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Multiple mechanisms of nitrate sensing by *Arabidopsis* nitrate transceptor NRT1.1

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In *Arabidopsis* the plasma membrane nitrate transceptor (transporter/receptor) NRT1.1 governs many physiological and developmental responses to nitrate. Alongside facilitating nitrate uptake, NRT1.1 regulates the expression levels of many nitrate assimilation pathway genes, modulates root system architecture, relieves seed dormancy and protects plants from ammonium toxicity. Here, we assess the functional and phenotypic consequences of point mutations in two key residues of NRT1.1 (P492 and T101). We show that the point mutations differentially affect several of the NRT1.1-dependent responses to nitrate, namely the repression of lateral root development at low nitrate concentrations, and the short-term upregulation of the nitrate-uptake gene *NRT2.1*, and its longer-term downregulation, at high nitrate concentrations. We also show that these mutations have differential effects on genome-wide gene expression. Our findings indicate that NRT1.1 activates four separate signalling mechanisms, which have independent structural bases in the protein. In particular, we present evidence to suggest that the phosphorylated and non-phosphorylated forms of NRT1.1 at T101 have distinct signalling functions, and that the nitrate-dependent regulation of root development depends on the phosphorylated form. Our findings add to the evidence that NRT1.1 is able to trigger independent signalling pathways in *Arabidopsis* in response to different environmental conditions.

Plants are sessile organisms that face dramatic fluctuations of external mineral nutrient availability. Thus, they have developed sophisticated nutrient sensing systems, which activate physiological and developmental responses that prevent nutrient deficiency or toxicity. To date, most of nutrient sensing systems remain unknown at the molecular level[1]. One exception is the sensing of nitrate (NO_3^-), which relies largely on 'transceptors', that is membrane NO_3^- carriers fulfilling a dual transport/sensing function[2–6]. The most documented NO_3^- transceptor is NRT1.1 of *Arabidopsis thaliana*[7–9], also called CHL1 (ref. 6) or NPF6.3 (ref. 10). Initially characterized as a dual-affinity transporter involved in root uptake and root-to-shoot translocation of NO_3^- (refs 11,12), NRT1.1 was later shown to mediate an impressive number of responses to NO_3^- (ref. 8), among which three have been extensively investigated. First, NRT1.1 is required for the 'primary nitrate response' (PNR), that is the short-term regulation of gene expression upon first NO_3^- supply. This includes the fast induction of many genes of the NO_3^- assimilation pathway such as *NRT2.1*, which encodes a main component of the root NO_3^- uptake system[13–15]. Second, NRT1.1 is also responsible in the longer term for an opposite response of some of these genes (for example *NRT2.1*) that are feedback repressed by high nitrogen provision[13,16]. Third, NRT1.1 was shown to promote root branching in response to NO_3^- (refs 17,18), in particular by repressing the emergence of lateral root primordia (LRP) at low NO_3^- availability[19].

How NRT1.1 can control such a large palette of responses to NO_3^- is not understood. Two different models have been proposed. On the one hand, the signalling role of NRT1.1 in the PNR was reported to be independent of the transport function of the protein. Indeed, the PNR is unaffected by P492L substitution (*chl1-9* mutation) that suppresses NRT1.1 NO_3^- transport activity[14]. Furthermore, the PNR is negatively affected by phosphorylation of NRT1.1 at the T101 residue[14], suggesting that it is the non-phosphorylated form that is predominantly active in NO_3^- signalling. On the other hand, the NRT1.1-dependent repression of LRP emergence at low NO_3^- availability was explained by auxin transport activity of NRT1.1[19]. We proposed that NRT1.1 mediates NO_3^--inhibited auxin transport that exports the hormone out of the LRPs at low NO_3^- concentration, thus preventing the auxin accumulation in these organs that is required for their proper development[20].

To date, there is no link between these two NRT1.1 signalling models. In addition, the mechanisms involved in the NRT1.1-dependent feedback repression of gene expression by high nitrogen remain totally obscure. Using *Arabidopsis* NRT1.1^{P492L} mutant and genotypes expressing the NRT1.1^{T101A} or NRT1.1^{T101D} mutants in an *NRT1.1* knockout mutant background[14], we show here that NRT1.1 modulates LRP development and triggers gene feedback regulation by high nitrogen through mechanisms that are independent of those involved in the PNR, and that the phosphorylated form of NRT1.1 also plays a crucial role in NO_3^- signalling and NO_3^--dependent auxin transport.

Results

NRT1.1-dependent regulation of lateral root development. The repression exerted by NRT1.1 on lateral root development at low external NO_3^- availability is evidenced by the markedly increased lateral root density and length in the *chl1-5* knockout mutant of *NRT1.1* compared with the Col wild type (WT) in NO_3^--free medium, whereas this difference is abolished or dramatically reduced on medium containing 1 mM NO_3^- (Fig. 1a–c). We have previously shown that it is NO_3^- *per se* that modulates the repressive action of NRT1.1, because supply of another nitrogen source such as glutamine does not mimic the effect of NO_3^- (ref. 19). Both genotypes *chl1-9* and T101A expressing the NRT1.1^{P492L} mutant (lacking NO_3^- transport activity[14]) or the non-phosphorylable NRT1.1^{T101A} mutant displayed a *chl1-5* phenotype, whereas T101D plants expressing the phosphomimetic NRT1.1^{T101D} mutant showed a WT phenotype (Fig. 1a–c). These results indicate that P492L and T101A but not T101D

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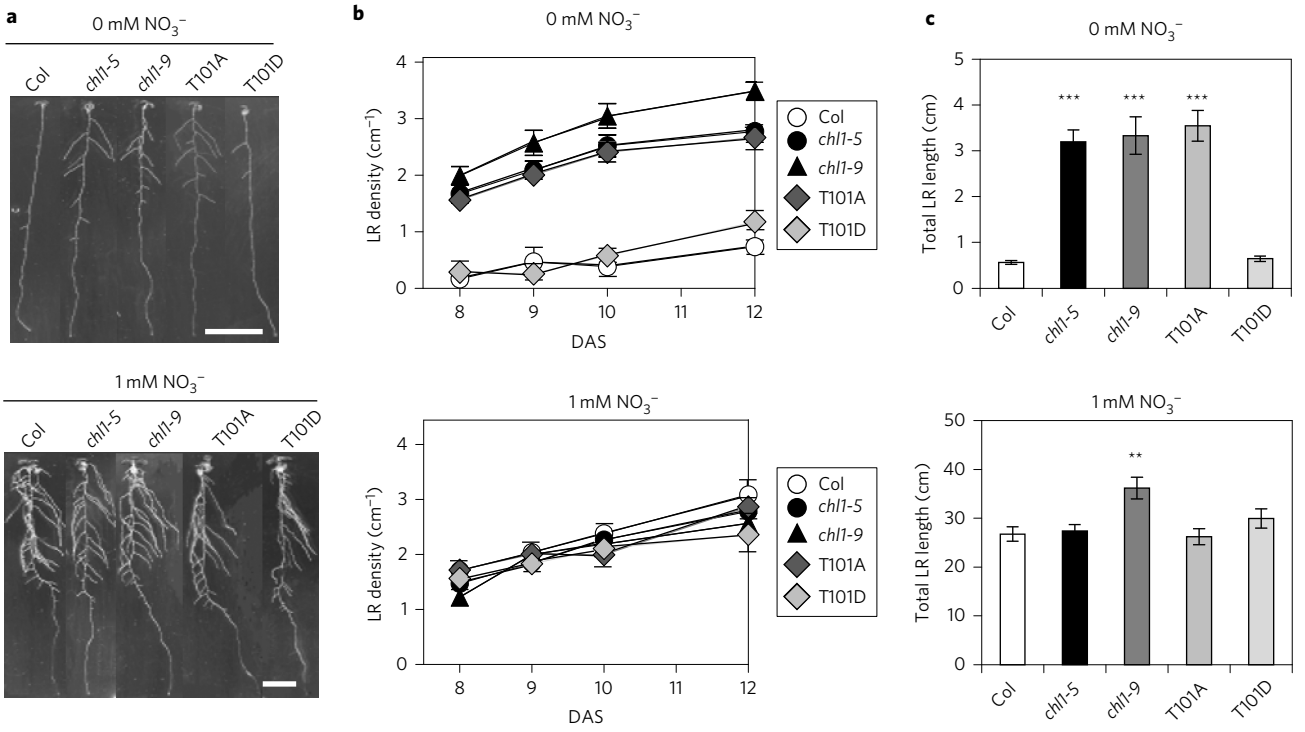

Figure 1 | Point mutations in the NRT1.1 nitrate transceptor differentially alter the nitrate regulation of lateral root growth. NRT1.1-dependent inhibition of lateral root development in the absence of NO_3^- is defective in *chl1-5*, *chl1-9* and T101A plants, but not in T101D plants. **a**, Plants grown for 12 days on NO_3^--free medium or on medium containing 1 mM NO_3^-. **b**, Lateral root density (number of visible lateral roots per cm of primary root) between 8 and 12 days after sowing (DAS). **c**, Total lateral root length at day 12. Data (n = 30–50) are mean ± s.e. from three independent experiments. Differences from the WT (Col) are statistically significant at $^{**}P < 0.01$ or $^{***}P < 0.001$. Scale bars, 1 cm.

substitutions prevented the repressive role NRT1.1 in lateral root development, thus indicating that it is the phosphorylated form of NRT1.1 that is responsible for the NRT1.1-dependent regulation of lateral root development.

NRT1.1^{T101D} transports auxin as the wild-type NRT1.1 protein. We have previously proposed that NRT1.1 represses LRP emergence through its auxin transport capacity[19]. The identification of point mutations in NRT1.1 that suppress this repression (such as P492L and T101A, Fig. 1) offers an important opportunity to validate this hypothesis. Therefore, the mutated forms of NRT1.1 were investigated for their auxin influx activity in *Xenopus* oocytes using 2,4-dichlorophenoxyacetic acid (2,4-D) as a substrate and a surrogate to indole-3-acetic acid (IAA)[21]. As reported previously with ^{3}H-IAA, oocytes injected with WT NRT1.1 cRNA displayed a 75% increase in ^{3}H-2,4-D accumulation compared with the control (Fig. 2a). Injection with NRT1.1^{T101D} cRNA yielded the same outcome, but injection with NRT1.1^{T101A} cRNA resulted in a markedly lowered ^{3}H-2,4-D accumulation, whereas expression of NRT1.1^{P492L} failed to increase it over that recorded in control oocytes (Fig. 2a). The facts that NRT1.1^{P492L} does not transport auxin and that NRT1.1^{T101D} has a markedly higher auxin transport capacity than NRT1.1^{T101A} were confirmed by ^{3}H-IAA uptake assays in tobacco BY-2 cell lines stably transformed with the corresponding genes under the control of an oestradiol-inducible promoter (Fig. 2b and Supplementary Fig. 1). Therefore, we confirm that the mutations preventing or dramatically reducing NRT1.1-mediated auxin transport (P492L and T101A substitution) are also those altering NRT1.1-dependent regulation of LRP emergence (Fig. 1). Furthermore, these data suggest that the P492L substitution not only suppresses transport of NO_3^- by NRT1.1 but also transport of auxin, and that it is the phosphorylated form of NRT1.1 that is predominantly active in auxin transport.

NRT1.1^{T101D} modulates auxin gradient in LRP as wild-type NRT1.1. Next, we investigated the effect of the point mutations on the auxin gradients in LRPs. Indeed, we have previously proposed that NRT1.1 represses emergence of LRPs because its auxin transport activity prevents auxin accumulation in these organs, in the absence but not in the presence of NO_3^- (ref. 19). To further document this point, we introduced the *DR5*::*GFP* auxin biosensor in the various mutant lines. As expected from the very low auxin transport capacity recorded for NRT1.1^{P492L} and NRT1.1^{T101A}, *chl1-9* × *DR5*::*GFP* and *T101A* × *DR5*::*GFP* plants behaved like *chl1-5* × *DR5*::*GFP* plants and exhibited strongly enhanced *DR5* activity (up to 300%) in LRPs compared with WT or T101D backgrounds in the absence, but not in the presence, of NO_3^- (Fig. 2c). Similar results were obtained with independent lines expressing the *DR5*::*GUS* transgene (Supplementary Fig. 2). These results are consistent with the hypothesis that NRT1.1^{P492L} and NRT1.1^{T101A} mutants are unable to prevent auxin accumulation in LRPs at low NO_3^-, and confirm that the effect of NRT1.1 on the local auxin gradients in LRPs is associated with, and most probably due to, its auxin transport activity.

P492L prevents plasma membrane localization of NRT1.1. Altogether, the above results are all in agreement with the conclusion that, due to its capacity to transport auxin, it is the phosphorylated form of NRT1.1 that actually represses LRP development at low NO_3^- by preventing auxin accumulation in these organs. To further understand why both NRT1.1^{P492L} and NRT1.1^{T101A} fail to fulfil this function, we checked whether P492L and T101A mutations led to lack of expression or mistargetting of the mutant proteins, by expressing oestradiol-inducible

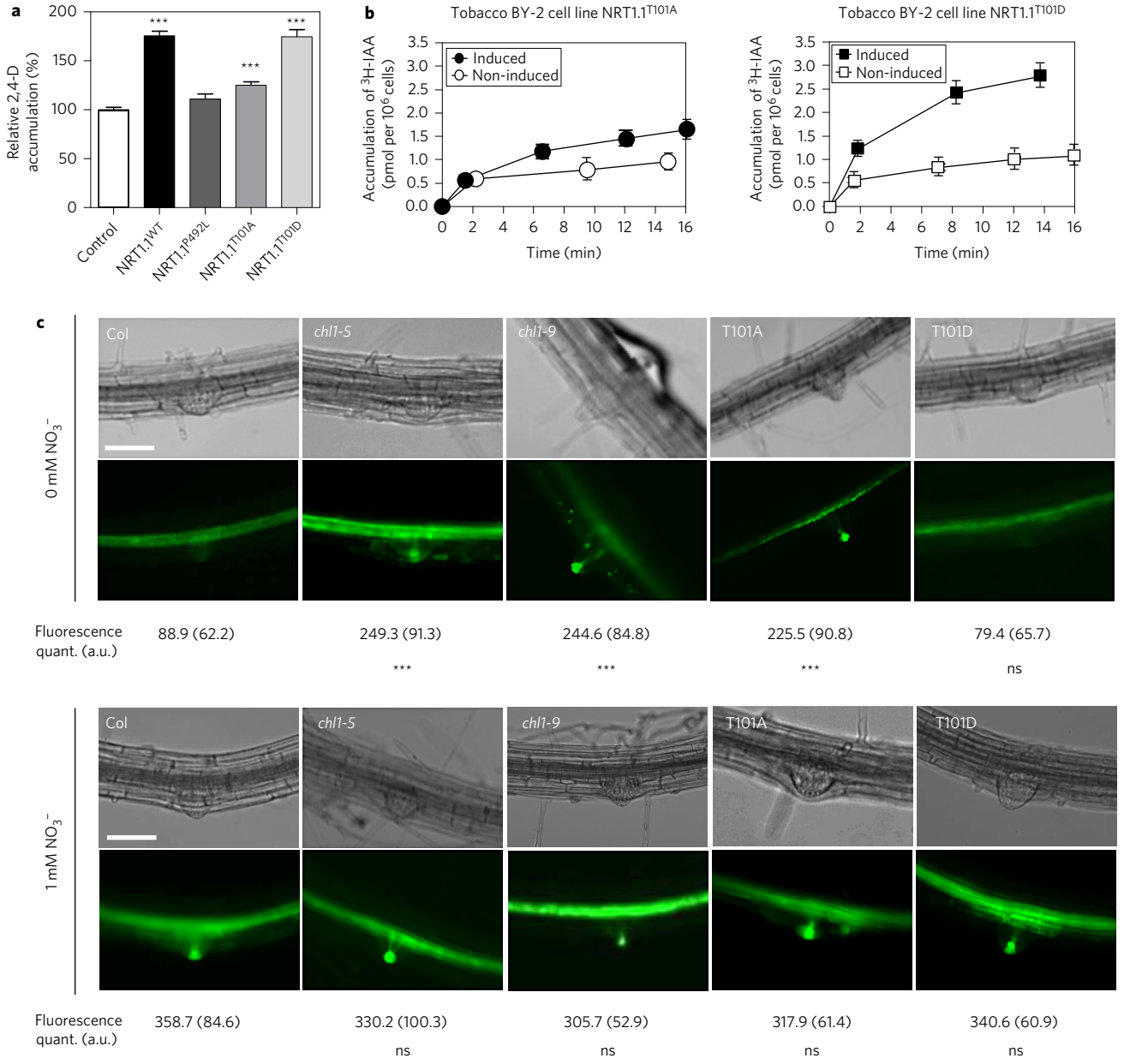

Figure 2 | P492L and T101A mutations alter the auxin transport activity of NRT1.1 and auxin gradients in lateral root primordia. a, ^{3}H-2,4-D uptake in NRT1.1^{WT}, NRT1.1^{P492L}, NRT1.1^{T101A} and NRT1.1^{T101D} cRNA- injected and control *Xenopus* oocytes supplied with 1 μM ^{3}H-2,4-D. The data ($n > 25$) (mean ± s.e.) are normalized to the control oocytes (uninjected) from three independent experiments. **b**, ^{3}H-IAA uptake in tobacco BY-2 cells expressing either NRT1.1^{T101A} or NRT1.1^{T101D} under the control of an oestradiol-inducible promoter and supplied with 2 nM ^{3}H-IAA. Data are the mean ± s.e. of four independent experiments. **c**, GFP expression pattern in LRPs of 8-day-old plants expressing *DR5::GFP* in Col-0, *chl1-5*, *chl1-9*, T101A or T101D genetic backgrounds, in the absence or presence of NO_3^- (1 mM). Scale bar, 100 μm. Fluorescence quantification values (n = 20–30 stage VI–VIII primordia) are the mean (with s.e. in parentheses) of 12–20 plants from two independent experiments. Differences with Col-0 are statistically significant at ***$P < 0.001$. ns, not significantly different.

NRT1.1–mCherry translational fusions in both *chl1-5* mutant plants and tobacco BY2 cells. All four proteins were expressed in LRPs at a similar level (Fig. 3a). However, unlike the other fusion proteins that were correctly addressed at the plasma membrane, NRT1.1^{P492L}–mCherry displayed a predominant intracellular localization in both root and BY2 cells (Fig. 3b,c). These data provide a relevant explanation for the lack of transport activity recorded for NRT1.1^{P492L} with both NO_3^- and auxin as substrates. They also indicate that reduced auxin transport by NRT1.1^{T101A} is not due to mistargetting, as also suggested by its high NO_3^- transport capacity[22], but is probably an intrinsic property of the non-phosphorylated form of the protein.

NRT1.1-dependent regulation of *NRT2.1* expression. It has been demonstrated[14] that both NRT1.1^{T101A} and NRT1.1^{P492L} can trigger NRT1.1-dependent short-term induction of *NRT2.1* expression by NO_3^- (illustrative of the PNR), whereas NRT1.1^{T101D} only activates a restricted response. Most surprisingly, we found that the other aspect of the NRT1.1-dependent regulation of *NRT2.1* expression, that is its long-term repression under high nitrogen supply, was affected by the point mutations of NRT1.1 in a totally different manner (Fig. 4). As previously shown[13,16], the *NRT2.1* mRNA level was much higher in the *chl1-5* knockout mutant than in Col when grown on 10 mM NH_4NO_3 (Fig. 4a). Both P492L and T101A substitutions

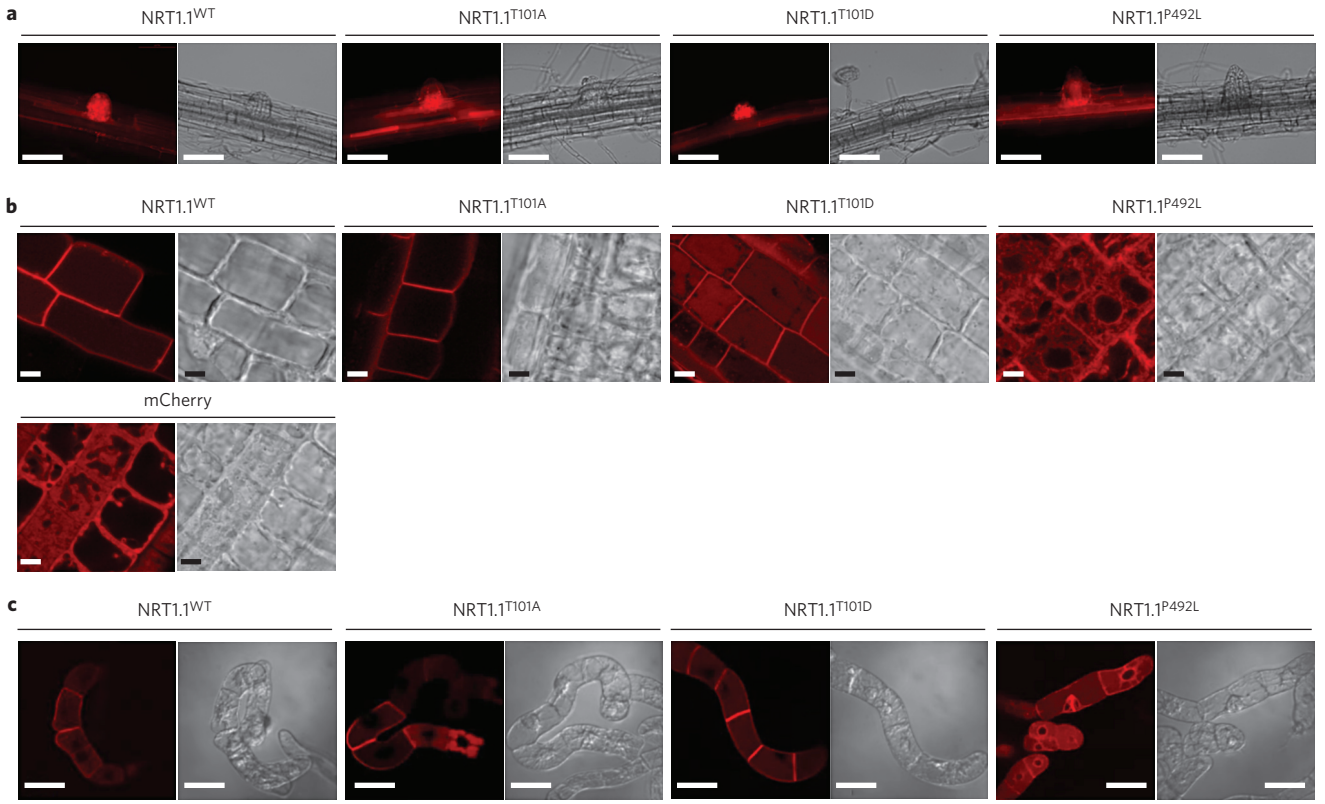

Figure 3 | The NRT1.1 point mutant proteins are expressed in lateral root primordia and properly addressed at the plasma membrane except for NRT1.1^{P492L}. **a**, mCherry expression pattern in lateral root primordia of transgenic lines expressing NRT1-1–mCherry, NRT1-1^{T101D}–mCherry, NRT1-1^{T101A}–mCherry or NRT1-1^{P492L}–mCherry protein fusions under the control of an oestradiol-inducible promoter in *chl1-5*. **b**, Subcellular localization of the different fusion proteins and mCherry protein in epidermal plant root cells. **c**, mCherry expression patterns of the different fusion proteins under the control of an oestradiol-inducible promoter in tobacco BY-2 cells. Scale bars, 100 μm (**a**), 10 μm (**b**) and 50 μm (**c**).

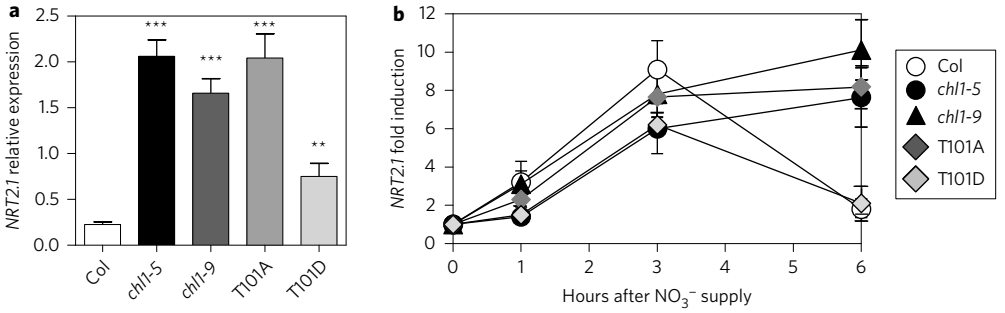

Figure 4 | Point mutations in the NRT1.1 nitrate transceptor differentially alter the regulation of *NRT2.1* expression in roots in response to nitrogen treatments. **a**, Long-term feedback repression of *NRT2.1* expression in roots by high nitrogen supply is lost in *chl1-5*, *chl1-9* and T101A, but not in T101D plants. Plants were grown for 8 days on 10 mM NH_4NO_3. The data are mean ± s.e. from five independent experiments. Relative *NRT2.1* mRNA levels are normalized to that of *CLATHRIN*. Differences with Col WT are statistically significant at $^*P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$. **b**, The feedback repression of *NRT2.1* is observed as early as 6 h after high NO_3^- supply in the WT and T101D plants, but not in *chl1-5*, *chl1-9* and T101A plants. Plants were grown for 12 days on a NO_3^--free medium containing 2.5 mM NH_4^+-succinate, and supplied with KNO_3 10 mM for 1 h, 3 h or 6 h. The data are mean ± s.e. from three independent experiments.

phenocopied knockout mutation of *NRT1.1* and prevented repression of *NRT2.1* expression by high nitrogen. In comparison, T101D mutation did not markedly alter this repression and only slightly increased *NRT2.1* mRNA level over that recorded in WT (Col) plants (Fig. 4a). To determine when the shift between induction and later feedback repression of *NRT2.1* occurs and how point mutations of NRT1.1 affect it, we performed time course studies of *NRT2.1* transcript accumulation following the first supply of NO_3^- at a high concentration (10 mM). In agreement with a previous study[14], induction of *NRT2.1* after 1 h of NO_3^- treatment was stronger in Col, *chl1-9* and T101A than in *chl1-5* or T101D (Fig. 4b). However, the relative difference in *NRT2.1* transcript accumulation between these genotypes was much lower after 3 h, and *NRT2.1* expression was markedly downregulated as early as 6 h after NO_3^- provision in both WT and T101D plants, but not in *chl1-5*, *chl1-9* and T101A plants

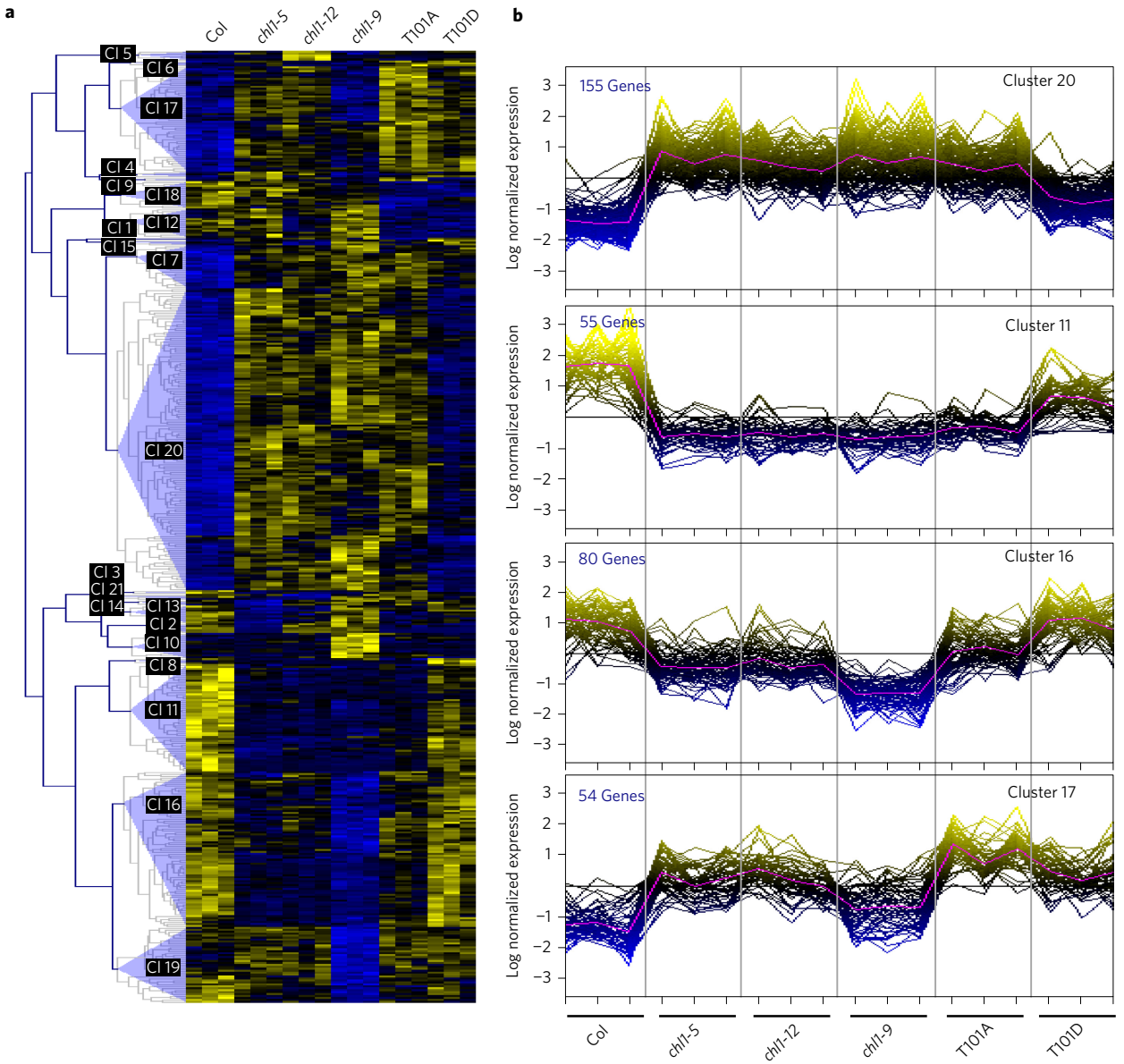

Figure 5 | Point mutations in the NRT1.1 nitrate transceptor affect different genome-wide responses to high nitrogen provision. Seedlings of the different lines (col, *chl1-5*, *chl1-12*, *chl1-9*, T101A, T101D) were grown on 10 mM NH_4NO_3 for 8 days and roots were collected to perform transcriptome analysis using ATH1 Affymetrix chips. The results were explored by a one-way ANOVA ($P < 0.001$) followed by a Tukey test ($P < 0.01$) and a clustering analysis. **a**, Clustering analysis of the differentially regulated probes. For each genotype, the three columns represent independent experiments. **b**, Transcript profiling for the genes of the four main clusters C20/C11/C16/C17 in the various genotypes. Each individual line plot represents one particular gene, with the three values recorded in three independent experiments for each genotype.

(Fig. 4b). This shows that NRT1.1-dependent repression of *NRT2.1* occurs soon after its initial induction, and that unlike the PNR, it requires the phosphorylated form of NRT1.1.

NRT1.1 regulation of transcriptome at high nitrogen. The unexpected finding that induction and repression of *NRT2.1* by NRT1.1 involves different mechanisms prompted us to get a genome-wide view of the transcriptome reprogramming triggered by the different forms of NRT1.1 under high nitrogen repressive conditions (10 mM NH_4NO_3). Three independent experiments were performed with the various genotypes (including *chl1-12*, an additional knockout mutant of *NRT1.1*). Statistical analysis highlighted 490 differentially regulated probes corresponding to 468 unambiguous genes (Supplementary Table 1, available at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?&acc=GSE49437), which could be organized in 21 clusters (Fig. 5 and Supplementary Fig. 3). Four clusters (11, 16, 17 and 20, Fig. 5b) were selected for further analysis on the basis of their size, expression pattern and over-represented functional categories. Cluster 20 gathers 155 genes that display *NRT2.1*-like expression, with upregulation in *chl1-5*, *chl1-12*, *chl1-9* and T101A genotypes compared with Col and T101D. Interestingly, the functional categories that are found to be enriched in this cluster relate to transport in general and to nitrogen, sulphur and selenium metabolisms (Supplementary Fig. 4). In particular, several nitrogen transport or assimilation genes, known to be nitrogen-regulated, such as *NRT1.7*, *NRT2.4*, *DUR3* and *GNL1.4*, belong to this cluster. Clusters 11 and 16 gather 135 genes that show a pattern of expression that is the opposite of *NRT2.1*, with downregulation in *chl1-5*, *chl1-12*, *chl1-9* and T101A genotypes compared with Col and T101D (Fig. 5b).

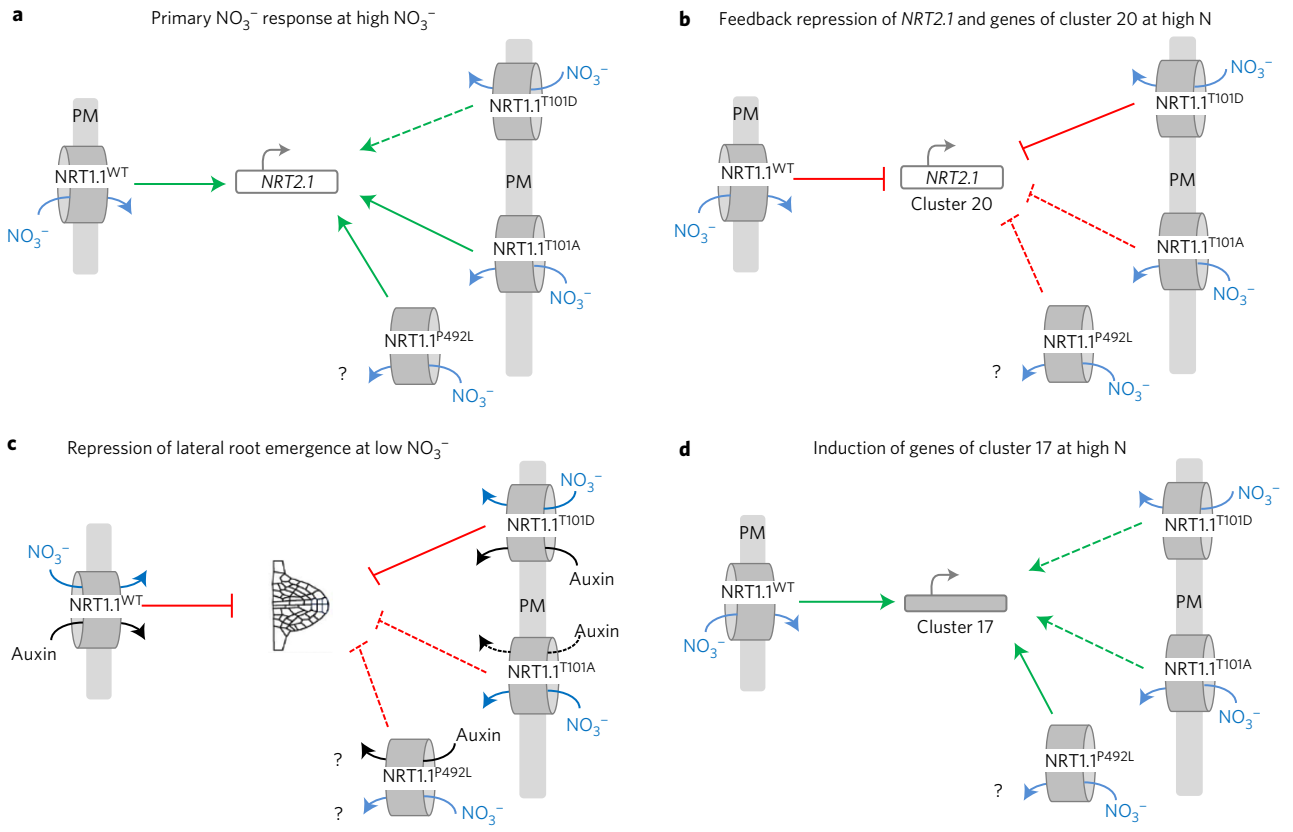

Figure 6 | Schematic representation of the four signalling 'modes' of NRT1.1. The four panels illustrate the NRT1.1-dependent responses to NO_3^- investigated in this work ($NRT1.1^{WT}$ is on the left side of each panel), and the effect of point mutations in NRT1.1 on these responses (right side of each panel). **a**, Primary nitrate response at high NO_3^- with induction of *NRT2.1* as a marker. **b**, Feedback repression of *NRT2.1* and genes of cluster 20 by high nitrogen concentration (the same pattern holds true for genes of clusters 11 and 16, but with induction instead of repression). **c**, Repression of lateral root emergence at low NO_3^-. **d**, Induction of genes of cluster 17 by high N (the same pattern holds true for genes of clusters 13, but with repression instead of induction). Green and red lines represent induction and repression, respectively. Plain lines represent normal responses as recorded for $NRT1.1^{WT}$. Dotted lines represent attenuated or suppressed responses as compared to $NRT1.1^{WT}$. $NRT1.1^{P492L}$ is not pictured in the plasma membrane to illustrate the predominant intracellular localization of the $NRT1.1^{P492L}$::mCherry protein. Auxin transport by NRT1.1 is only shown at low NO_3^- because this transport is inhibited at high NO_3^- (ref. 19).

Like cluster 20, clusters 11 and 16 show an over-representation of the transport functional category, but also in genes associated with environment interactions (Supplementary Fig. 4). Note that the NO_3^--inducible transcription factor gene *LBD39*, known to be a repressor of *NRT2.1* expression[23], was found in cluster 11. Finally, cluster 17 (54 genes) is striking because it displays an over-representation of genes related to extracellular matrix and secreted proteins as well as genes associated with oxydo-reduction reactions (Supplementary Fig. 4), and also because the pattern of expression has never been reported before, as it appears to correspond to a repressive effect of NRT1.1 that cannot be phenocopied by either $NRT1.1^{T101A}$ or $NRT1.1^{T101D}$ mutants. As such, this reveals a new role for NRT1.1 in the control of gene expression. Cluster 13 may also be representative of this role of NRT1.1 as it shows an expression pattern that is the opposite of cluster 17 (Supplementary Fig. 3).

We next investigated whether the main types of responses illustrated by the clusters shown in Fig. 5b could be associated with already known functions of NRT1.1 (for example regulator of NO_3^--responsive genes or auxin transport facilitator). Therefore, we quantified the overlap between clusters 11, 13, 16, 17 and 20 and transcriptome datasets reporting either the NO_3^- response in WT and nitrate reductase-null mutants[24], or the response to auxin[25]. Not unexpectedly, clusters 11, 16 and 20, which illustrate the same regulation behaviour (that is NRT1.1-dependent regulation phenocopied only by $NRT1.1^{T101D}$), display in common a highly significant overlap with the subsets of genes responding to NO_3^-, either in WT or NR-null mutants (Supplementary Table S1). Clusters 16 and 20 also show a significant overlap with the gene subset responding to auxin (the overlap is slightly above significance level for cluster 11). These data indicate that clusters 11, 16 and 20 are over-represented in genes regulated by signalling pathways known to be activated by NRT1.1. In contrast, clusters 13 and 17, illustrative of a totally different regulatory 'mode' (that is NRT1.1-dependent regulation not phenocopied by either $NRT1.1^{T101A}$ or $NRT1.1^{T101D}$ mutation), show no overlap with NO_3^--responsive genes, and no (cluster 13) or only a very weak overlap (cluster 17) with the gene subset regulated by auxin (Supplementary Table S1). This suggests that NRT1.1 acts on these two clusters through signalling mechanisms different from those described previously.

Discussion

In this work, we considered three different responses of the plant to NO_3^- that are dependent on NRT1.1 but occur under different experimental conditions: (1) the repression of lateral root development at low NO_3^- availability (Fig. 1), (2) the short-term induction of *NRT2.1* (Fig. 4b) that is representative of the PNR[14], and (3) the regulation of gene expression by high nitrogen that occurs in the longer term (Figs 4 and 5). Analysis of these responses in the various NRT1.1 point mutants unravels four different 'modes' of NRT1.1-dependent signalling (Fig. 6). Our data agree with those

reported previously[14], showing that the short-term induction of *NRT2.1* at high NO_3^- supply is negatively affected by T101D, but not by P492L and T101A substitutions (Fig. 6a). Furthermore, the long-term regulation of *NRT2.1* expression and of genes of clusters 20, 11 and 16 by high nitrogen (Fig. 6b), and the repression of lateral root emergence in the absence of NO_3^- (Fig. 6c) show the opposite pattern, with little or no effect of T101D substitution, and loss of signalling resulting from P492L or T101A mutations. Finally, clusters 13 and 17 (>60 genes), are under NRT1.1-dependent regulation that is suppressed by both T101A and T101D mutations, but not by P492L, indicating a yet uncharacterized signalling role of NRT1.1 (Fig. 6d).

The unexpected finding that P492L mutation prevents normal trafficking of NRT1.1, without affecting two of these regulatory 'modes' (PNR and regulation of the genes of clusters 13 and 17), suggests that NO_3^- signalling by NRT1.1 can partly occur independently of its localization and transport function at the plasma membrane. However, mistargetting of NRT1.1 due to P492L mutation raises the question of whether this substitution actually affects the intrinsic NO_3^- transport capacity of the protein. This point is important because apparent loss of NO_3^- transport by $NRT1.1^{P492L}$ was taken as evidence that transport and signalling functions of NRT1.1 can be uncoupled[14], thus supporting the transceptor model for this protein. Nevertheless, it is conceivable from our data that $NRT1.1^{P492L}$ can still transport NO_3^-, but that this ability does not lead to NO_3^- uptake into the cell, because $NRT1.1^{P492L}$ is not at the plasma membrane. In our opinion, these considerations are not sufficient to reject the transceptor model for NRT1.1, for at least two reasons. First, although nothing demonstrates that P492L supresses NO_3^- transport activity of NRT1.1, structural studies on this protein[26,27] provide some support for this hypothesis. Indeed, although P492 is not part of the NO_3^- recognition site, it is located in the short loop separating transmembrane domains 10 and 11. Therefore, its mutation is expected to alter the structural coordination of these two domains[26], and consequently the three-dimensional position of the E476 residue of transmembrane domain 10, which is crucial for the NO_3^- transport mechanism[27]. Second, P492L is not the only point mutation reported to uncouple transport and signalling functions of NRT1.1. Our own data (Fig. 6) and others[7] show that T101A substitution results in loss of NRT1.1-dependent NO_3^- signalling, although it does not prevent NO_3^- transport by NRT1.1[22].

The results obtained with the various mutants are fully consistent with our previous proposal that NRT1.1 regulates lateral root development through its auxin transport capacity[19]. Accordingly, point mutations of NRT1.1 that suppress or inhibit its auxin transport facilitation (P492L and T101A) (Fig. 2a,b) are also those leading to a misregulation of both *DR5* activity in LRPs (Fig. 2c) and emergence of LRPs in response to NO_3^- limitation (Fig. 1). Although it does not constitute a final proof, we believe this correlative evidence provides strong support for our proposal. In contrast, the mechanism involved in the NRT1.1-dependent long-term response to high nitrogen provision remains obscure, and there are no clues indicating that it is associated with the NO_3^-/auxin transport function of NRT1.1, or not. However, there is circumstantial evidence that the feedback repression of *NRT2.1* may be because NRT1.1 acts as an inducer of *LBD39*, a previously identified negative regulator of *NRT2.1* expression[23]. Accordingly, *GLN1.4*, which is also under negative control by LBD39 (ref. 23) is repressed by NRT1.1 in the same way as *NRT2.1* is. The validation of this hypothesis will nevertheless require further investigation.

Note that T101A and T101D mutations (that suppress and mimic T101 phosphorylation, respectively) inhibit different NRT1.1-dependent signalling responses (Fig. 6). This suggests that phosphorylated and non-phosphorylated forms of NRT1.1 have separate signalling roles. Previous studies have highlighted the crucial impact of T101 phosphorylation for switching between the high- and the low-affinity modes of the protein for NO_3^- transport[22]. Very recently, crystal structure studies have revealed that T101 phosphorylation controls dimerization and/or structural flexibility of NRT1.1[26,27]. Here, we show that T101 phosphorylation also directs the action of NRT1.1 towards the activation of specific responses (lateral root development and feedback regulation by high nitrogen), pointing out a crucial signalling function of the phosphorylated form of the protein (Fig. 6). The fact that neither $NRT1.1^{T101A}$ nor $NRT1.1^{T101D}$ can ensure the NRT1.1-dependent regulation of clusters 13 and 17 may appear highly surprising. However, several hypotheses may explain this observation. For instance, this regulation may be directly associated with the dynamic processes of phosphorylation/dephosphorylation of the T101 residue that are impaired by both T101A and T101D mutations. Alternatively, as NRT1.1 forms a dimer[27], it is theoretically possible that genes of clusters 13 and 17 are responsive to signalling by an NRT1.1 dimer with only one subunit phosphorylated, a structure that cannot be mimicked in T101A or T101D plants.

In conclusion, our work suggests that the wide range of responses to NO_3^- triggered by NRT1.1 may relate to different NO_3^- transduction mechanisms at the level of the NRT1.1 protein itself that can be separated by specific point mutations. This provides a generalization of observations made on transceptors in yeast[28]. For instance, the NH_4^+ transceptor Mep2 was reported to mediate two different signalling mechanisms, namely activation of the protein kinase A signalling pathway upon high NH_4^+ supply[29], and initiation of the so-called pseudo-hyphal growth in response to NH_4^+ limitation[30]. These two responses were uncoupled by point mutations in the protein that prevented pseudo-hyphal growth without affecting PKA activation[29]. Therefore, our work suggests that plants, like yeast, have developed versatile transceptors with multiple sensing/transduction mechanisms, able to activate independent responses, depending on the environmental conditions.

Material and Methods

Plant material. The *Arabidopsis thaliana* accession used was Columbia (Col-0). The various mutants and transformants were *chl1-5* (ref. 11) and *chl1-12* (SALK_034596) knockout *nrt1.1* mutants, *chl1-9* (ref. 14) a point mutant in which the proline 492 of NRT1.1 is replaced with a leucine, T101A and T101D transgenic lines expressing in *chl1-5* mutant background, modified forms of NRT1.1 in which the threonine 101 is mutated into alanine and aspartate[14]. Lines expressing *DR5*::*GFP* were obtained by crossing the *DR5*::*GFP* line[31] with *chl1-5*, *chl1-9*, T101A or T101D. Transgenic tobacco BY-2 cells and *Arabidopsis* lines expressing the various NRT1.1 forms fused with mCherry under the control of an oestradiol-inducible promoter are detailed in the Supplementary Material and Methods.

Plant culture and root growth analysis. Seedlings were grown and the root system architecture was analysed as described previously[19]. Basal culture medium was supplemented with the appropriate concentration of KNO_3, NH_4NO_3 or NH_4^+-succinate for each experiment as described in the figure captions.

RNA analysis. Total RNA was extracted from root samples using TRIzol reagent (Invitrogen Life Technologies SAS), and DNase was treated (Qiagen S.A.S. France) and purified using an RNeasy MinElute Cleanup Kit (Qiagen S.A.S. France). Reverse transcription was achieved with 4 µg of RNA in the presence of Moloney Murine Leukaemia Virus reverse transcriptase (Promega, France) after annealing with an anchored poly-$dT_{(18)}$ primer. Transcript accumulation 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 to the manufacturer's recommendations. Primers used were *CLATHRIN* F, 5′-AGCATACACTGCGTGCAAAG-3′; *CLATHRIN* R, 5′-TCGCCTGTGTCACATATCTC-3′; *NRT2-1* F, 5′-AACAAGGGCTAACGT-3′; and *NRT2-1* R, 5′-CTGCTTCTCCTGCTCATT-3′.

Microarray hybridization. Transcriptomes were realized using GeneChip Arabidopsis ATH1 Genome Array and the GeneChip 3′IVT Express kit (Affymetrix, Cleveland, OH, USA) according to the manufacturer's instructions. Further details are in the Supplementary Material and Methods.

Microscopy. Confocal imaging of *Arabidopsis* root cells expressing mCherry fusions were performed using a Leica SP8 Confocal Microscope (Leica, Germany). A laser (561 nm) was used for excitation. The emission light from 580 to 630 nm was

detected with a Leica 63× objective (HCX PL APO 1.4N.A). Green fluorescent protein (GFP) was excited at 470–510 BP filter and detected via a 500–535 BP filter (green) on an Olympus BH2 microscope. GFP quantifications were done with an Axiovert 200 M microscope (Zeiss, Germany) and coolsnapHQ camera (Photometrics Scientific, USA) and images analysed with MetaFluor V7.7.8.0 software (Molecular Device LLC Sunnyvale, USA). GFP was excited with a HC-495 filter and detected via a 525/45 BP filter (green). The data represent the fluorescence quantification values in a region of interest (corresponding to the primordium) from which the background fluorescence values measured in the part of the root not expressing DR5 were subtracted.

Auxin accumulation assays. Oocytes obtained from *Xenopus laevis* were treated and injected with cRNA ($NRT1.1^{WT}$, $NRT1.1^{T101A}$, $NRT1.1^{T101D}$, $NRT1.1^{P492L}$) as described previously[19]. Oocytes were incubated for 30 min in 1 ml of Ringer solution (pH 6) containing 1 µM 2,4-D (100 nM ^{3}H-2,4-D; ISOBIO, Fleurus, Belgium; diluted with 900 nM cold-2,4-D; Sigma-Aldrich, France) and treated and analysed as described previously[19]. Accumulation assay on BY-2 cells was performed[32] using ^{3}H-IAA (2 nM, radioactivity 20 Ci mmol^{-1}, American Radiolabelled Chemicals, USA).

Statistical analyses. Data are presented as mean ± s.e., and have been analysed using a one-way ANOVA, followed by a Tukey test.

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Author contributions

E.B., F.B., M.K., D.M., M.P., S.L., K.H. and P.N. performed experimental work; E.B., M.K., B.L., G.K., K.H., P.N. and A.G. performed data analysis; E.B., E.G., E.Z., P.N. and A.G. oversaw project planning; E.B., G.K., K.H., P.N. and A.G. wrote the paper.

Additional information

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Competing interests

The authors declare no competing financial interests.