**Tree Physiology** 24, 1073–1085


# Variation in transcript abundance during somatic embryogenesis in gymnosperms

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Received January 5, 2004; accepted March 13, 2004; published online August 2, 2004

**Summary** Somatic embryogenesis of Norway spruce (*Picea abies* L.) is a versatile model system to study molecular mechanisms regulating embryo development because it proceeds through defined developmental stages corresponding to specific culture treatments. Normal embryonic development involves early differentiation of proembryogenic masses (PEMs) into somatic embryos, followed by early and late embryogeny leading to the formation of mature cotyledonary embryos. In some cell lines there is a developmental arrest at the PEM–somatic embryo transition. To learn more about the molecular mechanisms regulating embryogenesis, we compared the transcript profiles of two normal lines and one developmentally arrested line. Ribonucleic acid, extracted from these cell lines at successive developmental stages, was analyzed on DNA microarrays containing 2178 expressed sequence tags (ESTs) (corresponding to 2110 unique cDNAs) from loblolly pine (*Pinus taeda* L.). Hybridization between spruce and pine species on microarrays has been shown to be effective (van Zyl et al. 2002, Stasolla et al. 2003). In contrast to the developmentally arrested line, the early phases of normal embryo development are characterized by a precise pattern of gene expression, i.e., repression followed by induction. Comparison of transcript levels between successive stages of embryogenesis allowed us to identify several genes that showed unique expression responses during normal development. Several of these genes encode proteins involved in detoxification processes, methionine synthesis and utilization, and carbohydrate metabolism. The potential role of these genes in embryo development is discussed.

*Keywords: embryo development, gene expression, hybridization, microarray, Picea abies, Pinus taeda.*

## Introduction

Plant embryogenesis begins with the division of the fertilized egg or zygote and culminates with the generation of a mature embryo, comprising an embryonic axis with shoot and root poles and cotyledon(s). In gymnosperms, the overall embryo development pathway can be divided in three distinct phases: proembryogeny, which includes stages before the elongation of a suspensor; early embryogeny, which initiates with the elongation of the suspensor and terminates with the appearance of the root meristem; and late embryogeny, which culminates with the maturation of the embryo (Singh 1978). Despite extensive knowledge on the physiological and molecular mechanisms regulating embryo maturation in angiosperms (Kermode 1990, Girke et al. 2000, Ruuska et al. 2002), little is known about the mechanisms governing the early stages of embryogenesis. In angiosperms, developmental arrest or aberrations at the later stages of embryonic and post-embryonic development are typical consequences of disturbed regulation during the early stages of embryo development (Dunn et al. 1997, Scanlon et al. 1997, Hamann et al. 1999, Heckel et al. 1999). The development of mutant analysis in *Arabidopsis* has identified several regulatory genes responsible for normal development of the suspensor (Vernon and Meinke 1994, Yadegari et al. 1994, Zhang and Sommerville 1997, Rojo et al. 2001) and embryo proper (reviewed by Jurgens 2001). In gymnosperms, long generation times make the selection of embryo-specific mutants practically impossible.

Somatic embryogenesis, the process in which embryos, similar in morphology to their zygotic counterparts, are induced to develop in culture from somatic cells, represents a suitable model system for investigating factors affecting embryo growth. Through this process, a large number of embryos at defined stages of development can easily be obtained. In

gymnosperms, the developmental pathway of somatic embryogenesis has been described for Norway spruce, consisting of a sequence of defined developmental stages corresponding to three specific treatments (Filonova et al. 2000*a*) (Figure 1A). Maintenance of the embryogenic potential through proliferation of proembryogenic masses (PEMs) occurs in the presence of the exogenously supplied plant growth regulators (PGR) auxin and cytokinin. Removal of these PGR from the medium reduces cell proliferation in PEMs and initiates early embryogeny through trans-differentiation of PEMs into somatic embryos. From this developmental stage on, the pathway of somatic embryogenesis is similar to zygotic embryo development. Late embryogeny requires abscisic acid (ABA) and involves establishment of root and shoot meristems and differentiation of cotyledons (Figure 1A). A critical step during the overall embryogenic process appears to be the trans-differentiation of PEMs into somatic embryos, requiring the execution of several physiological events, including programmed cell death (Filonova et al. 2000*b*, Bozhkov et al. 2002, Smertenko et al. 2003). Normal passage of PEMs to somatic embryos is precluded in some lines, resulting in a developmental arrest at the PEM stage (Filonova et al. 2000*a*, Smertenko et al. 2003). Preliminary studies on the molecular mechanisms regulating the early phases of somatic embryogenesis have revealed several genes with differentially regulated expression between embryogenic and non-embryogenic tissue (Bishop-Hurley et al. 2003, van Zyl et al. 2003). As an extension of this work and to contribute to the understanding of the molecular events associated with gymnosperm embryogenesis, this study compares the steady-state transcript levels of more than 2000 cDNAs from loblolly pine in three cell lines of Norway spruce. Of these lines, two were able to form somatic embryos, whereas the third line had a developmental block at the PEM–somatic embryo transition. The employment of a pine cDNA array for studies on gene expression in spruce has been documented previously (van Zyl et al. 2002) and implemented (Stasolla et al. 2003). The major objective of this work was to identify genes involved in physiological responses, which are required for the normal progression of embryo development.

## Materials and methods

### *Plant material*

Three lines of Norway spruce (*Picea abies* L. Karst.) were utilized: two normal lines, 61.21 (line $N_1$) and 88.17 (line $N_2$), both of which passed through the PEM–somatic embryo transition, and one developmentally arrested line, 88.1 (line B), where this transition was precluded (Figure 1A). All cell lines were stored in liquid nitrogen and thawed 5 months before starting the experiment. Maintenance of PEMs and initiation of embryo development were performed as previously reported (Bozhkov et al. 2002). Briefly, proliferation of PEMs was stimulated using half-strength LP medium (modified after Bozhkov and von Arnold 1998) supplemented with PGR auxin (9.0 μM 2,4-dichlorophenoxyacetic acid (2,4-D)) and cytokinin (4.4 μM $N^6$-benzyladenine (BA)). For maintenance of embryogenic potential, PEMs were sub-cultured weekly into fresh PGR-containing medium. Trans-differentiation of PEMs into somatic embryos was accomplished through two successive washings of 3-ml settled cell aggregates with 10-ml samples of half-strength LP medium devoid of PGR, followed by inoculation with 3 ml of washed cells into 47 ml of the same PGR-free medium for 1 week. Continuation of embryo development, i.e., generation of cotyledonary somatic embryos,

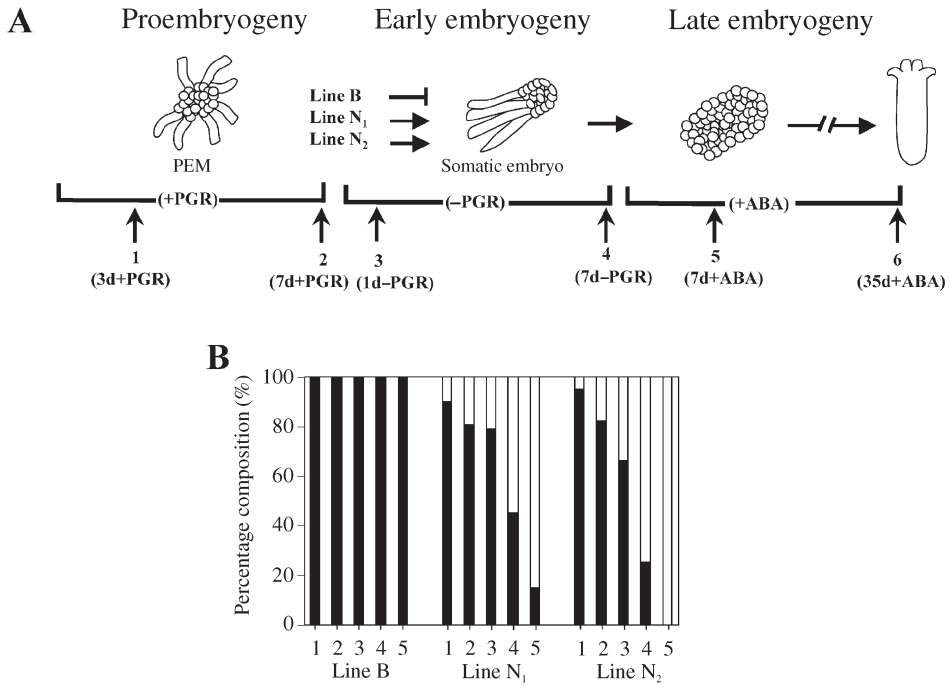


Figure 1. (A) Developmental pathway of somatic embryogenesis in Norway spruce (modified from Filonova et al. 2000*a*). In the two normal $N_1$ and $N_2$ lines, proembryogenic masses (PEMs) can successfully differentiate into early somatic embryos (ESEs) upon removal of plant growth regulators (–PGR), and mature into somatic embryos (SE) and cotyledonary embryos in the presence of abscisic acid (ABA). Trans-differentiation of PEMs into ESEs is precluded in the developmentally blocked B line. Tissue was collected for gene expression analysis at six stages of embryo development. (B) Percentage composition of PEMs (solid bars) and ESEs + SEs (open bars) in the three cell lines during the first five stages of development.

was carried out by plating 1 ml of settled cell aggregates onto Whatman No. 2 filter paper placed on solidified BMI-S1 maturation medium containing 30 μM ABA. Cotyledonary embryos were harvested after 35 days in culture. Sampling of cells for morphological observations and microarray experiments was performed during six stages of embryo development: Stage 1 (3d+PGR); Stage 2 (7d+PGR); Stage 3 (1d–PGR); Stage 4 (7d–PGR); Stage 5 (7d+ABA); and Stage 6 (35d+ABA) (see Figure 1A).

*Microarray procedure*

The elements on the array were selected from 55,000 expressed sequence tags (ESTs) grouped in 9000 contigs. These ESTs were obtained from five different cDNA libraries: NXNV (Xylem Normal-wood Vertical); NXCI (Xylem Compression-wood Inclined); NXSI (Xylem Side-wood inclined); ST (Shoot Tip); and PC (Pollen Cone) (http://web.ahc.umn.edu/biodata/nsfpine/contig_dir6). The cDNAs were selected closest to the 3′-end of the respective contig and were run on BLASTX against the database (http://mips.gsf.de/proj/thal/db/search/blast_arabi.html). The best hit from the BLAST search grouped the cDNAs into functional categories, as proposed for *Arabidopsis thaliana* (http://pedant.gsf.de; see Table 1). The selected cDNAs were transformed into *Escherichia coli* XL-1 blue competent cells and the plasmids were isolated using Qiagen kits. Probe preparation and printing were carried out as previously described (Stasolla et al. 2003). Clone identities were confirmed by re-sequencing for 86% of the cDNAs, including all the clones differentially expressed among lines.

*Target preparation*

For each stage of embryo development in the three cell lines ($N_1$, $N_2$ and B), RNA was extracted from 1 g of tissue (fresh mass), as described by Chang et al. (1993). The aminoallyl procedure developed by DeRisi (http://cmgm.stanford.edu/pbrown/protocols/index.html) was followed to label cDNA probes. The RNA from each sample was labeled with both Cy3 and Cy5 dyes and used for reciprocal hybridizations. Hybridization and stringency washes were performed applying the protocol from the Institute of Genomic Research (TIGR) (Hegde et al. 2000). The slides were scanned with a ScanArray 4000 Microarray Analysis System (GSI Lumonics, Ottawa, ON, Canada). Raw, non-normalized intensity values were collected with QUANTARRAY software (GSI Lumonics). Using the quantification option, spots were visually inspected for morphology and background. A few spots were flagged as unacceptable and excluded from further analysis.

*Experimental design and statistical analysis*

A fully balanced, incomplete loop experimental design was used for each of the three lines, as proposed by Kerr and Churchill (2001) and as employed in previous studies (Stasolla et al. 2003). Gene significance was estimated with a mixed model analysis of variance (ANOVA) as described by Wolfinger et al. (2001), Jin et al. (2001) and Chu et al. (2002). This modeling approach is highly sensitive and shows that changes of less than twofold in gene expression can be statistically significant. Two linear mixed models were applied in succession: the normalization model (1) and the gene model (2). The normalization model was applied for global normalization at the slide level, whereas the gene model was applied separately to each gene using residuals (observed minus fitted values) from model (1).

$$\log_2(Y_{ijkls}) = \theta_{ij} + D_k + S_l + DS_{kl} + \omega_{ijkls} \quad (1)$$

$$R^{(g)}_{ijkls} = \mu^{(g)}_{ij} + D^{(g)}_k + S^{(g)}_l + DS^{(g)}_{kl} + SS^{(g)}_{ls} + \varepsilon^{(g)}_{ijkls} \quad (2)$$

where $Y_{ijkls}$ represents the intensity of the $s$th spot in the $l$th

Table 1. Functional grouping of the expressed sequence tags present on the array and of those differentially expressed ($P < 0.05$) in three cell lines during the first five stages of embryo development. Numbers in brackets indicate percentage values.

| Category | Code | Array | Cell line B | Cell line $N_1$ | Cell line $N_2$ |
|---|---|---|---|---|---|
| Metabolism | M | 430 (19.7) | 16 (42.1) | 14 (15.2) | 5 (16.1) |
| Protein synthesis and destination | PS | 455 (20.9) | 6 (15.8) | 17 (18.5) | 5 (16.1) |
| Cell rescue | CR | 218 (10.0) | 3 (7.9) | 15 (16.3) | 9 (29.0) |
| Transcription | T | 196 (9.0) | 2 (5.3) | 3 (3.3) | 1 (3.2) |
| Cell growth | CG | 196 (9.0) | 2 (5.3) | 13 (14.1) | 2 (6.5) |
| Energy | E | 159 (7.3) | 2 (5.3) | 7 (7.6) | 1 (3.2) |
| Cellular biogenesis | CB | 137 (6.3) | 0 (0) | 6 (6.5) | 6 (19.4) |
| Cellular transport | CT | 109 (5.0) | 0 (0) | 5 (5.4) | 1 (3.2) |
| Transport facilitation | TF | 109 (5.0) | 3 (7.9) | 4 (4.3) | 0 (0) |
| Cellular communication | CC | 105 (4.8) | 2 (5.3) | 2 (2.2) | 0 (0) |
| Ionic homeostasis | IO | 15 (0.7) | 0 (0) | 0 (0) | 0 (0) |
| Transposable elements | TE | 15 (0.7) | 0 (0) | 0 (0) | 0 (0) |
| Development | D | 12 (0.3) | 0 (0) | 0 (0) | 0 (0) |
| Unknown function | U | 22 (1.0) | 2 (5.3) | 6 (6.5) | 1 (3.2) |
| Total | | 2178 (100) | 38 (100) | 92 (100) | 31 (100) |

slide with the $k$th dye applying the $j$th stage for the $i$th cell line. The mean effect of the $j$th stage in the $i$th cell line, the $k$th dye effect, the $l$th slide random effect and the random interaction effect of the $k$th dye in the $l$th slide is represented by $\theta_{ij}$, $D_k$, $S_l$, and $DS_{kl}$. The stochastic error term is $\omega_{ijkls}$ and $R^{(g)}_{ijkls}$ represents the residual of the $g$th gene from Model 1. Parameters $\mu^{(g)}_{ijkls}$, $D^{(g)}_k$, $S_l$ and $DS_{kl}$ represent similar roles as $\theta_{ij}$, $D_k$, $S_l$ and $DS_{kl}$, except that they are specific for the $g$th gene. The spot-by-slide random effect for the $g$th gene is represented by $SS_{ls}$. The stochastic error term is represented by $\varepsilon^{(g)}_{ijkls}$. All random effects were assumed to be normally distributed and mutually independent within each model. These models were fitted by using SAS Proc Mixed (SAS Institute, Cary, NC). In the gene model (2), $R^2$ values (representing the percentage of total variability explained by the model) were calculated to evaluate model fitness. Only two $R^2$ values < 0.9 among all 2178 ESTs were observed, implying that the models fit well. Also, nearly all of the standardized residuals from model (2) had absolute values < 3, indicating excellent array data quality. Using these fitted models, statistical comparisons of gene expression at neighboring stages within each cell line were achieved by testing the following hypothesis (3).

$$H_0\colon \mu^{(g)}_{j+1} = \mu^{(g)}_{j} \text{ vs. } H_1\colon \mu^{(g)}_{j+1} \neq \mu^{(g)}_{j},\ j+1 \neq j \qquad (3)$$

Results from these hypothesis tests are shown in volcano plots (Figure 2) generated with JMP software (SAS Institute). Differences in transcript levels between neighboring stages within each line were considered significant if higher than a Bonferroni cutoff, which conservatively controls the family-wise false positive rate across all hypothesis tests to be < 0.05. The horizontal line in the volcano plot represents the negative logarithm cutoff point ($-\log_{10}(0.05/2178/N)$, where 0.05 is the nominal significance level, 2178 is the number of ESTs on the array, and $N$ is the number of comparisons for one specific hypothesis test). To identify developmentally regulated genes involved in the progression of embryo development, we compared the transcript level response of each gene in the two normal lines $N_1$ and $N_2$ with that of the same element in the developmentally arrested B line by two-way ANOVA, as previously reported by van Zyl et al. (2003). We also used fitted means from the mixed models to construct expression profiles (see Figures 3–5). These were displayed both as colored heat maps and in parallel coordinate plots. The profiles were clustered hierarchically using Ward's distance metric as implemented in JMP.

The transcript levels of eight cDNAs, which appeared differentially expressed between the $N_1$ and B lines in the microarray experiments were confirmed by real time reverse transcription polymerase chain reaction (RT-PCR). Real time RT-PCR analysis was carried out as previously described (Stasolla et al. 2003).

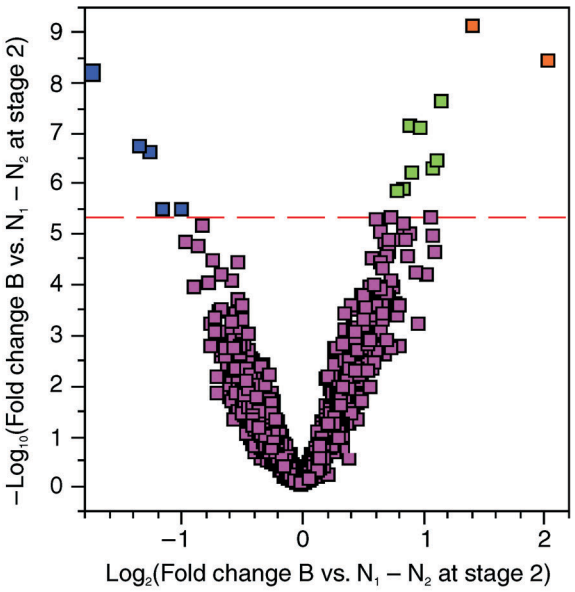


Figure 2. Volcano plot comparing the developmentally arrested B line to the normal lines $N_1$ and $N_2$ at Stage 2 of development (7d+PGR). Each value represents a gene. The $x$-axis is the estimated fold change (log 2 scale) and the $y$-axis is the $-\log_{10}(P\text{-value})$ of the comparison. The horizontal reference line was obtained from a Bonferroni correction across multiple hypothesis tests. Genes above this line are statistically significant under strict control of the false positive rate.

## Results

### *Developmental pathways of somatic embryogenesis in Norway spruce*

Time-lapse tracking experiments have revealed that the normal developmental pathway in Norway spruce can be conceptually divided in three distinct phases corresponding to regulatory treatments (Filonova et al. 2000*a*) (Figure 1A). In the presence of auxin and cytokinin, proliferating PEMs were visible. Removal of PGR triggered the trans-differentiation of PEMs into somatic embryos (Figure 1B), which were composed of an organized embryo proper subtended by an elongated suspensor. Further development of somatic embryos was promoted by ABA (for a detailed description, see Filonova et al. 2000*a*) (Figure 1A).

In the B line, withdrawal of PGR did not trigger the differentiation of PEMs into somatic embryos, thus precluding subsequent embryonic growth (Filonova et al. 2000*a*) (Figure 1A). Formation of somatic embryos, which was observed in increasing frequency during development of lines $N_1$ and $N_2$, was not observed in the B line (Figure 1B).

### *Functional grouping and hierarchical clustering of differentially abundant genes in the three cell lines*

Functional grouping of differentially abundant genes during the first five stages of development in the three lines and com-

parison with the percentages of genes belonging to distinct functional categories in the array are shown in Table 1. Compared to the developmentally arrested B line, the percentage of differentially abundant genes involved in metabolic processes was lower in both normal lines (42.1% in the B line, compared with 15.2 and 16.1% in the $N_1$ and $N_2$ lines, respectively). An opposite tendency was observed for genes included in the category of cell rescue (7.9% in the B line and 16.3 and 29% in $N_1$ and $N_2$) and cellular biogenesis (0% in the B line and 6.5 and 19.4% in $N_1$ and $N_2$). Altogether, more genes (92) were differentially expressed in the $N_1$ line compared with the other two lines (Table 1).

To assess typical transcript level patterns during development of the three lines, differentially abundant genes for each line were selected and grouped in hierarchical clusters. These genes were then divided into eight groups, having distinct transcription patterns during embryo development, and further classified in functional categories (Figures 3–5). A large number of genes of the developmentally arrested line (clusters A, B, D and F; Figure 3) had similar transcription patterns during early development, showing general up-regulation between Stages 1 and 2, followed by repression (Stages 2 and 3) and induction (Stages 3 and 4). In cluster B, more than half of the genes belonged to the category "Metabolism." In the lines showing a normal embryonic development, two clusters of genes (cluster D for $N_1$ and cluster H for $N_2$) showed a similar transcript level pattern characterized by an up-regulation between Stages 5 and 6 (Figures 4 and 5). The majority of the genes within both clusters belonged to the categories of "Metabolism" and "Cell rescue." Several other genes displayed stage-specific patterns of transcript levels, including those of clusters G and H for $N_1$ and cluster C for $N_2$, which were up-regulated between Stages 3 and 4 (Figures 4 and 5). Eight of these genes were involved in metabolic processes. Similarities in transcript abundance were also observed for clusters C and F, in lines $N_1$ and $N_2$, respectively. The transcript levels of these genes, mainly belonging to the categories "Cell rescue," "Cellular biogenesis" and "Protein synthesis and destination" decreased during the initial phases of development.

Validation of the microarray experiment was confirmed by RT-PCR studies of eight cDNAs that were differentially expressed between the $N_1$ line and the B line at Stages 1 and 5 of development. The same trends for up- and down-regulation were observed (Table 2), although variations in fold changes were obtained using two hybridization techniques, as reported previously (Stasolla et al. 2003).

### *Analysis of gene expression during proembryogeny and early embryogeny*

In the developmentally arrested line, only four genes were differentially abundant between Stages 1 and 2. This number increased during the subsequent phases and reached a maximum of 23 genes between Stages 3 and 4 before declining between Stages 4 and 5 (Figure 6A). The number of differentially ex-

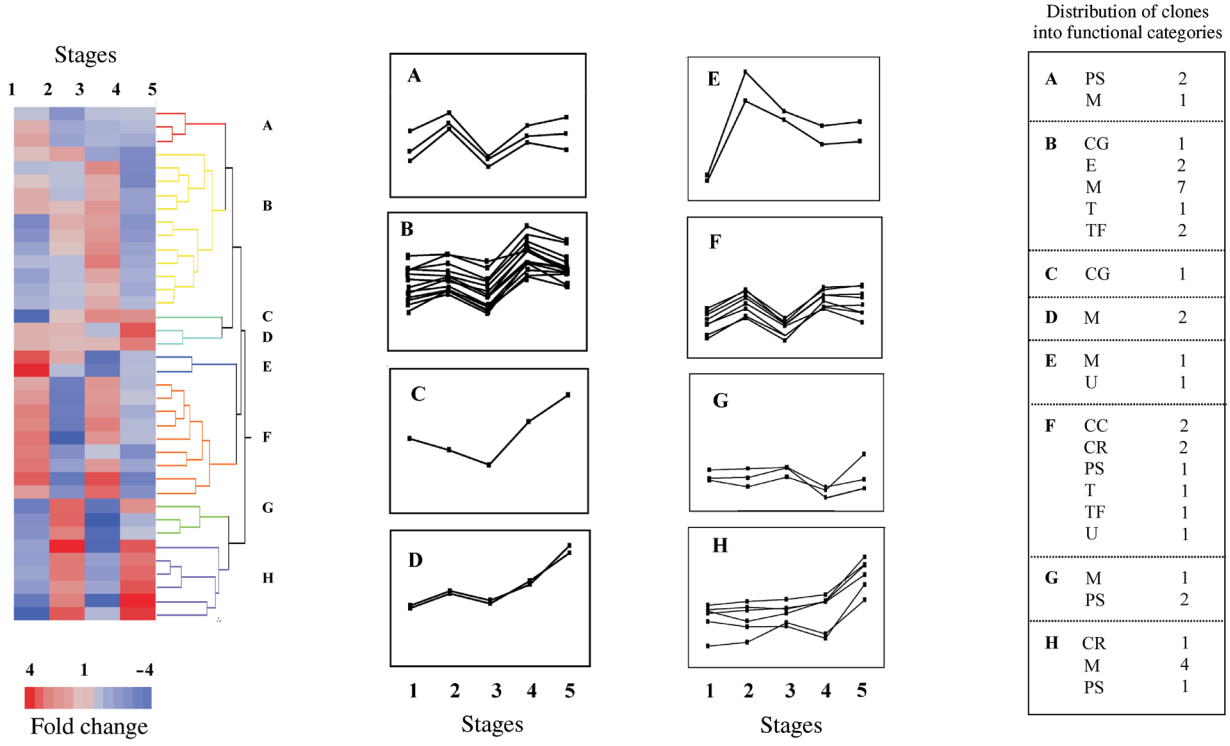


Figure 3. Expression profiles and hierarchical clustering of differentially expressed genes in the developmentally blocked (B) line. On the basis of the constructed tree (left), the genes were clustered in eight groups (A–H), based on their expression pattern during the first five stages of development (center), and classified in functional categories (right). See Table 1 for definitions of functional category codes.

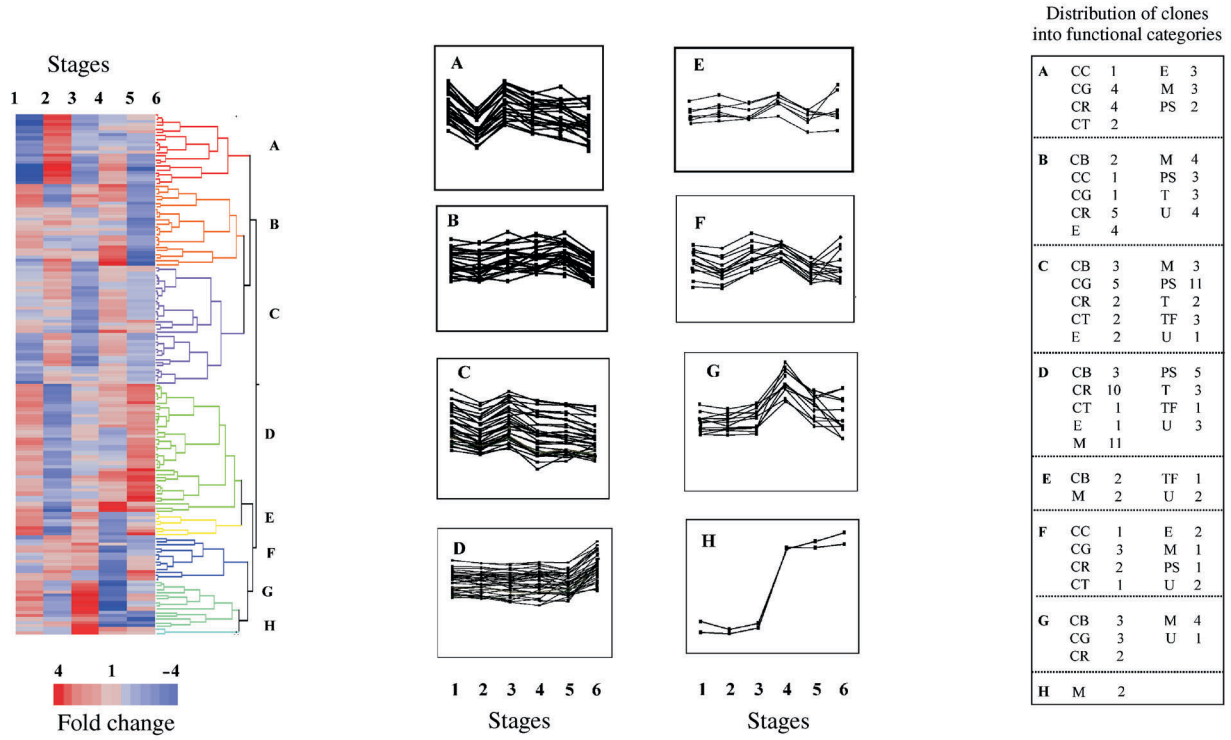


Figure 4. Expression profiles and hierarchical clustering of differentially expressed genes in the normal ($N_1$) line. On the basis of the constructed tree (left), the genes were clustered in eight groups (A–H), based on their expression pattern during the first six stages of development (center), and classified in functional categories (right). See Table 1 for definitions of functional category codes.

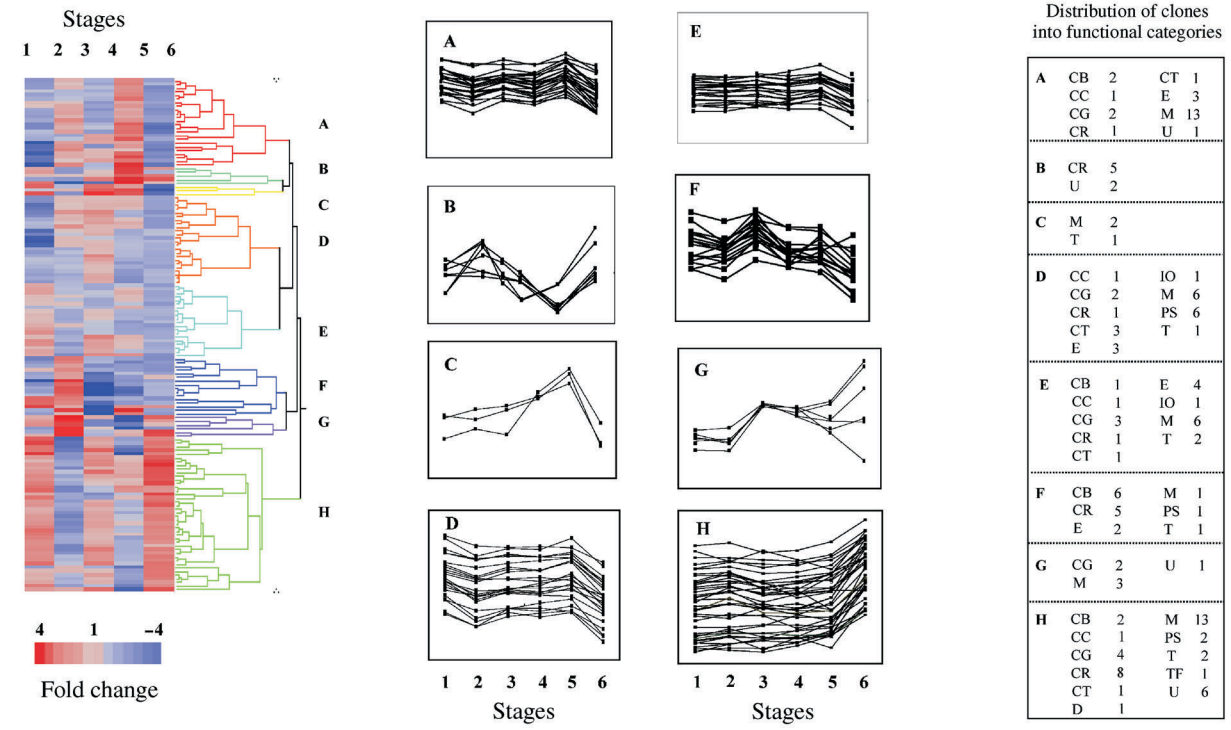


Figure 5. Expression profiles and hierarchical clustering of differentially expressed genes in the normal ($N_2$) line. On the basis of the constructed tree (left), the genes were clustered in eight groups (A–H), based on their expression pattern during the first six stages of development (center), and classified in functional categories (right). See Table 1 for definitions of functional category codes.

Table 2. Quality control of microarray experiments. Fold change differences of eight cDNAs, which resulted as differentially expressed between the normal ($N_1$) and the developmentally blocked (B) line at Stages 1 and 5 of development, were confirmed by reverse transcription polymerase chain reaction (RT-PCR). All RT-PCR reactions were repeated three times. Positive and negative signs indicate respective up-regulation and down-regulation in the $N_1$ compared to the B line.

| Clone ID | Function | Fold change ($N_1$ versus B) | |
|---|---|---|---|
| | | RT-PCR | Microarray |
| *Stage 1* | | | |
| NXNV_162_F07 | Cinnamyl alcohol dehydrogenase | +7.0 | +4.2 |
| NXCI_144_F06 | Steroid 5 alpha-reductase | +47.4 | +2.3 |
| NXSI_143_A04 | 60S ribosomal protein | +4.4 | +2.2 |
| NXCI_132_B11 | Proteasome p55 protein-like | +8.4 | +3.5 |
| *Stage 5* | | | |
| ST_21_E10 | Thioredoxin h | −11.2 | −5.3 |
| NXCI_002_G12 | Ascorbate peroxidase | −40.5 | −4.3 |
| ST_04_G06 | Hypothetical protein | +45.2 | +9.9 |
| ST_29_G11 | Putative protein | −3.9 | −4.9 |

pressed genes of the $N_2$ line was generally similar to that observed for the B line through development, except between Stages 3 and 4, where less than 10 genes were differentially expressed. A large magnitude of transcript abundance was observed during the normal developmental pathway of the $N_1$ line (Figure 6A).

To assess the overall pattern of transcript profiles during embryogenesis, the percentage of gene induction or repression was calculated for each line at each developmental stage. In the developmentally arrested B line, transcript accumulation was induced during the initial phases (Stages 1 and 2), repressed between Stages 2 and 3, and induced again during the subsequent phases (Figure 6B). An opposite tendency was observed during the normal developmental pathway. The early phases of embryo development in both $N_1$ and $N_2$ lines were characterized by an initial down-regulation of transcript level (Stages 1 and 2), followed by up-regulation (Stages 2 and 3). Variations in expression patterns were observed during the subsequent stages of embryonic development (Figure 6B).

To identify developmentally regulated genes involved in the normal progression of embryo development, the pattern of expression of particular genes was assessed by pair-wise comparisons among the three lines. A list of genes showing similar expression responses (induction or repression) during the normal developmental pathways of lines $N_1$ and $N_2$ is shown in Table 3. This response pattern was in marked contrast to the pattern of expression of the corresponding elements in the B line. Many ribosomal proteins are among the genes repressed during the initial stages of normal embryonic development

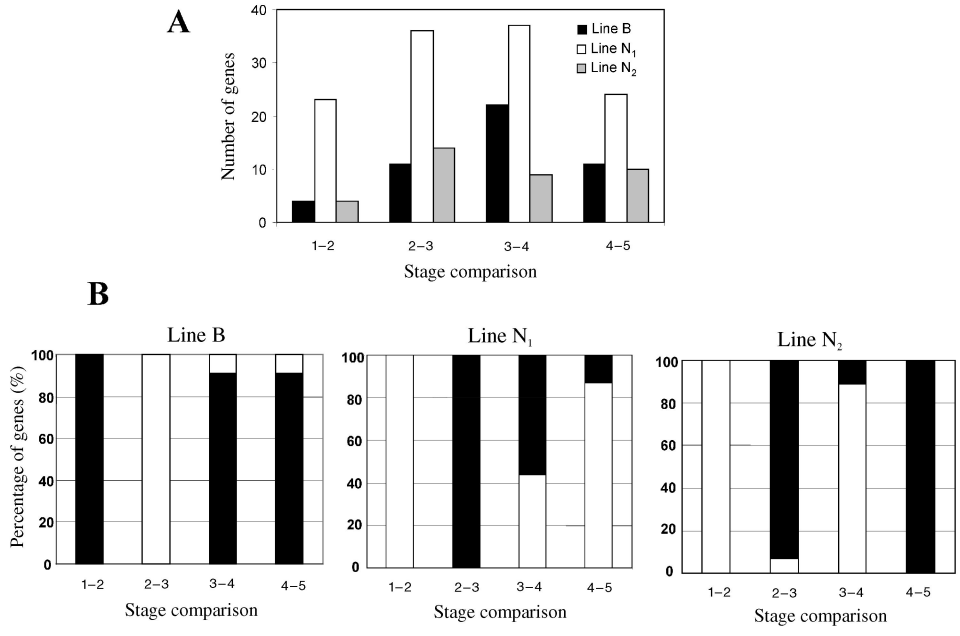


Figure 6. (A) Number of genes differentially expressed at a statistically significant level ($P < 0.05$) between neighboring stages of embryo development in the developmentally blocked (B) line and in the normal ($N_1$ and $N_2$) lines. (B) Percentage distribution of genes that are repressed (open bars) or induced (closed bars) between neighboring stages of embryo development in the B line and in the $N_1$ and $N_2$ lines.

Table 3. List of genes showing an expression response (repression = R and induction = I) during consecutive stages of embryo development in the normal lines $N_1$ and $N_2$. This pattern of expression is in marked contrast to that of the corresponding genes in the developmentally arrested line (two-way ANOVA $P = 0.05$).

| Function | Clone ID | E-value | Stages of development | | | |
|---|---|---|---|---|---|---|
| | | | 1–2 | 2–3 | 3–4 | 4–5 |
| *Protein synthesis and destination* | | | | | | |
| *At3g04920* ribosomal protein s24 | NXSI_136_B08 | 1.00E-31 | R | | | |
| *At3g04920* ribosomal protein s24 | NXSI_064_H06 | 9.00E-34 | R | | | |
| *At2g40010* 60S acidic ribosomal protein P0 | NXSI_058_B04 | 4.00E-42 | R | | | |
| *At2g09990* 40S ribosomal protein S16 | NXSI_055_E09 | 7.00E-49 | R | | | |
| *At4g36130* ribosomal protein L8 | NXSI_045_A04 | 4.00E-85 | R | | | |
| *At4g17390* 60S ribosomal protein L15 | NXSI_044_C06 | 2.00E-65 | R | | | |
| *At1g61580* 60s ribosomal protein L3 | NXSI_040_H10 | 9.00E-50 | R | | | |
| *At4g22380* ribosomal protein L7Ae-like | NXSI_036_B05 | 2.00E-08 | R | | | |
| *At1g69620* 60S ribosomal protein L34 | NXSI_024_H04 | 2.00E-27 | R | | | |
| *At5g45775* ribosomal protein L11-like | ST_23_F10 | 6.00E-31 | R | | | |
| *At4g00100* ribosomal protein S13 | NXNV_151_F01 | 3.00E-50 | R | | | |
| *At3g27850* 50S ribosomal protein L12-C | NXNV_103_G03 | 9.00E-06 | | | R | |
| *At5g56940* 30S ribosomal protein S16 | ST_19_D12 | 7.00E-24 | | I | | |
| *At3g56340* 40S ribosomal protein S26 homolog | ST_09_F05 | 2.00E-23 | R | | | |
| *At3g48930* cytosolic ribosomal protein S11 | ST_07_D12 | 2.00E-61 | R | | | |
| *At2g27530* 60S ribosomal protein L10A | NXNV_083_E06 | 4.00E-50 | R | | | |
| *At1g74270* ribosomal protein | AT_29_F11 | 7.00E-55 | R | | | |
| *At4g17390* 60S ribosomal protein L02 | NXNV_077_G03 | 4.00E-85 | R | | | |
| *At2g44120* 60S ribosomal protein L7 | NXNV_069_D04 | 9.00E-27 | R | | | |
| *At3g04400* ribosomal protein L17, putative | ST_07_A07 | 2.00E-64 | R | | | |
| *At4g39200* ribosomal protein S25 | NXNV_044_D09 | 1.00E-28 | R | | | |
| *At5g39850* 40S ribosomal protein S9-like | NXNV_022_C10 | 7.00E-18 | | I | | |
| *At2g39390* 60S ribosomal protein L35 | NXCI_152_B06 | 2.00E-37 | R | | | |
| *At5g45775* ribosomal protein L11 | NXCI_132_E06 | 1.00E-63 | R | | | |
| *At3g09680* 40S ribosomal protein S23 | NXCI_060_B03 | 2.00E-46 | R | | | |
| *At5g39740* ribosomal protein L5-like | ST_34_E12 | 5.00E-35 | R | | | |
| *At3g10610* putative 40S ribosomal protein S17 | ST_33_G08 | 2.00E-39 | R | | | |
| *At1g26880* 60s ribosomal protein L34 | ST_32_C07 | 2.00E-20 | R | | | |
| *At3g10410* carboxypeptidase type III | NXNV_063_B09 | 3.00E-44 | | I | | |
| *At1g17370* putative oligouridylate binding protein | NXSI_052_A11 | 1.00E-27 | | | R | |
| *At3g61140* fusca protein (FUS6) | NXNV_083_G07 | 2.00E-23 | | | R | |
| *At1g18540* hypothetical protein | ST_25_C01 | 5.00E-41 | R | | | |
| *At3g13920* initiation factor 4A-15 | NXSI_065_C08 | 4.00E-63 | R | | | |
| *At1g54270* initiation factor 4A-9 | NXSI_057_C07 | 1.00E-139 | | | R | |
| *Metabolism* | | | | | | |
| *At1g17880* hypothetical protein | NXSI_136_E08 | 4.00E-34 | R | | | |
| *At5g45880* SAH7 protein | NXSI_100_F12 | 1.00E-13 | | | R | |
| *At4g38440* cysteine protease inhibitor | NXSI_080_C08 | 2.00E-28 | | I | | |
| *At5g15490* UDP-glucose dehydrogenase | NXNV_132_G09 | 1.00E-165 | | | R | |
| *At2g36530* enolase | NXCI_122_A09 | 3.00E-72 | | | | R |
| *At3g03780* putative methionine synthase | NXCI_055_C01 | 7.00E-14 | | I | R | |
| *At4g13940* adenosylhomocysteinase | NXCI_053_F03 | 2.00E-23 | | I | | |
| *At1g20630* hypothetical protein | NXCI_044_F09 | 4.9 | R | | | |
| *At2g26080* glycine dehydrogenase | NXCI_026_F05 | 1.00E-159 | | | R | |
| *At3g51240* flavanone 3-hydroxylase (FH3) | ST_28_B11 | 1.00E-40 | R | | | |
| *At3g13790* beta-fructofuranosidase 1 | ST_27_A08 | 2.6 | R | | | |
| *At4g10960* UDP-galactose 4-epimerase-like protein | ST_21_A06 | 4.00E-63 | R | | R | |
| *At4g18590* pollen-specific protein-like | ST_15_D07 | 3.00E-16 | | | R | |
| *At1g78430* hypothetical protein | ST_07_D02 | 1.1 | R | | | |
| *At2g48070* unknown protein | ST_06_C04 | 2.00E-38 | R | | | |
| *At4g26450* putative protein | ST_04_F02 | 2.7 | R | | | |
| *At4g18590* pollen-specific protein-like | ST_03_D08 | 2.00E-11 | | | R | |
| *At3g57880* anthranilate phosphoribosyltransferase | NXSI_062_A03 | 1.00E-74 | | | R | R |

Continued on facing page.

Table 3 continued. List of genes showing an expression response (repression = R and induction = I) during consecutive stages of embryo development in the normal lines $N_1$ and $N_2$. This pattern of expression is in marked contrast to that of the corresponding genes in the developmentally arrested line (two-way ANOVA $P = 0.05$).

| Function | Clone ID | E-value | Stages of development | | | |
|---|---|---|---|---|---|---|
| | | | 1–2 | 2–3 | 3–4 | 4–5 |
| *Energy* | | | | | | |
| *At3g48000* aldehyde dehydrogenase | NXSI_067_F07 | 2.00E-75 | | | R | |
| *At1g63290* putative D-ribulose-5-phosphate | NXSI_025_H08 | 5.00E-31 | | I | | |
| *At1g79530* glyceraldehyde 3-P precursor | NXCI_044_H09 | 2.00E-26 | R | | | |
| *Cell rescue* | | | | | | |
| *At2g21320* putative CONSTANS-like | NXSI_065_C12 | 3.00E-28 | R | | | |
| *At3g51030* thioredoxin H | ST_21_E10 | 9.00E-33 | R | | | R |
| *At3g09640* cytosolic ascorbate peroxidase | NXNV_160_F07 | 6.00E-71 | R | | | |
| *At1g07890* L-ascorbate peroxidase | NXNV_123_H12 | 8.00E-47 | | I | | |
| *At5g56000* heat shock protein | NXNV_066_E07 | 2.00E-138 | R | | | |
| *At2g39700* putative expansin | ST_21_H02 | 4.00E-86 | R | | | |
| *Cell growth, biogenesis and communication* | | | | | | |
| *At5g56010* heat shock protein 90 | NXSI_116_B04 | 3.00E-174 | | | | R |
| *At4g14560* auxin-responsive protein IAA1 | NXNV 181 G06 | 0.19 | | | R | |
| *At2g36060* putative ubiquitin-conjugating enzyme | NXCI_097_A07 | 8.00E-16 | R | | | |
| *At3g05480* ubiquitin-conjugating enzyme | NXCI_005_A11 | 1.00E-51 | | I | | |
| *At4g28640* early auxin-inducible protein 11 (IAA11) | NXCI 027 F08 | 2.00E-08 | R | | R | |
| *At5g06600* ubiquitin carboxyl-terminal hydrolase | ST_30_A12 | 5.00E-31 | | | R | |
| *At1g72710* putative casein kinase I | ST_12_C12 | 2.00E-75 | R | | | |
| *At1g50010* putative tubulin alpha-2/alpha-4 chain | ST_08_B05 | 5.00E-52 | | | R | |
| *At1g01750* putative actin depolymerizing factor | ST_01_E09 | 4.00E-32 | | I | | |
| *At4g03290* putative calmodulin | NXNV_015_H07 | 1.00E-13 | | I | | |
| *At1g78900* vacuolar ATP synthase (subunit) | NXSI_121_D02 | 6.00E-59 | | | R | |
| *Transport facilitation* | | | | | | |
| *At4g35100* plasma membrane intrinsic protein | ST_38_B07 | 1.00E-28 | | | R | |
| *At3g54700* phosphate transport protein | ST_33_E11 | 2.00E-51 | | | R | |
| *Transcription* | | | | | | |
| *At3g12630* PVPR3 protein | NXSI_076_G02 | 3.00E-15 | | I | R | |
| *At3g51880* high mobility group protein 2-like | NXNV_073_E06 | 8.00E-18 | R | | | |
| *At1g20690* hypothetical protein | ST_23_D05 | 2.00E-15 | R | | | |
| *Unknown function* | | | | | | |
| *At3g07520* unknown protein | NXNV_017_D11 | 2.6 | R | | | |
| *At5g51000* putative protein | ST_35_G09 | 4.2 | R | | | |
| *At3g12030* hypothetical protein | ST_29_E01 | 8.00E-47 | R | | | |
| *At1g08200* hypothetical protein | ST_29_C11 | 1.00E-82 | R | | | |
| *At1g36940* hypothetical protein | ST_28_F04 | 4.7 | | | R | |
| *At3g52430* putative protein | ST_28_C03 | 2.6 | R | I | | |
| *At1g14450* hypothetical protein | ST_07_E03 | 3.00E-13 | R | | | |

(Table 3). Several other genes, belonging to the categories "Metabolism," "Energy," "Cell rescue," "Cell growth, biogenesis and communication," "Transcription" and "Unknown" were also repressed during these early phases. Upon further development (Stages 2 and 3), 13 genes were induced, including those encoding for a putative methione synthase and adenosylhomocysteinase. The decreased expression level of 21 genes delineated the progression of embryo development from Stage 3 to Stage 4. The majority of these genes belonged to the categories of "Metabolism" and "Cell growth, biogenesis and communication." Few genes (an enolase, a thioredoxin H, a heat shock protein 90 and an anthranilate phosphoribosyltransferase) were repressed during the late phases of early embryogeny (Stages 4 and 5) in the two normal lines, compared to the developmentally arrested culture (Table 3).

### *Analysis of gene expression during late embryogeny*

Analysis of gene expression during the late stages of embryo development (between Stages 5 and 6) was performed by comparing similar tendencies (increase or decrease) of transcript

levels between lines $N_1$ and $N_2$. As shown in Table 4, 31 genes showed similar expression patterns during the late events of embryo development in the normal lines. These included several stress-related proteins, such as five heat shock proteins (HSPs) and one late embryogenic abundant (LEA) protein, which had increased transcript levels in cotyledonary embryos, and other genes with different functions, e.g., methionine synthase, *S*-adenosylmethionine and malate dehydrogenase, which were repressed in cotyledonary embryos (Table 4).

## Discussion

Normal embryonic growth is accompanied by a defined pattern of transcript levels: down-regulation during the progression from Stage 1 to Stage 2, and up-regulation from Stage 2 to Stage 3, resulting in the formation of somatic embryos (Figures 1 and 6). This tendency appears to be critical for normal trans-differentiation of PEMs into somatic embryos, because it is reversed in the developmentally arrested B line, where this transition is precluded. A decrease in transcript levels toward the end of subculture in proliferation medium was also observed, albeit to a lesser extent than that seen in previous microarray work conducted on a similar system (van Zyl et al. 2003). A transcriptional repressive state, followed by active gene expression, is a pattern that has also been observed during early embryogenesis in a variety of animal systems. Perturbation of this pattern through experimental manipulations results in inhibition of further embryonic growth (Ma et al. 2001). Although the role of the initial transcriptional silencing is poorly understood, it may delay embryo differentiation until the appropriate stage (Forlani et al. 1998). A similar control may operate in Norway spruce where maximum competence of PEMs to differentiate into somatic embryos is reached when defined physiological and molecular conditions are met. Failure to meet these conditions may affect further development, as observed in the developmentally arrested line.

Comparisons of changes in transcript levels between normal and developmentally blocked lines during the transition from Stage 1 to Stage 2 revealed that, among the differentially expressed genes identified, several encode ribosomal proteins (Table 3). The down-regulation of these genes in the normal lines suggests that repression of protein synthesis may represent a physiological switch that initiates those morphogenic events, leading to proper embryo development. The activation of programmed cell death is well documented and crucial for the trans-differentiation of PEMs into somatic embryos (Filonova et al. 2000*b*).

Rapid cell proliferation and active aerobic metabolism, which occur mainly in the presence of auxin and cytokinin, are often associated with the production of reactive oxygen species, including hydrogen peroxide. In marked contrast to the developmentally arrested line, the transcript levels of one cytosolic ascorbate peroxidase and one thioredoxin H, both involved in detoxification mechanisms (Mouaheb et al. 1998, Potters et al. 2002), were repressed in the two normal lines between Stages 1 and 2 (Table 3). The higher numbers of these transcripts in the developmentally arrested line suggest that oxidative stress is high in this line, and may contribute to the embryonic block at the PEM stage. Besides their role in detoxification, both ascorbate peroxidase and thioredoxin have a major function in the control of cellular redox state, which is critical for normal development. Alterations in the activity of ascorbate peroxidase, for example, affect the ascorbate/dehydroascorbate ratio, which appears to have a regulatory role in the balance between cell proliferation and differentiation during embryogenesis of both gymnosperms (Stasolla and Yeung 2001) and angiosperms (Arrigoni et al. 1992). This finding is in line with the concept that the cellular redox state is a generic sensor that controls the early developmental events of embryogenesis (Stasolla et al. 2002) and may be critical in the trans-differentiation of PEMs into somatic embryos.

Generation of somatic embryos in the two normal lines is induced by removal of PGR and is accompanied by an increase of transcript levels. This tendency was reversed in the developmentally blocked line, where this transition was precluded. Two differentially abundant transcripts, induced in normal lines between Stage 2 and Stage 3, encode enzymes involved in methionine metabolism: adenosylhomocysteinase and methionine synthase (Table 3), responsible for the production of homocysteine and methionine, respectively. Both enzymes, together with *S*-adenosylmethionine (SAM) synthase, which showed higher transcript levels in normal lines than in the developmentally arrested line at Stage 3 (data not shown), are part of the SAM-dependent transmethylation system (Weretilnyk et al. 2001, Moffatt et al. 2002). In this system, high activity of these enzymes guarantees continuous production of methyl groups to be utilized by SAM-dependent methyltransferases. High levels of methylation during development have been implicated with numerous events, including chromatin modeling, selective gene expression and embryonic growth in both animals and plants. Mice embryos expressing low levels of the maintenance methyltransferase do not develop properly and abort prematurely (Razin and Shemer 1995). In carrot, normal progression of somatic embryo development is associated with increasing levels of endogenous SAM and high levels of DNA methylation (Munksgaard et al. 1995). Sustainability of the SAM-mediated transmethylation system is also strictly dependent on adenosine kinase, which through the removal of free adenosine, favors the conversion of *S*-adenosylhomocysteine to homocysteine (Weretilnyk et al. 2001, Moffatt et al. 2002). The high activity of adenosine kinase in white spruce tissue cultured in PGR-containing medium (Ashihara et al. 2001) further supports the importance of transmethylation events during the initial phases of embryogeny.

A possible increase in DNA methylation between Stages 2 and 3 may contribute to the transcriptionally repressive state observed for all differentially abundant transcripts during the subsequent stages of embryo development (i.e., Stages 3 to 4 and Stages 4 to 5) (Table 3). Active DNA methylation contributes to transcriptional repression both directly, by interfering with the binding of transcription factors to DNA, or indirectly,

Table 4. Fold changes of differentially expressed genes ($P < 0.05$) that show a similar expression pattern (up- or down-regulation) during late embryogenesis in both $N_1$ and $N_2$ lines. Positive and negative values indicate respective up-regulation and down-regulation of gene expression between Stages 5 and 6 of development.

| Function | Clone ID | E-value | Fold change | |
|---|---|---|---|---|
| | | | $N_1$ | $N_2$ |
| Plastid protein | ST_06_F07 | 2.00E-08 | +2.0 | +2.1 |
| Delta tonoplast integral protein (delta-TIP) | ST_07_A03 | 8.00E-43 | +1.6 | +1.4 |
| No hit | ST_18_B12 | 1.00E-32 | +1.7 | +1.7 |
| Late embryogenic abundant protein (LEA) | NXCI_021_G04 | 4.00E-30 | +1.9 | +1.5 |
| Heat shock protein | NXCI_002_G06 | 4.00E-31 | +2.3 | +2.3 |
| Translation initiation factor 3 subunit | NXCI_096_A09 | 1.00E-40 | +2.3 | +2.3 |
| Putative auxin transport protein | NXNV_129_F06 | 2.00E-31 | +1.5 | +1.5 |
| Heat shock protein | NXSI_011G01 | 4.00E-77 | +1.9 | +1.9 |
| Heat shock protein | NXSI_074_H11 | 9.00E-13 | +2.3 | +2.3 |
| Hypothetical protein | NXSI_076_G02 | 1.00E-17 | +2.0 | +2.7 |
| Putative isoprenylated protein | NXSI_085_B02 | 4.00E-23 | +1.7 | +1.7 |
| Myo-inositol-1-phosphate synthase | NXSI_103_A10 | 1.00E-45 | +2.1 | +2.3 |
| Heat shock protein | NXSI_116_B04 | 3.00E-57 | +1.6 | +1.6 |
| Phosphoenolpyruvate decarboxylase | NXSI_129_F07 | 1.00E-84 | +2.5 | +2.5 |
| Heat shock protein 18 | NXSI_139_G02 | 7.00E-33 | +1.7 | +1.5 |
| MADS box protein AGL 2 | ST_06_D02 | 3.00E-54 | –1.7 | –1.9 |
| Pollen specific protein-like | ST_15_D07 | 3.00E-16 | –1.5 | –1.7 |
| GAST 1-Like protein | ST_34_B04 | 2.00E-32 | –1.5 | –1.6 |
| Laccase | NXCI_046_E05 | 4.00E-34 | –1.5 | –1.6 |
| Putative methionine synthase | NXCI_055_C01 | 7.00E-14 | –2.4 | –3.0 |
| Farnesyl-pyrophosphate synthetase | NXCI_055_E11 | 5.00E-34 | –2.0 | –2.9 |
| Putative ubiquitin-conjugating enzyme | NXCI_097_A07 | 3.00E-29 | –1.4 | –1.4 |
| *S*-Adenosylmethionine | NXCI_133_B03 | 3.00E-29 | –1.9 | –2.1 |
| Histone H3 protein | NXCI_153_H12 | 7.00E-29 | –1.4 | –1.5 |
| 40S Ribosomal protein S9-like | NXNV_022_C10 | 7.00E-18 | –1.7 | –2.1 |
| Actin (ACT3) | NXNV_047_D05 | 2.00E-75 | –2.0 | –2.6 |
| UDP-Glucose dehydrogenase | NXNV_132_G09 | 2.00E-67 | –1.9 | –2.1 |
| Putative malate dehydrogenase | NXSI_048_D06 | 4.00E-68 | –1.5 | –2.7 |
| 14-3-3-Like protein | NXSI_048_D06 | 1.00E-33 | –1.5 | –1.5 |
| Ole I allergen | NXSI_100_F12 | 6.00E-06 | –1.4 | –1.5 |
| Putative cytochrome P450 | NXCI_104_E01 | 2.00E-80 | –1.5 | –1.4 |

by recruiting proteins that block access to other factors required for gene induction (see Curradi et al. 2002). Among the genes that showed a transcript reduction during normal development, several are involved in auxin metabolism. These include an anthranilate phosphoribosyltransferase, the enzyme which participates in the formation of the indole group during indole-3-acetic acid biosynthesis (reviewed by Bartel et al. 2001), and two auxin-regulated proteins (Figure 7). Auxin synthesis, transport and utilization are involved in a variety of regulatory pathways during embryo development. One critical event observed during early embryogenesis in plants, including gymnosperms, is the establishment of an apical–basal axis, which is dictated by the polar transport of auxin in developing embryos (Liu et al. 1993). In angiosperms, experimental inhibition of auxin flow both in vivo (Steinmann et al. 1999, Mayer et al. 1993) and in vitro (Ramesar-Fortner and Yeung 2001) has profound effects on the establishment of the embryonic polar axis. Although no related information is currently available for gymnosperms, it is tempting to speculate that a similar control, altered in the developmentally blocked line, is required for normal embryonic growth.

Proper embryo differentiation depends on the ability of the cells to differentiate according to their position and to give rise to a series of tissues organized into patterns. In several systems, including carrot (McCabe et al. 1997) and spruce (Egertsdotter and von Arnold 1995), cell differentiation, and ultimately cell fate, are affected by cell wall components. Thus, alterations in wall composition of the embryonic cells may be important for proper growth. During normal development, the repression of UDP-glucose dehydrogenase, the enzyme involved in the channeling of UDP-glucose to hemicellulose and pectin synthesis (Hertzberg et al. 2001), and UDP-galactose 4-epimerase, utilized in the biosynthesis of low molecular weight galactosides and cell wall polymers, (Joersbo et al. 1999) (Table 3) support this notion. A reorganization of cell wall architecture, possibly underlined by alterations in cytoskeletal organization, as suggested by the repression of a tubulin gene (Table 3), may be the effect of several physiological events, including alterations in auxin metabolism. The auxin-mediated effect on wall loosening and reorganization is well

established (Cosgrove 1997).

During the last phase of embryo development (Stages 5 and 6), a set of 31 genes showed similar transcription level patterns (up- or down-regulation) in both $N_1$ and $N_2$ lines and serve as molecular markers for late embryogenesis and embryo maturation (Table 4). Six of these elements, including one LEA protein and five HSPs, were induced in cotyledonary embryos. Accumulation of both LEA proteins and HSPs, which protect cellular components from severe dehydration, is often observed in fully developed embryos during the late stages of maturation (reviewed by Kermode 1990). An increase in the transcript levels of these classes of proteins was also observed in white spruce somatic embryos cultured in the presence of the osmoticum, polyethylene glycol (Dong and Dunstan 1996*a*, 1996*b*, Stasolla et al. 2003). Among the 31 marker genes, there are several participating in diverse cellular processes (Table 4), indicating that a large number of regulatory pathways contribute to the formation of the mature embryos.

This work provides detailed information on changes in transcript levels occurring during successive stages of embryo development in gymnosperms. Beside extending our knowledge on the molecular mechanisms governing gymnosperm embryogenesis, as the patterning of embryo formation in vivo is similar to that observed in vitro (Yeung et al. 1998, Filonova et al. 2000*a*), this work may assist in developing methods for the production of high-quality somatic embryos in conifers.

**Acknowledgments**

The authors thank Prof. E.C. Yeung, University of Calgary for valuable suggestions and discussions. The help of Mr. Matias Kirst is also acknowledged. This work was supported by a Natural Sciences and Engineering Research Council of Canada Grant to C.S., a National Science Foundation (USA) Grant DBI, 9975806 to R.S., and by the NCSU Forest Biotechnology Industrial Research Consortium.

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