

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.

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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 *NRT2.3* and *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 *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 *nia1 nia2* double mutant of Arabidopsis presents an impaired response to an avirulent strain of the bacteria *Pseudomonas syringae* [20]. The nitrate reductase (NR) enzyme, coded by two *Nia* genes in Arabidopsis was thought to produce a key signaling molecule, nitric oxide (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 *nia1 nia2* double mutant to this *Pseudomonas* strain did not result from a deficiency in amino acid content. Recently, the nitrate transporter 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 *nrt2-1* null mutant was found to be less sensitive to a virulent strain of *P. syringae* pv. tomato [23].

Additionally to its induction after infection by the tumorigenic *Agrobacterium tumefaciens* infection [24], *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, *Erwinia amylovora*, was shown to have an effect on the *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 *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 *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).

Results

The *NRT2.6* Gene is Weakly Expressed in Many Organs

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 *uidA* reporter gene under the control of *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 *uidA* expression is dependent on the anther developing stage (Figure 2C). To investigate more precisely the cellular localization of *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 *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) in *in vitro* grown young plantlets compared to sand conditions (Figure 1, A and B). The expression of *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 *NRT2.6* mRNAs levels were similar to those in low nitrate (1 mM) or in the absence of nitrogen. Thus, *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.

Is the NRT2.6 Protein Able to Transport Nitrate?

As NRT2.6 shares 67% of homology with the NRT2.1 protein [28], we asked the question of whether or not a deficiency in the 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 *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 *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 *nrt2.6-1* mutant. Plants were grown under hydroponic culture conditions during 35 short days and fed with 0.5 mM NH_4NO_3 and then 7 days with 0.2 mM NO_3^- before the uptake experiment. Two concentrations of ^{15}N were used: 0.2 and 6 mM $^{15}\text{NO}_3^-$ to measure nitrate uptake mediated by HATS and HATS + LATS, respectively. The *nrt2.6-1* mutant has exactly the same uptake as the Columbia wild-type genotype, whatever the concentration of $^{15}\text{NO}_3^-$ (Figure 3B).

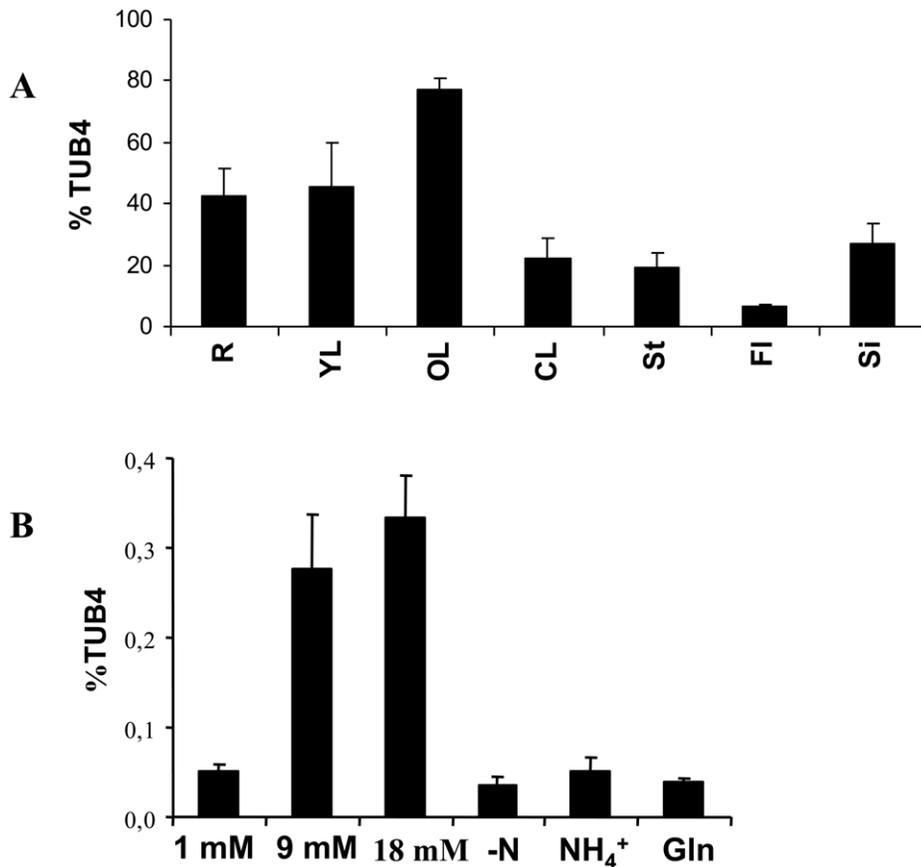


Figure 1. Transcriptional regulation of *NRT2.6* by nitrogen sources. **A:** *NRT2.6* expression in 37 day-old Columbia plants grown in greenhouse on sand with 10 mM nitrate as sole nitrogen source (R: Roots, YL: Young leaves, OL: Old leaves, CL: Cauline leaves, St: Stem, Fl: Flowers, Si: Siliques). The values are means \pm SD of three independent plants. **B:** *NRT2.6* expression in plantlets grown on culture medium containing 1, 9 or 18 mM NO₃⁻, 5 mM NH₄⁺ or 5 mM glutamine (Gln) as sole nitrogen source or without nitrogen (-N). The values are means \pm SD of 4 to 6 independent pools of plantlets.
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Thus, the loss of *NRT2.6* does not seem to modify NO₃⁻ 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 ¹⁵NO₃⁻ 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 ¹³NO₃⁻ 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 ¹⁵NO₃⁻ 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].

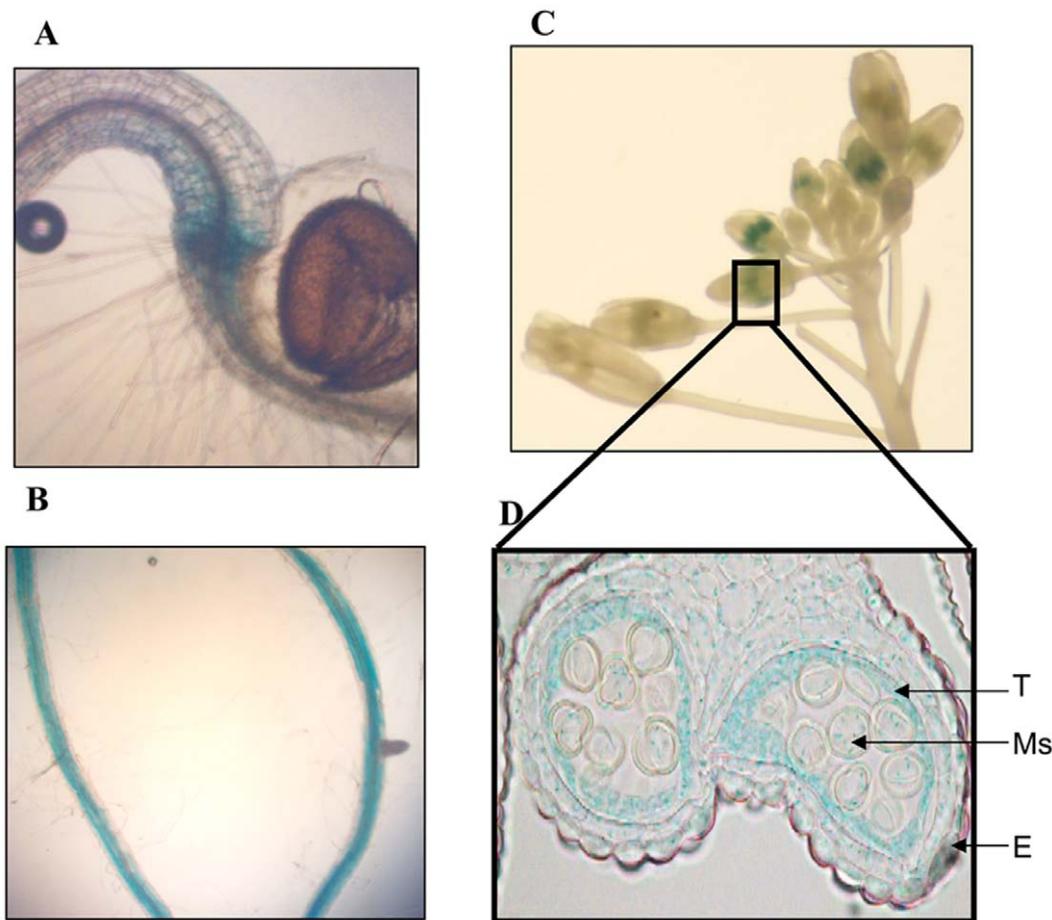


Figure 2. Transcriptional regulation of *ProNRT2.6::uidA* in different plant tissues. **A:** GUS staining of 7 day-old plantlets grown in vitro on culture medium with 9 mM NO_3^- . **B:** GUS staining of lateral roots of 25 day-old plants grown in greenhouse with 10 mM NO_3^- . **C:** GUS staining in inflorescences of plants grown in greenhouse with 10 mM NO_3^- . **D:** Longitudinal cross section of anther : T: tapetum, Ms: meiocytes, E: epidermis. doi:10.1371/journal.pone.0042491.g002

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 H_2O_2 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 H_2O_2 than Col whereas the H_2O_2 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

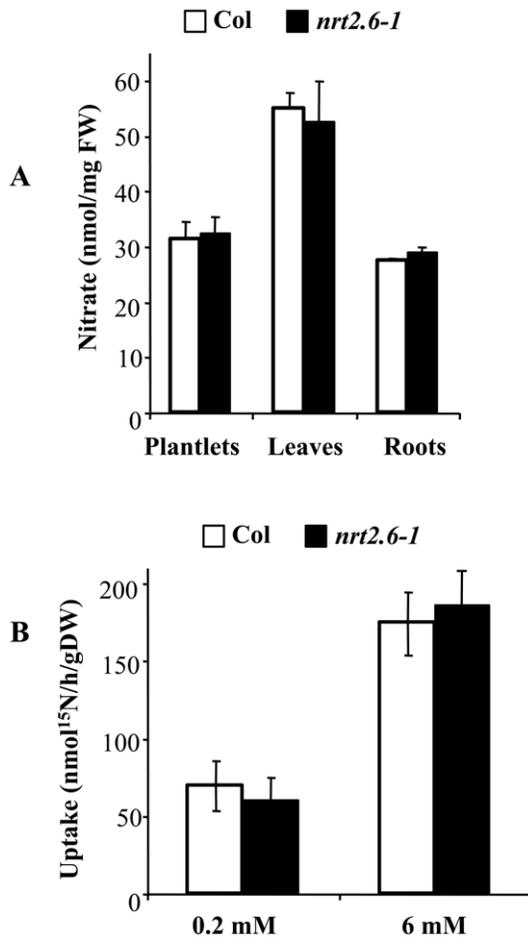


Figure 3. Nitrate contents and root nitrate influx in Col and *nrt2.6-1* mutant. **A:** Nitrate contents of plants were grown either *in vitro* with 9 mM NO₃⁻ (plantlets) or on sand with 10 mM NO₃⁻ (leaves and roots). The values are means ±SD of 5 to 6 plants. **B:** Nitrate influx of plant roots. Plants were grown in hydroponic culture on 0.5 mM NH₄NO₃ for 42 days and then transferred for 1 additional week to 0.2 mM NO₃⁻. Root influx was measured at both 0.2 and 6 mM ¹⁵NO₃⁻ to provide estimation of HATS and HATS + LATS activities, respectively. The values are means ±SD of 5 to 6 plants. doi:10.1371/journal.pone.0042491.g003

amount of H₂O₂ is decreased in the mutant and increased in the complemented mutants.

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 H₂O₂ 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 DCFD-DA 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 H₂O₂.

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* (At3g45060) 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 *NAXT1*) 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].

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

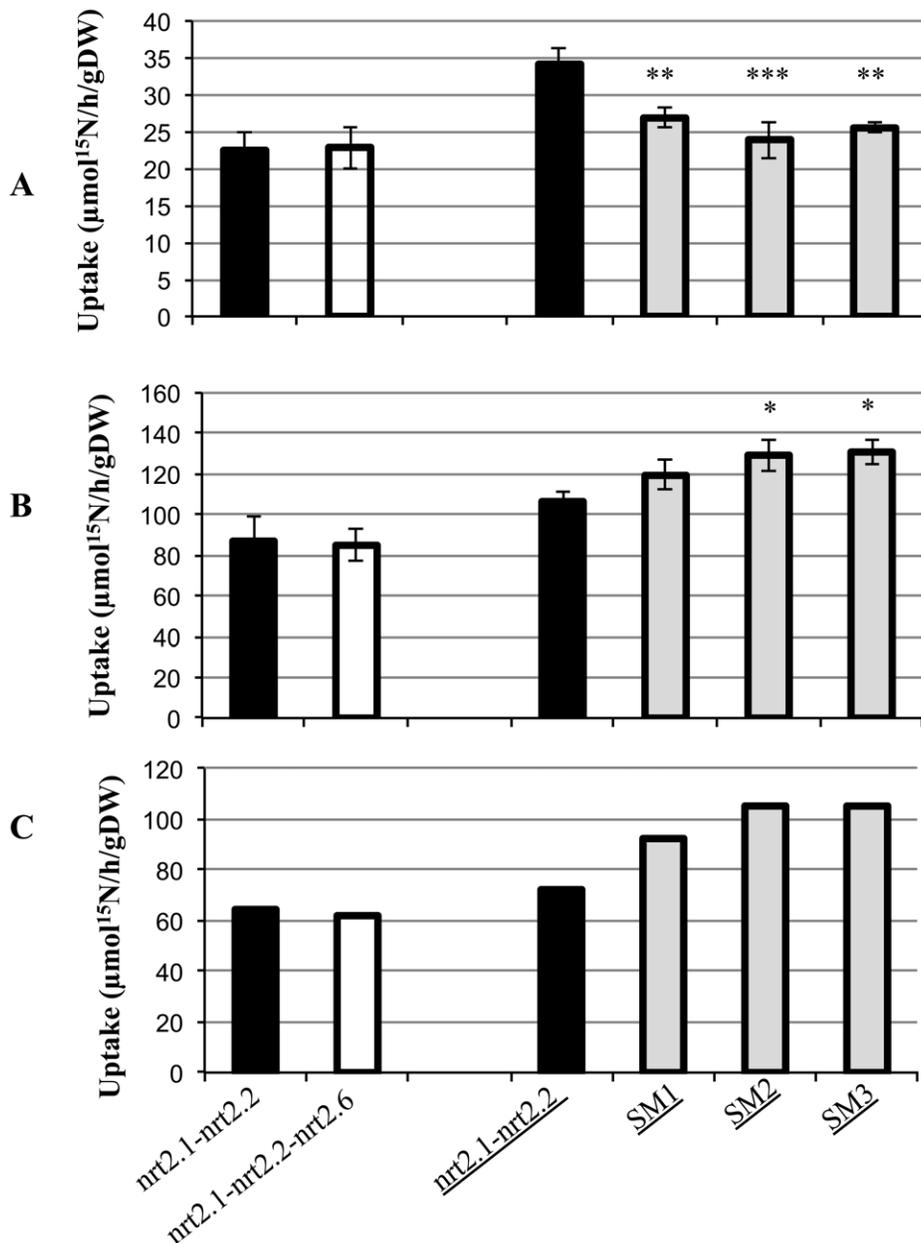


Figure 4. Nitrate uptake in different genotypes. Mutants and supplemented mutants (SM) in Col or Ws (underlined) genetic backgrounds were grown in hydroponic culture on 0.5 mM NH_4NO_3 and transferred for 1 additional week to 0.2 mM NO_3^- . Root influx was measured at both 0.2 and 6 mM $^{15}\text{NO}_3^-$ 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 (*nrt2.1-nrt2.2* mutants) (Test Fisher * $P < 0.05$, ** $p < 0.01$, *** $P < 0.001$). doi:10.1371/journal.pone.0042491.g004

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 *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 *nrt2.6-1* mutant, showing that it is still able to recognize *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_2O_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 *E. amylovora* infection (stronger symptoms and higher levels of bacteria). The level of plant sensitivity to bacteria can be compensated by the overexpression of *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 *E. amylovora*.

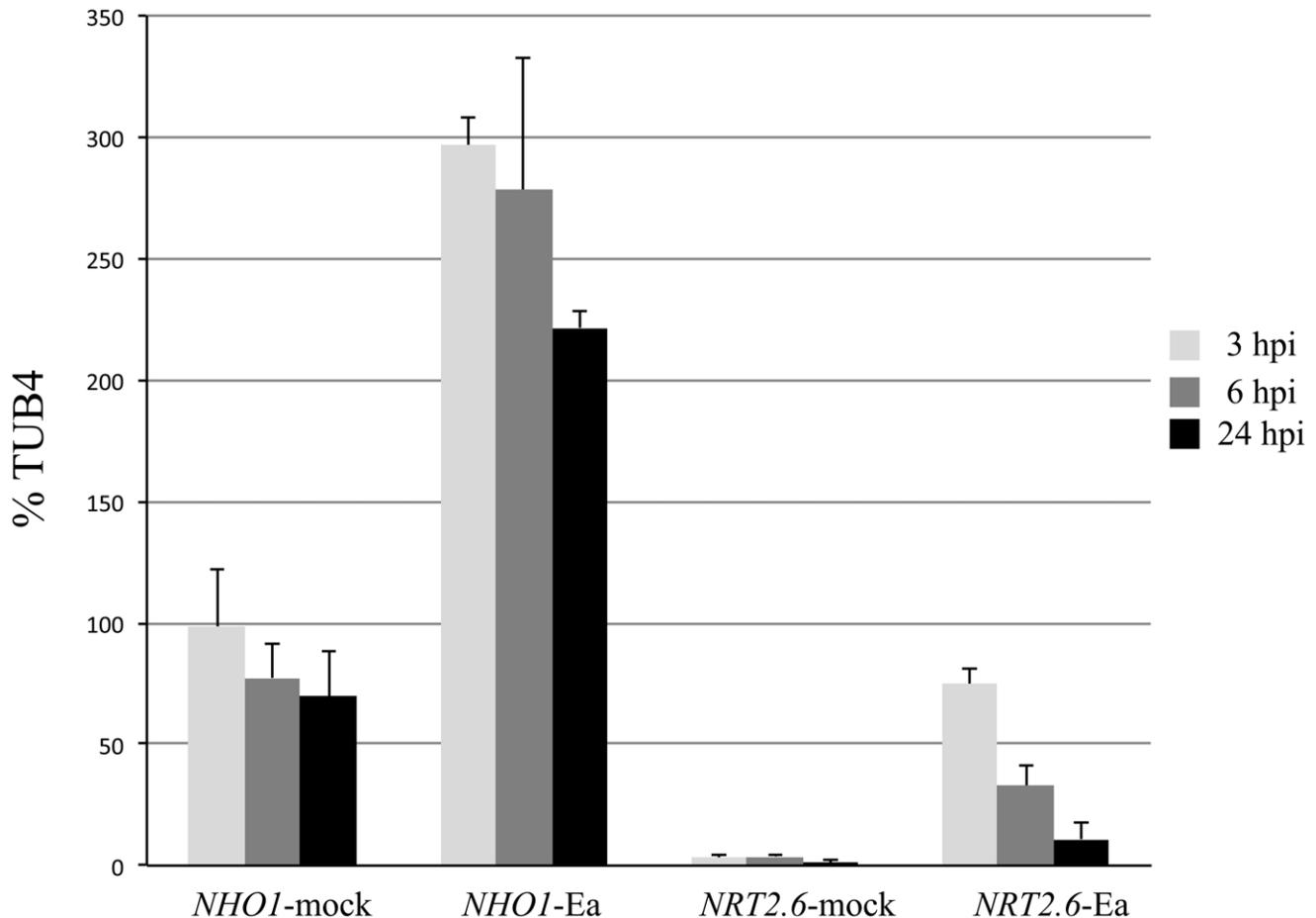


Figure 5. Time course of *NHO1* and *NRT2.6* expression following *E amylovora* inoculation. Plants were grown in culture chamber and leaves were inoculated with wild type *E. amylovora* strain (Ea) or with water (mock) as described in Materials and Methods. RT-qPCR analyses were performed on inoculated leaves 3, 6 or 24 h post inoculation (hpi). doi:10.1371/journal.pone.0042491.g005

Is *NRT2.6* Involved Only in H_2O_2 Accumulation?

H_2O_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_2O_2 balance, either the expression of genes involved in H_2O_2 production is enhanced or the expression of genes involved in H_2O_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 cryptogin 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_2O_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_2O_2 production may have led to an elevated level of H_2O_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'-TCAAACCACTATTCATTTCGCTAAACC-3') and

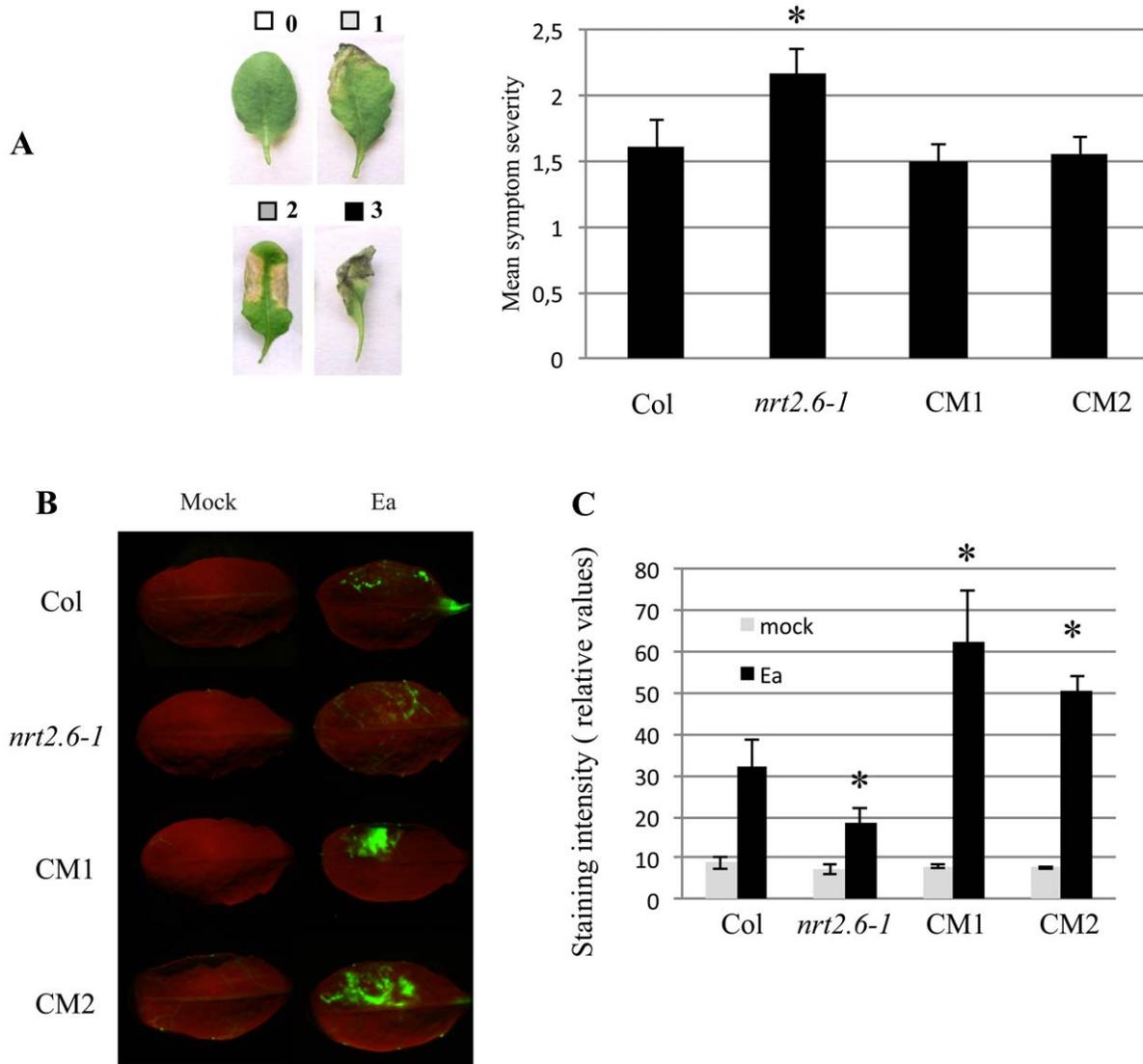


Figure 6. Responses of different genotypes to *E. amylovora* inoculation. Col: Columbia wild-type, *nrt2.6-1*: *NRT2.6* mutant, CM1 and CM2: *nrt2.6-1* mutant complemented by 35S::NRT2.6 construct. **A:** Mean symptom intensities induced on *A. thaliana* leaves 48 h after *E. amylovora* inoculation. **B:** Accumulation of ROS detected 18 h after *E. amylovora* inoculation by DCFH-DA coloration and microscope imaging. **C:** Quantitative analysis of staining intensities. The asterisk indicates means that are statistically different from wild-type according to Mann and Whitney's test (P value < 0.05).

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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 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Basic medium without nitrogen contained: 0.8 % Bromo-Cresol Purple (BCP), 0.07% 2-(N-Morpholino) ethanesulfonic (MES) acid pH 6, 2.5 mM KH_2PO_4 , 2 mM MgSO_4 , 5 mM KCl, 2 mM CaCl_2 , 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 H_3BO_3 , 14 μM MnCl_2 , 0.5 μM CuSO_4 , 10 μM NaCl, 1 μM ZnSO_4 , 0.01 μM CoCl_2 , 0.2 μM NaMoO_4 , 5 % Iron ammoniac citrate, 0.7% agarose (Kalys) and 1% sucrose. In the 1 mM NO_3 medium, KCl and CaCl_2 were lowered to 4.5 mM and 1.75 mM, respectively, and 0.5 mM KNO_3 and 0.25 mM $\text{Ca}(\text{NO}_3)_2$ were added. For the 9 mM and

18 mM NO_3 media, KCl was replaced by 5 mM and 10 mM KNO_3 , and CaCl_2 was replaced by 2 mM and 4 mM $\text{Ca}(\text{NO}_3)_2$, respectively. For the NH_4^+ and Gln media, 5 mM $(\text{NH}_4)_2$ 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 NO_3^- containing 5 mM KNO_3 , 2.5 mM $\text{Ca}(\text{NO}_3)_2$, 0.2 mM NaCl, 0.25 mM KH_2PO_4 , 0.25 mM MgSO_4 , 10 mg/L Fer-EDTA, 243 μM $\text{Mo}_7\text{O}_{24}(\text{NH}_4)_6$, 0.4 μM H_3BO_3 , 118 μM SO_4Mn , 10 μM de SO_4Cu and 34.8 μM SO_4Zn . 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 synthesised according to Daniel-Vedele and Caboche [50] using M-MLV reverse transcriptase

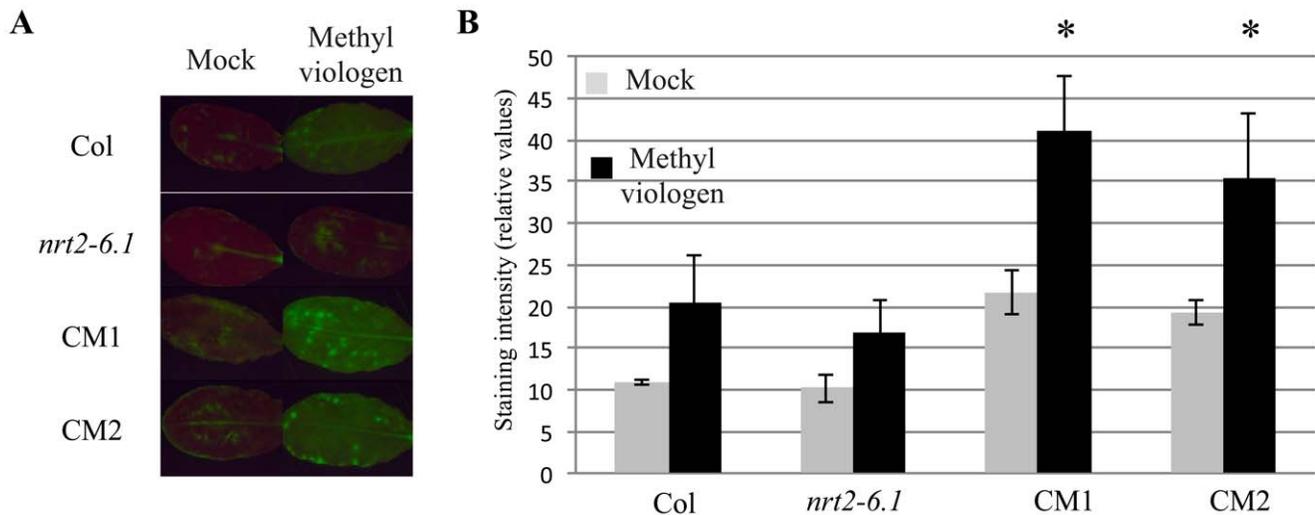


Figure 7. Responses of different genotypes to spray of methyl viologen. Leaves of 5 week-old plants grown in the greenhouse were sprayed with a solution containing 0.1 μM methyl viologen or with water (mock). Col: Columbia wild-type, *nrt2-6.1*: *NRT2.6* mutant, CM1 and CM2: *nrt2-6.1* mutant complemented by 35S::*NRT2.6* construct. **A:** Representative pictures of ROS accumulation detected by DCFH-DA 3 h after treatment. **B:** Quantitative analysis of staining intensities. The asterisks indicate means that are statistically different from the mutant according to Mann and Whitney's test (P value <0.05).

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(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.

Nitrate Measurements

Nitrate was extracted in water from frozen aliquots of plant material. The anion detection was performed with a colorimetric method based on the detection of a chromophore obtained by reduction of nitrate to nitrite by Vanadium (III), adapted from Miranda [51].

Influx Experiments

Plants were grown for 42 days under hydroponic culture conditions on 0.5 mM NH_4NO_3 as previously described [52] (8 h light at 21°C, 150 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ /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 NO_3^- , and the solution was changed daily. After 42 days, the plants were transferred first to 0.1 mM CaSO_4 for 1 min, then to basic nutrient solution containing 0.2 mM $^{15}\text{NO}_3^-$ (atom% ^{15}N :99%) or 6 mM $^{15}\text{NO}_3^-$ for 5 min and finally to 0.1 mM CaSO_4 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 $^{15}\text{NO}_3^-$ was calculated from the total N and ^{15}N 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'-AAAAAAGCAGGCTAAA-GACCATCCCAGATGAAAG-3') and NRT2.6 PGW3' (5'-AA-GAAAGCTGGGTGTAAAGTTGGAGAAGATGAG-3'). Amplification 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 Somerville 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'-GGAGATAGAAC-CATGGCTCACAAACCATTCTAATG) and AttB2-NRT2.6end-stop (5'-TCCACCTCCGGATCAGACATGAGCCGGA-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'-AGATTGGGGAC-CACTTTGTACAAGAAAGCTGGGTCTCCACCTCCG-GATC-3') and U5 primers (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG-3') to create the recombinant site AttB. The product of recombination reactions (BP reactions) was used to transform competent *Escherichia coli*, strain TOP10 (Invitrogen), by heat shock. LR clonase reactions to transfer T-DNA fragments from the entry clone 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

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 NO₃⁻ as nitrogen source during 5 weeks. Inoculations were performed using a blunt syringe with the bacterial wild-type strain *E. amylovora* CFBP1430 [59] and the inoculum density was adjusted at 0.1 O.D. in water (10⁷ 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 Degraeve et al [30]. Bacterial growth was analyzed 24 h after inoculation as described by Degraeve 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 1p 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 NO₃⁻ as sole nitrogen source. T-DNA or native gene was detected by PCR analyses. **B:** Pollen viability measured by Alexander test.

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Alexander test was performed on opened flowers from wild type and mutant [61].

(TIF)

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

(TIFF)

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.

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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 value < 0.05).

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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.

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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 value < 0.05). No statistical differences were found between wild-type and *nrt2.6-1* mutant.

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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.

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