How do plants sense their nitrogen status?

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Abstract

The primary processes that contribute to the efficient capture of soil nitrate are the development of a root system that effectively explores the soil and the expression of high-affinity nitrate uptake systems in those roots. Both these processes are highly regulated to take into account the availability and distribution of external nitrate pools and the endogenous N status of the plant. Whilst significant progress has been made in elucidating the early steps in sensing and responding to external nitrate, there is much less clarity about how the plant monitors its N status. This review specifically addresses the questions of what N compounds are sensed and in which part of the plant, as well as the identity of the signalling pathways responsible for their detection. Candidates that are considered for the role of N sensory systems include the Target of Rapamycin (TOR) signalling pathway, the General Control Non-derepressible 2 (GCN2) pathway, the plastidic PII-dependent pathway and the family of Glutamate-Like Receptors (GLRs). However, despite significant recent progress in elucidating the function and mode of action of these signalling systems, there is still much uncertainty about the extent to which they contribute to the process by which plants monitor their N status. The possibility is discussed that the large GLR family of Ca^{2+} channels, which are gated by a wide range of different amino acids and expressed throughout the plant, could act as amino acid sensors upstream of a Ca^{2+}-regulated signalling pathway such as the TOR pathway to regulate the plant's response to changes in N status.

Key words: amino acids; GCN2; glutamate receptors; nitrate uptake; PII; root development; signal transduction; target of rifampicin.

Abbreviations: 2-OG, 2-oxoglutarate; AspRS, aspartyl-tRNA synthetase; BABA, β-aminobutyric acid; eIF2α eukaryotic translation initiation factor alpha; GCN2, general amino acid control non-derepressible 2; GLR, glutamate-like receptor; GS, glutamine synthetase; hVps34, human vacuolar protein sorting 34; iGluR, ionotropic glutamate receptor; NAGK, *N*-acetyl-L-glutamate kinase; NR, nitrate reductase; PP2A, protein phosphatase 2A; TAP46, 2A phosphatase-associated protein of 46 kDa; TORC, target of rapamycin complex.

Introduction

It has long been appreciated that plants are able to regulate their nutrient uptake to match their demand for those nutrients. As far back as 1906, J. F. Breazeale demonstrated that wheat plants that were nitrogen starved for the first 15 d after germination subsequently showed much higher capacities for absorbing nitrate than plants that had received sufficient N (Breazeale, 1906). Developmental processes in both roots and shoots are also known to be modified by signals related to the N status of the plant (Forde and Lorenzo, 2001). As there is no evidence that plants have evolved separate N sensing mechanisms for controlling N uptake and plant development, this review will consider the most recent evidence relating to the control of both physiological and developmental processes, focusing specifically on how plants sense their internal N status. To learn more about the downstream components of N signalling pathways in plants the reader is referred to a number of excellent reviews that have taken a broader view of the topic (Krapp, 2015; Li *et al.*, 2014; Liu *et al.*, 2015; Medici and Krouk, 2014; O'Brien *et al.*, 2016; Ruffel *et al.*, 2014; Sirohi *et al.*, 2016; Vidal *et al.*, 2015).

What is sensed and where is it sensed?

An ability to respond to changes in the internal N status implies the existence of mechanisms that can monitor the abundance of one or more key effector molecules that provide reliable information on the availability of N in specific plant tissues. In molecular terms we would expect to find molecules (usually proteins), that act as sensors by binding to specific N-containing compounds and transmitting that information to downstream components of a signal transduction pathway. We will begin by considering what the key effector molecules might be and in which parts of the plant their abundance is likely to be monitored.

Glutamine, as the product of the first step in the pathway of N assimilation in bacteria and fungi as well as in plants (Lea *et al.*, 1990), is the organic form of N that has been most commonly considered to be a candidate for the key effector in the sensing of the intracellular N status in many organisms. In *Aspergillus nidulans* and other filamentous fungi, when glutamine levels are high, pathways responsible for assimilating energetically unfavourable N sources (like nitrate) are down-regulated through a process called *nitrogen metabolite repression* (Crawford and Arst, 1993). However, although much is known about the complex genetic control of nitrogen metabolite repression, the precise mechanism by which glutamine is sensed has not been determined (Tudzynski, 2014), which leaves open the possibility that it is not glutamine itself that is the effector. In mammals and the budding yeast

Saccharomyces cerevisiae, the evolutionarily conserved Target Of Rapamycin Complexes (TORCs) are involved in sensing amino acid-derived signals to stimulate a variety of metabolic processes. However, glutamine sensing appears to be only part of the story (Fumarola *et al.*, 2005; Nakajo *et al.*, 2005), with other amino acids such as leucine and arginine also being implicated in some of the multiple pathways of TORC activation (Kingsbury *et al.*, 2015; Shimobayashi and Hall, 2016). For example, SLC38A9, a solute carrier family protein with a proposed role in transporting glutamine and arginine into the mammalian lysosome, has recently been identified as an arginine sensor upstream of mammalian TORC1 (mTORC1) (Shimobayashi and Hall, 2016) and two distinct leucine sensors operating in this pathway leucyl-tRNA synthetase (Han *et al.*, 2012) and Sestrin2 (Wolfson *et al.*, 2016) have also been uncovered. Another important mechanism for monitoring the internal N status in yeast and mammals is the General amino acid Control Non-derepressible 2 (GCN2) pathway, which does not sense amino acids *per se* but rather the uncharged tRNAs that accumulate during amino acid deprivation (Chantranupong *et al.*, 2015).

In bacteria, it is the glutamine:2-oxoglutarate (2-OG) ratio that is the key indicator of N status, regulating as it does glutamine synthetase activity through a PII-mediated mechanism (discussed below). In *E coli*, the PII proteins are encoded by the related GlnB and GlnK genes and their main function is to regulate glutamine synthetase (GS) activity (Arcondeguy *et al.*, 2001). The PII-modifying enzyme GlnD, which uridylylates and deuridylylates PII proteins, is inhibited by binding to glutamine and has the role of glutamine sensor, while GlnB itself binds to 2-OG and acts as a 2-OG sensor.

In plants, the question of the signalling role of glutamine in feedback regulation of nitrate uptake has often been asked, but without producing a definitive answer. It has long been thought likely that phloem-mediated shoot-to-root signalling is responsible for regulating NO_3^- uptake activity to match the plant's demand for N (Forde and Clarkson, 1999; Imsande and Touraine, 1994). Amino acids, as significant components of phloem sap, have generally been considered strong candidates for the relevant phloem transmissible compounds (Cooper and Clarkson, 1989). However, attempts to identify glutamine or other individual amino acids as key effectors in this pathway have proved inconclusive. For example, when individual amino acids were indirectly loaded into the phloem through the cut surface of soybean cotyledons, 8 of the 14 amino acids tested were effective in down-regulating nitrate uptake in the roots, but glutamine was significantly less potent than arginine or alanine (Muller and Touraine, 1992). Despite these and other reports indicating a negative correlation between the amino acid content of the phloem and the rate of nitrate uptake, there is also evidence from experiments with split-root

systems that feedback regulation of nitrate uptake can occur independently of any change in the amino acid content of the phloem (Tillard *et al.*, 1998). Furthermore, it has been found that the local amino acid content of the root itself is not correlated with nitrate uptake rates (Lainé *et al.*, 1995) and that feedback regulation of the expression of the *NRT2.1* nitrate transporter in roots is dependent on the global N status of the plant and not the local supply of N to the root (Gansel *et al.*, 2001). These pieces of evidence support the idea of long distance shoot-to-root signals that communicate the plant's N status to the root nitrate uptake system and but argue against those signals being amino acids. A detailed discussion of the identity of alternative long-distance signals is beyond the scope this review, but includes a variety of phloem-mobile molecules that have been implicated in N signalling, including auxin (Forde, 2002), microRNAs (Zeng *et al.*, 2014) and small peptides (Araya *et al.*, 2014). Very relevantly in this context, a recent report identified Arabidopsis ELONGATED HYPOCOTYL5 (HY5), a bZIP transcription factor, as a shoot-to-root phloem-mobile signal that stimulates both root growth and nitrate uptake in response to illumination of the shoot (Chen *et al.*, 2016). This is particularly significant for the integration of N and C metabolism because, as well as acting as a hub for multiple hormonal and abiotic signalling networks, HY5 is also known to regulate C fixation in the shoot (Lau and Deng, 2010).

In terms of the identity of the key metabolite(s) directly relevant to N sensing, candidates other than glutamine have been identified. A detailed transcriptomics and metabolomics analysis in Arabidopsis found evidence that leucine abundance was strongly correlated to the expression of a set of several hundred genes (Hannah *et al.*, 2010), leading to the suggestion that in plants, as in yeast and mammals, leucine could be a key regulator of gene expression. There is also evidence that tissue nitrate concentrations can also contribute to the plant's perception of its N status. Using tobacco lines with varying levels of nitrate reductase (NR) activity it was found that accumulation of high concentrations of nitrate in the shoot led to stimulation of organic acid metabolism, repression of starch synthesis and inhibition of root growth (Scheible *et al.*, 1997a; Scheible *et al.*, 1997b; Stitt and Feil, 1999). These are responses associated with high N status, yet were seen even though the NR-defective plants were severely deficient in organic N. Similarly, early lateral root development in an NR-deficient mutant was found to be more sensitive than the wild-type to the inhibitory effects of high nitrate concentrations (Zhang *et al.*, 1999), rather than less sensitive as would have been expected if assimilation of nitrate into organic forms of N was required for feedback repression. Thus we must consider the possibility that nitrate sensors present in the shoot have an important role to play in global N-status sensing.

It is of course over-simplistic to consider N status sensing in isolation since the plant must integrate signals from a wide range of other metabolites, most notably those related to carbon metabolism (Nunes-Nesi *et al.*, 2010; Zheng, 2009). The PII regulatory pathway in bacteria, which senses the glutamine:2-oxoglutarate ratio (Arcondeguy *et al.*, 2001), is a good example of how sensing of the C/N balance can be achieved. However, the mechanisms by which nitrogen and carbon signalling are integrated in plants are poorly understood and likely to be much more complex than in bacteria, so will not be dealt with in detail here. We have already noted above the important identification of HY5 as a novel regulatory molecule that has the ability to integrate C and N metabolism both within and between distant plant organs (Chen *et al.*, 2016). In the following sections, we review the most recent advances in our understanding of the multiple mechanisms by which N-containing compounds are sensed in plants, as candidates for the role of sensing global N status.

Glutamine sensing in plastids by PII proteins

PII proteins belong to one of the most evolutionarily conserved families of signalling proteins, being widely distributed in bacteria and plants as well as in many species of the archaeal kingdom (Forchhammer and Luddecke, 2016). However, despite their conservation at the amino acid sequence level, the signalling roles of PII proteins are diverse. In plants, the plastid-localized PII protein (encoded by the nuclear-localized *GlnB* gene) has been shown to interact with *N*-acetyl-L-glutamate kinase (NAGK) (Burillo *et al.*, 2004; Sugiyama *et al.*, 2004), which catalyses an important rate-limiting step in arginine biosynthesis, and with acetyl-CoA carboxylase (Bourrellier *et al.*, 2010), a key enzyme in fatty acid biosynthesis. More recently it has been discovered that glutamine binds to a plant-specific C-terminal extension of the PII protein and it is only after binding that the PII protein is able to form a complex with and activate NAGK (Chellamuthu *et al.*, 2014). By this means plants have evolved a simplified glutamine-sensing mechanism for the PII regulatory pathway, circumventing the need for covalent modification of PII by a glutamine-sensitive uridyl transferase as occurs in *E. coli*. It has been proposed that the low-affinity binding site for glutamine on PII allows for activation of NAGK only at high plastidic glutamine concentrations, enabling increased biosynthesis of arginine (which can be used for N storage) under conditions of N over-supply (Chellamuthu *et al.*, 2014). Although the glutamine-binding motif in PII is highly conserved in plants, it is surprisingly missing in Arabidopsis and other members of the *Brassicaceae* family (Chellamuthu *et al.*, 2014), yet two PII knock-out mutants of Arabidopsis showed strongly reduced accumulation of arginine when supplied with ammonium after N starvation

(Chellamuthu *et al.*, 2014; Ferrario-Mery *et al.*, 2006). Therefore, it appears that Arabidopsis must use an alternative mechanism for PII-dependent regulation of arginine metabolism.

Despite the significance of PII signalling in plastids, and evidence that over-expressing PII in a leguminous plant leads to a decline in rates of N-fixation in nodules (D'Apuzzo *et al.*, 2015), its primary function would appear to be limited to the regulation of the arginine biosynthesis and fatty acid metabolism and therefore a more global role in sensing the shoot's N status does not currently appear likely.

TOR Signalling

Arabidopsis has a single TOR kinase gene (*AtTOR*) (Menand *et al.*, 2002) and also carries homologues of many, but not all, of the genes encoding components of the TORC1 complex in mammals (Xiong and Sheen, 2014). TOR kinase is known to have diverse roles in regulating plant growth and development and to act as an integrator of multiple signalling networks to coordinate growth and developmental transitions (Dobrenel *et al.*, 2016; Xiong and Sheen, 2014). The TOR complex is therefore ideally placed to play a key role in N status sensing in plants, as it does in yeast and mammals (discussed above). There are several lines of evidence indicating that the TOR complex in Arabidopsis is involved in regulating N metabolism as well as in coordinating N and C metabolism. A number of studies that have investigated the metabolic effects of down-regulating TOR itself, or of down-regulating genes encoding TOR-associated proteins, have observed major shifts in metabolism that include an accumulation of amino acids and additional effects on the abundance of starch and sugars (Caldana *et al.*, 2013; Deprost *et al.*, 2007; Moreau *et al.*, 2012). Because TOR is a positive regulator of protein synthesis and a negative regulator of protein turnover, this accumulation of amino acids in TOR-deficient plants could be accounted for by a combination of a decline in the rate of protein synthesis and an up-regulation of the rate of protein degradation (Caldana *et al.*, 2013). However, other factors may additionally be at play because, in Arabidopsis lines that are defective in expression of either the *TOR* or *TAP46* (*2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 kDa*) genes, effects are seen at the mRNA level that could contribute to the changes in N metabolism, namely down-regulation of nitrate assimilatory genes and an induction of genes involved in amino acid recycling (Ahn *et al.*, 2011). TAP46 is a protein phosphatase 2A (PP2A)-associated protein that regulates PP2A activity in Arabidopsis and that has

been identified as a downstream effector of the TOR complex (Ahn *et al.*, 2011). This implies that TOR, acting at the transcriptional level through TAP46 and PP2A, may be important for regulating the balance between primary N assimilation and N recycling to accommodate changes in the plant's N status. However, while there is evidence that TOR activity in plants is regulated by intracellular sugar availability (Dobrenel *et al.*, 2016), there is currently no corresponding information in plants on the mechanism of N sensing upstream of TOR, or on which N metabolites are sensed.

The GCN2 protein kinase

The GCN2 protein kinase is a key component of the general amino acid control mechanism in yeast, which is responsible for suppressing global protein synthesis under conditions of N deficiency to help maintain amino acid homeostasis (Schneper *et al.*, 2004). During N starvation, the hyperaccumulation of uncharged tRNAs directly stimulates the kinase activity of GCN2 which then phosphorylates its target, the eukaryotic translation initiation factor eIF2α. Phosphorylation of eIF2α stimulates the translation of a specific set of mRNAs (e.g. the mRNA for the GCN4 transcription factor in yeast) whilst reducing the efficiency of translation initiation for most mRNAs, leading to a general decline in protein synthesis. Plants possess genes for GCN2 and two types of eIF2α kinases, although no plant GCN4 homologue has been identified. In yeast, activation of the kinase activity of GCN2 involves binding of the uncharged tRNAs to a histidyl-tRNA synthetase-related domain located at its C-terminus. Despite the plant homologue of GCN2 having a truncated version of the C-terminal domain, it has recently been demonstrated using *in vitro* assays that it too is activated by binding to uncharged tRNAs and is then able to phosphorylate both of the eIF2α homologues present in Arabidopsis (Li *et al.*, 2013b).

A recent insight into the role and mechanism of amino acid sensing in plant defence has come from studies into the mechanism of β-aminobutyric acid (BABA)-induced priming of the plant immune response. A screen for Arabidopsis mutants defective in BABA-induced priming revealed that plant perception of BABA is mediated by an aspartyl-tRNA synthetase (AspRS) encoded by the *Impaired in BABA-induced Immunity 1* (*IBI1*) gene (Luna *et al.*, 2014). As well as priming the defence response in leaves, BABA also inhibits plant growth and the same study found evidence that this response to BABA (but not the priming response) operates through a GCN2-dependent pathway. This has led to a model in which amino acid uptake by a parasitizing plant pathogen leads to a decline in cellular aspartate

levels, reducing the canonical AspRS activity of IBI1 and thereby activating a secondary IBI1 activity to prime the defence response. In this model, R-BABA (the active enantiomer of BABA, which has stereochemical similarity to L-Asp) acts by blocking the binding of L-Asp to IBI1 and thereby 'tricks' the protein into sensing low L-Asp levels. Thus, it appears that an enzyme whose primary function is in protein synthesis (AspRS) has been co-opted to serve a role in assisting the plant to mobilise its defences to pathogen attack. It has been proposed that activation of GCN2 by BABA arises from its inhibition of AspRS activity, leading to accumulation of the uncharged tRNAs (Luna *et al.*, 2014). Subsequent GCN2-catalysed phosphorylation of eIF2α would then trigger downstream responses such as inhibition of plant growth and presumably other eIF2α-regulated responses to amino acid deficiency.

GSI-like genes

In addition to the well-studied cytoplasmic and plastidic glutamine synthetases (GSs) that are so important for N metabolism, plants also carry another form of GS that is more closely related to prokaryotic GS (GSI-type) than to these eukaryotic forms (GSII-type). Most plant GSI-type genes encode a protein with an N-terminal aminohydrolase domain related to the nodulin 6 protein (Doskocilova *et al.*, 2011) and hence are referred to as NodGS. The first fusion protein of this form to be identified was the fungal FluG protein, and in *Aspergillus* sp. FluG has a regulatory role as a morphogenetic factor that stimulates asexual sporulation under conditions of N starvation (but not C starvation), a role that was shown to reside in the GSI-like domain of the protein (D'Souza *et al.*, 2001). In Arabidopsis, downregulation of NodGS by RNAi led to multiple developmental effects, including a shortened primary root and disruption of the root cap, suggesting a possible regulatory role in root morphogenesis (Doskocilova *et al.*, 2011). The model legume *Medicago truncatula* has two NodGS genes, *MtGSIa* and *MtGSIb*, which are preferentially expressed in roots and root nodules and whose expression is down-regulated by externally applied amino acids (Silva *et al.*, 2015). Neither FluG nor the plant NodGSs so far analysed possess significant GS enzymatic activity (D'Souza *et al.*, 2001; Doskocilova *et al.*, 2011; Silva *et al.*, 2015), leading to speculation that their GSI-like domains might perform a different function, perhaps in the production of some form of signalling molecule. The lack of GS activity in FluG/NodGS is associated with the lack of conservation of two key amino acid residues in the active site (Doskocilova *et al.*, 2011), but the overall sequence conservation of the protein indicates that it could still provide binding sites for NH_4^+, glutamate and/or glutamine and therefore

potentially perform a role as some form of N sensor. The general idea of a sensory role for a GS enzyme is supported by a study in the rice fungal pathogen *Fusarium fujikuroi* (Wagner *et al.*, 2013), which found evidence that the GSII-type enzyme in that species has a regulatory function involving NH_4^+ sensing that could be separated from its catalytic activity by site-directed amino acid substitutions.

The GLR family of glutamate receptor-like proteins

Plants possess a large family of membrane proteins with homology to the ionotropic glutamate receptor (iGluR) family that was first identified and characterised in mammals (Price *et al.*, 2012). Mammalian iGluRs are primarily known for their role as fast excitatory neurotransmitters in the central nervous system where they act as glutamate-gated cation channels, selective for Na^+, K^+ and Ca^{2+} ions. In Arabidopsis there are 20 *GLR* (*Glutamate-Like Receptor)* genes encoding proteins with the same modular structure as their mammalian homologues: an N-terminal domain, a ligand-binding domain, a transmembrane domain that includes a pore region, and a C-terminal domain (Davenport, 2002). Since their discovery in 1998 (Lam *et al.*, 1998) there has been considerable interest in what role the GLR proteins might play in plants, where a nervous system is obviously lacking. At the molecular level the accumulated evidence indicates that GLRs act as amino acid-gated Ca^{2+} channels and that members of the family are located in different cell membranes, including the plasma membrane, the inner and outer chloroplast membranes and the mitochondrion (reviewed by Weiland *et al.* 2016). However, while the iGluRs are largely glutamate-specific (with only aspartate acting as an alternative, weak agonist (Flores-Soto *et al.*, 2012), plant GLRs appear to be gated by a broad range of different amino acids. Evidence for this has come from studies using both *GLR* knockout mutants (Michard *et al.*, 2011; Qi *et al.*, 2006; Stephens *et al.*, 2008; Tapken *et al.*, 2013) and heterologous expression (Tapken *et al.*, 2013; Vincill *et al.*, 2012). For example, when expressed in Xenopus oocytes the Arabidopsis AtGLR1.4 protein was found to be gated to varying degrees by methionine, tryptophan, phenylalanine, leucine, tyrosine, asparagine and threonine, but not by L-glutamate or other proteinogenic amino acids (Tapken *et al.*, 2013), while the AtGLR3.4 protein expressed in Human Embryonic Kidney 293 cells was sensitive to asparagine, L-serine and glycine, but not to L-glutamate, alanine, cysteine or phenylalanine (Vincill *et al.*, 2012). These findings are consistent with the evidence from various Arabidopsis knock-out mutants (Michard *et al.*, 2011; Qi *et al.*, 2006; Stephens *et al.*, 2008; Tapken *et al.*, 2013) that, in contrast to their mammalian homologues, the plant GLRs are relatively promiscuous in their ligand specificity. These experimental data are further supported by homology modelling studies which not only indicate

271 that all members of the AtGLR family could potentially bind amino acids but also that the sequence
272 diversity that exists in their ligand-binding domains would be consistent with them being gated by a
273 diverse set of agonists (Tapken *et al.*, 2013). In addition, there is the possibility that amino acids or
274 other metabolites could allosterically regulate the plant GLRs by binding to sequences in their extended
275 N-terminal domains (Weiland *et al.*, 2016). Some mammalian iGluRs have a similarly long N-terminal
276 domain that allows both positive and negative allosteric regulation of the receptor by a variety of small
277 molecules and ions (Kumar and Mayer, 2013). Should the N-terminal domain in plant GLRs serve a
278 similar function it would greatly amplify the potential of this family of receptors to integrate a multitude
279 of metabolic signals.

280 Of the candidates for a role in sensing N status the GLRs are amongst the most compelling. As
281 a family they are ubiquitously expressed throughout the plant, their products are located on both the
282 plasma membrane and organellar membranes, collectively their activity is gated by a diverse set of
283 amino acids, and their ability to control the movement of Ca^{2+} ions across these membranes potentially
284 provides a direct link to multiple Ca^{2+} signalling pathways (Dodd *et al.*, 2010). Experimental evidence
285 for their physiological role comes primarily from two early papers that described the pleiotropic effects
286 of disrupting the expression of the *AtGLR1.1* gene on aspects of carbon, nitrogen and hormone
287 metabolism (Kang *et al.*, 2004; Kang and Turano, 2003). It was reported that germination of *AtGLR1.1*
288 antisense lines on N-free full-strength Murashige and Skoog medium was inhibited by the presence of
289 3% sucrose (but not by other C sources) and that the inhibitory effect of sucrose could be overcome by
290 the inclusion of 5 mM NO_3^- (but not 5 mM NH_4^+) (Kang and Turano, 2003). Other aspects of the
291 pleiotropic phenotype of the *antiAtGLR1.1* line included effects on the expression of some enzymes of
292 C and N metabolism (Kang and Turano, 2003) and an increased sensitivity of germination and root
293 growth to external abscisic acid (ABA) treatment (Kang *et al.*, 2004). Taken together with additional
294 effects on the expression of some genes related to ABA signalling and biosynthesis (Kang *et al.*, 2004)
295 these observations were interpreted as indicating a role for AtGLR1.1 in linking changes in C/N status
296 to ABA signalling and other metabolic and developmental responses.

297 There are three clades of *GLR* genes in plants and most studies into the physiological role of
298 plant GLRs have focussed on members of clade 3 (principally AtGLR3.3, AtGLR3.4 and AtGLR3.5)
299 (Weiland *et al.*, 2016). Diverse functions that have been assigned to these GLRs on the basis of the
300 phenotype of knock-out mutants or over-expressing lines have so far included roles in stomatal closure,
301 root branching, maintenance of the primary root meristem, gravitropism, pollen tube signalling and the

defence response (reviewed Weiland *et al.* 2016). However, there is surprisingly little experimental evidence directly linking any of these phenotypes to activation of a GLR by its known agonist(s). One notable exception is a study of the role of the *AtGLR3.3* gene in the immune response (Li *et al.*, 2013a), where it was found that cysteine and the tripeptide glutathione (both of which were identified as AtGLR3.3 agonists) were each able to suppress growth of the bacterial pathogen *Pseudomonas syringae* pv tomato DC3000 in Arabidopsis leaves and that this response was defective in an *atglr3.3* knock-out mutant. A more recent paper reported that L-glutamate was able to trigger stomatal closure in Arabidopsis leaves and that this was dependent on a functional *AtGLR3.5* gene (Yoshida *et al.*, 2016). The difficulty in achieving this degree of definition in our understanding of the physiological role of most of the GLRs is likely to be due to a combination of genetic redundancy in the large gene family and the multiplicity of agonists that can activate them. Nevertheless, based on what we know so far, it seems plausible that many or all the diverse and pleiotropic phenotypes caused by disruption or overexpression of members of the *GLR* family are due, directly or indirectly, to perturbations in amino acid (or small peptide) sensing. If this is the case then it points to the GLR family having important roles throughout the plant in monitoring changes in amino acid distribution (between different cellular and extracellular compartments), amino acid composition and overall amino acid abundance. It remains to be established how important the Ca^{2+} signals generated as a result of this GLR activity are in initiating the effects on gene expression and root development that are symptomatic of changes in the plant's N status.

Conclusions

In this review, we have attempted to assess the most recent advances in our understanding of how plants monitor their N status. The main candidates for this N sensing role belong to signalling pathways that have mostly been chosen for investigation on the basis of their homology to nutrient sensing systems previously identified in other organisms (bacteria, yeast or mammals). The exception to this rule is the GLR family of glutamate receptor-like proteins, whose homologues in mammals are primarily (but not exclusively) associated with the nervous system and the process of neurotransmission. Despite the progress that has been made in recent years, particularly in elucidating the TOR-, PII- and GCN2-mediated pathways in higher plants, there is still little clarity about which N-compounds are being monitored to maintain amino acid homeostasis or the identity of the molecular sensors for those

compounds. In the absence of hard evidence, some speculation may be permissible. Is it possible that members of the GLR family, whose credentials for the role of ubiquitous amino acid sensors have been outlined above, are the upstream components required by a TOR-mediated amino acid signalling pathway? Although yeast has no GLR/iGluR homologues, there is a precedent for this in mammals where there is evidence that iGluRs belonging to the NMDA group are able to regulate mTOR signalling activity in neurons (Burket *et al.*, 2015; Gong *et al.*, 2006; Huang *et al.*, 2007). Some experimental support for this hypothesis exists in the form of the phenotype of the *AtGLR1.1* antisense line described above, involving changes in the expression of enzymes involved in C and N metabolism and ABA signalling (Kang *et al.,* 2004; Kang and Turano, 2003), which is not dissimilar to phenotypes described for lines defective in components of the TOR signalling pathway (Dobrenel *et al*., 2016).

It is also worth noting that there are examples in both mammals and yeast of interactions between Ca^{2+} signalling and TOR signalling (Deutsch *et al.*, 2014; Mulet *et al.*, 2006) and a study using human cell lines found evidence that amino acids activate the mTOR complex *via* a pathway involving Ca^{2+}-dependent activation of the human vacuolar protein sorting 34 (hVps34), a type III phosphatidylinositol 3-kinase (Gulati *et al.*, 2008). To date there are no reports of a role for Ca^{2+} signalling in the activation of the TOR complex in plants, however Arabidopsis does have a homologue of hVp34 (AtVPS34) in which the Ca^{2+}-dependent lipid-binding domain is conserved between plants and humans (Welters *et al.*, 1994). Thus we consider that the possibility of a link between Ca^{2+} influx through amino acid-gated members of the GLR family and an AtVPS34-dependent TOR signalling pathway in plants is one that is worthy of investigation.

Fig. 1 illustrates how amino acid signalling through a panel of GLR complexes with multiple and differing ligand specificities could, *via* a Ca^{2+}-dependent signalling pathway, provide a mechanism for regulating the TOR complex in response to changes in the overall amino acid pool. The multiplicity of the amino acids that are able to act as ligands for plant GLRs could help to explain why, as discussed above, previous research has failed to pinpoint any particular amino acid(s) for the role of effector in N status sensing. Overcoming genetic redundancy amongst GLR family members to test the postulated link between GLRs and TOR in amino acid signalling will be a challenge, but as previously discussed in more detail (Forde, 2014), opportunities may arise through the application of chemical genetic approaches if small molecule agonists or antagonists can be identified that target specific clades or sub-groups of the GLRs. The mechanism by which plants monitor their N status is so fundamental to

how they regulate the processes of N acquisition, N metabolism and N storage that a better understanding of these is expected to be important for future efforts to improve the efficiency with which crop plants capture and utilize soil nitrate.

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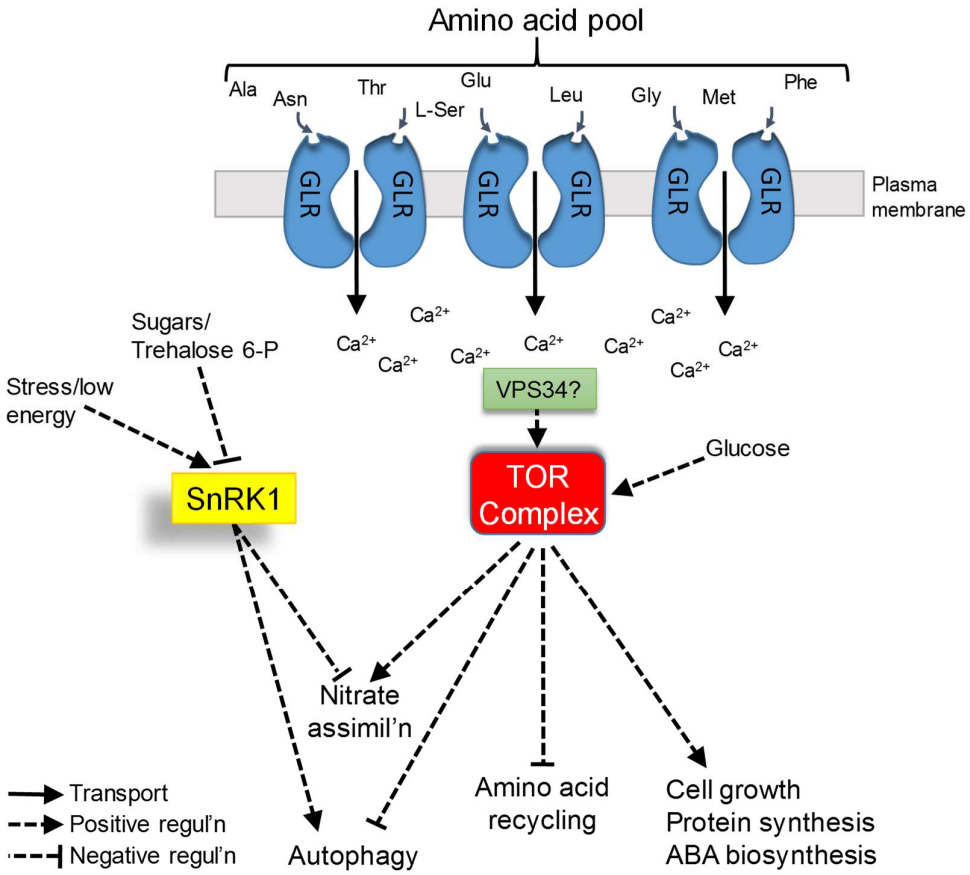
Legends

Fig. 1. Hypothetical model for activation of the TOR complex by amino acid sensing through the family of GLR glutamate receptor-like Ca^{2+} channels present in plants. The TOR complex plays a central role in regulating plant growth and metabolism in response to changes in nutrient availability, working in conjunction with the Snf1-related kinase (SnRK1) which largely acts antagonistically to TOR (Dobrenel *et al.*, 2016; Robaglia *et al.*, 2012). The diagram depicts a cell in which a diverse set of GLRs are expressed, each activated by a variety of different amino acid ligands (only a proportion of which are indicated). Collectively this panel of amino acid sensors would be able to respond to changes in the overall amino acid pool, therefore potentially providing an accurate reflection of the N status of the tissue. Although the GLRs are shown as located in the plasma membrane, there is evidence that they are also to be found on internal (plastidic) membranes (Teardo *et al.*, 2011), so that their sensory role would not be restricted to amino acids in the apoplast. Ligand binding by plant GLRs is known to trigger Ca^{2+} influx (Dietrich *et al.*, 2010) and it is proposed that this could be linked to activation of the TOR complex, perhaps through a pathway involving the Ca^{2+}-dependent regulation of VPS34 and the downstream production of phosphatidic acid which is an activator of TOR in animals (Gulati *et al.,* 2008). See text for further details.

Fig. 1


Amino acid pool
Ala
Asn
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L-Ser
Glu
Leu
Gly
Met
Phe
GLR
Plasma membrane
Ca2+
Sugars/
Trehalose 6-P
Stress/low
energy
VPS34?
Glucose
SnRK1
TOR
Complex
Nitrate
assimil'n
Transport
Positive regul'n
Negative regul'n
Autophagy
Amino acid
recycling
Cell growth
Protein synthesis
ABA biosynthesis