Chapter 12
Protein Phosphatases in N Response and NUE in Crops

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12.1 Introduction

Nitrogen is an essential macronutrient for plant growth and crop yield, as most crop plants barring N-fixing legumes depend on reactive N compounds such as urea, ammonium salts, or nitrates from manures or fertilizers as their main nitrogen source. Fertilizers are the world's largest anthropogenic source of reactive nitrogen, with annual global consumption at approximately 119.40 million tons and annual growth of 1.4% (FAO 2018). Due to the poor N use efficiency (NUE) of most crops at a global average of <30%, unused reactive N compounds from fertilizers and other sources are a major source of pollution of land, water, and air, affecting human and animal health, biodiversity, and climate change (Sutton and Bleeker 2013; Sutton et al. 2019).The fourth UN Environment Assembly held in March 2019 has adopted a resolution to work toward sustainable nitrogen management (https://sdg.iisd.org/events/fourth-session-of-the-un-environment-assembly-unea-4/). Therefore, the development of N use efficient crops is an extremely important goal, not only for sustainable agriculture but also for environmental sustainability and mitigation of climate change (Raghuram et al. 2007; Raghuram and Sharma 2019).

NUE can be defined in several ways (Raghuram and Sharma 2019), but it is best understood as yield per unit N, or the maximal output with minimal N input. NUE is a complex genetic trait involving several genes and their interactions with the environment, which are yet to be fully characterized (Mandal et al. 2018). NUE involves a combination of processes such as N uptake, retention, assimilation, and remobilization of internal N reserves. Plants possess families of transporters to take up urea, ammonium, and nitrate ions (NO_3^-), of which NO_3^- ions not only acts as nutrient but also play a very important role in signal transduction and regulation of

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one or more of the above processes (Raghuram et al. 2006; Pathak et al. 2008, 2011; Chakraborty and Raghuram 2011; Mandal et al. 2018; Raghuram and Sharma 2019).

The progress on the precise characterization of the biological avenues for improvement for NUE has been painfully slow, despite some occasional advances on phenotype (Sun et al. 2014; Sharma et al. 2018). Indeed, even though low nutrient stress is known to be an important abiotic stress for crops in developing countries, the interface of N and stress has not been explored in great detail (Jangam et al. 2016). While this has hampered crop improvement by forward genetics, recent advances in genomics and functional genomics of several crops have opened the opportunity for using reverse genetics to address NUE using candidate gene approach. To understand the physiological and molecular basis of N response under normal and low supply of nitrogen, transcriptomic and proteomic studies have identified a large number of differentially expressed genes in crops like rice (Yang et al. 2015;Waqas et al. 2018; Sinha et al. 2018), sorghum (Gelli et al. 2014), maize (Jiang et al. 2018), and barley (Quan et al. 2016, 2019).

Among the multitude of pathways and processes that constitute N response, protein kinases and phosphatases as well as transcription factors and other signaling intermediates regulated by phosphorylation emerged significant. Protein kinases as well as protein phosphatases have been widely studied in rice, Arabidopsis, wheat, maize, etc. (Singh et al. 2010; Xue et al. 2008; Bradai et al. 2018; Trevisan et al. 2011). PP2Cs are a major class of phosphatases and play an important role in stress in plants (Moorhead et al. 2009; Singh et al. 2010, 2015). In this chapter, we discuss the role of protein phosphatases in N-sensing and signaling and their role in carbon and nitrogen metabolism and phosphatases involved in N-response and NUE with enhancement potential for further validation of phosphatases.

12.2 Phosphatases in N Uptake and Primary Nitrate Response

For optimal growth as well as ion balance and homeostasis of plant, adjustment of uptake of ammonium (NH_4^+) and NO_3^- is essential as compared to other major ions (Haynes 1990). Phosphorylation and dephosphorylation play major roles in regulating transport of these ions by transporter protein through plasma membrane (Straub et al. 2017). An important target of such regulation is NRT1.1/CHL1/NPF6.3, a dual-affinity nitrate transporter. It works under high as well as low NO_3^- condition. PP2C plays a major role in dephosphorylation of NRT1.1 by binding to the calcineurin B-like-interacting protein kinase (CIPK). CIPK consists of two domains; one is the conserved N-terminal kinase catalytic domain, and the other is the highly variant C-terminal regulatory domain having NAF/FISL motif and a phosphatase interaction motif (Guo et al. 2001). In response to low NO_3^- condition, NO_3^- binds to a high affinity site, which activates CIPK23 to phosphorylate CHL1 at T101. The phosphorylated CHL1 prevents higher primary nitrate response to low

NO_3^- concentration. At high NO_3^- concentrations, NO_3^- binds to a low nitrate affinity site, which dephosphorylates CHL1 at T101 and generates a high primary nitrate response (Ho et al. 2009; Vert and Chory 2009; Straub et al. 2017). In low-affinity phase of (NO_3^-), CIPK8 is activated by an unknown CBL, which phosphorylates CHL1 at a residue different from T101 (Hu et al. 2009). CIPK23 acts as a negative regulator of high-affinity NO_3^- response, while it acts as a positive regulator of primary nitrate response in low-affinity phase of NO_3^- response.

12.3 PP2Cs Are Negative Regulators of ABA Signaling in NO_3^- Sensing

ABA signaling pathway includes a type 2C protein phosphatase (PP2C; a negative regulator) and a SNF1-related protein kinase 2 (SnRK2; a positive regulator) (Umezawa et al. 2009). ABA can inhibit or stimulate the functioning of the root meristem and modulate root growth depending on its concentration (Cheng et al. 2002). Development of root architecture is strongly regulated by the concentration of NO_3^- in the root environment, as well as its distribution. If the environment surrounding the root system is uniformly high in NO_3^-, lateral root growth is generally inhibited (Zhang and Forde 2000; Walch-Liu et al. 2005). NO_3^- locally stimulates lateral root elongation and in some species initiation, when NO_3^- presents only in patch (Zhang and Forde 1998). In Arabidopsis, this local stimulation of lateral root elongation by patches of NO_3^- requires ABA signaling (Signora et al. 2001). In Arabidopsis, PP2C proteins such as ABA-insensitive 1 (ABI1), ABI2, and Hypersensitive to ABA 1 (HAB1) have been found to function in regulation of root development. ABA-induced signaling functions under both biotic and abiotic stresses by interacting with SnRK2s and PYR/PYL/RCARs.

12.4 Phosphatases: Key Players in Carbon and Nitrogen Balance

In addition to the independent utilization of carbon and nitrogen metabolites, their ratio (known as C/N balance) is more important for the regulation of plant growth. The signaling mechanism underlying C/N balance is not clear till date (Sulpice et al. 2013; Lu et al. 2015). In leaves, NO_3^- and nitrite are reduced to ammonia and then to glutamate (Glu) via the glutamine synthetase (GS)-glutamine-2-oxoglutarate aminotransferase (GOGAT) pathway. Glutamate is a source of C and N for the biosynthesis of most other amino acids (Forde and Lea 2007). GOGAT is found in two isoforms (FdGOGAT form and NADH-GOGAT) and located in the chloroplast in higher plants. Among these, Fd-GOGAT was found to be very active in chloroplast of photosynthetic tissues (Nigro et al. 2014). It plays a very important role in

re-assimilation of ammonia released during photorespiration and is potential target to improve NUE (Zeng et al. 2017). Phosphatases and kinases both play very important role in posttranslational modification of chloroplast protein in regulating distribution of light energy between the photosystem I and photosystem II (Michelet et al. 2013; Rochaix 2013; Grabsztunowicz et al. 2017). The PSII core proteins D1 and D2, inner antenna protein CP43, and a minor PSII subunit PsbH are targets for light-dependent Thr phosphorylation catalyzed mainly by the STN8 kinase (Fristedt and Vener 2011), while the PSII core phosphatase is responsible for the dephosphorylation (Samol et al. 2012). Dephosphorylation of light harvesting complex (LHC) by the PPH1/TAP38 (chloroplast protein phosphatase/thylakoid-associated phosphatase of 38 kDa) protein phosphatase results in redistribution of excitation energy toward PSII (Pribil et al. 2010; Shapiguzov et al. 2010).

Ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) plays a central role in CO_2 assimilation of all photosynthetic organisms. It catalyzes the photosynthetic CO_2 fixation by the carboxylation of ribulose-1,5-bisphosphate (RuBP) in C3 photosynthesis. Rubisco is a slow enzyme, and its large amount (up to 25% of leaf N and 50% of leaf-soluble protein) is needed for photosynthesis (Parry et al. 2013; Whitney et al. 2015). In a study, it has been shown that in antisense plants by decreasing Rubisco content up to 15–20%, nitrogen demand reduced by 10% without negatively affecting photosynthetic carbon fixation (Sitt and Schulze 1994; Parry et al. 2013). Thus, increasing CO_2 fixing capacity by changing Rubisco and/or Rubisco activase function, i.e., more carbon fixed for same amount of leaf protein, would increase nitrogen use efficiency. Rubisco has been reported as a target of reversible phosphorylation in many plant species (Wang et al. 2014; Roitinger et al. 2015). Two subunits of Rubisco, RBCL (Rubisco large chain gene) and RBCS (Rubisco small chain gene), contain multiple phosphorylation sites (Cao et al. 2011; Wang et al. 2014). Phosphorylation of the highly conserved RBCL residues Ser208, Thr246, Tyr239, and Thr330, located in the close proximity to RuBP binding site, might affect the catalytic activity of the enzyme (Hodges et al. 2013). Dephosphorylation of RBCL has been found to dissociate Rubisco holocomplex and decreased activity of the enzyme by affecting the interaction between Rubisco and Rubisco activase (RA) (Chen et al. 2011; Hodges et al. 2013).

12.5 PP2A-TOR in Regulation of Nitrate Metabolism

Target of rapamycin (TOR) is a serine/threonine kinase and is highly conserved among all eukaryotes. TOR is activated by both nitrogen and carbon metabolites and promotes cell division, mRNA translation, and repressing nutrient remobilization through autophagy (Dobrenel et al. 2016). It is a master regulator of growth and development including transcription, ribosome biogenesis, autophagy, nutrition recycling, and cellular metabolism in plants (Bakshi et al. 2019). The plant TOR complex consists of TOR, Raptor, and LST8. The TOR complex promotes cell growth and translation through the regulation of S6K and its substrate ribosomal

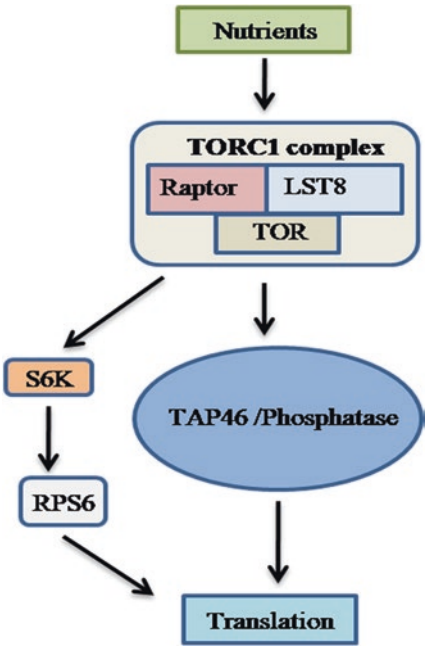

Fig. 12.1 Schematic representation of TOR signaling in plants in response to nutrients (Modified from Ahn et al. 2011)

protein S6(RPS6). It has been reported that TAP46 and its associated phosphatases (PP2Ac and PP2Ac-like subunits) play a critical role in mediating TOR signaling, leading to the promotion of protein translation, the repression of autophagy, and nitrate metabolism (Fig. 12.1). TAP42-phosphatase complex associates with TOR complex 1 (TORC1), under normal condition, while in nutrient deprivation condition or rapamycin treatment, TAP42-phosphatase complex dissociates and is released to cytosol. Interaction of TAP42 with PP2A or PP2A-like phosphatases modulates the activity of downstream effectors and regulates transcription and translation of starvation-responsive genes (Ahn et al. 2011). In Arabidopsis transgenic plants, overexpression of TAP46 leads to upregulation of genes related to nitrogen metabolism and nitrate assimilation and also increased TOR activity, whereas TAP46-silenced plants exhibited a reduction in N-assimilating enzymes (Ahn et al. 2015).

12.6 Protein Phosphatases: Fine-Tuning of Nitrate Reductase

Phosphatases are not only passive partners of kinases, rather they play a dynamic regulatory role in several metabolic processes. They activate or deactivate themselves through posttranslational modification (Heidari et al. 2011). Posttranslational regulation of nitrate reductase (NR) activity through phosphorylation/dephosphorylation

by PP2A (protein phosphatase 2A), CDPK (calcium-dependent protein kinase), and SNRK (SNF/sucrose nonfermenting-related kinase) is light dependent (Raghuram and Sopory 1995; Huber et al. 1996). Activation of NR is triggered by photosynthesis, but the signaling cascades from chloroplasts to cytosol, where NR is located, are not clear. Okadaic acid and microcystin-LR are known inhibitors of the protein phosphatase families including protein phosphatase 1 (PP1), PP2A, PP4, PP5, PP6, and PPP-Kelch, and they prevent light activation of NR in plant system (Lillo et al. 1996; Ali et al. 2007). PP2C also plays a major regulatory role in different stress signalings in plants (Moorhead et al. 2007; Singh et al. 2015). Dephosphorylation of NR is essential for activation of NR.14-3-3 which is highly conserved family of proteins found in all eukaryotes. Depending on the developmental and environmental requirements, 14-3-3 activity could direct carbon either into sucrose and storage carbohydrate synthesis or, via inactivation of carbon metabolism and activation of nitrogen assimilation, divert carbon skeletons into the synthesis of amino acids. Plants could use the various possible combinations of 14-3-3 homo- and heterodimers for fine-tuning (Comparot et al. 2003). In a study in rice, dephosphorylation of SnRK and 14-3-3 by PP2C led to the activation of nitrate reductase for increasing NUE and assimilating efficiency of plants (Waqas et al. 2018). In extract of spinach leaf, okadaic acid and microcystin were found to inactivate NR activity, but inhibitor 2 (known inhibitor of PP1 family) showed no effect on NR. Mammalian PP2A activates NR *in vitro*, and it further suggested the role of other phosphatases including PP2A in this process (Mackintosh 1992). BSL1, BSL2, and BSL3 are expressed in leaf and can be considered as potential candidate genes for dephosphorylating NR (Mora-García et al. 2004). Heidari et al. (2011) showed that by knocking down of all three subunits of PP2A in Arabidopsis, NR activity was negatively regulated. This provides a confirmation of involvement of PP2A in NR activation in light-dark transition in plants.

12.7 Phosphatases Identified in N Response/NUE

Global gene expression and comparative analysis of genotypes contrasting for NUE allow the enrichment of candidate genes for NUE. Considerable literatures are now available on transcriptomics, proteomics, as well as on quantitative trait loci (QTL) affecting N response and NUE (Sinha et al. 2018). However, only limited studies explored the regulatory role of different classes of phosphatases in N responses and NUE (Waqas et al. 2018; Sinha et al. 2018; Xiong et al. 2019).

A compilation of the different phosphatases reported to be involved in plant N response and/or NUE is provided in Table 12.1. In a study conducted by Sinha et al. (2018), two contrasting rice genotypes IR64 and Nagina22 were used for genome-wide transcriptome analysis under optimal and chronic starvation of nitrogen from 15-day-old root and shoot tissues. Two genes, *LOC_Os01g71420* and *LOC_Os10g25430* belonging to Ser/Thr phosphatase family, are reported as N-responsive genes.In transgenic line, over expression of PP2C9TL has been reported to improve

Table 12.1 List of genes encoding phosphatase for N response/NUE

Organism	Gene symbol/locus id	Description	References
Rice	LOC_Os01g47580	Lipid phosphate phosphatase 2	Hsieh et al. (2018)
Rice	LOC_Os09g15670	Protein phosphatase 2 C 68 (PP2C68)	Hsieh et al. (2018)
Rice	LOC_Os05g02110	Protein phosphatase 2 C 46 (PP2C46)	Hsieh et al. (2018)
Rice	LOC_Os04g33080	Protein phosphatase 2 C 39 (PP2C39)	Hsieh et al. (2018)
Rice	LOC_Os01g71420	Ser/Thr protein phosphatase family protein, putative, expressed	Sinha et al. (2018)
Rice	LOC_Os10g25430	Ser/Thr protein phosphatase family protein, putative, expressed	Sinha et al. (2018)
Rice	LOC_Os08g33370.2	14-3-3 protein	Waqas et al. (2018)
Rice	LOC_Os05g11550.1	Ser/Thr protein phosphatase5	Waqas et al. (2018)
Rice	LOC_Os07g32380.1	Protein phosphatase 2C	Waqas et al. (2018)
Rice	LOC_Os09g06230.1	Ser/Thr protein phosphatase 16	Waqas et al. (2018)
Rice	LOC_Os04g56450.1	Protein phosphatase 2C	Waqas et al. (2018)
Barley	hv_10051	T6P phosphatase (TPP)	Fataftah et al. (2018)
Maize	GRMZM2G010855	Protein phosphatase 2c (pp2c)	Liu et al. (2011)
Maize	GRMZM2G152447	Purple acid phosphatase 1	Jiang et al. ((2018)
Maize	GRMZM2G134054	Ser/Thr protein phosphatase	Jiang et al. (2018)
Wheat	Traes_6BS_143FEF476	Bifunctional protein-serine/threonine kinase/phosphatase	Xiong et al. (2019)
Sorghum	Sb08g019110	Phosphatases	Gelli et al. (2014)
Poplar	POPTR_0007s05670	Haloacid dehalogenase-like hydrolase (HAD) superfamilyprotein/trehalose-phosphatase family protein	Plavcova et al. (2013)
Poplar	POPTR_0010s11510	UDP-glycosyltransferase/trehalose-phosphatase familyprotein	Plavcova et al. (2013)
Poplar	POPTR_0008s13590	Glycosyl hydrolase 9C2	Plavcova et al. (2013)
Poplar	POPTR_0001s19180	HAD superfamily, subfamily IIIB acid phosphatase	Plavcova et al. (2013)
Poplar	POPTR_0004s16720(Pt-PAP.2)	Purple acid phoshatase 10	Plavcova et al. (2013)
Arabidopsis	TAP46	2A phosphatase-associated protein of 46 kDa	Ahn et al. (2015)
Arabidopsis	ABI2	ABA-insensitive 2	Leran et al. (2015)
Arabidopsis	BSL1	BSU-like phosphatase 1	Heidari et al. (2011)
Arabidopsis	BSL2	BSU-like phosphatase 2	Heidari et al. (2011)
Arabidopsis	BSL3	BSU-like phosphatase 3	Heidari et al. (2011)

(continued)

Table 12.1 (continued)

Organism	Gene symbol/locus id	Description	References
Arabidopsis	PBCP	PSII core phosphatase	Samol et al. (2012)
Arabidopsis	PPH1/TAP38	Chloroplast protein phosphatase/ thylakoid-associated phosphatase of 38 kDa	Samol et al. (2012), Pribil et al. (2010), Shapiguzov et al. (2010)

NUE due to higher activity of NR by downregulation of SnRK and 14-3-3 (Waqas et al. 2018). The higher activity of NR leads to more production of nitric oxide, which then increases N uptake by enhancing the lateral root development. Besides NR, phosphoenolpyruvate carboxylase (PEPC) was also found to be downregulated due to overexpression of PP2C, which dephosphorylates PEPC. Upregulation of PSII and Rubisco increases photosynthetic rate under low N (Waqas et al. 2018). PP2C68 and lipid phosphatase 2 have been reported as upregulated N-responsive phosphatases, while PP2C39 and PP2C46 have been reported to be downregulated in root under N starvation condition (Hsieh et al. 2018). Purple acid phosphatase 1 and serine threonine phosphatase have been found to be nitrate-responsive gene under low nitrate condition in maize (Jiang et al. 2018). Two nitrogen-responsive genes (trehalose-6-phosphate, T6P) in barley have been found to be downregulated under low N condition (Fataftah et al. 2018). These genes along with other genes reverse reaction from pyruvate back to phosphoenolpyruvate during N starvation as an efficient pathway for the remobilization of N sources. In wheat, Traes_6BS_143FEF476 encoding bifunctional protein-serine/threonine kinase/ phosphatase was found to be a candidate gene providing tolerance to low N (Xiong et al. 2019).

12.8 Conclusions and Future Prospects

Understanding the genetic basis of tolerance to low N in crops is important for the development of NUE improvement strategies. Protein phosphatases are emerging as an important gene family among the candidate genes/families identified from different plants, as they regulate N uptake, assimilation, and remobilization. While functional genomic approaches have provided several leads in this regard, their potential in the improvement of NUE needs further validation through mutant/transgenic lines, before using them as targets in crop breeding programs for NUE. In the meantime, integration of all the available information at the molecular, physiological, and genetic level will be useful to mine SNPs through genome-wide association studies (GWAS) and marker development using candidate gene approach. We can expect to see some developments in this direction in the years ahead.

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