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REVIEW

Signaling mechanisms integrating carbon and nitrogen utilization in plants

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Abstract **Carbon (C) and nitrogen (N) are two essential nutrients affecting plant growth and development. Plants are non-motile organisms and have evolved highly sophisticated and complex sensing and signaling mechanisms to respond to the dynamic changes of C and N nutrients in their surroundings. C and N metabolism are tightly coordinated to maintain intracellular C/N homeostasis. However, the regulatory mechanism underlying C/N coordination and balancing in plants remains to be elucidated. It has been suggested that C and N metabolism are modulated by the interaction of C signaling with N signaling or by C/N ratio signaling. This review focuses on cell signaling studies that provide insight into the regulation mechanism of C/N balancing in plants.**

Keywords carbon, nitrogen, balancing, coordination, signaling

Introduction

Plants are non-motile and therefore they have evolved a highly sophisticated and complex sensing and signaling mechanisms to respond to the dynamic changes of their surrounding environments. Carbon (C) and nitrogen (N) are two essential macronutrients for all organisms. Plants acquire C and N nutrients in a unique manner. Aboveground green plant shoot tissues fix C through photosynthesis into carbohydrate that enhances root growth. By contrast, nitrogen is taken up from the soil by the underground root tissue and promotes shoot growth. C and N nutrients therefore, modulate plant growth and development via coordinated metabolism and signaling pathways.

In plants, the assimilation of C and N nutrients is tightly linked to almost every biochemical pathway (Stitt and Krapp, 1999). Two routes were identified in plant kingdom for generation of carbon skeletons (2-OG), energy and reducing equivalents that are required for nitrogen assimilation (Foyer et al., 2011). In addition to the common tricarboxylic acid (TCA) route in all organisms, the genus *Oryza* has a unique route in using chloroplastic phospho*enoyl*pyruvate carboxylase (PEPC) to provide organic acids for ammonium assimilation (Hanning and Heldt, 1993; Masumoto et al., 2010). N metabolism is severely impaired when photosynthesis rate is decreased (Joy, 1988). On the other hand, photosynthesis rate is significantly reduced when N availability is limited (Longstreth and Nobel, 1980). Therefore, balanced C and N nutrient provisions are critical to ensure maximal use efficiency and to maintain an appropriated shoot:root ratio for normal plant growth and development.

In addition to their physiologic functions as nutrients to modulate metabolism, C and N nutrients and their derived metabolites act as signaling molecules to regulate genome-wide gene expression and protein activities (Vidal and Gutiérrez, 2008; Smeekens et al., 2010). The observation that the N content of a plant does not need to be modified before a growth modification suggests that plant growth, at least in the first hours of the environmental change, is controlled by nutritional signals rather than by modifications of the overall nutritional status (Walch-Liu et al., 2000). Plant growth, however, creates a sink for nutrient needs, which in turn stimulates the uptake of the nutrients or re-allocation of

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the nutrients from source tissues (Li et al., 2008).

Key enzymes that participate in C and N metabolism and their regulation by C and N nutrients are well understood. Many enzymes are co-regulated by both C and N signals. However, the molecular mechanisms underlying the co-regulation remain elusive. C/N balancing may be adjusted by the cross-talk between C and N signaling pathways, a mechanism supported by increasing evidence. It has also been suggested that plants may sense the C/N balance or the C/N ratio and several genes have been reported to be involved in such processes (Malamy and Ryan, 2001; Kang and Turano, 2003; Gao et al., 2008; Zheng, 2009). Given the central role of coordinated C/N utilization in all organisms, numerous studies have been recently performed to identify the regulators of C-N interaction. Several comprehensive and excellent reviews on individual C or N sensing and signaling exist (Rolland et al., 2006; Krouk et al., 2010; Nunes-Nesi et al., 2010; Xu et al., 2012). This review provides an overview of the latest studies on the regulatory links between C and N metabolism in plants. The complex interacting networks of the identified regulators and their potential regulation mechanism responsible for balancing C and N metabolism are discussed.

Regulatory links between C and N signaling

C and N are not only important energy sources and structural components, but also central regulatory molecules controlling gene expression in living organisms (Jang et al., 1997). C and N signaling pathways are relatively well understood. Glucose, sucrose, trehalose and α-ketoglutarate (α-KG) are suggested to act as C signals (Rolland et al., 2006; Paul et al., 2008). Nitrate and N metabolites including nitrite, ammonium and amino acids, act as N signals to regulate global gene expression in plants (Stitt M, 1999). Although sensors of glucose and nitrate and some important signaling components downstream of these sensors have been identified, the mechanisms of cross-talk between C and N signaling pathways are still poorly understood. The identified players interconnecting C and N regulation are overviewed below and summarized in Fig. 1.

PII is a highly conserved small homotrimeric signal transduction protein in archea, bacteria and plants (Ninfa and Jiang, 2005). In bacteria and archea, PII proteins serve as the central processing unit (CPU) for the integration of carbon and nitrogen status to control nitrogen assimilation (Ninfa and

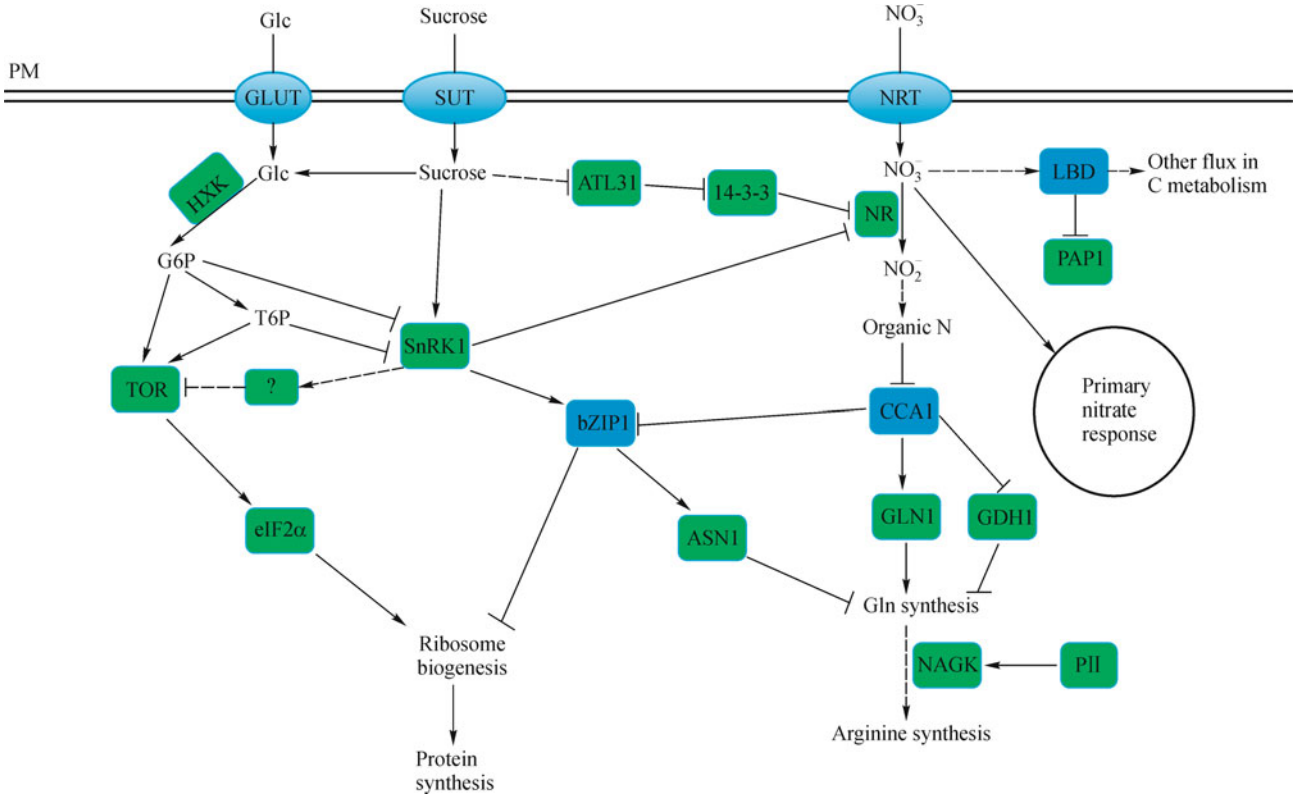

Figure 1 Simplified model for interactions between C and N signaling in plants. NRT is the transporter and sensor of nitrate. NR, bZIP, CCA1, SnRK1 and TOR play pivotal roles in regulating C and N metabolism. They are regulated by both C and N signals directly or indirectly and control their metabolisms accordingly. Dashed lines indicate multiple or hypothetical connections. See text for details.

Atkinson, 2000). The key signals of cellular N and C status in *E. coli* appear to be glutamine and α-KG respectively (Ninfa and Jiang, 2005). In *E. coli*, the uridyltransferase/uridyl-removing enzyme (UTase/UR) responds to the N status of the cell depending on the ratio of glutamine to α-KG. In the presence of a low glutamine to α-KG ratio, UTase adds a UMP group to PII, which promotes deadenylylation of glutamine synthesis (GS) by adenyltransferase (ATase), resulting in the active form of GS (Sanchez and Demain, 2002). α-KG regulates the activity of *E. coli* PII by directly binding to it. In higher plants, PII is encoded by a nuclear gene and the protein localizes to the chloroplast (Hsieh et al., 1998). Despite serving as a central integrator for C and N metabolism in many prokaryotes, PII appears to have evolved a secondary and more specialized role in higher plants. New bioinformatics insights revealed that in higher plants, PII might possess a tissue-specific role to regulate storage protein accumulation in developing seeds (Uhrig et al., 2009). In addition, PII in photosynthetic leaf tissue have also been proposed to regulate NO_2^- import into the chloroplast (Ferrario-Mery et al., 2008). Although PII regulates multiple interacting targets in prokaryotes, only one partner (NAGK) was characterized in higher plants (Sugiyama et al., 2004). PII forms a complex with NAGK to alleviate feedback inhibition by high arginine concentrations, suggesting PII is a major player in regulating the arginine biosynthetic pathway in plants (Chen et al., 2006).

SnRK1 (SNF1-related protein kinase-1) is a plant protein kinase closely related to the yeast SNF1 (sucrose non-fermenting-1) and mammalian AMPK (AMP-activated protein kinase) (Halford et al., 2003). SnRK1 kinase activity is controlled by the phosphorylation of a conserved threonine in the "T-loop" of its catalytic subunit, but an upstream kinase or phosphatase has not been yet identified in plants. SnRK1 is activated by high cellular sucrose and low levels of glucose, but inhibited by glucose 6-phosphate and trehalose 6-phosphate (Toroser et al., 2000; Halford et al., 2003; Zhang et al., 2009). SnRK1 is involved in re-allocating carbon by modulation the gene expression of sucrose synthase, ADP-glucose pyrophosphorylase and alpha-amylase (Laurie et al., 2003; McKibbin et al., 2006). The SnRK1 signaling pathway could also regulate nitrogen assimilation and amino acid biosynthesis through both the phosphorylation and inactivation of nitrate reductase (NR), and the regulation of asparagine synthase gene expression (Baena-Gonzalez et al., 2007). These observations suggest a potential route by which carbon availability could influence nitrogen assimilation or vice versa, through SnRK1.

TOR (target of rapamycin) is a serine/threonine protein kinase that functions as a central regulator of yeast and animal cell growth in response to nutrient and growth factors (Schmelzle and Hall, 2000). In the presence of amino acids, Rag GTPases promote the translocation of mTORC1 to the lysosomal surface, where the mTORC1 is activated by v-ATPase (Zoncu et al., 2011). Mammalian Rac1, a member of the Rho family of GTPases, binds directly to the C-terminal of mTOR to mediate TORC1 and TORC2 localization to specific membranes in response to growth-factor stimulation (Saci et al., 2011). It was suggested that mTORC2 signals to the actin cytoskeleton through a small Pho-type GTPase (ROP) (Jacinto et al., 2004). Studies of TOR in plants lag well behind parallel studies in yeast and animal systems due to the insensitivity of plant TOR to rapamycin treatment and embryo lethality of *TOR* mutant plants (Menand et al., 2002). However, considerable progress has been recently made using inducible *TOR* RNAi transgenic plants or higher doses of TOR inhibitors. Glucose strongly activates TOR activity in *Arabidopsis* seedlings. The retarded seedling growth by rapamycin treatment resembles that of the inducible *tor* mutant grown in the presence of glucose (Xiong and Sheen, 2012). These findings demonstrate the involvement of TOR in glucose signaling. Silencing of TOR in *Arabidopsis* leads to the inhibition of translation initiation (Deprost et al., 2007). A protein kinase called general control non-derepressible-2 (GCN2) reduces protein synthesis through the phosphorylation of the eukaryotic translation initiation factor-2 (eIF2α) in response to decreases in amino acid levels. TOR and SnRK1 act in opposite ways in the regulation of nutrient-driven processes. It was hypothesized that TOR links SnRK1 with general control non-derepressible-2 (GCN2), which is another potential route through which carbon metabolic signaling interacts with nitrogen signaling in living cells (Hey et al., 2010).

UPS (ubiquitin-proteosome system) is a unique protein degradation mechanism conserved in eukaryotic cells (Hershko and Ciechanover, 1998). Ubiquitination and the subsequent degradation of target proteins by the 26S proteasome is involved in almost all aspects of plant growth, development, and response to biotic and abiotic stresses (Sato et al., 2011b). Recent studies reveal that the UPS plays an essential role in the adaptation to C and N availability in plants. The ubiquitin ligases ATL31 and ATL6 from *Arabidopsis* were found to be positive regulators of C and N responses (Maekawa et al., 2012). Loss of function mutants for ATL31 and ATL6 showed hypersensitivity to high C/N stress conditions. The 14-3-3 protein was identified as the target of ATL31 and ATL6 (Sato et al., 2011a). Ubiquitinized 14-3-3 protein is degraded in a proteasome-dependent manner. Overexpression of the 14-3-3 protein results in hypersensitivity to C/N stress conditions, which is enhanced by the absence of ATL31 and ATL6 activity (Sato et al., 2011b). These data indicate that ATL31 regulates the C/N response by degrading the 14-3-3 protein via ubiquitination followed by proteasome-mediated degradation. 14-3-3 proteins are considered master regulators of many signal transduction cascades in eukaryotes. Particularly, plant 14-3-3 proteins have been reported to play major roles as regulators of carbon and nitrogen metabolism by direct interaction with essential enzymes such as the plasma membrane H^+-ATPase, nitrate reductase, sucrose phosphate

synthase, ADP-glucose pyrophosphorylase and glutamine synthase (Ferl et al., 1999). Extensive novel roles for 14-3-3 proteins in the TCA cycle regulation were determined recently (Diaz et al., 2011). 14-3-3 proteins were demonstrated to interact with isocitrate dehydrogenase and malate dehydrogenase, the key enzymes of TCA cycle. Levels of malate and citrate were decreased in 14-3-3 overexpressing transgenic plants, due to the reduced activities of isocitrate dehydrogenase and malate dehydrogenase.

Several transcriptional regulators are implicated in coordinating C-N metabolism. Dof1 (DNA binding with one finger) is an activator for expression of multiple genes associated with organic acid metabolism, including the expression of phosphoenolpyruvate carboxylase (PEPC) gene (Yanagisawa S, 2000). In *Arabidopsis,* the overexpression of *Dof1* improved growth under low-nitrogen supply, accompanied by upregulation of multiple genes encoding for carbon-skeleton production. The transgenic *Arabidopsis* possessed a marked increase of amino acid contents, and a reduction of the glucose level (Yanagisawa et al., 2004). In addition, the introduction of the *ZmDof1* gene in rice also enhanced C and N assimilation under low-nitrogen conditions (Kurai et al., 2011). The master clock control gene *CCA1* was found to be regulated by Glu or Glu-derived metabolite, which in turn regulates the expression of key N-assimilatory genes (Gutierrez et al., 2008). ASN1 is a predicted target of the transcription factor bZIP1, which is a predicted target of CCA1. Because bZIP1 is also regulated by carbon (Price et al., 2004), bZIP1 and CCA1 may function as integrators of C and N signaling to regulate N-assimilation in *Arabidopsis.*

In addition to the above-mentioned systems involved in the interaction between C and N signaling, a putative methyltransferase named OSU1 was reported to be a critical modulator in balancing C and N nutrient response in *Arabidopsis* (Gao et al., 2008). *osu1* mutants are more sensitive than wild-type to both of the imbalanced C/N conditions, high C/low N and low C/high N. However, under the balanced C/N conditions (low C/low N or high C/high N), the *osu1* mutants demonstrate similar phenotype as wild-type. Under high C/low N conditions, *osu1* mutants have higher anthocyanin accumulation. The root length of the *osu1* mutant is much shorter under low C/high N condition. It was determined that OSU1 regulates two operating pathways to respond to imbalanced C/N condition, including the low C/high N pathway suppressing *ASN1* expression, and the high C/low N pathway affecting the expression of MYB75 transcription factor (Gao et al., 2008). The direct target of OSU1 is yet to be determined.

C/N nutrient signals integrate with phytohormone to control C and N utilization

C and N nutrients play key roles in governing growth and morphology of the plant. Many developmental processes respond to C and N provisions, such as germination, architecture of the root system, leaf growth, flowering and seed development (Vidal and Gutierrez, 2008). The role of nutrients in stimulating plant growth is not only due to metabolic effects, but also to the fact that plants modulate their growth according to the perception of the resources available in the soil. Among the major plant growth regulators, cytokinin (CK) and abscisic acid (ABA) have been demonstrated to play roles in signaling C and N status in plants. In a meta-analysis involving 75 ATH1 chips from different laboratories, a large proportion of genes under the control of NO_3^- was shown to be also controlled by hormonal signaling, mainly CK and ABA (Nero et al., 2009). Interestingly, genes regulated by both NO_3^- and hormones are more responsive to NO_3^- than genes controlled by NO_3^- alone (Nero et al., 2009), suggesting that hormones are involved in and enhance NO_3^- signaling.

Involvement of CK in C/N regulation of plant growth and development

Evidence shows that cytokinin (CK) has a central role in signaling the intracellular N status in plant (Inoue et al., 2001). The concentration of active CK in plants changes in parallel with the change of N supply, for example, increasing when additional N is supplied, due to the induction of key enzyme of CK biosynthesis and adenosine phosphate-isopentenyltransferase 3 (IPT3) in the roots (Takei et al., 2004). Compelling evidence supports the hypothesis that nitrate regulates leaf growth through a tight connection between nitrate and CK signaling. Indeed, nitrate stimulates both CK biosynthesis in roots and their translocation to the shoot (Kiba et al., 2011). Since CK promotes cell division in the shoot apical meristem, CK is proposed to act as a long distance signal or a second messenger indicating nitrate availability in the roots to directly and rapidly stimulate shoot growth. The reduction of shoot biomass production by limited nitrate supply was classically attributed to metabolic limitations. However, it now appears that the slowing of shoot growth under nitrate limitation is at least partly mediated by nitrate signaling, as an important adaptive response to prevent internal N deficiency (Rahayu et al., 2005).

GNC (GATA, nitrate-inducible, carbon metabolism-involved) and CGA1 (cytokinin-responsive GATA1) are highly expressed in green tissues, and their expression is inducible by nitrate and CK (Bi et al., 2005; Naito et al., 2007). Throughout their life span, *GNC* T-DNA insertion mutants showed green pale leaves with significantly reduced chlorophyll content and they were sensitive to exogenous glucose, while GNC overexpressing transgenic plants were shown to be less sensitive to exogenous glucose. Transcription profiling comparison of *GNC* mutants with wild type revealed a large number of repressed genes involved in carbon metabolism (Bi et al., 2005), demonstrating a pivotal role of GNC and CGA1 in nitrate induced C-metabolism. In

addition to the regulation of chlorophyll production, the Schaller group also found that GNC and CGA1 serve as two of the master transcriptional regulators of chloroplast biogenesis, acting downstream of CK and mediating the development of chloroplasts from proplastids and enhancing chloroplast growth and division in specific tissues (Chiang et al., 2012). These observations indicate the possibility that CK mediates the nitrate enhancement of photosynthesis by stimulating chloroplast biogenesis via GNC and CGA1.

ABA coordinates C/N signaling pathway during seed development and germination

High amounts of starch and moderate amounts of storage proteins are synthesized in the endosperm of maturing seeds. Carbon metabolite precursors must be properly allocated for protein and starch synthesis respectively, to ensure a normal seed development. The *RisØ16* mutant with affected cytosolic ADP-Glc formation and starch synthesis accumulates higher levels of free sugars hexose and sucrose but lower amounts of starch and storage protein in mature seeds (Faix et al., 2012). Decreased levels of ABA in *RisØ16* endosperm were observed. In barley, endosperm development and maturation is controlled by ABA, which stimulates cell differentiation and grain filling (Sreenivasulu et al., 2010). These findings suggest a relationship between starch synthesis, storage protein synthesis and ABA biosynthesis. ABA might play critical roles in coordinating starch and protein synthesis during seed development.

Seed germination of *antisense AtGLR1.1* (a putative glutamate receptor in *Arabidopsis*) lines was strongly inhibited by 3% sucrose due to the elevated ABA content, while WT seeds germinated normally (Kang and Turano, 2003; Kang et al., 2004). The higher levels of ABA in the seeds of antisense *AtGLR1.1* lines compared to WT seeds indicate that AtGLR1.1 is a negative regulator of ABA biosynthesis during seed germination under high sucrose conditions. Interestingly, glucose does not demonstrate an inhibitory effect on seed germination of antisense *AtGLR1.1* lines, and only NO_3^- but not NH_4^+ can reverse this inhibition. ABA is accumulated in cucumber plants grown under low N conditions (Oka et al., 2012). Thus, AtGLR1.1 might function as a sensor of intracellular sucrose and NO_3^- status. A high C and low N status in plants might stimulate the biosynthesis and accumulation of ABA.

Roles of GABA shunt in balancing C and N metabolism

γ-Aminobutyric acid (GABA) is a conserved four-carbon non-protein amino acid (Bown and Shelp, 1997). In plants, GABA is mainly metabolized via a short GABA-shunt pathway composed of the cytosolic enzyme glutamate decarboxylase (GAD), the mitochondrial enzyme GABA transaminase (GABA-T) and succinic semi-aldehyde dehydrogenase (SSADH) (Fig. 2) (Bown and Shelp, 1997). GABA shunt is associated with various physiologic responses, including carbon fluxes into the TCA cycle and nitrogen metabolism. It was proposed that GABA could be involved in the control of C/N balance either by controlling glutamate availability (metabolic role) or via a direct interaction with glutamate receptors (signaling role) (Bouche and Fromm, 2004).

GABA modulates the activity of enzymes involved in primary nitrogen metabolism and nitrate uptake. Supplementing 1/8 strength MS medium with 50 mM GABA enhanced the activities of several enzymes involved in nitrogen and carbon metabolism including nitrate reductase (NR), glutamine synthetase (GS), glutamate synthase (NADH-GOGAT), and phospho*enol*pyruvate carboxylase (PEPCase) in *Arabidopsis* seedlings (Barbosa et al., 2010). Different from GAD in other organisms, plant GAD has been shown to bind calmodulin (CaM) (Baum et al., 1996). Dry seeds of transgenic *Arabidopsis* expressing a truncated *GAD* lacking the CaM binding domain accumulated considerable amounts of GABA, higher protein content, but lower amounts of the intermediates of glycolysis, and total fatty acids, indicating that GAD-mediated conversion of glutamate to GABA plays an important role in balancing C and N metabolism (Fait et al., 2011). However, molecular mechanisms underlying the regulation of C-N metabolism by GABA remain obscure. Investigating the endogenous and exogenous factors regulating the binding of CaM to GAD should provide insights for the functions of GABA shunt in regulating plant primary metabolism.

GABA is a neurotransmitter in animals with a clearly defined role in signaling (Storm-Mathisen, 1974). Hypothesis about the signaling role of GABA in controlling plant primary metabolism is supported by increasing evidences. Both GABA and glutamate (at 5 or 10 mM) stimulated growth of *Lemna*, while other amino acids had no effect (Kinnersley and Lin, 2000). In roots of *Brassica napus*, 100 μM GABA treatment induced a significant increase in abundance of mRNA for the BnNRT2 nitrate transporter, while other amino acids had either no effect or (in the case of Gln) had the opposite effect (Beuve et al., 2004). These results demonstrate that exogenous GABA has specific effects that extend beyond its role as N source. Although the contrasting effects on growth of duckweed by known agonists and antagonists of animal GABA receptors suggest the possible presence of GABA-responsive receptors in plants (Kinnersley and Lin, 2000), proteins highly homologous to the animal GABA receptors are not present in the *Arabidopsis* genome. It was speculated that GABA binds and modulates the activity of AtGLRs, because they possess a domain known to bind modulatory ligand molecules, which share structural homology with domains of several receptors including the $GABA_B$ receptors (Bouche and Fromm, 2004). Indeed, AtGLR1.1 regulated several key enzymes involved in the control of the

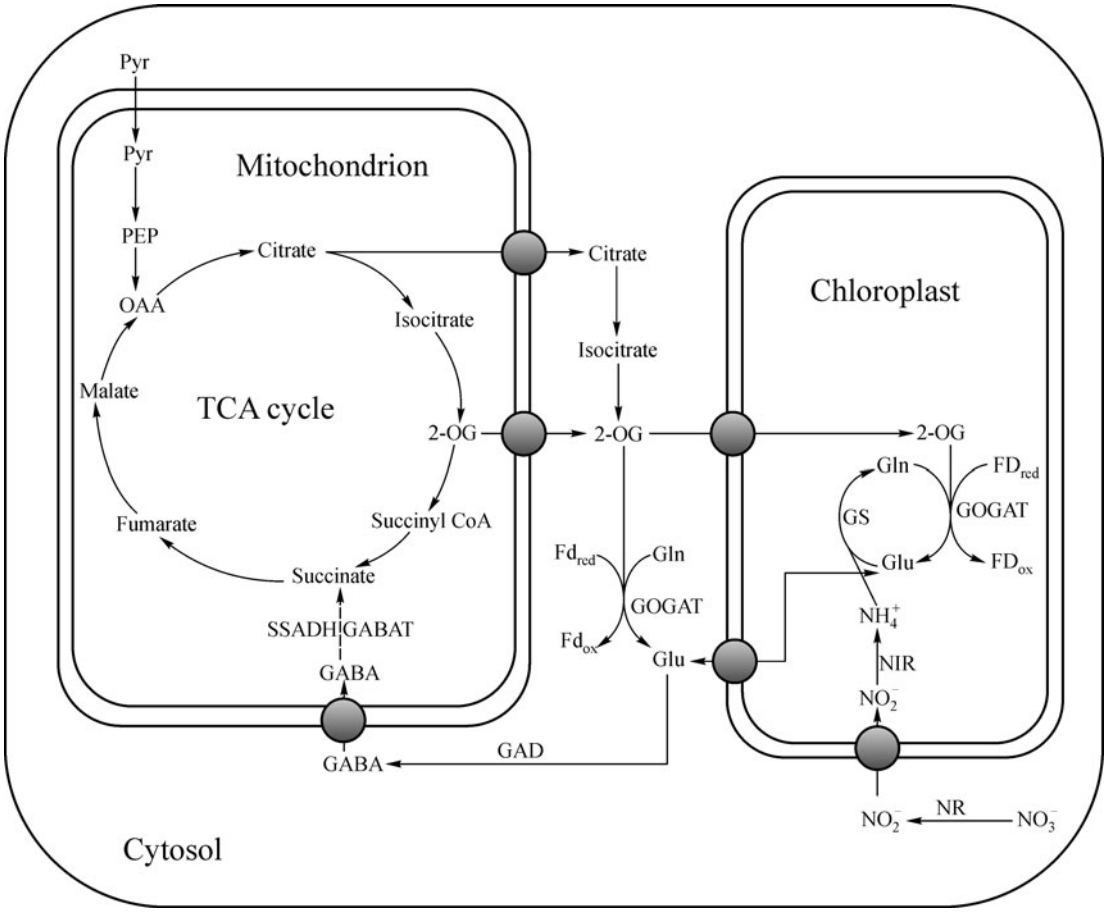

Figure 2 GABA shunt pathway is critical in coordinating C and N metabolism. Carbon skeletons used for N assimilation are mainly from TCA cycle in mitochondria, while chloroplast is the place in which N is assimilated into glutamate. Glutamate is decarboxylated into GABA by GAD in cytosol. GAD is the key limitation enzyme in the GABA shunt pathway. Regulation of GAD might be critical in balancing C and N metabolism.

C/N balance (Kang and Turano, 2003). However, more studies need to be performed to determine whether AtGLRs are GABA receptors.

Perspectives

Carbon (C) and nitrogen (N) metabolism are intimately linked. N deficiency decreases C assimilation, while C starvation reduces N utilization in plants. To engineer crops and cereals with higher photosynthesis rate under low N conditions, it is very necessary to understand the molecular mechanisms underlying the coordination between C and N metabolism. Though many integrators between C and N metabolism were identified, an integrated view of how plants control C and N metabolism coordinately, especially at the whole plant level, is still lacking. Emerging "omics" system analysis of plants treated with various C/N ratios should allow us to identify novel players or gene modules correlated to changes in C and N status. The identification and characterization of mutants with higher C/N ratio should shed new light on mechanism underlying high C assimilation under low N conditions. This knowledge will provide us molecular basis in engineering crops with higher yields under low N conditions.

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