<0.05). (b) Other irons except N were detected by ICP-MS. n=5.
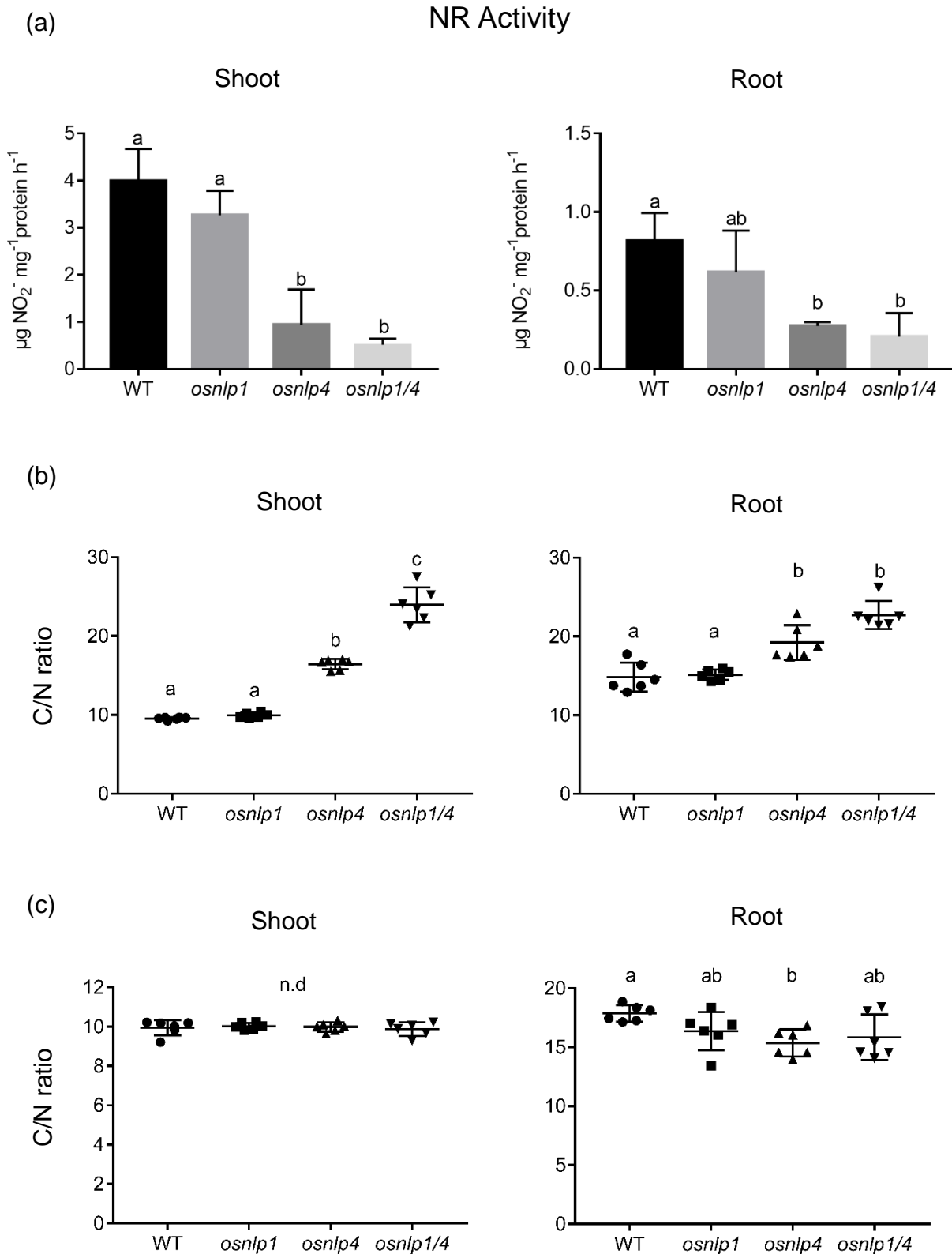
Figure 2-6. The NR activity and C/N ratio in the *osnlp1 and osnlp1/4* mutant. (a) Nitrate reductase activity of WT and *osnlp1, osnlp4 and osnlp1/4* mutants under nitrate condition. n=3. The carbon to nitrogen ratio of plants in nitrate condition (b) or ammonium condition (c). Error bars, s.d, n=6 (REGWQ test, *$p<0.05$).

(a) Nitrate Absorption Rate
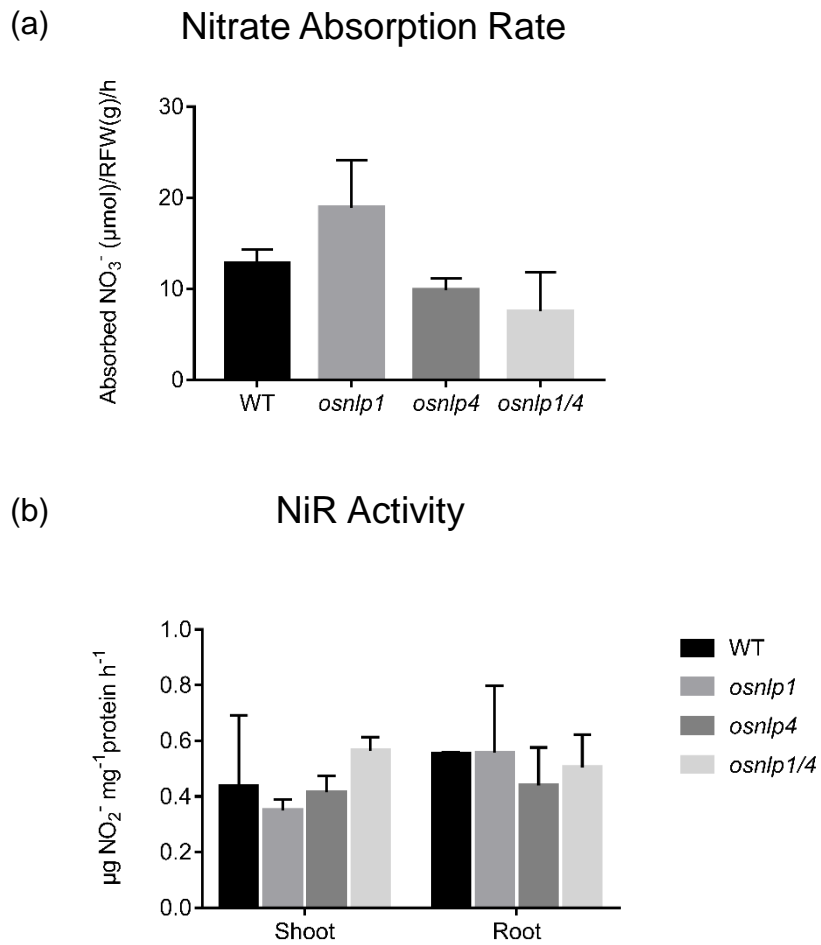
(b) NiR Activity

Figure 2-7. The nitrate uptake speed and NiR activity of all mutants. (a) Nitrate absorption rate of 15-day WT, *osnlp1, osnlp4 and osnlp1* under continuous nitrate condition. (c) Nitrite reductase activity. Error bars, s.d, n=3 (REGWQ test, *$p<0.05$).
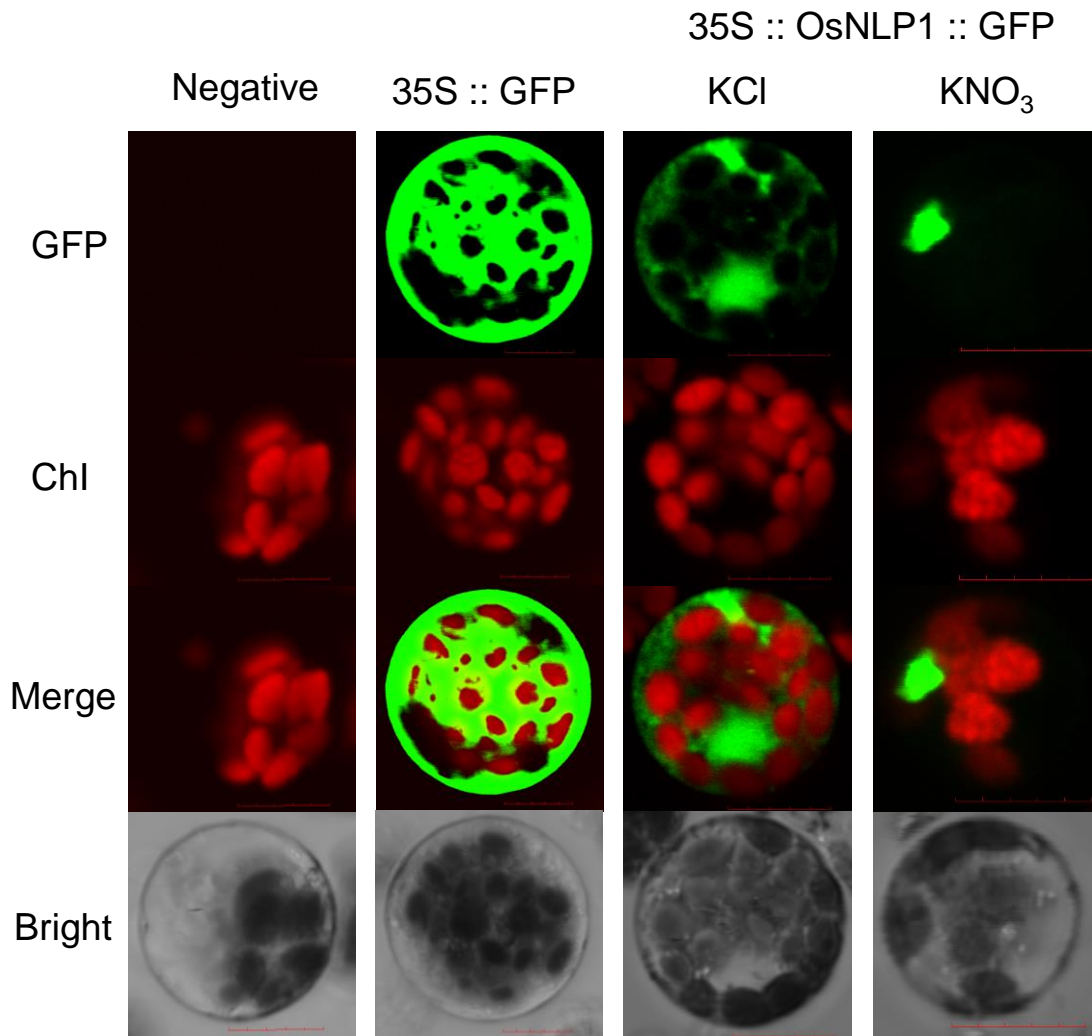
Figure 2-8. Subcellular localization of OsNLP1-GFP in the KCl- or KNO₃- treated rice protoplasts. The pMDC83 vector was used as a negative control and 35S :: GFP was used as a positive control of GFP signal. After PEG-mediated transformation, added KCl or KNO$_3$ to the protoplast incubation medium at 2 mM final concentration. Chl : chloroplasts autofluorescence. A bar indicates 10 μm. Images are representative of 6 protoplasts.
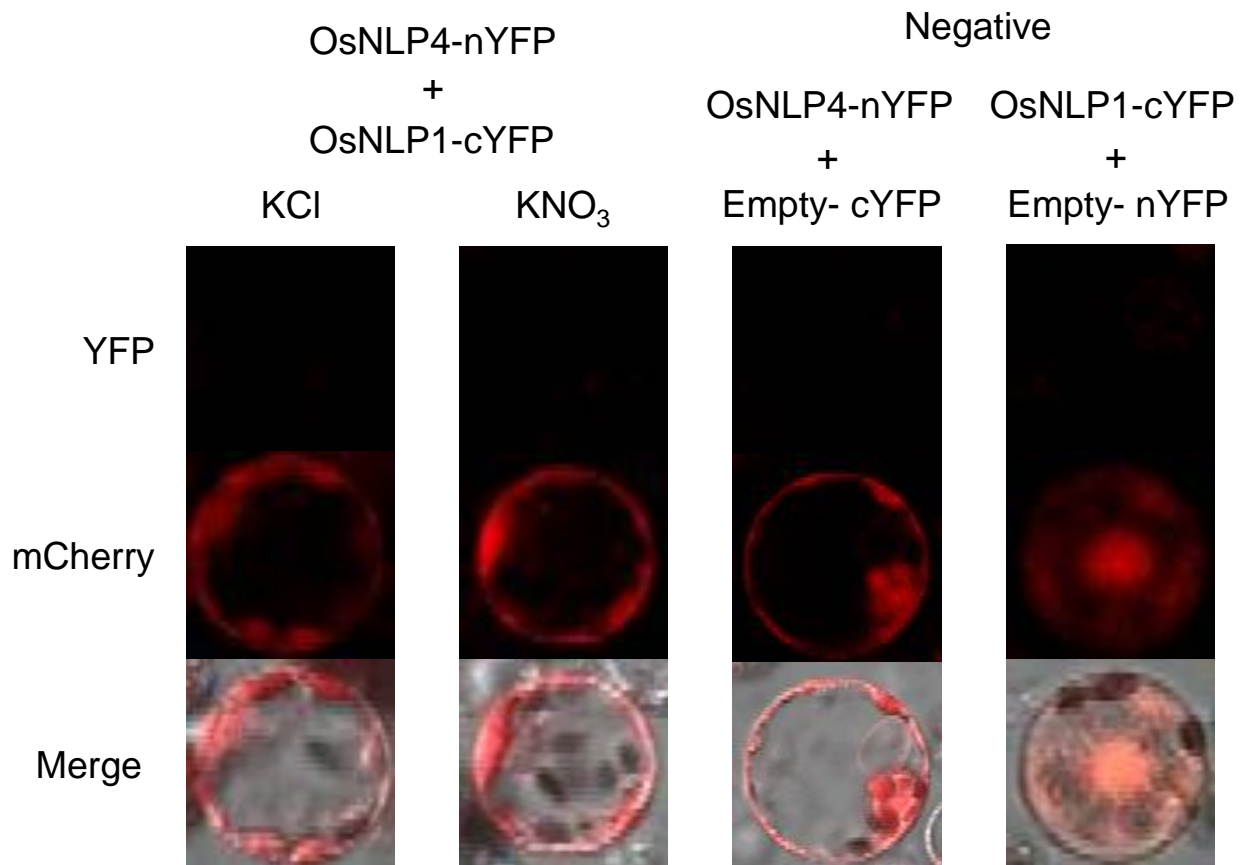
Figure 2-9. Protein-protein interaction between OsNLP4 and OsNLP1. The red mCherry was used as a control to show successful transient expression. BiFC was conducted in rice protoplasts in N-starvation and nitrate treatment. Images are representative of 5 protoplasts.
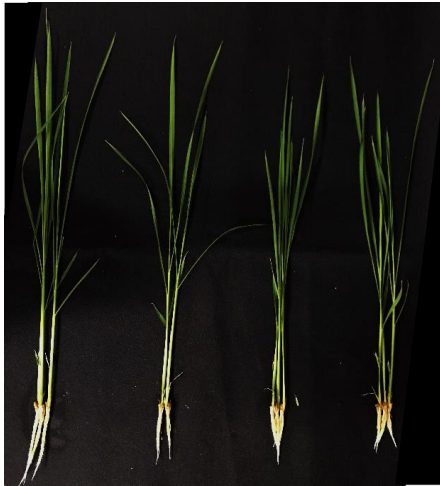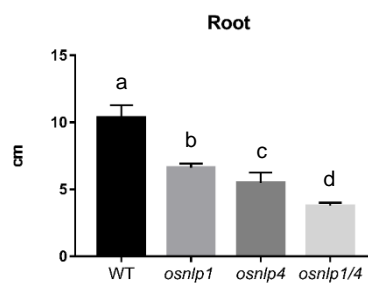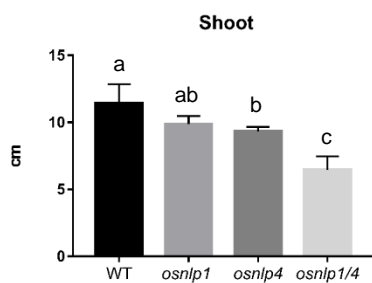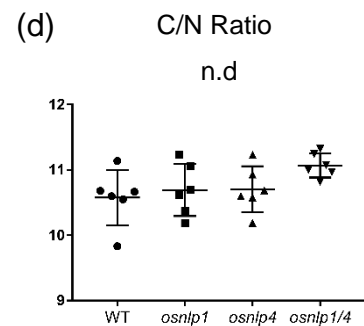
Figure 2-10 Morphological and physiological phenotype of WT and the *osnlp1*, *osnlp4*, *osnlp1/4* under nitrite condition. (a) Plants grown in hydroponic culture for 15 days on 2 mM nitrite (KNO_2) modified KimuraB solution with of a density of 8 seeds/L. (b) Plants grown in agar medium containing 2 mM nitrite for 7 days. (c) The shoot length and root length of the *osnlp1, osnlp4* and *osnlp1/4* compared to wild type for plants grown as in (b). (d) The carbon to nitrogen ratio of shoot of plants grown as in (a). Error bars represent the standard deviation for 6 plants (REGWQ test, *$p < 0.05$).
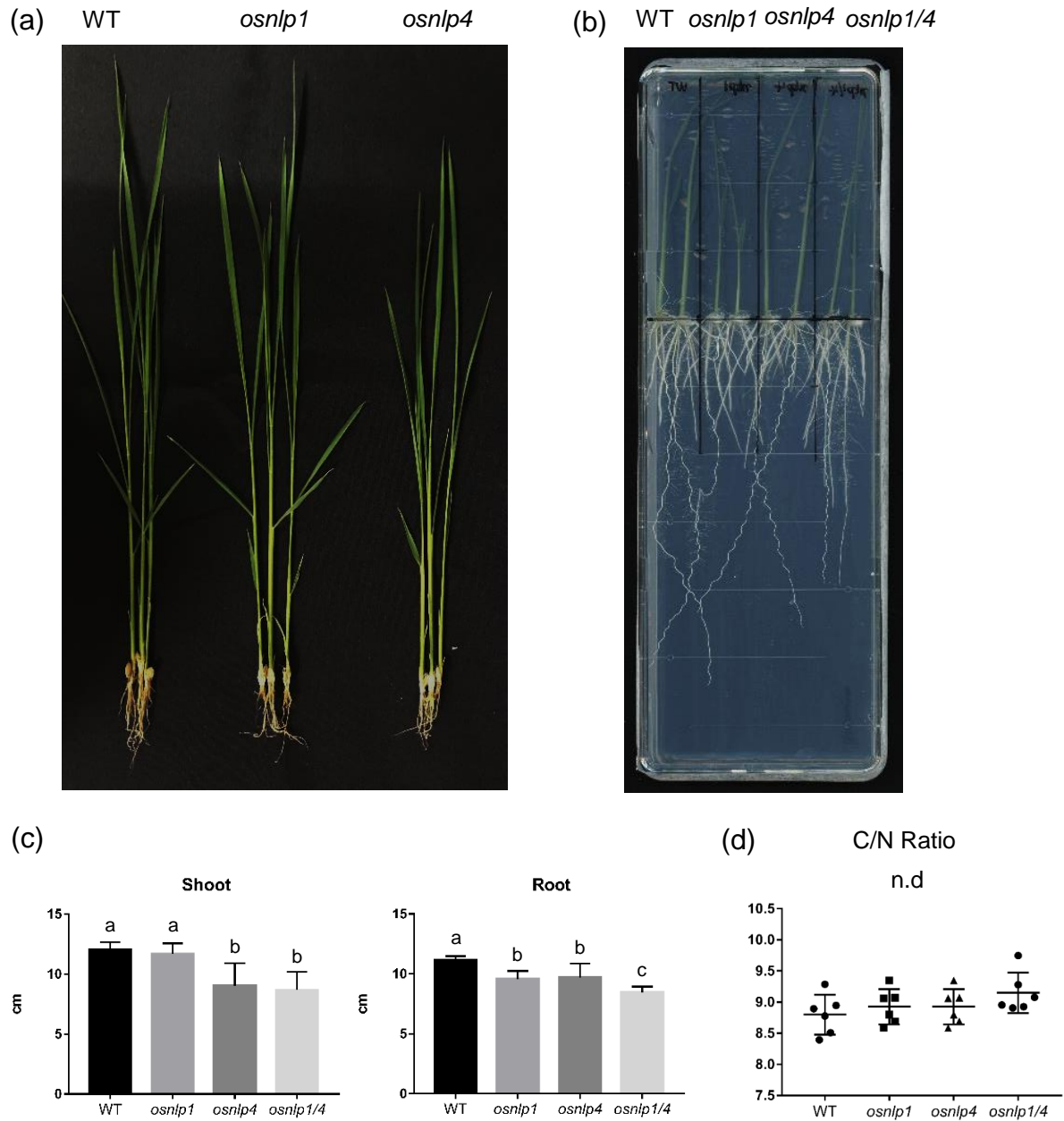
Figure 2-11 Morphological and physiological phenotype of the WT and the *osnlp1, osnlp4, osnlp1/4* **under glutamine condition.** (a) Plants grown in hydroponic culture for 15 days on 2 mM glutamine modified KimuraB solution with of a density of 8 seeds/L. (b) Plants grown in agar medium containing 2 mM glutamine for 7 days. (c) The shoot length and root length of the *osnlp1, osnlp4* and *osnlp1/4* compared to wild type for plants grown as in (b). (d) The carbon to nitrogen ratio of shoot of plants grown as in (a). Error bars represent the standard deviation for 6 plants (REGWQ test, *$p < 0.05$).
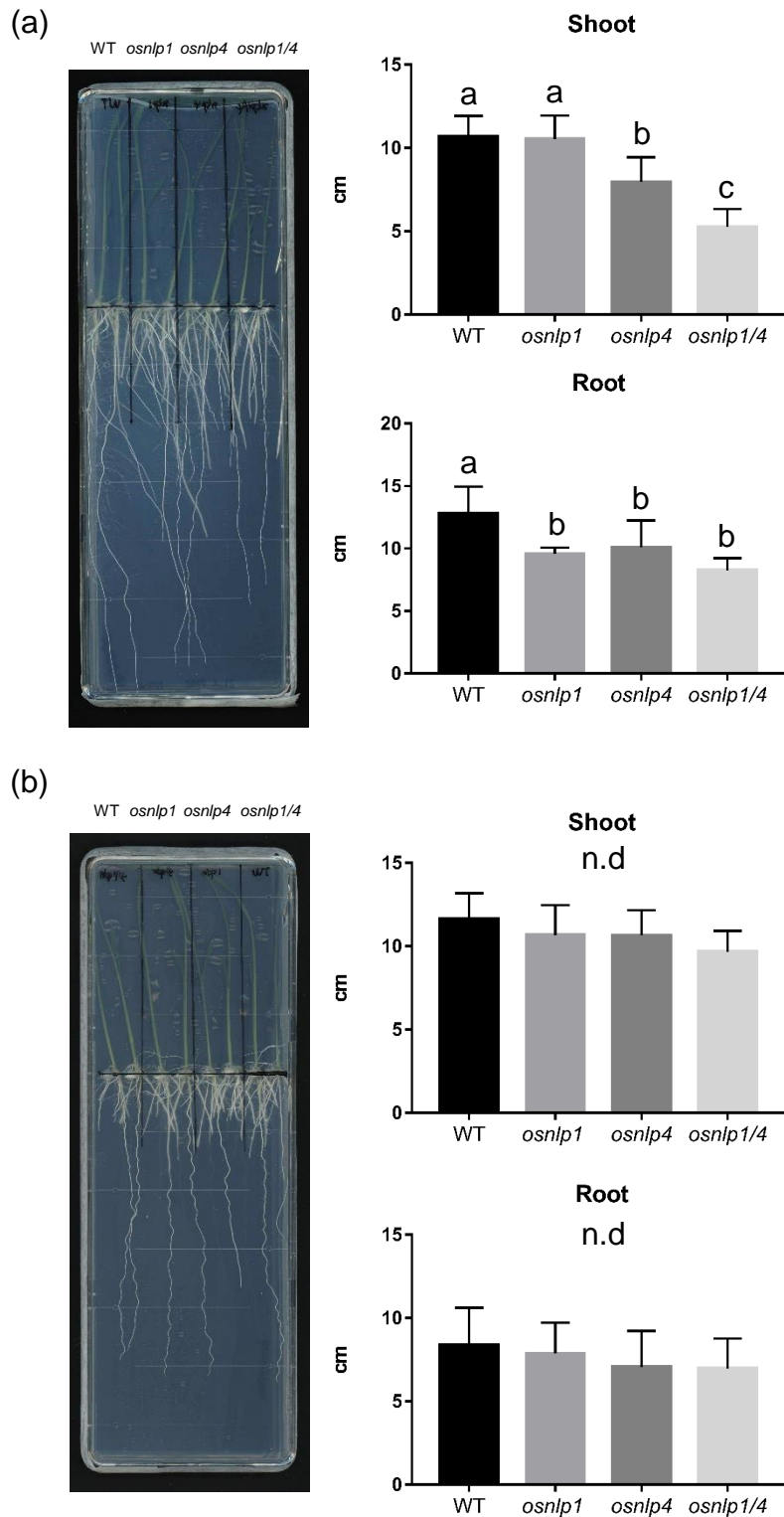
Figure 2-12. Morphological and physiological phenotype of WT and the *osnlp1*, *osnlp4*, *osnlp1/4* in agar medium containing nitrate and ammonium. Plants grown in agar medium containing 2 mM nitrate (a) or 2 mM ammonium (b) for 7 days. The shoot length and root length of the *osnlp1, osnlp4* and *osnlp1/4* were compared with wild type. Error bars represent the standard deviation for 6 plants (REGWQ test, *p<0.05).
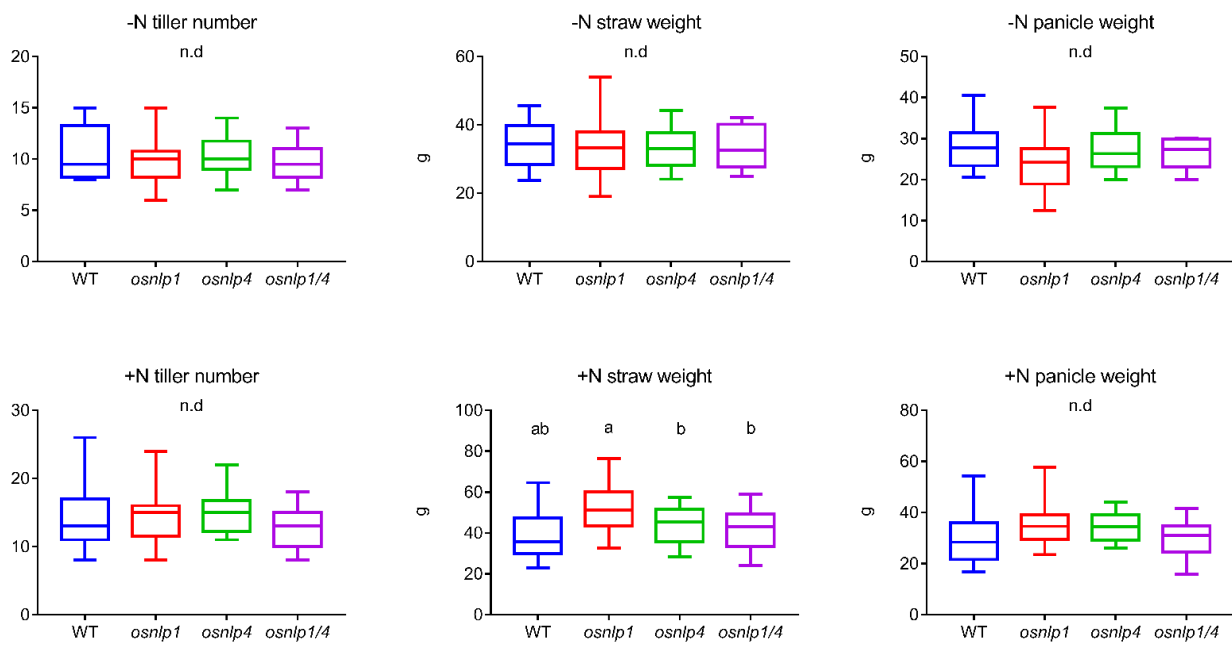
Figure 2-13 Agronomic traits of the *osnlp1, osnlp4* and *osnlp1/4* in paddy field (2018). Tiller number, straw weight and panicle weight per plant were determined for the *osnlp1, osnlp4* and *osnlp1/4* in comparison to WT in a field trial conducted in Sendai, Japan in 2018 (+ N, Fertilizer paddy field; -N, No-fertilizer paddy field).n=30 (REGWQ test, *p<0.05).
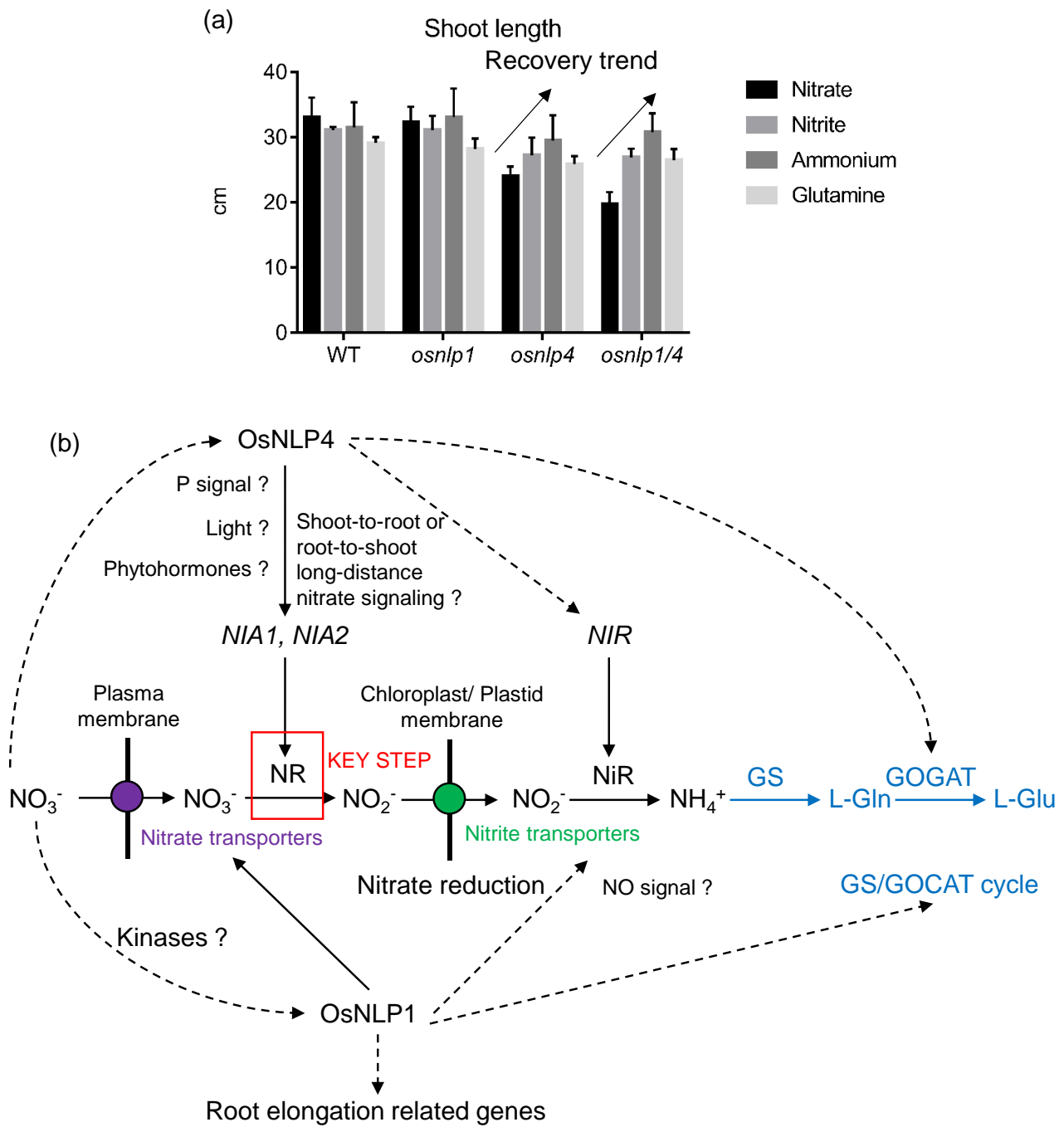
Figure 2-14 The hypothesis of differential roles of *OsNLP1* and *OsNLP4* in nitrate pathway. (a) The recovery trend of *osnlps* mutants in shoots in a range of nitrogen conditions. (b) A schematic model of proposed OsNLP1 and OsNLP4 regulation network in rice.

Table 2-1. Primer pairs used for constructing plasmids and sequencing.

Name	Primer Name	Sequence (5'-3')
35S::*OsNLP1*::*GFP*	*OsNLP1*-Slice-F	CAATTCAGTCGACTGGATCCGATGGAGCAGAAGCCGTCGCCCCGCCG
	OsNLP1-Slice-R	GAAAGCTGGGTCTAGATACCCAGACAGACCAGTTTGACCGAAGG
35S::*OsNLP4*::*GFP*	*OsNLP4*-Slice-F	CAATTCAGTCGACTGGATCCGATGGAAGAGGGAGACCCCCAGCC
	OsNLP4-Slice-R	GAAAGCTGGGTCTAGATACCCTGAGAAACCAGTGTGACCAATGG
OsNLP1 sequencing primers	*OsNLP1*-A1	CGCCTCCGCGCGGCTGCGTT
	OsNLP1-A2	CTACCGGACGGTGTCGATGA
	OsNLP1-A3	GCTTGATGCCAGGTTGTTGA
	OsNLP1-A4	GCTCTGTAGTGGTATTAATG
	OsNLP1-A5	GCAATAGATCCAACTGAAAG
OsNLP4 sequencing primers	*OsNLP4*-S2	GTTCAGGGAGGTTTCGACAA
	OsNLP4-A2	TTGTCGAAACCTCCCTGAAC
	OsNLP4-A6	TGTGCATGGTTGATCCTAGG
	OsNLP4-S7	CTGGAGTACCCATTGTCTCA
	OsNLP4-A3	TTGGCAAACACTTCTCATGG
	OsNLP4-S4	AAAGTTCCGATTCAAGCCCT
	OsNLP4-A4	AGGGCTTGAATCGGAACTTT
	OsNLP4-A8	GGACTTTGAGACATGTGCTG
	OsNLP4-S10	ACCAGCTTAACAGTGCTTCCT

Chapter 3. The phenotypes of overexpression lines of *OsNLP1*, *OsNLP4* and *OsNLP6* in a range of nitrogen conditions

Abstract

Recently, the homozygous overexpression lines of *OsNLP1*, *OsNLP4* and *OsNLP6* were obtained. In this short chapter, the phenotypes of these lines in a range of nitrogen conditions were initially discussed. The most interesting phenotype is that all ox-lines showed better growth of both shoot and root than WT in varying degrees only in nitrogen free condition but not in nitrogen supply conditions. In nitrogen supply conditions, these ox-lines also had some different phenotypes with WT. For example, the shorter root were observed in nitrate condition. Absolutely, the phenotypes should be confirmed further using more independent lines or nutrient conditions. The possibility was debated in this chapter.

Result

I constructed the overexpression transgenic lines of *OsNLP1*, *OsNLP4* and *OsNLP6* in the Nipponbare background by traditional *Agrobacterium*-mediated transformation. First, the mRNA expression level of each gene was checked in independent ox-*OsNLP1,* ox-*OsNLP4* and ox-*OsNLP6* lines, respectively. In ox-*OsNLP1* line, the mRNA level of *OsNLP1* was more than 4 folds of WT. In ox-*OsNLP4* line, *OsNLP4* expression level was almost 7 times of WT. In ox-*OsNLP6* line, the mRNA level of *OsNLP6* was twice of WT, though the expression level was still very low in nitrate condition (Figure 3-1).

The most interesting thing is that in nitrogen free condition, the growth of all ox-lines was better than WT, which means longer shoots and roots and higher dry weights (Figure 3-2a, c). The shoot dry weight of the ox-*OsNLP6* line was about 25 mg, almost twice of WT, while the ox-*OsNLP4* line showed the highest root dry weight at around 10 mg (Figure 3-2a). But the difference in C/N ratio among WT and ox-lines was not significant (Figure 3-2b).In contrast, in nitrate condition, the root elongation was impaired in all ox-lines compared with WT, though no obvious change was in root dry weight (Figure 3-3a). Only the ox-*OsNLP1* line had relatively lower shoot dry weight (Figure 3-3a). The difference in C/N ration was also not meaningful (Figure 3-3b). In ammonium condition, there was no difference in all testing factors between WT and ox-lines, in addition to ox-*OsNLP1* that had slightly reduced root length (Figure 3-4a, b). In nitrite condition, the ox-*OsNLP1* and the ox-*OsNLP4* had reduced shoot- and root dry weight (Figure 3-5a). But the root of ox-*OsNLP4* line was quit longer than WT and other ox-lines with lower C/N ratio in shoot (Figure 3-5a, b).

In conclusion, the overexpression lines of *OsNLP1* or *OsNLP4* showed nitrogen-responding phenotypes in a range of nitrogen conditions. However, the OsNLP6 overexpression line did not show any obvious phenotype under nitrogen supply conditions in my case, while the growth of it was much healthier than WT in nitrogen free condition.

Discussion

The overexpression lines of *OsNLP1*, *OsNLP4* and *OsNLP6* had better growth than WT in no N condition (Figure 3-2). Rice seedlings utilize the nutrients stocked in seeds first. In addition, the ox-lines showed shorter root length than WT in normal nitrate or ammonium condition without difference in C/N ratio (Figure 3-3 & Figure 3-4). Usually, excessive nitrogen inhibits root growth and promotes shoot growth in rice (Ericsson, 1995, Fichtner and Schulze, 1992). Probably, overexpression of *OsNLPs* can improve NUE. On the other way, this hypothesis can elucidate rice only accumulate *OsNLP1* and *OsNLP4* at mRNA level because WT didn't grow better than

osnlps mutants in nitrogen free condition (Figure 1-5 & Figure 2-1c). And the mRNA expression level of *OsNLP6* was still very low even I used strong promoter 35S to promote its expression in ox-*OsNLP6* line (Figure 3-1). Taking together, the post-transcriptional regulation or post-translational modification must exist in functional OsNLPs. Frankly, I also constructed CRISPR lines of *OsNLP1* and *OsNLP6*. In the case of *osnlp6*, I obtained four homozygous independent lines with 1 bp deletion in the first exon. But the four lines finally died in our green house after around one month (data not shown). It is possible that OsNLP6 is quit essential for specific growth period in rice, although the expression level is not high.

The overall efficiency of the root system depends on not only the root architecture but also on the availability of C provided by photosynthesis (Little et al., 2005, Remans et al., 2006). Larger roots take away more C from the shoots (Coque et al., 2008). It might be the reason ox-*OsNLP4* line had longer root with lower C/N ratio in shoot (Figure 3-5).

To fully assess the functions the OsNLP family in rice, more work is needed to prove binding regions, expression tissues and phosphorylation of proteins *et al.* For economically and environmental friendly use the valuable N resources in paddy field, the research about OsNLPs may provide an idea to understand nutrient balance in rice and cultivate rice seedlings with maximizing NUE for crop improvement.

Materials and Methods

The 35S::*OsNLP1/OsNLP4/OsNLP6* overexpression constructs were made by inserting the coding region of *OsNLP1/OsNLP4/OsNLP6* amplified by PCR into pMDC83 vector via Slice system. The *OsNLP1/OsNLP4/OsNLP6*-overesrpression transgenic lines were obtained by *Agrobacterium*-mediated transformation in the Nipponbare background and homozygous lines were identified by PCR and TAIL-PCR (Liu and Chen, 2007, Liu and Whittier, 1995) with specific primers. All the primers used are listed in Table 3-1. The plant growth conditions, hydroponic culture and analysis of C/N ratio were same with Chapter One.
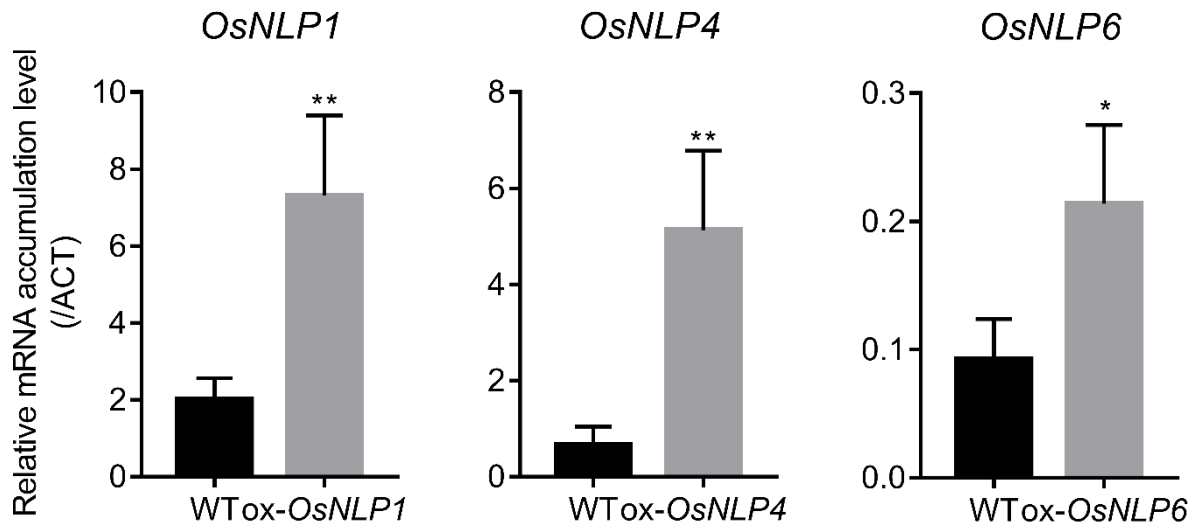
Figure 3-1. The mRNA expression levels of *OsNLPs* in relative overexpression lines of *OsNLP1*, *OsNLP4* and *OsNLP6* in nitrate condition. RNA was extracted from plants grown in hydroponic culture for 15 days in KimuraB solution containing 2 mM nitrate with of a density of 8 seeds/L. Relative expression of *OsNLP*s was analyzed by qRT-PCR and normalized to the expression of *ACT*. Each gene had three biological replicates and the assays were repeated twice. Error bars, s.d (t-test, *$p<0.05$, **$p<0.01$).

(a)

Shoot length

Root length

Shoot dry weight

Root dry weight

(b)

Shoot

Root

(c)

WT ox-OsNLP1 ox-OsNLP4 ox-OsNLP6   WT ox-OsNLP1 ox-OsNLP4 ox-OsNLP6

10 cm   10 cm
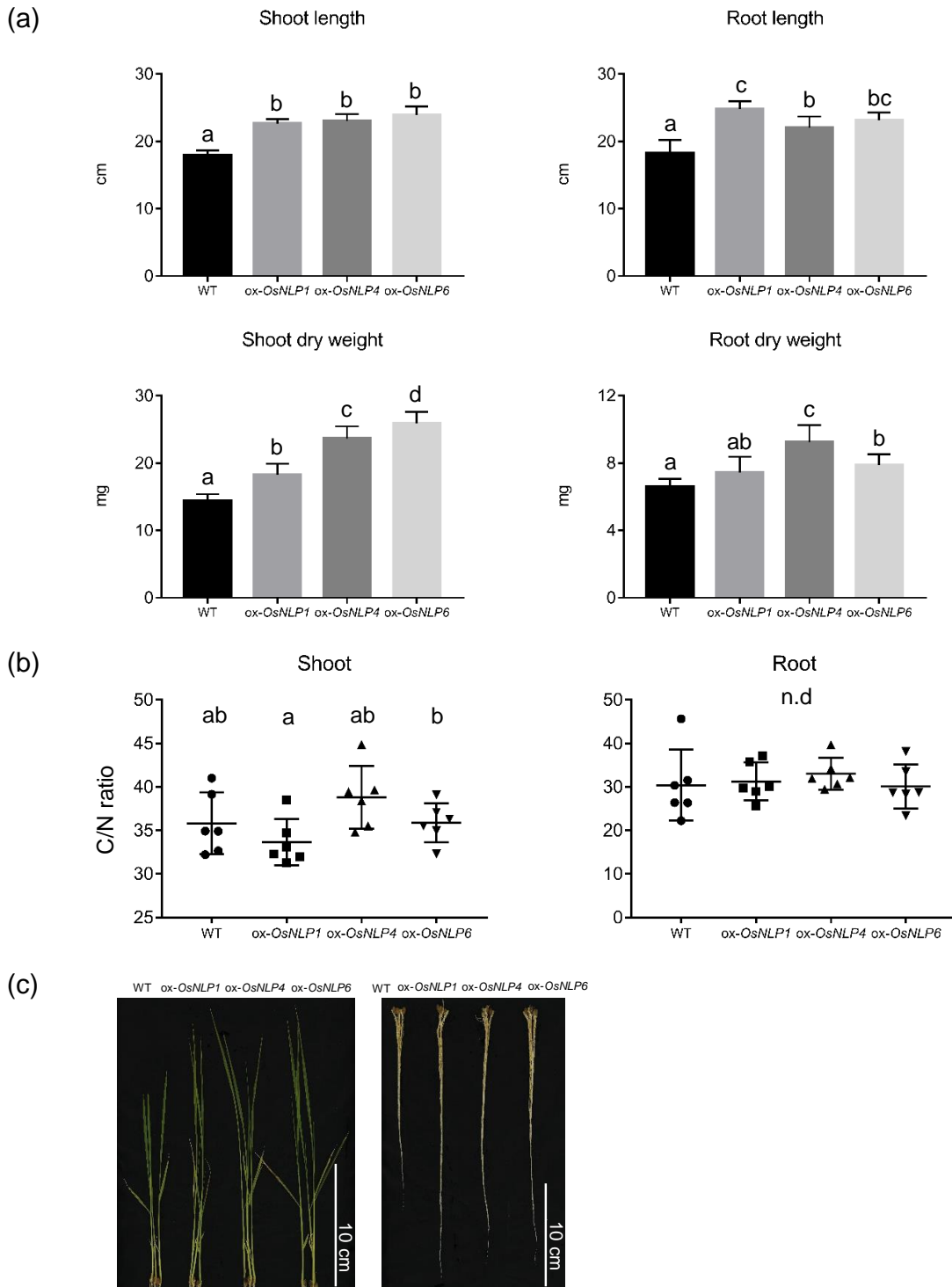
Figure 3-2. Morphological and physiological phenotype of ox-*OsNLP1*, ox-*OsNLP4* and ox-*OsNLP6* lines in nitrogen free condition. (a) The shoot length, root length, shoot dry weight and root dry weight of the overexpression lines were compared to wild type. Plants were grown in hydroponic culture for 15 days on modified KimuraB solution without nitrogen with of a density of 8 seeds/L. Error bars represent the standard deviation for 6 plants. (b) Carbon to nitrogen ration. (c) Photos of WT and the *osnlp4-1* in nitrate condition. Differences are statistically significant (REGWQ test, *p<0.05).
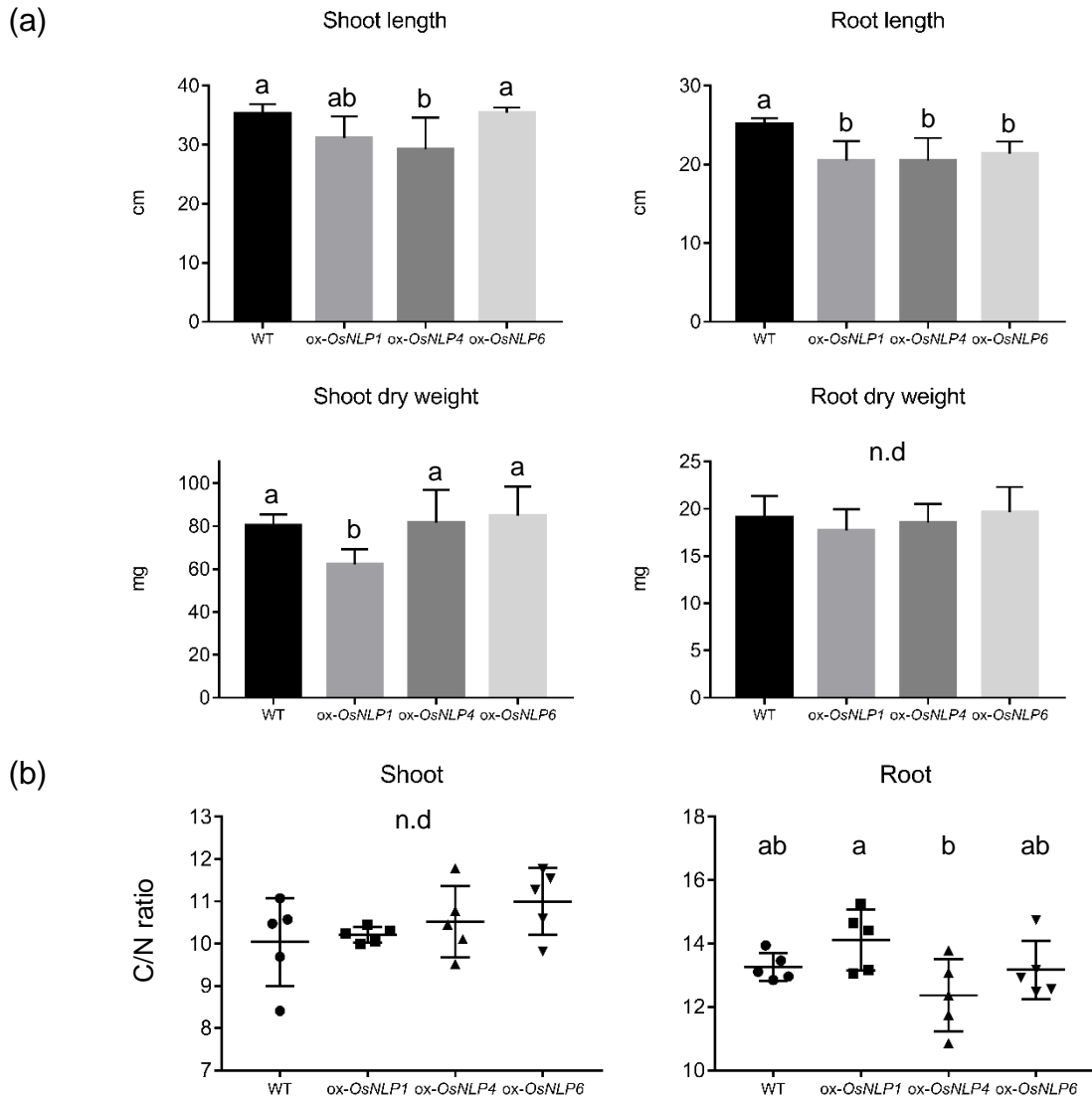
Figure 3-3. Morphological and physiological phenotype of ox-*OsNLP1*, ox-*OsNLP4* and ox-*OsNLP6* lines in nitrate condition. (a) The shoot length, root length, shoot dry weight and root dry weight of the overexpression lines were compared to wild type. Plants were grown in hydroponic culture for 15 days on modified KimuraB solution without nitrogen with of a density of 8 seeds/L. Error bars represent the standard deviation for 6 plants. (b) Carbon to nitrogen ration. Differences are statistically significant (REGWQ test, *p<0.05).
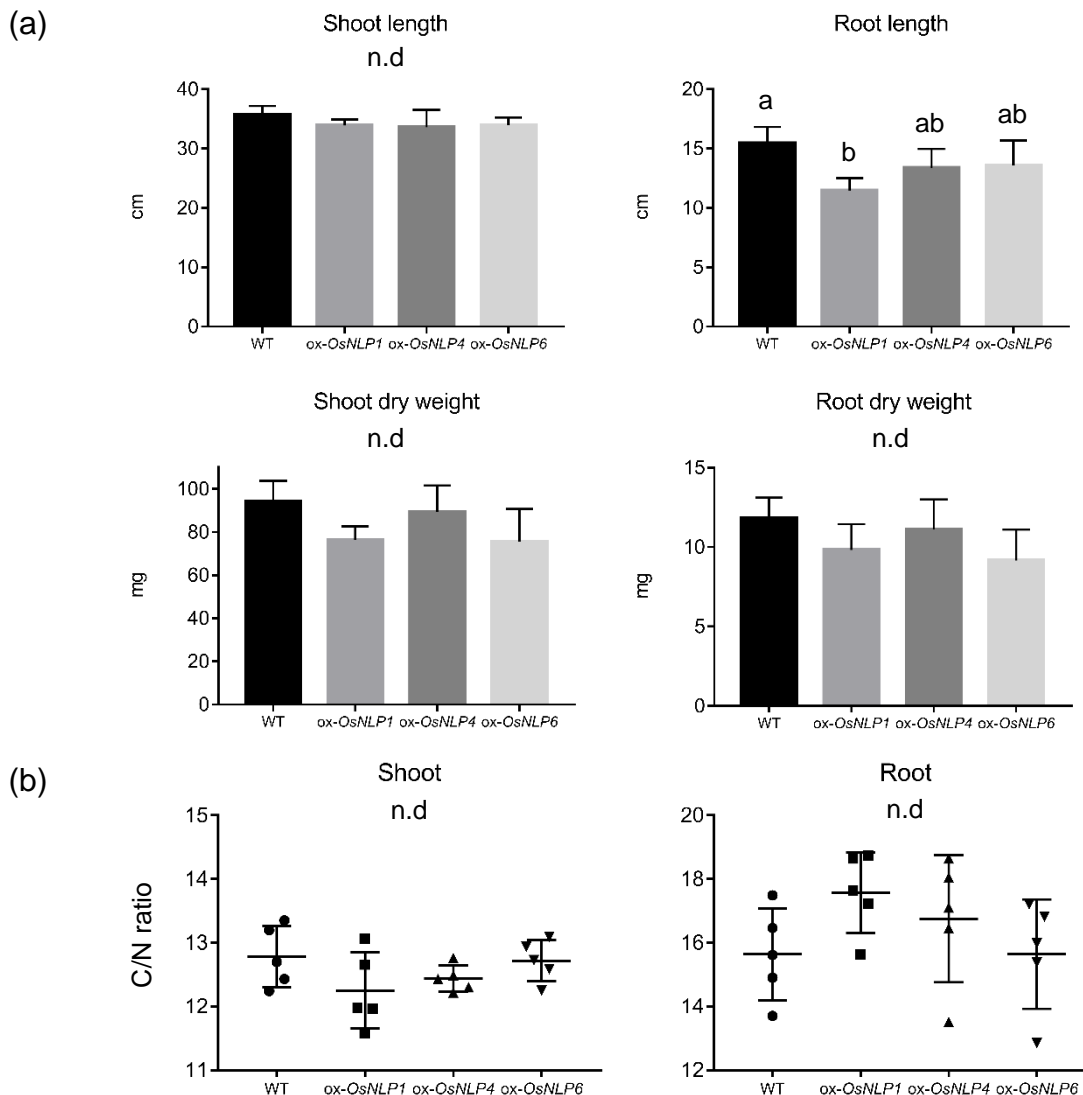
Figure 3-4. Morphological and physiological phenotype of ox-*OsNLP1*, ox-*OsNLP4* and ox-*OsNLP6* lines in ammonium condition. (a) The shoot length, root length, shoot dry weight and root dry weight of the overexpression lines were compared to wild type. Plants were grown in hydroponic culture for 15 days on modified KimuraB solution without nitrogen with of a density of 8 seeds/L. Error bars represent the standard deviation for 6 plants. (b) Carbon to nitrogen ration. Differences are statistically significant (REGWQ test, *p<0.05).

(a)
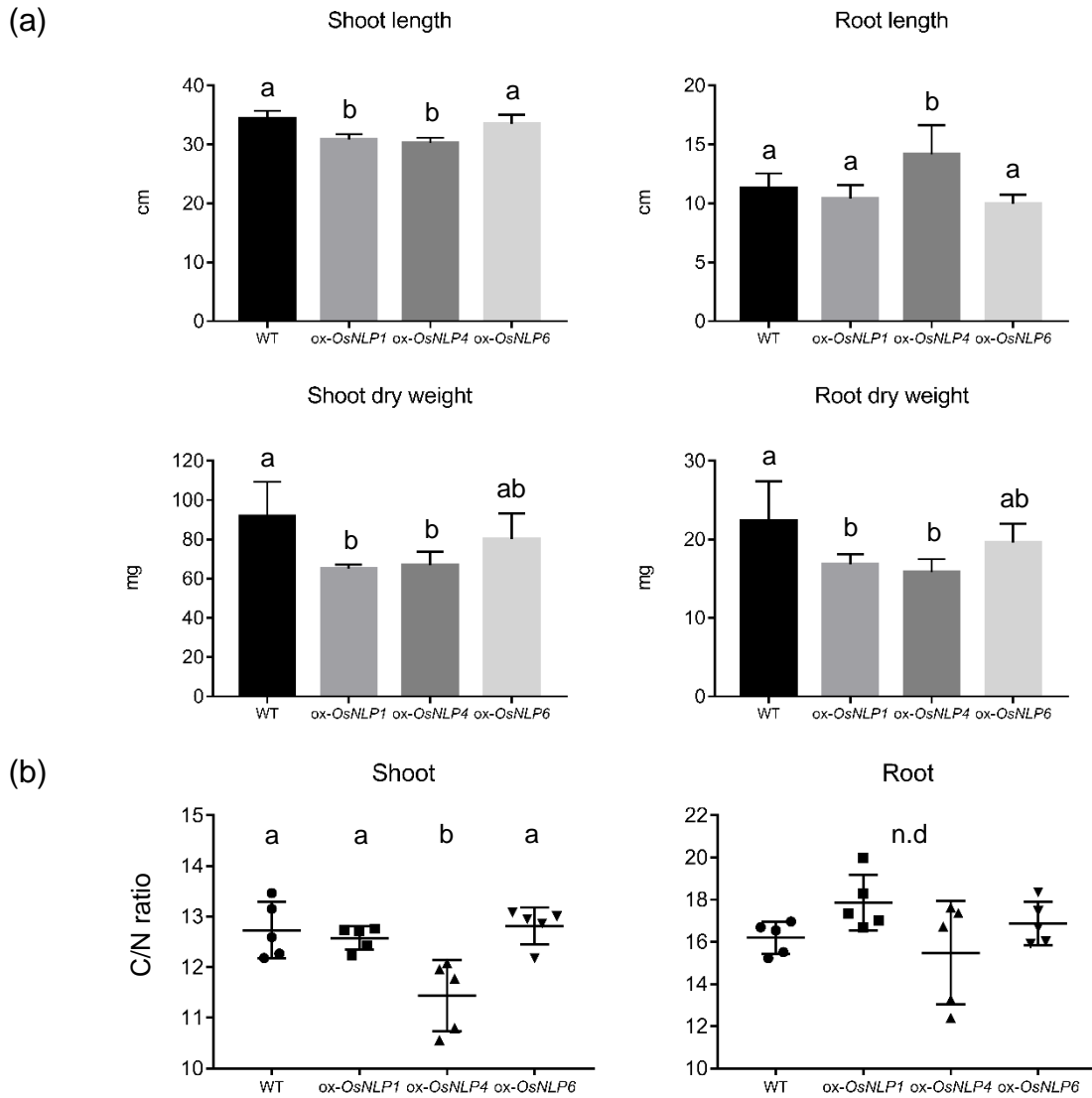
Figure 3-5. Morphological and physiological phenotype of ox-*OsNLP1*, ox-*OsNLP4* and ox-*OsNLP6* lines in nitrite condition. (a) The shoot length, root length, shoot dry weight and root dry weight of the overexpression lines were compared to wild type. Plants were grown in hydroponic culture for 15 days on modified KimuraB solution without nitrogen with of a density of 8 seeds/L. Error bars represent the standard deviation for 6 plants. (b) Carbon to nitrogen ration. Differences are statistically significant (REGWQ test, *$p<0.05$).

Table 3-1. Primer pairs used for constructing plasmids and sequencing.

Name	Primer Name	Sequence (5'-3')
35S::*OsNLP6*::*GFP*	*OsNLP6*-Slice-F	CAATTCAGTCGACTGGATCCGATGGAGCGCGT TGTTGGCGACTTC
	OsNLP6-Slice-R	GAAAGCTGGGTCTAGATACCCATCTGAGCTAG CGCAGGAACTCCCG
OsNLP6 sequencing primers	*OsNLP6-1*	CGACGCGACGGCGCCGGAGT
	OsNLP6-2	CCGCCGGCGCGCCGTTCCAC
	OsNLP6-3	GTTGGCAAGAACAAGACGAA
	OsNLP6-4	GGCCAGCCACAGAGGTGACA
osnlp1 line	*OsNLP1*_CRISPR_Guide9_F	GTTGTTGTCGGAGTTCTGGCATCC
	*OsNLP1*_CRISPR_Guide9_R	AAACGGATGCCAGAACTCCGACAA
osnlp6 line	*OsNLP6*_CRISPR_Guide1_F	GTTGCCTACACGGACGTTCACCGG
	*OsNLP6*_CRISPR_Guide1_R	AAACCCGGTGAACGTCCGTGTAGG
TAIL-PCR primers	pMDC83-RB-0a	CATGTTGACCTGCAGGCACGCCAAGCTTGG
	pMDC83-RB-1a	CTTGGCACTGGCCGTCGTTTTACAACGTCG
	pMDC83-RB-2a	CGCCTTGCAGCACATCCCCCTTTCGCCAGC
	LAD1-1	ACGATGGACTCCAGAGCGGCCGCATCTCTGG AA
	LAD1-3	ACGATGGACTCCAGAGCGGCCGCAGTCTAGC CAA

Summary and future prospects

In this research, I have identified the fundamental functions of *OsNLP1* and *OsNLP4* genes and hypothesized the networks of both genes in nitrate signaling pathway. Through the study on *OsNLP1* and *OsNLP4* genes, all these findings brought together show the importance of NLPs family in nitrate-dependent growth in rice and OsNLPs have separate roles in nitrate signaling or other signaling. In the future, I intend to work on these overexpression lines and construct crispr lines of other *OsNLPs* to extend our understanding of OsNLPs involvement in the nitrate signaling system. The way OsNLPs sensing the nitrate signaling, the binding sites in downstream-regulated genes and the crosstalk with other signaling will also be examined. Our ox-lines indeed showed better growth than wild type in nitrogen free condition. These studies will shed light on improving rice annual yield in field in an environmentally-friendly way.

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