CEPR1 REGULATES *ARABIDOPSIS THALIANA* ROOT ARCHITECTURE BY MODULATING AUXIN PRODUCTION THROUGH *NIT1*

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Dedication: This thesis is dedicated to Clara, who helped me grow in the early stages of my career, to my family who always believed in my ability to pursue great things, and to my group of lifelong friends, who are an amazing support system that never left my side.

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Abstract

Like all organisms, plants must make decisions about growth that ultimately lead to their conservation or expenditure of energy. Carbon and nitrogen are both critical macronutrients required for growth and survival, and plants must be able to sense their internal abundance of both to ensure that there is enough to either commit to growth or avoid wasting resources on growth when environmental conditions are suboptimal. In *Arabidopsis thaliana*, the receptor-like kinases *CEPR1* and *CEPR2* are involved in a regulatory pathway that comprises a systemic signaling network that can influence root system architecture depending on the availability of both carbon and nitrogen. Here, we present evidence that *CEPR1* can integrate nitrogen and carbon status to influence lateral root growth through genetic interactions with the auxin biosynthetic enzyme, *NITRILASE 1 (NIT1)* and that interactions between *CEPR1* and *NIT1* can affect auxin levels in the primary root. Additionally, we show that mutations in *NIT1* can suppress an infertility phenotype associated with mutants deficient for *CEPR1.* Overall, our results suggest a model that *CEPR1* regulates root development under different amounts of carbon and nitrogen by modulating auxin production via *NIT1*.

Introduction

For terrestrial plants, survival on land requires a coordinated response to the perception and integration of many environmental cues. How plants make decisions to respond to multiple environmental signals simultaneously, or to a single cue that drastically limits plant growth has recently been the subject of much scientific research. As a result, there is increasing evidence that the root and the shoot, which experience vastly different stresses and environmental inputs, communicate with each other to coordinate optimal responses to balance the use of their internal resources and ensure growth and survival (reviewed in Li et al., 2021). Among the many environmental factors that influence plant growth is the availability of the two most critical nutrients required for plant growth to occur: carbon and nitrogen.

Carbon and Nitrogen: two mutually dependent macronutrients

Carbon is used by plants for energy and to make the structural components to generate organs like stems, leaves, and roots. In the atmosphere, carbon is most abundant in the form of the gas CO_2. Plants fix CO_2 through photosynthesis and downstream metabolic processes and convert carbon into usable forms like sucrose, glucose, fructose, or cellulose. Glucose is the main sugar used by plants for cellular respiration and cellulose is the main constituent of the plant cell wall. Thus, carbon is critical in plants for normal metabolism and for the biogenesis of new organs.

Additionally, nitrogen is a major limiting factor for plant growth, as it is present in many important macromolecules and is required for any metabolic processes to occur- including carbon fixation and photosynthesis. Nitrogen is ubiquitous in the atmosphere; however, few forms of nitrogen are usable by plants which rely on abiotic and biotic sources like lightning or various species of microbes in the soil to convert atmospheric N_2 to ammonium and nitrate. A key difference between carbon and nitrogen uptake is the location in the plant where these two processes occur. Carbon fixation and photosynthesis occur in the shoot while nitrogen is taken up exclusively by the roots. But both processes are interdependent, as carbon is required for cell wall biogenesis and root growth to occur, and nitrogen uptake is dependent on a functional root system. Because of this, a substantial degree of communication would be expected between the root and the shoot to evaluate both the external and internal abundance of carbon and nitrogen and to optimize growth under normal and stressed conditions.

Since CO_2 is evenly distributed throughout the lower atmosphere, a plant's shoot is typically exposed to a homogeneous source of carbon. However, the root system can be present in soil that contains a heterogeneous distribution of nutrients, including nitrogen. This predicts that the root system must be able to perceive and integrate multiple different nitrogen status signals from different regions of the soil and determine whether the energy required to continue growing in that area is worth expending.

Regulation of carbon homeostasis: sugar sensing and signaling

Although plants rarely have limited access to CO_2, it is possible for them to grow in conditions where light is limited. Reduced light levels would result in an impaired ability for a plant to convert CO_2 into sugars because the light-dependent reactions of photosynthesis are what provide the energy for the light-independent reactions involved in carbon fixation (Calvin cycle) to occur.

In all organisms, sugar is critical for growth and basic metabolic processes like cellular respiration. Because of this, some mechanisms to respond to low energy during times when sugar is scarce evolved early on and are conserved across major lineages of eukaryotes. In *Arabidopsis thaliana*, one major component involved in responding to energy scarcity is the protein complex *SUCROSE NON-FERMENTING-1-RELATED KINASE 1* (*SnRK1*), which functions as a heterotrimer of α (catalytic), β and γ (regulatory) subunits and plays a central role in energy sensing and signaling (reviewed in Broeckx et al., 2016, Wurzinger et al., 2018), and is tightly linked to sugar metabolism. *SnRK1* is the plant orthologue of the *SUCROSE NON-*

FERMENTING 1 (SNF1) protein complex in yeast and *AMP-ACTIVATED PROTEIN KINASE (AMPK)* in mammals (Broeckx et al., 2016).

In *Arabidopsis thaliana*, *SnRK1* has been shown by numerous groups to be inhibited by excess energy in the form of sugar phosphates (Toroser et al., 2000, Zhang et al., 2009, Nunes et al., 2013), hinting at its role in responding to the abundance of energy. Oppositely, *SnRK1* is activated in response to energy deficiencies by promoting catabolic pathways that produce ATP while simultaneously inhibiting metabolic pathways that consume ATP (Baena-Gonzalez et al., 2007, Wurzinger et al., 2018). Plant overexpression experiments demonstrate that *KIN10*, one of the α subunits of *SnRK1*, promotes transcription of catabolic genes and inhibits transcription of anabolic genes (Baena-Gonzalez et al., 2007). Additionally, null mutations in both *KIN10* and *KIN11,* another α subunit of *SnRK1*, abolish transcriptional responses to darkness and impair starch mobilization at night (Baena-Gonzalez et al., 2007). A phosphoproteomics experiment revealed that *KIN10* can differentially regulate various proteins through phosphorylation, including transcription factors, metabolic enzymes, and chloroplast-localized proteins (Nukarinen et al., 2016). This verified the findings of earlier *in-vitro* studies in other plant species reporting *SnRK1* phosphorylation of enzymes that are important for sucrose synthesis such as *SUCROSE PHOSPHATE SYNTHASE* (*SPS*) (Sugden et al., 1999), carbon partitioning (*FRUCTOSE-6-PHOSPHATE 2-KINASE/FRUCTOSE-2,6-BISPHOSPHATASE, F2KP)*(Kulma et al., 2004), and trehalose metabolism (*TREHALOSE 6-PHOSPHATE SYNTHASE*, *TPS*) (Harthill et al., 2006). This highlights how *SnRK1* can influence metabolism both through transcriptional regulation as well as directly by regulating the activity of enzymes involved in metabolism.

Aside from responding to energy deficiency, *SnRK1* has also been implicated in regulating nitrogen assimilation because of its demonstrated ability to phosphorylate *NITRATE REDUCTASE 1* (*NR1*) both *in-vitro* and *in-vivo* (Sugden et al., 1999, Cho et al., 2016, Nukarinen et al., 2016). *NR1* regulates the first step of nitrogen assimilation, whereby nitrate is reduced to nitrite and later converted to organic nitrogen. By phosphorylating *NR1, SnRK1* has been shown to inhibit its activity (Sugden et al., 1999) which fits with its role in energy scarcity, as nitrate reduction is very energy intensive and would be otherwise unnecessary during times when energy is scarce.

Maintenance of energy homeostasis is critical for the growth of all organisms and must be tightly regulated to avoid wasting further resources during times when energy cannot be generated. In plants, carbon/energy scarcity would result in root growth inhibition regardless of the nutrient conditions the roots are present in. This is because the plant would not have the energy to assimilate any nutrients or the structural components to generate new roots to uptake nutrients. Overall, this underlines an important need for signaling between organs because the availability of carbon in the shoot would dictate whether roots can grow. Meanwhile, the root system needs to communicate its nutrient status to the shoot for the plant to decide if it’s worth enhancing root growth in those conditions.

Long-distance signaling in plants

One way that plants coordinate their growth and development is through long-distance signaling. Long-distance signals in plants can exist in the form of RNA, peptides, lipids, and other small molecules and many examples exist where long-distance signaling is involved in regulating aspects of development or for responding to environmental stress. For example, flowering is a developmental transition from a vegetative phase to a reproductive phase that requires tight coordination between many components. In *Arabidopsis thaliana,* one classic example of a long-distance signal involved in this phase transition is *FLOWERING LOCUS T* (*FT*). *FT* is a protein signal required to induce flowering that moves from the leaves to the shoot apical meristem via the phloem, where it can interact with other proteins to promote the developmental transition to flowering (Corbesier et al., 2007, Notaguchi et al., 2008, Yoo et al., 2013).

Besides regulating their normal growth and development, plants must also respond to a myriad of different biotic and abiotic stresses throughout their lives. Many of the ways plants respond to these stresses also rely on long-distance signaling. One major example of this is in systemic acquired resistance to pathogens. Plant defenses such as PAMP-triggered or effector-triggered immunity are activated locally in tissues experiencing a pathogen invasion, which triggers acquired immunity in distant parts of the plant. This response relies on long-distance signaling of several small molecules to trigger immunity in uninfected tissues of the plant. These small molecules include metabolites such as methyl salicylate, pipecolic acid, glycerol-3-phosphate, and azelaic acid (reviewed in Shah & Zeier et al., 2013).

Drought is one of the most severe stress factors for plants and it is critical for them to be able to respond to it. Long-distance signaling has been shown to play a role in orchestrating responses to drought stress as well. In *Arabidopsis thaliana* plants experiencing dehydration, a small peptide called *CLE25,* which is a member of the *CLAVATA3/EMBRYO-SURROUNDING REGION*-related (*CLE*) family genes, has been shown to be expressed in the vasculature of roots and leaves, but is upregulated in roots experiencing drought stress (Takahashi et al., 2018). Induction of *CLE25* during drought leads to an increase in abscisic acid (ABA) biosynthesis specifically in leaves, which mediates stomatal closure and enhanced drought tolerance. Takahashi et al., 2018, have shown this to be due to *CLE25* moving from roots to leaves, where it can interact with the two receptor-like kinases, *BARELY ANY MERISTEM 1* and *3* (*BAM1, BAM3*) to promote expression of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*)*,* an important ABA biosynthetic enzyme. These examples highlight the remarkable communication that exists between different parts of the plant that may be exposed to different environments, where one organ experiencing stress can signal to another unaffected organ to prime it for dealing with stress rapidly.

CEP*s and their receptors, *CEPR1* and *CEPR2

In addition to the long-distance signaling pathways discussed above, other signals in the form of small peptides that travel between the root and the shoot have been identified in *Arabidopsis thaliana* and *Medicago truncatula*. These appear to be involved in regulating root or root nodule growth in heterogeneous soil, depending on the abundance of internal carbon and nitrogen resources (reviewed in Oldroyd & Leyser, 2020). In *Arabidopsis thaliana,* these signals are C-Terminally Encoded Peptides (*CEPs*), which are a family of peptides encoded by 15 genes with distinct expression patterns that are induced by different environmental cues (Delay et al., 2013). *CEP* genes encode proteins that are post-translationally modified and cleaved into small peptides of <20 amino acids (Murphy et al., 2012, Roberts et al., 2013). Several *CEP* genes are expressed in primary and lateral roots (Roberts et al., 2013) and some of these are upregulated under low nitrogen conditions (Tabata et al., 2014). These small peptides can act as ligands for the leucine rich repeat receptor-like kinases (LRR-RLKs), *C-TERMINALLY ENCODED PEPTIDE RECEPTOR 1* & *2* (*CEPR1* and *CEPR2*). In general, *CEP*s can be perceived by *CEPR1* and *CEPR2* locally in the root tissues, which leads to repression of lateral root growth (Roberts et al., 2016, Dimitrov & Tax, 2018). They can also be secreted into xylem vessels and mobilized to leaves, where they can be perceived by shoot *CEPR1* and *CEPR2* (Tabata et al., 2014). Upon *CEP* perception in the shoot, a downward shoot-to-root signal occurs, where two mobile polypeptides, encoded by two members of the CC-type glutaredoxin (ROXY) family, *CEP DOWNSTREAM 1* and *2* (*CEPD1/2),* translocate to the roots. Induction of this downward signal is dependent upon *CEP* signaling through *CEPR1* in the shoot (Ohkubo et al., 2017). Additionally, a third signal, *CEPDL2*, which is upregulated in the shoot in response to *trans*-Zeatin (tZ, a cytokinin) that is produced in roots exposed to nitrogen rich conditions and transported from root-to-shoot, integrates with *CEPD1* and *CEPD2* in the roots to upregulate nitrate transporter expression in roots exposed to high nitrogen conditions (Ota et al., 2020). Cytokinins, classic plant hormones, are known to inhibit lateral root development but are primarily involved in promoting proper shoot growth. In the context of this signaling network, *trans*-zeatin may serve as a signal to shoots to indicate that a supply of nitrogen is available because its biosynthesis is only upregulated in roots exposed to high nitrogen conditions, and shoot growth is dependent on the amount of nitrogen the plant can access.

CRA2* - A *CEPR1* ortholog in *Medicago truncatula

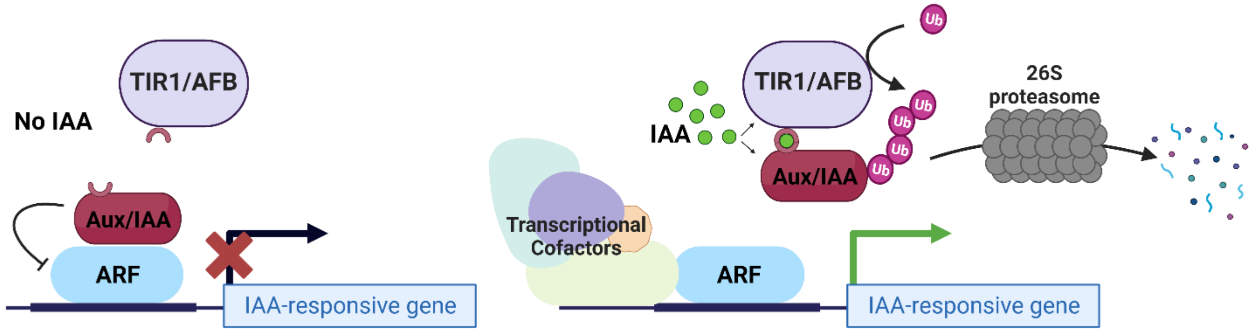
In *Medicago truncatula*, a model legume, *COMPACT ROOT ARCHITECTURE 2* (*CRA2*) is an ortholog of *CEPR1* that is involved in inhibiting lateral root development. However, in this species, *CRA2* simultaneously promotes root nodule development. In addition to lateral roots, plants in the family Fabaceae can produce root nodules, which give them the unique ability to create mutualistic associations with nitrogen fixing bacteria (*Rhizobia spp.)* and as such, can promote nitrogen fixation in their roots (Oldroyd et al., 2011). Nitrogen fixation is very energy intensive for *Rhizobia* and so this mutualism is only beneficial to the plant in situations where nitrogen availability is low, but enough carbon (photosynthate) is available to provide the bacteria with energy (Ballhorn et al., 2016). This mutualism is also costly to the plant and this underlines the importance that sensing carbon-nitrogen balance would have on mediating these associations.

Similar to *CEP*s in *Arabidopsis thaliana, CEPs* in *Medicago truncatula* are also produced under low nitrogen conditions and grafting experiments have revealed that they move from the root to the shoot, where they interact with *CRA2* (Imin et al., 2013, Hualt et al., 2014). Interactions between *CEPs* and *CRA2* have been shown to positively regulate nodulation through the induction of *miR2111* in the shoot, which moves back to the roots and inhibits transcripts of *TOO MUCH LOVE 1 & 2* (*TML1*), negative regulators of nodulation (Tsikou et al., 2018, Gautrat et al., 2020). In contrast, in ample nitrogen, *CLE12* and *CLE13* are produced in nodulated roots and are transported to the shoot, where they interact with *SUPER NUMERIC NODULE* (*SUNN*), an LRR-RLK related to *BAM1/3* and inhibitor of *miR2111* expression (Schnabel et al., 2005, Mortier et al., 2010). This ultimately leads to inhibition of nodulation. The ability of *M. truncatula* to enhance nodulation in low nitrogen and inhibit nodulation when ample nitrogen levels have been reached has been termed autoregulation of nodulation.

Overall, this pathway in *Medicago truncatula* is similar in *Arabidopsis thaliana,* where *CEPR1/CRA2* can modulate root development depending on the status of both internal carbon and nitrogen, highlighting the tight link between the two nutrients and the importance of balance between them.

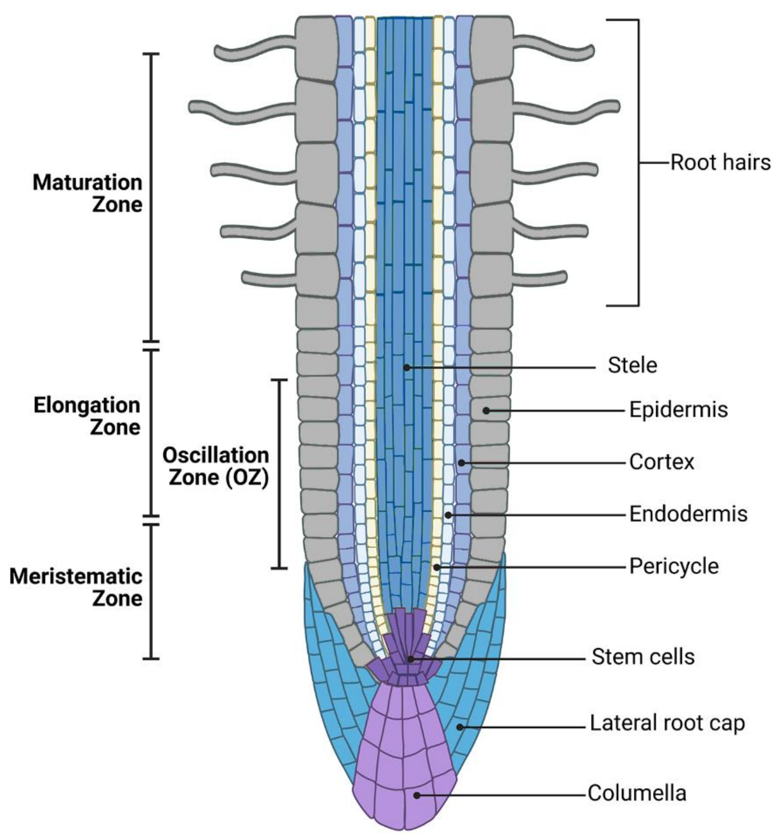
Auxin and Lateral Root Development

Auxin, or indole-3-acetic acid (abbreviated as IAA), is a developmentally critical plant hormone that acts as a morphogen and controls many aspects of plant development, from embryogenesis to post-embryonic organogenesis (De Smet & Jürgens, 2007, Vanneste & Friml, 2009). Decades of research have revealed that IAA signaling causes large changes in gene expression in the tissues where it is present (Abel et al., 1994, Paponov, 2008) and that its signaling capacity can be regulated in many ways, including through the rate of its biosynthesis and its transport throughout the plant (reviewed in Stewart & Nemhauser, 2010). IAA signals intracellularly through its nuclear receptor, *TRANSPORT INHIBITOR RESISTANT 1/AUXIN SIGNALING F-BOX* (*TIR1/AFB)* (Kepinski & Leyser, 2005, Dharmasiri et al., 2005). The *TIR1/AFB* receptor proteins interact with two other proteins to form a functional SCF E3 ubiquitin ligase complex called SCF^{TIR1}. Binding of IAA to *TIR1* increases the affinity of *TIR1* to *Aux/IAA* proteins, which are IAA co-receptors and transcriptional repressors of IAA-responsive genes (Tan et al., 2007), and stabilizes their interaction. The interaction between *TIR1/AFB*s and *Aux/IAA*s leads to *Aux/IAA* ubiquitination (Maraschin et al., 2009) and subsequent degradation in the 26S proteasome (Gray et al., 2001, Zenser et al., 2001). This degradation releases the inhibition of AUXIN RESPONSE FACTOR (ARF) transcription factors, which can then interact with other various cofactors to initiate transcription of IAA-responsive genes.



Lateral Root Priming

Root development is among the plethora of processes that auxin is involved in. In *Arabidopsis thaliana* lateral root development, the role of IAA begins very early, when it accumulates near the primary root tip in an oscillatory fashion. This leads to the formation of evenly spaced pre-branch sites where future lateral roots may continue to develop and eventually emerge as the primary root continues to grow (DeSmet et al., 2007, Moreno-Risueno et al., 2010, Van Norman et al., 2013). Throughout the development and eventual emergence of lateral root primordia, auxin continues to play



a major role. The primary root is divided into radially arranged rings of different cell types that surround the stele, a cylinder of cells where vascular tissue is present. The cell layer that surrounds the stele is known as the pericycle, which has two subsets of cells called the xylem and phloem pericycle poles (XPP and PPP, respectively), because they are directly adjacent to the xylem and phloem vascular tissues within the stele. The oscillatory accumulation of IAA occurs in a broad region of the root basal meristem that overlaps with sections of the meristematic and elongation zones and has been named the oscillation zone (OZ), due to recurrent and periodic increases in the activity of the synthetic auxin-responsive promoter *DR5 (DIRECT REPEAT 5*) that can be observed in that region of the root (DeSmet et al., 2007, Moreno-Risueno et al., 2010). The amplitude of these auxin oscillations in the basal meristem, which is important for determining pre-branch site formation, is controlled in part through spatiotemporal regulation of IAA biosynthesis in the lateral root cap (Xuan et al., 2015) and its transport to the basal meristem (Blilou et al., 2005, Chen et al., 2023, reviewed in Laskowski & Tusscher, 2017), highlighting the dynamic regulation of auxin in controlling root development.

IAA accumulation in the OZ is involved in lateral root pre-patterning and thus the region where it accumulates overlaps with where XPP cells are respecified into lateral root founder cells (Dubrovsky et al., 2008), which maintain steady *DR5* activity even after an oscillation has ended. Auxin activity in these founder cells abolishes the inhibition of *IAA28* on *ARF5, ARF6, ARF7, ARF8,* and *ARF19* to promote expression of *GATA23*, which encodes a member of the GATA factor family of zinc finger transcription factors and controls the acquisition of founder cell identity (De Rybel, 2010). Interestingly, while only one or two XPP cells that exit the OZ become specified as lateral root founder cells, up to 12 pericycle cells can be present within the OZ during an oscillation (Van Norman et al., 2013), indicating that auxin alone is not sufficient for XPP re-specification to occur and that other signals are involved. Indeed, genes that are required for lateral inhibition to prevent an excess of founder cells from forming have been identified and will be discussed in the following section. Another pathway that possibly acts independently of auxin that appears to be involved in the re-specification of XPP cells to lateral root primordia includes the early lateral root initiation marker *MEMBRANE-ASSOCIATED KINASE REGULATOR4 (MAKR4)*, as *makr4* loss-of-function mutants have fewer lateral roots but maintain the same number of pre-branch sites as wild type (Xuan et al., 2015).

Lateral root founder cells, in summary, are cells that are competent to become lateral roots but have not yet become initiated, which requires oscillations of auxin in the primary root meristem to promote founder cells to become division-competent and undergo an asymmetric cell division. However, the frequency of *DR5* oscillations in the OZ has been observed to be higher than the frequency by which lateral roots initiate, highlighting the likelihood that founder cell specification and lateral root initiation are independent developmental events (Van Norman et al., 2013). Despite this, auxin continues to play a pivotal role throughout lateral root initiation and emergence, and tight spatiotemporal regulation of both auxin production and transport remain critical for lateral root development to progress.

Lateral Root Initiation and Emergence

Following lateral root founder cell specification, the first event of lateral root initiation occurs in the differentiation zone of the primary root, where the nuclei of both founder cells migrate towards the cell wall dividing the two (De Smet et al., 2007, De Rybel et al., 2010). This migration is largely mediated by the auxin-dependent regulation of *LATERAL ORGAN BOUNDARIES-DOMAIN (LBD)* transcription factors (reviewed in Torres-Martínez et al., 2022). Of these, *LBD18* and *LBD33* have been shown to regulate the transcription factor *E2Fa*, a member of the *E2F* family of transcription factors that regulate cell cycle, which is essential for lateral root initiation to occur (Berckmans et al., 2011). Additionally, auxin has been shown to inhibit *SOLITARY ROOT(SLR)/IAA14* and release its inhibition on *ARF7* and *19* to promote nuclear migration (Fukaki et al., 2002, De Rybel et al., 2010). Nuclear migration to the common cell wall between two founder cells precedes a formative cell division, which is followed by a series of anticlinal asymmetric cell divisions. During these anticlinal cell divisions, a rotation in the cell division plane takes place, followed by periclinal cell divisions that give rise to a second layer of cells and a new growth axis for the new developing lateral root primordia. Following the first asymmetric cell divisions, an auxin response maximum forms (due to an oscillation) in the tip of the new lateral root primordia and high levels of auxin are maintained throughout lateral root emergence (Benková et al., 2003).

As stated in the previous section, up to 12 XPP cells can be present within the OZ during an oscillation while typically only one or two cells become respecified into lateral root founder cells. For this to occur, a signal must be preventing too many founder cells from gaining division competence to prevent the initiation of an excess of lateral root primordia. One way this is known to occur is through the auxin-response module *MONOPTEROS/ARF5-BODENLOS/IAA12,* which has been shown to be essential for the development of lateral root primordia with optimal spacing in between them, as disruption of this module results in closely spaced or fused emerged lateral roots (De Smet et al., 2010). Additionally, *LBD16,* an auxin-responsive gene, has been shown to induce expression of *TARGET OF LBD SIXTEEN2 (TOLS2),* an 11 amino acid secreted peptide that is expressed in more mature lateral root founder cells undergoing initiation. *TOLS2* was revealed to bind *RECEPTOR-LIKE KINASE7 (RLK7),* a leucine-rich-repeat receptor like kinase, in adjacent lateral root founder cells (Toyokura et al., 2019). *TOLS2-RLK7* interaction in these cells inhibited founder cell identity and future lateral root initiation by inducing expression of *PUCHI,* a gene encoding a member of the ethylene response factor transcription factor family (Hirota et al., 2007). The mechanism by which *PUCHI* inhibits founder cell identity and lateral root initiation is unknown, however, *puchi* mutants have increased LR density (Hirota et al., 2007, Kang et al., 2013) and an increase in the frequency of founder cells that form in adjacent pairs (Toyokura et al., 2019), indicating an inhibitory role in founder cell priming and initiation.

In summary, there are a multitude of genes that are known to control lateral root initiation and emergence, including many that weren't discussed here. Many of these genes integrate to regulate the signaling processes that ensure proper lateral root initiation and emergence, such as lateral inhibition, which creates the spacing between emerged lateral roots.

NITRILASE 1 (_NIT1_), its role in auxin production, and other possible functions

In 2016, through collaboration with the Steven Clouse Proteomics Group (North Carolina State University), a co-immunoprecipitation of *CEPR1* from *Arabidopsis thaliana* revealed 45 proteins that are predicted to interact with *CEPR1* either directly or within a larger complex (unpublished data). Of these 45 co-immunoprecipitating proteins, 14 are involved in processes related to carbon metabolism, such as sucrose synthesis, gluconeogenesis, carbon-nitrogen balance, or sugar transport, highlighting the potential role *CEPR1* has in regulating carbon-nitrogen balance and sugar transport. Additionally, six proteins may be involved in controlling levels of the plant hormone auxin, which is well-known to play a pivotal role in many stages of lateral root development. However, some of these interactors function at metabolic branch points between auxin and glucosinolate biosynthesis. Understanding the role that auxin plays in the regulation of root growth by *CEPR1* is important because the specific mechanism by which *CEPR1* modulates root growth under variable nitrogen and sugar conditions has remained elusive.

To test whether *CEPR1* regulates auxin production in variable nitrogen conditions, we chose to focus on one of the auxin-related enzymes that co-immunoprecipitated with *CEPR1*, *NITRILASE 1 (NIT1*). *NIT1,* along with *NIT2* and *NIT3,* is a member of the *NIT1*-subfamily of enzymes specific to Brassicaceae*.* These three genes are thought to have emerged from *NIT4,* an ancient enzyme involved in the hydrolysis of organic cyanides that has representatives across all land plants, via gene duplication (Janowitz et al., 2009). The *NIT1, 2,* and *3* genes appear to have undergone neofunctionalization (Janowitz et al., 2009) because their spatial and temporal expression patterns differ (Klepikova et al., 2016), they can be induced differently under specific conditions (Kutz et al., 2002, Klepikova et al., 2016), and they have differing levels of activity against a variety of different substrates that come from glucosinolate catabolism (Vorwerk et al., 2001).

NIT1 was originally identified from an *Arabidopsis thaliana* cDNA expression library and shown to convert indole-3-acetonitrile (IAN) into indole-3-acetic acid (IAA) *in vitro* (Bartling et al., 1992). Later, a genetic screen to isolate mutants that are resistant to the auxin-like effects of IAN revealed that *NIT1* can also catalyze the conversion of IAN to IAA *in vivo* (Normanly et al., 1997). When wild type (Col-0) and *nit1* mutants were treated with 30 µM IAN, dramatic primary root inhibition resulted in the wild-type plants while *nit1* mutants were insensitive to the treatment. This result indicates that *NIT1* can catalyze the conversion of IAN to IAA because IAA overaccumulation inhibits primary root growth. Treatment of wild type and *nit1* mutants with 1 µM IAA resulted in primary root inhibition in both, demonstrating that IAA sensitivity is not altered in *nit1* mutants and that IAN is the required substrate for *NIT1* to make IAA. More recent literature has continued to highlight that *NIT1*, as well as its close relatives *NIT2* and *NIT3,* can synthesize IAA from IAN. Lehmann et al., 2017 demonstrated that free IAA (the signaling form of auxin) is significantly elevated in *NIT1*-overexpression lines while *nit1-3* and *NIT2* RNAi lines, which knock down the entire *NIT1* subfamily of enzymes, had significant reductions in total auxin (free IAA + conjugates).

In the context of root development, mutations in *NIT1* have not been described to cause any phenotype except for when it’s overexpressed. *NIT1* overexpression has been reported to lead to inhibition of primary root growth and formation of more lateral roots (Lehmann et al., 2017) which is consistent with the phenotypes wild-type plants exhibit when treated with exogenous auxin. Additionally, *NIT1* has been shown to be expressed specifically in the region of the primary root maturation zone where lateral root primordia development occurs (Winter et al., 2007, Brady et al., 2007). *NIT1* has also been shown to be highly expressed in a root bending assay used to identify expression of genes that are potentially involved in lateral root initiation and emergence (Winter et al., 2007, Lavenus et al., 2015).

While the research discussed above makes a strong case that *NIT1* does make IAA from IAN and that the amounts of auxin can be perturbed due to mutations in *NIT1*, other pieces of evidence have called into question the primary role for *NIT1* in basal auxin production and point to a possible alternative function. First, as mentioned previously, *NIT1*, *NIT2,* and *NIT3* are specific to Brassicaceae, and their expression can be modulated under various environmental conditions. Glucosinolates, which are sulfur and nitrogen-containing secondary metabolites found mainly in plants of the family Brassicaceae, are often involved in the response to the different growth conditions that induce expression of *NIT1, NIT2, and NIT3*. To elaborate, when tissue is damaged by insect herbivory, pathogen invasion, or mechanical wounding, intact glucosinolates are cleaved by proteins called myrosinases. This cleavage results in the release of different metabolites that can be further metabolized into compounds that are toxic to fungi, bacteria, insects, and other herbivores (Matile, 1980, Chhajed et al., 2019). Many of these metabolites are nitriles, including IAN. Glucobrassicin, or indol-3-ylmethylglucosinolate, is the most abundant glucosinolate in *Arabidopsis thaliana* (Pfalz et al., 2009) and is a precursor of IAN and other nitriles and thio- and isothiocyanates (Janowitz et al., 2009). Interestingly, *NIT1* expression is known to be upregulated by mechanical wounding (Klepikova et al., 2016, Sullivan et al., 2019) or *Plasmodiophora brassicae* infection (Grsic-Rausch et al., 2000). Additionally, *NIT1* assembles into filaments in response to wounding or herbicide-induced cell death (Cutler & Somerville, 2005), making a case for the conformational regulation of *NIT1* activity and pointing to a possible role in glucosinolate metabolism during tissue wounding.

Secondly, the *NIT1, NIT2,* and *NIT3* enzymes all display relatively weak activity on IAN when compared to other nitrile-containing substrates (Vorwerk et al., 2001). This indicates that *NIT1, NIT2,* and *NIT3* may primarily function in detoxifying many different glucosinolate metabolites. However, it is possible that the variable activity against different metabolites could be due to different conformations of the *NIT* proteins. A recent publication has demonstrated the importance of the helical twist in *Capsella rubella NIT* protein filaments in determining substrate specificity due to changes in the size of the substrate binding site (Woodward et al., 2018). Thus, *A. thaliana NIT1* could potentially have modular specificities for different compounds depending on how *NIT1* is being regulated.

Lastly, the possible function of *NIT1* in glucosinolate metabolite detoxification with respect to *CEPR1* is compelling because *CEPR1* interacts with several other proteins that are either known to or could potentially be involved in glucosinolate metabolism. Among these interactors are *CYP83A1 (REF2), CYP79F1 (BUS1), BETA-GLUCOSIDASE 24 (BGLU24), METHIONINE SYNTHASE 2 (MS2),* and *S-ADENOSYLMETHIONINE SYNTHASE (MTO3).* Additionally, glucosinolates have been shown to accumulate in specialized cells that are adjacent to phloem vessels (Koroleva et al., 2000, Koroleva et al., 2010) which is interesting in the context of *CEPR1,* as it is expressed in phloem (Bryan et al., 2012).

Considering the above, it is possible that *CEPR1* regulates *NIT1* to detoxify nitriles during glucosinolate metabolism. However, aside from tissue wounding, multiple different stress conditions have been reported to cause changes in glucosinolate levels, including salinity (Martinez-Ballesta et al., 2015), drought (Ren et al., 2009), sulfur deficiency (Falk et al., 2007, Jeschke et al., 2019), and excess ammonium (Marino et al., 2016). Additionally, experiments performed by Jeschke et al., 2019 revealed that growth on different levels of exogenous sulfur, nitrogen, and carbon all have profound effects on levels of endogenous glucosinolates. In these experiments, *Arabidopsis* plants grown on +S/-N have significantly less glucosinolate accumulation than plants grown on -S/+N conditions. While plants grown on -S/-N have the most substantial reduction in glucosinolate accumulation, the fact that the absence of nitrogen in the presence of sulfur affects glucosinolate accumulation more than the presence of sulfur in the absence of nitrogen clearly indicates that exogenous available nitrogen plays a greater role in *de novo* glucosinolate biosynthesis. However, when grown on ample nitrogen and sulfur, plants accumulate substantially more glucosinolates than the other three conditions. Additionally, the amounts of sucrose supplied to plants in the media also impacted glucosinolate accumulation, as plants grown on 29.2 mM (1%) sucrose had a large reduction in glucosinolates after 13 days when compared to plants grown on media without sucrose. Altogether, these results suggest a role for glucosinolates in regulating metabolism and growth, especially under normal metabolic conditions which lead to higher glucosinolate accumulation.

Since glucosinolates contain nitrogen, sulfur, and glucose, it would be important for their synthesis to be coordinated with the uptake and metabolism of sulfur and nitrogen as well as the production of sucrose from photosynthesis. Considering that the abundance of exogenous sulfur, nitrogen, and carbon can affect the levels of endogenous glucosinolates (Jeschke et al., 2019), there is a case for glucosinolates potentially being used as a way for Brassicaceae members to sequester nitrogen, sulfur, and carbon. Under nutrient limiting conditions, glucosinolates could be metabolized to free up nutrients for use in other processes. This would require recruitment of glucosinolate metabolic proteins like *NIT1* to hydrolyze glucosinolates and detoxify their downstream metabolic products. This would lead to auxin production wherever glucosinolates are being mobilized to and metabolized. Thus, depending on the abundance of nutrients a plant has access to, the activity of *NIT1* would need to be modulated.

Indeed, potential mechanisms of regulation of *NIT1* under various stress conditions have been identified. As discussed previously, *NIT1* mRNA levels can be regulated differently under various conditions. However, aside from transcriptional regulation, it is possible that the activity of *NIT1* is post-translationally regulated by *CEPR1* through phosphorylation. Multiple widescale phosphoproteomic studies have uncovered that *NIT1* can become phosphorylated at multiple sites, most notably S330 (PhosPhAt 4.0, Heazlewood et al., 2008, Durek et al., 2010). Six separate groups identified this site as becoming phosphorylated under drought conditions (Bhaskara et al., 2017, Umezawa et al., 2013), abscisic acid treatment (Umezawa et al., 2013), and irradiation (Roitinger et al., 2015). Additionally, a project investigating the dynamics of the chloroplast phosphoproteome identified phosphorylated S330 on the *NIT1* peptide (Reiland et al., 2009). In a later study comparing the phosphoproteome between wild-type plants and mutants of the chloroplast thylakoid kinase *STATE TRANSITION 8* (*STN8),* the same group also identified phosphorylated S330 from *NIT1* in a *stn8* mutant (Reiland et al., 2011). Gene Ontology analysis for *STN8* (TAIR) suggests that *STN8* plays a role in photosystem II stabilization, indicating a possible role in carbon metabolism.

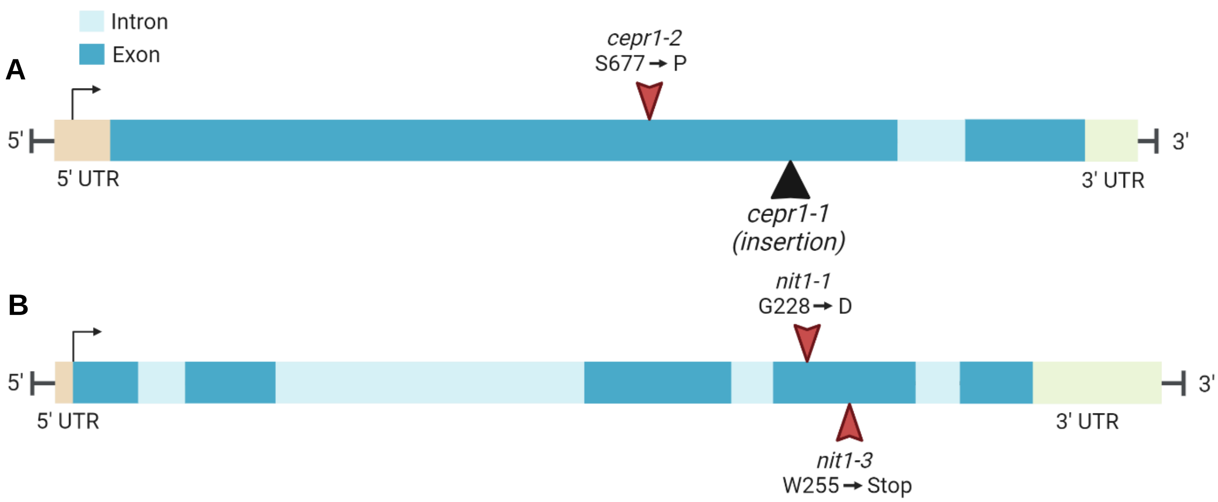
While S330 is the site with the most evidence for becoming phosphorylated under various conditions, *NIT1* peptides with other phosphorylated residues have also been identified. In *A. thaliana* plants exposed to ionizing radiation, *NIT1* peptides with phosphorylated T14 were found (Roitinger et al., 2015), and peptides with three other phosphorylated sites at the N terminus of *NIT1* have also been reported (Al-Momani et al., 2018). In brief, *NIT1* can be phosphorylated in multiple different ways depending on the environmental conditions, indicating that its activity could potentially be modified according to the environment.

Methods

Mutant generation and genotyping

Arabidopsis thaliana nit1-1, nit1-3, cepr1-1, cepr1-2, and *cepr2-2* mutants were published previously (Normanly et al., 1997, Bryan et al., 2012, Tabata et al., 2014, Dimitrov & Tax, 2018). Plants carrying the *R2D2* transgene (Liao et al., 2015) were obtained from the Nottingham Arabidopsis Stock Centre (NASC ID N2105637). TAIL-PCR, as described in Liu & Chen, 2007, was used to determine the location of the *R2D2* transgene to design primers for genotyping. Multiple TAIL-PCR products of various sizes were cleaved, gel purified, sequenced, and aligned to an intragenic region of AT1G03820. Crosses between *nit1-1* or *nit1-3* and *R2D2* were performed, and homozygotes for both the mutants and the transgene were selected in the F2 generation. *Cepr1-1*, *cepr1-2*, and *cepr2-2* were then crossed into *nit1-1;R2D2* independently. Single and double mutants of each combination in the *R2D2* background were selected (*nit1-1;cepr1-1;R2D2, nit1-1;cepr1-2;R2D2* and *cepr1-1;R2D2, cepr1-2;R2D2,* and *cepr2-2;R2D2*) in the F2 or F3 generations.

Since the *nit1-1* and *cepr1-2* alleles are both single base pair changes, PCR products for each allele were amplified with PCR and subjected to a restriction digest to determine the copy number of each mutant allele in the segregating populations. The *nit1-1* mutation creates an *MboII* restriction site while the *cepr1-2* mutation disrupts an *HpaII/MspI* restriction site. The *nit1-3* allele changes an amino acid to a stop codon; however, the mutation does not disrupt or introduce a known restriction enzyme recognition site.



<u>Figure 3. Gene models.</u> Representative gene models of (A) *CEPR1* and (B) *NIT1* showing the location of mutations in each mutant. Red ticks represent point mutations and black ticks represent insertion mutations.

Plants homozygous for the *nit1-3* allele were confirmed with Sanger sequencing (Arizona Genetics Core) and then crossed into *nit1-1;cepr1-2;R2D2*, *nit1-1;cepr1-1;R2D2*, and *nit1-1;cepr2-2;R2D2* homozygous plants. The *nit1-3* allele was confirmed to be present in subsequent generations by analyzing the copy number of the *nit1-1* allele. In the F2 and F3 generations, the presence of the *nit1-3* allele in the mutants was verified using the Derived Cleaved Amplified Polymorphic Sequences (dCAPS) assay, which utilizes a primer with a single base mismatch to introduce a restriction site into either the PCR products of wild-type or mutant amplicons. The *nit1-3* dCAPS mismatch primer was designed to incorporate a BsrI (BseNI) restriction site into wild-type (control) samples but not *nit1-3* samples. After digestion with BseNI, the products were run on a 4% agarose gel at a low voltage (50V) to visualize the slight size separation between mutant and wild-type PCR products.

Primers

Allele	Primer name	Primer sequence
nit1-1	nit1-1F	5'-CTGAAGTAGCAAGATTGGCTG-3'
	nit1-1R	5'-GATGACTATCAAATGAGAGTGT-3'
nit1-3	nit1-3_dCAPS-F	5' CCTGACTACTTGTTTACCGACT-3'
	nit1-3_dCAPS-R	5'- GACGAGACCCTCTGATTCAAAG-3'
R2D2	AT1G03820_F	5'-GATGACTATCAAATGAGAGTGT-3'
	AT1G03820_R	5'-TAACACATACTCGTCGCTGG-3'
	R2D2_RB72	5'-GTCTTCCATTAGCTTGGGCT-3'
cepr1-1	cepr1-1F	5'-CAGCTCACTCAAAGGCGGTAATACG-3'
	cepr1-1R	5'-GTCCGCAACTTTAGGCTGATAG-3'
	DS5-1	5'-GAAACGGTCGGGAAACTAGCTCTAC-3'
cepr1-2	cepr1-2F	5'-CGCATAAGTTTCGACCAAAGG-3'
	cepr1-2R	5'-GGTAACTTATGGGACGCTCTTC-3'
cepr2-2	cepr2-2F	5'-GAGAGTAAAGATAGATCATTTGC-3'
	cepr2-2R	5'-CAGAGCTTGTTTCTCAACG-3'
	pROK2	5'-ATTTTGCCGATTTCGGAAC-3'

Conditional nitrogen media and growth conditions

Mutant and wild-type (*R2D2*) plants were grown on 1% agar plates under six conditions containing different concentrations of nitrogen with and without 1% sucrose- low (3 mM N), normal (30 mM N), and high (60 mM N). To normalize the concentration of all other nutrients to typical ½ MS media (Murashige & Skoog, 1962), modified MS media without nitrogen (BioWORLD) was used. Nitrogen in the form of potassium nitrate and ammonium nitrate was added separately to create the final concentration of nitrogen in ½ MS for the normal nitrogen conditions and the concentration of nitrogen in 1X MS in the high nitrogen conditions. 1 mM of each N source was added to the low nitrogen conditions for a final concentration of 3 mM total nitrogen. Each condition was made in batches of 2 liters, separated into two 1 L flasks, and sucrose was added to one of the liters. The ratio of nitrate:ammonium was maintained at roughly 2:1 for each condition while the amount of potassium chloride was adjusted across each condition to normalize the final concentration of potassium at 30 mM per condition. Ingredients for each condition are listed below:

	Low Nitrogen (3 mM)		**Normal Nitrogen (30 mM)**		**High Nitrogen (60 mM)**	
	w/v (g/L)	Conc.	w/v (g/L)	Conc.	w/v (g/L)	Conc.
NH_4NO_3	0.08 g	1 mM	0.825 g	10.3 mM	0.825 g	10.3 mM
KNO_3	0.10 g	1 mM	0.95 g	9.4 mM	3.03 g	30 mM
KCl	2.16 g	29 mM	1.49 g	20 mM	-	0 mM
NH_4Cl	-	0 mM	-	0 mM	0.53 g	10 mM
Modified MS Salts	0.39 g	0.5X MS	0.39 g	0.5X MS	0.39 g	0.5X MS

Nitrogen source	**Resulting concentration (Low N)**	**Resulting concentration (Normal N)**	**Resulting concentration (High N)**
NO_3^-	2 mM	20.7 mM	40.3 mM
NH_4^+	1 mM	10.3 mM	20.3 mM

1 g/L of MES hydrate was added as a buffer, and the pH of each solution was adjusted to 5.7 ± 0.1 with 5M KOH. 10 g/L (1%) agar (Thermo-Fisher) was added to each solution, media was autoclaved, and 50 mL was poured into square petri dishes (100mm x 100mm, Fisherbrand).

For plating, seeds were surface sterilized for 15 minutes in 500 uL of a solution containing 70% ethanol and 0.01% Triton-X, and then rinsed three times in 95% ethanol. Plates were placed upright in a Conviron growth chamber with a 16h/8hr light/dark cycle at 22°C.

Lateral root density analysis

To calculate the estimated lateral root density of mutant and wild-type plants grown on variable nitrogen conditions, plants grown for 8 days were image-scanned on plates, wet-mounted on slides, and visualized using a Zeiss Axioplan epifluorescence microscope with a 20X objective. Since the *R2D2* cassette is under the control of the *RPS5a* promoter, *mDII-tdTomato* signal can be visualized in proliferating cells of the primary root, including un-emerged lateral root primordia at all stages, making *R2D2* a valuable tool for thorough analysis of lateral root density. For visualization of un-emerged lateral root primordia, *tdTomato* was excited at 554 nm and detected at 581 nm and un-emerged lateral root primordia and emerged lateral roots were counted. The sums of emerged and un-emerged lateral root primordia were then divided by the length of the primary roots. The lateral root density data across experiments were combined and the density phenotypes of each genotype were compared to wild-type *(R2D2)* phenotypes.

Primary and lateral root length analysis

To calculate lateral root length, mutant and wild-type plants grown under the conditions above were imaged on agar plates with an EPSON Perfection 2400 photo scanner at 1000 dpi. ImageJ was used to measure the primary and lateral root lengths of plants growing on each condition after 10 days. For all genotypes, lateral roots less than 0.1 cm in length were not measured or included in the data. If no emerged lateral roots were seen in a sample, the root length was scored as zero and included in the data. For statistical analysis, we performed a Kruskal-Wallis test to compare the overall root growth of each genotype across all three nitrogen conditions to wild type. To evaluate sensitivity of lateral root growth of each genotype to the different nitrogen conditions, we performed a Friedman test to assess significance between the lateral root growth of each individual genotype across the three nitrogen conditions tested.

Confocal imaging and quantification of *R2D2*

Wild-type and mutant plants were grown vertically under lights on normal (30 mM) nitrogen conditions with 1% sucrose for 8 days. Z-stacks (1.8 µm slices) of primary root tips were imaged in 8-bit format at 1024x1024 resolution on a Zeiss LSM880 NLO Upright confocal microscope with a Plan-Apochromat 20x/0.8 NA objective. *DII-Venus* was excited at 488 nm at 11% power and detected at 493-556 nm. *mDII-tdTomato* was excited at 561 nm at 4% power and detected at 566-691 nm. Between 6-9 samples were imaged per genotype/condition and experiments were duplicated. All samples were unstained and mounted on glass slides in distilled water. The same microscope settings were used to image all samples.

To quantify the fluorescence intensity of *DII-Venus* and *mDII-tdTomato*, maximum intensity projections of three images from confocal Z-stacks were generated using ZEN lite (Zeiss). Fiji (Schindelin et al., 2012) was used to quantify the fluorescence intensity of *mDII-tdTomato* and *DII-Venus* in the first nine consecutive cells of the cortex on both the right and left sides of the root. The fluorescence intensities (gray values) of *mDII-tdTomato* and *DII-Venus* in all 18 nuclei were divided and an average was taken. Mean values were min/max normalized.

Results

***CEPR1* and *NIT1* interact to control the rate of lateral root initiation**

To test whether *NIT1* plays similar roles in lateral root development as *CEPR1*, two mutants of each were chosen for phenotypic analysis for growth on varying levels of nitrogen and carbon. The *cepr1-1* mutation is an insertion and null mutation in the kinase domain of *CEPR1* (Bryan et al., 2012, Tabata et al., 2014, Dimitrov & Tax, 2018) and mutants are characterized by having an increased emerged lateral root density when compared to wild-type plants (Dimitrov & Tax, 2018). The *cepr1-2* mutation has also been characterized and contains an amino acid substitution in the kinase domain (Bryan et al., 2012, Roberts et al., 2016). *CEPR1* transcripts have been recovered from *cepr1-2* mutants (Bryan et al., 2012) and in contrast to *cepr1-1*, *cepr1-2* mutants have a reported lateral root density phenotype that is lower than wild type (Roberts et al., 2016), suggesting that the *cepr1-2* allele could be a gain-of-function mutation.

The *nit1-3* mutation encodes a nonsense mutation and *nit1-3* plants do not produce a recoverable protein product (Cutler & Somerville, 2005). Additionally, *nit1-3* mutants are unable to synthesize auxin from IAN via *NIT1* (Normanly et al., 1997). Similarly, the *nit1-1* mutant cannot synthesize auxin through *NIT1* but likely still makes a transcript. No notable lateral root phenotype is associated with *nit1* mutants under normal conditions besides their insensitivity to IAN (Normanly et al., 1997).

To test if *NIT1* is required to initiate lateral roots in response to different concentrations of nitrogen, *nit1 and cepr1* single mutants were grown on increasing levels of nitrogen. Using the internal control signal of *R2D2 (mDII-tdTomato)* as a marker for lateral root primordia, the sum of unemerged and emerged lateral roots from wild-type *R2D2* plants and mutants in the *R2D2* background was determined and divided by their respective primary root lengths. Under these growth conditions with 1% sucrose, wild-type *R2D2, nit1-1,* and *nit1-3* mutants were generally insensitive to changes in the amount of nitrogen, except for *nit1-3* mutants that were grown on high nitrogen, which had a reduced lateral root density when compared to wild type ($P<.001$) (Fig. 4A, 4C). In contrast, *cepr1-1* mutants had a significantly higher lateral root density than wild-type plants under all nitrogen conditions (P=.001 in low nitrogen, $P<.001$ in normal and high nitrogen), and the density increased with higher concentrations.

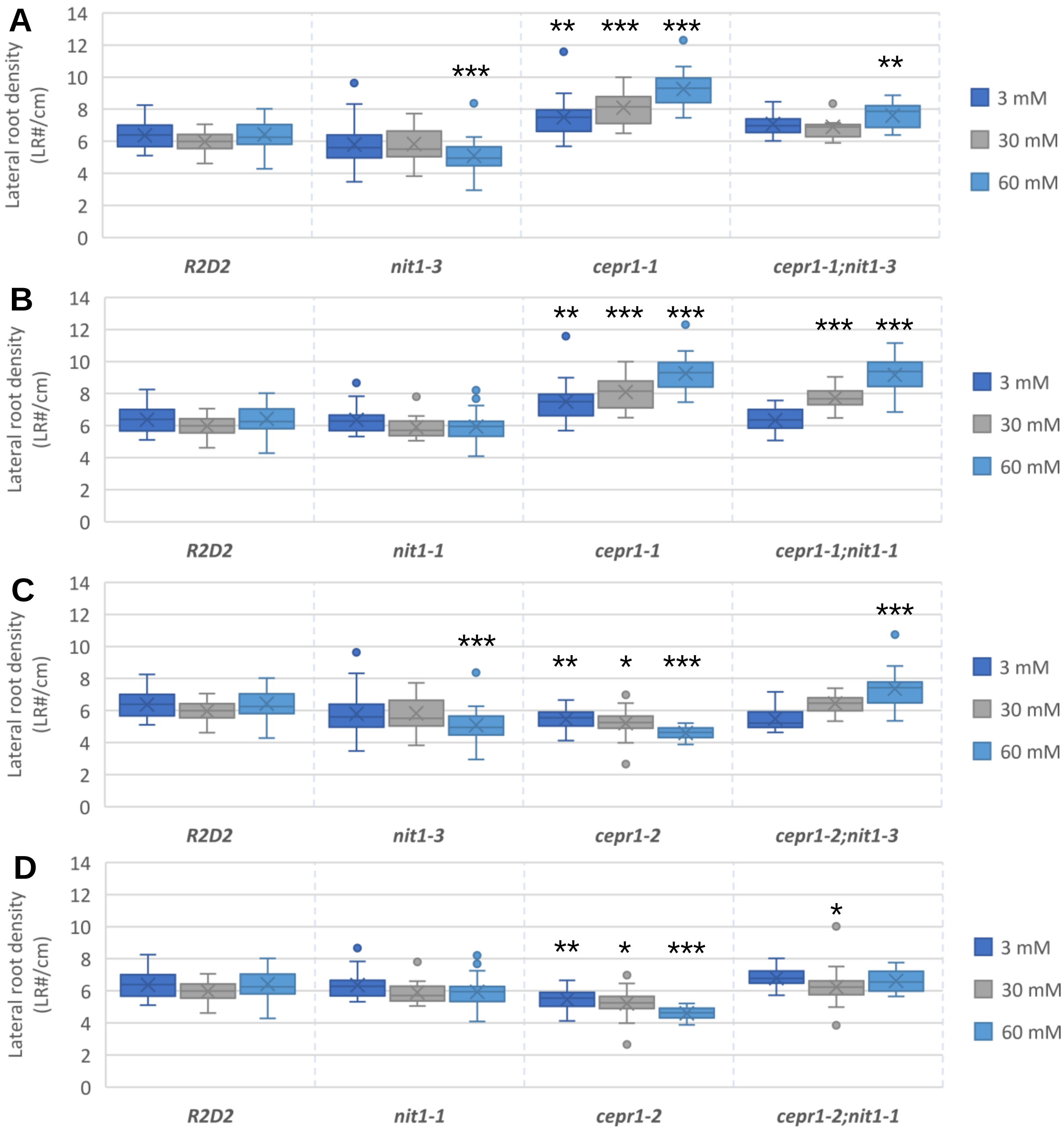


Figure 4. *NIT1* is required for *CEPR1* to inhibit LR development with increasing nitrogen. Comparison of the average lateral root density between wildtype (*R2D2*) and *nit1-3* (A), or *nit1-1* (B) *cepr1-1,* and double mutants. (C-D) Comparison of the average lateral root density of wildtype and *nit1-3* (C) or *nit1-1* (D), *cepr1-2*, and double mutants. Significance was determined by two-way ANOVA and Bonferroni post hoc analysis. Asterisks indicate level of significance when compared to the wild type under same condition. * *P*<.05, ** *P*<.01, *** *P*<.001. n=15-31 for each genotype.

One possible interpretation of the immunoprecipitation result is that *CEPR1* binds and phosphorylates *NIT1*, regulating its activity. To test if *NIT1* is required for *CEPR1* to initiate more lateral roots under different amounts of nitrogen, we examined roots from a *nit1-3;cepr1-1* double mutant. Our results show that the *nit1-3* mutation partially suppresses the enhanced sensitivity of *cepr1-1* to increasing levels of nitrogen (Fig. 4A). Under the same conditions, *nit1-1* mutants were insensitive to increasing nitrogen, similar to wild-type controls. However, in contrast to *nit1-3* suppressing the sensitivity of *cepr1-1* to increasing nitrogen, *nit1-1;cepr1-1* mutants remain sensitive to increasing nitrogen concentrations and have similar densities when compared to *cepr1-1* single mutants, except for in low nitrogen (Fig 4B).

The *nit1-1* and *nit1-3* mutations were also tested in combination with the *cepr1-2* allele (Fig. 4C, 4D). Under our conditions, *cepr1-2* mutants had significantly lower lateral root densities in high nitrogen (*P*<.001), and slightly lower densities in low and normal nitrogen, similar to what has been reported previously (Roberts et al., 2016). The *cepr1-2* mutant plants were also sensitive to increasing nitrogen, however, unlike the *cepr1-1* mutants which have higher lateral root densities with more abundant nitrogen, the lateral root density of the *cepr1-2* plants decreases with increasing nitrogen. Although *cepr1-2* has an opposite phenotype to *cepr1-1, nit1-3* was able to suppress the sensitivity of *cepr1-2* to increasing nitrogen, however, the density of *cepr1-2;nit1-3* increases with the concentration of nitrogen (Fig. 4C). In contrast, *nit1-1* was able to suppress the *cepr1-2* phenotype, where the lateral root densities are more comparable to wild type.

***NIT1* inhibits LR extension**

Our results show that mutations in *NIT1* can suppress the lateral root initiation phenotypes of *cepr1* mutations. To test if *NIT1* is also required for lateral root extension, we grew mutants on variable nitrogen conditions for 10 days and evaluated their total lateral root growth. The lengths of all lateral roots were measured for each plant and the average sum of lateral root lengths for each genotype was calculated. We observed that wild-type plants exhibited an expected sensitive foraging response where total lateral root growth was significantly different across the three nitrogen conditions tested (*P*<.001, by Friedman test) and the most dramatic growth was observed in low nitrogen (3 mM) but became restricted as nitrogen levels increase (Fig. 5A). A similar response was seen in *cepr1-1*; however, these mutants had reduced sensitivity to different levels of nitrogen (Fig. 5A). Additionally, *cepr1-1* mutants had significantly longer lateral roots than wild type in normal and high nitrogen (*P*=.008 and *P*<.001, respectively).

Under all the conditions, *nit1-3* mutants also had significantly more lateral root growth than wild type (*P*=.001 for low nitrogen, *P*<.001 for normal nitrogen and high nitrogen)(Fig. 5A), suggesting a general role for *NIT1* in restricting lateral root extension. The *nit1-3* mutation had only a minor effect on the overall *cepr1-1* lateral root growth phenotype on different conditions, but root growth was slightly suppressed in the *cepr1-1;nit1-3* double mutant on low nitrogen when compared to *cepr1-1* single mutants (*P*=.022). The *nit1-3* allele did have an influence on the insensitive phenotype of *cepr1-1* mutants, and sensitivity to the different conditions was restored in the *cepr1-1;nit1-3* double mutant (*P*=.035) due to the reduced growth in low nitrogen when compared to *cepr1-1* single mutants.

Under our variable nitrogen conditions, *cepr1-2* mutants were sensitive to the different conditions (*P*<.001) and had significantly shorter lateral roots than wild type in low nitrogen (*P*=.005) but had similar lateral root growth phenotypes in normal and high nitrogen. The *cepr1-2;nit1-3* growth phenotype in low nitrogen more closely resembled the phenotype of *nit1-3* plants, indicating that *nit1-3* can suppress the gain-of-function phenotype of *cepr1-2* plants, and suggesting a role for *NIT1* in suppressing lateral root extension, especially in nitrogen-stress conditions like low nitrogen.

We also evaluated the lateral root lengths of *cepr2-2* and *cepr2-2;nit1-3* mutants. In general, *cepr2-2* single mutants had a similar phenotype as wild-type plants across our conditions and were sensitive to the different nitrogen conditions (*P*=.005). The *nit1-3* mutation slightly enhanced lateral root growth in the *cepr2-2* background across our conditions, and the root growth phenotype of the *cepr2-2;nit1-3* double mutants was more like the growth phenotypes of *nit1-3* single mutants, suggesting that *CEPR2* and *NIT1* do not interact to regulate lateral root growth.

Lastly, we quantified the root growth of *nit1-1* single and double mutants with *cepr1-1* (Fig. 5B). In general, the *nit1-1* mutation had a less dramatic impact on lateral root growth than the *nit1-3* mutation. Across our different nitrogen conditions, lateral root growth of *nit1-1* single mutants did not significantly differ from wild type, and the sensitivity to the different conditions was also similar to wild type. Interestingly, in the *cepr1-1;nit1-1* double mutant, the *nit1-1* mutation suppressed the *cepr1-1* lateral root growth phenotype almost identically to *nit1-3*, and root growth in normal and high nitrogen was significantly higher than wild type (*P*<.001 for both conditions). The sensitivity of *cepr1-1;nit1-1* to different levels of nitrogen was also similar to *cepr1-1;nit1-3.* Additionally, like the *cepr1-1;nit1*-3 plants, the *cepr1-1;nit1-1* double mutants had suppressed growth in low nitrogen when compared to *cepr1-1* (*P*=.039). Overall, our results with *nit1-1* and *nit1-3* are consistent with *CEPR1* acting upstream of *NIT1* and suggest a role for *NIT1* in inducing a foraging response in low nitrogen conditions.

When evaluating the *cepr2-2;nit1-1* mutant, we observed a somewhat similar growth response to the different nitrogen conditions as *cepr2-2;nit1-3*, however lateral root growth under normal nitrogen was lower in *cepr2-2;nit1-1* and more similar to wild type*.* In general, the *nit1-1* allele did not significantly enhance overall lateral root growth beyond what was observed in *cepr2-2* single mutants or wild-type plants. This result again suggests that *NIT1* and *CEPR2* do not interact to regulate lateral root growth, at least in our conditions with excess sucrose.

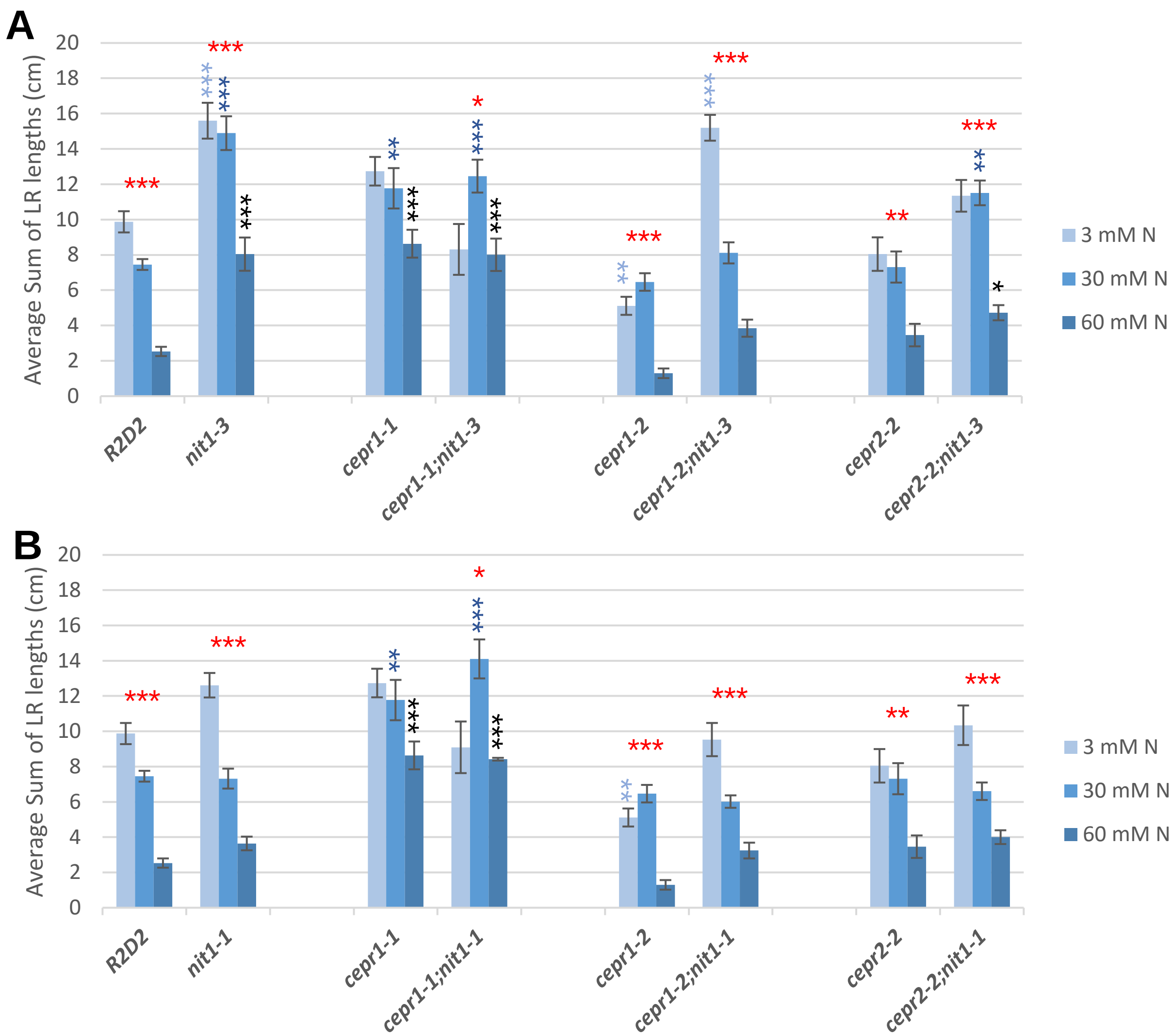


Figure 5. *Nit1* mutations suppress *cepr1-1* and *cepr1-2* lateral root growth phenotypes

(A) Average sum of lateral root (LR) lengths of *nit1-3* single mutants and double mutants in *cepr1-1*, *cepr1-2, and cepr2-2* backgrounds. (B) Average sum of LR lengths of *nit1-1* single mutants and double mutants in *cepr1* and *cepr2* backgrounds. The significance between the lateral root growth of each genotype on each individual condition when compared to wild type or other genotypes was assessed by the Kruskal-Wallis test and a Dunn-Bonferroni post hoc test. Significance between the growth of individual genotypes across each nitrogen condition, to evaluate the sensitivity of each genotype to different conditions (red asterisks), was determined by a Friedman test. Error bars represent SE. n=10-18 samples per genotype, per condition. * *P*<.05, ** *P*<.01, *** *P*≤.001.

Sucrose enhances lateral root growth and is required for a sensitive response to different nitrogen conditions in *cepr1-2* and *nit1-3* mutants

Since *cepr1-1* mutants have been previously shown to require sucrose to enhance the growth of lateral roots (Dimitrov & Tax, 2018, Chapman et al., 2019), we chose to evaluate total lateral root growth in different levels of nitrogen but in the absence of sucrose to investigate the role that *CEPR1* and *NIT1* might have in responding to sucrose (Fig. 6). In general, all genotypes including wild type had greatly reduced and more variable root growth in the absence of excess sucrose, and only a few of the genotypes we tested significantly differed from wild type on certain levels of nitrogen. However, we observed a difference in the sensitivity of wild-type plants and several mutants to different nitrogen conditions, where growth was enhanced in normal nitrogen more than in low and high nitrogen. There was still a significant difference between the lateral root growth of wild-type plants across the different conditions (P=.001), indicating that they remain sensitive to different nitrogen levels in the absence of sucrose, but that excess sucrose is at least required for a linear sensitive response to increasing levels of nitrogen. We observed the same response to different conditions in *nit1-3* mutants, which also had longer lateral roots in normal nitrogen than in low and high nitrogen (Fig. 6A), suggesting that in the absence of excess sucrose these genotypes preferentially optimize lateral root growth in normal nitrogen conditions and restrict growth when nitrogen is limited or when there is a large surplus.

The *cepr1-1* mutants had lateral roots that were a similar length to wild-type plants, but these mutants were insensitive to different levels of nitrogen, similarly to when grown on the same conditions but with excess sucrose (Fig. 5). This suggests a role for *CEPR1* in orchestrating a sensitive root growth response to different levels of nitrogen, but not necessarily in regulating overall root growth. In the *cepr1-1* background, *nit1-3* slightly suppressed the insensitive phenotype of *cepr1-1* across the conditions by enhancing growth in normal nitrogen, but overall,

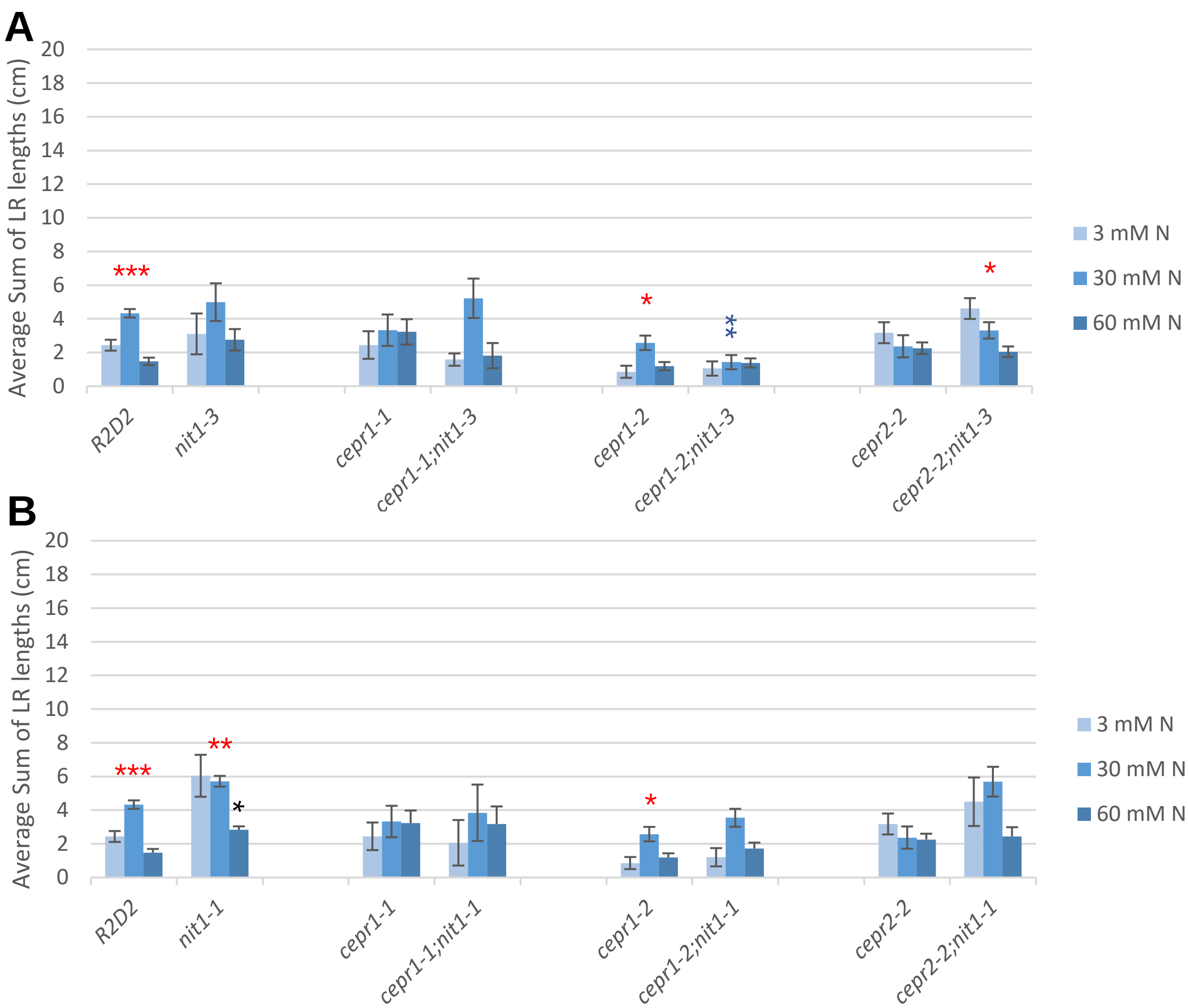


Figure 6. *Cepr1-1 and nit1-3* mutants are insensitive to changes in nitrogen concentration in the absence of sucrose

(A) Average sum of lateral root (LR) lengths of *nit1-3* single mutants and double mutants in *cepr1-1*, *cepr1-2, and cepr2-2* backgrounds. (B) Average sum of LR lengths of *nit1-1* single mutants and double mutants in *cepr1* and *cepr2* backgrounds. The significance between the lateral root growth of each genotype on each individual condition when compared to wild type or other genotypes was assessed by the Kruskal-Wallis test and a Dunn-Bonferroni post hoc test. Significance between the growth of individual genotypes across each nitrogen condition, to evaluate the sensitivity of each genotype to different conditions (red asterisks), was determined by a Friedman test. Error bars represent SE. n=10-18 samples per genotype, per condition. * *P*<.05, ** *P*<.01, *** *P*≤.001.

the difference in growth of *cepr1-1;nit1-3* across the three conditions was not statistically significant, and there were no significant differences between the growth of *cepr1-1* and *cepr1-1;nit1-3.* This result is again consistent with *CEPR1* acting upstream of *NIT1,* because we would expect that non-functional *CEPR1* would be unable to regulate a functional version of *NIT1.*

We also evaluated the lateral root growth of *cepr1-2* mutants in the absence of sucrose, which had slightly but not significantly reduced lateral root growth than wild type. The *cepr1-2* mutants were sensitive to different nitrogen conditions (*P*=.012) and had very suppressed growth in low and high nitrogen and slightly enhanced growth in normal nitrogen. Interestingly, and unlike in excess sucrose conditions, the *nit1-3* mutation in the *cepr1*-2 background prevented enhancement of lateral root growth in normal nitrogen (which we observed in wild type, *nit1-3*, *cepr1-1;nit1-3*, and *cepr1-2*) and completely abolished sensitivity to the different conditions.

When evaluating *cepr2-2* mutants, we observed that they exhibited a similarly insensitive lateral root growth phenotype as *cepr1-1* mutants across the different nitrogen conditions. The *nit1-3* mutation in the *cepr2-2* background restored sensitivity (*P*=.011) and these double mutants exhibited an expected linear growth response to increasing nitrogen that was observed in wild-type plants growing in excess sucrose (Fig 5), but not in the absence of sucrose (Fig 6). Overall, growth did not differ significantly between *cepr2-2;nit1-3* and *cepr2-2* or *nit1-3* under any of the conditions tested.

Finally, we quantified the total lateral root growth of *nit1-1* single and double mutants (Fig 6B). The *nit1-1* mutants had significantly more lateral root growth than wild-type plants in high nitrogen (*P*=.01) and were sensitive to different levels of nitrogen (*P*=.004). In the *cepr1-1* and *cepr1-2* backgrounds, *nit1-1* had no effect on growth when compared to wild type or to the respective *cepr1* single mutant. However, both *cepr1-2* and *cepr1-2;nit1-1* had shorter roots than *nit1-1* single mutants in low nitrogen (*P*=.013 and *P*=.006, respectively) and normal nitrogen (*P*=.001 and *P*=.022, respectively), suggesting that the *cepr1-2* protein's regulatory activity can be influenced by the levels of sucrose available to the plant, as both *cepr1-2* and *nit1-1* both likely generate functional protein products. We also evaluated root growth of *cepr2-2;nit1-1* and interestingly, the *nit1-1* allele slightly enhanced root growth in the *cepr2-2* background in normal nitrogen (*P*=.009), but the *nit1-1* single mutant, when compared to *cepr2-2,* also had significantly more root growth in normal nitrogen (*P*=.001), and there was no significance between the growth of *cepr2-2;nit1-1* and *nit1-1* under the same condition, again suggesting that *CEPR2* and *NIT1* do not interact to regulate root growth. It is likely that the *cepr2-2* allele does not affect lateral root extension, and differences in growth can be attributed to the *nit1-1* allele in the *cepr2-2* background.

Cepr1-2 **mutants make more auxin through** ***NIT1***

Our genetic interaction data indicate that *CEPR1* acts on *NIT1* to regulate lateral root initiation and extension. To test whether this is because of their effect on auxin levels in the roots, we used the auxin reporter, *R2D2* (Liao et al., 2015)*,* to measure the relative abundance of auxin in the primary roots of wild type, *nit1-3, cepr1* mutants, and double mutants grown normal nitrogen (30 mM). Surprisingly, nearly every mutant observed had similar levels of auxin when compared to wild-type controls. However, *cepr1-2* mutants had significantly higher levels of auxin when compared to wild type (*P*<.001)(Fig. 7G). We observed that *nit1-3;cepr1-2* mutants had similar auxin levels as wild type, consistent with the hypothesis that *cepr1-2* mutants make more auxin through *NIT1.*

The high variability in the auxin levels of *cepr1-2* can be explained due to variation in the intensity of fluorescence of *mDII-tdTomato*. Since auxin mediates the degradation of *DII*, mutant roots expressing *R2D2* that have higher levels of auxin will have a reduced or completely degraded *DII-Venus* signal. Because the intensity of *DII-Venus* was so low in *cepr1-2* samples due to elevated auxin levels, even slight differences in the intensity of *mDII-tdTomato* resulted in very different ratios of *mDII/DII* intensity. Despite this caveat, a majority of the *cepr1-2* samples

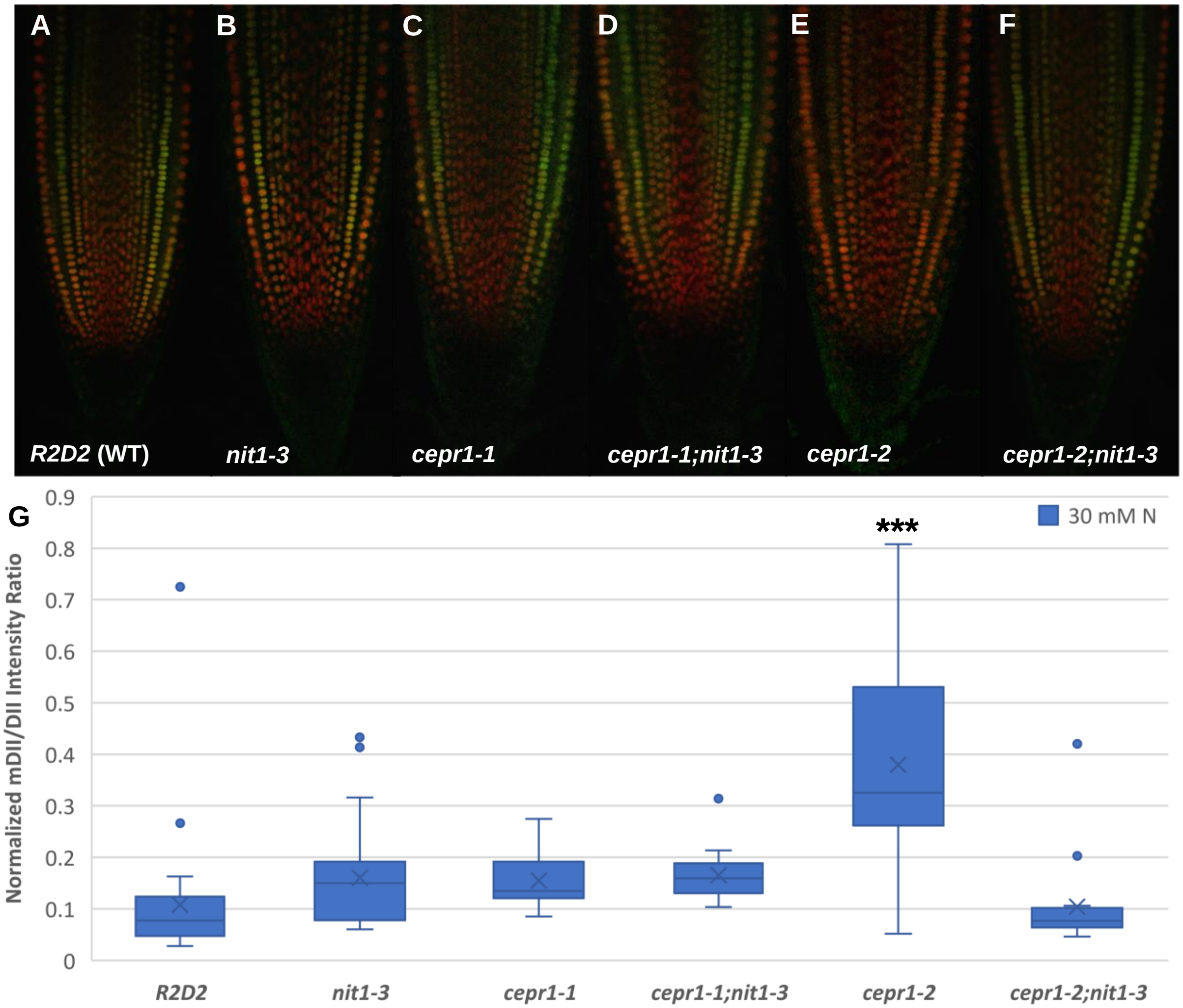


Figure 7. ***CEPR1* modulates auxin production via *NIT1*.** Maximum intensity projections of three optical slices from Z-stacks of primary roots of (A) wild-type *R2D2*, (B) *nit1-3,* (C) *cepr1-1,* (D) *cepr1-1;nit1-3,* (E) *cepr1-2,* and (F) *cepr1-2;nit1-3* mutants in the *R2D2* background. (G) Comparison of relative auxin levels in different mutants in the *R2D2* reporter background grown on 30 mM nitrogen reveals similar levels of auxin except for in *cepr1-2,* which has significantly higher levels. In the *cepr1-2;nit1-3* double mutant, auxin levels are at the same level as in wild type. Significance between the mDII/DII ratio of wild-type *R2D2* and mutants was determined by independent *T*-test. * $P<.05$, ** $P<.01$, *** $P\leq.001$. n=14-33 for each sample. Experiments were performed twice and the data for each genotype across experiments was combined.

observed had a diminished *DII-Venus* signal, suggesting that these mutants generally overproduce auxin.

Mutations in *NIT1* suppress *CEPR1* infertility

Because *nit1* mutations can suppress the root phenotypes of *CEPR1* mutants, we tested if *nit1* mutations could also suppress other lateral organ phenotypes associated with *CEPR1*. To test if the *nit1-1* and *nit1-3* alleles can suppress the infertility phenotype we observe in our *cepr1-1* mutant, we measured the average silique lengths of wild-type and mutant plants when the silique closest to the rosette (the first silique to develop) began to lose green coloration.

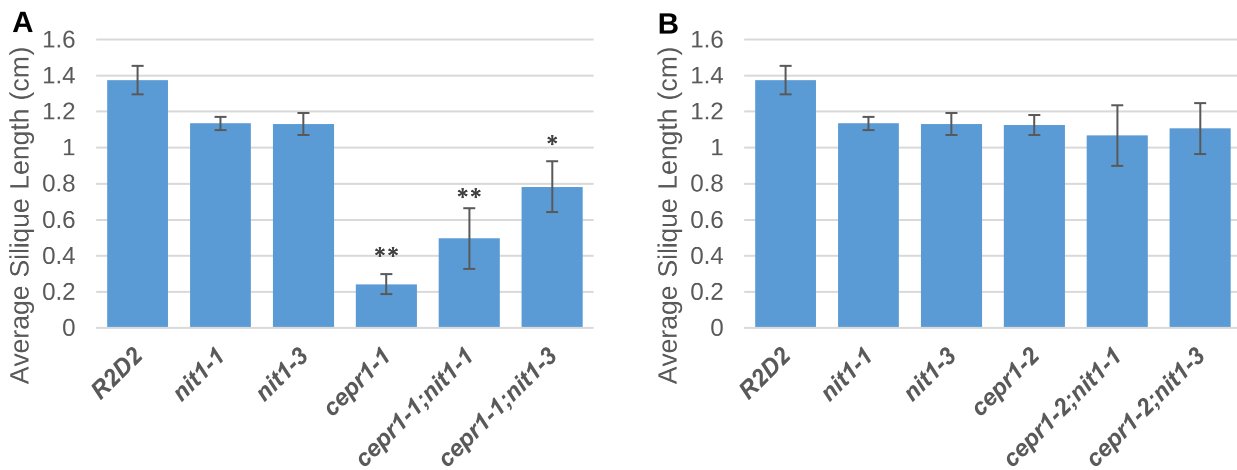


Figure 8. Both mutant *nit1* alleles suppress *cepr1-1* infertility.

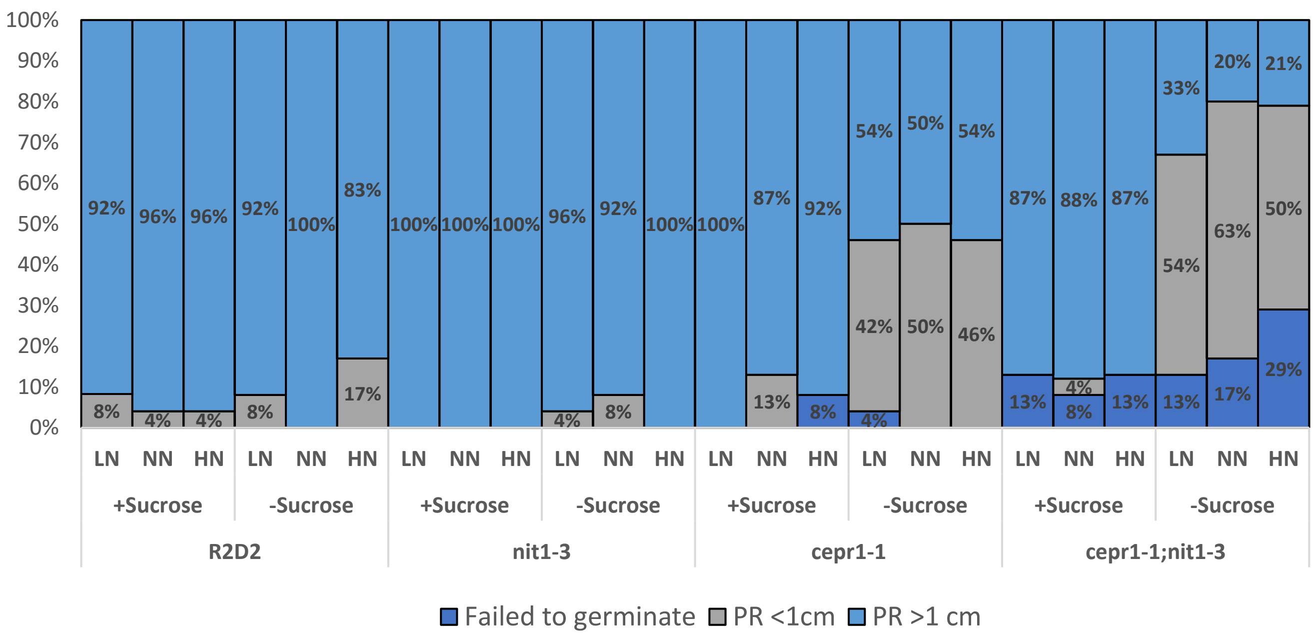
(A) Average silique length of *nit1-1* and *nit1-3* mutants in the *cepr1-1* background. $P<.001$ by Kruskal-Wallis test. A Dunn-Bonferroni test was performed for post hoc analysis to determine significance between the mean lengths of wild-type and mutant siliques. Asterisks indicate level of significance when compared to wild type, * $P<.01$, ** $P<.001$. Error bars represent SD.

(B) Average silique length of *nit1-1* and *nit1-3* mutants in the *cepr1-2* background.

We observed that *nit1-1* and *nit1-3* both have slightly shorter siliques than wild type, while *cepr1-1* mutants have siliques that are approximately 7-fold shorter than wild type ($P<.001$)(Fig. 8A. Interestingly, both *nit1-1* and *nit1-3* alleles suppress this phenotype in the *cepr1-1* background, with *nit1-3* having a more dramatic effect. In the *cepr1-2* background, siliques were approximately the same length as *nit1-1* and *nit1-3* single mutant siliques, and both *nit1* alleles had no significant effect in the *cepr1-2* background.

Double mutants of *cepr1-1;nit1-3* require sucrose for primary root elongation post-germination

Our results with *cepr1* and *nit1* single and double mutants suggest that nitrogen and carbon in the media are both important for lateral root growth. In our phenotypic evaluation of root growth, we noticed that in the absence of sucrose, *cepr1-1* and *cepr1-1;nit1-3* plants had germination and primary root extension phenotypes. We first observed that nearly half of *cepr1-1* and a majority of *nit1-3;cepr1-1* mutants failed to extend primary roots only when grown on media without sucrose. To quantify this, wild type, *nit1-3*, *cepr1-1*, and *nit1-3;cepr1-1* mutants were grown for 8 days on low (3 mM), normal (30 mM), and high (60 mM) nitrogen conditions with and without 1% sucrose, and their growth was evaluated.



<u>Figure 9. The majority of *nit1-3;cepr1-1* fail to extend primary roots or germinate.</u>

The proportion of plants of the indicated genotype grown on different nitrogen conditions with and without sucrose that extended their roots beyond 1 cm, failed to extend their roots beyond 1 cm, or failed to germinate (n=23-25 for each genotype/condition).

All wild-type and *nit1-3* mutant seeds germinated and had wild-type levels of primary root extension. As expected, a majority of *cepr1-1* mutants (87-100%, depending on the condition) extended their primary roots beyond 1 cm when grown on 1% sucrose, however, 42-50% failed to do so in the absence of sucrose, hinting at a potential role of *CEPR1* in sucrose perception and early root development. Interestingly, this growth inhibition phenotype was slightly enhanced in *nit1-3;cepr1-1*, of which 77-87% extended their primary roots when grown on 1% sucrose but only 20-33% did in the absence of sucrose. Additionally, more *nit1-3;cepr1-1* plants failed to germinate than wild type or other mutants, but these germination rates were similar in 1% sucrose (87-92%) and no sucrose conditions except for in high nitrogen without sucrose, where only 71% germinated.

Discussion

Overall, our results provide evidence that is consistent with a model that *CEPR1* regulates *NIT1* during development, including during two independent stages of lateral root development, and in the growth of siliques. We also provide evidence that this occurs by modulating auxin production.

***CEPR1* can suppress LR initiation through *NIT1* in a nitrogen-dependent manner**

We hypothesize that *CEPR1* coordinates with and negatively regulates *NIT1* to maintain a steady rate of lateral root initiation in homogeneous nitrogen conditions. In the *cepr1-1* plants, we observed a higher lateral root density that increased with higher levels of nitrogen, and this effect was suppressed in the *cepr1-1;nit1-3* double null mutant (Fig. 4A). Our hypothesis is based on the evidence that both mutations are nulls: *cepr1-1* does not produce detectable full-length mRNA (Bryan et al., 2012) and *nit1*-3 encodes a nonsense mutation and does not produce a detectable protein (Cutler & Somerville, 2005).

Although we only saw this suppression in *cepr1-1;nit1-3* and not *cepr1-1;nit1-1,* there are a few explanations for why we observe this. It is possible that unlike the *nit1-3* allele, the *nit1-1* missense mutant still produces a protein product, which could possibly preferentially make other metabolites, such as indole-3-acetamide (IAM), that can be converted into IAA by other proteins. IAM is a precursor of IAA and has been reported to be an additional reaction product of *NIT1* (Pollmann et al., 2002). As such, the *nit1-1* protein product could be capable of directly and indirectly contributing to auxin production but could still remain under regulation by *CEPR1.* In a *cepr1-1* mutant background, inhibition would be lifted, and the *nit1-1* protein product could indirectly contribute to auxin production possibly by converting IAN to IAM. Further investigation into this would be required to draw conclusions about the indirect effects of the *nit1-1* protein product on auxin production, such as *in-vitro* experiments to test if purified *nit1-1* protein can convert IAM (instead of IAN) to IAA.

Another possibility is that since the *nit1-1* allele encodes a full-length protein, it could still form filamentous heterocomplexes with other *NIT* proteins, such as *NIT2* and *NIT3.* As discussed previously, *NIT1* has been shown to assemble into filaments (Cutler & Somerville, 2005) and it has been reported that *NIT1* can form complexes with *NIT2 in-vivo* (Doskocliva et al., 2013). Protein filamentation or protein complex assemblies are typically associated with regulation of enzyme activity. In the case of *NIT1*, assembly into filaments, as well as single amino acid mutations, have been reported to alter substrate specificity and enzyme activity (Woodward et al., 2018, Mulelu et al., 2019). If *nit1-1* is still capable of making certain metabolites besides IAA and *CEPR1* can inhibit its activity but it can still form filaments with other *NIT* proteins, it could be possible that these interactions upregulate the activity of other *NIT* proteins, which can also make IAA from IAN (Normanly et al., 1997).

An alternative possibility is that other metabolites besides auxin are affecting root development, such as glucosinolates or their downstream catabolic products that are formed in part due to *NIT1* function. In the absence of both a functional *CEPR1* and *NIT1*, it’s conceivable that many of the downstream glucosinolate catabolites that would not be detoxified or inactivated could thus influence growth.

Overall, while we do see differences in the lateral root densities of *nit1* mutants in the *cepr1* background when compared to single *cepr1* mutants, it’s possible that *NIT1* does not have a major role in lateral root initiation, as evidenced by the lack of a dramatic phenotype in *nit1* mutants, but mutations in *NIT1* could possibly still perturb auxin homeostasis in the root in a *cepr1* background, leading to suppression of the *cepr1* phenotypes in double mutants.

CEPR1 and _NIT1_ together regulate lateral root extension and sensitivity to nitrogen levels

Aside from having a clear effect on lateral root initiation, our results indicate that *NIT1* and *CEPR1* are also involved in regulating lateral root extension. We suspect that the foraging response (inhibition of growth under higher nitrogen conditions) we observed in wild-type plants (Fig. 5A) is due to different levels of auxin in lateral roots, because *DR5* expression has been reported to be higher in lateral roots exposed to higher levels of nitrogen when compared to low nitrogen (Mounier et al., 2014) and we expect that auxin can inhibit lateral root extension similarly to how it inhibits primary root extension. The *nit1-3* null mutant had significantly more lateral root growth than wild-type plants across all three conditions (P=.001 in low nitrogen, P<.001 in normal and high nitrogen) and *cepr1-1* null mutants had significantly more lateral root growth in normal (P=.008) and high nitrogen (P<.001), which is consistent with what we would expect if there was less auxin present in the lateral roots. This is also consistent with *CEPR1* acting upstream of *NIT1* and suggests a role for *CEPR1* in inhibiting lateral root extension through *NIT1*. However, the *nit1-1* mutants did not have significantly longer lateral roots than wild type in any of the conditions, but this could be due to the ability of the *nit1-1* protein to indirectly affect auxin levels, as discussed previously. The *nit1-1* allele did, however, suppress the *cepr1-1* lateral root growth phenotype. This is also consistent with *CEPR1* acting upstream of *NIT1,* because whether the *nit1-1* protein is functional or not, *CEPR1* is not functional in the *cepr1-1;nit1-1* double mutant and thus would not be able to regulate *nit1-1.*

We also observed a suppressed lateral root growth response (a suppressed foraging response) in *cepr1-1* mutants (Fig. 5A), which did not have significantly different lateral root growth phenotypes across the different levels of nitrogen, suggesting a role for *CEPR1* in mediating a foraging response in lower levels of nitrogen and suppressing growth in higher nitrogen conditions. In the *cepr1-1* background, both *nit1-3* and *nit1-1* slightly restore a sensitive response to different levels of nitrogen, however the response in these double mutants was not linear, and lateral root growth was enhanced in normal nitrogen but suppressed in low and high nitrogen. This indicates that wild-type *CEPR1* mediates the foraging response in part through *NIT1* inhibition, and the degree by which lateral root growth is regulated by *CEPR1* depends on the levels of nitrogen in the environment.

In the *cepr1-2* mutants, which have a phenotype that is consistent with a gain-of-function mutation, overall growth in normal and high nitrogen conditions was not significantly different than wild type, but we did observe significantly less lateral root growth in *cepr1-2* on low nitrogen when compared to wild type (*P*=.005). This suggests that *CEPR1,* when more active, can inhibit lateral root growth to a larger degree in lower nitrogen conditions because *cepr1-2;nit1-3* plants had much higher lateral root growth in low nitrogen than *cepr1-2.* Aside from this observation, our overall lateral root growth data indicates that *NIT1* generally inhibits lateral root growth independent of the levels of nitrogen present in the environment but that *CEPR1* ultimately orchestrates a sensitive response to different conditions. This could be in part through *NIT1*, as *nit1-3* mutants remained sensitive to different levels of nitrogen (*P*<.001) and the *nit1-3* allele only slightly restored sensitivity in the *cepr1-1* background.

By enhancing growth in normal nitrogen and suppressing growth in more abundant nitrogen conditions, the *cepr1-2* mutants appear to optimize growth more effectively in variable nitrogen, and our data implies that *cepr1-2* mediates this inhibitory effect through *NIT1*, because the *cepr1-2;nit1-3* double mutants had substantially more lateral root growth in low nitrogen, and slightly more growth in high nitrogen, when compared to the *cepr1-2* single mutants ($P<.001$ for low nitrogen, $P=.007$ for high nitrogen).

Considering this, we propose that *NIT1* can be regulated by *CEPR1* under specific environmental conditions and during specific developmental stages to modulate lateral root growth. This would make sense as an evolutionary strategy to conserve energy and resources, considering that many resources need to be redirected and allocated to roots to induce lateral root initiation and enhance extension. In ample nitrogen, a plant does not need to extend its lateral roots and it would be a waste of resources to do so if its roots already have access to nitrogen. On the contrary, in low nitrogen, it may be worth allocating extra resources to enhance lateral root extension if pockets of excess nitrogen are nearby but not in immediate proximity to where the roots are.

Sucrose in the media enhances sensitivity to different levels of nitrogen through *CEPR1*

Our results show that sucrose is required to enhance sensitivity in responding to different levels of nitrogen. In the absence of sucrose, *CEPR1* is still required to respond sensitively to different levels of nitrogen. Excess sucrose is also required for wild type plants to elicit an expected foraging response to different levels of nitrogen (required for wild type to respond linearly to increasing levels of nitrogen), suggesting that without excess energy, plants cannot respond appropriately to more extreme nitrogen conditions.

Considering that *cepr1-2* mutants have an opposite phenotype as *cepr1-1* and behave like gain-of-function mutants, we expected that they would not have an insensitivity to different levels of nitrogen. Indeed, *cepr1-2* mutants responded to increasing nitrogen conditions similarly to the way wild-type plants did in the absence of sucrose, but average lateral root growth was reduced, though not significantly.

We observed that the *nit1-3* mutation could slightly suppress the insensitivity of *cepr1-1* mutants, specifically by enhancing growth in normal nitrogen. In the *cepr1-2* background, the *nit1-3* mutation caused a more severe insensitive phenotype, which the *cepr1-2* single mutants did not have. On the contrary, the *nit1-1* mutation had little to no effect on growth phenotypes in either the *cepr1-1* or *cepr1-2* backgrounds. Overall, this suggests that *NIT1* is regulated by *CEPR1,* even in the absence of sucrose, to modulate root growth according to the levels of nitrogen. As mentioned previously, the *nit1-1* mutants still can make a transcript which could encode a protein product that can't synthesize auxin but could possibly be involved in generating other metabolites that can affect root growth. This could be why the *nit1-1* allele has a very minimal effect in either *cepr1* mutant background. In contrast, the *nit1-3* allele does not produce a protein product, and thus would be unable to be regulated by *CEPR1*. This can be seen in the different phenotypes of the *cepr1-2* single mutant and *cepr1-2;nit1-3* mutant, where it appears that *NIT1* is required by *cepr1-2* to respond sensitively to different levels of nitrogen in the absence of excess sucrose (Fig. 6A). We did not observe this effect in *cepr1-2;nit1-3* in the presence of excess sucrose (Fig. 5A), which suggests that carbon abundance is a major factor in determining the regulatory activity of *CEPR1,* and especially the *cepr1-2* protein product.

In the *cepr2-2* background the *nit1-3* alleles reduced the insensitivity that *cepr2-2* single mutants have to different levels of nitrogen. This could simply be due to the resulting phenotype of each *nit1* mutation in an otherwise phenotypically silent *cepr2-2* background, as *cepr2-2* mutants only appeared to differ from wild type plants in normal nitrogen conditions, and it is unknown whether *NIT1* interacts with *CEPR2* as well as *CEPR1,* however we do not have evidence that *NIT1* and *CEPR2* interact.

Overall, our data indicates that sucrose plays a major role in the ability of *CEPR1* to orchestrate an appropriate root growth response to different levels of nitrogen, because the *cepr1-1* null mutant was insensitive to different conditions in both the presence and absence of excess sucrose, while the *cepr1-2* mutants were still able to respond sensitively. *NIT1* also appears to play a role in regulating root growth accordingly depending on the conditions. It's likely that sucrose has a stimulatory effect on the regulatory activity of *CEPR1,* and depending on the level of sucrose, *NIT1* can be regulated differently by *CEPR1.*

The *cepr1-2* mutant is possibly gain-of-function and generates excess auxin through *NIT1*

Given that *cepr1-2* plants have opposite lateral root initiation and extension phenotypes when compared to the *cepr1-1* null mutant, we believe that *cepr1-2* is a gain-of-function mutant. C*epr1-2* is a missense mutation, and these mutants are predicted to produce a full-length protein product with an amino acid substitution in the kinase domain (Bryan et al., 2012). As such, this mutant could possibly generate more auxin through interactions with *NIT1*, as *NIT1* overexpression lines have been reported to have significantly less primary root elongation (Lehmann et al., 2017), which is indicative of auxin overaccumulation.

Using the *R2D2* auxin reporter (Liao et al., 2015) we observed that *cepr1-2* has higher levels of auxin in the primary root tip than other mutants (Fig. 7). This high-auxin *R2D2* phenotype is suppressed by the *nit1-3* null mutation in a *nit1-3;cepr1-2* double mutant, suggesting that *cepr1-2* mutants make more auxin directly through *NIT1.* We also observed that the *cepr1-2* mutants have a significantly lower lateral root density than wild-type plants (Figs. 4C, 4D) and we attribute this lower lateral root density and reduced lateral root extension phenotype to the higher levels of auxin in the roots of *cepr1-2* mutants, which we predict is because the *cepr1-2* protein inhibits *NIT1* more strongly than wild-type *CEPR1*.

High levels of exogenously applied auxin are well-known to inhibit primary root growth and promote lateral root initiation. Several auxin-overproducing mutants have been reported to have higher lateral root densities than wild-type plants (Ivanchenko et al., 2010), indicating that endogenous high levels of auxin also enhance lateral root initiation. While Ivanchenko et al., 2010 report that one auxin overproducing mutant, *iaaM-OX,* has a lower lateral root density than wild type, this is only observed when density is measured as the number of initiations per 100 cells, rather than per length of primary root. We analyzed the lateral root densities of our mutants by counting the number of lateral root initiations per centimeter of primary root. The lateral root phenotype we observed in *cepr1-2* is not consistent with the phenotypes reported for other auxin overproducing mutants*.* However, auxin activity and function are very tightly and complexly regulated through transport and biosynthesis. Although we see high levels of auxin in the primary root of *cepr1-2* mutants, which have a reduced lateral root density, it is possible that with this mutant being gain-of-function that a multitude of different components of root development could become dysregulated, which could influence auxin perception, regulation, transport, or even crosstalk with other hormones.

Another possibility is that *CEPR1* and *NIT1* function to increase the supply of auxin during critical developmental windows, like lateral root initiation, emergence, or extension. In wild-type plants, *CEPR1* could regulate *NIT1* to fine-tune lateral root development in response to environmental conditions by supplying additional IAA from a root-specific source of IAN during lateral root initiation, depending on the amount of nitrogen that is accessible. A similar mechanism has been described where indole-3-butyric acid (IBA, an auxin intermediate) can be converted to IAA in the root cap, which contributes to the amplitude of oscillatory auxin accumulation in the primary root (Strader et al., 2010, Xuan et al., 2015). Depending on where the IAN to IAA conversion occurs in the root, *NIT1* could affect lateral root development by locally increasing the concentration of auxin during each oscillation (increasing the amplitude of the oscillations) or contributing to the maintenance of auxin production during each oscillation and throughout lateral root initiation.

We propose that *cepr1-2* mutants post-translationally upregulate the activity of *NIT1*, which is why we observe higher levels of auxin in the primary root of *cepr1-2* mutants. The role of *NIT1* in this is clear, given our observation that the high auxin phenotype is abolished in the *cepr1-2;nit1-3* double mutant, which we observed to have wild-type levels of auxin.

Cepr1-1 and *nit1-3;cepr1-1* have growth defects in the absence of sucrose

In experiments where plants were grown in the absence of sucrose, we noticed severe post-germination root growth defects in *cepr1-1* and *cepr1-1;nit1-3* mutants, but not other mutants. Our results show that in the absence of sucrose, most wild-type plants and *nit1-3* mutants germinate and elongate their primary roots at a normal rate. About half of the *cepr1-1* mutants failed to extend their primary roots but did not have a germination defect, while more than half of the *cepr1-1;nit1-3* mutants tested failed to either germinate or extend their primary roots. We believe that this could be due to the inability of *CEPR1* to properly regulate *NIT1* during germination and primary root extension in situations where carbon resources are low, such as in low light. This could be due to disrupted coordination of auxin production during this critical developmental window.

During imbibition, *NIT1* expression is upregulated (Nakabayashi et al., 2005) suggesting a role for *NIT1* in germination, as imbibition causes metabolism to resume and ultimately promotes germination. Upregulation of *NIT1* begins 48 hours after imbibed seeds are moved under lights and expression is maintained during germination (Nakabayashi et al., 2005, Winter et al., 2007). Throughout seed development, *NIT1* is expressed in different tissues of the developing seed, including the peripheral endosperm during early seed development and throughout the entire embryo when seeds mature (Le et al., 2010, Winter et al., 2007). However, although *NIT1* is expressed throughout seed development, *nit1-3* single mutants did not have a seed or germination defect and the *cepr1-2;nit1-3* seeds still had a ~71-87% germination rate, depending on the nitrogen conditions. In the double mutants, it was the combined effect of the *cepr1-1* and *nit1-3* mutations that led to most plants restricting primary root growth post-germination, indicating that at least both are required for germination and primary root extension to occur. Interestingly, *CEPR1* is also expressed consistently throughout seed development, specifically in the chalazal seed coat (Le et al., 2010, Winter et al., 2007), which is where the transfer of sugar and nutrients into the seed is mediated. If this process is perturbed in *cepr1-1* single mutants, it would make sense why nearly half fail to extend their primary roots in the absence of sucrose, as *cepr1-1* seeds likely have less stored resources than wild-type seeds and would not be able to support growth during a heterotrophic developmental phase like post-germination seedling development. Germinating in conditions where transpiration and photosynthesis are hindered (our experimental growth conditions in agar plates) could lead to growth defects post-germination, however the role of *NIT1,* which is expressed in other tissues of the developing seeds but not the chalazal, in this process remains unknown.

Alternatively, it's possible that during germination, *NIT1* is involved in detoxifying products from glucosinolate catabolism. After germination, *A. thaliana* seeds have higher levels of glucosinolates than any other organs, and that after germination, the levels decrease dramatically (Petersen et al., 2002, Brown et al., 2003, Barth and Jander, 2006), suggesting that during germination, glucosinolates are catabolized. This would result in the production of toxic compounds, which could negatively affect plant growth and development in the absence of a functional *NIT1.* Although single *nit1-3* mutants do not have a post-germination root growth phenotype like *cepr1-1;nit1-3* mutants do, it's possible that with functional *CEPR1* being active, it can regulate other redundant *NIT* proteins that can serve the same or a similar function as *NIT1*.

Glucosinolate catabolism during germination could serve several purposes, including ensuring that seedlings are protected from herbivory during the most vulnerable time in their development. However, glucosinolate catabolism during germination could also serve as a way for the plant to make sequestered nutrients such as nitrogen and carbon accessible during early development when the plant cannot forage or produce its own energy and relies on maternal resources stored in the seed to develop. This would ensure that during germination when the primary root is undeveloped, the plant would have access to the resources it needs to develop into a plant that can forage on its own.

Mutations in *NIT1* enhance fertility in a *cepr1-1* background

Our results show that both mutant alleles of *nit1* can suppress an infertility phenotype associated with *cepr1-1* mutants. Like roots, fruits are a carbon sink that compete with other tissues for the allocation of carbon resources, like sucrose and starch. Throughout development, the regulation of carbon partitioning is incredibly important, because during different stages of development the carbon demands of different tissues changes, including the demands of sink tissues such as roots or seeds. For example, during vegetative development, most carbon resources are allocated to developing leaves and roots (reviewed in Wardlaw, 1990, Durand et al., 2018) to promote growth of the organs that forage for resources and generate energy. However, during the reproductive phase, the proportion of carbon allocated to leaves decreases, but increases in the stem (Durand et al., 2018), which doesn't emerge until the reproductive phase and thus does not act as a competing tissue during early vegetative development. During silique ripening, the amount of biomass partitioning to the root slightly decreased, while biomass partitioning in the stem increased (Durand et al., 2018), suggesting that carbon resources are redirected from the root towards the growth of new, competing tissues. Additionally, in related experiments observing translocation of radiolabeled sucrose, the amount of sucrose transported to roots decreases dramatically after the transition from vegetative phase to the reproductive phase, which could be associated with the decrease in biomass partitioning observed during the transition to and throughout the reproductive phase.

Considering the apparent role of *CEPR1* in carbon partitioning and the general effect of *NIT1* mutations on lateral organ development, it's again plausible in this case that *CEPR1* regulates *NIT1* under certain environmental conditions and during specific developmental phases to optimize growth when resources are abundant and inhibit growth when they are not. It is possible that the reduced fertility and short silique phenotype associated with *cepr1-1* could be due to an inability to properly regulate the partitioning and transport of sugars. In the case of *cepr1* mutants, which typically have a smaller rosette, smaller siliques, but more root growth, it's likely that an abundance of carbon resources are being transported to the root even throughout developmental transitions, leaving fewer resources for the development of other tissues. Based on our results, it's likely that *NIT1* is negatively regulated by *CEPR1* to prevent silique elongation. Lastly, it's possible that in *cepr1-1* single mutants, *NIT1* is in an active state and inhibits silique elongation because *CEPR1* is not functional in this background.

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