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Identification of genes regulated by ammonium availability in the roots of maritime pine trees

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Abstract Conifers have a preference for ammonium over nitrate as the main inorganic nitrogen source. However, it is unknown how changes in nitrogen nutrition may affect transcription profiles. In this study, microarray analysis and suppressive subtraction hybridization were used to identify differentially expressed genes in the roots of maritime pine exposed to changes in ammonium availability. A total of 225 unigenes that were differentially regulated by changes in ammonium nutrition were identified. Most of the unigenes were classified into seven functional categories by comparison with sequences deposited in the databases. A significant proportion of these genes were encoded for ammonium-regulated proteins of unknown functions. The differential expression of selected candidate genes was further validated in plants subjected to ammonium excess/deficiency. The transcript levels of representative genes were compared in maritime pine roots, 1, 15 and 35 days after nutritional treatments. Gene expression patterns suggest the existence of potential links between ammonium-responsive genes and genes involved in amino acid metabolism, particularly in asparagine biosynthesis and utilization. Functional analyses and exploration of the natural variability in maritime pine populations for a number of relevant genes are underway.

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Introduction

Nitrogen is the most important macronutrient in plants and its availability in soils affects many aspects of plant growth and development (Lea and Morot-Gaudry, 2001). During their life cycle, plants need to cope with a range of physiological situations, including conditions of nitrogen excess or deficiency. When the nutritional demand exceeds the supply, there is a deficiency that affects plant growth and requires readaptation of metabolic pathways. When more nitrogen is available than required for sustained growth, the excess is absorbed to a certain extent and stored in the form of amino acids or proteins (Lea and Morot-Gaudry 2001).

Nitrate and ammonium are the predominant inorganic sources of nitrogen available in soil. The regulatory mechanisms that control nitrate acquisition and signaling in roots are relatively well known (Forde 2002). Thus, it has been well established that nitrate acts as a signaling molecule in the regulation of its own assimilation pathway, root architecture and also systemic responses of the plant nitrogen status (Forde 2002; Miller et al. 2007; Gutiérrez et al. 2007). However, much less is known about the molecular regulation of ammonium acquisition and how plants respond to ammonium availability at the molecular level. It was recently found in Arabidopsis that the regulation of ammonium uptake takes place at the local level in roots, and adaptive responses and systemic signaling observed in nitrate-fed pants are absent (Ruffel et al. 2008). These findings indicate that ammonium uptake and

metabolism possibly involve regulatory steps that are different from nitrate acquisition.

Nitrogen availability is usually very low in forest ecosystems; however, industrial activities, atmospheric contamination and intensive agricultural practices have increased nitrogen deposition in the ecosystems (Gruber and Galloway 2008). Conifers dominate large ecosystems in the Northern Hemisphere and include tree species of major economic and ecological importance worldwide. In conifer forests, low pH, high residual content of lignin and other secondary plant products in the soil limit nitrification. Consequently, ammonium is the predominant source of nitrogen for conifer tree nutrition, and it is well documented that conifers, unlike herbaceous plants, have a preference for ammonium over nitrate as an inorganic nitrogen source (Kronzucker et al. 1997). The uptake of inorganic nitrogen and growth of pine seedlings have been examined. Thus, it has been previously reported that the ability of Scots pine roots to incorporate ammonium is greater than for nitrate; however, large amounts of ammonium lead to nutrient imbalance and decreased growth that possibly reflects ammonium toxicity (Ohlünd and Näsholm 2001). The primary assimilation of ammonium and the biosynthesis of the major amino acids in conifers have been recently reviewed (Cánovas et al. 2007).

Research efforts in our laboratory are focussed on improving the understanding of the response of maritime pine trees to ammonium availability and the transcriptional control of ammonium assimilation into amino acids. The identification of ammonium-responsive genes is a first step in the functional characterization of key genes that determine the response of conifers to nutritional stress. Conifer trees are largely undomesticated, and the molecular and functional characterization of key genes that govern adaptive traits of commercial interest may contribute to the selection and breeding of superior trees for plantation forestry.

Materials and methods

Plant material

Eight-month-old seedlings of *Pinus pinaster* Ait were provided by the forest nursery "Lugar Nuevo" (Consejería de Medio Ambiente of Junta de Andalucía, Jaén, Spain). Before starting the nutritional treatments, seedlings were subjected to an acclimation period of 3 weeks in a controlled growth chamber (Ibercex H-900-B) at 23°C, with 16-h light/8-h dark cycles. Then, seedlings were grown for 5 weeks in a growth chamber with the following nutrient solution (mg/L): Na_2HPO_4 (20), KCl (40), $CaCl_2$ (40),

$MgSO_4.7H_2O$ (20), chelated Fe (5.6), $NaMoO_4.7H_2O$ (0.001), $CuSO_4.7H_2O$ (0.006), H_3BO_3 (0.09), $ZnSO_4.7H_2O$ (0.1) and $MnCl_2.4H_2O$ (0.7). Two groups of seedlings were irrigated with the same nutrient solution supplemented with either 3 or 10 mM ammonium in the form of $(NH_4)_2SO_4$. The pH of all nutrient solutions was adjusted to 4.5 with hydrochloric acid. Seedlings were watered individually with 50 mL of the appropriate nutrient solution every week. The root samples were harvested 1, 14 and 35 days after the first irrigation with the nutrient solution and immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

Total RNA isolation

Total RNA was isolated from roots following the method of Liao et al. (2004). Approximately, 1 g of frozen tissue was pulverized in liquid nitrogen and used for total RNA isolation. RNA concentration and purity were determined spectrophotometrically, and only samples with an A260/A280 nm ratio of 1.9–2.1 and an A260/A230 nm ratio of more than 1.7 were used for subsequent experiments. RNA quality was further checked by agarose gel electrophoresis.

cDNA synthesis and construction of suppression subtractive hybridization (SSH) libraries

Equal quantities (1 μg) of total RNA isolated from root samples were used as the templates to synthesize double stranded cDNA, according to the manufacturer's instructions described in the SMART PCR cDNA Synthesis kit (Clontech, Palo Alto, CA).

Four different subtractive libraries were constructed using the PCR-Select cDNA Subtraction Kit (Clontech). The cDNA samples from 3 and 10 mM ammonium seedlings were used to construct two libraries (forward and reverse) for the excess ammonium conditions. The cDNA samples from ammonium-deprived and 3 mM ammonium seedlings were used to construct two libraries (forward and reverse) for the ammonium-deficient conditions.

The subtracted PCR products generated by SSH (sizes ranged from approximately 0.2–1.2 kb with an average length of 600 bp) were purified using the NucleoSpin Extract II kit (Macherey–Nagel, Düren, Germany) and cloned into the pGEM-T easy vector (Promega). *Escherichia coli* XL1 Blue competent cells were transformed by electroporation with the ligated products using a Micro-Pulser electroporation apparatus (Bio-Rad España, Madrid, Spain). The transformed bacteria were plated onto LB agar plates containing 100 μg/mL ampicillin, 1 mM IPTG and 80 μg/mL X-gal, and the plates incubated at 37°C overnight. The white colonies were manually picked

and cultured in 200 μL of LB containing ampicillin in 96-well plates. After overnight culture, glycerol was added to a final concentration of 10% (v/v) and then the plates were stored at −80°C.

Screening of the SSH libraries

Differentially expressed cDNAs were selected using the PCR-Select Differential Screening Kit (Clontech). First, 1 μL of each recombinant clone was transferred from the 96-well plates to 384-well plates containing ampicillin (100 μg/mL)-supplemented LB freezing medium [25 g/L LB broth, 6.3 g/L K$_2$HPO$_4$, 1.8 g/L KH$_2$PO$_4$, 0.5 g/L sodium citrate, 1 g/L MgSO$_4$, 0.9 g/L (NH$_4$)$_2$SO$_4$ and 4.4% glycerol] and incubated at 37°C overnight. Clones were transferred onto nylon membranes (Schleicher & Schuell, Dassel, Germany) using a 384-pin replicator and then placed on solid LB medium with 100 μg/mL ampicillin. After overnight incubation at 37°C, colonies were denatured by placing the membranes onto Whatman 3 MM paper saturated with denaturation solution (0.5 M NaOH and 1.5 M NaCl) for 4 min and then neutralized for 4 min on Whatman 3 MM paper saturated with neutralization solution (1.5 M Tris–HCl at pH 7.5 and 1.5 M NaCl). After air drying, membranes were UV-crosslinked (70,000 μJ/cm^2) and stored at −80°C until hybridization. Prehybridization, hybridization, washes and the analysis of results were done following the instructions of the PCR-Select Differential Screening Kit (Clontech).

Sequence analysis

After differential screening, the positive clones were grown in 96-well plates. One microliter from each positive clone was used as the template for PCR reactions with the SP6 and T7 primers. Extraction of plasmids by the standard alkaline lysis method was performed when no insert was recovered after the PCR reactions. Automated fluorescence sequencing was performed using the GenomeLab DTCS Quick Start Kit (Beckman Coulter) on a CEQ 8000 Genetic Analysis System (Beckman Coulter) using a PCR product or plasmid as the template.

Vector and adapter sequences were deleted using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Only sequences that were longer than 100 bp were analyzed. All sequences were compared to the NCBI database using the BLASTN and BLASTX algorithms (http://www.ncbi.nlm.nih.gov/BLAST), and the cDNAs were named according to the homologous sequence with the lowest BLAST e-value found in the database. No homology was considered when e-values were higher than 1e–4.

Array construction, sample labeling, hybridization and data analysis

The cDNA libraries used as the source of ESTs as well as the experimental conditions for microarray (PINARRAY) construction with a set of 3,346 unique cDNAs were as described elsewhere (Villalobos et al. submitted). ESTs from three pine cDNA libraries were preprocessed and assembled in unigenes before proceeding to functional annotation (Cantón et al. 2003; Alonso et al. 2007). ArrayControl Sense Oligo Spots (spikes) (Ambion Inc., Austin, TX) were also included in the microarray.

As much as 1 μg of total RNA extracted from root samples 35 days after treatment was amplified using the Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion) following the manufacturer's instructions. The antisense amplified RNA (aRNA) was labeled with the CyDye Post-Labeling Reactive Dye Pack (GE Healthcare, Barcelona, Spain) and 2 μg of each labeled sample (target and control) were dissolved in 50 μL of the Pronto! Long Oligo/cDNA Hybridization Solution (Corning, Lowell, MA). Samples from 3 mM ammonium were used as the controls, and ammonium-deprived or 10 mM ammonium as the target samples. Microarray slides were prehybridized, hybridized and washed according to the Pronto! Universal Hybridization Kit protocol (Corning). Microarray hybridization was performed in the Genetix Hybridization Chamber at 42°C for 16 h. For each comparison, one dye swap was performed as a technical replication. Hybridized slides were scanned at 5 μm resolution and their signal intensities were detected by Q-Scan (Genetix, New Milton, UK). The raw data were subsequently analyzed using PreP + 07 (Martín-Requena et al. 2009), using the print-tip Lowess normalization procedure and z test to identify differentially expressed genes.

Colony arrays were performed on a nylon membrane using a 96-pin replicator. A total of nine membranes were printed and hybridized with a mix of different radioactively labeled cDNA. From each sample (ammonium deprived, 3 mM and 10 mM ammonium at 1, 14 and 35 days after treatment), 50 μg of total RNAwere labeled and purified using the LabelStar Array kit (Qiagen gmbH, Hilden, Germany). Hybridization was carried out by incubation at 65°C overnight in a hybridization oven, followed by serial stringent washing (15 min in 2× SSC and 0.5% SDS, and 15 min in 0.2× SSC and 0.5% SDS at 65°C) to remove excess radioactivity. Filters were exposed to a phosphor-imaging screen (Fuji Imaging plate BAS-MS 2040) for 24 h at room temperature and scanned using an FLA-7000 Imaging System (Fujifilm). Hybridization signals were quantified using the ImageJ software (http://www.rsbweb.nih.gov/ij/). The local mean background was subtracted from the intensity of each spot to calculate the net

signals and then was normalized to an internal control (40S ribosomal protein S3A).

Real-time quantitative PCR (qPCR)

Prior to reverse transcription, 1 μg of total RNA was treated with RQ1 RNase-Free DNase (Promega Biotech Ibérica, Madrid, Spain) according to the manufacturer's instructions to remove any traces of genomic DNA. Complementary DNA (cDNA) was synthesized from 500 ng of total RNA with the PrimeScript RT reagent Kit (Takara, Shiga, Japan). Reverse-transcribed products were diluted to a final concentration of 5 ng/μL (RNA equivalent) and then stored at −20°C for qPCR. Gene-specific primers were designed with Web-based software Primer3Plus (Untergasser et al. 2007).

qPCR was performed in an Mx3000P Real-Time PCR System (Stratagene). The qPCR reaction was performed in 20 μL with a primer concentration of 0.3 μM, 10 ng of cDNA and the Quantimix Easy SYG kit (Biotools, Madrid, Spain). Each sample was measured in triplicate using the following protocol: 2 min at 95°C and 35 cycles of 10 s at 95°C, 20 s at 60°C and 30 s at 72°C. The fluorescence signal was captured at the end of each extension step, and melting curve analysis was performed from 60 to 95°C. The PCR products were verified by melting point analysis at the end of each experiment and during protocol development by gel electrophoresis.

The baseline calculation and starting concentration (N_0) per sample of the amplification reactions were estimated directly from the raw fluorescence data using the LinReg 11.3 program (Ruijter et al. 2009). The relative expression levels were obtained from the ratio between N_0 of the target gene and the normalization factor. We used the geometric mean of three reference genes (actin, 40S ribosomal protein and elongation factor-1-alpha) to calculate the normalization factor (Vandesompele et al. 2002).

Results

Identification of genes that respond to changes in ammonium nutrition

A schematic representation of the general experimental approach used to study the response of pine roots to ammonium availability is outlined in Fig. 1. Young maritime pine trees (8-months old) were subjected to conditions of ammonium excess (10 mM) or ammonium deficiency (0 mM) for a period of 35 days. Pine trees treated with a sufficient supply of ammonium (3 mM) were considered as nutritional controls in our experiments, according to the data previously reported by Ohlünd and Näsholm (2004) in

Scots pine. Total intact RNA was isolated from pine roots, and differentially expressed genes in response to ammonium excess/deficiency were identified by transcriptomic analyses. As the full sequence of the pine genome is still not available, two combined experimental approaches were used in the present work: microarray analysis and sequencing of clones isolated by suppressive subtractive hybridization (SSH). Candidate genes putatively regulated by ammonium availability were identified and validated by hybridization analysis. Differentially expressed genes were further selected and their steady-state levels were determined by qPCR. This experimental approach provided relevant genes for detailed functional analyses and investigation of the natural variability in pine populations.

Analysis of differentially expressed genes using microarrays

In the first set of experiments, intact RNA was extracted from the roots of plants and microarray analysis was performed to compare the excess/deficient conditions with the nutritional control plants. RNA was labeled with CyDye and hybridized to slides of PINARRAY. Four replicates were sampled and a dye swap for each biological sample was performed. Supplemental Table 1 shows that a total of 29 genes were identified as candidates that respond to changes in the ammonium availability. Four of these candidates had no evident similarity with proteins described in the databases and six of them encoded proteins of unknown functions. There were eight cDNAs that were differentially expressed in conditions of ammonium deficiency, including proteins that show similarity to cellulose synthase, heat-shock proteins, a water-channel protein and a protein of unknown function. A higher number of cDNAs (21) were differentially expressed in conditions of excess ammonium including genes encoding stress-related proteins, such as an antimicrobial peptide, a universal stress protein, aluminum-induced protein, catalase, enzymes of nitrogen metabolism such as asparagine synthetase, asparaginase and enzymes related to carbohydrate metabolism, such as a member of the aldose 1-epimerase family, transaldolase and a granule-bound starch synthase.

Construction of SSH libraries and selection of differentially expressed clones in response to ammonium

As the gene probes included in the microarray (PINARRAY) only represent a fraction of the maritime pine transcriptome, we decided to complement our studies by constructing and screening SSH libraries to identify genes regulated by changes in ammonium availability. Intact
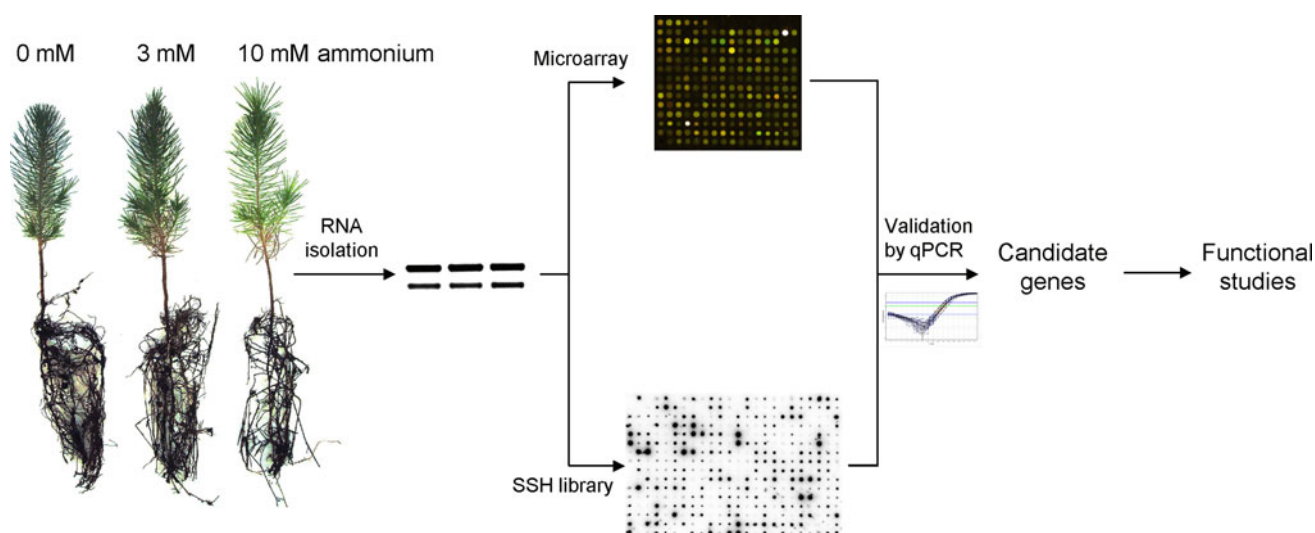
Fig. 1 Schematic representation of the experimental approaches used to identify genes regulated by ammonium availability in maritime pine roots

mRNAs were isolated from pine roots and retrotranscribed, and the resulting cDNAs from plants under ammonium excess/deficiency were used as testers, while cDNAs from control plants were used as drivers. A range of putative ammonium-regulated cDNAs between 200 and 1,200 bp were selected for molecular cloning with the majority of fragments around 600 bp in size. Four subtractive libraries were constructed under ammonium excess/deficiency. A total number of 2,304 clones (576 colonies per library) were picked and arrayed for further analysis. Through the differential screening of the SSH libraries, individual signals corresponding to downregulated and upregulated genes were identified (Fig. 2a). A total number of 339 clones (140 for ammonium excess and 199 for deficiency) were selected and fully sequenced. The resulting sequences were compared to gene and EST databases using the appropriate bioinformatic tools. Finally, 196 unigenes that were differentially expressed in response to changes in ammonium availability were identified (Supplemental Table 2).

About 18% of the sequences that were differentially expressed under excess ammonium, and 22% of those observed under ammmonium deficiency showed no homology to mRNAs or proteins deposited in the databases (Fig. 2b). These data are consistent with the methodological approach used for the subtractive library construction and could be explained in part by the relatively small size of the SSH cDNAs, which could lead to over-representation of non-coding fragments that show no homology in the databases. However, the higher percentage of differentially expressed genes observed in the deficient condition further suggests that a number of the sequences could correspond to novel ammonium-responsive genes. The unique sequences
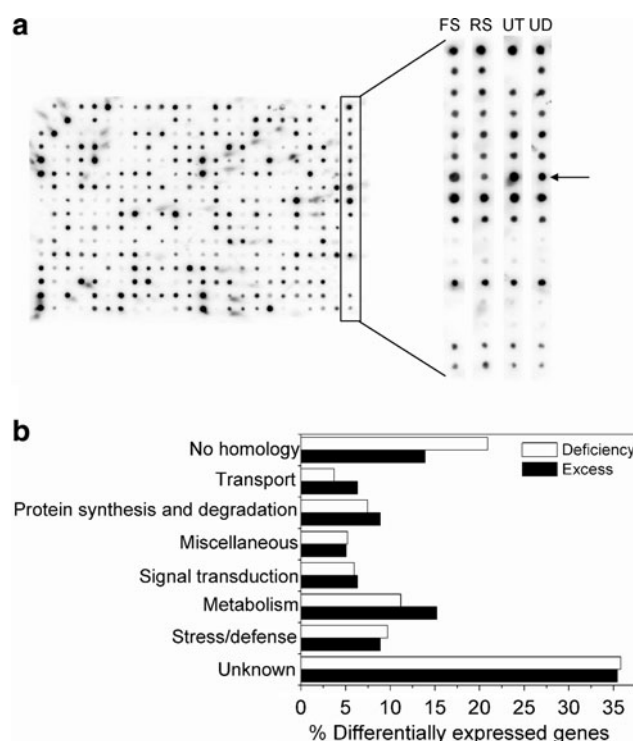

Fig. 2 Identification and functional classification of genes regulated by ammonium availability. An example of the screen used to identify the differentially expressed genes (**a**). A colony array was hybridized with forward subtracted probe (*FS*). The *rectangular box* shows in detail three additional hybridizations with reverse subtracted probe (*RS*), unsubtracted tester probe (*UT*) and unsubtracted driver probe (*UD*). The *arrow* indicates a positive clone. Distribution of genes in functional categories (**b**). The putative functions of positive clones were determined by sequence comparison with the GenBank database using BLASTX/BLASTN algorithm. The *white* and *black bars* represent the percentage of differentially expressed genes in ammonium deficiency or excess, respectively

that exhibited significant similarity to proteins present in the databases were grouped into seven functional categories (Fig. 2b). Interestingly, about 35% of the genes that were differentially regulated by ammonium in pine roots encoded proteins with unknown functions. The percentages of differentially expressed unigenes under ammonium excess or deficiency were quite similar in most of the functional categories established, except for genes related to nitrogen transport and metabolism, which were significantly higher in proportion under conditions of excess ammonium (Fig. 2b). In this nutritional condition, an increase in carbon skeletons is required for the incorporation of ammonium into amino acids. Genes related to carbon metabolism were differentially regulated, including triose phosphate/phosphate translocator, phosphoglycerate kinase, citrate synthase, malate synthase family, fructose-bisphosphate aldolase-like protein and starch synthase. Another group of genes related to abiotic and biotic stress, including aluminum-induced protein, universal stress protein and antimicrobial peptide were also identified, as well as genes for enzymes involved in asparagine metabolism. Under nitrogen deficiency, several stress-related genes were differentially regulated, such as pathogenesis-related protein, heat-shock proteins, chaperonin, thioredoxin, catalase and peroxidase. Genes for carbon/nitrogen metabolite transporters, such as a sugar transporter or a glutamine dumper, were also identified.

## Validation analysis of maritime pine genes regulated by ammonium availability

To confirm the validity of the findings from the SSH library screen, the expression profiles of 88 randomly selected clones from all functional categories were examined by colony printing in a macroarray and hybridization with ^{32}P-labeled probes for ammonium excess/deficient conditions (data not shown). Hybridization signals were not observed for a significant amount of the clones included in the array, in all nutritional conditions examined, indicating that the corresponding genes were expressed at very low levels in maritime pine roots. Although this group of genes deserves further attention and should be characterized in much more detail, we decided to concentrate our initial studies in the group of highly expressed targets. Differential expression was clearly observed for 47 clones included in the analysis, which showed different patterns of upregulation/downregulation during the 35 days of treatment with different levels of available ammonium. Based on this experimental approach, 12 genes were selected for quantitative determination of gene expression by qPCR, most of which were identified in the substractive libraries and encoded antimicrobial peptide (AMP), a pathogenesis-related

protein (PR10), malate synthase (MS), an aluminum-induced protein (AIP), a glutamine dumper (GDU), a late embryogenesis abundant protein (LEA), a thioredoxin (TH), a RING-H2 zinc-finger protein (RING-H2 ZF) and an unknown protein. The other three genes encoding an embryo-abundant protein (EAP), a member of the aldose 1-epimerase family (A1-E) and a granule-bound starch synthase (GBSS) were identified in the microarray analysis (Supplemental Table 1). Sense and antisense primers were designed for the specific amplification of these genes, and the corresponding sequences are presented in Supplemental Table 3. The expression levels of these candidate genes were examined at 1, 15 and 35 days of treatment with excess or deficient ammonium, and the results were compared with those observed in the normal ammonium condition (3 mM). As shown in Fig. 3, significantly different expression patterns were observed for the selected candidate genes. A1-E transcripts accummulated at the end of the treatment with excess ammonium. However, other genes such as EAP and AMP exhibited a sustained activation in response to ammonium excess. PR10 was apparently repressed by deficiency and activated by excess ammonium at the end of the treatment period. MS was clearly activated by both ammonium deficiency and excess. In contrast, AIP and GDU were repressed early by excess ammonium, whereas LEA and TH showed sustained repression by excess ammonium. RING-H2 ZF exhibited sustained repression by ammonium excesss and deficiency. Sustained activation by deficiency and repression by excess ammonium was observed for the GBSS gene. Interestingly, a gene of unknown function showed early activation by deficiency and repression by excess ammonium.

Co-regulation of genes in response to ammonium availability and genes involved in nitrogen assimilation

In our experimental approach, no major changes in the expression of genes involved in nitrogen assimilation and amino acid metabolism were found in response to ammonium excess/deficiency with the exception of the genes encoding asparagine synthetase (AS) and asparaginase (ASPG). We were interested in confirming these findings by examining in detail the expression levels of a range of genes that were previously characterized in our laboratory (Cánovas et al. 2007; de la Torre et al. 2007; Cañas et al. 2008; Pascual et al. 2008; unpublished data). The same samples from which the 12 candidate genes were previously analyzed (Fig. 3) were used to determine the steady-state levels of transcripts for the enzymes GS1b, NADH-dependent glutamate synthase (NADH-GOGAT), NADP$^+$-dependent isocitrate dehydrogenase (ICDH), aspartate aminotransferase (AAT), arginase (ARG) and ornithine

Fig. 3 Time-coursse expresssion profiles of selected candidate genes regulated by ammonium availability in maritime pine roots. Total RNA was isolated from maritime pine roots 1, 14, and 35 days after nutritional treatments, and mRNA levels were determined using qPCR. The values represent the $\log_2$ of the ratio between the relative expression levels in either ammonium deficiency or excess and in control samples. The relative expression level of a target gene was normalized using a geometric mean of three reference genes. Fold-change values are represented as the mean $\pm$ SEM ($n = 3$). Student's t test (two-sample, unpaired) was performed to test the significance of upregulation or downregulation for each gene between treated and control samples ($P < 0.01$). Significant changes are indicated with an *asterisks*
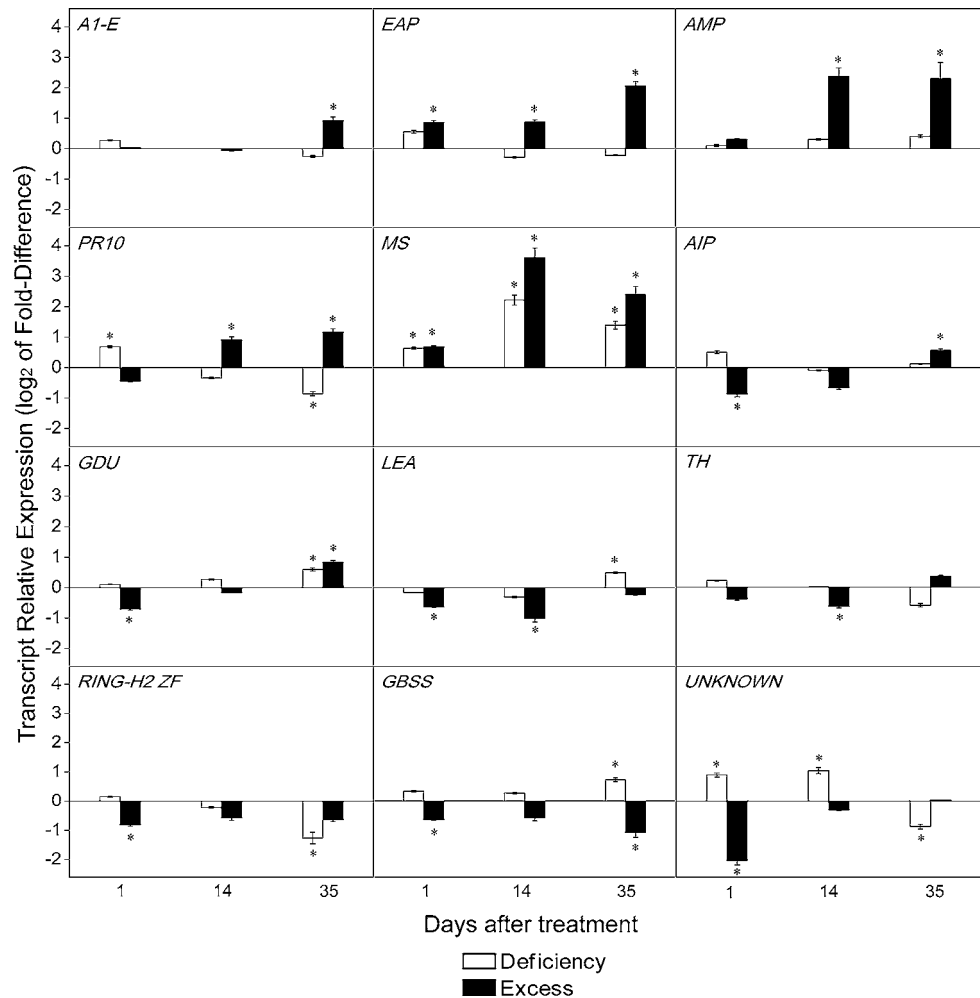
δ-aminotransferase (δ-OAT). As shown in Fig. 4, three genes, *NADH-GOGAT*, *AS* and *ASPG* were differentially expressed in response to changes in ammonium availability, and major changes in gene expression were observed at the end of the period of nutritional stress. *NADH-GOGAT* expression was repressed by ammonium deficiency. In contrast, *ASPG* transcripts accumulated during ammonium stress. Interestingly, *AS* expression was regulated by both, ammonium deficiency and excess (Fig. 4).

We were particularly interested in examining whether or not the expression of ammonium-responsive candidate genes could be coordinately regulated. Hierarchical cluster analysis of the expression profiles showed the existence of three different expression patterns in maritime pine roots in response to ammonium availability (Fig. 5). Cluster I included the *AIP*, *GDU*, *TH*, *UNKNOWN* and *GBSS* genes. In the cluster II, genes related to stress/defense (*AMP*, *EAP* and *PR10*), carbon (*A1-E* and *MS*) and asparagine (*AS* and *ASPG*) metabolism were grouped. Interestingly, genes for enzymes in nitrogen metabolism, except for *AS* and *ASPG*, were included in cluster III.

Discussion

In this study, microarray analysis and suppressive subtraction hybridization (SSH) were used to identify differentially expressed genes in the roots of maritime pine exposed to changes in ammonium availability. A complex response was observed in our study with significant changes in the transcriptome in response to ammonium excess or deficiency as discussed below. A total of 225 unigenes were identified as differentially regulated by ammonium availability. The differential expression of 88 selected genes was validated by colony array hybridization with cDNAs isolated from plants under ammonium stress/deficiency. A variety of expression patterns were observed in response to ammonium excess/deficiency and, based on the data, 12 genes were further selected for accurate quantitation of gene expression by qPCR. Differential expression was confirmed for all selected candidate genes and differences in the temporal response to ammonium availability were observed.
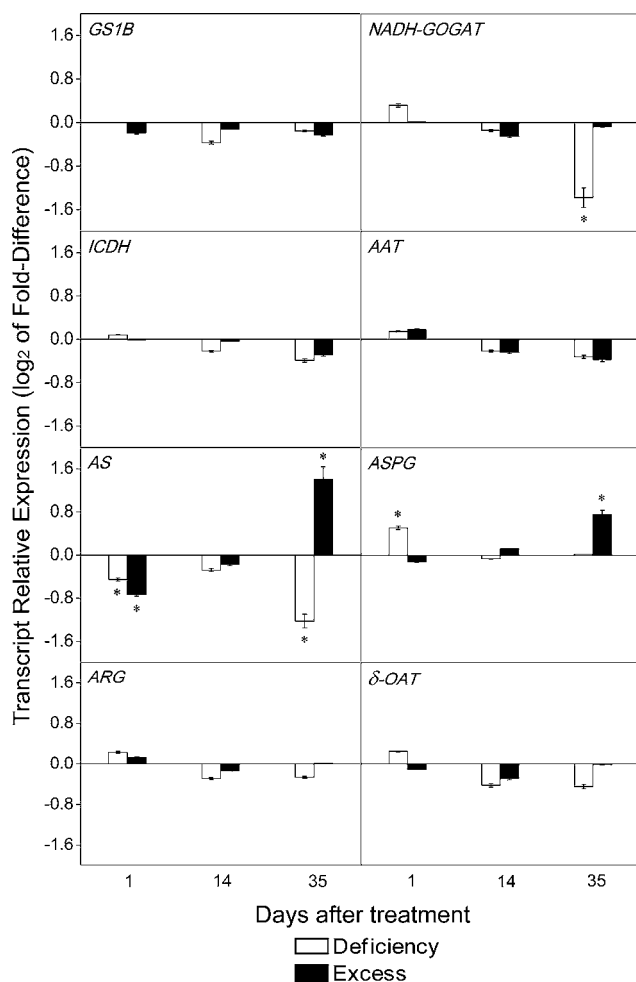
Fig. 4 Time course expression profiles of genes involved in nitrogen metabolism in response to ammonium availability. Total RNA was isolated from maritime pine roots 1, 14, and 35 days after nutritional treatments, and mRNA levels were determined using qPCR. The values represent the $\log_2$ of the ratio between the relative expression levels in either ammonium deficiency or excess and in control samples. The relative expression level of a target gene was normalized using a geometric mean of three reference genes. Fold change values are represented as the mean $\pm$ the SEM ($n = 3$). Student's t test (two-sample, unpaired) was performed to test significance of upregulation or downregulation for each gene between treated and control samples ($P < 0.01$). Significant changes are indicated with an *asterisks*

Genes for enzymes involved in carbohydrate metabolism, *A1-E* and *MS* were upregulated, whereas *GBSS* was repressed by excess ammonium. These findings may reflect the requirement of carbon skeletons for nitrogen assimilation in conditions of high ammonium.

The *AMP*, *PR10* and *EAP* genes were upregulated in the high ammonium condition. All of these genes encode conifer proteins of small sizes (10–15 kDa) that have shown activities related to stress/defense (Ekramoddoullah et al. 2006; Liu and Ekramoddoullah 2006; Wang and Ng 2000). AMP and EAP are positively charged proteins

secreted in the apoplasm where they are thought to function. It has been described that aquaporins and other permeases may have a role in low-affinity ammonium transport and accumulation in roots (Loqué et al. 2005). It is tempting to speculate that these positively charged proteins could interact with the plasma membrane to affect permeability and/or the activity of channels involved in ammonium inter- or intracellular transport. In fact, antimicrobial activity of these proteins appears to involve their interaction with the negatively charged membrane of the invaders (Takeuchi et al. 2004). In addition to the role as antimicrobial agents and in response to ammonium excess reported here, AMP, PR10 and EAP may also have roles in plant development.

A gene with homology to *GLUTAMINE DUMPER1 (GDU)* was also differentially expressed in response to ammonium availability. Overexpression of *GDU* in Arabidopsis leads to hypersecretion of glutamine and increases in the amino acid contents of the plant (Pilot et al. 2004). GDU may play a role in the intercellular distribution of glutamine in conditions of ammonium excess/deficiency. Another related gene, *AIP*, has been identified in the response of wheat to aluminum stress (Snowden and Gardner 1993). *AIP* genes also encode small proteins (about 10 kDa) that contain a glutaminase domain related to members of the Ntn (N-terminal nucleophile) hydrolase superfamily, which is also found in the GOGAT or AS enzymes (Snowden and Gardner 1993). It remains to be determined whether these proteins could in some way be involved in ammonium/glutamine regulation in pine roots. How and to what extent the other selected candidate genes, such as *LEA*, *TH* and RING-H2 *ZF* could be involved in the response to ammonium is much more difficult to interpret. Finally, it should be highlighted that the expression pattern of *UNKNOWN* suggests that this particular gene is a good candidate as an early molecular marker in the response to changes in the ammonium availability. These data reinforce the importance of further characterization of the large number of genes with unknown function that were identified in the present study.

It has been previously reported that differential gene expression is highly dependent of the inorganic nitrogen source. In Arabidopsis roots, a large number of genes were differentially regulated by nitrate availability (Wang et al. 2003; Scheible et al. 2004; Ruffel et al. 2008). In contrast, the number of differentially expressed genes and the intensity of the variations observed in response to ammonium nutrition were much lower than those observed in nitrate-fed roots (Ruffel et al. 2008). In fact, the accumulation of transcripts for ammonium transporters or for enzymes involved in ammonium assimilation was unaffected in the roots of ammonium-fed plants (Ruffel et al. 2008). Similar results were found in the present study; no

Fig. 5 Hierarchical clustering of the expression patterns of ammonium-responsive and nitrogen metabolism genes. Complete-linkage hierarchical clustering with centered Pearson correlation as a similarity metric was done using Gene Cluster 3.0 software with qPCR data. Three groups (I, II and III) have been made for the genes showing similar expression patterns
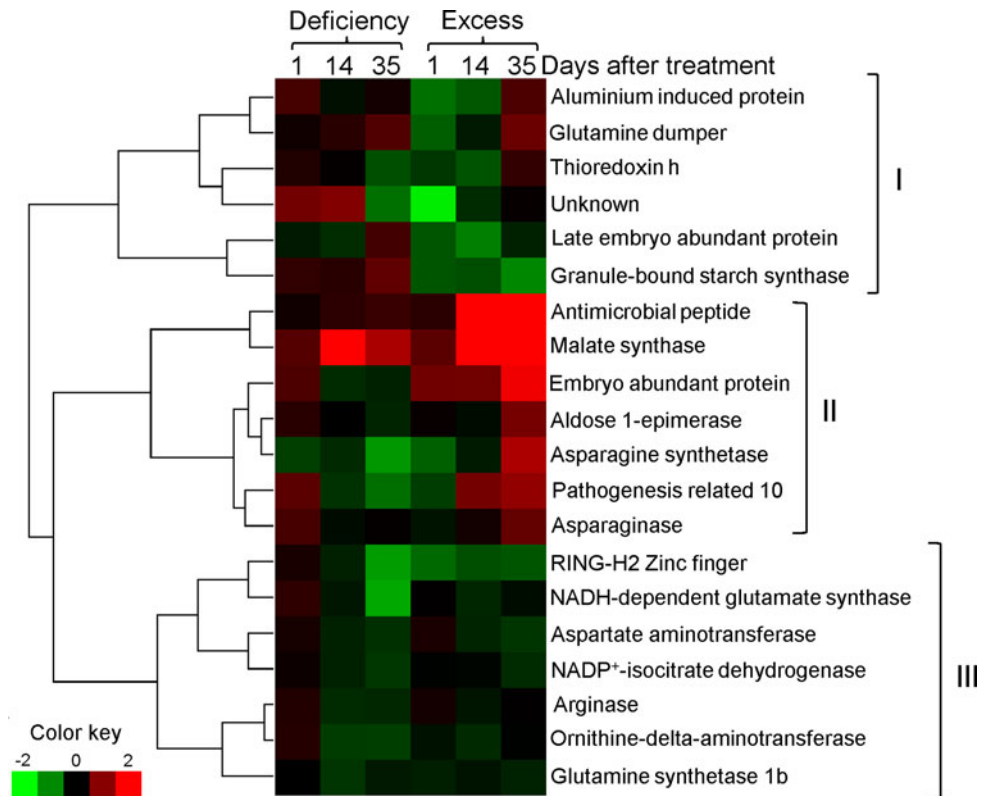
differential expression of genes for ammonium transporters or genes involved in ammonium assimilation was observed in response to ammonium availability, except for *NADH-GOGAT,* which was repressed during ammonium deficiency. It should be noted that ammonium was the unique source of nitrogen for young pine trees, but is also released in many metabolic activities. In fact, the strong preference of conifer seedlings for ammonium as a source of inorganic nitrogen may be related to their capacity to assimilate and store it in the roots. Thus, in Norway spruce seedlings supplied with external ammonium, in vivo ^{14}N-NMR studies have provided experimental evidence for the accumulation of large amounts of ammonium in the root vacuoles, possibly to protect the plant against ammonium toxicity (Aarnes et al. 2007). In addition, it has been proposed that tolerance to ammonium is related to the plant's capacity to maintain high levels of inorganic nitrogen assimilation in the roots (Cruz et al. 2006; El Omari et al. 2010). A balance between ammonium assimilation by GS and storage in the root vacuoles would prevent its transport to the shoot where it would be more toxic. Therefore, the maintenance of ammonium homeostasis may require that the regulation of ammonium acquisition and metabolism in roots is not finely tuned and possibly controlled by the levels of glutamine synthetase to maintain a sustained glutamine biosynthesis. In fact in conifers, the reaction catalyzed by cytosolic glutamine synthetase (GS1) is a key step that controls the plant's capacity to assimilate and

recycle nitrogen reserves (Cánovas et al. 1998, 2007). On the other hand, it has been reported that endogenous ammonium levels, or an increase in glutamine concentration in Scots pine roots, downregulate the uptake of ammonium in response to increased nitrogen supply (Ohlünd and Näsholm 2004). These data and data reported here suggest that amino acids could act as a signal of satiety for the control of ammonium acquisition in pine roots. In fact, our data indicate that asparagine metabolism may play a relevant role in response to ammonium excess/deficiency. Thus, the steady-state levels of transcripts for the asparagine metabolic enzymes, AS and ASPG, were significantly affected by ammonium availability. There are a number of reports that describe the accumulation of asparagine and increases in *AS* expression in response to plant biotic and abiotic stresses, including pathogen attack, carbon starvation, cold and salt stress or mineral deficiencies (Herrera-Rodríguez et al. 2007; Lea et al. 2007). Induction of *AS* gene expression has also been observed in response to the supply of high levels of ammonium (Herrera-Rodríguez et al. 2004; Wong et al. 2004). These findings suggest that nitrogen derived from high external ammonium concentrations and nitrogen remobilization is accumulated in the form of asparagine. In fact, coordinated regulation of *GS* and *AS* has been reported in tomato leaves infected with *Pseudomonas syringae* (Olea et al. 2004). The expression pattern of *AS* has been closely correlated with the acumulation of asparagine in pine seedlings

(Cañas et al. 2006), suggesting that excess ammonium leads to asparagine accumulation as an innocuous, easily utilizable and transitory store of nitrogen. The enhanced expression of *AS* and *ASPG* in these conditions does not imply the existence of a futile asparagine cycle, since both genes are expressed in different cell types (Cañas et al. 2007). Furthermore, the expression of *AS* and *ASPG* is temporally coordinated to control the reallocation of nitrogen in pine seedlings (Cañas et al. 2007). Taken together, these results suggest that the interplay between glutamine and asparagine biosynthesis is critical for the response of maritime pine roots to changes in ammonium availability. The repression of glutamate and asparagine biosynthesis during deficiency, when the ammonium/glutamine levels should be low, and the induction of asparagine metabolism in conditions of high ammonium supply strongly support the above hypothesis. The *AS* and *ASPG* genes appear to be co-regulated with genes related to stress/defense (*AMP*, *EAP*, and *PR10*) and carbon metabolism (*A1-E* and *MS*). This supports the idea that the products of these genes may have related functions in the response of maritime pine roots to ammonium availability. All of them have been selected as relevant genes for detailed functional analysis and are currently being used as molecular markers to explore the natural diversity in maritime pine populations.

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