Characterization of the Nrt2.6 Gene in Arabidopsis thaliana: A Link with Plant Response to Biotic and Abiotic Stress

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Abstract

The high affinity nitrate transport system in Arabidopsis thaliana involves one gene and potentially seven genes from the NRT1 and NRT2 family, respectively. Among them, NRT2.1, NRT2.2, NRT2.4 and NRT2.7 proteins have been shown to transport nitrate and are localized on the plasmalemma or the tonoplast membranes. NRT2.1, NRT2.2 and NRT2.4 play a role in nitrate uptake from soil solution by root cells while NRT2.7 is responsible for nitrate loading in the seed vacuole. We have undertaken the functional characterization of a third member of the family, the NRT2.6 gene. NRT2.6 was weakly expressed in most plant organs and its expression was higher in vegetative organs than in reproductive organs. Contrary to other NRT2 members, NRT2.6 expression was not induced by limiting but rather by high nitrogen levels, and no nitrate-related phenotype was found in the nrt2.6-1 mutant. Consistently, the over-expression of the gene failed to complement the nitrate uptake defect of an nrt2.1-nrt2.2 double mutant. The NRT2.6 expression is induced after inoculation of Arabidopsis thaliana by the phytopathogenic bacterium Erwinia amylovora. Interestingly, plants with a decreased NRT2.6 expression showed a lower tolerance to pathogen attack. A correlation was found between NRT2.6 expression and ROS species accumulation in response to infection by E. amylovora and treatment with the redox-active herbicide methyl viologen, suggesting a probable link between NRT2.6 activity and the production of ROS in response to biotic and abiotic stress.

Introduction

Nitrate uptake and nitrate distribution through the whole plant has been intensively studied during the last decade, particularly in the model plant Arabidopsis thaliana [1,2]. Physiological studies have led to the separation of the uptake process into two systems: the high affinity transport system (HATS) and the low affinity transport system (LATS), operating at low (<1 mM) or high (>1 mM) external nitrate concentrations, respectively [3]. The molecular organization of the uptake is however much more complex. Indeed, each system combines components inducible or non-inducible by nitrate and each component in turn is encoded by several genes belonging to multigenic families.

The NRT2 family includes 7 members in Arabidopsis that encode potential transporters with high affinity for nitrate. Of these 7 genes, NRT2.1 and NRT2.2 are mainly expressed in the root and participate in the high affinity influx of nitrate from soil into root cells [4]. Reverse genetics studies have shown the importance of NRT2.1 whereas NRT2.2 activity is only noticeable in the absence of NRT2.1 [5]. Their expression is strongly induced by low concentrations of nitrate [6] and at least NRT2.1 is also positively regulated by photosynthesis products [7]. On the other hand, nitrate uptake and NRT2.1 expression are severely inhibited when reduced nitrogen sources are provided such as ammonium or glutamine [8,9]. The NRT2.1 protein is localized on the plasma membrane [10,11]. The NRT2.4 protein is also localized on the plasma membrane and is thought to play a role in nitrate transport activity in the very high affinity range in both roots and shoots under N starvation [12]. In contrast to these transporters, the NRT2.7 gene is expressed very specifically in the seed, showing a peak of expression during later stages of seed maturation. The protein is localized on the tonoplast and seems to be responsible for the accumulation of nitrate in the vacuoles of seeds [13]. Currently, there is no functional data on other genes of the NRT2 family, NRT2.3–5–6. Expression analysis showed that the NRT2.5 gene is regulated in an opposite way to that of NRT2.1: as NRT2.4, it is expressed in the absence of nitrate and repressed by an additional exogenous nitrogen source, either as nitrate or ammonium [14]. This kind of regulation suggests that it could play a role in nitrate retrieving in response to limiting nitrogen supply. Indeed, the NRT1.7 gene coding for a low-affinity nitrate transporter, is positively regulated by nitrogen starvation and null
mutants showed growth retardation under starvation [15]. In contrast, the expression of the \textit{NRT2.3} and \textit{NRT2.6} is not affected in response to either nitrogen starvation or nitrogen re-supply, in roots and shoots [14].

Once entered the plant cell, nitrate is directed towards the vacuole to be stored or reduced into nitrite by the cytosolic enzyme nitrate reductase (NR). Then nitrite is translocated to the chloroplast where it is reduced into ammonium by the nitrite reductase (NIR), ammonium which is further incorporated into amino acids by the glutamine synthetase/glutamine synthase cycle. The link between nitrogen assimilation and plant response to microorganisms has been shown in symbiotic as well as in pathogenic interactions [16]. High concentrations of nitrogen often increase susceptibility of plants to disease and even the form of nitrogen available to plants and pathogens can affect the severity of the disease [17]. At the molecular level, bacterial and fungal genes that are induced in planta during infection are also induced \textit{in vitro} under nitrogen limiting conditions [18]. Among the complex defense mechanisms set up by plants in response to pathogen attacks [19], one major gene of the nitrate assimilation pathway has been shown to play also a key role in plant-pathogen interactions. The \textit{nia1 nia2} double mutant of Arabidopsis presents an impaired response to an avirulent strain of the bacteria \textit{Pseudomonas syringae} [20]. The nitrate reductase (NR) enzyme, coded by two \textit{Nia} genes in Arabidopsis was thought to produce a key signaling molecule, nitric oxyde (NO), through its associated nitrite-reducing activity [21]. However, it was further demonstrated that the NR activity was not essential for NO synthesis but as an important source of nitrite, through nitrate reduction, for subsequent NO production and plant resistance [20]. Another N-metabolite modified in the double mutant, the L-arginine, can also be used as endogenous substrate for NO synthesis but Oliveira and co-workers [22] showed that the susceptibility of \textit{nia1 nia2} double mutant to this \textit{Pseudomonas} strain did not result from a deficiency in amino acid content. Recently, the nitrate transporter \textit{NRT2.1} was also shown to play a role in the resistance against pathogens, linking further nitrate metabolism and plant resistance to biotic stress. Indeed, an \textit{nrt2.1} null mutant was found to be less sensitive to a virulent strain of \textit{P. syringae} pv. tomato [23].

Additionally to its induction after infection by the tumorigenic \textit{Agrobacterium tumefaciens} infection [24], \textit{NRT2.6} mRNAs accumulate also in response to interactions with a plant growth-promoting rhizobacterium (PGPR), which triggers beneficial effects both on plant growth and health [25]. A third bacterium, \textit{Erwinia amylovora}, was shown to have an effect on the \textit{NRT2.6} expression after inoculation in Arabidopsis leaves (CATdb database, urgv.evry.inra.fr/cgi-bin/projects/CATdb/catdb_index.pl). All these results prompted us to characterize the function of \textit{NRT2.6} as a nitrate transporter and its role in the plant, particularly in response to bacterial pathogens. We thus analyze precisely the expression profile of \textit{NRT2.6} and show that it is unable to complement a mutant affected in nitrate transport. However, we uncover an important role of the gene in plant response to pathogen attacks, possibly through the accumulation of reactive oxygen species (ROS).

\section*{Results}

\textbf{The NRT2.6 Gene is Weakly Expressed in Many Organs}  
\textit{NRT2.6} expression profile was detected by quantitative RT-PCR (Figure 1A). Plants were grown in the green house and fed with a standard nutrient solution [26]. The gene is weakly expressed in all plant organs but its expression seems to be slightly higher in vegetative parts (roots and rosette leaves) than in reproductive parts (cauline leaves, stems, siliques and flowers). To confirm these results, we transformed plants with a construct containing the \textit{uidA} reporter gene under the control of \textit{NRT2.6} promoter (Figure 2). Despite the weak expression found by RT-qPCR in all organs, the blue coloration is readily detectable only in lateral roots (Figure 2B) and in the collar of young plantlets, the region between radicle and hypocotyl where roots hair grow (Figure 2A). The gene is also expressed in inflorescences but the level of \textit{uidA} expression is dependent on the anther developing stage (Figure 2C). To investigate more precisely the cellular localization of \textit{NRT2.6} mRNAs, we performed histochemical studies of anthers and found that the blue coloration is very specific of the specialized tapetal cells (Figure 2D). This layer surrounding the locule is transient and allows pollen development [27]. This highly specific localization led us to investigate the potential effect of a mutation in the \textit{NRT2.6} gene on the transmission of male gametophyte to the progeny and pollen viability. However, no difference concerning these two parameters was detected between a null mutant (see below) and the wild-type (Figure S1).

In order to investigate the effect of nitrogen sources on gene expression, we grew plantlets during 4 days on different culture media containing nitrate, ammonium or glutamine as sole nitrogen sources or without nitrogen (Figure 1B). The gene expression was significantly lower (100 times less) \textit{in vitro} grown young plantlets compared to sand conditions (Figure 1, A and B). The expression of \textit{NRT2.6} was weak and similar in the absence of nitrogen and in low nitrate condition (1 mM). This expression increased as nitrate availability increased but the expression level was the same whether in full or very high nitrate supply (9 or 18 mM, respectively). When the nitrogen source was provided by ammonium or glutamine the \textit{NRT2.6} mRNAs levels were similar to those in low nitrate (1 mM) or in the absence of nitrogen. Thus, \textit{NRT2.6} expression seems to be induced by high levels of nitrate supply but, in contrast to other NRT2 members, is not sensitive to reduced nitrogen forms.

\textbf{Is the NRT2.6 Protein Able to Transport Nitrate?}

As \textit{NRT2.6} shares 67\% of homology with the \textit{NRT2.1} protein [28], we asked the question of whether or not a deficiency in the \textit{NRT2.6} could have also an effect on nitrate uptake. Among all the T-DNA collections, only one T-DNA insertion line was available. The \textit{nrt2.6-1} mutant in Columbia accession was obtained from the NASC center (NASC ID: N121890). The mutant was isolated from an insertional mutagenesis based on the maize En/Spm element [29]. In this mutant, one T-DNA copy was inserted in the beginning of the second exon (Figure S2). No expression of full-size cDNA was detected by RT-PCR (data not shown), demonstrating that it is a null mutant.

We first measured the nitrate contents in both genotypes. As shown in Figure 3A, the mutant accumulates the same nitrate contents than the wild-type whether it is grown \textit{in vitro} with 9 mM nitrate as the sole nitrogen source or on sand in the greenhouse with 10 mM nitrate (see Materials and Methods). We then measured the nitrate uptake capacity of the \textit{nrt2.6-1} mutant. Plants were grown under hydroponic culture conditions during 35 short days and fed with 0.5 mM NH\textsubscript{4}NO\textsubscript{3} and then 7 days with 0.2 mM NO\textsubscript{3}\textsuperscript{-} before the uptake experiment. Two concentrations of \textsuperscript{15}N were used: 0.2 and 6 mM \textsuperscript{15}NO\textsubscript{3}\textsuperscript{-} to measure nitrate uptake mediated by HATS and HATS + LAT5, respectively. The \textit{nrt2.6-1} mutant has exactly the same uptake as the Columbia wild-type genotype, whatever the concentration of \textsuperscript{15}NO\textsubscript{3}\textsuperscript{-} (Figure 3B).
Thus, the loss of NRT2.6 does not seem to modify NO$_3^-$ import by roots. The NRT2.1 protein is the main actor of root nitrate uptake mediated by the HATS and could prevent the detection of a smaller contribution. We thus crossed the nrt2.6-1 with the nrt2.1–nrt2.2 (Col) double mutant, isolated in the Columbia genetic background and affected in the AtNRT2.1 and AtNRT2.2 genes, and performed comparative analyses of influx capacities. As shown in figure 4, the triple mutant, nrt2.1-nrt2.2-nrt2.6 had the same HATS and LATS capacities as the nrt2.1-nrt2.2 double mutant. As the NRT2.6 is expressed only weakly in roots (Figure 1), we then overexpressed the protein in the nrt2.1-nrt2.2 (Ws) double mutant isolated in Ws genetic background [4] with the NRT2.6 coding sequence under the control of a strong promoter (35S) (Figure 4, A, B and C, underlined genotypes). Following 0.2 mM 15NO$_3^-$ supply, the three supplemented lines (SM) showed a significant decrease of HATS-mediated nitrate uptake in roots, about 21% in SM1, 30% in SM2 and 27% in SM3, in comparison to nrt2.1-nrt2.2. Conversely, with 6 mM 15NO$_3^-$ supply, two of the three supplemented mutants, SM2 and SM3, show a slight significant increase in HATS + LATS activities of 22% in comparison to nrt2.1-nrt2.2 genotype. When LATS activity was measured as the difference between root 15NO$_3^-$ influx measured at 6 mM and 0.2 mM, a slight increase was observed for SM genotypes in comparison to the mutant (Figure 4C). Thus, the NRT2.6 gene does not seem to be able to complement the nitrate uptake defect of nrt2.1-nrt2.2 mutant even when it is overexpressed in roots. Moreover, this overexpression led to a slight decrease in HATS capacity.

The Expression of NRT2.6 is Induced Upon E. amylovora Infection

CATMA microarray data from the public resource of Arabidopsis expression database CATdb (urgv.evry.inra.fr/cgi-bin/projects/CATdb/catdb_index.pl) show that the NRT2.6 gene responds to few stimuli. The strongest induction is found in response to E. amylovora infection. E. amylovora is a pathogenic bacterium causing fire blight disease on members of the rosaceae family such as apple and pear trees. Several of the host reactions have been also found in Arabidopsis in which E. amylovora triggers a type three secretion system (T3SS)-dependent cell death [30]. This interaction leads to leaf necrosis, which is correlated with bacterial growth (M Fagard, unpublished data). To confirm the public CATMA data, we checked the expression of NRT2.6 following E. amylovora inoculation (Figure 5). The NRT2.6 expression was induced by the bacteria as soon as 3 h post inoculation (hpi), and subsequently decreased. This short-term response can be compared with the expression of an early responsive gene to pathogen attack like Non-Host 1 [NHO1, 31].

Figure 1. Transcriptional regulation of NRT2.6 by nitrogen sources. A: NRT2.6 expression in 37 day-old Columbia plants grown in greenhouse on sand with 10 mM nitrate as sole nitrogen source (R: Roots, YL: Young leaves, OL: Old leaves, CL: Cauline leaves, St: Stem, Fl: Flowers, Si: Siliques). The values are means ±SD of three independent plants. B: NRT2.6 expression in plantlets grown on culture medium containing 1, 9 or 18 mM NO$_3^-$, 5 mM NH$_4^+$ or 5 mM glutamine (Gln) as sole nitrogen source or without nitrogen (−N). The values are means ±SD of 4 to 6 independent pools of plantlets.
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Role of NRT2.6 in Plant Response to E. amylovora

We further investigated the potential role of NRT2.6 during the plant’s response to E. amylovora inoculation using three different NRT2.6–related genotypes. As there was only one mutated allele in the Arabidopsis mutant libraries, we complemented the nrt2.6-1 mutant with the NRT2.6 coding sequence under the control of a strong promoter (35S). Two complemented mutants, CM1 and CM2, accumulating NRT2.6 mRNAs 126 and 238 times more than the wild-type respectively (Figure S3), were then analysed. Two days after E. amylovora inoculation, nrt2.6-1 exhibited significantly stronger symptoms than the wild-type (Col). These symptoms were correlated with a higher bacterial multiplication in the mutant than in the wild-type (Figure S4). In contrast to nrt2.6-1, the CM lines displayed wild-type levels of necrotic symptoms in response to the pathogen (Figure 6A). Therefore, the altered phenotype of the nrt2.6-1 mutant in response to E. amylovora infection can be attributed to the loss of NRT2.6 function since its overexpression in the two complemented mutant lines is able to reverse the phenotype.

It is known that in apple leaves, the infection by E. amylovora is associated with the activation of the expression of defense genes [32,33]. The same defense signaling pathways take place after E. amylovora inoculation in Arabidopsis [30]. To dissect the response of nrt2.6-1 mutant and CM lines to E. amylovora infection, we chose to study the expression of the following marker genes: AtrbohD (Respiratory Burst Oxidase Homologue D), known to be involved in ROS production, SID2 (Salicylic acid Induction Deficient 2), coding for an isochorismate synthase of the salicylic acid (SA) synthesis pathway, PR2 (Pathogenesis Related protein 2) also called BGL2 (β-Glucanase 2) which has been associated with programmed cell death (PCD), PR1 (Pathogenesis Related protein 1), an SA–induced gene and NHO1 (Non-Host 1). Figure S5 shows that the expression of NHO1, AtrbohD, PR2 and SID2 were found to be similar in all the genotypes, including the wild-type, 24 h post E. amylovora inoculation.

As the expression of defense genes did not change after E. amylovora inoculation in our genotypes, we measured cellular defense responses like callose deposition (Figure S6), nitric oxide (NO) production and ROS accumulation. We did not detect any significant difference between our genotypes for the first two traits. But, when we analyzed the H₂O₂ production at 18 hpi in inoculated leaves by DCFH-DA (2,7-Dichlorodihydrofluorescein diacetate) coloration (Figure 6B), a significant difference appeared between wild-type, mutant and CM lines (Figure 6C). The nrt2.6-1 mutant accumulated less H₂O₂ than Col whereas the H₂O₂ accumulation was stronger in the CM lines as compared to wild-type.

Therefore, although the expression of defense responsive genes was not modified in our genotypes following E. amylovora inoculation, one of the plant defense responses is modified: the...
in vitro with 9 mM NO\(_3^-\) complemented mutants. The values are means ± SD of 5 to 6 plants.

**Discussion**

Among the seven Arabidopsis NRT2 genes, the roles of NRT2.1 [5], NRT2.2 [5], NRT2.4 [12] and NRT2.7 [13] are clearly established and we were interested in the role of the other family members, particularly the NRT2.6. The closest gene to NRT2.6 (At5g45060) is NRT2.3, which shares 91% of nucleotide identity [28], but nothing is known on its nitrate transport capacity and its potential role in planta.

The NRT2.6 Protein is Unable to Complement a Nitrate Uptake Deficient Mutant

A double mutant with a well-characterized nitrate uptake deficiency mutant is a valuable tool to study the capacity of a protein to participate in the nitrate uptake process. For example, the activities of the very close proteins NRT2.1 and NRT2.2 were deciphered by transformation of the corresponding double mutant by a single gene (NRT2.1) [5]. The lack of mutant phenotype of nrt2.6-1 compared to wild-type for nitrate content and nitrate uptake could be easily explained by the very low expression of the gene in the root cells (Figure 1). However, in planta experiments using the nrt2.1-nrt2.2 double mutant failed to demonstrate a direct role in the nitrate transport process for the NRT2.6 protein. Indeed, over expression of NRT2.6 in the nrt2.1-nrt2.2 mutant did not bring any evidence of even partial complementation. Rather it led to a decrease in HATS activity (Figure 5), which could suggest a role in nitrate efflux. Two members of the NRT1 (NRT1.5 and NRT1.6) family have been shown to participate to nitrate efflux at the root plasma membrane [35,36]. The activity of NRT2.1 protein depends on the presence of a NAR2/NRT2.1 two-components complex at the plasma membrane [37] and one could imagine that the nitrate transport activity mediated by the NRT2.6 protein depends on a partner protein that would not be expressed enough in nrt2.1-nrt2.2 mutant background to ensure an efficient transport capacity. However, NAR2.1 expression level in the nrt2.1-nrt2.2 mutant is similar to wild-type level [38]. We also could not exclude that NRT2.6 protein might be involved in the transport of other molecules than nitrate, as it is the case for some NRT1 family members [39].

**Role of NRT2.6 in Plant Response to Oxidative Stress**

In addition to being produced during plant pathogen interactions, active oxygen species can be produced and accumulate after certain drug treatments. For example, methyl viologen, used as an herbicide, is a redox-active compound that generates superoxide anions in chloroplasts [34]. To test the response of the NRT2.6 modified genotypes to methyl viologen, we performed leaf inoculation of different drug concentrations (0.05, 0.1 and 0.25 μM) by syringe injections or reagent spraying. We found that H\(_2\)O\(_2\) was produced as soon as 30 min after inoculation in a methyl viologen dose-dependent manner and that spraying instead of syringe inoculation led to more reproducible results when compared to water-treated plants (data not shown). Eight leaves corresponding to 4 independent plants of each genotype were sprayed with either 0.1 μM of methyl viologen or water and DCFDA coloration was performed 3 h later as described in Materials and Methods (Figure 7A).

For all four genotypes, no more than one leaf showed faint fluorescence signals after water spraying. In contrast, in response to methyl viologen treatment, 25% and 12.5% of wild-type or mutant leaves showed high accumulation of ROS, respectively, while at least 50% of overexpressor leaves exhibited strong fluorescence after DCFH-DA coloration. These results were quantified by measuring staining intensities (Figure 7B) and statistically significant differences appeared between the two CM complemented lines and the nrt2.6-1 mutant.

Altogether, our data suggest that the function of NRT2.6 is positively correlated with the accumulation of H\(_2\)O\(_2\).

**NRT2.6 is Involved in Arabidopsis Response to E. amylovora**

The recognition of bacterial pathogens by receptors leads to MAP kinase activation, defense gene induction, callose deposition, synthesis of the defense hormone SA and production of ROS [40]. Arabidopsis is naturally resistant to E. amylovora: bacterial cells are only able to multiply weakly and transiently in Arabidopsis leaves and do not colonize non-inoculated tissue as they do in host plants [30]. However, E. amylovora is able to induce in inoculated leaves necrotic symptoms, which are correlated with bacterial growth [31,41], this work. Except for
MAP kinase activation which was not tested in this plant-pathogen interaction, all the mechanisms identified on host plants have been shown to take place in the Arabidopsis/E. amylovora interaction [30]. The \textit{NRT2.6} gene is expressed very early after bacteria inoculation but is not involved in the subsequent cascade of defense gene induction. Callose deposition was also not altered in the \textit{nrt2.6-1} mutant, showing that it is still able to recognize \textit{E. amylovora} and to set up a partial defense response.

ROS are known to play an important role in plant-pathogen interactions during which they are involved in both signaling and direct antimicrobial activities [42]. We used DCFH-DA to detect H$_2$O$_2$ and we found a negative correlation between ROS accumulation and bacterial multiplication as well as associated necrotic symptoms. Indeed, ROS accumulation was significantly reduced in the mutant, which showed an increased sensitivity to \textit{E. amylovora} infection (stronger symptoms and higher levels of bacteria). The level of plant sensitivity to bacteria can be compensated by the overexpression of \textit{NRT2.6} in the mutant background, demonstrating the role of the gene in the mutant phenotype. Therefore, our data suggest that ROS production detected by DCFH-DA in Arabidopsis could be correlated with defense against \textit{E. amylovora}.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure4.png}
\caption{Nitrate uptake in different genotypes. Mutants and supplemented mutants (SM) in Col or Ws (underlined) genetic backgrounds were grown in hydroponic culture on 0.5 mM NH$_4$NO$_3$ and transferred for 1 additional week to 0.2 mM NO$_3$$. Root influx was measured at both 0.2 and 6 mM $^{15}$NO$_3$ to provide estimation of HATS (A) and HATS + LATS (B) activities, respectively. LATS activities (C) were calculated as the difference between HATS + LATS and HATS. The asterisks indicate a statistically significant difference between the SM genotypes and their controls (\textit{nrt2.1-nrt2.2} mutants) (Test Fisher *$p$<0.05, ** $p$<0.01, ***$p$<0.001). doi:10.1371/journal.pone.0042491.g004}
\end{figure}
Is NRT2.6 Involved Only in H$_2$O$_2$ Accumulation?

H$_2$O$_2$ accumulation in nrt2.6-1 complemented mutant lines occurred in response to ROS generating treatments, whether they are biotic or abiotic such as methyl viologen spraying. Due to its toxic aspect, the steady-state levels of ROS must be tightly regulated in wild-type plants by a fine tuning between ROS-scavenging and ROS-producing proteins. In Arabidopsis, at least 152 genes are involved in this equilibrium [43]. For example, to disrupt the H$_2$O$_2$ balance, either the expression of genes involved in H$_2$O$_2$ production is enhanced or the expression of genes involved in H$_2$O$_2$ scavenging is inhibited. However, our current knowledge does not allow us to favor one of the two possibilities concerning the potential role of NRT2.6 regarding ROS homeostasis.

One can ask the question of whether or not NRT2.6 is involved in other ROS species accumulation? In particular, NO acts as endogenous mediator in different biological processes [44]. For example, the accumulation of NO-related species has been shown to occur intra- and extra-cellularly in tobacco cells in response to cryptogein exposure [45]. Although the understanding of the biosynthesis of NO in plants is still incomplete [46], the best characterized pathway of NO production in plants is through the activity of nitrate reductase (NR). Indeed, the NR deficient nia1 nia2 double mutant shows reduced NO [20]. We measured by DAF-2DA coloration method, which has been developed as a specific indicator for this molecule, the NO produced during E. amylovora/Arabidopsis interactions and found no significant difference between our different genotypes (data not shown). On the contrary, a clear difference appeared with the DCFH-DA coloration test, which allows the detection of intracellular H$_2$O$_2$ but also the detection of peroxynitrite, a toxic derivative of NO, in vitro [47]. In response to a transient NO-burst, the cross talk between NO and H$_2$O$_2$ production may have led to an elevated level of H$_2$O$_2$ and of peroxynitrite as well [48].

To further investigate the relationship between nitrate transporters and plant response to pathogens, it will be very interesting on one hand to test if other NRT2 members could be also involved in Arabidopsis-E. amylovora interaction. On the other hand, we would like now to explore the potential role of NRT2.6 protein in Arabidopsis interaction with other pathogens like P. syringae as it has been performed for NRT2.1 [23].

Materials and Methods

Plant Material and Growth Conditions

The nrt2.6-1 mutant (SM_3.35179) was obtained from the NASC center among a mutagenized population (SM lines transposon) of the Col8 Arabidopsis accession [29]. Homozygous mutant plants were identified by PCR using the forward primer SM-upper (5'-TCAAGCCACTATCATCGCTAAACC-3') and the reverse primer SM-lower (5'-ATCCCTCTCATGATAGCAGC-3').

Figure 5. Time course of NHO1 and NRT2.6 expression following E. amylovora inoculation. Plants were grown in culture chamber and leaves were inoculated with wild type E. amylovora strain (Ea) or with water (mock) as described in Materials and Methods. RT-qPCR analyses were performed on inoculated leaves 3, 6 or 24 h post inoculation (hpi). doi:10.1371/journal.pone.0042491.g005
the reverse specific SM lines transposon primer Spm32 (5′-TACGAATAAGAGCGTCCATTTTAGAGTGA-3′). The mutant was backcrossed two times with the wild type.

Plants were grown in vitro (16 h light/8 h dark) with a constant temperature of 25°C and a light intensity of 80 μmol m⁻² s⁻¹. Basic medium without nitrogen contained: 0.8% Bromo-Cresol Purple (BCP), 0.07% 2-(N-Morpholino) ethanesulfonic (MES) acid pH 6, 2.5 mM KH₂PO₄, 2 mM MgSO₄, 5 mM KCl, 2 mM CaCl₂, 554 μM myo-inositol, 2.1 μM calcium pantothenate, 8.12 M nicotinic acid, 5.9 μM pyridoxine-HCl, 3.32 μM thiamine-HCl, 0.4 μM biotin, 70 μM H₂BO₃, 14 μM MnCl₂, 0.5 μM CuSO₄, 10 μM NaCl, 1 μM ZnSO₄, 0.01 μM CoCl₂, 0.2 μM NaMoO₄, 5 μM Iron ammoniac citrate, 0.7% agarose (Kalys) and 1% sucrose. In the 1 mM NO₃ medium, KCl and CaCl₂ were lowered to 4.5 mM and 1.75 mM, respectively, and 0.5 mM KNO₃ and 0.25 mM Ca(NO₃)₂ were added. For the 9 mM and 18 mM NO₃ media, KCl was replaced by 5 mM and 10 mM KNO₃, and CaCl₂ was replaced by 2 mM and 4 mM Ca(NO₃)₂, respectively. For the NH₄⁺ and Gln media, 5 mM (NH₄)₂ succinate or 5 mM glutamine, respectively, were added to the basic medium without nitrogen.

In the greenhouse, plants were grown on sand [49] and fed with a solution of 10 mM NO₃⁻ containing 5 mM KNO₃, 2.5 mM Ca(NO₃)₂, 0.2 mM NaCl, 0.25 mM KH₂PO₄, 0.25 mM MgSO₄, 10 mg/L Fe-EDTA, 243 μM MoO₇(OH)₆, 0.4 μM H₃BO₃, 118 μM SO₄Mn, 10 μM de SO₄Cu and 34.8 μM SO₄Zn. Plants were sub-irrigated over 2 h three times per week.

RNA Extraction and Quantitative PCR (qPCR)

Total RNA was isolated using the Trizol Reagent procedure (Invitrogen). First-strand were synthesized according to Daniel-Vedele and Caboche [50] using M-MLV reverse transcriptase.
which N was supplied as 0.2 mM NO₃ during
the experiment, the plants were transferred to basic medium in
Nutrient solution was renewed every 2 d and, during the 2 first
changed daily. After 42 days, the plants were transferred first to
0.1 mM CaSO₄ for 1 min, then to basic nutrient solution
with primers NRT2.6 PGW5’ (5’-AAAAAGCAGGCTAAA-
GACCATCCCCATGAAAG-3’) and NRT2.6 PGW3’ (5’-AA-
GAAAAGCTGGTTGTAAATGGGAAGATGAG-3’). Am-
plification was performed using the Expand high-fidelity PCR
system (Roche), and the amplified fragment was cloned in front of
the GUS coding sequence in the pBI101 derived gateway vector
[54]. The binary plasmids were transferred to Agrobacterium
tumefaciens strain C58C1 (pMP90) by triparental mating. Wild
type Arabidopsis plants, were transformed according to the
in planta method using the surfactant Silwet L-77 [55]. Transgenic
plants were selected on Estelle and Sommerville media [56]
containing 50 mg.L⁻¹ of kanamycin.

The transgenic plants carrying the ProNRT2.6::uidA construct
were grown on horizontal plates [56] at 25 °C under long-day
conditions or in the greenhouse. The plants were observed under
a light microscope (Axioplan 2; Zeiss) after GUS staining [57]. For
histological analysis, samples were embedded in resin as already
described [58] and blocks were sectioned at 4 µm thickness using
a Leica RM 2165 microtome.

Generation of Complemented Lines
First primers AttB1-NRT2.6start (5’-GGAGATAGAAGAC-
CATGGCTCAACACCATTCTAATG) and AttB2-NRT2.6end-
stop (5’-TCCACCTCCCCGATGACATGAGCGGCCGGA-
GATCC-3’) were used to amplify a complete NRT2.6 cDNA from
roots of 33 day-old Columbia plants. PCR products were
obtained with the iProof High Fidelity PCR kit (Bio-Rad) and
amplified with the universal U3endstop (5’-AGATTTGGGAC-
CATTGTGACAAAGAAGTGGGTCTCCACTCCGGG-
GATCC-3’) and U5 primers (5’-GGGGAGTATTGTTGA-
AAAAAAGCGCTTCGAAAAGAGTATGAC-3’) to create the recombinant site AttB. The product of recombination reactions (BP reactions) was used to transform competent
Escherichia coli, strainTOP10 (Invitrogen), by heat shock. LR
clonase reactions to transfer T-DNA fragments from the entry
cloning site to the destination binary vector pMDC32 [53] were
performed. The vector pMDC32::NRT2.6 was generated and the
binary vector, containing the Pro35S::NRT2.6 construct was
(Gibco-BRL) and oligo (dT) 15 primers. The qPCR was
performed on a Mastercycler Realplex instrument (Eppendorf)
with the MESA FAST qPCR MasterMix Plus (Eurogentec). Each
reaction was performed on a 1/20 dilution of the first cDNA
strands in a total reaction of 20 µL. The primers used for qPCR
are listed in Table S1.

Influx Experiments
Plants were grown for 42 days under hydroponic culture
conditions on 0.5 mM NH₄NO₃ as previously described [52]
(8 h light at 21 °C, 150 µmol.m⁻².s⁻¹/16 h dark at 17 °C).
Nutrient solution was renewed every 2 d and, during the 2 first
weeks, used at half-strength. At the age of 5 weeks and 7 d before
the experiment, the plants were transferred to basic medium in
which N was supplied as 0.2 mM NO₃⁻, and the solution was
changed daily. After 42 days, the plants were transferred first to
0.1 mM CaSO₄ for 1 min, then to basic nutrient solution
containing 0.2 mM ¹⁵NO₃⁻ (atom% 15N:99%) or 6 mM
¹⁵NO₃⁻ for 5 min and finally to 0.1 mM CaSO₄ for 1 min. After
homogenization, an aliquot of the frozen powder was dried
overnight at 80 °C to calculate the total N and ¹⁵N content of the roots.

GUS Construction and Staining
Binary vectors containing uidA fusions with the NRT2.6 promoter were obtained using Gateway technology [53]. A
genomic Arabidopsis NRT2.6 region, starting from position -2000 bp upstream of the translation initiation site and terminating before the ATG codon, was amplified from Ws accession by PCR
with primers NRT2.6 PGW5’ (5’-AAAAAGCAGGCTAAA-
GACCATCCCCATGAAAG-3’) and NRT2.6 PGW3’ (5’-AA-
GAAAAGCTGGTTGTAAATGGGAAGATGAG-3’). Am-
plification was performed using the Expand high-fidelity PCR
system (Roche), and the amplified fragment was cloned in front of
the GUS coding sequence in the pBI101 derived gateway vector
[54]. The binary plasmids were transferred to Agrobacterium
tumefaciens strain C58C1 (pMP90) by triparental mating. Wild
type Arabidopsis plants, were transformed according to the
in planta method using the surfactant Silwet L-77 [55]. Transgenic
plants were selected on Estelle and Sommerville media [56]
containing 50 mg.L⁻¹ of kanamycin.

The transgenic plants carrying the ProNRT2.6::uidA construct
were grown on horizontal plates [56] at 25 °C under long-day
conditions or in the greenhouse. The plants were observed under
a light microscope (Axioplan 2; Zeiss) after GUS staining [57]. For
histological analysis, samples were embedded in resin as already
described [58] and blocks were sectioned at 4 µm thickness using
a Leica RM 2165 microtome.

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¹⁵NO₃⁻ for 5 min and finally to 0.1 mM CaSO₄ for 1 min. After
homogenization, an aliquot of the frozen powder was dried
overnight at 80 °C and analyzed using the ANCA-MS system
(PDZ Europa). Influx of ¹⁵NO₃⁻ was calculated from the total N
and ¹⁵N content of the roots.

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sequenced before transformation of A. tumefaciens. The nrt2.6-1 and nrt2.1-nrt2.2 double mutants were transformed with the pMDC32/NRT2.6 constructs by the in planta method using the surfactant Silwet L-77 [55] and transformants were selected on 20 mg L\(^{-1}\) of hygromycin B.

**E. amylovora Inoculations, Methyl-viologen Treatments and ROS Detection**

Arabidopsis plants were grown in the greenhouse as described [30] in an 8 h light/16 h dark cycle, at 19°C, with 70% relative humidity, using 10 mM NO\(_3\)- as nitrogen source during 5 weeks. Inoculations were performed by bluing a spray with the bacterial wild-type strain *E. amylovora* CFBP1430 [59] and the inoculum density was adjusted at 0.1 O.D. in water (10\(^2\) c.f.u.mL\(^{-1}\)). Symptom severity was scored according to a visual scale from 0 (no apparent necrosis) to 3 (necrosis of the whole leaf) as described in Degrave et al [30]. Bacterial growth was analyzed 24 h after inoculation as described by Degrave et al [30].

For methyl viologen treatments, two rosette leaves by plant were inoculated by one spray with a water solution containing different concentrations of methyl viologen (Acros-Organics, from 0.05 µM to 0.25 µM). Water atomization was used as a control and, 30 min after treatment, leaves were subjected to DCFH-DA coloration.

ROS detection method was adapted from Zhang et al [60]. At 18 h following half-leaf infiltration with *E. amylovora* or water, leaves were immersed in a 300 µM DCFH-DA (2,7-Dichlorodihydrofluorescein-diacetate) solution and vacuum-infiltrated. Whole leaf images were taken using an Olympus SZX12 binocular magnifier. Green fluorescence was detected with an HQ510 lp emission filter. Experiments were repeated twice and quantitative measurements were done by measuring mean gray levels of the green channel of each image by using ImageJ v1.46f (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011).

**Analysis of Callose Accumulation**

For callose detection, the leaves were inoculated as described above and collected 8 hpi. Callose deposits were detected using aniline blue as described in [30]. Experiments were repeated twice with similar results. Representative pictures are shown. The number of callose deposits per picture was determined using ImageJ (National Institute of Health, Bethesda, MD, U.S.A.) and compared using Mann and Whitney’s test (\(\alpha = 0.05\)). We analyzed 25 to 30 pictures corresponding to more than five independent leaves for each treatment.

**Supporting Information**

**Figure S1** Role of NRT2.6 in the tapetum. A: Transmission of T-DNA to the progeny. Mother plants grown in the greenhouse were fed with 10 mM nitrate until bolting and then 0.2, 2, 10 and 50 mM nitrate until seed maturation. Seeds were sown on agar medium containing basic medium with 9 mM NO\(_3\)- as sole nitrogen source. T-DNA or native gene was detected by PCR analyses. B: Pollen viability measured by Alexander test. Alexander test was performed on opened flowers from wild type and mutant [61].

**Figure S2** Structure of the transposon insertion in the nrt2.6-1 mutant.

**Figure S3** Levels of NRT2.6 expression in two complemented lines. Plants were grown under standard conditions in the greenhouse and transgene NRT2.6 expression was measured by RT-qPCR as described in Materials and Methods. An arbitrary value of 1 was given to NRT2.6 expression in Col.

**Figure S4** The nrt2.6-1 mutant supports higher bacterial multiplication of *E. amylovora* cells than wild-type plants. Bacterial count of *E. amylovora* in wild-type (Col) and mutant (nrt2.6-1) plants. The number of CFU present in leaf extracts was counted 24 h post inoculation. The asterisk indicates that the means are statistically different according to Mann and Whitney’s test (\(P \text{ value} < 0.05\)).

**Figure S5** Defense gene expression in response to infection by *E. amylovora*. Expression of marker genes was measured 24 h after *E. amylovora* inoculation. A 100% arbitrary value was affected to expression levels in Col.

**Figure S6** Callose accumulation in response to *E. amylovora* is not affected in the nrt2.6-1 mutant. Analysis of callose deposits in *E. amylovora*-inoculated wild-type (Col) and mutant (nrt2.6-1) plants. Leaves were collected 8 hpi and stained with aniline blue as described previously [31]. No significant difference in callose deposition could be observed between wild-type and mutant plants. A: Representative images are shown for each treatment. B: Experiments were repeated twice with similar results. The asterisks indicate that the means are statistically different between mock and Ea treatments according to Mann and Whitney’s test (\(P \text{ value} < 0.05\)). No statistical differences were found between wild-type and nrt2.6-1 mutant.

**Table S1** Sequences of oligonucleotides used in RT-PCR reactions. This table summarizes the sequences of oligonucleotides used in this study. For each targeted gene, the sequences of forward (F) primer and reverse (R) primer are given.

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**Author Contributions**

Conceived and designed the experiments: AK MF FDV. Performed the experiments: JD OP. Analyzed the data: JD MF FDV. Contributed reagents/materials/analysis tools: JD OP MF. Wrote the paper: JD AK MF FDV.

**References**

