Nitrite and nitric oxide are important in the adjustment of primary metabolism during the hypersensitive response in tobacco

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Abstract

Nitrate and ammonia deferentially modulate primary metabolism during the hypersensitive response in tobacco. In this study, tobacco RNAi lines with low nitrite reductase (NiR) levels were used to investigate the roles of nitrite and nitric oxide (NO) in this process. The lines accumulate NO₂−, with increased NO generation, but allow sufficient reduction to NH₄⁺ to maintain plant viability. For wild-type (WT) and NiR plants grown with NO₃−, inoculation with the non-host biotrophic pathogen Pseudomonas syringae pv. phaseolicola induced an accumulation of nitrite and NO, together with a hypersensitive response (HR) that resulted in decreased bacterial growth, increased electrolyte leakage, and enhanced pathogen resistance gene expression. These responses were greater with increases in NO or NO₂− levels in NiR plants than in the WT under NO₃− nutrition. In contrast, WT and NiR plants grown with NH₄+ exhibited compromised resistance. A metabolomic analysis detected 141 metabolites whose abundance was differentially changed as a result of exposure to the pathogen and in response to accumulation of NO or NO₂−. Of these, 13 were involved in primary metabolism and most were linked to amino acid and energy metabolism. HR-associated changes in metabolism that are often linked with primary nitrate assimilation may therefore be influenced by nitrite and NO production.

Keywords: Amino acid metabolism, nitrate, nitric oxide, nitrite, nitrite reductase.

Introduction

Nitric oxide (NO) has well-established roles in plant physiology and metabolism (Mur et al., 2013a). Key early reports focused on the role of NO in contributing to the hypersensitive response (HR) form of pathogen-elicited programmed
cell death (Delledonne et al., 1998; Durner et al., 1998). Subsequent work has placed NO as playing an integral role in the network of host–pathogen elicitory events that govern the outcome of this interaction. NO is not only involved in HR as a part of effector-triggered immunity (ETI) (Delledonne et al., 1998) but it is also induced by pathogen-associated molecular patterns (PAMPs) (Zeidler et al., 2004), and it is involved in controlling the formation of cell-wall appositions (papillae) in host resistance to penetration (Prats et al., 2005).

With regards to mechanisms of NO generation in plants, the importance of nitrate reductase (NR) functioning as a nitrite reductase is now well established (Gupta et al., 2011). This places NO generation within the context of a series of reactions including NAD(P)H-dependent NO3 reduction to NO2 by NR, and subsequently via nitrite reductase (NiR) through to NO. An alternative step mediated by NiR diverts the nitrogen (N) flow towards NO and away from NO3 (NO3 → NO2 → NO). NO is not the only signalling molecule within the N economy in plants. NO3 signaling can potentiate its own as- 

Material and methods

Plant material

Seeds of tobacco (Nicotiana tabacum) cv. Gatersleben (wild-type, WT) were germinated on vermiculite under a 14/10 h day/night regime at 24/20 °C, a relative humidity of 80%, and 350–400 µE m−2 s−1 photosynthetically active radiation (PAR). This study also included the Gatersleben transgenic RNAi transgenic line 271 (NiRr), which is deficient in NiR (Morot-Gaudry-Talarmain et al., 2002). After 3 weeks, the plants were transferred to hydroponic culture for an additional 5 weeks. Plastic pots, each containing 1.8 l nutrient solution, were kept in a growth chamber with artificial illumination (HQI 400W; Schreder, Winterbach, Germany) at a PAR of 300 µmol m−2 s−1 with 16-h daily light periods. The day/night temperature regime of the chamber was 24/20 °C. The NO3− nutrient solution (pH 6.3) contained 3 mM KNO3, 1 mM CaCl2, 1 mM MgSO4, 0.025 mM NaFe-EDTA, 0.5 mM KH2PO4, 1 mM KH2PO4, and trace elements. The NO2− nutrient solution (pH 6) contained 3 mM NH4Cl, 1 mM CaCl2, 2 mM MgSO4, 25 µM NaFe-EDTA, 0.5 mM K2HPO4, 1 mM KH2PO4, and trace elements. The nutrient solutions were changed three times a week.

Growth of plant pathogen and inoculation

Pseudomonas syringae pv. phaseolicola strain PspH 1448A was grown at 28 °C in King’s B medium containing the appropriate antibiotics (Zeier et al., 2004). Overnight log-phase cultures were washed three times with 10 mM MgCl2 and diluted to a final concentration of 106 cells ml−1. The bacterial suspensions were infiltrated from the abaxial side into leaves using a 1 ml syringe without a needle. Control inoculations were performed with 10 mM MgCl2 alone. The inoculations were performed when plants had been growing in the nutrient solutions for 4 weeks, and in each case a fully mature leaf was inoculated. In planta bacterial growth was assessed using disks taken from the infiltrated areas of three leaves from different plants at 24 h after inoculation. The leaf disks were homogenised in 1 ml of 10 mM MgCl2, platting appropriate dilutions on King’s B medium, and counting colony numbers after incubating the plates at 28 °C for 2 d. Comparable samples were also taken from control plants.
Estimation of electrolyte leakage

Loss of membrane integrity was estimated by electrolyte leakage in 1-cm diameter leaf disks taken from the inoculated areas of leaves from three different plants as described by Mur et al. (1997). Kinetics of cell death were determined by this method.

Measurement of nitrite content

Leaf disks (1-cm diameter) were taken from three different inoculated and uninoculated plants. Each sample (100 mg) was extracted in 1 ml buffer that contained 50 mM HEPES, pH 7.2, and 100 µM MgCl₂, and 100 µl of extract was added to a reaction mixture containing: 600 µl sulfuricamide (1%), 600 µl N-(1)-(Naphthyl)ethylenediaminedihydrochloride (0.02%), and 125 µl zinc acetate (0.5 mM). After incubation for 25 min at 24 °C the samples were centrifuged at 16 000 g for 5 min, and the NO₃⁻ content of the supernatant was determined spectrophotometrically (Hageman et al., 1980).

RNA extraction and gel blot hybridisation analysis

Leaf samples were harvested after infiltration of leaves with Psph or with the control, the infiltrated areas with MgCl₂. Total RNA was extracted with PEQ Gold RNPure™ reagent (PEQ LAB, Erlangen, Germany) according to the manufacturer’s instructions. The RNA obtained was separated on 1.5% agarose gels containing 5.5% formaldehyde, and blotted into nylon membranes. The nucleic acids bound to the membrane were cross-linked by UV-irradiation for 2 min. The expression of Pathogenesis related protein 1 (PR1) provides a marker for NO-elicited production of the defence hormone salicylic acid (Mur et al., 2000, 2013b). PR1 probes were labelled to high specific activity using the Random Primer DNA labeling system with 40 µCi [α-³²P] dCTP (ICN Biomedicals, Eschwege, Germany) according to the supplier’s instructions. Hybridisation was performed at 65 °C for 15 h in 1% BSA, 1 mM EDTA, and 0.5 mM Na₂HPO₄. The membrane was washed with 2× saline sodium citrate (SSC), 0.1% SDS at 65 °C for 30 min, and afterwards with 0.2× SSC, 0.1% SDS for 15 min at 65 °C prior to autoradiography. The air-dried membrane was exposed to Kodak X-omat DS film (Stuttgart, Germany) with an intensifying screen at −80 °C and the film was developed after appropriate time intervals.

NO measurement using a quantum cascade laser

The use of a quantum cascade laser (QCL) to detect NO has been described previously (Mur et al., 2011). The system allows on-line NO measurements with a detection limit of 0.8 ppbv in 1 s (Mandon et al., 2016). Detached tobacco leaves were placed in a glass cuvette (200 ml volume) with an air inlet and outlet, and NO production was monitored at a controlled continuous flow rate of 1 l h⁻¹. Multiple cuvettes could be monitored in sequence, each being measured for ~13 min. The laser light emitted by the QCL (at ~1850 cm⁻¹) passed through a multi-pass absorption cell where the NO molecules are transported via the airline. The intensity of the transmitted laser light is strongly attenuated due to NO absorption in the multi-pass cell (effective path length=76 m), following the Beer–Lambert law. The detected signal depends of the laser intensity before the multi-pass cell, the absorption length, and the absorption coefficient of NO at the given wavelength. The NO concentration was calculated by measuring the attenuation of the light coming into the cell relative to the transmitted light (after the cell). Following measurement, the fresh weight of the leaves inside the cuvettes was determined.

Metabolite profiling

Gas chromatograph–mass spectrometry (GC-MS) analysis was performed as described previously (Cuadros-Inostroza et al., 2009). Six replicates each consisting of six pooled plants obtained from two independent experiments were subjected to GC-MS analysis. Metabolite levels were determined in a targeted fashion using the TargetSearch software (Cuadros-Inostroza et al., 2009). Metabolites were selected by comparing their retention indices (+/− 2 s) and spectra (similarity >85%) against the compounds stored in the Golm-Metabolome-Database (GMD) (Kopka et al., 2005), resulting in the identification of 141 metabolites. Each metabolite is represented by the observed ion intensity of a selected unique ion, which allows for a relative quantification between groups. Metabolite data were log₁₀-transformed. In addition, total amino acids were measured by HPLC described previously by Mahmood et al. (2002). Data were subjected to ANOVA using Minitab v.14. Comparisons between treatments were performed using Tukey multiple pairwise comparisons. Time-course data were compared using ANOVA. Principal component analyses and hierarchical cluster analyses were performed using the MetaboAnalyst software (Xia et al., 2015).

Results

Effects of N source on NO₂⁻, NH₄⁺, total amino acids, and NO production

Wild-type (WT) and NiR⁺ tobacco plants (n=6 per genotype) were grown hydroponically and supplied with NO₃⁻ or NH₄⁺ nutrient solutions for a 5-week period, after which leaves were sampled. At this stage the leaves were viable and apparently fully green, although in line with a previous report the NiR⁺ plants were smaller (Morot-Gaudry-Talarmain et al., 2002). In plants fed with NO₃⁻ there was a substantial and significant accumulation of NO₂⁻ (>5-fold compared to the WT) due to lower rates of reduction to NH₄⁺ (Fig. 1A). When fed with NH₄⁺, WT plants exhibited negligible levels of NO₂⁻ as the NR-dependent N assimilation pathway was circumvented. The NH₄⁺ content in plants fed with NH₄⁺ did not significantly differ between the WT and NiR⁺ (Fig. 1B). The lower amounts of NH₄⁺ in WT plants fed with NO₃⁻ presumably reflected the effects of N assimilation. The total amino acid contents of WT and NiR⁺ plants significantly differed according to the type of N nutrition (Fig. 1C). We next assessed NO production using a QCL-based system and the results indicated...
negligible production in WT and NiRr plants fed with NH4+ (Fig. 1D; note the log scale). However, feeding NO3– to NiRr resulted in >100-fold increase in NO production compared to the equivalent WT plants.

**Effects of N source on the hypersensitive response to a non-host pathogen**

Leaves of WT and NiRr plants fed with either NH4+ or NO3– were inoculated with the non-host pathogen *P. syringae pv phaseolicola* (Psph), or with a control solution of 10 mM MgCl2 and the resulting phenotypes were assessed after 24 h. No symptoms were seen in control WT or NiRr plants (Fig. 2A) but some signs of HR-associated necrosis were observed in WT plants fed with NO3– following inoculation with Psph. Necrotic symptoms were much more evident in leaves of NiRr plants fed with NO3– (Fig. 2B). In line with HR-elicited NO production being dependent on the reduction of NO3– and NO2– (Gupta et al., 2011), in the WT only plants fed with NO3– exhibited increased NO levels (Fig. 2C). The increase in NO production was substantially greater in NiRr plants. Some increased NO production was found in NiRr plants fed with NH4+, which probably reflects the residual NO3– concentrations observed in this genotype (Fig. 1).

The patterns of NO generation were broadly in line with other observed defence parameters. We assessed electrolyte leakage as a quantitative measure of cellular stress and death, and the most rapid increase was observed NiRr plants fed with NO3– (Fig. 3A). Increases in electrolyte leakage were also seen in the other treatments, but there was a notably slower and lower response in WT plants fed with NH4+. This was consistent with some increased stress that might be linked to the ultimate formation of a HR (Gupta et al., 2013). The delayed deployment of defences in WT plants fed with NH4+ was associated with increased numbers of Psph bacteria compared to plants fed with NO3– (Fig. 3B). A similar pattern was observed in NiRr plants but it was not so pronounced, presumably due to the effects of NO production in this genotype (Fig. 2C).

**Effects of N source on the metabolomic impact of a non-host pathogen**

Principal component analysis (PCA) was initially employed to describe the effects of the N regimes on the plant responses to inoculation with Psph. This revealed that challenge with Psph had relatively minor impacts on the metabolomes with respect to background genotypic effects (Fig. 4A). The delayed deployment of defences in WT plants fed with NH4+ was associated with increased numbers of Psph compared to plants fed with NO3– (Fig. 3B). A similar pattern was observed in NiRr plants but it was not so pronounced, presumably due to the effects of NO production in this genotype (Fig. 2C).
Fig. 2. Phenotypic responses and NO production in wild-type (WT) and transgenic tobacco plants with suppression of nitrite reductase (NiR) following inoculation with *Pseudomonas syringae* pv *phaseolicola* (Psph) in 10 mM MgCl$_2$. Plants were subject to hydroponic growth with either N form solutions prior to inoculation. Lesion development at 24 h after inoculation in leaves of (A) the WT and (B) NiR plants. Plants in the mock control were inoculated with 10 mM MgCl$_2$ only. Symptoms were more frequent and severe in NiR plants than in the WT when grown with NO$_3^-$, although phenylalanine levels were broadly maintained or increased following Psph inoculation. This may reflect prioritisation for phenylalanine biosynthesis to feed defence-associated biosynthesis of phenylpropanoids and flavonoids. For pyruvate and TCA-derived metabolites, each was increased or, in the case of α-ketoglutarate, maintained in response to Psph in NiR plants. Of the pyruvate-derived amino acids, there was an increase in alanine on infection at 12 hpi under both feeding regimes in NiR plants, was although levels declined with NH$_4^+$ at 24 hpi. Levels of valine in NiR plants showed no increase associated with Psph and declined in plants fed NO$_3^-$. The α-ketoglutarate derivatives glutamate and glutamine did not vary significantly in NiR plants whilst proline levels increased in plants fed with NH$_4^+$ to match those seen following feeding with NO$_3^-$. The oxaloacetate route demonstrated the greatest variety of responses. Aspartate and methionine transiently increased in NiR plants fed with either N form at 12 hpi. In the case of asparagine, this transient increase was only seen in - NiR plants fed with NO$_3^-$. For lysine, a similar increase at 12 hpi was also observed but only in the WT lines, irrespective of the N-feeding regime. Threonine gave the most distinct pattern with NiR plants fed with NH$_4^+$ showing increases following inoculation with Psph.

Other aspects of primary metabolism were visualised using a heat map (Fig. 7). This indicated two classes of response, namely metabolites that were induced in NiR plants or those that were induced in WT plants. Most metabolites showed only marginal changes following challenge with Psph. As with uninfected plants, glyceraldehyde-3-phosphate, fumarate, and malate appeared to be elevated whenever NO production was increased, suggesting a coordinated response between amino acid biosynthesis and the bioenergetic pathways in response to alterations in N nutrition.

**Discussion**

*NiR transgenic lines facilitate analyses of the effects of *N* forms on plant physiology*

Far from only acting as components in the nitrogen assimilation pathway, NO$_3^-$ and NO$_2^-$ (and possibly NH$_4^+$)
are emerging alongside NO as signalling components that influence important facets of primary metabolism. NO$_3^-$ signaling events have already been well characterised at the molecular level (Castaings et al., 2009; Xu et al., 2016). Whilst studies on NO$_2^-$ are not so advanced, transcriptomic experiments have established some overlapping effects of NO$_3^-$ and NO$_2^-$ on gene expression in Arabidopsis (Wang et al., 2007). NH$_4^+$ has been considered to be toxic at high levels and the bacterial tabtoxin produced by P. syringae pv. tabaci inhibits glutamine synthetase in plants, elevating NH$_4^+$ and initiating chlorosis (Langston-Unkefer et al., 1987). However, we have previously found that feeding with NH$_4^+$ can also bias N metabolism towards the production of GABA (Gupta et al., 2013), a nutrient source for some pathogens (Solomon and Oliver, 2001; Borrero et al., 2012). In contrast, feeding plants with NO$_3^-$ shifts metabolism towards increased resistance that correlates with elevated NO and polyamine production (Gupta et al., 2013). Crucially, these effects could reflect contributions from NO$_3^-$/NO or NO$_2^-$/NO, or a combination of all three N forms. Our current study aimed to address the question as to the relative involvement of these forms of nitrogen.

The difficulty in investigating the roles of any particular N form lies in their facile reduction, or in some cases oxidation, to other forms. Thus, although Kasten et al. (2016) treated plants with NO$_2$ gas, this resulted in the generation of variety of N forms. Wang et al., (2004) addressed this problem by generating NR null mutants, which could not reduce NO$_3^-$ to NO$_2^-$. In this current work, we exploited a previously characterised transgenic line that is suppressed in NiR (NiR$^r$), which would allow NO$_2^-$ accumulation relative to WT lines. Our initial assessment of the accumulation of different forms of N in NiR$^r$ plants were in line with the previous observations of Morot-Gaudry-Talarmain et al., (2002). Thus, the NiR$^r$ plants are able to highlight any NO$_2^-$/NO-mediated effects that were hidden in our previous analyses based on feeding of WT tobacco with NO$_3^-$ (Gupta et al., 2013). However, our approach had some difficulties as NO would also accumulate in NiR$^r$ plants fed

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**Fig. 3.** Effects of inoculation with *Pseudomonas syringae* pv *phaseolicola* (Psph) on wild-type (WT) and transgenic tobacco plants with suppression of nitrite reductase (NiR). Plants were subject to hydroponic growth with either NO$_3^-$ or NH$_4^+$ nutrient solutions prior to inoculation. (A) Electrolyte leakage in leaves 0–24 h after inoculation. (B) Bacterial growth on leaves measured at 24 h after inoculation. Data are means (±SE) of n=6 replicates. Different letters indicate significant differences between means as determined using ANOVA (P<0.05). (C) *PR1* gene expression in response to Psph infection. Representative data from one of three biological replicates are shown.
with NO$_3^-$, so we could not dissect NO$_2^-$ from NO effects. NiR$^+$ plants fed with NH$_4^+$ could indicate effects of this particular form of N, but we did note some very minor accumulation of NO$_2^-$ (Fig. 1A) and some minimal generation of NO (Fig. 1D). This NO$_2^-$ accumulation could reflect contributions of N from plant-associated microbes. Interestingly, low levels of NH$_4^+$ were detected in NiR$^+$ plants even with NO$_3^-$, most likely arising from residual activity of NiR (Fig 1C). This low level of NH$_4^+$ seemed to be able to maintain the viability of NiR$^+$ plants when feeding with NO$_3^-$.

**Fig. 4.** Metabolomic assessment of the impact of *Pseudomonas syringae pv. phaseolicola* (Psp) inoculation on wild-type (WT) and transgenic tobacco plants with suppression of nitrite reductase (NiR$^+\,$). Plants were subject to hydroponic feeding with either NO$_3^-$ or NH$_4^+$ nutrient solutions prior to inoculation. Samples for analysis were taken from leaves at 12 h and 24 h after inoculation and also from control mock inoculated leaves (n=6 replicates). (A) Principal component analysis (PCA) of 141 metabolites as detected by gas chromatography–mass spectrometry. (B, C) Sub-analysis of the same dataset focusing only on the responses of (B) NiR$^+$ and (C) WT plants. The shaded areas indicate 95% confidence intervals for each experimental class.
Discrete roles for NO₂⁻/NO and NH₄⁺ during a non-host HR in tobacco

The results obtained from the NiR⁻ line could potentially be artefacts, and therefore we aimed to concentrate on situations where NH₄⁺ and NO₂⁻ would be accumulating in WT plants so that their specific effects could be examined. NH₄⁺ accumulation is considered to be toxic and there appear to be no non-pathological situations under which it accumulates. Exogenous application has been shown to confer resistance against P. syringae in tomato, most likely through the initiation of oxidative stress to confer resistance (Fernandez-Crespo et al., 2015). By contrast, we have previously found that NH₄⁺ contributes to susceptibility to P. syringae in tobacco, possibly by diverting metabolism towards GABA as an N nutrition source (Gupta et al., 2013). The concentration of applied NH₄⁺ was similar in our study to that of Fernández-Crespo et al., (2015) except that we used NH₄Cl instead of (NH₄)₂SO₄. There are well-established situations where NO₂⁻ can accumulate, especially where substantial NO production is required during a HR (Gupta et al., 2011). The HR is therefore a situation where both NO and NO₂⁻ could have discrete effects from other NO forms. Kasten et al., (2016) also found the NO₂⁻ could

![Fig. 5. Changes in defence-associated metabolites in response to Pseudomonas syringae pv. phaseolicola inoculation on wild-type (WT) and transgenic tobacco plants with suppression of nitrite reductase (NiR). Plants were subject to hydroponic feeding with either NO₃⁻ or NH₄⁺ nutrient solutions prior to inoculation. Samples for analysis were taken from leaves at 0, 12h24 h after inoculation (n=6 replicates). Data show means (±SE) of relative accumulation of metabolites (see Methods).]
be linked to programmed cell death. Thus, our approach based on NiR\(^{-}\) plants allowed us to assess how far NO\(_{2}^-\) / NO could be influencing the HR as opposed to NO\(_3^-\) / NH\(_4^+\). Furthermore, the challenge with Psph would produce a stress situation in which amino acid catabolism could predominate over anabolism (Less and Galili, 2009).

Assessments of broad parameters linked to the HR confirmed the influence of NO/NO\(_2^-\) effects on the kinetics of

Fig. 6. Changes in amino acid accumulation in response to Pseudomonas syringae pv. phaseolicola inoculation on wild-type (WT) and transgenic tobacco plants with suppression of nitrite reductase (NiR\(^{R}\)). Plants were subject to hydroponic feeding with either NO\(_3^-\) or NH\(_4^+\) nutrient solutions prior to inoculation. Samples for analysis were taken from leaves at 0–24 h after inoculation (n=6 replicates). Data show means (±SE) of relative accumulation of amino acids and providers of amino-acid carbon skeletons, are plotted against the schematic diagram used in Fig. 6.
cell death, resistance to PspL, and PR1 expression as a biomarker for salicylic acid accumulation (Fig. 3). These are all well established as being linked to NO-mediated events (Delledonne et al., 1998). We chose to focus on the impact of N form on the metabolome in order to align with our previous study (Gupta et al., 2013; Mur et al., 2017). Wang et al. (2004, 2007) used a transcriptomic approach to demonstrate that nitrite at micromolar concentrations is able to induce changes in expression linked to primary metabolism, especially bioenergetic pathways and amino acid biosynthesis. As such pathways are also under allosteric control that would not be reflected in gene expression profiles, in our current study we adopted a metabolomic approach in order to reveal actual changes in key metabolites (as also suggested by Kusano et al., 2011) and thus to improve our understanding of nitrogen metabolism.

Our current study clearly indicated that our previous observed effects on GABA and spermidine (Gupta et al., 2013) were linked to NO$_2^-$/NO effects rather than to NO$_3^-$. Increases in putrescine were again linked to NH$_4^+$ application (Fig. 1B, Fig. 5) and seemed not to be linked to any antioxidant/stress hormone effect (Fig. 5). Further work is needed to establish the mechanistic basis of such NH$_4^+$ effects. More broadly, and in line with the studies of Wang et al. (2004, 2007), our metabolomic approach showed that major sources of variation between plants fed with the different forms of N were in metabolites associated with bioenergetic pathways (glycolysis, TCA cycle, and pentose phosphate pathways) and linked to amino acid biosynthesis (Supplementary Table S1 at JXB online). Many studies have linked the HR to metabolic pathways that drive increased carbon flux through the TCA cycle, and this has been presumed to serve the energetic demands of defence, and to be linked to the HR form of programmed cell death (Bolton et al., 2009; Balmer et al., 2018). Our data from NiR$^-$ plants would suggest that these effects on primary metabolism are influenced by NO$_2^-$/NO. Indeed, several of the steps previously linked to NO$_3^-$, particularly in the pentose phosphate and glycolytic pathways (Wang et al., 2000), appeared to be influenced by NO$_2^-$/NO in our study.

Given the obvious links with N assimilation and NO, much of our analyses focused on amino acid accumulation (Galili et al., 2016). A series of bioinformatic analyses have indicated that genes involved in amino acid catalysis are the most responsive to stress conditions (Less and Galili, 2008, 2009). Complementary to this, Less and Galili (2009) identified a ‘Met metabolism module’ with the asparate family network being the most active during growth. Beyond differential gene expression, several amino acid enzymes are subject to allosteric regulation, often by the end-products of various branches of the amino acid biosynthetic pathways. Our examination of the differential patterns of amino acid accumulation suggested changes that could be associated with the different bioenergetic pathways acting as the source of the carbon–skeleton precursors (Fig. 6). However, the patterns in changes that we found in amino acid metabolism bore no similarities to the ‘metabolism modules’ as defined by the transcriptomic analyses of Less and Galili, (2009).

Examination of amino acid metabolism during the interaction with PspL indicated a range of responses in the NiR$^-$ line. We separated the amino acids based on their source of carbon skeletons (Fig. 6) and this demonstrated a range of responses to N forms, with increases (pyruvate) or no significant changes (α-ketoglutarate, serine) on infection under both
feeding regimes. In some cases (tryptophan, tyrosine, asparagine, isoleucine, valine) changes were only seen with NiRr plants fed with NO3−, which would reflect regulation by NO3−/NO. The accumulation of these amino acids was dramatically reduced with Psph infection, and in the case of tryptophan accords with a report that NO can reduce the expression of genes involved in tryptophan–dependent auxin biosynthesis (Elhiti et al., 2013). Assuming that NO was exerting such an effect on the transient increases seen following Psph inoculation, asparagine was slightly differentially regulated as NiRr plants fed with NO3− exhibited a transient increase at 12 hpi before showing a reduction. Whether the regulatory mechanisms involve NO suppression of biosynthetic enzyme gene expression and/or increased amino acid catalysis remains to be established.

For oxaloacetate, methionine there were transient increases in both N-feeding regimes. This was a perplexing observation, perhaps indicating overlapping and general effects of various N forms. Equally, we observed NO3−/NO effects in plants fed with NH4+ so these could reflect sensitive responses to NO3−/NO. We also found that accumulation of oxaloacetate, asparagine, and threonine was increased more following inoculation with Psph in NiRr plants fed with NH4+ than in those fed with NO3−. This could be the result of interactions between NH4+ and NO3− accumulation, or of NO-mediated reduction of biosynthetic gene expression/increased catalysis. The picture is further complicated by glutamate and glutamine where NiRr plants showed no increases, suggesting either increased catalysis or reduced accumulation/accelerated utilisation.

Whilst our data suggest a role for NO3− acting either alone or via NO, they may not reflect a direct role of either on plant metabolism. Thus, changes in NO/NO2− in NiRr plants clearly have impacts on the redox status and, either directly linked to these or in parallel, there is an accumulation of defence signals (Fig. 5). In this context, NO has already been linked to changes in antioxidants and these can lead to accumulation of salicylate (Durner et al., 1998; Begara-Morales et al., 2016). However, this ignores the signaling roles of glutamate (Forde, 2014). Although not fully characterised, exogenous application of glutamate affects root growth and branching (Walch-Liu et al., 2006), causes stomatal closure (Yoshida et al., 2016), and induces genes involved in nitrogen uptake in roots (Kan et al., 2015), and several glutamate-like receptors (Kong et al., 2015; Singh et al., 2016) and signalling modules have been proposed (Forde, 2014). We found that glutamate accumulation (and therefore any linked signalling) only occurred in situations where NO3− accumulation was absent (Fig. 6). Thus, elevated NO3−/NO could function in an negative-feedback mechanism to suppress potentially excessive N assimilation, as has been suggested by ourselves and others (Mur et al., 2013b; Frungillo et al., 2014).

One question that remains outstanding from our work concerns the potential differential regulatory role of NO versus NO3− in influencing metabolism, and this will be addressed in our future work. However, taken together, our observations have provided clear evidence that N forms influence patterns of metabolism, tailoring them to particular situations through the relative levels of NO3−, NO2−/NO, and NH4+. These patterns serve precise bioenergetic needs for particular development or defence requirements.

## Supplementary data

Supplementary data are available at JXB online.

Table S1. Major sources of variation resulting from differential feeding of WT and NiRr tobacco plants with NO3− or NH4+

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### Author contributions

JGK, LM, WMK, and ARF conceived the ideas described in this paper; AK provided technical support and aided in reviewing and editing the final manuscript; JGK and LM wrote the manuscript and prepared the figures; YB prepared the metabolomic profiles; YB, AK, ARF, and LM analysed the metabolomic profiles; JZ undertook some preliminary bacterial infection experiments; JM, SMC, LM, and FH undertook NO measurements using the Quantum Cascade system.

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