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# Identification of genes differentially expressed during adventitious shoot induction in *Pinus pinea* cotyledons by subtractive hybridization and quantitative PCR

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**Summary** As part of a study aimed at understanding the physiological and molecular mechanisms involved in adventitious shoot bud formation in pine cotyledons, we conducted a transcriptome analysis to identify early-induced genes during the first phases of adventitious caulogenesis in *Pinus pinea* L. cotyledons cultured in the presence of benzyladenine. A subtractive cDNA library with more than 700 clones was constructed. Of these clones, 393 were sequenced, analyzed and grouped according to their putative function. Quantitative real-time PCR analysis was performed to confirm the differential expression of 30 candidate genes. Results are contrasted with available data for other species.

*Keywords: caulogenesis, conifers, cytokinins, organogenesis, SSH, stone pine.*

## Introduction

In conifers, adventitious shoot formation is induced by culturing cotyledon explants in the presence of cytokinins (Thorpe 1993). Shoot organogenesis, also known as caulogenesis, from detached pine (*Pinus* spp.) cotyledons is direct, and histological studies of differentiating cotyledons show that shoot primordia develop de novo from sub-epidermal layers of the cotyledonary surface (Villalobos et al. 1985, López et al. 1996). These explants are fully competent to respond to inductive hormones and do not require a de-differentiation phase as is necessary in other models, such as *Arabidopsis thaliana* (Flinn et al. 1988).

The caulogenesis model established for *Pinus pinea* L. cotyledons is a well characterized system for studying the control of in vitro shoot organogenesis in conifers at the physiological level (Valdés et al. 2001, Moncaleán et al. 2005). In the *P. pinea* model, the caulogenic process is induced by benzyladenine (BA) as the sole hormone in the shoot-induction medium and the caulogenic response is highly efficient and synchronous. Shoot determination and shoot development phases have been determined by performing medium transfer experiments and analyzing the uptake and metabolism of BA (Moncaleán et al. 2005). Metabolism of BA in *P. pinea* cotyledons shows three phases. During the first phase, BA is absorbed from the medium, reaching a peak concentration at around 16 h, which precedes the beginning of cell proliferation. From Day 2 to 8, endogenous BA concentration tends to match the BA concentration in the medium. After eight days of culture no significant changes in BA metabolism occur. The BA peak at 16 h indicates the time when the cells become determined as shoot meristem precursors. The measure of cell determination relates to the average state of determination for the entire explant (Hicks 1994). The cells involved in organ initiation are included within a mass of non-responding cells, which hampers the study of the organogenic process in finer detail (Thorpe 1980).

Previous studies on organogenesis in conifers have taken a physiological approach (Flinn et al. 1989, Valdés et al. 2001, Moncaleán et al. 2005). However, a molecular approach has been lacking, and no studies have described the initial switch in the developmental program of non-meristematic cells leading to the formation of buds in response to exogenous application of cytokinin. In angiosperms, molecular interactions have been found among cytokinin signal transduction, the cell cycle and shoot meristem development (Rupp et al. 1999, Riou-Khamlichi et al. 2000), but always in regeneration systems that require an initial de-differentiation stage in an auxin-rich medium.

A model integrating cytokinins and the diverse gene path-

ways involved in shoot development has been proposed (Howell et al. 2003, Zhang et al. 2004), in which the cytokinin signal is perceived by three membrane-located sensor histidine kinases (Heyl and Schümilling 2003). The signal is further transmitted by a multi-step phospho-relay system via histidine phospho-transfer proteins to nuclear response regulators, which can activate or repress transcription. Several regulatory genes have been identified during shoot meristem development, including maize *KNOTTED1* (*KN1*; Vollbrecht et al. 1991), its orthologue in *Arabidopsis KNAT1*, *SHOOT MERISTEMLESS (STM*; Long et al. 1996), *WUSCHEL* (*WUS*; Laux et al., 1996) and *CLAVATA1–3* (*CLV1–3*; Leyser and Furner 1992, Kirsten et al. 2005). These genes alone cannot sustain ectopically formed shoot meristems (Gallois et al. 2002, Howell et al. 2003).

In this paper, we describe the application of the suppression subtractive hybridization (SSH) technique (Diatchenko et al. 1996) to identify early-induced genes during the first phases of adventitious caulogenesis in *P. pinea* cotyledons treated for 16 h with BA (Moncaleán et al. 2005). A subtractive cDNA library was constructed, and selected expressed sequence tags (ESTs) were clustered according to their putative function. Differential expression of several candidate genes was confirmed by quantitative real-time PCR (RT-PCR) analysis. Based on this approach, we discovered genes that were preferentially expressed in *P. pinea* cotyledons 16 h after induction with BA. Most of them have not been reported previously to be associated with adventitious shoot development.

## Materials and methods

### *Explant source*

One-year-old mature seeds from open-pollinated stone pine (*Pinus pinea* L.) trees growing in a natural stand (Meseta Norte region, Spain) were provided by the "Servicio de Material Genético del Ministerio de Medio Ambiente" (Spain). After removal of the seed coat, seeds were surface sterilized by immersion in 7.5% $H_2O_2$ for 45 min, followed by three rinses in double-distilled water. Seeds were then imbibed on moistened sterile paper for 48 h at 4 °C to facilitate dissection of the embryos according to Humara et al. (1999).

### *Bud induction*

After removal of the megagametophyte, cotyledons were excised from the embryos and placed horizontally in baby food jars containing 20 ml of bud induction medium (1/2LP), with or without 44.4 µM of BA (Moncaleán et al. 2005). The bud induction medium consisted of half-strength Le Poivre medium (1/2LP), as modified by Aitken-Christie et al. (1988), with 0.8% (w/v) agar (Roko S.A., A Coruña, Spain) and 3% (w/v) sucrose (Humara et al. 1999). Before autoclaving, the pHs of all media were adjusted to 5.8. Cultured cotyledons were maintained for 16 h in a growth chamber at 25 ± 1 °C, weighed and immediately frozen in liquid nitrogen and stored at –70 °C.

### *Subtractive cDNA library construction*

For each experiment, about 500 (2 g fresh mass) from frozen BA-treated and untreated *P. pinea* cotyledons were powdered in a mortar with a pestle and total RNA was extracted by the LiCl precipitation method (Chang et al. 1993). The RNA concentration was determined by spectrophotometry and its quality assessed by denaturing fomaldehyde–agarose gel electrophoresis. Double-stranded cDNA was produced from about 2 µg of total RNA using the template switch mechanism at the 5′ end (Smart) and long-distance PCR (LD-PCR) (BD Biosciences, Clontech Laboratories, Palo Alto, CA). To preserve the original relative transcript abundance, the optimal LD-PCR cycle number was empirically determined for each RNA sample to ensure that the cDNA remained in the exponential phase of amplification. We performed SSH with the PCR-Select cDNA Subtraction Kit (BD Bioscience Clontech). Briefly, cDNA samples were separately digested with *Rsa*I to generate short blunt-ended molecules. Tester cDNA was produced from RNA isolated from BA-treated *P. pinea* cotyledons, and the driver cDNA population was made with RNA from untreated cotyledons. A tester population with specific adaptors was made, but no adaptors were ligated to the driver cDNA. Subsequent hybridization steps were carried out to equalize the sequence populations and enrich for tester-specific sequences, yielding templates for PCR amplification. A subtracted cDNA pool, theoretically enriched in up-regulated cDNAs from the RNA population of BA-treated cotyledons was obtained.

The SSH library was constructed by ligation of the subtracted cDNAs into the pGEM-T Easy vector (Promega, Madison, WI). The ligation mixture was transformed into competent JM 109 *Escherichia coli* cells and plated onto LB agar containing 100 µg $ml^{-1}$ ampicillin, 1 mM isopropyl-D-thiogalactopyronoside (IPTG) and 80 µg $ml^{-1}$ X-gal, and incubated at 37 °C overnight. White colonies, containing recombinant cDNA, were selected and cultured in 100 µl of LB broth containing ampicillin in 96-well plates. After overnight culture, 50% glycerol was added and the plates were gently shaken and then stored at –70 °C.

### *cDNA sequencing, EST generation and analysis*

To generate an EST collection from BA-treated *P. pinea* cotyledons, aliquots from the bacterial stocks arranged in 96-well plates were used as template for PCR reactions with T7 and SP6 primers. Amplification products were purified with GFX PCR purification kit (Amersham Biosciences, U.K.) before sequencing. Automated fluorescence sequencing was performed at the Oviedo University DNA Analysis Facility, using the BigDye 3.1 Terminator chemistry on an ABI PRISM 3100 Genetic Analyzer platform (Applied Biosystems, Foster City, CA). Sequences were edited manually to remove contaminants from the vector, primer sequences and polyA tails. Sequences of less than 200 bp were excluded from further analysis.

Similarity searches were performed with the BLASTX program (Altschul et al. 1997) against two public databases: National Center for Biotechnology Information and The Institute

for Genomic Research *Pinus* Gene Index. The degree of sequence similarity between the cDNA clone and a known sequence is represented by the BLASTX probability E-value. Alignments with E-values $< e^{-10}$ and scores > 65 bits were considered to have significant similarity to other genes in the database. The database hit with the lowest E-value for each EST was manually assigned as the putative identity and cellular function of the EST. The functional categories are based on the MIPS standard (Schoof et al. 2002) and the TAIR GO annotation search (http://arabidopsis.org).

### *Quantitative real-time-PCR analysis*

To confirm the differential expression of several candidate genes, quantitative RT-PCR analyses were performed with BA-treated and untreated cotyledons from five independent induction experiments. To minimize mRNA loss and avoid DNA contamination, isolated polyA+ RNA was used as a template for first-strand synthesis before RT-PCR. Poly-adenylated ($A^+$) RNA was prepared using Dynabeds oligo$(dT)_{25}$ (Dynal, Oslo, Norway) following the manufacturer's instructions. The cDNA first strand synthesis was performed with 350 ng of polyA+ RNA at 55 °C for 1 h with Superscript III RT (Invitrogen) and oligo-dT as a primer, and then diluted 6-fold for subsequent amplification of each sample. Real-time PCR was carried out on a Bio-Rad iCycler (Bio-Rad Laboratories) under the following conditions: 95 °C for 15 min (1 cycle), 95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s (45 cycles). Individual reactions were assembled with oligonucleotide primers (0.30 mM each), 10 µl of 2× QuantiTec SYBR Greeen PCR Master Mix (Quiagen, Valencia, CA) plus 1 µl of diluted cDNA in a final volume of 20 µl. Data analysis was partially carried out by the iCycler software, which produced threshold cycle values (Cts) for each gene. A melting curve (55–95 °C with heating rate of 0.5 °C per 10 s and a continuous fluorescence measurement) was recorded at the end of every run to assess amplification product specificity (Ririe et al. 1997). Gene-specific primers were designed based on ESTs (primer sequences available on request). For each EST, three reactions were carried out. All reactions were performed at least twice. The Bio-Rad gene expression macro version 1.1 software (Bio-Rad Laboratories) was used to calculate relative gene expression by means of the comparative cycle threshold method (Pfaffl 2001, Livak et al. 2001) with normalization of data to the geometric average of three internal control genes (Vandesompele et al. 2002). The controls were amplified with primers designed with sequences from *Pinus pinaster* Ait. ubiquitin (Accession no. AF461687), *Pinus taeda* L. actin, (Accession no. AY172979) and *Pinus taeda* a-tubulin (Accession no. AY670521).

### *Accession Numbers*

The sequences reported in this paper have been deposited in the dbESTs database of the National Center for Biotechnology Information (http://www.ncbi.nih.gov/dbEST/index.html; Accession nos. EC428477-EC428747 and EC611867-EC611-884).

## Results

### *EST sequencing and analysis*

The EST collection was generated from *Pinus pinea* cotyledons treated with BA for 16 h, coinciding with the first peak of BA uptake (Moncaleán et al. 2005). After the construction of the SSH library, 700 subtracted cDNA clones were randomly selected for analysis. After amplification and gel electrophoresis analysis, the sizes of the inserts ranged from 150 to 2100 bp, with most fragments between 500 and 1000 bp. After discarding short fragments (< 200 bp), cDNAs from 393 clones were sequenced and compared to identify genes putatively involved in shoot organogenesis in *P. pinea* cotyledons. The comparison showed that 240 of 393 ESTs were unique or non-redundant (39% redundancy). The deduced amino acid sequences of 155 (64.5%) showed high homology (E-values $< 1e^{-10}$ and Blast score > 65 bits) to sequences in public databases, whereas 85 (35.5%) showed no significant similarity to any available sequence.

BlastX results for the 155 sequences with significant homologies are grouped by their putative function in Table 1. The ESTs were named Pp (for *P. pinea*) followed by the code corresponding to the clone in the SSH library. BlastX revealed that some of the subtracted cDNAs had similarities to several previously reported shoot-induction or development-related genes, including *CLAVATA1* receptor kinase (Kirsten et al. 2005), *PASTICCINO1* (*PAS1*; Vittorioso et al. 1998), Enhancer of Shoot Regeneration (*ESR1*; Banno et al. 2001), No Apical Meristem (*ATAF-1/NAM*; Takada et al. 2001), small nuclear ribonucleoprotein complex U1 (*U1 snRNPc*; Golovkin et al. 1998) and phosphoglycerate mutase (PGM; Mazarei et al. 2003). In addition, we identified a number of shoot-induction-related ESTs putatively encoding various zinc-finger-like proteins, several histone coding genes, ras-related GTP binding proteins, senescence associated proteins, the transcription factors WRKY and MYC, and splicing factors.

The proportion of ESTs belonging to each functional category is shown in Table 1. The largest set of sequences (20%) was assigned to the translation category. The ESTs putatively involved in transduction mechanisms (13.5%) and cell defence, rescue and aging (13.5%) formed the second and third largest groups.

Thirty-one cDNAs were classified in a functional category for translation: most were ribosomal proteins. However, we also annotated two translation initiation factors (eIF3d and eIF4a), a nucleoside diphosphate kinase, a threonyl-tRNA synthetase, a nascent polypeptide associated complex alpha chain and one QM-like protein.

The signal transduction mechanism category contained a large number of diverse sequences (13.5%). These included deduced amino acid sequences similar to a G-protein coupled receptor, three ras-related GTP-binding proteins, TGF-beta receptor-interacting protein, NEP1-interacting protein, ras-related GTP-binding protein, CLAVATA1-like LRR receptor kinase, one OGT protein, FK506-binding protein/PASTICCINO, a histidine-containing phospho-transfer protein 2 (hpt2

Table 1. List of the 155 expressed sequence tags isolated by supression subtractive hybridization showing significant homology (BLASTX E-values < $1e^{-10}$) with sequences available in the public databases of the National Center for Biotechnology Information and The Institute for Genomic Research Pinus Gene Index (*). Functional categories are based on the MIPS standard and the TAIR GO. The percentage of clones associated with each category is shown after its title.

| Clone | Accession no. | Homology | Homology Accession no. | BLASTX E-value |
|---|---|---|---|---|
| *Translation, ribosomal structure and biogenesis (20%)* | | | | |
| Pp1D6 | EC428480 | Ribosomal protein L11, cytosolic (*Arabidopsis thaliana*) | AY086300 | 1e-35 |
| Pp1H7 | EC428495 | Ribosomal protein S24e (*Arabidopsis thaliana*) | AY086479 | 1e-31 |
| Pp2E2 | EC428512 | Eukaryotic translation initiation factor 4A (eIF4a) (*Triticum aestivum*) | Z21510 | 1e-56 |
| Pp2H2 | EC428523 | 60S Ribosomal protein L6 (*Arabidopsis thaliana*) | AAF98420 | 2e-65 |
| Pp2H5 | EC428526 | 60S Ribosomal protein L13E (*Picea abies*) | AJ132537 | 4e-63 |
| Pp2C6 | EC428504 | 40S Ribosomal protein (*Oryza sativa*) | XM_479106 | 2e-85 |
| Pp2D10 | EC428511 | Ribosomal L9-like protein (*Oryza sativa*) | AY323481 | 6e-84 |
| Pp5C2 | EC428615 | RNA binding/exonuclease (*Arabidopsis thaliana*) | NM_100218 | 6e-59 |
| Pp3F2 | EC428552 | 60S Ribosomal protein L36 (*Oryza sativa*) | NM_190535 | 2e-35 |
| Pp3H10 | EC428571 | Ribosomal protein YL16 (*Mesembryanthemum crystallinum*) | X69378 | 3e-67 |
| Pp3F12 | EC428559 | 60s Ribosomal protein L7 (*Solanum tuberosum*) | BF154094 | 1e-55 |
| Pp4A2 | EC611882 | Ribosomal protein L28 (*Arabidopsis thaliana*) | AY079378 | 3e-22 |
| Pp4A6 | EC611883 | Acidic ribosomal protein P1a (*Oryza sativa*) | XM_479786 | 2e-21 |
| Pp4H5 | EC611881 | 40S Ribosomal protein S20 (*Oryza sativa*) | XM_476305 | 4e-12 |
| Pp4C6 | EC428581 | Nucleoside diphosphate kinase I (*Mesembryanthemum crystallinum*) | AF072289 | 6e-65 |
| Pp4B11 | EC428580 | Ribosomal protein (PETRP) (*Petunia × hybrida*) | AF307336 | 1e-45 |
| Pp5F1 | EC428626 | Eukaryotic translation initiation factor (eIF3d) (*Oryza sativa*) | AAU10658 | 5e-15 |
| Pp5E2 | EC428622 | 60S Ribosomal protein L24 (*Prunus avium*) | AF298827 | 4e-26 |
| Pp5E6 | EC428623 | RL5 Ribosomal protein (*Medicago sativa*) | X78284 | 4e-84 |
| Pp5G6 | EC428633 | Ribosomal protein S13 (*Amaryllis belladonna*) | AF315223 | 3e-49 |
| Pp5B7 | EC428612 | 60s Ribosomal protein L13a (*Picea mariana*) | AF051212 | 2e-94 |
| Pp5H11 | EC428639 | QM-Like protein (Ribosomal protein L10) (*Elaeis guineensis*) | AF295636 | 2e-34 |
| Pp7E2 | EC428719 | Ribosomal protein S12 (*Fragaria × ananassa*) | U19940 | 1e-71 |
| Pp7H2 | EC428742 | RNA-Binding like protein (*Arabidopsis thaliana*) | CAB16795 | 2e-21 |
| Pp7G4 | EC428737 | S18.A Ribosomal protein (*Arabidopsis thaliana*) | AY064680 | 5e-44 |
| Pp7A5 | EC428694 | 40S Ribosomal protein (*Oryza sativa*) | XM_479106 | 6e-51 |
| Pp7B9 | EC428704 | Ribosomal protein L17-1 (*Poa secunda*) | AF264022 | 2e-28 |
| Pp6H10 | EC428689 | Threonyl-tRNA synthetase (*Oryza sativa*) | Y14368 | 1e-94 |
| Pp6F1 | EC428666 | 40S Ribosomal protein S8 (*Prunus armeniaca*) | AF071889 | 4e-45 |
| Pp6C3 | EC428652 | 40S Ribosome protein S7 (*Avicennia marina*) | AF056316 | 2e-53 |
| PpA3 | EC611877 | Nascent polypeptide associated complex alpha chain (*Pinus taeda*) | AF220200 | 3e-32 |
| *Signal transduction mechanisms (13.5%)* | | | | |
| Pp1F3 | EC428487 | Similar to G protein-coupled receptor 107 (*Danio rerio*) | XP_684244 | 2e-51 |
| Pp1H12 | EC428498 | FUSCA Protein FUS6 (*Arabidopsis thaliana*) | AY051056 | 1e-120 |
| Pp7E6 | EC428722 | Delta-COP (*Zea mays*) | AF216852 | 4e-36 |
| Pp2C10 | EC428507 | TGF-Beta receptor-interacting protein 1 (*Phaseolus vulgaris*) | AF335551 | 1e-81 |
| Pp2C9 | EC428506 | NEP1-Interacting protein (*Arabidopsis thaliana*) | AJ400897 | 2e-25 |
| Pp3F5 | EC428554 | *Pinus taeda* EST similar to UP\|Q9C7C6 Ras-related GTP-binding protein | TC75123* | 1.8e-174 |
| Pp3G7 | EC428562 | *Pinus taeda* EST similar to UP\|Q94GN3 Putative receptor-associated protein | TC57698* | 9.6e-46 |
| Pp3D2 | EC428545 | High mobility group protein 2 HMG2 (*Ipomoea nil*) | U39747 | 7e-25 |
| Pp4C3 | EC611869 | Membrane protein COV (*Arabidopsis thaliana*) | AY170845 | 5e-85 |
| Pp4F6 | EC428597 | Cullin 1A (*Nicotiana tabacum*) | AJ344533 | 3e-82 |
| Pp4C12 | EC428586 | Nuclear RNA binding protein A (*Oryza sativa*) | XM_476001 | 9e-10 |
| Pp7D8 | EC428716 | Piwi domain containing protein (*Oryza sativa*) | XP_468898 | 1e-20 |
| Pp7H4 | EC428744 | *Pinus taeda* EST similar to UP\|Q40521 Ras-related protein Rab11B1 | TC58552* | 1.2e-125 |
| Pp6B6 | EC428647 | Protein serine/threonine kinase (*Arabidopsis thaliana*) | AAO42877 | 3e-70 |
| Pp6F6 | EC428669 | CLAVATA1 receptor kinase (*Medicago truncatula*) | AAW71475 | 1e-49 |
| Pp6G12 | EC428683 | *O*-Linked GlcNAc transferase (*Arabidopsis thaliana*) | *NP_187074* | 1e-49 |
| Pp6D1 | EC428655 | RNA Polymerase sigma factor-like (*Oryza sativa*) | XP_475997 | 4e-41 |
| Pp4E12 | EC428596 | *Pinus taeda* EST homologue to UP\|O04834 GTP-binding protein SAR1A | TC74543* | 1e-118 |
| Pp6G2 | EC428676 | FK506-Binding protein/PASTICCINO (*Arabidopsis thaliana*) | NP_566762 | 1e-53 |
| Pp3F7 | EC428556 | CBS1 (*Hyacinthus orientalis*) | AAT08729 | 4e-43 |
| Pp7H8 | EC428746 | Glycine-rich RNA-binding protein PsGRBP (*Pisum sativum*) | NM_123660 | 2e-30 |

Continued on facing page.

Table 1 Cont'd. List of the 155 expressed sequence tags isolated by supression subtractive hybridization showing significant homology (BLASTX E-values < $1e^{-10}$) with sequences available in the public databases of the National Center for Biotechnology Information and The Institute for Genomic Research Pinus Gene Index (*). Functional categories are based on the MIPS standard and the TAIR GO. The percentage of clones associated with each category is shown after its title.

| Clone | Accession no. | Homology | Homology Accession no. | BLASTX E-value |
|---|---|---|---|---|
| *Transcription factor (10.3%)* | | | | |
| Pp4D10 | EC428590 | Brassinosteroid signalling positive regulator-related (*Arabidopsis thaliana*) | NM_106517 | 2e-27 |
| Pp3H4 | EC428567 | WRKY Transcription factor 28 (*Arabidopsis thaliana*) | AAL35286 | 1e-23 |
| Pp3G11 | EC428564 | Zinc finger (C2H2 type) family protein (*Arabidopsis thaliana*) | NP_565854 | 9e-47 |
| Pp4B1 | EC611884 | TIP1 (TIP GROWTH DEFECTIVE 1) (*Arabidopsis thaliana*) | NM_122042 | 5e-41 |
| Pp6G11 | EC428682 | *Pinus taeda* EST similar to UP\|Q6H7H6 AP2-related transcription factor | TC61836* | 7.2e-95 |
| Pp4H8 | EC428606 | Putative zinc finger transcription factor ZFP216 (*Oryza sativa*) | XM_476740 | 5e-111 |
| Pp7A7 | EC428696 | MYC Transcription factor (*Solanum tuberosum*) | CAF74710 | 5e-14 |
| Pp6A11 | EC428642 | DNA Binding protein ACBF (*Nicotiana tabacum*) | AAC49850 | 2e-60 |
| Pp7G3 | EC428736 | *Pinus taeda* EST similar to UP\|Q9ZTM6 zinc finger protein PGPD14 | TC65086* | 2.2e-47 |
| Pp7F2 | EC428727 | *Pinus taeda* EST homologue to UP\|Q40203 RAB1C | TC65017* | 3.4e-46 |
| Pp7E1 | EC428718 | Zinc finger protein-like (*Oryza sativa*) | BAD53623 | 5e-35 |
| Pp7E3 | EC428720 | Putative leucine zipper protein (*Lycopersicon esculentum*) | Z12127 | 1e-46 |
| Pp4D4 | EC611872 | Putative G10 protein (*Oryza sativa*) | AL606445 | 1.8e-92 |
| Pp7G8 | EC428739 | SNF2 Domain-containing protein, putative helicase (*Oryza sativa*) | XM_463462 | 1e-58 |
| Pp7A4 | EC428693 | *Pinus taeda* EST similar to UP\|O65061 ATAF1-like protein | TC76830* | 6.9e-92 |
| Pp6F3 | EC428667 | Putative PHD-type zinc finger protein (*Arabidopsis thaliana*) | AY087820 | 2e-49 |
| *Transcription (3.9%)* | | | | |
| PpB11 | EC611880 | Putative splicing factor, arginine/serine-rich (*Oryza sativa*) | XM_483049 | 9e-41 |
| Pp2H3 | EC428524 | DNA-Directed RNA polymerase; ATRPAC42 (*Arabidopsis thaliana*) | NP_849833 | 2e-54 |
| Pp2C6 | EC428504 | Maturase (*Pinus pinea*) | AB019859 | 2e-40 |
| Pp2G6 | EC428520 | Putative RNA polymerase I subunit (*Oryza sativa*) | AAK18841 | 2e-34 |
| Pp3H2 | EC428566 | Putative U1 small nuclear ribonucleoprotein C (*Oryza sativa*) | BAD27897 | 6e-34 |
| Pp6H7 | EC428688 | DNA-Directed RNA polymerase IIb (*Nicotiana tabacum*) | AF153278 | 4e-44 |
| *Transposable elements, viral and plasmid proteins (2.6%)* | | | | |
| Pp1B5 | EC428477 | Gypsy-like retroposon DNA IFG7 (*Pinus radiata*) | AJ004945 | 1e-175 |
| Pp6H3 | EC428685 | Gypsy/Ty3 element polyprotein (*Arabidopsis thaliana*) | AAG51464 | 3e-43 |
| Pp3F6 | EC428555 | Hypothetical protein (Onion yellows phytoplasma OY-M) | NP_950744 | 1e-15 |
| Pp3C12 | EC428544 | Putative retrotransposon polyprotein (*Oryza sativa*) | NM_190881 | 4e-10 |
| *Cell division and chromosome partitioning (5.8%)* | | | | |
| Pp1G12 | EC428493 | Beta-tubulin 1 (*Hordeum vulgare*) | Y09741 | 1e-107 |
| Pp3D6 | EC428546 | Putative histone H4 (*Pinus pinaster*) | AJ306724 | 2e-43 |
| Pp3H12 | EC428572 | Beta-tubulin 2 (*Triticum aestivum*) | U76745 | 1e-176 |
| Pp4F10 | EC428600 | Tubulin alpha-5 chain (*Arabidopsis thaliana*) | A32712 | 4e-72 |
| Pp5B6 | EC428611 | Proliferating cell nuclear antigen (PCNA) (CYCLIN) (*Catharanthus roseus*) | X55052 | 7e-66 |
| Pp5G7 | EC428634 | Gonidia forming protein GlsA (*Lilium longiflorum*) | AB106868 | 2e-28 |
| Pp7F3 | EC428728 | Histone H3 (*Oryza sativa*) | M15664 | 3e-19 |
| Pp3G12 | EC428565 | Diadenosine 5',5'''-P1,P4-tetraphosphate hydrolase (*Lupinus angustifolius*) | U89841 | 2e-27 |
| Pp2G8 | EC428521 | 21 kDa Subunit of the Arp2/3 protein complex (*Arabidopsis thaliana*) | AY084614 | 7e-71 |
| *Cell envelope biogenesis, outer membrane (1.9%)* | | | | |
| Pp3C3 | EC428538 | Putative UDP-glucose 4-epimerase (*Oryza sativa*) | XM_479925 | 2e-49 |
| PpA10 | EC611878 | Non-repetitive nucleoporin family protein (NUP155) (*Arabidopsis thaliana*) | NM_101354 | 4e-27 |
| Pp3H6 | EC428569 | Xyloglucan endotransglycosylase XET2 (*Asparagus officinalis*) | AF223420 | 1e-72 |
| *Cell rescue, defence, aging (13.5%)* | | | | |
| Pp4C1 | EC611867 | Senescence-associated protein-like (*Oryza sativa*) | XM_481260 | 2e-64 |
| Pp1E5 | EC428484 | Heat shock cognate protein HSC70 (*Brassica napus*) | AF035414 | 2e-35 |
| Pp2G12 | EC428522 | Putative disease resistance response protein (*Arabidopsis thaliana*) | AAD29806 | 7e-21 |
| Pp2D5 | EC428509 | *Pinus taeda* weakly similar to UP\|O22341 Annexin | TC66545* | 8.6e-43 |
| Pp3B2 | EC428534 | Similar to senescence-associated protein (*Arabidopsis thaliana*) | AK175156 | 5e-63 |
| Pp7B8 | EC428703 | Peroxidase (*Picea abies*) | AJ566201 | 1e-33 |
| Pp3B12 | EC428536 | Ascorbate peroxidase (*Zantedeschia aethiopica*) | AF159254 | 2e-33 |
| Pp4E1 | EC611874 | CuZn Superoxide dismutase (*Pinus sylvestris*) | X58578 | 6e-83 |

Continued overleaf.

Table 1 Cont'd. List of the 155 expressed sequence tags isolated by supression subtractive hybridization showing significant homology (BLASTX E-values < $1e^{-10}$) with sequences available in the public databases of the National Center for Biotechnology Information and The Institute for Genomic Research Pinus Gene Index (*). Functional categories are based on the MIPS standard and the TAIR GO. The percentage of clones associated with each category is shown after its title.

| Clone | Accession no. | Homology | Homology Accession no. | BLASTX E-value |
|---|---|---|---|---|
| Pp4E6 | EC428593 | Heat shock protein HSP81-1 (*Arabidopsis thaliana*) | 1908431A | 1e-41 |
| Pp4G7 | EC428601 | PDR5-Like ABC transporter (*Spirodela polyrhiza*) | Z70524 | 3e-58 |
| Pp4E10 | EC428594 | Putative heat shock protein (*Oryza sativa*) | NM_189556 | 8e-79 |
| Pp7C5 | EC428708 | *Pinus taeda* EST similar to PIR\|C86285 probable oxidoreductase F9L1.8 | DR097395* | 7e-47 |
| Pp3G7 | EC428562 | Cationic peroxidase (*Vigna angularis*) | JQ2252 | 3e-30 |
| Pp5H9 | EC428638 | Ferritin (*Pinus taeda*) | AF028072 | 2e-53 |
| Pp7G2 | EC428735 | Manganese superoxide dismutase (*Digitalis lanata*) | AJ278863 | 3e-37 |
| Pp4C8 | EC428583 | Putative blue copper protein precursor (*Oryza sativa*) | XM_467895 | 1e-10 |
| Pp5E9 | EC428624 | Carboxylesterase-like (*Oryza sativa*) | BAD68856 | 6e-59 |
| Pp7F9 | EC428734 | Probable glutathione *S*-transferase (*Picea mariana*) | AF051238 | 6e-72 |
| Pp6G8 | EC428680 | Antimicrobial peptide 1 (*Pinus sylvestris*) | AF410952 | 2e-55 |
| Pp6B12 | EC428649 | Plant viral-response family protein-like (*Oryza sativa*) | BAD38317 | 2e-40 |
| Pp4E2 | EC611875 | NADPH-Cytochrome P450 reductase(ATR1) (*Pseudotsuga menziesii*) | Z49767 | 1e-115 |
| *Energy production and conversion (9%)* | | | | |
| Pp2F6 | EC428516 | Phosphoenolpyruvate carboxykinase (*Lycopersicon esculentum*) | AY007226 | 4e-15 |
| Pp2H12 | EC428528 | Vacuolar ATP synthase subunit E (*Citrus limon*) | AF165939 | 3e-61 |
| Pp2C7 | EC428505 | Putative succinate dehydrogenase flavoprotein subunit (*Arabidopsis*) | AK119142 | 6e-40 |
| Pp3G3 | EC428560 | Short-chain alcohol dehydrogenase-like protein (SAD) (*Arabidopsis*) | CAB63154 | 2e-74 |
| Pp3A6 | EC428529 | Putative hydroxypyruvate reductase (*Oryza sativa*) | XM_463779 | 1e-120 |
| Pp4E4 | EC611876 | Acetyl-CoA C-acyltransferase (*Cucurbita sp*) | S72532 | 7e-93 |
| Pp4A9 | EC428575 | Non-symbiotic hemoglobin class 1 (*Gossypium hirsutum*) | AF329368 | 1e-14 |
| Pp6B3 | EC428644 | *Picea abies* EST similar to NADH dehydrogenase subunit 1 (*Petunia hibrida*) | TC11501* | 1.2e-62 |
| Pp4A11 | EC428577 | Phosphoglycerate mutase (*Zea mays*) | Z33611 | 2e-89 |
| Pp5B5 | EC428610 | Carbonic anhydrase (*Populus tremula* × *Populus tremuloides*) | U55837 | 4e-54 |
| Pp7F5 | EC428730 | NADH-Ubiquinone oxidoreductase (*Pinus sylvestris*) | X86217 | 1e-47 |
| Pp7E7 | EC428723 | Alpha-amylase isozyme 3A precursor (*Oryza sativa*) | BAD38366 | 2e-10 |
| Pp6G9 | EC428681 | Glyceraldehyde-3-phosphate dehydrogenase (*Pinus sylvestris*) | L07501 | 3e-84 |
| Pp6D3 | EC428656 | Succinyl-CoA-ligase beta subunit (*Arabidopsis thaliana*) | AJ001808 | 1e-39 |
| *Posttranslational modification (1.3%)* | | | | |
| Pp2F12 | EC428518 | GDP-Fuc:Gal-beta-1,3GlcNAc-R alpha1,4-fucosyltransferase (*Beta vulgaris*) | AJ315848 | 1e-56 |
| Pp2F11 | EC428517 | Dolichyl-diphosphooligosaccharide-protein glycosyltransferase (*Oryza*) | XP_477102 | 7e-78 |
| *Protein fate (2.6%)* | | | | |
| Pp4D2 | EC611871 | AAA-Type ATPase-like (*Oryza sativa*) | XM_467382 | 3.7e-122 |
| Pp4D5 | EC611873 | Putative secretory carrier membrane protein (*Oryza sativa*) | NP_911223 | 2e-80 |
| Pp7C6 | EC428581 | *Pinus taeda* EST similar to UP\|Q8H768 33-kDa secretory protein | TC57157* | 2.8e-48 |
| Pp6F9 | EC428671 | Polyubiquitin (*Elaeagnus umbellata*) | AF061807 | 1e-89 |
| *Lipid metabolism (2.6%)* | | | | |
| Pp4D12 | EC428592 | GDSL-Motif lipase/hydrolase-like protein (*Arabidopsis thaliana*) | BAB10602 | 4e-44 |
| Pp7F6 | EC428731 | *Pinus taeda* EST similar to UP\|Q8S2Q4 Lysophospholipase-like | TC75261* | 1.3e-105 |
| Pp5C12 | EC428619 | Nonspecific lipid transfer protein (*Pinus taeda*) | U10432 | 1e-34 |
| Pp6H5 | EC428686 | Beta-oxyacyl-(acyl-carrier protein) reductase (*Brassica napus*) | AJ243091 | 2e-39 |
| *Amino acid transport and metabolism (1.3%)* | | | | |
| Pp4C2 | EC611868 | Proline dehydrogenase (*Glycine max*) | AY492003 | 1e-73 |
| Pp2A3 | EC428499 | Putative quinone reductase (*Arabidopsis thaliana*) | BT000937 | 2e-21 |
| *Carbohydrate metabolism (1.3%)* | | | | |
| Pp3C6 | EC428540 | Putative aldo/keto reductase (*Arabidopsis thaliana*) | BT004098 | 8e-35 |
| Pp4C9 | EC428584 | Plastidic aldolase (*Solanum brevidens*) | AY155602 | 8e-41 |
| *Nitrogen metabolism (0.6%)* | | | | |
| Pp4B9 | EC428579 | NFU2 Protein (*Arabidopsis thaliana*) | NM_124372 | 2e-21 |
| *Unknown function (9.7%)* | | | | |
| Pp3F3 | EC428553 | DNA-Binding protein (*Arabidopsis thaliana*) | NM_202461 | 9e-51 |

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Table 1 Cont'd. List of the 155 expressed sequence tags isolated by supression subtractive hybridization showing significant homology (BLASTX E-values < $1e^{-10}$) with sequences available in the public databases of the National Center for Biotechnology Information and The Institute for Genomic Research Pinus Gene Index (*). Functional categories are based on the MIPS standard and the TAIR GO. The percentage of clones associated with each category is shown after its title.

| Clone | Accession no. | Homology | Homology Accession no. | BLASTX E-value |
|---|---|---|---|---|
| Pp4G8 | EC428602 | Fertilization independent endosperm development protein (*Eucalyptus grandis*) | AY150283 | 1e-38 |
| Pp2D4 | EC428508 | GYF Domain-containing protein (*Arabidopsis thaliana*) | NM_123660 | 2e-20 |
| Pp3F10 | EC428558 | Translocon-associated protein beta (TRAPB) (*Solanum tuberosum*) | ABB87132 | 1e-50 |
| Pp5G2 | EC428631 | Hypothetical protein FLJ23451 (methylttransferase) (*Homo sapiens*) | NM_024766 | 8e-30 |
| Pp3B8 | EC428535 | Unknown protein related to GNS1/SUR4 family (*Arabidopsis thaliana*) | NM_106157 | 1e-44 |
| Pp2B12 | EC428503 | Hypothetical protein (*Lotus corniculatus*) | AB185449 | 4e-62 |
| PpA12 | EC611879 | Putative gag-pol polyprotein (*Oryza sativa*) | AAQ56388 | 2e-34 |
| Pp4D1 | EC611870 | Syringomycin biosynthesis enzyme-like protein (*Arabidopsis thaliana*) | BAB03049 | 8e-44 |
| Pp6B8 | EC428648 | Putative potassium transporter (*Oryza sativa*) | BAD46101 | 3e-27 |
| Pp1E6 | EC428485 | TCTP-Like protein (*Pseudotsuga menziesii*) | AJ012484 | 1e-70 |
| Pp4E11 | EC428595 | *Pinus taeda* EST homologue to UP\|Q5YJK5 Farnesyl diphosphate synthetase | TC66694* | 1.6e-58 |
| Pp7D1 | EC428713 | *Pinus taeda* EST similar to endoplasmatic reticulum retrieval protein Rer1B | TC66516* | 1.6e-61 |
| Pp7F1 | EC428726 | PIR7A Protein (*Oryza sativa*) | XP_479728 | 1e-59 |
| Pp7C9 | EC428712 | Calcium-dependent protein kinase 3 (*Nicotiana tabacum*) | CAC82999 | 1e-56 |

gene) and FUS6 protein among others.

Twenty cDNAs were found to be involved in cell rescue, defence and aging. Two senescence-associated proteins, three heat shock proteins, two superoxide dismutases, an ascorbate peroxidase and three proteins associated with the plant disease resistance response represent some of them.

Ten percent of the differentially expressed cDNAs in our library correspond to ESTs with deduced amino acid sequence similarity to transcription factors. Among them, five zinc finger proteins (including members of the PGF, C2H2 and PHD families), an SNF2 domain-containing protein, G10 protein, DNA binding protein ACBF, brassinoesteroid signalling positive regulator, an AP2 domain protein closely related to ERF/EREBP class, an NAM/ATAF1 protein and one WRKY transcription family. Among those related to transcription, U1 small ribonucleoprotein C seems to be the most relevant (Golovkin et al. 1998).

About 9% (14) of the differentially expressed cDNAs in the SSH were involved in energy production and conversion, including genes encoding phosphoglycerate mutase, hydroxypyruvate reductase, acetyl-CoA-acyltransferase, vacuolar ATP synthase, phosphoenolpyruvate carboxykinase and glyceraldehyde-3-phosphate dehydrogenase.

Only 5% of the total ESTs with a putative function are related to lipid, amino acid and carbohydrate metabolism. Several cDNAs involved in protein fate, such as AAA-type ATPase and a secretory carrier membrane protein that are related to transposable elements (gypsy-like retroposon IFG7), posttranslational modification (fucosyltranserase) or cell envelope and outer membrane (XET2), were present but did not represent more than 3% of the total cDNAs differentially expressed. Finally, 9% of ESTs with a putative function correspond to proteins with unknown biological function in plants such as PIR7A, TCTP, a protein related to GNS1/SUR4 family, or a GYF domain-containing protein.

### *Verification of differential expression by quantitative real-time PCR*

To exclude false-positive clones and to provide further data on the relative expression levels of the cloned cDNAs, quantitative RT-PCR was undertaken. Thirty ESTs, selected for their similarity to a gene involved in developmental processes, signal transduction or transcriptional regulation were analyzed. Data for the 30 ESTs assayed (Table 2) are presented as fold-induction relative to untreated control cotyledons and normalized to the internal control values of ubiquitin, actin and tubulin.

Of the 30 ESTs analyzed, 66% were differentially expressed (fold change > 2) in BA-treated cotyledons. Pp1F3, Pp1H12, Pp6G12 and Pp6F6, belonging to the signal transduction mechanism category, showed increased transcript abundance. Within the transcription category, 15 ESTs were screened by RT-PCR to confirm their differential expression. Of these, nine with similarity to transcription factors showed differential expression, of which Pp6G11, Pp3H4, Pp6A11, Pp4B1, Pp4H8, Pp7E1, Pp7G8 and Pp4D4 were up-regulated, whereas Pp4D10 was down-regulated. Pp2H3 and Pp3H2 were also expressed more than twofold in BA-treated cotyledons compared with untreated cotyledons. Pp2E2, included in the translation functional category, Pp3B8, Pp2B12 and Pp7F1 with homology to proteins with unknown biological function were also up-regulated in BA-treated cotyledons.

## Discussion

The complexity of adventitious bud induction suggests that a large number of genes are involved in the process. This is especially true during initiation, when some cells have the ability to recognize a cytokinin signal that commits them to the caulogenic program. In this study, we used the SSH strategy to isolate early-induced genes involved in de novo shoot formation

Table 2. Normalized expression of thirty expressed sequence tags belonging to the suppression subtractive hybridization library as determined by quantitative real-time PCR. Relative gene expression was measured by the comparative cycle threshold method with normalization of data to the geometric average of three internal control genes.

| Clone | Normalized fold expression | Homology | BlastX E-value |
|---|---|---|---|
| Pp1F3 | 2.38 ± 0.42 | Similar to G protein-coupled receptor 107 (*Danio rerio*) | 2e-51 |
| Pp1H12 | 2.22 ± 0.30 | FUSCA Protein FUS6 (*Arabidopsis thaliana*) | 1e-120 |
| Pp2B12 | 4.01 ± 1.36 | Hypothetical protein (*Lotus corniculatus*) | 4e-62 |
| Pp2E2 | 2.30 ± 0.37 | Eukaryotic translation initiation factor 4A (eIF4a) (*Triticum aestivum*) | 1e-56 |
| Pp2H3 | 4.59 ± 2.03 | DNA-directed RNA polymerase; ATRPAC42 (*Arabidopsis thaliana*) | 2e-54 |
| Pp2G12 | 1.41 ± 0.15 | Putative disease resistance response protein (*Arabidopsis thaliana*) | 7e-21 |
| Pp2G8 | 0.76 ± 0.04 | 21 kDa Subunit of the Arp2/3 protein complex (*Arabidopsis thaliana*) | 7e-71 |
| Pp3H6 | 1.41 ± 0.10 | Xyloglucan endotransglycosylase XET2 (*Asparagus officinalis*) | 1e-72 |
| Pp3D2 | 1.41 ± 0.10 | High mobility group protein 2 HMG2 (*Ipomoea nil* ) | 7e-25 |
| Pp3B8 | 2.14 ± 0.15 | Unknown protein related to GNS1/SUR4 family (*Arabidopsis thaliana*) | 1e-44 |
| Pp3G11 | 1.15 ± 0.13 | Zinc finger (C2H2 type) family protein (*Arabidopsis thaliana*) | 9e-47 |
| Pp3H2 | 2.22 ± 0.28 | Putative U1 small nuclear ribonucleoprotein C (*Oryza sativa*) | 6e-34 |
| Pp3H4 | 2.00 ± 0.31 | WRKY Transcription factor 28 (*Arabidopsis thaliana*) | 1e-23 |
| Pp4B1 | 2.41 ± 0.20 | Ribosomal protein (PETRP) (*Petunia* × *hybrida*) | 1e-45 |
| Pp4C3 | 0.78 ± 0.08 | Membrane protein COV (*Arabidopsis thaliana*) | 5e-85 |
| Pp4D4 | 2.22 ± 0.34 | Putative G10 protein (*Oryza sativa*) | 1.8e-92 |
| Pp4D10 | 0.14 ± 0.03 | Brassinosteroid signalling positive regulator-related (*Arabidopsis thaliana*) | 2e-27 |
| Pp4H8 | 2.02 ± 0.29 | Putative zinc finger transcription factor ZFP216 (*Oryza sativa*) | 5e-111 |
| Pp6A11 | 8.30 ± 2.77 | DNA Binding protein ACBF (*Nicotiana tabacum*) | 2e-60 |
| Pp6B6 | 0.57 ± 0.04 | Protein serine/threonine kinase (*Arabidopsis thaliana*) | 3e-70 |
| Pp6G11 | 2.37 ± 0.44 | *Pinus taeda* EST similar to UP\|Q6H7H6 AP2-related transcription factor | 7.2e-95 |
| Pp6G12 | 2.14 ± 0.50 | *O*-Linked GlcNAc transferase (*Arabidopsis thaliana*) | 1e-49 |
| Pp6F6 | 5.28 ± 1.03 | CLAVATA1 Receptor kinase (*Medicago truncatula*) | 1e-49 |
| Pp7A7 | 0.60 ± 0.18 | MYC Transcription factor (*Solanum tuberosum*) | 5e-14 |
| Pp7D8 | 1.27 ± 0.06 | Piwi domain containing protein (*Oryza sativa*) | 1e-20 |
| Pp7G8 | 2.28 ± 0.26 | SNF2 Domain-containing protein, putative helicase (*Oryza sativa*) | 1e-58 |
| Pp7E1 | 2.93 ± 0.90 | Zinc finger protein-like (*Oryza sativa*) | 5e-35 |
| Pp7F1 | 2.07 ± 0.23 | PIR7A Protein (*Oryza sativa*) | 1e-59 |
| Pp7H2 | 1.11 ± 0.3 | RNA-Binding like protein (*Arabidopsis thaliana*) | 2e-21 |
| PpA3 | 0.84 ± 0.27 | Nascent polypeptide associated complex alpha chain (*Pinus taeda*) | 3e-32 |

from *Pinus pinea* cotyledons treated for 16 h with BA. Some of the genes (e.g., *CLAVATA1*) have been related to caulogenesis in monocots and dicots (Bommert et al. 2005), suggesting the conservation of this process in gymnosperms and angiosperms.

One quarter of the ESTs found in our library are in the signal transduction and transcription factor categories, highlighting the complexity of adventitious bud induction. Several of these genes are overexpressed in BA-treated stone pine cotyledons and are related directly or indirectly with the regulation of transcription and signal transduction by cytokinins such as Pp6G2. The latter EST was found to be similar to *PAS1* (E-value of $3e^{-54}$), a gene that encodes an immunophilin-like protein (FK506) involved in the determination of the sensitivity of cell division to cytokinin (Faure et al. 1998, Vittorioso et al. 1998).

Organogenesis at the shoot meristem requires a delicate balance between stem cell specification and differentiation. Clone Pp6F6 of our SSH library has high similarity ($1e^{-49}$) with *CLV1* a gene encoding a leucine-rich receptor kinase from *Medicago truncatula*. In *Arabidopsis thaliana*, the *CLV1*, *CLV2* and *CLV3* loci appear to promote differentiation by repressing *WUSCHEL* (*WUS*), a key factor promoting stem cell identity expression (Kirsten et al. 2005). According to Cary et al. (2002), *CLV1* is expressed during shoot development in *Arabidopsis* root explants, and before any organization of the shoot apical meristem is evident. Gallois et al. (2002) observed its expression only in *Arabidopsis* hypocotyls and in cotyledons in the initial phases of ectopic organ formation and proposed it as an apical meristem central zone marker. *Pinus pinea* cotyledons cultured in shoot-inducing medium for 16 h showed a fivefold increased expression of Pp6F6 (Table 2). This differential expression may indicate that meristematic cell identity is established in the first hours of culture with BA.This is the first time that an increase in *CLV1* expression has been directly linked to in vitro adventitious shoot induction.

Other ESTs isolated with sequence similarity to transcription factors included Pp7G8, Pp4D4, Pp3H4 and Pp7A4. Pp7G8 is similar (E-value of $1e^{-58}$) to a gene encoding a transcription factor containing the SNF2 domain and displayed a twofold increase in expression in our system in response to BA induction of caulogenesis (Table 2). Transcription factors containing the SNF2 domain are involved in chromatin remodel-

ing, regulating the access of trans-acting transcriptional regulators or are components of the general transcription machinery to the packaged genome (Emerson 2002, Kadam and Emerson 2002). Because this process is essential for the reprogramming of transcription associated with development and cell differentiation, it seems to be related to induction or determination events or both. Moreover, *AtBRM*, a member of this family in *Arabidopsis*, is primarily expressed in meristems, organ primordia and tissues with active cell division and is directly related to shoot development (Farrona et al. 2004).

We found ESTs with homology to genes related to transcription machinery and splicing. Clone Pp7A4 has a high similarity (E-value of $6.9e^{-92}$) to *ATAF1* (*Arabidopsis Transcription Activator Factor-1*) an apparent orthologue of *NO APICAL MERISTEM* (*NAM*) gene in petunia. *ATAF1* seems to be similar in its expression pattern and developmental roles to *CUC1* and *CUC2* (Souer et al. 1996), which have been shown to be essential for meristem formation (Aida et al. 1997, Takada et al. 2001), in particular, overexpression of *CUC1* is enough to induce ectopic shoot formation in transgenic plants (Takada et al. 2001). Because *NAM*/*ATAF*1 expression is limited to a ring around the developing stem apical meristem (SAM), the presence of a clone with a sequence identity of 70% with an ATAF1 domain containing protein in our SSH library seems to validate our experimental system and suggests the induction of SAM primordia after 16 h of induction in the presence of BA.

Another EST upregulated at the onset of shoot organogenesis is Pp6G11; its transcript levels increase twofold after 16 h of BA-treatment in *P. pinea* cotyledons (Table 2). It contains a domain with a high (E-value of $7.2e^{-95}$) sequence similarity to the AP2/ERBP domain found in a family of transcription factors in higher plants (Chang and Shockey 1999, Riechmann et al. 2000). Whereas AP2/ERBP domain of Pp6G11 is most similar to the AP2-related transcription factor from *Pinus taeda*, Pp6G11 does not display sequence homology with any known protein outside of the AP2 domain. One of the proteins containing the AP2/ERBP domain is Enhancer of Shoot Regeneration 1 (ESR1) described by Banno et al. (2001). ESR1 has a key role during in vitro shoot formation by regulating *CUC1* transcription. Whether Pp6G11 is an orthologue of ESR1 requires further investigation.

Finally, one of the most differentially expressed clones is Pp6A11 ($8.3 \pm 2.8$), which has a BLASTX similarity (E-value of $1e^{-60}$) to a gene encoding a DNA-binding protein ACBF from *Nicotiana tabacum*. The deduced amino acid sequence of ACBF contains a long repeat of glutamine residues characteristic of a certain type of transcription factor. Séguin et al. (1997) were the first to describe this protein and demonstrate its capacity to bind to the AC-rich region of the *cis* regulatory elements of the promoter. Besides, they reported that ACBF mRNA was present in all tissues examined, but the highest transcript accumulation occurred in stem tissues.

These findings suggest that ACBF could bind to more than one promoter element involved in the transcriptional regulation of specific genes during shoot induction. Although, ACBF has been associated with petal development and xylogenesis (Laitinen et al. 2005), a sequence with similarity to ACBF has never been related to caulogenesis.

Most of the genes mentioned above are involved in developmental regulation, but there are few reports of their involvement in adventitious shoot induction in conifers. The absence of genomic data, the difficulty of developmental induction in conifers and the lack of molecular tools especially designed for gymnosperms hampers gene regulation studies in these species. In this work we tried to establish a basis for further studies on the genetic regulation of adventitious bud formation in conifers. Expression profiling with microarrays (now in progress) together with in situ hybridization, would cast light on the process that leads to the formation of a meristem from a group of undifferentiated cells.

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