

Transcriptome analysis of barley anthers: effect of mannitol treatment in microspore embryogenesis

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Abstract

Carbohydrate starvation is an efficient stress treatment for induction of microspore embryogenesis. Transcriptome analysis of anthers response to mannitol treatment using the 22k Barley1 GeneChip revealed large changes in gene expression. Statistical analysis and filtering for 4-fold or greater changes resulted in 2,673 genes, of which 887 were up-regulated and 1,786 down-regulated. Great differences in some metabolic pathways, accompanied by a multi-dimensional stress response were found. Analysis of transcription factors showed that most of the down-regulated transcription factors were related to growth and development, and the up-regulated with abiotic and biotic stress responses and changes in developmental programs. Interestingly, the expression of most cell cycle related genes did not change significantly. Transcriptome analysis provided a

successful approach to identify genes involved in mannitol treatment, essential for triggering microspore embryogenesis.

Introduction

Microspore embryogenesis is the most efficient method for production of barley doubled haploid (DH) lines. DH lines are important tools for plant research; reducing the time to release new cultivars in breeding programs, and contributing to the development of genetic linkage maps, quantitative trait loci (QTL) analysis and marker assisted selection (Forster and Thomas 2005).

Microspore embryogenesis is based on the switch of microspores from their normal pollen development towards an embryogenic pathway, which can be induced by different stress treatments. The highest DH frequencies are obtained by temperature shock (cold or heat), carbohydrate starvation, carbohydrate starvation together with nitrogen starvation, or chemical inducers (for example colchicine, hydroxynicotinic acid) (Zorinants et al. 2005). In barley, carbohydrate starvation alone or in combination with cold is the most efficient treatment (Cistué et al. 1999, Kasha et al. 2001). This treatment is based on incubation of anthers in a medium with a non-metabolizable carbohydrate such as mannitol. Carbohydrate starvation occurs naturally along the plant life cycle during environmental changes such as darkness, dormancy and senescence. Extensive metabolic changes result in the recycling of cellular components, activation of mechanisms to prevent severe damage and maintenance of important biochemical pathways (Yu 1999).

Previously, differential gene expression analysis identified several genes putatively involved in early stages of microspore embryogenesis. Some examples are genes encoding an ABA-responsive cysteine-labelled metallothionein (EcMt), an arabinogalactan-like protein (AGP), small heat shock proteins (HSP), a AP2/ERF transcription factor (BABY BOOM), and endosperm-specific proteins ZmAE and ZmAE3 (for review see Maraschin et al. 2005a). Other genes have been associated with the stress treatment in *Nicotiana tabacum* such as a gene encoding phosphoprotein (NtEPc) (Kyo et al. 2000) and a stressed microspore N10 protein (NtSM10) (Hosp et al. 2005).

Few reports have taken a broader approach to the transcriptome during microspore embryogenesis. Boutillier et al. (2005), used an 1800 cDNA macroarray to analyze the transcriptome of heat-stressed Brassica microspore cultures and described induction of genes involved in transcription, chromatin remodeling, protein degradation and signal transduction. As far as we know, only one report has analyzed the transcriptome of barley microspores response to mannitol treatment (Maraschin et al. 2005b). This study described the induction of genes related to sugar and starch hydrolysis, proteolysis, stress response, inhibition of programmed cell death and signaling, and a down-regulation of genes involved in starch biosynthesis and energy production by using a 1,421 cDNA macroarray containing genes from developing caryopsis 1-15 days after flowering. Despite these studies, the mechanisms that control induction of microspore embryogenesis have only been partially explored.

In the present work we analyzed the anther transcriptome before and after four days of mannitol treatment using the 22k Barley1 GeneChip which allows the study of

approximately 22,000 genes (Close et al. 2004). This study provided new insights to the gene expression changes accompanying the reprogramming of microspores from the gametophytic to the sporophytic pathway.

Materials and methods

Genetic materials and growth conditions

The barley (*Hordeum vulgare* L.) doubled haploid line 46 (DH46) was used in this study. This line was obtained by anther culture from a cross between the winter/spring six-rowed cv. Dobra and the winter two-rowed cv. Igri. The anther culture response of DH46 was previously characterized (Chen et al. 2006), having 78.26 % of anthers responding to mannitol treatment, 2,644 divisions per 100 anthers, 18.84 % of embryos per 100 dividing microspore and 97.65% of albino plants. Anthers extracted from the spikes under a stereoscopic microscope were inoculated in a treatment medium containing 0.7 M mannitol, 40 mM CaCl₂, 8 g/l agarose, and kept at 25°C in the dark for 4 days (Cistué et al. 2003). Samples were collected before and after 4 days in mannitol medium. Three samples from each step were harvested and used for microarray analysis.

RNA isolation and array hybridization

Total RNA was isolated using TRIzol Reagent (Gibco BRL), and passed through RNeasy columns (Qiagen) for further clean up. Double-stranded cDNA was synthesized from the poly(A)⁺ mRNA present in the isolated total RNA (8.5 µg total RNA starting

material each sample reaction) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA) and poly (T)-nucleotide primers that contained a sequence recognized by T7 RNA polymerase. The cDNA was used to generate biotin tagged cRNA which was fragmented and hybridized to Affymetrix Barley1 GeneChips followed by washing and staining; these steps were done according to standard protocols (Affymetrix GeneChip[®] Expression Analysis Technical Manual available at www.affymetrix.com). The Barley1 Genechip contains 22,840 probe sets of which most are composed of 11-pairs of 25-mer oligos (Close et al. 2004). Different types of probe sets results from the probe selection process (i) probes in a unique probe set do not cross-hybridize to any other sequences, (ii) probes in a gene family probe set all cross-hybridize to a set of sequences that belong to the same gene family and (iii) probes in a mixed probe set contain at least one probe that cross-hybridizes with other sequences. The Barley1 GeneChip contain, 18,100 unique, 3,600 gene family and 1,100 mixed probe sets. This information can be obtained from the HarvEST database (<http://harvest.ucr.edu>).

Data analysis

Scanned images were analyzed with GCOS 1.2 (Affymetrix, Inc., Santa Clara, CA). Expression estimates was calculated using gcRMA implemented in GeneSpring 7.1 (Silicon Genetics, Redwood City, USA). We used the flags “present” as an indicator of whether or not a gene was expressed. Only probe sets with a present call in all three replicates were considered to be expressed. Statistical analysis was done with t-Test and for multiple testing correction we used the Benjamini and Hochberg algorithm. Analysis was done using false discovery rate (FDR) adjusted p-values of 0.01 as the cut off

followed by filtering for two and four fold or greater changes. The mannitol responsive genes were compared with Venn diagrams to genes expressed statistically significantly higher or exclusively in specific tissues (Supplemental Figure 1). Comparisons were done to expression data from caryopsis 5 days after pollination (DAP), caryopsis 10 DAP, embryo 22 DAP and endosperm (22 DAP) (Druka et al. 2006).

For annotation purposes blastx (e-value cutoff = e^{-10}) data was exported from HarvEST:Barley version 1.32 (<http://harvest.ucr.edu>) (Note from TC: should be using version 1.34 or later for improved annotations). Gene ontology classification of barley unigenes was obtained by transferring annotation data from corresponding Arabidopsis proteins. The Arabidopsis International Resource Gene Ontology (TAIR-GO) web site (<http://www.arabidopsis.org/tools/bulk/go/index.jsp>) and the Munich Information Center for Proteins Sequences *Arabidopsis thaliana* Database (MIPS) (http://mips.gsf.de/proj/funcatDB/search_main_frame.html) were used for functional classification. Transcription factor classification was made also considering additional data files from Honys and Twell (2004).

Results and discussion

Mannitol treatment alters the expression of over 4,300 genes

We used the Barley1 GeneChip to analyze the transcriptome of anthers before and after four days of mannitol treatment. At this stage the anthers are composed of a vascular bundle, a complex anther wall including the tapetum and uninucleate vacuolated microspores. After mannitol treatment, microspores that survive are surrounded by a

degenerated anther wall (Huang and Sunderland 1982). Microarray data from three independent experiments were analyzed to find genes with statistically significant changes (t-test). We found 4,288 probe sets (28.4%) with statistically significant differences when using an FDR adjusted p-value cutoff of 0.01 and by filtering for 2-fold or greater changes. These numbers reflect the large changes on the anther transcriptome associated to mannitol treatment. Due to the high number of genes selected, a more stringent approach with a filtering ≥ 4 fold was adopted. In this case the selection resulted in 2,673 genes (17.6% of the total number of genes), of which 887 were up-regulated and 1,786 down-regulated (Supplemental Table I). The high number of down-regulated genes indicated that differences were mainly due to decrease of gametophytic information.

The distribution in functional categories of the 2,673 mannitol treatment responsive genes based on MIPS Database revealed that forty percent of the genes belonged to the “unclassified proteins” category and that the number of down-regulated genes exceeded up-regulated in a majority of the functional categories, except for “cell cycle” and “protein synthesis” (Fig. 1). Of the classified categories more than 50% of the responsive genes belonged to “Metabolism” and “Energy”, with 38.6% and 13.6% respectively. Four functional classes (transcription, transport facilitation, cell rescue, defense and virulence; biogenesis of cellular components; and defense and virulence) constituted around 40% of the changes (Fig. 1). For further characterization we focused on categories with special relevance to the effect of mannitol, including “Metabolism”, “Energy”, “Cell rescue, defense and virulence”, transcription factors and “Cell cycle”.

Mannitol treatment affects central carbon metabolism

During mannitol treatment there is a decrease of nutrient availability due to reduction of photosynthesis in the dark, degradation of the tapetum and lack of a metabolizable carbohydrate source in the medium. This situation is clearly reflected in some metabolic and energetic pathways (Fig. 2). Around 50% of metabolism-related genes belong to “C-compound and carbohydrate metabolism”, whereas “Glycolysis and gluconeogenesis” (39%) is the group with most genes in the “Energy” category. These results indicate that mannitol treatment mainly affected central carbon metabolism.

Starch breakdown is the first source of nutrient under dark and sugar-deficient conditions (Yu 1999). Repression of starch biosynthesis after the first pollen mitosis has been associated with induction of microspore embryogenesis (Touraev et al. 1997). Surprisingly, after mannitol treatment we observed an induction of starch biosynthesis genes (starch synthase I, starch branching enzyme class II and glucose-1-phosphate adenylyltransferase) and catabolism genes (starch debranching enzyme, beta-amylase, alpha glucosidase). The activation of the biosynthetic pathway may be due to the presence of microspores that do not respond to the treatment and accumulate starch before dying, and/or to the high albino rate of DH46, as a tendency of starch accumulation in albino genotypes during microspore embryogenesis has been described (Caredda et al. 2000). However, all starch catabolism related genes have a higher fold change than starch biosynthetic genes indicating a predominant use of starch in the system. In particular, an anther specific beta-amylase increased 2,363-fold whereas tissue-ubiquitous beta-amylases and a chloroplast beta-amylase were 59-fold and 44-fold down regulated, respectively.

Sucrose metabolism is vital not only for carbon resources but also for the initiation of hexose-based sugar signals (Koch 2004). After mannitol treatment regulation of sucrose metabolism is modified by the down-regulation of genes encoding a sucrose synthase, a sucrose-phosphate synthase and a cell wall invertase, whereas a vacuolar invertase gene was up-regulated. This change could provide a greater energetic capacity, stimulate specific sugar sensors and play an osmotic role in cellular expansion (Koch 2004). Other genes associated with sucrose import and signaling, such as the sucrose transporter *SUC3* (Meyer et al. 2004), were also up-regulated. The increase in gene expression of hexokinase, UDP-glucose pyrophosphorylase and phosphofructokinase genes might indicate the need of hexose-6-phosphates for the glycolytic process, and a change in sugar signaling.

A few genes associated with glycolysis, tricarboxylic acid (TCA) cycle and electron transfer/oxidative phosphorylation were up-regulated after mannitol treatment, some of which represent different isoforms of down-regulated genes. Induction of some genes involved in the lipolysis and glyoxylate cycle such as lipase (class3), phospholipase D, aconitase and malate synthase indicates a tendency to replenish intermediate compounds.

Taken together, our expression data confirm reorganization of central carbon metabolism during induction of microspore embryogenesis to flexible use of carbon skeletons from different sources, as was described in carbohydrate depletion (Contento et al. 2004). In this process regulation of a beta-amylase, and a vacuolar invertase gene could play a major role.

Mannitol treatment triggers a multi-dimensional stress response

During carbohydrate starvation a coordinate mechanism between metabolic adaptations and the induction of general stress responses including osmotic stress, reactive oxygen-scavengers (ROS) and disease resistance was proposed (Contento et al. 2004). Different stress-related proteins have been associated with the reprogramming of cellular metabolism in barley microspores, like glutathione S-transferases (GST), heat shock proteins (HSP) and alcohol dehydrogenases (ADH) (for review see Maraschin et al. 2005a).

Accordingly, around 11% of the differentially expressed genes belonged to “Cell rescue, defense and virulence” (Fig. 1). These genes were related to water deficit, osmotic stress, phosphate starvation, oxidative stress, cold response, pathogen response and response to wounding. Therefore, a multi-dimensional stress response was observed, which is in agreement with the existence of complex networks in abiotic and biotic stress responses (Cheong et al. 2002).

Surprisingly, genes associated with a general response to osmotic stress and pollen dehydration such as proline and glycinebetaine biosynthesis genes and the pollen coat proteins aquaporins and dehydrins were constitutively expressed or down-regulated. Few genes related to water stress as lipoxygenase (*LOX2*), *LEA3*, and the cysteine proteinases *RD21* and *RD22* were up-regulated. It is well known that abscisic acid (ABA) integrates environmental constraints, particularly to water status, with metabolic and developmental programs. ABA was implicated, in microspore embryogenesis as

different genes modulated by ABA were activated by mannitol treatment (Maraschin et al. 2005b). Increasing levels of ABA was also associated with higher regeneration efficiencies in mannitol treatment (Hoekstra et al. 1997). After mannitol treatment we observed no up-regulation of ABA biosynthesis genes but a change in the regulation of the ABA response as negative regulators (*ROP6*, *PP2C*, *CIPK3* and *SAD*) were down-regulated and the positive regulator *PKABA* was up-regulated (Himmelbach et al. 2003). A key regulatory role in stress signal transduction during mannitol treatment could be mediated by up-regulation of the mitogen-activated protein kinase (*MPK3*) due to its function in osmotic stress, ABA transduction and developmental arrest (Lu et al. 2002).

Carbohydrate starvation also generates oxidative stress. Interestingly, most of the antioxidant system genes such as superoxide dismutase (*SOD*), catalases and peroxidases were constitutively expressed or down-regulated. However, a large number of glutathione-S-transferases (*GSTs*) were up-regulated representing 2% of the up-regulated genes, at the same time, a large number of other isoforms of *GSTs* were down-regulated. *GSTs* are implicated in oxidative stress response, pathogen attack and heavy-metal toxicity (Marrs 1996) and up-regulation of members of the *GST* family during the initial steps of microspore embryogenesis was previously described (Vrinten et al. 1999, Maraschin et al. 2005b).

Members of the *HSP* family have been reported to be highly expressed during androgenesis induced by carbohydrate starvation and heat but its role on microspore embryogenesis has been discussed (Zarsky et al. 1995, Symkal and Pechan 2000, Zhao et al. 2003). In our analysis, minor changes on expression of the *HSP* family were observed. Only four *DnaJ HSPs* and one heat shock transcription factor (*HSF7*) were

up-regulated, however thirty other HSP family members, were constitutively expressed and thirteen were down-regulated indicating that the implication of HSP on microspore embryogenesis could be mediated by other controls than transcriptional. The eukaryotic DnaJ play an essential role in the regulation of the 70 kDa stress protein (HSP70) leading to stable binding of substrates and contributing to its chaperone function (Wang et al. 2004). HSF7 is a member of the class B HSF factors involved in controlling delayed functions in the stress response (Busch et al. 2005).

Several genes related to protein metabolism (protein folding, turnover, and biosynthesis, and ubiquitin-dependent catabolism) that have a fundamental role in post-translational regulation of stress response were mainly constitutively expressed or in some cases down-regulated. The up-regulation of some RING domain-containing proteins, that participated in the substrate recognition component of the ubiquitin-proteasome pathway (Stone et al. 2005), suggested a change in the target-specific proteolysis after mannitol treatment.

Several disease resistance genes were up-regulated by mannitol treatment. Among them the plant defensin-fusion gene *PDF2.4* was up-regulated 2,939 fold. Also genes involved in systemic acquired resistance (SAR) (Eulgem 2005) were up-regulated: for example a basic endochitinase (100 fold change), and a pathogenesis-related gene 1 (*PR-1*) (44 fold change) that was associated to cell death protection. The gene coding for the integral membrane protein MLO-1 that plays a broad role in defense, cell death protection, and stress response processes (Piffanelli et al. 2002) was up-regulated 27 fold.

The constitutive expression of several cold-inducible genes in *Arabidopsis* pollen after cold stress was described by Lee and Lee (2003) and the inability to induce the cold-inducible genes was linked to the high cold sensitivity of pollen. Pollen is also very sensitive to other environmental stresses and this fact could be the consequence of a general inability to induce common responses to stress as was observed in this study, in this sense the identification of new candidate genes for pollen stress tolerance could guide studies on mapping of tolerance genes to abiotic stress (Tondelli et al. 2006).

Mannitol treatment changes transcriptional regulation

Although developmental programs are controlled largely at the level of transcriptional regulation, so far no key regulatory genes have been associated with the differentiation induction treatment. We identified 340 genes encoding putative transcription factors on the Barley1 GeneChip (Table 1). Of these, 269 genes (around 75%) were expressed in anthers before treatment. The C3H family represented the highest number of genes followed by C2C2, bZIP and AP2/EREBP whereas a low representation was observed for MADS, WRKY, GARP, and NAC families (Table 1).

After mannitol treatment 79 putative transcription factor genes were down-regulated. Three families decreased their members more than 80%: EIL, ARF and Aux/IAA (Table 1, Supplemental Table 2). ARFs and auxin regulated proteins (Aux/IAA) play a critical role in most growth responses controlling auxin responsive genes. EIL participates in the ethylene signaling pathway, and its inhibition has been associated with cell growth inhibition and accelerated senescence. Four families represented more

than 50% of the 27 up-regulated genes: MYB, bHLH, C2H2 and C3H. Interestingly, members of the smaller classes of MYB-like, ARID and PcG E(z) were over-represented among the up-regulated transcription factors.

The up-regulated transcription factor genes can be divided into two groups according to the processes they regulate: abiotic and biotic stress responses and changes in developmental programs. The first group included: ICE1, a MYC-like b-HLH transcriptional activator associated with cold and dehydration response (Chinnusamy et al. 2003); ATAF2-like, a NAC family member (group I) induced by wounding, methyl jasmonate, salicylic acid and by dehydration (Delessert et al. 2005); WRKY46, a factor of the WRKY family (group IIIb) related to pathogen infection and salicylic acid response (Kalde et al. 2003); AtbZIP60, regulating the endoplasmic reticulum stress response (Iwata and Koizumi 2005); and HSF7, a member of heat stress transcription factor family associated with heat shock response (Busch et al. 2005). The second group included: YABBY5, a member of the YABBY family probably related to meristem development and maintenance of stamens and carpels (Jang et al. 2004), ZIML2, a C2C2 factor associated with cell wall modification during elongation (Shikata et al. 2004); and CURLY LEAF, a PcG E(z) factor involved in the repression the ABC control model of floral identity (Goodrich et al. 1997). The CURLY LEAF activation was accompanied by the down-regulation of ABC model genes *APETALA1* (*AP1*), *APETALA3* (*AP3*), *AGAMOUS* (*AG*) and *AGL9/SEPALLATA3* (*SEP3*).

A member of the AP2/ERF family BABY BOOM (BBM) was associated with microspore embryogenesis induction as determinant of cell totipotency after a period of differentiation (Boutilier et al. 2002). However, out of the 20 members of this family

represented on the Barley1 GeneChip none was up-regulated after mannitol treatment. The transcription factor expression patterns indicated that after mannitol treatment microspores end in an undifferentiated state. No other evidence in progression of embryogenesis pathway was observed in comparison with barley reference data (Supplemental Figure 1) (Druka et al. 2006).

Mannitol treatment conserves the cell cycle machinery

Mid to late uninucleate stage microspores is considered the best stage for efficient microspore embryogenesis in barley. In this study, most of the microspores were at late uninucleate stage corresponding to G2 phase before stress treatment, according to Sim and Kasha (2003) (Fig. 3A). A slow progression of the cell cycle was observed along treatment, since a high percentage of the microspores had completed an asymmetric first division (Fig. 3B, C). This slow progression contrasted with the high rate of division after 3 days of culture, resulting in a multinuclear structure (Fig 3D). In barley, mannitol treatment at 4°C suspended the progression of the microspore cell cycle, whereas cold treatment and mannitol treatment at 24°C allowed its progression to a symmetrical division (Sim and Kasha 2003). Our induction system is similar the one described in tobacco for microspores in G2 phase during starvation and heat treatment (Touarev et al. 2001), where microspores underwent an asymmetric division.

For further characterization, the effect of mannitol treatment on cell cycle was determined by analyzing core cell cycle genes expression. Based on the 81 core cell cycle genes of *Arabidopsis* (Menges et al. 2005) we identified 27 barley homologues

that are represented on the Barley1 GeneChip. Of these, 23 were constitutively expressed and only three (*CDKC;1*, *CKL10* and *CDC25*) increased their expression level. The role of *CDKC;1* and *CKL10* in cell cycle regulation is not clear (Menges et al. 2005). *CDKC;1* was not directly involved in cell cycle regulation in alfalfa (Magiar et al. 1997), but recently was described as a positive regulator of transcription in plants (Fülop et al. 2005). *CKL10* is a novel class of plant CDK-likes (*CKL1-15*) identified in *Arabidopsis*, whose expression was not described in flowers and pollen (Menges et al. 2005). A gene homologous to yeast *CDC25* was recently identified in plants (Landrieu et al. 2004). In yeast, this gene is known to compete with *WEE1* kinase in the control of transition from G2 to M phase (Dewitte and Murray 2003). After mannitol treatment, induction of *CDC25* together with the absence of expression of *WEE1*, suggests that the cell cycle is not arrested in G2 phase. These data agree with the observed division of the microspores during mannitol treatment (Fig. 3B, C). E2Fa transcription factor is one of the key regulators in the transition from G1 to S phase. In our study E2Fa as well as E2F target genes including DNA replication and origin factors, DNA repair and genes encoding structural proteins of chromatin (Vandepoele et al. 2005), were constitutively expressed.

The constitutive expression of most of the core cell cycle and E2F target genes, represented on the Barley1 GeneChip, might reflect the maintenance of the cell cycle machinery in a steady-state during mannitol treatment, and ready for reactivation in culture. Similar results have been described by Schrader et al. (2004) during cambial meristem dormancy in trees, whereas cell division cessation was associated with sugar starvation in suspension cells (Contento et al. 2004).

Concluding remarks

The application of a stress treatment is essential for reprogramming of the microspore from their normal pollen development towards an embryogenic pathway. Transcriptome analysis of mannitol treated anthers revealed dramatic changes on the transcriptome. Most of the mannitol treatment responsive genes were observed in the central metabolism, with major reorganization of metabolic pathways as a consequence of nutrient deprivation. This was accompanied by a modification of stress related genes expression that resulted in a complex but surprising response since common protection systems was not induced. Transcription factor analysis indicated that transcriptional control relied on the protective roles of stress and the suppression of the gametophytic development pathway, but without any sign of the switch to the embryogenic pathway, leading to a transition developmental phase. Evidences of the progression but not of the arrest of the cell cycle arrest were found.

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Table 1. Expression of transcription factor family members in anthers before (Control) and after 4 d of mannitol treatment at 25°C in the dark. “% genes” refer to the percentage of family members represented on the Barley1 GeneChip.

<i>Transcription factor family</i>	<i>Barley1Gene Chip</i>	<i>Control anthers</i>		<i>Down-regulated mannitol treatment</i>		<i>Up-regulated mannitol treatment</i>	
		<i>gene</i>	<i>gene</i>	<i>% genes</i>	<i>gene</i>	<i>% genes</i>	<i>gene</i>
MYB	18	14	77.8	7	38.9	3	16.5
MYB-like	4	4	100.0	2	50.0	1	25.0
AP2/EREBP	20	17	85.0	4	20.0	0	
bHLH	24	14	58.3	4	16.7	3	12.5
NAC	17	11	64.7	1	5.9	2	11.7
C2H2	23	17	72.7	3	13.0	3	13.0
Homeobox	25	16	64.0	7	28.0	2	8.0
MADS	10	7	70.0	3	30.0	0	
bZIP	24	18	78.3	3	12.5	2	8.3
WRKY	15	10	66.6	3	20.0	1	6.7
GARP	12	11	91.6	2	16.7	0	
C2C2	25	21	84.0	7	28.0	2	8.0
CCAAT	11	9	81.8	2	18.2	0	
GRAS	9	8	88.9	2	22.2	1	11.1
Trihelix	9	7	77.7	2	22.2	0	
HSF	8	5	62.5	1	12.5	1	12.5
TCP	2	2	100.0	0		0	
ARF	9	8	88.9	7	77.8	0	
C3H	32	29	90.3	5	15.6	4	12.5
SBP	3	3	100.0	0		0	
NIN-like	4	4	100.0	0		0	
ABI3/VP1	3	3	100.0	1	33.3	0	
TUBBY	5	4	80.0	1	20.0	0	
E2F/DP	1	1	100.0	0		0	
CPP	4	4	100.0	2	50.0	0	
Alfin-like	4	4	100.0	0		0	
EIL	2	2	100.0	2	100.0	0	
LEAFY	0	0		0		0	
Aux/IAA	10	10	100.0	8	80.0	0	
HMG-box	1	1	100.0	0		0	
ARID	1	1	100.0	0		1	100.0
JUMONJI	2	2	100.0	0		0	
PcG E(z)	2	1	50.0	0		1	50.0
PcG Esc	1	1	100.0	0		0	
Total	340	269		79		27	

Fig. 1. Distribution of functional categories of the differentially expressed mRNAs after mannitol treatment.

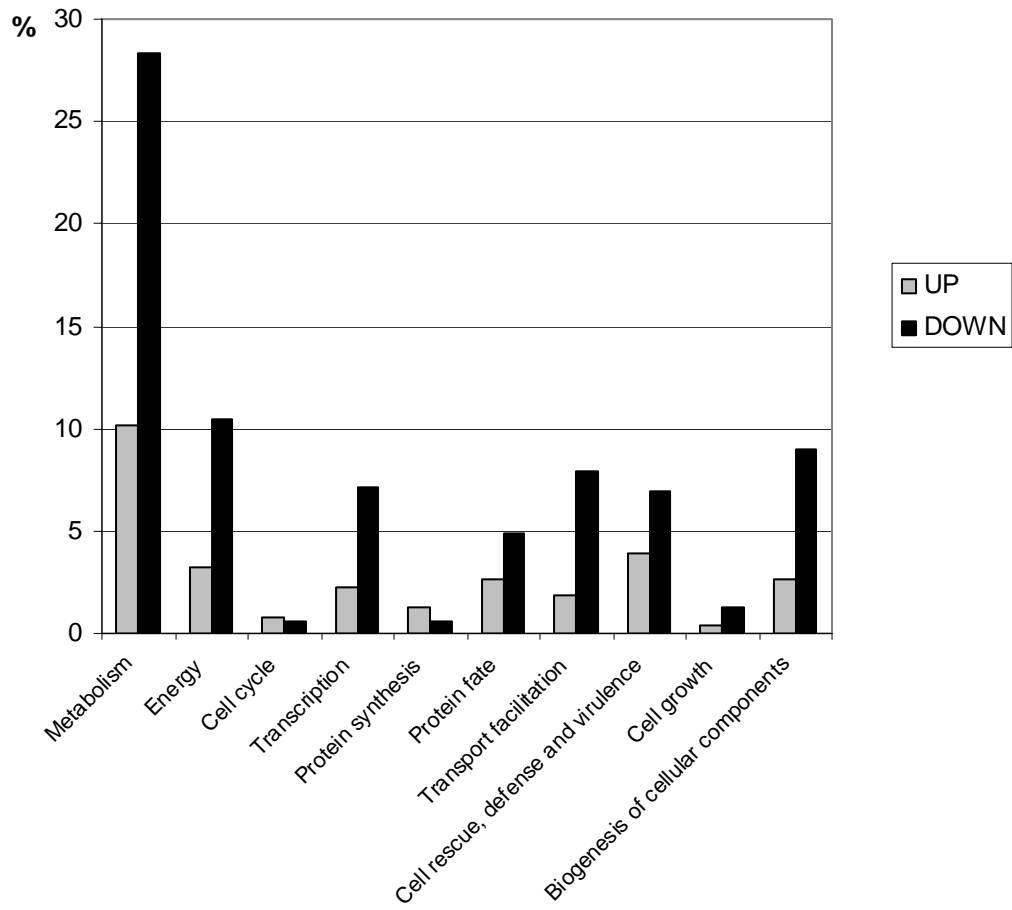


Fig. 2. Distribution of functional sub-categories in the “Metabolism” and “Energy” categories of differentially expressed mRNAs after mannitol treatment.

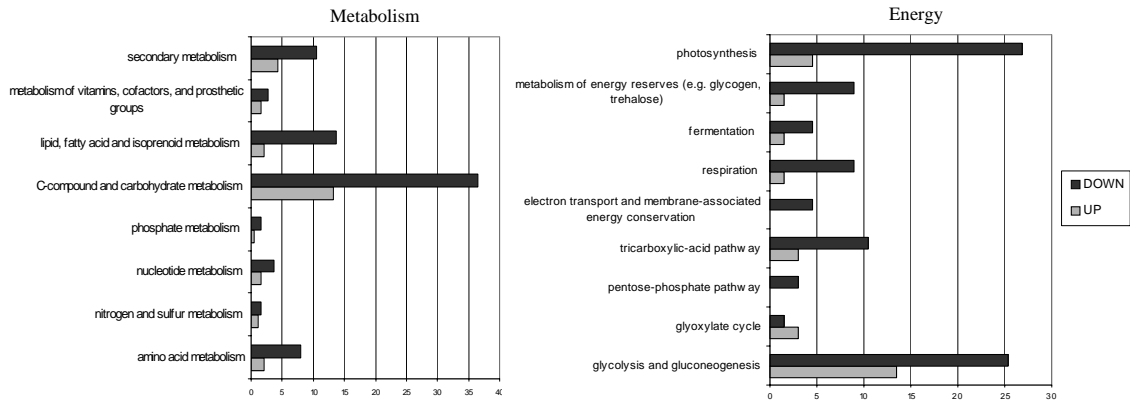


Fig. 3. Progress of microspore division during mannitol treatment (DAPI staining). (A) Late uninucleate microspores before treatment. (B) Microspore on the second day of mannitol treatment. (C) Binucleate microspores after mannitol treatment. (D) Multinuclear embryogenic structure with 8 nuclei after 3 d in culture medium.

