



# Signalling Overlaps between Nitrate and Auxin in Regulation of The Root System Architecture: Insights from the *Arabidopsis thaliana*

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**Abstract:** Nitrate ( $\text{NO}_3^-$ ) and auxin are key regulators of root growth and development, modulating the signalling cascades in auxin-induced lateral root formation. Auxin biosynthesis, transport, and transduction are significantly altered by nitrate. A decrease in nitrate ( $\text{NO}_3^-$ ) supply tends to promote auxin translocation from shoots to roots and vice-versa. This nitrate mediated auxin biosynthesis regulating lateral roots growth is induced by the nitrate transporters and its downstream transcription factors. Most nitrate responsive genes (short-term and long-term) are involved in signalling overlap between nitrate and auxin, thereby inducing lateral roots initiation, emergence, and development. Moreover, in the auxin signalling pathway, the varying nitrate supply regulates lateral roots development by modulating the auxin accumulation in the roots. Here, we focus on the roles of nitrate responsive genes in mediating auxin biosynthesis in *Arabidopsis* root, and the mechanism involved in the transport of auxin at different nitrate levels. In addition, this review also provides an insight into the significance of nitrate responsive regulatory module and their downstream transcription factors in root system architecture in the model plant *Arabidopsis thaliana*.

**Keywords:** nitrate; auxin; signalling crosstalk; nitrate responsive genes; root system architecture; *Arabidopsis*; primary root; lateral root

## 1. Introduction

Roots are crucial for the anchorage of plants in the soil and facilitate the translocation of water and mineral nutrients. Thus, plant roots play an essential role in plant metabolism [1]. The root system is composed of primary root (embryonic roots) and postembryonic roots (lateral roots). Root system architecture (RSA) is the general spatial arrangement of individual parts of the root system and enables the plant to acquire adequate resources from the soil. The rate at which individual parts of the root system develop and grow can be reformed by their response to environmental signals, including changes in water, nutrient, and oxygen availability, or pathogens and pests [2,3].

Nitrogen (N) is in two major forms nitrate ( $\text{NO}_3^-$ ) and ammonium ( $\text{NH}_4^+$ ), which plays a vital role in the root growth and development of the plant. It was suggested that lateral roots (LRs) formation is regulated by  $\text{NO}_3^-$  and  $\text{NH}_4^+$  ratio via modification of the polar auxin transport [4].  $\text{NH}_4^+$  ions were found to dramatically suppress *Arabidopsis* root growth in the absence of  $\text{K}^+$ , even though  $\text{NO}_3^-$  is available [5]. The ammonia transporter gene *AtAmt1.1* has also been found to play a critical role in restructuring LRs architecture under N-starvation [6,7]. However, the majority of the plants preferentially uptake nitrogen in the form  $\text{NO}_3^-$  in aerobic soil [8], and by contrast, only a few grow

well when taking N only from  $\text{NH}_4^+$  [4]. This uptake system of N by the roots is under the synchronized control of nutrients availability and hormonal signalling [2,3].

Nitrate ( $\text{NO}_3^-$ ) plays a signal regulatory role in many physiological processes including root growth. The nutrient uptake efficiency depends on root system architecture in the soil [9], and these processes are controlled by several gene-transcript levels, which are regulated by  $\text{NO}_3^-$  [10].  $\text{NO}_3^-$  regulates root branches under various signalling pathways, such as *NRT1.1* (dual-affinity  $\text{NO}_3^-$  responsive gene), which is assumed to participate in the nitrate-sensing system [11,12], and also governed by auxin [13].

Signalling communication is a general rule rather than an omission. For instance, it is cited that about 1545 genes were the nutrient-related signals of  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , or both nitrogen forms. Also, 982 (64%) genes were controlled by hormonal signals [14]. Several studies reveal that hormone biosynthesis, transport, and transduction are significantly influenced by several mineral nutrients [11]. Hormones play a key role in the root-growth adaptation to  $\text{NO}_3^-$  readiness [13]. Numerous studies have also shown that  $\text{NO}_3^-$  regulation of root system architecture (RSA) entails an overlap between  $\text{NO}_3^-$  and auxin signalling pathways [15,13]. IAA is the most researched and best naturally occurring active auxin [12], and it plays a specific role in the control of systemic inhibition of fresh lateral roots (LRs) developments in response to the sufficient supply of nitrate [16]. Both external  $\text{NO}_3^-$  and IAA supply significantly influence the auxin concentration in the tiller nodes [17]. As part of the root system, lateral root not only depends on the external  $\text{NO}_3^-$  supply, but also on the amount of internal  $\text{NO}_3^-$  supplied to the plant root system [18].

Several studies on transporter/channels as well as nutrient responses have been reported for many years. However, at the more basic level, important mechanisms of the signalling overlap between nitrate and auxin in lateral root initiation, emergence, and development in *Arabidopsis* are still poorly understood. In this review, we focus on an in-depth role of  $\text{NO}_3^-$  in auxin-induced signalling, and the relationship between  $\text{NO}_3^-$  and IAA transcript levels in the regulation of lateral root initiation, emergence, and development in the model plant *Arabidopsis thaliana*.

## 2. Classification of Nitrate Responsive Genes

Root growth and development are influenced by the plants' nutritious status and the external availability of the nutrients [19,20].  $\text{NO}_3^-$  is taken up by the root cell is considered to be a key constituent of the N metabolism. Moreover, the  $\text{NO}_3^-$  can be subsequently excreted from the root cell by the primary extrusion from the external medium or unloading in the xylem vessel to reach the aerial organs of the plant [21]. For such nitrate transport through the cellular membrane,  $\text{NO}_3^-$  influx is an active process [21]. The recognizable proof of the proteins and the transport process of  $\text{NO}_3^-$  inside the plants are essential to understand the components that control  $\text{NO}_3^-$  retention and distribution within the entire plant. Here, we classify the genes that potentially involved in  $\text{NO}_3^-$  responses [20,22], as short-term and long-term responsive (Table 1–3), which are described in detail below.

**Table 1.** Short Term Primary Nitrate Responsive Genes.

| Gene        | Gene Family                                  | Treatment | Transcriptionally NO <sub>3</sub> <sup>-</sup> Responsive | Effect On RSA                                   | Target Genes                                | Root Expression Profile          | Subcellular Localization                                                              | Refs    |
|-------------|----------------------------------------------|-----------|-----------------------------------------------------------|-------------------------------------------------|---------------------------------------------|----------------------------------|---------------------------------------------------------------------------------------|---------|
| NLP6        | Plant regulator RWP-RK family protein        | low       | no                                                        | support root meristem growth under N starvation | NRT2.1, NRT2.2, and NIA                     | stele, root hair                 | nucleus                                                                               | [23,24] |
| LBD39,37,38 | LBD                                          | high      | yes                                                       | cell cycle progression of the pericycle         | NIA<br>NRT1.1, NRT2.1, NRT2.2, and NRT2.5   | root meristem                    | mitochondrion                                                                         | [25–27] |
| SPL9        | SPL                                          | medium    | yes                                                       | LR and PR induction                             | NiR, NIA2, and NRT1.1 (potential)           | unknown                          | cytoplasm /nucleus                                                                    | [28,29] |
| NIGT1s      | NIGT                                         | low       | yes                                                       | primary root growth                             | NRT2.1, NRT2.4                              | expressed in roots (lower panel) | nucleus                                                                               | [30–32] |
| CIPK23      | CBL-interacting protein kinase               | low       | yes                                                       | Primary root growth                             | Phosphorylating NRT1.1                      | guard cells and root hairs       | cytosole, plastid, chloroplast, cytoplasm, cytosol, nucleus, plasma membrane, plastid | [33–35] |
| NRG2        | Putative bZIP                                | unknown   | no                                                        | unknown                                         | regulating NRT1.1 and interacting with NLP7 | stele, root tip                  | nucleus                                                                               | [36]    |
| CPSF30      | Polyadenylation specificity factor           | unknown   | no                                                        | PNR/ Nitrate accumulation in roots              | alternative polyadenylation                 | unknown                          | nucleus                                                                               | [26]    |
| FIP1        | Factor interacting with poly(A) polymerase 1 | unknown   | no                                                        | PNR in the root                                 | polyadenylation of NRT1.1                   | unknown                          | nucleus, cytosole, mitochondria, vacuole, golgi,                                      | [29]    |
| NRT1.1      | NPF                                          | dual      | yes                                                       | LRs inhibition and stimulation                  | regulating CIPK8, CIPK23, TGA1/4            | root cap and root tip            | plasma membrane                                                                       | [37,38] |

**Table 2.** Long Term and Short Term Nitrate Responsive Genes.

| Gene.  | Gene Family                             | Treatment | Transcriptionally NO <sub>3</sub> <sup>-</sup> Responsive | Effect On RSA                   | Target Genes                 | Root Expression Profile                | Subcellular Localization | Refs    |
|--------|-----------------------------------------|-----------|-----------------------------------------------------------|---------------------------------|------------------------------|----------------------------------------|--------------------------|---------|
| TGA1/4 | bZIP                                    | high      | yes                                                       | LRs induction (initiation)      | NRT2.1 and NRT2.2            | pericycle and root hair                | nucleus                  | [39]    |
| CIPK8  | CBL-interacting                         | high      | yes                                                       | Primary root growth             | unknown                      | guard cells and root hairs             | cytosol, nucleus         | [40,41] |
| NLP7   | RWP-PK Subgroup                         | low/high  | no                                                        | LRs and primary root repression | NRT2.1, NiR, NRT2.2, and NIA | stele, root hair, endodermis, root tip | nucleus                  | [42,41] |
| CPK10  | Ca <sup>2+</sup> -sensor protein kinase | low       | yes                                                       | Primary root growth             | phosphorylate NLP7           | unknown                                | nucleus                  | [43]    |

**Table 3.** Long Term Nitrate Responsive Genes.

| Gene      | Gene Family                   | Treatment | Transcriptionally NO <sub>3</sub> <sup>-</sup> Responsive | Effect On RSA                                          | Target Genes                       | Root Expression Profile               | Subcellular Localization                    | Refs    |
|-----------|-------------------------------|-----------|-----------------------------------------------------------|--------------------------------------------------------|------------------------------------|---------------------------------------|---------------------------------------------|---------|
| ANR1      | MADS-box                      | low       | yes                                                       | LRs induction (elongation)                             | regulating NRT1.1                  | LRs (primordia, base and apex), stele | nucleus                                     | [44]    |
| NRT2.1    | NPF                           | high      | yes                                                       | repressor of LRs initiation                            | unknown                            | root cortical and epidermal cells     | plastid, nucleus                            | [45–47] |
| ARF8      | Auxin response factors        | high      | yes                                                       | LRs induction (initiation)                             | unknown regulating                 | pericycle, root cap stele,            | nucleus                                     | [48]    |
| miR167    | microRNA                      | high      | yes                                                       | LRs induction (initiation)                             | regulating the expression of ARF8  | unknown                               | nucleus                                     | [43]    |
| miR393    | microRNA                      | high      | yes                                                       | LRs formation                                          | specifically cleaving AFB3         | unknown                               | nucleus                                     | [42]    |
| AFB3      | Auxin receptor                | high      | unknown                                                   | LRs induction and PRs suppression                      | unknown                            | unknown                               | nucleus                                     | [49]    |
| NAC4      | NAM/ATAF/CUC                  | high      | yes                                                       | LRs induction                                          | OBP4                               | unknown                               | nucleus                                     | [49]    |
| CLE       | CLAVATA3/SR-related XI        | low       | unknown                                                   | LRs repression (development and emergence)             | CLV1                               | expressed in the pericycle            | extracellular region of the cell            | [50]    |
| CLV1      | XI LRR-RLKs                   | low       | unknown                                                   | LRs repression (development and emergence)             | feedback regulation of CLE         | expressed in the pericycle            | plasma membrane                             | [51]    |
| CEP       | CEP Component                 | unknown   | unknown                                                   | Inhibits LRs formation                                 | unknown                            | LRs primordial and meristem region    | extracellular, golgi, endoplasmic reticulum | [52]    |
| HIN9/IWS1 | Component of RNAPII complexes | high      | no                                                        | regulation of root NO <sub>3</sub> <sup>-</sup> uptake | NRT2.1                             | unknown                               | nucleus                                     | [53]    |
| TCP20     | TCP                           | low       | no                                                        | LRs development                                        | NRT1.1, NIA, NRT2.1, and NiR Roles | stele, root cap                       | nucleus                                     | [54]    |

### 2.1. Short Term Primary Nitrate Response (PNR) Genes

In *Arabidopsis*, *NRT1.1* is one of the chief low-affinity nitrate transporter gene, which affects the root growth in nitrate-responsive signalling, and toggles from the low nitrate condition to high nitrate condition by its phosphorylation of at the Thr101 by CIPK23 kinases (Table 1). *NRT1.1* activates a specific nitrate signalling pathway, thereby regulating RSA, and subsequently stimulating LR growth under low and high  $\text{NO}_3^-$  supply [55]. It is also evident that auxin might be a secondary signal, triggering the regulatory action of *NRT1.1* on LR growth and development. *NRT1.1* transports nitrate and also encourages the uptake of auxin. In contrast,  $\text{NO}_3^-$  also inhibits *NRT1.1*-mediated IAA uptake, which revealed that nitrate signal transduction alters the transport of auxin via *NRT1.1*. Moreover, *NRT1.1* functions in phosphorylation and de-phosphorylation in response to diverse nitrate conditions [56,57] (Table 1).

In addition to *NRT1.1*, lateral organ boundaries domain (LBD) *LBD37/38/39* have been recently identified to be essential nitrate regulatory genes that suppress several N responsive genes required for  $\text{NO}_3^-$  uptake and assimilation, and influence  $\text{NO}_3^-$  content [58,59]. LBDs regulates EXPANSINA 17 (*EXPA17*), a gene encoding cell wall loosening factor by coupling with *EXPA17* to enhance lateral root emergence. Overexpression of *EXPA17* increases LRs emergence density in the presence of auxin compared to wild type (WT) [60]. Studies have also shown that *LBD18* regulatory gene EXPANSINA 14 (*EXPA14*) expression is involved in the apical tissue of LRs primordium. Collectively, these findings confirm that *LBD18* up-regulates a subset of EXPAs genes to enhance cell division which promotes LR emergence in *Arabidopsis* [61]. Moreover, LBDs act downstream of the auxin influx carrier *AUX1* and *Lax1*, regulating LR initiation and development [62,63]. These transient developmental phases are further synchronized by the action of microRNA (miRNA) families miRNA156 and miRNA172 [20]. It was revealed that miRNA156 targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) genes *SPL3*, *SPL9*, and *SPL10*, which are involved in the suppression of lateral root growth. This further suggests the function of both miR156 and SPLs in LR development by their response to auxin signalling [22]. *SPL9* was identified to be a sentinel short-term nitrate-responsive gene [57], which is targeted by miR156, while *rSPL9* (overexpression plants of *SPL9*) are resistant to the degradation of miR156 [64]. Thus, the miR156-resistant transgenic plants were identified as the *rSPL9* mRNA, generated from the modified gene resistant to degradation by miR156. This demonstrated the role of the *SPL9* in the regulation of genes involved in the primary  $\text{NO}_3^-$  response. It was further demonstrated that the role of *SPL9* over-expression on the transcription factor (TF) levels of genes in the network over time leads to a significant increase in *NRT1.1*, *NIA2*, and *NIR1* in response to  $\text{NO}_3^-$  [57] (Table 1). Moreover, *TAA1* and *TAR2* are involved in auxin biosynthesis, keeps the appropriate root auxin concentration, and the preservation of root stem cell niches (<https://www.uniprot.org/>).

From the genome-wide association study (GWAS) analysis, it was revealed that *JR1* (JOSMONATE RESPONSIVE 1) was related to LR length in low and high  $\text{NO}_3^-$  condition. Compare to high  $\text{NO}_3^-$  condition, there is a specific defect in the average length of the LRs under low  $\text{NO}_3^-$  condition. This is an indication that *JR1* functions in regulating LRs length under low N condition [58]. Subsequently, the two genes JASMOANTE RESPONSE (*JR1*) and D AMINO ACID RACEMASE2 (*DAAR2*) are involved in lateral root growth under low nitrate conditions [58,59].

Under  $\text{NO}_3^-$  deficient conditions, *NRT2.4* and *NRT2.5* are down-regulated in the *chl7* mutants, and therefore accumulate lower  $\text{NO}_3^-$  content compared to WT plants, and subsequently inhibit the root development upon nitrate starvation in *chl7* [65], which indicates that *CBL7* enhances root development when expressed in the roots of the young seedlings, which is induced by  $\text{NO}_3^-$  starvation [65]. In addition, short-term responsive gene family NITRATE-INDUCABLE GRAP-TYPE TRANSCRIPTIONAL REPRESSOR (NIGT1s) transcription factors, incorporated in nitrate starved condition and act as a downstream sensor and transporters of *NPF6.3/NRT1.1* to repress primary root growth in the nitrate sufficient condition [66]. The transcriptase and co-transfection analysis showed that NLPs auto-regulate and control the NIGTs [67]. It is also suggested that *NRT2.1* is independently regulated by NIGTs and *NLP7* because of their different binding sites. Moreover, the *NLP-NIGT1*

transcriptional cascade controls the targeted genes together by NLP7-mediated activation, while it independently induces repression [67].

The CBL-interacting protein kinases, CIPK8 and CIPK23, are involved in short-term primary nitrate response. Similarly, the subcellular  $\text{Ca}^{2+}$ -dependent protein kinases (CPKs), such as CPK10, CPK30, and CPK32, are necessary for instant nitrate-stimulated cellular and metabolic responses and nitrate-induced root to shoot growth. Under sufficient nitrate conditions,  $\text{Ca}^{2+}$ -sensor CPKs move to the nucleus, whereas CPK10 targets NLP7S205 phosphorylation to hold NLP7 inside the nucleus in a nitrate-dependent manner [68,69]. Furthermore, nitrate regulatory gene 2 (NRG2) also plays a critical regulatory role in plant primary nitrate responses (PPNR) based on their binding affinity for nitrate [70]. In addition to NRG2, FIP1 genes (factor interacting with poly (A) polymerase 1) enforce a specific role in nitrate signalling in *Arabidopsis*. FIP1 is an important part of the polyadenylation factor complex. FIN219-interacting protein 1 (FIP219) have also been demonstrated to be rapidly stimulated by auxin and confined consistently in the cytosol without fluctuation in subcellular localization by light [71]. FIN219 was exhibited to be a silencer of COP1 [71], thus demonstrating the role of FIN219/JAR1 as a significant regulator in modulating auxin-mediated phytohormone signalling. However, its physiological function in light signalling and plant development remains unclear [28]. It was also found that FIP1 interacts with cleavage and polyadenylation specificity factor 30-L (CPSF30-L), which is also an indispensable player in  $\text{NO}_3^-$  signalling [29]. CPSF30 significantly reduced the primary root length and exhibits fewer LR when CPSF30 mutant *opt6* was grown on medium containing IAA [72].

## 2.2. Short and Long Term Nitrate Response Genes

As stated earlier that CIPK8 functions as a positive regulator under high and low nitrate conditions [30,40]. The *cipk8* mutant was repressed mainly in  $\text{NO}_3^-$  limited condition, and its response to both high and low-affinity nitrate signalling system is suggested to be genetically distinct. This indicates that CIPK8 is involved in low nitrate conditions. Similarly, CIPK8 also functions in long-term nitrate modulated primary root growth and nitrate-induced expression of a vascular malate transporter [73,30].

Furthermore, the transcription factors belonging to the bZIP family, namely TGA1 and TGA4, are stimulated by  $\text{NO}_3^-$  in the *Arabidopsis* root [31]. TGA1 and TGA4 regulate the expression of  $\text{NO}_3^-$  transporter genes NRT2.1 and NRT2.2 [31]. The stimulation of both TGA1 and TGA4 is repressed in *chl1-5* and *chl1-9* mutants after  $\text{NO}_3^-$  application, which indicated that both TGA1 and TGA4 are regulated by nitrate transport, rather than the signalling function of NRT1.1 [31]. These findings suggest that TGA1 and TGA4 act as a primary nitrate responsive genes (Table 2).

Another short-term and long-term nitrate responsive transcription factor, NLP7 plays a prominent role in the LR growth in response to nitrate, promotes the LR density in high  $\text{NO}_3^-$  condition, acts as nitrate sensing and assimilation, suggested to be short-term PNR gene. However, the *nlp7* mutant exhibited lateral roots inhibited phenotypes on split-root plates in both high and low nitrate conditions, further suggesting that NLP7 may mediate local nitrate response [42,23]. Conversely, the present findings indirectly strengthen the possible function of the NLP7 in regulating the LR growth in varying nitrate conditions, which suggests the role of NLP7 as long-term nitrate response gene [50]. In addition to NLP7, the transcription factor that regulates the expression of the prototypical  $\text{NO}_3^-$  response gene is SPL9 [57].

## 2.3. Long Term Nitrate Response Genes

When the supply of nitrogen becomes limited, the long-term high-affinity type transporters enabling the nitrate uptake, are expressed in the root to enhance the efficacy of nitrogen acquisition [45,46] (Table 3). Among them, the nitrate inducible MADS family protein ANR1 promotes lateral root in response to nitrate [60]. NRT1.1 and its downstream MADS-box transcription factor ANR1 mediate local signalling [74,32]. In the long-term mechanism, ANR1 functions as a positive regulator in the signalling pathway of nitrate-induced LR proliferation [75,76].

Another long-term high-affinity nitrate regulatory gene *NRT2.1* involved in the  $\text{NO}_3^-$  uptake, are responsible for the accumulation and induction of the transcript in the root under low nitrate conditions [77]. Similarly, microRNA (miRNA) emerged as a key regulator in nitrate-induced root growth and development. miR167 targets and regulates the expression of auxin response factor (*ARF8*), and both *ARF8* and miR167 are expressed in the root cap [78,43]. *ARF8* is accumulated in the pericycle by inhibition of miR167 under nitrate availability and subsequently display an increased ratio of initiation vs. emerging LR in response to  $\text{NO}_3^-$  treatments [42]. Moreover, miR393 was activated in response to nitrate supply, mainly by binding the auxin *AFB3* transcript and subsequently modulating the accumulation of *AFB3* mRNA in the root in response to nitrate treatments [79].

In *A. thaliana* plants, *CLE41*, a homolog of the phloem-derived secretory peptide tracheary element differentiation inhibitory factor (TDIF) [45], and the *CLE41* peptides (*CLE41p*) stimulated the proliferation of vascular cells, despite the delay in its division into phloem and xylem cell lineages. *CLE19/41/44/TDIF* are localized in the vascular stem cells and function in the maintenance of LR emergence [80,81]. In response to high nitrate availability, *HNI9/AtHWS1* (HIGH NITROGEN INSENSITIVE9) plays an important role in the systemic regulation of root  $\text{NO}_3^-$  uptake in *Arabidopsis* [32], and functions as an inhibitor of *NRT2.1* transcription in the root [82]. Moreover, it plays a significant role in N signalling by regulating hundreds of  $\text{NO}_3^-$  responsive genes in the root [82]. Among other, long-term nitrate responsive TFs, that function in lateral root development of *A. thaliana*, *CLEs-CLVs* related peptides function as a regulatory module in the nitrate signalling pathway and negatively regulates LR growth under limited nitrate supply [80,83]. Similarly, CEP hormones, 15-amino-acid peptides, act as negative regulatory hormones in growth and development. *CEP1* is a candidate for a novel peptide plant hormone, mainly expressed in the lateral root primordial, and its overexpression significantly inhibits root growth [80]. These polypeptides are imported into the roots to activate nitrate transporter genes in the rhizosphere under a sufficient supply of  $\text{NO}_3^-$  [81]. In addition, *AtTCP20* is also another nitrate responsive transcription factor, which modulates LR in nitrate dependent manner, and is studied in chrysanthemum and heterologous overexpression in *Arabidopsis*. The overexpression of *CmTCP20* significantly increased the number and average length of lateral root (LRs) compared with the wild type in chrysanthemum and *Arabidopsis* [78], indicating that *TCP20* is regulated by  $\text{NO}_3^-$ , supplied to N starved roots.

Members of the bZIP transcription factor family, *TGA1* and *TGA2*, are induced by nitrate in the roots [31]. Transcriptome analysis of roots showed that differentially expressed numbers of genes regulated by nitrate availability suggest *tga1tga4* as a double mutant. Moreover, *TGA1* and *TGA4* significantly decrease the induction of its target genes *NRT2.1* and *NRT2.2*. Further findings showed that *TGA1* could bind *NRT2.1* and *NRT2.2* and regulates their expression [42,84]. As stated earlier, adequate  $\text{NO}_3^-$  suppresses the level of miR167a to allow the accumulation of *ARF8* transcript in the pericycle upon nitrogen treatments, while under low  $\text{NO}_3^-$  condition miR167a targets *ARF8* [23]. Similarly, the *AFB3* transcriptional factor plays a significant role in coordinating LR and primary roots under adequate nitrate supply. Nitrate also activates *NAC4*, as downstream of *AFB3*, which is an essential part of regulating the nitrate-responsive system, the mutants of *NAC4*, *nac4* showed altered LR growth. Meanwhile, the primary root growth remains normal under nitrate state. Thus, *AFB3* regulatory network confers changes in LR development in response to nitrates [49], which confirms the long-term role of these transcripts in response to  $\text{NO}_3^-$ .

Similarly, the complex overlap between nitrate and phytohormone auxin signalling pathways are regulated by genomic distinctiveness. This induces the allelic variety of genes. ROOT SYSTEM ARCHITECTURE 1 (*RSA1*) and PHOSPAHT TRANSPORTER 1 (*PHO1*) are the two genes regulating allometric parts of root system architecture (RSA), however the mechanisms through which their  $\text{NO}_3^-$  and IAA signalling pathways regulate the root architecture remains to be understood [82].

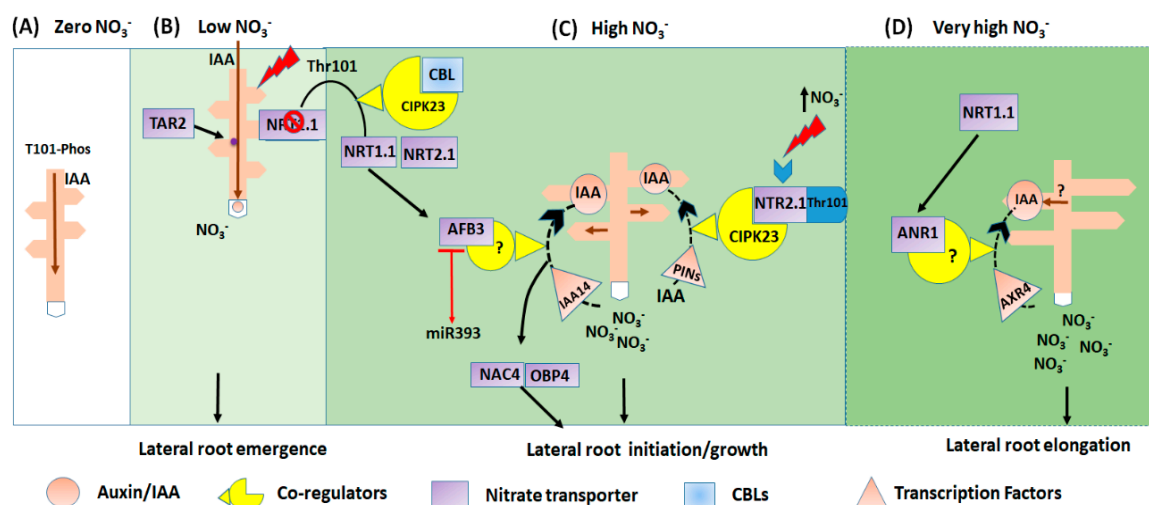
### 3. Nitrate Responsive Genes Enhances Auxin Activity in The Roots

Nitrate ( $\text{NO}_3^-$ ) supply can alter auxin biosynthesis and its transport [40,85,86]. Indole-3-acetic acid (IAA) is an essential plant native auxin [53], which is required for plant growth and development under

diverse environmental conditions [87]. In *Arabidopsis* roots, the decay of auxin cannot be achieved by the translocation of auxin from shoot to root, which indicated that shoot localized auxin alone is not adequate for supporting the root initiation, elongation, and development [84]. Auxin transportation has been hypothesized to be crucial intercellular and intracellular IAA distributions in the plant. Inside the plant cells, it was reported that for the directional anionic auxin and formation of the polar flow, it needs transporters and their uneven localization [88]. This evidence revealed that roots nitrate transcriptionally regulate the transport and biosynthesis of auxin [89].

To date, few transcription factors that contribute to  $\text{NO}_3^-$ -dependent auxin efflux have been studied. For instance, nitrate responsive genes which include *NRT1.1*, *NRT2.1*, *NRT2.2*, *NIA1*, *NIA2*, *NIR*, and Arabidopsis Nitrate Regulated (*ANR1*) [75]; *TAR2* (tryptophan aminotransferase related 2) [90]; NIN like Protein 6 (NLP6) [91]; NIN-Like Protein 7 (NLP7) [92]; LOB Domain-Containing proteins (LBD37/38/39) [62]; Squamosa Promoter Binding Protein-Like 9 (SPL9) [93]; Basic Leucine-Zipper 1 (*bZIP1*) [94]; NAC Domain Containing Protein 4 (NAC4) [49]; *TGA1/TGA4* [31], Teosinte Branched1/Cycloidea/Proliferating Cell Factor 20 (*TCP20*) [78]; and Nitrate Regulatory Gene 2 (*NRG2*) [70]. Moreover, the link between *NLP6* & 7, *TGA1*, *bZIP1*, and *TCP20* with the promoter of the genes of interest was also confirmed [87].

It has been reported that in the roots *TAR2* expression is significantly increased compared to *TAA1*, which is induced moderately under low  $\text{NO}_3^-$  condition. However, in shoots, both genes were repressed. *TAR2* was expressed in the root pericycle and also the vasculature of the root maturation zone close to the root tip [85] (Figure 1). The mild  $\text{NO}_3^-$  deficiency positively affects LR formation in *Arabidopsis* which required an auxin biosynthesis gene *TAR2*. A change L-Trp to indole-3-pyruvic acid (IpyA) by *TAR2* is the first step in the IpyA pathway branching from a Trp-dependent auxin pathway [90]. Under low  $\text{NO}_3^-$  source, the *TAR2* expression was up-regulated, resulting in an increase in IAA levels in the developing LR. Nevertheless, the *tar2-c* null mutants exhibit much shorter total LR length and fewer visible LR numbers, demonstrating that mutation of *TAR2* reduced the LR formation [85]. Therefore, low  $\text{NO}_3^-$ -stimulated LR emergence depended on root-synthesized auxin in a *TAR2*-dependent manner [50]. So far, all the NRTs-dependent hormone transporters are involved in the local redistribution of the hormones within the plant [40].  $\text{NO}_3^-$  transporter enhances the redistribution and transport of auxin at a short distance [95,96]. For instance, *NRT1.1* transports auxin (IAA), and consequently overlaps nitrate and auxin (IAA) signalling pathways [40]. *NRT1.1* transports the auxin from the lateral root primordial under low nitrate condition, thus suppressing the development of LR primordia and fresh LR. However, at optimum nitrate condition, the repressed *NRT1.1*-dependent IAA transport was activated, which result in subsequent accumulation of auxin in the LR and promotion of the primordium growth [81,52]. Under the low N condition, the *nrt2* mutant suppresses the lateral root emergence and the IAA storage in the LR primordia [85].



**Figure 1.**  $\text{NO}_3^-$  regulates auxin activity, and consequently controls the  $\text{NO}_3^-$  assimilation pathway and transport. The model represents the root morphology subjected to four different nitrate conditions. (A) The deficient (zero)  $\text{NO}_3^-$  stimulates auxin translocation from shoot to roots. (B) This mechanism is utilized under low  $\text{NO}_3^-$  conditions, either *NRT1.1* transport auxin from shoot to root, causing LR emergence, or reduced the auxin storage in primordia and new lateral root tip, and subsequently inhibits LR emergence and elongation. Thus considered as the principal regulator for the auxin biosynthesis in the root promotes lateral root growth. (C) Under the high nitrate  $\text{NO}_3^-$  condition; *NRT1.1* induces the expression of *AFB3*, and the *AFB3-NAC4-OBP4* signalling network expressed their protein in the root pericycle of the cell. The *NAC4* and *OBP4* part of the pathway is probably regulated by AUX/IAA protein IAA14. The black dotted arrow indicates the effect of *AFB3* on the IAA accumulation, promotes lateral root initiation under high nitrate condition. (D) Under the excessive supply of  $\text{NO}_3^-$ ; *NRT1.1* stimulates the ANR1-dependent signalling pathways that modulate lateral root elongation. The black dotted arrow indicates the effect of *NRT1.1* on the IAA accumulation and expression of *ANR1* in the LR primordia, and lateral root development. The question mark represents the unconfirmed effect of *NRT1.1* nitrate transport activity on the expression of *ANR1* [97].

*NLP7* is the primary auxin-efflux protein's carrier localized in the plasma membrane [44]. *NLP7*-induced auxin efflux activates *PIN7* [88]. Further studies have shown that *NLP7* encodes 851 genes under optimum nitrate conditions. In addition, new nitrate regulatory factor NITRATE REGULATORY GENE 2 (*NRG2*), disrupts the stimulation of  $\text{NO}_3^-$  sensor genes in the *nrg2* mutant when subjected to nitrate treatments. This indicates the physical interaction of *NRG2* with *NLP7* in the nucleus [87]. Similarly, *NIGT1s* expression is auto-regulated and controlled by NLPs. Therefore, *NIGT1s* inhibit the *NRT2.1* stimulation by *NLP7* [67]. *NLP7* regulates the fluctuation in local auxin by controlling the auxin-efflux to build up and sustain the root primordium and determine the LR number [88]. Genome-wide transcriptional profiling data revealed that *NLP7* and *TAR2* are among the top *NLP7*-activated genes, in addition to the nitrate inducible genes such as *NiR*, *NIA1*, *FNR2*, and *NRT2.1* [78,32]. A transcriptomic study demonstrates that an auxin bio-module containing auxin carrier 9 (including among others *PIN1*, *PIN2*, *PIN4*, and *PIN7*) is specifically regulated by  $\text{NO}_3^-$  [98]. Furthermore, it suggests TCP20-NLP6/7 complexes as the upstream target of the Auxin-ROP2-TOR-E2Fa/b signalling pathway [89].

In *Arabidopsis* root, a nitrate responsive miR393/*AFB3* regulatory module, which assimilates nitrate and auxin signals control both primary and LR growth [49], and is capable of stimulating the nitrate (5 mM) signal to promote root growth in response to auxin. Independent of nitrate metabolism and transport, *AFB3* induces auxin signalling, shows sensitivity and response to  $\text{NO}_3^-$ , subsequently regulate LR growth and functions as downstream of nitrate signalling [49]. Family of no apical meristem (NAM)/activating transcription factors/cup-shaped cotyledon (ATF/CUC) proteins, and its targeted genes *OBP4*, acts as a downstream target of *AFB3* gene. The interactive signalling network of *AFB3-NAC4-OBP4* with their existing protein expression in the pericycle cell of the root is required for the nitrate inducible LR initiation and emergence (Figure 1). The part of *NAC4-OBP4* signalling pathways is possibly regulated by AUX/IAA proteins, IAA14 [49,99] (Figure 1).

Under nitrate deficient condition, the AGL17-clade MADs-box gene, *AGL21*, and auxin promote longer LR in *Arabidopsis*. However, the overexpression (OE) of *AGL21* significantly up-regulates the auxin biosynthesis genes *YUC5*, *YUC8*, and *TAR3*, and is down-regulated in *agl21* mutants. *Ag121* mutants showed a decrease in the rate of LR elongation in low nitrate supply, demonstrating that *AGL21* promotes the local auxin biosynthesis in the LR primordia and LR, thus positively regulating LR development [100].

Studies have shown that *SPL* may function in the auxin homeostasis. To this end, OE of *SPL* led to the downregulation of auxin reporter DR5-GUS, and subsequently down-regulate several auxin-responsive genes in the leaves of *spl-D*. Further studies have shown that the two auxin biosynthesis genes, *YUC2* and *YUC6* were significantly suppressed in *spl-D* plants. Both genomic and phenotypic studies on *spl-D/yuc6-D* double mutant revealed that *SPL* might control auxin homeostasis by suppressing the transcription of *YUC2* and *YUC6* and contributing to lateral organ morphogenesis [48].

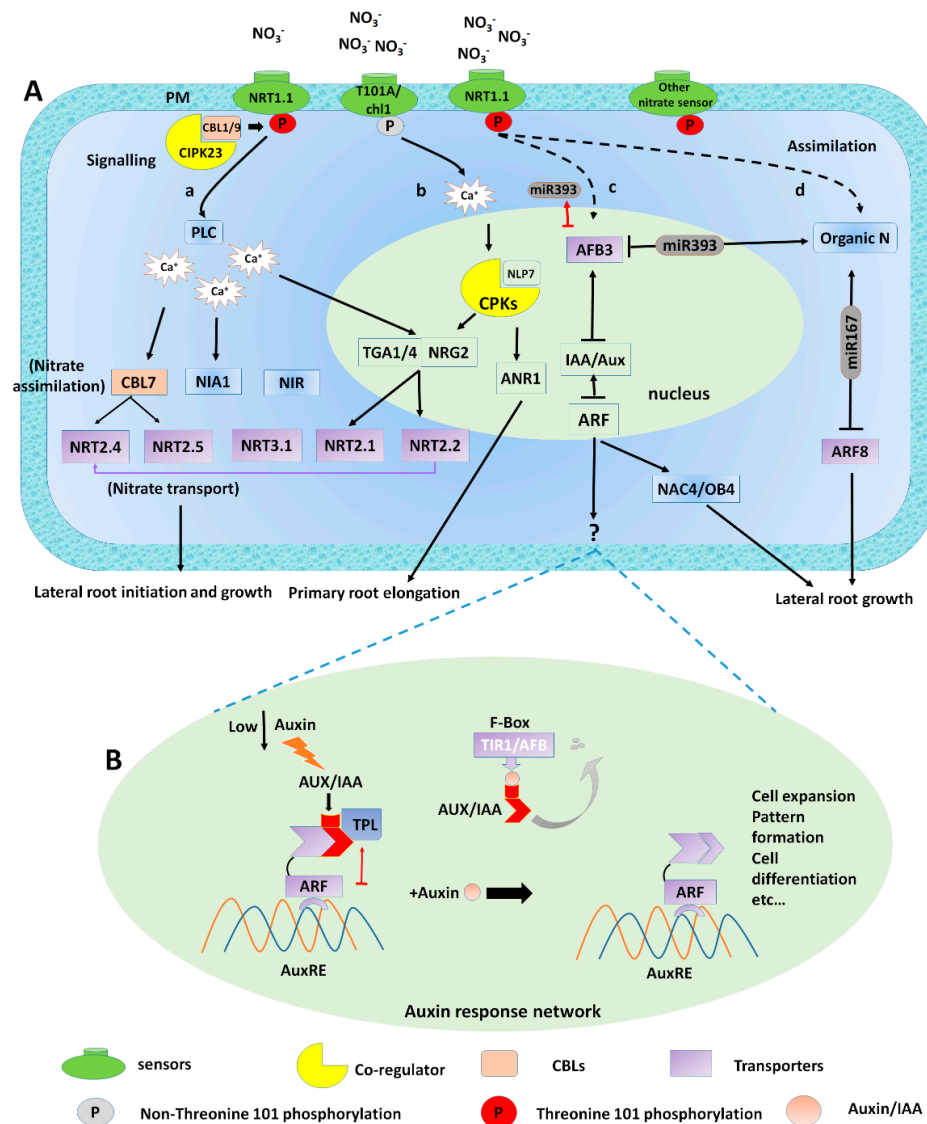
The report of Cui-Hui Sun showed that under low  $\text{NO}_3^-$  condition, the nitrate signalling pathway has a specific stimulatory effect on the LR growth. The molecular players' complexes involved in these pathways regulate various stages of LR development and also influence auxin biosynthesis and transport [50]. These findings demonstrate that nitrate ( $\text{NO}_3^-$ ) signalling constituents function upstream of auxin biosynthesis, transport, and control of LR development.

#### 4. Nitrate Signalling Pathways and Auxin Response

The  $\text{NO}_3^-$  and IAA cross-talk was first identified by Goerge S. Avery and Louise Pottorf in the 1940s. They demonstrated a direct relationship between  $\text{NO}_3^-$  and IAA supply [101]. Numerous studies have also revealed that the interaction between  $\text{NO}_3^-$  and the auxin signalling pathways that regulate RSA [39]. Presently, the molecular mechanism of  $\text{NO}_3^-$  signalling transduction has been revealed in *Arabidopsis* [43]. NRT1, NRT2, CLC (Chloride Channel), and SLAC1/SLAH (slow anion channel 1/SLAC1 homolog) are the four nitrate transporters families that have been characterized in *Arabidopsis* [102].

##### 4.1. Nitrate Uptake

*NRT1.1* is phosphorylated by the CBL1/9-CIPK23 complex [102,103]. Nitrate is absorbed into the root cell by plasma membrane-localized transporter family, NRT1 and NRT2 [104]. Under limited nitrate condition, *CBL1/9* interacts with *CIPK23* to form the CBL1/9-CIPK23 complex, which phosphorylates *NRT1.1* at the threonine site T101, for efficient  $\text{NO}_3^-$  transport [104,105] (Figure 2). This phosphorylation promotes *NRT1.1* enrolment into functional membrane microdomains at the plasma membrane (PM). This activity enhances the NRT1.1-dependent auxin flux, and as a result, drains the auxin level from the LR tips and limits growth. When the  $\text{NO}_3^-$  supply was increased, the non-phosphorylated *NRT1.1* shows oligomerization, reduced lateral mobility at the PM and exhibits inducible endocytosis. This activity promotes LR growth, induces NRT1.1-auxin transport activity at the PM, and stimulates  $\text{Ca}^{2+}$ -ANR1 signalling from the endosomes [54].



**Figure 2.** Signalling mechanism of nitrate mediated auxin modulation induces lateral root growth; (a) *NRT1.1* initially senses, taken up and transport by the *NRT1.1* transporter, thereby changing its uptake affinity and modulating phosphorylation to activate signalling pathway. In low nitrate conditions, the phosphorylation switches *NRT1.1* into the high-affinity system. This sensing ability of  $\text{NO}_3^-$  altering *NRT1.1* phosphorylation causes calcium ( $\text{Ca}^{2+}$ ) efflux by the activation of phospholipase C (PLC). This results in varying the expression of (*TGA1/4*\*) and nitrate transporter genes (*NRT2.1*, *NRT2.2*, *NRT3.1*) and nitrate assimilation genes (*NIA1* and *NiR*). (b) *T101A/chl1* mutant triggers  $\text{Ca}^{2+}$ -ANR1 signalling from the endosomes under high  $\text{NO}_3^-$  supply. (c) Under adequate  $\text{NO}_3^-$  supply, *AFB3* regulates *NAC4* and *OBP4* expression, which as a result affect the root renovation. (d) Finally, as a result of nitrate assimilation organic nitrogen is produced, this system includes miR167 and miR393, which regulates *AFB3* and *ARF8*, respectively (B) In Auxin response network; Class ARF directly connects to the AuxRE (red arrow). Under low nitrate conditions, AUX/IAs and TPLs repress transcriptional activation by class-ARFs. The degradation of Aux/IAA released this inhibition by connecting auxin and TIR1/AFB. (See text for further details).\**TGA1/4* regulatory factors mediate nitrate response inside *Arabidopsis* root, and while the link of *TGA4* to PLC is not approved. Other transcription factors, for instance, *NRG2*, *NLP7*, connection to  $\text{Ca}^{2+}$  signalling is currently unknown.

The phosphorylation activates the high-affinity nitrate transporter *NRT2.1* [106], and stimulation of *NRT1.1*, *NRT2.1*, *NRT2.2*, and *NRT2.4* was reported under nitrate starved seedlings after nitrate supply and all the nitrates assimilation genes were up-regulated [107,108]. Studies have identified the

roles of the  $\text{Ca}^{2+}$  in the nitrate signal transduction and stimulate nitrate-inducible regulation of the genes expressed in *Arabidopsis*. It was observed that nitrate treatments significantly increased cytoplasmic  $\text{Ca}^{2+}$  levels in the roots and the seedlings of the plants [109] (Figure 2). *NRT1.1* is governed by two modes of phosphorylation at Th-101 residue to regulate LR growth, modulating  $\text{NO}_3^-$ -dependent basipetal auxin transport and  $\text{NO}_3^-$  induced signal transduction. By using the two *Arabidopsis* phosphomimetic *NRT1.1T101A* and non-phosphorylatable *NRT1.1T101D* mutants respectively [54], *NRT1.1T101D* has presented the fast lateral mobility and membrane partitioning that enabled auxin flux under low-nitrate conditions while In contrast *NRT1.1T101A* mutants showed low lateral mobility and oligomerized at the plasma membrane (PM), where it induced endocytosis under high  $\text{NO}_3^-$ . These activities stimulate LR development by suppressing *NRT1.1*-mediated auxin transport on the PM and inducing  $\text{Ca}^{2+}$ -ANR1 signalling network from the endosomes [54] Figure 2b. To this end, phosphorylation mediates the calcium flux in the PM by  $\text{NO}_3^-$  transporter *NRT1.1*, subsequently regulates auxin flux and nitrate signalling in lateral roots development [54]. These studies indicated that  $\text{NO}_3^-$ -mediated  $\text{Ca}^{2+}$  flux modulating auxin response and polar auxin transport in the root growth and development.

#### 4.2. Auxin Response Network

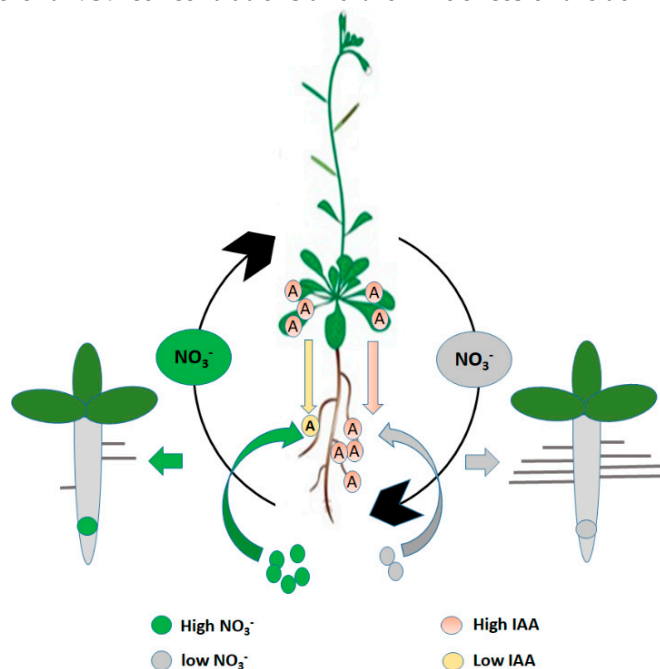
It was previously reported that auxin- $\text{NO}_3^-$  pathway by identifying a passive feed-forward mechanism consisting of auxin receptor auxin signalling F-box 3 (AFB3) and microRNA miR393 [79]. The expression of AFB3 protein in response to  $\text{NO}_3^-$ , indicating  $\text{Ca}^{2+}$ -independent pathways that regulate the nitrate-sensitive genes [109] (Figure 2c). Under high nitrate conditions, *AFB3* is stimulated, whereas, under lower  $\text{NO}_3^-$ , miR393 induced by nitrate reduction and assimilation which produces N metabolites, and subsequently *AFB3* suppressed by miR393 [79] (Figure 2c). Furthermore, it was established that nitrate-AFB3-NAC4-OBP4 mediated perception, signalling and response of auxin in a nitrate-dependent manner is mainly regulated by multiple signalling mechanisms and their coordination under unfavorable nitrate condition [68]. Nitrate-specific induction of *AFB3* inside the root may regulate a specific signalling network of Aux/IAA and ARF factors that modulate *NAC4* activation. AFB3 regulates the transcription of the IAA-responsive gene by promoting the degradation of the Aux/IAA transcriptional repressor of auxin [110] (Figure 2c). The microRNA, miR167, and its target auxin-responsive factor *ARF8* mRNA [42] function to regulate several genes connected via a network and activate the lateral root initiation and inhibition of elongated roots in response to  $\text{NO}_3^-$  [42] (Figure 2d).

Auxin stabilizes the communication among TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALLING F-BOX proteins (TIR1/AFB3) and Domain II of AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional co-regulators to stimulates the ubiquitin-dependent breakdown of AUX/IAA protein in the 26S proteasome [111] (Figure 2B). When the auxin concentration is low, individuals from the AUXIN/IAA-INDUCIBLE (AUX/IAA) family of transcriptional repressors bind with DNA-binding protein of ARF [112,113], which exactly possess auxin-response promoter elements (AuxREs) in various auxin-regulated genes [114,115]. AUX/IAA protein inhibits the ARF function either by passively sequestering ARF protein away from their specific target promoters [116], or by interacting AUX/IAA with the co-repressor TOPLESS (TPL) to stimulate chromatin inactivation and silencing of ARF target genes [117]. The auxin concentration is increased by the auxin-stimulated module of co-receptor complexes, which include TIR1/AFBs family and an AUX/IAA member [118]. TIR1/AFBs specifically allows subunit of nuclear S-PHASE KINASE ASSOCIATED PROTEIN 1-CULLIN-F-BOX PROTEIN (SCF)-type E3 ubiquitin-protein ligases (SCFTIR/AFB) and stimulate the recognition of substrate. The auxin response is initiated by connecting the hormones to the TIR1/AFB receptor. The auxin receptor is a constituent of the SCFTIR1/AFB ubiquitin ligase complex [119]. This complex binds auxin to its receptor TIR1/AFB to activate the formation and breakdown of the polyubiquitination of AUX/IAA repressor, and subsequently activates ARF that induces auxin-responsive gene transcription [120–122] and represents the pivotal role of auxin signalling (Figure 2).

In a nutshell, auxin-initiated AUX/IAA removal regulates ARF repression and activates the transcription of primary genes. [123]. The abundance of Aux/IAA-ARF modules chronologically generate new LR and control LR development in *Arabidopsis* model plant.

### 5. Relationship between Nitrate Level and Auxin Translocation between The Root and The Shoot

A decrease in  $\text{NO}_3^-$  supply tends to promote auxin translocation from shoots to roots [124] (Figure 3). Here, we discuss the mechanism involved in the transportation of the auxin in response to different  $\text{NO}_3^-$  concentrations and the influences of the auxin concentration on the root growth.



**Figure 3.** The schematic model represents  $\text{NO}_3^-$  dependent shoot-root auxin transport and accumulation inside the *Arabidopsis* root. “A” represents auxin (IAA).

#### 5.1. Auxin Translocation in Response to Nitrate Level

High  $\text{NO}_3^-$  concentration (50 mM) negates the lateral root growth [121]. These responses are linked with an auxin transport inhibitor. Previous studies have established that the internal supply of nitrate reduced shoot to root auxin transport and subsequently decrease root auxin concentration to a significant level for lateral root growth. Therefore, for the stimulation of the lateral root growth, change in the root auxin concentration alone is not adequate, as nitrate concentration is also a determinant factor [125,121]. There is a decrease in the level of auxin in the root when there is an adequate supply of nitrogen because the adequate supply of  $\text{NO}_3^-$  seems to inhibit auxin transport activity from the shoots to the roots. Further reports have shown that the external IAA concentration partially lowers the stimulatory effect of localized nitrate (Figure 3). This inverse relationship between nitrate and auxin concentration disrupts the LR development [126].

As stated earlier, the auxin level in the roots is reduced by an adequate supply of nitrate ( $\text{NO}_3^-$ ) which disrupted LR development [126]. Moreover, The tissue auxin fixation was estimated in the roots 24 h after transferring the *Arabidopsis* seedlings from higher to lower nitrate medium [16]. Results indicated that the restricted root growth induced by 50 mM  $\text{NO}_3^-$  is replenished back to its normal growing state, 24–48 h after reducing the high nitrate ( $\text{NO}_3^-$ ) concentration. It is worthy to note that if the auxin plays a role in controlling this process, its concentration in the root may depend on the time before activating the inhibited lateral root. Further revealed that, a 50% increase was observed in the IAA content of the root when moved to 1 mM  $\text{NO}_3^-$  compared to that of 50 mM  $\text{NO}_3^-$  [127]. Similarly, the seedlings grown under 1 mM  $\text{NO}_3^-$  has a root auxin concentration that was four-fold more than the plants grown on 8 mM  $\text{NO}_3^-$  condition [128]. This is also evident that high  $\text{NO}_3^-$  (8 mM) concentration

significantly reduces the root auxin content [128]. Meanwhile, the high nitrate supply reduces the IAA concentration in the phloem exudates. Thus, the suppression of root growth by high nitrate could be linked to the reduced IAA level in the roots, particularly in the root tip region. It is speculated that the inhibitory effects of high nitrate concentration on the restricted root growth might be related to the decline in auxin content of the root [126].

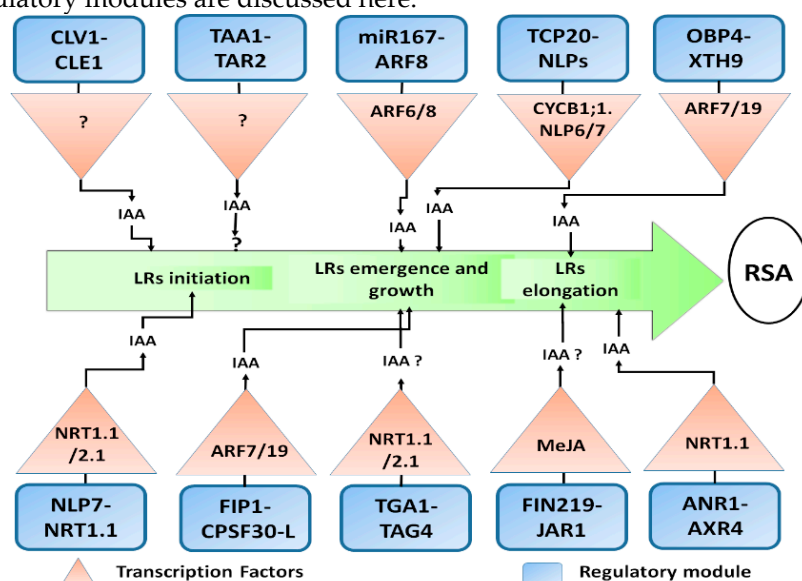
## 5.2. Influence of Auxin Concentration on Root Growth

Recent studies demonstrated that auxin accumulation in the LR primordia overlaps with the low nitrate-stimulated expression of *TAR2* in the root [16,85]. The application of the 2-4-carboxyphenyl-4, 4, 5, 5-tetramethylimidazole-1-oxyl-3-oxide (cPTIO) under  $\text{NO}_3^-$  supply, initiates auxin (IAA) level in the root [112]. In contrast to wild-type (WT), the *ospin1b* mutant has lower auxin level in their root, fewer LRs and shorter seminal root (SRs). These results demonstrate the effect of  $\text{NO}_3^-$  on LR arrangement and seminal root (SR) elongation by regulating auxin transport and  $\text{NO}_3^-$  [112].  $\text{NO}_3^-$  and IAA regulate the expression of the rice adenosine phosphate isopentenyltransferase (*OsIPT*) genes, limit the cytokinin (CTKs) biosynthesis in the tiller nodes, thereby regulating the development of tiller bud in rice [17]. Similarly, it is revealed that there was no increase in LRs elongation of auxin resistant *axr4* when subjected to low nitrate conditions [113]. Reversed-phase ultra-performance liquid chromatography (RP-UPLC) results on the plants showed that LR auxin concentration was increased while the nitrogen level decreased, hence the tea plant LRs formation might be stimulated by low nitrogen level via IAA biosynthesis and accumulation [129].

Auxin exhibits either a stimulatory or inhibitory effect on the primary and lateral roots of the plants depending on auxin concentration in the plants [130]. A reduced IAA (auxin) level in the roots inhibits LRs development [90], which is an indication of the  $\text{NO}_3^-$ -regulated auxin transport in the root. It has been inferred that  $\text{NO}_3^-$  increase in the shoot may repress the transition of auxin to the root, resulting in auxin-transport for LRs development [130]. However, in the auxin signalling pathway, high nitrate enhances LR development by influencing auxin levels in the roots. For example, at high external nitrate fixation, the LRs elongation in maize was repressed, due to a decrease of auxin translocation in phloem from shoot to root [90].

## 6. Nitrate Responsive Regulatory Module Initiates Root Growth

Nitrate modulates universal gene expression [131] (Figure 4), and some of the nitrate responsive regulatory modules are discussed here.



**Figure 4.** Molecular players involved in nitrate signalling regulating the primary and lateral roots; Schematic presentation of a regulatory module involved in the nitrate-mediated auxin response in *Arabidopsis* root. For

clarity purposes, all players and their secondary transcription factors (both upstream and downstream) are presented with blue and black colors respectively, which might not be the case in a plant cell. Auxin fluxes and accumulations contribute to primary and lateral root growth are indicated with the black arrow. However, the detailed mechanism and other transcripts are still unknown.

#### 6.1. TAA1 and TAR2 Regulatory Module

Analysis of *TAA1* and paralogues revealed a link between assimilation of local auxin, tissue-specific ethylene effect, and organ development. Indole-3-pyruvate (IPA) processed auxin synthesis is key to synthesize auxin response to light and environmental cues [36]. *TAA1* and *TAR2* are interlinked with auxin synthesis, required for keeping the suitable root auxin fixation and also required for the protection of root stem cell niches (<https://www.uniprot.org/>).

#### 6.2. ANR1 and AXR4 Regulatory Module

There is an interaction between the *ANR1*-dependent pathway and auxin signalling [113]. However, it remains unclear whether there is a relationship between the *ANR1* and the miR393/AFB3 module. It has been reported that *NRT1.1* functions upstream of *ANR1*, as it regulates LR elongation, obviously by its role as  $\text{NO}_3^-$  sensor [55]. In addition,  $\text{NO}_3^-$  signal activated by  $\text{NO}_3^-$  sensor (targeted at the PM), is transmitted by pathways that consist of the products of *ANR1* and *AXR4* genes. The possible function of *AXR4* and whether it lies upstream or downstream of *ANR1* in a signal transduction pathway remains unclear [96].

#### 6.3. TGA1 and TAG4 Regulatory Module

Using the integrative bioinformatics approach, *TGA1* and *TGA4* TFs were shown to mediate  $\text{NO}_3^-$  responses in *Arabidopsis* roots. The ATH1 Affymetrix microarray analysis showed that *tga1/tga4* double mutants have different responses to nitrate compared to wild type. This implies that TFs regulate genes that participate in the  $\text{NO}_3^-$  transport and metabolic processes. Also, ChIP analysis indicates that the TFs control the expression of high nitrate affinity transporters *NRT2.1* and *NRT2.2* by binding to their promoters. This reveals that *TGA1/TGA4* & *NRT2.1/NRT2.2* regulates the lateral root growth induced by nitrate signalling in *Arabidopsis* [31].

#### 6.4. CLV1 and CLE1 Regulatory Module

This signalling module consisting of the nitrogen responsive (CLAVATA3/ESR-related) and leucine-rich occurring receptor-like kinase CLAVATA1 (CLV1) are expressed in the root vasculature of *Arabidopsis* root [132]. *CLV1* and *CLE1* regulate the elongation of the root system under limited nitrogen conditions [132]. *CLE1*, -3, -4 and -7 stimulated by limited internal  $\text{NO}_3^-$  in the root, and mainly expressed in the cells of root pericycle [133]. In *A. thaliana* plants, the *CLE41* peptides (*CLE41p*) promote the proliferation of vascular cells. This proliferation is dependent on auxin binding since it was improved by the exogenous application of synthetic auxin. This report established that vascular architecture is regulated by various *CLE* peptides related to hormonal signalling [74].

#### 6.5. FIN219-JAR1 Regulatory Module

*FIN219/JAR1* plays an exemplary role in regulating the combination of phytohormones in an auxin-dependent manner [28]. Specifically, Since *FIN219* contains far-red (FR) light and methyl jasmonate (MeJA), that control various TFs including 94 basic helix-loop-helix (*bHLH*) TFs. Some of the loss of function mutant *bHLH* influenced by *FIN219* demonstrate an altered response to MeJA in the regulation of the hypocotyl and root elongation. Thus, *FIN219/JAR1* is specifically controlled by exogenous MeJA and interacts with different plant hormones to modulate the hypocotyl and root elongation of *Arabidopsis* seedlings, likely by regulating a group of TFs [118].

#### 6.6. FIP1 and CPSF30-L Regulatory Module

It is reported that *FIP1* interacts with the cleavage and polyadenylation specificity factor 30-L (CPSF30-L), which is also a fundamental player in nitrate signalling [29]. To study the expression of *CPSF30* in the LR by qRT-PCR plant poly (A) signal (PAS) in *oxt6* was performed. The results demonstrated that in the absence of auxin the expression of *ARF7* and *ARF19* was found lower in *fip1* compared to WT, which may be one of the reasons for less LR in *oxt6*. However, in *oxt6*, there was a significantly greater increase in the expression of *ARF7* and *ARF19* when exogenous IAA was applied than that of WT. This further validates the role of *CPSF30* in auxin regulation of LR growth and development to a greater extent through *ARF19* [134].

#### 6.7. TCP20-NLPs Regulatory Module

The branched1/cycloidea/proliferating cell factor1-20 (*TCP20*) and NIN-like protein (NLP) NLP6 & NLP7, which functions to induce nitrate assimilatory genes, target the promoter upstream of nitrate reductase genes *NIA1* [135,136]. *TCP20-NLP6* and 7 heterodimers aggregate in the nucleus, and its overlaps mediate up-regulation of  $\text{NO}_3^-$  assimilation genes, and down-regulates G<sub>2</sub>/M cell-cycle marker gene *CYCB1;1* [136]. In a likewise manner, *TCP20-NLP6* & 7 supports root meristem development under N deficiency. This report elucidates mechanisms through which plants facilitate accessibility to nitrate responses, subsequently interconnecting  $\text{NO}_3^-$ -assimilation and signalling with cell-cycle progression [136]. It was revealed that *Chrysanthemum morifolium TCP20* (*CmTCP20*) positively influence auxin accumulation in the LR, partially by improving auxin biosynthesis, transport, and response, and thereby stimulating LR growth [78].

#### 6.8. Nitrate-responsive OBP4-XTH9 Regulatory Module

Xyloglucan endotransglucosylases (XTHs) plays a crucial role in cell wall biosynthesis of group genes 1 *XTHs* gene, e.g., *XTH1-11*. *XTH9* is highly expressed in the LR primordial. Consistently, *xth9* mutant shows fewer LR compared to the OE *XTH9* exhibits more LR. This indicating the potential role of *XTH9* in regulating LR growth [122]. Genetic analysis revealed that this function of *XTH9* depend on auxin-mediated *ARF7/19* and the downstream *AFB3* in response to nitrogen signals, stimulates the expression of *XTH9* to enhance the LR [122].

#### 6.9. Nitrate Responsive NLP7 and NRT1.1 Module

It was reported by [137], that the expression of a transceptor *NRT1.1* is modulated by NIN-like protein 7 (NLP7). Genetic and molecular analyses indicated that *NLP7* functions as an upstream of *NRT1.1* in the regulation of nitrate when  $\text{NH}_4^+$  is present, while in absence of  $\text{NH}_4^+$ , it functions in nitrate signalling without being mediated by *NRT1.1*. However, in the *nlp7* mutant, the expression of *NRT1.1* initiates a partial or complete nitrate signalling [137]. More convincing evidence from ChiP and EMSA analyses indicated that *NLP7* may bind to the particular region of the promoter of *NRT1.1*. Hence, *NLP7* gene plays a significant role in  $\text{NO}_3^-$  signalling by regulating *NRT1* [137].

### 7. Conclusion and Future Perspective

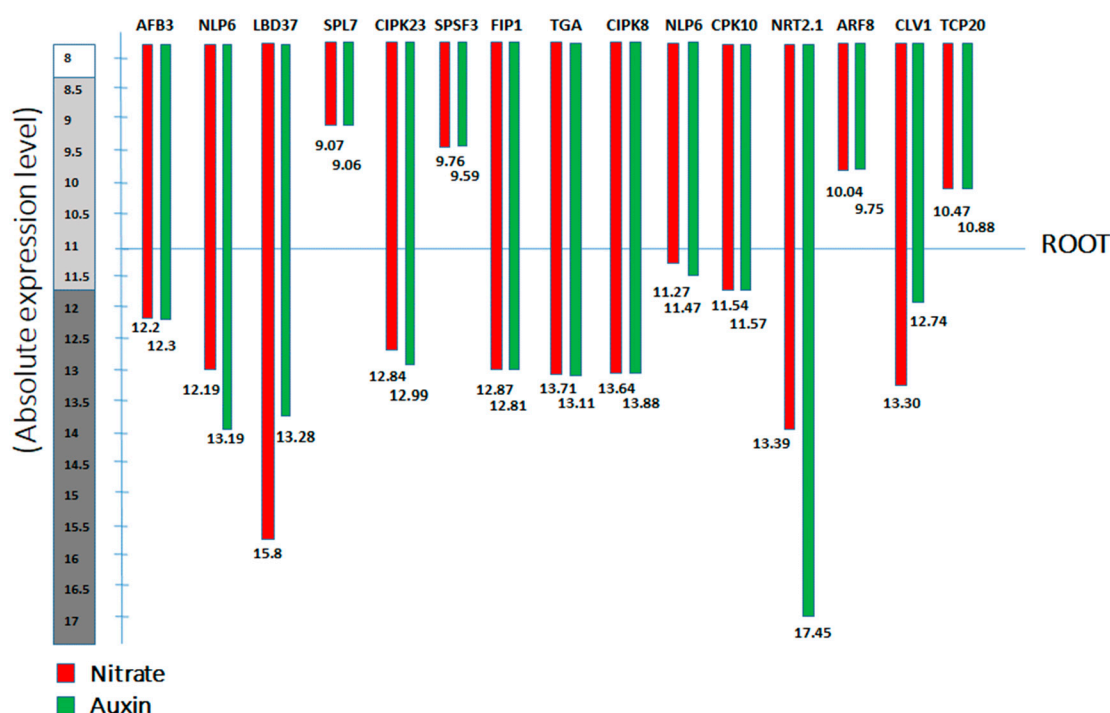
Recently, the system biology approach was established to distinguish the constituents and the crosstalk between nitrate and hormonal signal influenced by nitrate-signalling [138].  $\text{NO}_3^-$  and IAA contribute to the cascade of events involved in the signal transduction towards the auxin-induced adventitious and LR formation [139,140].

Several nitrate responsive genes involved in nitrate signalling promotes lateral root growth. And these genes are characterized in terms of nitrate regulatory genes, which build up signalling overlap between nitrate and auxin, and subsequently induce LR initiation, emergence, and development [50]. We distinguished these genes as short-term and long-term nitrate responsive transcription factors, highlighted their possible roles and also elucidate how they mediate auxin biosynthesis to initiate LR in *Arabidopsis* at different nitrate levels (Figure 1) (Table 1-4). To further demonstrate this signalling crosstalk, here, we have presented the absolute expression by collecting information from

“Genevigator database” (Table S1 and Figure 5), namely the Affymetrix *Arabidopsis* ATH1 genome microarray analysis of root, which further presents each gene’s absolute expression in response to nitrate and auxin treatment respectively. Moreover, a refined set of genes was designated to be regulated by both nitrate ( $\text{NO}_3^-$ ) and auxin signalling. We found that these genes have significantly higher levels of baseline expression to  $\text{NO}_3^-$  and auxin (Figure 5).

**Table 4.** Unknown Affinity Nitrate Responsive Genes.

| Gene  | Gene Family                                | Treatment | Transcriptionally NO <sub>3</sub> <sup>-</sup> Responsive | Effect On RSA                            | Target Genes | Root Expression Profile                | Subcellular Localization                                       | Refs |
|-------|--------------------------------------------|-----------|-----------------------------------------------------------|------------------------------------------|--------------|----------------------------------------|----------------------------------------------------------------|------|
| TAR2  | Tryptophan Aminotransferase Related 2      | low       | unknown                                                   | maintenance of the root stem cell niches | unknown      | root meristem and root vascular system | endoplasmic reticulum membrane, integral component of membrane | [36] |
| JR1   | Mannose-binding lectin superfamily protein | low       | unknown                                                   | root allometry                           | unknown      | root                                   | nucleolus, vacuole, etc.                                       | [58] |
| DAAR2 | Phenazine biosynthesis PhzC/PhzF protein   | low       | unknown                                                   | LR induction                             | unknown      | root                                   | cytosol, nucleus                                               | [58] |
| RSA   | ATP/GTP-binding protein family             | high      | unknown                                                   | LRs induction (elongation)               | unknown      | root                                   | cytoplasm, cytosol, nucleus                                    | [82] |
| PHO1  | Phosphate 1                                | high      | unknown                                                   | root allometry                           | unknown      | root                                   | golgi apparatus, golgi membrane, cytoplasm,                    | [82] |



**Figure 5.** Affymetrix *Arabidopsis* ATH1 genome microarray analysis of the root; The schematic model represents the absolute expression level of the different nitrate transcription factors in the root of *Arabidopsis*, under nitrate ( $\text{NO}_3^-$ ) and auxin treatment, the red bar represents the gene expression for nitrate treatment, while the green bar represents the expression level for auxin treatments. (See text for further detail).

Thus, our results demonstrate the identical regulation of these transcription factors for both nitrate and auxin treatments in the root of *Arabidopsis thaliana*. These discoveries lead to new speculation that nitrate plays a vital role in enhancing the effect of auxin signal. Additionally, cis-regulatory elements (CREs) in the promoters of these genes imply their role as candidates that might stimulate the  $\text{NO}_3^-$  regulated genes expression. This review highlights the ongoing innovation about the several regulatory modules controlling  $\text{NO}_3^-$  uptake and promoting root growth in response to mutual nitrate and auxin signalling in the model plant *Arabidopsis thaliana*.

However, limited studies assessed the role of auxin in nitrate induced lateral root growth. There has been an outstanding achievement in our understanding of  $\text{NO}_3^-$  signalling pathways. With the schematic diagram, we have presented the nitrate mediated auxin biosynthesis regulates LR's growth. This regulation is via co-signalling induced by the primary nitrate transporter *NPF6.3/NRT1.1* and its downstream TFs, including *NIA1*, *NIA2*, *NiR*, *NR*, and *NRT2.1* (Figure 2), which mediate IAA biosynthesis induced lateral root growth (Figure 1). However, distinctive transcription factors such as *CBL7*, *NLP7*, *TGA1/TGA4*, *TCP20*, *NRG2*, *RSA1*, *CLV1/CLE1*, *RSA1*, *NAC4/OBF4* (downstream of *AFB3*), *SPL9*, *CLE/CLV1*, *LBDs*, *JR1*, *PHO1*, *DAAR2* are nitrate responsive, also may target *NPF6.3/NRT1* and *NRT2.1*. However, how these transcription factors interact to build up a signalling module to regulate the LR root growth of *Arabidopsis thaliana* in response to nitrate is still questionable. Furthermore, some nitrate responsive genes are yet to be characterized and their interaction with auxin is yet unknown. However, they are the fundamental contributor genes in the regulation of LR development.

The  $\text{NO}_3^-$  and IAA signalling overlap in the *Arabidopsis* root system open up a huge research gap for each identified effect on root growth. Hence, the functional identification and characterization of various players associated with  $\text{NO}_3^-$  signalling pathways and their possible functional interaction with auxin in regulating LR's of *Arabidopsis* is the following step to comprehend the N responses in the plant. Moreover, it is also of much interest to uncover evidence to support the auxin signalling pathways that are self-regulated as they are involved

in the feedback regulation of nitrate transport and assimilation to induce lateral root growth and development, which is fundamental to the field of crop biotechnology.

**Supplementary Materials:** The following are available online at [www.mdpi.com/1422-0067/21/8/2880/s1](http://www.mdpi.com/1422-0067/21/8/2880/s1), Table S1. Affymetrix Arabidopsis ATH1 Genome Microarray of Anatomical Part: Root.

**Author Contributions:** M. Asim and Q. Wang has jointly developed the conceptual structure of the manuscript. M. Asim wrote the manuscript, including all the Tables and Figures. O. Aluko assisted in further modification of the manuscript. Z. Ullah corrected the grammatical mistakes and revised the manuscript. H. Liu has provided critical feedback, revised, and approved it for publication. All authors have read and agreed to the published version of the manuscript.

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## Abbreviation

|             |                                                                                                  |
|-------------|--------------------------------------------------------------------------------------------------|
| RSA         | Root system architecture                                                                         |
| LBD         | Lateral Organ Boundaries Domain                                                                  |
| SPL         | SQUAMOSA PROMOTER BINDING PROTEIN-LIKE                                                           |
| NIGT1       | NITRATE-INDUCIBLE GRAP-TYPE TRANSCRIPTIONAL REPRESSOR1                                           |
| FIP1        | Factor interacting with poly (A) polymerase 1                                                    |
| ARF         | Auxin Response Factor                                                                            |
| HNI9        | HIGH NITROGEN INSENSITIVE9                                                                       |
| NLP         | NIN-like protein                                                                                 |
| TCP20       | The branched1/cycloidea/proliferating cell factor1-20                                            |
| XTH         | Xyloglucan endotransglucosylases                                                                 |
| JR1         | JOSMONATE RESPONSIVE 1                                                                           |
| DAAR2       | D AMINO ACID RACEMASE2                                                                           |
| HNI9/AtIWS1 | HIGH NITROGEN INSENSITIVE9                                                                       |
| PHO1        | PHOSPAHT TRANSPORTER 1                                                                           |
| RSA1        | ROOT SYSTEM ARCHITECTURE 1                                                                       |
| NRG2        | NITRATE REGULATORY GENE 2                                                                        |
| TIR1/AFB    | TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALLING F-BOX                                             |
| AUX/IAA     | AUXIN/ INDLOE-3-ACETIC ACID                                                                      |
| ARF         | AUXIN RESPONSE FACTOR                                                                            |
| CPSF30-L    | cleavage and polyadenylation specificity factor 30-L                                             |
| SCFTIR/AFB  | S-PHASE KINASE ASSOCIATED PROTEIN 1-CULLIN-F-BOX PROTEIN (SCF)-type E3 ubiquitin-protein ligases |
| MeJA        | Methyl jasmonate                                                                                 |
| EMSA        | Electrophoretic mobility shift analysis                                                          |

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