

Molecular Physiology of Nitrate Sensing by Roots



L. Ravazzolo, S. Trevisan, and S. Quaggiotti

Abstract Nitrogen (N) is needed by plants in great quantities. Besides being a nutrient, it also acts as a signal, regulating many downstream processes. Understanding the physiological and molecular processes regulating nitrogen use efficiency (NUE), particularly the below-ground traits related to root architecture, is crucial to reducing N loss and improving the efficacy of N fertilisation. Nitrate is the predominant source of nitrogen in aerobic agricultural soils and many studies have investigated the molecular mechanisms underlying the root response to nitrate, especially in *Arabidopsis*, one of the best studied model plants in plant biology. Maize is a very important crop, and its root apparatus is quite different from and more complex than that of *Arabidopsis*. Elucidating the molecular events underlying nitrate regulation of the root architecture in both these species is a crucial step towards improving technology transfer in the field. Auxin has been shown to play a prominent role in the transduction process leading to root architecture adjustments in response to nitrate availability in both *Arabidopsis* and maize, but the two plants differ in many other specific molecular components of this response.

Keywords *Arabidopsis* · Auxin · Maize · Nitrate · Nitrogen use efficiency · Root · Strigolactones · Transition zone

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1 NUE and Roots for a Second Green Revolution

In the twentieth century, novel technological innovations introduced during the Green Revolution led to massive changes in crop productivity worldwide. For the most part, success was achieved by a combination of high rates of investment in crop research (e.g., genetic improvement of crops to obtain high-yielding varieties) and in public services, market development and appropriate policy assistance (Pingali 2012). A central role was also played by the wide use of chemical fertilisers, in particular artificial nitrogenous fertilisers, made possible by the development of the Haber–Bosch process: almost 50% of people still rely on this process today (Godfray et al. 2010).

Nitrogen (N) is the essential macronutrient most needed by plants. It is a building block for nucleic acids, proteins, enzymes, and some metabolic components, such as chlorophyll, ATP and phytohormones (Andrews et al. 2013; Gojon 2017). Since it comprises almost 2% of plant dry matter and nearly 16% of total plant protein (Frink et al. 1999), unavailability limits plant development, crop yield and global primary production (Gutiérrez 2012). Besides its importance as a crucial plant nutrient, N also acts as a signalling molecule by regulating many plant processes, such as resistance to biotic and abiotic stresses, root development, dormancy, flowering time, leaf expansion, seed germination and hormone signalling (Bouguyon et al. 2012; O'Brien et al. 2016; Guan 2017; Izmailov and Nikitin 2020). Terrestrial plants can absorb N from the soil in two forms: inorganic compounds, such as nitrate (NO_3^-) and ammonium (NH_4^+), or organic compounds, such as amino acids, peptides, urea and proteins (Miller et al. 2007). Of these, nitrate (NO_3^-) is the preferred source in aerobic agricultural soils, while ammonium (NH_4^+) is critical in acidic and anaerobic environments.

Under most cropping situations, reduced N availability in the soil limits plant productivity (Dechorgnat et al. 2018). Furthermore, when the soil is subjected to massive synthetic N fertilisation, only half of the N fertiliser applied is absorbed by plants, while the remainder can have negative consequences on both the environment and human health (Gruber and Galloway 2008). As a result, understanding the physiological and molecular processes regulating nitrogen use efficiency (NUE) in plants is now crucial.

NUE is an important but complex concept that can be defined in terms of the total biomass or yield (e.g., of grain) produced per unit of N fertiliser applied to the soil (Xu et al. 2012). At each step of N metabolism, NUE is controlled by multiple interacting genetic and environmental factors, such as the nature of the N source, its interactions with microorganisms, soil type and management, and climate (Moll et al. 1982), and also by the efficiency of N uptake, remobilisation and assimilation (Hirel et al. 2007; Chen et al. 2020). In order to increase crop yield while decreasing N fertilisation, more sustainable agricultural practices along with controlled genetic manipulation and breeding strategies to improve crop NUE should be the new targets of a second green revolution (Han et al. 2015; Hirel and Lea 2018). For instance, a crop plant optimised for NUE should not just have a high rate of N uptake from

the soil and N incorporation into organic forms, but also be highly efficient in N use, recycling and remobilisation into grains (Omara et al. 2019). In this scenario, below-ground traits related to root architecture, N uptake and N fixation are crucial elements in defining NUE, and improving them is a key step towards a second green revolution (Den Herder et al. 2010). For example, the increased growth rate and biomass accumulation in maize, which correlated with the increase in yield in the USA Corn Belt, was very much dependent on changes in the root system architecture (RSA) (Hammer et al. 2009).

Root plastic development is pivotal in determining soil exploration and nutrient acquisition. In particular, the lateral spread and depth of root foraging are primary traits for the acquisition of soluble nutrients such as nitrate (Lynch 2007). Ground anchorage, water seeking and uptake, and establishing an advantageous relationship with symbiotic organisms are also highly dependent on the root system. Improving NUE in crops must, therefore, be based on an understanding of the physiological, molecular and signalling mechanisms directing root development in response to N fluctuations.

2 Root Morphology: Maize Root Versus Arabidopsis Root

Arabidopsis is one of the best studied model plants in plant biology, particularly the root system because of its transparent, simple organisation and its invariant cell lineage that can be traced back to a few founder cells (Benfey and Scheres 2000). The Arabidopsis root system comprises a single primary root (PR) and several lateral roots (LR) that remain active during the whole plant life cycle (Benfey and Schiefelbein 1994). It has no shoot-borne root system, and the number of epidermal cells that will form root hairs is quite predictable (Hochholdinger and Zimmermann 2008). Moreover, a single tiny layer of 8 cells forms the cortex, and just four cells form the quiescent centre (QC), while maize has as many as 800–1200 cells in the QC and 10–15 cortical cell layers (Smith and De Smet 2012) (Fig. 1).

Maize (*Zea mays* L.) is one of the most important cereal crops for both human and animal consumption. In 2019 global production reached 1124 million tons of grain over a cultivation area of 197 million ha (FAOSTAT 2019). In the developed world, maize is mainly used to feed livestock and produce biofuel, while in many developing countries it is primarily grown and consumed directly as food. Maize is particularly important in the diets of the people of sub-Saharan Africa and Latin America (Shiferaw et al. 2011). A simultaneous rise in demand and decline in productivity of the crop has been predicted, which will incur an annual cost of US\$30 billion by 2050 (Rosegrant et al. 2009).

The maize root system is organised into an embryonic and a post-embryonic system. The embryonic root system derives directly from the seed to produce a primary root (PR) and seminal roots (SR). The PR elongates rapidly and forms many lateral roots (LR), although these usually do not persist throughout the maize plant's life (Feldman 1994). The post-embryonic root system, also called the shoot-borne

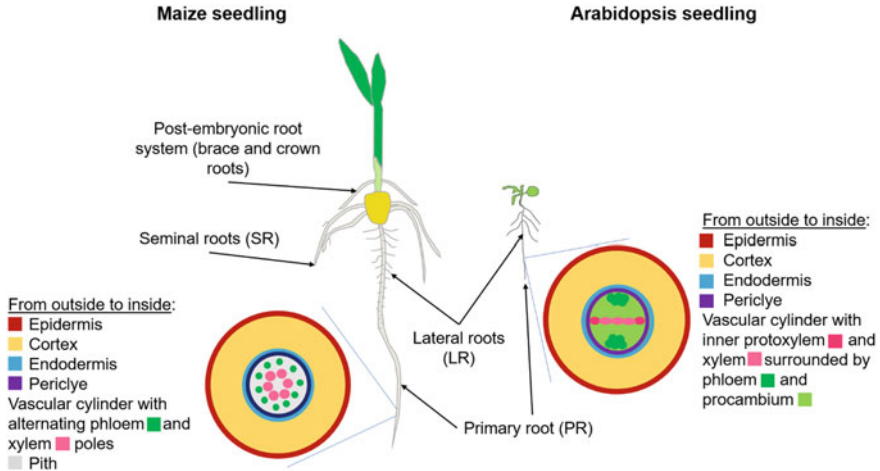


Fig. 1 Comparison of maize and Arabidopsis root systems with diagrams of the transverse sections of their primary roots. While the maize seedling has both an embryonic (PR: primary root; SR: seminal root) and a post-embryonic root system (brace and crown roots), Arabidopsis forms only a primary root (PR) with some lateral roots (LR) throughout its development

system, derives from the shoot and produces crown roots (CR) below the soil, and brace roots (BR) above the soil. Both CR and BR have many LR. Although LR were formerly considered part of the post-embryonic system, they may represent the link between the embryonic and post-embryonic systems, growing on PR, SR, CR and BR (Yu et al. 2015).

Maize PR and LR have a cylindrical structure with a distal extremity called the root apex (Alarcón et al. 2014). From outside to inside, the maize root consists of the epidermis, the cortex and the vascular cylinder (Alarcón et al. 2014). While the epidermis is uniseriate, the cortex comprises 6–10 layers of parenchymatous tissue, the innermost being the endodermis, the outermost the exodermis, both of which have highly specialised cells. The vascular cylinder consists of an outer layer, the pericycle, within which is the typical alternating organisation of xylem and phloem poles. In maize, the LR originate from pericycle and endodermis cells located opposite the phloem poles, while in eudicots, such as Arabidopsis, they originate from pericycle cells opposite the protoxylem poles (Hochholdinger et al. 2004; Casimiro et al. 2003; Jansen et al. 2012). Moreover, while Arabidopsis has only two protoxylem poles, maize can have ten or more phloem poles, resulting in a highly radial root branching phenotype (Smith and De Smet 2012).

Finally, roots can also be divided longitudinally into four consecutive zones (Baluška et al. 2010), namely the meristem (M, the first 2 mm up from the root tip), the transition zone (TZ, the next 2 mm above the M), the elongation zone (EZ, the next 4 mm above the TZ) and the maturation zone (MZ, from the EZ up to the seed). The TZ is a crucial root zone that integrates external and internal stimuli

into adaptative responses, and will therefore be described in some detail in the last paragraph.

3 Main Molecular Actors for Nitrate Sensing in Arabidopsis Root

Exogenous signals directly control plant growth and development by activating a large number of regulatory networks. Endogenous cellular sensors are able to quantitatively measure environmental fluctuations, which can arise in a very short time (milliseconds) and can last for several hours. This sensing/signalling pathway optimises the plants' adaptation to changing environments and variations in nutrient availabilities. Plants are able to sense NO_3^- in their environment and can rapidly respond to fluctuations in its availability. Nitrate sensors are thus elements that perceive alterations in the environment through ion binding, and transduce those alterations to an output.

Nitrate sensing systems have been partly identified in Arabidopsis (Fig. 2). They consist of membrane transporters that have already been shown to be involved in the uptake of NO_3^- into root cells. This dual ability gave rise to the term 'transceptor' (transporter/receptor). Transceptors have been described in yeast and animals. Interestingly, their function as sensors is independent of their function as transporters in plants (Ho et al. 2009).

Among the nitrate transporters, NITRATE TRANSPORTER 1 (NRT1.1)/PEPTIDE TRANSPORTER FAMILY 6.3 (NPF6.3) is representative of this class of transceptors (Bouguyon et al. 2015), acting also as a nitrate sensor (Muños et al. 2004).

NRT1.1/NPF6.3 participates in nitrate-dependent regulation of gene expression that leads to root development (Muños et al. 2004; Bouguyon et al. 2015). The activity of NRT1.1/NPF6.3 in N sensing is governed by Thr-101 (T101) phosphorylation. NRT1.1 acts as a dual-affinity transporter and nitrate sensor and responds to changes in soil nitrate concentrations by switching between phosphorylated and dephosphorylated forms (Liu et al. 1999; Liu and Tsay 2003; Ho et al. 2009; Lérán et al. 2014).

Nitrate uptake and the related transceptor NRT1.1 activation induces phospholipase C (PLC) activity, which in turn increases cytoplasmic calcium (Riveras et al. 2015). CBL-INTERACTING SERINE/THREONINE-PROTEIN KINASE 23 (CIPK23), CALCINEURIN B-LIKE PROTEINS 1 AND 9 (CBL1 and CBL9), and the protein phosphatase ABSISSIC ACID INSENSITIVE 2 (ABI2) are then involved in decoding the calcium signal and triggering a phosphorylative cascade.

Under low nitrate conditions, phosphorylation of T101 promotes recruitment of NRT1.1 into functional membrane microdomains at the plasma membrane. This facilitates NRT1.1-dependent auxin flux and reduces auxin concentrations in the

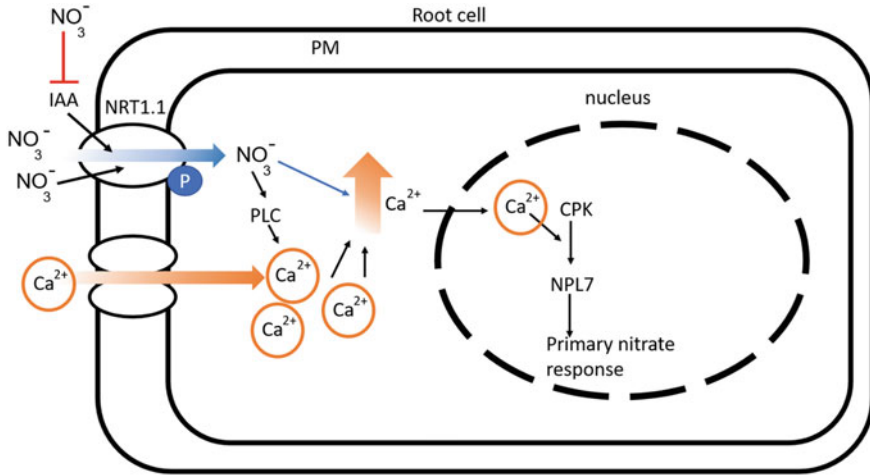


Fig. 2 Nitrate sensing in the Arabidopsis root cell. Nitrate is absorbed into the root cell by the plasma membrane-localised nitrate transporter family. In low nitrate conditions, the phosphorylation mechanism switches NRT1.1 into the high-affinity system. This sensing of NO_3^- alters the phosphorylation state of NRT1.1 causing calcium (Ca^{2+}) efflux through the activation of phospholipase C (PLC), which increases the cytosolic IP₃ levels. The increased IP₃ concentration in the cytosol induces the opening of Ca^{2+} channels and the accumulation of cytosolic Ca^{2+} . This calcium-dependent signalling results in variation in the expression of nitrate transporter genes. This activation stimulates LR growth, induces NRT1.1-auxin transport activity at the PM, and stimulates Ca^{2+} -ANR1 signalling from the endosomes. The signals resulting from the increased Ca^{2+} can be sensed by CPK10/30/32, which then phosphorylates NLP7. The phosphorylated NLP7 is retained in the nucleus to activate primary nitrate responsive genes. NRT1.1 also transports auxin, and nitrate-dependent auxin transport is an integral part of nitrate sensing, allowing root growth to adjust locally to variations in the nitrate supply in the soil

lateral root primordia (LRP) inhibiting their outgrowth. If nitrate increases, the non-phosphorylated NRT1.1 oligomerises and lateral mobility at the plasma membrane decreases, resulting in faster, inducible endocytosis. These processes could promote LR development by suppressing NRT1.1-auxin transport activity on the plasma membrane and initiating Ca_2^+ -ANR1 (Ca_2^+ -ARABIDOPSIS NITRATE REGULATED 1) signalling from the endosome (Zhang et al. 2019). ANR1 is the nuclear target of the nitrate- Ca^{2+} signalling cascade.

Once nitrate is sensed by NRT1.1, this signal needs to be sent to the nucleus to transduce its action on gene expression. NLP7 plays a prominent role in transmitting the nitrate signal from the membrane to the nucleus (Liu et al. 2017).

The nitrate-dependent calcium signal is necessary for changes in gene expression for the primary response which involves the regulation of several genes, such as *NRT2.1* and *TGA1*. This nitrate-dependent calcium influx activates the protein kinases CPK10/30/32, which in turn promotes phosphorylation of the transcription factor NLP7 (NIN-LIKE PROTEIN 7). The calcium signalling cascade can then influence other transcription factors (TFs) to control and coordinate additional responses,

which could ultimately regulate nitrate uptake by the roots and/or modulate the root architecture.

Nevertheless, NRT1.1 is also able to transport auxin, thereby generating nitrate-dependent auxin transport. The resulting auxin reallocation allows root growth to locally adjust to variations in the nitrate supply in the soil (Krouk et al. 2010b). When nitrate availability to the plant is low, CHL1/NRT1.1 functions as an auxin transporter to remove auxin from the LR primordia, thereby blocking their development (Krouk et al. 2010b; Mounier et al. 2014).

Nitrate perception triggers rapid transcriptional reprogramming (<5 min), suggesting that several TFs are involved in this regulation (Coneva et al. 2014; Obertello et al. 2015; Alvarez et al. 2020). For example, the aforementioned ANR1 would induce cell proliferation in LR tips and thus LR growth.

Several gene regulatory networks have been identified in which different TFs play a major role, including NLP7 (reviewed in Wang et al. 2018).

NLP7 directly regulates the nitrate-dependent transcriptional response (Alvarez et al. 2020; Marchive et al. 2013) and nitrate-assimilation processes (Marchive et al. 2013; Castaings et al. 2009; Konishi and Yanagisawa 2013). NLPs belongs to the family of RWP-RK transcription factors (Schäuser et al. 2005; Chardin et al. 2014). *NLPs* genes are closely related to the leguminous *NIN* (nodule inception) genes. When the transceptor NRT1.1 perceives nitrate at the plasma membrane, the consequent calcium influx triggers nuclear retention of NLP7, which then binds with and regulates the transcription of its downstream target genes (Liu et al. 2017; Alvarez et al. 2020). The resulting transcription cascade enables the plant to respond to nitrate within minutes without requiring de novo protein synthesis (Wang et al. 2018; Marchive et al. 2013).

NRT2 proteins are members of the major facilitator superfamily (MFS) of transporter and transporter-like proteins (Pao et al. 1998) and their expression has been shown to be related to repression of LR initiation, suggesting their role either as nitrate sensors or signal transducers to coordinate the development of the root system using nutritional cues (Little et al. 2005).

Sensors need to be capable of responding to and reporting spatially-delimited signalling processes that might be restricted to specific organs, tissues, cells, organelles, or even to a subregion of the cytosol. Spatially-delimited sensing can be achieved by cell type expression of endogenous sensors and subcellular targeting of proteins. Endogenous sensors are often restricted to specific regions of the cytosol by tethering to membranes or other signalling components.

With regard to spatial localisation of nitrate sensing, it is tempting to suggest that root tips are central in N perception: they would rapidly sense N signals and send a signal to the shoots via the stele. A systemic signal would then come back to the roots in a root-shoot-root interplay, as described by Ruffel et al. (2011).

4 Regulation of Root Development by Nitrate Availability: Maize Versus Arabidopsis

As mentioned above, the effects of the N source on the root system are complex and depend on several factors, such as its concentration and form in the soil, the N endogenous status of the plant and the responsiveness of the different genotypes. Nitrate (NO_3^-) is the main N source for crops in aerobic soils (Vidal et al. 2020) and it became evident in the late 1990s that it was involved in root response (Zhang et al. 1999). Studies on root system architecture (RSA) and morphology are critical, given that RSA determines the ability of plants to explore the soil for water and nutrients, including nitrate (Asim et al. 2020).

The primary root (PR) is the first root to emerge in both dicots and monocots (Smith and De Smet 2012). The effect of nitrate on PR growth is controversial, but appears to vary greatly according to NO_3^- concentration, and temporal and spatial factors (Andrews et al. 2013; Vidal et al. 2015; Ruffel and Gojon 2017). PR growth in *Arabidopsis* is typically found to be relatively insensitive to or even induced by moderate nitrate availability, but it can also be inhibited by high nitrate supply (Vidal et al. 2013; Tian et al. 2014). For example, many *A. thaliana* accessions were found to have a nitrate stimulatory effect on PR growth after nine days of NO_3^- exposure at concentrations ranging from 0.05 to 5 mM (Walch-Liu and Forde 2008). A similar effect was obtained by Gifford et al. (2013) after twelve days of exposure to NO_3^- at concentrations ranging from 0 to 20 mM. In contrast, Zhang and Forde (1998) observed no changes in PR length with nitrate concentrations ranging from 0.01 to 100 mM (fourteen days of exposure), nor were any effects found by Signora et al. (2001) after seven days of exposure to NO_3^- in concentrations ranging from 0.1 to 10 mM. These last authors, however, reported an inhibitory effect at concentrations higher than 50 mM. Moreover, long-term exposure (17/18 days) to NO_3^- at a low concentration (0.01/1 mM) significantly inhibited PR elongation (Linkohr et al. 2002), while Naulin et al. (2020) recently reported that provision of 5 mM nitrate for 3–14 days stimulated PR growth, further suggesting increased meristem activity due to the involvement of cytokinin (CK) in nitrate signalling. Overall, these results indicate that the regulation of PR growth in response to nitrate in the model species *Arabidopsis thaliana* is highly complex and not entirely clear.

In maize, a consistent inhibitory effect on PR length was observed by Tian and co-authors (2005) after twelve days of growth at a nitrate concentration of 20 mM. These authors subsequently showed that after twelve days of treatment, nitrate concentrations lower than 0.5 mM had no effect on the elongation of primary, seminal and crown roots, while concentrations above 5 mM affected root elongation more significantly (Tian et al. 2008). A similar inhibitory effect was also observed after growing maize seedlings under two different NO_3^- concentrations (0.1 and 10 mM) for seven days, then exposing them, respectively, to 0.1 and 1 mM NO_3^- for 48 h (Zhao et al. 2007). More recently, maize PR growth was monitored over 48 h of NO_3^- provision (1 mM) to seedlings previously starved of N, revealing a dual effect: PR growth was stimulated after 2 h of nitrate provision and inhibited after 24–48 h (Manoli et al.

2016; Ravazzolo et al. 2019). The maize primary root would therefore seem able to adapt its pattern of growth according to variations in nitrate concentrations and length of exposure.

Unlike nitrate regulation of PR growth, which is in many ways still unclear, more is known about the molecular and morphological mechanisms governing lateral root (LR) formation and development in response to nitrate. LR create an extraordinary, extensive underground branching network (Atkinson et al. 2014) and perform fundamental functions of soil exploration, water and nutrient uptake, and establishment of beneficial symbioses. LR are generally more sensitive to variations in the nitrogen source than PR (Tian et al. 2014; Hachiya and Sakakibara 2017), but the role played by nitrate is complex and is bound by both genetics and the environment (Sun et al. 2017). LR development is usually still stimulated under mild nitrate deficiency, but is inhibited under severe N shortage (Krouk et al. 2010a). High nitrate supply, on the other hand, always exerts an inhibitory effect on LR growth.

According to Malamy and Benfey (1997), LR development generally unfolds in four stages: LR initiation, LR primordia (LRP) formation, LR outgrowth and emergence, LR elongation.

In maize, LR initiate from a few pericycle cells at the phloem poles, called founder cells, which undergo de-differentiation and proliferation to produce the LRP. Founder cell priming involves asymmetric cell division by cell cycle reactivation and auxin accumulation at the quiescent centre (Jung and McCouch 2013). Arabidopsis LRs, on the other hand, originate exclusively from pericycle founder cells positioned at opposite xylem poles (Dolan et al. 1993) and their initiation is positively regulated by auxin, gibberellins (GA) and jasmonate (JA), but negatively regulated by CK, abscisic acid (ABA) and high concentrations of ethylene (ET) (Péret et al., 2009; Guan et al. 2017; Vega et al. 2019). For instance, it has been hypothesised that ABA and CK reverse the auxin effect by reducing its polar transport (Shkolnik-Inbar and Bar-Zvi 2010), while JA promote both LR initiation and emergence (Raya-González et al. 2012). In addition, LR development and emergence are stimulated by treatments that raise ethylene production in the root (Ivanchenko et al. 2008). In maize, auxin modulates LR initiation, thereby determining pericycle cell length (Alarcón et al. 2019) as well as LR development (Ravazzolo et al. 2021). Furthermore, strigolactones (SLs) negatively regulate LR development in Arabidopsis (Ruyter-Spira et al. 2011) and rice (Sun et al. 2014, 2019), while inhibition of SL in maize seems to reactivate auxin signalling, so it is, at least in part, responsible for stimulating LR development by nitrate (Ravazzolo et al. 2021). Finally, a negative effect of ET and CK on maize LR initiation has also been hypothesised (Alarcón et al. 2014).

Two proteins involved in high affinity nitrate transport, NRT2.1 and NAR2.1, have been shown to act as positive regulators of the stimulatory effect of LR initiation by low nitrate (0.5 mM) in Arabidopsis (Remans et al. 2006b; Orsel et al. 2007). The aforementioned ANR1 (ARABIDOPSIS NITRATE-REGULATED1), a MADS-box transcription factor specifically expressed in LRP, and the NO₃⁻ “transceptor” NRT1.1/NPF6.3 have been identified as further key actors in LR development in response to nitrate in this model plant (Remans et al. 2006a). Transgenic Arabidopsis lines, in which *ANR1* expression was down-regulated or even

suppressed, gave rise to an Arabidopsis phenotype that was less responsive to localised NO_3^- . Consequently, it has been suggested that *ANRI* acts downstream of *NRT1.1/NPF6.3* in the signalling pathway stimulating LR initiation in response to low nitrate (Remans et al. 2006a). The Arabidopsis auxin biosynthetic gene *TAR2* has been reported to play a role in the stimulatory effect of low nitrate on LR development (Ma et al. 2014). *TAR2* encodes a tryptophan aminotransferase-related protein 2 and its expression in the pericycle and vasculature of developed roots close to the root tip is enhanced under low nitrogen conditions, but *tar2* null mutants do not display N-stimulated auxin accumulation in the root tip. Recently, it has also been shown that NRT1.1 phosphorylation in Ser 101 plays a key role in this response by facilitating auxin flow under low-nitrate conditions (Zhang et al. 2019). As previously reported, the nitrate “transceptor” NRT1.1/NPF6.3 transports both auxin and nitrate (Krouk et al. 2010b) and negatively regulates LR emergence at low nitrate concentrations by promoting auxin lateral basipetal transport out of the LR. High nitrate levels, on the other hand, inhibit NRT1.1/NPF6.3-dependent basipetal auxin transport leading to auxin accumulation in LR tips and stimulating their growth.

It has also been suggested that miR167 and its target AUXIN RESPONSE FACTOR 8 (ARF8) (Gifford et al. 2008) and miR393 and the auxin receptor AFB3 (AUXIN SIGNALING F-BOX PROTEIN 3) (Vidal et al. 2010) are additional important players in the regulation of LR initiation and LR outgrowth in Arabidopsis. MiR167/ARF8 is a module that regulates the ratio between LR initiation and development (Gifford et al. 2008), while the miR393/AFB3 regulatory module has been studied for its modulatory effect on both LR and PR growth in response to nitrate by integrating nitrate and auxin signalling (Vidal et al. 2010, 2013). In Arabidopsis, miR393 is encoded by the two loci MIR393a and MIR393b, and post-transcriptionally regulates mRNAs for the ubiquitin protein ligase SCFTIR1/AFB, auxin receptors TIR1 (Transport Inhibitor Response Protein 1), AFB1 (Auxin Signalling F-box Protein 1), AFB2 and AFB3 (Parry et al. 2009). The TIR1/AFBs constitute a small subset of F-box-containing auxin receptors and mediates proteasomal degradation of Aux/IAA transcriptional repressors to release the activities of auxin response factors (ARFs), thus promoting the transcription of auxin-responsive genes (dos Santos et al. 2009). In particular, AFB3 was found to be the only auxin receptor transcriptionally induced by nitrate and subsequently post-transcriptionally repressed by miR393. These studies suggest that, besides modulating auxin gradients in roots through NRT1.1/NPF6.3 activity (Krouk et al. 2010b), nitrate can also increase root auxin sensitivity by affecting *AFB3* expression (Bouguyon et al. 2016).

In cereals, such as maize, the molecular regulation of LR development in response to nitrate is complex (Bray and Topp 2018, and references therein) and, indeed, only a few lateral root mutants have been described in these crops, generally those related to auxin pathways (Hochholdinger and Tuberosa 2009; Atkinson et al. 2014; Yu et al. 2019). One example is the maize *rum1*, which encodes an Auxin/indole-3-acetic acid (Aux/IAA) protein called RUM1 (ROOTLESS WITH UNDETECTABLE MERISTEM 1) (von Behrens et al. 2011). Aux/IAA protein degradation leads to the release of ARFs (Auxin Response Factors), which can then bind to the promoters of downstream auxin-responsive genes involved in lateral and seminal root formation

(reviewed in Taylor-Teeple et al. 2016). *rul1* (*rum1-like1*) is the homolog of *rum1*, originating from an ancient maize genome duplication (Zhang et al. 2016). Both RUM1 and RUL1 have the canonical four-domain structure of Aux/IAA proteins and nuclear localisation, and they interact in vivo with ZmARF25 and ZmARF34 (Zhang et al. 2016), probably blocking LR formation in non-precursor pericycle cells (von Behrens et al. 2011). Moreover, RUM1 can directly bind to the promoter of *lrp1* (*lateral root primordia 1*) which encodes an auxin-inducible transcriptional activator (Zhang et al. 2015). In Arabidopsis, *AtLRP1* encodes a member of the SRS (short internodes-related sequence) family with a zinc finger motif and is involved in early lateral root formation (Smith and Fedoroff 1995). Maize *lrp1* expression is localised in the root meristem and emerging lateral root primordia (LRP), and is repressed by binding with RUM1, suggesting the involvement of LRP1 in maize auxin signal transduction downstream of *rum1* (Zhang et al. 2015). RUM1 can specifically interact also with RAP1 (RUM1 ASSOCIATED PROTEIN 1) (Zhang et al. 2016), while no interaction was observed between RUL1 and RAP1. The RAP1 family includes six other members, called RAP1-like: RAL1, RAL2, RAL3, RAL4, RAL5, RAL6 (Zhang et al. 2016). RAP1 is the homolog protein of AtSPR1 (SPIRAL 1), a nitrilase-associated microtubule-localised protein in Arabidopsis involved in the directional control of rapidly expanding cells (Nakajima et al. 2004).

Besides LR development, regulation of the shoot-borne roots of maize is also crucial (Taramino et al. 2007). The paralogous LOB-domain maize proteins RTCS (ROOTLESS CONCERNING CROWN AND SEMINAL ROOTS) and RTCL (RTCS-Like) are important for development of the crown root (Xu et al. 2015). In particular, the LBD (LATERAL ORGAN BOUNDARIES Domain) protein family plays a role in defining organ borders and in many other plant developmental processes (Majer and Hochholdinger 2011). As mentioned above, maize LBD-dependent signalling in root development includes *RTCS* and *RTCL* expression, both of which have auxin-responsive elements and are preferentially expressed in roots. Their expression is activated through binding with auxin-induced *ZmARF34*. Consequently, RTCL and RTCS bind to the promoters of the genes operating downstream by acting as transcription factors (Xu et al. 2015). In maize, RTCS is the closest homolog of AtLBD29, and *rtcs* acts upstream of *rtcl*. Although *AtLBD19*, *AtLBD16* and *AtLBD29* have redundant functions in LR emergence in Arabidopsis (Okushima et al. 2007), the maize *rtcs/rtcl* double mutants did not reduce LR density. Hence, LBD proteins in maize seem to be involved only in shoot-born root formation (Xu et al. 2015).

As with Arabidopsis, the establishment of auxin response maxima in LR initiation is also crucial in maize (Atkinson et al. 2014; Ötvös et al. 2021). Auxin transport is fundamental to the generation of these local auxin maxima, and PIN transporters determine polar auxin transport (PAT). It has been shown that where LR develop, the monocot-specific *PIN9* can modulate auxin efflux to pericycle cells at the phloem poles, thereby activating the cell cycle (Yu et al. 2015).

5 Maize Transition Zone and Nitrate Sensing

The transition zone (TZ) is the part of the root between the meristem and the elongation zone. It is a distinctive zone that translates several endogenous and exogenous signals into adaptive differential growth (Baluška and Mancuso 2013; Trevisan et al. 2014). It has been known for a long time that TZ cells undergo a series of crucial changes in their cytoarchitecture and are characterised by a complex system of polar auxin transport circuits that make them highly responsive to auxin (Baluška et al. 2010).

These characteristics make the TZ a dynamic sensor that can reorganize root growth in response to various stimuli, such as gravity (Masi et al. 2015), touch and extracellular calcium (Ishikawa and Evans 1992; Baluška et al. 1996), osmotic stress (Baluška and Mancuso 2013), hypoxia (Pucciariello and Perata 2017; Manrique-Gil et al. 2021), oxidative stress and auxin (Mugnai et al. 2014) and heavy metal stress (Sivaguru et al. 2013; Yang et al. 2014; Sahay and Gupta 2017; Kong et al. 2018; Wei et al. 2020). In addition, the TZ seems to be the designated nitrate responsive region in maize (Manoli et al. 2014; Trevisan et al. 2014).

Coordinated spatio-temporal regulation of the expression of genes encoding a nitrate reductase (NR) and a non-symbiotic haemoglobin (nsHb) soon after nitrate provision to N-depleted roots has been observed in the root epidermal TZ cells of maize (Trevisan et al. 2011). Nitrate perception triggers a sudden rise in NO production by NR and concomitant activation of nsHbs to rapidly detoxify high intracellular NO concentrations, thus protecting the cell from oxidative stress. This process was explored further by Manoli et al. (2014), who demonstrated *in vivo* NR-dependent NO production soon after nitrate supply using confocal microscopy. Remarkably, the TZ appeared to be the elected zone for NO production, making it one of the maize root regions most responsive to nitrate. Together, these studies present a new perspective on the contribution of nitric oxide (NO) to the root response to nitrate (Trevisan et al. 2011, 2015; Manoli et al. 2014). NO is a general bioactive plant signalling molecule involved in many physiological and developmental processes, and regulates both biotic and abiotic stress responses and hormonal crosstalk (Arora et al. 2016; Kolbert et al. 2019; Sánchez-Vicente et al. 2019). It has been reported to be required for RSA development (Prakash et al. 2020), particularly in PR growth (Fernández-Marcos et al. 2012; Manoli et al. 2014), adventitious root formation (Pagnussat et al. 2003), LR formation (Wang et al. 2010) and root hair formation (Lombardo and Lamattina 2018). It has been suggested that auxin and NO act together in the process of LR development (Correa-Aragunde et al. 2015) and regulation of the stem-cell niche (Sanz et al. 2014). It has also been demonstrated that the NO produced after nitrate provision triggers auxin and PIN1 re-localisation in TZ cells, which favours cell expansion over cell division and guides root apex elongation (Manoli et al. 2016). Detailed RNA-sequencing has subsequently revealed the transcriptional signature of the TZ cells in response to nitrate supply, and many crucial transcripts have been identified (Trevisan et al. 2015). Strigolactone (SL) biosynthesis and signalling has

emerged from this study as a further pivotal element regulating the response to N availability in TZ cells.

SLs are carotenoid-derived phytohormones that regulate plant development in response to various environmental stimuli and in concert with many other regulators through their action as both endogenous and exogenous signalling molecules (Machin et al. 2020). Since the interplay of NO and auxin is important in regulating multiple aspects of root biology (Sami et al. 2018) and a connection between SLs and NO has been shown (Kolbert et al. 2019; Oláh et al. 2020, 2021), the role of SLs in the pathway where NO acts as a coordinator of nitrate and auxin signalling to regulate the overall maize root response is interesting. A recent survey (Ravazzolo et al. unpublished data) suggests that the observed shutdown of SL production in response to nitrate might occur independently of NO production, leading us to suppose that there are two distinct signalling pathways controlling NO production and SL inhibition in response to nitrate provision.

Many studies have shown that soil nutrient deficiencies, particularly phosphate starvation, induce increased SL biosynthesis, which in turn influences the root architecture (Kohlen et al. 2011; Koltai, 2015; Ito et al. 2016; Marzec and Melzer 2018). Recently, it has been shown that a lack of nitrogen could be more effectual than phosphorous deficiency in stimulating SL exudation in maize root, while nitrate availability rapidly switched off SL exudation (Ravazzolo et al. 2019). In the same study, it was also suggested that the shutdown of SL production by nitrate could play a role in the complex LR developmental pathway. The negative regulation of LR by SLs has already been documented in *Arabidopsis* (Ruyter-Spira et al. 2011) and rice (Sun et al. 2014, 2019). Indeed, a lower number of lateral root primordia (LRP) were found in both these species when plants were treated with a racemic mixture of an SL analogue (*rac*-GR24). Moreover, the impact of SLs on root development in response to nutrient deprivation appeared to be dependent on auxin levels (Omoarelojie et al. 2019), and some studies on rice (Zhang et al. 2010), pea (Ligerot et al. 2017) and *Arabidopsis* (de Jong et al. 2014) have focused more specifically on the interaction between SLs and auxin (Rameau et al. 2019). With regard to maize root, a more recent systemic molecular study based on RNA-sequencing highlighted some genes whose transcription is regulated in response to nitrate and is dependent on auxin, SLs, or both (Ravazzolo et al. 2021). Four independent clusters of transcripts regulated by nitrate and dependent on either auxin or SLs, or on both, or independent of both of them have been described in maize root. Each cluster holds several putative molecular candidates potentially attributable to these alternative transduction pathways. They represent a useful starting point for broadening current knowledge of the entire process.

A transcriptomic study on TZ cells (Trevisan et al. 2015) has also enabled identification of further previously unknown players contributing to nitrate perception by this root region. Particular attention should be paid to the coordinated opposite regulation of the transcription of *ZmUPBI* and *ZmPRX112* that occurs in TZ cells and that seems to affect the overall balance between H_2O_2 and $O_2^{\bullet-}$ in the first mm of the primary root and the equilibrium between cell division and elongation that in turn drives PR growth in response to nitrate in maize (Trevisan et al. 2019). It is known

that differences in superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) accumulation in the root tip significantly affect PR growth and differentiation (Dunand et al. 2007) and the transition of cells from a zone of cell division to a zone of cell elongation and differentiation (Tsukagoshi et al. 2010). *ZmUPBI* orthologue expression in the TZ is highly repressed by nitrate supply but induced by N deficiency (Trevisan et al. 2015).

From the above, it appears that the perception of nitrate is very high in the TZ of maize, and that this region is a promising reservoir of useful information for studying and modelling the process leading to the adaptive response of roots to nitrate availability. A sophisticated interplay of highly specific events, including NO and ROS homeostasis regulation, and hormonal (auxin and SLs) accumulation and signalling seems to characterise the early stages of nitrate perception in these dedicated root cells, which in turn activate a transduction pathway to the whole root (Fig. 3).

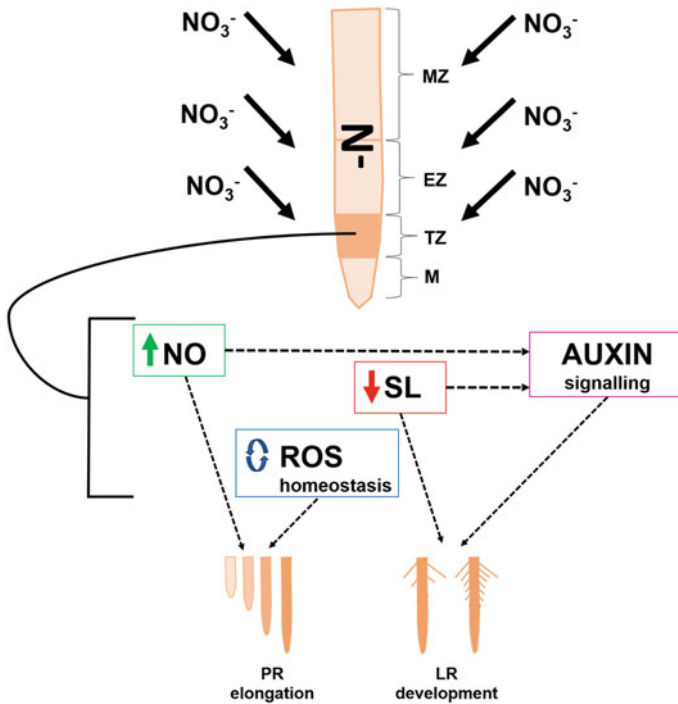


Fig. 3 Proposed model of nitrate response in the maize root transition zone (TZ). After experiencing nitrate deficiency (-N), the maize root perceives the nitrate (NO_3^-) supply and many transduction pathways from the transition zone (TZ) are activated leading to an adaptive response. Some key signals are represented by nitric oxide (NO) and reactive oxygen species (ROS), which are implicated in primary root (PR) elongation, while strigolactones (SL) and auxin are involved in lateral root (LR) development. *Abbreviations:* MZ, maturation zone; EZ, elongation zone; TZ, transition zone; M, meristem zone

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