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ORIGINAL ARTICLE

Buthionine sulfoximine (BSO)-mediated improvement in cultured embryo quality in vitro entails changes in ascorbate metabolism, meristem development and embryo maturation

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Abstract Applications of buthionine sulfoximine (BSO), an inhibitor of GSH (reduced glutathione), which switches the cellular glutathione pool towards the oxidized form GSSG, positively influences embryo quality by improving the structure of the shoot apical meristem and promoting embryo maturation, both of which improve the post-embryonic performance of the embryos. To investigate the mechanisms underlying BSO-mediated improvement in embryo quality the transcript profiles of developing *Brassica napus* microspore-derived embryos cultured in the absence (control) or presence of BSO were analyzed using a 15,000-element *B. napus* oligo microarray. BSO applications induced major changes in transcript accumulation patterns, especially during the late phases of embryogenesis. BSO affected the transcription and activities of key enzymes involved in ascorbate metabolism, which resulted in major fluctuations in cellular ascorbate levels. These changes were related to morphological characteristics of the embryos and their post-embryonic performance. BSO applications also activated many genes controlling meristem formation and function, including *ZWILLE*, *SHOOTMERISTEMLESS*, and *ARGONAUTE 1*. Increased expression of these genes may contribute to the improved structural quality of the shoot poles observed in the presence of BSO. Compared to their control counterparts, middle- and late-stage BSO-treated embryos also showed increased accumulation of transcripts associated with the maturation phase of zygotic embryo development, including genes encoding ABA-responsive proteins and storage- and late-embryogenic abundant (LEA) proteins. Overall these transcriptional changes support the observation that the BSO-induced oxidized glutathione redox state allows cultured embryos to reach both morphological and physiological maturity, which in turn guarantees successful regeneration and enhanced post-embryonic growth.

Keywords *Brassica napus* · Buthionine sulfoximine · Canola · Meristem · Transcript levels

Abbreviations

ASC	Reduced ascorbate
APX	Ascorbate peroxidase
BSO	Buthionine sulfoximine
DHA	Dehydroascorbate
DHAR	Dehydroascorbate reductase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
MDEs	Microspore-derived embryos
MDHA	Monodehydroascorbate
MDHAR	Monodehydroascorbate reductase
LEA	Late embryogenic abundant

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Introduction

One of the best examples of cellular totipotency, proposed by Haberlandt (1902), is the ability of plant cells to generate embryos in culture. This phenomenon was first reported in the late 1940s by Levine (1947) using carrot cells, and subsequently extended to a variety of plant species (see Dunstan et al. 1995; Thorpe and Stasolla 2001). Despite the improvement in tissue culture conditions achieved for many species in the last decades, a recurrent problem encountered during in vitro embryogenesis is the low conversion rate of the embryos into seedlings. Attempts to enhance the regeneration frequency of the embryos have mainly focused on optimizing the culture environment during the early phases of embryo development, with the assumption that once initiated the embryogenic pathway is completed successfully. Although often true, this notion cannot be generalized as cellular and tissue deterioration leading to embryo abortion have been reported in both angiosperms (Yeung et al. 1996; Belmonte et al. 2006) and gymnosperms (Kong and Yeung 1992). Such abnormalities mainly develop during mid- to late-embryogenesis and specifically affect the integrity of the shoot apical meristem. Structural studies have revealed that fully formed shoot meristems degenerate due to the formation of intercellular spaces, which lead to separation of the meristematic cells. This is often followed by the loss of meristematic identity by the apical cells, which acquire parenchymous features (reviewed by Yeung and Stasolla 2001). It is therefore apparent that culture conditions during the advanced phases of embryo development are not optimal.

In vitro embryogenesis can be achieved through two processes: somatic embryogenesis, the formation of embryos from somatic cells, or gametophytic embryogenesis, the generation of haploid embryos from cells of the male or female gametophyte. The latter process is advantageous in that the endogenous genetic variation can be efficiently fixed through the recovery of diploid homozygous plants following artificial chromosome doubling by colchicine treatment (Yao et al. 1997). In general, the most efficient system for gametophytic embryogenesis is androgenesis, in which immature pollen grains (microspores) are used as the starting material to generate microspore-derived embryos, MDEs. Androgenesis has been obtained in a variety of systems through the development of optimized protocols and is routinely used as an integrated tool in many breeding programs. Over the past years the canola *Brassica napus* MDE system has received increasing attention as a model to study embryogenesis in plants for a variety of reasons (Yeung 2002; Boutilier et al. 2005). First, a large numbers of synchronized embryos can be rapidly and efficiently produced. Second, the embryogenic pathway is initiated in the absence of hormones and a callus phase, thereby allowing studies from the few-celled embryo stage onward. Third, the morphogenic events occurring during MDE development closely mimic those observed during zygotic embryogenesis (Yeung et al. 1996). Finally, the genetic similarities between *Brassica* and *Arabidopsis* can be exploited to apply the genomic information available in the latter for improving and understanding the mechanisms regulating *Brassica* embryogenesis. Due to these advantages, it is not surprising that the *B. napus* MDE culture system has been extensively utilized to investigate anatomical, physiological, and molecular events occurring during embryo development in vitro (reviewed in Yeung 2002; Boutilier et al. 2005). Recent transcriptome and proteome analyses have focused on the molecular events delineating the induction of microspore embryogenesis, that is the initial transition from gametophytic to embryonic cell state (Joosen et al. 2007; Malik et al. 2007). Both studies have revealed the existence of robust markers and pathways associated with the early phases of embryo development. To date however, there is no information documenting the molecular pathways active during the middle and late phases of MDE development, when cellular deterioration affects the integrity of the shoot apical meristems and compromises embryo regeneration.

Alterations in the cellular redox status have been shown to control embryo development in several systems (Earnshaw and Johnson 1987; Gardiner et al. 1998; Shi et al. 2000; Stasolla et al. 2004a; Belmonte et al. 2006). In recent studies on *B. napus*, Belmonte et al. (2006) show that the endogenous glutathione redox status [defined as the ratio of the concentration of the reduced form (GSH) to the concentration of the oxidized plus reduced forms (GSSG + GSH)] can be easily manipulated in culture through applications of buthionine sulfoximine (BSO). If applied in the medium after 7 days of culture, BSO induces an oxidized glutathione environment by inhibiting GSH synthesis (Griffith and Meister 1979), thereby lowering the cellular GSH/GSH + GSSG ratio. This metabolic switch improves embryo quality, as measured by the enhanced conversion frequency, and induces major structural changes in the developing embryos (Belmonte et al. 2006). Histological analyses revealed that compared to their control counterparts, BSO-treated embryos form well organized meristems and accumulate storage products in a similar pattern to that observed in seed embryos. Such characteristics, which can be reversed if the glutathione redox status is shifted towards a reduced state through exogenous applications of GSH, are also observed when BSO is applied to other plant culture systems (Belmonte et al. 2007). The enhanced embryonic development resulting from the imposition of a BSO-induced oxidized cellular environment is likely the result of a complex genetic network that is not properly executed under standard tissue culture conditions.

Gene expression profiling is a powerful tool for dissecting the molecular mechanisms of relevant developmental processes, including embryogenesis. Over the past few years cDNA microarray studies have revealed the existence of transcript patterns associated with embryo development in both angiosperms (Girke et al. 2000; Thibaud-Nissen et al. 2003; Joosen et al. 2007; Malik et al. 2007) and gymnosperms (Stasolla et al. 2003, 2004b). Most importantly, these studies have revealed the existence of key genes able to affect the embryogenic process if their expression level is experimentally altered (Tahir et al. 2006; Belmonte et al. 2007). Here we use microarray technology to elucidate the effect of a BSO-mediated oxidized environment on global changes of gene expression during the middle and late phases of *Brassica* MDE development. We propose that the morphological and physiological improvements in embryo quality observed after BSO treatment can be attributed to underlying transcriptional changes regulating antioxidant responses, and mechanisms controlling meristem formation and embryo maturation.

Materials and methods

Brassica napus embryo development and BSO treatments

Induction of embryo development from *B. napus* cv Topas DH4079 microspores and applications of BSO were carried out exactly as reported by Belmonte et al. (2006). For this experiment days in culture were counted after the imposition of the heat shock treatment. Globular embryos were treated at day 7 with 0.1 mM BSO (Belmonte et al. 2006). Control and BSO-treated embryos were harvested at day 7 after the heat shock treatment (at the same time BSO was added to the culture medium), 14, 21 and 35, frozen in liquid nitrogen and subsequently stored at −80°C. Samples were referred to as stage 0 (day 7) stage 1_C, 2_C, 3_C (control embryos collected at day 14, 21, and 35, respectively), and stage 1_B, 2_B, 3_B (BSO-treated embryos collected at days 14, 21, and 35). For some of the studies, embryos were also treated with 0.2 mM GSH (Belmonte et al. 2006).

Histological analyses and ascorbate metabolism

Structural studies during embryo development were conducted exactly as reported by Yeung (1999). Measurements of endogenous ascorbate and the enzymatic reactions of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR) were carried out according to Stasolla and Yeung (2001) and Belmonte et al. (2006).

Microarray development

The *B. napus* 50-mer oligo array (see supplementary Table 1 for a complete list of the oligos) was developed by Agriculture and Agri-Food Canada using the collection of approximately 150,000 3′ and 5′ *B. napus* ESTs derived from cDNA libraries representing a range of vegetative tissues of the homozygous line DH12075. A custom unigene set of sequences identified from the collection was used for oligo design with the aim of developing a resource with the potential to detect differential expression between members of closely related multi-gene families within the polyploid *B. napus* genome. A full description of the development of the set of 15,000 50-mer oligos will be published separately (Sharpe et al., in preparation). The oligos were synthesized by Illumina (San Diego) and printed onto epoxide slides (Corning Inc. Lovell, MA, USA) using a 48-pin GeneMachines arrayer at the University of Alberta Microarray Facility (Edmonton, AB, Canada) Microarray Facility. Each one of the 48 sub-arrays were designed with 26 columns × 25 rows for a total capacity of 31,200 features. Each of the 15,000 oligos were replicate spotted within each sub-array. The Stratagene Alien oligo set (La Jolla, CA, USA) and phosphate buffer blanks were added as controls.

Experimental design

Three biological replicates were collected for each of the four control samples (stage 0, 1_C, 2_C, 3_C) and the three BSO treated samples (1_B, 2_B, 3_B). Each replicate sample was hybridized on the same slide with a common reference sample comprising equal amounts of amplified RNA derived from one biological replicate of each of the seven samples. This experimental design provided information on gene expression during the progression of embryo development in control (stage 0, 1_C, 2_C, 3_C) and BSO treatments (stage 0, 1_B, 2_B, 3_B), i.e. time course comparison, as well as between time points across treatments (stage 1_C vs. 1_B, stage 2_C vs. 2_B, and stage 3_C vs. 3_B), i.e. direct comparison. A swapped-dye experiment (1_C vs. common reference) was also conducted to verify the effect of the dye on the hybridization outcome.

Microarray hybridization and scanning

Total RNA was extracted from control and BSO-treated embryos using a guanidium isothiocyanate method described by Ouellet et al. (1992). Total RNA (1 μg) was amplified, labeled with fluorescent Cy3 or Cy5 dyes, and fragmented, using an RNA ampULSe:Amplification and Labeling kit (Kreatech Biotechnology, Amsterdam, The Netherlands) following the manufacturer's instructions.

The integrity of the amplified RNA and the degree of labeling was determined using gel electrophoresis and by measuring Cy5 and Cy3 emissions on a Molecular Imager FX Pro Plus scanner (BioRad, Munich, Germany http://www.bio-rad.com).

Microarray hybridizations were carried as described below. After a pre-hybridization treatment in a buffer containing 5× SSC, 0.1% SDS, and 0.1 mg/ml BSA at 37°C for 1 h, the slides were washed three times in 0.1× SSC at 22°C for 5 min and once in water at 22°C for 30 s. The slides were dried by centrifugation at 1,000×*g* for 2 min and stored until hybridization. The slides were then hybridized using 200 ng of Cy5-labeled target and 400 ng of Cy3-labeled target added to warm (37°C) hybridization solution containing 25% formamide, 5× SSC, 0.1% SDS, and 0.1 mg/ml salmon sperm. Hybridization was carried out for 16 h at 37°C in a hybridization station (Perkin Elmer HybArray12™, Perkin-Elmer, Foster City, CA, USA). Post-hybridization washes were performed by immersing the slides in a solution composed of 2× SSC, 0.1% SDS for 15 min at 37°C, followed by two washes in 1× SSC for 2 min at 22°C, and three washes in 0.1× SSC for 2 min at 22°C. The slides were dried by centrifugation and fluorescence levels of the Cy dyes were determined using a ScanArray 3000 laser scanner (Perkin-Elmer) with ScanArray software version 3.1 (Packard Bioscience).

Data analysis

The microarray data, expressed in $\log_2$ ratios, were first normalized using a Lowess normalization (Cleveland 1979) and then subjected to the following criteria: (1) the mean value of any stage was dismissed if more than one of the three replicates was missing, and (2) in the time course analysis, a value was dismissed if two or more time points were missing across the series of control or BSO-treated embryos. Based on these two criteria 3,023 probes (which included more than 1,000 blanks) were eliminated from further analysis. The remaining probes were analysed with analysis of variance (ANOVA). An *F*-probability (*P* value) and an least significant difference (LSD value) were calculated for each clone on the array and then adjusted for multiple comparisons according to the procedure of Benjamini and Hochberg (1995) to test for significant differences among samples. The adjusted *P* values were used to verify if a difference observed between stages and treatments was statistically significant. The LSD was calculated based on the standard deviation among the replicates and used to identify patterns of gene expression among elements on the array that showed significant differences among samples. A first selection was performed by comparing the expression ratios of signal intensities at different stages or treatments, and selecting only those probes having an adjusted *P* value ≤ 0.05 and an absolute difference in expression ratios higher than the LSD. The stringency of the analysis was further increased by considering differentially expressed probes as being only those having a difference in $\log_2$ ratios between two point comparisons greater than 1.5 in at least one of the two duplicate spots of the same probe, with the other spot showing a similar behavior (Pearson correlation ≥ 0.8). The presented values are therefore expressed as average signals over the two replicate probes. To exclude possible dye effects, all probes exhibiting an opposite behavior in the swapped-dye experiment were eliminated from the final analysis.

The normalized $\log_2$ ratios of the probes were imported in GeneMaths (GeneMaths XT (Applied Maths, Sint-Martens-Latem, Belgium http://www.applied-maths.com/genemaths/genemaths.htm) and used to perform hierarchical cluster analysis, cluster analysis by self-organizing maps (SOMs), and principal component analysis (PCA).

Validation of microarray data and expression analysis

Microarray data were validated by quantitative real-time (RT)-PCR according to Stasolla et al. (2003) for selected genes that showed differential expression patterns during embryo development. The relative level of gene expression was determined with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) using actin (accession number AF111812) as a reference and the common reference (combined pooled of the seven sample stages) as the calibrator. The primer sequences are shown in supplementary Table 2.

BnZWILLE was isolated from MDEs using RACE PCR based on sequence information obtained from *Arabidopsis*.

Results

Effects of BSO on the endogenous glutathione pool and embryo structure

Applications of BSO in the culture medium shifted the glutathione redox state towards its oxidized form, i.e. GSSG (Fig. 1). Compared to control embryos, the GSH/GSH + GSSG ratio declined quickly in BSO-treated embryos over the course of the culture period, reaching its minimum value in fully mature stage 3 embryos. A decline in this ratio during the late phases of embryogenesis was also observed in vivo (Fig. 1). Structural studies were conducted to investigate the effects of BSO treatments on embryo morphology. BSO was added to the embryo culture medium at day 7 (stage 0), when the MDE culture mainly comprised early globular-stage embryos (Fig. 2). After a week (day 14, stage 1) embryos increased in size and the apical meristem and a pair of cotyledons were visible at the

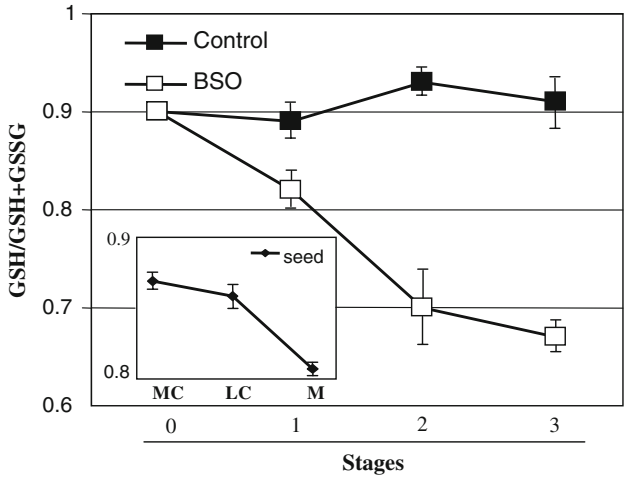


Fig. 1 Glutathione redox state (GSH/GSH + GSSG) during the different stages (0–3) of *Brassica napus* microspore-derived embryos cultured in the absence (control) or presence of 0.1 mM BSO. The glutathione redox state of *B. napus* seeds containing mid- (*MC*) and late cotyledon stage (*LC*) embryos, as well as mature (*M*) embryos is also shown as an *inset*

apical pole of each embryo (Fig. 2). At day 21 (stage 2) the cotyledon-stage embryos had completed their histodifferentiation program. The shoot apical meristem was fully formed, but signs of deterioration, as estimated by the appearance of small intercellular spaces, became visible in control embryos (Fig. 2). These abnormalities were not observed in stage 2 BSO-treated embryos (Fig. 2), which are able to regenerate viable plants at a higher frequency (46% compared to 33% of control) (Belmonte et al. 2006). Compared to control embryos, where meristem deterioration became more pronounced upon prolonged culture (day 35, stage 3) (Fig. 2), the apical pole of BSO-treated embryos remained intact (Fig. 2). Overall, stage 3 BSO-treated embryos displayed an improved post-embryonic growth (76% conversion frequency compared to 11% of control embryos) (Belmonte et al. 2006).

Transcriptome analysis during embryo development

Transcriptome analysis was conducted to compare mRNA profiles during the progression of embryo development in the absence or presence of BSO (time course comparison), as well as to identify genes that are differentially expressed between treatments at each stage of development (direct comparison). A custom *B. napus* oligo microarray composed of 15,000 unique gene-specific probes, each of which was spotted in duplicate, was used for these analyses. The arrays were hybridized to targets generated from control and BSO-treated embryos harvested at stage 0, 1, 2, and 3. Each of the amplified targets was then hybridized against a common reference composed by pooling amplified RNA samples from all stages (see "Material and methods"). The reliability of the microarray data was confirmed by monitoring the expression level of seven differentially expressed probes using quantitative real-time (RT)-PCR. The expression ratios of the selected probes using RT-PCR was very similar to the ratios derived from the microarray experiment and the Pearson correlation coefficient between the two data sets was always higher than 0.8 (supplementary Table 3).

A principal component analysis (PCA) plot was used to visualize overall differences in expression patterns between the control and BSO-treated samples. As shown in Fig. 3, the three biological replicates for each stage of development and treatment clustered into distinct groups indicating little variation among replicated samples. Overall differences in global transcript levels between treatments, i.e. control (C) and BSO (B), were apparent after stage 1 and increased markedly during the following phases of development (Fig. 3). These results correlated closely with the morphological studies showing that major structural differences in the apical poles of control and BSO-treated embryos became apparent at stage 2 and increased at stage 3 when control embryos exhibited extreme cellular deterioration in the meristematic region (Fig. 2).

Time course analysis

Using the ANOVA test statistic (*P* value) and LSD values, we identified 446 elements (see supplementary Table 4) which had a significant difference in $\log_2$ ratio between two neighboring stages of embryo development greater than 1.5 for at least one of the two duplicated probes, with the other probe exhibiting a similar behavior (Pearson coefficient ≥ 0.8). The number of probes showing differential expression between progressive stages of embryo development in the absence (control) or presence of BSO is shown in Fig. 4a. During the transition from stage 0 to stage 1 about 200 probes were differentially expressed in both control and BSO-treated embryos. This number decreased markedly from stage 1 to stage 2 before increasing again during the last phases of development, but only in the presence of BSO. Approximately 250 probes were differentially expressed between stages 2 and 3 in BSO-treated embryos, compared to less than 75 probes between stages 2 and 3 in control embryos (Fig. 4a). This divergent expression profile during late embryogeny was also visualized using hierarchical cluster analysis (compare the signal intensity between 2_C vs. 3_C and 2_B vs. 3_B in Fig. 4b). Self organizing maps (SOMs) were further generated to identify clusters of genes showing similar expression profiles during the progression of embryo development. The most representative SOMs are shown in Fig. 5. Five SOMs (A–E) included genes exhibiting an almost identical transcription pattern during the development of both control and BSO-treated

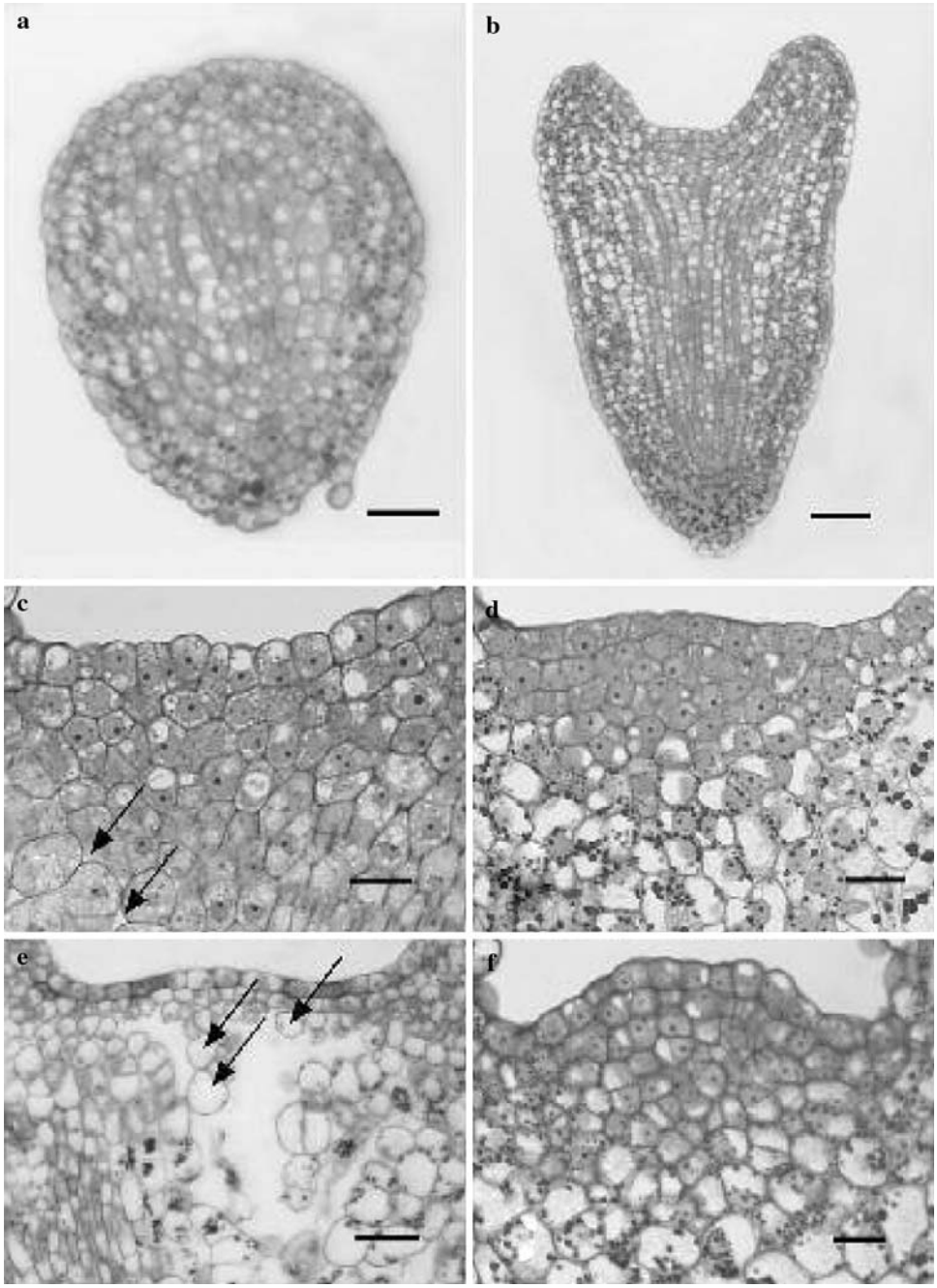


Fig. 2 Structural features of control and BSO-treated developing embryos. **a** Day 7 (stage 0) microspore-derived embryo cultures are characterized by the presence of globular stage embryos. **b** Upon further development (day 14, stage 1) the embryos increase in size and two cotyledons emerge from the apical pole of the embryos delineating the early cotyledonary stage of development. Structural differences between control and BSO treatments become visible after 21 days (stage 2) in culture, especially in the shoot apical poles of the embryos. **c** Small intercellular spaces (*arrows*) appear in the sub-apical region of the shoot apical meristems of stage 2 control embryos. **d** No structural abnormalities are visible in the apical pole of BSO-treated embryos at stage 2. The apical pole is generally dome-shaped and composed of highly cytoplasmic cells. **e** Cellular deterioration continues in the shoot apical meristems of stage 3 control embryos, which, in extreme cases, results in the complete physical separation of the meristematic cells. These cells also lose their "meristematic features" as they enlarge and become vacuolated (*arrows*). **f** A well organized architecture is retained in stage 3 embryos cultured in the presence of BSO. *Scale bars* **a** and **e** (5 μm), **b** (2.5 μm), **c**, **d** and **f** (2 μm)

embryos. The list of genes extracted from these SOMs is shown in Table 1. The majority of these genes are involved in basic cellular metabolic processes ranging from nitrogen metabolism (nitrate reductase, glutamine synthetase, and aspartate aminotransferase), to lipid metabolism (fatty acid elongase 1 and several lipid transfer proteins). Of interest is the large number of photosynthesis-related genes grouped in SOM C that show a pronounced increase in transcript levels during the initial phases of embryo development, i.e. from stage 0 to stage 1 (Fig. 5; Table 1). The remaining SOMs (F–M) include genes that show differential behavior between treatments. These include an ascorbate peroxidase and a CLAVATA3/ESR-related 27 gene (SOM f, Fig. 6), both of which are up-regulated in BSO-treated embryos during early embryogeny (stage 1), several genes involved in a variety of developmental (*LOB 41*, *NO APICAL MERISTEM*) and metabolic (fructose-6-*P*-phorphotransferase, glutathione-*S*-transferase, and aspartate aminotransferase) processes, which are induced by BSO during middle embryogeny (stage 2), and other genes up-regulated in BSO-treated embryos during late embryogeny (stage 3). Among the latter are several genes encoding transcription factors (a myb-related protein, Hap5a and a homeodomain leucine-zipper protein), hormone-regulated proteins (an

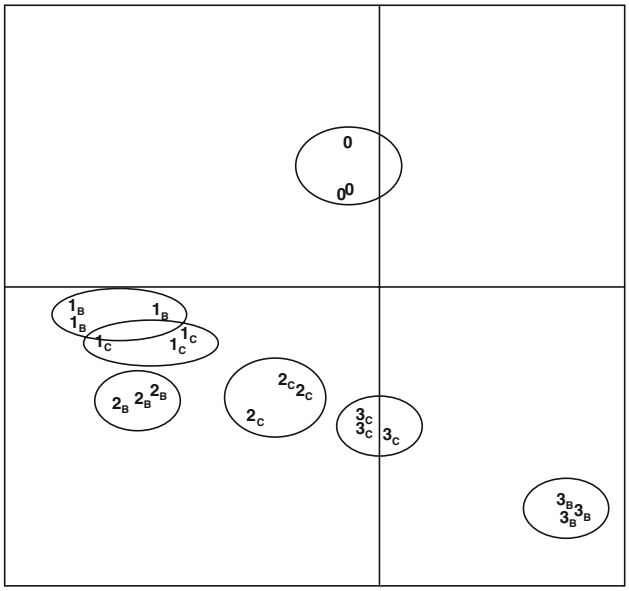


Fig. 3 PCA plot of gene expression profiles during different stages (0–3) of *Brassica napus* MDE development in control (*C*) and BSO-treated (*B*) embryos. *Zero* 7 day embryos; *1* day 14 embryos, *2* day 21 embryos, *3* day 35 embryos

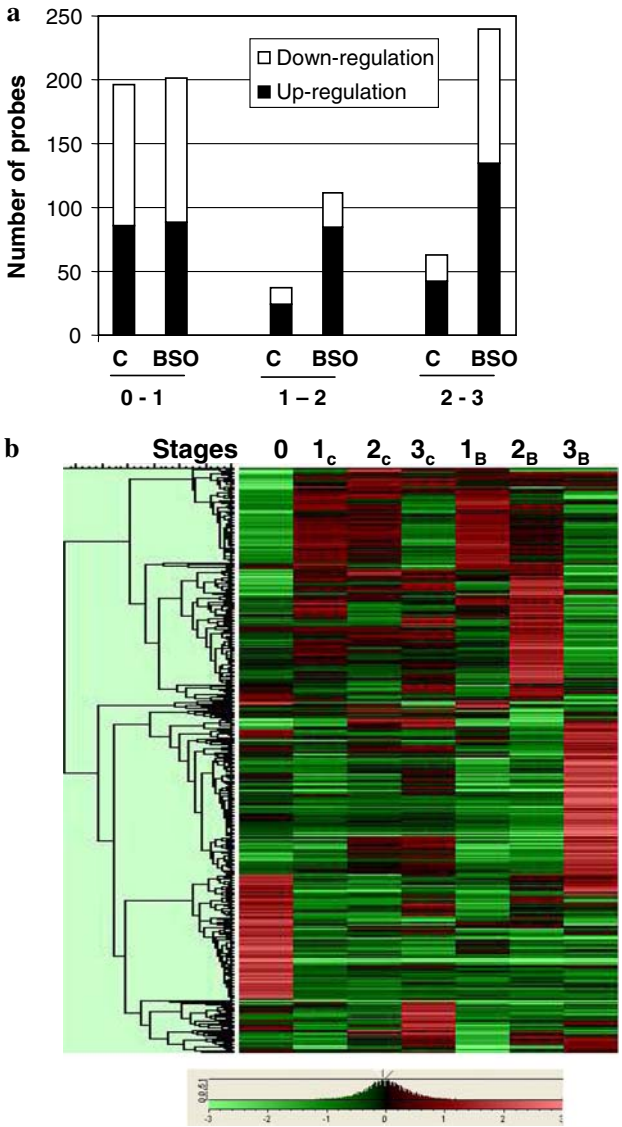


Fig. 4 **a** Number and behavior (up-or down-regulation) of probes which are differentially expressed during neighboring stages (0 vs. 1, 1 vs. 2, and 2 vs. 3) of development in control (*C*) and BSO treated embryos. **b** Hierarchical cluster analysis of 446 probes differentially expressed during different stages of development (0–3) in control (*C*) and BSO-treated (*B*) MDEs. *Colors* indicate probes which are either up-regulated (*red*) or down-regulated (*green*) compared to the common reference. The *color* scale depicts the fold change ($\log_2$ scale) in expression ratios between samples

auxin-responsive and a gibberellin-regulated protein), and late-embryogeny abundant (LEA) and storage proteins (SOM j, Fig. 6). A complete list of probes in the SOMs shown in Fig. 5 is compiled in supplementary Table 5.

Direct comparison analysis

To identify differentially expressed probes between control and BSO-treated embryos at each stage of development, we used a similar strategy employed in the time course comparisons. Selected probes had a $\log_2$ ratio between treatments at the same stage of development greater than 1.5 for at least one of the two duplicated probes, with the other probe exhibiting a similar behavior (Pearson coefficient ≥ 0.8). The stringency of the selection process was further increased by selecting only probes for which the $\log_2$ ratio average of the two duplicated probes was greater than 1.5. Based on the above strategies, we compiled a list of 334 differentially expressed probes (supplementary Table 6). The number of probes that were differentially expressed between treatments increased during development reaching a maximum (219) in stage 3 embryos (Fig. 7). To facilitate our analysis we grouped these probes in categories based on their putative functions.

Representative probes falling in these categories are shown in Fig. 8. Several probes implicated in antioxidant responses encode proteins involved in ascorbate and glutathione metabolism. An ascorbate peroxidase and a monodehydroascorbate reductase were induced respectively, in the presence of BSO at stage 1 and stage 3. Several glutathione-*S*-transferase encoding probes were induced by BSO at stage 2. Many probes clustered within the "Development" category were up-regulated in BSO-treated embryos at stage 2; these included ZWILLE, AGO1, a putative AGO7, and a LOB 41-encoding protein. Differences between treatments were also observed for probes encoding hormone-related proteins (among which are three ABA-responsive probes) and storage/LEA proteins. This latter set of probes was up-regulated in stage 3 embryos cultured in the presence of BSO (Fig. 8).

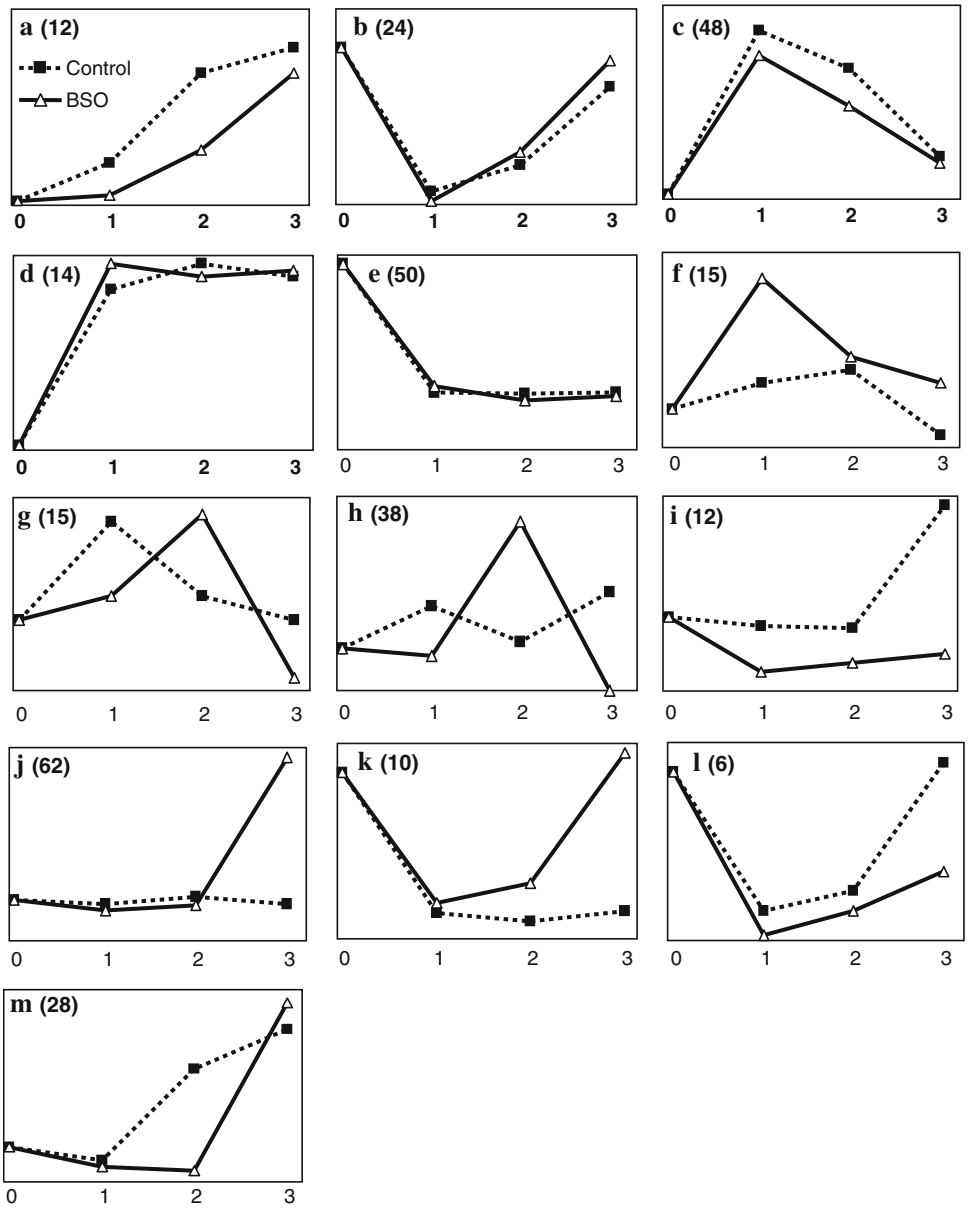


Fig. 5 Selected self-organizing maps (SOMs) showing expression profiles of probes during different stages of development (0–3) in control (*C*) and BSO-treated MDEs. The number of probes in each self organizing map is represented in *brackets*

Differential expression between treatments during middle (stage 2) and late (stage 3) embryogeny was also observed for several probes corresponding to genes involved in transcription-translation, signal transduction, methionine and nucleotide metabolism, and lipid metabolism (Fig. 8).

Ascorbate metabolism in developing embryos

We observed that genes encoding several antioxidant enzymes were differentially expressed between BSO and control treatments (Fig. 8). We therefore determined whether differences in glutathione-ascorbate metabolism (Fig. 9a) could be detected in developing control and BSO-treated embryos by measuring endogenous ascorbate and glutathione levels, as well as the activity of several key regulatory enzymes, namely ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). In agreement with the gene expression studies, the activities of both APX and MDHAR increased in BSO-treated embryos at stages 1 and 3, respectively. This differential regulation was mainly ascribed to the imposition of the BSO-induced oxidized environment, as GSH applications, which switch the glutathione pool towards a reduced state (Belmonte et al. 2006), had an opposite effect. The activities of both APX and MDHAR in embryos treated with BSO + GSH were comparable to those measured in control embryos (Fig. 9b). BSO did not induce any change in the activity of DHAR, as was also observed at the gene expression level in the microarray experiments. The activity of another key enzyme, GR, which recycles GSSG to

Table 1 List of selected genes extracted from the self-organizing maps (SOMs) A–E shown in Fig. 5, exhibiting a similar expression profile during development of control and BSO-treated embryos

SOM A	
At1g54000.1	Myrosinase-associated protein
At4g34520.1	Fatty acid elongase 1 (FAE)1
At3g10340.1	Phenylalanine ammonia-lyase (putative)
At5g51210.1	Glycine-rich protein/oleosin
At1g62510.1	Lipid transfer protein (LTP)
At1g77760.1	Nitrate reductase 1
At4g09760.2	Choline synthase
SOM B	
At3g17390.1	*S*-adenosylmethionine synthetase
At3g03640.1	Glycosyl hydrolase family 1 protein
At1g03880.1	12S seed storage protein
At4g01610.1	Cathepsin B-like cysteine protease
At4g21990.1	5′-adenylsulfate reductase (APR3)/ PAPS reductase homolog
At4g11650.1	Osmotin-like protein (OSM34)
At3g17820.1	Glutamine synthetase (GS1)
At3g10985.1	Wound responsive protein
At1g07600.1	Metallothionein-like protein
SOM C	
At2g28950.1	Expansin (EXP6), putative
At4g28250.1	Expansine (EXPB3)
At1g20020.1	Ferredoxin-$NADP^+$ reductase
At4g37870.1	Phosphoenolpyruvate carboxykinase
At1g29930.1	Chlorophyll A-B binding protein 2
At3g08940.2	Chlorophyll A-B binding protein LHCII type 1
At3g61470.1	Chlorophyll A-B binding protein LHCA2
At2g30570.2	Photosystem II reaction center W
At1g61520.1	Chlorophyll A-B binding protein LHCI type 3
At4g21280.1	Oxygen evolving enhancer protein 3
At4g28750.1	Photosystem I reaction center subunit IV
At2g37630.1	myb transcription factor (MYB91)
SOM D	
At3g28200.1	Peroxidase
At3g24503.1	Aldehyde dehydrogenase
At3g21560.1	UDP-glucosyltransferase
At4g31990.2	Aspartate aminotransferase
At5g35630.1	Glutamine synthetase (GS2)
At3g21560.1	UDP-glucosyltransferase, putative
SOM E	
At4g39330.1	Mannitol dehydrogenase, putative
At3g51600.1	Lipid transfer protein 5 (LTP5)
At5g59320.1	Lipid transfer protein 3 (LTP3)
At5g59310.1	Lipid transfer protein 4 (LPT4)
At1g10370.1	Glutathione-*S*-transferase

GSH, was reduced at stages 2 and 3 by BSO applications. Addition of GSH to the embryo culture medium increased the activity of this enzyme.

Compared to the control embryos, the cellular level of total ascorbate (ASC + DHA + MDHA) was lower at all stages of development in BSO-treated embryos, especially at stage 3, where DHA and MDHA became the most predominant forms within the overall ascorbate pool. Application of GSH had opposite effects to those described for BSO. The endogenous ascorbate pool in embryos cultured with BSO + GSH was comparable to that of control embryos (Fig. 9c).

Cell measurements

Buthionine sulfoximine altered APX activity and the cellular ascorbate pool, both of which are implicated in cell wall loosening (reviewed by Potters et al. 2005). We therefore hypothesized that cell elongation was affected in BSO-treated embryos. To test this hypothesis, we measured cortical cells length in stage 3 MDEs and fully mature zygotic embryos. Compared to control MDEs, the average length of cortical cells of middle hypocotyl sections was lower in BSO-treated embryos and zygotic embryos. Applications of GSH to MDEs increased cell elongation to values above those observed in control embryos (Fig. 10).

Expression studies of meristem and embryo marker genes

As BSO altered embryo morphology (Fig. 2) and induced the expression of several genes related to developmental processes (Fig. 8), we decided to further investigate the role of this compound on meristem and embryo formation by conducting additional expression studies on several development-related genes that were not represented on the array. For this purpose we chose two meristem-expressed and four embryo-expressed marker genes. The two meristem-expressed genes correspond to a *B. napus ZWILLE* homolog (*BnZWILLE*, accession number EU329719) and a *B. napus* homolog of the *Brassica oleracea SHOOTMERISTEMLESS-1* (*BroSTM-1*) gene. *BroSTM-1* has been shown to be a reliable meristem-specific marker gene (Teo et al. 2004). The four embryo-expressed markers correspond to *B. napus LEAFY COTYLEDON1 and 2* (*BnLEC1* and *BnLEC2*) and two *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes, *BnWOX2 and BnWOX9*, all of which have been shown to be unequivocal MDE markers (Malik et al. 2007). Expression studies were conducted by quantitative real-time (RT)-PCR using sets of gene specific primers (supplementary Table 2). Of the six genes tested only three; *BroSTM-1*, *BnZWILLE*, and *BnLEC1* showed differential expression between control and BSO treatments (Fig. 11). BSO induced the expression of *BroSTM-1* at stage 3, *BnZWILLE* at stage 2, and *BnLEC1* at stages 2 and 3. These changes in transcript levels were only due to the BSO-induced oxidized environment as they were not observed

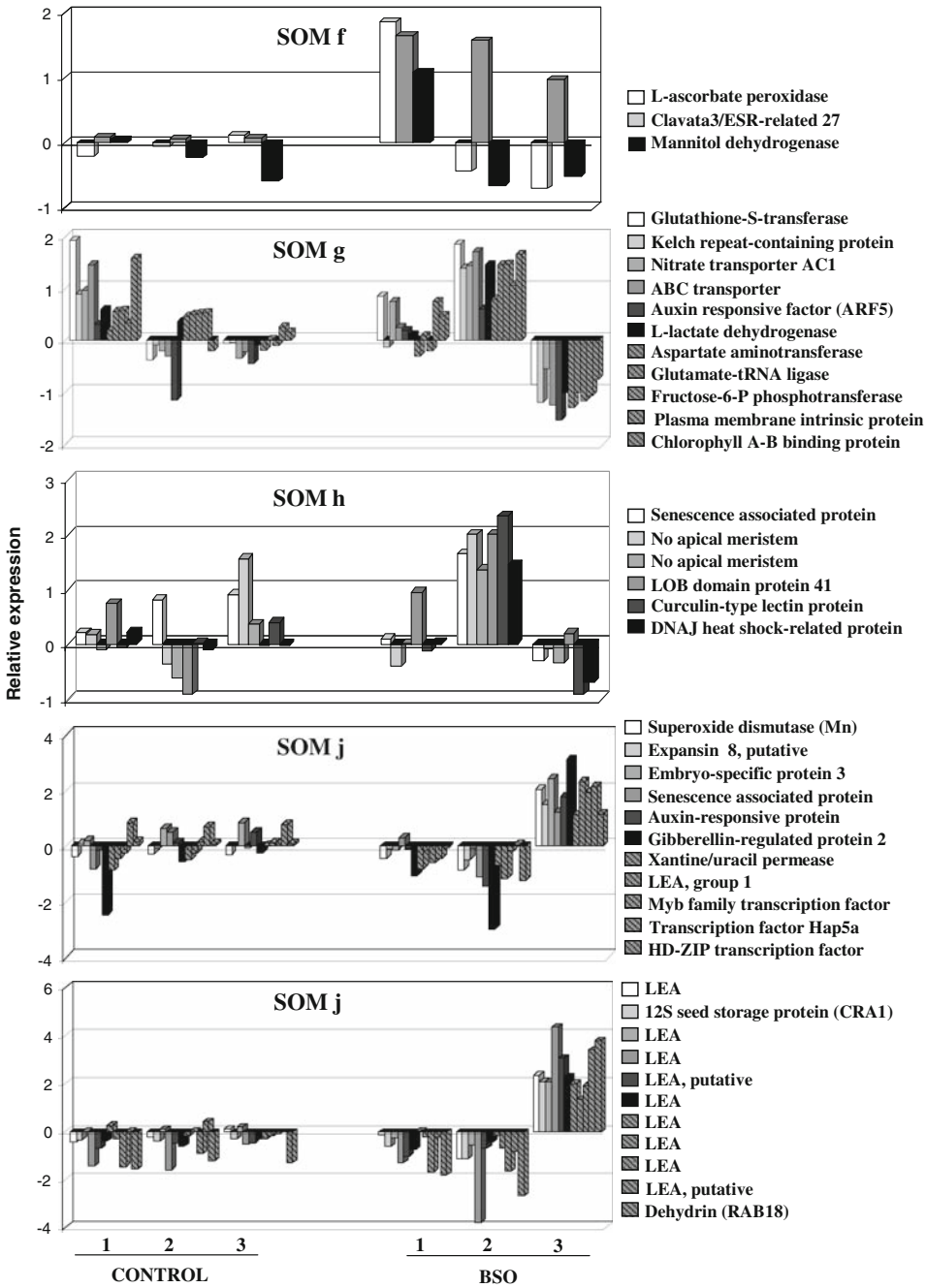


Fig. 6 Profiles of selected probes that exhibit differential expression patterns between control and BSO-treated embryo development. The probes belong to several self-organizing maps (SOMs) shown in Fig. 4. Expression values (*y* axis) were normalised to those of day 7 embryos (stage 0), which were set at 0

in embryos cultured with either GSH or BSO + GSH (Fig. 11).

Discussion

Due to the difficulty of isolating and manipulating zygotic embryos, investigations on various aspects of embryo development have primarily made use of embryos derived from in vitro culture systems. The establishment of "healthy" immature embryos is critical for initiating proper development, although it does not guarantee the successful completion of the embryogenic process. Cellular and tissue deterioration are often observed in culture where they disrupt the architecture of developing MDEs leading to poor post-embryonic performance and in extreme cases embryo abortion (Yeung et al. 1996; Belmonte et al. 2006). The fact that these abnormal phenomena are not observed in vivo underlines the importance of improving culture conditions during the middle and late phases of embryogenesis as well as the need for understanding the molecular mechanisms which regulate the progression from immature to fully mature embryos.

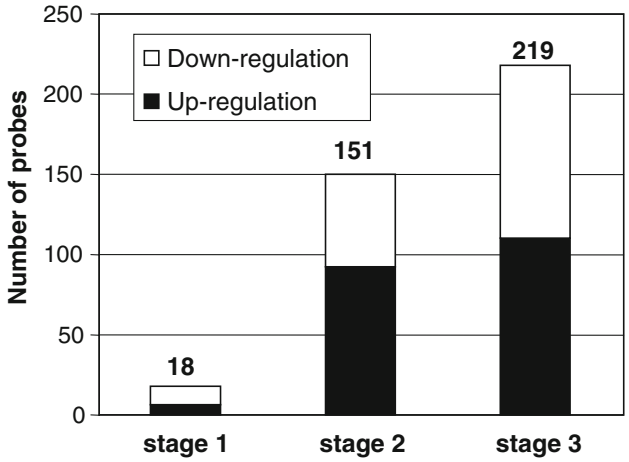


Fig. 7 Number of probes which are differentially expressed between the same stage of development (1–3) in control and BSO-treated embryos. Probes up-regulated or down-regulated in BSO embryos compared to their control counterparts are represented by *closed* and *open bars*, respectively

The glutathione redox state, established by the endogenous balance of GSH and GSSG, plays a key role in a variety of developmental processes (Tommasi et al. 2001; Yeung et al. 2005), including embryogenesis (Arrigoni et al. 1992; Belmonte et al. 2006; Belmonte and Stasolla 2007). As observed in this study (Fig. 1) a shift of the glutathione pool towards its oxidized form, GSSG, occurs in developing seeds to ensure the proper execution of the developmental pathway (Arrigoni et al. 1992). The imposition of an oxidized environment, which can be achieved in culture through exogenous applications of the GSH-biosynthetic inhibitor BSO (Griffith and Meister 1979) (Fig. 1) prevents cellular deterioration in the apical poles of the embryos (Fig. 2), thereby improving their quality. Belmonte et al. (2006) described a more "normal" or "zygotic like" appearance of embryos treated with BSO and a respective increase in conversion frequency of 28 and 85% for stages 2 and 3 MDEs, when this compound was added into the medium. These observations allowed us to dissect gene expression differences in a comparison between the abnormal structure observed in control embryos and the enhanced development of BSO-treated embryos. For clarity we will refer to "early response" as those developmental events affected by BSO at stage 1 and "late response" those affected by BSO later in culture.

The "early response"

The developmental transition from globular embryos (stage 0) to early cotyledonary embryos (stage 1), characterized by major histodifferentiation events required for proper cell and tissue patterning (Yeung et al. 1996), is accompanied by major changes in gene expression (more than 200 probes, Fig. 4a). The molecular regulation of these events does not seem to be greatly affected by BSO, as only 18 probes were differentially expressed between treatments during this transition phase (Fig. 7). However, among these probes are several encoding proteins related to developmental processes, including ARGONAUTE 1 (AGO1), a protein that is closely related to AGO7, and CLAVATA3 ESR-related 27 (CLE 27) (Fig. 8). The ARGONAUTE family are large proteins characterized by conserved PAZ and PIWI domains that are thought to be important for protein–protein interaction (Cerrutti et al. 2000). AGO proteins, in addition to their well characterized role in RNA interference (reviewed by Carmell et al. 2002), have been shown to regulate stem cell identity and cell fate decision in both animal and plant systems (Bohmert et al. 1998; Moussian et al. 1998; Cox et al. 1998; Kataoka et al. 2001). Genetic analyses suggest that AGO1 is required for both meristem formation and identity (Lynn et al. 1999; Kidner and Martienssen 2005), as in *ago1* mutant plants the shoot apical meristem is often replaced by a pin-like organ (Lynn et al. 1999). AGO1-mediated regulation of the apical meristem may be due to the interaction of this gene within several pathways, which include proper accumulation and localization of SHOOTMERISTEMLESS, specification of CUPSHAPED COTYLEDONS 1 and 2 boundaries, and expression of several homeodomain-leucine zipper (HD-ZIP) family members (Kidner and Martienssen 2005). Recent work (Nagasaki and Sato 2007) has showed that mutations in rice *SHL4/SHOOT ORGANIZATION2* (*SHO2*), which encodes an ortholog of *Arabidopsis* AGO7, result in abnormal meristem formation during embryogenesis. Taken together these results suggest that the BSO-activation of several *ARGONAUTE* genes between stage 0 and stage1 may be required to ensure proper meristem formation. Another developmentally regulated probe that is induced by BSO treatments during the early embryogenic phases encodes a CLE 27 protein. The *Arabidopsis* genome contains 31 *CLE* genes encoding 26 short peptides. Although some of the *CLE* genes, have been shown to unequivocally regulate meristem size (Fletcher et al. 1999; Fiers et al. 2005), not much is known about the functions of the other members. Recent work (Ito et al. 2006) suggests that CLE 27 may play a similar role in meristem development.

The "late response"

Unlike the early stages, late stages (2 and 3) control and BSO-treated embryos are characterized by profound differences in gene expression patterns (Fig. 7). Several differentially expressed probes falling in this "late response" were clustered within the categories of "Antioxidant response", "Developmental response", "Hormone-related", and

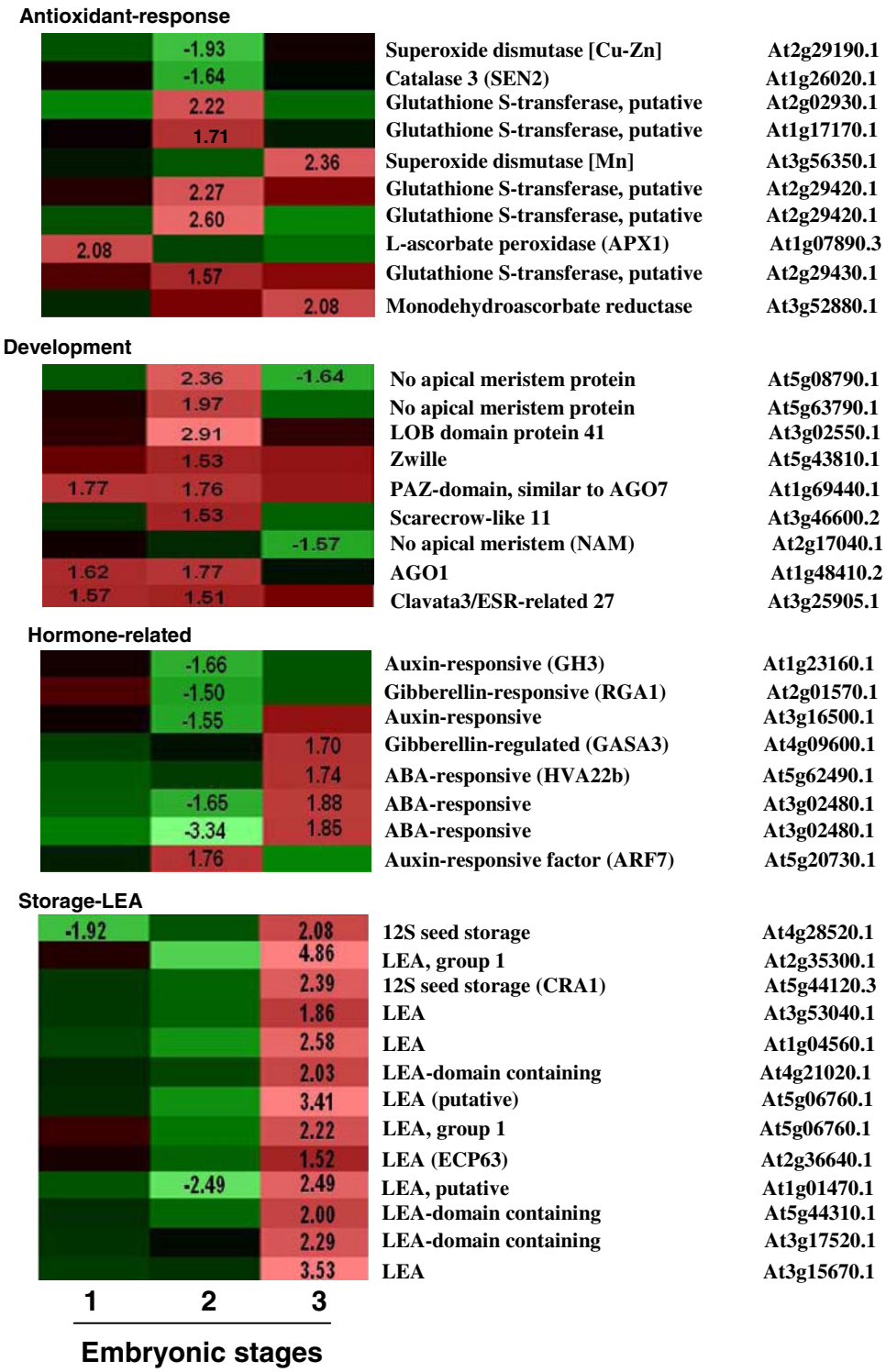


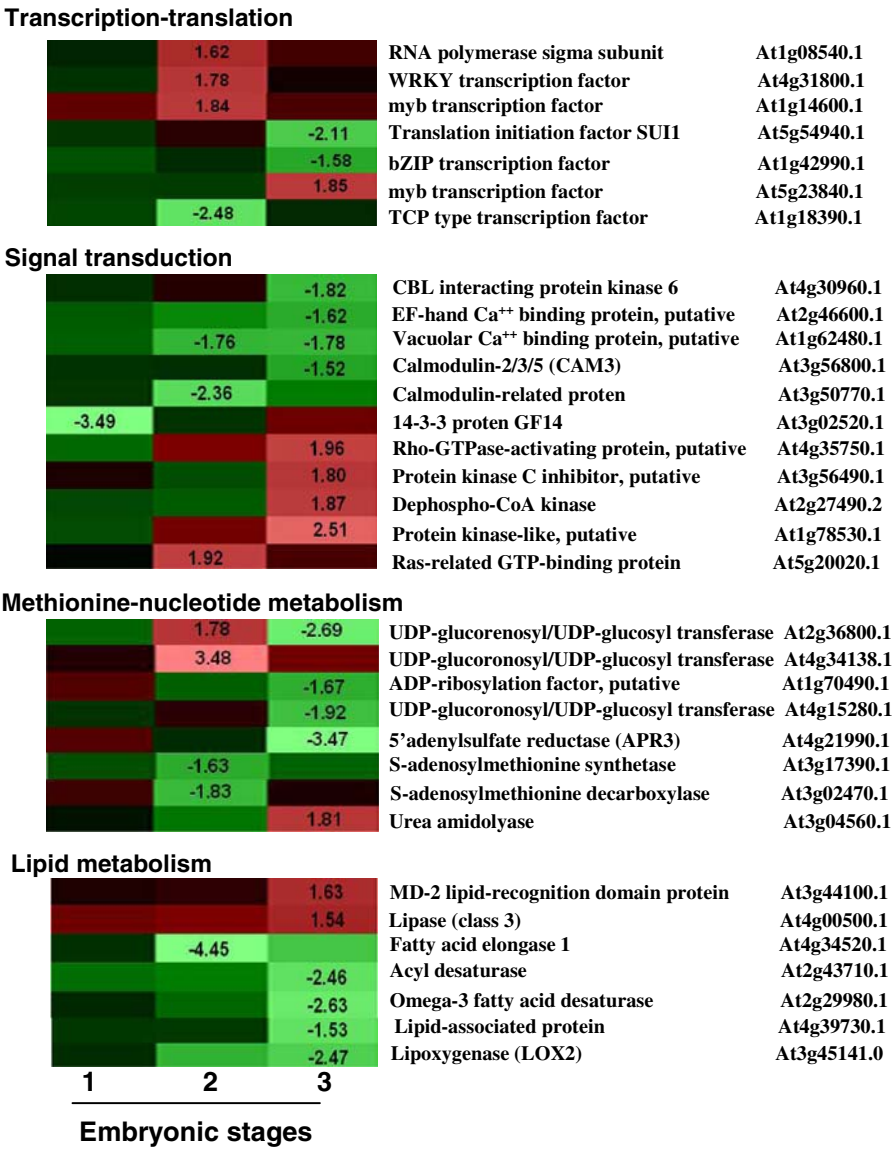
Fig. 8 Expression differences of probes involved in several cellular processes between control and BSO-treated embryos at stage 1, 2, and 3 of development. *Colors* indicate probes which are either up-regulated (*red*) or down-regulated (*green*) in BSO embryos compared to control. The *color* scale shown in Fig. 4b depicts fold changes in the expression ratios between samples. The *number* in each box indicates fold-change values that are statistically significant using the criteria outlined in "Material and methods". The gene identifier for the most closely related *Arabidopsis* gene is indicated for each *B. napus* probe

"Storage proteins and LEAs" and are discussed in the following sections.

Antioxidant response

Several probes encoding antioxidant-related proteins were differently expressed between treatments at stages 2 and 3 (Fig. 8). Five probes (two of them representing different *B. napus* orthologues corresponding to the same *Arabidopsis* gene) encoding glutathione-*S*-transferase (GST) proteins are up-regulated in stage 2 BSO-treated embryos. GSTs are homo- or hetero-dimeric proteins involved in detoxification processes through the conjugation of glutathione to a variety of substrates including reactive oxygen species. These

Fig. 8 continued



proteins are generally found in several cells and tissue types, although they are preferentially present during intensive active growth. In animal systems GSTs are expressed especially during embryonic and fetal growth where they support normal growth and development by reducing the levels of toxic organic hydroperoxides (Raijmakers et al. 2001). A similar role may also be exercised during plant embryo development, as this ubiquitous group of proteins has been shown to increase during embryo development in a variety of culture systems (Vrinten et al. 1999; Galland et al. 2001; Maraschin et al. 2006). BSO-mediated up-regulation of several GSTs may therefore be required for protecting the developing embryos against stress conditions, thereby ensuring successful growth and normal development.

Since glutathione metabolism is closely linked and dependent upon ascorbate synthesis and utilization in MDEs, it is not surprising that BSO affects several key ascorbate enzymes, including ascorbate peroxidase (APX), and monodehydroascorbate reductase (MDHAR). Ascorbate peroxidase is a well characterized ASC-consuming enzyme (De Gara and Tommasi 1999). The higher APX activity in BSO-treated embryos during early development may have a double function; it may have a major role in decreasing the toxic levels of H_2O_2 generated during active embryonic growth, as the transcript levels of a catalase probe is lower in these embryos, and may also maintain cell wall plasticity by reducing the pool of H_2O_2 utilized by cell wall peroxidases in cross-linking cell wall polymers (De Gara et al. 1996). A reduction in cell wall-linking peroxidase activity may be beneficial for embryo development and shoot meristem formation (Stasolla and Yeung 2006). The increased APX activity observed in BSO-treated embryos can be reversed by applications of GSH, suggesting

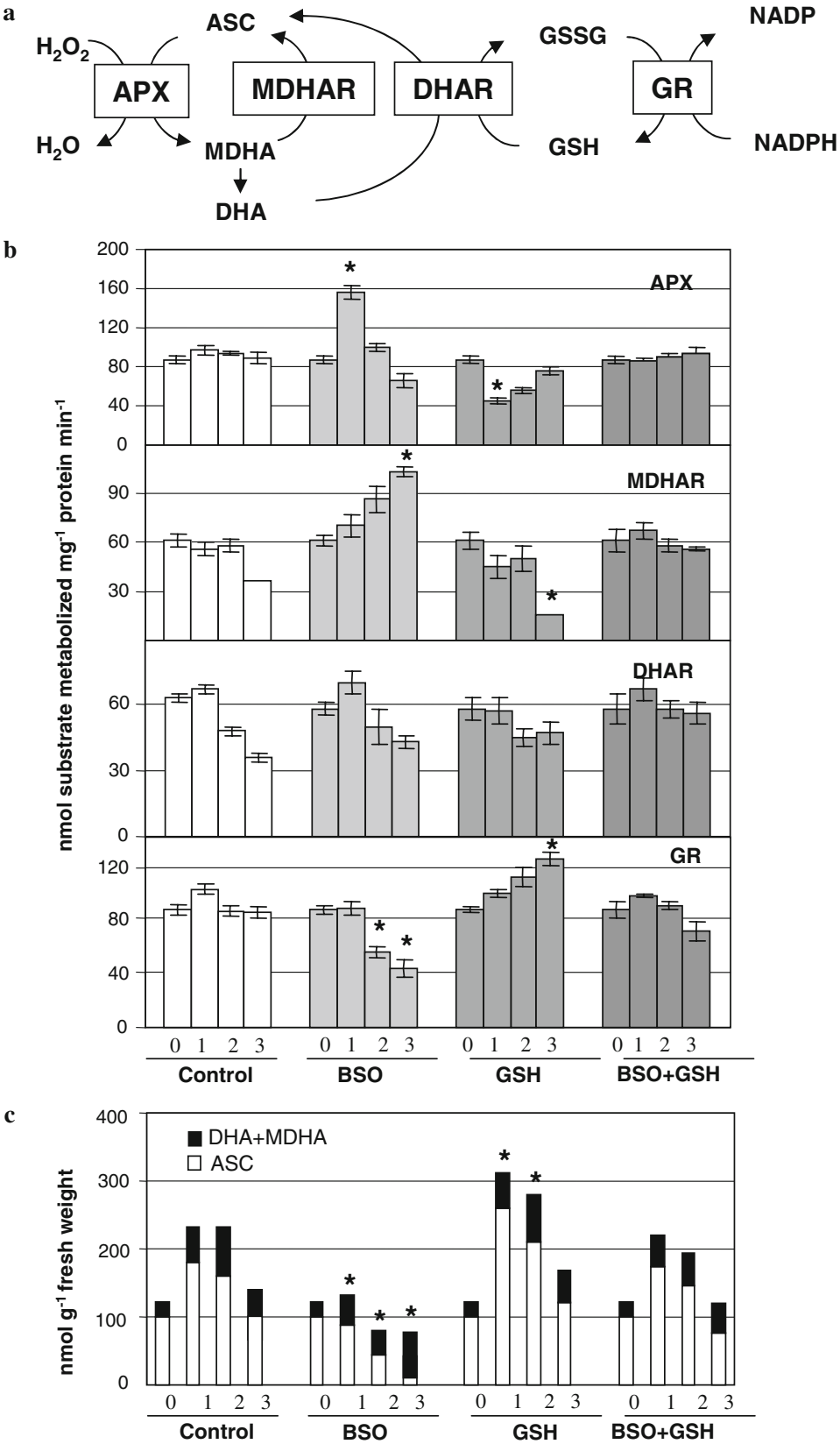


Fig. 9 **a** Schematic diagram of the ascorbate-glutathione system. *ASC* reduced ascorbate, *MDHA* monodehydroascorbate, *DHA* dehydroascorbate, *GSH* reduced glutathione, *GSSG* oxidized glutathione, H_2O water, H_2O_2 hydrogen peroxide, $NADP^+$ nicotinamide adenine dinucleotide phosphate oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form, *MDHAR* monodehydroascorbate reductase, *APX* ascorbate peroxidase, *DHAR* dehydroascorbate reductase, *GR* glutathione reductase. **b** Enzymatic activity of *APX*, *MDHAR*, *DHAR*, and *GR* during the different stages (0–3) of untreated embryo and embryos treated with *BSO*, *GSH*, and *BSO* + *GSH*. Values ± SE represent the mean of 3 or more independent experiments. *Stars* above each *bar* indicate values which are significantly different from control values ($P \leq 0.05$) at the same sampling time. **c** Endogenous ascorbate pool measured in developing embryos subjected to the treatments indicated in (**b**)

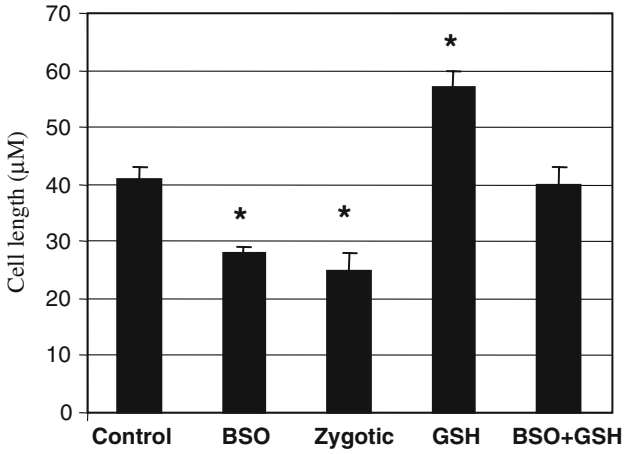


Fig. 10 Cortical cell elongation in zygotic embryos, untreated (*control*) microspore-derived embryos and MDEs treated with *BSO*, *GSH*, and *BSO* + *GSH*. Cortical cells in the middle section of the hypocotyl were measured at stage 3 (and fully mature zygotic embryos). Values ± SE represent the mean of three independent experiments. *Stars* above each *bar* indicate values that are significantly different from control values ($P \leq 0.05$) at the same sampling time

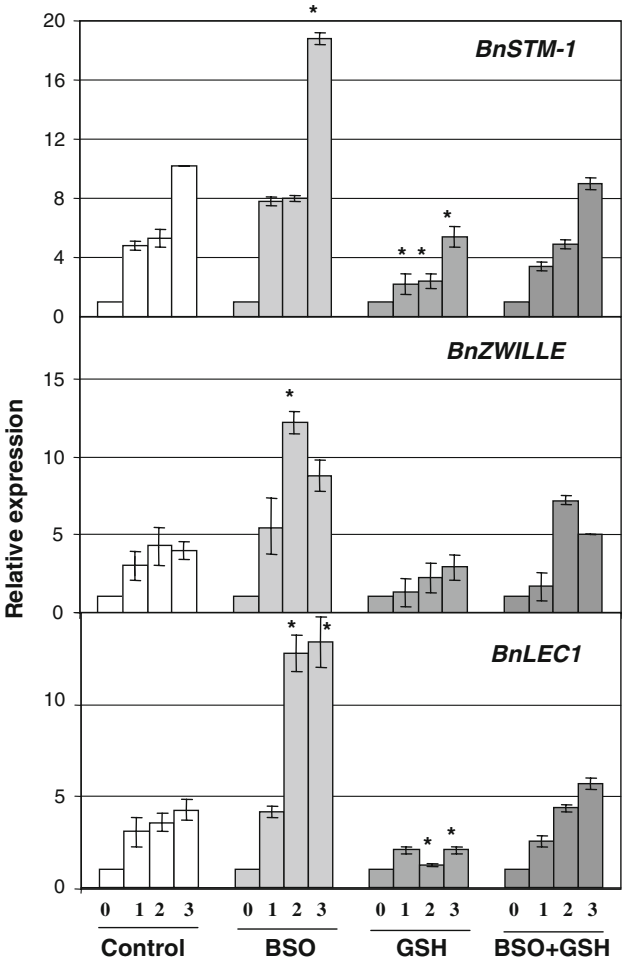


Fig. 11 Expression profiles of *B. napus STM–1*, *ZWILLE*, and *LEC1* during the four stages of development (0–3) in untreated embryos and embryos cultured in the presence of *BSO*, *GSH*, and *BSO* + *GSH*. The relative expression is based on comparisons to transcript levels in stage 0 embryos, which are set at 1. The values represent the average of at least three independent experiments. *Stars* above each *bar* indicate values which are significantly different from control values ($P \leq 0.05$) at the same sampling time

that the regulation of this enzyme is controlled by the cellular glutathione redox-state. High levels of APX during the early development of BSO-treated embryos also reduce the total cellular ascorbate pool. This would explain why applications of BSO reduce the overall size of MDEs (Belmonte et al. 2006). In agreement with previous studies, which show a correlation between ascorbate levels and cell division and elongation (reviewed by Potters et al. 2005), low ascorbate levels in BSO-treated MDEs also correlate with a reduction in cell elongation, although no effect on cell division was observed (data not shown).

During the late phases of development the BSO-induced oxidized glutathione environment affects the transcription and activity of MDHAR, as well as the composition of the total ascorbate pool, which becomes enriched with the oxidized forms, i.e. DHA and MDHA. The high activity of the ASC recycling enzyme MDHAR and the increasing levels of MDHA and DHA in BSO-treated embryos may be a strategy to efficiently regenerate ASC at the onset of germination, prior to the complete reactivation of the de novo ASC biosynthetic pathway. These metabolic alterations, documented during normal embryonic growth in vivo and in vitro (De Gara et al. 1997; Stasolla and Yeung 2001), are not observed in control embryos. The potential to quickly regenerate ASC from MDHA in the presence of an oxidized glutathione environment may explain the improved conversion frequency of BSO-treated embryos (Belmonte et al. 2006). High levels of ASC during the early phases of germination are required for meristem reactivation and the successful continuation of post-embryonic development in vitro (Stasolla and Yeung 2006).

Developmental response

Many probes encoding genes directing developmental processes were up-regulated in BSO-treated embryos at stage 2 (Fig. 8). These included those participating in the "early response", such as *AGO1* and *CLE 27*, as well as *ZWILLE*. Both the microarray and RT-PCR data showed that *ZWILLE* is up-regulated at stage 2 in BSO-treated embryos (Figs. 8, 11). In *Arabidopsis ZWILLE* plays a key role in maintaining an undifferentiated group of cells in the shoot meristem during late embryogenesis (Moussian et al. 1998). Mature *zll* embryos have disrupted shoot apical meristems in which the stem cells differentiate and fail to generate new organs during post-embryonic growth (Moussian

et al. 1998). *ZWILLE* has been suggested to specify the expression pattern of SHOOTMERISTEMLESS (STM), which is also involved in the maintenance of the apical pole (Long et al. 1996). Our RT-PCR data indicate that expression of an *STM* homolog increased in BSO-treated embryos at stages 3. These changes in expression patterns, due to the BSO-induced oxidized cellular environment as they are generally reversed in embryos treated with GSH, suggest that BSO improves the formation and maintenance of the apical poles through the activation of meristem-specific genes. Reduced or misexpression of these genes in the apical pole of control embryos may contribute to the structural deterioration observed during late embryogeny leading to stem cell differentiation.

We further investigated whether BSO controls other general embryonic processes by conducting RT-PCR analyses on a set of "embryo marker" genes identified by Malik et al. (2007). Among the genes tested, only *Bn LEC1* is affected by BSO during stages 2 and 3 (Fig. 11). LEC1 is a transcription factor involved in several developmental processes ranging from cotyledon identity specification and proper completion of embryo growth (Lotan et al. 1998). Its over-expression in vegetative tissue has been shown to induce somatic embryogenesis (Lotan et al. 1998). West et al. (1994) showed that *lec1* embryos are intolerant to desiccation, lack protein bodies in their cotyledons, and initiate post-germinative development prematurely. Together, these results suggest that the up-regulation of *LEC1* in BSO-treated embryos contributes to the acquisition of maturity and improved post-embryonic performance.

Hormone-related mechanisms

Buthionine sulfoximine treatments alter the expression patterns of several hormone-responses/regulated probes (Fig. 8). Of interest are three probes encoding two ABA-responsive proteins that are up-regulated by BSO at stage 3. This observation is in line with previous studies (Belmonte et al. 2006) showing that BSO alters ABA synthesis and catabolism during embryo development. We suggested that the beneficial effect of BSO during embryo growth was possibly due to an increased level of ABA, which plays a key role during in vivo and in vitro embryo maturation (reviewed by Kermode 1995; Thorpe and Stasolla 2001). Application of ABA in the MDE culture mimicked the effects of BSO, whereas a reduction of cellular ABA through fluoridone addition had a negative effect (Belmonte et al. 2006).

Ethylene is a gaseous plant growth regulator which often accumulates in culture under stress conditions (Gaspar et al. 1996), causing pronounced structural abnormalities including the formation of intracellular air-spaces observed in control MDEs (Fig. 2). Synthesis of ethylene is initiated by conversion of *S*-adenosylmethionine into 1-aminocyclopropane-1-carboxylate (ACC) which is subsequently converted to ethylene by the enzyme ACC oxidase (reviewed by Gaspar et al. 1996). In BSO-treated embryos a probe encoding *S*-adenosylmethionine synthase, the enzyme generating *S*-adenosylmethionine from methionine, is down-regulated at stage 2 (Fig. 8). Down-regulation of this enzyme may reduce ethylene production and accumulation in BSO-treated embryos, thereby preventing cellular deterioration in the shoot meristem. In support of this observation, spruce somatic embryos treated with BSO produce less ethylene (Belmonte et al. 2005).

Seed storage and LEA proteins

Storage product and late embryo-abundant (LEA) protein deposition during in vitro embryo development is a highly orchestrated process which is required for successful post-embryonic performance (Yeung 1995). While storage products are needed as an energy source for the germinating embryo, the highly hydrophilic LEA proteins protect many cellular components from the severe dehydration accompanying the later phases of seed/zygotic embryo development (reviewed by Kermode 1995). The up-regulation of many probes encoding storage products and LEAs during the late phases of development of BSO-treated embryos (Fig. 8) indicates a more complete "maturation state" reached by embryos cultured in an oxidized glutathione redox environment. These events, lacking in control embryos, suggest that under control conditions stage 3 embryos are morphologically ready to undergo post-embryonic growth but are not physiologically prepared to do so. This notion is also confirmed by Belmonte et al. (2006) who showed that compared to their control counterparts, BSO-treated embryos have a "zygotic-like" storage deposition pattern as they preferentially accumulate lipids and proteins, and to a lesser extent starch. The increased accumulation of storage and LEA proteins observed in the presence of BSO may be the result of transcriptional or post-translational events. BSO may promote transcription of these proteins by increasing the synthesis of ABA (Belmonte et al. 2006), as shown by Kermode (1995). Alternatively, the BSO-induced oxidized glutathione environment may stimulate post-translational mechanisms which facilitate folding and assembling of newly synthesized storage proteins, as suggested by De Gara et al. (2003). Increased accumulation of LEAs and storage products would improve the post-embryonic performance of the embryos.

In conclusion, the imposition of an oxidized environment, effected by BSO applications is beneficial for microspore-derived embryogenesis. Compared to their control counterparts, BSO-treated embryos show improvements in the structure of the apical meristems, as well as major

changes in transcript accumulation patterns. Many genes involved in a variety of functions, such as antioxidant response and developmental processes, are differentially expressed in the presence of BSO. Besides elucidating the effects of this beneficial compound during embryogenesis, this work has important implications for the identification of target genes which can be used to improve embryo production and quality via genetic engineering.

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