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HIGHLIGHT

The NRT1.1 transceptor controls both upstream auxin biosynthesis for lateral root primordia emergence and downstream action of auxin for lowering the mechanical resistance of the primary root tissues allowing LRP outgrowth.

ABSTRACT

In agricultural systems, nitrate is the main source of nitrogen available for plants. Besides its role as a nutrient, nitrate has been shown to act as a signal molecule for plant growth, development and stress responses. In *Arabidopsis*, the NRT1.1 nitrate transceptor represses lateral root (LR) development at low nitrate availability by promoting auxin basipetal transport out of the LR primordia (LRPs). In addition, our present study shows that NRT1.1 acts as a negative regulator of the *TAR2* auxin biosynthetic gene expression in the root stele. This is expected to repress local auxin biosynthesis and thus to reduce acropetal auxin supply to the LRPs. Moreover, NRT1.1 also negatively affects expression of the LAX3 auxin influx carrier, thus preventing cell wall remodeling required for overlying tissues separation during LRP emergence. Both NRT1.1-mediated repression of *TAR2* and LAX3 are suppressed at high nitrate availability, resulting in the nitrate induction of *TAR2* and LAX3 expression that is required for optimal stimulation of LR development by nitrate. Altogether, our results indicate that the NRT1.1 transceptor coordinately controls several crucial auxin-associated processes required for LRP development, and as a consequence that NRT1.1 plays a much more integrated role than previously anticipated in regulating the nitrate response of root system architecture.

Key words: auxin signaling, development, lateral root primordia, nitrate, NRT1.1, transceptor

INTRODUCTION

Nitrogen (N) is an essential element that plants can acquire from various inorganic or organic sources in the soil, but nitrate (NO_3^-) is the main form taken up and assimilated by most species (Nacry *et al.*, 2013). Soil NO_3^- concentration is often a major factor limiting the growth and yield of crop plants as it fluctuates dramatically in both time and space (Crawford and Glass 1998; Miller *et al.*, 2007). To cope with this, plants are able to quickly modulate their NO_3^- acquisition efficiency according to changes both in their own internal N status and in the external NO_3^- availability (Forde 2002; Nacry *et al.*, 2013; Kiba and Krapp 2016; O'Brien *et al.*, 2016). In addition to the regulation of root NO_3^- transport systems (Kiba and Krapp 2016; Bellegarde *et al.*, 2017; Jacquot *et al.*, 2017), modification of root system architecture (RSA) is another key component of this response (Nacry *et al.*, 2013; Forde, 2014; Giehl and von Wirén, 2014; Kiba and Krapp, 2016; O'Brien *et al.*, 2016). In more detail, NO_3^- has a dual effect on RSA in many species. On the one hand, high NO_3^- provision to the plant has a systemic repressive effect (Zhang and Forde, 1998; Giehl and von Wirén, 2014), and as a consequence mild NO_3^- limitation results in a general stimulation of root growth for improved foraging of the soil (Forde, 2002; Hermans *et al.*, 2006; Giehl and von Wirén, 2014). On the other hand, NO_3^- supply locally stimulates growth of the roots in contact with a high NO_3^- concentration, therefore contributing to preferential colonization and exploitation of the NO_3^--rich areas of the soil (Drew, 1975; Forde, 2002; Ruffel *et al.*, 2011, Nacry *et al.*, 2013). In dicotyledonous species where the root system is generally constituted of a primary root (PR) originating from the embryo, onto which lateral roots (LRs) develop post-embryonically, NO_3^- was generally reported to markedly affect LR development and growth, and to a much lesser extent PR growth (Forde, 2014). Furthermore, many responses of RSA to changes in NO_3^- availability were shown to be due to a signaling rather than a nutritional effect, pointing out the crucial role of specific NO_3^- sensing and signaling systems (O'Brien *et al.*, 2016; Guan, 2017; Sun *et al.*, 2017).

Compelling evidence indicates that the membrane NO_3^- transporter NRT1.1 (CHL1/NPF6.3), first identified as an influx carrier participating in the root uptake of NO_3^- (Tsay *et al.*, 1993; Liu and Tsay, 2003), is a central component of the NO_3^- sensing and signaling systems in *Arabidopsis thaliana* (Gojon *et al.*, 2009, 2011; Nacry *et al.*, 2013; Forde, 2014; Bouguyon *et al.*, 2015). This led to the proposal that it acts as a NO_3^- transceptor (transporter/receptor), with a dual transport/sensing function (Muños *et al.*, 2004; Walch-Liu and Forde, 2008; Ho *et*

al., 2009; Wang *et al.*, 2009; Gojon *et al.*, 2011). In particular, NRT1.1 is a master player in the NO_3^- regulation of RSA as it governs many responses of LR growth to NO_3^- (Remans *et al.*, 2006; Krouk *et al.*, 2010; Bouguyon *et al.*, 2015; O'Brien *et al.*, 2016; Sun *et al.*, 2017). In *Arabidopsis*, LRs originate from pericycle founder cells that divide and differenciate in order to initiate a LRP (Stage I), which has to develop across overlying tissues to eventually emerge out of the parent root (Stage VIII) (Malamy and Benfey, 1997). Through this series of well-defined stages, auxin plays a central role in controlling many steps of LRP development (Dubrovsky *et al.*, 2008; Benkova *et al.*, 2003; Bhalerao, 2002; Fukaki *et al.*, 2007; Ljung *et al.*, 2001). Within the LRP, an auxin gradient is progressively established with a maximum at the apex that is required for proper growth of the LRP (Benkova *et al.*, 2003). This gradient is created by the coordinated activity of efflux proteins (encoded by the *PIN* and *ABCB* gene families) that facilitate movement out of cells and influx transporters (encoded mostly by the *AUX/LAX* gene family) that facilitate auxin entry into cells (Petrasek and Friml, 2009; Vieten *et al.*, 2007. A "fountain model" was proposed for auxin flow in LRP based on auxin accumulation and auxin transporters expression patterns (Benkova *et al.*, 2003). According to this model, an acropetal flow (involving PIN1/7 located in the inner cell layers) allows auxin to come from the PR stele and to reach the LRP apex where it accumulates, while a basipetal flow (mediated by PIN3/4 in the outer cell layer) redirects the hormone to the PR. In addition, AUX1, ABCB transporters and LAX3 have been shown to be involved in LRP development: AUX1 facilitates LRP initiation and emergence (Marchant *et al.*, 2002; Swarup and Péret, 2012) and ABCB1 and ABCB19 physically interact with PIN1 as well with AUX1 for normal LR initiation and development (Mravec *et al.*, 2008). The influx/efflux auxin transporter ABCB4 (Yang and Murphy, 2009; Wu *et al.*, 2010; Kubes *et al.*, 2012) was also identified as a negative regulator of LRP initiation (Santelia *et al.*, 2005) through a mechanism remaining to be elucidated. Finally, LAX3 plays an important role in LR emergence process by increasing the level of auxin in LRP overlying cells, thus leading to a remodeling of cortical/epidermal cell walls that facilitates their separation and allows the outgrowth of the LRP (Swarup *et al.*, 2008).

Within this general scheme, we recently proposed a model for the mechanism by which NRT1.1 regulates LR growth in response to NO_3^- (Krouk *et al.*, 2010; Bouguyon *et al.*, 2015; Bouguyon *et al.*, 2016). Using heterologous expression systems, we found that NRT1.1 is not only able to transport NO_3^- but also auxin. The auxin influx activity of NRT1.1 is however inhibited by NO_3^- in a concentration-dependent manner (Krouk *et al.*, 2010). Furthermore, we

observed that NRT1.1 is expressed in LRPs only in the absence or at low NO_3^- availability (Bouguyon *et al.*, 2016). Taking into account the perfect match between the localization of the NRT1.1 protein with that of the basipetal transport route for auxin (outer cell layer of LRP), we postulated from the above observations that NRT1.1 mediates auxin influx into the outer cell layer of the LRPs, and thus facilitates the basipetal export of auxin out of these organs in the absence of NO_3^- or at low NO_3^- concentration (Krouk *et al.* 2010). Accordingly, in the absence of NO_3^- or at low NO_3^- concentration NRT1.1 lowers auxin accumulation/signaling (assessed by *DR5* activity) in the LRPs, which in turn leads to an inhibition of the emergence of the LRPs (Krouk *et al.*, 2010; Bouguyon *et al.*, 2015; Bouguyon *et al.*, 2016; Zhang *et al.*, 2019). At higher (>0.5 mM) NO_3^- supply, both NRT1.1 expression in LRPs and NRT1.1-dependent auxin transport activity are inhibited. This results in an increased auxin accumulation/signaling in LRPs, which favors their emergence. Therefore, the local stimulation of LR growth by NO_3^- is explained by the relief of the repressive action of NRT1.1. Accordingly, null mutants for *NRT1.1* (*chl1* mutants) have a much higher LR density than wild-type plants in the absence, but not in the presence of NO_3^- (Krouk *et al.* 2010, Bouguyon *et al.* 2015, Bouguyon *et al.* 2016)

An important point that remains unclear in this model is to know if, in addition to its direct effect on auxin transport in the LRPs, NRT1.1 also acts indirectly through modified expression/activity of other molecular actors determining auxin distribution in these organs. As matter of fact, auxin transport in LRP also involves the action of up to ten other auxin carriers, *i.e.*, PIN1-4, PIN7, ABCB1, ABCB4, ABCB19, AUX1 and LAX3 (see Petrášek and Friml, 2009). Interestingly, earlier transcriptome studies at the whole root or whole plant level have revealed that several auxin transporter genes are responsive to NO_3^- (Gutierrez *et al.*, 2007) in an NRT1.1-dependent manner (c Furthermore, there are reports indicating that responses of RSA to nutrients, including N, can be accounted for by changes in expression of *AUX* and *PIN* genes (Li *et al.*, 2011; Giehl *et al.*, 2012; Sun *et al.*, 2017; Naz *et al.*, 2019). Nevertheless, these considerations do not allow any conclusion on a putative role of NRT1.1 in regulating other auxin carriers for modulating LR development because little is known on the regulation of PIN, ABCB and AUX/LAX expression by NO_3^- signaling in LRPs. In addition, recent evidence highlights the importance of not only auxin transport, but also local auxin biosynthesis in determining the changes in auxin accumulation in plant tissues in response to external factors (Brumos *et al.*, 2018; Zhao *et al.*, 2018). This suggests that the nutrient regulation of RSA may also rely on a control exerted on auxin biosynthesis pathways. Of particular interest is the observation that the *Arabidopsis TAR2* gene, encoding

a Tryptophane amino transferase catalyzing the first step of the Trp-dependent auxin biosynthetic pathway, is required for stimulation of LR growth in response to mild N limitation (Ma *et al.*, 2014). However, nothing is known on the role of this gene in the local NO_3^- stimulation of LRP development in connection with NRT1.1-dependent signaling.

In the current work, we thus aimed at investigating how NO_3^- regulates the expression of the major auxin transporters (PINs, ABCBs, AUX1/LAX3) and of the TAR2 auxin biosynthetic enzyme during LRP development, and at determining if this regulation may account for an indirect effect of NRT1.1 on LR growth, through its NO_3^- signaling action on auxin transporter or auxin biosynthetic enzyme expression. As shown by the results detailed below, our work revealed that NRT1.1 has a much more integrated role in regulating LRP development than anticipated before. Indeed, our data indicate that NRT1.1 not only participates in auxin transport in LRPs, but also controls both the upstream biosynthesis of the hormone as a source of auxin for the LRPs, and the downstream action of auxin for lowering the mechanical resistance of the PR tissues to LRP outgrowth.

MATERIALS & METHODS

Plant material

The *Arabidopsis thaliana* accessions used in this study were Columbia (Col-0) and Wassilewskija (WS). The following GFP-tagged lines were used and crossed with the *chl1-5* mutant (Tsay *et al.*, 1993): *pDR5::VENUS* (Brunoud *et al.*, 2012), *pPIN1::PIN1-GFP* (Benkova, 2003), *pPIN2::PIN2-GFP* (Xu and Scheres, 2005), *pPIN3::PIN3-GFP* (Zadnikova *et al.*, 2010), *pPIN4::PIN4-GFP* (Vieten, 2005), *pPIN7::PIN7-GFP* (Blilou *et al.*, 2005; Mravec et al., 2008), *pAUX1::AUX1-YFP* (Swarup, 2004), *pLAX3::LAX3-YFP* (Swarup *et al.*, 2008), *DR5::GFP* (Ulmasov *et al.*, 1997), p*TAR2::GUS* and *tar2-c* (Ma *et al.*, 2014). The following GFP-tagged lines were used and crossed with the *chl1-10* mutant (Munos *et al.*, 2004): *pABCB1::ABCB1-GFP* (Mravec *et al.*, 2008), *pABCB4::ABCB4-GFP* (Cho *et al.*, 2007), *pABCB19:: ABCB19-GFP* (Bailly *et al.*, 2008). Homozygous plants for both *chl1-5* mutation and genes encoding tagged proteins were screened on F2 and F3 offsprings by PCR for *chl1-5* deletion as described in Mounier *et al.*, (2014) and fluorescence. Transgenic Arabidopsis line expressing *NRT1.1-GFPLoop* in WT background were obtained as described by Bouguyon et al., 2016.

Plant culture and root growth analysis

Seedlings were grown for 12 days in vertical petri dishes according to Krouk *et al.*, (2010). Basal culture medium without N was supplemented with the appropriate concentration of KNO_3, or Gln for each experiment as described in figure's caption. For analysis of root system architecture, the petri dishes were scanned using a Epson Perfection V700 PHOTO scanner (24-bits-color; resolution 600 ppp). The root system parameters such as primary root length, lateral roots length and number as well as lateral root density were measured by the Neuron J pluging from Image J software. Root system pictures for all genotypes were taken using Nikon digital camera D90. To analyze root growth parameters, Student T Test's was used to show statistical difference between genotypes and treatments.

RNA analysis

Root samples were frozen in liquid nitrogen and disrupted for 1 min at 30 s^{-1} in a Retch mixer mill MM301 homogenizer. Total RNA was extracted using TRIzol reagent (Invitrogen), DNase treated (Qiagen) purified using an RNeasy MinElute Cleanup Kit (Qiagen) and reverse transcription was achieved with 4 μg of RNAs with ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis (Invitrogen) using an anchored oligo-$dT_{(20)}$ primer. Accumulation of transcript was measured by quantitative real-time PCR (LightCycler 480; Roche Diagnostics) using the Light Cycler 480 SYBR Green 1 Master kit (Roche Diagnostics). All steps were performed according manufacturer's recommendations. The gene expression was normalized using *CLATHRIN* as an internal standard. The specific primers used are summarized in table 1. In order to examine whether gene expression was significantly different between treatments or genotypes, ANOVA followed by a Turkey test or Student T Test's were applied.

Gus staining

Samples were prefixed for 45 minutes at room temperature in a solution containing 50 mM NaPO4 pH7, 1,5% formaldehyde and 0,05% Triton X100. Then plants are washed 3 times in 50 mM NaPO4 pH7 with 0.05% Triton X100. Thereafter, samples were stained in 50 mM NaPO4 pH7, 0,5 mM Ferricyanide, 0,5 mM Ferrocyanide, 0,05% Triton X100 and 1 mM X-Gluc at 37°C for 48h in the dark. Afetr GUS staining, plants were washed in 50 mM NaPO4 pH7 for 5 minutes and chlorophyll was removed by EtOH increasing concentrations solutions.

The samples were mounted in 20% EtOH and were imaging by using x10 N.A. 0,3 or x40 N.A. 0,75 objectives on an Olympus BX61 microscope equipped with a camera Jenoptik Progres.

Confocal microscopy

For confocal microscopy observation, plant samples were mounted in osmosed water and viewed on a Leica SP8 Confocal Microscope (Leica). GFP/YFP were excited at 488nm by an Argon source and re-emitted light was filtered by a passing band of 505-530nm. Laser intensity was adjusted for each line and pinhole was settled at 1 unit, resolution at 1024 x 1024 to compare expression and accumulation of reporter proteins. Quantification of fluorescence in roots was performed using Image J software. The data represent the mean pixel values in a ROI.

RESULTS

Regulation of auxin carriers mRNA levels in response to NO_3^-.

To determine how auxin transporter genes respond to NO_3^- and whether this response is dependent on NRT1.1, we measured by quantitative RT-PCR the transcript levels of ten auxin transporters in roots of both WT and *chl1-5* (*NRT1.1* null mutant) plants grown for 8 days on either N-free medium or on medium containing 1mM KNO_3. The ten auxin transporter genes reported to contribute to auxin traffic in LRs (*PIN1-4*, *PIN7*, *ABCB1*, *ABCB4*, *ABCB19*, *AUX1* and *LAX3*) were sorted in three different groups according to their expression patterns (Figure 1). The first one is constituted by *PIN2*, *PIN3* and *ABCB1*, for which mRNA levels are not significantly influenced either by NO_3^- supply or by *chl1-5* mutation (Figure 1A). The second group gathers *ABCB4*, *ABCB19*, *PIN1*, *PIN4*, and *PIN7*, for which mRNA levels are modulated by NO_3^-. Among these genes, *ABCB4* is the only one to be repressed upon NO_3^- supply, whereas the others are induced (Figure 1B). The NO_3^--responses of these genes are the same in both WT and *chl1-5* plants, suggesting that NRT1.1 plays no role in these responses. The last group contains *AUX1* and *LAX3* (Figure 1C). Both genes show higher expression in presence of NO_3^- as compared to N-free medium in WT plants, but the difference is suppressed or strongly attenuated in *chl1-5* mutant. This is due to the up-regulation of these two genes in the *chl1-5* mutant as compared to the WT in N-free

condition, but not on 1 mM NO_3^-. Therefore, NRT1.1 appears to act as a negative regulator of the expression of both *AUX1* and *LAX3* in the absence, but not in the presence of NO_3^-. Altogether, these data show that although the majority of the auxin carrier genes investigated (7 out of 10) respond to NO_3^- provision in the roots, only two of them display a NO_3^- response that seems to involve NRT1.1 as a regulator.

Protein level and localization of auxin carriers in response to NO_3^-.

The response to NO_3^- of NRT1.1 and of the ten auxin transporters was evaluated at the protein level in LRPs using reporter-tagged transgenic plants (translational fusions with GFP or YFP) in WT or *chl1* mutant genetic background. Confocal sections through roots of transgenic plants grown in similar conditions to those used for the quantitative RT-PCR measurements were performed to compare protein accumulation and localization in LRPs in different conditions and at different developmental stages.

As reported previously (Bouguyon *et al.*, 2016), the protein expression of NRT1.1 in LRPs is strongly regulated by NO_3^-. Indeed, the GFP signal in roots of plants expressing a *NRT1.1::GFP* transgene under the control of the *NRT1.1* promoter in *chl1-5* background indicates that NRT1.1 is present in pre-emerged LRPs in the absence, but not in the presence of 1 mM NO_3^- in the medium (Supplemental Fig. 1). In NO_3^-–fed plants, NRT1.1 expression is only observed in LRPs after emergence. Most auxin transporters (8 out of 10) do not show any significant NO_3^--dependent change either in protein accumulation or localization in the context of LRP development and emergence. In our conditions and regardless the presence of NO_3^-, ABCB4 and PIN4 are not detectable in LRPs and PIN7 appears to be only transiently expressed at stages I and II but then rapidly disappears as LRPs progress to next phase (data not shown). Moreover, as previously shown (Benkova *et al.*, 2003), PIN2 is not expressed in LRPs before emergence (Supplemental Fig. 2). Neither the localization nor the protein abundance of PIN1, ABCB19 and AUX1 are significantly affected by NO_3^- or *chl1* mutation (Supplemental Figs 3, 4 and 5). Finally, although the GFP signal associated with the ABCB1-GFP fusion protein seemed slightly increased in the presence of NO_3^-, this was not found to be statistically significant after quantification (Supplemental Fig. 6).

In contrast, PIN3, and LAX3 are responsive to NO_3^- treatments. In WT plants and in presence of NO_3^-, PIN3-GFP spatio-temporal expression pattern in LRPs is similar to that previously

shown (Benkova *et al.*, 2003). Indeed, PIN3 is expressed from the first developmental stage to emergence and its early widespread localization is progressively restricted to the meristematic zone (Figure 2A- a-c right panels). However, on N-free medium, PIN3-GFP signal becomes undetectable in LRPs from stage IV-V up to emergence (Figure 2A b-c left panel). To determine whether this is due to a NO_3^- specific effect, PIN3-GFP expression was investigated in plants grown on 0.5 mM glutamine as an alternative N source (Figure 2A, central panels). Supply of glutamine results in a PIN3-GFP expression similar to that recorded with NO_3^-, indicating that lack of expression on N-free medium is not due to the absence of NO_3^- *per se*, but most probably results from N-deficiency. In agreement with the hypothesis that PIN3-GFP is not specifically regulated by NO_3^-, the effect of N on this protein is NRT1.1-independent since the same pattern is observed in a WT or a *chl1* mutant background (Figure 2B, 2C).

As previously shown (Swarup *et al.*, 2008), LAX3-YFP signal in WT background is detected from stage II to stage VII in mature stele and in cortical or epidermal cells overlaying LRPs under NO_3^- supply (Figure 3A- a to c right panels). However, LAX3-YFP signal is strongly decreased in cells overlying LRPs, regardless of the LRP developmental stage, in response to either N-starvation (Figure 3A- a to c left panel) or supply of glutamine as a N source (Figure 3A, middle panel). Interestingly, this down-regulation of LAX3-YFP expression is not recorded in *chl1-5* mutant background (Figure 3B, 3C). These data show that NRT1.1 acts as a negative regulator of LAX3 expression in the absence of NO_3^-. Interestingly, the *chl1-5* mutation restores LAX3-YFP signal in LRP overlying cells only after LRPs have reached stage IV (Figure 3A and B-compare a with b and c stages), indicating that NRT1.1 is responsible for LAX3 extinction at late, but not at early stages of LRP development. This is fully consistent with the observation that NRT1.1 starts repressing LRP development at stages IV/V (Krouk *et al.*, 2010; Bouguyon *et al.*, 2016). In conclusion, the pattern of LAX3-YFP expression around LRPs in WT and *chl1* mutant background is in agreement with that of *LAX3* transcript accumulation in the roots (*i.e.*, both are up-regulated by NO_3^- in WT plants and by *chl1* mutation in the absence of NO_3^-).

It has been reported that the upregulation of *LAX3* expression that facilitates LRP outgrowth is triggered by the LBD29 transcription factor (Swarup *et al.*, 2008; Porco *et al.*, 2016). To test whether LBD29 responds to NO_3^- in a NRT1.1-dependent manner, its transcript accumulation in roots was investigated in roots of Col or *chl1-5* plants grown on N-free

medium or on 1 mM NO_3^-. The data indicate that expression of *LBD29* is strongly induced by NO_3^- supply as compared to N-free conditions in WT plants, and that it is markedly overexpressed in the *chl1-5* mutant as compared with the WT on N-free medium, but not in the presence of NO_3^- (Figure 3D). This is fully consistent with the expression patterns of both *LAX3* gene (Figure 1) and LAX3-GFP protein (Figure 3), which therefore suggests that LAX3 response to NO_3^- is mediated by the NRT1.1-dependent regulation of *LBD29* expression.

The TAR2 auxin biosynthesis enzyme is up-regulated by NO_3^- in a NRT1.1-dependent manner.

The *TAR2* gene was reported to be N-responsive and to contribute to the regulation of LR development by NH_4NO_3 supply (Ma *et al.*, 2014). However, its putative role in the specific response to NO_3^- in connection with the NRT1.1-dependent pathway is not known. Therefore, we first investigated the effect of the three N regimes (N-free, 0.5 mM glutamine or 1 mM NO_3^-) on *TAR2* mRNA accumulation in the roots of WT and *chl1-5* plants, as well as in those of the *tar2c* knock-out mutant of *TAR2*. Although *TAR2* mRNA accumulation was previously shown to be down-regulated by increasing NH_4NO_3 concentrations (Ma *et al.*, 2014), it was in our conditions markedly induced in the WT by N supply as compared with N-free condition, with a much more pronounced response on NO_3^- as compared with glutamine (Figure 4A). This NO_3^- induction was fully lost in the *chl1-5* mutant because *TAR2* expression was already high in this line on N-free medium or on gln. As expected, no significant *TAR2* mRNA level was recorded in the *tar2c* knock-out mutant.

Both the induction of *TAR2* expression by NO_3^-, and its up-regulation in N-starved *chl1-5* plants as compared to WT were confirmed by histological staining of GUS activity in *pTAR2::GUS* plants (Figure 4B). As shown previously (Ma *et al.*, 2014), *TAR2* expression in roots is predominantly localized in the stele, and we did not observe *pTAR2* activity in endodermis, cortex or epidermis (Figure 4B and 4C). In presence of either 0.5 mM gln or 1 mM NO_3^-, strong GUS staining was also observed inside the vast majority (>90%) of young LRPs (stages I-III) in both WT and *chl1-5* plants (Figure 4C). On N-free medium, GUS staining in LRPs was only visible in ca~60% of the young LRPs of *chl1-5* plants, and at a lower intensity than that recorded in plants fed with either gln or NO_3^- (Figure 4C). In all cases, *pTAR2::GUS* expression in LRPs was no more recorded as soon as these LRPs

evolved to later stages of development (stages IV-V onwards). Altogether, the above results show that in the absence of NO_3^-, NRT1.1 acts as a strong negative regulator of *TAR2* expression in the stele and to a lower extent in the young LRPs. This indicates that NRT1.1 may control LRP development not only through its auxin transport facilitation within the LRPs (Krouk *et al.*, 2010), but also by modulating auxin biosynthesis in the stele. In addition, these results also show that *TAR2* expression may not only be responsive to NRT1.1-mediated NO_3^- signaling but also to another N stimulatory effect that can be activated by gln supply.

TAR2 is required for the stimulation of LR development by NO_3^- and its regulation by NRT1.1

The induction of *TAR2* expression in the roots by NO_3^- suggests that increased auxin biosynthesis is associated with NO_3^- regulation of RSA. To determine whether TAR2 may contribute to the NO_3^- stimulation of LR development by locally increasing auxin provision to LRPs, expression of *DR5::GFP* was investigated in both WT and *tar2c* mutant backgrounds in the roots of plants grown on 1 mM NO_3^- (Figure 5). The GFP signal was predominantly found in the stele and LRPs of both genotypes, but with a strongly reduced intensity in *tar2c* as compared to WT (Figure 5). The negative effect of *TAR2* mutation on *DR5::GFP* expression in LRPs was found at almost all stages of development, with the exception of stage VII, just prior to emergence (Figures 5B and 5C). This indicates that TAR2-dependent auxin biosynthesis in the stele and/or the LRPs is required for increasing auxin accumulation/signaling in LRPs in response to NO_3^-. Note however that although important, the presence of a functional *TAR2* gene is not strictly required to generate *DR5::GFP* expression in the root. This expression is still significant in the stele and in LRPs at all developmental stages in the *tar2c* mutant (Figures 5A, 5B and 5C), suggesting that auxin partly comes from another origin than the TAR2-dependent biosynthesis pathway.

TAR2 mutation alone (*tar2c* mutant) had only very limited effect, if any, on the growth of the primary root in the three conditions tested (Figures 6A and 6B). However, it very significantly decreased (by nearly 50%) the density of LRs in the plants grown on NO_3^-, but not in those supplied with either glutamine of N-free medium (Figures 6A and 6C). This demonstrates that TAR2 participates in the stimulation of LR development by NO_3^-, in agreement with the observations that *TAR2* expression is induced by NO_3^- and that its

mutation lowers *DR5* activity in LRPs (Figures 4 and 5). Combining *NRT1.1* and *TAR2* mutations (double *chl1-5tar2c* mutant) had no additional effect on LR development as compared to the single *TAR2* mutation, but strikingly affected it as compared to the single *NRT1.1* mutation (Figures 6A and 6C). Indeed, the dramatic increase of LR density classically observed in *chl1-5* as compared to the WT on NO_3^-–free medium (Krouk *et al.*, 2010; Bouguyon *et al.*, 2015) was totally abolished in the double *chl1-5tar2c* mutant. These data indicate that the up-regulation of *TAR2* expression in response to *NRT1.1* mutation (Figure 4) is most likely one key response required to trigger enhanced LR development in *chl1-5* plants.

DISCUSSION

Nitrate regulation of auxin carriers in lateral root primordia

One striking conclusion arising from our work is that, with the noticeable exception of LAX3, NRT1.1 has little effect on the expression of the auxin carriers involved in LRP development. However, as suggested by previous reports on *Arabidopsis* (Li *et al*., 2011) and rice (Naz *et al.*, 2019; Sun *et al.*, 2017), our data show that the root expression of key auxin carriers is responsive to N supply in WT plants. Although not dramatic, changes in mRNA levels in the whole root were found in response to NO_3^- for 7 out of the 10 auxin carriers investigated (Figure 1). When considering more precisely the expression at the protein level in or around the LRPs of WT background plants, only 2 of them (namely, PIN3 and LAX3) displayed a visible increase in the fluorescence from GFP or YFP fusion proteins on 1 mM NO_3^- as compared to N-free medium (Figures 2 and 3). Most importantly, for PIN3 this effect was not specific of NO_3^- since the same increase in fluorescence was observed upon provision of 0.5 mM gln as a N source (Figure 2). This suggests that the response to NO_3^- of PIN3 is not specifically due to NO_3^- signaling, but either to a signaling action of an N metabolite downstream of NO_3^- assimilation, or to more general effect of N nutrition. As a matter of fact, organic forms of N, and in particular amino acids, have been shown to specifically control several mechanisms involved in root growth and development in *Arabidopsis* (Walch-Liu *et al.*, 2006; Gifford *et al.*, 2008; Vidal *et al.*, 2010). Our data indicate that none of the auxin carriers expressed in LRPs (PIN1-4, PIN7, ABCB1, ABCB4, ABCB19 and AUX1) displays a regulation pattern correlated with that previously reported for *DR5* activity in LRPs (*e.g.*, repression in the absence of NO_3^- that is suppressed in the

chl1-5 mutant, (see Krouk *et al.*, 2010). This supports the hypothesis that NRT1.1 modulates auxin accumulation in LRP directly through its auxin transport capacity, and not indirectly through modified expression of other auxin carriers. However, it cannot be ruled out that NRT1.1 may act on these carriers at a regulatory level that was not investigated in our study, by triggering posttranslational modifications affecting transport activity for instance. Furthermore, although not predominantly expressed in LRPs, ABCB21 has recently been reported to markedly affect *DR5* activity in these organs (Jenness et al., 2019), but its regulation by NO_3^- is unknown. Therefore, further investigation is needed to conclude whether the above listed auxin carriers are regulated by NRT1.1, or not.. In contrast, our data on LAX3 mRNA accumulation and of LAX3-YFP fusion protein expression clearly indicate that the expression of this carrier in overlying tissues of the LRPs is under control by NRT1.1-dependent NO_3^- signaling. First, the stimulation by NO_3^- of LAX3-YFP expression in cortical cells cannot be mimicked by gln supply (Figure 3). Second, both the increases in *LAX3* gene and LAX3-YFP protein expression in response to NO_3^- were suppressed in the *chl1-5* mutant (Figures 1 and 3). This is further supported by the fact that the gene expression of LBD29, the direct positive regulator of LAX3 (Porco *et al.*, 2016), displays the same pattern of regulation (Figure 3). In agreement with what was previously recorded for LR density and for auxin accumulation/signaling in LRP (*DR5* activity), the stimulatory effect of NO_3^- was suppressed because LAX3 and *LBD29* expression were already high in the *chl1-5* mutant in the absence of NO_3^-, indicating a negative regulatory role of NRT1.1 under this condition (Figures 1 and 3). The mechanisms by which NRT1.1 may lead to repression of *LBD29* and LAX3 expression are not known. However, the stimulation of both LBD29 and LAX3 expression in cells overlying LRPs was proposed to be due to the induction of *LBD29* and *LAX3* genes by the auxin diffusing from the LRP to the overlying tissues (Swarup *et al.*, 2008; Porco *et al.*, 2016). Therefore, it is conceivable that the NRT1.1-mediated repression of auxin accumulation in LRPs in the absence of NO_3^- (Krouk *et al.*, 2010; Bouguyon *et al.*, 2015; Bouguyon *et al.*, 2016; Zhang et al., 2019), may lead to a lowered auxin diffusion out of the LRPs, which may in turn limit induction of both *LBD29* and *LAX3* genes.

Auxin biosynthesis is a target of NRT1.1-dependent NO_3^- signaling in roots

The importance of local auxin biosynthesis for controlling plant development has been recently reconsidered, and shown to be important for responses to internal or external signals (ethylene, light, temperature and pathogens, Ljung *et al.*, 2001; Brumos *et al.*, 2018; Zhao *et al.*, 2018). Data concerning RSA responses to nutrients are scarce on this point (Guan *et al.*, 2017; Olatunji *et al.*, 2017), with the exception of the report by Ma et *al.*, (2014), indicating that the *TAR2* auxin biosynthetic gene contributes to the root foraging response triggered by mild N-limitation in *Arabidopsis*. This response is considered to be due to a repression exerted by high N supply, which is not specific to NO_3^-, and may involve systemic signaling (Forde, 2014; Giehl and von Wirén, 2014). Our data confirm a key role of TAR2 in the N regulation of LR growth, but in a different context that is the local stimulation of LRP development by NO_3^- signaling. Indeed, *TAR2* expression is induced by NO_3^- (Figure 4), and *tar2* mutants display a markedly reduced increase in LR density in response to NO_3^- supply as compared with WT plants (Figure 6). This is correlated with reduced *DR5* activity in both the stele and LRPs of plants with *tar2* as compared to WT background (Figure 5). This suggests that the TAR2 biosynthetic pathway is an important source of auxin for ensuring proper accumulation of the hormone in LRPs and for yielding full stimulation of LR development by NO_3^-. However, because neither *DR5* activity nor LR emergence is totally suppressed in the *tar2* mutant, it is likely that another auxin source, coming from acropetal transport from the shoot, other biosynthesis and/or conjugation pathway (Bhalerao *et al.*, 2002; Petrasek and Friml, 2009; Olatunji *et al.*, 2017), also contributes to supply auxin to the LRPs. As it is the case for LR density and key factors associated with it (*DR5* activity in LRPs, LBD29/LAX 3 expression), the stimulation of *TAR2* expression by NO_3^- in the stele is actually mostly due to the relief of the repression exerted by NRT1.1 in the absence of NO_3^-. This is illustrated by the upregulation of *TAR2* in the *chl1-5* mutant on N-free medium (Figure 4). This *TAR2* upregulation appears to be crucial for the strongly increased LR growth phenotype of the *chl1-5* plants because the double *chl1-5tar2c* mutant did not differ in LR density from the WT in NO_3^-–free media (Figure 4). The mechanism associated with the NRT1.1-dependent repression of *TAR2* expression in the stele in the absence of NO_3^- is unclear. One hypothesis is that the stimulation of basipetal auxin transport out of the LRPs by NRT1.1 (Krouk *et al.*, 2010) may locally increase auxin levels in the stele. Because the TAR2 auxin biosynthetic pathway has been recently reported to be feedback repressed by auxin (Suzuki *et al.*, 2015; Takato *et al.*, 2017), this may explain why *TAR2* expression is

reduced in the stele of WT plants deprived of NO_3^- (Figure 4). Alternatively, because NRT1.1 has been shown to be also expressed in the central cylinder of mature portions of the PR, where it contributes to xylem loading of NO_3^- (Léran *et al.*, 2013), it is also possible that NRT1.1 may act on *TAR2* expression directly in the stele when NO_3^- is absent from the medium.

Interestingly, *TAR2* was found to be expressed in a N-stimulated manner in young LRPs at stages I-III (Figure 4C), *i.e.*, at earlier developmental stages than those (IV-V) where NRT1.1 starts to act as a regulator of LRP emergence (Bouguyon *et al.*, 2016). Because *TAR2* expression in young LRPs is responsive to both gln and NO_3^-, this suggests that N can modulate early LRP development by controlling auxin biosynthesis *in situ* independently of NRT1.1. This is in line with the previous proposal that N can act on various developmental check points of LR development through different mechanisms (Forde, 2014; Giehl and von Wirén, 2014).

A more integrated model for NRT1.1-dependent regulation of LR development by NO_3^-.

Our previous working model for explaining the NRT1.1-dependent repression of LR growth in the absence or at low availability (<0.5 mM) of NO_3^- almost entirely relied on the hypothesis that NRT1.1 inhibits LRP outgrowth (more specifically at developmental stage IV) because it facilitates the basipetal auxin transport out of the LRP, therefore preventing the hormone accumulation at the LRP tip (Krouk *et al.*, 2010; Bouguyon *et al.*, 2015). This hypothesis was supported by the auxin transport capacity of NRT1.1 recorded in heterologous systems (Krouk *et al.*, 2010; Bouguyon *et al.*, 2015; Wei *et al.*, 2018 ; Zhang *et al.*, 2019), and by its largely predominant localization in the outer cell layer of the LRP (Krouk *et al.*, 2010), which corresponds to the basipetal auxin transport route (Benkova *et al.*, 2003). Because increasing NO_3^- concentrations (>0.5 mM) results both in an inhibition of the NRT1.1-mediated auxin transport facilitation (Krouk *et al.*, 2010) and in a repression of the NRT1.1 protein expression in LRPs (Bouguyon *et al.*, 2016), we proposed that the local stimulation of LR growth by NO_3^- is due to a relief of the repression exerted by NRT1.1. The data reported here strengthen our previous conclusions, but show that NRT1.1 affects other processes in addition to auxin distribution within the LRP, such as the biosynthesis of the hormone and the mechanical resistance of the PR tissues to LRP outgrowth. As summarized in Figure 7, the repressive role of NRT1.1 on LRP development can now be extended to three separate but synergistic actions. Indeed, NRT1.1 prevents auxin accumulation in the LRP not

only by favoring the basipetal transport of the hormone, but also by triggering the repression of *TAR2* expression in the stele. As a consequence of reduced auxin biosynthesis, it is likely that the acropetal flux channeling the hormone to the LRP tip is also lowered, therefore contributing to slowing down LRP development and emergence. In addition, NRT1.1 acts as a negative regulator of the expression of LAX3 in the PR tissues overlying the LRPs. Given the proposed function of LAX3 in LRP development (Swarup *et al.*, 2008), this is expected to prevent the cell wall relaxation in the LRP overlying tissues that is required to lower the mechanical resistance to LRP emergence (Lucas *et al.*, 2013).

Beyond the repression of LRP development at low NO_3^- availability summarized in Figure 7, NRT1.1 has also been reported to favor LR elongation in NO_3^-–rich patches of the external medium (Remans *et al.*, 2006), and to trigger the induction of the *AFB3* auxin receptor gene by NO_3^- that results in repressed PR growth and stimulated LR initiation (Vidal *et al.*, 2010; Vidal *et al.*, 2014). By interfering with auxin biosynthesis, transport, signaling, and thus by controlling various key checkpoints of LR development, our studies reveal that NRT1.1 has a much more integrated function in governing the NO_3^- regulation of RSA in *Arabidopsis* than previously anticipated (Vidal *et al.*, 2014; Bouguyon *et al.*, 2015). It may therefore be expected that all aspects and mechanisms of this integrated function are not fully unraveled yet.

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FIGURE LEGENDS

Fig. 1: Relative mRNA accumulation of auxin transporters in roots of WT (Col) and *chl1-5* seedlings grown in presence or absence of NO_3^-.

Wild-type and *chl1-5* seedlings were grown for 8 days in vertical agar plates on basal medium supplemented with 1 mM NO_3^- or not. Transcript levels were quantified in the roots by quantitative RT-PCR using *CLATHRIN* as an internal standard. The ten auxin transporter genes investigated are grouped into three different classes, according to the response of their mRNA levels to NO_3^- and *NRT1.1* mutation:

A) Auxin transporters not regulated by NO_3^-

B) Auxin transporters regulated by NO_3^- independently of *NRT1.1*.

C) Auxin transporters regulated by NO_3^- in a *NRT1.1*-dependent way.

The data are the mean ± se of 2 replicates from 3 independent experiments.

Statistically significant differences between genotypes or N treatments at $P < 0.05$ are shown by different letters and ANOVA followed by a Turkey Test were used to show these significant differences.

Fig. 2: PIN3-GFP expression in lateral root primordia is repressed by N starvation both in WT (Col) and *chl1-5* genetic backgrounds.

Seedlings expressing PIN3-GFP in Col (A) or in *chl1-5* (B) genetic background were grown for 8 days in vertical agar plates on basal medium deprived of N (left panel) or supplemented with either 0.5mM glutamine (Gln ; middle panel) or 1mM NO_3^- (right panel). Primary root portions with lateral root primordia at various developmental stages were observed under confocal microscope.

a = stage I-III, b = stage IV-V, c = stage VI-VII. White bar represents 100µm. The pictures shown are representative of >20 primordia from >10 plants of 3 independent experiments.

(C) Fluorescence quantification in different root tissues of 8-day-old plants expressing PIN3-GFP in *WT* or *chl1.5* lines in the absence or presence of NO_3^- or glutamine (Gln). Fluorescence was measured in unmerged primordia stage IV to VIII, and emerged primordia. Values are the mean of 5–10 plants from 2 independent experiments. P values were analyzed by Student's T Test and differences with N treatments are statistically significant at ***P , 0.001.

Fig. 3: LAX3-YFP expression in tissues overlaying lateral root primordia is repressed in the absence of NO_3^- in a *NRT1.1*-dependent manner.

Seedlings expressing LAX3-YFP in Col (A) or in *chl1-5* (B) genetic background were grown for 8 days in vertical agar plates on basal medium deprived of N (left panel) or supplemented with either 0.5mM glutamine (middle panel) or 1mM NO_3^- (right panel). Primary root portions with lateral root primordia at various developmental stages were observed under confocal microscope.

a = stage I-III, b = stage IV-V, c = stage VI-VII. White bar represents 100µm. The pictures shown are representative of >20 primordia from >10 plants of 3 independent experiments.

(C) Fluorescence quantification in different root tissues of 8-day-old plants expressing LAX3-YFP in *WT* or *chl1.5* lines in the absence or presence of NO_3^- or glutamine (Gln). Fluorescence was measured in cortical/epidermal cells surrounding unmerged primordia stage IV to VIII. Values are the mean of 5–10 plants from 2 independent experiments. P values were analyzed by Student's T Test and differences with N treatments are statistically significant at ****P , 0.0001.(D) Relative mRNA accumulation of LBD29 in roots of Col and *chl1-5* plants grown for 12 days in agar plates on basal medium deprived of N or supplemented with 1 mM NO_3^-. The expression was quantified by RT-qPCR and normalized by *CLATHRIN*.

The data are the mean ± se from 3 independent experiments. P values were analyzed by Student's T Test and differences between genotypes are statistically significant at *P < 0.005.

Fig.4: *TAR2* expression in the roots is repressed in the absence of NO_3^- in a *NRT1.1*-dependent manner.

Wild-type, *chl1-5* and *tar2-c* seedlings were grown for 12 days in vertical agar plates on basal medium deprived of N, or supplemented with either 1 mM NO_3^-, or 0.5 mM L-glutamine.

(A) Relative accumulation of *TAR2* transcripts in roots. The data are the mean ± se of 2 replicates from 3 independent experiments. P values were analyzed by Student's T Test and differences between genotypes are statistically significant at **P < 0.01.

(B) Histochemical staining of GUS activity in roots of plants expressing a *pTAR2::GUS* transgene in either wild type (Col) or *chl1-5* genetic background. Black bar represents 0.5 cm.

(C) Histochemical staining of GUS activity in the root stele and lateral root primordia of plants expressing a *pTAR2::GUS* transgene in either wild type (Col) or *chl1-5* genetic

background. The pictures shown display root portions with lateral root primordia at stages I-III. The pictures shown are representative of 10 primordia from >10 plants of two independent experiments. Black bar represents 0.5 cm.

Fig. 5: Mutation of *TAR2* represses DR5::GFP expression in LRPs and in the stele under NO_3^- supply.

(A) DR5::GFP expression in mature root zone of plants with WT (left panel) or *tar2c* (right panel) genetic background. The seedlings were grown for 12 days on basal medium supplemented with 1mM NO_3^-. White bars = 100 µm.

(B) DR5::GFP expression in lateral root primordia of plants with WT (upper panel) or *tar2c* (lower panel) genetic background. The seedlings were grown for 12 days on basal medium supplemented with 1mM NO_3^- . The pictures display lateral root primordia at different developmental stages (from stage II to VI). The asterisk visualizes the location of the primordium. The pictures are representative of 10 plants of 2 independent experiments. White bars = 100 µm.

(C) Fluorescence quantification in lateral root primordia at different developmental stages of 12-d-old plants expressing DR5::GFP in WT or *tar2c* background. Plants were grown on basal medium supplemented with 1mM of NO_3^-. Data represent mean ± se of 2 independent experiments (n= 10). P values were analyzed by Student's T Test and differences between genotypes are statistically significant at *, $P < 0.05$; **, $P < 0.01$) ***, $P < 0.001$.

Fig. 6: Regulation of lateral root development by NO_3^- and NRT1.1 relies on the TAR2 auxin biosynthesis enzyme.

Root system architecture of Col, *chl1-5*, *tar2c* and *chl1-5tar2c* plants grown for 12 days in vertical agar plates on a basal nutrient medium deprived of N or supplemented with either 1 mM NO_3^- or 0.5 mM Gln.

(A) Representative pictures of Col, *chl1-5*, *tar2-c* and *tar2-c✗ chl1-5* plants. White bar represents 0,5 cm.

(B) Mean length the primary root. Results (n=100) are representative of 5 independent experiments. P values were analyzed by Student's T Test and differences between mutant and wild-type genotypes are statistically significant at ***, P < 0.001 (t test).

(C) Mean density of visible lateral roots (> 0,5 mm). Results (n=100) are representative of 5 independent experiments. P values were analyzed by Student's T Test and differences between mutant and wild-type genotypes are statistically significant at ***, $P < 0.001$ (t test).

Fig. 7: Schematic model of the integrated repressive role of NRT1.1 on lateral root development.

NRT1.1 coordinately controls three separate auxin-related processes for repressing LRP development at low NO_3^- availability. First, NRT1.1 facilitates the basipetal transport of auxin out of the LRP (Krouk et al. 2010). Second NRT1.1 represses local auxin biosynthesis by downregulation the expression of the *TAR2* auxin biosynthetic gene, which reduces the acropetal flux of auxin to the LRP. These two actions are expected to lower auxin accumulation at the LRP apex that is required for normal LRP development and growth. Third, NRT1.1 maintains a mechanical resistance to the LRP outgrowth by repressing LAX3 in the tissues overlying the LRP, thereby preventing cell wall remodeling in these tissues. All three repressive effects of NRT1.1 are suppressed by high NO_3^- availability (>0.5 mM), leading to the local NO_3^-–induced stimulation of LRP development and emergence.

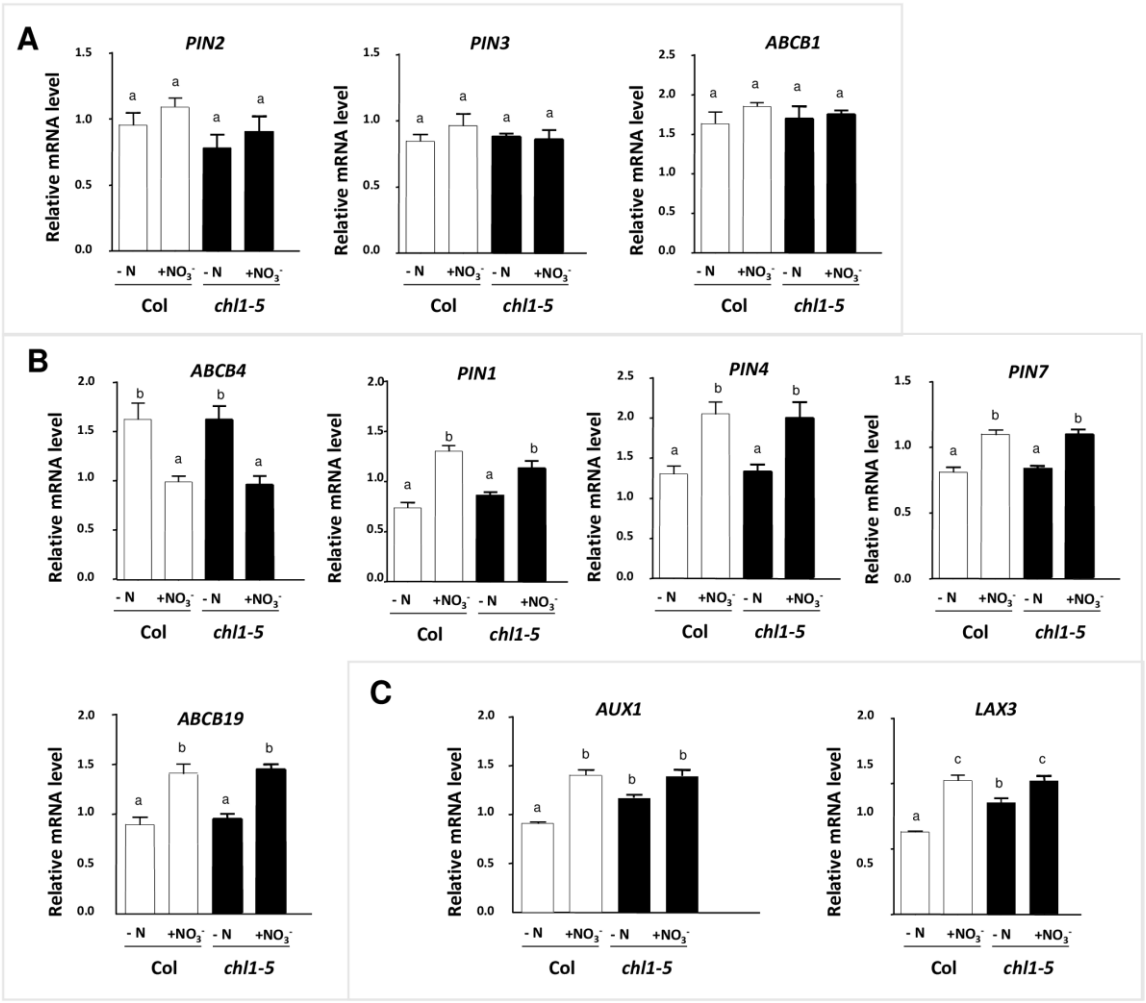

Figure 1.

Figure 2

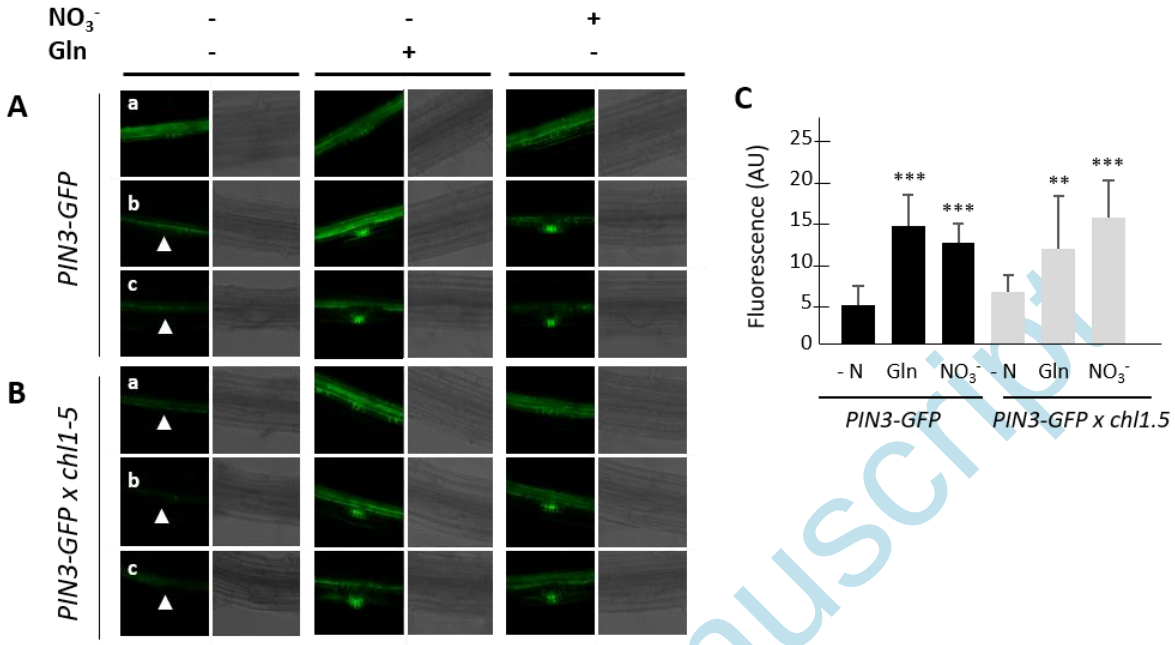

Figure 3

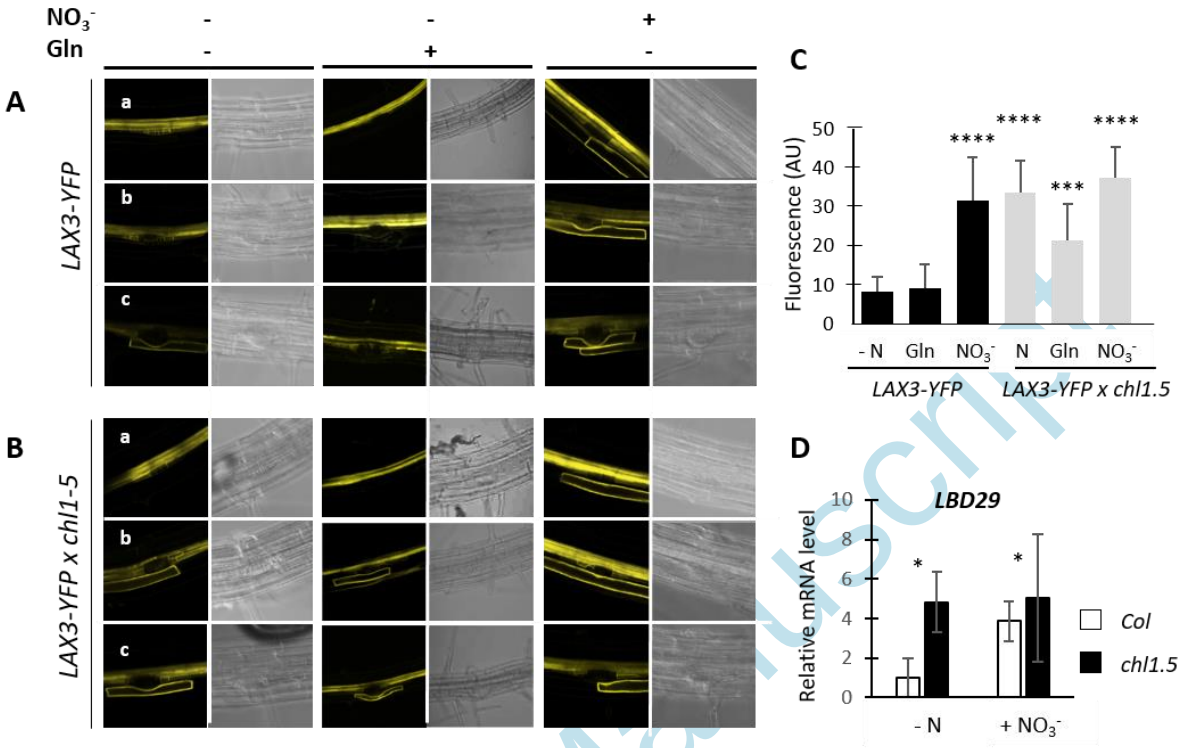

Figure 4

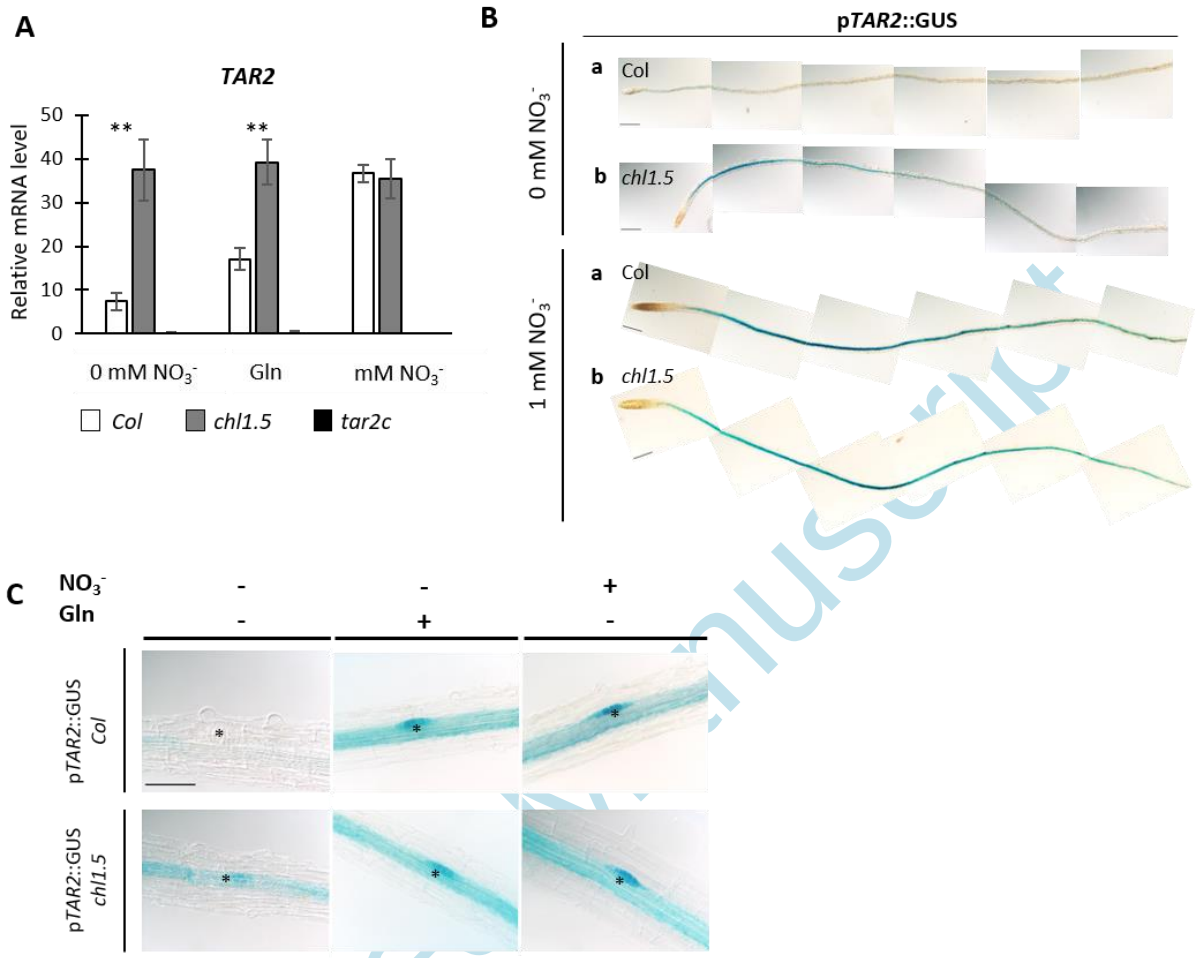

Figure 5

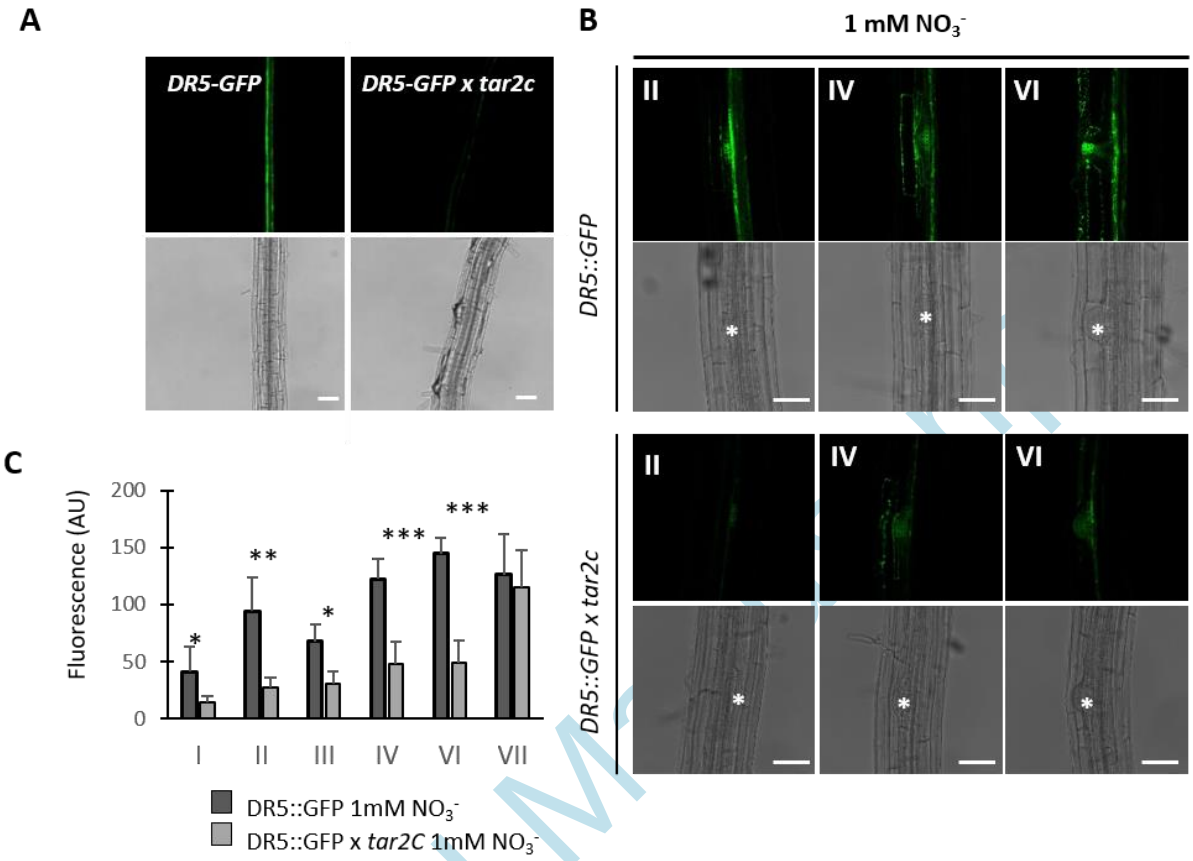

Figure 6

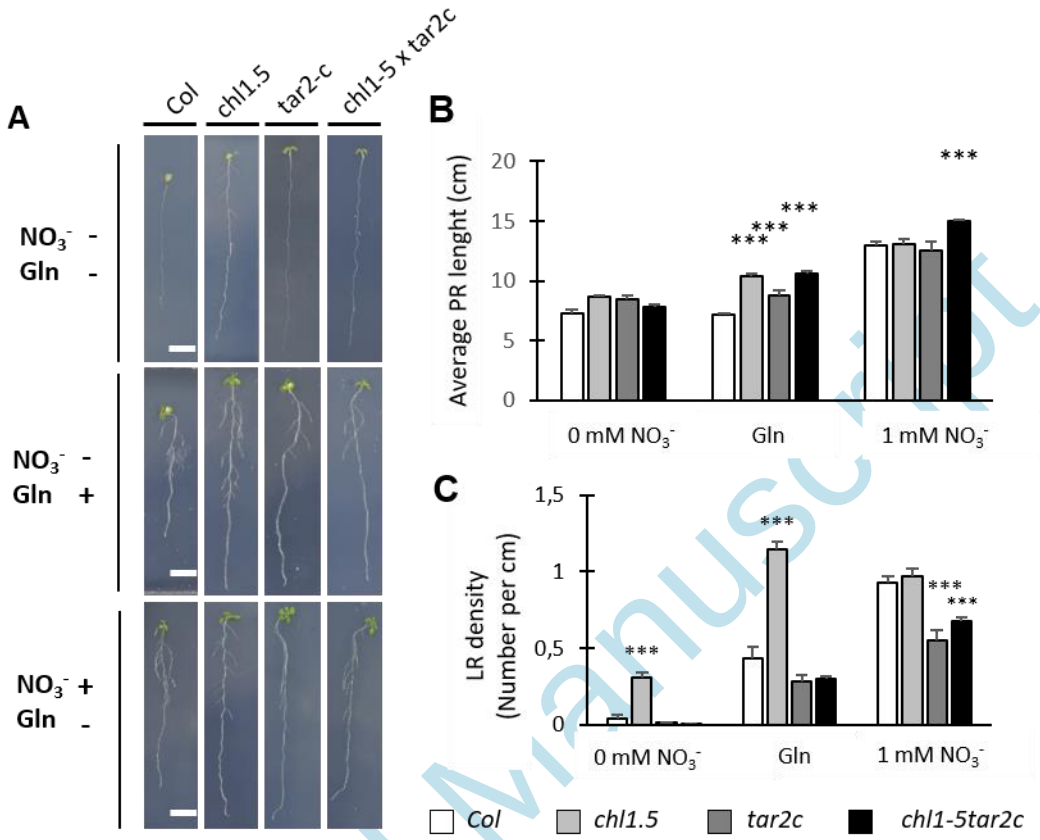

Figure 7

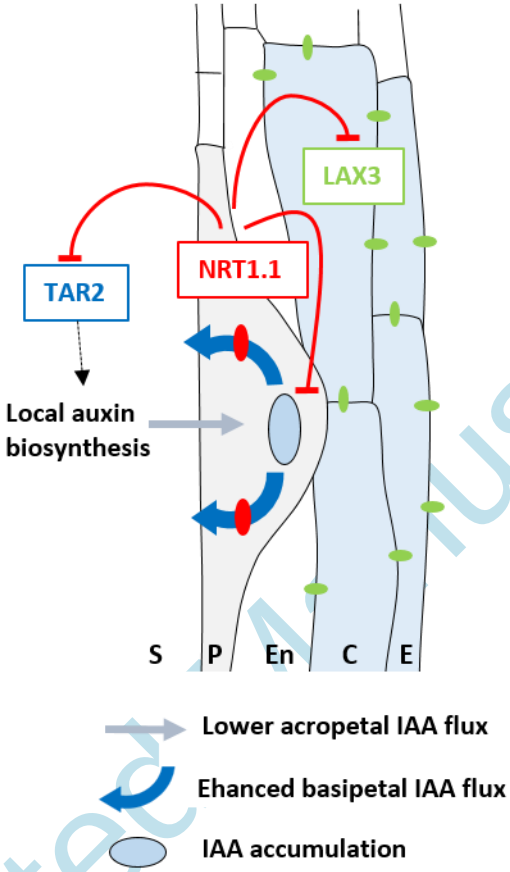

Table 1. Primers used for qRT-PCR.

Description	AGI	Primer	Sequence (5'-3')
CLATHRIN	AT4G24550	Forward	AGCATACACTGCGTGCAAAG
		Reverse	TCGCCTGTGTCACATATCTC
AUX1	AT2G38120	Forward	GAAGCCACCGTTCTTTATGC
		Reverse	GCTGCAGCTGGTTTACATTG
LAX3	AT1G77690	Forward	TTGTGAGGAGGGCATTGGTA
		Reverse	GGAGAGGCCACCGAGAGT
ABCB1	AT2G36910	Forward	CGACGGGAAAGACATAAGGA
		Reverse	CTGGTAGCGCGGATATGAAT
ABCB4	AT2G47000	Forward	CGTGTCGTGCTTTCCTTTCT
		Reverse	ATCCAAAGGTGTTGCTGCTC
ABCB19	AT3G28860	Forward	CCAGCAAGAGGGTCGTAAAA
		Reverse	GAGACGATCCGTGGAGACAT
PIN1	AT1G73590	Forward	TACTCCGAGACCTTCCAACTACG
		Reverse	TCCACCGCCACCACTTCC
PIN2	AT5G57090	Forward	CCTCGCCGCACTCTTTCTTTGG
		Reverse	CCGTACATCGCCCTAAGCAATGG
PIN3	AT1G70940	Forward	GAGGGAGAAGGAAGAAAGGGAAC
		Reverse	CTTGGCTTGTAATGTTGGCATCAG
PIN4	AT2G01420	Forward	TTGTCTCTGATCAACCTCGAAA
		Reverse	ATCAAGACCGCCGATATCAT
PIN7	AT1G23080	Forward	CGGCTGATATTGATAATGGTGTGG
		Reverse	GCAATGCAGCTTGAACAATGG
LDB29	AT3G58190	Forward	GCACATTGGCATTAGGCTTT
		Reverse	CTCAATCCCCACCTCAGCTA
TAR2	AT4G24670	Forward	CAGATTTGGCTTACTATTTGGCCACAG
		Reverse	GTCTTTCACCAAAGCCCATCCCAATC