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REVIEW ARTICLE

Role of protein phosphatases in the regulation of nitrogen nutrition in plants

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Abstract The reversible protein phosphorylation and dephosphorylation mediated by protein kinases and phosphatases regulate different biological processes and their response to environmental cues, including nitrogen (N) availability. Nitrate assimilation is under the strict control of phosphorylation—dephosphorylation mediated post-translational regulation. The protein phosphatase family with approximately 150 members in Arabidopsis and around 130 members in rice is a promising player in N uptake and assimilation pathways. Protein phosphatase 2A (PP2A) enhances the activation of nitrate reductase (NR) by deactivating SnRK1 and reduces the binding of inhibitory 14–3–3 proteins on NR. The functioning of nitrate transporter NPF6.3 is regulated by phosphorylation of CBL9 (Calcineurin B like protein 9) and CIPK23 (CBL interacting protein kinase 23) module. Phosphorylation by CIPK23 inhibits the activity of NPF6.3, whereas protein phosphatases (PP2C) enhance the NPF6.3-dependent nitrate sensing. PP2Cs and CIPK23 also regulate ammonium transporters (AMTs). Under either moderate ammonium supply or high N demand, CIPK23 is bound and inactivated by PP2Cs. Ammonium uptake is mediated by nonphosphorylated and active AMT1s. Whereas, under high ammonium availability, CIPK23 gets activated and phosphorylate AMT1;1 and AMT1;2 rendering them inactive. Recent reports suggest the critical role of protein phosphatases in regulating N use efficiency (NUE). In rice, PP2C9 regulates NUE by improving N uptake and assimilation. Comparative leaf proteome of wild type and PP2C9 over-expressing transgenic rice lines showed 30 differentially expressed proteins under low N level. These proteins are involved in photosynthesis, N metabolism, signalling, and defence.

Keywords Nitrogen · Protein phosphatase · Protein kinase · Post-translational regulation · Nitrogen use efficiency

Introduction

Various kinds of environmental cues regulate plant growth and development. The signals for environmental cues are perceived by receptors located on the plasma membrane, cytoplasm or nucleoplasm. The downstream signalling pathways are activated that helps the plant to cope up with stress, either by adaptation or tolerance mechanisms. Post-translational modification of proteins through kinases or phosphatases is one of the essential mechanisms, which will either activate or inactivate the signalling intermediates by adding or removing a phosphate group. They play an indispensable role in various physiological, developmental, and stress tolerance mechanisms. In *Arabidopsis*, more than 36,000 phosphorylation sites were predicted from nearly 3600 phosphoproteins (van-Wijk et al. 2014). Protein phosphorylation is a reversible post-translational

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alteration in which protein kinases (PKs) transfer the donor ATP's -phosphoryl group to the acceptor protein side chains. In contrast, protein phosphatases (PPs) dephosphorylate the phosphoproteins. The human protein kinome and phosphatome contain 518 PKs and 189 PPs, respectively, whereas Arabidopsis has roughly 1125 PKs and 150 PPs (Bheri et al. 2021). In silico analysis of rice revealed nearly 1900 serine/threonine kinases and 130 protein phosphatases (Singh et al. 2010). The eukaryotic PPs are classified into phosphoprotein phosphatases (PPP), metallo-dependent protein phosphatases (PPM), protein tyrosine (Tyr) phosphatases (PTP), and aspartate (Asp)-dependent phosphatases. The plant PPPs and PPMs are serine(Ser)/threonine(Thr)-specific phosphatases (STPs). DsPTPs/DSPs are enzymes that dephosphorylate all three phosphoresidues: Ser, Thr, and Tyr. The PP2C phosphatases- pyruvate dehydrogenase phosphatase, and other magnesium (Mg^{2+})/manganese (Mn^{2+})-dependent STPs are closely related but lack sequence homology with PPP, creating a separate PPM family (Xuan et al. 2017; Pflüger et al. 2018; Bheri et al. 2021).

The plant PPPs show significant homology among different members and is further divided into four subgroups PP1, PP2A, PP2B, and PP2C (Cohen 1989). PP2B and PP2C are regulated by Ca^{2+} and Mg^{2+}, respectively, whereas PP2A and PP1 do not require divalent cations for activity. A group of drugs, including okadaic acid, calyculin A, and cantharidin, helps to distinguish the different members of PP1 and PP2-type enzymes. For example, both okadaic acid and calyculin A potently inhibit the activity of PP1 and PP2A but are not effective against PP2B and PP2C. Cantharidin inhibits only PP2A but not others (Li and Casida 1992). The PTP superfamily has low sequence homology among different members. All PTPs contain an active-site signature motif—[I/V]HCxAGxxR[S/T]G with a catalytic cysteinyl residue involved in the formation of phosphoenzyme reaction intermediate (Guan and Dixon 1990).

Apart from water, nitrogen (N) is the second most important input for crop growth, constituting about 0.7% of dry plant biomass (Zhang et al. 2014). Nitrogen acts as a building block of protein, DNA, and many other metabolites whose normal activity is determined by optimum C/N ratio (Huerta et al. 2013). Deficiency of N leads to increased photoinhibition and lipid peroxidation, decreased carboxylation, antioxidant enzyme activity, yield, and quality (Lu and Zhang 2000; Huang et al. 2004). Metabolism of N occurs via four components, viz., uptake, transport, assimilation, and remobilization. Plants takes up N in nitrate and ammonium form via different membrane transport proteins (Lee and Rudge 1986). The activities of N uptake and assimilatory proteins and/or their regulatory proteins are controlled by phosphorylation and dephosphorylation events which involve kinases and phosphatases, respectively (Fig. 1).

Protein phosphatases in nitrate sensing and signalling

Nitrogen is an essential macronutrient that is absorbed by plants in two oxidation states, The oxidized and foremost form utilized by plants is nitrate, while ammonium is the reduced form. Plants adapted to aerated soils prefers nitrate, while plants adapted to waterlogged soils takes up ammonium (Xuan et al. 2017). In addition to their role in mineral nutrition, the N forms also act as important signalling molecules regulating plant growth and metabolism (Undurraga et al. 2017). Earlier research has shown that kinase and phosphatase activities are required for changes in gene expression in response to nitrate treatments. Treatments with inhibitors of calmodulin-dependent protein kinases suppress the nitrate regulated activation of genes encoding nitrate assimilatory enzymes including Nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase 2 (GS2), and ferredoxin glutamate synthase (Fd-GOGAT) in maize leaves. On the other hand, protein phosphatase inhibitors prevented the nitrate response of NR, NIR, and GS2 (Sakakibara et al. 1997). In another investigation, pharmacological inhibitors of STPs and tyrosine protein kinases suppressed the nitrate-induced accumulation of NR and NiR transcripts in barley leaves (Sueyoshi et al. 1999). Changes in protein phosphorylation status were significant for regulating gene expression in response to nitrate treatments (Vega et al. 2021). Phosphorylation status can affect the formation of homo or heterodimers, protein stability, and protein localisation (Hunter 1995; Olsen et al. 2006). Nitrate regulates root system architecture traits such as primary root growth, lateral root initiation, lateral root elongation, and root hair proliferation. These changes involve nitrate transceptors, calcium signalling components, calcium-binding proteins, kinases, phosphatases and transcription factors (Riveras et al. 2015). In bacteria, nitrate signalling occurs via a His-to-Asp phosphorelay system, but the evidence for such a relay system is scarce in plants (Stewart 1994). In plants, pieces of evidence suggest that nitrate drives transcriptional and post-transcriptional regulation of an array of genes. Nitrate signalling involves two interrelated components: Primary Nitrate Response (PNR) and N Starvation Response (NSR). PNR refers to rapid (within 20 min) activation of nitrate specific genes (*NR*, *NiR*, *G6PDH*, *HRS1*) without additional protein synthesis (Ho et al. 2009). It is regulated by Chlorate Resistance 1 (CHL1/AtNRT1.1), Calcium-sensor protein kinase (CPKs), Calcineurin B-Like protein (CBLs), CBL-interacting protein kinase (CIPKs), and phosphatases. Expression of PNR

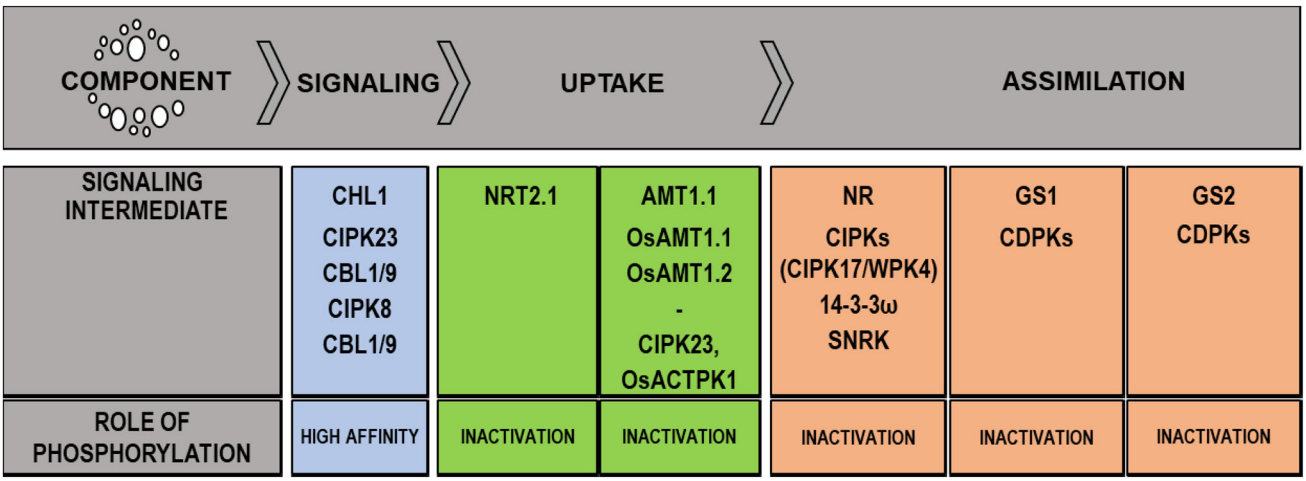

Fig. 1 Regulatory role of protein phosphorylation in nitrogen metabolism and signalling

genes is directly proportional to nitrate concentrations; low (< 1 mM) and high concentrations (> 1 mM) evokes an equivalent magnitude of PNR gene expression (Hu et al. 2009). The CHL1 is a dual affinity transporter that acts as a nitrate sensor (transceptor) in plants. In contrast to PNR, NSR occurs after 24–48 h of N starvation which includes a slow activation of high-affinity nitrate transporters belonging to NRT2 family and other important genes like *CBL7*, *miR169*, *HRS1*, *HRS1 HOMOLOG* (*HHO*), *LBD36*, *LBD37*, and *NFYA* (Wang et al. 2018). The phosphorelay is shown to have physiological outcomes too. The phosphorylated form of NRT2.1 represses the lateral root emergence under low nitrate concentration, while the dephosphorylated state of NRT2.1 induces the primary root growth (Bouguyon et al. 2015). A previous study established that under low nitrate, CHL1 gets phosphorylated and upregulates the expression of NRT2.1 and repress the auxin transport into the developed lateral root primordia and young lateral roots. This response will retard the root growth under low external nitrate (Krouk et al. 2010).

The results from protein phosphatase and kinase inhibitor studies suggested the role of protein phospho/dephosphorylation in nitrate signalling (Sakakibara et al. 1997; Sueyoshi et al. 1999). A study with okadaic acid and calyculin A inhibitors showed the role of type 1 and type 2A STPS in nitrate signalling (Sueyoshi et al. 1999). Reduction in nitrate response was observed with inhibitors of tyrosine kinase viz., genistein, quercetin, and curcumin in barley leaves (Sueyoshi et al. 1999). In maize leaves, inhibitors of kinases and phosphatases have prominent effects on transcript expression of *NR*, *NiR*, and *GS*2. In contrast, the pre-treatment with kinase inhibitor, namely W-7, inhibited the nitrate induced expression of nitrate assimilatory genes *NR* (34%), *NiR* (34%), *GS*2 (63%) and *Fd-GOGAT* (59%) (Sakakibara et al. 1997). Such a response was not observed in transcript level of NR and NiR when Ca^{2+}/calmodulin dependent protein kinase was inhibited with W-7 and trifluoperazine antagonist in barley leaves (Sueyoshi et al. 1999). A study with N starvation in rice roots showed rapid induction of six protein kinases and one protein phosphatase gene. The cell wall-associated receptor kinases (WAKs) regulate plant cell wall functions such as pathogen response, binding to pectin to control cell expansion, morphogenesis, and development (Oliveira et al. 2014). The expression of *WAK125* (*Os12g0478400*) and *WAK37* (*Os04g0365100*) was rapidly induced by N starvation in rice roots (Hsieh et al. 2018). A transcriptomic study showed downregulation of *PP2C40* gene (*At3g16560*) under nitrate starvation (3 h) in *Arabidopsis* (Menz et al. 2016) as well as in Nodule Inception (*NIN*) mutants of *Medicago* (homolog *Medtr4g11983)* (Liu et al. 2019). The physiological role of the gene is yet to be understood.

Protein phosphatases in nitrate transport

Phosphorylation status of nitrate transporters in the plasma membrane and tonoplast regulate nitrate transport and signalling (Liu and Tsay 2003; Migocka et al. 2013). When external nitrate concentration is low, CBL1/9 and CIPK23 phosphorylate NRT1.1 at threonine 101, located in the intracellular side between second and third transmembrane helices (Liu and Tsay 2003; Ho et al. 2009; Hu et al. 2009). Phosphorylation at threonine 101 leads to a shift in the property of NRT1.1 into a high-affinity nitrate carrier (Liu and Tsay 2003). Low nitrate conditions also reduce the PNR and weak upregulation of high-affinity nitrate transporter NRT2.1 (Filleur et al. 2001; Ho et al. 2009). It was observed that CIPK8, along with CBL1/9, is involved in the positive regulation of NRT1.1, maintaining it as a low-

affinity transporter under high nitrate conditions (Hu et al. 2009). X-ray crystallographic study showed that dephosphorylated form of NRT1.1 forms a homodimer and works as low-affinity transporter, whereas phosphorylation leads to a decoupling of dimer and shifting into high-affinity transporter (Parker and Newstead 2014; Sun et al. 2014). In nitrate signalling, the role of ABA-sensitive Protein Phosphatase 2C, namely ABA INSENSITIVE 2 (ABI2), is also unravelled. ABI2 inhibits the phosphorylation of NRT1.1 by preventing the phosphorylation of CIPK23. The crosstalk with ABA signalling shows that abiotic stresses influence nitrate uptake via ABA (Léran et al. 2015).

Nitrate sensing by CHL1 transmits signals to the nucleus and controls the expression of several genes. Calcium signalling components and other transcription factors like NLP7, NLP6, TGA1, TGA4, bZIP1, LBD37, LBD38, TCP 20, and NAC4 are shown to be the regulators of nitrate metabolism related target genes (O'Brien et al. 2016). CHL1 mediated signalling was shown to upregulate the expression of high-affinity nitrate transporter NRT2.1 in *Arabidopsis* (Ho et al. 2009). Independent of its uptake mechanism, nitrate inhibits lateral root initiation, but the underlying mechanism is unknown (Little et al. 2005). Phosphorylation at serine 501 of C terminus of NRT2.1 will inhibit the protein activity (Jacquot et al. 2020). Recently (Ohkubo et al. 2021), a type 2C protein phosphatase called CEPD-induced phosphatase (CEPH) was reported to be the phosphatase activator that dephosphorylates Ser501 of NRT2.1.

The P-type H^+-ATPase 2 (AHA2) supplies protons required for nitrate transport (two protons/nitrate molecules). AHA2 has nine phosphorylation sites, of which phosphorylation at Thr881 and 947 increases its activity, while at Ser899 and 931 decrease its activity (Haruta et al. 2015). The concomitant requirement of Mg^{2+} for dephosphorylation indicates the role of type2C protein phosphatase in *Vicia faba.* AHA2 activity is reduced by PP2C-D1 which dephosphorylate at T947. It was also reported that PP2C-D1 activity is negatively regulated by direct interaction with SMALL AUXIN UP-RNA 19 (SAUR19) protein (Spartz et al. 2014).

The anion channel SLAC1 homolog 3 (SLAH3) alleviates ammonium toxicity when nitrate concentration is low. SLAH3 activity in guard cells is determined by CPK21 and protein phosphatase ABI1 (Lind et al. 2015). Recently it has been observed that (Sun et al. 2021) SnRK1.1 phosphorylates the C-terminal of SLAH3 at the site S601 and inhibits the activity. At high-ammonium/low-pH, SnRK1.1 shifts the localisation from the cytosol to nucleus, releases SLAH3 from inhibition and thus alleviate ammonium toxicity by SLAH3-mediated nitrate efflux.

Protein phosphatases in ammonia sensing and signalling

The post-transcriptional regulation ammonium transporters prevents ammonium toxicity. Several distinct ammonium receptors or transceptors have been identified from various kingdoms. For instance, Methylammonium (MA) permease 2 (Mep2) from fungi *S. cerevisiae* (Berg et al. 2016); Ammonium transporter 5 (Ks-Amt5) from anammox bacteria (Pflüger et al. 2018), and AMT1;1, AMT1;2, AMT1;3, etc. from several plant species undertakes sensory and/or channel function for ammonium (Chen et al. 2020). AMT is not the only ammonium sensor in plants. It is also regulated by ammonium-dependent sensory kinases and phosphatases at its Cytosolic C-terminal region (CTR) domain. CIPK15 and CIPK23 of Arabidopsis are ammonium sensory proteins that phosphorylate the CTR domain of AMTs to prevent its uptake function at toxic cytosolic levels (Chen et al. 2020). Another protein, CPK32, mediates the phosphorylation of AMT1;1 at Ser450 in Arabidopsis to activate its ammonium transport function (Qin et al. 2020). In another study, the dephosphorylation at T464 and T494 of AMT1;3 led to the 'turning on' of the transport function in Arabidopsis. The property was confirmed by dephosphorylation mimics T464A and T494A in AMT1;3 (Wu et al. 2019). The ammonia signalling exhibits specific morpho-physiological roles in plant roots. AMT1;3 triggers lateral root branching by sensing the ammonium levels in a nutrient medium (Lima et al. 2010). The phosphatase sensor which interacts with the transporters has not been discovered from plants.

Protein phosphatases in ammonia transport

Studies from the model plant *Arabidopsis* have shown that under low external ammonium conditions, AMT1;1 and AMT1;2 get dephosphorylated at T460 and 472 residues near C terminus and helps in the ammonium uptake (Lanquar et al. 2009). Excess ammonium leads to phosphorylation of these transporters, which prevents toxic uptake. In support of this, the dephospho variant of $AMT1;1^{T460A}$ resulted in a constitutively active transporter (Loqué et al. 2007; Neuhäuser et al. 2007). Arabidopsis CIPK23 directly interacts and phosphorylates AMT1;1 (Straub et al. 2017). In rice, the phosphoregulation of AMT1;1 occurs at T446 and 456 residues (Zhu et al. 2015). An ACT domain and protein kinase domain-containing protein (OsACTPK1) was found to be involved in phosphorylation of OsAMT1;1 and OsAMT1;2. Under ammonium deficiency, the transcript level of *OsACTPK1* is reduced, which will render the AMTs to remain

dephosphorylated. This process will promote the uptake of more ammonium from soil (Beier et al. 2018). Treating rice with the Ser/Thr protein phosphatase inhibitor FK506 led to a significant reduction in ammonium uptake (Yang et al. 2015). It was observed that INDETERMINATE DOMAIN10 (IDD10) encoding C2H2 zinc finger protein transcriptionally upregulates the AMT1;2 expression by binding to its promoter in rice (Xuan et al. 2013). ammonium treatment leads to dephosphorylation of IDD10 at S313 residue. It was also found that there is increased phosphorylation of PP2C9 and dephosphorylation of PP2C19 (Zhu et al. 2015), but their association with IDD10 activity remains unclear at present. In rice, treatment with either FK506 (a calcium-regulated serine/threonine-specific protein phosphatase inhibitor) or genistein (a selective inhibitor of tyrosine-specific protein kinases) reduced OsAMT1.1-mediated ammonium absorption by at least 30% (Yang et al. 2015). Resupply of nitrate, but not ammonium, was found to cause rapid dephosphorylation of T494 in the CTR of AtAMT1.3, resulting in increased transport activity of AtAMT1.3 and hence increased overall ammonium uptake in Arabidopsis (Wu et al. 2019). The phosphatase involved in the dephosphorylation of the T494 position in AtAMT1.3, on the other hand, is unknown.

Protein phosphatases for the regulation of N assimilation

Studies have shown that phosphoregulation of protein activity is prominent in plants compared to animals as it ensures a prompt response to environmental signals and helps in survival (Liu and Tsay 2003). Proteins involved in N assimilation are also regulated by phosphorylation. Nitrate Reductase is activated by dephosphorylation (Huber et al. 1992), and the same response was also observed for GS2 (Lima et al. 2006). Conversely, phosphorylation state leads to activation of GS1 (Finnemann and Schjoerring 2000) and Glutamate Dehydrogenase (GDH) (Lin and Reeves 1994).

Nitrate reductase (NR)

Nitrate reductase, the primary key enzyme involved in nitrate assimilation, is highly regulated at transcriptional and post-transcriptional levels, mainly from plastid signals, but the mechanistic details are partially deciphered (Lillo 2008; Nesi et al. 2010; Krouk et al. 2010). It was earlier known that protein dephosphorylation leads to activation of NR, which needs active photosynthesis (Kaiser and Behnisch 1991). The protein phosphatase inhibitor studies confirmed the role of dephosphorylation in the activation of NR. Inhibitors microcystin-LR and okadaic acid suppressed the light-dependent activation of NR in spinach and barley (MacKintosh 1992), but inhibitor 2 (a known inhibitor of the PP1 family) did not affect the activation of NR in leaves extracts. An *in-vitro* study has shown that mammalian PP2A could activate NR, confirming the role of phosphatases in NR activation except for PP1 (MacKintosh 1992). PP2As are heterotrimeric proteins consisting of 36–38 kD catalytic (C), 65 kD structural (A), and 48–74 kD regulatory (B) subunits. The structural subunits include A2, A3, and ROOTS CURL IN NAPHTHYLPHTHALAMIC ACID1 (RCN1), with regulatory subunits, namely B55α and β (Heidari et al. 2011). Various studies have demonstrated in Arabidopsis that the A subunit is required for root morphology (Zhou et al. 2004) and auxin transport (Michniewicz et al. 2007). These three A subunits show overlapping function, and the triple mutant was lethal (Zhou et al. 2004; Michniewicz et al. 2007). One of the A subunits is RCN1, which masks the function of PP2AA2 and PP2AA3 and has a cardinal role in regulating phosphatase activity (Zhou et al. 2004). Activation of NR by PP2A was revealed from an inducible knockdown mutant of all three subunits using miRNAs lines targeting all three subunits of PP2A (Michniewicz et al. 2007). The B subunit regulates substrate specificity and subcellular localization of PP2A in mammals (Bheri et al. 2021). It is assumed that the B subunit has the same role in plants (Matre et al. 2009).

The presence of light and nitrate induces the expression of NR and NiR, whereas dark adaptation leads to the degradation of these proteins in plants. Though the expression of NR is high, the enzyme activity may not be the same due to post-translational regulation. NR activity is induced within minutes in response to light, CO_2, sugar, nitrate, and anoxia (Huber et al. 1992, 1996). Cytosolic pH, carbon metabolism, and coordination of plastidic and cytosolic metabolism regulate NR activation. Under dark conditions, NR is inactivated by a two-step mechanism involving phosphorylation by calcium-dependent protein kinase (CDPK; CPK17 in case of Arabidopsis) and sucrose non-fermented related kinase (SnRK). This phosphorylation site lies between the heme and molybdenum cofactor-containing domains (Lambeck et al. 2012) at S543 in Spinach and S534 in Arabidopsis at hinge I region. Illumination leads to dephosphorylation at these sites by protein phosphatase and activation of the enzyme (Huber et al. 1992; MacKintosh et al. 1995). This dephosphorylation was proposed to be performed by PP2A (MacKintosh et al. 1995) as the inhibitors of PP2A (okadaic acid and microcystin) resulted in delayed light-induced activation of NR (MacKintosh 1992). In short, NR is activated by PP2A involving the structural subunits A2, A3, and RCN1 and regulatory subunits B55α and β (Heidari et al. 2011).

However, neither the ability of PP2A to dephosphorylate NR nor the location of the dephosphorylation site was explored further. Furthermore, AtNIA2 has been shown to physically interact with Mitogen-Activated Protein Kinase 6 (MPK6), resulting in AtNIA2 phosphorylation at Ser627 and enhanced NR activity (Wang et al. 2010).

Experiments with ^{32}P labelling and kinase assay showed that phosphorylation status of NR leads to change in NR activity during light/dark cycles in spinach leaves (Huber et al. 1992; MacKintosh 1992). Studies with peptide antibodies showed that phosphorylation occurs at serine 543 residue that initiates its inhibition (Weiner and Kaiser 2001). The enzyme activity is attenuated by 14–3-3 protein binding to phosphorylated NR and divalent cations like Mg^{2+} (Bachmann et al. 1996; Athwal and Huber 2002).

A full-scale proteomic study showed that nitrate starvation and resupply affect the abundance of about 170 proteins and phosphorylation status of 36 proteins. Starvation of nitrate leads to increased abundance of stress-responsive proteins and those involved in catabolism with a collateral decrease in abundance of the biosynthetic proteins. Starvation of 48 h leads to downregulation of NiR, Carbonic Anhydrase 2 (CA2), Carbamoyl Phosphate Synthase (CARB), and Arginosuccinate Synthase (AS) (Wang et al. 2012). It was also observed that protein phosphorylation status changes with the resupply of N. These include short term responses associated with plasma membrane-associated proteins AHA1 and AHA2 subunits, medium-term responses associated with cytosolic proteins, and long term responses associated with nuclear or cell interior proteins (Engelsberger and Schulze 2012). The most rapid change in phosphorylation status was observed in high-affinity nitrate transporter NRT2.1, where dephosphorylation occurs within 3 min of N resupply. A relationship between Ca^{2+} signalling and phosphorylation was previously known in animal systems where it was observed that activity of some PI-PLCs (Phosphatidyl Inositol–Phospholipase C) was modulated by phosphorylation, which established the link between phosphorylation and phospholipase activation (Gresset et al. 2010). Mass spectrometric and bioinformatics predictions showed the presence of phosphorylation sites in many plant PI-PLCs (Durek et al. 2010). The phosphoproteomic study showed that nitrate resupply leads to the phosphorylation of proteins from the phosphatidylinositol pathway. Such a response was not observed when N was supplied in the ammonium form, showing that N-dependent phosphorylation is nitrate specific (Engelsberger and Schulze 2012).

Ammonia assimilatory enzymes

Glutamine synthetase, another key enzyme in N assimilation in plants, has two octameric isoforms: GS1 encoded by *GLN1* localized in cytosol and GS2 encoded by *GLN2* localized in plastids. The radiolabeling by [γ-P^{32}]ATP, followed by immunodetection, revealed that GS1 and 2 are phosphoproteins, out of which phosphorylation leads to activation of GS1 and inactivation of GS2 (Finnemann and Schjoerring 2000; Lima et al. 2006). This phosphorylation status is maintained by 14–3-3 protein in both isoforms. Dephosphorylation by Ser/Thr phosphatases PP2A and/or PP1 leads to the inactivation of GS1. The role of PP2A and/PP1 was confirmed by an inhibitor study with microcystin (a specific inhibitor of PP2A and PP1) (Finnemann and Schjoerring 2000). Phosphorylation leads to inactivation of GS2 by making it prone to binding of 14–3–3 binding followed by protein degradation in a mechanism similar to that of NR. CDPKs perform the phosphorylation at Ser97 in *Medicago truncatula* and rice (Lima et al. 2006). In *Arabidopsis*, the phosphoregulatory site in GS1.1, 1.2, and 1.3 was detected at the second residue of N terminus. In contrast, in GS2 (At5g35630), it was found to be TIEKP-VEDP(pS)ELPK at the N terminal region, proximal to transit peptide (Engelsberger and Schulze 2012; Hodges et al. 2013). Plant glutamate synthase occurs in two forms; chloroplastic ferredoxin (Fd)-dependent and cytosolic NADH-dependent. Previous reports have highlighted the absence of phosphorylation sites in GOGAT (Hodges et al. 2013), but Zhu et al. (2015) reported an ammonium led phosphorylation at serine residue in rice roots. This was accompanied by increased phosphorylation of PP2C19 and dephosphorylation of PP2C9 at Ser and Thr residue under ammonium treatment (Zhu et al. 2015). Sugar starvation sensed by ATP levels leads to activation of GDH. GDH was also repressed by ATP and activated by AMP and is further proposed to be regulated by phosphorylation (Athwal et al. 1997). Autoradiography studies in *E. coli* with ^{32}P-labeled monosodium phosphate provided evidence for the ATP-dependent phosphorylation of $NADP^{+}$-specific GDH (Lin and Reeves 1994). Further studies in macroalga showed that NADP-GDH activity of chloroplast decreases, while that of cytosol/mitochondria increases by ATP and cAMP-dependent phosphorylation (Inokuchi and Okada 2001).

In chloroplasts, there exist two forms of the antiporter dicarboxylate transporter, namely DiT1 and DiT2. DiT1 acts as an importer of 2-oxoglutarate in exchange for malate, while DiT2 helps export glutamate in exchange for malate. It was observed that phosphorylation occurs at two serine residues in the N terminus of DiT1 while at the threonine residue of DiT2.2. These antiporters' activity is inhibited by dephosphorylation, preventing the decarboxylate uptake into the chloroplast. Extracts from N-starved cell cultures resupplied with nitrate or ammonium showed the presence of DiT2.2. It is important for ammonium assimilation in the chloroplast as this protein

Table 1 Examples of protein kinase and phosphatases differentially regulated by nitrogen treatments in plants

Sl. No	Gene/Locus ID	Regulation	Description	Experiment	References
1	Os09g0325700/ LOC_Os09g15670	Up regulation of gene expression	Protein phosphatase 2C 68 (PP2C68)	Nitrogen deprived rice seedling roots	Hsieh et al. (2018)
2	Os07g0678300/ LOC_Os07g48090	Up regulation of gene expression	CBL-interacting protein kinase 29 (CIPK29)		
3	Os12g0478400/ LOC_Os12g29430	Up regulation of gene expression	Wall-associated receptor kinase 125 (WAK125)		
4	Os03g0812400/ LOC_Os03g59770	Up regulation of gene expression	Calmodulin-like protein 2		
5	Os02g0198200/ LOC_Os02g10470	Up regulation of gene expression	Calcium-binding protein CML21		
6	Os04g0517500	Up regulation of gene expression	PPCK3		
7	Os02g0120100/ LOC_Os02g02780	Down regulation of gene expression	Serine/threonine-protein kinase STY46		
8	Os12g0113500/ LOC_Os12g02200	Down regulation of gene expression	CBL-interacting protein kinase 14 (CIPK14)		
9	Os05g0111800/ LOC_Os05g02110	Down regulation of gene expression	Protein phosphatase 2C 46 (PP2C46)		
10	Os04g0403701/ LOC_Os04g33080	Down regulation of gene expression	Protein phosphatase 2C 39 (PP2C39)		
11	Os01g0208700/ LOC_Os01g11054	Down regulation of gene expression	Phosphoenolpyruvate carboxylase 4 (PPC4), chloroplastic		
12	Os01g0191700/ LOC_Os01g09570	Down regulation of gene expression	ATP-dependent 6-phosphofructokinase 6 (PFK01)		
13	AT1G22280.1	Phosphorylated in response to Nitrate resupply	Protein phosphatase 2C	Nitrate and ammonium resupplied to nitrogen-starved Arabidopsis seedlings	Engelsberger et al. (2012)
14	AT1G34750.1	Phosphorylated in response to Nitrate resupply	Protein phosphatase 2C		
15	AT5G02400.1	Phosphorylated in response to Nitrate resupply	PLL2 (POL-LIKE 2)		
16	AT3G56760.1	Phosphorylated in response to ammonia resupply	Calcium-dependent protein kinase		
17	AT2G42810.1	Phosphorylated in response to ammonia resupply	PP5.2 (PROTEIN PHOSPHATASE 5.2)		
18	AT5G23720.1	Phosphorylated in response to ammonia resupply	PHS1 (PROPYZAMIDE-HYPERSENSITIVE 1)		
19	AT5G53000.1	Phosphorylated in response to ammonia resupply	TAP46 (2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 KD)		
20	AT1G10940.1	Phosphorylated in response to Nitrate resupply	SnRK2.4		
21	AT5G07070.1	Phosphorylated in response to Nitrate resupply	CIPK2		
22	AT5G35410.1	Phosphorylated in response to ammonia resupply	CIPK24		
23	AT3G20410.1	Phosphorylated in response to ammonia resupply	CPK9		
24	AT3G56760.1	Phosphorylated in response to Nitrate resupply	CDPK		
25	AT4G04700.1	Phosphorylated in response to ammonia resupply	CPK27		

Table 1 continued

Sl. No	Gene/Locus ID	Regulation	Description	Experiment	References
26	At3g16560.1	Up regulation of gene expression in nitrate versus ammonia treatment	Protein phosphatase 2C family protein	Early nitrogen-deprivation responses in Nitrate and ammonium -starved Arabidopsis seedlings	Menz et al. (2016)
27	At2g26980.4	Up regulation of gene expression in nitrate versus ammonia treatment	CBL-interacting protein kinase 3		
28	At2g25090.1	Up regulation of gene expression in nitrate versus ammonia treatment	CBL-interacting protein kinase 16		
29	AB011670	Up regulation of gene expression in nitrogen starvation	WPK4-SnRK3/CIPK protein kinase	Nitrogen starvation in wheat	Luang et al. (2018)
30	–	Upregulation by N deficiency	PP2C	Nitrogen starvation in Potato	Tiwari et al. (2020)

exists at the gateway between carbon and N metabolism (Schneidereit et al. 2006; Hodges et al. 2013).

Omics of protein phosphatase mediated N stress adaptation and improvement in NUE

Recent evidence posits that nitrate sensed by NRT1.1 activates a phospholipase C activity necessary for increased cytosolic calcium levels in the nitrate signalling pathway. The nitrate-elicited calcium increase presumably activates calcium sensors, kinases, or phosphatases, resulting in changes in the expression of PNR genes. Consistent with this model, nitrate treatments elicit proteome-wide changes in phosphorylation patterns in a wide range of proteins, including transporters, metabolic enzymes, kinases, phosphatases, and other regulatory proteins. Transcriptomics studies from potatoes have shown that N deficit stress led to a significant reduction of Tartrate-resistant acid phosphatases/Purple acid phosphatases (TRAcPs/PAPs) in leaves (Table 1). In roots, a member of the PP2C family and high-affinity nitrate transporter (NRT) was highly upregulated under stress (Tiwari et al. 2020). A phosphoproteome study in *Arabidopsis* under nitrate supply showed that 127 phosphoproteins were upregulated and 52 were downregulated. Among the dephosphorylated proteins, the PIN2 protein was also identified. Nitrate regulates the dephosphorylation of PIN2 at Ser539, and this change is responsible for its altered membrane localisation, the reduced primary root growth, and the enhanced lateral root growth (Vega et al. 2021).

Nitrogen starvation followed by supplementation of nitrate or ammonium also resulted in specific phosphorylation patterns, mainly signalling proteins, transcription factors, and transporters (Engelsberger and Schulze 2012). Hsieh et al. (2018) recognized around six protein kinase and one protein phosphatase genes in rice induced by N deficiency in root tissue (Table 1). These include wall-associated receptor kinases (WAKs) such as WAK125 (Os12g0478400) and WAK37 (Os04g0365100); WAK125 is reported to be an early glutamate-responsive gene. Similarly, the expression of PPCK3, a PEPC kinase gene (Os04g0517500), was also rapidly induced by N starvation in rice roots. CIPK29 gene (Os07g0678300) was also up-regulated by N deficiency, whereas K deficiency down-regulated its expression. Expression of rice PP2C68 (Os09g0325700), a homolog of Arabidopsis HAI1/2/3 (highly ABA-induced PP2C protein 1/2/3), was rapidly induced by N starvation. Expression of 2 phosphatase genes was quickly repressed by N starvation in roots of rice seedlings, Os04g0403701 (PP2C39) and Os05g0111800 (PP2C46) (Table 1). However, their roles in the regulation of N response are yet to be characterized. Waqas et al. (2018) observed that the protein phosphatase (PP2C9) has a regulatory role in improving rice NUE by enhancing N uptake and assimilation. PP2C9TL showed higher NUE than WT due to higher NR activity. Wheat Kinase- Phosphatase-14–3–3 interactome was unravelled by Luang et al. (2018). They observed that the ABA-responsive bZIP2 was

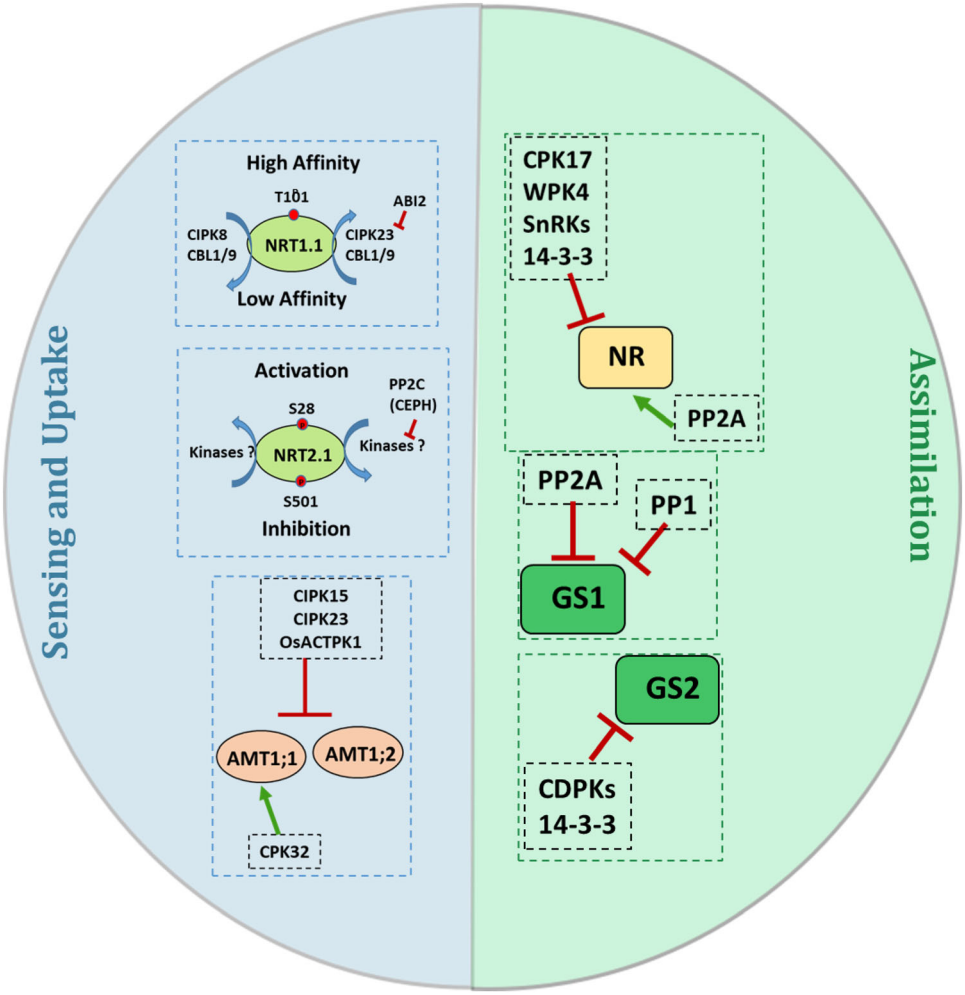

Fig. 2 Illustration depicting phosphorylation and dephosphorylation of nitrate uptake, sensing and nitrogen metabolism. The regulatory phosphatases are shown. NRTs mediate nitrate sensing and uptake, while NR and GS mediate nitrogen assimilation

under the negative regulation of a 14–3–3 protein, TaWIN1 and the positive regulation of SnRK3/CIPK protein kinase WPK4, nutrient starvation responsive kinase. These studies provide new insights into the role of protein phosphatases which may be necessary for developing robust strategies to increase NUE in crop plants.

Kumari and Raghuram (2020) recently published a complete list of phosphatases implicated in crop N-response and NUE. Based on meta-analysis (Kumari et al. 2021), nine phosphatases regulating NUE were identified. The discovered phosphatases are Fructose-1,6-bisphosphatase, Type II inositol-1,4,5-trisphosphate 5-phosphatase 12, Type I inositol-1,4,5-trisphosphate 5-phosphatase CVP2, Vacuolar pyrophosphatase, Fructose-1,6-bisphosphatase class 1/Sedoheputulose-1,7-bisphosphatase etc. (Kumari et al. 2021). Six of the identified phosphatase genes were up-regulated by N, whereas the other three were down regulated. These N responsive phosphatases can improve NUE (Hsieh et al. 2018; Kumari et al. 2021) I crop plants.

Conclusion

Protein kinases and phosphatases are known to have a key role in NUE and N-response in plants. Protein phosphatase 2A bind with NR and activate its nitrate-reduction activity. The canonical nitrate-signalling route of Arabidopsis roots comprises NRT1.1/NPF6.3, calcium, and kinases/phosphatases (Fig. 2). The phosphatases associated with NRT2.1 was recently discovered. Further studies on identifying phosphtase counterparts associated with PNR, NSR and N metabolism may provide additional insights and targets for improving NUE of crop plants.

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Declarations

Conflict of interest The authors have no competing interests to declare that are relevant to the content of this article.

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