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REVIEW PAPER

Emergence of a new step towards understanding the molecular mechanisms underlying nitrate-regulated gene expression

Mineko Konishi and Shuichi Yanagisawa*

Biotechnology Research Center, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

* To whom correspondence should be addressed. E-mail: asyanagi@mail.ecc.u-tokyo.ac.jp

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Abstract

Nitrogen is one of the primary macronutrients of plants, and nitrate is the most abundant inorganic form of nitrogen in soils. Plants take up nitrate in soils and utilize it both for nitrogen assimilation and as a signalling molecule. Thus, an essential role for nitrate in plants is triggering changes in gene expression patterns, including immediate induction of the expression of genes involved in nitrate transport and assimilation, as well as several transcription factor genes and genes related to carbon metabolism and cytokinin biosynthesis and response. Significant progress has been made in recent years towards understanding the molecular mechanisms underlying nitrate-regulated gene expression in higher plants; a new stage in our understanding of this process is emerging. A key finding is the identification of NIN-like proteins (NLPs) as transcription factors governing nitrate-inducible gene expression. NLPs bind to nitrate-responsive DNA elements (NREs) located at nitrate-inducible gene loci and activate their NRE-dependent expression. Importantly, post-translational regulation of NLP activity by nitrate signalling was strongly suggested to be a vital process in NLP-mediated transcriptional activation and subsequent nitrate responses. We present an overview of the current knowledge of the molecular mechanisms underlying nitrate-regulated gene expression in higher plants.

Key words: NIN-like protein (NLP), nitrate, nitrate-responsive gene expression, post-translational regulation, transcription factor, nitrate signalling.

Introduction

Nitrogen (N), like phosphorus (P) and sulphur (S), is a mineral element that is required in abundance for plant growth and is covalently integrated into organic compounds. Plants take up inorganic forms of N (mainly nitrate and ammonium) and S (sulphate) in soils and assimilate them for the biosynthesis of various N-containing and S-containing organic compounds, whereas phosphate, the inorganic form of P that also comes from soils, is directly incorporated into organic compounds, such as nucleic acids, phosphoproteins, and ATP. In soils, anion forms of these elements show different mobility; the mobility is highest for nitrate, followed by sulphate and then phosphate. Nitrate thus is available for uptake by plants but also easily leaches from soils, whereas most of the phosphate is unavailable to plants because it is strongly adsorbed in soils and forms precipitates (Johnson and Cole, 1980; Vance, 2001). Due to their sessile nature, higher plants need to adapt their growth and metabolism to the availability of these elements in soils, particularly N and P. Modulations of gene expression play a pivotal role in plant adaptation to the varying availability of these elements. A number of previous studies have already revealed that transcript levels of genes involved in the uptake and utilization of these mineral

Abbreviations: N, nitrogen; NIN, NODULE INCEPTION; NiR, nitrite reductase; NIR1, *Arabidopsis* NiR gene; NLP, NIN-like protein; NR, nitrate reductase; NRE, nitrate-responsive *cis*-element; OPP pathway, oxidative pentose phosphate pathway; P, phosphorus; S, sulphur.

nutrients are modified in response to N, P, and S availability (Hammond *et al.*, 2003; Hirai *et al.*, 2003; Wang *et al.*, 2003, 2004, 2007; Wu *et al.*, 2003; Scheible *et al.*, 2004; Hirai *et al.*, 2005; Misson *et al.*, 2005; Bi *et al.*, 2007; Morcuende *et al.*, 2007), and they have also disclosed differences in plant responses to availability of these elements. In the case of P and S, starvation induces the expression of genes associated with promoting the acquisition and utilization of these elements, suggesting that internal demand for these nutrients dictates the plant's response (Lai *et al.*, 2007). In contrast, in the case of N, not only internal demand for N (Gansel *et al.*, 2001; Ruffel *et al.*, 2011) but also nitrate provision induces a plant's response. Nitrate application immediately induces the expression of many genes associated with nitrate uptake and assimilation (Wang *et al.*, 2003; Krouk *et al.*, 2010). Nitrate itself (the major source of inorganic N for land plants in most cases) is presumably a signalling molecule that induces this response to nitrate provision, since tobacco and *Arabidopsis* mutants that are deficient in nitrate reductase (NR) activity and therefore cannot assimilate nitrate still retain the ability to respond to exogenously applied nitrate (Scheible *et al.*, 1997; Wang *et al.*, 2004). On the other hand, since application of N-containing metabolites such as glutamine and glutamate also modifies gene expression, such metabolites might be signalling molecules to mediate the internal demand for N (Vincentz *et al.*, 1993; Vidmar *et al.*, 2000; Nazoa *et al.*, 2003; Takei *et al.*, 2004; Gifford *et al.*, 2008; Gutierrez *et al.*, 2008; Rubin *et al.*, 2009).

Nitrate is an oxidized form of N. For N assimilation using nitrate, nitrate must be initially reduced into ammonium prior to assimilation into amino acids. Thus, nitrate assimilation requires additional energy and is costly compared with N assimilation using ammonium absorbed directly from soils. Probably for this reason, utilization of nitrate as an N source is only an alternative strategy in microorganisms. Nitrate assimilation-related genes are therefore expressed in microorganisms only when the microorganisms sense environmental nitrate under ammonium starvation conditions (Marzluf, 1997; Luque-Almagro *et al.*, 2011; Ohashi *et al.*, 2011). However, most land plants prefer to use nitrate as an N source, probably because land plants need to adapt to life in terrestrial ecosystems, which are oxidative environments where reduced forms of N are easily oxidized. Actually, unlike in microorganisms, in higher plants the expression of nitrate assimilation-related genes is induced by nitrate even in the presence of ammonium (see, for example, Ho *et al.*, 2009; Konishi and Yanagisawa, 2013b). Thus, nitrate is one of the critical N response-related signals in higher plants, and plants appear to have developed ingenious regulatory mechanisms to operate nitrate-regulated gene expression. In such regulatory mechanisms, transcription factors for the primary nitrate response must exist prior to the sensing of nitrate. In fact, the presence of cycloheximide, an inhibitor of protein synthesis, does not affect gene expression that is immediately induced by nitrate treatment (Gowri *et al.*, 1992; Sakakibara *et al.*, 1996; Price *et al.*, 2004). Recently, these transcription factors were found to be NODULE INCEPTION (NIN)-like proteins (NIN-like proteins, NLPs). This discovery has allowed us to enter into a new stage of understanding the molecular mechanisms underlying nitrate-regulated gene expression in higher plants. Here, we summarize recent findings and provide an overview of the regulatory mechanisms for nitrate-regulated gene expression. Although it has been shown that several nitrate-inducible transcription factors are involved in a plant's response to nitrate (Rubin *et al.*, 2009; Krouk *et al.*, 2010; Vidal *et al.*, 2010; Sawaki *et al.*, 2013), this review focuses on the primary response to nitrate provision and roles of NLPs in the primary response.

The *cis*-element that confers nitrate-inducible gene expression

The first essential step towards unravelling the mechanisms underlying nitrate-regulated gene expression is the identification of nitrate-responsive *cis*-elements (NREs), which should be located contiguous to and/or within nitrate-inducible genes. Nitrate induces the expression of nitrate transporter genes whose expression is concomitant with increases in nitrate uptake activity (Liu *et al.*, 1999; Zhuo *et al.*, 1999; Filleur *et al.*, 2001), genes encoding enzymes for nitrate reduction, NR and nitrite reductase (NiR) (Cheng *et al.*, 1986, 1991; Back *et al.*, 1988; Kramer *et al.*, 1989; Friemann *et al.*, 1992; Gowri *et al.*, 1992; Kronenberger *et al.*, 1993; Sander *et al.*, 1995), and genes encoding enzymes involved in ammonium assimilation, such as *GLT1* (encoding glutamine-2-oxoglutarate aminotransferase) and *ASN2* (encoding asparagine synthetase) (Wang *et al.*, 2003). The mRNA levels of some genes encoding enzymes in the oxidative pentose phosphate pathway (OPP pathway; which provides reducing power for nitrate assimilation) increase during nitrate treatment, which is accompanied by increases in the activity of these enzymes (Redinbaugh and Campbell, 1998; Wang *et al.*, 2003). The list of nitrate-inducible genes has been expanded greatly by recent transcriptome analyses, and the current list includes genes involved in glycolysis, trehalose metabolism, and iron acquisition, and several genes encoding putative regulatory proteins (Wang *et al.*, 2003; Price *et al.*, 2004; Scheible *et al.*, 2004; Bi *et al.*, 2007; Krouk *et al.*, 2010). However, efforts to identify NREs have focused mainly on promoter sequences of very familiar nitrate-inducible genes, NR and NiR genes, and the gene for a major high affinity nitrate transporter in *Arabidopsis* (*NRT2.1*). Analyses of NiR gene promoters from various higher plant species including *Arabidopsis*, spinach, tobacco, bean, and birch have revealed that the proximal regions of these promoters are responsible for nitrate-inducible expression of NiR genes (Sander *et al.*, 1995; Rastogi *et al.*, 1997; Dorbe *et al.*, 1998; Sivasankar *et al.*, 1998; Warning and Hactel, 2000; Konishi and Yanagisawa, 2010). Subsequent analysis focusing on a sequence conserved in the proximal regions of the NiR gene promoters (Fig. 1A, B) established that the conserved sequence is an authentic NRE (Konishi and Yanagisawa, 2010, 2011b). A gain-of-function experiment using the minimal 35S promoter fused to four copies of a conserved sequence from the promoter of the *Arabidopsis* NiR gene (*NIR1*) indicated that this sequence

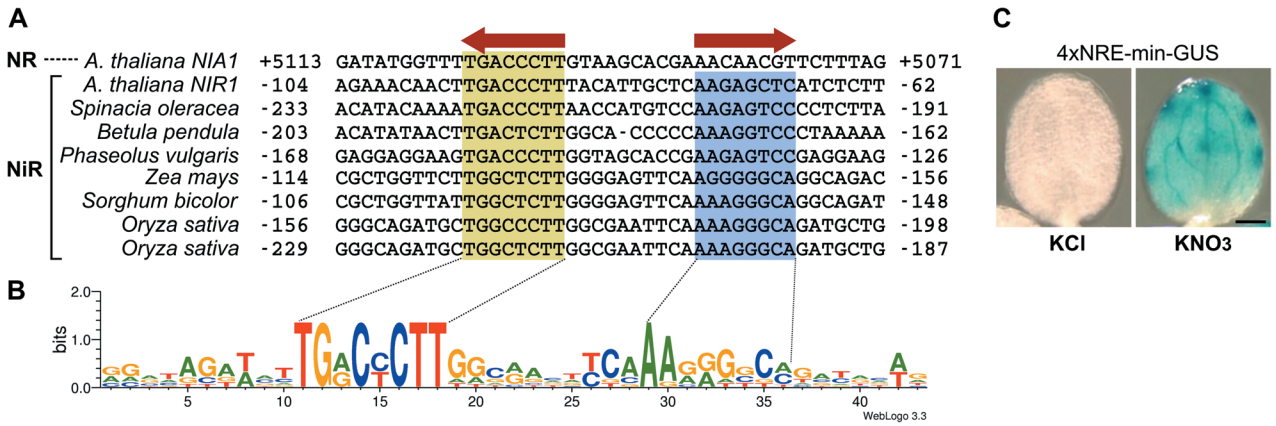

Fig. 1. Nitrate-responsive *cis*-element (NRE). (A) Nucleotide sequences of NRE and related sequences found in the flanking regions of genes encoding NR and NiR. The NRE found in the *Arabidopsis NIR1* promoter is pseudo-palindromic, and the two half-sites are indicated by yellow and blue. Accession numbers for the sequences for the flanking regions of NiR genes from *Spinacia oleracea*, *Betula pendula*, *Phaseolus vulgaris*, *Zea mays*, *Sorghum bicolor*, and *Oryza sativa* are X17031, X60093, U10419, GRMZM2G079381, Sb04g034160, and Os01g0357100, respectively, in the GenBank, MaizeSequence.org, or Gramene database. (B) The consensus sequence of NRE displayed using the sequence logo generation program WebLogo (Crooks *et al.*, 2004). (C) Nitrate-specific induction of GUS reporter activity under the control of four copies of NRE. Scale bar=200 μm.

is sufficient for nitrate-inducible gene expression (Fig. 1C). On the other hand, disruption of the conserved sequence in the native *NIR1* promoter diminishes the nitrate-responsive activation of this promoter (Konishi and Yanagisawa, 2010). Furthermore, corresponding sequences within the NiR gene promoters from several other plant species confer nitrate-inducible gene expression in transgenic *Arabidopsis* plants. Hence, the conserved sequence is an authentic NRE that is necessary and sufficient for nitrate-inducible expression of NiR genes (Konishi and Yanagisawa, 2011b).

The conserved sequence consists of a highly conserved motif, TG(A/G)C(C/T)CTT, and a less conserved motif (Fig. 1A, B). In the case of the *Arabidopsis NIR1* promoter, the sequence of the second motif is 'AAGAGCTCA', which is similar to the sequence of the first motif in a palindromic orientation, although the second motif contains an additional nucleotide (Fig. 1A, B). Thus, the NRE of the *Arabidopsis NIR1* promoter is a pseudo-palindromic sequence with a 10 bp spacer between the first and second motifs. Mutations within the first and second motifs make the *NIR1* promoter less sensitive to nitrate signalling, and the disruption of both motifs completely diminishes the nitrate responsiveness of the *NIR1* promoter (Konishi and Yanagisawa, 2010), suggesting that both motifs are necessary for full induction of the *NIR1* promoter. However, since the NRE of an *Arabidopsis* NR gene contains only the first motif (as discussed below) and the second motif is not strongly conserved even among the NiR gene promoters, the role of the second motif in the functioning of the NRE is still unclear.

In the case of NR genes, the NRE sequence was found in a relatively unexpected region. Although initial surveys of NREs for NR genes were performed with the promoter regions of *Arabidopsis* NR genes (*NIA1* and *NIA2*) (Lin *et al.*, 1994; Hwang *et al.*, 1997), the NRE responsible for nitrate-inducible *NIA1* expression was identified in the 3′-flanking region of this gene (~1.5 kb downstream of the site corresponding to the polyadenylation site) (Konishi and Yanagisawa, 2011a, 2013a, *b*). In spite of its location, this NRE is the most dominant regulator of nitrate-inducible *NIA1* expression because mutations within this NRE mostly diminish nitrate-inducible *NIA1* expression. The NRE for *NIA1* expression is identical to the first motif of the NRE found in the *NIR1* promoter, but this NRE lacks the second motif (Fig. 1A).

On the other hand, analysis of the *NRT2.1* promoter revealed that a 150 bp sequence in the proximal region of this promoter is involved in both nitrate-induced expression and high N status-induced repression of this gene (Girin *et al.*, 2007). Since this 150 bp sequence contains a sequence similar to the NRE found in the *NIR1* promoter (Supplementary Table S1 available at *JXB* online), this sequence may function as an NRE. Furthermore, we could find NRE-like sequences in 5′- and 3′-flanking regions of several nitrate-inducible genes as putative NREs (Supplementary Table S1). However, it is necessary to examine experimentally whether these sequences really function as NREs, because currently it is difficult to predict variations of the NRE due to a very limited number of experimentally identified NREs. It is worth noting that there is still the possibility that currently unidentified sequences regulate nitrate-inducible expression of some genes.

NLPs are transcriptional activators that interact with the NRE and govern nitrate-responsive gene expression

Identification of transcription factors that interact with the NRE is an absolutely necessary step towards revealing the regulatory machinery controlling nitrate-responsive gene expression. Recently, yeast one-hybrid screening using the NRE found in the *NIR1* promoter revealed that NLPs are NRE-interacting transcription factors (Konishi and Yanagisawa, 2013a). NLPs are homologous to NIN, which was genetically identified as an essential factor for root nodule formation in *Lotus japonicus* (Schauser *et al.*, 1999), but NLPs are present

in both non-leguminous and leguminous plants (Schauser *et al.*, 2005). Since NIN is unique to leguminous plants but NLPs are not, NIN and NLPs probably play different roles *in planta*. A previous study with the *Arabidopsis* mutant of *NLP7* (one of nine genes encoding NLPs in the *Arabidopsis* genome) indicated impaired nitrate-inducible expression of *NIA1*, *NIA2*, and nitrate transporter genes (*NRT2.1* and *NRT2.2*) in this mutant, implying the involvement of NLP7 in nitrate-inducible gene expression (Castaings *et al.*, 2009). Furthermore, in a previous study in which a genetic screen was performed to identify regulatory factors involved in the nitrate response, the mutation on *NLP7* has been shown to influence the activity of a nitrate-inducible promoter containing promoter fragments from the *NIA1* and *NIR1* promoters (Wang *et al.*, 2009). Accordingly, the phenotypes of the *nlp7* mutant are consistent with the fact that NLPs interact with an NRE.

NIN and NLPs contain an RWP-RK domain that was previously proposed to be a putative DNA-binding domain (Ferris and Goodenough, 1997; Schauser *et al.*, 1999), as well as a putative protein–protein interaction domain (the PB1 domain) in their C-termini (Sumimoto *et al.*, 2007) and an N-terminal region that is highly conserved among NLPs but partially conserved in NIN (Schauser *et al.*, 2005). In agreement with the previous proposal, the RWP-RK domains of all *Arabidopsis* NLPs examined (NLP1, NLP2, NLP3, NLP5, NLP6, NLP7, and NLP9) function as DNA-binding domains that interact with the NRE in *in vitro* binding and yeast assays. Furthermore, all NLPs tested (NLP1, NLP2, NLP5, NLP6, NLP7, and NLP9) function as transcription factors that promote NRE-dependent transcription in *Arabidopsis* leaf cells, suggesting that *Arabidopsis* NLPs are transcriptional activators with similar DNA-binding properties (Konishi and Yanagisawa, 2013a).

Due to high homologies among all NLPs of *Arabidopsis*, it was speculated that NLPs might play redundant roles in controlling nitrate-regulated gene expression *in planta*. Thus, transgenic *Arabidopsis* plants expressing a chimeric repressor, instead of *nlp* mutants, were employed to investigate the roles of NLPs *in planta* (Konishi and Yanagisawa, 2013a). Because NLP6 fused to the transcriptional repression domain of SUPERMAN (SUPRD), which has the ability to convert transcriptional activators into transcriptional repressors (Hiratsu *et al.*, 2003; Kubo *et al.*, 2005; Heyl *et al.*, 2008; Eklund *et al.*, 2010), could strongly repress NRE-dependent transcription in protoplasts, the NLP6–SUPRD chimera repressor was expected to repress the expression of target genes of NLPs *in planta*. Interestingly, repression of NLP function through the expression of NLP6–SUPRD resulted in small plant body size regardless of the inorganic form of N used as the N source (Fig. 2A), probably because NLPs play roles in the regulation of nitrate assimilation as well as other metabolic or regulatory processes that are under the control of the nitrate signalling pathway (Konishi and Yanagisawa, 2013a). We recently

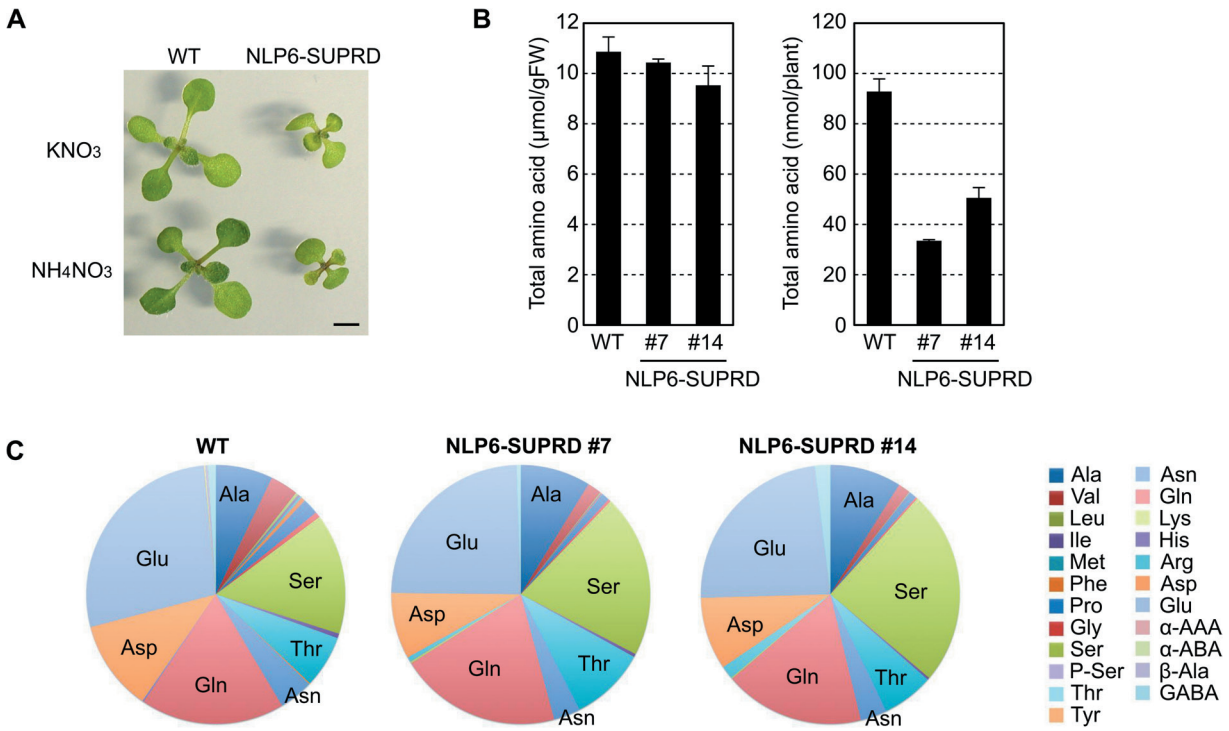

Fig. 2. Effects of reductions in NLP activity on growth and amino acid contents. (A) Images of wild-type (WT) and transgenic *Arabidopsis* plants expressing modified NLP6, which represses endogenous NLP activity (NLP6-SUPRD). Plants were grown on potassium nitrate or ammonium nitrate medium for 9 d. Scale bar=2 mm. (B) Total amount of amino acids in the WT and two NLP6-SUPRD lines. Amounts per gram fresh weight (left panel) and per plant (right panel) are shown. (C) Amino acid compositions of WT and NLP6-SUPRD lines. P-Ser, phosphoserine; α-AAA, α-aminoadipic acid; α-ABA, α-aminobutyric acid; β-Ala, β-alanine; GABA, γ-aminobutyric acid. In (B) and (C), amino acid contents of plants grown on agar medium containing 10 mM potassium nitrate as the N source for 14 d under continuous light were analysed (n=3). The complete data for amino acid contents are shown in Supplementary Table S2 at *JXB* online.

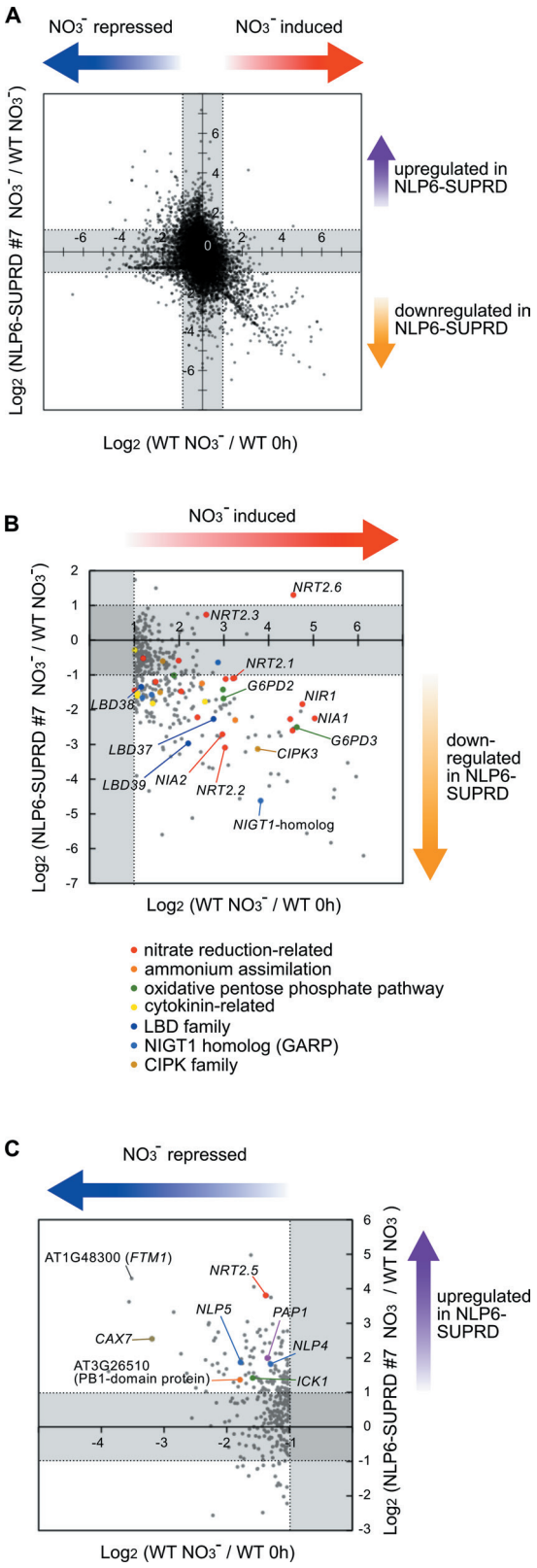

Fig. 3. Down-regulation of nitrate-responsive gene expression by repressing the function of NLP. (A) Scatter plots of nitrate-induced fold levels versus fold changes in the NLP6-SUPRD plants against the wild type (WT). Nitrate-induced fold levels of the expression of individual genes were calculated as values of expression levels in nitrate-treated WT seedlings against those in non-treated WT seedlings, and $\log_2$ values of nitrate-induced fold levels were plotted on the *x*-axis. Fold changes were calculated as values of expression levels in nitrate-treated NLP6-SUPRD plants (line 7) against those in nitrate-treated WT samples, and $\log_2$ values were plotted on the *y*-axis. Enlarged views indicating only genes that were induced >2-fold (B) or repressed less than half (C) by nitrate in the WT (n=3, P<0.05) are shown. *G6PD2* and *G6PD3* encode glucose-6-phosphate dehydrogenases in the OPP pathway. Seedlings grown in liquid medium containing 2.5 mM ammonium succinate as the N source were treated with 10 mM potassium nitrate for 1 h. Whole seedlings were used for analysis. The microarray data were deposited in the GEO database (accession no. GSE53852).

investigated the amino acid content of the transgenic lines expressing NLP6–SUPRD (NLP6-SUPRD lines) and found that repressing NLP function drastically decreases the amino acid content per plant. However, the amino acid content per fresh weight of tissue was only reduced in the NLP6-SUPRD lines to a small extent due to the reduced body sizes of the NLP6-SUPRD lines (Fig. 2B). Furthermore, there was little difference in amino acid composition between the wild-type and NLP6-SUPRD lines (Fig. 2C; Supplementary Table S2; Supplementary methods at *JXB* online). Thus, although the inhibition of NLP function impedes nitrogen utilization and/or some other processes, the metabolic balance is still maintained in the NLP6-SUPRD lines through a reduction in body size. Although the serine content apparently increased in the NLP6-SUPRD lines, the physiological relevance of this is currently unknown. Such increases in the serine content have been reported in plants grown on media with a high ammonium/nitrate ratio (Hachiya *et al.*, 2012; Sato and Yanagisawa, 2014), and plants that constitutively mimic the nitrate starvation condition due to the triple knockout of high affinity nitrate transporter genes have been shown to display unaltered metabolic balance and reduced body sizes (Takatani *et al.*, 2014).

On the other hand, our recent transcriptome analysis revealed that the expression of almost all nitrate-inducible genes (Wang *et al.*, 2003; Scheible *et al.*, 2004) was diminished in the NLP6-SUPRD lines (Fig. 3A; Supplementary methods at *JXB* online), suggesting that NLP activity is associated with nitrate responsiveness of almost all nitrate-inducible genes. The nitrate-induced expression levels of nitrate transporter genes including *NRT2.1* and *NRT2.2*, genes encoding enzymes for nitrate reduction (*NIA1*, *NIA2*, and *NIR1*), genes associated with ammonium assimilation (*GLT1* and *ASN2*) (Wang *et al.*, 2003), the LBD37/38/39 transcription factor genes (Scheible *et al.*, 2004; Rubin *et al.*, 2009), and genes encoding homologues of the rice nitrate-inducible NIGT1 transcriptional repressor from the GARP family (Scheible *et al.*, 2004; Krouk *et al.*, 2010; Sawaki *et al.*, 2013) were reduced in the NLP6-SUPRD lines. Furthermore, the nitrate-inducible expression of genes involved in the OPP pathway (Wang *et al.*, 2003), cytokinin biosynthesis (*IPT3*) (Takei *et al.*, 2004), and signal transduction (A-type ARR genes), and genes encoding a Ser/Thr protein kinase associated with a calcineurin B-like calcium sensor (*CIPK3*) was also strongly reduced by reductions in NLP activity (Fig. 3B). In addition to nitrate-induced genes, a number of nitrate-repressed genes were up-regulated in the NLP6-SUPRD plants (Fig. 3A). These genes include *PAP1* that encodes a

transcription factor for anthocyanin biosynthesis (Scheible *et al.*, 2004; Tohge *et al.*, 2005), *NRT2.5* the expression of which is down-regulated by nitrate provision (Okamoto *et al.*, 2003; Scheible *et al.*, 2004), two NLP genes, *NLP4* and *NLP5*, a gene for a cyclin-dependent kinase inhibitor, *ICK1* (Wang *et al.*, 1997, 2000; Cheng *et al.*, 2013), and a gene for a calcium exchanger, *CAX7* (Fig. 3C). Because NLP6–SUPRD only represses the expression of target genes, the genes up-regulated in the NLP6-SUPRD plants are probably indirect targets of NLPs. Since many regulatory genes are under the direct or indirect control of NLPs, NLPs probably function as master regulators governing many biological processes through transcriptional cascades, hormone (cytokinin) synthesis, protein phosphorylation, and so on, and therefore regulation by NLPs is widespread soon after plants sense nitrate. Such time-dependent effects, together with direct reductions in the expression levels of genes associated with ammonium assimilation, may lead to the inhibition of growth of NLP6-SUPRD lines even on medium containing ammonium.

By the combination of chromatin immunoprecipitation experiments using *Arabidopsis* plants expressing green fluorescent protein (GFP)-tagged NLP7 and transcriptome analysis of *nlp7* mutants, it has been shown that NLP7 directly binds to the flanking sequences of a number of nitrate-inducible genes and regulates their expression (Marchive *et al.*, 2013). Even if a transcription factor binds to a site on a chromosome *in vivo*, the binding does not always indicate a functional interaction that modulates gene expression (MacQuarrie *et al.*, 2011; Paris *et al.*, 2013). Nevertheless, most of the observed binding of NLP7 to sites in the flanking sequences of nitrate-inducible genes probably represents functional interactions because these interactions are consistent with the physiological function of NLPs. Among 36 genes that were identified as target genes of NLP7, 24 genes (67%) are down-regulated in the NLP6-SUPRD lines (Supplementary Table S3 at *JXB* online), suggesting a significant overlap of the target genes of NLP7 and NLP6–SUPRD. Furthermore, several sequences similar to the NRE within the *NIR1* promoter are found in the regions identified as NLP7-binding regions by the ChIP-chip analysis (Supplementary Table S1; Marchive *et al.*, 2013). Moreover, very recently, NLPs were also found to promote the expression of nitrite transporter genes (*NITR2;1* and *NITR2;2*) through interactions with DNA sequences similar to the NRE in the *NIR1* promoter (Supplementary Table S1; Maeda *et al.*, 2014). However, due to limited information on DNA recognition of NLPs, it is difficult at this stage to predict NLP-binding sites exactly. Thus, precise identification of NLP-binding sites regulating the expression of nitrate-inducible genes would be necessary to clarify the role of NLPs as master regulators of nitrate-regulated gene expression.

Post-translational regulation of NLPs is a key step in the nitrate response

Transcriptional activation that is immediately induced by nitrate treatment is not blocked by the inhibition of *de novo* protein synthesis (Gowri *et al.*, 1992; Sakakibara *et al.*, 1996; Price *et al.*, 2004; Konishi and Yanagisawa, 2011b). Thus, transcription factor(s) involved in primary nitrate-responsive gene expression must exist prior to the sensing of the nitrate signal, and their activity must be induced in a post-translational manner. Domain-swapping experiments have demonstrated that NLP6 satisfies this diagnostic criterion for transcription factors that regulate primary nitrate-responsive gene expression (Konishi and Yanagisawa, 2013a). A chimeric transcription factor containing the RWP-RK DNA-binding domain of NLP6 and the amino acid sequence for the transcriptional activation domain of the herpes virus protein VP16 transactivates the NRE-containing promoter independent of nitrate signalling, indicating that the activity of the RWP-RK DNA-binding domain itself is not under the control of nitrate signalling. However, when the amino acid sequence N-terminal to the RWP-RK domain of NLP6 (residues 1–546) fused to a nuclear localization signal and the bacterial DNA-binding protein LexA (which does not show transcriptional activation activity in plant cells) was expressed under the control of a constitutive promoter (the 35S promoter) in *Arabidopsis*, the chimeric transcription factor transactivated a LexA-binding site-containing promoter in a nitrate-dependent manner. Hence, the N-terminal region of NLP6 possesses a transcriptional activation domain and a domain that receives nitrate signalling and is involved in the post-translational regulation of NLP activity, although it is currently unknown whether these domains are separable (Konishi and Yanagisawa, 2013a). Another important result was recently reported by Marchive *et al.* (2013). Using NLP7–GFP fusion protein, they showed that the nuclear retention of NLP7 is regulated in response to nitrate signalling. By a nuclear export inhibitor experiment, they also suggested that nitrate signalling regulates nuclear export rather than nuclear import of NLP7. Furthermore, our unpublished data indicate that nitrate signalling does not affect the protein levels of NLP6, which precludes the possibility that the stability of NLP proteins is regulated by nitrate signalling. Accordingly, the current most likely hypothesis for the mechanism underlying primary nitrate-responsive transcriptional activation is as follows. Nitrate signalling modulates nuclear export of NLPs via a nitrate signal-responsive domain located within their N-terminal regions, and thereby NLPs accumulate in the nucleus in the presence of nitrate. The accumulated NLPs interact with NREs contiguous to and/or within nitrate-inducible genes and induce nitrate-responsive gene expression (Fig. 4). In this hypothesis, the induction of NLP activity in a post-translational manner is a key step connecting the nitrate signalling pathway with nitrate-responsive transcriptional regulation, and therefore a much deeper characterization of this process has emerged as an important topic of study.

RWP-RK domain-containing proteins in higher plants and *Chlamydomonas reinhardtii*

The RWP-RK domain is unique to plants (Riechmann *et al.*, 2000) and is found in other plant proteins in addition to NLP

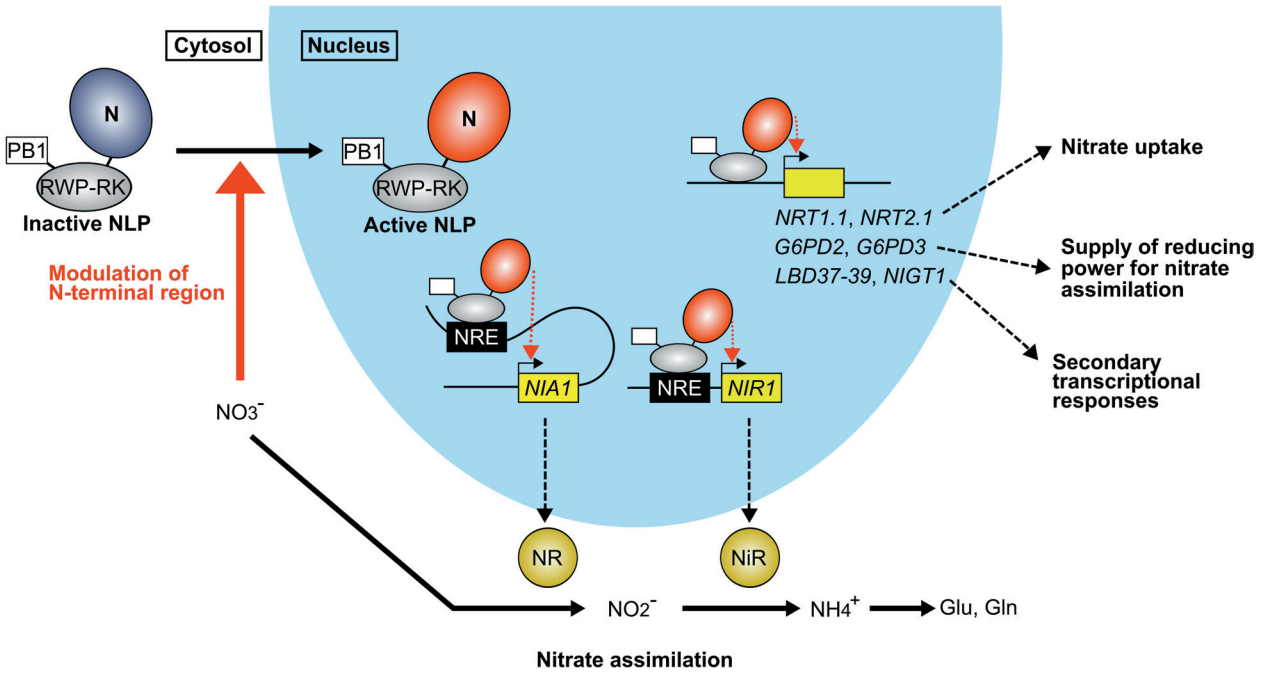

Fig. 4. A current model for nitrate-inducible gene expression in *Arabidopsis*. Nitrate signalling modulates nuclear accumulation of NLPs through their N-terminal regions (N), although it is unknown where NLPs receive the nitrate signal. Accumulated NLP binds to nitrate-responsive elements (NREs) and activates the expression of nitrate-inducible genes, including *NIA1* and *NIR1*. Up-regulation of nitrate transporter genes (*NRT1.1* and *NRT2.1*) and OPP pathway-related genes (*G6PD2* and *G6PD3*) also contributes to the promotion of nitrate assimilation. Two classes of transcription factor genes, *LBD37-39* and *NIGT1*, may also be targets of NLPs, and they might trigger secondary transcriptional events in response to nitrate. Binding sites of NLPs at the *NRT1.1*, *NRT2.1*, *G6PD2*, *G6PD3*, *LBD37-39*, and *NIGT1* loci have not been identified yet. PB1, the PB1 domain; RWP-RK, the RWP-RK DNA-binding domain; NR, nitrate reductase; NiR, nitrite reductase.

and NIN (Fig. 5). RWP-RK domain-containing proteins in higher plants are currently classified into two subgroups, the NLP family and the RKD (RWP-RK domain-containing) family, based on the similarity in amino acid sequences of their RWP-RK domains (Fig. 5A, B) and their overall protein structures (Fig. 5C). Amino acid sequences in the RWP-RK domains of NLPs are highly homologous to one another, but their homologies to those of RKD proteins are much lower (Fig. 5A). Thus, the NLP and RKD families apparently form different clades in the phylogenetic tree of the RWP-RK domain (Fig. 5B). The low homologies observed between the RWP-RK domains of NLPs and RKD proteins imply that NLPs and RKD proteins may bind to different DNA sequences and regulate different biological processes. In fact, NLPs are probably devoted to regulating nitrate-responsive gene expression, while RKD proteins are involved in gametogenesis and embryogenesis (Jeong *et al.*, 2011; Koszegi *et al.*, 2011; Waki *et al.*, 2011). Furthermore, unlike NLPs, RKD proteins do not possess known protein domains other than the RWP-RK domain. Thus, the N-terminal conserved region and the PB1 domain characterize NLPs. The difference in the N-terminal regions of NLP and RKD proteins is in agreement with the notion that the N-terminal regions of NLPs are involved in receiving nitrate signals. The PB1 domain of NLPs may be linked to NLP function as well, although this has not yet been clarified.

Based on current knowledge, NIN is thought to be a unique member of the NLP family (Fig. 5B), although NIN was the first RWP-RK domain-containing protein to be described in higher plants. *NIN* was originally identified as a causative gene for an *L. japonicus* mutant defective in nodulation (Schauser *et al.*, 1999), and its homologues probably play the same role in other legumes, i.e. *Medicago truncatula* and *Pisum sativum* (Borisov *et al.*, 2003; Marsh *et al.*, 2007). Furthermore, LjNIN and MtNIN were recently shown to be transcriptional activators for the expression of *NF-YA* and *NF-YB* in *L. japonicus* (Soyano *et al.*, 2013) and the pectate lyase gene in *M. truncatula* (Xie *et al.*, 2012), respectively. Due to the high homologies among the RWP-RK domains of NINs and NLPs (Fig. 5A), NINs were grouped within the NLP family in the phylogenetic tree of the RWP-RK domain (Fig. 5B). Consistent with this notion, LjNIN was recently found to be capable of binding to the NRE *in vitro* and activating transcription from an NRE-containing promoter *in vivo* (Suzuki *et al.*, 2013). However, NINs display an interesting characteristic: in spite of the high similarity in overall protein structure between NLPs and NINs, NINs possess only an incompletely conserved N-terminal region (Fig. 5C) (Schauser *et al.*, 2005; Suzuki *et al.*, 2013). Unlike the N-terminal region of NLP6 that exhibited transactivation activity only in the presence of nitrate, the N-terminal region of NIN transactivated transcription even in the absence of nitrate (Suzuki *et al.*, 2013). Hence, NIN is likely to have descended from an NLP protein that lost nitrate-responsiveness in an ancestral leguminous plant, and has been recruited to regulate nodule development. As nodules are effectively formed under N-deficient conditions and the presence of nitrate inhibits this process (Carroll *et al.*, 1985;

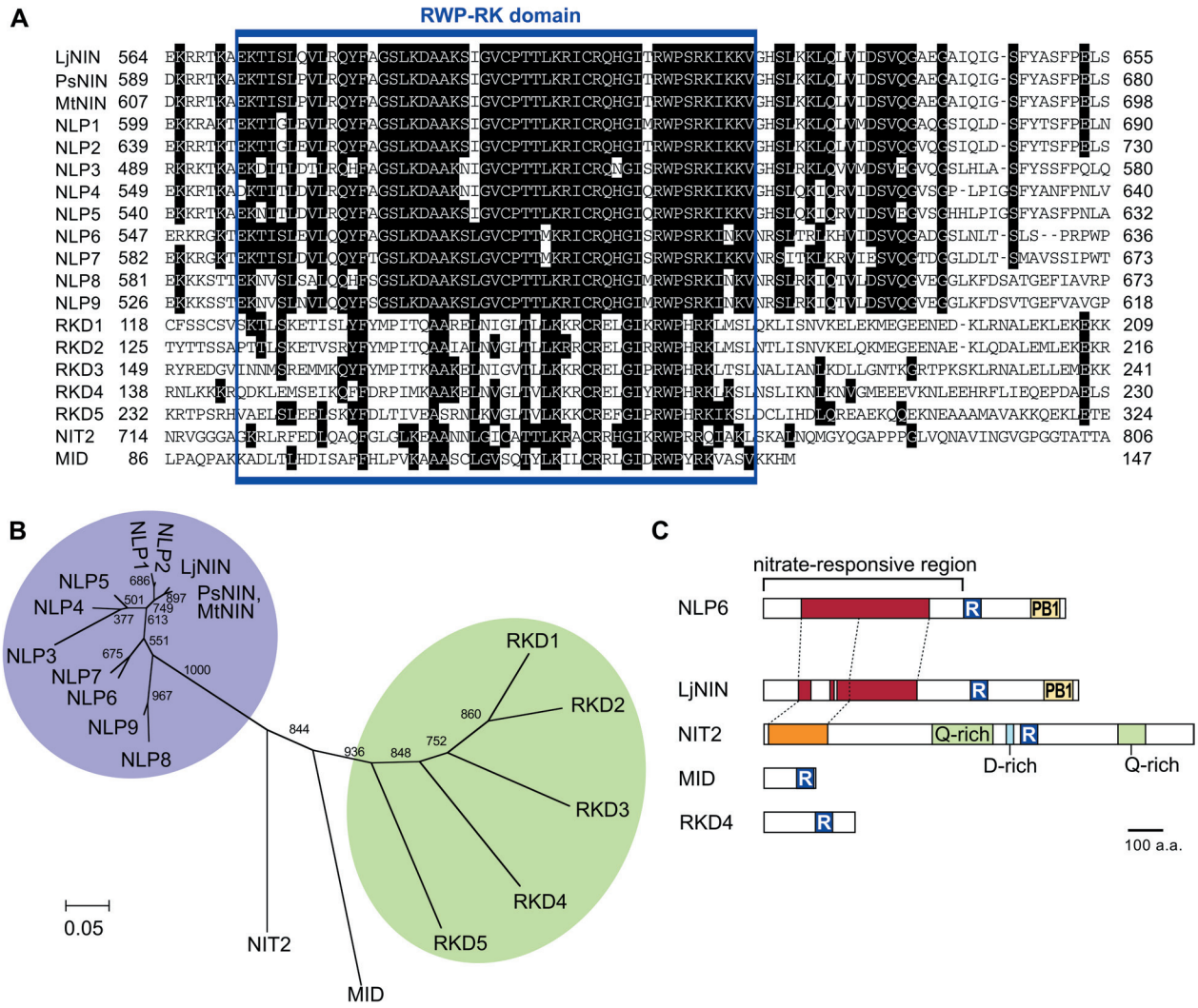

Fig. 5. RWP-RK domain-containing proteins. (A) Alignment of the amino acid sequences of RWP-RK domains of RWP-RK domain-containing proteins from *Arabidopsis* (NLP1-9 and RKD1-5), NINs from legumes (*L. japonicus*, *M. truncatula*, and *P. sativum*), and RWP-RK domain-containing proteins from *C. reinhardtii* (NIT2 and MID). The NIN proteins from *L. japonicus* (Schauser *et al.*, 1999), *M. truncatula* (Marsh *et al.*, 2007), and *P. sativum* (Borisov *et al.*, 2003) have been experimentally confirmed to regulate nodule formation. (B) Phylogenetic tree of the RWP-RK domain. Bootstrap values of 1000 calculations are indicated. (C) Schematic representation of RWP-RK domain-containing proteins. The structures of NLP6, LjNIN, and RKD4 are shown as representatives of NLPs, NINs, and RKD proteins, respectively. The RWP-RK domain (R), the PB1 domain (PB1), and the glutamine-rich (Q-rich) and aspartate-rich (D-rich) regions in NIT2, which may act as a transactivation domain (Triezenberg, 1995), are indicated. Dark red boxes indicate the regions conserved among the N-terminal regions of NLPs and NINs. A putative GAF domain in NIT2 is indicated by an orange box. The position of the N-terminal region that displays the ability for both transcriptional activation and mediation of nitrate signalling is indicated above the structure of NLP6.

Streeter, 1985; Barbulova *et al.*, 2007), the nitrate-independent, constitutive activity of NIN may be necessary for this protein to function in nodule development.

In addition to RWP-RK domain-containing proteins in higher plants, two RWP-RK domain-containing proteins in the unicellular green algae *Chlamydomonas reinhardtii* have been characterized to date. The first is MID (minus dominance), which determines the *minus* mating type of gametes by activating *minus*-specific genes and repressing *plus*-specific genes (Ferris and Goodenough, 1997). The expression of the *MID* gene is activated during gametogenesis that is induced by nitrogen starvation (Ferris and Goodenough, 1997). The other RWP-RK domain-containing protein is NIT2. The *nit2* mutant was first identified as being defective in NR activity due to the loss of the regulation of NR synthesis (Fernandez and Matagne, 1984; Schnell and Lefebvre, 1993), and NIT2 was subsequently shown to bind specifically to the promoter-proximal region of the NR gene promoter and to activate the expression of the NR gene in the presence of nitrate (Camargo *et al.*, 2007). Thus, NIT2 in *C. reinhardtii* and NLPs in higher plants play similar physiological roles in nitrate-responsive gene expression. Consistent with their similar physiological roles, NIT2 rather than MID is located near the NLP family in the phylogenetic tree of the RWP-RK domain. However, the amino acid sequences of the RWP-RK domains of both NIT2 and MID are quite different from those of NLPs in higher plants (Fig. 5A), consistent with the fact that the sequences responsible for the nitrate-inducible expression of the *C. reinhardtii* NR gene (Loppes and Radoux, 2002; Camargo *et al.*, 2007), which are probably NIT2-binding sites, are completely different from NREs found in *Arabidopsis*.

Interestingly, NIT2 contains an amino acid sequence that displays a low but significant similarity to the N-terminal half of the amino acid sequences in the N-terminal conserved regions of NLPs (Camargo *et al.*, 2007). This region, which is composed of ~170 amino acid residues (Fig. 5C), was previously annotated as a GAF domain (Camargo *et al.*, 2007), although such annotation has been deleted from the recent database. We recently found that the corresponding region of NLP6 is predicted to fold into a GAF-related structure, as determined using the PHYRE automatic fold recognition server for predicting the structures of protein sequences (Kelley and Sternberg, 2009). It is still unclear whether these regions truly form a GAF domain. However, considering that the corresponding regions in NINs are mutated and cannot respond to nitrate signals (Fig. 5C), the regions for putative GAF domains may be important for mediating nitrate signalling. This notion, together with the speculation that the functioning of NIT2 is also regulated post-transcriptionally by nitrate (Camargo *et al.*, 2007), suggests that similar mechanisms involving proteins that contain both GAF-related and RWP-RK domains may control nitrate-inducible gene expression in *C. reinhardtii* and in higher plants. However, since ammonium deficiency rather than the presence of nitrate is a dominant factor that induces the functioning of NIT2, the mechanisms underlying NLP-mediated regulation in higher plants and NIT2-mediated regulation in *C. reinhardtii* may be similar but not identical. Furthermore, since very little homology is present among the RWP-RK domains of NLPs and NIT2, NLPs and NIT2 probably recognize different nucleotide sequences as binding sites.

Conclusions and perspectives

In light of recent findings, the NLP-mediated pathway has emerged as a central mechanism controlling nitrate-regulated gene expression in higher plants. Nitrate-inducible gene expression occurs in bacteria, fungi, and algae, as well as in higher plants. In bacteria, a two-component regulatory system consisting of nitrate sensor-histidine kinases (NarX and NarQ) and transcription factors (NarL and NarP) operates the nitrate-inducible expression of the genes encoding enzymes for dissimilatory nitrate reduction (Stewart, 1994), and another two-component system composed of a nitrate sensor-histidine kinase (NasS) and a transcription anti-terminator protein (NasT) regulates the expression of NR and NiR genes and nitrate assimilation in some bacteria (Luque-Almagro *et al.*, 2011, 2013). In fungi, another mechanism controls nitrate-inducible expression of the genes involved in nitrate utilization. In this system, GAL4-type transcription factors not present in higher plants, including NirA in *Aspergillus nidulans* and NIT4 in *Neurospora crassa* (Riechmann *et al.*, 2000), function as transcription factors that mediate nitrate signalling. As NirA translocates from the cytosol to the nucleus upon nitrate application, controlling the nuclear accumulation of GAL4-type transcription factors may be a key step that occurs in fungi (Yuan *et al.*, 1991; Marzluf, 1997; Berger *et al.*, 2006; Bernreiter *et al.*, 2007). Hence, the mechanism that regulates nitrate-responsive gene expression in higher plants is completely different from the mechanisms employed in bacteria and fungi. However, it appears to be partially similar to the system in *C. reinhardtii*, since RWP-RK domain-containing proteins play a pivotal role in both higher plant and algal systems.

The fact that expression of most nitrate-inducible genes is greatly diminished by the inhibition of NLP function indicates that the NLP-mediated regulatory pathway is largely responsible for nitrate-inducible gene expression. Although the nitrate transporter NRT1.1 was shown to function as a nitrate sensor in *Arabidopsis* (Ho *et al.*, 2009), the NLP-mediated regulation is independent of NRT1.1 activity, since nitrate-dependent and NRE-mediated activation of the *NIR1* promoter occurs even in the *nrt1.1* mutant (Konishi and Yanagisawa, 2010). Hence, there are at least two distinct pathways in higher plants for nitrate-regulated gene expression, namely the NLP-mediated and NRT1.1-mediated pathways. Furthermore, since nitrate-inducible expression is attenuated in the presence of chemicals that inhibit calcium uptake, for example EGTA and La^{3+}, as well as the protein phosphatase inhibitors okadaic acid and calyculin A (Sakakibara *et al.*, 1996, 1997; Sueyoshi *et al.*, 1999) and a CBL-interacting protein kinase, CIPK8, also functions in the modulation of nitrate-inducible gene expression (Hu *et al.*, 2009), the regulatory mechanisms for nitrate-regulated gene expression might also involve calcium signalling and protein phosphorylation. Thus, new challenges based on the current knowledge include identifying the remaining components involved in the mechanisms underlying nitrate-regulated gene expression, clarifying the different roles of the NLP-mediated and NRT1.1-mediated pathways in controlling nitrate-regulated gene expression, and revealing how the NLP- and NRT1.1-mediated regulation and possible calcium signalling- and protein phosphorylation-mediated regulation are integrated into the system controlling nitrate-regulated gene expression and nitrate responses. Significant progress made in recent years will form an invaluable basis towards understanding the molecular mechanisms underlying nitrate-regulated gene expression in higher plants.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. Representatives of putative NREs at nitrate-inducible gene loci.

Table S2. Amino acid contents in NLP6-SUPRD plants.

Table S3. Genes that are direct targets of NLP7 and also down-regulated in NLP6-SUPRD plants.

Supplementary methods.

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