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Searching for Nitrogen Sensing Systems in Higher Plants

Man-Wah Li[1] • Hon-Ming Lam[1,2*]

[1] Molecular Biotechnology Program, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China
[2] Department of Biology, The Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China

Corresponding author: * **honming@cuhk.edu.hk**

ABSTRACT

Nitrogen is an essential element of all life forms. With limited mobility, most plants cannot actively hunt for additional nitrogen resources apart from the substratum they rooted. To efficiently utilize the finite nitrogen resource, plants need to adopt a set of nitrogen sensing systems to manage the nitrogen status from time to time. To cope with diverse situations, different systems were evolved to perceive different form of signals (from inorganic nitrogen to organic nitrogen) at different levels (from intracellular to the whole plant). The integrated effects of these signals ensure efficient uptake, transportation, assimilation, utilization, and storage of important nitrogen resources. Several nitrogen sensing systems were documented in microorganisms and animals. In this review, we summarized the recent progress of similar systems found in plants, including: (i) PII-mediated nitrogen regulatory system; (ii) general amino acid control; (iii) ionotropic glutamate receptors; (iv) cytokinin His-Asp phosphorelay system; and (v) NIT2 system.

Keywords: cytokinins His-Asp phosphorelay, general amino acid control, glutamate receptors, nitrogen signaling, PII, signal transduction

CONTENTS

INTRODUCTION

Plants are sessile living organisms which depend on the limited nutrients in the confined substratum where they rooted. Changes of nutrient availability may happen either as a long-term subtle change or an unpredictable rapid event. Coordinated adjustment to nutrient status is the key of success in the community.

Nitrogen (N) is one of the major constituents of life. It is essential for the biosynthesis of important biomolecules such as amino acids, nucleic acids, chlorophyll, phytohormones as well as other N-containing metabolites. Though nitrogen gas (N_2) makes up 78% of the Earth's atmosphere, it cannot be directly used by plants. Plants mainly acquire N in form of nitrate from the soil and assimilate the acquired N resources into amino acids via reduction processes. Some plants can undergo symbiotic N fixation to "fix" N_2 into amino acids. Both processes are extremely energy demanding. The ultimate energy source is coming from respiratory oxidation of photosynthetic products while, on the other hand, N is an essential constituent of the photosynthetic machinery. An efficient coordination between N (acquisition, assimilation, and catabolism) and carbon (C) (photosynthesis and respiration) pathways is essential for the survival of plants.

Monitoring systems for N availability is therefore strategically important. N sensing systems were first reported in microorganisms and animals, including: (i) PII-mediated nitrogen regulatory system in *Escherichia coli* and cyanobacteria sensing the balance between C and N metabolites; (ii) general amino acid control (GAAC) in yeast sensing amino acid starvation conditions; (iii) ionotropic glutamate receptors (iGLRs) in animal nervous system responsive to organic N in the form of amino acids; (iv) cytokinin His-Asp phosphorelay system in bacteria mediates signal initiated by inorganic nitrate; and (v) NIT2 system responsive to both organic and inorganic forms of N signals.

Although the plants have evolved a unique mode of living, increasing evidences suggest that plants have adopted N sensing systems homologous to the systems in bacteria, yeast and animals described above. In this review, we summarized some current developments in this aspect.

PII-mediated nitrogen regulatory system

PII-mediated nitrogen sensing has been thoroughly studied in *E. coli* and cyanobacteria and identified as a system to regulate cellular C:N balance.

In the glutamine synthetase/glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle, ammonium (NH_4^+) generated via nitrate/nitrite reduction is first incorporated into glutamate (Glu) to form glutamine (Gln). The Gln will then react with 2-oxoglutarate (2KG) to form two Glu molecules. Therefore, NH_4^+ deficiency will lead to an accumulation of 2KG (N-limiting) while high level of Gln will be built up with surplus of NH_4^+ (N-rich). The PII system regu-

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Invited Review

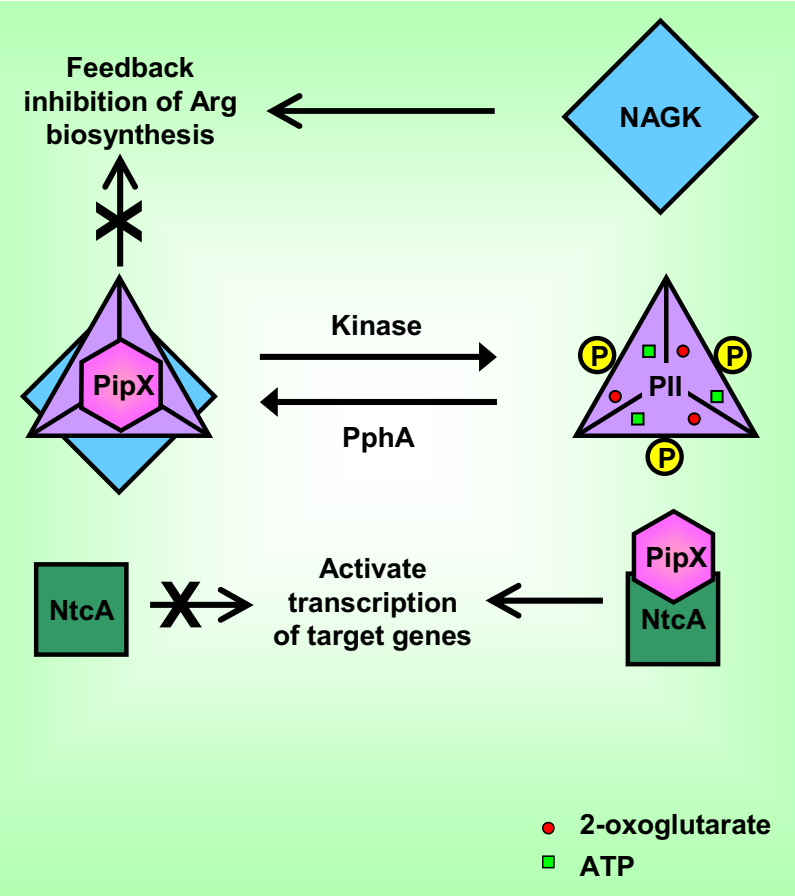

Fig. 1 Model of PII-mediated nitrogen regulatory system in cyanobacteria. When N is not limiting (signified as low ATP and 2-oxoglutarate (2KG) levels), PII interaction protein X (PipX) and N-acetyl-L-glutamate kinase (NAGK) bind to unphosphorylated PII. PII bound NAGK becomes non-responsive to the feedback inhibition by arginine (Arg). When N is limiting (elevated ATP and 2KG levels), PII will be phosphorylated. Dephosphorylation of PII by PphA is prevented by the binding of ATP (and 2KG). PipX and NAGK will be released from PII. PipX will then interact with NtcA and activate transcription of target genes involved in N uptake, metabolism, and regulation while free NAGK is the target for feedback inhibition in the Arg biosynthetic pathway. Plant homologues of PII and NAGK have been identified and proven to be involved in the regulation of Arg biosynthesis.

lates the C:N balance largely base on this equilibrium.

In *E. coli*, N-limiting conditions (high 2KG, low Gln) will stimulate binding of 2KG and ATP to PII and stimulate PII uridylation by the uridylytransferase/uridylyl-removing enzyme (UTase/UR) at Tyr-51. PII-UMP will attempt to (i) deadenylylate the inactive adenylylated glutamine synthetase (GS) by stimulating adenylyltransferase (ATase); (ii) stimulate NRI phosphorylation by removing PII from NRII (NRI kinase) to activate the transcription of the *glnA* gene (encoding GS) (Ninfa and Atkinson 2000).

Distinct from PII of *E. coli*, PII in the photosynthetic cyanobacteria (**Fig. 1**) is post-translationally regulated by phosphorylation on serine-49 (Ser-49) instead of uridylylation. When Ser-49 is substituted with alanine (Ala), PII-mediated regulation of nitrate and nitrite uptake will be lost (de Marsac *et al*. 2001).

When organic N become limiting (high 2KG, absence of NH_4^+, presence of NO_3^- or NO_2^-), cyanobacterial PII will be phosphorylated upon synergistic binding of ATP and 2KG (Forchhammer and Demarsac 1994; Forchhammer and Hedler 1997; Kobayashi *et al*. 2005). Under such conditions, dephosphorylation of PII is prevented (Ruppert *et al*. 2002) to keep the switch in the "ON" state. Dephosphorylation of PII-P from *Synchococcus* sp. strain PCC6803 is catalyzed by a type-2C protein phosphatase (phosphor-PII (PII-P) phosphatase: PphA) under low ATP concentration independent of 2KG (Ruppert *et al*. 2002). PII and PphA are also important for the regulation of NO_3^- uptake in *Synechocystis* sp. strain PCC6803 when the reducing power is low, under low light conditions (Kloft and Forchhammer 2005).

Under normal growth conditions, the PII interaction protein X (PipX) binds to the unphosphorylated PII. However, N deprivation triggers phosphorylation of PII to release the PipX protein from the protein complex. Favored by the presence of 2KG, the free PipX will then bind to the transcriptional factors NtcA (Espinosa *et al*. 2006) and up-regulate the expression of *glnB* (encoding PII: Lee *et al*. 1999), *glnA* (encoding GS: Vazquez-Bermudez *et al*. 2002), and *nirA-nrtABCD-narB* (encoding nitrite reductase, high affinity-transporter permease, nitrate reductase) in the *nir operon* (Luque *et al*. 1992) as well as *ntcA* itself.

N-acetyl-L-glutamate kinase (NAGK) is an enzyme that involved in arginine (Arg) biosynthetic pathway using Glu as a precursor. NAGK is the key target enzyme for feedback inhibition by excessive Arg. In *Syuchococcus elongates* strain PCC7972, when N is not limiting, unphosphorylated PII binds NAGK and greatly reduces the feedback inhibition of Arg biosynthesis. Conversely, when N is insufficient, NAGK will be released from phosphorylated PII and the feedback inhibition was restored (Maheswaran *et al*. 2004). Such control is useful in manipulating C:N balance since Arg is the amino acid having the lowest C:N ratio.

Plant PII homologues were first identified in *Arabidopsis thaliana* (encoded by *AtGLB1*) and *Ricinus communis* (castor bean) and exhibit more than 50% homology to PII proteins from *E. coli* and cyanobacteria (Hsieh *et al*. 1998). Subsequent EST search revealed the presence of PII-like proteins in over 20 plant species including *Oryza sativa* (rice), *Lycopersicon esculentum* (tomato), *Glycine max* (soybean), *Medicago sativa* (alfalfa) and so on (Moorhead and Smith 2003). The plant PII proteins are nuclear-encoded and targeted to chloroplast (Hsieh *et al*. 1998). Resembling the PII proteins in *E. coli* and cyanobacteria, the plant PII-like proteins are homotrimers with subunit of around 16.8kDa (Smith *et al*. 2003).

In *A. thaliana* (Burillo *et al*. 2004) and rice (Sugiyama *et al*. 2004), NAGK is a potential interacting partner of plant PII. Recent studies have further proved that the biosynthesis of Arg in *A. thaliana* is also regulated by the interaction between NAGK and PII, similar to that in cyanobacteria (Ferrario-Mery *et al*. 2006) while PII-NAGK crystal structure revealed structurally how NAGK is regulated by PII and Arg (Mizuno *et al*. 2007). In addition, plant PII proteins can bind to 2KG or ATP at their chloroplastic physiological concentrations (Smith *et al*. 2003). Overexpression of *AtGLB1* impairs plant ability in sensing and metabolizing Gln, leading to an unusual accumulation of anthocyanin in seedlings grown on medium containing high sucrose and using Gln as the sole N source (Hsieh *et al*. 1998). Moreover, *A. thaliana* PII mutants show increased sensitivity to

nitrite toxicity and become defective in monitoring C:N balance under N-limiting or excessive NH_4^+ conditions (Ferrario-Mery *et al*. 2005). All these findings support the involvement of PII in the N sensing in plants.

Despite these advances, there are still many black boxes that have not been unveiled in the plant PII-mediated N regulatory system. First, the NRI/NRII two-component system of *E. coli* and the NtcA system of cyanobacteria have not been discovered in plants. The mechanism of how plant PII proteins convert the N depletion signal into transcription control remained unknown. Moreover, phosphorylation/dephosphorylation has yet to be demonstrated in plant PII proteins. Although the Ser residue needed for phosphorylation is conserved in the T-loop of *A. thaliana* PII protein, crystal structure reveals that the Ser residue is not expose to the surface (Smith *et al*. 2003). Such exposure is important for protein-protein interactions to take place.

General amino acid control

General amino acid control (GAAC) refers to the phenomenon of cross-pathway transcription regulation of amino acid biosynthesis upon amino acid starvation in *Saccharomyces cerevisiae* (yeast) (**Fig. 2**). Details of the yeast GAAC can be found in a comprehensive review by Hinnebusch (2005).

Uncharged tRNAs, which accumulated as a result of amino acid starvation, will bind to the histidyl-tRNA synthetase (HisRS) related domain of the GCN2 (general control non-repressible 2) protein to activate the juxtaposed kinase domain. The binding of uncharged tRNA to GCN2 is facilitated by the interaction of GCN2 with the GCN1/GCN20 (two elongation factor 3 (EF3)-like proteins) complex. The activated GCN2 phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF2α), leading to a subsequent global inhibition of protein translation and reprogramming of transcriptome.

The eIF2 consists of α, β, and γ subunits. GTP-bound eIF2 together with methionyl initiator tRNA (Met-$tRNA_i^{Met}$) to form a ternary complex (TC: eIF2/GTP/ Met-$tRNA_i^{Met}$). The TC assembles 40S ribosomal protein to form the 43S pre-initiation complex which scans along the mRNA from the 5'cap for the AUG start codon. At which, GTP will be hydrolysed by eIF5 to GDP during the formation of the 48S complex. Translation will begin after recruitment of the 60S ribosomal subunit by the 48S complex. GDP-bound eIF2 will be converted back to the GTP-bound form by the guanine-nucleotide exchange factor (eIF2B) to initiate the next round of translation.

After phosphorylation by GCN2 during amino acid starvation, eIF2α will inhibit the exchange of GDP with GTP and reduce the availability of eIF2-GTP for TC formation, resulting in a halt of the global translation. In contrast, such condition will activate the translation of the transcriptional factor GCN4 (Hinnebusch 2005). Four upstream open reading frames (uORFs) are found located in the 5′ untranslated region (5'UTR) of *GCN4* mRNA. Translation of these four

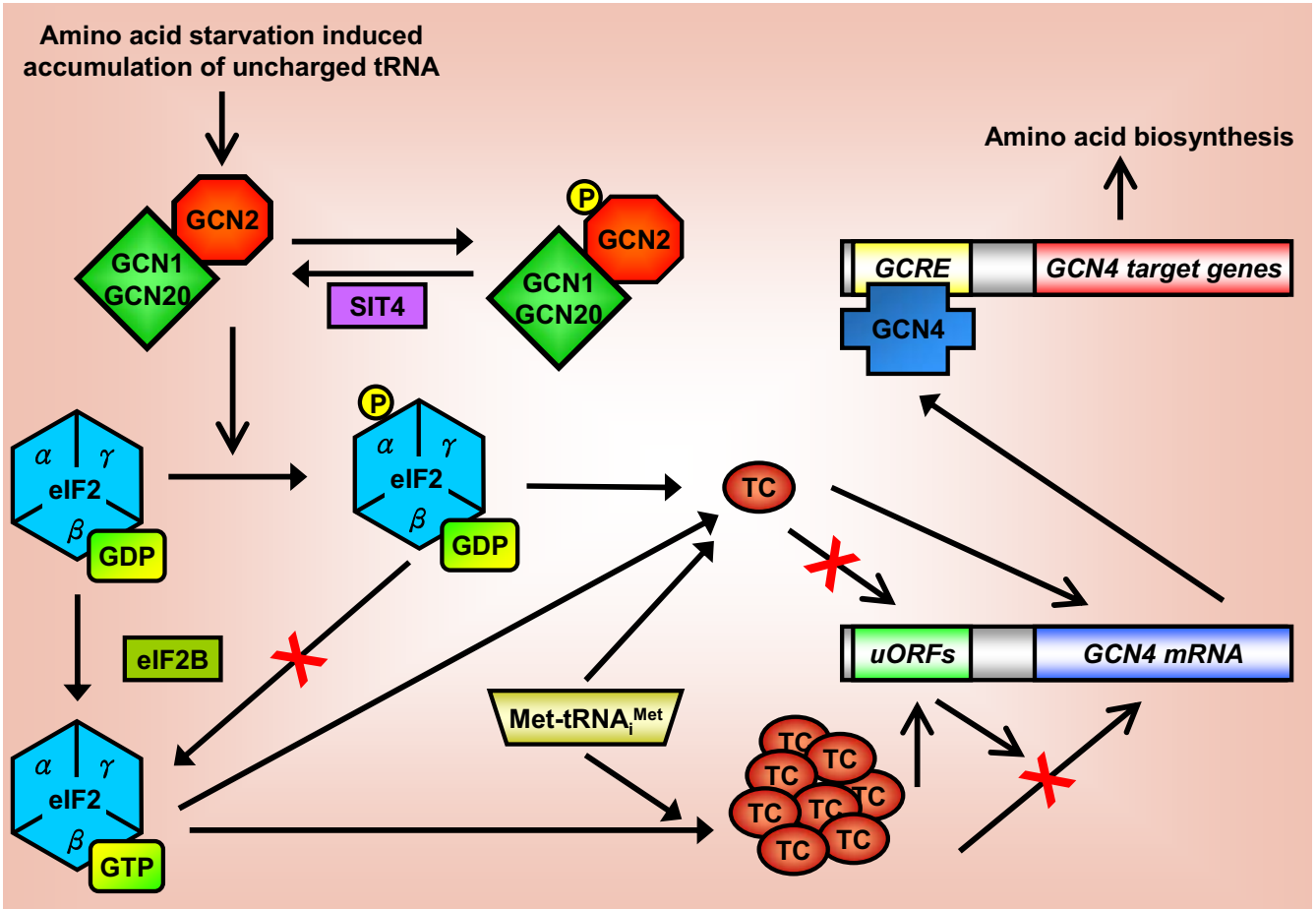

Fig. 2 Model of general amino acid control in yeast. The kinase activity of GCN2 is activated by binding to uncharged tRNAs accumulated upon amino acid starvation. The binding of uncharged tRNAs is facilitated by the binding of GCN1/GCN20 complex to GCN2. The activated GCN2 phosphorylates the α subunit of eukaryotic translation initiation factor-2 (eIF2α) and inhibits the exchange of GDP on the eIF2β with GTP. This will reduce the availability of eIF2-GTP for the formation of ternary complex (TC) involved in translation initiation. When there is no starvation, TC formation is not limited by the phosphorylated eIF2α, abundant TC allows translation of the upstream open reading frame (uORFs) of GCN4 which leads to pre-mature disintegration of the ribosome complex before translating the *GCN4 ORF*. Reduction of TC during amino acid starvation allows the ribosome to bypass the uORFs of GCN4 and allows the translation of the *GCN4 ORF*. GCN4 protein is a transcription factor acting on *GCRE* motif in the promoter of target genes. GCN4 regulates the expression of several hundreds of genes, including a number of amino acid biosynthetic genes. When N supply is scarce, GCN2 is dephosphorylated by SIT4 which is a component of the TOR pathway. Homologues of GCN1, GCN2, GCN20, eIF2α, *GCRE*-like motif and genes with uORFs have been found in plants.

uORFs will lead to the pre-mature release of ribosomes and make the translation of *GCN4* ORF impossible. Shortage of TC during starvation allows ribosomes to bypass uORF4 before rebinding to TC, enabling the translation of the ORF of *GCN4*.

GCN4 is a transcription factor that regulates the expression of a large number of genes to deal with adverse conditions. Microarray analysis revealed at least 539 GCN4 target genes including those involved in 12 different amino acid biosynthetic pathways, amino acid precursor biosynthesis, vitamin/cofactor biosynthesis, peroxisome biogenesis, glycogen metabolism, purine biosynthesis and so on (Natarajan *et al*. 2001). GCN4 regulates the expression of these gene by acting on the GCN4-responsive element (GCRE: 5′TGACTC3′) in their promoters (Arndt and Fink 1986).

The expression of *GCN2* itself is also upregulated by overexpressing *GCN4* (Roussou *et al*. 1988). Other proteins including GCN5 (a histone acetyltransferase), ADA2 (a transcriptional adaptor protein), MBF1 (multiprotein bridging factor 1), and TBP (TATA-box binding protein) also participate in gene expression regulation mediated by GCN4 (Barlev *et al*. 1995; Takemaru *et al*. 1998; Kuo *et al*. 2000; Swanson *et al*. 2003).

On the other hand, GCN2 is also under the regulation of the TOR (target of ripamycin) pathway. Under non-starvation conditions, GCN2 is phosphorylated at Ser-577 to reduce its affinity towards uncharged tRNAs. At the same time, the downstream effector TAP42 of the TOR pathway binds to SIT4 (a type-2A protein phosphatase), preventing it from dephosphorylating GCN2. In contrast, during starvation conditions, SIT4 will be released from TAP42 and dephosphorylates GCN2 to restore GCN2 affinity towards uncharged tRNAs (Hinnebusch 2005).

In *A. thaliana*, blockage of histidine (His) biosynthesis with the herbicide IRL1803 induced expression of 8 genes involved in the biosynthesis of aromatic amino acid, lysine, purine and His (Guyer *et al*. 1995). This result suggests the existence of GAAC-like cross-pathway regulation in plants. Other studies using different herbicides or mutants defective in the biosynthesis of certain amino acids support the same notion (Zhao *et al*. 1998; Noutoshi *et al*. 2005).

The discovery of homologues of yeast GAAC components provides further evidence for the presence of GAAC in plants. Wheat eIF2α, which can functionally complement the yeast *sui2* mutant (*SUI2* encodes yeast eIF2α) under GCN4 derepressing conditions, is phosphorylated at Ser-51 by the yeast GCN2 (Chang *et al*. 1999, 2000). A GCN2 homologue was identified in *A. thaliana* which can complement a yeast *gcn2* mutant (Zhang *et al*. 2003). Although the *in planta* eIF2α kinase activity toward plant GCN2 has yet to be proven, it has been demonstrated that plant eIF2α can be phosphorylated by a plant double-stranded RNA-dependent kinase (pPKR: Langland *et al*. 1996).

Putative homologues of GCN1 and GCN20 involving in GCN2 activation were also found in *A. thaliana* and the interaction between these two proteins was demonstrated using the yeast-two-hybrid system (Kato *et al*. 2004).

Analysis of plant genome sequences does not reveal the present of GCN4 homologues. Discovery of possible functional analogs of GCN4 may shed light on this missing link. For instance, Opaque2 (ZmO2) is a maize transcriptional factor that is translationally regulated by uORFs (Lohmer *et al*. 1993), similar to the case of GCN4. ZmO2 acts on GCRE-like elements on the promoter regions of its target genes (Wu *et al*. 1998) and interacts with maize GCN5 (ZmGCN5) and ADA2 (ZmADA2: Bhat *et al*. 2004). Furthermore, ZmO2 can functionally complement the yeast *gcn4* mutant (Mauri *et al*. 1993). GCRE-like motifs have also been reported in the promoter sequences of plant genes involved in N metabolism (Muller and Knudsen 1993; Wu *et al*. 1998).

Homologues of other players in the yeast GAAC system were also found in plants, including GCN5 and ADA homologues from *A. thaliana* (Stockinger *et al*. 2001) and maize (Bhat *et al*. 2004); MBF homologues from *A. thaliana* (AtMBF1: Tsuda *et al*. 2004) which binds to yeast GCN4 and TBP and functionally complements the yeast *mbf1* mutant.

Although several plant homologues of the GAAC components have been discovered, there is still no direct evidence linking up each component to verify their roles in the plant GAAC system. Further investigation is needed to assemble these pieces of puzzle.

Ionotropic glutamate receptors

There are two classes of animal Glu receptors (i) the ionotropic (ion-conducting) Glu receptors (iGLRs), which are ligand-gated ion channels and (ii) metabotropic Glu receptors (mGLRs) or trans-l-aminocyclopentane-l,3-dicarboxylate (tACPD) receptors, which are G-protein-linked receptors (Dingledine *et al*. 1999). Mammalian iGLRs can be subdivided into 3 pharmocological types: (i) N-methyl-D-aspartate (NMDA) type; (ii) α-amino-3-hydroxy-5-methyl-4-isoxazole propionate and kainite (AMPA/KA) type; and (iii) Delta type (Dingledine *et al*. 1999). iGLRs present on neurons allow rapid synaptic transmissions to deal with the fast changing environment.

Despite the absence of a nervous system in plant, a gene family encoding 20 putative iGLRs is present in the genome of *A. thaliana* (Lam *et al*. 1998; Lacombe *et al*. 2001; Chiu *et al*. 2002), supporting the notion that iGLRs appeared before the divergence of plant and animal (Chiu *et al*. 1999). By the percentage identity of amino acid, the 20 iGLRs from *A. thaliana* can be clustered into 3 clades similar to those in animals (Chiu *et al*. 2002). Subsequent studies reveal the possible roles of these in plants (**Fig. 3**).

Several amino acids (alanine, asparagine, cysteine, glutamate, glycine and serine) were shown to trigger rapid and drastic changes in cytosolic Ca^{2+} concentration, using the *A. thaliana* aequorin reporter lines (Dennison and Spalding 2000; Dubos *et al*. 2003; Qi *et al*. 2006). In addition, impaired Ca^{2+} utilization and increased sensitivity to ionic stress by the constitutive expression of *AtGLR3.2* in *A. thaliana* (Lacombe *et al*. 2001) suggests the existence of amino acid-gated Ca^{2+} channels. Further studies revealed the absence of amino acids induced membrane depolarization in *glr3.3* knockout lines, supporting the relationship between plant iGLRs and the Ca^{2+} influx (Qi *et al*. 2006). Consecutive Glu treatment of *A. thaliana* at pH 7.7 leads to desensitization (Qi *et al*. 2006), similar to the regulation of synaptic Ca^{2+} signaling in the animal central nervous system (Jones and Westbrook 1996). This further supports the ligand-receptor relationship of plant iGLRs.

To test if plant iGLRs participate in signal transduction as their counterparts in animals, *A. thaliana* was subjected to the animal iGLR antagonist 6,7-dinitroquinoxaline-2, 3-dione (DNQX: Lam *et al*. 1998) and agonist S(+)-β-diaminopropionic acid (BMAA: Brenner *et al*. 2000) treatments. DNQX treatment blocked the light induction of chlorophyll synthesis and light inhibition of hypocotyl elongation, while BMAA treatment caused hypocotyl elongation and inhibited cotyledon opening.

Using amino acids as the signaling molecules, it is likely that plant iGLRs can take part in regulation C and N metabolisms. Underexpression of *AtGLR1.1* alters the C/N sensitivity of the plant during seed germination (Kang and Turano 2003). Reduced transcript levels of genes encoding C and N metabolic enzymes including cytosolic glutamine synthetase (*GS1*), aspartate aminotransferase (*AAT2*), nitrate reductase (*NR1*), nitrite reductase (*NiR*), nitrate transporter (*CHL1*), NADP-dependent isocitrate dehydorgenase (*ICDH*), and hexose kinase 1 (*HXK1*) were observed in the *antiAtGLR1.1* line (Kang and Turano 2003), suggesting a regulatory role of AtGLR1.1 in C and N metabolisms.

Abscisic acid (ABA) is a plant hormone that regulates germination and stomatal closure and can mediate the regulatory effects of nitrate on root branching in *A. thaliana*. Underexpression of *AtGLR1.1* results in the upregulation of

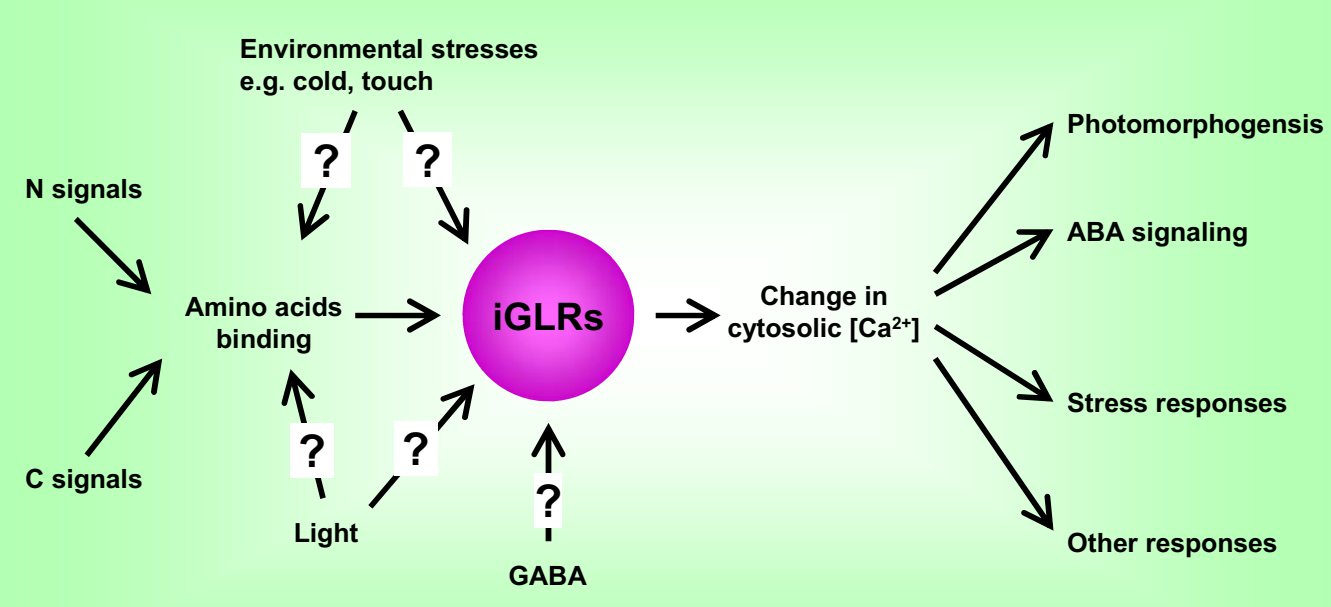

Fig. 3 Stimulus and responses of ionotropic glutamate receptors mediated signal transduction in plants. Amino acids bind to iGLRs and trigger the change in transmembrane Ca^{2+} transport to regulate cytosolic Ca^{2+} concentration. This will eventually lead to changes in ABA biosynthesis and expression of C/N metabolic genes. iGLRs also perceive signals like light, environmental stresses (cold, touch), and GABA to induce related responses. It is still unclear whether iGLRs perceive these signals directly or through the binding to amino acids.

ABA biosynthetic gene (zeaxanthin epoxidase: *ABA1*) while DNQX treatment leads to an accumulation of ABA and a reduction in germination rate (Kang and Turano 2003). Kang and Turano (2003) proposed a working model in which N signals (NO_3^- or amino acids) act as the positive signals stimulating AtGLR1.1 to inhibit the ABA biosynthesis and ease the ABA induced physiological changes, while C signals (sucrose) act negatively toward AtGLR1.1 to reduce the expressions of C and N metabolic genes and elevate ABA biosynthesis which in term inhibits seed germination.

Decomposition of organic matter in the soil, plant root exudates and microbial actions on roots release amino acids into the rhizosphere. The growth of primary roots but not lateral roots is strongly inhibited by Glu in the medium (Filleur *et al*. 2005). All 20 iGLRs from *A. thaliana* are strongly expressed in roots while 5 of them are root specific (Chiu *et al*. 2002). iGLRs in roots may take part in sensing the change of amino acid composition in soil (Filleur 2005; Qi 2006), leading to the adjustment of root architecture for colonizing the nutrient-rich patch of soil (Filleur *et al*. 2005).

γ-aminobutyric acid (GABA) is recently found to be a long-distance signal in up-regulation of nitrate uptake in *Brassica napus* L. (Beuve *et al*. 2004). However, the presence of plant GABA receptors is yet to be confirmed. Sequence analysis showed that the N-terminal region of plant iGLRs are related to the $GABA_B$ receptors, suggesting that plant iGLRs may also participate in shoot-to-root signaling mediated by GABA (Turano *et al*. 2001).

Other than N sensing, plant iGLRs may also play roles in environmental stress responses. For examples, *AtGLR3.4* can be induced through touch, cold, and the applications of GABA, aspartate, malate as well as membrane-permeable weak acids (Meyerhoff *et al*. 2005). Treatment of either the Ca^{2+} channel blocker gadolinium or the iGLR antagonist 2-amino-5-phosphonopentanoate can eliminate the effects of Glu, resembling the toxicity of Al^{3+} on cortical microtubules depolymerization and root growth inhibition (Sivaguru *et al*. 2003).

Cytokinins His-Asp phosphorelay

Cytokinins are a type of important plant hormones that involved in the regulation of diverse biological processes such as cell division and differentiation, chloroplast biosynthesis, senescence and signal transduction. Naturally occurred cytokinins are adenine-type cytokinins derived from adenine and can be further classified into isoprenoid cytokinins and aromatic cytokinins.

Cytokinins may also mediate N signal between root and leaf (**Fig. 4**). Replenishment of nitrate to maize trigger accumulation of certain cytokinins species in roots by recycling of the stored form and *de novo* synthesis (Takei *et al*. 2001). This is supported by the observation that a cytokinin biosynthetic gene, isopentenyl transferase 3 gene (*ATIPT3*), was induced by nitrate replenishment (Miyawaki *et al*. 2004). Mass flow in xylem induced by absorption of nitrate brings along the accumulated cytokinins to the leaves (Takei *et al*. 2001) at which the signal will be perceived by the His-Asp phosphorelay.

The His-Asp phosphorelay was first identified in bacteria which comprises of two components, the histidine protein kinase (HPK) and the response regulator (RR) (Stock *et al*. 1989). Upon stimulation, HPK will autophosphorylate a highly conserved His residue within the transmitter domain using an ATP, and then pass the phosphoryl group to a conserved Asp residue on the receiver domain of RR, which will in turn trigger the downstream responses (Stock *et al*. 2000). Multi-step His-Asp phosphorelay was later found to have an extra component, the histidine-phosphotransfer protein (HPT), which helps relaying the phosphoryl group between the HPKs and RRs (Appleby *et al*. 1996). Hybrid HPKs having extra (one or more) receiver domains fused to the C-terminus of the transmitter domain were also found such that the phosphoryl group in the multi-step His-Asp phosphorelay must pass through several rounds of phosphorylation to reach the RRs (Appleby *et al*. 1996).

In plants, multi-step His-Asp phosphorelay is adopted to perceive the cytokinins signals. The N-terminal of the plant HPKs consists of multiple transmembrane regions forming a cyclases/histidine kinase-associated sensory extracellular (CHASE) domain as the docking site of cytokinins (Anantharaman and Aravind 2001).

Cytokinin-responsive HPKs have been identified in *A. thaliana* (AHK2, AHK3, AHK4/CRE1/WOL: Inoue *et al*. 2001; Ueguchi *et al*. 2001), maize (ZmHK1, ZmHK2, ZmHK3a/3b: Yonekura-Sakakibara *et al*. 2004), and rice (OHK2-5: Ito and Kurata 2006). AHK4 is responsive to only *trans*-zeatin (Inoue *et al*. 2001) while ZmHK1 is responsive to zeatin in both of its *cis* and *trans* forms (Yonekura-Sakakibara *et al*. 2004). Furthermore, AHK3 and

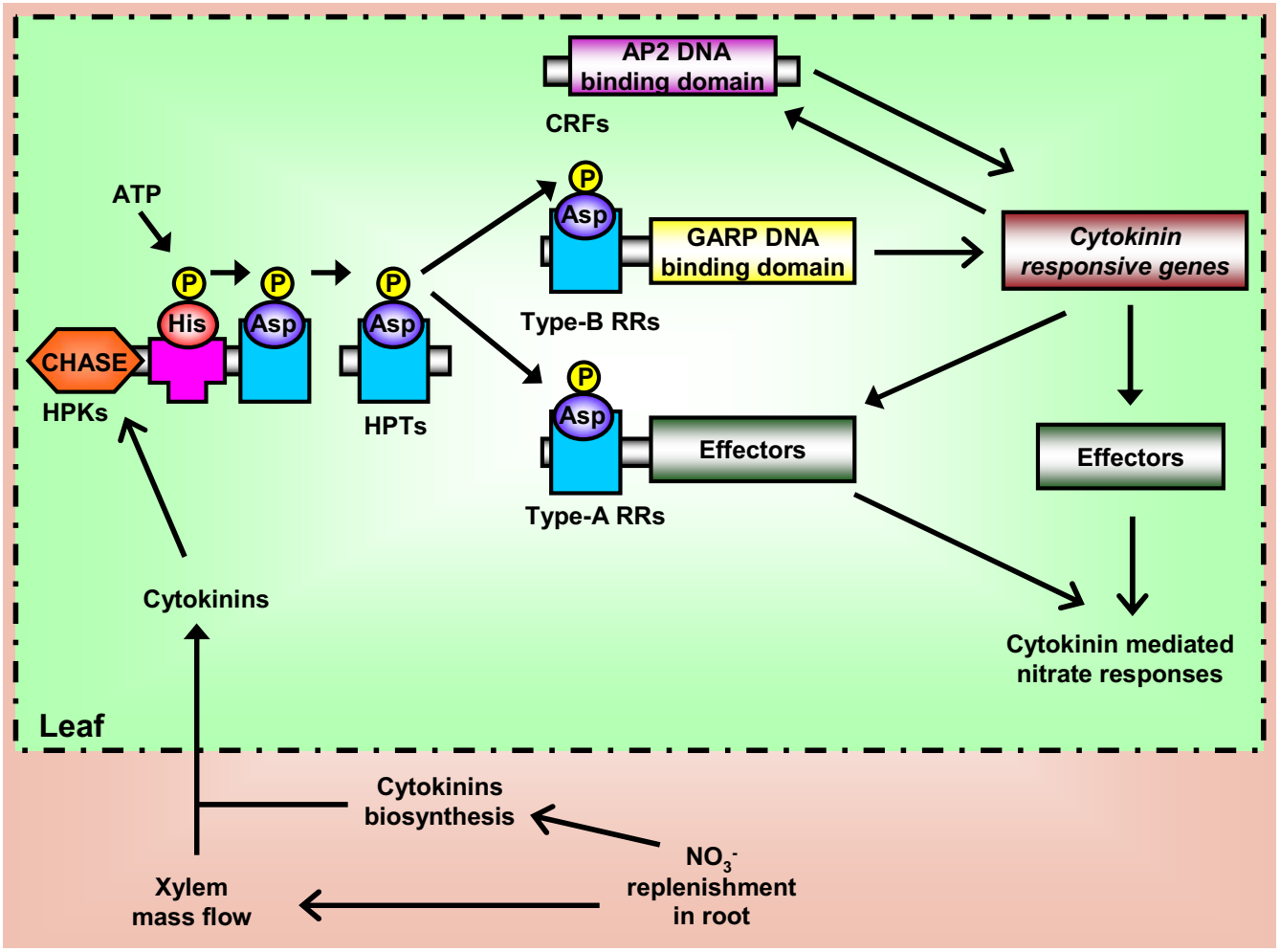

Fig. 4 Model of cytokinins His-Asp phosphorelay mediated N sensing in plants. Cytokinins biosynthesis and xylem mass flow are triggered by the replenishment of nitrate (NO_3^-) in the soil. Cytokinins are transported to the leaves along with the xylem mass flow. Cytokinins stimulate the CHASE domain of the histidine protein kinases (HPKs) thus triggers the autophosphorylation of a conserved histidine (His) residue on the transmitter domain using ATP. The phosphoryl group is further relayed to an aspartate (Asp) on the receiver domain of the same HPK and then a His on the histidine-phosphotransfer protein (HPT) and at last to the Asp on the response regulator (RR). Type-B RRs are transcription factors that activate transcription of cytokinins responsive genes including those encoding type-A RRs and cytokinin response factors (CRFs). CRFs will further activate transcription of cytokinins responsive genes while type-A RRs regulate other downstream effectors.

AHK4 exhibit differential binding affinity toward different cytokinins and such preference is pH-, salt-, and temperature dependent (Romanov *et al*. 2006). Together, these data implicate that different plant HPKs may have diverse specificities towards different cytokinins and may function in response to different kinds of environment stimulations.

Multiple HPTs are also present in different plant species including *A*. *thaliana* (AHP1-5: Imamura *et al*. 1999), rice (OHP1-2: Ito and Kurata 2006), and maize (ZmHP1-3: Sakakibara *et al*. 1998; Sakakibara *et al*. 1999; Asakura *et al*. 2003). AHPs relocalize into nucleus upon cytokinins treatment (Hwang and Sheen 2001) without an upregulation in transcription (Tanaka *et al*. 2004), suggesting that plant HPTs act as the shuttles to pass the cytokinins signals from HPKs on the membrane to the RRs and cytokinins response factors (CRFs) (see below) in the nucleus. High-order but not single *ahp* mutations reduce the plant sensitivity toward cytokinins (Hutchison *et al*. 2006). AHP1-3,5 are proposed to be the positive regulators of the cytokinins response. On the other hand, AHP4 is a negative regulator since cytokinins sensitivity reduction in high-order *ahp* mutants can be slightly reverted with an additional *ahp4* mutation (Hutchison *et al*. 2006). Pseudo-HPTs lacking the conserved His residue for phosphorylation (APH6: Mahonen *et al*. 2006) and alternatively spliced AHP5 (AHP5L) (Hradilova and Brzobohaty 2007) are also putative negative regulators in the cytokinins responses.

Downstream of HPTs are the RRs and CRFs. Multiple RRs are present in *A*. *thaliana* (ARRs: Hwang *et al*. 2002), rice (OsRRs: Ito and Kurata 2006), and maize (ZmRRs: Sakakibara *et al*. 1998; Sakakibara *et al*. 1999). CRFs are recently reported to be components of the cytokinins His-Asp phosphorelay in *A*. *thaliana* (Rashotte *et al*. 2006).

Based on sequence homology, physiological functions and modes of induction, RRs are further divided into type-A RRs and type-B RRs. Type-B RRs are transcriptional factors containing the GARP DNA-binding domain which activate genes with upstream GARP motif (Riechmann *et al*. 2000). Targets of type-B RRs are involved in diverse regulatory functions. For example, the targets of ARR1 (a type-B RR) include genes encoding type-A RRs, CRFs, cytokinins metabolic enzymes, putative disease resistance response proteins, and structural proteins (Taniguchi *et al*. 2007).

Functions of type-A RRs may be quite varied. For example, ARR4 from *A*. *thaliana* can bind to seemingly unrelated targets such as DNA-binding proteins (Yamada *et al*. 2004) and phytochrome B (Sweere *et al*. 2001). GARP motif is commonly found on the promoter of type-A RRs (Sakai *et al*. 2001). In *A*. *thaliana*, type-A RRs are transcriptionally induced by Type-B RRs in response to cytokinins and nitrate (Taniguchi *et al*. 1998). Despite that the expression of type-A RRs can be induced by nitrate in maize plants, expression of type-A RRs in detached leaves can only be induced by cytokinins but not nitrate (Sakakibara *et al*. 1998; Sakakibara *et al*. 1999). This supports the notion that nitrate signal is transferred to effectors through the His-Asp phosphorelay mediated by cytokinins.

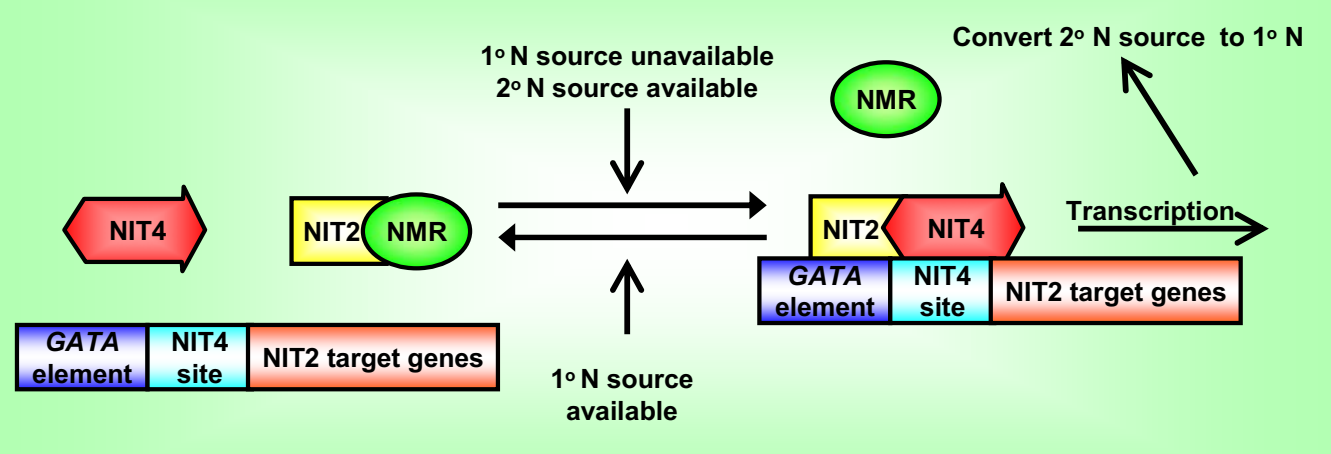

Fig. 5 Model of NIT2 system in *Neurospora crassa*. In the presence of primary N sources (ammonium, glutamine, and glutamate), NMR (nitrogen metabolite repression) binds to NIT2. This prevents NIT2 from binding to the *GATA* element. On the other hand, when secondary N sources (nitrate and nitrite) instead of the primary N sources are available, NIT2 will be released from NMR and bind to the *GATA* element in the promoter of NIT2 target genes. Concurrently, NIT4 will bind to NIT4 binding site and interacting with NIT2 to enhance the production of enzymes such as nitrate reductase (NR) and nitrite reductase (NiR) for efficient utilization of the available N sources. Plant NIT2-like protein (NLT1) and NIT2 bind site (*GATA* element) on the promoter of plant genes have been identified.

CRFs belong to the family of AP2 transcription factors and are transcriptionally induced by cytokinins in a type-B RRs dependent manner. Like the HPTs, CRFs also relocalize to the nucleus upon cytokinins treatment (Rashotte *et al*. 2006). Loss of function analysis showed that CRFs control mainly the development of cotyledons, leaves, and embryos while microarray data revealed that a large portion of genes regulated by CRFs are overlapping with genes induced during early cytokinins transcriptional response and a portion of genes that are regulated by type-B RRs.

Redundancy and overlapped functions have been shown in every steps of the His-Asp phosphorelay in *A. thaliana* by knockout assays, including AHKs (Higuchi *et al*. 2004; Nishimura *et al*. 2004; Riefler *et al*. 2006), AHPs (Hutchison *et al*. 2006), type-A RRs (To *et al*. 2004), type-B RRs (Mason *et al*. 2005); and CRFs (Rashotte *et al*. 2006). Such high redundancy may help to fine-tune the cytokinins His-Asp phosphorelay signaling pathway toward different situations (Hutchison *et al*. 2006).

Existence of HPTs and hybrid HPKs containing additional receiver domains may help in the signal transduction by: (i) providing multiple points of regulation such as transcription control of each unlinked components or cross-talk with other signaling pathways (Appleby *et al*. 1996); and (ii) increasing tolerance to noise ratio without affecting the rapid response toward the actual stimulation (Kim and Cho 2006).

Correlation between N signals and responses of cytokinins His-Asp phosphorelay has not yet been concretely proven. Meanwhile, gene induction overlapped in nitrate response, cytokinins response, RRs induced response, and CRFs induced response does not seem to relate directly to primary N metabolism. However, cytokinins His-Asp phosphorelay signal is induced upon sufficient supply of N that signifies a proper time for growth and development. Induction of a whole array of genes could therefore be a preparation for growth under rich nutrient supply.

NIT2 system

The major nitrogen regulatory protein NIT2 is a GATA transcription factor in *Neurospora crassa* (Chiang and Marzluf 1994). NIT2 contains a single zinc finger DNA-binding domain (Fu and Marzluf 1990) that regulates the expression of many structural genes as shown in **Fig. 5**, including *nit-3* (nitrate reductase; NR) and *nit-6* (nitrite reductase; NiR) which are important for N metabolism (Marzluf 1997).

When primary N sources (NH_4^+, Gln and Glu) are available, the negative regulator NMR (nitrogen metabolite repression) will interact with NIT2 to neutralize its DNA-binding activity, leading to the loss of transcription activation (Xiao *et al*. 1995; Pan *et al*. 1997). Either deletion or mutation of the NMR binding regions on NIT2 will result in the loss of N repression (Pan *et al*. 1997). In contrast, when primary N sources are limiting, NIT2 will bind to the NIT2 sites (GATA elements) upstream of the target genes (Marzluf 1997). At the same time, the pathway-specific regulatory factor NIT4 will act on the NIT4 sites located adjacent to the NIT2 sites and interact with NIT2 to derepress the gene expression (Feng and Marzluf 1998; Mo and Marzluf 2003), resulting in a shift to utilize secondary N resources (nitrate and nitrite). However, the exact N sensing mechanism of NIT2 is still unclear.

Most plants obtain N in the form of nitrate from soil which is than converted into NH_4^+ by the sequential reduction reactions by NR and NiR before assimilated into amino acids. The NIT2-like regulatory system may be involved in the regulation of NR and NiR genes. *In vivo* DMS footprinting and electrophoretic mobility shift assays using the *Neurospora* NIT2 revealed the existence of putative nitrate-responsive NIT2 binding site on the promoter of the spinach *NiR* gene (Rastogi *et al*. 1997). The plant homologue of NIT2 (NTL1: NIT2-Like protein), exhibiting a 60% homology to the zinc finger DNA-binding domain of NIT2, was identified in *Nicotiana plumbaginifolia* by cross-hybridization (Danielvedele and Caboche 1993). However, the function of NTL1 has not been fully elucidated.

CONCLUSION

In this review, we have discussed five putative plant N sensing systems, responding to different signals. iGLRs are receptors using amino acids as ligands while GAAC is activated during amino acid depletion. Cytokinin His-Asp phosphorelay mediates signals upon replenishment of N (N sufficient) while PII responses to low C:N ratio (N deficient). Despite that the function of the plant NIT2 system is still largely unknown, the mode of its activation seems to be quite distinct from the other four systems. Previously, the search of plant N sensing systems based heavily on the identification of components homologous to that found in bacteria, fungi, and animals. Individual N sensing system was often investigated independently. With the rise of system biology and the gradual revealing of missing links of each system, the future focus of researches will switch to the interactions between systems, giving rise to a detailed and macroscopic view of the N sensing mechanisms in plants. The VirtualPlants (http://virtualplant-prod.bio.nyu.edu/cgi-bin/virtualplant.cgi) system recently developed pro-

vides a sophisticated tool and platform to integrate every single system *in silico* to reveal the interactions and cross-talks therein.

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