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## Redox metabolism: the hidden player in C and N signaling?

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### Statement

This review highlights and discusses the role of redox metabolism in the regulation of nitrogen metabolism in response to both carbon and nitrogen in plants.

## Abstract

While decades of research have considered redox metabolism as purely defensive, recent results show that ROS are necessary for growth and development. Strikingly, close relationships were found between the regulation of nitrogen (N) metabolism and ROS in response to both carbon (C) and N availability. Root  $\text{NO}_3^-$  uptake and N metabolism have been shown to be regulated by a signal coming from the Oxidative Pentose Pathway in response to C signaling. As a major source of NADP(H), OPPP is critical to maintain redox balance under stress situations. Furthermore, recent results suggest that at least part of the regulation of root  $\text{NO}_3^-$  transporter by N signaling is also linked to the redox status of the plant. This leads to the question of a more general role of redox metabolism in the regulation of N metabolism by C and N. This review will (i) highlight the role of OPPP in C signaling and redox metabolism and (ii) the interaction between redox and N metabolism. We will then discuss how redox metabolism could be an important player in the regulation of N metabolism in response to C/N interaction and the implication for plants' adaptation to extreme environment and the development of future crops.

**Key words:** ROS, redox metabolism, nitrate uptake, carbon signaling, nitrogen signaling, carbon/nitrogen interaction

## Introduction

Nitrogen (N) metabolism in plants is tightly regulated by N and carbon (C) signaling. These signaling pathways target many genes of N transport and metabolism, and account (i) for the modulation of N use efficiency by both changes in external N availability, and variations of the organic N demand of the plant for growth (Crawford and Glass, 1998; Gojon *et al.*, 2009) and (ii) for the balance between photosynthate supply with N uptake and assimilation to ensure the production of amino acids (Vincentz *et al.*, 1993; Oliveira and Coruzzi, 1999; Stitt *et al.*, 2002; Lejay *et al.*, 2008). This interplay is nicely illustrated in the case of  $\text{NO}_3^-$  reductase (NR), the first step of the  $\text{NO}_3^-$  assimilation pathway, which catalyses the reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . NR is considered as a controlling step in  $\text{NO}_3^-$  assimilation and is a complex enzyme that is regulated by a number of different processes. Its expression is induced by  $\text{NO}_3^-$  and repressed by amino acids (Vincentz *et al.*, 1993), and the inductive action of  $\text{NO}_3^-$  is completely abolished during C starvation (Klein *et al.*, 2000; Matt *et al.*, 2002). The potential complexity underlying this scenario is illustrated by the fact that this enzyme is also subject to a complex regulation at the level of translation, protein degradation and protein phosphorylation (Lillo, 2008).

During the past decade, these regulatory mechanisms have been mainly studied at the level of root  $\text{NO}_3^-$  transporters and they depend on both local and long-range signaling pathways. On one hand,  $\text{NO}_3^-$  acts as a localized positive signal and relates to what is called the  $\text{NO}_3^-$  primary response (NPR). It is characterised by a rapid induction of root  $\text{NO}_3^-$  uptake and is associated with an increase in the expression of the root  $\text{NO}_3^-$  transporters *NRT2.1* and *NPF6.3* shortly after  $\text{NO}_3^-$  treatment (Medici and Krouk, 2014; Zhao *et al.*, 2018). On the other hand, the metabolites resulting from  $\text{NO}_3^-$  assimilation in leaves are involved in feedback repression that down-regulates root  $\text{NO}_3^-$  uptake and the  $\text{NO}_3^-$  transporters *NRT2.1*, *NRT2.2*, *NRT2.4* and *NRT2.5* under N satiety conditions (Lejay *et al.*, 1999; Zhuo *et al.*, 1999; Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). This repression is relieved when plants experience N limitation. For *NRT2.1*, split-root experiments have demonstrated that this regulation relies on systemic signaling pathways (Gansel *et al.*, 2001). Since C skeletons are essential for the incorporation of inorganic N into amino acids, the regulation of root  $\text{NO}_3^-$  uptake is linked to the production of sugar through photosynthesis in the leaves (Delhon *et al.*, 1996; Lejay *et al.*, 1999; Lejay *et al.*, 2003). It is characterized by a diurnal rhythm of  $\text{NO}_3^-$  uptake, with a peak during the light period and a minimum in the dark. But the dark-dependent decrease of  $\text{NO}_3^-$

uptake can be reversed by addition of sucrose. Moreover,  $\text{NO}_3^-$  uptake is enhanced by the  $\text{CO}_2$  level. At the molecular level, the regulation of root  $\text{NO}_3^-$  uptake by light and sugars has been correlated with changes in the expression of *NRT2.1*, *NRT2.4* and *NPF6.3* (Lejay *et al.*, 2008). Altogether, these regulations enable plants to activate genes involved in N-assimilation when C skeletons are abundant and internal levels of organic N are low, or to inhibit N assimilation when levels of photosynthate are low or internal levels of organic N are high. These data emphasize the importance of C/N ratio signaling in the regulation of N metabolism.

Concerning the molecular components of these signaling mechanisms, major breakthroughs have been made on local  $\text{NO}_3^-$  sensing and components of the long distance signaling of N and C status have also been found during the past few years. Interestingly, for both C and N long distance signaling, it is starting to reveal a possible close relationship with ROS and the oxidative status of the plant. Generally, the implication of ROS in cellular lifecycle can typically be divided in two different ways. First, ROS can accumulate to such levels that they generate a highly imbalanced redox status, leading to oxidative stress due to molecular and cellular damages (Schieber and Chandel, 2014). In another way, a more recent view of ROS homeostasis supports a major role for ROS in a large number of signaling pathways (Noctor *et al.*, 2018). However, these two aspects of ROS homeostasis, both leading to functional regulation of biological processes, may not be that easy to distinguish, especially in the case of the interaction with N metabolism or signaling pathways.

In this review, we highlight recent discoveries concerning the regulatory mechanism involved in long distance signaling for the regulation of N metabolism by C and N in connection with redox-related mechanisms. We then discuss how the oxidative status of the plant could be an important player to explain C/N signaling interactions and how it could help to explain plants' adaptation to extreme environment.

## **1. Regulation of N metabolism by C signaling**

### **Sugar sensing pathways for the regulation of N metabolism**

Sugars produced from plant photosynthesis are important signals controlling many aspects of plant metabolism and development (Rolland *et al.*, 2006; Eveland and Jackson, 2012). Although sucrose is the main sugar for systemic transport from source to sink in plants (Zhang *et al.*, 2014), many of the sugar responses observed in plants are channeled through invertases or sucrose synthases (Barratt *et al.*, 2009; Ruan, 2014) to generate glucose and

other signaling sugars. It then triggers signal transduction via direct perception by sensors or indirect signaling by energy and metabolite sensors. In plants, at least three different glucose signaling pathways have been documented:

(i) An hexokinase (HXK) dependent pathway, in which the regulation of gene expression by glucose requires HXK signaling function independently of its enzymatic activity (Moore *et al.*, 2003; Cho *et al.*, 2006; Granot *et al.*, 2014). Indeed, HXKs were the first demonstrated intracellular glucose sensors in plants and there is much available evidence that plant hexokinase-dependent glucose metabolism can be separated from hexokinase-dependent glucose signaling. For example, overexpressing yeast HXK2 in Arabidopsis causes a dominant negative effect by elevating HXK catalytic activity but reducing glucose repression of gene expression (Jang *et al.*, 1997). It shows that HXK is a dual-function enzyme with a distinct regulatory function not interchangeable between plants and yeast.

(ii) An HXK-independent pathway, in which glucose induction and repression of genes is observed independently of glucose metabolism, i.e. independently of sense and antisense overexpression of hexokinase (Price *et al.*, 2004; Rolland *et al.*, 2006). This pathway could be linked to RGS1 (regulator of G-protein signaling), a seven-transmembrane domain protein on the plasma membrane, which has been proposed to play a critical role as external glucose sensor in plants (Grigston *et al.*, 2008; Urano *et al.*, 2012).

(iii) A glycolysis-dependent pathway, that is this time dependent on the enzymatic activity of HXK, but not on its signaling function. It could involve an unknown metabolite(s) downstream HXK in the glycolytic pathway (Xiao *et al.*, 2000).

In addition to glucose, ample evidence indicates that sucrose itself is also perceived as a sugar signal, which cannot be substituted by glucose. The best-known example is the sucrose-specific down-regulation of *bvSUT1* (*Beta vulgaris* Sucrose Transporter 1), encoding the phloem-located proton-sucrose symporter, which is neither elicited by hexoses nor affected by mannoheptulose, a hexokinase inhibitor (Vaughn *et al.*, 2002). However, the mechanisms of sucrose perception remains unknown.

Concerning the regulation of N metabolism, specific investigations of sugar sensing have been conducted at the level of root  $\text{NO}_3^-$  transporters in Arabidopsis using *NRT2.1* as a model gene. It revealed that the regulation of root  $\text{NO}_3^-$  transporters by light and sugar was not related to HXK-sensing activity, but was dependent on a glycolysis-dependent pathway downstream HXK, as described above (Lejay *et al.*, 2003). To gain further insight on this sugar-signaling pathway, *NRT2.1* and *NPF6.3* were used as model genes to look for possible correlations between the level of C metabolites downstream of the HXK step in glycolysis

and gene expression across different treatments (Lejay *et al.*, 2008). It showed that upregulation of the  $\text{NO}_3^-$  transporters *NRT2.1* and *NPF6.3* is related to the concentration of glucose 6-phosphate (G6P) in the roots, a metabolite involved in the upper part of glycolysis. G6P is located at the cross road between three metabolic pathways, Glycolysis, Oxidative Pentose Pathway (OPPP) and Trehalose metabolism (Fig. 1). Trehalose 6-phosphate (T6P) levels has emerged as an essential signal metabolite in plants, linking growth and development to C availability (Lunn *et al.*, 2014). However, the addition of trehalose in the nutrient solution is not able to mimic the inductive effect of sucrose in the dark, while 6-aminonicotinamide (6-AN), an inhibitor of the OPPP has a strong inhibitory effect on *NPF6.3* and *NRT2.1* expression (Lejay *et al.*, 2008). These results strongly suggested that T6P is not involved and that sustained C flow through OPPP is required for sugar induction of both *NRT* genes. This result was confirmed by de Jong *et al.* (2014) and the role of OPPP in C signaling has then been extended to the regulation of N metabolism. Indeed, the use of a knock-down mutant for *PGL3*, the plastidic-localized OPPP enzyme 6-phosphogluconolactonase 3, revealed that *NR* and *NiR* genes expression are no more induced by sugar in this mutant compared to wild-type plants (Bussell *et al.*, 2013). Interestingly, the link in roots was found between OPPP and the regulation by sugars of transporters involved in N or S acquisition, two metabolisms requiring the reducing power produced by the OPPP for nitrite reductase, GOGAT and the assimilation of  $\text{SO}_4^{2-}$  into Cys (Oji *et al.*, 1985; Bowsher *et al.*, 1989; Bowsher *et al.*, 1992; Neuhaus and Emes, 2000; Yonekura-Sakakibara *et al.*, 2000; Kopriva and Rennenberg, 2004). As a consequence, OPPP and N or S assimilation are tightly coordinated processes. In particular, genes encoding OPPP enzymes are among those most affected by  $\text{NO}_3^-$  signaling in Arabidopsis roots (Wang *et al.*, 2000; Wang *et al.*, 2003) and  $\text{NH}_4^+$  can induce an isoform of G6P dehydrogenase in barley (*Hordeum vulgare*) roots (Esposito *et al.*, 2001). Altogether, these results support the existence of a common OPPP-dependent sugar signaling mechanism for regulation of N and S metabolism, which would coordinate the availability of all three amino acid components (C, N and S) for adequate amino acid synthesis.

### **Candidate signaling mechanisms linked to the Oxidative Pentose Phosphate Pathway**

The OPPP is an ubiquitous pathway present in all eukaryotes and most bacteria (Kruger and von Schaewen, 2003). It divides into an oxidative and non-oxidative branch. The oxidative branch is highly active in most eukaryotes and converts G6P into carbon dioxide, ribulose 5-phosphate and as a byproduct  $\text{NADP}^+$  is reduced to NADPH. The non-oxidative branch



metabolizes the glycolytic intermediates fructose 6-phosphate and glyceraldehyde 3-phosphate as well as sedoheptulose sugars, yielding ribose 5-phosphate for the synthesis of nucleic acids and sugar phosphate precursors for the synthesis of amino acids (Stincone *et al.*, 2015) (Fig. 2). Whereas the oxidative branch is considered unidirectional, the non-oxidative branch can supply glycolysis with intermediates derived from ribose 5-phosphate and *vice versa*, depending on the biochemical demand. The initial reactions of the OPPP are carried out by glucose-6P dehydrogenase (G6PDH) producing 6-phospho-glucono lactone, which is then hydrolyzed by a 6-phosphogluconolactonase (6PGL) to 6-phosphogluconic acid. Then, the phosphogluconic acid dehydrogenase (6PGDH) detaches a CO<sub>2</sub> molecule, reducing a NADP<sup>+</sup> to NADPH, thus forming ribose-5P (Esposito, 2016). In plants, the OPPP subcellular localization suggests a complex network of coordination of C pathways in cells. Although the cytosolic OPPP cycle represents the major part of the measured activity, the existence of a complete OPPP confined in the plastidial compartment has been widely demonstrated (Esposito, 2016).

Concerning the signaling mechanism linked to the OPPP three hypothesis can be made: (i) one of the C metabolites generated through the OPPP could play the role of a signal molecule; (ii) an enzyme of the OPPP could generate a signal like HXK in glycolysis and (iii) the reducing power produced by the OPPP could be involved in redox regulation of N metabolism via, for example, a NADPH-dependent signaling pathway. The results obtained by de Jong *et al.* (2014), showing that OPPP metabolism is required for Glc-mediated *NRT2.1* expression and can be rescued by OPPP-derived amino acid precursor metabolites, such as pyruvate, in an HXK mutant, suggest that C-metabolites or an enzyme of the OPPP could be involved. However, more recently, *NRT2.1* expression has been shown to be regulated by the redox status of the plant suggesting that the production of reducing power by OPPP could be involved in the C signaling pathway (Bellegarde *et al.*, 2019). This mechanism could ensure the coordination of root NO<sub>3</sub><sup>-</sup> uptake with the production of reducing equivalents required for assimilating N into amino acids. Interestingly, the tight relationship between OPPP and N metabolism in plants has been found mainly at the level of G6PDH. Indeed, the promoter sequences of NiR and G6PDH all present the same NIT-2 motif, a N metabolism regulating factor (Neuhaus and Emes, 2000), while GOGAT activity in roots is clearly supported by plastidic G6PDH, which is able to satisfy the increased request of reducing power upon ammonium assimilation (Bowsher *et al.*, 1992; Esposito *et al.*, 2001; Esposito *et al.*, 2003; Esposito *et al.*, 2005). In the OPPP, G6PDH is considered as the rate-limiting enzyme and it determines the level of NADPH by controlling the metabolism of glucose via OPPP. The



importance of the NADPH-producing function of the OPPP is critical to maintain redox balance under stress situations. As such, it has been suggested that this pathway represent a true metabolic sensor during response to oxidative stress and it has been implicated in several human diseases such as neurodegeneration (Alzheimer's disease) and cancer (Kruger *et al.*, 2011; Stincone *et al.*, 2015). In plants, the possible physiological and biochemical roles played by OPPP and namely G6PDH have been demonstrated upon cold stress (Van Heerden *et al.*, 2003; Lin *et al.*, 2013), heat (Gong *et al.*, 2012), metal pollution (Xu *et al.*, 2003; Devi *et al.*, 2007; Pérez-Chaca *et al.*, 2014; Corpas *et al.*, 2016), drought (Liu *et al.*, 2013; Landi *et al.*, 2016) and salinity (Cardi *et al.*, 2015). In yeast, it has been shown that oxidative treatments induce OPPP enzymes on transcript and protein levels (Godon *et al.*, 1998; Shenton and Grant, 2003; Chechik *et al.*, 2008). The benefits of this metabolic transition are explained by the production of NADPH in the OPPP, the primary redox-cofactor for glutathione synthesis and for enzymes of the antioxidative machinery (Juhnke *et al.*, 1996; Pollak *et al.*, 2007; Grant, 2008). Cells with an increased influx to the OPPP have a higher NADPH/NADP<sup>+</sup> ratio and are more resistant to oxidants (Ralsler *et al.*, 2007). On the opposite, deletion of G6PDH causes hypersensitivity to multiple oxidants in various organisms (Juhnke *et al.*, 1996; Ho *et al.*, 2000).

Concerning the implication of the OPPP in the regulation of root NO<sub>3</sub><sup>-</sup> uptake, the impact of 6-AN on *NPF6.3* and *NRT2.1* regulation could indicate a role of G6PDH and thus of the redox status of the plant in this regulatory mechanism (Lejay *et al.*, 2008). Indeed, 6-AN is an inhibitor of both G6PDH and 6PGDH, the two steps producing NADPH in the OPPP (Kohler *et al.*, 1970; Rawat *et al.*, 2012). For the regulation of root NO<sub>3</sub><sup>-</sup> transporters by C, the implication of the redox status of the plant is also supported by the recent discovery of the role of the transcription factor Elongated Hypocotyl 5 (HY5) in the regulation of *NRT2.1* expression. HY5 is a shoot-to-root phloem-mobile signal and it mediates the regulation of root growth and NO<sub>3</sub><sup>-</sup> uptake by light (Chen *et al.*, 2016). In the shoot, HY5 promotes C assimilation and translocation, whereas in the root it mediates the activation of *NRT2.1* expression, supporting the fact that it coordinates plant nutrition and growth in response to fluctuating light environments. Furthermore, HY5 regulates the sucrose metabolism and sucrose movement into phloem cells for shoot-to-root translocation by increasing the expression levels of *SWEET11* and *SWEET12*, two genes encoding sucrose efflux transporters required for sucrose phloem loading (Chen *et al.*, 2012; Chen *et al.*, 2016). The signaling pathways involving HY5 remain largely unknown but it has been shown that HY5 can directly bind the G-box element present in the promoters of ROS-responsive genes to regulate

deetiolation in response to light and ROS (Chen *et al.*, 2013). It revealed that HY5 can mediate crosstalk between light and ROS-signaling and thus could be involved in a cross-talk between sugar and the redox status of the plant for the regulation of *NRT2.1* by C through OPPP. However, the regulatory mechanisms involving the OPPP could be more complex. Indeed, Bussel *et al.* (2013) found that the induction of N assimilation genes by sucrose is affected when the step after G6PDH is mutated in the knock-down mutant *6pgl3*. Furthermore, G6PDH activity was found higher in *6pgl3* compared to the WT and no significant differences were detected in the NADPH/NADP<sup>+</sup> ratio (Xiong *et al.*, 2009). Altogether, these results suggest that for N assimilation genes, the OPPP-derived signal is independent of G6PDH and of the production of reducing power. Unfortunately, the conditions used by Bussel *et al.* (2013) did not allow to determine the impact of *6pgl3* mutation on the induction of root NO<sub>3</sub><sup>-</sup> transporters by sugars. However, it cannot be excluded that several signaling pathways linked to OPPP co-exist in plants. Indeed, as demonstrated in yeast, the deletion of a nonoxidative PPP enzyme decreased oxidant sensitivity in a yeast strain that cannot use the PPP for NADP<sup>+</sup> reduction. *Vice versa*, increased flow into the non-oxidative PPP via the expression of a foreign enzyme, which generates the PPP intermediate sedoheptulose-7P from the non-PPP metabolite sedo-heptulose, increased yeast's H<sub>2</sub>O<sub>2</sub> tolerance (Kruger *et al.*, 2011). Altogether, these results support the existence of a second, NADPH-independent function of the PPP in the stress response.

## 2. Regulation of N metabolism and signaling by redox-related mechanisms

N metabolism in plants is central and limiting for growth, biomass accumulation and development. It is therefore connected to many facets of primary and secondary metabolisms in plants and it is finely tuned by the N demand of the whole organism (Ruffel *et al.*, 2014). Over the past few years some components of the systemic N signaling pathway involved in the regulation of root NO<sub>3</sub><sup>-</sup> transporters have started to be identified and are related to redox signaling mechanisms.

A forward genetic screen identified HIGH NITROGEN INSENSITIVE 9 (HNI9), a chromatin modifier, as a factor involved in the repression of *NRT2.1* expression and NO<sub>3</sub><sup>-</sup> uptake under high N provision (Widiez *et al.*, 2011). Indeed, *NRT2.1* expression is partly repressed in a *hni9* mutant background under high N provision. Interestingly, it has been now demonstrated that HNI9, in cooperation with the transcription factor ELONGATED HYPOCOTYL 5 (HY5), positively regulates the expression of a set of detoxification genes

induced under high N, which suggests that a high N environment leads to an increase in ROS content. Indeed, ROS strongly accumulate in *hni9* and *hy5* mutants due to the mis-expression of detoxification genes (Fig. 3A) (Bellegarde *et al.*, 2019). Further experiments using ROS treatments or ROS scavenging molecules showed that *NRT2.1* level of expression is in fact correlated with a higher level of ROS (Jung *et al.*, 2018).

It is known for decades that N deprivation leads to the accumulation of ROS, especially in the roots (Shin *et al.*, 2005; Kong *et al.*, 2013). Indeed, starvation in most of the major nutrients induces an elevation in the production of H<sub>2</sub>O<sub>2</sub>, but the ROS accumulation might be the highest in the case of plants that are deprived of N (Shin *et al.*, 2005). The accumulation of ROS in the roots of N-deprived plants occurs mainly in epidermis and cortical cells, which are interestingly the site of N uptake and N root assimilation, and is visible from the elongation zone to more mature zones of the root. The origin and the function of this accumulation of ROS under N starvation is not clearly understood, although it may be tempting to make a link between the role of N in growth and development, and the fact that ROS can mediate signaling events to regulate growth and development, especially in roots (Foreman *et al.*, 2003). More recently, several reports have linked ROS accumulation, as well as effectors of ROS metabolism, with downstream signaling events of the N starvation response. Indeed, treatment of Arabidopsis N-starved plants with ROS scavenging molecules inhibits the induction of *NRT2.4* and *NRT2.5*, which are molecular markers of the N starvation response in plants, and essential actors of plant survival under N starvation (Safi *et al.*, 2018). ROS accumulation during N starvation response in plants might be, at least partly, under the control of the HHO family of transcription factors, which induce the expression of a set of genes related to ROS homeostasis under N satiety. Another study revealed that shoot-derived polypeptides named CEPD1 and CEPD2 act as descending long-distance mobile signals for the regulation of *NRT2.1* (Ohkubo *et al.*, 2017). They upregulate the expression of the NO<sub>3</sub><sup>-</sup> transporter gene *NRT2.1* in roots specifically when NO<sub>3</sub><sup>-</sup> is present in the rhizosphere and Arabidopsis plants deficient in this pathway show impaired systemic N acquisition response along with N-deficiency symptoms. Interestingly, the genes coding for CEPD1 and CEPD2 are assigned to the plant-specific class III glutaredoxins (Grxs) family, which are involved in redox regulation (Rouhier *et al.*, 2006). Grxs are small oxidoreductase enzymes that can reduce disulfite and Cys-glutathione bonds within target proteins, and are thus potential actors in detoxification pathways due to their redox buffering capacities (Rouhier *et al.*, 2008). The Grx isoforms belonging to class III are specific to land plants and from genetic analyses, it appears that class III Grxs isoforms, also named CC-type Grxs or

ROXY, play a role in plant development and in pathogen defense mechanisms (Meyer *et al.*, 2012; Couturier *et al.*, 2013). Several class III GRXs are transcriptionally regulated by  $\text{NO}_3^-$  variations, and several members of this family of 21 homologues emerged as important factors of the plant responses to  $\text{NO}_3^-$  variation. *AtGRXS3/4/5/8* expression is induced in response to  $\text{NO}_3^-$  resupply after starvation, and their induction leads to the repression of primary root growth (PRG), which corresponds to one of the developmental events, aiming at reshaping root system architecture under  $\text{NO}_3^-$ -sufficient soil environment (Patterson *et al.*, 2016). In addition, expression of others *AtGRXs*, among them *CEPD2* (also known as *AtGRXC13* and *ROXY9*), is up-regulated by  $\text{NO}_3^-$  starvation (Jung *et al.*, 2018). Interestingly, the functions of CC-type GRXs have been related to their ability to interact with TGA transcription factors, likely regulating their redox states. In  $\text{NO}_3^-$  metabolism, a functional connection between *AtGRXS3/5/5/8* (*ROXY11* to *ROXY15*) with TGA1 and TGA4 seems likely to regulate primary root growth in response to  $\text{NO}_3^-$  (Patterson *et al.*, 2016). Furthermore, since TGA1 and TGA4 have been involved in the regulation of *NRT2.1* in response to  $\text{NO}_3^-$ , TGAs and Grxs could be a module involved in both the regulation of root  $\text{NO}_3^-$  transporters and root development in response to N availability (Alvarez *et al.*, 2014). Collectively, these studies shed light on Grxs as small oxidoreductase enzymes acting in different facets of N signaling. Although a formal role involving their redox buffering properties is still required, it supports a close interaction between the plant redox properties and the regulation of growth and development through N availability (Fig. 3B).

Therefore, the association between ROS and N metabolism appears under different physiological condition, reinforcing the idea that N metabolism and ROS homeostasis are intrinsically linked, especially for the regulation of  $\text{NO}_3^-$  uptake genes. A correlation between ROS levels and  $\text{NO}_3^-$  assimilation has also been made, as ROS levels increase in an *Arabidopsis nia1 nia2* mutant showing a complete absence of  $\text{NO}_3^-$  reductase activity (Pan *et al.*, 2019). Altogether, these observations tend to conclude that ROS homeostasis is just as much affected by N metabolism that the latter is modulated by ROS levels or signaling. Indeed, N starvation and N excess both leads to ROS accumulation, and normal physiological conditions for cellular ROS levels take place in between, in intermediate nutritional conditions. This consideration is in fact not restricted to plants, as in animals limited or excessive nutrition generate abnormal ROS accumulation, which can be considered as pathologic in certain situations (Gorlach *et al.*, 2015).

### 3. ROS signaling at the nexus of C/N regulations

In plants, C and N metabolisms need to be tightly coordinated for C-N-containing metabolite synthesis at the biochemical level and for long distance signaling for the C/N balance. Indeed, the process of N assimilation, especially when  $\text{NO}_3^-$  is its main source, is energetically costly and requires large amount of ATP and C-skeletons produced from the C metabolic processes (Foyer *et al.*, 2011). On the other hand, large amounts of N are essential for plant photosynthetic machinery and for  $\text{CO}_2$  assimilation. If the existence of interactions between C and N signaling has been well described for the regulation of gene expression, the molecular mechanisms involved remain mostly unknown (Palenchar *et al.*, 2004; Gutierrez *et al.*, 2007). Interestingly, the recent advances on long distance signaling for C and N presented above bring more and more evidence that modulation of the redox status of the plant might be an important signaling mechanisms for the coordination between C and N assimilation. Indeed, all the elements presented are involved in redox signaling and HY5, beyond its role in C and redox signaling, has been found to regulate many N related genes in a N concentration dependent manner (Huang *et al.*, 2015). These recent results build on several reports indicating that the redox status plays an important role in the integration of C and N metabolism. For example, studies of the *Nicotiana sylvestris* CMSII mutant, which lacks complex I functionality, demonstrated that the adjustment of respiratory pathways was associated with alterations of foliar CN balance, as well as with increases in  $\text{NAD}^+$  and  $\text{NADH}$  levels. This indicates that  $\text{NADH}$  availability is a critical factor influencing  $\text{NO}_3^-$  assimilation and in coordinating ammonia assimilation with the production of C skeletons (Dutilleul *et al.*, 2005). Another study, wherein plants were subjected to different levels of N and varying atmospheric  $\text{CO}_2$  concentrations, revealed that  $\text{NAD}^+$  and  $\text{NADH}$  levels were highly integrated with those of glutamate, glutamine, asparagine and arginine in N metabolism, suggesting a potential link between nutritional status and  $\text{NAD}$ -dependent signaling (Hager *et al.*, 2010).

There is a longstanding recognition that in addition to being an intrinsic part of metabolism, redox status plays an active role in metabolic regulation (Geigenberger and Fernie, 2014). However, despite intense research activities, the exact mechanism underlying the signaling and its specificity is still unclear. ROS have received great attention and accumulating evidences suggest that  $\text{H}_2\text{O}_2$  is an active signaling molecule and that its accumulation through redox sensing leads to variety of cellular responses (Bhattacharjee, 2005; del Rio *et al.*, 2006; Halliwell, 2006). However, if  $\text{H}_2\text{O}_2$  accumulates at a high level it becomes toxic and a



sensitive regulation of its level is therefore important for its signaling function and detoxification (Apel and Hirt, 2004). The ascorbate (Asc)/glutathione (GSH) cycle forms the main H<sub>2</sub>O<sub>2</sub>-detoxification system in plants and it uses NADPH, produced in the roots by the OPPP, to regenerate the oxidized form of GSH (Noctor and Foyer, 1998). Interestingly, the increased level of H<sub>2</sub>O<sub>2</sub> has been recently associated with the induction of *NRT2.1* in response to high N in the *hni9* mutant (Bellegarde *et al.*, 2019). Furthermore, treatments with Asc cause a decrease in *NRT2.1* expression, while H<sub>2</sub>O<sub>2</sub> leads to an increase in *NRT2.1* transcript level, like what is observed in the Asc-deficient mutant, *vtc2* (Bellegarde *et al.*, 2019). Altogether, these results could explain a potential link between the NADPH produced by OPPP in the roots, which is regulated by both C and N level (Esposito, 2016) and the H<sub>2</sub>O<sub>2</sub>-detoxification system for the regulation of *NRT2.1* by C/N signaling and ROS production. Unfortunately, this potential “sensing” mechanism cannot explain the specificity of the regulations linked to C and N signaling. Indeed, GSH and Asc are considered to be unspecific reducer molecules because of their small molecular masses and the Asc/GSH cycle is induced by all kind of stresses affecting ROS level. However, plants have also complex enzymatic antioxidant systems composed of thioredoxins (TRX) and glutaredoxins (GRX), known under the name of redoxins (RX) (Serrato *et al.*, 2013). On the contrary to GSH and Asc, surface topology of RX allows specific target interactions. The major function of RX is to reduce disulfide bridges in target proteins so that its own active site becomes oxidized to a disulfide. For a new catalytic cycle, oxidized RXs are then reduced in a reaction catalyzed by NADPH-dependent thioredoxin reductases (NTR) for TRX or NADPH-dependent glutathione reductases (GR) for GRX (Meyer *et al.*, 2012). In non-green tissue, these reactions depend on the NADPH produced by the OPPP. TRX and GRX are able to reduce different types of oxidized thiols and generally use distinct reduction mechanisms. However, although certain targets are highly specific, TRX and GRX targets overlap, thereby enabling the development of interaction networks and strong buffering capacity. In Arabidopsis, the number of TRX, TRX-like proteins or proteins with TRX-domains have risen to 44 members (Meyer *et al.*, 2012), many of them without any assigned function. TRX are classified, according to their subcellular location and sequence similarity, into 15 subgroups (Meyer *et al.*, 2012). The classification of vascular plant GRX is more difficult to establish than that of the TRX. In Arabidopsis, there are 31 GRX genes that can be classified into three distinct subgroups based on the amino acid sequences at their active sites: the CPYC, CGFS and CC-type GRX classes (Meyer *et al.*, 2012). Out of the 31 GRXs in Arabidopsis, 21 GRXs belong to the CC-type, now named ROXY, which is specific for land plants, while the other two GRX classes,

CPYC and CGFS, are common to eukaryotes and prokaryotes (Rouhier *et al.*, 2006; Jung *et al.*, 2018). Although the roles of GRXs have been associated mainly with oxidative stress, there is growing evidence that GRXs and especially CC-type GRXs also play important roles in cell signaling and development. Interestingly, several CC-type glutaredoxins (ROXY) have already been involved in plant response and signaling under  $\text{NO}_3^-$  starvation as described above (Ohkubo *et al.*, 2017; Jung *et al.*, 2018). Furthermore,  $\text{H}_2\text{O}_2$  treatments have been shown to differentially regulate the expression of the ROXYs suggesting also the involvement of ROS in response to  $\text{NO}_3^-$  deficiency, in agreement to what was found for *NRT2.1* regulation (Jung *et al.*, 2018; Bellegarde *et al.*, 2019). Interestingly, TRXs are also involved in the regulation of C allocations by C signaling through the redox regulation of AGPase, a key enzyme for starch synthesis. Indeed, the redox regulation of AGPase is mediated, in roots, by the bifunctional protein NADP-thioredoxin reductase C (NTRC) (Michalska *et al.*, 2009). NTRC is a bimodular enzyme directly catalyzing the transfer of reducing groups from NADPH to TRX and from TRX to target proteins. Based on the biochemical properties of NTRC, including its high affinity for NADPH produced from sugars by the OPPP and the presence of a TRX domain at the C terminus, it has been proposed that NTRC in roots act as a redox switch able to convert reducing power in the form of NADPH into a redox signal through the thiol groups of its TRX domain (Kirchsteiger *et al.*, 2012). Thus NTRC may constitute a direct pathway for redox homeostasis in heterotrophic plastids and is a very interesting candidate to explain the link between C/N signaling, the level of ROS and NADPH produced by the OPPP for the regulation of root  $\text{NO}_3^-$  transporters. There is now a need for focused genetic studies to dissect the role of the different members of the thioredoxin and glutaredoxin gene families in the regulation of CN metabolism. In particular, the identification of specific targets of NTRC and the different TRXs or root plastids will help to establish the metabolic processes that depend on these enzymes. It is also interesting to note that transcription factors, such as HY5, can induce different genes depending on the signal leading to ROS accumulation. Indeed, HY5 has been involved in the control of ROS homeostasis in response to light and cold treatments but the set of genes induced by HY5 to balance ROS levels seems to differ from one stimulus to another (Catala *et al.*, 2011; Chen *et al.*, 2013; Chai *et al.*, 2015). Furthermore, Bellegarde *et al.* (2019) found that the genes involved in the ROS detoxification network in response to high N was dependent on HY5 but independent on the presence of a HY5 binding site in their promoter. It suggests that HY5 may also function as an indirect regulator of other transcription factors, which could specify the response associated to the level of ROS according to the environmental signals.



#### 4. Conclusions and perspectives

The recent developments we discussed in this review show that the redox status clearly plays a central role in the regulation of N metabolism and especially of root  $\text{NO}_3^-$  transporters in response to C and N signaling. These results offer new hypotheses for a comprehensive understanding of the regulatory mechanisms associated to C/N interactions. Indeed, as summarized in Fig. 4, the level of ROS could be a way to integrate C/N level and regulate root  $\text{NO}_3^-$  transporters through either (i) the activation of the OPPP and/or the production of NADPH, which could activate a signaling pathway linked to NTRC or (ii) the direct activation, by other yet unknown mechanisms of transcription factors such as HY5 and/or GRXs such as the CEPDs. However, further work is now required to resolve the network of redox signaling linked to CN balance in order to get a comprehensive understanding of both the control of redox by C and N and conversely, the redox control of N metabolism. A major question also concerned the targets of candidates GRXs and TRXs for C and N signaling. This will require genetic and metabolomics approaches as well as the application of techniques to analyze redox states at the subcellular level.

The role of redox metabolism in the regulation of N metabolism has far reaching implications in the context of climate change. Indeed, future crop plants will need to be more performant under increasingly frequent and severe growth conditions, which will likely impact the redox status of the plants. Furthermore, the use of fertilizer along with water and pesticide will be increasingly constrained. In this context, plants adapted to extreme environments such as deserts become an interesting tool. Indeed, despite extreme water deficit, nutrient poor soils and high light radiation diverse plant species are able to grow in those environments. The identification of the genes and mechanisms that allow these “extremophiles” to grow could help us to understand how redox metabolism has adapted and would allow new strategies for the selection of future crops.

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## Figure Legends

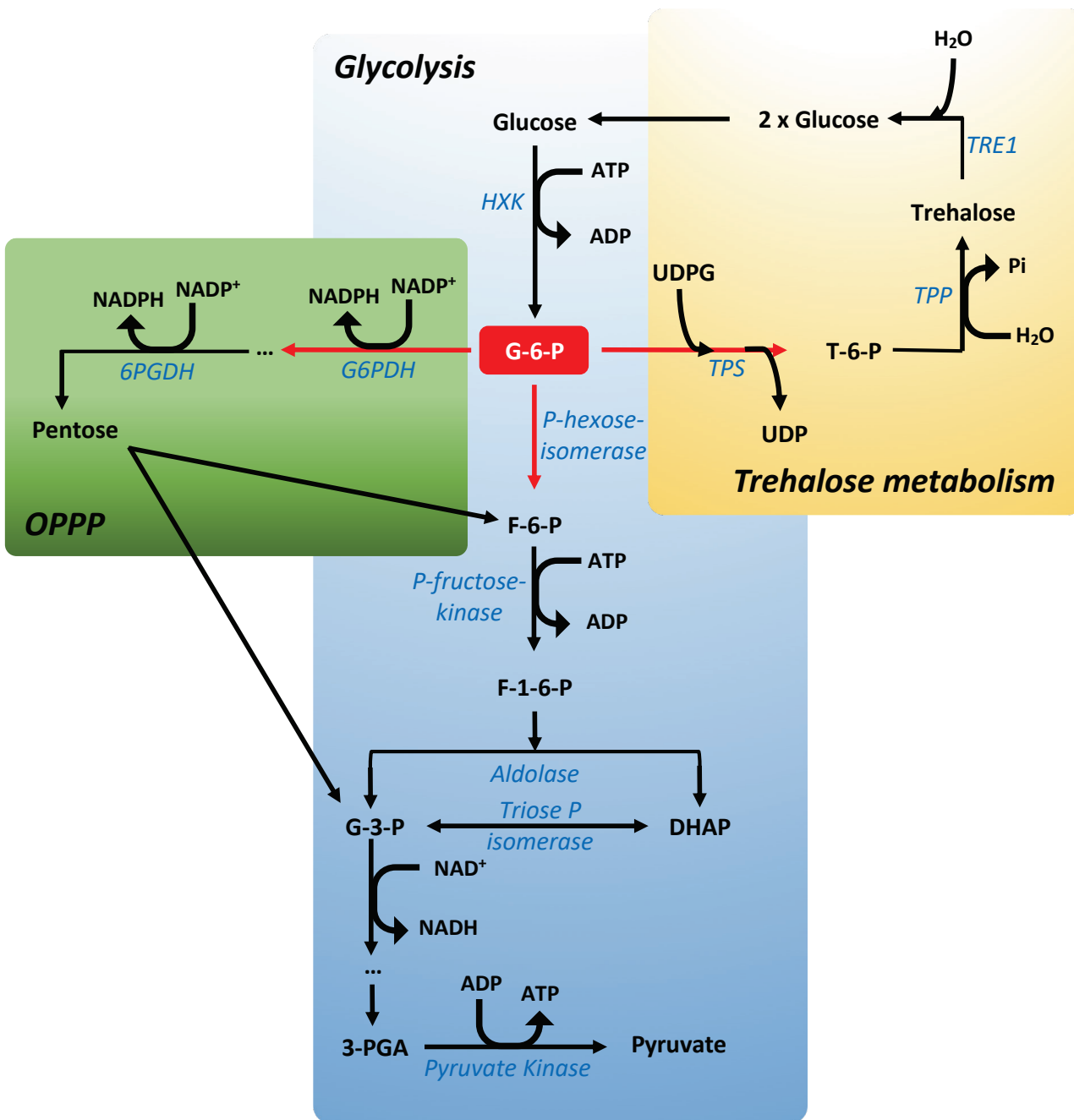
**Fig. 1.** Schematic representation of the fate of G6P in the OPPP, glycolysis and trehalose metabolism. Abbreviations are as follow: TPS, trehalose-6-phosphate synthase, TPP, trehalose-6-phosphate phosphatase, TRE1, Trehalase, G6PDH, Glucose-6-phosphate dehydrogenase, 6PGDH, 6-phosphogluconate dehydrogenase, F6P, Fructose-6-phosphate, F-1-6-P, Fructose-1-6-biphosphate, G3P, Glyceraldehyde-3-phosphate, DHAP, Dihydroxyacetone phosphate, 3-PGA, 3-phosphoglycerate, T6P, Trehalose-6-phosphate.

**Fig. 2.** Schematic representation of the pentose phosphate pathway (PPP). The oxidative phase of PPP is represented in blue and the non-oxidative phase of PPP is represented in gray. The oxidative phase transforms glucose into pentose and produces two molecules of NADPH. The non-oxidative phase transforms pentose into C metabolites able to feed-back glycolysis. The activity of G6PDH is regulated by the ratio between NADPH and  $\text{NADP}^+$ , which depends on the level of ROS. The blue arrow and red line represent positive and negative control, respectively. Abbreviations are as follow: G6P, glucose-6-phosphate, G6PDH, glucose-6-phosphate dehydrogenase, 6PGL, 6-phosphogluconolactonase, 6PGDH, 6-phosphogluconate dehydrogenase.

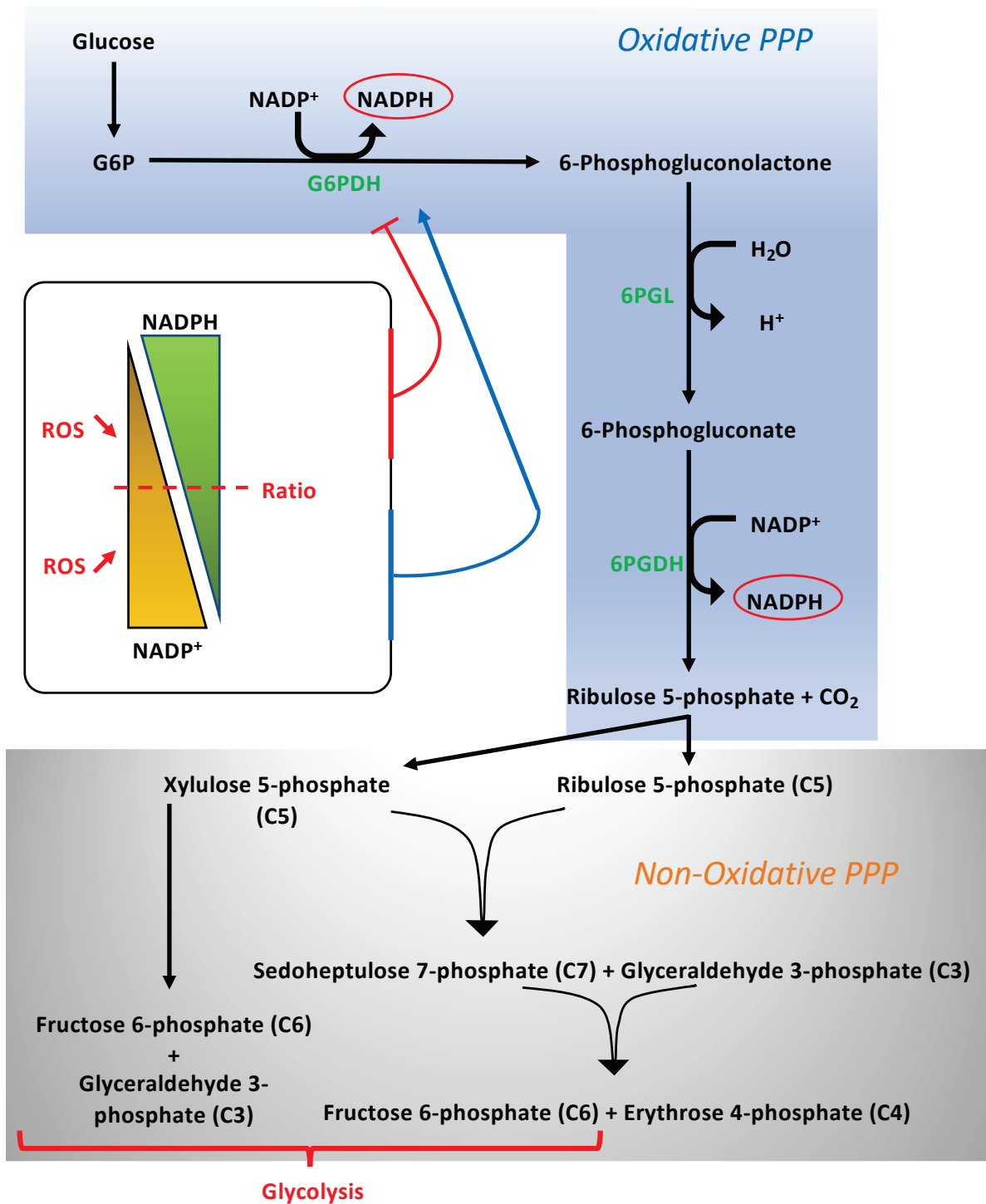
**Fig. 3.** Schematic representation of possible signaling pathways for the regulation of root *NRT2* transporters and root growth by N and ROS. (A) High N conditions ( $10\text{mM NH}_4\text{NO}_3$ ) stimulates the accumulation of ROS, which is involved in the induction of *NRT2.1* expression. However, in the same time the increase of ROS accumulation and/or  $\text{NH}_4\text{NO}_3$  induce the expression of *HY5* as well as its possible interaction with *HNI9*, which are both involved in the induction of the expression of detoxification genes. Thus, collectively, these regulations maintain *NRT2.1* expression at a low level in response to high N (Bellegarde *et al.*, 2019). (B) Low N conditions also induce ROS accumulation. It results in the induction of the activity of specific glutaredoxins (*GRXS* or *GRXC*) involved in the regulation of primary root growth (PRG) (Patterson *et al.*, 2016) and of *NRT2.1* expression (Jung *et al.*, 2018). This regulatory mechanisms could also be involved in the regulation of *NRT2.4* and *NRT2.5* by ROS accumulation in response to low N conditions. Blue arrows and red lines represent positive and negative control, respectively. Plain lines represent regulatory mechanisms, which have already been described, while dashed lines are hypothesis based on the analysis of published data.

**Fig. 4.** Model for the regulation of root  $\text{NO}_3^-$  transporters by C/N interactions through the level of ROS. Plain lines represent regulatory mechanisms, which have already been described, while dashed lines are hypothesis based on the analysis of published data. Abbreviations are as follow: OPPP, oxydative pentose phopshate pathway, NTRC, NADPH thioredoxin reductase C, Trxs, thioredoxins, CC-Grxs, CC-glutaredoxins, TFs, transcription factors.

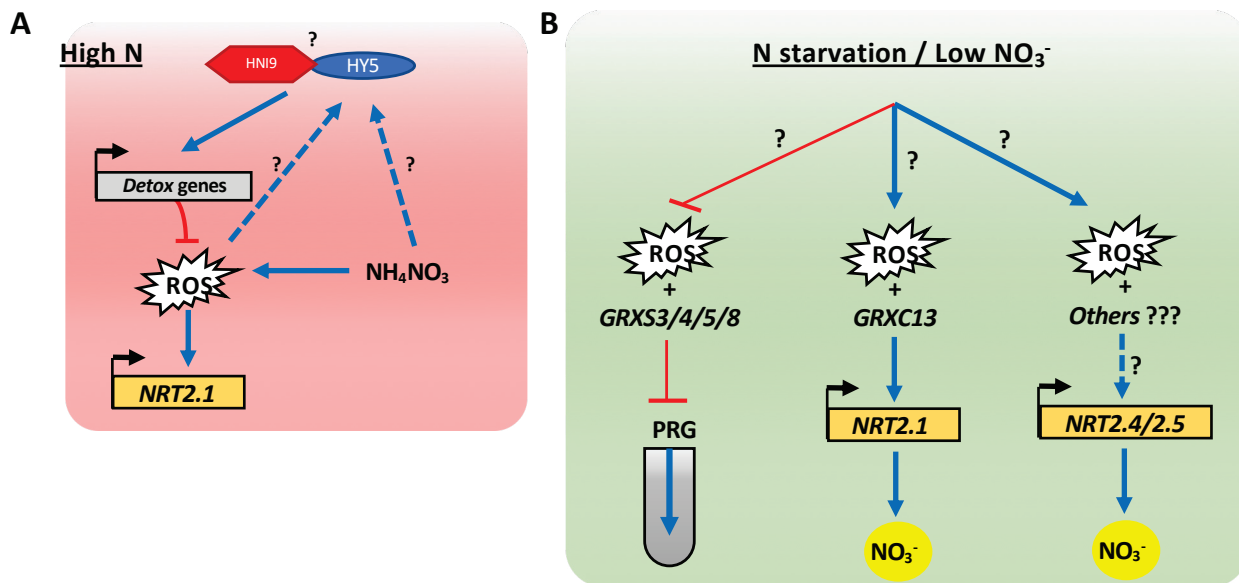
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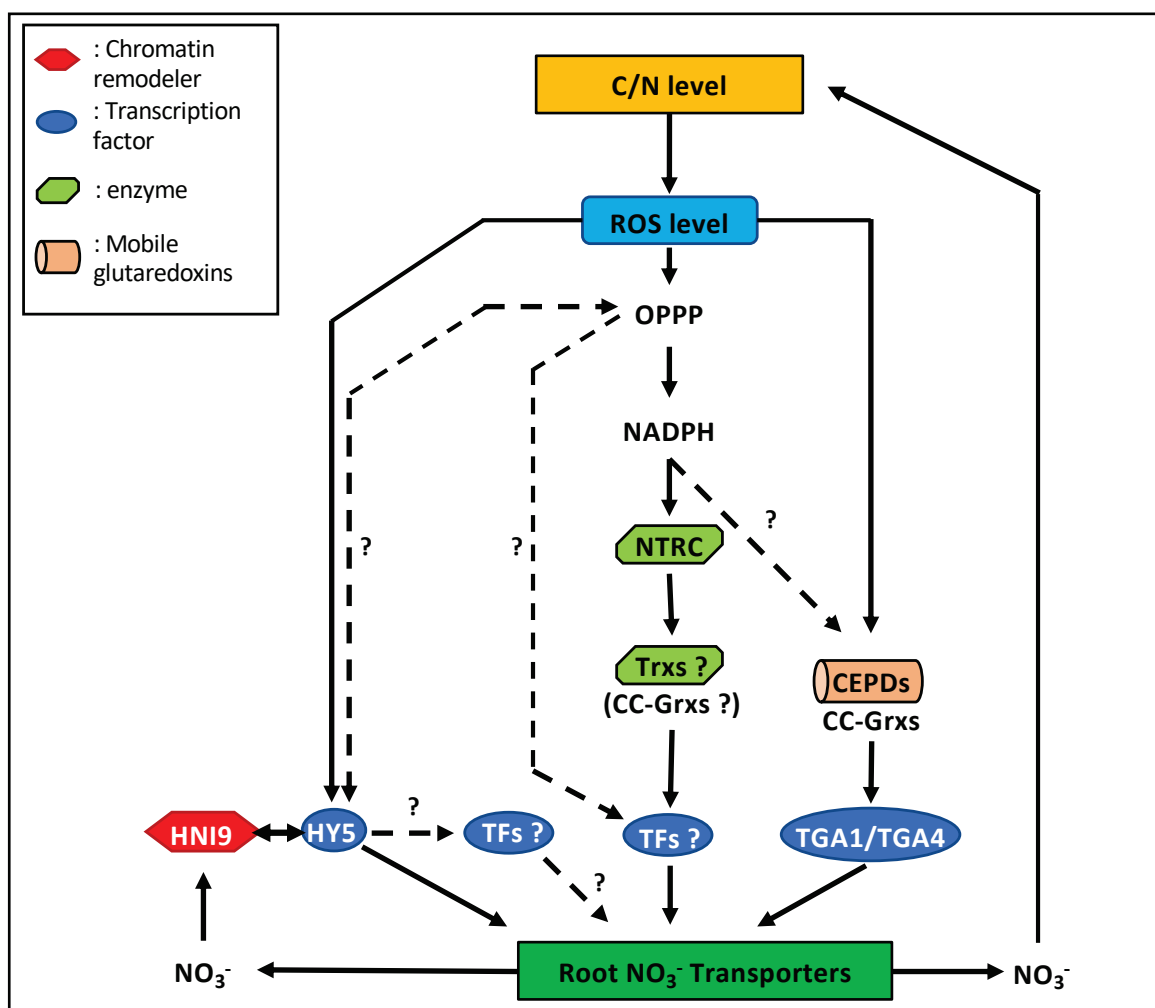
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