Review

Nitrate Signaling, Functions, and Regulation of Root System Architecture: Insights from Arabidopsis thaliana

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Abstract: Root system architecture (RSA) is required for the acquisition of water and mineral nutrients from the soil. One of the essential nutrients, nitrate (NO$_3^-$), is sensed and transported by nitrate transporters NRT1.1 and NRT2.1 in the plants. Nitrate transporter 1.1 (NRT1.1) is a dual-affinity nitrate transporter phosphorylated at the T101 residue by calcineurin B-like interacting protein kinase (CIPKs); it also regulates the expression of other key nitrate assimilatory genes. The differential phosphorylation (phosphorylation and dephosphorylation) strategies and underlying Ca$^{2+}$ signaling mechanism of NRT1.1 stimulate lateral root growth by activating the auxin transport activity and Ca$^{2+}$-ANR1 signaling at the plasma membrane and the endosomes, respectively. NO$_3^-$ additionally functions as a signal molecule that forms a signaling system, which consists of a vast array of transcription factors that control root system architecture that either stimulate or inhibit lateral and primary root development in response to localized and high nitrate (NO$_3^-$), respectively. This review elucidates the so-far identified nitrate transporters, nitrate sensing, signal transduction, and the key roles of nitrate transporters and its downstream transcriptional regulatory network in the primary and lateral root development in Arabidopsis thaliana under stress conditions.

Keywords: nitrate; nitrate transporters; primary response; phospholipase C; root system architecture; lateral roots; primary roots

1. Introduction

Nitrogen significantly influences plant growth and development. Plants adopt numerous strategies to modulate the uptake capacity of their roots to cope with spatial and temporal fluctuations in N availability [1]. In plants, the root architecture adjusts to these environmental fluctuations [2,3] and synchronizes the NO$_3^-$ supply and demand inside the plants by the coordination of the systemic signal required to deal with root NO$_3^-$ acquisition [4].

The regulatory pattern of root NO$_3^-$ uptake simplifies the root transport system in two ways; The first is the rapid uptake after the NO$_3^-$ provision, which requires de novo protein synthesis [5,6], and the other is the root NO$_3^-$ efflux, strongly upregulated by N deficiency or low availability and downregulated by high nitrate supply [7,8]. An important hypothesis arising from the recently
identified dissimilar NO$_3^-$ influx and efflux and the low- and high-affinity NO$_3^-$ transporters has revealed that several diverse carrier proteins are involved in the root nitrate (NO$_3^-$) transport system. Studies on Arabidopsis thaliana suggest that at least two transporters, NRT1.1 and NRT2.1, are involved in NO$_3^-$ sensing [9].

NRT1.1 activates four signaling mechanisms [10]. Firstly, the primary nitrate response (PNR) [11], the long-term response of NRT1.1. Secondly, this then acts as feedback repression of NRT2.1 under a high NO$_3^-$ supply [12]. Thirdly, the promotion of lateral root (LR) branching by NRT1.1 in response to NO$_3^-$, inhibiting the emergence of LR primordia at low NO$_3^-$ availability [13], and finally, the induction of genes at high NO$_3^-$ conditions [10]. After nitrate uptake via NRT1s and NRT2s, the part of the NO$_3^-$ influx into the cell is reduced and thereby assimilated as amino acid through a series of enzymes such as nitrate reductase (NR), nitrite reductase (NiR), glutamine synthesis (GS), and glutamate synthase (GOGAT). These NO$_3^-$-mediated developmental processes are governed by a complex network of kinases and phosphatase [14], influencing the primary nitrate response (PNR) [15]. Further studies on sensitive Ca$^{2+}$ biosensors have revealed that NO$_3^-$ treatment upgrades Ca$^{2+}$ concentration in the cytoplasm and nucleus of the protoplast from the mesophyll cells in the tip, pericycle, and stele of the intact roots. In-gel kinase assays have demonstrated that the activity of protein kinases (CPKs) is stimulated by NO$_3^-$ treatment in protoplasts. Previous studies on protoplasts have distinguished subgroup III of the CPKs as regulators of NO$_3^-$ responsive genes [16]. This further confirms the function of NO$_3^-$ as an important signal that regulates gene expression, plant growth, and development [17].

The contribution of the nitrate transporter signaling pathway in the regulation and patterning of root system architecture (RSA) is momentous. This review discusses the significant milestones in the early response signaling and phosphorylation status of NO$_3^-$ in Arabidopsis root, with greater emphasis on the signal transduction pathways that shape the architecture of the root in response to altered NO$_3^-$ supply.

2. The Nitrate Signaling Mechanism in RSA

2.1. Nitrate as Early Response Sensing

The molecular identification and the functional characterization of the genes encoding the NO$_3^-$ transporters in plants began in the mid-1990s and is still an active field of research [18]. The molecular mechanism of NO$_3^-$ signaling transduction has been discovered in Arabidopsis. Nitrate transporter1/peptide transporter family (NRT1/NPF), nitrate transporter 2 (NRT2), chloride channel (CLC), and slowly activating anion channel (SLAC/SLAH) are the four nitrate transporter families that have been characterized in Arabidopsis [19].

Nitrate transporter 1.1 (NRT1.1), also called CHL1/NPF6.3, belongs to the NRT1/PTR family (NPF) [20]. As a dual-affinity nitrate transporter, NRT1.1 functions in both low and high nitrate affinity states [21,22], subsequently controlling root architecture by acting as a potential nitrate sensor [23,24] and triggering nitrate-dependent changes in gene expression. Moreover, its nitrate uptake function regulates the expression of key nitrate assimilatory genes. Its affinity state changes according to the phosphorylation status of the T101 residue [14,25]. NRT1.1 is capable of triggering independent signaling pathways in response to nitrate in Arabidopsis roots. Different NRT1.1 mutant alleles exhibit distinct responses to nitrate at the transcriptome level as well as the repression of LR development [10]. However, in NRT1.1 (CHL1/NPF6.3), the mutant’s chl1-9 allele, where proline 292 replaces leucine, shows imperfect NO$_3^-$ affinity but exhibits a biphasic initial NO$_3^-$ response for NRT2.1 [14]. Different studies have revealed that both chl1-9 and chl1-5 (deletion mutant of NRT1.1) are identical to the long-term suppression of NRT2.1 expression and LR development without nitrate [10].

Both the primary and secondary NO$_3^-$ responses accomplished by transcriptomic studies indicate that the Affymetrix ATH1 chip has a significant impact on gene expression within 20 min after NO$_3^-$ treatments. These changes were more apparent in roots than in the shoot, with the root having 1176...
affected transcripts and only 183 affected transcripts in the shoot [26]. Another study revealed that after NO$_3^-$ supply in nitrate-starved conditions, the NO$_3^-$ transporters NRT1.1, NRT2.1, NRT2.2, and NRT2.4 were stimulated [27].

Hence, an additional sensing system may exist for NO$_3^-$ influx or efflux with distinctive or overlapping signaling functions related to NRT1.1 [28]. For instance, under low NO$_3^-$ conditions, the CBL-interacting protein kinases 23 (CIPK23) phosphorylates at threonine residue 101 (T101) by toggling NRT1.1 to a high-affinity nitrate transport system [29]. In the early NO$_3^-$ response system, Ca$^{2+}$ is a versatile signaling modulator in various regulatory pathways [30,31]. Ca$^{2+}$ signaling is associated with NO$_3^-$ responsive regulatory genes in Arabidopsis roots [16,32]. One should think of how the calcium signal is being triggered. There are some interesting viewpoints about this inquiry, elucidating that NRT1.1 is regulated by CIPK/CBL proteins, which are also themselves being regulated by calcium [14]. However, the mechanism behind the toggling of CPKs in the nucleus in response to nitrate is unknown [15].

2.2. Nitrate and Protein Kinases

The calcineurin B-like protein kinase, CIPK8, is rapidly activated by NO$_3^-$ and downregulated in chl1-5 mutants. To study the function of CIPK8, the two independent T-DNA insertion mutants (cipk8-1 and cipk8-2) were isolated and a reduction in the cipk8 mutant was apparent via the induction of nitrate-responsive genes NRT1.1, NRT2.1, NIA1, and NiR. This then clearly demonstrated that CIPK8 functions as a positive regulator of the primary NO$_3^-$ response in the low-affinity system [25]. Another protein kinase complex, CIPK23-CBL1/CBL9 (CIPK, CBL-interacting protein kinase; CBL, calcineurin-B-like protein), has been associated with dual-affinity transition changes of NRT1.1 via phosphorylation [14,33]. Further studies have also shown that FIP1 (factor interacting with poly (A) polymerase 1) adversely regulates the expression of CIPK8 and CIPK23 associated with NO$_3^-$ signaling. In the fip1 mutant, the increased expression of CIPK23 may affect NO$_3^-$ uptake and subsequently reduce NO$_3^-$ content. Molecular genetics suggest that FIP1 and CPSF30-L operate similar NO$_3^-$ signaling pathways. FIP1-induced NO$_3^-$ signaling interacts with CPSF30-L and is regulated by CIPK8 and CIPK23 [34,35].

The role of the subgroup III protein kinases (CPKs) CPK10, -30, -32 in NO$_3^-$ regulated root growth was examined [15]. The NO$_3^-$-induced LR primordial density was reduced and LR elongation was significantly hindered in icpk [15], thus associating the inhibition of nitrate–CPK-stimulated genes with transcription, metabolism, and transport activities [15]. The activity of the CPKs can be enriched within 10 min in response to nitrate. These CPKs have been distinguished as the primary regulators that coordinate the essential NO$_3^-$ response [15] and modulate various essential cell and metabolic functions instantly triggered by NO$_3^-$ [36,37].

CPK10 and CPK30 have also been shown to be associated with the abscisic acid (ABA) responsiveness of the mesophyll protoplasts, which is a promising avenue of research on the coregulation of NO$_3^-$ and ABA pathways. Both have been speculated to contribute to the regulation of the root growth and gene expression [37]. For instance, ABI2 (ABA-insensitive 2) phosphatase is a fundamental component of the ABA sensing system [38]. Besides the CIPK23–CBL9 complex functioning in the dual affinity transition changes of NRT1.1, ABI2 and CBL1 also interact with phosphorylated CIPK23, which is recognized as an additional segment of this regulation process. NO$_3^-$ sensitivity instigates a rapid increase in the cytoplasmic Ca$^{2+}$ level downstream of NRT1.1 in a PLC-dependent manner [28].

In short, nitrate-mediated CPK signaling phosphorylates transcription factors to regulate the expression of downstream genes that affect nitrogen assimilation, carbon/nitrogen metabolism, and proliferation [15]. However, it is possible that additional NO$_3^-$ sensors and NRT1.1-independent pathways could be involved in the Ca$^{2+}$ influx and other signaling measures [28,39] (Figure 1). An increase in Ca$^{2+}$ initiates a change in the protein phosphorylation status while controlling the movement of the key component of the NO$_3^-$ signaling pathway. CPK10, 30, and 32 work as regulators...
of the essential NO₃⁻ response, linking the Ca²⁺ influx with the phosphorylation of the target proteins. CPK activation could also be linked with NRT1.1-dependent pathways [28].

3. Nitrate Signaling and Calcium

Previous studies have revealed that nitrate treatments abruptly raise cytoplasmic Ca²⁺ levels in the roots as well as in the entire seedling [16] (Figure 1). This confirmed that the function of Ca²⁺ in nitrate signaling originates from early research on corn and barley, where EGTA or LaCl₃ alters the expression of NO₃⁻-responsive genes. The potential role of Ca²⁺ as a second messenger was thus indicated [32,40].

![Figure 1. Summary of early responses in nitrate signaling and assimilation. NO₃⁻ signaling pathway switches its affinity via phosphorylation (modified from Undurraga [41]). Nitrate-responsive genes are depicted in light green, transcription factors in purple, and microRNAs in grey. For clarity purposes, the cell nucleus is shown. Phosphatidylinositol-specific (PI-PLC) and Ca²⁺-dependent pathways. At Low NO₃⁻ condition, protein kinases CBL1/9–CIPK23 complex phosphorylates NRT1.1 and changes it into a high-affinity transporter, which activates PLC and results in calcium influx (Ca²⁺ acts as a second messenger). This cascade mediates changes in the expression of transcription factors (TGA1/4 *) and genes involved in nitrate transport (NRT2.1, NRT2.2, and NRT3.1) and nitrate assimilation (NIA1 and Nir). Nonphosphorylated form of NRT1.1-induced signaling. Nitrate-induced Ca²⁺-ANR1 signaling that promotes lateral root (LR) initiation is assumed to be a nonphosphorylated form of NRT1.1 signaling after the supply of nitrate in limited-nitrate conditions. (C) PI-PLC and Ca²⁺-independent pathways. Conversely, AFB3 is regulated by nitrate in a phospholipase C (PLC)- and calcium-independent manner. AFB3 modulates the expression of NAC4 and OBP4 with subsequent effects on root remodeling. Finally, nitrate assimilation produces organic N, which induces miR393 and represses miR167 (grey) and regulates the abundance of AFB3 and ARF8, respectively. * TGA1 and TGA4 are redundant regulatory factors that mediate nitrate responses in Arabidopsis roots. However, the interaction between TGA4 and the PLC–calcium pathway has not been experimentally validated. 

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Ca\(^{2+}\) sensor proteins perceive changes in the \((\text{Ca}^{2+})_{\text{cyt}}\) and subsequently transduce downstream signaling cascades to stimulate alteration of enzymatic activity, cytoskeleton orientation, phosphorylation, and gene expression [42,43]. This was further confirmed by the pretreatment of seedlings with phospholipase C inhibitors or Ca\(^{2+}\) channel blockers, which severely affected \(\text{NO}_3^-\)-responsive gene expression in Arabidopsis, indicating the function of Ca\(^{2+}\) as a secondary messenger in \(\text{NO}_3^-\) signaling pathways. A model was therefore suggested, where the \((\text{Ca}^{2+})_{\text{cyt}}\) level increases by \(\text{NRT}1.1\) and phospholipase C activity in response to \(\text{NO}_3^-\), which is required for changes in the prototypical \(\text{NO}_3^-\)-responsive gene expression [16]. Taken together, both \(\text{NRT}1.1\) and phospholipase activity are mandatory for \(\text{NO}_3^-\)-mediated increase in cytoplasmic Ca\(^{2+}\) levels and IP3 (Figure 1) [16].

PLC enzymes are membrane-associated, resulting in the remodeling of lipid membranes by the breakdown of phospholipids and the subsequent production of multiple secondary messengers [16]. In plants, two classes of PLCs exist, and they are distinguished based on their substrate specificity. One is phosphatidylinositol-specific (PI-PLC) and the other is non-specific (NPC). Plant NPCs share homology with bacterial PLCs. NPCs can incline either phosphatidylcholine-specific phospholipase C (PC-PLC), phosphatidylethanolamine (PE-PLC), or phosphatidylserine (PS-PLC). However, PI-PLC is the most considered class of PLC, which hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP2) from the plasma membrane to create IP3 and diacylglycerol (DAG) [44]. The nitrate signaling and phosphatidylinositol-specific PI-PLC links were found in Arabidopsis. Nitrate triggers Ca\(^{2+}\) and inositol 1, 4, 5- triphosphate (IP3), which were not witnessed in the plant’s pretreatment with PLC inhibitor U73122. For instance, the \(\text{NRT}1.1\) mutants, \(\text{chl}1\) and \(\text{chl}9\), revealed that this was an \(\text{NRT}1.1\)-based response. The associated rise in IP3 after \(\text{NO}_3^-\) treatment also suggested that the activity of phospholipase C (PLC) was associated with this signaling pathway [16].

In Arabidopsis thaliana, expression analysis of different PI-PLC genes demonstrated that PLC isoforms were differentially expressed in different plant organs [45–47] and that the expression of \(\text{AtPLC}1\), 2, 3, 4, 5, and 9 were root-specific [48,49].

3.1. Nitrate-Induced Ca\(^{2+}\) and PI-PLC-Dependent Signalling

Phosphatidylinositol-specific phospholipase C (PI-PLC) is the major part of nitrate signaling and transport, modulated by the phosphorylation/dephosphorylating process. Both plasma membrane and tonoplast nitrate transport activity are regulated by phosphorylation [27,29]. In Arabidopsis, Ca\(^{2+}\) has a definite role in plant signal transduction and is also significant for the \(\text{NO}_3^-\)-mediated signaling of gene expression. As stated earlier, \(\text{NO}_3^-\) treatment rapidly increased the cytoplasmic Ca\(^{2+}\) level in the roots [27,29] (Figure 1) and nitrate is absorbed in the root cell by plasma-membrane-localized nitrate transporter families, NRT1 and NRT2 [22]. \(\text{NRT}1.1/\text{CHL}1\) is a low-affinity transporter that switches to a high-affinity transport system when \(\text{NRT}1.1\) is phosphorylated at the threonine residue 101(T101) by protein kinase CBL1/9-CIPK23 [9]. The protein complex CIPK23–CBL9 (CBL-interacting protein kinase (CIPK); calcineurin-B like protein (CBL)) and CIPK8 have been implicated in the dual-affinity transition changes of \(\text{NRT}1.1\) through phosphorylation [33]. More recent studies have revealed that a protein phosphatase 2C (PP2C) family member, \(\text{ABI}2\) (ABA-insensitive 2), and the calcium sensor \(\text{CBL}1\) were distinguished as supplementary constituents that modulate \(\text{NRT}1.1\) transport functions and \(\text{NRT}2.1\) expression in root growth \(\text{NO}_3^-\) responses [38] (Figure 1).

Hence, the phosphorylation activates a weak upregulation of high-affinity nitrate transporter \(\text{NRT}2.1\) [14], and subsequently induces \(\text{NRT}1.1\), \(\text{NRT}2.1\), \(\text{NRT}2.2\), and \(\text{NRT}2.4\) under nitrate-starved seedlings after nitrate supply, while upregulating all the nitrate assimilatory genes [27,50]. CPK phosphorylates the NLP TFs, particularly \(\text{NLP}7\), which interact with \(\text{CPK}20\) in the nucleolus under \(\text{NO}_3^-\) availability. Besides \(\text{NPL}7\), more TFs, such as \(\text{TCP}20\), also contribute to the \(\text{NO}_3^-\)-induced transcriptional changes and systemic signaling. In contrast, \(\text{TGA}1/4\) controls the genes which participate in the PNR, transport, metabolic, and developmental processes [28].
Under limited-nitrate conditions, the NRT1.1 is, therefore, phosphorylated at the T101 in order to stimulate NRT1.1 association with membrane microdomains at the plasma membrane (PM). When nitrate supply is increased, the nonphosphorylated NRT1.1 shows oligomerization and low structural mobility at the PM, thereby initiating rapid inducible endocytosis. These activities could promote LR growth by switching NRT1.1-auxin transport activity on the PM and stimulating Ca^{2+}-ANR1 signaling from the endosomes (discussed in detail in Section 3.2.1, nonphosphorylated nitrate signaling) [51].

3.2. Differential Phosphorylation State of Nitrate Transporters

NRT2.1 is firmly induced by the nonphosphorylated form of NRT1.1, which transports NO$_3^-$ at low affinity. After prolonged exposure to NO$_3^-$ treatment, NRT2.1 is repressed by phosphorylated NRT1.1. The NO$_3^-$ transport capacity under this condition remains obscure [52].

However, after the point mutation at the plasma membrane, the mode of NRT1.1T101 phosphorylation may be different in both NO$_3^-$ uptake and signaling. Transgenic plants of T101A, which mimic the NRT1.1/CHL1 dephosphorylation, exhibits only low-affinity NO$_3^-$ uptake, but can also sense NO$_3^-$ at high-affinity range, with the high-affinity for NO$_3^-$ being comparatively less than the wild-type (WT) [14]. These properties propose that WT NRT1.1 and the T101A mutant may have two NO$_3^-$-binding sites; high affinity and low affinity. It is worth noting that only the low-affinity binding site of the T101A mutant can be transported over the plasma membrane (PM). Unlike NO$_3^-$ uptake, NO$_3^-$ binding to both sites of T101A mutants could trigger the NO$_3^-$ response. This could justify the reason why the CHL1T101A mutant still exhibits a biphasic primary response [14]. In contrast, T101D-expressing transgenic plants that mimic phosphorylated NRT1.1/CHL1 displayed only high-affinity NO$_3^-$ uptake activity and are activated only at a high-affinity primary NO$_3^-$ response. This suggests that T101D can only bind NO$_3^-$ with a high-affinity uptake system [14]. Subsequently, it could be possible that binding sites with low affinity could be blocked by T101 phosphorylation [14] (Figure 2).

![Figure 2](image-url)

**Figure 2.** The schematic diagram describes the differential phosphorylation status of NRT1.1.1T101 at plasma membrane (PM) in the Arabidopsis root, modified by [52]. The layout represents the two binding sites’ low affinity (LA) and high affinity (HA) of T101A. The T101A mutant at the LA binding site follows the NRT1.1-ANR1 signaling pathway upon prolonged exposure to the NO$_3^-$ under low-nitrate conditions, resulting in LR elongation. This is a nonphosphorylated form of NRT1.1-induced signaling that promotes LRs. In the inserted graph, the grey line represents the weak upregulation of NRT2.1 under low nitrate, and the blue line represents the strong upregulation of NRT2.1 under high nitrate. The graphs on the left and right represent the NRT2.1 induction; see text for more details.
The two NO$_3^-$ binding sites depicted here (Figure 2) have two adaptations of a single binding site. Taken together, these findings suggest that at the low level of NO$_3^-$ sensing, T101 phosphorylation keeps the PNR, whereas, for uptake and substrate-binding, T101 phosphorylation may repress the low-affinity NO$_3^-$ binding and is then required to use the high-affinity transport system [14].

3.2.1. Non-Phosphorylating Form of NO$_3^-$-Induced Signaling

*NRT1.1* contributes to the NO$_3^-$-mediated auxin transport, regulates auxin storage, and subsequently influences LR development [53]. The signaling network comprising of Ca$^{2+}$-, Ca$^{2+}$-protein kinases (CPKs), and NIN-like protein (NLPs) interacts with NO$_3^-$ via primary transcription to regulate LR growth [23,45]. In addition to this Ca$^{2+}$-, ARABIDOPSIS NITRATE REGULATED1 (ANR1), a transcription factor functioning downstream of *NRT1.1* and NLP7, has been involved in LR elongation under high NO$_3^-$ (HN) conditions [54]. In a plant developmental network, nitrate-induced Ca$^{2+}$-ANR1 signaling is a nonphosphorylated form of *NRT1.1* signaling, promoting LR growth. NO$_3^-$ triggers a unique Ca$^{2+}$-CPKs-NLPs signal, acting as downstream segments of NLP and ANR1, subsequently controlling LR elongation [16] (Figure 1).

*NRT1.1* phosphoryl influences cytoplasmic Ca$^{2+}$ levels in the epidermal cells of the LRs, which was measured by using Fluo-4 dye in various genotypes [52]. In view of the pseudocolor and kymograph pictures of wild-types (WTs), after NO$_3^-$ stimulation, (Ca$^{2+}$)$_{cyt}$ signaling was screened at the proposed 60-second period. This was previously depicted by [16]. The researcher found that NO$_3^-$ explicitly induced Ca$^{2+}$ signature in the WT but not in *chll-1-5* mutant seedlings. Under both HN and LN conditions, *T101A* seedlings exhibited a transient increase in (Ca$^{2+}$)$_{cyt}$ [51], while *T101D* seedlings displayed a decrease in (Ca$^{2+}$)$_{cyt}$. Concomitantly with (Ca$^{2+}$)$_{cyt}$ accumulation, HN-stimulated expression of *ANR1* in LRs is sensed in *T101A*, but not in *T101D*. In the light of these findings, it is suggested that a nonphosphorylated form of *NRT1.1* could activate the Ca$^{2+}$-CPKs-NLPs signaling pathway to induce the expression of *ANR1*, and subsequently control LR elongation [51]. It was analyzed that intracellular transport of *T101A* and *T101D* in LR cells showed that differential phosphorylation of *NRT1.1* enhanced the implementation of *NRT1.1*-stimulated signal transduction in LR growth [51]. Phosphorylated *NRT1.1* takes up the sparingly accessible NO$_3^-$ from the soil at high affinity and induces the *NRT2.1* expression to a lower extent compared to the low-affinity state [52] (Figure 2). Under high NO$_3^-$ conditions, *NRT1.1*-induced auxin transport is inhibited, and shortly after NO$_3^-$-treatment, the dual affinity modes of the *NRT1.1* are regulated at Thr-101(T101) phosphorylation [52].

As mentioned earlier, under low NO$_3^-$ conditions, phosphorylation at T101 stimulates *NRT1.1* association with a functional membrane microdomain at PM [51], confirming the *NRT1.1*-mediated auxin flux, and subsequently repressing their growth by reducing the LRP auxin level. With an increased NO$_3^-$ level, nonphosphorylated *NRT1.1* shows oligomerization and low lateral mobility at the PM and rapid inducible endocytosis. This activity may stimulate LR development by supporting *NRT1.1*-auxin transport activity on the PM to induce Ca$^{2+}$-ANR1-signaling from the endosome [51]. Further studies have shown that seedlings of *T101A* had much higher LR density than that of *T101D* when grown under low NO$_3^-$ conditions (0.2 mM), whereas in high NO$_3^-$ conditions (1 mM), no significant difference was observed in the LR density of the mutants compared to WT plants [51]. These findings confirm that *T101A* and more nonphosphorylated WT *NRT1.1* promote LR growth in LN by suppressing basipetal auxin transport, and subsequently accumulating auxin in the LR tips [51].

3.3. Nitrate-Induced Ca$^{2+}$ and PI-PLC-Independent Signaling

Ca$^{2+}$ and PI-PLC are not affected by the expression of NO$_3^-$ responsive auxin signaling F-Box3 (AFB3) protein, indicating that beyond Ca$^{2+}$ and PI-PLC, there is a PI-PLC-independent pathway that controls the regulation of the nitrate-sensitive genes [16,55] (Figure 1). Hence, *NRT1.1* toggles within the phosphorylation status of a critical threonine residue from low- to high-affinity states. This residue is amongst the second and third transmembrane helices of *NRT1.1* located in the intracellular side [14,25].
In *Arabidopsis* root, Ca$^{2+}$ and PI-PLC-independent miR393/AFB3 regulatory modules are recognized as nitrate responsive genes, which assimilate nitrate and auxin signaling [56]. Nitrate induced LRs are dependent on miR167, and its target auxin-responsive factor *ARF8* mRNA [57] plays a distinctive role in regulating several genes connected via a network to promote the stimulation of LR initiation and inhibition of elongated roots in response to N [57] (Figure 1). This earlier identified regulatory module, controlled by miR393 microRNA and the AFB3 auxin receptor, stimulates LRs in response to external and internal NO$_3^-$ applications [51,58]. AFB3 is induced by NO$_3^-$ and repressed by miR393, whereas nitrate reduction and assimilation produced N metabolites, which induces miR393 [59] (Figure 1). Furthermore, AFB3 coregulates NAC4 and OBP4, and this coregulation is confirmed by using the green fluorescence protein (GFP)-expressing lines after 2 h, in response to nitrate. AFB3, activated in the pericycle, indicated that the AFB3-NAC4-OBP4 complex might build a regulatory module that controls LR growth in a NO$_3^-$-dependent manner [56].

Nitrate-stimulated AFB3 induced in the root might be a specific signaling network of Aux/IAA and ARF factors to modulate NAC4 activation and LR growth. The abundant Aux/IAA-ARF modules chronologically generate new LRs and control LR development in *Arabidopsis*. The lateral root basal meristem (the zone between meristem and elongation) depends on IAA28 and ARF proteins, which include transcription factors *ARF5*, *ARF6*, *ARF7*, *ARF8*, and *ARF19* [13,53]. In plant RSA, the LR initiation and emergence of the AFB3 overexpression line and the *afb3* mutant line have emerging roles compared to wild-types and display increased growth of LRs under nitrate-sufficient conditions. Additional findings revealed that the transcription factor NAC4, which functions downstream of AFB3, might be involved in two dependent pathways of RSA regulation [52,58]. Following AFB3, NAC4 acts downstream in the pericycle cell to alter LR density in nitrate treatments [9,51].

**Auxin Response Network**

Auxin signaling is primarily passed over by transcriptional pathways for morphogenesis and developmental processes, which include TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) proteins, AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional co-regulators and AUXIN RESPONSE FACTOR (ARF) transcription factors [60–62]. At low levels of auxin concentration, members of the transcriptional inhibitor family AUXIN/INDUCIBLE (AUX/IAA) interact with the DNA-binding protein of ARF [56,57], while the ARF proteins function to detect the auxin-response promoter elements (AuxREs) in various auxin-regulated genes to activate or suppress their expression [63,64]. AUX/IAA protein inhibits the ARF function either by passively inhibiting ARF proteins from their target promoters [65] or by binding ARF with the corepressor TOPLESS (TPL) for inactivation of the chromatin and silencing of ARF target genes [56,59,66]. An increase in auxin concentration by an auxin-induced module of the coreceptor complex consists of F-box protein from the TRANSPORT INHIBITOR RESPONSE 1 (TIR1)/AUXIN SIGNALING F-BOX PROTEIN (AFB) family and is an Aux/IAA member [60,67,68]. TIR1/ABFs, a subunit of nuclear S-PHASE KINASE ASSOCIATED PROTEIN 1-CULLIN-F-BOX PROTEIN (SCF)-type E3 ubiquitin-protein ligases (SCFTIR1/AFB), stimulate the recognition of substrates. The auxin response is initiated by connecting hormones to the TIR1/AFB receptor. The auxin receptor is part of the SCFTIR1/AFB ubiquitin ligase complex [69,70]. Binding of auxin to its receptor TIR1/AFB activates the information and breakdown of the polyubiquitination of the Aux/IAA inhibitor, which subsequently releases the inhibition of ARF transcription factors, which induce the transcription of auxin-responsive genes [71,72]. This represents the pivot of auxin signaling.

In a simpler form, auxin-initiated AUX/IAA removal relieves ARF inhibition and activates the transcription of primary genes. Remarkably, the auxin response network is enough to reconstitute the AuxRE-dependent activation of reporter genes in yeast [73]. Hence, in *Arabidopsis* root, a miR393/AFB3 regulatory module is recognized as nitrate-responsive, which assimilates nitrate and auxin signaling to promote root growth [56].
4. The Effects of Nitrate on RSA

4.1. Effects on Primary Root Growth and Development

Generally, the primary root (PR) growth in *Arabidopsis* is typically found to be relatively insensitive to or even induced by the normal range of NO$_3^-$ concentration [24,74,75]. It could be inhibited under some culture conditions by moderately high NO$_3^-$ supply [56]. It was presumed that AFB3 controls LR initiation and PR development by two distinct pathways, of which one is NAC3-dependent, while the other is NAC3-independent [76]. However, studies on the effect of amino acid and peptide on root growth and branching have gained little attention [77]. At a low concentration of glutamate (<50 mM), the PR tip has a unique and differential effect on root architecture, inhibiting PR growth and subsequently stimulating LR growth [78] (Figure 3).

![Figure 3](https://example.com/figure3.png)

**Figure 3.** The schematic diagram presents the multiple pathways regulating the root system architecture (RSA; lateral and primary root) response to the localized and high nitrate conditions in *Arabidopsis*. Only those pathways discussed in the present review are depicted. The green arrows indicate systemic transport and assimilation, the black arrows indicate positive signaling as a stimulatory effect, red lines indicate negative signaling as an inhibitory effect, the orange lines depict the unknown positive and negative signaling pathways, and dotted lines represent the unconfirmed nitrate-mediated signaling pathways. The low nitrate and severely low nitrate conditions have been reported to have a stimulatory and inhibitory effect on LR development, respectively, while high NO$_3^-$ supply has an inhibitory effect on LR growth [24] (see text for further information). External NO$_3^-$ regulates primary root growth in *Arabidopsis*. The receptor for the external glutamate signal is shown as a glutamate-gated Ca$^{2+}$ channel because these are known to be activated at root tips [79]. However, its specific role in this signaling pathway is unconfirmed (see text for further information).
To this end, this response is glutamate-specific in *Arabidopsis* since an ongoing study of the impact of 17 other proteinogenic amino acids on the architecture of the roots found none that could produce its distinctive effect on root architecture [77]. By using a chemical genetic approach, the MEKK1 MAP kinase gene has since been investigated as part of the glutamate signaling pathways in PR tips [80]. MEKK1 functions mainly as a distinctive immune system and its expression was demonstrated to be profoundly receptive to a variety of abiotic factors [81]. Nitrate exhibits a strong signal to stimulate the primary root development by enhancing the activity of the meristem and cytokinin signaling. Cytokinin sensing and biosynthesis mutants showed shorter roots compared with wild-type when subjected to NO$_3^-$ treatments, especially when NO$_3^-$ is the primary source [82]. Histological studies of the root tip revealed reduced cell division and elongation in the cytokinin receptor double mutant *ahk2ahk4* (histidine kinase) compared with WT plants under adequate NO$_3^-$ supply. It is worth noting that as NO$_3^-$-mediated restriction in the root growth was observed between 5 and 6 days after planting, the WT plants had the potential to recover from the growth-restricted condition, whereas cytokinin signaling or biosynthesis mutants were most certainly not capable of recovering [82].

In addition, the transcriptomic analysis indicated that genes associated with both cell division and elongation are possibly significant for PR development in response to NO$_3^-$, thereby indicating the interaction between nitrate and cytokinin signals in regulating PR development in *Arabidopsis* [82].

### 4.2. Effects on Lateral Root Growth and Development

The growth of lateral roots is strongly affected by the concentration of N in the growth environment. For instance, in low NO$_3^-$ soil, patches of high NO$_3^-$ have a localized stimulatory impact on LR development, which varies in different plant species [2,74], whereas under high NO$_3^-$ conditions (with no restricted growth), LR development is repressed [83]. Further studies also revealed that NO$_3^-$ plays a prominent role in regulating LRs. Generally, low NO$_3^-$ has a dual effect on the LRs, such as stimulatory as well as inhibitory effects, whereas high NO$_3^-$ supply only exhibits an inhibitory effect on LR growth and development of LRs [4]. In other words, there are two clear morphological adaptations. Under N-deficient conditions, the LRs are significantly stimulated; however, when exposed to more severe N deficiency, the entire LR length reduces and LR formation disappears [13]. This is initiated by the signaling impact of NO$_3^-$ itself, rather than downstream metabolites [2].

#### 4.2.1. Stimulatory Effect of Low Nitrate on LR Growth

The low NO$_3^-$-stimulated *Arabidopsis* LR development depends on the role of the auxin biosynthetic gene *TAR2* (tryptophan aminotransferase related 2; Figure 3), which is expressed in the pericycle and vasculature of developed roots close to the root tip and is stimulated under low-nitrogen conditions. In WT plants, the low NO$_3^-$ restored auxin accumulation in the primordial of the nonemerged LRs, with an additional three cell layers and LR emergence. On the other hand, these low N-stimulated auxin accumulation and root developmental responses were disrupted in *tar2* null mutants [4,51]. Subsequently, *TAR2* is required for restructuring the root architecture in response to low N conditions. Another nitrate responsive gene, *BBX16* (bobby sox homolog), belongs to the constans-like zinc finger family. The *bbx16-1* mutant affects lateral root length (LRL) in response to NO$_3^-$, with longer LRs by 1 mM KNO$_3^-$ as low nitrate treatment. The *bbx16-1* mutants produce larger LRs under NO$_3^-$ limitation [48] (Table 1).

When the NO$_3^-$-deficient condition becomes severe, the *Arabidopsis* *AGL17*-clad MADs-box gene *AGL21* is induced by N shortage and auxin to promote LRs in *Arabidopsis*, whereas *agl21* mutants exhibit a reduction in LR elongation in response to low NO$_3^-$ treatments. Furthermore, the auxin biosynthesis genes *YUC5*, *YUC8*, and *TAR3* are significantly upregulated in overexpressing (OE) lines and downregulated in *agl21* mutants, demonstrating that *AGL21* enhances the local auxin activity in the LR primordial, and thus substantially influencing LR growth regulation [77,80,88].
Table 1. Transcription factors of genes associated with nitrogen signaling and nitrogen-associated processes in *Arabidopsis thaliana*.

| Transcription Factors | Family                                      | Transcriptionally Associated with NO<sup>3</sup>− Signalling | Tissue Expression                                      | Molecular Function                                      | Effect on Root                                      | Localization                              | Refs   |
|-----------------------|---------------------------------------------|-------------------------------------------------------------|--------------------------------------------------------|---------------------------------------------------------|----------------------------------------------------|------------------------------------------|--------|
| CEPD2                 | CC-type glutaredoxin (ROXY) family          | yes                                                        | Root, root endodermis, root vascular system            | Cellular response to nitrogen starvation                | Regulate the efficiency of root N acquisition     | cytoplasm, nucleus                        | [84]   |
| AtGRXS3/4/5/8/ROXY11  | CC-type glutaredoxin (ROXY) family          | yes                                                        | Root and other tissue                                  | Cell redox homeostasis                                  | Increased primary root length                   | cytoplasm, nucleus                        | [85]   |
| ERF4                  | Subfamily B-1 of ERF/AP2 transcription factor family | yes                                                        | Root and other tissue                                  | Transcription regulatory region DNA binding             | Antagonizes JA inhibition of root elongation       | nuclear body, nucleus                     | [48,86]|
| RAV2                  | Ethylene-responsive element-binding protein family | yes                                                        | Root and other tissue                                  | Transcription regulatory region DNA binding             | Genotype based Shorter LRL to both high and low NO<sup>3</sup>− | nucleus                                 | [48]   |
| VIP1                  | VIRE2-interacting protein 1                 | yes                                                        | Root and other tissue                                  | unknown                                                | unknown                                            | cytosol, nucleus                          | [48]   |
| ERF070                | Ethylene-responsive element-binding protein family | yes                                                        | Root and other tissue                                  | Regulation of transcription                            | unknown                                            | nucleus                                  | [48]   |
| HMGB15                | AT-rich interaction domain-containing transcription factor family | yes                                                        | Root and other tissue                                  | Glucosinolate metabolic process,                      | Larger LRs response to nitrate deprivation        | nucleus, pollen tube                      | [48]   |
| PAP2/MYB90            | MYB domain transcription factor family       | yes                                                        | unknown                                                | Regulation of transcription,                           | Trichome and root hair organogenesis              | nucleus                                  | [49,87]|
| BBX16                 | Constans-like zinc finger family            | yes                                                        | unknown                                                | Positive regulation of transcription                   | Total LRs length (LRL)                           | nucleolus, nucleus                        | [48]   |
Previous studies about rice have revealed that the AtNRT2.1 homolog OsNAR2.1 knock-out mutant initiates the inhibition of LRs under low NO$_3^-$ concentration by reducing PIN protein levels in the roots [89]. NRT2.1 positively regulates LRs by influencing the polar transport of auxin under low NO$_3^-$ conditions. The impact of NRT2.1 on LR growth is possible by a combination of NO$_3^-$ uptake and signaling. NRT2.1 cannot function independently as a NO$_3^-$ transporter. Hence, NRT2.1 might act as a key factor in this signaling pathway [4]. It was thus demonstrated that OsNRT2.1 could be involved in the nitrate-dependent pathway of root elongation by regulating auxin transport to the roots under low NO$_3^-$ conditions [90]. Apart from the aforementioned pathways comprising both transcriptional factors and hormonal signals, nitric oxides (NOs) have been accounted for as a significant NO$_3^-$-mediated signal which regulates RSA in plants [79,82]. In rice, NO produced by NR could enhance the inadequate production of N by developing LR initiation under partial NO$_3^-$ availability [91,92]. To this end, LRs are significantly stimulated by mild NO$_3^-$ deficiency. Different molecular players are involved in the regulation of different stages of plant growth and development.

4.2.2. Inhibitory Effects of Severely Low Nitrate on LR Growth

Earlier studies have found that the impact of NO$_3^-$ was related to the ability of the localized NO$_3^-$ supply to stimulate LR elongation [23,83]. Experimental estimation of using a limited, rather than uniform, NO$_3^-$ treatment initiates the specific effects of the external NO$_3^-$ on LR development, and this can be observed under conditions where the systemic effects, due to changes in the N status of the plant, can be limited to a greater extent [2,4]. Under severe N deficiency, both LR formation and length are repressed in plants [93].

A recent investigation [4] featured the vital role of the peptide-receptor signaling module, which comprises N-responsive CLE (CLV3/ENDOSPERM SURROUNDING REGION (ESR))-related peptides and the CLAVATA1 (CLV1) leucine-rich repeat receptor-like kinase regulatory module, in regulating LR growth of Arabidopsis thaliana. CLE1, −3, −4, and −7 are expressed in root pericycle cells of Arabidopsis roots. Under NO$_3^-$ deficient conditions, overexpression (OE) of CLE genes results in the repression of LR emergence from the PR. This inhibitory action of the CLE peptides also affected LR development required for the feedback function of CLV1 expressed in the phloem of the root companion cells, indicating that the downstream signal is transmitted via phloem for the systemic regulation of RSA [4]. An additional system, downstream of CLV1 feedback, regulates the transcript level of the N-responsive CLE genes in the roots for fine-tuning of the signal amplitude [4,89]. In other words, CLEs-CLV acts as a regulatory module in NO$_3^-$ signaling pathways, and it also antagonistically controls the growth of LRs under limited N conditions [4,94].

Similarly, one member of the CEP (C-TERMINALLY ENCODED PEPTIDE) gene family has been shown to arrest root growth [95]. The analysis of OE-lines of several CEP genes demonstrates their distinctive function. It was reported that CEPs have an antagonistic effect on LR growth while initiating a delay in PR and LR growth [95].

Another mechanism of the systemic inhibition of LR growth is associated with the inhibition of LRs in response to NO$_3^-$. Limited NO$_3^-$ supply significantly increases abscisic acid (ABA) accumulation, as this ABA accumulation inactivates its coreceptor ABI2 (ABA-insensitive 2) and protein phosphate 2C (PP2C) [96] (Figure 3). The ABI2 then co-interacts with Ca$^{2+}$-sensor subunit CBL1 and the kinases (CBL1-CIPK23) complexes, with their substrate being NRT1.1/NPF6.3. Hence, under low NO$_3^-$ conditions, the protein kinase CIPK23 phosphorylates NRT1.1 to sustain movement at low NO$_3^-$ concentrations [14] (Figure 3). This hypothetical pathway, reconfirmed in recent studies, has revealed that alteration in the ABI2 status promotes the activation of the CBL1–CIPK23 complex, and subsequently reduces root NO$_3^-$ uptake by inhibiting NRT1.1 transport activity under NO$_3^-$-deficient conditions [38]. However, the downstream constituents of this pathway are still unknown. It is thus still unclear whether the antagonist effect of ABA on the LRs, subjected to low N conditions, is a consequence of the disrupted NO$_3^-$ signaling pathway or physiological function of ABA itself.
Moreover, irrespective of the NO$_3^-$ activity, NRT2.1 functions as a NO$_3^-$ sensor or signaling component to inhibit LR initiation under low-NO$_3^-$ conditions [25,93]. However, their exact underlying mechanism is still unclear. The negative effect of NRT1.1/NRT2.1 on LR growth indicates the distinct systemic pathways under limited NO$_3^-$ supply [4]. Taken together, NRT1.1/NRT2.1 has a negative role in LR growth and possibly clarifies the inhibitory effect of high NO$_3^-$ on L development. NRT1.1/NRT2.1 functions negatively and also have an inverse effect on these signaling pathways to control LR growth and development under limited NO$_3^-$ conditions. The action of each pathway depends on the level of the N deficiency in plants or their specific ecological conditions [4].

4.2.3. Systemic Inhibitory Effect of High External Nitrate on LR Growth

The LRs of Arabidopsis exhibited two different responses to high NO$_3^-$. High NO$_3^-$ (10 mM) conditions decreased the entire root system, whereas, when plants are subjected to low NO$_3^-$ concentrations (10 μM), the PR part exposed to high NO$_3^-$ triggered the local induction of LR elongation [2,83]. However, the global inhibitory effect of NO$_3^-$ appeared to be as a result of prolonged exposure of plants to ample NO$_3^-$ supply. The LR elongation under this condition was also suppressed in the areas of the root system that were subjected to the state of low NO$_3^-$ conditions [4,97].

As reported earlier, the AFB3 receptor gene is strongly induced by NO$_3^-$, and the LR initiation is specifically diminished in afb3 mutants [59]. Research on the nitrate reductase (NR)-null mutants has revealed that NO$_3^-$ itself was the main stimulator of AFB3. AFB3 expression feedback is regulated by nitrate-assimilatory products, such as miR393, a micro RNA that targets AFB3 transcript for degradation. This pathway has further confirmed the findings that nitrate (NO$_3^-$) induced NAC4 and OBP4 transcription factors, functioning downstream of AFB3. Taken together with the results obtained from nac4 mutants, the afb3 mutant displays an apparent reduction in LR growth in response to NO$_3^-$ [56]. Similarly to this was the influence of the myb29-1 allele on lateral root length (LRL) when subjected to diverse NO$_3^-$ conditions, exhibiting shorter lateral root length (LRL) at high NO$_3^-$ (10 mM KNO$_3^-$) treatments [48]. However, the rvo2-1 and erf107-1 alleles, which are genotype-dependent, exhibited reduced lateral root length (LRL) when subjected to both 1 and 10 mM KNO$_3^-$ conditions [48] (Table 1). Recent studies have demonstrated that high-affinity NO$_3^-$ transporter AtNRT2.1 may be involved in the inhibition of LR initiation at high C: N ratios [98]. Also, the involvement of the ABA affecting LR growth, in response to NO$_3^-$, might be connected to the recently identified ABA receptor [98]. Nitrate reductase (NR)-lacking mutants display sensitivity to this systemic inhibitory effect, indicating that NO$_3^-$ concentration in the tissue of plant cells may function in inhibitory signal induction. Thus, this model defines root branching, as modulated by inhibitory signals via internal N status and external NO$_3^-$ supply [83].

Furthermore, ABA, which is associated with the systematic inhibitory effect of high NO$_3^-$ on LR growth, might be connected with the recently identified ABA receptor FLOWERING CONTROL LOCUS A (FCA). In addition, root architecture response to the recently identified external L-glutamate conceivably provides a significant tool for studying biological functions of plant glutamate receptors and amino acid signaling [98]. It was also reported that FCA possibly acts as a receptor for ABA. The loss of function mutant fca displays low sensitivity to the inhibitory effect of ABA on LRs, indicating that FCA might be a constituent in signaling transduction pathways associated with high NO$_3^-$ ABA-mediated inhibition of LRs [4,99,100].

It has been genetically proven that inhibition by ABA and NO$_3^-$ is mediated by the same signaling mechanism. For instance, the LABI (lateral roots ABA-insensitive) is characterized based on the LR production affinity when exposed to 0.5 μM, which is less sensitive to the high NO$_3^-$-induced LR inhibition [4] (Figure 3). The identification of LABI genes could give indepth information about the signaling mechanism underlying this inhibition [98]. Interestingly, all the mutants produced shorter primary roots phenotypes, which indicated that LR development could be intrinsically correlated with PR growth. It was reported that the presence of the PR meristem is required for high NO$_3^-$ and ABA-induced inhibition; however, this inhibition could be eliminated by the removal of the PRs [4].
Furthermore, root architecture response to glutamate may give an essential experimental framework to study glutamate signaling in plants and to elucidate the possible roles of the glutamate receptor [98]. Recent studies have shown that high NO$_3^-$ supply (30 mM) stimulated ABA accumulation in the emerging root tips by discharging it from the inactive stores via ER-localized β-GLUCOSIDASE1 (BG1) to regulate root development. This information provides a system for NO$_3^-$-induced root development via the regulation of ABA accumulation in the root tips. It was hypothesized that there is a close association between ABA and NO$_3^-$ signaling to coregulate LR growth [81]. A recent study has also shown that myb29-1 mutants increased the LR length, LR density, and total length under adequate NO$_3^-$ supply in a genotype-dependent manner [48] (Table 1).

5. Coordinated Regulation of Nitrate and Other Messengers on RSA

Root foraging for NO$_3^-$ involves both local and systemic signaling. NO$_3^-$-auxin-CK regulation could also be a key constituent of N systemic signaling, which coordinates nutritional requirements among various organs at different growth stages [101,102].

5.1. Nitrate-Mediated Auxin Allocation

A systemic regulation that includes the inhibition of auxin translocation from the shoot to root suppresses LR initiation and development and subsequently affects NO$_3^-$ use efficiency in plants [103]. In such a situation, growing Arabidopsis thaliana on a nitrate medium was observed to have reduced auxin contents in the roots, while increasing the auxin content in the shoots. These findings have demonstrated that high NO$_3^-$ inhibits the translocation of auxin from the shoots to the roots [78].

In addition, nitric oxide (NO) was found to be a key nitrate-related signal that regulates plant RSA and the signaling cascade of lateral root formation induced by auxin [104]. It can be deduced from the previous observation that a decrease in NO$_3^-$ provision tends to promote auxin translocation from shoot to root. The high NO$_3^-$-inhibited root growth is a consequence of condensed cell elongation, and also probably due to the changes in meristematic length. Higher NO$_3^-$ supply diminished the IAA concentration in the phloem exudates. The NO$_3^-$-induced inhibited root growth was closely associated with the reduction of auxin in the roots, especially in the regions close to the root tips. The regrowth of PRs by external NAA and IAA under high NO$_3^-$ levels confirms that this inhibitory effect via high NO$_3^-$ might be partially associated with the reduced IAA level in the roots [42].

However, the effect of NO$_3^-$ on root growth could be complicated by the fact that high NO$_3^-$ concentration (50 mM) triggers complete inhibition of LR development [105]. It has been experimentally confirmed that these responses are linked to an auxin transport inhibitor. To this end, the local supply of nitrate reduced the transport of auxin from shoot to root, and this subsequently resulted in decreased root auxin concentration to a level more appropriate for lateral root growth. However, for the stimulation of LRs, a change in the root auxin concentration only is not adequate. Regardless of these models, few ideas concerning the transcriptional gene regulatory system are known [106].

Furthermore, under available nitrate conditions, the auxin level in the root decreased compared to low NO$_3^-$ conditions, and nitrate application seemed to inhibit auxin transport from shoot to root. In many cases, the external IAA partially lowers the stimulatory effect of localized nitrate. High nitrate supply reduces the IAA concentration in the phloem exudates; thus, suppression of root growth by high nitrate is mainly dependent on the reduction of IAA levels in the roots, specifically in the root tip region. It could be deduced that the inhibitory impact of high nitrate concentration on the restricted root growth may be associated with the decline in auxin content in the roots [42].

The currently accessible information leading to a potential connection between nitrate and auxin accumulation influences the rate of auxin biosynthesis, transport, and allocation of auxin from root to shoot [107].
5.2. Nitrate-Mediated Cytokinin Allocation

Cytokinin (CK) affects intercellular auxin transport by regulating the expression of numerous auxin transport components, and thus balances the auxin distribution to regulate the size of root meristem [108]. Findings have also shown that the NO\textsubscript{3}−-CK shoot–root dependent system exhibits the NO\textsubscript{3}− demands of the whole plant, which affects root growth in NO\textsubscript{3}− rich patches of the soil [109]. Since CK could be widely distributed throughout the entire plant cell, CK-induced root–shoot coordination is a proposed model of systemic signaling for nutritional status [110]. CK activity could be closely associated with NO\textsubscript{3}− accessibility. Apart from the downstream metabolites of NO\textsubscript{3}−, NO\textsubscript{3}− has been known to initiate rapid de novo CK synthesis and accumulation in Arabidopsis roots [111]. The CK biosynthesis occurs in different parts of the plant tissue, where the adenosine phosphate-isopentenyltransferase (IPT) is expressed. IPTs are the primary enzymes that mainly influence the rate of CK biosynthesis, such as the prenylation of adenosine 5′ phosphates and ATP and ADP at the N\textsuperscript{6}- terminal with dimethyl diphosphate (DMAPP) [112].

In Arabidopsis, IPT3 is regulated in a NO\textsubscript{3}−-dependent manner. The expression of IPT3 with several Arabidopsis response regulators 3, 5, 6 (ARR3, 5, 6) are induced by NO\textsubscript{3}− during the PNR. Moreover, IPT3 is highly induced in the roots and weakly induced in the shoots in both WT and NR-null mutant plants during the PNR, partially mediated by NRT1.1 [35]. During the PNR, NLA is among the highly inducible genes; thus, NO\textsubscript{3}− firmly controls CK biosynthesis via activation of IPT3. This indicates that IPT3 is the fundamental determinant of short-term NO\textsubscript{3}−-dependent CK biosynthesis, specifically in the roots, in response to immediate variation in the soil NO\textsubscript{3}− [111]. In addition, the type-A ARR genes, including ARR3, 5, and 7, similar to the CK metabolism genes, were found to respond to NO\textsubscript{3}− but not to NH\textsubscript{4}+. CYTOKININ RESPONSE FACTORS (CRFs) which are also highly inducible by NO\textsubscript{3}− [15], are known to be transcriptionally activated by CK and its disruption influences the basal expression of a significant number of CK-regulated genes, including type-A ARRs. CRFs are involved in promoting plant growth and leaf senescence [113]. The close regulation of the CYP735A2 and IPT3 by NO\textsubscript{3}− could be a major factor shaping NO\textsubscript{3}−-dependent spatio-temporal CK distribution in plants, and also regulating root system architecture in response to several abiotic stresses [114]. In short, nitrate and two hormonal mediators, CK and its antagonistic partner, auxin, act in synergy to modulate CK biosynthesis for root development.

6. Role of NO\textsubscript{3}− Transporters in Mitigating Plant Stress

Nitrate transporters are ultimately responsible for the absorption of NO\textsubscript{3}− from the soil and translocation of NO\textsubscript{3}− to various aerial parts of the plant [115]. NO\textsubscript{3}− transporter NRT1.1 acts as a positive growth regulator of vegetative and reproductive organs [116]. Studies have shown that AtNRT1.1/AtNPF6.3/CHL1 might be involved in the tolerance of the plant to proton toxicity; further studies on chl1 mutants, however, have revealed a reduced proton tolerance when compared with WT [117]. Moreover, the accumulation of sodium (Na\textsuperscript{+}) in the plant was found to be defective on npf6.3/nrt1.1 mutants, thus npf6.3/nrt1.1 functions in drought tolerance in the presence of NO\textsubscript{3}− [118]. The downregulation of NRT1.5 and the upregulation of NRT1.8 were observed in the root of the plant on exposure to cadmium (Cd\textsuperscript{2+}). Thus, increased NO\textsubscript{3}− accumulation in the root [119] indicates that NRT1.1 and NRG2 function downstream from NRT1.1 to regulate Cd\textsuperscript{2+} stress and also to stimulate NO\textsubscript{3}− distribution to the root [119]. AtNPF7.3/ATNRT1.5 is highly expressed in the root and highly inducible by phosphate starvation. The ATNRT1.5 mutant atnrt1.5 exhibits longer PRs, with reduced LR density under Pi-deficient conditions, compared with WT. This is an indication that a reduction in the morphological variation by ethylene synthesis antagonizes CO\textsubscript{2} [120].

In addition to the transporters stated earlier, npf6.4 mutants exhibit increased resistance to polyamine [115]. AtNPF2.12/AtNRT2.6 positively regulated seed abortion under NO\textsubscript{3}−-deficient conditions in Arabidopsis [121]. Moreover, AtNPF2.5 and AtNPF2.3 induced chloride (Cl−) efflux from Arabidopsis roots and subsequently contributed to NO\textsubscript{3}− translocation [122]. AtNRT1.8/AtNPF7.2 tolerates Cd\textsuperscript{2+} and salt stress. However, its knock-out mutants exhibited sensitivity to abiotic stress [123].
AtNPF3.1 transported ABA and GA (gibberellic acid) in vitro [124]. The interaction between NO$_3^-$ and NRT-mediated NO$_3^-$ uptakes on exposure to Pb in Arabidopsis via NRT-related mutants [125] demonstrates a new strategy for plant tolerance to lead (Pb) contamination [125].

Under low NO$_3^-$ conditions, an NRT2 member, AtNRT2.1, contributes to iHATS (inducible high-affinity transport system) and plays a crucial role in the RSA, while AtNRT2.4 contributes to plant biomass production. AtNRT2.5 also stimulates mature plants under NO$_3^-$-deficient conditions [126]. AtNRT2.6 expression is induced after phytopathogenic bacterium inoculation. Hence, plants with low NRT2.6 expression show lower tolerance to pathogenic attacks [127]. Interestingly, there is a correlation between NRT2.6 expression and reactive oxygen species (ROS) accumulation in response to E. amylovora infection and treatments with the redox-active herbicide methyl viologen. This indicates a probable link between NRT2.6 activity and the production of ROS response to biotic and abiotic stresses [127].

In the chloride channel family (CLC), AtCLC accumulates anions in the vacuole when stomata are open, and also facilitates anion release during stomatal closure in response to stress hormones like abscisic acid (ABA) [128]. In addition to the NO$_3^-$ transporter, the NO$_3^-$-associated transcription factor, phloem-mobile CEPD-like 2 (CEPDL2)-polypeptide contributes to NO$_3^-$ acquisition, along with CEPD1 and CEPD2, which mediate root N status, and the loss of each of these three proteins severely impair N homeostasis in the plants. A similar study showed that shoots of the CEPDL2/CEPD1/2 genotype characterize a high-affinity NO$_3^-$ uptake duration in the roots, thereby indicating a systematic regulation of root N acquisition [84]. ANTHOCYANIN PIGMENT1 (PAP1) and its homolog PAP2/MYB90 were strongly stimulated by NO$_3^-$ [129]. Recent research has demonstrated that three LBDs regulate anthocyanin synthesis via repression of PAP1 and PAP2. MYB and bHLH (basic helix-loop-helix) proteins form complexes with TTG1 (TRANSPARENT TESTA GLABRA1) WD40-repeat protein in Arabidopsis to modulate several other epidermal gene expressions such as anthocyanin regulation, proanthocyanin, and mucilage biosynthesis in the seed coat or trichome and root hair organogenesis [49].

7. Conclusions

RSA response of the plant to NO$_3^-$ accessibility represents a prominent model to study developmental plasticity; however, the underlying mechanism remains highly obscure [130]. One of the most important discoveries in the past few years has been the involvement of NO$_3^-$ transporters NRT1.1 and NRT2.1 in early response signaling, and their effects on the morphological adaptation of the plant RSA. Despite their roles as transporters and in signaling response, NRT1.1 cannot fully explain the complete mechanism of the NO$_3^-$ responses observed in plants [43]. However, some findings have supported the previous speculation that NO$_3^-$ transporters could act as early NO$_3^-$ sensors [98]. This provides critical insights into understanding the ability to sense NO$_3^-$ as well as other nutrients [52].

In this review, we have summarized in depth the characterization of the nitrate transporters NRT1.1 and NRT2.1 in Arabidopsis (Figure 1), delivering clues on how NO$_3^-$ is sensed, taken up, and mobilized, and their modification by phosphorylation at the T101 residue has also been well demonstrated. In addition, the influences of physiological growth on RSA under low and high NO$_3^-$ conditions, and the underlying molecular players, including TFs and N metabolites, are hypothesized and are associated with the transcriptional control of significant NO$_3^-$-responsive genes, which include NIA1, NIA2, Nir, NR, NRT2.1, -2.2, -2.4,-2.5, and NRT3.1. However, the fact is that different TFs, NLP7, TGA1/4, and TCP20, can regulate the expression of the same target gene, NRT2.1 (Figure 1). These TFs co-interact in response to NO$_3^-$ to regulate root growth.

Despite the development of multiple NO$_3^-$ signaling pathways regulating RSA and the characterization of primary Ca$^{2+}$-induced responses elucidated in the present review, many important inquires on how PLCs are implicated in nitrate signaling and the specificity of the protein kinases that switch the different constituents of PLCs are yet to be answered. Moreover, the speculated nonphosphorylated form of the NRT1.1-signaling Ca$^{2+}$-CPKs-NLPs pathway has received trivial
experimental attention. Additionally, PLC- and Ca²⁺-independent nitrate signaling pathways have another component, as evidenced by AFB3 expression and its downstream TFs, which lead to the possibility that there might be another second messenger involved in nitrate responses.

There are more nitrate regulatory modules in existence, with no clues about their signaling pathways and components; however, they are the fundamental contributors controlling LR development. Hence, functional identification and characterization of the various players associated with this and other NO₃⁻ signaling pathways and their possible functions in the root architecture of Arabidopsis is the next step to try and comprehend the NO₃⁻ responses that will facilitate crop genetics improvement.

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Abbreviations

NRT1 Nitrate transporter1
CLC Chloride Channel
CIPK CBL-Interacting Protein Kinase
FIP1 Factor interacting with poly (A) polymerase 1
CPSF30-L Cleavage and Polyadenylation Specificity Factor 30-L
ABI2 ABA-insensitive 2
AFB3 AUXIN RECEPTOR F-BOX3
NAC4 NAC-domain containing protein 4
OBP4 OBF Binding Protein 4
TGA1/4 Targets the activation sequence1/4
EGTA Ethylene glycol tetraacetic acid
CEP C- TERMINALLY ENCODED PEPTIDE
LaCl₃ Lanthanum (III) chloride hydrate
PT-PLC Phosphatidylinositol-specific phospholipase C
TCP20 Teosinte branched1/cycloidea/proliferating cell factor1-20
ANR1 ARABIDOPSIS NITRATE REGULATED1
ARF AUXIN RESPONSE FACTOR
SCFTIR/AFB S-PHASE KINASE ASSOCIATED PROTEIN 1-CULLIN-F-BOX PROTEIN (SCF)-type E3 ubiquitin-protein ligases
MEKK1 Mitogen-Activated Protein Kinase Kinase 1
BBX Bobby sox homolog
ROXY Floral glutaredoxins
CEPD Phloem-specific peptides
ERFs Ethylene-responsive element binding factors
RAV2 Regulator of V-ATPase in vacuolar membrane protein 2

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