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Physiologia Plantarum 127: 535–550. 2006

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Received 31 July 2005; revised 27 November 2005

doi: 10.1111/j.1399-3054.2006.00673.x

Different aspects of androgenesis induction have been studied in detail, but little is known about the molecular mechanisms associated with this developmental switch. We have employed macroarrays containing 1421 expressed sequence tags covering the early stages of barley zygotic embryogenesis to compare the gene expression profiles of stress-induced androgenic microspores with those of uninucleate microspores as they progressed into binucleate stage during pollen development. Principal component analysis defined distinct sets of gene expression profiles that were associated with androgenesis induction and pollen development. During pollen development, uninucleate microspores were characterized by the expression of cell division-related genes and transcripts involved in lipid biosynthesis. Progress into binucleate stage resulted in the significant increase in the level of transcripts associated with starch biosynthesis and energy production. These transcripts were downregulated in androgenic microspores. These results indicate that stress blocks the expression of pollen-related genes. The induction of androgenesis by stress was marked by the upregulation of transcripts involved in sugar and starch hydrolysis, proteolysis, stress response, inhibition of programmed cell death, and signaling. Further expression analysis revealed that the induction of genes encoding alcohol dehydrogenase 3, metalloprotease FtsH, cysteine protease 1 precursor, phytepsin precursor (aspartic protease), and a 26S proteasome regulatory subunit was associated with the androgenic potential of microspores, whereas the induction of transcripts involved in signaling and cytoprotection was associated with stress responses. Taken together, these expression profiles represent 'bio-markers' associated with the androgenic switch in microspores, providing a substantial contribution toward understanding the molecular events underlying stress-induced androgenesis.

Introduction

Pollen development follows a controlled sequence of events that can be divided into two major processes, microsporogenesis and microgametogenesis (Bedinger 1992). In barley, microsporogenesis begins with the meiosis of pollen mother cells and ends with the formation of haploid microspores. The first pollen mitosis marks the initiation of microgametogenesis,

where an asymmetric division gives rise to a condensed generative cell embedded in the large vegetative cytoplasm. Although the generative cell undergoes another mitosis to produce two sperm cells, the vegetative cell becomes arrested in the G_1 phase of the cell cycle and fills up with starch grains and other storage products (McCormick 1993). The events that take place during the transition between microsporogenesis and microgametogenesis represent a critical point in the commitment to the pollen developmental pathway, because a stress treatment applied around the first pollen mitosis is sufficient to switch the microspores to an embryogenic route of development, a process called androgenesis (Touraev et al. 1997). Owing to the haploid nature of pollen cells, androgenesis is a valuable tool to generate double haploids for breeding purposes (Wang et al. 2000). Recent molecular and biochemical approaches have demonstrated that microspore, somatic, and zygotic embryos share the expression of several transcription factors and key regulatory proteins (Perry et al. 1999, Vrienten et al. 1999, Baudino et al. 2001, Boutilier et al. 2002). Androgenesis represents, in this context, a convenient model system to address questions concerning plant embryogenesis (Matthys-Rochon 2002). For both applied and fundamental research, it is of uttermost importance to understand how a highly specialized cell such as the developing pollen grain can be reprogrammed to become embryogenic.

Several types of stress treatments are known to efficiently trigger androgenesis. They usually consist of subjecting whole plants in vivo or tillers, buds, anthers, and isolated microspores in vitro to carbon and nitrogen starvation, heat, cold, or osmotic shock (Touraev et al. 1997). Upon stress, the microspores enlarge and the cytoplasm is characterized by the lack of starch and lipid accumulation, the presence of organelle-free regions in the cytoplasm, and an overall decrease in the number of ribosomes (Rashid et al. 1982, Hoekstra et al. 1992, Telmer et al. 1995, Maraschin et al. 2005). The nucleus re-enters the cell cycle as DNA replication takes place during stress treatment (Touraev et al. 1996). Both the de-repression of cell cycle arrest and the inhibition of pollen differentiation have been pointed out as important features of androgenesis induction (Touraev et al. 1997). At the molecular level, the induction of several genes marks the reprogramming of microspore toward the androgenic pathway. Stress proteins, such as members of the chaperone family of heat shock proteins (HSPs), are induced in rapeseed (*Brassica napus* L.) and tobacco (*Nicotiana* spp.) microspores upon heat shock, starvation, and colchicine (Zarsky et al. 1995, Smykal and Pechan 2000). In wheat (*Triticum aestivum* L.), an ABA-responsive, early cysteine-labeled class II metallothionein gene has been identified as a marker for acquisition of embryogenic potential, suggesting a role for ABA in androgenesis induction (Reynolds and Crawford 1996). In rapeseed, androgenesis-related marker genes were first identified in heat-stressed microspores and encode a subfamily of napin genes (Boutilier et al. 1994). It is also from rapeseed microspores that the *BABY BOOM* (*BBM*) gene, a member of the AP2/ERF family of transcription factors, has been identified by Boutilier et al. (2002). Its ectopic expression in *Arabidopsis* and rapeseed suggests that *BBM* plays a role in vegetative-to-embryonic transition, being the first androgenic-related marker to date to exhibit a putative function in microspore embryogenesis induction. However, holistic approaches to characterize the stress-induced gene expression programs during androgenesis induction have not yet been explored, and there is nearly no information available on the transcriptome associated with this developmental switch.

With the recent development of high-throughput techniques allowing the expression analysis of thousands of expressed sequence tags (ESTs), the analysis of complex networks governing developmental and metabolic processes has become possible (Lee et al. 2002). In an attempt to identify gene expression profiles associated with androgenesis induction in barley (*Hordeum vulgare* L.), macroarrays containing 1421 barley ESTs isolated from a cDNA library covering the first 15 days of seed development were used (Michalek et al. 2002, Potokina et al. 2002, Sreenivasuslu et al. 2002). Efficient androgenesis in barley is induced by a combination of starvation and osmotic stress, which is achieved via a mannitol treatment of anthers containing microspores at the mid-late to late (ML-L) uninucleate stage, just prior to the first pollen mitosis (Hoekstra et al. 1992). Following mannitol stress treatment, embryogenic potential is displayed by a population of highly vacuolated, enlarged cells which can be isolated by means of a sucrose gradient (Maraschin et al. 2003). Principle component analysis (PCA) based on array expression data revealed the gene expression profiles that were associated with normal pollen development as ML-L microspores developed into binucleate pollen and their reprogramming during androgenesis induction. Our results provide a comprehensive overview of the molecular events unfolding in the microspores during their reprogramming and identify cellular processes that have never been so far described in the context of androgenesis.

Materials and methods

Plant material, androgenesis induction, and microspore isolation

Donor plants of barley (*H. vulgare* L. cv Igri, Landbouw Bureau Wiersum, the Netherlands) were grown in a phytotron under conditions described previously (Hoekstra et al. 1992). For array experiments, three microspore populations representing different developmental stages were assayed: (1) ML-L uninucleate microspores, (2) early to mid-binucleate pollen, and (3) enlarged microspores after androgenesis induction. For the isolation of developmental stages 1 and 2, microspores were separated from the anther tissues by gently blending anthers in 8.5% (w/v) maltose solution. The microspore suspensions were then sieved through appropriate nylon meshes for the collection of microspores as previously described (Maraschin et al. 2003). For isolation of developmental stage 3, anthers containing microspores at the ML-L uninucleate stage were incubated for 4 days in the dark at 25°C in 0.37 *M* mannitol in cell and protoplast washing (CPW) basal salt buffer (440 mOsm kg^{-1}; van Bergen et al. 1999). After androgenesis induction, microspores were separated from the anther tissues by gentle blending (Maraschin et al. 2005), and the enlarged microspore fraction was isolated by a sucrose gradient (Maraschin et al. 2003). Only the fraction composed of viable, enlarged microspores was used for array experiments. Alternatively, two additional pretreatment solutions with different osmolarities were used to induce androgenesis: anther pretreatment in CPW basal salt buffer without the addition of mannitol (50 mOsm kg^{-1}) and anther pretreatment in deionized water alone (0 mOsm kg^{-1}; van Bergen et al. 1999). Following stress treatment, enlarged microspores were isolated as described for developmental stage 3. All microspore samples were immediately frozen in liquid nitrogen after isolation.

Total RNA isolation

Total RNA was isolated from microspore samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions.

Macroarray hybridization and data analysis

For array analysis, polyA + RNA purified from 20 µg of total RNA from developmental stages 1, 2, and 3 was used for the preparation of ^{33}P-labeled cDNA probes as previously described (Sreenivasulu et al. 2002). Radioactive-labeled probes were hybridized onto Nylon membranes containing 1421 ESTs selected from barley cv. Barke cDNA libraries of developing caryopsis 1–15 days after flowering (Michalek et al. 2002, Potokina et al. 2002, Sreenivasuslu et al. 2002). Sequence-related clone information are available at the IPK Website http://www.pgrc.ipk-gatersleben.de. For each microspore stage, two hybridizations were carried out using RNA from biological duples, totalizing six hybridizations. After quantitative removal of the probe, macroarrays were re-used up to three times as described by Sreenivasulu et al. (2004). Radioactive signals on the cDNA macroarrays were detected using a phosphoimager (Fuji BAS, 2000, Fuji Photo Film Co., Ltd, Tokyo, Japan), and the intensities of individual spots and the corresponding local backgrounds were quantified (Array-Vision, Imaging Research, St Catherine's, ON, Canada). The data were exported to a spreadsheet program for further processing. The local background signals were subtracted from spot-signal intensities. Normalization of spot-signal intensities of individual hybridizations was performed based on the total amount of radioactivity bound to the array for each different hybridization. Because each EST was spotted twice per macroarray filter, and two independent filter hybridizations were performed per biological condition, gene filtering was performed based on the average spot-signal intensities of four values obtained per EST ($n = 4$). Only the gene data with averaged spot-signal intensities equal or greater than five times the average background and a standard error (standard deviation/average spot-signal intensity) smaller than 0.3 in at least one of the six hybridizations were further analyzed.

Principle component analysis

The expression data of the ESTs which showed signals above background level were analyzed by PCA (Matlab version 6, The MathWorks, Inc., Natick, MA; PLS toolbox version 2, Eigenvector Research, Inc., Manson, WA). For PCA, mean centering and level scaling were used to normalize the average spot-signal intensities of the ESTs. Level scaling has been chosen so that up- or downregulations of similar relative level will get similar weight in the PCA model. The frequency distribution of the ESTs according to the sum of the square root of the average loadings from PC1 and PC2 (distance to origin on the loading plot) was used to identify the ESTs which were differentially expressed. The average sample scores of PC1 and PC2 were used to calculate the optimal orientation of the samples on the two-dimensional plots. The loading plot was subsequently divided in six areas defined by the average between the sample optimal orientations. These criteria were

used to assign the ESTs comprised in each area to six different groups. The derived vector components (factor spectra) of the sample loadings of PC1 oriented in the optimal direction of developmental stages 1, 2, and 3 were used to quantify the relative contribution of the ESTs to each of the developmental stages. The expression dynamics of each EST was calculated as a ratio between the maximum and the minimum average spot-signal intensities within the three populations.

cDNA annotation and functional classification of genes

For annotation and functional classification, the sequences of the differentially expressed ESTs were compared with the SwissProt database (Apweiler et al. 2004) using BlastX (Altschul et al. 1997). Protein hits in the SwissProt database with e-value equal or greater than E-15 were classified according to their putative functions.

Northern blot analysis

Ten micrograms of total RNA sample was separated electrophoretically along with a RNA marker (GiboBRL, Gaithersburg, MD) in a 1.5% (w/v) agarose gel containing 2% (v/v) formaldehyde, 20 m*M* 3-(*N*-morpholino) propanesulphonic acid, 8 m*M* sodium acetate, 10 m*M* EDTA, pH 7.0, blotted onto nitrocellulose membranes and hybridized with ^{32}P-labeled cDNA probes as described previously (Menke et al. 1999). The cDNA clones corresponding to the ESTs HY10G20, HY08C24, HY06G09, HY06J08, HY01C15, HY01O02, HY10N13, HY01B24, HK03G06, HY10O06, HK04B02, HW01H17, HY03O15, HW01K08, and HY06J20 were used as probes. The corresponding cDNA inserts were excised from pBK-CMV plasmids by enzymatic digestion with *Bam*HI/*Xho*I and purified from agarose gels using Qiaquick Gel Extraction kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. cDNA probes were labeled using Rediprime II kit and purified using Microspin S-200 columns (Amersham Biosciences, Piscataway, NJ) according to manufacturer's instructions.

Results and discussion

Classification of microspore developmental stages

Three cell populations representing defined developmental stages during pollen and androgenic development were assayed for array analysis: ML-L uninucleate microspores, binucleate pollen, and enlarged microspores after androgenesis induction (Fig. 1, stages 1–3). ML-L uninucleate microspores represent the responsive stage for androgenesis induction. Morphologically, they are characterized by a large central vacuole and an undifferentiated cytoplasm, being at the verge of mitosis (Fig. 1, stage 1). Binucleate pollen represents the stage when ML-L microspores further develop for 4 days in the mother plant, being characterized by a small generative cell embedded in the starch-rich cytoplasm of the vegetative cell (Fig. 1, stage 2). Enlarged microspores represent the stage when ML-L microspores are treated in 0.37 *M* mannitol solution for 4 days (Fig. 1, stage 3). This stress treatment is able to switch 50% of the enlarged microspores toward the androgenic pathway, as indicated by the formation of star-like microspores and multicellular structures. Nevertheless, half of the enlarged microspores are not induced to undergo androgenesis, as they accumulate starch and eventually die in culture (Fig. 1; Maraschin et al. 2005). Owing to the heterogeneity of the microspore population at stage 3, comparisons between the gene expression profiles in the transition from stage 1 to 2 (Fig. 1, bold arrow) and between stages 1 and 3 (Fig. 1, open arrow) were needed in order to distinguish genes related to pollen and androgenic development.

PCA of gene expression in microspores at different developmental stages

polyA + RNA isolated from stages 1, 2, and 3 was used to synthesize ^{33}P-labeled cDNA probes. ^{33}P-labeled cDNA probes were hybridized to macroarrays containing 1421 barley ESTs (Michalek et al. 2002, Sreenivasuslu et al. 2002). From the 1421 ESTs, 509 ESTs showed signal above background level, from which 418 displayed a standard error smaller than 0.3. The complete data set from six hybridizations comprising the normalized signal intensities from the 418 ESTs was subjected to PCA. The six hybridizations resulted in up to six principle components (PCs) that describe the total variance in the gene expression data. The first PCs are the most important, as the amount of variance captured decreases with an increasing PC number. Each PC is based on a data trend or 'gene expression profile' that is expressed in the loading vector of that PC. For each sample, a score is assigned by the algorithm for this data trend, representing how important that the profile of ESTs is to that respective sample. Differences between samples can therefore be visualized by plotting the scores, whereas the underlying data trends can be shown in the loading plots.

Fig. 2A reveals the major differences between the gene expression data from individual hybridizations. This score plot indicates that the first two components

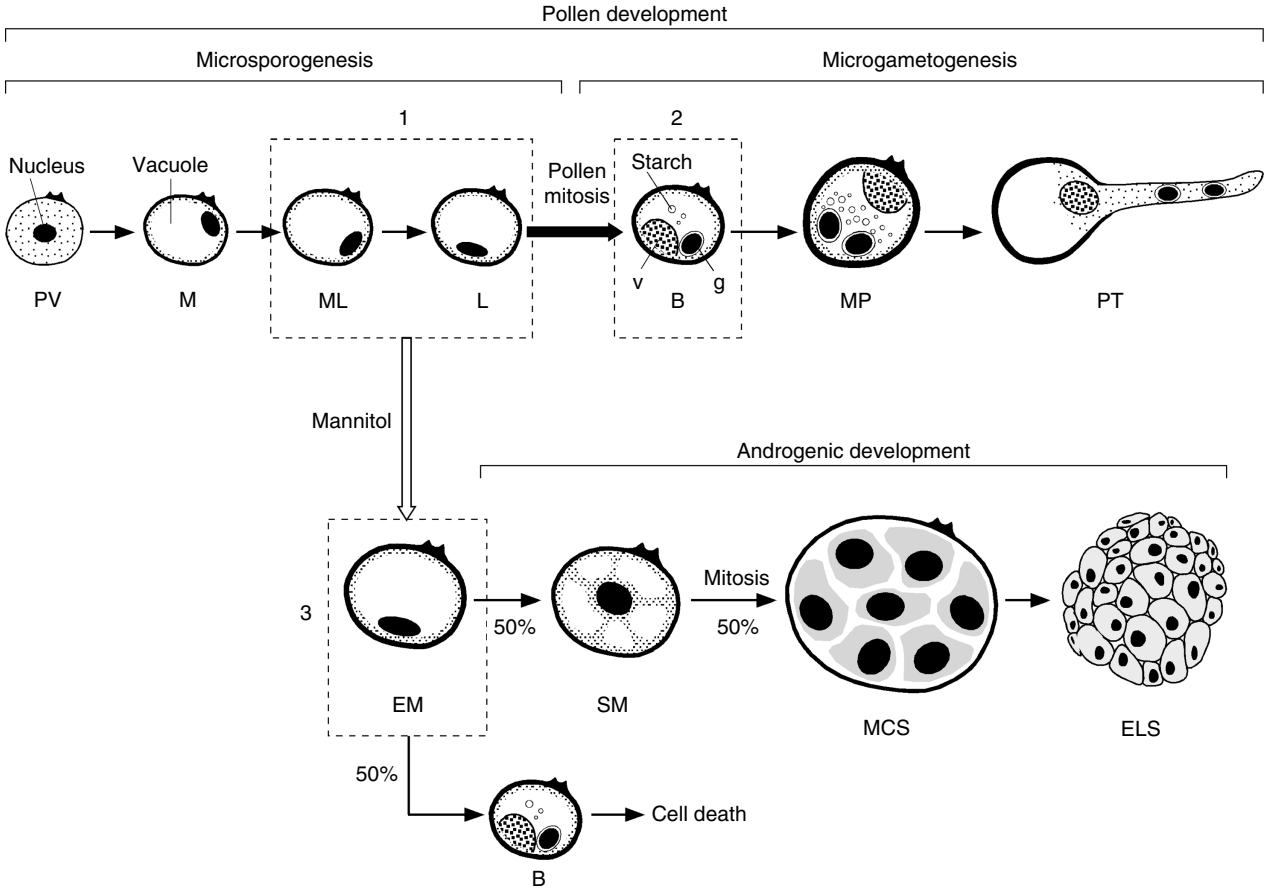

Fig. 1. Pollen and mannitol-induced androgenic development in barley. Developmental stages contained by boxes were assayed for array analysis: (1) ML-L uninucleate microspores, (2) early mid-binucleate pollen, and (3) enlarged microspores after mannitol treatment. The relative conversion of enlarged microspores into star-like and multicellular structures, as well as the frequency of enlarged microspores that are still committed to the pollen developmental pathway, is indicated (Maraschin et al. 2005). Bold arrows indicate transition between stages 1 and 2, whereas open arrows indicate transition between stages 1 and 3. B, binucleate pollen; ELS, embryo-like structure; EM, enlarged microspores; L, late uninucleate microspore; M, mid-uninucleate microspore; MCS, multicellular structure; ML, mid-late uninucleate microspore; MP, mature tricellular pollen; PT, pollen tube growth; PV, prevacuolate microspore; SM, star-like microspore.

of the expression data (PC1 and PC2) corresponded to the differences in the stage and in the developmental pathway of the cells, respectively, and together accounted for 82% of the variances found. The relative distances between the biological duples and between stages 1, 2, and 3 illustrate that the variances between biological duples were relatively small compared with the variances between developmental stages, indicating a high reproducibility between biological duples. We interpret the PCA results as an indicative of reprogramming of gene expression during the commitment of ML-L uninucleate microspores to the pollen developmental pathway (transitions 1–2) or their switch toward the androgenic route (transitions 1–3).

To reveal which ESTs were primarily responsible for the variances found between the three developmental stages, the 418 ESTs were analyzed based on their average PC1 and PC2 loadings (Fig. 2B). This plot illustrates the contribution of each of the 418 ESTs to the differences between the three developmental stages. In the loading plot, EST distance and orientation from the origin of the plot can be regarded as indicatives for the degree of correlation of each EST to one of the three developmental stages according to their optimal directions as deduced from the sample scores (Fig. 2A, B). To reduce the amount of weakly correlated genes, ESTs most close to the origin were discarded (Fig. 2B, outlined by circle). This was performed by creating a histogram of the distribution of the ESTs according to their distance to origin in the loading plot (Fig. 2C). Because ESTs that were 0.07 units far from the origin were observed more frequently than it would be expected

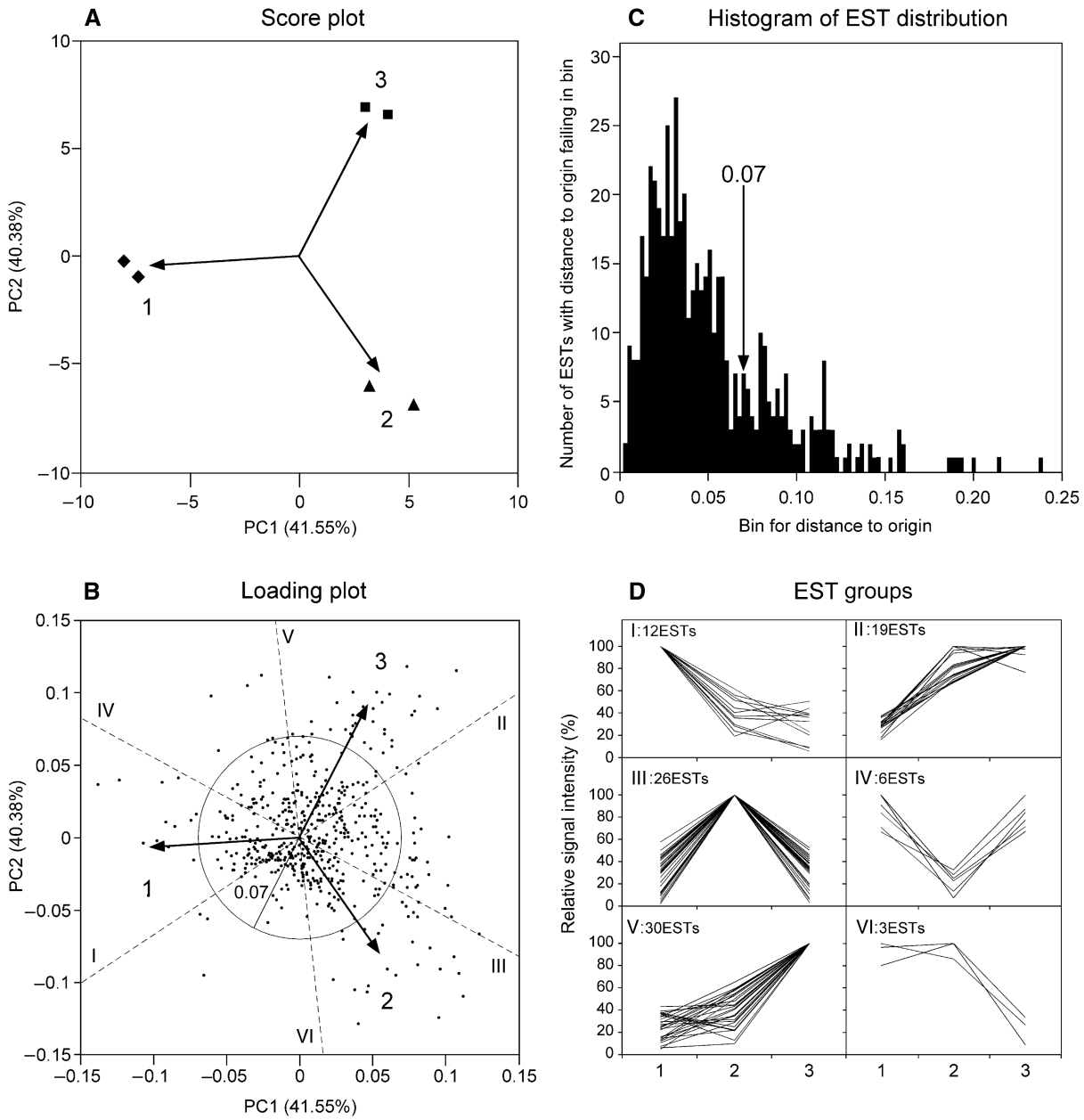

Fig. 2. Principle component analysis of gene expression in microspores at different developmental stages. (A) Score plot based on gene expression data sets of three developmental stages: (1) ML-L uninucleate microspores, (2) early mid-binucleate pollen, and (3) enlarged microspores after mannitol treatment. Biological duples are represented for each developmental stage. Arrows pointing to 1, 2, and 3 indicate the optimal vector orientation of the three developmental stages based on the average PC1 and PC2 scores between duple hybridizations. (B) Loading plot displaying the 418 individual expressed sequence tags (ESTs) that contributed to the variances between different developmental stages. The ESTs displaying distance to origin greater or equal to 0.07 were considered to be differentially expressed (96 ESTs, out of the circle). Dashed lines indicate the average distance between the optimal orientation of the different cell populations (indicated by arrows) and were used to divide the loading plot into six areas. (C) Histogram of the ESTs distribution according to their distance to origin in the loading plot. (D) Clustering of the 96 differentially expressed ESTs into six different groups representing different expression profiles.

based on the histogram shape for a normal distribution, they were considered to be differentially expressed between the three developmental stages. The reduced data set was composed of 96 ESTs, whose expression dynamics varied by a minimum of 2.0-fold and a maximum of 52-fold. The loading plot was subsequently divided in six areas defined by the average between the sample optimal orientations (Fig. 2B, dashed lines).

These criteria were used to group the ESTs comprised in each of the six areas of the loading plot. The ESTs grouped in the same area showed a similar expression profile (Fig. 2D, I–VI). This indicates that the position of the ESTs in the loading plot correlates with their expression profiles. The ESTs plotted in the positive orientation of developmental stages 1, 2, and 3 were specifically induced in these cell populations (Fig. 2B, D, I, III, V), whereas the ESTs plotted in the opposite direction were downregulated (Fig. 2B, D, II, IV, VI). The relative correlation of each EST to a given group was quantified with the factor spectra of the derivative of the loading of PC1 oriented in the optimal directions of stages 1, 2, and 3 (data not shown). These factor spectra were used to list the ESTs in descending order according to their degree of relevance to each group as summarized in Table 1, along with their putative functions based on BlastX hits in the SwissProt database and their expression dynamics between the three developmental stages.

Gene expression profiles associated with ML-L microspores

In total, 12 ESTs were higher expressed in stage 1 as compared with stages 2 and 3 (Fig. 2D, I; Table 1, I). These ESTs correspond to the class of early genes (Mascarenhas 1990), which are preferentially expressed in uninucleate microspores prior to the first pollen mitosis. Based on their putative functions, the ESTs in group I were involved with two main processes: synthetic activity prior to the first pollen mitosis and cell division.

Synthetic activity in stage 1 is evidenced by the expression of ESTs homologous to nucleoside diphosphate kinase I (NDK I), fibrillarin, peroxisomal multifunctional enzyme type II, and cytochrome b5 (Table 1, I). NDK I is involved in the biosynthetic pathway of nucleotides. This kinase is responsible for the phosphorylation of nucleosides into nucleotides, which are building blocks for several primary and secondary products and nucleic acids and serve as an energy source. In cell-culture systems, an increase in nucleotide biosynthesis is a prerequisite for the initiation of cell division, where intense DNA and RNA synthesis takes place (Stasolla et al. 2003). Fibrillarin is a component of a small nuclear ribonucleoprotein particle (snRNP) localized in coiled bodies, which are nuclear subdomains thought to participate in pre-rRNA processing machineries. rRNA genes are known to be actively transcribed in microspores prior to pollen mitosis (Mascarenhas 1990), indicating that the expression of fibrillarin may be related to pre-rRNA processing in stage 1. Peroxisomal multifunctional enzyme type II and cytochrome b5 function in lipid biosynthesis by their association with fatty acid beta-oxidation and desaturation, respectively (Smith et al. 1992). An isoform of cytochrome b5 has been reported to be induced in anther tapetum and in microspores at the verge of mitosis (Martsinkovskaya et al. 1999). After the first pollen mitosis, pollen cells start to accumulate significant amounts of storage products (Bedinger 1992), and the cytoplasm of barley binucleate pollen is characterized by the presence of several lipid bodies (Huang 1986). Our results point out that genes involved with lipid biosynthesis are expressed prior to the first pollen mitosis in barley, thus preceding the stage of intensive lipid biosynthesis. Stage 1 was further characterized by the expression of an EST homologous to a centromere/microtubule binding protein (CBF 5; Table 1, I), a gene that is involved in mitotic chromosome segregation (Winkler et al. 1998).

Dynamic of gene expression profiles associated with pollen development

Based on PCA, ESTs associated with pollen development (transitions 1–2) were represented by the ESTs that were induced specifically in stage 2 (Fig. 2D, III), as well as by those specifically downregulated in 3, indicating that the latter were probably important for the transitions 1–2, but not for stage 3 (Fig. 2D, VI).

Group VI was composed of three ESTs that encoded cobalamin-independent methionine synthase, rubisco subunit binding protein, and a protein with no significant homology (Table 1, VI). On the other hand, group III was composed of 26 ESTs (Table 1, III). Based on the putative functions and on the factor spectra of the ESTs in group III, the induction of an EST coding for inositol-3 phosphate synthase (IPS; 16-fold) and of ESTs involved with carbohydrate and energy metabolism were highly correlated with the stage 2. Most of the ESTs associated with carbohydrate metabolism encode enzymes involved with sucrose and starch biosynthesis: sucrose synthase 1 (SS1), phosphoglucomutase (PGM), UDP-glucose 4-epimerase, glucose-1-phosphate adenylystransferase (AGPase B), UTP-glucose-1-phosphate uridylyltransferase (UGPase), and granule-bound starch synthase 1 (GBSS1). Our results are in agreement with Datta et al. (2001, 2002), whose findings indicate that the major transcriptional changes after the first pollen mitosis are the upregulation of genes involved in the biosynthesis of starch. ESTs involved in energy production were represented by phosphoglycerate kinase, ubiquinol-cytochrome c reductase, cytochrome c oxidase, and ATP synthase (Table 1, III). Although the first is involved in glycolysis, the others are part of the five protein complexes that are involved with electron transport and oxidative phosphorylation. These results

Table 1. List of the 96 expressed sequence tags (ESTs) differentially expressed between the three microspore developmental stages. ESTs are listed in descending order according to their relative contribution to the EST groups (I–VI) as determined by the factor spectra of the derived vector component of PC1 loading oriented in the optimal direction of stages 1, 2, and 3. [a]EST from the 5′ or 3′ of the clone. [b]Information about EST-ID can be found at the IPK Website (http://www.pgrc.ipk-gatersleben.de). [c]Similarity between ESTs and hit proteins was considered significant when the BlastX e-value was equal or greater than E-15. [d]LM, lipid metabolism; MCL, metabolism of complex lipids, AAM, amino acid metabolism; CM, carbohydrate metabolism; MCC, metabolism of complex carbohydrates; NM, nucleotide metabolism; EM, energy metabolism; BSM, biosynthesis of secondary metabolites. [e]Dy, dynamics of EST expression between developmental stages 1, 2, and 3. *Number of ESTs showing homology to the same hit proteins.

GenBank accession number[a]	IPK EST ID[b]	e-Value and BlastX homology[c]	Putative functions[d]	Dy[e]
Group I				
AL510785	HY05P23	9e-15 Peroxisomal multifunctional enzyme type II	LM	11
AL511296	HY07J18	5e-69 Nucleoside diphosphate kinase I, NDK I**	NM	16
AL503494	HW02F11	2e-55 Vacuolar invertase, VI	CM, MCC	3.3
AL510698	HY05L18	4e-65 Cytochrome b5	LM	5.2
AL512102	HY10G13	3e-51 20-kDa chaperonin	Cochaperone	4.8
AL506360	HY02N20	2e-58 Centromere/microtubule binding protein, CBF5	Cell division	3.1
AL512252	HY10O19	5e-27 Fibrillarin	rRNA processing	2.7
AL511960	HY09O09	6e-16 PPLZ12 protein	Unknown	2.7
AL511726	HY09A06	No significant homology	Unknown	2.5
AL507915	HY07D14	5e-53 Glutamate dehydrogenase	EM, AAM	2.6
AL510697	HY05L17	No significant homology	Unknown	2.5
Group II				
AL510870	HY06E02	No significant homology	Unknown	6
AL507111	HY05I10	e-44 Probable ATP synthase 24-kDa subunit	EM	6.6
AL511334	HY07L16	No significant homology	Unknown	4.6
AL506101	HY02A11	e-22 ATP synthase delta chain	EM	4.8
AL510976	HY06J15	e-109 Glutamate decarboxylase	AAM, CM	3.6
AL511258	HY07H20	No significant homology	Unknown	3.6
AL511115	HY07A18	2e-22 C-4 methyl sterol oxidase	Unclassified	3.6
AL506696	HY03P14	5e-47 Dehydrogenase/reductase SDR family member 4	MCL	3.4
AL506640	HY03M17	6e-57 S-adenosylmethionine decarboxylase proenzyme	AAM	3.6
AL510968	HY06J04	No significant homology	Unknown	3.7
AL509234	HY01C04	No significant homology	Unknown	3.7
AL499671	HK01I16	No significant homology	Unknown	3.3
AL506048	HY01L10	3e-40 Elongation factor-1 alpha	Protein synthesis	3.7
AL511059	HY06N18	e-101 14-3-3-like protein A (14–3-3A)	Protein interaction	3.2
AL506065	HY01M23	8e-96 l-ascorbate peroxidase	CM	3.5
AL506970	HY04M13	e-20 Transmembrane protein PFT27	Unclassified	3.4
AL507833	HY06P18	3e-45 DnaJ-like protein	Unclassified	2.7
AL506613	HY03L07	No significant homology	Unknown	2.7
Group III				
AL511500	HY08E09	2e-89 Inositol-3-phosphate synthase IPS	MCL, BSM	16
AL503638	HW02O11	3e-17 UDP-glucose 4-epimerase	NM, MCC	6.7
AL511879	HY09J04	e-68 ATP synthase beta chain	EM	13
AL508462	HY08N11	2e-74 Gluc 1-phosphate adenylyltransferase, AGPaseB***	MCC	9.4
AL506807	HY04E21	e-75 Actin 1	Cytoskeleton	8
AL511872	HY09I18	No significant homology	Unknown	3.7
AL511706	HY08P05	9e-97 ADP/ATP carrier protein**	EM	4.5
AL506567	HY03I21	e-100 Granule-bound starch synthase I, GBSS1	MCC	3.4
AL511708	HY08P09	e-110 Sucrose synthase 1, SS1	MCC	7.7
AL507123	HY05I22	6e-64 Ferritin 1	Iron storage	4.3
AL511309	HY07K08	7e-16 Tyramine *N*-feruloyltransferase	Unclassified	4.4
AL511268	HY07I08	e-23 Cytochrome c oxidase	EM	4
AL502305	HW07C09	6e-15 UTP-gluc-1-phosphate uridylyltransferase, UGPase	CM, MCC, NM	2.6
AL511218	HY07F23	e-102 Phosphoglycerate kinase	EM, CM	4
AL506698	HY03P16	4e-97 Phosphoglucomutase, PGM	MCC, CM, BSM	2.7
AL511357	HY07M21	No significant homology	Unknown	3.5
AL508466	HY08N15	6e-34 RAC-like GTP binding protein RHO1	Signaling	3.1

Table 1. Continued

GenBank accession number[a]	IPK EST ID[b]	e-Value and BlastX homology[c]	Putative functions[d]	Dy[e]
AL507096	HY05G15	e-113 Phospho-2-dehydro-3-deoxyheptonate aldolase 1	AAM	2.6
AL512172	HY10K07	7e-57 Guanine nucleotide-binding protein alpha-1 subunit	Signaling	2.9
AL510976	HY05M10	9e-55 Glutamate decarboxylase 1	AAM, CM	2.8
AL508740	HY09K13	e-81 Cytosolic monodehydroascorbate reductase	CM	2.8
AL506589	HY03J22	2e-81 Ubiquinol cytochrome c reductase**	EM	2.6
Group IV				
AL507141	HY05K18	e-40 Glutathione *S*-transferase, GST	Stress response	3.7
AL507411	HY05H18	2e-82 Alpha glucosidase precursor, maltase	CM, MCC	2.9
AL507431	HY05J18	2e-83 Ubiquitin-specific protease, UBP	Proteolysis	2.7
AL506437	HY03C01	7e-88 Catalase 1	Stress response	2.7
AL511519	HY08F08	8e-38 Hypothetical protein At1g60740	Unknown	2.7
AL508576	HY09C18	6e-82 Ubiquitin-conjugating enzyme, Ub-E2	Proteolysis	2.6
Group V				
AL507319	HY01O02	e-102 Alcohol dehydrogenase 3, ADH3	LM, MCL, AAM	52
AL503324	HW01K08	e-40 Glutathione *S*-transferase, GST	Stress response	18
AL505972	HY01C15	4e-75 Glutathione *S*-transferase 1, GST class-phi	Stress response	19
AL510913	HY06G09	No significant homology	Unknown	5.3
AL499908	HK04B02	No significant homology	Unknown	5.7
AL511473	HY08C24	3e-53 Bax inhibitor-1 (BI-1)**	PCD inhibitor	3.9
AL507702	HY06J20	e-112 Glyceraldehyde-3-phosphate dehydrogenase, GAPD	EM, CM	3.3
AL510971	HY06J08	e-104 20S proteasome subunit alpha-5	Proteolysis	3.5
AL512108	HY10G20	e-105 Ras-related protein RIC2	Signaling	4.9
AL509162	HY10O06	e-66 Phytepsin precursor (aspartic protease)	Proteolysis	3.3
AL511054	HY06N11	8e-53 Fructose-bisphosphate aldolase	EM, CM	2.9
AL505964	HY01B24	2e-18 26S proteasome regulatory subunit 8	Proteolysis	3.2
AL499780	HK03G06	2e-30 Cysteine protease 1 precursor**	Proteolysis	3.4
AL511847	HY09H06	e-31 20S proteasome subunit alpha-2	Proteolysis	2.6
AL509296	HY01F11	No significant homology	Unknown	2.6
AL503287	HW01H17	2e-56 Cell wall invertase, CWI	CM, MCC	3.5
AL510859	HY06D13	3e-24 Tryptophanyl-tRNA synthetase	AAM	2.5
AL507218	HY01D11	6e-46 60S ribosomal protein L26A	Protein synthesis	2.4
AL510656	HY05J22	No significant homology	Unknown	2.3
AL511801	HY09E20	No significant homology	Unknown	2.6
AL510614	HY05H16	No significant homology	Unknown	2.5
AL512228	HY10N13	e-90 Filamentous temperature-sensitive protein, FtsH	Proteolysis	3.1
AL506676	HY03O15	4e-86 Dihydrodipicolinate synthase 2	AAM	3.3
AL506034	HY01J20	e-116 Hypothetical protein yiiG	Unknown	2.6
AL508862	HY10A14	e-76 Phospholipid hydroperoxide glutathione peroxidase	Stress response	2
AL511543	HY08G13	8e-42 TGF beta-inducible nuclear protein 1	Unclassified	2.7
AL509039	HY10I17	e-54 Hypothetical UPF0204 protein At2g03800	Unknown	2.6
AL508611	HY09E11	No significant homology	Unknown	2.3
Group VI				
AL506629	HY03M03	No significant homology	Unknown	13
AL507142	HY05K19	2E-97 Cobalamin-independent methionine synthase	AAM	3.9
AL511072	HY06O11	3e-78 Rubisco subunit-binding protein	Unclassified	3

indicate that the induction of transcripts involved in electron transport and oxidative phosphorylation might be needed to match the demand of ATP for starch biosynthesis and to fulfill the role of the vegetative cell as a 'powerhouse' to drive further pollen maturation and pollen tube germination (McCormick 1993). This is further evidenced by the induction of two ESTs coding for an ADP/ATP carrier protein in stage 2 (Table 1, III).

Overlapping gene expression profiles in pollen and androgenic development

Group II was represented by 19 ESTs that were induced both in the transition from stages 1–3 and in the transitions 1–2 (Fig. 2D, II). These ESTs represent therefore the gene expression profiles that are common to both pollen development and androgenesis induction. ESTs within this group were mainly involved in oxidative

phosphorylation and energy production, amino acid metabolism, carbohydrate metabolism, metabolism of complex lipids, and protein biosynthesis (Table 1, II). Because a considerable amount of stage 3 microspores still display the characteristics of stage 2 during the initial stages of microspore culture (Maraschin et al. 2005; Fig. 1), it is likely to assume that these ESTs were induced in those microspores that were still committed to the pollen developmental pathway. Therefore, they may represent ESTs that are associated with pollen development rather than with the acquisition of androgenic potential. However, one cannot exclude the possibility that the induction of these ESTs is needed for both pollen development and the induction of androgenesis.

Dynamics of gene expression profiles associated with androgenesis induction

Based on PCA, ESTs associated with androgenesis induction (transitions 1–3) were represented by the ESTs that were induced in stage 3 (Fig. 2D, V), as well as by those ESTs that were specifically downregulated in stage 2 (Fig. 2D, IV).

The six ESTs included in group IV are of particular interest because their induction was not important for pollen development. Therefore, they represent putative candidates for playing a role in androgenesis induction. They encode glutathione *S*-transferase (GST), alpha-glucosidase precursor (maltase), ubiquitin-specific protease (UBP), catalase 1, ubiquitin-conjugating enzyme (Ub-E2), and a hypothetical protein (Table 1, IV). Maltase is the enzyme responsible for the breakdown of maltose in starch granules (Tibbot et al. 1998). This suggests that the breakdown of storage products, such as starch, might be an important feature of androgenesis induction. GST and catalase 1 are involved in protecting the cell against the harmful effect of reactive oxygen species (ROS). Because many types of biotic and abiotic stresses are known to cause oxidative stress, it has been proposed that ROS might be the common secondary stress factor responsible for the induction of antioxidant enzymes (Scandalios et al. 1997). The promoter region of many plant *GST* genes contains elements responsive to ROS (Chen and Singh 1999, Garretón et al. 2002). However, in our system, *GST* and *CATALASE 1* were expressed both in stage 1 during microspore development and in stage 3 during the initiation of androgenesis by stress. Therefore, it is likely that these genes are developmentally regulated in microspores. In agreement with this hypothesis, *CATALASE* and *GST* genes have been reported to be spatially and temporally regulated during plant development (Marrs 1996, Bailly et al. 2004).

The ESTs coding for Ub-E2 and UBP (Table 1, IV) are components of the ubiquitin/26S proteasome proteolytic pathway. Proteins subjected to degradation are marked with ubiquitin tags and are subsequently targeted to the degradative action of the 26S proteasome (Hellmann and Estelle 2004). Ub-E2 functions in the enzymatic cascade involved in the conjugation of ubiquitin to target proteins. On the other hand, UBPs belong to a family of proteins involved in deubiquitination of proteins and therefore have a role in regulating a protein's half-life by reversing the ubiquitin reaction (Smalle and Vierstra 2004). The regulation of the cell cycle involves the ubiquitin-mediated degradation of mitotic cyclins and the control of the half-life of regulatory factors, which are important for mitotic progression (Harper et al. 2002). In plants, Ub-E2 proteins participate in the formation of the anaphase-promoting complex (APC). Mutations affecting *APC* genes in *Arabidopsis* have demonstrated that the APC is essential for cell cycle progression in plants (Blilou et al. 2002, Capron et al. 2003). In our system, higher mRNA levels of Ub-E2 and UBP in stages 1 and 3 suggest that these ESTs might play a role in stress-induced microspore division by controlling their re-entry into mitosis.

The 30 ESTs comprised in group V represented the most interesting ESTs, because they were upregulated exclusively during androgenesis induction (Table 1, V). Therefore, their expression profiles can be regarded as molecular markers for the induction of androgenesis in barley. Based on their factor spectra, the sharp induction of *ALCOHOL DEHYDROGENASE 3* (*ADH 3*; 52-fold) and two *GST* genes (18/19-fold) showed the highest correlation with the stage 3. Other gene expression profiles correlated with this stage were associated with the inhibition of programmed cell death (PCD; bax inhibitor, BI-1), ubiquitin-mediated proteolysis, and protein degradation (filamentous temperature-sensitive protein, FtsH; 20S proteasome subunit alpha-5 and alpha-2; 26S proteasome regulatory subunit 8; cysteine protease 1; and phytepsin precursor), and carbohydrate metabolism (glyceraldehyde-3-phosphate dehydrogenase, GAPD; cell wall invertase, CWI; and fructose-biphosphate aldolase). In addition, stage 3 was characterized by the induction of seven ESTs, which encoded proteins with no known homologies, along with ESTs involved in various functions, such as Ras-related protein RIC2, TGF beta-inducible nuclear protein 1, tryptophanyl-tRNA synthetase, 60S ribosomal protein L26A, and dihydripicolinate synthase 2.

Validation of array data

Because the ESTs grouped in group V showed the most interesting expression profiles with regard to

androgenesis induction, we wanted to validate their expression profiles obtained by macroarrays using Northern blot analysis. To do so, the expression of the 15 ESTs that were induced in stage 3 by a minimum of three-fold (Table 1, V) was further analyzed. Fig. 3 illustrates the expression of 10 of the 15 most dynamic ESTs. These results show that all 10 ESTs representing BI-1, RIC2, ADH3, FtsH, phi-GST, cysteine protease 1 precursor, phytepsin precursor, 20S proteasome subunit alpha-5, 26S proteasome regulatory subunit 8, and clone HY06G09 were induced in stage 3 as compared with stages 1 and 2, indicating that there was a high consistency between the expression profiles obtained by macroarray and Northern blot analysis.

Gene expression associated with microspore androgenic potential

To explore the possibility that these 10 ESTs were associated with the embryogenic potential of enlarged microspores treated by a mannitol stress, we further investigated their expression in enlarged microspore populations that had been treated under optimal and suboptimal conditions for androgenesis induction. Optimal regeneration efficiency in barley (which was set to 100%) is obtained by a combination of starvation and osmotic shock, achieved by an anther treatment in 0.37 *M* mannitol in CPW basal salt buffer. However, starvation alone is sufficient to trigger androgenesis at lower frequencies. The regeneration efficiency drops to 57% of the optimal when mannitol is omitted in the CPW basal salt buffer during stress treatment. The reduction is even more drastic when the CPW salts are not present, and microspores are treated in deionized water alone, resulting in a drop to 37% of the optimal (van Bergen et al. 1999).

Fig. 4 shows that there were mainly two groups of ESTs based on their expression in enlarged microspores subjected to mannitol, CPW, or water treatment. In the first group, upregulation was independent of the regeneration efficiency. The EST HY06G09 and the ESTs encoding the PCD inhibitor BI-1, the small GTPase RIC2 involved in signaling, a class phi-GST, and the subunit alpha-5 of the 20S proteasome belonged to the first group (Fig. 4, I). Taken together, these results indicate that the induction of genes involved in cytoprotection (*GST, BI-1*; Kampranis et al. 2000, Bolduc and Brisson 2002), signal transduction (*RIC2*; Bischoff et al. 1999), and ubiquitin-mediated proteolysis (20S proteasome subunit alpha-5) are probably not related to the acquisition of androgenic potential but rather might play a role in stress signaling and/or stress responses. On the other hand, in the second group of ESTs, the induction

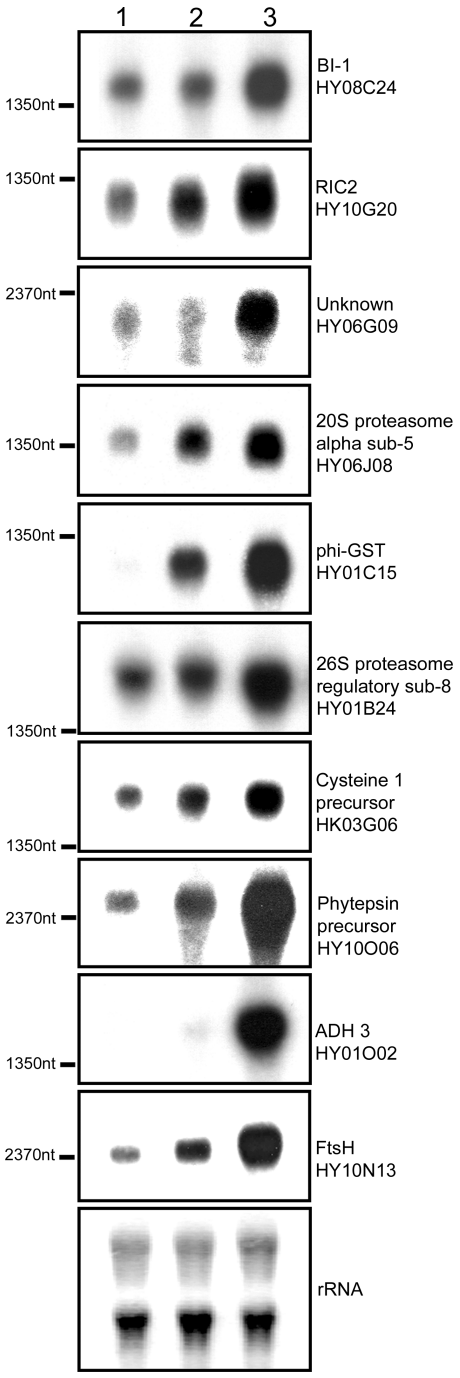

Fig. 3. Validation of expression profiles obtained by macroarray using Northern blot analysis. Expression is shown for 10 expressed sequence tags (ESTs) whose expression was induced in stage 3 as compared with stages 1 and 2 by a minimum of three-fold. Ethidium bromide staining of rRNA shows equal RNA loading. Lane 1, ML-L uninucleate microspores; lane 2, early mid binucleate pollen; lane 3, enlarged microspores after mannitol treatment.

of gene expression was positively correlated with the regeneration efficiency. ESTs in group II are represented by 26S proteasome regulatory subunit 8,

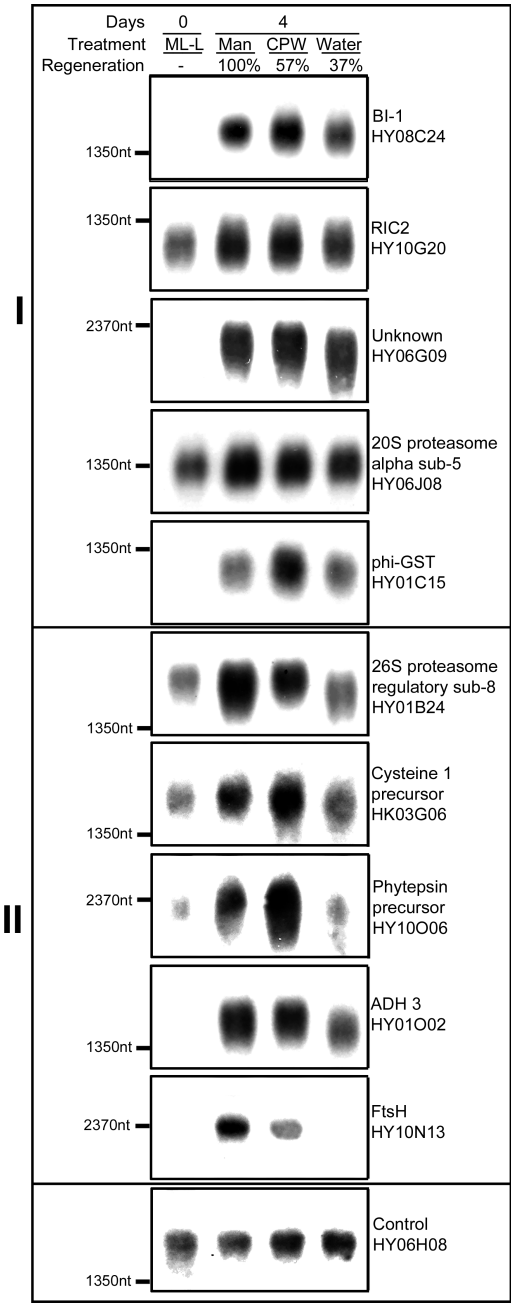

Fig. 4. Northern blot analysis of expressed sequence tags (ESTs) upregulated by mannitol stress under optimal and suboptimal treatments for androgenesis induction. Group I, upregulation upon stress is independent of regeneration efficiency; group II, upregulation shows a positive correlation with regeneration efficiency. The EST HY06H08 was equally expressed between the three developmental stages based on array analysis and was used as a control for RNA loading on Northern blots. ML-L, ML-L uninucleate microspores; Man, microspores treated in 0.37 *M* mannitol in CPW basal salt buffer; CPW, microspores treated in CPW basal salt buffer alone; water, microspores treated in deionized water. The regeneration efficiencies associated with each microspore treatment and the duration of treatment are indicated on each lane (van Bergen et al. 1999).

metalloprotease FtsH, cysteine protease 1 precursor, phytepsin precursor (aspartic protease), and ADH3 (Fig. 4, II). These results indicate that the expression of this second group of genes marks the commitment of microspores to androgenesis and suggests a possible role for protein degradation in the acquisition of androgenic potential.

The 26S proteasome is involved in many different aspects of cellular regulation, including stress and hormonal responses, cell cycle control, nutrient remobilization, and organ differentiation, being composed of the 20S proteasome multicatalytic complex and the 26S regulatory subunits. The ubiquitin/26S proteasome proteolytic pathway is regulated at both the transcriptional and post-translational levels, and one important mechanism of regulation is known to involve the 26S regulatory subunits (Hellmann and Estelle 2004). In our system, although the induction of the subunit alpha-5 of the 20S proteasome was independent of the embryogenic potential (Fig. 4, I), the upregulation of the 26S proteasome regulatory subunit 8 was positively correlated with the high embryogenic potential of mannitol-treated microspores (Fig. 4, II). This regulatory subunit shows homology to the regulatory particle ATPase subunit 2 from rice (*RPT2*). *RPT* genes are known to confer ATP dependence and specificity for ubiquitinated substrates to the 26S proteasome (Fu et al. 1999). This indicates that the regulation of ubiquitin-mediated proteolysis may differ in embryogenic microspores that have been treated by mannitol, CPW, or water stress treatments, pointing out to a role for proteolysis in the acquisition of androgenic potential.

Further evidence to support a role for proteolysis in androgenesis induction is demonstrated by the higher expression of a cysteine and an aspartic protease upon mannitol and CPW treatment as compared with water (Fig. 4, II). Cysteine and aspartic proteases have been implicated in protein maturation, execution of PCD, germination, and tissue remodeling (Beers et al. 2004). The induction of cysteine proteases correlates with somatic embryogenesis induction (Thibaud-Nissen et al. 2003, Mitsuhashi et al. 2004), and several proteases are induced during zygotic embryogenesis (Dong et al. 2004, Sreenivasulu et al. 2004). During androgenesis induction, several studies have reported that one of the main changes brought by stress is represented by an overall decrease in total protein in the microspores (Kyo and Harada 1990, Garrido et al. 1993, Ríhová et al. 1996). Morphologically, stress leads to the marked repression of gametophytic differentiation, characterized by the degradation of the pollen cytoplasm (Dunwell and Sunderland 1975, Rashid et al. 1982). Based on these evidences, it has been postulated that

androgenesis induction may involve the proteolytic degradation of pollen-specific proteins. Our gene expression data represent the first molecular evidence to show that proteolysis may take part of the stress responses leading to the acquisition of microspore embryogenic potential.

The *FtsH* gene encodes a protein that displays both metalloprotease and chaperone activities. In plants, *FtsH* has been demonstrated to be the protease involved in degrading photosystem II reaction center D1 protein upon its irreversible photo-oxidative damage (Lindahl et al. 2000). The *FtsH* EST induced in barley microspores showed homology to the *Arabidopsis FtsH2* isoform, which is needed for the formation of normal, green chloroplasts (Yu et al. 2004). Chloroplast biogenesis is an important factor for the production of green plants derived from microspores. The treatment of barley anthers in mannitol has been reported to induce not only higher regeneration efficiencies, but also higher green/albino ratios among microspore-derived plants (Caredda et al. 1999). The association between higher levels of *FtsH* expression with mannitol-treated microspores indicates that this protein might play a role in chloroplast biogenesis during the induction of barley androgenesis.

In addition, the induction of *ADH3* was correlated to the higher regeneration efficiencies of mannitol- and CPW-treated microspores (Fig. 4, II). ADH catalyzes the reversible reaction of an aldehyde or ketone into alcohol in a NAD-dependent manner. *ADH* expression has been implicated in the shift from the oxidative pathway to a fermentative one, leading to the induction of glycolytic and fermentative genes (Dat et al. 2004). There are three ADH isoforms in barley, which are mainly regulated at the transcriptional level (Hanson et al. 1984). Gene expression of the barley *ADH1* gene has been demonstrated to be induced by ABA (Macnicol and Jacobsen 2001). The promoter of the *ADH1* gene contains sequence motifs that closely resemble the ABA-responsive elements found in the *Arabidopsis ADH* promoter (de Bruxelles et al. 1996). ABA is important for mediating plant tolerance to many environmental stresses, and ABA increases with the level of osmotic stress (Zeevaart and Creelman 1988), being correlated with mannitol-induced androgenesis induction in barley (Wang et al. 1999). Although it is not yet known whether the *ADH3* promoter contains ABA-responsive elements, *ADH3* gene expression was correlated with increasing osmotic stress, indicating that this gene might be an indicative for stress responses associated with the high osmotic pressure because of mannitol treatment in barley androgenesis induction.

Concluding remarks

In this study, macroarrays were used to gain insight into the gene expression profiles active during the stress-induced reprogramming of microspores from pollen toward androgenic development. The main transcriptional changes associated with this developmental switch were represented by the downregulation of genes involved in starch biosynthesis, and the induction of genes involved in sugar and starch hydrolysis, cytoprotection, signal transduction, stress responses, and proteolysis. PCA based on gene expression data has proved to be a useful tool to identify 'bio-markers' for androgenesis induction, which represent multiple gene activities with diverse pathways. Further research should focus on the role of proteolysis in the regulation of stress-induced androgenesis. This issue is attracting increasing attention as an important regulatory mechanism in cell differentiation and cell cycle progression in plant cells (Geschink et al. 1998, Yanagawa et al. 2002, Ahn et al. 2004, Hellmann and Estelle 2004). In addition, the development of microspore stage-specific cDNA arrays will help extending our current knowledge of transcript profiles associated with androgenesis induction.

Acknowledgements – The authors are grateful to Dr Nils Stein for the support and help in facilitating the array hybridizations at the IPK, Germany, Ying Zhang for technical assistance, and Dr Sylvia de Pater for critical reading of the manuscript.

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Edited by V. Hurry