Mitochondrial respiratory pathways modulate nitrate sensing and nitrogen-dependent regulation of plant architecture in *Nicotiana sylvestris*

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Summary

Mitochondrial electron transport pathways exert effects on carbon–nitrogen (C/N) relationships. To examine whether mitochondria–N interactions also influence plant growth and development, we explored the responses of roots and shoots to external N supply in wild-type (WT) *Nicotiana sylvestris* and the cytoplasmic male sterile II (CMSII) mutant, which has a N-rich phenotype. Root architecture in *N. sylvestris* seedlings showed classic responses to nitrate and sucrose availability. In contrast, CMSII showed an altered ‘nitrate-sensing’ phenotype with decreased sensitivity to C and N metabolites. The WT growth phenotype was restored in CMSII seedling roots by high nitrate plus sugars and in shoots by gibberellic acid (GA). Genome-wide cDNA-amplified fragment length polymorphism (AFLP) analysis of leaves from mature plants revealed that only a small subset of transcripts was altered in CMSII. Tissue abscisic acid content was similar in CMSII and WT roots and shoots, and growth responses to zeatin were comparable. However, the abundance of key transcripts associated with GA synthesis was modified both by the availability of N and by the CMSII mutation. The CMSII mutant maintained a much higher shoot/root ratio at low N than WT, whereas no difference was observed at high N. Shoot/root ratios were strikingly correlated with root amines/nitrate ratios, values of <1 being characteristic of high N status. We propose a model in which the amine/nitrate ratio interacts with GA signalling and respiratory pathways to regulate the partitioning of biomass between shoots and roots.

Keywords: nitrogen signalling, cellular redox state, mitochondria, abscisic acid, gibberellic acid, root:shoot ratios.

Introduction

Plant architecture is orchestrated throughout development by environmental and internal nutritional factors (Enquist and Niklas, 2002; Reinhardt and Kuhlemeier, 2002). Final

organ size and dimensions are governed by cell division, the length of time over which cell division is sustained and the amount of expansion of the cells after they have

ceased to divide (Reinhardt and Kuhlemeier, 2002). These parameters are controlled by hormones, particularly auxin, abscisic acid (ABA) and cytokinins, but also by carbon (C) and N status (De Smet *et al.*, 2003; Forde, 2002; Signora *et al.*, 2001; Vlieghe *et al.*, 2003). Plant architecture is thus the result of a complex web of interactions between phytohormone and nutritional/metabolic signalling pathways (Steffens *et al.*, 2005).

The formation of lateral roots (LRs) is a major post-embryonic developmental event and plays a critical role in determining the architecture and spatial arrangement of the root system (Malamy and Benfey, 1997a,b). The patterning of lateral organ formation is directly related to auxin patterning and/or distribution (Casimiro *et al.*, 2003; Reinhardt *et al.*, 2003). Environmental factors alter various aspects of auxin homeostasis, including auxin redistribution, via effects on the expression of *PIN* genes which mediate polar auxin transport (Pasternak *et al.*, 2005). Other hormones such as ethylene retard or stimulate growth in response to a range of environmental stresses. Gibberellic acid (GA) and ABA are also important in regulating growth in stressful environmental conditions. Thus, for example, while auxin is required for LR initiation, further development is regulated by ABA-dependent components which perceive C and N signals (De Smet *et al.*, 2003; Signora *et al.*, 2001). Gibberellic acid regulates elongation growth and related stress responses via degradation of the nuclear-localized growth-repressing DELLA proteins (Sun and Gubler, 2004), which integrate responses to independent hormonal and environmental signals, particularly in stressful environments (Achard *et al.*, 2006). The growth restraint conferred by DELLA proteins is beneficial and promotes survival, permitting a flexible and appropriate modulation of plant growth responses to changes in environmental conditions (Achard *et al.*, 2006).

Nitrogen is one of the most important external factors modulating plant growth, architecture and inter-organ allocation of resources. Plants grown with low N (LN) not only accumulate less overall biomass but they exhibit decreased shoot/root ratios compared to plants with an optimal or high N (HN) supply (Kruse *et al.*, 2002). Thus, N is one of several environmental factors that impact on the developmental programme to influence resource allocation between shoots and roots (Deak and Malamy, 2005; Raghothama, 1999; Stitt *et al.*, 2002). Nitrogen also influences the extent of root branching (Scheible *et al.*, 1997), notably through its effects on LR formation (Forde, 2002). During root branching developmental and environmental controls of cell cycle regulation are crucial. Perhaps not surprisingly, this regulation is reciprocal and the cell cycle influences the expression of a large number of genes encoding enzymes of primary N assimilation and associated metabolism. Evidence of extensive metabolic crosstalk has been provided between DNA replication and N

assimilation linked to the production of nucleotides (Menges *et al.*, 2002; Vlieghe *et al.*, 2003).

Despite the impact of the availability of N on plant development, much remains to be discovered. One outstanding question concerns which N compounds are most important in controlling LR growth and shoot/root ratios. Metabolites that have been implicated in root/shoot signal transduction include amino acids and nitrate (Forde, 2002; Foyer *et al.*, 2003, 2006; Scheible *et al.*, 1997; Stitt *et al.*, 2002). Nitrate reductase (NR) mutants were used to show that the shoot nitrate content is an important signal regulating shoot/root N allocation (Scheible *et al.*, 1997). Nitrogen signals interact intimately with C signals, particularly sucrose, glucose and trehalose, as well as organic acids such as 2-oxoglutarate (Finkelstein and Gibson, 2002; Finkelstein and Lynch, 2000; Leon and Sheen, 2003; Palenchar *et al.*, 2004). Sucrose and hexose-specific signalling mechanisms link source metabolism to N signalling and to hormone signalling pathways.

Tobacco (*Nicotiana* spp.) has been one of the most intensively studied model systems for understanding N assimilation and its impact on shoot/root partitioning (Foyer *et al.*, 1994; Geiger *et al.*, 1999; Hansch *et al.*, 2001; Knowles *et al.*, 1998; Kruse *et al.*, 2002; Scheible *et al.*, 1997; Vincentz and Caboche, 1991). Despite the wealth of data generated from studies on tobacco, relatively few studies have been carried out on N and C signalling during seedling establishment. Most of what is known comes from studies on *Arabidopsis thaliana* seedlings. This work has allowed important C and N signal transduction components to be identified through the use of molecular genetic techniques allied to *in vitro* studies of seedlings under controlled conditions (Zhang and Forde, 1998). In the present study, we have addressed the regulation of N-dependent control of root architecture and shoot/root ratios in *Nicotiana sylvestris*. This species ('woodland tobacco') has a rosette habit like *Arabidopsis*, and bolts after 10 weeks. In order to analyse the role of different respiratory pathways, and attendant changes in N assimilation, we have exploited the *N. sylvestris* mutant CMSII (cytoplasmic male sterile II). This mutant carries the only well characterized stable homoplasmic mitochondrial DNA mutation in any plant species that results in alteration of the respiratory electron transport chain (Gutierrez *et al.*, 1997). Respiratory pathways could be important in C/N sensing because both C metabolism and N assimilation depend on respiration. We have recently shown that modification of respiratory pathways in CMSII *N. sylvestris* mutants lacking mitochondrial complex I has significant consequences for leaf resource allocation between C and N metabolism (Dutilleul *et al.*, 2005), markedly influencing the *in vivo* efficiency of nitrate assimilation and producing a metabolic signature characterized by increased ammonia and amino acids when plants were grown under

non-limiting N conditions (Duttilleul *et al.*, 2005). Here we establish how modified respiratory pathways impact on the capacity of plants to perceive and respond to changes in nutritional status by examining the interplay between external N supply, plant C/N status and key N-regulated developmental responses in *N. sylvestris*.

Results

Respiration has distinct roles in different organs and at different stages of development. To examine the influence of modified respiratory pathways on N signalling we therefore compared the responses of wild-type (WT) and CMSII *N. sylvestris* to N availability during two developmental processes known to be N regulated. First, to establish whether N signalling pathways are modified in CMSII, we analysed root architecture during seedling establishment on agar. Second, we examined the consequences of modified

respiratory pathways for resource allocation between shoots and roots in mature plants.

Effect of respiratory pathways on N signalling and the control of root architecture

Increasing the availability of nitrate enhanced primary root growth in *N. sylvestris* seedlings but this effect was saturated at 1 mM (Figure 1b). Primary root growth was also stimulated by increasing sucrose from 0.5% to 2% at 1 and 5 mM nitrate (Figure 1b). Under all conditions, primary root growth was lower in the CMSII mutant than the WT. At low sucrose, the mutant response to nitrate was qualitatively similar to the WT, i.e. stimulation of primary root growth was saturated at 1 mM nitrate (Figure 1). When sucrose was increased, however, nitrate stimulation of root growth no longer saturated at 1 mM but was significantly increased by 5 mM nitrate (Figure 1b). Therefore, CMSII roots responded

Figure 1. The effect of KNO₃ availability on primary root growth in wild-type (WT) and CMSII mutant seedlings.

Primary root lengths in WT (black circles) and CMSII (white circles) at different times after sowing. Each data point in (b) is a mean value for between 30 and 45 seedlings. At each point the error bar is smaller than the symbol. Each experiment was repeated at least three times with similar numbers of seedlings.

Figure 2. The frequency of lateral root formation in wild-type (WT, black circles) and CMSII mutant (white circles) seedlings as a function of N concentration at different times after sowing on agar.

Note that N or chloride is expressed as $\log_{10}[\mu\text{M}]$ and that the scale takes into account the presence of two moles N per mole NH_4NO_3 . Each data point in (b) is a mean value for between 30 and 45 seedlings. At data points where the error bars are not obvious, the standard error values were smaller than the symbol. Each experiment was repeated at least three times with similar numbers of seedlings.

less than WT roots to nitrate and the extent of the difference between WT and CMSII root growth was condition-dependent.

In *Arabidopsis*, LR development requires an optimal nitrate concentration and inhibition of LR growth at supra-optimal nitrate can be partly overcome by high sucrose (Zhang and Forde, 2000). Wild-type *N. sylvestris* plants produced virtually no LRs in the absence of a N source in the medium during the first 14 days after sowing (Figure 2j–l). Nitrate strongly stimulated LR production in WT with an optimum of 1–5 mM (Figure 2d–f). Increasing sucrose antagonised the inhibition at high nitrate (Figure 2g–i) while NH_4NO_3 allowed LR production at much lower concentrations than KNO_3 (Figure 2a–c).

In sharp contrast to the WT, CMSII plants produced no LRs during the first 7 days of seedling establishment regardless of the N or sugar source available (Figure 2a,d,g,j). Even after 14 days, nitrate alone did not promote LR formation in CMSII (Figure 2f). Lateral root formation was conditional upon the presence of both nitrate and high sucrose (Figure 2h,i) or ammonium as well as nitrate (Figure 2c). Strikingly, CMSII was able to produce just as many LRs as the WT over the range 20 μM to 1 mM NH_4NO_3 but this was delayed to 14 days (Figure 2c). Elongation of LRs showed a similar dependence on C/N compounds to LR initiation except on NH_4NO_3 where poor extension growth was observed (Figure 3).

The above observations show that growth differences between WT and CMSII are conditioned by the environment and that the modified respiratory pathways in CMSII influence the C/N signalling network controlling root growth architecture.

To examine whether modified respiratory pathways also influence the development of the shoot we studied the responses of the two genotypes to exogenous gibberellic acid (GA; Figure 4). Stimulation of shoot growth by GA was about 2.5-fold in WT but 3.5-fold in CMSII (Figure 4). Consequently, CMSII shoot elongation was about 50% that of the WT in the absence of exogenous GA, whereas GA treatment allowed CMSII shoots to achieve 75% of WT shoot length (Figure 4). Thus, GA treatment caused CMSII plants to show similar shoot growth to the WT and repressed elaboration of the root system in both genotypes to a similar extent (Figure 4).

To investigate whether cytokinin signalling pathways might be involved in the CMSII phenotype seedlings were exposed to different concentrations of zeatin (Figure 5). Root growth was inhibited by zeatin in both WT and CMSII. For example, a concentration of 1 μM zeatin was sufficient to decrease root growth to about 10% of the control values in both genotypes (Figure 5).

The influence of modified respiratory pathways on N signalling in mature plants

To further dissect the interaction between modified respiratory pathways and N signalling, we analysed mature WT and CMSII plants grown on vermiculite with either 5 mM NH_4NO_3 (HN) or 0.1 mM NH_4NO_3 (LN). Following the observed effects of exogenous GA on the CMSII phenotype (Figure 4), we investigated GA metabolism in the WT and the CMSII mutant under these conditions. Since ABA acts antagonistically to GA in the regulation of several developmental process,

Figure 3. Lateral root growth at different NH_4NO_3 and KNO_3 and sucrose regimes in wild-type (WT, black circles) and CMSII mutant (white circles) seedlings at different times after sowing.

Each data point in (b) is a mean value for between 30 and 45 seedlings. At data points where the error bars are not obvious, the standard error values were smaller than the symbol. Each experiment was repeated at least three times with similar numbers of seedlings.

and because ABA is implicated in the control of root branching (Signora *et al.*, 2001), contents of this phytohormone were also quantified. Whereas N supply had a significant effect on leaf ABA content, root ABA was similar for both N regimes (Figure 6a). Similarly, no differences were found between CMSII and WT, either in shoots and roots at HN or LN (Figure 6a).

Nitrogen-dependent changes in GA metabolism were assessed by quantitative (q)RT-PCR analysis of transcripts encoding three oxidases involved in GA synthesis and turnover. While GA20ox was largely unaffected by N abundance in the WT (Figure 6c), GA3ox and GA2ox transcripts were greatly enhanced at LN compared with HN (Figure 6d,e). When abundance was compared between WT and CMSII, significant decreases in GA3ox and GA20ox (Figure 6c,d) and a significant increase in GA2ox (Figure 6e) were observed in the mutant specifically at LN. In contrast, at HN, the abundance of GA20ox was not affected in CMSII (Figure 6c), though both GA3ox and GA2ox were higher in the mutant (Figure 6d,e).

The technical difficulties of GA analysis preclude its ready determination in plants grown at LN. However, the effect of deficiency of complex I on GA metabolism was further assessed at HN by measuring the abundance of all extractable GAs. While little change in the abundance of the bioactive GAs (GA_1 and GA_4) was detected, substantial

effects on other forms of GA, particularly GA_8 , GA_{19} , GA_{20} and GA_{29} , were observed (Figure 6f,g).

The interaction between modified respiratory pathways and N signalling was further explored by transcript profiling. We performed a genome-wide cDNA-amplified fragment length polymorphism (AFLP) analysis that monitored the expression of 10,440 non-redundant transcripts in WT and CMSII plants grown under HN or LN. K-means clustering of the 223 differentially expressed transcripts was used to reveal major trends in gene expression (Figure 6). Clusters 1 and 2 show N-independent genotypic (WT versus CMSII) differences and clusters 3 and 4 represent N-dependent, genotype-independent changes. Genotypic differences that are affected by N nutrition are shown in clusters 5 and 6. Two-way ANOVA analysis revealed that 156 transcript fragments were significantly modulated in response to the modified respiratory pathways in CMSII. Of these, 105 were upregulated and 51 downregulated. Of the 142 transcripts modified in response to LN, 76 were upregulated and 66 downregulated. Importantly, 71 transcripts were differently expressed in response to LN in a genotype-specific manner (Table S1).

Of the 223 differentially expressed transcripts, 124 fragments were successfully sequenced (Table 1). Multiple transcript fragments for mitochondrial genes were

Figure 4. Effect of gibberellic acid (GA) on wild-type (WT) and CMSII mutant architecture at 5 days after sowing.

Seeds were germinated on basic medium supplemented with 1 mM NH_4NO_3 in the absence or presence of 10 μM GA. The lengths of the hypocotyl and primary root of 20 seedlings were measured per experiment. Data in the lower figure are the means $\pm$ the standard errors for 20 seedlings. Each experiment was repeated at least three times with similar numbers of seedlings. Black bars, shoots. White bars, roots.

upregulated in response to the modified respiratory pathways in CMSII (including 2-oxoglutarate dehydrogenase, an acyl carrier protein and four mitochondrially encoded genes). Transcript levels of genes associated with photosynthesis and photorespiration [a chlorophyll-binding protein, a photosystem II (PSII) reaction centre protein, an unknown thylakoid protein and mitochondrial serine hydroxymethyltransferase] were lower in CMSII leaves. Transcript that were differently expressed at low N included three glutathione-S-transferases and transcripts involved in proline synthesis (delta-1-pyrroline-5-carboxylate synthetase) and catabolism (mitochondrial proline dehydrogenase), nucleotide transport (two purine transporters), protein turnover (ubiquitin, two proteases and a protease inhibitor), starch synthesis (ADP-glucose pyrophosphory-

Figure 5. Effect of zeatin on wild-type (WT) and CMSII mutant architecture at 14 days after sowing on basic medium supplemented with 1 mM NH_4NO_3 and different concentrations of zeatin (shown on the left). Twenty seedlings were measured per experiment but typical examples only are shown in the figure for clarity.

lase beta subunit) and cell wall organisation (two arabinogalactan proteins).

Of the 71 transcripts that were differentially expressed in a genotype-specific manner in response to LN, transcript fragments with similarity to NAC transcription factors and embryo-abundant proteins were induced in CMSII only at low N. Conversely, a pseudo-response regulator-like transcript and four metal transporters were downregulated in WT by the LN treatment, but did not respond significantly in CMSII.

The nutritional status of WT and CMSII grown at LN and HN was first analysed by measuring total tissue C/N ratios (Figure 8). Shoot C/N ratios were increased 2.5- to 3-fold at LN but were not significantly altered in response to the modified respiratory pathways in CMSII (Figure 8). Strikingly, however, root C/N was lower in the mutant than in the WT under both growth conditions. Indeed, the root C/N ratio of the mutant at LN was not significantly different from that of the WT at HN (Figure 8).

Lower root C/N ratios in CMSII are consistent with our previous observations that the mutant is N-rich, based on profiling of leaf C and N metabolites (Dutilleul *et al.*, 2005). Because of the importance of soluble (i.e. non-protein) N compounds in signalling, these were analysed in roots and shoots of the two genotypes grown at HN and LN (Table 2). At HN conditions, both nitrate and total free amines were enriched in both CMSII shoots and roots compared to WT. At LN, however, the only enriched soluble N pool in CMSII was the root amine pool (Table 2). In both genotypes, biomass accumulation in roots and shoots was much greater in plants grown on HN than on LN (Figure 8b). Under both nutrient regimes, the mutant had lower biomass than the WT.

However, the difference in biomass between CMSII and WT was dependent on N availability. At HN, CMSII shoots

Figure 6. Interactions between the CMSII mutation, N availability, and phytohormone metabolism. (a, b) Free ABA content in shoots and roots (five biological replicates were performed per experiment). (c–e) Transcripts involved in gibberellic acid (GA) synthesis and metabolism (three biological replicates were performed per experiment). For (a–e) each frame shows high nitrogen (HN, 5 mM NH₄NO₃) on the left and low nitrogen (LN, 0.1 mM NH₄NO₃) on the right. (f, g) Abundance of different gibberellins in the wild type (WT, two replicates) and CMSII mutant (three replicates) at HN. Data are the means ± the standard errors for the number of replicates indicated above.

and roots were about 3.5-fold smaller than WT on both FW and DW bases (Figure 9a). In contrast, at LN, where root biomass was also much smaller in CMSII than in the WT (about 2.5-fold less), there were negligible differences in shoot biomass between the two genotypes (Figure 9b).

The data presented in Figure 10 provide evidence that the changes in C/N relations driven by modified respiratory pathways (Figure 8, Table 2) have consequences for resource allocation between shoots and roots. Despite the lower biomass in CMSII compared to WT grown at HN

(Figure 9), shoot/root ratios were conserved between the genotypes under this condition (Figure 10a). LN significantly decreased the shoot/root ratio in WT plants but the effect was much less marked in CMSII, where a relatively high ratio was maintained (Figure 10a). It has been previously been shown in tobacco WT and NR-deficient lines that the shoot/root ratio shows a positive linear correlation with leaf nitrate pools (Scheible *et al.*, 1997). Figure 10(b) shows that in the present study the most evident correlation with shoot/root ratios was observed for the amine/nitrate ratio, and this was particularly marked for the root amines/nitrate ratios. The data show that the root amines/nitrate ratio has a much stronger influence on shoot/root ratios than the shoot nitrate content alone, particularly in situations where plants are in an HN state.

Discussion

The CMSII mutant has modified respiratory pathways compared with WT leaves (Sabar *et al.*, 2000). As a consequence, CMSII leaves have enhanced rates of primary N assimilation and are enriched for shoot N compounds under HN growth conditions (Dutillieul *et al.*, 2005). This feature makes CMSII an intriguing genetic system in which to investigate the links between modified respiratory pathways and internal C/N status, and to identify how these affect N-influenced developmental processes. An analysis of the results presented in this paper allows the following conclusions to be drawn.

Nitrogen availability modulates the abundance of a small number of transcripts in N. sylvestris

The N-dependent regulation of gene expression has been described previously in *Arabidopsis* (Palenchar *et al.*, 2004). Many such studies have involved time-courses in response to withdrawal or increased supply of N. Our analysis of tobacco focused on long-term effects during growth at different N availabilities and has identified the following principal genotype-independent effects. Low N caused an increased accumulation of transcripts involved in purine transport, ammonia metabolism (phenylalanine ammonia-lyase), proline degradation (proline dehydrogenase) as well as protein turnover. In contrast, several transcripts involved in secondary metabolism and cell wall synthesis/structure were downregulated at LN (two arabinogalactan proteins and a pectin methyl esterase).

Transcripts associated with primary metabolism and metal transport are influenced by deficiency of complex I

The results of the genome-wide cDNA-AFLP analysis of *N. sylvestris* presented here demonstrate that modified respiratory pathways affect transcripts that are mostly targeted to the mitochondria. The data hence provide little

Table 1 List of differentially expressed transcript fragments in wild-type (WT) versus CMSII plants at high nitrogen (HN; 5 mM NH₄NO₃) and low nitrogen (LN; 0.1 mM NH₄NO₃)

Gene ID	Annotation	Normalized expression values				Solana EST e-value	(re) BLAST e-value
		WT		CMSII			
		HN	LN	HN	LN		
Cluster 1							
NS-BC1M41-385	Ribosomal protein 60S	-3.22	-2.93	1.22	0.52	1E-110	4E-119
NS-BC2M34-580	Subtilisin-like protease	-1.67	-0.26	0.68	0.32	1E-144	7E-116
NS-BC3M22-143	Mitochondrial transcription termination factor	-1.34	-1.54	0.86	0.54	2E-40	1E-08
NS-BT4M22-165	Similar to FAD-binding domain-containing protein	-1.23	-0.92	0.64	0.57	3E-27	1E-48
NS-BC1M22-225	Putative AAA ATPase	-1.25	-1.77	0.59	0.84	6E-82	1E-128
NS-BC1M14-216	DEAD box RNA helicase	-1.28	-0.91	0.50	0.71	2E-43	1E-49
NS-BT2M34-287	Mitochondrially encoded hypothetical protein orf138b	-0.85	-1.00	0.88	0.14	4E-99	5E-39
NS-BT4M12-221	Putative senescence-associated protein	-0.96	-1.56	0.75	0.55	5E-14	1E-35
NS-BT2M31-255	Nicotianamine synthase	-0.80	-1.98	0.89	0.40	2E-44	2E-140
NS-BT3M34-181	Mitochondrial acyl carrier protein mtACP-1	-1.13	-1.29	0.17	1.01	9E-46	2E-41
NS-BT1M22-154	60S ribosomal protein	-0.86	-1.20	0.28	0.84	5E-41	9E-95
NS-BC4M13-142	Mitochondrial DNA hypothetical protein	-0.63	-1.53	0.38	0.77		2E-10
NS-BT1M21-170	Tetratricopeptide-like protein	-0.86	-1.35	0.09	1.00	2E-28	1E-28
NS-BT1M13-206	Glutathione-S-transferase	-0.85	-1.98	0.07	1.10	9E-65	9E-94
NS-BT2M44-301	RBX1-like protein	-0.46	-1.06	0.29	0.65	2E-94	3E-55
NS-BC3M24-082	Aldose-1-epimerase-like protein	-0.51	-1.30	0.24	0.77	2E-05	2E-98
NS-BT4M32-300	Glutathione-S-transferase	-0.50	-1.44	0.18	0.84	2E-54	2E-71
		-1.14	-1.51	0.56	0.67		
Cluster 2							
NS-BT4M43-290	Similar to unknown protein	-0.12	1.33	-2.27	-1.45	3E-97	6E-95
NS-BC4M11-092	Mitochondrial serine hydroxymethyltransferase	0.31	0.88	-1.06	-1.16	1E-24	3E-174
NS-BC4M13-100	Photosystem II reaction centre W protein	0.75	0.31	-0.41	-1.59	3E-22	8E-32
NS-BC2M32-435	Chlorophyll a/b-binding protein	0.37	0.64	-0.55	-1.11	1E-175	2E-145
NS-BC2M11-525	Similar to chloroplast thylakoid protein	0.24	0.68	-0.53	-0.95	1E-174	0E+00
		0.57	0.61	-1.28	-1.57		
Cluster 3							
NS-BT1M24-252	Mitochondrial proline dehydrogenase	-1.45	0.81	-2.82	0.80	1E-101	4E-41
NS-BC1M43-302	Patatin homolog	-1.51	0.58	-0.26	0.40	1E-71	3E-75
NS-BT4M12-244	Plastidic purine transporter	-1.10	0.72	-2.00	0.71	2E-82	3E-30
NS-BT4M22-114	Superoxide dismutase [Cu-Zn]	-0.71	0.98	-0.92	-0.18	7E-20	2E-83
NS-BC1M23-261	Similar to ATP-dependent Clp protease	-0.98	0.71	-0.65	0.28	1E-60	6E-04
NS-BC2M42-313	ADP-glucose pyrophosphorylase beta subunit	-1.46	0.22	-0.71	0.90	1E-123	2E-101
NS-BT3M32-137	Haloacid dehalogenase-like hydrolase	-0.71	0.70	-1.36	0.46	6E-06	0E+00
NS-BT4M32-428	Putative protease	-0.81	0.35	-1.41	0.83	0E+00	2E-84
NS-BT4M21-228	Similar to phenylalanine ammonia-lyase	-1.16	-0.04	-1.09	1.08	9E-03	2E-26
NS-BT4M32-242	Purine transporter	-0.51	0.52	-1.49	0.59	9E-04	1E-09
NS-BT4M41-511	HAP2-related transcription factor	-0.66	0.01	-0.98	0.89	1E-146	0E+00
NS-BT2M23-270	Putative cullin 1	0.02	0.46	-1.37	0.30		5E-48
		-1.04	0.46	-1.32	0.67		
Cluster 4							
NS-BC3M23-300	Similar to chloroplast gene ORF124	0.96	-2.35	0.73	-2.30	3E-84	2E-23
NS-BT2M34-271	Fasciclin-like arabinogalactan cell adhesion protein	0.88	-1.53	0.54	-1.45	4E-09	1E-42
NS-BC2M32-116	Peptidylprolyl isomerase	1.11	-0.83	-0.42	-0.92	7E-12	1E-55
NS-BC4M14-331	Putative peroxidase	0.39	-0.97	0.86	-1.47	2E-20	6E-65
NS-BC3M24-406	Endochitinase B	0.24	-1.09	0.81	-0.76	1E-138	5E-90
NS-BC3M14-794	Wound inducible lipoxigenase	0.47	-0.48	0.54	-1.19	0E+00	1E-66
NS-BT2M12-165	Leucine rich repeat protein	0.58	-0.36	0.49	-1.67	9E-30	5E-75
NS-BC2M31-208	Chloroplast fructose-bisphosphate aldolase	0.49	-0.34	0.48	-1.29	4E-39	0E+00
NS-BT3M42-181	Pectin methylesterase	-0.25	-1.03	1.10	-0.92	2E-56	2E-169
NS-BC3M12-181	Similar to arabinogalactan protein	0.35	-0.23	0.80	-2.96	7E-59	3E-05
NS-BC1M34-177	Non-specific lipid-transfer protein 2 (LTP 2)	-0.80	-0.96	1.27	-1.03	4E-51	2E-42
NS-BC3M23-120	Aspartyl protease family protein	-0.34	-0.31	0.93	-0.99	3E-35	2E-87
		0.40	-0.95	0.65	-1.47		

Table 1 Continued

Gene ID	Annotation	Normalized expression values				Solana EST e-value	(re) BLAST e-value
		WT		CMSII			
		HN	LN	HN	LN		
Cluster 5							
NS-BC1M22-150	Protease inhibitor/seed storage/lipid transfer protein (LTP)	-2.92	0.10	-0.68	1.12	5E-31	3E-21
NS-BT1M43-243	Similarity to SMAP1 (SMALL ACIDIC PROTEIN 1)	-1.84	-1.58	-0.40	1.39	1E-44	3E-06
NS-BT2M44-119	NAC domain jasmonic acid 2	-0.57	-0.63	-0.69	1.04	7E-07	4E-114
NS-BT1M44-130	Similar to protease inhibitor	-0.81	-1.76	-0.40	1.25	1E-22	2E-33
NS-BT2M12-175	Mitochondrially encoded hypothetical protein orf138c	-1.13	-0.96	-0.21	1.11	4E-08	7E-40
NS-BT4M33-207	RNAse	-0.75	-1.26	-0.21	1.09	2E-56	2E-56
NS-BC1M44-504	Embryo-abundant protein	-0.67	-0.81	-0.29	0.99	0E+00	4E-31
NS-BT4M24-152	Glutathione S-transferase	-1.87	-1.51	-0.01	1.25	1E-44	7E-31
NS-BC1M12-750	Ubiquitin precursor	-2.07	-0.46	-0.09	1.07	0E+00	0E+00
NS-BT1M22-374	2-Oxoglutarate dehydrogenase	-0.73	-0.88	0.09	0.84	1E-61	1E-142
NS-BT1M22-277	18S mitochondrial rRNA	-1.59	-0.78	0.20	0.95	1E-102	0E+00
NS-BT2M14-159	Mitochondrially encoded putative tRNA	-1.52	-0.76	0.41	0.79	2E-39	0E+00
		-1.37	-0.90	-0.30	1.08		
Cluster 6							
NS-BT4M22-212	Delta 1-pyrroline-5-carboxylate synthetase	0.27	-2.17	0.77	-0.20	8E-13	0E+00
NS-BC1M24-151	Similar to unknown protein	-0.44	-2.77	0.77	0.50	2E-30	4E-02
NS-BC4M11-162	Nicotianamine synthase	0.19	-1.82	0.59	0.09	2E-56	2E-39
NS-BT2M11-229	Similar to metal transporter	-0.43	-2.36	0.57	0.66	8E-13	2E-36
NS-BC1M14-165	Similar to lipid transfer protein	0.33	-1.59	0.64	-0.24	1E-22	1E+00
NS-BC4M31-171	Nicotianamine synthase	0.20	-1.67	0.56	0.09	8E-40	2E-39
NS-BC2M43-287	Mitochondrially encoded hypothetical protein orf138b	0.63	-1.22	-0.23	0.23	0E+00	0E+00
NS-BC4M43-122	Putative glucan phosphorylase	0.57	-1.26	0.26	-0.14	0E+00	0E+00
NS-BC2M44-110	Similar to pseudo-response regulator transcription factors	0.20	-1.54	0.51	0.12	1E-18	4E-18
NS-BT2M33-193	ZIP family metal transporter	-0.08	-1.71	0.75	0.09	4E-54	4E-56
NS-BC1M14-209	Pathogenesis-related protein STH-2	-0.30	-1.75	0.87	0.09	3E-74	1E-66
NS-BT2M22-304	RBX1-like protein	-0.17	-1.59	0.59	0.35	1E-109	3E-55
		0.01	-2.13	0.67	0.10		

Only transcript fragments with similarities to known genes are shown (BLAST e-values are indicated). Normalized expression values were clustered into six groups by *K*-means clustering (cf. Figure 7) and average expression values per cluster are shown. Clusters 1 and 2 show N-independent genotypic differences. Clusters 3 and 4 represent N-dependent genotype-independent changes. Clusters 5 and 6 show genotypic differences that are dependent on N nutrition.

Figure 7. *K*-means clustering of differential transcript fragments in wild-type (WT) and CMSII mutant plants under high nitrogen (HN, 5 mM NH_4NO_3) and low nitrogen (LN, 0.1 mM NH_4NO_3) conditions.

Log_2 -transformed averaged expression values of the 223 differential transcript fragments determined by cDNA-AFLP transcript profiling were clustered into six groups. Coloured bars represent the average value per cluster per sample.

evidence for large global pleiotropic effects of the mutation at the level of the transcriptome. Despite the marked phenotypic changes caused by the loss of complex I, only 156 of

10 440 assessed transcript fragments were differentially expressed in CMSII compared with WT plants under HN conditions. This demonstrates that many of the changes

Figure 8. C/N ratios in shoots and roots of wild-type (WT) and CMSII mutant plants grown at either low nitrogen (LN, 0.1 mM NH_4NO_3) or high nitrogen (HN, 5 mM NH_4NO_3) for 10 weeks. Data are the means $\pm$ the standard errors of five replicates in each case.

Table 2 The shoot and root content of N compounds in wild-type (WT) and CMSII tobacco grown under high (5 mM NH_4NO_3) and low (0.1 mM NH_4NO_3) nitrogen (HN, LN)

	LN (0.1 mM NH_4NO_3)		HN (5 mM NH_4NO_3)	
	Shoot	Root	Shoot	Root
WT				
Nitrate	3.0 $\pm$ 0.6	2.8 $\pm$ 0.2	10.2 $\pm$ 2.3	5.2 $\pm$ 0.6
Total amines	3.7 $\pm$ 1.1	1.3 $\pm$ 0.1	25.6 $\pm$ 1.2	7.0 $\pm$ 0.9
Soluble protein	3.1 $\pm$ 0.4	0.8 $\pm$ 0.1	16.2 $\pm$ 2.0	2.2 $\pm$ 0.3
CMSII				
Nitrate	4.4 $\pm$ 0.9	3.5 $\pm$ 0.5	37.9 $\pm$ 7.7*	13.4 $\pm$ 2.2*
Total amines	5.1 $\pm$ 0.3	3.4 $\pm$ 0.3**	68.7 $\pm$ 15.5**	17.3 $\pm$ 0.7***
Soluble protein	4.4 $\pm$ 0.7	1.8 $\pm$ 0.6	11.2 $\pm$ 0.2*	4.9 $\pm$ 0.2***

* $P < 0.5$, ** $P < 0.1$ and *** $P < 0.001$.

Values are means of three or four independent extracts of different plants sampled 10 weeks after germination. Nitrate and amines are given in $\mu\text{mol g}^{-1}$ fresh weight (FW). Protein is given in $\text{mg g}^{-1}\text{FW}$. For CMSII values, significant difference from the corresponding WT value is indicated by asterisks.

occur at the level of metabolism rather than in the transcriptome, as already suggested by Dutilleul *et al.* (2005). Nevertheless, the transcript profiling described here reveals further insights into the effects of the complex I-deficient genotype on plant development under both HN and LN conditions. Firstly, several transcripts encoding mitochon-

drial proteins were altered in abundance in CMSII, underlining the impact of loss of complex I on mitochondrial function. Some of these transcripts, such as 2-oxoglutarate dehydrogenase, responded to LN in a genotype-specific manner. Thus, the way the tricarboxylic acid (TCA) cycle responds to N status is modified when the electron transport chain can no longer oxidize NADH through complex I.

Transcripts for the mitochondrial photorespiratory enzyme serine hydroxymethyl transferase were also altered (Table 1). Previous observations suggest that photorespiration is modified in CMSII (Dutilleul *et al.*, 2003; Priault *et al.*, 2006). Transcripts encoding chloroplast proteins were also affected by the loss of complex I, several being downregulated in an N-independent manner (Table 1). Even though modified respiratory pathways do not affect the photosynthetic capacity of CMSII at HN, these transcriptomic changes further support the importance of functional complex I in optimizing chloroplast-mitochondrion interactions (Dutilleul *et al.*, 2003).

Four transcripts encoding putative metal transporters were significantly downregulated in WT plants at LN, but showed little or no change in CMSII under these conditions. The reasons for possible alterations in metal homeostasis at LN is unclear but they may be linked to pH modifications caused by changes in N content. For example, it has recently been shown that cellular pH and N affect iron availability and homeostasis (Zhao and Ling, 2007).

Deficiency of complex I affects transcription factors involved in N signalling

The cDNA-AFLP analysis of tobacco reveals that several transcription factors responded to N in a genotype-dependent manner, indicating that deficiency of complex I activates signalling pathways that feed directly into the N-signalling network controlling growth and metabolism. For example, a HAP2-related transcription factor is induced by LN in both genotypes. However, it is expressed more strongly when complex I is absent, suggesting that modified respiratory pathways influence N-dependent transcriptional regulation. Several other transcripts showed genotype-specific responses to the LN treatment. A NAC domain transcription factor was strongly induced in CMSII under LN, but its expression remained low in the WT. Conversely, a pseudo-response regulator-like transcription factor was strongly downregulated in WT under LN, but it remained largely unchanged in CMSII. Both these transcription factors are therefore candidates for positive regulators of shoot/root ratios at LN. Strikingly, RBX1, a ring-finger E3 component of the SCF^{TIR} ubiquitin ligase complex that was recently shown to be an auxin receptor (Dharmasiri *et al.*, 2005; Kepinski and Leyser, 2005), was specifically downregulated in WT at LN. Another transcript possibly involved in

Figure 9. Comparisons of N-dependent effects on the wild-type (WT) and CMSII phenotypes.

(a) Rosette phenotype of plants grown at either low nitrogen (LN, 0.1 mM NH₄NO₃) or high nitrogen (HN, 5 mM NH₄NO₃) for 8, 9 and 10 weeks.

(b) Total shoot and root biomass (fresh and dry weight per plant) at 10 weeks. Black bars, shoot. White bars, root. Data are the means ± the standard errors for five replicates in each case.

auxin signalling, coding for a SMAP1-related protein (Rahman *et al.*, 2006), was only upregulated in CMSII at LN. The altered expression of both genes suggests that deficiency of complex I opposes the repression of auxin signalling at LN.

Modified respiratory pathways dampen the responses of LR development to N

The C and N responses of LR formation in *N. sylvestris* WT seedlings were similar to those in *Arabidopsis* (Signora

Figure 10. Comparisons of shoot/root ratios in CMSII and wild-type (WT) plants grown at high nitrogen (HN) and low nitrogen (LN).

(a) Shoot/root ratios calculated for fresh weight (black columns) or dry weight (white columns). Data are the means $\pm$ the standard errors for five replicates in each case.

(b) Shoot/root ratios (in fresh weight) plotted against organic N parameters (nitrate, left; amines, centre; amines/nitrate, right; top, leaf contents; bottom, root contents). White triangles, WT at LN. White circles, CMSII at LN. Black triangles, WT at HN. Black circles, CMSII at HN.

et al., 2001; Zhang *et al.*, 1999). With respect to the model of Zhang and Forde (2000), however, the observed differences between KNO_3 and NH_4NO_3 are intriguing in that ammonium or its products stimulate LR formation even at very low concentrations. The marked effect of ammonium on LR numbers in both WT and CMSII, particularly in 14-day-old seedlings, suggests that this N metabolite can be a powerful signal for LR formation at certain stages of root development. This behaviour is consistent with the established preference of plants for ammonium when provided with both ammonium and nitrate. Indeed, ammonium becomes an increasingly important substrate on ammonia-fertilized soils or on poorly drained, acidic soils where nitrification by micro-organisms is limited.

Models of N signalling and LR formation developed in *Arabidopsis* postulate that nitrate is the most important external signal, whereas a second level of regulation comes from internal C/N status-mediated through tissue amines and carbohydrates (Zhang and Forde, 2000). In this model, high contents of amines or certain amino acids reflect N sufficiency and act as a brake on excessive LR growth. At

both LN and HN, the CMSII mutant has an enhanced root amine content, correlated with poor LR growth and no stimulation by external KNO_3 , except at high sucrose. This suggests that the high internal N/C status of the mutant represses the formation of LRs. The partial alleviation of repression by high sucrose is consistent with this view. Indeed, on this basis CMSII could have been identified as a nitrate-sensing mutant in classical selection screens for C/N reciprocal sensing.

The N-rich state of CMSII drives shoot investment even at limiting N

Plants grown on LN accumulate less biomass and have lower shoot/root ratios than plants on HN, the rationale being that more investment in root biomass is required to ensure maximum capture of available nutrients, particularly N. While the mature CMSII plants had similar shoot/root ratios to the WT under the HN regime, they maintained a much greater shoot/root ratio at LN. Indeed, at LN, accumulation of shoot biomass was similar in WT and CMSII. The

mutant continued to invest heavily in shoot biomass when faced with LN, suggesting that it is less sensitive to the LN signal that leads to increased root investment in the WT.

Amino acids and sugars form part of the repertoire of signals controlling shoot/root ratios (Hansch *et al.*, 2001). Metabolite profiling of CMSII and WT grown at LN and HN using gas chromatography-time-of-flight mass spectrometry (GC-TOF-MS) shows that the most dramatic genotype-dependent changes are in leaf amino acids, with other compounds being less affected (TKP, RDP, GN, CHF, unpublished results). Data presented by Scheible *et al.* (1997) support a model in which shoot nitrate is the major control over shoot/root ratios in *N. tabacum*. Here, we show that even though CMSII shoots are enriched in nitrate and amines under the HN regime, this enrichment is much diminished at LN, where differences between CMSII and WT in shoot/root ratio are in fact most evident (Table 2). Indeed, the increase in shoot amino acids does not occur at LN (TKP, RDP, GN, CHF, unpublished results), and as shown here CMSII and WT have similar overall shoot C/N ratios (Figures 8 and 10). In contrast, the C/N ratio in the roots was lower in CMSII than in WT under both HN and LN, and at LN the key difference between CMSII and WT is the root amine pool (Table 2). This implicates a root signal downstream of nitrate in the control of shoot biomass. Specifically, our data indicate that the root nitrate/amine ratio could be an important factor. At LN, the nitrate/amine ratio of WT roots increases to 2.15 (from 0.75 in HN plants). In contrast, CMSII maintains a HN signature, with a root nitrate/amine ratio of around 1, independent of N availability. The root plays a particularly important role in nitrate reduction at LN in many species (Hansch *et al.*, 2001) and consequently the abundance of reduced root N metabolites may fulfil a useful signalling role, particularly under these conditions. Furthermore, it is probable that the transcription factors showing differential genotypic responses to the LN treatment (see above) are intermediates in the root nitrate/amine ratio-dependent regulation of biomass partitioning between roots and shoots.

Modified respiratory pathways influence the C/N-dependent control of hormone action in plant growth and development

Since hormones are important in controlling plant growth and development, the roles of several key phytohormones were examined by physiological, transcriptomic or biochemical approaches. Root and shoot ABA contents were increased in plants grown at LN compared with HN but no differences in tissue ABA were observed between WT and CMSII plants in either condition. Thus, while ABA is involved in C/N signalling (Signora *et al.*, 2001), it does not seem to be an important factor in mediating changes in growth and development linked to deficiency of complex I. Likewise, treatment with zeatin resulted in similar reductions in root length in CMSII and WT seedlings, suggesting that CMSII

does not have an impaired response to cytokinins. However, three types of data point to a role for GA signalling in regulating shoot and root development in response to modified respiratory pathways. Firstly, treatment with GA stimulated shoot growth such that CMSII seedlings almost recovered the WT phenotype in the presence of GA. Secondly, the cellular composition of GAs was altered in a genotype-specific manner under HN. Thirdly, the abundance of key transcripts involved in GA synthesis and metabolism confirms the interaction at HN and suggests that the role of GA is even more important at LN.

The response of transcripts involved in GA metabolism implicates GA in N signalling and reveals that this role is strongly affected by the absence of complex I. In the WT, the availability of N modified GA2ox and GA3ox transcripts, but not those encoding GA20ox. This suggests that N can modulate GA metabolism and thus influence growth. In Arabidopsis, transcripts involved in GA synthesis have previously been shown to be affected by N supply in seedlings (Scheible *et al.*, 2004), while the effect of phosphate on root growth and architecture has recently been shown to be mediated via GA and DELLA proteins (Jiang *et al.*, 2007). The final steps in the synthesis of bioactive GAs are catalysed by GA20ox and GA3ox, and the abundance of their transcripts is known to be highly sensitive to GA concentration (Thomas *et al.*, 1999). These transcripts can therefore be considered as indirect markers of GA abundance. In contrast, GA2ox is involved in the further metabolism of bioactive GAs, and transcripts are induced by high GA concentrations (Thomas *et al.*, 1999). Thus, the GA transcript profiles of CMSII at LN strongly suggest that GA is enhanced in this condition, in which CMSII and WT shoots show similar biomass accumulation. Figure 11 integrates these observations into a model to explain the interactions between N availability, GA signalling and respiratory pathways.

The data presented here illustrate the complexity of the network of integrated metabolic, hormonal and environmental signals that control plant growth. They demonstrate that as well as modifying overall plant growth, mitochondrial respiratory pathways exert a major effect on the partitioning of biomass between roots and shoots. Moreover, they show that modified respiratory pathways ('MRP' in Figure 11), such as those operating in CMSII, exert control over the GA biosynthetic pathway. Finally, we note that the cDNA-AFLP analysis reveals that other hormone signalling pathways, such as those involving auxin, may contribute to this network.

Experimental procedures

Analysis of seedling growth

Basic medium and growth conditions. Seeds of *N. sylvestris* WT and the CMSII mutant (Gutierrez *et al.*, 1997) plants were ger-

Figure 11. Model for the regulation of shoot growth by N and the impact of modified respiratory pathways (MRP). The scheme is based on importance of the degradation of growth-repressing DELLA proteins by gibberellic acid (GA) in controlling shoot growth (Achard *et al.*, 2006; Sun and Gubler, 2004). (a) In wild-type (WT) plants, high nitrogen (HN) perceived through signals such as a high root amines/nitrate ratio favours high GA which maintains low DELLA levels and promotes production of shoot biomass. (b) In WT plants at low nitrogen (LN), lower GA allows DELLA proteins to inhibit growth. (c, d) In CMSII, MRP promotes N enrichment (left) and inhibits overall growth through an unknown mechanism (right). At HN (c), despite metabolic N enrichment, the root amines/nitrate ratio is similar in CMSII to the WT. The GA signalling in CMSII is thus not greatly enhanced and so the inhibitory mechanism predominates, causing a smaller shoot biomass than in the WT [compare parts (a) and (c)]. At LN (d), MRP promotes a root amines/nitrate ratio similar to plants at high N [compare parts (a) and (d)], and this efficiently counterbalances the inhibitory growth effect to allow shoot growth that is similar to the WT at low N [compare parts (b) and (d)].

minated and grown on agar for 15 days. The basic growth medium consisted of 100 μM KCl, 40 μM MgSO_4 , 20 μM CaCl_2 , 22 μM NaH_2PO_4 , 0.9 μM MnSO_4 , 0.09 μM KI, 0.97 μM H_3BO_3 , 0.14 μM ZnSO_4 , 2 nM CuSO_4 , 20.6 nM Na_2MoO_4 , 2.1 nM CoCl_2 , 3.6 μM Fe-EDTA, 0.5 g L^{-1} 2-(N-morpholino)ethanesulphonic acid (MES) pH (5.7) and 1% agar-agar. Seedlings were grown in a controlled-environment chamber at 25°C with a 16-h light/8-h dark regime. Germination and primary and lateral root growth were followed during 15 days, for 20 seedlings of each genotype per treatment per experiment. For hormone treatments seedlings were germinated with 20–25 ml basic medium supplemented with 1 mM NH_4NO_3 and 10 μM GA or varying levels of zeatin.

C and N supplementation. Application of C and N compounds was carried out as previously described (Signora *et al.*, 2001; Zhang and Forde, 2000; Zhang *et al.*, 1999). Sterilized seeds were placed in 9-cm Petri dishes with 20–25 ml basic medium supplemented with different concentrations of KCl (control), KNO_3 or NH_4NO_3 (10 or 20 μM or 1, 20, 50 or 100 mM, respectively) and either 0.5% sucrose or 2% sucrose for the specified periods. Germination frequency was

noted and primary and lateral root lengths of 20 individual seedlings were measured directly using a ruler.

Monitoring of early lateral root development. Lateral root formation was defined according to the classification of Zhang *et al.* (1999). The relative frequency of LR_s at developmental stages C (post-emergence to <5 mm) and D (>5 mm) within segments of the primary root was calculated.

Effect of high and low N supplies on shoot and root biomass in mature plants. Seedlings were grown on Petri dishes for 15 days on the basic medium supplemented with 10 μM NH_4NO_3 as described above. Seedlings were then transferred to vermiculite, first in 6-cm square pots and thereafter in pots appropriate for their size. Plants were provided with the following nutrient solution: 1.5 mM KH_2PO_4 , 0.5 mM MgSO_4 , 25 μM KCl, 0.5 mM CaSO_4 , 5 μM Fe-EDTA, 2 μM MnSO_4 , 0.5 μM CuSO_4 , 2 μM ZnSO_4 , 25 μM H_3BO_3 , 0.5 μM MoO_3 supplemented with either 0.1 or 5 mM NH_4NO_3 . Nutrient solution was given twice per week for the first 4 weeks and thereafter every 2 days. Plants were grown in the greenhouse with supplemental lighting to achieve a 16-h photoperiod at 25°C (day)/20°C (night). Shoot and root biomass was determined in each condition and for each genotype, at the time points and the sample numbers indicated on the figures. At 10 weeks, leaf and root samples were harvested, frozen in liquid N_2 , and stored for metabolite and total C and N analysis.

Analyses of N compounds. Nitrate and total amines were measured in samples ground to a fine powder in liquid N_2 then in 100 mM HCl and appropriate amounts of insoluble polyvinylpyrrolidone (PVP). Following clarification by centrifugation (12 000 g), nitrate was measured by the salicylic acid method, where absorbance is measured at 410 nm against a blank containing everything but the sample. Total amines were measured by a ninhydrin colour assay as in Ferrario-Méry *et al.* (2002). Protein was measured in the pellet using the Bio-Rad (<http://www.bio-rad.com/>) colour reagent kit. Total N and C was measured by combustion in a Leco CNS 2000 Carbon Nitrogen Sulphur analyser (Leco Instruments Ltd, <http://www.leco.com/>).

Analyses of gibberellins and abscisic acid. Ten-week-old HN-grown WT (two independent replicates) and CMSII (three replicates) plants were freeze dried and 0.5 g dry weight was analysed for GA content as described by Coles *et al.* (1999) with modifications according to Griffiths *et al.* (2006). After methanol extraction and sample preparation GA methyl esters were resolved by reverse phase HPLC (Croker *et al.*, 1990) and pooled fractions were analysed as methyl ester trimethylsilyl ethers on a ThermoFinnigan GCQ mass spectrometer (<http://www.thermo.com/>).

The ABA content of root and shoot samples of five replicates per treatment was determined in aqueous extracts of freeze-dried tissue using a direct radio-immunoassay based on Quarrie *et al.* (1988) with modifications as described in Trouverie *et al.* (2004).

Complementary DNA-AFLP analysis. Complementary DNA-AFLP analysis was performed using RNA harvested from plants grown under HN and LN conditions for 10 weeks, as described by Queval *et al.* (2007) except that 128 *Bst*YI+1/*Mse*I+2 primer combinations were used. In total, 10 440 sequence transcript fragments were scored and subjected to two-way ANOVA ($P < 0.01$). Transcript fragments were expressed in the pairwise combina-

tions (WT HN vs. WT LN; CMSII HN versus CMSII, WT HN versus CMSII LN; WT LN versus CMSII LN), and analysed using the Student's *t*-tests ($P < 0.05$ and three-fold change). After an additional visual on-gel assessment 223 transcript fragments were retained, excised from gel and reamplified with *Bst*YI+1/*Mse*I+1 primers as described by Vuylsteke *et al.* (2007). Sequences were obtained for 124 transcript fragments and compared with nucleotide and protein sequences in the public databases by BLAST sequence alignments (Altschul *et al.*, 1997). Nucleotide BLASTs were also performed against Solanaceae expressed sequence tags (ESTs), followed by BLAST against public protein databases. A K-means clustering method with six groups using the Pearson correlation coefficient was applied on the log2-transformed averaged expression values in TMEV4 (The Institute of Genomic Research, <http://www.tigr.org/>).

Transcript analysis. After 10 weeks' growth under the HN and LN conditions described above, three replicates were harvested and immediately frozen in liquid nitrogen. Total RNA was extracted from 100 mg of frozen leaf material using 1 ml of TRIzol (Invitrogen, <http://www.invitrogen.com/>) according to the manufacturer's instructions. Following resuspension the RNA was further cleaned up using RNeasy Mini Spin Columns (Qiagen, <http://www.qiagen.com/>) according to protocol. After removing residual DNA with DNaseI, amp grade (Gibco-BRL; <http://www.invitrogen.com/>) RNA was reverse transcribed using 0.5 µg oligo(dT)12–18 (Gibco-BRL) and 0.5 µl random primers (Gibco-BRL) with 200 units SuperScript II (Gibco-BRL) at 50°C.

Quantitative PCR was performed on an Applied Biosystems 7500 real-time PCR system (<http://www.appliedbiosystems.com/>) using SYBR green JumpStart Kit (Sigma-Aldrich, <http://www.sigmaaldrich.com/>) following the manufacturer's instructions. The expression of the genes of interest was normalised with three endogenous controls NTPP2A, GAPDH and tubulin. Accessions and primer sequences were as follows: *N. sylvestris* GA20ox1 (AF494087; 5'-GACTTGTTGGTGAAGCATGTGCG-3'; 5'-CGCTAATCTCATCTGCA-ACG-3'); *N. sylvestris* GA3ox1 (AF494089; 5'-TGGACAATATGGAG-GAAGCTGG-3'; 5'-ATTGAAGGCTGCTCGTTCTGC-3'); *N. sylvestris* GA20x1 (AY242858; 5'-CGAACCCTCACAACAGCCAA-3'; 5'-TGTA-GAGGAATGCGAGTTGCC-3'); *N. tabacum* NTPP2A (X97913; 5'-TGCCCTTGTTGAGGAAAAAC-3'; 5'-TGGCAATGGCTGAAGAGC-TT-3'); *N. tabacum* GAPDH (M14419; 5'-TGTGGACCTTACCGTA-AGACTAGAGA-3'; 5'-CCCTCCGATTCTCTCTTGA-3'); *N. tabacum* tubulin BA1 (AJ421411; 5'-TGATCCTCGCCATGGAAAGT-3'; 5'-TG-ACATCCTTTGGCACAACATC-3').

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Supplementary Material

The following supplementary material is available for this article online:

Table S1. Differentially expressed transcript fragments in wild type (WT) and CMSII plants at high and low nitrogen (HN, LN).

This material is available as part of the online article from <http://www.blackwell-synergy.com>.

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