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ORIGINAL PAPER

Gene expression profiling in the stem of young maritime pine trees: detection of ammonium stress-responsive genes in the apex

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Abstract The shoots of young conifer trees represent an interesting model to study the development and growth of conifers from meristematic cells in the shoot apex to differentiated tissues at the shoot base. In this work, microarray analysis was used to monitor contrasting patterns of gene expression between the apex and the base of maritime pine shoots. A group of differentially expressed genes were selected and validated by examining their relative expression levels in different sections along the stem, from the top to the bottom. After validation of the microarray data, additional gene expression analyses were also performed in the shoots of young maritime pine trees exposed to different levels of ammonium nutrition. Our results show that the apex of maritime pine trees is extremely sensitive to conditions of ammonium excess or deficiency, as revealed by the observed changes in the expression of stress-responsive genes. This new knowledge may be used to precocious detection of early symptoms of nitrogen nutritional stresses, thereby increasing survival and growth rates of young trees in managed forests.

Keywords Conifers · Pine development · Nitrogen · Ammonium nutrition · Transcriptional regulation

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Introduction

Forests are essential components of the ecosystems, and they play a fundamental role in the regulation of terrestrial carbon sinks. Coniferous forests dominate large ecosystems in the Northern Hemisphere and include a broad variety of woody plant species, some ranking as the largest, tallest, and longest living organisms on Earth (Farjon 2010). Conifers are the most important group of gymnosperms and have evolved very efficient physiological adaptation systems after the separation from angiosperms, which occurred more than 300 million years ago. Conifer trees are also of great economic importance, as they are major sources for timber, oleoresin, and paper production.

Maritime pine (*Pinus pinaster* Aiton) stands are distributed in the southwestern area of the Mediterranean region. *P. pinaster* dominates the forest scenario in France, Spain and Portugal, where this is the most widely planted species in about 4 million hectares. The maritime pine is particularly tolerant to abiotic stresses showing relatively high-levels of intra-specific variability (Aranda et al. 2010). The maritime pine is also the most advanced conifer model species for genomic research in Europe, and a large number of genomic resources and phenotypic data have been generated in the last few years and are available for the conifer research community (http://www.scbi.uma.es/pindb/; https://www4.bordeaux-aquitaine.inra.fr/biogeco/Ressources/BDD). Furthermore, results on this conifer

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species can be easily transferred to the closely related *Pinus* species and other economically and/or environmentally important gymnosperm species.

Our laboratory is interested in studying the effects of abiotic stress factors such as drought, extreme temperatures and nutrient excess/deficiency in the transcriptome of young maritime pine trees. The identification and molecular analysis of genes responding to these stresses could provide a better understanding of the cellular processes that are. Nitrogen is the most important macronutrient in plants and its availability affects many aspects of plant growth and development (Lea and Morot-Gaudry 2001). The balance between nitrogen availability in soil and metabolic utilization is of particular importance in trees, which are able to cope with seasonal periods of growth and dormancy (Suárez et al. 2002; Cánovas et al. 2007).

In nature, inorganic and organic forms of nitrogen are present in the soils of forests, although they are usually available only at low levels. In fact, trees have developed adaptation mechanisms to cope with mineral stresses, including mycorrhizal associations to increase the efficiency of nitrogen acquisition and metabolic assimilation (Martin et al. 2007). However, human activities alter nitrogen recycling in natural ecosystems to a great extent, contributing to the deposition of high amounts of nitrogen as a result of the utilization of fossil fuels, agricultural intensification with a massive use of fertilizers and an increased concentration of chemical industries releasing high levels of atmospheric pollutants (Driscoll et al. 2003). In coniferous forests, a low pH, a 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 many herbaceous plants, have a preference for ammonium over nitrate as a nitrogen source (Kronzucker et al. 1997).

In Europe, coniferous forests have shown clear signs of stress over the past 30 years, including needle loss, limp hanging branches, and chlorosis in the older needles (Galiano et al. 2010). Increased nitrogen deposition appears to be one of the most critical factors responsible for these symptoms triggering the imbalance in tree nutrition, alterations in nitrogen metabolism and tree growth (Hornung and Langan 1999). Ammonium is easily taken up by the roots as well as through the needles and Nihlgard (1985) and Mohr (1986) have proposed a correlation between excessive ammonium supply in forest ecosystems and the forest dieback.

Because pine trees live in forest ecosystems where ammonium ions are the main source of available inorganic nitrogen, we were particularly interested in the assimilation of this nutrient (Cánovas et al. 2007) and have initiated functional genomic approaches to study the molecular changes in response to ammonium stress (Cánovas et al. 2009). In a recent paper, we investigated the effects of ammonium availability on the transcriptome of maritime pine roots using a combination of microarray analysis and suppressive subtraction hybridization (Canales et al. 2010).

In this study, we have investigated developmental gene expression from the apical shoot tip enriched in meristematic cells to the shoot base enriched in lignified cells. We were interested to know whether the pattern of gene expression along the stem would be altered by changes in the ammonium availability. Our results show that the apex of maritime pine trees is extremely sensitive to conditions of ammonium excess or deficiency.

Materials and methods

Plant material

Seeds of *P. pinaster* Ait were germinated and the seedlings were grown under natural environmental conditions at the nursery "Lugar Nuevo" (Consejería de Medio Ambiente de la Junta de Andalucía, Jaén, Spain). Eight-month-old trees were selected for experimental analysis and subjected to acclimatization for a period of 3 weeks in a controlled growth chamber (Ibercex H-900-B) at 23°C, with 16 h light/8 h dark cycles. Illumination was provided by fluorescent lamps (Sylvania F-48T12/CW/WHO, Koxka, Pamplona, Spain) at a flux density of 150 μmol/cm^2/s^1. The seedlings were grown in plastic pots (0.15 L) using unfertilized peat (Sphagnum, pH 5.5, conductivity 0.85 ms/cm) as substrate. Needles and branches were removed from plants and then 20 stems were collected and divided into five sections of equal length with a razor blade. The stem sections were immediately frozen in liquid nitrogen and stored at −80°C until further analysis.

The ammonium treatments were defined as described by Ohlünd and Näsholm (2004) in Scots pine. For the nitrogen treatments, plants were grown for 5 weeks in a controlled growth chamber with the following nutrient solution (mg/L): Na_2HPO_4 (20), KCl (40), $CaCl_2$ (40), $MgSO_4{\cdot}7H_2O$ (20), EDTA-Fe (5.6), $NaMoO_4{\cdot}7H_2O$ (0.001), $CuSO_4{\cdot}7H_2O$ (0.006), H_3BO_3 (0.09), $ZnSO_4{\cdot}7H_2O$ (0.1) and $MnCl_2{\cdot}4H_2O$ (0.7). This solution was supplemented with 3 or 10 mM ammonium. Plants were watered individually with 50 mL of the appropriate nutrient solution every week, and always at the same time, early in the morning. Stem samples were collected 24 h, 1 and 5 weeks after the first irrigation with nitrogen solution. Samples of tissues were collected in all cases at a fixed time (mid-day), in order to minimise potential circadian variations in the levels of gene expression. Twenty plants per treatment were collected and analysed.

Tissue sectioning and staining

Small pieces (approximately 4–10 mm^3) were cut out from the central region of each stem section and then fixed following the freeze substitution protocol described by Regan et al. (1999). After fixation, tissue pieces were paraffin-embedded as described by Cantón et al. (1999). Three independent trees were sampled and 10–15 sections were prepared from each stem. The embedded tissues were sectioned at a 10-µm thickness with a microtome (Leica), mounted on poly-L-lysine-coated glass slides (Menzel-Gläser) and stained with hematoxylin and eosin for microscopic examination.

Microarray construction

The cDNA libraries used as the source of ESTs for PINARRAY were described elsewhere (Cantón et al. 2003; Alonso et al. 2007). To construct the microarray, 3,346 unique cDNAs were printed in duplicate onto ULTRA gaps II-coated slides (Corning, NY, USA) using a Qarray2 (Genetix Ltd, Queensway, UK) with a telechem printing head and 16 split pins (Biorobotics, Cambridge, UK). ArrayControl Sense Oligo Spots (spikes) (Ambion Inc., Austin, TX) were also included in the microarray.

RNA extraction, sample labelling and microarray hybridisation

Total RNA was isolated following the method described by Liao et al. (2004). Pooled samples of frozen tissues from 20 plants were ground to a fine powder in liquid nitrogen and approximately 1 g of homogenised tissue was used for total RNA isolation. RNA concentration and purity was determined spectrophotometrically (NanoDrop ND-1000A UV-Vis spectrophotometer); only samples with a 1.9–2.1 A260/A280 nm ratio and higher than a 1.9 A260/A230 nm ratio were used for subsequent experiments. RNA quality was further checked by agarose gel electrophoresis.

One microgram of total RNA was amplified using the Amino Allyl Message Amp II aRNA amplification kit (Ambion) following the manufacturer's instructions. The amplified antisense RNA (aRNA) was labelled using the CyDye post-labelling reactive dye pack (GE Healthcare), and 2 µg of each labelled sample (target and control) were dissolved in 50 µL of Pronto! long oligo/cDNA hybridisation solution (Corning). Microarray slides were prehybridised, hybridised and washed according to the Pronto! universal hybridisation kit protocol (Corning). Microarray hybridisation was performed in a Genetix hybridisation chamber at 42°C for 16 hours. Hybridised slides were scanned with a 5 micron resolution and their signal intensities were detected by Q-Scan (Genetix).

Microarray data analysis

Three independent biological replicates were sampled and dye-swaps for each biological sample were analysed. Spots flagged below 0 using GenePix v6.0 software as well as those whose signal intensity did not surpass 2× their background signal in both channels were discarded. Background correction was performed with the "normexp" method of the limma library (Ritchie et al. 2007). Expression ratios were computed as the ratio between the background-corrected foreground intensities of the Cy3 and Cy5 channels. M values were defined as the base two logarithms of the expression ratios. Raw expression data were normalised for all sources of systematic variation using the print-tip loess method (Yang et al. 2002), with the common assumption that the whole microarray expression data set was invariant. Scaling between arrays was not needed. Gene significance was then estimated using a robust linear model corrected by a moderated t test (empirical Bayes), which is more powerful than the conventional t test, especially in cases where only a few measurements are available (Smyth 2004). This adjustment takes into account differences among biological and technical replicates. The multitesting effect was corrected by adjusting P values using the Benjamini and Hochberg method (1995). A gene was considered significantly up- or down-regulated if it met these two criteria: (1) adjusted $P \leq 0.05$; and (2) a fold change ≥ 1.5 (that is, $|M| > 0.585$). The Biobase v 2.0.1 program of the Bioconductor package (Gentleman et al. 2004) was installed under R version 2.7.1 for all statistical analysis, and the limma v 2.14.5 (Smyth 2005) and marray v 1.18.0 libraries of the Bioconductor package were used in most cases.

Quantification of gene expression by real-time PCR

Prior to reverse transcription, 1 µg of total RNA was treated with RQ1 RNase-free DNase (Promega) according to the manufacturer's instructions to remove any traces of genomic DNA. Complementary DNA (cDNA) was synthesised from 500 ng total RNA using the PrimeScript RT reagent kit (Takara). RT products were diluted to a final concentration of 5 ng/µL and then stored at −20°C for quantitative real-time PCR determinations (qPCRs). Gene-specific primers were designed with the web interface software Primer3Plus (Untergasser et al. 2007) and the sequences are described in Supplemental Table 1.

qPCR was conducted using an Mx3000P real-time PCR system (Stratagene). The qPCR reaction was performed in 20 µL of a primer concentration of 0.3 mM and 10 ng cDNA and Quantimix Easy SYG kit (Biotools). Each sample was measured in triplicate using the following protocol: 2 min at 95°C, 35× (15 s at 95°C, 30 s at 60°C,

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 raw fluorescence data using the LinReg 11.3 program (Ruijter et al. 2009). The relative expression levels were obtained from the ratio between the N_0 of the target gene and the normalisation factor. We used the geometric mean of three control genes (actin, 40S ribosomal protein and elongation factor 1 alpha) to calculate the normalisation factor (Vandesompele et al. 2002). Reference genes were selected based on their stable expression in the microarrays. Furthermore, these genes were stably expressed in all conditions and tissue portions examined as determined by statistical analysis using Normfinder (Andersen et al. 2004).

Results and discussion

Differential gene expression between the apex and the base of maritime pine shoots

The differential gene expression was analysed in maritime pine stems using microarrays. Intact total RNA was extracted from the apex and the basal part of the stems, labelled with CyDye and hybridised to slides of PINARRAY, a maritime pine microarray constructed in our laboratory. Microarray data were lowess normalised to account for intensity-dependent differences between channels. After normalisation, the dye-swap replicates did not show strong deviations from linearity, proving a low dye bias. The comparisons between replicates showed a high degree of reproducibility, with Pearson's correlation coefficients of approximately 0.98. Similar transcriptomic analyses have been previously performed in Sitka spruce (Friedmann et al. 2007). Microarray analyses were also used for transcript profiling in differentiating xylem of loblolly pine and white spruce (Yang et al. 2004; Pavy et al. 2008).

Genes differentially expressed at the apical and the basal parts of the maritime pine stem were identified by bioinformatic analysis of hybridisation signals in the microarray, using a cut-off t test p value < 0.05 and a fold change >1.5, as shown in a volcano plot (Fig. 1). With these parameters, 44 and 26 unigenes were identified as differentially expressed in the apex and the base, respectively.

A list of selected genes with enhanced gene expression levels in the apex is shown in Table 1. Transcripts for genes encoding photosynthetic proteins, including those located in the thylakoid membranes involved in the photosystems I and II, light-harvesting complexes, as well as soluble proteins of the plastid stroma such as the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco SSU; EC 4.1.1.39), were particularly abundant. This part of the stem contains the shoot apical meristem which drives stem growth and develops new needles requiring the biosynthesis of proteins for the photosynthetic machinery. Also abundant were transcripts for lipid transfer proteins (LPT), metallothionein-like proteins (MT) and stress proteins such as an antimicrobial peptide (AMP), a putative dehydrin and a late embryogenesis abundant protein. The expression of stress-related genes has also been reported in the apical shoot meristem of Sitka spruce where they may be involved in the protection of meristematic cells against mechanical wounding or insect attack (Ralph et al. 2006). Interestingly, a number of genes involved in lignin biosynthesis and cell wall formation were also upregulated in the apical part of the maritime pine stem. These included a putative cinnamoyl-CoA reductase (EC 1.2.1.44), a serine-hydroxymethyltransferase (EC 2.1.2.1), xyloglucan endotransglycosylases (EC 2.4.1.207), an endo-1,4-β-mannosidase (EC 3.2.1.78), a putative proline-rich arabinogalactan and a germin-like

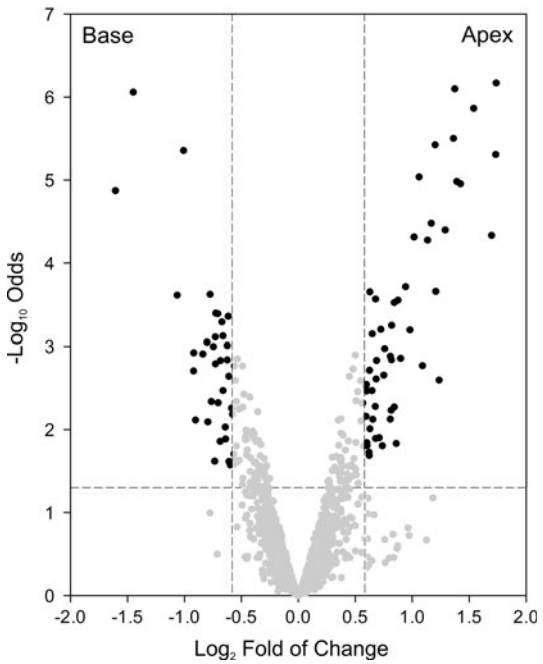

Fig. 1 Graphical representation of the microarray data analysis. Microarray normalised data were analysed by moderate t test statistics. Logarithms of the probability of the t test were represented as a function of the logarithm of the fold change for each gene. *Horizontal and vertical dot lines* represent the p value and fold change cuts-off, respectively, for the selection of differentially expressed genes, represented as *black circles*

Table 1 Genes over-expressed at the apical part of the maritime pine stem which were identified by microarray analysis

Clone Id	Accession number	Putative function	*E* value BLASTX
Photosynthesis			
5D08	AM983062	Chlorophyll *a–b*-binding protein type II member 1B (*Pinus sylvestris*)	5.00E−150
4A07	AM982985	Chlorophyll *a–b*-binding protein type II 1B (*Pinus sylvestris*)	5.00E−150
3D11	AM982945	Chloroplast photosystem II light-inducible protein (*Pachysandra terminalis*)	2.00E−40
7F07	AM983232	Ferredoxin NADP reductase (*Arabidopsis thaliana*)	2.00E−36
2A04	AM982868	Lhca4 (*Pinus sylvestris*)	2.00E−144
7G05	AM983238	Oxygen-evolving complex of photosystem II (*Cucumis sativus*)	4.00E−60
4F04	AM983017	Photosystem I reaction center subunit V (*Arabidopsis thaliana*)	8.00E−44
5B10	AM983045	Photosystem I reaction center subunit XI (*Spinacia oleracea*)	1.00E−51
3C01	AM982931	Photosystem I reaction center subunit XI (*Spinacia oleracea*)	1.00E−51
7G07	AM983240	Photosystem II 22 kDa protein (*Solanum lycopersicum)*	2.00E−41
9G08	AM983379	Plastocyanin (*Spinacia oleracea*)	3.00E−46
3 E7	AM982951	Ribulose bisphosphate carboxylase SSU (*Pinus thunbergii*)	1.00E−88
1A10	AM982823	Thylakoid membrane phosphoprotein (*Arabidopsis thaliana*)	2.00E−27
4H01	AM983026	Type 1 chlorophyll a /b-binding protein (*Pinus sylvestris*)	7.00E−118
Lipid transport			
3F04	AM982958	Non-specific lipid transfer protein (*Pinus taeda*)	9.00E−08
2C04	AM982880	Non-specific lipid-transfer protein (*Lens culinaris)*	1.00E−26
7B02	AM983192	Non-specific lipid-transfer protein (*Lens culinaris*)	2.00E−26
8H02	AM983320	Non-specific lipid-transfer protein (*Lens culinaris*)	1.00E−26
Metal binding			
7F06	AM983231	Metallothionein-like protein (*Picea abies*)	4.00E−18
Pp020C11	BX249127	Metallothionein-like protein (*Picea glauca*)	1.00E−26
7F03	AM983228	Metallothionein-like protein (*Picea glauca*)	2.00E−24
1 E09	AM982845	Metallothionein-like protein (*Picea abies*)	5.00E−14
Stress			
10 E10	AM983430	Antimicrobial peptide 3 (*Pinus sylvestris*)	2.00E−58
Pp054 H11	BX251756	Putative dehydrin (*Picea glauca*)	3.00E−52
Pp031B01	BX249989	Late embryogenesis abundant protein (*Picea glauca*)	9.00E−60
Lignin biosynthesis			
Pp050G11	BX251529	Xyloglucan endotransglycosylase precursor (*Populus tremula x Populus tremuloides*)	5.00E−89
9A06	AM983330	Xyloglucan endotransglucosylase (*Sagittaria pygmaea*)	8.00E−94
1G09	AM982858	Serine-hydroxymethyltransferase (*Medicago truncatula*)	2.00E−91
2C12	AM982885	Putative cinnamoyl-CoA reductase (*Oryza sativa*)	9.00E−14
Cell-wall related and carbohydrate metabolism			
Pp043E02	BX250962	Endo-1,4-beta-mannosidase protein 2 (*Prunus persica*)	3.00E−120
5 E02	AM983067	Glyceraldehyde-phosphate dehydrogenase (*Pinus sylvestris*)	8.00E−40
Pp053E03	BX251647	Putative proline-rich arabinogalactan protein 4 (*Pinus pinaster*)	5.00E−30
Pp112E05	BX255189	Beta-galactosidase (*Raphanus sativus*)	3.00E−51
6D07	AM983139	Germin-like protein (*Chimonanthus praecox*)	6.00E−64
Miscellaneous			
8A03	AM983256	Ribosomal protein S7 (*Pinus thunbergii*)	8.00E−59
Pp021H10	BX249257	MAP kinase kinase 3 (*Arabidopsis thaliana*)	2.00E−44
Q1K19	X69822	Glutamine synthetase 1a (*Pinus sylvestris*)	0.0
Pp093A05	BX254079	Myo-inositol oxygenase 1 (*Arabidopsis thaliana*)	5.00E−63
Unknown			
Pp045C04	BX251096	Unknown (*Picea sitchensis*)	2.00E−36
Pp103A03	BX254708	No match	

Table 1 continued

Clone Id	Accession number	Putative function	*E* value BLASTX
3 E10	AM982953	Unknown (*Picea sitchensis*)	9.00E−41
Pp108C01	BX254959	No match	
Pp058D04	BX252000	No match	
Pp059H07	BX252110	No match	

protein. These genes may have an important role in the processes of cell division and elongation that take place in the shoot apex of young conifer trees and in primary vascular development (Friedmann et al. 2007). Recent studies carried out in the crown of adult pine trees such as *P. radiata* (Cato et al. 2006) and *P. pinaster* (Paiva et al. 2008) revealed the existence of much more complex patterns of gene expression as a consequence of the phenotypic differences between young and adult trees.

In Table 2, a selection of genes with enhanced gene expression levels in the base of shoots is presented. Genes involved in lignin biosynthesis and deposition, as well as those involved in secondary-cell wall development were strongly upregulated. These included genes such as those encoding for phenylalanine ammonia lyase (PAL, EC 4.3.1.5), 4-coumarate: CoA ligase (4CL, EC 6.2.1.12), 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (EC 4.1.2.15), laccase (EC 1.10.3.2), serine hydroxymethyltransferase (EC 2.1.2.1), phosphoenolpyruvate-carboxykinase (PEPCK, EC 4.1.1.32) and sucrose synthase (SuSy EC 2.4.1.13). Additional genes that were preferentially expressed in these secondary-xylem enriched tissues were encoding a small heat-shock protein (HSP), aquaporin, aspartyl-protease (EC 3.4.23) and various transcription factors. This expression pattern is associated with the existence of lignified secondary xylem and bark tissue in the base of the stem (Friedmann et al. 2007).

Validation of differentially expressed genes in the stem by quantitative real time PCR (qPCR)

To validate the differences in gene expression levels determined by the microarray analyses we selected a number of relevant genes to examine by qPCR for accurate expression level analysis. For these experiments, the maritime pine stem was divided into five different sections that might represent a gradient of development along the stem, from the bottom to the top. To confirm that this assumption was correct, the sections were fixed, embedded in paraffin, cut, stained and examined by light microscopy (Fig. 2). The stained sections clearly showed how meristematic tissues and young vascular bundles in the apex (Fig. 2, section 1) develop into parenchymatic cells and a continuous vascular cylinder, with a particular enrichment of xylem cells in the basal part (Fig. 2, sections 4 and 5). We extracted intact total RNA from sections 1 to 5, and the steady-state levels of transcripts for reference housekeeping genes were examined (Supplemental Fig. 1). This represented the first step to quantitatively determine the expression levels of selected genes by qPCR.

The genes selected for validation as preferentially expressed genes at the apex were as follows: *GS1a* (glutamine synthetase a), *Rubisco SSU*, *Lhcb2* (light harvesting chlorophyll-binding protein), *AMP*, *LTP* and *MT*. Sense and antisense primers were designed for the specific amplification of the above selected genes. The sequences of the primers are depicted in Supplemental Table 1. The expression patterns of these genes were examined along the stem from the apex (section 1) to the base (section 5) and the results are shown in Fig. 3. The transcript levels for all genes examined were higher in the apex than in the base, thus confirming the differential gene expression revealed by the microarray analysis. *GS1a* was expressed at high levels along the stem, except at the base where the transcript levels were about 20% of those observed in the apex. *Rubisco SSU* expression was also high in the apical part of the stem and decreased gradually to the basal part, where transcripts were present at much lower levels. A coordinated expression of *GS1a* and *Rubisco SSU* has been reported in green tissues of pine seedlings (Cantón et al. 1999), indicating that the GS1a enzyme is tightly associated with nitrogen metabolism and chloroplast development/activity.

Unexpectedly, transcripts for *Lhcb2* were not only abundant in the apex but also present at relatively high levels in all sections of the stem, including the base. These results are consistent with the presence of significant amounts of chlorophyll in the same tissues (results not shown) and suggest the existence of functional chlorophyll–protein complexes along the maritime pine stem. The highest levels of *AMP* transcripts were present in the apex, with lower abundances in other sections, except for a peaking bulk in section 4, which represented about 30% of the maximal observed expression. *AMP1* is a member of the plant AMP β-barrelin family and could have a role in the regulation of ammonium nutrition in maritime pine roots (Canales et al 2010, 2011). In contrast, the *LPT* and *MT* genes were almost exclusively expressed in the upper

Table 2 Genes upregulated at the basal part of the maritime pine stem which were identified by microarray analysis

Clone Id	Accession number	Putative function	*E* value BLASTX
Lignin biosynthesis			
Pp081A07	BX253310	4-Coumarate-CoA ligase enzyme (*Pinus taeda*)	5.00E−77
Pp022C11	BX249292	DAHP synthase (*Vitis vinifera*)	1.00E−119
5B02	AM983038	Phenylalanine ammonia lyase (*Pinus pinaster*)	4.00E−69
Pp021G09	BX249246	Laccase (*Pinus taeda*)	6.00E−107
Pp086F08	BX253670	Phenylalanine ammonia lyase (*Pinus pinaster*)	0.0
Pp094C06	BX254161	Serine hydroxymethyltransferase (*Populus tremuloides*)	0.0
Cell-wall related			
Pp045A07	BX251077	Phosphoenolpyruvate carboxykinase (*Oryza sativa*)	4.00E−66
Pp066B03	BX252531	Sucrose synthase (*Pinus taeda*)	0.0
Pp034H06	BX250288	Cellulose synthase-like A1 (*Pinus taeda*)	5.00E−51
Unknown			
Pp083H07	BX253472	Unknown (*Picea sitchensis*)	2.00E−82
Pp075G07	BX252964	Unknown (*Physcomitrella patens*)	2.00E−16
Pp109C03	BX255010	No match	
Pp057G05	BX251958	No match	
Pp082D08	BX253373	Unknown (*Picea sitchensis*)	8.00E−47
Pp102C09	BX254664	No match	
Pp035E09	BX250331	Unknown (*Picea sitchensis*)	2.00E−29
Pp086E02	BX253653	Unknown (*Picea sitchensis*)	1.00E−50
Miscellaneous			
Pp040G11	BX250752	Calcium binding protein (*Pinus taeda*)	4.00E−78
Pp046B05	BX251164	Transcription factor E2F (*Chenopodium rubrum*)	4.00E−76
Pp045D02	BX251102	Small heat-shock protein (*Pseudotsuga menziesii*)	2.00E−55
Pp103G12	BX254751	NC domain-containing protein (*Oryza brachyantha*)	1.00E−57
Pp021G08	BX249245	Aquaporin PIP2 (*Arabidopsis thaliana*)	2.00E−60
Pp110H09	BX255105	SEC14 cytosolic factor family protein (*Arabidopsis thaliana*)	5.00E−41
Pp092G02	BX254056	Aspartyl protease family protein (*Arabidopsis thaliana*)	2.00E−13
Pp070G03	BX252788	Blue copper protein (*Arabidopsis thaliana*)	7.00E−31
Pp107H12	BX254947	Ring box-1-like protein (*Petunia inflata*)	1.00E−52
Pp020A12	BX249104	Photoassimilate-responsive protein (*Arabidopsis thaliana*)	5.00E−35

part of the stem, suggesting that they are mainly associated with the function of meristematic cells.

Likewise, another group of six genes preferentially expressed in the base were validated: *PAL*, *4CL*, *SuSy*, *CLS*, *Small HSP* and *PEPCK*. The levels of their transcripts along the stem from the apex (section 1) to the base (section 5) are shown in Fig. 4. Gene expression levels for all genes determined by qPCR analysis were lower in the apex than in the base, again validating the gene expression data derived from the microarrays. *PAL* and *4CL* exhibited a coordinated pattern of gene expression; transcripts for these particular genes were expressed in all sections, but their relative levels increased gradually from the tip to the basal part of the stem, reaching about twice the observed values in the base than in the apex. These results are consistent with the increased lignin biosynthesis associated with the differentiation of secondary xylem from the top to the base of the stem. A group of three genes, *SuSy*, *CLS* and *PEPCK*, also exhibited a related pattern of gene expression along the stem: their transcript levels increased from the apical to the basal part with an inflexion point observed in section 4. The over-expression of genes related to carbohydrate and lignin biosynthesis has been reported in the base of adult pine trees (Cato et al. 2006; Paiva et al. 2008). These results suggest, therefore, that there are strong similarities in gene expression between young and adult trees. The relative abundance of transcripts for the small HSP protein increased sharply about fourfold from the apex to section 4, decreasing significantly in the last section of the stem examined. The role of this stress-related gene appears

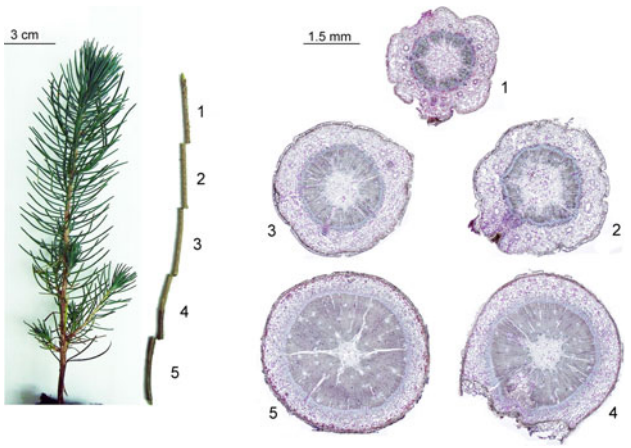

Fig. 2 Sectioning and histochemical analysis along the maritime pine stem. The numbers in the given order (*1*, *2*, *3*, *4*, *5*) correspond to different sections taken from the apex to the base. Sections were stained with hematoxylin–eosin and examined by light microscopy

to be associated with the development of lignified tissues in the base of the stem and is clearly unrelated to small HSP proteins accumulating in the crown of maritime pine trees (Paiva et al. 2008).

Changes in gene expression in response to excess/deficiency of ammonium nutrition

We were interested to know how changes in ammonium nutrition could affect the developmental patterns of gene expression in the maritime pine stem. Young trees were grown for 30 days under conditions of severe deficiency (0 mM), sufficient-supply (3 mM) or excess of ammonium levels (10 mM). Intact RNA was extracted from the apical and basal parts of stems, and microarray analyses were performed comparing plants exposed to ammonium excess/deficiency with control plants that were supplied with sufficient nitrogen (3 mM).

Small changes in gene expression were observed in the basal part of the stem; however, a number of genes were over-expressed or repressed at the apical part of the stem in response to ammonium stress (Fig. 5). Interestingly, many of these genes were "stress responding genes", such as those encoding chaperones, small HSPs and the HSPs 70, 82, 90 and 101. This altered expression pattern was observed in response to both, ammonium-excess (10 mM) and ammonium-deprivation treatments, although the observed differences were more pronounced under

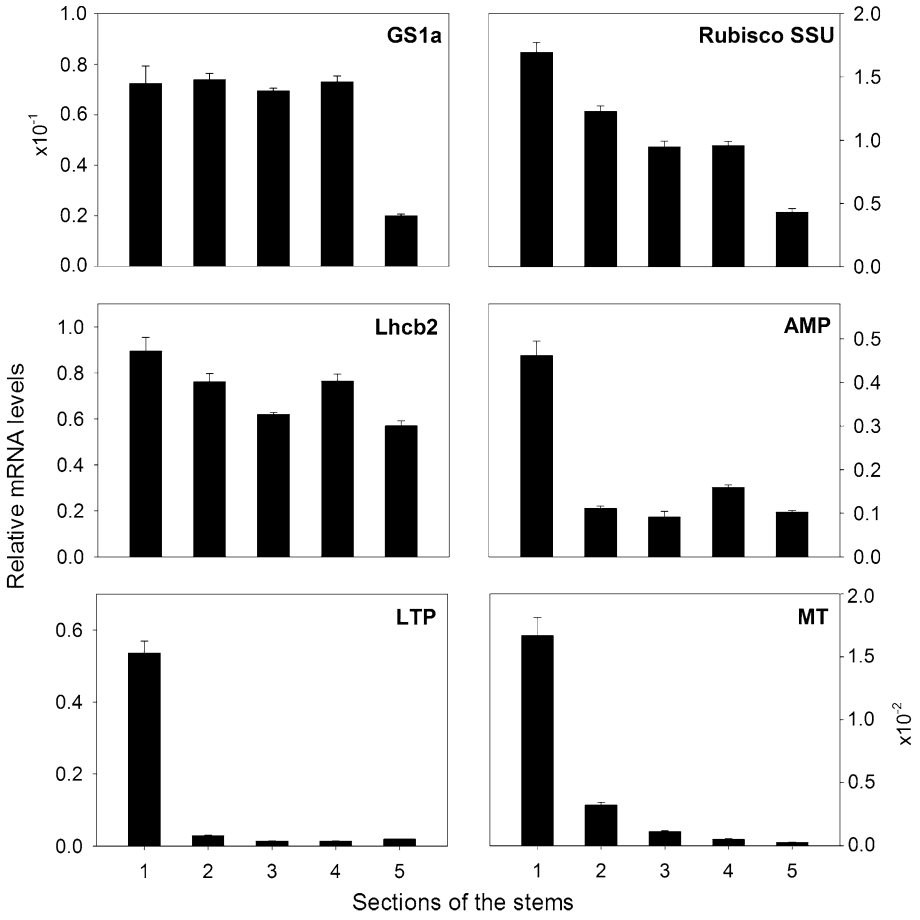

Fig. 3 Expression patterns along the maritime pine stem of six genes that were identified as differentially expressed in the apex. The relative abundance of each transcript was quantified in comparison to the expression levels of three genes of reference (*EF1α*, *40S* and *actin*). The histograms represent the mean values of three independent experiments with standard deviations. *GS1a* cytosolic glutamine synthetase a, *Rubisco SSU* small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase, *Lhcb2* light-harvesting chlorophyll-binding-protein of photosystem II, *AMP* antimicrobial peptide, *LTP* lipid transfer protein, *MT* metallothionein

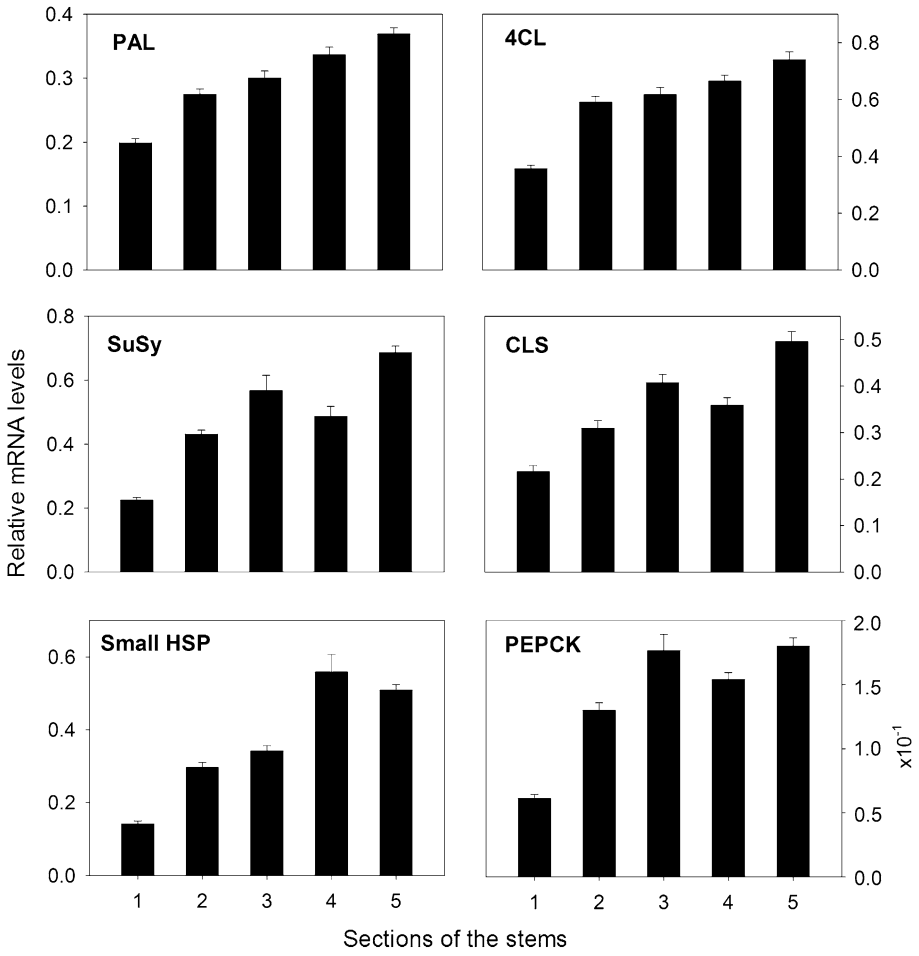

Fig. 4 Expression patterns along the maritime pine stem of six genes that were identified as differentially expressed in the base. The relative abundance of each transcript was quantified in comparison to the expression levels of three genes of reference (*EF1α*, *40S* and *actin*). The histograms represent the mean values of three independent experiments with standard deviations. *PAL* phenylalanine ammonia-lyase, *4CL* 4-coumarate: CoA ligase, *SuSy* sucrose synthase, *CLS* cellulose synthase, *small HSP* small heat-shock protein, *PEPCK* phosphoenolpyruvate-carboxykinase

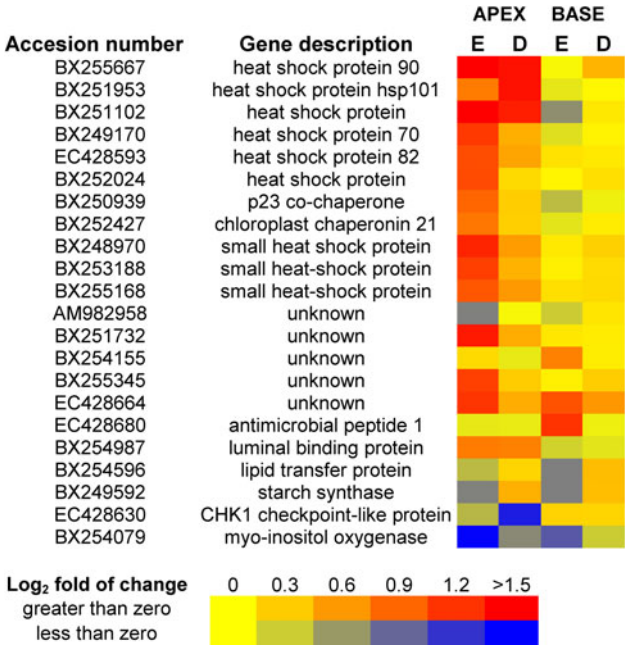

Fig. 5 Genes differentially expressed in maritime pine stems in response to ammonium excess (E) or deficiency (D) identified by microarray analysis. Log expression ratio values from each treatment were represented as heatmaps

ammonium excess. We have previously reported that ammonium excess and deficiency trigger changes in the transcriptome of maritime pine roots (Canales et al. 2010). The differential expression patterns of a number of representative genes suggested the existence of potential links between ammonium-responsive genes and genes involved in amino acid metabolism, particularly in asparagine biosynthesis and utilisation (Canales et al. 2010). The results reported here indicate that the metabolic changes observed in roots are transmitted to the stem apex. This fact implies the existence of a systemic signal that may represent a part of the response of maritime pine seedlings to nutritional stress by ammonium. The nature of this systemic signal is presently unknown; however, we can speculate that altered levels of organic nitrogen in the form of asparagine may be involved. High-levels of this amino acid accumulate in pine hypocotyls and a role of asparagine in nitrogen reallocation has been proposed (Cañas et al. 2006). In fact, asparagine is a vehicle for nitrogen transport in plants and it is well known that there is a stress-induced asparagine accumulation in response to mineral deficiencies, drought or pathogen attack (Lea et al. 2007).

Conclusion

The availability of genomic resources for transcriptomic analysis in maritime pine enabled us to initiate studies of gene expression profiling in the stem of young maritime trees. The data reported in the present work complement recent studies of gene expression in conifers and specifically address where and how gene expression patterns along the maritime pine stem are altered by changing levels of ammonium availability. Specifically, our results show that the apex of young stem trees are particularly sensitive to ammonium excess or deficiency, as revealed by the observed changes in the expression of stress-responsive genes. This new knowledge may be used to detect early effects of nitrogen nutritional stresses through the development of a range of molecular markers and strategies. Young trees are more sensitive than adult trees to a variety of abiotic stress factors that alone or in a combination can lead to reduced tree growth and eventually death, severely affecting the survival and maintenance of the forest landscape.

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