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A deletion in the nitrate high affinity transporter *NRT2.1* alters metabolomic and transcriptomic responses to *Pseudomonas syringae*

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A deletion in the high affinity nitrate trasporter *NRT2.1* in Arabidopsis results in a reduced susceptibility to *Pseudomonas syringae* by two different mechanisms, the SA priming and an interference in the effector triggered susceptibility. In the present research we further characterized the metabolic and genetic profiles of the mutant *nrt2* in the interaction with *P. syringae*. Despite the priming found in the SA-dependent pathway, the metabolic changes in *nrt2* compared with wild-type plants are more remarkable prior infection. This is associated mainly to a pre-existing over representation of signals attributed to aromatic amino acids and phenylpropanoids in the *nrt2*. Genomic analysis confirms the implication of aromatic aminoacids and phenylpropanoids, but additionally, suggests a new role in ribosomal proteins as the major changes observed in *nrt2* upon infection by the bacterium.

Introduction

Increasing evidences are shown suggesting additional effects of nitrate transporters. We recently demonstrated that *NRT2.1* may act as a transceptor. This term comprises a dual role as a high-affinity nitrate transporter and also as a signal deliverer in order to coordinate other metabolic plant features. *NRT1.1* and *NRT2.1* can both perceive changes in the external amounts of nitrate and transmit signals throughout the plant to coordinate growth and nutrition.[1] Additionally, *NRT2.1* represses responses to biotrophyc pathogens probably favoring abiotic stresses resistance, probably as an attempt to save energy for the plant. Consequently, *nrt2* mutant displays reduced sensitivity to the bacterial pathogen *P. syringae*, and this phenotype is attributed to faster responses of the SA-dependent signaling and a reduced sensitivity to the bacterial effector coronatine.[2]

The implication of hormonal regulated pathways in the basal and primed defense of Arabidopsis against *P. syringae* have been widely studied.[3,4] Despite these advances, recent metabolomic approaches have revealed an implication of indolic and phenolic compounds in the basal defense of Arabidopsis against the bacterium.[5] In addition to this metabolite families, rapid alterations in the abundance of amino acids and other nitrogen-containing compounds, as well as glucosinolates, disaccharides and molecules that influence the prevalence of reactive oxygen species have been related to plant responses against *P. syringae*. However, among these last set of compounds, these research also comprises the superimposed effect of pathogen's effectors on defense suppression. Pathogens have been shown to reconfigure host metabolism through Effector Triggered Susceptibility to provide the adequate environment in the plant apoplast to sustain growth of bacterial populations.[6] Therefore, it must be considered that most metabolomic analysis may have a contribution of the response of the plant and the host manipulation by the pathogen. In this regard, a complementary transcriptomic study in resistant genotypes is very helpful to discriminate responses of the plant contributing to resistance.

Pre-existing Metabolic Changes in *nrt2* Mutant May Contribute of its Reduced Sensitivity

We showed previously that SA-dependent responses are primed in *nrt2* upon *P. syringae* infection, this response is critical to hold a less susceptible phenotype since the mutant *nrt2-sid2* losses the resistance showing wild-type levels again.[2] Some previous reports by Fan et al. showed that pre-existing enhanced ABA levels in *cds1* compromise its resistance against biotrophs while contributes positively to resist *A. brassicicola*. Interestingly, in a LC-Q-TOF analysis of the metabolome of *nrt2* vs. Ws, major differences in *nrt2* compared with Ws are found in the absence of infection. Therefore some responses in the mutant can be attributed to pre-existing changes. This is observed clearly in the heatmap and also in pc-1 and pc-2 components of the PCA analysis in both positive and negative Electrospray ionization (ESI) analysis (**Fig. 1A and B**). Additionally, 48 h after infection, the metabolic changes in Ws and *nrt2* merge to a similar profile although still cluster differently in both positive and negative as it is shown in the PCA analysis (**Fig. 1B**).

In a tentative approach to identify the main metabolic changes we used the exact mass data for a search in the metabolic database

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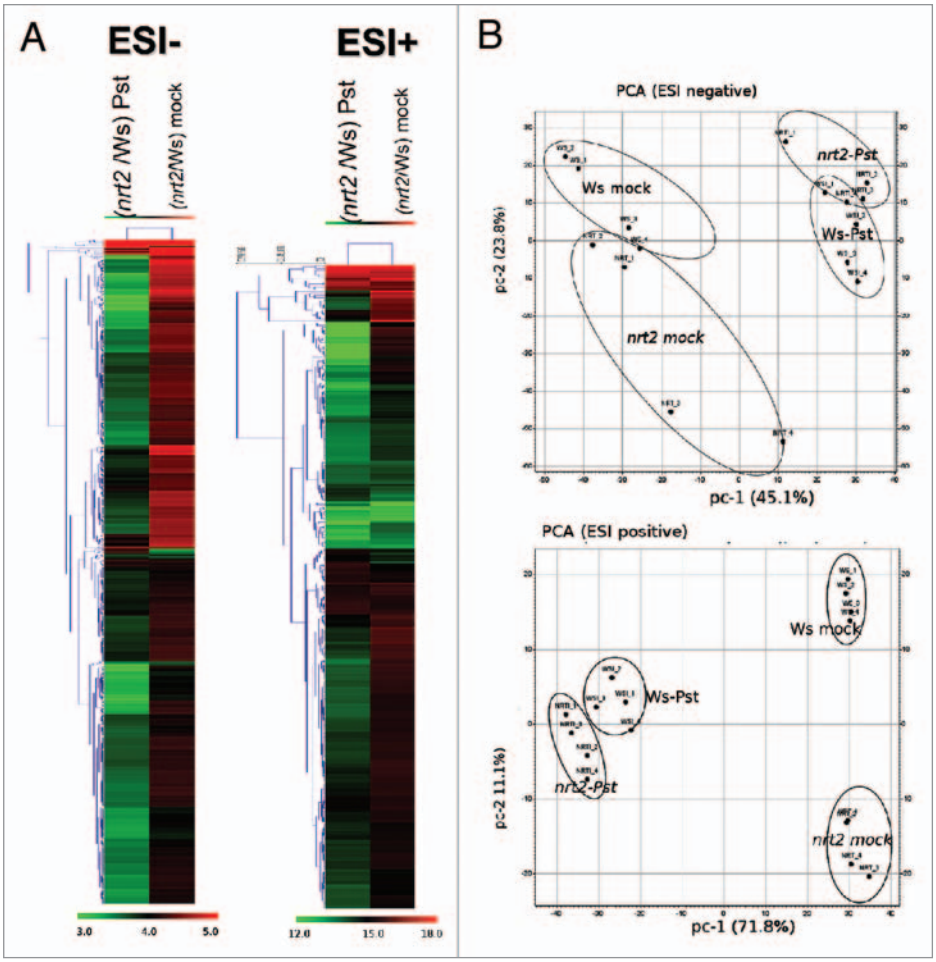

Figure 1. The multivariate analyses applied include hierarchical cluster analysis (HCA) and principal component analysis (PCA). (A) Heatmaps of *nrt2* vs. Ws either in the presence or in the absence of the infection. Heatmaps were generated by using Multi-experiment Viewer[14] after a t-test by applying a standard Bonferoni correction and a hyerarchical clustering. Data are represented in a $\log_2$ scale. Detection of peaks was performed in positive or negative electrospray (ESI) ionization mode and subsequent separation of the ions in a Quadrupole-Time-of-Flight (model Premier of Waters). (B) PCA plot were generated to specifically identify m/z changes with infection or plant genotype. PCA plots were constructed for *P. syringae* infected material for each genotype (WS and *nrt2*). Ginkgo Analysis System (1.7) (http://biodiver.bio.ub.es/ginkgo/index.html) was used to obtain PCA score plots of the LC-MS in negative and positive mode. PCA plot obtained showing two major sources of variability among Ws and *nrt2* after LC-MS. Percentage of variability is given on each axis.

METLIN (metlin.scripps.edu/). Four main metabolite families are overrepresented in *nrt2* vs. Ws upon infection; they belong to the aromatic aminoacids, nucleotides and phenylpropanoids. In the other hand, a metoxiflavone, a dinucleotide and glucosilated flavonoids are less represented in the mutant upon infection. This suggests that both priming of SA and aromatic aminoacids and phenylpropanoids may prepare the plant to perform better once the pathogen is present.

Trasncriptomic Profiling of *nrt2* Infected with *Pseudomonas syringae*

To further characterize the molecular response of *nrt2* to *P. syringae*, whole-genome transcriptional profiles of Ws and *nrt2* mock treated or infected with *P. syringae* were obtained using Arabidopsis ATH1 array of Affimetrix. Bioinformatic analysis was performed by Progenika Co., using the PartekGenomics Suite, dChip (www.dchip.org) and the software Affy and affyPLM form the consortium BioConductor (www.bioconductor.org). As we showed previously there are sets of genes differentially expressed in the mutant that were not changed in Ws, while many genes were up or downregulated in Ws and remained unaltered in *nrt2* as a consequence of infection. A gene ontology (GO function analysis) by categories of differentially expressed genes of Ws vs. *nrt2* plants upon infection showed that at the Biological Process level, most genes belong to response to biotic or abiotic stress or stimulus and defense (**Fig. 2A**). At the molecular function level, main changes have been observed for oxidoreductase enzymes, ribosomal proteins and logically in membrane transporters (**Fig. 2B**).

Kegg pathway analysis has shown that infection with *P. syringae* causes alterations of two major pathways in *nrt2* compared with Ws (**Fig. 2C**). These are the synthesis of phenolic amino acids and modification in the gene expression codifying for 15 ribosomal proteins. The biosynthetic pathway of Phe and Tyr and the indolic amino acid Trp have a clear link to defense signaling since the synthesis of SA has a second branch in which phenylalanine ammonia lyase has been proposed as a key enzyme. In addition, some Trp derivatives such as glucosinolates are also relevant mediators of PAMP-triggered callose accumulation[8] and defense against bacterial pathogens.[9] This is in agreement with the results observed in the metabolome since aromatic aminoacids are also overrepresented in the mutant compared with the wild type. Contrastingly, much less is known about the implication of ribosomal proteins in plant disease resistance. The silencing of two ribosomal proteins (L12 and L19) in *Nicotiana benthamiana* suppresses the non host resistance to bacterial pathogens but does not affect the host resistance.[10] Recent findings in, proteomic analysis of rice have demonstrated that several ribosomal proteins are upregulated after a *Nilaparvata lugens* infection.[11] Interestingly, the L10 ribosomal protein is upregulated in *nrt2* after *P. syringae* infection compared with Ws. This protein has been shown to be a component in the nuclear shuttle protein-interacting kinase of Arabidopsis, that participates in

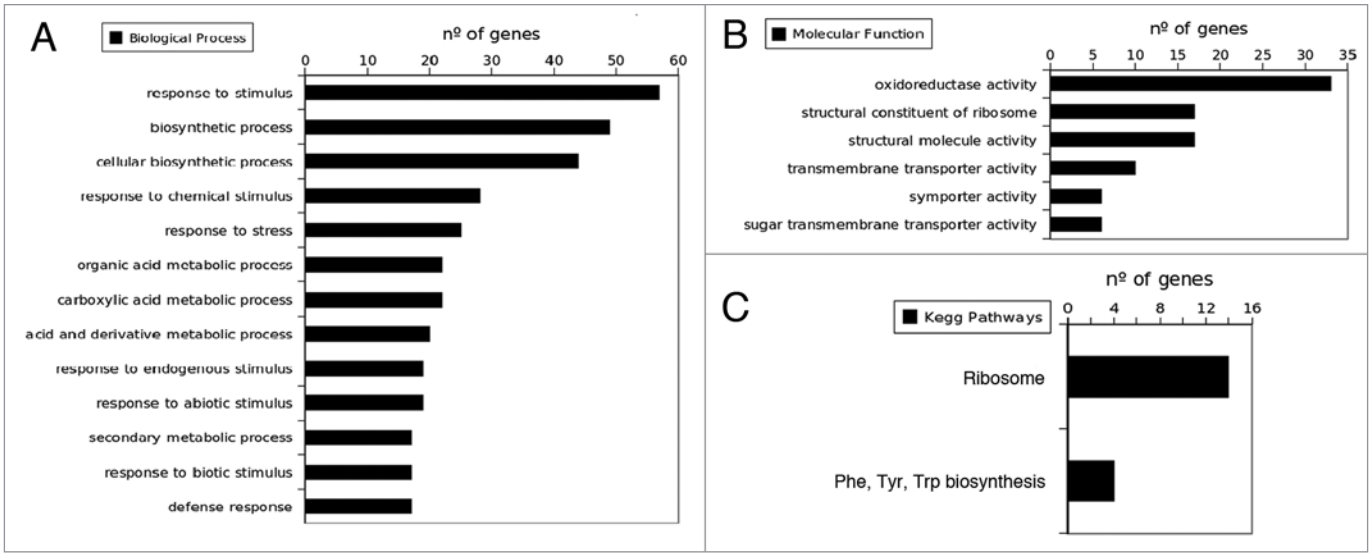

Figure 2. Microarray analysis and Kegg pathway and Go categorization of wild-type and *nrt2* genes differentially expressed after inoculation with *P. syringae* using PartekGenomics Suite. (A) Number of genes differentially expressed in *nrt2* vs. wild-type upon infection categorized by metabolic pathways. (B–D) Number of genes differentially expressed in *nrt2* vs. wild-type upon infection categorized by biological process, cellular component and molecular function. Percentages relate to total number of genes with an ontology at each analysis. Only categories with higher percentages of total number of genes are shown.

antiviral signaling.[12] Although there are many other factors that could contribute to the enhanced resistance of the mutant, our results suggest a putative implication of ribosomal proteins in resistance against bacterial diseases in Arabidopsis.

Interestingly, a study of gene vs. stimulus performed with the Genevestigator application (www.genevestigator.org),[13] has shown a reasonably good correlation in the response to stimulus between the *NRT2.1* (At1g08090) and *NRT2.2* (At1g08100). Stimuli such as iron deficiency, OPDA, SA and MeJA downregulate all these genes but on the contrary cold, nematodes, the elicitors Hrpz, LPS and FLG22 strongly upregulate their expression in a coordinated manner. This suggests that they act coordinately not only under nitrogen deprivation, but also upon different abiotic and biotic stimuli. Finally, another study of response to stimuli performed on the Genevestigator database of the gene *NRT2.1* compared with some marker genes of SA, JA and ABA signaling pathways, has shown a low correlation in the induction or repression. This may indicate that perception of stimulus activates differentially *NRT2.1* and hormonal controlled defense pathways.

Unfortunately, the metabolic and genomic analysis of *nrt2* infected by *P. syringae*, still has not revealed a definitive explanation to its lower sensitivity to the bacterial effector coronatine, which is also contributing to the *nrt2* reduced susceptibility to the pathogen.[2]

Concluding, it is clear that the alteration in the *NRT2.1* gene produces global changes, affecting other plant responses that are not solely linked to nutrient transport rather than to responses to environmental changes, indeed this include responses to pathogens. Therefore these observations reinforces the hypothesis that the *NRT2.1* may be acting also as a transceptor, coordinating perception of external signals with plant responses to abiotic and biotic stresses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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