An RNA Sequencing Transcriptome Analysis Reveals Novel Insights into Molecular Aspects of the Nitrate Impact on the Nodule Activity of *Medicago truncatula*[1][W]

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The mechanism through which nitrate reduces the activity of legume nodules is controversial. The objective of the study was to follow *Medicago truncatula* nodule activity after nitrate provision continuously and to identify molecular mechanisms, which down-regulate the activity of the nodules. Nodule H_2 evolution started to decline after about 4 h of nitrate application. At that point in time, a strong shift in nodule gene expression (RNA sequencing) had occurred (1,120 differentially expressed genes). The most pronounced effect was the down-regulation of 127 genes for nodule-specific cysteine-rich peptides. Various other nodulins were also strongly down-regulated, in particular all the genes for leghemoglobins. In addition, shifts in the expression of genes involved in cellular iron allocation and mitochondrial ATP synthesis were observed. Furthermore, the expression of numerous genes for the formation of proteins and glycoproteins with no obvious function in nodules (e.g. germins, patatin, and thaumatin) was strongly increased. This occurred in conjunction with an up-regulation of genes for proteinase inhibitors, in particular those containing the Kunitz domain. The additionally formed proteins might possibly be involved in reducing nodule oxygen permeability. Between 4 and 28 h of nitrate exposure, a further reduction in nodule activity occurred, and the number of differentially expressed genes almost tripled. In particular, there was a differential expression of genes connected with emerging senescence. It is concluded that nitrate exerts rapid and manifold effects on nitrogenase activity. A certain degree of nitrate tolerance might be achieved when the down-regulatory effect on late nodulins can be alleviated.

Nitrate, a major form of inorganic nitrogen in the soil solution, exerts complex effects on plant growth and development (Forde and Walch-Liu, 2009). In addition to functioning as a mineral nutrient, nitrate changes plant architecture through influencing hormone balances (Bouguyon et al., 2012; Wang et al., 2012). The growth of lateral roots is locally induced by nitrate (Walch-Liu et al., 2006; Gan et al., 2012), while the shoot/root ratio tends to increase with increasing soil nitrate availability (Kruse et al., 2002).

Nitrate profoundly affects gene expression in plant tissues. Prominent among the known primary receptors and transmitters of nitrate availability is the dual affinity nitrate transporter AtNRT1.1 (Tsay et al., 1993). In addition to its transporter activity, this protein senses nitrate outside the root cell and induces several different molecular responses (Wang et al., 2012). These attributes mean that this protein classifies as transceptor. A related protein has been recently described for *Medicago truncatula* (Morère-Le Paven et al., 2011).

Various kinases (e.g. calcineurin B-like-interacting protein kinase8 and calcineurin B-like-interacting protein kinase23) and the nodule inception-like protein NLP7 have been shown to induce gene expression responses downstream of nitrate in Arabidopsis (*Arabidopsis thaliana*). A transcriptome study revealed that 550 genes were differentially expressed in the roots of this plant within 20 min after nitrate application (Krouk et al., 2010a). Estimates based on summarizing microarray data from various studies have revealed that, depending on the overall experimental context, up to approximately one-tenth of the whole genome of Arabidopsis can be influenced by the presence of nitrate (Krouk et al., 2010b).

Nitrate inhibits nodule formation in legumes (Streeter and Wong, 1988; Ferguson et al., 2010). The underlying molecular mechanism is largely understood (Okamoto et al., 2009) and resembles the mechanism governing

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the autoregulation of nodulation (Reid et al., 2011). It has also been clearly shown that nitrate strongly inhibits the activity of existing nodules (Vessey and Waterer, 1992). Within a few days after nitrate exposure, nodule activity is almost completely lost (Schuller et al., 1988) and the nodules become senescent (Matamoros et al., 1999).

Uncoupling nitrate assimilation and nodule activity, at least to a certain extent, would most probably enhance legume yield. Under field conditions, legumes depending on nitrogen fixation tend to keep their nitrogenase activity at the lowest necessary level and the C/N ratio in their leaves constant (Schubert, 1995). A more soil-N independent nitrogen fixation would most probably be beneficial in increasing the yield of leguminous plants (e.g. protein content or vegetative matter). To achieve such a goal, the understanding of the underlying mechanisms of nitrate effects on nodules is pivotal. However, despite decades of research (Vessey and Waterer 1992), the mechanism of nitrate's impact on the activity of existing nodules is not fully understood and remains controversial as there are several competing theories. The nitrate ion itself, nitrite, or nitric oxide has been implicated by either blocking leghemoglobin (Kanayama et al., 1990; Kato et al., 2010) or by triggering a regulatory network at the gene expression level (Neill et al., 2008; Wilson et al., 2008; Meilhoc et al., 2011). A further hypothesis is that the effect of nitrate on legume nodules is mediated by a closure of the oxygen diffusion barrier or a reduction in oxygen permeability facilitated by unspecified mechanisms (Vessey and Waterer, 1992; Minchin, 1997). In addition to the fact that the functioning of the oxygen diffusion barrier remains elusive, no clear time course study is available that shows that a decrease in nodule O_2 uptake precedes and induces a decline in nitrogenase activity. For the argon-induced decline in nitrogenase, Fischinger and Schulze (2010a) could show that the actual decline in oxygen uptake of nodules did not precede but rather followed the beginning of the decline in nitrogenase activity. Certainly, it must be also borne in mind that the decreased respiration and lowered oxygen influx into the nodule after nitrate provision might also be the result of a decline in nitrogenase activity induced by a different mechanism.

Another reason for the reduction in nitrogenase activity under nitrate impact might be found in an either local or systemic assimilate diversion. Fujikake et al. (2003) could show that the growth of emerging soybean (*Glycine max*) nodules immediately and completely stopped after nitrate application (within minutes to hours). Moreover, by applying ^{14}C-labeled carbon dioxide, it was shown that carbon allocation within the plant was profoundly influenced by the nitrate, with less going to the nodules. However, although the amount of carbon allocated to the nodules might decline, there is no unequivocal proof that this is the primary factor inducing the decline in nodule activity. Various studies on the concentration of free sugars and starch in nodules after nitrate application do not support the hypothesis that the decline in activity is a result of assimilate shortage. The activity of Suc synthase is an indicator for carbon sink strength. Gordon et al. (1999) have shown that under several stress conditions, there was a coincidental decline in both Suc synthase and nitrogenase activity. An antisense down-regulation of the nodule-enhanced Suc synthase in *M. truncatula* resulted in impaired plant growth, especially when the plants were totally dependent on nitrogen fixation (Baier et al., 2007). However, a detailed study on the relationship of soybean Suc synthase activity and nitrogen fixation after nitrate application revealed that the decreased Suc synthase activity was the result of, rather than the reason for, the decreased nitrogen fixation (Gordon et al., 2002). Overall, the results are contradictory with respect to the question of whether the altered assimilate allocation after nitrate provision is the primary reason for the decline in nodule activity.

In addition, there may be a shoot-mediated mechanism of down-regulating nitrogen fixation dependent on a phloem mobile signal reflecting the nitrogen satiety status of the leaves (Naudin et al., 2011). Such a signal might contain nitrogen as nitrogen travels quickly from leaves to nodules (less than 15 min; Fischinger et al., 2006). However, neither the nature of such a signal nor the related mechanism of down-regulating nodule activity have been shown unambiguously.

Our hypothesis was that reasons for the nitrate-induced decline in nodule activity would be mirrored in the transcriptome when the decline begins after exposure to nitrate. A further transcriptome profiling when a low level of nodule activity was reached would additionally allow distinguishing between effects of nitrate versus effects resulting from the cessation of ammonium influx in the cytosol.

The objective of this study was to firstly establish a high-resolution time course of the decline in nitrogenase activity after the application of a sufficient amount of nitrate that would fully cover the plant's N demand. To achieve this, we planned to apply a method that allows the nitrogenase activity to be followed continuously and noninvasively at a high resolution. Moreover, using this approach, we would be able to determine two points in time after the application of nitrate at which 1) an adequate amount of nitrate for the plant's needs would have been taken up and the nodule activity decline had begun and 2) a significant decline in nitrogenase had occurred. At each of these two points in time, we then performed a comparative nodule RNA sequencing (RNA-seq) transcriptome profiling.

To date, RNA-seq based on the next-generation deep-sequencing technique (Mardis, 2008) is the most powerful tool for comparative transcriptome profiling (Wang et al., 2009; Nookaew et al., 2012). In addition to a precise and extensive covering of the expression of known genes, this method can potentially provide information on possibly regulated RNA which produced unmapped reads or the importance of alternative splicing events. RNA-seq has already been successfully applied to *M. truncatula* tissue including nodules (Boscari et al., 2013).

RESULTS

Time Course of Nodule Activity after Nitrate Application

A typical time course of the nitrate-induced decline in nitrogenase activity is shown in Figure 1. The amount of nitrate-N taken up by the plants exceeded more than 10-fold that what the control plants fixed during the experimental period (28 h), assuming a constant electron allocation coefficient (EAC) of 0.58 as measured before the treatment. Individual plant uptake was not measured; however, total nitrate uptake of the treatment groups was estimated from data of parallel plants by measuring the nitrate depletion of the nutrient solution. The plants had taken up about 70% of the nitrate in the first 2 h. At the end of the measurement time (28 h after the beginning of nitrate exposure), the nitrate in the nutrient solution was depleted to a concentration of about 0.5 mM.

Nodule H_2 evolution remained unaffected during a period of about 3 h after nitrate exposure. This was followed by a slight and more or less continuous decrease until the beginning of the dark period at 11 h after nitrate provision (Fig. 1). At that point in time, the nodule H_2 evolution had declined to approximately 83% of the level before the nitrate application. With the beginning of the dark period, a sharp decline in H_2 evolution occurred independent of the treatment. This decline was largely parallel to the preset lower temperature in the climate chamber during the dark period. During the subsequent dark period, the nitrate-induced decline abated after about 2 h and the H_2 evolution recovered, reaching the control level at the end of the second dark period. During the subsequent light period, the H_2 evolution in the nitrate treatment remained at the level of the control for about 4 h, after which there was a steep and rapid decrease. A new and more or less constant level of about 41% of the pretreatment H_2 evolution was reached at about 28 h after nitrate exposure.

We measured the EAC on parallel plants three times as indicated by the arrows in Figure 1. The EAC was not significantly altered during the nitrate-induced decline. At 4 and 28 h after nitrate exposure, nodules were harvested for a comparative RNA-seq transcriptome profiling. The two points in time were chosen, as, firstly, a significant amount of nitrate had been taken up at 4 h and so any mechanism triggering the nitrate-induced decline in nodule activity should be mirrored in transcriptome changes found at this time. Secondly, as the nodule activity had plateaued off at 28 h after nitrate exposure, it was considered that the changes found at this time would additionally reveal the effects of the lower nitrogenase activity on nodule gene expression.

Changes in Nitrate Reductase and Gln Synthetase Expression in Leaves after Nitrate Application

To determine the site of nitrate reduction and the intensity of ammonium incorporation, the expression of two genes for nitrate reductase (NR1 [Medtr3g073150.1] and NR2 [Medtr5g059820.1]) and Gln synthetase (Medtr3g065250.1) were measured in leaves at 0.5, 4, 24.5, and 28 h after nitrate exposure (Fig. 2). Gln synthetase as well as NR1 and NR2 activity in the leaves was increased already 0.5 h after nitrate application.

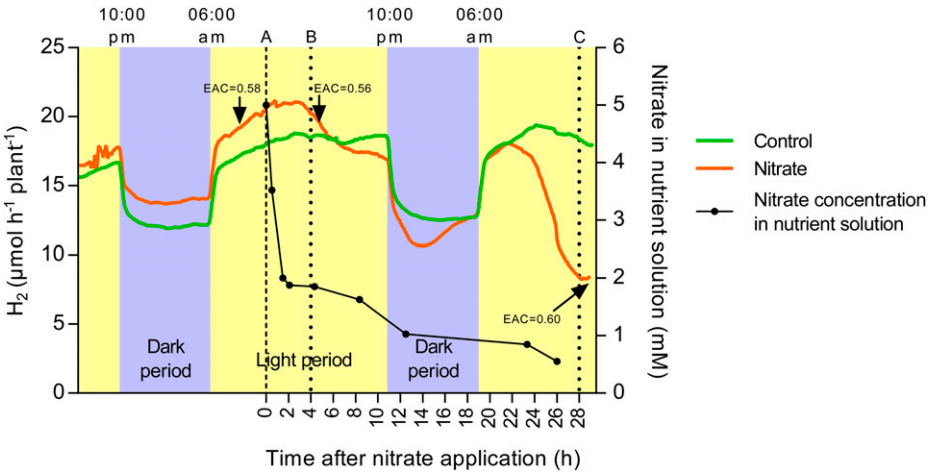

Figure 1. Time course of whole-plant ANA affected by nitrate application. Nitrogenase activity was measured as H_2 evolution per plant with measurements of H_2 concentration in the continuous airflow from the root/nodule compartment taken every 5 min. Data are means of six replicates. The dotted line (A) indicates the point in time when the nitrate was added to the nutrient solution (5 mM). Lines B and C indicate the points in time when nodules were harvested for transcriptome analysis. The black line shows the depletion of nitrate in the nutrient solution; the black dots indicate when the samples were taken for nitrate analysis (data are means of four replicates). Nitrate depletion was analyzed in a separate experiment. The black arrows indicate the point in time when the EAC was determined by replacing N_2 by Ar for 5 min (separate experiment, four replicates, values were not significantly different among measurements, $P < 0.05$).

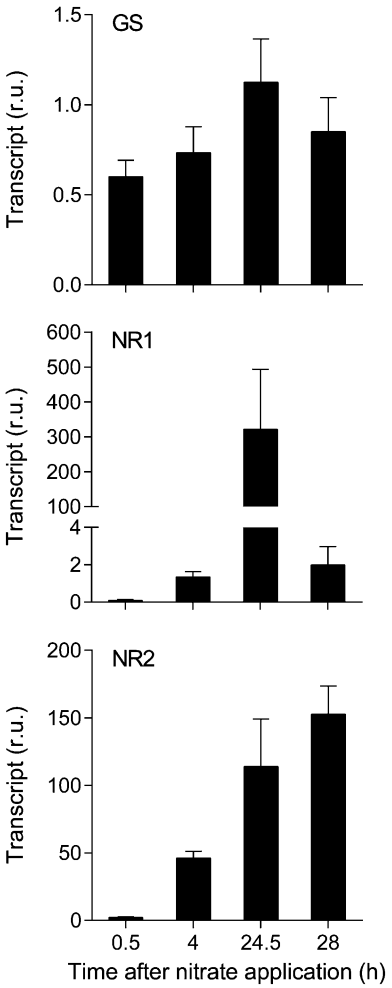

Figure 2. Relative expression of Gln synthetase (GS) and NR1 and NR2 in the leaves of *M. truncatula* at the indicated points in time after nitrate application measured by quantitative PCR (qPCR). The transcript levels are expressed as relative units (r.u.). Data are means of four biological independent replicates ± SE.

The actual increase in Gln synthetase was slight, but it should be born in mind that the controls exhibited a very high expression of this gene. By contrast, the increase in the expression of both NR1 and NR2 was high; however, the expression of these two genes was very low in the controls.

qPCR Validation of the RNA-Seq Data

A quantitative real time PCR validation proved the reliability of the RNA-seq data (Supplemental Table S1). Fourteen genes were tested, using the same RNA pools that had been previously used for the next-generation sequencing. The PCR results were significantly correlated to the RNA-seq data at both 4 and 28 h (r^2 = 0.85 and 0.91, respectively). The slope of the regression line was in both cases close to one (4 h = 0.94; 28 h = 0.91; see figure in Supplemental Table S1).

Transcriptome Changes after Nitrate Provision

The RNA-seq yielded between 16 and 25 million mapped reads per replicate and thus achieved the necessary depth for our analyses. A cluster dendrogram analysis showed that the RNA pools (biological replicates) were similar within but differed among the control and treatment groups (Fig. 3). In the control, 15,129 genes were expressed in the nodules (≥20 'unique hit' counts [DEseq]), representing 31.8% of the genes annotated in the Mt3.5v3 gene model. The total number of expressed genes was only slightly altered in response to the nitrate exposure (15,023 or 15,165 after 4 or 28 h, respectively). About 93% to 94% of the genes were expressed in both the control and treatment groups. A total of 1,120 genes were differentially regulated at 4 h when only those that had a fold change (FC) greater than two and were significantly different in expression with a false discovery rate (FDR) of less than 0.01 were considered (Fig. 4). The number of differentially regulated genes almost tripled at 28 h. The number of down-regulated genes exceeded those up-regulated by about 20% at both points in time of the transcriptome profiling (Fig. 4). There were a significant number of genes that were unaffected at 4 h, though they were up- or down-regulated at 28 h, and vice versa (Fig. 4). Six genes were down-regulated at 4 h and up-regulated at 28 h; for nine genes, the regulation occurred the other way around (Fig. 4). The majority of the differentially regulated genes showed a 2- to 4-fold change in expression ($\log_2$ 1–2), with some scattered cases being down-regulated with a FC up to about $\log_2$ –8, or up-regulated with a FC up to approximately $\log_2$ 6.

Overrepresentation Analysis

Differentially expressed genes of various metabolic processes were over- or underrepresented among all the genes annotated in the gene model Mt3.5v3 (Supplemental

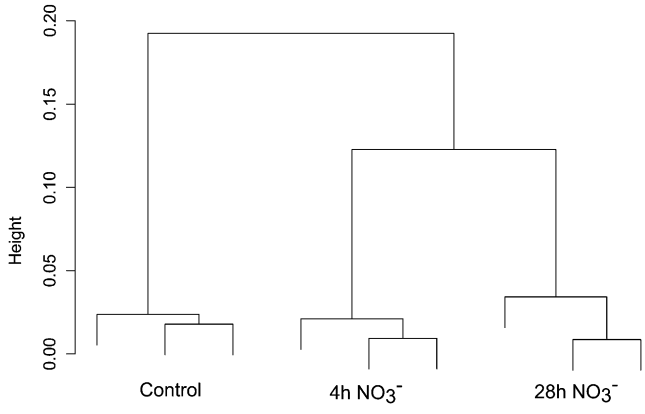

Figure 3. Dendrogram analysis of similarity among treatments and replicates of *M. truncatula* nodules. One biological replicate stands for an RNA pool from the nodules of three plants; 25 healthy appearing nodules were harvested per plant. The nitrate induced no visible senescence (no greenish color) within the 28 h of nitrate exposure.

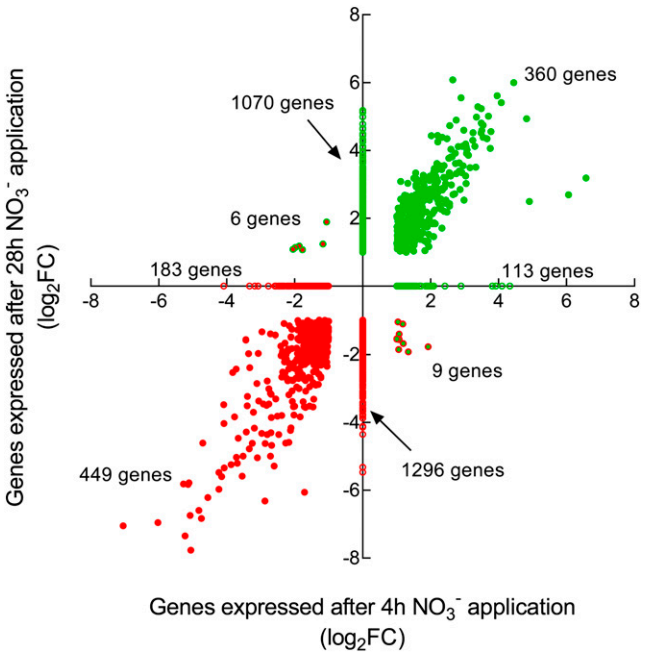

Figure 4. Scatterplot analysis of differential gene expression in *M. truncatula* nodules after nitrate application. A green dot stands for one up-regulated gene and a red dot for one down-regulated gene. Genes were considered as being expressed and differentially regulated when they complied with the following criteria: at least 20 unique hit counts (DEseq) in treatment and/or control, FDR < 0.01 and FC $>$ [2]. Data were taken from three biological replicates.

Fig. S1). For example, in contrast to 4 h after nitrate exposure, at 28 h, the up-regulated genes for protein synthesis were strongly underrepresented. By contrast, the up-regulated genes for nitrate transporters were overrepresented at both points in time. Up-regulated genes were also overrepresented in most groups of genes responsible for secondary metabolism and hormone metabolism at 28 h. In addition, stress-related genes were strongly overrepresented among the up-regulated genes and underrepresented among the down-regulated genes.

Expression of Sentinel Genes

Twelve genes known to be strongly affected by nitrate are listed in Table I. All of these genes already showed a clear reaction at 4 h after nitrate exposure. Up-regulation occurred in the genes for the nitrate transporters of the NRT/peptide transporter protein families 1 and 2 (Forde, 2000; Wang et al., 2012), nitrate and nitrite reductase (Horchani et al., 2011), siroheme synthase, the Numerous Infections and Polyphenolics/Lateral Root-Organ Defective (NIP/LATD) gene (Bagchi et al., 2012), and a gene involved in the biosynthesis of molybdenum-containing cofactors (Molybdopterin biosynthesis calnexin1; Schwarz et al., 1997). As expected, the gene for the nodule inception protein was down-regulated (Castaings et al., 2009). The two high-affinity nitrate transporters were the two most up-regulated genes after 4 h. The recently described gene for MtNRT1.3 (Medtr5g093170.1; Morère-Le Paven et al., 2011), which functionally resembles the AtNRT1.1 nitrate transceptor, appears not to be expressed in the nodules (zero reads per kilobase pair transcript length per million total reads [RPKM], control and treatment groups).

DISCUSSION

Overall Impact of Nitrate on Nodules

The impact of nitrate on mature nodules proved to be rapid and of a complex nature at the molecular level (Supplemental Tables S2 to S4). The per plant nitrogen fixation was substantially reduced, and strong shifts in metabolism occurred within 1 d of nitrate exposure. Between 4 and 28 h of nitrate application, the number of differentially expressed genes almost tripled ($\geq$20 unique hit counts [DEseq] in the treatment and/or control groups, FDR < 0.01 and FC $\geq$ [2]). These 3,190 genes represent about 20% of all the genes expressed in the treatments and/or control, or 6.7% of all the genes annotated in Mt3.5v3. In comparison, a meta-analysis of numerous microarray studies in Arabidopsis revealed that about 10% of the whole genome is potentially affected by nitrate (Krouk et al., 2010a).

In our experiment, in several cellular systems and metabolic pathways (e.g. mitochondria, secondary metabolism, etc.), the intensity of the effects visible at 4 h increased until 28 h, not only in the sense of higher FCs of already affected genes, but also by the number of genes affected within a given gene family (e.g. cytochrome P450: 15 genes affected at 4 h, 37 at 28 h) or metabolic pathway (e.g. the mitochondrial electron transport chain; Fig. 5). However, there were also other effects that emerged at 28 h. In particular, there was a recognizable shift of the nodules' metabolism toward senescence and cessation of growth. For example, a senescence-associated protein (Medtr5g023170.1) was strongly induced and genes for histones H2A, H2B, H3, and H4, in conjunction with the gene for nucleosome assembly (Medtr4g108570.1), were down-regulated. The production of various ribosomal proteins, in particular those of the large subunit 60, was decreased. Concurrently, nine autophagy-related genes were up-regulated. In addition, several genes for DNA and RNA polymerase subunits were down-regulated (Supplemental Table S3).

Overall, although the quantitative data produced by this study cannot be compared unambiguously with microarray studies, the number and relative proportion of affected genes appears to be high when compared to previous studies on root tissue. For example, Ruffel et al. (2008) identified a total of about 2,000 genes in the roots of *M. truncatula* that were affected by nitrate application, depending on the length and molar concentration of the nitrate treatment. The particularly strong impact of nitrate on nodules is most certainly due, in part, to the fact that in addition to the direct nitrate-related effects, the substantial ammonium influx

Table I. *Expression of sentinel genes in nodule tissue after 4 h of nitrate exposure*

Data are taken from three biological replicates and are given as DEseq normalized counts. Comparison between control and treatment was done using the DEseq method.

IMGAG Annotation	Function	Control	4 h NO_3	FC $\log_2$	FDR
Medtr5g012290.1	Nitrate transporter	11.9	386.7	4.91	<0.001
Medtr2g085510.1	High-affinity nitrate transporter	3.7	312.9	6.06	<0.001
AC233663_23.1	High-affinity nitrate transporter	5.5	615.2	6.57	<0.001
Medtr3g073150.1	Nitrate reductase (NADH dependent)	2436.3	6653.6	1.45	<0.001
Medtr3g073180.1	Nitrate reductase (NADH dependent)	2552.6	9729.9	1.93	<0.001
Medtr5g059820.1	Nitrate reductase	3114.7	11607.7	1.90	<0.001
Medtr5g012290.1	Nitrate transporter	11.9	386.7	4.91	<0.001
Medtr4g086020.1	Ferredoxin-nitrite reductase	3725.3	13559.8	1.86	<0.001
Medtr5g087490.1	Molybdopterin biosynthesis CNX1	1216.5	2003.8	1.65	<0.001
Medtr1g009200.1	NIP/LATD	750.6	1091.6	0.54	<0.01
Medtr3g026780.1	Siroheme synthase	786	1298.7	0.72	<0.001
Medtr4g068000.1	Nodule inception protein	6602.6	2350.7	–1.49	<0.001

into the cytosol abates over time and the nodule as a plant organ loses its functional significance for the whole plant.

Ammonium Transporter/Channel in the Symbiosome Membrane

It is an unresolved question as to which protein(s) catalyze the movement of ammonia/ammonium through the peribacteroid membrane (Udvardi and Poole, 2013). However, the genes coding for proteins of that function might be affected when much less ammonium is formed in the symbiosome space 28 h after nitrate exposure. In addition to the symbiotic ammonium transporter (Medtr4g009110.1), we found four other members of the ammonium transporter family expressed in the nodules (Medtr1g045550.1, Medtr7g069640.1, Medtr7g098930.1, Medtr8g095040.1). One of these genes (Medtr8g095040.1) showed a significant down-regulation between 4 and 28 h, the period of time of a concurrent significant reduction in ammonium production in the symbiosome (Supplemental Table S3). However, this occurred at a low expression level (3.19 RPKM in the controls, Supplemental Table S5). It is not known whether the proteins from these genes are peribacteroid membrane targeted or not.

A further group of proteins potentially involved in the conduit of ammonium are the potassium channels of the KUP family. A member of the group 2 type (Elumalai et al., 2002) of these transporters (Medtr5g071860.1) was among the most strongly down-regulated genes. This effect was already present at 4 h after nitrate exposure ($\log_2$ FC 4 h = –3.72, 28 h = –5.03).

Aquaporins are another group of proteins that are implicated as possible alternative proteins facilitating the movement of ammonium ions through the peribacteroid membrane (Hwang et al., 2010). Along with other types of proteins with various functions, aquaporins are abundant in the peribacteroid membrane (Wienkoop and Saalbach, 2003; Colditz and Braun, 2010). There is convincing, albeit indirect, evidence for their at least partial involvement in ammonium transport. This is indicated by the fact that the cytosolic form of glutamine synthetase in soybean nodules interacts with a domain at the C-terminal end of nodulin 26 (Masalkar et al., 2010). While at 4 h, only one Plasma membrane Intrinsic Protein aquaporin was down-regulated and two Tonoplast Intrinsic Protein aquaporins were up-regulated, a total of 15 aquaporin-related genes were differentially expressed at 28 h. Two genes (Medtr8g087710.1, Medtr8g087720.1) for a Nodule-26-Like Intrinsic Protein aquaporin on chromosome 8 are implicated to be involved in ammonium transport, judging from their level of expression and extent of their down-regulation between 4 and 28 h.

The Effect on Nodulins

Any nitrate-induced effect that is causative for the decline in nodule activity should be reflected in the transcriptome after 4 h of nitrate exposure, as according to our measurements this was the point in time when a decline in nitrogenase activity began. At this point in time, nitrate application had affected the expression of 1,120 genes (≥20 'unique hit' counts [DEseq] in treatment and/or controls, FDR < 0.01 and FC $\geq$ [2]). The reaction to nitrate occurred in what might be called a 'module-like' fashion, meaning that a number of genes in a dynamic and flexible gene network shifted the metabolism in a particular direction (Krouk et al., 2010a, 2010b).

Such a module-like shift occurred in the early down-regulation of the majority of all the expressed nodulins already at 4 h. The most strongly affected gene was the gene for early nodulins 93 (ENOD93; $\log_2$ FC = –5.23). Given its strong expression in our mature nodules (874 RPKM), the classification of this gene as an early nodulin according to Kouchi and Hata (1993) might be questionable. However, the role of ENOD93 in mature nodules is unclear (Okubara et al., 2000).

In addition to the gene for ENOD93, the most strongly affected group of genes among nodulins were those for nodule-specific cysteine-rich peptides (NCRs). Out

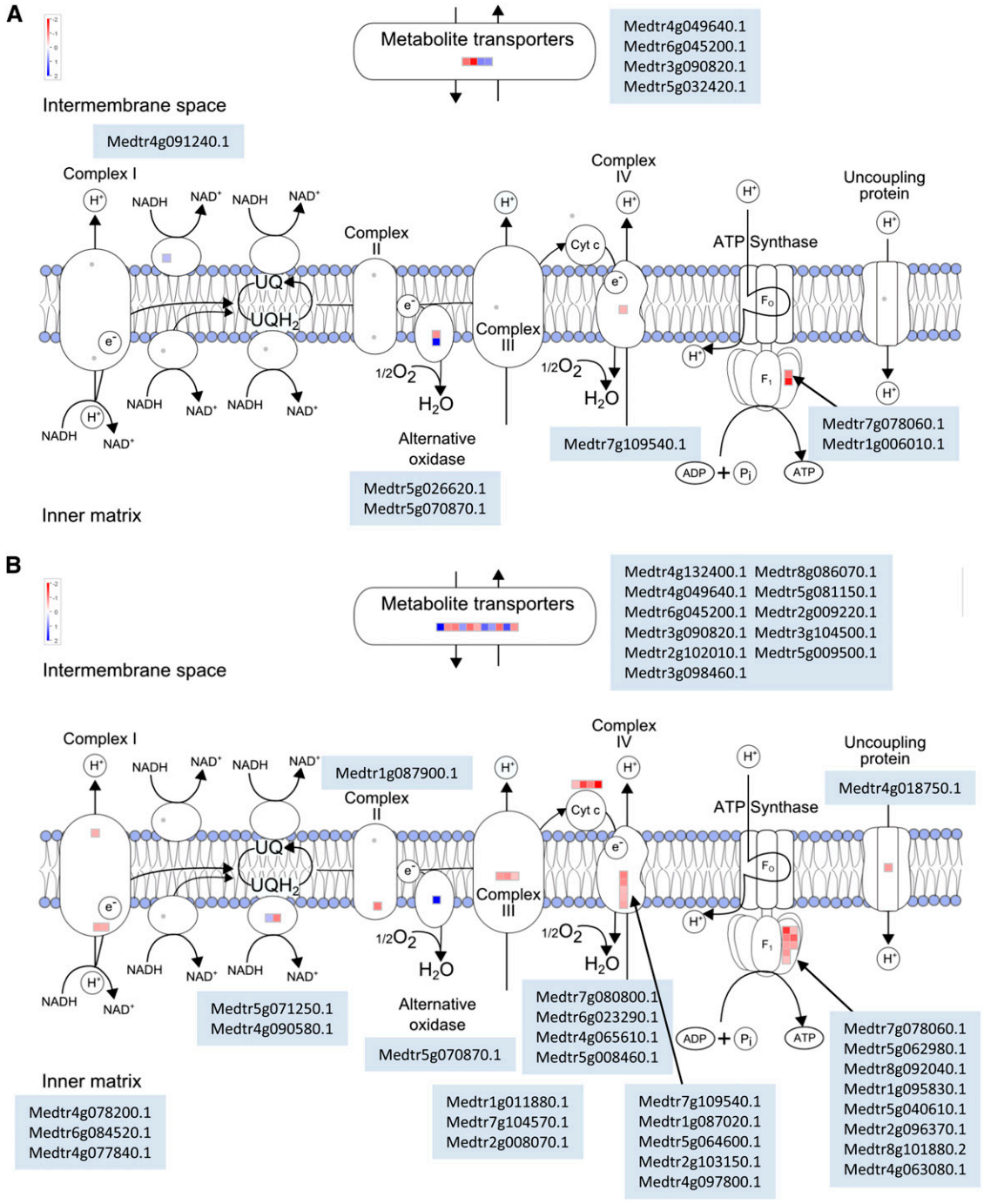

Figure 5. Differentially expressed genes involved in the mitochondrial electron transport chain at 4 h (A) and 28 h (B) after nitrate exposure. Red bins represent down-regulated genes and blue bins up-regulated genes. The data analysis was performed using the MapMan software (Thimm et al., 2004). The individual bins are identified using the International Medicago Genome Annotation Group (IMGAG) annotation. The precise extent of the expression change can be found in Supplemental Tables S2 and S3. Genes were considered as being expressed and differentially regulated when they complied with the following criteria: at least 20 'unique hit' counts (DEseq) in treatment and/or control and FDR < 0.01. Data were taken from three biological replicates. A and B are adapted from the MapMan pathway figures and from Jones et al. (2013).

of 238 genes for such peptides annotated in Mt3.5v.3, 204 were expressed in the control nodules (≥1 RPKM) with an overall high transcript abundance (on average, 348 RPKM). At 4 h after nitrate exposure, 127 of these genes were significantly down-regulated (FDR < 0.01; average $\log_2$ FC of –1.13). At 28 h, the number increased

to 186 genes, (average $\log_2$ FC of –1.45). NCRs are known to be symbiosome targeted (Wang et al., 2010) and are involved in the terminal transformation of *Rhizobium* spp. bacteria into bacteroids in legumes of the inverted repeat-lacking clade (IRLC; Van de Velde et al., 2010). These peptides are related to defensins, which are antimicrobial peptides found in human, animal, and plant bodies (Gachomo et al., 2012). We found three genes for defensin expressed in the nodules (Medtr8g075980.1, Medtr8g095370.1, Medtr8g023090.1); two of which were down-regulated at 4 h. At 28 h, the third gene (Medtr8g023090.1) was strongly down-regulated from a high level of expression (563 RPKM).

Furthermore, at 28 h, two components of the signal peptidase complex (Medtr3g027890.1, Medtr7g113740.1) and the catalytic core of the complex (Medtr2g103570.1) were strongly down-regulated. The strongest reaction was shown by Medtr3g027890.1 with a $\log_2$ FC of –1.71 from a high level of expression (1,062 RPKM). Plants with mutation in this gene are known to show a fix$^-$ phenotype (*defective in nitrogen fixation1*; Wang et al., 2010).

NCR and the related symbiosome-targeting machinery are part of the plant's defense reaction to bacteria that turns an infection into a beneficial symbiosis in the case of the *Rhizobium*-legume interaction (Kereszt et al., 2011). The physiological bottom line of the transformation into bacteroids is an overall slowdown of the bacteria's metabolism and an oxygen concentration-coupled, intensive expression and functioning of nitrogenase (Becker et al., 2004). Thus, a continuous flow of these peptides might be necessary for a high expression of nitrogenase in the symbiosome. This may mean that nitrate targets the bacteroid metabolism primarily through these peptides.

Four hours after nitrate exposure, all nine of the expressed genes for leghemoglobin were down-regulated from the high level of expression shown in the controls (up to 5,347 RPKM for Medtr5g081000.1). This down-regulation reached a level of on average $\log_2$ –1.46 FC for these genes. Leghemoglobins have two functions that are pivotal for nodule activity. Firstly, they are part of a variety of measures that maintain low free oxygen levels in the nodule-infected zone (Minchin, 1997). Secondly, they facilitate the oxygen flux to the bacteroids necessary for their respiration. RNA interference-based silencing of leghemoglobin genes has been shown to result in a loss of nitrogenase activity and increased oxygen levels inside the nodules of *Lotus japonicus* (Ott et al., 2005). In the case of nitrate, in addition to it causing this down-regulation of the gene expression, existing leghemoglobin protein might be blocked through nitrosylation.

Although, according to our data, the nodules were sites of intensive nitrate uptake and nitrate reduction shortly after the nitrate application, it is not clear whether a significant amount of nitrate would have reached the nodule-infected zone when the first posttreatment samples were taken at 4 h. This is because there are contradictory reports about the time needed for nitrate to reach the nodule's interior. Cesar et al. (1998) could measure significant amounts of nitrate in the infected zone of soybean nodules 2 d after exposure. However, at this point in time, the nodule had most probably lost most of its nitrogen fixation activity. By contrast, Becana et al. (2010) did not find any nitrate in the infected zone before 7 to 10 d after nitrate exposure. As yet, there have been no detailed time course studies done to show that the blocking of leghemoglobin precedes the decline in nitrogenase activity. Nevertheless, Cesar et al. (1998) found that after 8 d of nitrate exposure, 83% of nodule leghemoglobin activity is lost. Taking all this information into consideration, it appears safe to assume that the strong and concerted down-regulation of leghemoglobin genes, together with a possibly progressive nitrosylation of the existing leghemoglobin protein, is another very early impact on nodules caused by nitrate. This would probably result in a transient increase in free oxygen levels and a subsequent hampering of the flow of leghemoglobin-bound oxygen to the high-affinity cytochrome oxidase proteins present in the bacteroid.

Iron Allocation in Nodules

In addition to the high nodule nitrate and nitrite reduction activity and the reduced expression of genes for leghemoglobin and other nodulins, among the most strongly down-regulated genes at 4 h after nitrate application was nicotianamine synthase (Medtr1g084050.1; $\log_2$ FC –4.7). This is even more remarkable, as the gene in Arabidopsis is considered to be a sentinel gene for nitrate, however, one reacting with increased expression to nitrate application. Nicotianamine is a key player in intracellular Fe allocation (Hofmann, 2012). The down-regulation of nicotianamine synthase was induced from a high level of expression (367 RPKM in the controls) and occurred in conjunction with a down-regulation of a ferric-chelate reductase (Medtr7g038510.1). Furthermore, the metal-nicotianamine transporter *YELLOW STRIPE-LIKE2* (Medtr1g007500.1) was tendencially also down-regulated, although, with an FDR value of 0.059, this did not reach a significant difference according to our chosen threshold.

Iron is necessary for nitrogen fixation, not only as a major constituent of nitrogenase, but as an indispensable component of leghemoglobins and ferredoxin as well. To fulfill these functions, the iron ion needs to cross both the plasmalemma and the peribacteroid membrane. Further evidence that the intracellular allocation of iron is affected by nitrate was given by the fact that a vacuolar iron transporter (Medtr8g105790.1) was up-regulated after both 4 and 28 h, while the genes for ferritin formation (Medtr5g083170.1) and ferredoxin (Medtr4g131820.1, Medtr4g053120.1) were up- and down-regulated, respectively.

Effects on Nodule Respiration

Another early effect of nitrate on nodule function was a down-regulatory impact on genes of the mitochondrial electron transport chain and mitochondrial

ATP synthase (Fig. 5A). In addition, at 4 h, there was a significant up-regulation of a gene for an alternative oxidase (AC235677_8.1) and of one putatively for an alternative oxidase (Medtr2g025160.1), as well as of an external NADH-ubiquinone oxidoreductase gene that circumvents complex I (Medtr4g091240.1). This is consistent with various previous reports indicating a shift of the ATP/ADP ratio in the nodules after nitrate application and a slow or nondetectable effect on nodule Suc content (Kanayama and Yamamoto, 1990). The latter effect was confirmed in our study, as although two genes for Suc synthase (Medtr5g076830.1, Medtr6g081120.1) were down-regulated at 4 h, a MapMan analysis showed that glycolysis and the citric acid cycle remained largely unaffected after this time.

A number of genes involved in shifts in mitochondrial function were differentially expressed. The mitochondrial Rho GTPase (Medtr5g034580.1) was up-regulated, whereas four genes for a mitochondrial chaperone (Medtr2g030420.1, Medtr6g009520.1, Medtr6g009720.1, Medtr4g128260.1) were down-regulated. The chaperone is involved in the assembling of complex III and interacts with the Leucine Zipper EF-Hand Containing Transmembrane Protein1 (LETM1) complex in the human body (Tamai et al., 2008). The LETM1 gene (Medtr7g080610.1) itself was strongly up-regulated.

The reduction in processes for ATP synthesis was much stronger at 28 h than at 4 h, not only because of a stronger impact on the mitochondria electron transport chain (Fig. 5B), but also due to an effect on several aspects of sugar breakdown and oxidation. For example, five genes of the citric acid cycle were significantly down-regulated at this time (Fig. 6).

ATP produced by the plant's mitochondria is required for the uptake of malate into the symbiosome. Therefore, the reduction in ATP synthesis after the exposure to nitrate may also impair malate transport. One indication of such an impairment in the symbiosome uptake of malate might be the fact that a tonoplast malate transporter (Medtr4g133230.1) was strongly up-regulated at 28 h. The peribacteroid membrane targeted malate carrier in *M. truncatula* still has to be characterized (Udvardi and Poole, 2013). However, proteins of the aluminum activated malate transporter (AMLT) family have been shown to fulfill various functions in plants (Liu et al., 2009; Ligaba et al., 2012). The annotated gene for an AMLT (Medtr8g104120.1) in Mt3.5v.5 was already strongly down-regulated at 4 h and might be involved in malate import into the symbiosome. In conclusion, the apparent shortage of ATP developing in the nodules after nitrate application might slow down nitrogen incorporation and malate uptake of the symbiosome.

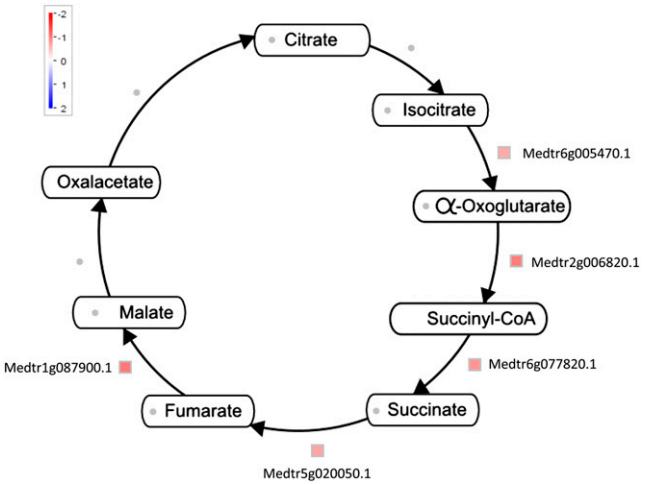

Figure 6. Differentially expressed genes involved in the citric acid cycle at 28 h after nitrate exposure. Red bins represent down-regulated genes. The data analysis was performed using the MapMan software (Thimm et al., 2004). The individual bins are identified by the IMGAG annotation. The gray dots indicate those genes that remained unchanged. The precise extent of the expression change can be found in Supplemental Table S3. Genes were considered as being expressed and differentially regulated when they complied with the following criteria: at least 20 unique hit counts (DEseq) in treatment and/or control and FDR < 0.01. Data were taken from three biological replicates.

Nodule Oxygen Permeability

Various studies have shown that the nitrate impact on nodules is connected with reduced oxygen permeability of the nodule (Vessey et al., 1988; Denison and Harter, 1995). The question of whether this is the primary effect is connected to two unanswered problems. Firstly, it is not known whether the decline in nitrogenase activity is the cause for or the result of a decrease in nodule oxygen permeability, as there is no time course study available that unambiguously shows the true situation. Both scenarios are conceivable, because nitrogenase activity needs intensive nodule respiration. Secondly, the precise functioning of the variable oxygen diffusion barrier remains elusive. A very quick and effective change in nodule oxygen permeability (within minutes) has been shown as a reaction to changing oxygen pressure around nodules (Hunt et al., 1989).

However, there are other clear and strong long-term impacts affecting nodule morphology and physiology that can influence oxygen permeability. Among them, the formation of various proteins, in particular glycoproteins, that 'clog' the nodule water-free apoplast regions has been indicated in lupine nodules (Delorenzo et al., 1993; Iannetta et al., 1993; Iannetta et al., 1995). Our data strongly support the occurrence of such processes for a time as early as 4 h after nitrate application. We found a strong up-regulation of various genes for albumin (5), germin-like proteins (10), patatin-like protein (1), thaumatin-like proteins (2), and stem 28 kD glycoproteins (2) at this time. None of these proteins have any obvious function in nodule metabolism. Nevertheless, albumin, in addition to its possible role in clogging intercellular spaces, is also involved in the colloid osmotic pressure in human and animal cells, and it is thought that part of the regulation of oxygen

diffusion in nodules is brought about by a shrinking or swelling of their cortex cells (Denison and Kinraide, 1995). Along with the up-regulation of the aforementioned genes for (gluco)proteins, numerous genes for proteinase inhibitors that contain a Kunitz domain were also strongly up-regulated. This could then possibly lead to a reduction in the degradation of proteins that clog the oxygen pathways, thereby facilitating such clogging.

CONCLUSION

Nitrate induces shifts in nodule metabolism that can be divided into early effects that down-regulate nitrogenase activity and later ones that cause a slowdown in nodule growth and induce senescence. The down-regulatory effects on nitrogenase occur in a module-like fashion with several genes and gene families being affected that are concertedly aimed at reducing the formation of the nitrogenase complex and causing a decrease in mitochondrial ATP formation. Thus, rather than inducing a more or less indirect reduction of the oxygen supply, or diversion of the assimilate flow away from the nodule, the nitrate ion seems to strike at the very heart of the N_2 reduction process, i.e. the formation of the nitrogenase complex itself and ATP generation. Given the complex character of the early nitrate effects and the numerous genes, gene families, and metabolic pathways involved, it appears unlikely that a total uncoupling of nitrate availability and symbiotic nitrogenase activity in nodules is possible. A gradual reduction in nitrate sensibility might be achieved by targeting those processes that reduce nitrogenase formation.

MATERIALS AND METHODS

Plant Growth

Seeds of *Medicago truncatula* 'Jemalong A17' were submerged in H_2SO_4 (96% [v/v]) for 5 min for chemical scarification, sterilized with 5% (v/v) sodium hypochlorite for 3 min, and rinsed several times with deionized water. The seeds were subsequently kept at 4°C for 12 h in darkness and submerged in tap water. The next step was a 2- to 4-day slight shaking of the submerged seeds at 25°C and continuous light. When the seeds had developed an approximately 20-mm-long primary root, they were transferred to small growth boxes (170 mm × 125 mm × 50 mm) filled with aerated nutrient solution. Each box contained 20 seedlings, which were fixed by small x-shaped cuts in adhesive tape on the upper side of the boxes.

The plants were grown for 2 weeks in these boxes in a growth chamber with a 16-h/8-h light/dark cycle at 25°C/18°C, respectively. The nutrient solution level in the boxes was maintained by the addition of an appropriate amount of nutrient solution every other day. The light intensity at plant height was approximately 400 μmol m^{-2} s^{-1}. Immediately after being transferred to the growth boxes, the seedlings were inoculated with 1 mL per box of a stationary *Sinorhizobium meliloti* (102F51) yeast extract-mannitol culture, with an approximate cell density of 10^9 mL^{-1}. The *S. meliloti* strain induced good nodulation with the first macroscopic nodules visible after about 7 to 10 d. This strain does not contain an uptake hydrogenase (Blumenthal et al., 1997).

After 2 weeks, the plants were transferred to glass tubes that allowed the separate measurement of root/nodule H_2 and CO_2 evolution. The system has been described by Fischinger and Schulze (2010a, 2010b). We enhanced the set-up by connecting a group of six plants through the lower side of the glass tubes to a 20-L nutrient solution container. Thus, using gravity, the nutrient solution level in the glass tubes depended on the height of the container, and losses via plant transpiration could be adjusted by the addition of nutrient solution to the container, thereby not interfering with the measurements in the root/nodule compartment. In addition, a pump in the container drove a nutrient solution flow of about 10 mL min^{-1} into the upper part of each individual glass tube. Each tube contained about 150 mL nutrient solution, which was replaced every 15 min. The nutrient solution in each glass tube was individually aerated with a gas stream of 200 mL min^{-1} (N_2/O_2; 80:20, v/v). The nutrient solution contained macronutrients (mM) K_2SO_4,(0.7), $MgSO_4$ (0.5), and $CaCl_2$ (0.8) and micronutrients (μM) H_3BO_3 (4.0), Na_2MoO_4 (0.1), $ZnSO_4$ (1.0), $MnCl_2$ (2.0), $CoCl_2$ (0.2), $CuCl_2$ (1.0), and FeNaEDTA (30). The pH was adjusted to 6.4 using KOH and buffered with 0.25 mM MES. Phosphorus was added daily as KH_2PO_4 to give a concentration of 5 μM P. Furthermore, after transfer to the growth boxes, the nutrient solution was adjusted once to a 0.5 mM NH_4^+ concentration by the addition of NH_4SO_4, as low concentrations of ammonium support nodule formation in *M. truncatula* (Fei and Vessey, 2009). During their growth in the glass tubes, the plants depended solely on nitrogen fixation for their N nutrition.

The nutrient solution was changed every week. During this procedure, the pump in the container was switched off and the backflow from the glass tubes to the container was blocked. Using this method, the ongoing measurements in the root/nodule compartment were not affected. The procedure of replacing the nutrient solution in the container took about 10 min, after which the nutrient solution turnover system was set back to normal.

Root/Nodule Gas Exchange Measurement

The system for measuring nodule H_2 evolution, including the determination of apparent nitrogenase activity (ANA), total nitrogenase activity, and the calculation of EAC, has been described by Fischinger and Schulze (2010a). We measured the ANA continuously during a 2-d period. The H_2 concentration data in the continuous gas stream were taken every 5 min. Total nitrogenase activity was measured on parallel plants at various points in time during the experiment.

qPCR

Total RNA was isolated from leaf material using the TRIzol reagent (Invitrogen) as described by Hummon et al. (2007; TRIzol/chloroform method). The integrity of the RNA was tested by gel electrophoresis. After DNase I (Invitrogen) treatment, the complementary DNA (cDNA) construction from 2 μg total RNA was made following the instructions of the High-Capacity RNA-to-cDNA Kit (Applied Biosystems) and using the StepOne thermo cycler (Applied Bioscience). The cDNA was diluted 1:2.5 and stored at –20°C.

For the qPCR, gene-specific primers were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The primer sequences of the tested genes and the reference genes are listed in Supplemental Table S1. For validation of the RNA-seq results, RNA from the extracted RNA pools for Illumina sequencing was used. The samples and reference genes were run in triplicate using the Fast SYBR Green Master Mix protocol (Applied Biosystems) on a StepOne Real-Time PCR System (Applied Biosystems) following the manufacturer's recommendations.

qPCR was performed in a 20-μL reaction mix containing 10 μL Fast SYBR Green Master Mix, 4 μL distilled, deionized water, 2 μL forward primer (2 pmol μL^{-1}), 2 μL reverse primer (2 pmol μL^{-1}), and 2 μL template cDNA (100 ng). The PCR conditions according to the Fast SYBR Green Master Mix protocol were 20 s of predenaturation at 95°C, 45 cycles of 3 s at 95°C, and 30 s at 60°C, followed by steps for the dissociation curve generation (15 s at 95°C, 60 s at 60°C, and a stepwise increase of 0.3°C up to 95°C).The StepOnePlus software (Applied Biosystems) was used for data collection. Relative transcript levels were obtained using the comparative cycle threshold C_T method.

RNA Extraction, cDNA Library Preparation, and RNA-Seq

Total RNA was purified from 27 samples ([untreated controls, 4 h nitrate, 28 h nitrate] × [three biological replicates] × [nodules from three plants to be pooled for one biological treatment]) using the TRIzol protocol (Invitrogen). The RNA was digested with RNase-Free DNase (Qiagen) and checked for integrity by capillary gel electrophoresis (Bioanalyzer, Agilent Technologies).

Library preparation for RNA-seq was performed using the TruSeq RNA Sample Preparation Kit (Illumina, Cat. N°RS–122–2002) starting from 500 ng

of total RNA. Accurate quantitation of cDNA libraries was performed by using the QuantiFluor dsDNA System (Promega). The size range of the final cDNA libraries was determined applying the DNA 1000 chip on the Bioanalyzer 2100 (Agilent; 280 bp). The cDNA libraries were amplified and sequenced by using the cBot and HiSeq2000 from Illumina.

The sequence images were transformed with Illumina software BaseCaller to bcl files, which were demultiplexed to fastq files with CASAVA version 1.8.2. The quality checks were done via fastqc (version 0.10.0, Babraham Bioinformatics). The sequences were aligned by Bowtie2 (version 2.0.2, Johns Hopkins University Baltimore) to the gene model Mt3.5v3.

Gene Expression Analysis

For the gene expression analysis, the expression level of each gene in each library was calculated by quantifying the number of Illumina reads that mapped to the Mt3.5v3 gene model using the Bowtie program counting only 'unique hits.' We use the term 'gene expression,' although what is measured by RNA-seq is a net result of expression and decay intensity of mRNA. The raw gene expression counts were normalized using the RPKM method (Mortazavi et al., 2008). RPKM values were used for comparing gene expression within one treatment. Genes showing differential expression were identified using the DEseq method for pairwise differential expression analysis, as the DEseq method has proven to be the most reliable way for RNA-seq-based differential expression analysis (Dillies et al., 2012). Differentially expressed genes identified by DEseq were required to have an FDR value of less than 0.01. Additionally, either control or treatment tissues had to have a DEseq value above 20 (Supplemental Tables S2-S4) before a gene was considered as having been expressed. The dendrogram was made with R. Graphics and the scatterplot figure was generated using GraphPad Prism (GraphPad Software). The overrepresentation analysis and part of the analyses of patterns of differentially expressed genes were done using MapMan (Thimm et al., 2004).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Overrepresentation analysis of genes differentially expressed after nitrate exposure.

Supplemental Table S1. qPCR validation of the RNA-seq data and sequences of all used primers.

Supplemental Table S2. Transcripts differentially expressed between untreated nodules and treated nodules after 4 h nitrate exposure.

Supplemental Table S3. Transcripts differentially expressed between untreated nodules and treated nodules after 28 h nitrate exposure.

Supplemental Table S4. Transcripts differentially expressed between treated nodules after 4 and 28 h nitrate exposure.

Supplemental Table S5. RPKM values for all genes annotated in Mt3.5v3 in untreated and treated nodules after 4 or 28 h nitrate exposure.

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