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RESEARCH PAPER

Modulation of embryo-forming capacity in culture through the expression of *Brassica* genes involved in the regulation of the shoot apical meristem

Mohamed Elhiti, Muhammad Tahir, Robert H. Gulden, Khalil Khamiss and Claudio Stasolla*

Department of Plant Science, University of Manitoba, Winnipeg, R3T 2N2, Manitoba, Canada

* To whom correspondence should be addressed. E-mail: stasolla@ms.umanitoba.ca

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Abstract

Somatic embryogenesis in *Arabidopsis* is achieved by culturing bending-cotyledon embryos on a 2,4-D-containing induction medium for 14 d followed by a transfer on to a hormone-free development medium. Several genes orthologous to *Arabidopsis SHOOTMERISTEMLESS* (*STM*), *CLAVATA 1* (*CLV1*), and *ZWILLE* (*ZLL*) were isolated from *Brassica oleracea* (*Bo*), *B. rapa* (*Br*), and *B. napus* (*Bn*), and ectopically expressed in *Arabidopsis* to assess their effects on somatic embryogenesis. Ectopic expression of *BoSTM*, *BrSTM*, and *BnSTM* increased the number of somatic embryos, whereas a different effect was observed in lines overexpressing *BnCLV1* in which somatic embryo formation was severely repressed. The introduction of *BnZLL* did not have any effects on *Arabidopsis* somatic embryogenesis. The increased embryo-forming capacity observed in lines overexpressing *Brassica STM* was associated with a lower requirement for the inductive signal 2,4-D, and a higher expression of *WUSCHEL* (*WUS*) which demarcates the formation of embryogenic cells. This was in contrast to the *35S::BnCLV1* lines which showed the highest requirement for exogenous 2,4-D and a reduced *WUS* expression. Microarray studies were conducted to monitor global changes in transcript levels during *Arabidopsis* somatic embryogenesis between the wild-type (WT) line and a *BoSTM*-overexpressing line, which showed the most pronounced enhancement of somatic embryo yield. The introduction of *BoSTM* affected the expression of many genes involved in hormone perception and signalling, as well as genes encoding DNA methyltransferases and enzymes of glutathione metabolism. Pharmacological experiments performed to confirm some of the microarray results showed that *Arabidopsis* somatic embryogenesis is encouraged by a global hypomethylation of the DNA during the induction phase and by a switch of the glutathione pool towards an oxidized state during the subsequent development phase. Both events occurred in the *35S::BoSTM* line, but not in the WT line. Altered expression of *Brassica STM* also had profound effects on *B. napus* microspore-derived embryogenesis. The yield of microspore-derived embryos increased in lines overexpressing *BnSTM* and significantly decreased in antisense lines down-regulating *BnSTM*.

Key words: Apical meristem, *Arabidopsis*, *Brassica*, embryogenesis, gene expression, glutathione.

Introduction

Embryogenesis in higher plants is initiated by the fusion of the sperm cell with the egg, resulting in the formation of a diploid zygote (Willemsen and Scheres, 2004). An important event during embryogenesis is the establishment of the shoot apical meristem (SAM), which in *Arabidopsis* is regulated by several genes including *SHOOTMERISTEMLESS* (*STM*), *ZWILLE* (*ZLL*), and *CLAVATA 1* (*CLV1*). *STM* encodes a knotted-like homeobox protein present throughout the vegetative and floral meristems (Barton and Poethig, 1993). Genetic studies (Barton and Poethig, 1993; Endrizzi *et al.*, 1996) demonstrate the requirement for STM for the formation of embryonic meristems and for their post-embryonic maintenance by suppressing differentiation and maintaining an indeterminate cell fate within the SAM.

Abbreviations: *CLV*, *CLAVATA*; GSH, reduced glutathione; GSSG, oxidized glutathione; MDE, microspore-derived embryo; SAM, shoot apical meristem; *STM*, *SHOOTMERISTEMLESS*; *WUS*, *WUSCHEL*; *ZLL*, *ZWILLE*.

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As also observed for *stm* mutants, *zll* embryos produce terminally differentiated cells instead of stem cells at the site of the SAM (McConnell and Barton, 1995; Moussian *et al.*, 1998; Lynn *et al.*, 1999). However *zll* seedlings were able to form adventitious SAMs originating between the cotyledons and to generate shoots *in vitro*. These observations are consistent with the notion that ZLL, a member of the ARGONAUTE family, is also required for the formation of the primary embryonic SAM, but, unlike STM, it is not needed for post-embryonic meristem function (McConnell and Barton, 1995; Moussian *et al.*, 1998; Lynn *et al.*, 1999).

Another key player in SAM regulation is CLV1, a leucine-rich repeat receptor kinase protein (Clark *et al.*, 1997), which promotes cell differentiation of the meristematic cells by limiting the expression of the *WUSCHEL* (*WUS*) domain through an elaborated signalling model involving other CLV members (reviewed by Dodsworth, 2009). A mutation in *CLV1* disrupts the balance between cell division and differentiation within the SAM culminating in enlarged meristems as a result of an atypical expansion of the *WUS* expression domain (Schoof *et al.*, 2000). Therefore, in contrast to STM which maintains stem cells in an undifferentiated state by preventing meristematic cells from adopting an organ-specific cell fate, the role of CLV1 is to limit the expansion of the undifferentiated stem cell population in the SAM and promote differentiation. Insights into the competitive role of STM and CLV1 in meristem activity have been documented by genetic studies (Clark *et al.*, 1996).

Embryogenesis can also occur *in vitro* via somatic embryogenesis or androgenesis (Thorpe and Stasolla, 2001). In *Arabidopsis*, somatic embryogenesis can be induced from immature zygotic embryos (Mordhorst *et al.*, 2002; Bassuner *et al.*, 2007). This system is very effective in providing a large number of embryos through an intervening callus phase, and represents an attractive 'proof of concept' model to examine the molecular events associated with the transition of somatic cells into embryos (Mordhorst *et al.*, 2002; Su *et al.*, 2009).

An alternative propagation system is androgenesis which uses immature pollen grains, or microspores, to generate microspore-derived embryos (MDEs). In recent years *Brassica napus* androgenesis has received increasing attention as a model to investigate embryogenesis in plants especially because of the genetic similarities shared between *Brassica* and *Arabidopsis*, and for the absence of a callus phase which allows studies during the initial phases of embryo development. These characteristics have been exploited for the identification of transcripts delineating the initial transition of gametophytic cells into embryogenic cells (Malik *et al.*, 2007), and the subsequent development of the MDEs (Stasolla *et al.*, 2008). Despite the increasing interest in *B. napus* microspore-derived embryogenesis, no information is currently available on genes involved in the formation and maintenance of the SAM. This is unfortunate as this system has been proved to be extremely suitable for examining the physiological factors required for proper meristem development (Belmonte *et al.*, 2006; Stasolla *et al.*, 2008). Thus the identification of *Brassica* SAM molecular markers is critical for elucidating the mechanisms governing meristem development during microspore-derived embryogenesis.

Over the past few years attempts have been made to determine if genes regulating SAM activity *in vivo* are also involved in the formation of embryonic cells during *in vitro* embryogenesis. While the majority of studies have used loss-of-function mutants (Ogas *et al.*, 1999; Mordhorst *et al.*, 1998, 2002), very few deal with overexpression experiments (Zuo *et al.*, 2002). The aim of the present work is to characterize *Brassica* genes orthologous to *Arabidopsis STM*, *ZLL*, and *CLV1*, and to investigate the effects of their ectopic expression on embryogenesis *in vitro*. The results indicate that (i) somatic embryogenesis in *Arabidopsis* is affected by the introduction of *Brassica STM* and *CLV1*, but not by *Brassica ZLL*; (ii) overexpression of *BnSTM* and *BnCLV1* has antagonistic effects on *Arabidopsis* somatic embryo formation, with *BnSTM* having a promoting role and *BnCLV1* a repressing role; (iii) the increased *Arabidopsis* somatic embryo production in tissue ectopically expressing *Brassica STM* entails profound changes in the expression of genes involved in different functions; and (iv) besides improving *Arabidopsis* somatic embryogenesis (an indirect embryogenic system requiring a callus phase), the introduction of *Brassica STM* also facilitates microspore-derived embryogenesis in *B. napus* (a direct embryogenic system without an intervening callus phase).

Materials and methods

Culture treatments

Production of *B. napus* cv. Topas DH4079 MDEs and *Arabidopsis* somatic embryos was achieved by following the method outlined in Belmonte *et al.* (2006) and Bassuner *et al.* (2007). Briefly, *Brassica* plants with flower buds were transferred to 12 °C day/7 °C night temperature cold treatment until bud collection. Flower buds (2–3 mm long) were collected and suspended in NLN medium (Lichter, 1982) with 13% sucrose (pH 5.8) where they developed into embryos after the imposition of a heat shock treatment at 32 °C for 72 h. Fully developed MDEs were observed after 25 d in culture (Belmonte *et al.*, 2006).

Arabidopsis somatic embryogenesis was induced by culturing bending-cotyledon zygotic embryos on a 2,4-D containing induction medium for 14 d followed by transfer onto a hormone-free development medium in which somatic embryos developed. This procedure was identical to that described by Bassuner *et al.* (2007).

Isolation of BnSTM, BnCLV1, BnZLL-1, *and* BnZLL-2, *and expression studies*

Total RNA extracted from *B. napus* MDEs after 28 d in culture was utilized for cDNA synthesis using a SuperScript™ II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). PCR-based amplification of full-length *BnSTM* cDNA (GU480584) was obtained using primers (5′-ATGGAAAAGTGGTTCCAACA-3′ and 5′-ATCCGGGACAATGCTTTGA-3′) designed from the sequence of *Brassica oleracea BoSTM* (AF193813) identified by Teo and Swarup (1999). *BnZLL-1* (EU329719) and *BnZLL-2* (GU731230) were isolated using primers (5′-ATGCCGATTAGGCAAATGAA-3′ and 5′-GAGTCATGTTTTACTGCTAA-3′) based on the sequence of *Arabidopsis* ZLL (NM 123748). *Brassica napus BnCLV1* (GU480585) was isolated by 5′ and 3′ RACE [rapid amplification of cDNA ends; First Choice RLM-RACE kit (Ambion, Austin, TX,

USA)] using a partial *CLV1* sequence (AY283519) from the same species.

Expression studies of *AtSTM*, *AtCLV1*, and *AtZLL* in different tissue of 28-d-old *Arabidopsis* plants and *BnSTM*, *BnCLV1*, *BnZLL-1*, and *BnZLL-2* in 40-d-old *Brassica* plants were performed by semi-quantitative reverse transcriptase-PCR (RT-PCR). Briefly, total RNA was extracted using a QIAGEN RNeasy® Plant mini Kit (catalogue no. 74904) and used to generate cDNA with Invitrogen SuperScript® II Reverse Transcriptase (catalogue no. 19064-002). For RT-PCR studies in *Brassica* the following primers were used: *BnSTM* (5′-TGATGGTCCGATGTGTCCTA-3′ and 5′-GCACCAGAGGAAGGAGAACA-3′), *BnCLV1* (5′-CGGCGGGGACTATTCACTA-3′ and 5′-AAGGAAGTGTCGTGAAGACTAGG-3′), *BnZLL-1* (5′-CGACGACGACGCCGCCAGCTCAGA-3′ and 5′-CAGACTCTTTGTATAGTCTCACTA-3′), and *BnZLL-2* (5′-GCCAAGCTTCTTCACCTTCTCCTC-3′ and 5′-CAGACTCTTTGTATAGTCTCACTA-3′). As an internal control actin (AF111812) was amplified using primers 5′-TAAAGTATCCGATTGAGCATGGTAT-3′ and 5′-GACATTAAAGAGAAGCTTGCCTACG-3′.

For *Arabidopsis* the following primers were used: *AtSTM* (5′-TGTCAGAAGGTTGGAGCACCA-3′ and 5′-TTTGTTGCTCCGAAGGGTAA), *AtCLV1* (5′-CTTAAATACCTCTCTTTCGGTGGA-3′ and 5′-ATCTCTATGCAAGATCAATGGTGA), and *AtZLL* (5′-CTGGTAAACGGGCAGATTGT-3′ and 5′-TTTTTCTTTCCCGTTCTCGTGATAC-3′). As an internal control ubiquitin (NM_178968) was amplified using the primers 5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′ and 5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGC-3′. The PCR products (during the exponential phase of amplification) were separated on 1% (w/v) agarose gels, and the intensities of ethidium bromide-stained bands were detected using ImageJ software (1.36b National Institutes of Health).

Light microscopy and localization studies

Histological studies and localization of *BnSTM*, *BnCLV1*, *BnZLL-1*, and *BnZLL-2* were performed by RNA *in situ* hybridization using the procedure outlined in Belmonte *et al.* (2006). For histological examination, the tissue was stained with periodic acid–Schiff (PAS) reagent for total carbohydrates and counterstained with toluidine blue (TBO) for general histological organization.

Transformation studies and in vitro regeneration

Arabidopsis transformation experiments were carried out using Gateway technology (Invitrogen, Carlsbad, CA, USA) as reported by Karimi *et al.* (2002). Seeds of the *Arabidopsis* ecotype Columbia were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH, USA).

Full-length *BoSTM*, *BrSTM*, *BnSTM*, *BnCLV1*, *BnZLL-1*, and *Bn-ZLL-2* cDNAs were inserted in the Gateway entry clone vector pDONR™221 (Invitrogen) according to the instruction manual and then transferred into the pK2GW7 (for sense transformation) or pK2WG7 (for antisense transformation) vectors carrying the 35S promoter and the 35S terminator (http://www.psb.ugent.be/gateway/index.php). The constructs were introduced into the *Agrobacterium tumefaciens* strain GV3101 which was utilized to spray transform wild-type (WT) *Arabidopsis* plants via standard *Agrobacterium*-mediated transfection techniques (Weigel and Glazebrook, 2002). Many transformed plants were generated and the level of the transgene was measured by quantitative RT-PCR (Supplementary Fig. S1 available at *JXB* online).

Brassica transformation with *BnSTM* in sense or antisense orientation was performed as described by Bhalla and Singh (2008). Briefly, surface-sterilized seeds were germinated for 5 d on 1/2 MS-B5 medium supplemented with 1% sucrose. Hypocotyls (2–3 mm) were inoculated on co-cultivation medium [MS-B5 medium supplemented with 3% sucrose and 2 mg l^{-1} benzyladenine (BA)] for 4 d, and then incubated with the same *Agrobacterium* strain used for *Arabidopsis* transformation, harbouring the pK2GW7 (for sense transformation) or pK2WG7 (for antisense transformation) vector for 2 min. Following a 4 d co-cultivation period, the explants were plated on shoot induction medium (MS-B5 supplemented with 5 mg l^{-1} $AgNO_3$, 2 mg l^{-1} BA, 3% sucrose, 20 mg l^{-1} timentin, and 50 mg l^{-1} kanamycin) for 8 weeks. The emerging shoots were first transferred on elongation medium (MS-B5 supplemented with 3% sucrose, 0.1 mg l^{-1} GA_3, and 1 mg l^{-1} BA) for 4 weeks and then placed on rooting medium [MS-B5 supplemented with 1% sucrose, 2 mg l^{-1} 1-naphthaleneacetic acid (NAA)] for 4 weeks. Rooted shoots were transferred onto soil to generate fully mature plants (F_0). Seeds from F_0 plants were germinated on 1/2 MS basal medium with 1% sucrose and 50 mg l^{-1} kanamicin, and the resulting F_1 generation was screened for the presence of the transgene by RT-PCR using a forward 35S promoter primer (5′-TGGACCCCCACCCACGAG-3′) and a reverse gene-specific primer (5′-GCACCAGAGGAAGGAGAACA-3′). Microspores harvested from the positive F_1 plants were cultured to produce haploid MDEs which were germinated on 1/2 MS medium supplemented with 1% sucrose and 70 mg l^{-1} kanamycin for 4 weeks. The haploid seedling were then treated with 0.2% colchicine for 6 h in order to generate homozygous (double haploid) plants. After checking the ploidy level by chromosome counting and confirming the presence of the transgene by RT-PCR, selected plants were used as a source of seeds to generate the next (F_2) generation. The expression level of *BnSTM* in these plants was measured by quantitative RT-PCR (Supplementary Fig. S2 at *JXB* online).

Microarray development: experimental design

Differences in global changes in transcript levels during somatic embryogenesis of WT *Arabidopsis* plants and plants ectopically expressing *BoSTM* (*35S::BoSTM*) were measured using the full genome *Arabidopsis* oligo microarrays [Quiagen-Operon Arabidopsis Ready Oligo Set (AROS) version 3.0] containing 29 000 elements and available through the University of Arizona. The experimental design was identical to that utilized in previous studies (Stasolla *et al.*, 2008). Tissue during *Arabidopsis* somatic embryogenesis was collected at the beginning of the induction period (stage 0), at the end of the induction period (stage 1), and after 3 d and 9 d in development medium (stages 2 and 3, respectively). Three biological replicates were used for each of the four WT samples and four *35S::BoSTM* samples. 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 eight samples. This experimental design provided information on gene expression during the progression of somatic embryo development (stage 0, 1, 2, and 3) in the WT and *35S::BoSTM* lines, i.e. time course experiment, as well as between stages of development (WT versus *35S::BoSTM* at stage 0, 1, 2, and 3), i.e. direct comparison analysis. A swapped-dye experiment (stage 1 versus common reference) was also conducted to verify the effect of the dye on the hybridization outcome (Stasolla *et al.*, 2008).

Microarray hybridization and scanning

Total RNA extracted from developing somatic embryos produced by the WT and *35S::BoSTM* lines was amplified, labelled with fluorescent Cy3 or Cy5 dyes, and fragmented, using an RNA ampULSe: Amplification and Labelling kit (Kreatech Biotechnology, Amsterdam, The Netherlands) following the manufacturer's instructions. The integrity of the amplified RNA and the degree of labelling were determined by gel electrophoresis and by measuring Cy5 and Cy3 using a Nano-Drop ND-1000 spectrophotometer (Nano Drop Technologies, Thermo Scientific).

Microarray hybridization was performed as described at http://ag.arizona.edu/microarray/methods.html. Briefly, after a pre-hybridization treatment in a buffer containing 5× SSC, 0.1% SDS,

and 0.1 mg ml^{-1} bovine serum albumin (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 1000 *g* for 2 min and stored until hybridization. The slides were then hybridized using 200 ng of Cy5-labelled target and 350 ng of Cy3-labelled target added to warm (42 °C) hybridization solution containing 2% formamide, 5× SSC, 0.1% SDS, and 0.1 mg ml^{-1} salmon sperm DNA. Hybridization was carried out for 16 h at 42 °C in a hybridization oven. 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 an Axon GenePix 4000B laser scanner (MDS Analytical Technologies) with GenePix® Pro. Software Version 6.

Data analysis

Differences in gene expression were determined using a mixed model approach developed by Wolfinger *et al.* (2001) and used in previous studies (Stasolla *et al.*, 2004). Prior to mixed model analysis of normalized log ratio expression data, flagged and error-tagged observations were removed. To improve normality of the data, outliers were removed based on studentized residuals (Lund, 1975). Although the experimental design was a two-way factorial treatment structure with main factors stage (0–3) and lines (WT versus *35S::BoSTM*), the data were analysed as a one-way treatment structure. This reduced the impact of missing data points on the key comparisons that were required. Data were analysed within each gene considering treatment as the fixed effect and array as the random effect. Within each analysis, *a priori* expression differences were examined between 10 pairs of treatments. Differences were evaluated with four direct comparisons of WT versus transformed tissue at each time point (WT versus *35S::BoSTM* at stages 0, 1, 2, and 3), and changes in gene expression over time were evaluated with three comparisons among successive time points (stage 0 versus 1, 1 versus 2, and 2 versus 3) within WT and *35S::BoSTM* lines. To limit the experiment-wise type 1 error rate, experiment-wise (i.e. all genes combined) *P*-values were adjusted using the FDR (false discovery rate) adjustment option in the MULTITEST procedure in SAS (e.g. Saama *et al.*, 2006). The FDR option adjusts *P*-values based on the Benjamini and Hochberg step-up procedure (Benjamini and Hochberg, 1995). The FDR adjusted *P*-values were used to verify whether a difference observed between stages or lines was statistically significant. A first selection was performed by comparing the expression ratios of signal intensities at different stages or between WT and *35S::BoSTM* lines, and selecting only those probes having an adjusted *P*-value $\leqslant 0.01$ and an absolute difference in expression ratios >4. Furthermore, in order to exclude possible dye effects, all probes exhibiting an opposite behaviour in the swapped-dye experiment were eliminated from the final analysis as per Stasolla *et al.* (2008).

The normalized expression ratios of the differentially expressed probes were imported in Tree View (http://sourceforge.net/projects/jtreeview/) and used to perform hierarchical cluster analysis, cluster analysis by self-organizing maps (SOMs), and principal component analysis (PCA).

Validation of microarray data

Microarray data were validated by quantitative real-time RT-PCR according to Stasolla *et al.* (2003) for several selected genes (Supplementary Table S1 at *JXB* online) that showed differential expression patterns during embryo development. The relative level of gene expression was analysed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001) using actin (AY139999) as a reference.

Glutathione metabolism

Measurements of glutathione content, glutathione reductase (GR) activity, and glutathione applications were performed according to Belmonte *et al.* (2006).

Methylation assays

DNA methylation was estimated using coupled restriction enzyme digestion and random amplification (CRED-RA), as reported by Leljak-Levanic *et al.* (2004). Briefly, the DNA extracted from WT and *35S::BoSTM* lines at stage 1 of somatic embryogenesis was digested with *Hpa*II and *Msp*I endonucleases (which cut the sequence 5′-C/CGG-3′ with different sensitivity to cytosine methylation; *Msp*I cuts if the inner C is methylated, whereas *Hpa*II cannot cleave in the presence of methyl groups), and then used as a template in a PCR employing six random 10-base primers: 5-GACTGCACAC-3 (primer 1), 5-AGGCGGGAAC-3 (primer 2), 5-ACGATGAGCC-3 (primer 3), 5-ACCGCCTGCT-3 (primer 4), 5-AGGCCGGTCA-3 (primer 5), and 5-CAACCGGTCT-3 (primer 6). The PCRs were performed in a total volume of 25 µl using: 1 µl (50 ng) of digested DNA, 1 µl of 10 nM primer, 0.5 µl of 10 nM $MgCl_2$, 11.25 µl of nuclease-free H_2O, and 11.25 µl of 2× BioMix master mix (GE Healthcare UK Limited, Amersham, UK). The PCR conditions and measurements of the methylated bands were carried out exactly as described by Leljak-Levanic *et al.* (2004).

Results

Isolation and characterization of Brassica napus BnSTM, BnCLV1, BnZLL-1, *and* BnZLL-2

Using the available nucleotide sequence of *STM* from *Arabidopsis* and of *CLV1* and *ZLL* from other *Brassica* species, full-length *B. napus BnSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* were isolated through a PCR-RACE approach. The four genes encode proteins with a high degree of amino acid sequence identity to their respective *Arabidopsis* orthologues (91% for *Bn*STM, 88% for *Bn*CLV1, and 96% for *Bn*ZLL-1 and *Bn*ZLL-2) and with all the conserved domains. These include the KNOX1, KNOX2, ELK, and a homeodomain for *Bn*STM, a serine/threonine kinase domain for *Bn*CLV1, and the PIWI and PAZ domains for *Bn*ZLL-1 and *Bn*ZLL-2 (Supplementary Fig. S3 at *JXB* online). Phylogenetic analyses further confirm the similarities of the *Brassica* genes to their *Arabidopsis* orthologues (Supplementary Fig. S4).

The relative expression of the *B. napus* genes in *B. napus* plants was measured in different tissues and compared with the expression pattern of the *Arabidopsis* orthologues measured in *Arabidopsis* plants (Fig. 1). Like *AtSTM* in *Arabidopsis* plants, the highest levels of *BnSTM* expression in *B. napus* plants were observed in shoot tips, flowers, and siliques. Transcripts of *BnCLV1*, *BnZLL-1*, and *BnZLL-2* were detected in all those tissues where their respective *Arabidopsis* orthologues were also expressed (Fig. 1). Both *BnSTM* and *BnCLV1* were expressed in the SAM of *B. napus* zygotic embryos and MDEs (Fig. 2). The combined signal of *BnZLL-1* and *BnZLL-2* (the high degree of similarity in the sequences of the two genes did not allow for the design of specific probes) was first localized in the vascular tissue of immature zygotic embryos and then extended to the SAM of fully developed cotyledonary

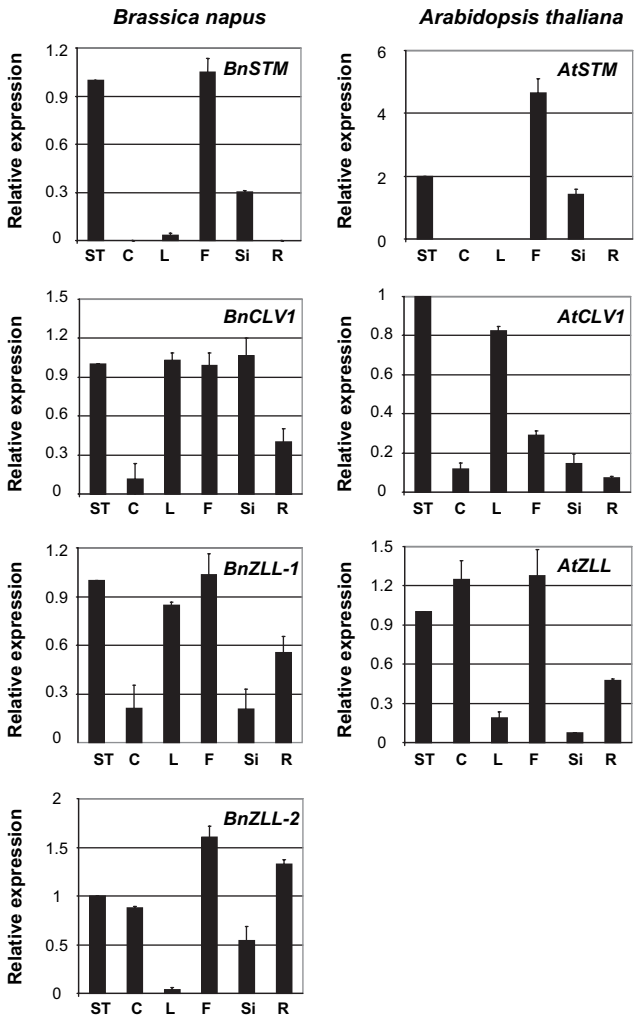

Fig. 1. (A) Expression studies by semi-quantitative RT-PCR of *BnSTM*, *BnCLV1*, *BnZLL-1*, and *BnZLL-2* in *Brassica napus* tissues, and *AtSTM*, *AtCLV1*, and *AtZLL* in *Arabidopsis* tissues. Shoot tips (ST), cotyledons (C), leaves (L), flowers (F), siliques (Si), and roots (R) harvested from *B. napus* (40-d-old plants, with the exception of C harvested from 10-d-old plants) and *Arabidopsis* (28-d-old plants with the exception of C harvested from 10-d-old plants). Values ±SE (n=3) were normalized to the expression level in ST set at 1.

embryos. A similar localization pattern to that observed for zygotic embryos was also observed in MDEs (Fig. 2). The expression domains of the *Brassica* genes in *B. napus* zygotic embryos and MDEs coincided perfectly with those described for the *Arabidopsis* orthologues, which are also expressed in the SAM of *Arabidopsis* embryos (reviewed by Dodsworth, 2009).

Effects of ectopic expression of Brassica *genes on* Arabidopsis *somatic embryogenesis*

Given the very low efficiency of *B. napus* Topas (DH4079 embryogenic line) transformation, the effects of ectopic expression of the four *Brassica* genes during *in vitro* embryogenesis were first analysed in *Arabidopsis*. Gene(s) promoting somatic embryogenesis were then introduced in sense and antisense orientation in *B. napus* to verify if their effects were also retained during microspore-derived embryogenesis.

Independently transformed *Arabidopsis* lines ectopically expressing *BnSTM*, *BnCLV1*, *BnZLL-1*, or *BnZLL-2* were generated (Supplementary Fig. S1 at *JXB* online) and utilized to produce somatic embryos by first culturing bending-cotyledon zygotic embryos on a 2,4-D-enriched induction medium for 14 d and then transferring them onto a hormone-free development medium (Fig. 3A). Differences in the initial explants, i.e. bending-cotyledon zygotic embryos, were observed among the transformed lines (Fig. 3B). Compared with WT embryos, the introduction of *BnCLV1* decreased the size of the SAM and induced the accumulation of storage products in the subapical cells. This was in contrast to *35S::BnSTM* zygotic embryos which showed well-defined, dome-shaped SAMs (Fig. 3B).

The number of explants (bending-cotyledon zygotic embryos) producing somatic embryos doubled in three *35S::BnSTM* lines (2–4) analysed (Fig. 3C). This prompted the ascertainment of whether these results could be replicated, and possibly further improved, by overexpressing the *STM* orthologues from *B. oleracea* (AF193813) and *B. rapa* (GU480585) (denoted as *BoSTM* and *BrSTM*, respectively), which are the progenitor species of *B. napus*. The two cDNAs were isolated using a PCR-based approach followed by RACE. Protein sequence alignment revealed a high degree of identity between *Bn*STM and the two *Brassica* orthologues (97% for *Br*STM and 99% for *Bo*STM). Ectopic expression of both *BoSTM* and *BrSTM* (Supplementary Fig. S1 at *JXB* online) increased the response of the explants to the culture conditions (Fig. 3C). The number of embryo-forming explants in the *35S::BnZLL-1* and *35S::BnZLL-2* lines was similar to control levels, although a small increment was observed for one *35S::BnZLL-2* line. All the *35S::BnCLV1* lines analysed showed a decrease in the number of explants able to generate embryos (Fig. 3C).

Arabidopsis somatic embryo composition was further examined in all transformed plants, and the results of representative lines are shown in Fig. 3C. The number of embryos produced by each responsive explant increased in all lines overexpressing the *Brassica* (*Bo*, *Bn*, and *Br*) *STM* gene especially in line 5 (*35S::BoSTM*) in which almost 90% of the responsive explants generated ⩾5 embryos. No appreciable differences in embryo composition were observed between the *35S::BnZLL-1* and *BnZLL-2* lines and the WT counterpart, whereas the number of explants producing only one somatic embryo almost doubled in all *35S::BnCLV1* lines.

Effects of BnSTM *overexpression or down-regulation on* B. napus *MDE*

To evaluate if the beneficial effects of *Brassica STM* overexpression were only restricted to embryogenic systems with an intervening callus phase, such as *Arabidopsis* somatic embryogenesis, microspore-derived embryogenesis was induced in *B. napus* lines overexpressing or down-regulating

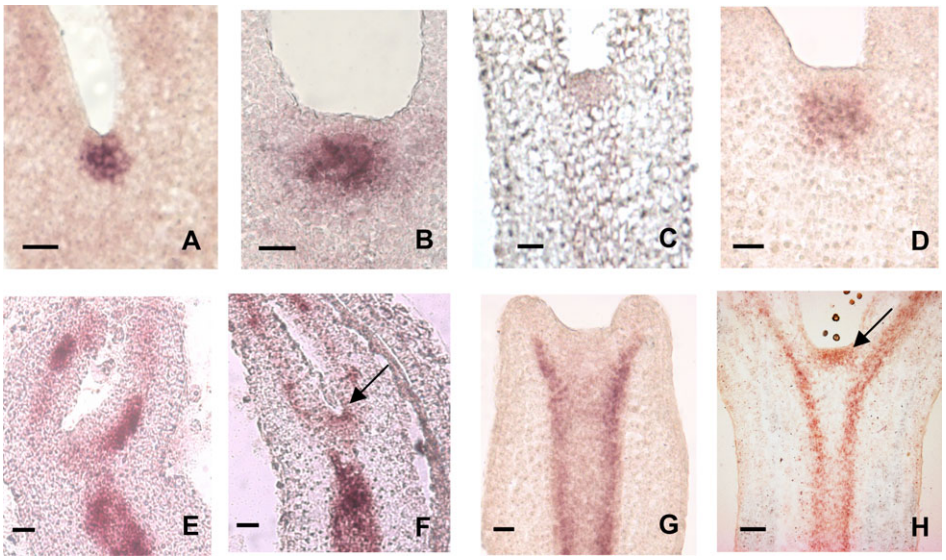

Fig. 2. RNA *in situ* hybridization of *BnSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* in *Brassica napus* zygotic embryos at the late cotyledonary stage (A, C, and F) or at the middle cotyledonary stage (E) of development, and in microspore-derived embryos (MDEs) cultured for 15 d (G) or 25 d (B, D, and H). The localization patterns of both *BnSTM* (A, B) and *BnCLV1* (C, D) are restricted to the apical pole of both zygotic embryos and MDEs. The combined expression of Bn*ZLL-1* and *BnZLL-2* (the high degree of similarity in the sequences of the two genes did not allow for the design of specific probes) is first localized in the vascular tissue of both middle cotyledonary zygotic embryos (E) and 15-d-old MDEs (G), and then extended to the apical regions (arrows) of late cotyledonary zygotic embryos (F) and 25-d-old MDEs (H). All scale bars=40 μm

BnSTM (Supplementary Fig. S2 at *JXB* online). In this system microspores develop directly into embryos (MDEs) without the formation of callus. Differentiation of microspores into MDEs increased significantly in sense lines overexpressing *BnSTM*, whereas an inhibition of embryo-forming capacity was observed in antisense lines with reduced *BnSTM* levels (Fig. 4). The down-regulation of *BnSTM* resulted in profound morphological abnormalities (Fig. 5). While MDEs produced by the WT line were characterized by two distinct cotyledons and an elongated embryonic axis, MDEs with reduced *BnSTM* expression were shorter and often exhibited fused cotyledons. No morphological alterations were observed in MDEs produced by lines overexpressing *BnSTM* (Fig. 5).

Transcriptome analysis during Arabidopsis *somatic embryogenesis*

Microarray analysis was conducted during stages of *Arabidopsis* somatic embryos produced by the WT line and the *Brassica STM*-overexpressing line showing the most pronounced enhancement of somatic embryogenesis, i.e. *35S::BoSTM* (line 5). This comparison would allow identification of genes responsive to the introduction of the *Brassica STM* gene and important to the formation of somatic embryos.

In the WT line, 885 probes were differentially expressed (fold change >4 and $P \leqslant 0.01$) between stage 0 and 1, and this number declined during the successive comparisons (315 between stage 1 and 2, and 56 between stage 2 and 3). A similar decline in expression profile was also observed in the *35S::BoSTM* line, despite a larger number of probes being expressed during the first two stage comparisons (1334 between stage 0 and 1, and 1032 between stage 1and 2). To facilitate the analysis, the differentially expressed elements were grouped into categories based on their putative function, and a list of representative probes is shown in Table 1. Many probes implicated in hormone synthesis and signalling showed different behaviour, whereas probes involved in meristem function (*STM*, *WUS*, *PINHEAD/ZWILLE*, *AGO1*, and *FIDDLEHEAD*) were induced only in the *35S::BoSTM* line between stage 0 and 1. Differences between WT and *BoSTM*-overexpressing lines were also observed for GR and glutathione synthase (GSH2), both repressed in the transformed line during the first days of development (stage 1 versus 2; Table 1). Two probes encoding DNA (cytosine-5)-methyltransferases were also repressed in the *35S::BoSTM* line during the induction phase (stage 0 versus 1; Table 1).

A direct comparison analysis was also performed to identify differentially expressed probes between WT and *35S::BoSTM* lines at each stage of somatic embryo development. The most significant differences in expression between lines occurred at stage 1, when many ribosomal proteins and enzymes participating in the glycolytic and tricarboxylic acid (TCA) cycle were repressed in the WT line (Supplementary Fig. S5A at *JXB* online). A similar tendency was also observed for several transcription factors, including the lateral organ boundary (LOB) genes 12, 16, and 41 (LOB 1, 2, and 3) and two homeobox (HB) genes *WUS* and *REVOLUTA* (HB 6 and 7) (Supplementary Fig. S5B). Different profiles were detected for probes clustered in the categories of disease and defence (D&D), metabolism (M), and hormonal control. A probe (M 11) coding for

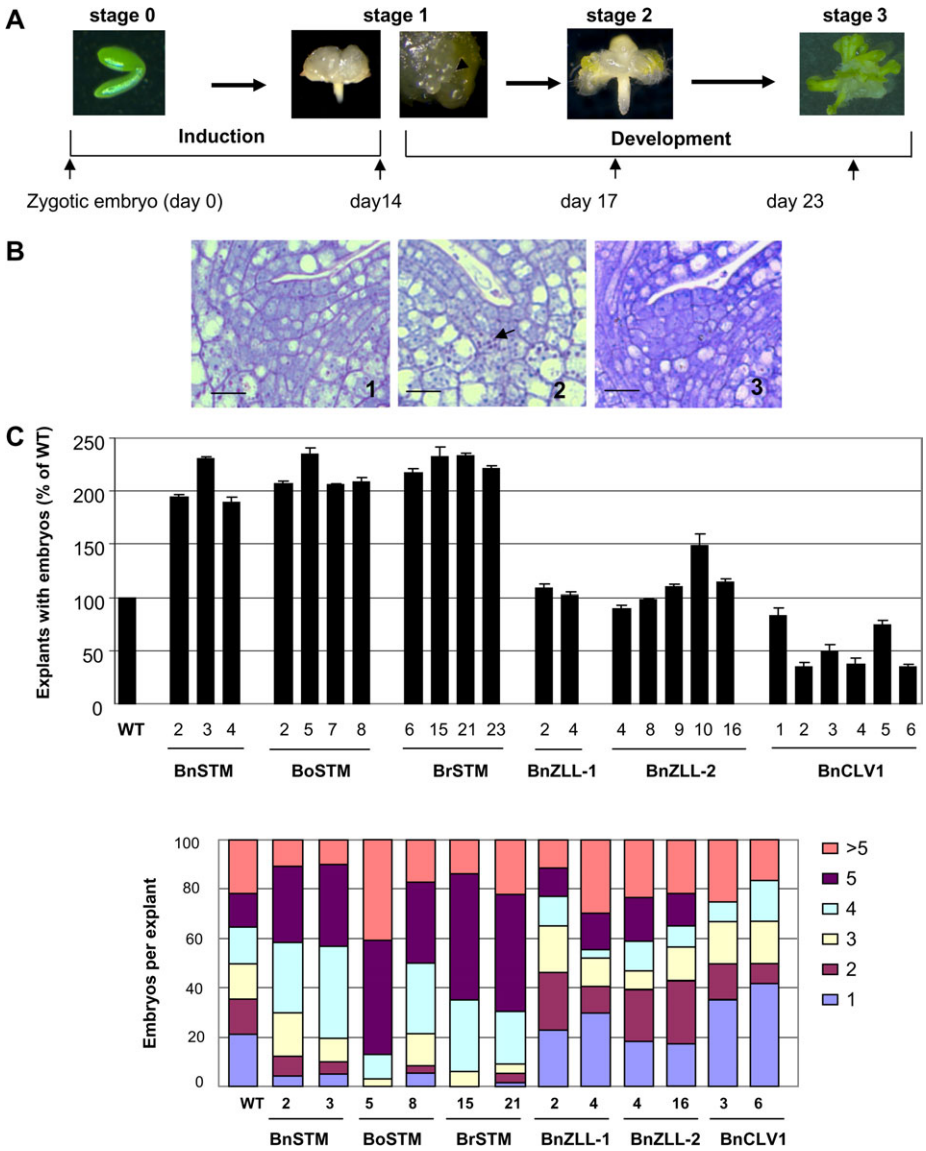

Fig. 3. (A) Schematic representation of the somatic embryogenic process in *Arabidopsis* and stages, with relative time points, used for the microarray experiment. Bending-cotyledon zygotic embryos were cultured on induction medium for 14 d and then transferred on development medium. Embryogenic cells formed from the adaxial sides of the cotyledons during the induction phase and meristemoids (arrowhead) appeared on the surface of the callus. Growth of the meristemoids resulted in the production of fully developed somatic embryos (stage 3). (B) Structure of the apical pole in *Arabidopsis* bending-cotyledon zygotic embryos used as explants to induce somatic embryogenesis. Compared with WT embryos (B1), the SAM of *35S::BnCLV1* embryos (B2) was smaller in size and was characterized by the accumulation of storage products (arrow) within the subapical cells. Dome-shaped SAMs were observed in *35S::BnSTM* embryos (B3). Sections were stained with periodic acid–Schiff (PAS) reagent which stains carbohydrates red and subsequently counterstained with toluidine blue (TBO) for general histological organization. All scale bars=15 μm. (C) Effects of the ectopic expression of *BnSTM*, *BoSTM*, *BrSTM*, *BnCLV1*, *BnZLL-1*, and *BnZLL-2* on the number of *Arabidopsis* explants with somatic embryos and on the number of somatic embryos originating from each explant. Values ±SE are averages of at least three independent experiments.

S-adenosylmethionine synthase was significantly repressed in the WT line (Supplementary Fig. S5C)

WUS *expression and localization during* Arabidopsis *somatic embryogenesis*

Expression of *WUS* was measured by semi-quantitative RT-PCR at stage 1 of *Arabidopsis* somatic embryogenesis (Fig. 3A) in the WT line as well as in lines overexpressing *BoSTM*, *BnSTM*, *BrSTM*, and *BnCLV1* (Fig. 6). Compared with the WT line, *WUS* expression increased in all lines overexpressing *Brassica STM* (confirming the microarray data, Table 1 and Supplementary Fig. S5B), whereas it decreased in the two *BnCLV1* lines (Fig. 6). In order to assess if these alterations in expression levels were associated with changes in localization patterns, RNA *in situ* hybridization was also performed. In the WT line (stage 1) *WUS* signal was restricted to small clusters of cells

originating from the callus on the adaxial side of the cotyledons (Fig. 6B1) and to the meristemoids (Fig. 6B2). *WUS* was expressed in the meristematic cells which stained blue with TBO (Fig. 6B3, 4). In the *35S::BoSTM* line (line 5) the localization of WUS was extended to all apical and subapical cells of the callus (Fig. 6B5). Compared with the subtending parenchyma cells, the *WUS*-expressing cells were smaller and highly cytoplasmic (Fig. 6B6). The extent of *WUS* expression and the formation of meristematic cells at stage 1 correlated to the number of somatic embryos produced by the explants at the end of the culture period, i.e. stage 3 (Fig. 6B7, 8). The expression of *WUS* in the *35S::BnCLV1* line (line 3) at stage 1 of somatic embryogenesis was often limited to very small clusters of cells (Fig. 6B9), although in many instances no expression level was detected (Fig. 6B10).

Auxin requirement for the induction of Arabidopsis *somatic embryogenesis*

The requirement for auxin during the acquisition of embryogenic competence in the induction phase (stage 0–1, Fig. 3A) of *Arabidopsis* somatic embryogenesis was evaluated in the WT line and lines ectopically expressing *BoSTM* (line 5), *BnSTM* (line 2), *BrSTM* (line 15), and *BnCLV1* (lines 3 and 6). Somatic embryo production in the WT line more than halved when the level of 2,4-D was reduced from the optimal concentration of 4.5 μM to 2.5 μM and declined further at lower auxin levels (Fig. 7). The introduction of *STM* attenuated the decline of somatic embryo formation in a low auxin environment. In some lines (*35S::BoSTM* line 5 and *35S::BnSTM* line 2) embryo formation was still observed when 2,4-D levels were lowered to 0.75 μM. The highest requirement for auxin for the induction of somatic embryogenesis was observed in the lines ectopically expressing *BnCLV1*. The introduction of this gene caused a substantial decrease in embryo formation when the level of 2,4-D was lowered from 4.5 μM to 3.5 μM (Fig. 7).

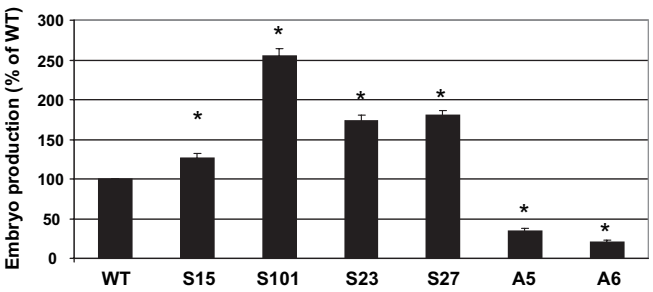

Fig. 4. Production of microspore-derived embryos (MDEs) from *Brassica napus* plants with altered *BnSTM* expression. Antisense lines (A, line 5 and 6) with lower *BnSTM* expression or sense lines (S, line 15, 101, 23, and 27) with increased *BnSTM* expression were utilized to generate MDEs. For each experiment 40 000 microspores were plated and embryo number was counted after 25 d in culture. Values ±SE (n=3) are expressed as a percentage of the WT. Asterisks above each bar indicate values which are significantly different from the WT value ($P \leqslant 0.05$).

DNA methylation and analysis of glutathione metabolism during Arabidopsis *somatic embryogenesis*

To ascertain whether the down-regulation of two DNA (cytosine-5)-methyltransferases observed in the *35S::BoSTM* line during the induction phase (stage 0–1, Table 1) of *Arabidopsis* somatic embryogenesis affected the pattern of DNA methylation, a CRED-RA was performed in the WT line and the *35S::BoSTM* line 5 at stage 1 of somatic embryogenesis. As shown in Fig. 8, the degree of methylation of cytidine residues was lower in the transformed line. The role of DNA methylation during somatic embryogenesis was further tested to assess if 5-azadeoxycytidine (azadC), a hypomethylating agent which inhibits DNA methyltransferase activity (Sano *et al.*, 1989), would increase the somatic embryo-forming capacity of the WT line. Inclusions of this compound at different days during the induction phase favoured the production of WT somatic embryos. An opposite trend was observed if azadC was added in the development medium (Fig. 8B).

Transcriptional studies showed that during the initial days in the development medium (stage 1–2) GR and GSH2 are repressed in the *35S::BoSTM* line (Table 1). This observation prompted the investigation of the role of glutathione metabolism during *Arabidopsis* somatic embryogenesis. Compared with the WT line, the endogenous level of reduced glutathione (GSH) in the *35S::BoSTM* line 5 decreased

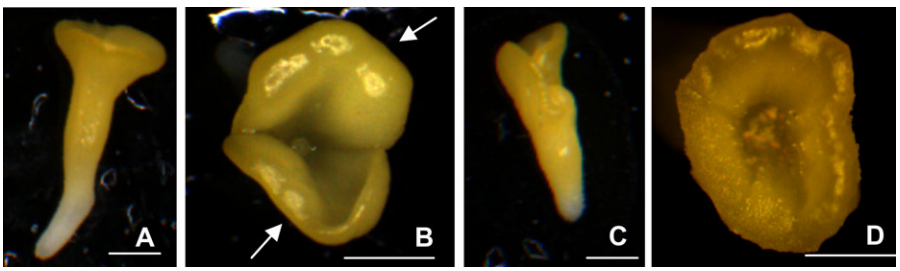

Fig. 5. Morphology of microspore-derived embryos (MDEs) produced by *B. napus* lines with altered *BnSTM* expression. MDEs were collected after 25 d in culture. MDEs obtained from the WT line (A) were characterized by two distinct cotyledons (arrows) (B). MDEs produced by antisense lines and with lower *BnSTM* expression were smaller (C) and often produced fused cotyledons (D). No morphological differences were observed in MDEs produced by lines overexpressing *BoSTM*. All scale bars=0.3 mm.

Table 1. List of selected genes showing differential expression (fold change >4 and $P \leqslant 0.01$) during progressive stages (1–3) of *Arabidopsis* somatic embryogenesis in the wild-type (WT) line and the *35S::BoSTM* line

Positive values indicate an up-regulation of gene expression between two adjacent stages.

	WT			***35S::BoSTM***		
	Stage 0–1	**Stage 1–2**	**Stage 2–3**	**Stage 0–1**	**Stage 1–2**	**Stage 2–3**
Cytokinin						
At3g23630 Adenylate isopentenyltransferase 7/IPST7	–	–	–	+6.5	–	–
At3g48100 Response regulator 5 (ARR5)	–	–	–	+40	–	–
Ethylene						
At5g03280 Ethylene-insensitive 2 (EIN2)	–	–	–	–7.6	–	–
At5g25190 Ethylene-responsive element	–10.9	–	–	–33.5	+5.6	–
At4g29100 Ethylene-responsive element	–	–	–	–	–12.9	–
Gibberellins						
At5g07200 Gibberellin 20-oxidase	+8.6	–	–	+16.9	–	–
At3g02885 Gibberellin-regulated protein 5 (GASA5)	–	–	–	–16.7	–	–
Abscisic acid						
At3g19290 ABA-responsive element-binding protein 2 (AREB2)	–	–	–	–	–7.4	–
Auxin						
At1g70940 Auxin transport protein (PIN3)	–6.0	–	–	–	–	–
At1g19850 MONOPTEROS transcription factor	–9.8	–	–	–50.1	+8.7	–
At2g33860 Auxin-responsive factor (ARF3)	–	–6.2	–	–7.4	–	–
At4g14560 IAA-induced protein 1 (IAA1)	–13.3	+4.3	–	+45.2	+6.7	–
At2g22670 IAA-induced protein 8 (IAA8)	–	–	–	+13.7	–9.4	–
At5g65670 IAA-induced protein 9 (IAA9)	–	–	–	+7.4	–22.8	–
At3g15540 IAA-induced protein 19 (IAA19)	–	+24.8	–	–	+41.2	–
At5g25890 IAA-induced protein 28 (IAA28)	–	–	–	+4.3	–16.4	–
Brassinosteroids						
At4g39400 Brassinosteroid-insensitive 1 (BR1)	–	–	–	–6.7	–	–
At3g50750 Brassinosteroid signalling-positive regulator	–	–	–	–7.1	–	–
Signal transduction						
At5g19280 KAPP	–	–	–	+4.9	–	–
Transcription						
At1g62630 SHOOTMERISTEMLESS (STM)	–	–	–	+5.2	–	–
At2g17950 WUSCHEL (WUS)	–	–	–	+4.6	–	–
At5g17430 Ovule development protein (homologue to BABY BOOM)	–	–	–	+7.5	–	–
Post-transcription						
At5g43810 PINHEAD/ZWILLE	–	–	–	+6.8	–	–
At1g48410 ARGONAUTE 1 (AGO1)	–	–	–	+7.5	–	–
Disease and defence						
At4g35000 L-Ascorbate peroxidase 3 (APX3)	–	–	–	–6.3	–	–
At5g21105 L-Ascorbate oxidase	–8.3	–	–	–25.2	–	–
At3g27820 Monodehydroascorbate reductase	–	–	–	–9.7	–	–
At3g10920 Superoxide dismutase [Mn]-mitochondrial	–	–	–	–	–6.1	–
At3g56350 Superoxide dismutase [Mn]-putative	–4.5	+8.0	–	–	+9.6	–
At5g27380 Glutathione synthetase (GSH2)	–	–	–	–	–9.3	–
At3g24170 Glutathione reductase (GR)	–	–	–	–	–6.7	–
Metabolism						
At2g26250 β-KetoacylCoA synthase (FIDDLEHEAD)	–	–	–	+13.7	–	–
At3g17820 Glutamine synthetase (GS1)	–	–	–	–	–9.0	–
At5g35630 Glutamine synthetase (GS2)	–	–	–	–8.0	–	–
At5g49160 DNA (cytosine-5)-methyltransferase	–	–	–	–4.5	–	–
At5g25480 DNA (cytosine-5)-methyltransferase	–	–	–	–9.2	–	–

sharply within the first 3 d in the development medium (from stage 1 to stage 2) and remained low during the following days in culture (Fig. 9A). This profile correlated with the reduced activity of GR in the *35S::BoSTM* line after stage 1. No differences in the endogenous content of oxidized glutathione (GSSG) were observed between lines during somatic embryogenic (Fig. 9A).

Based on this observation, it was hypothesized that the somatic embryo-forming capacity of the WT line could be modulated by alterations of the glutathione pool, with an

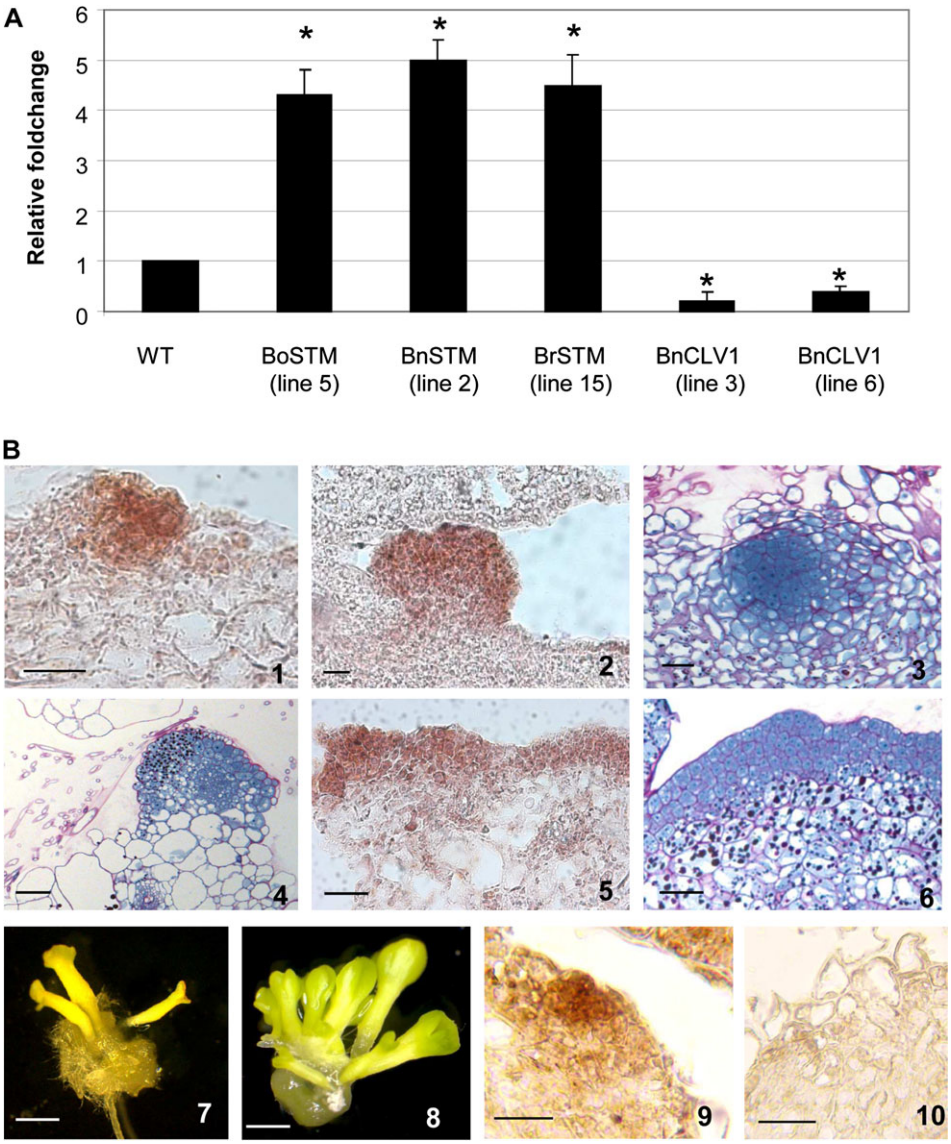

Fig. 6. *WUSCHEL* (*WUS*) expression (measured by semi-quantitative RT-PCR) and localization (by digoxigenin-labelled RNA *in situ* hybridization) at the end of the induction period (stage 1) of *Arabidopsis* somatic embryogenesis in the WT line and in lines overexpressing *BoSTM* (line 5), *BnSTM* (line 2), *BrSTM* (line 15), and *BnCLV1* (line 3 and 6). Values ±SE are means of three independent experiments and are normalized to the value of the WT line set at 1. Asterisks indicate values which are significantly different from the WT value ($P \leqslant 0.05$) (A). In the WT *Arabidopsis* line at stage 1 of somatic embryogenesis *WUS* expression (red stain) was initially localized in distinct clusters of cells on the adaxial side of the cotyledons of the explant (B1) and throughout the developing globular embryos (B2). *WUSCHEL* expression demarcated the formation of embryogenic cells which stained blue with toluidine blue (TBO) (B3, 4). In the line overexpressing the *BoSTM*, the *WUS* domain was extended to the surface of the cotyledons (B5) corresponding to apical and subapical layers occupied by embryogenic cells (B6). The extent of the *WUS* domain at stage 1 of somatic embryogenesis correlated with the number of somatic embryos produced by the WT line (B7) and *35S::BoSTM* line (B8) at the end of the culture period (stage 3). In lines overexpressing the *Brassica CLV1*, the *WUS* signal at stage 1 of somatic embryogenesis was either localized in a very small cluster of cells (B9) or completely absent (B10). Scale bars=40 μm (1–6 and 9, 10) and 1 mm (7, 8).

oxidized environment (low GSH/GSSG ratio) favouring somatic embryo development and a reduced environment (high GSH/GSSG) having the opposite effect. This hypothesis was tested by supplementing the induction and/or the development medium of the WT line with exogenous GSH or GSSG (which switch the cellular glutathione pool towards a reduced or oxidized glutathione environment; Belmonte *et al.*, 2006) or BSO (DL-buthionine-[*S*,*R*]-sulphoximine), a specific inhibitor of GSH synthesis (Griffith and Meister, 1979). Applications of GSH in the development medium (after stage 1 of somatic embryogenesis) decreased both the number of explants able to form somatic embryos and the number of embryos produced by each explant (Fig. 9B). The inhibitory effect of GSH was reversed by the

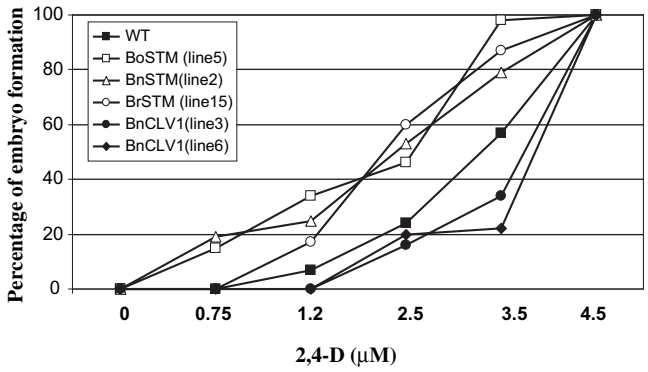

Fig. 7. Auxin requirement for the induction of *Arabidopsis* somatic embryogenesis. The WT line and lines ectopically expressing *BoSTM* (line 5), *BnSTM* (line 2), *BrSTM* (line 15), and *BnCLV1* (lines 3 and 6) were cultured on the induction medium with different levels of 2,4-D. Values (n=3) are presented as a percentage of the embryo production occurring at the optimal 2,4-D level (4.5 μM).

combined application of GSH+BSO. Somatic embryo formation in the WT line was encouraged by the application of BSO or GSSG in the development medium (Fig. 9B).

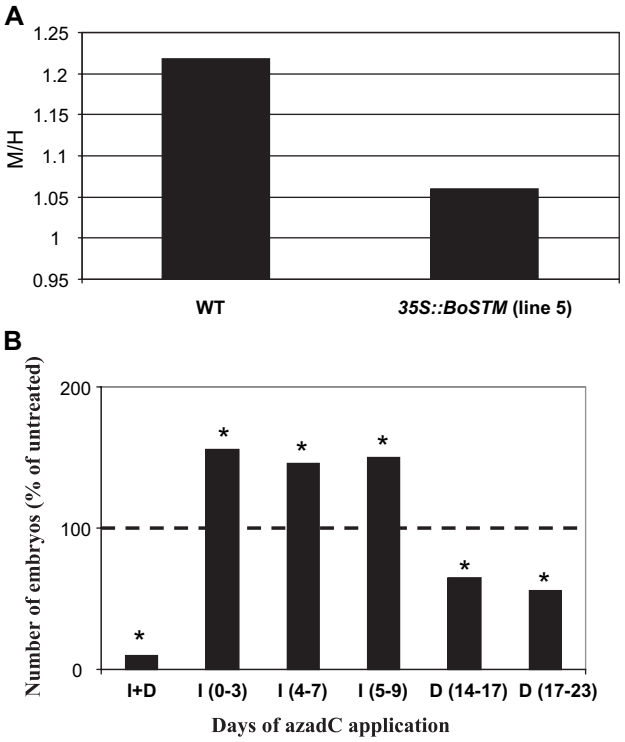

Fig. 8. (A) DNA methylation analysis in the WT line and in the *35S::BoSTM* line 5 at stage 1 of *Arabidopsis* somatic embryogenesis. Ratios of consistently amplified bands following digestion with *Msp*I (M) and *Hpa*II (H). DNA was extracted at stage 1, digested with *Hpa*II and *Msp*I endonucleases (which cut the sequence 5'-C/CGG-3' with different sensitivity to cytosine methylation; *Msp*I cuts if the inner C is methylated, whereas *Hpa*II cannot cleave in the presence of methyl groups), and then used as a template in a PCR employing six random 10-base primers (Leljak-Levanic *et al.*, 2004). (B) Effects of the DNA-hypomethylating agent 5-azadeoxycytidine (azadC) on the somatic embryo-forming capacity of the WT line. Data are expressed as percentage changes in somatic embryo production following applications of azadC on different days in the induction (I) or development (D) medium. AzadC was also added throughout the culture period (I+D). Values (n=3) are expressed as percentages of the value of untreated tissue (set at 100%). Asterisks indicate values which are significantly different from the untreated value ($P \leqslant 0.05$).

Discussion

Characterization of B. napus *SAM genes*

The putative orthologues of *Arabidopsis STM*, *CLV1*, and *ZLL* were isolated from *B. napus* tissue and denoted as *BnSTM*, *BnCLV1*, and *BnZLL-1* and *BnZLL-2*. The *Brassica* genes displayed high levels of sequence identity with their respective *Arabidopsis* orthologues with which they share all the conserved domains (Supplementary Figs S3, S4 at *JXB* online). Unique features of *Bn*STM are the four conserved regions KNOX1, KNOX2, ELK, and the homeodomain, which also characterize *AtSTM* (Scofield and Murray, 2006). Besides occupying the central region of the SAM during both zygotic and microspore-derived embryogenesis (Fig. 2), as also observed for *AtSTM* (Long and Barton 1998), a down-regulation of *BnSTM* in *B. napus* plants induces SAMs with a determined fate (data not shown). These observations suggest that like *At*STM, *Bn*STM is involved in the establishment and maintenance of a functional SAM.

The *B. napus BnCLV1* contains all the signatures of its respective *Arabidopsis* orthologue *AtCLV1* receptor kinase, including the conserved C-terminal serine/threonine kinase domain (Supplementary Fig. S3 at *JXB* online). Similarities among the two genes (*BnCLV1* and *AtCLV1*) were also observed in their localization patterns during embryogenesis (Fig. 2 and Clark *et al.*, 1997) and expression during post-embryonic development (Fig. 1). The termination of the SAMs due to differentiation of the meristematic cells observed in *Arabidopsis* seedling overexpressing *BnCLV1* (data not shown) suggests that *Bn*CLV1 regulates SAM activity through mechanisms analogous to those ascribed to *At*CLV1, which limits the accumulation of undifferentiated or uncommitted cells and promotes differentiation (reviewed by Dodsworth, 2009).

The two products of the *BnZLL* genes, denoted as *Bn*ZLL-1 and *Bn*ZLL-2, share a high degree of amino acid sequence identity (96%) with *At*ZLL and also contain two conserved regions, the PIWI and PAZ domains, which are unique features of the AGO members, including *At*ZLL (Cerruti *et al.*, 2000). Additional similarities shared by *Bn*ZLL-1 and *Bn*ZLL-2 and the *Arabidopsis At*ZLL include the two distinct expression domains (vascular tissue and SAM) during embryogenesis. The localization of *Bn*ZLL-1 and *Bn*ZLL-2 in the vascular tissue of immature embryos and in the SAM of fully developed cotyledonary embryos

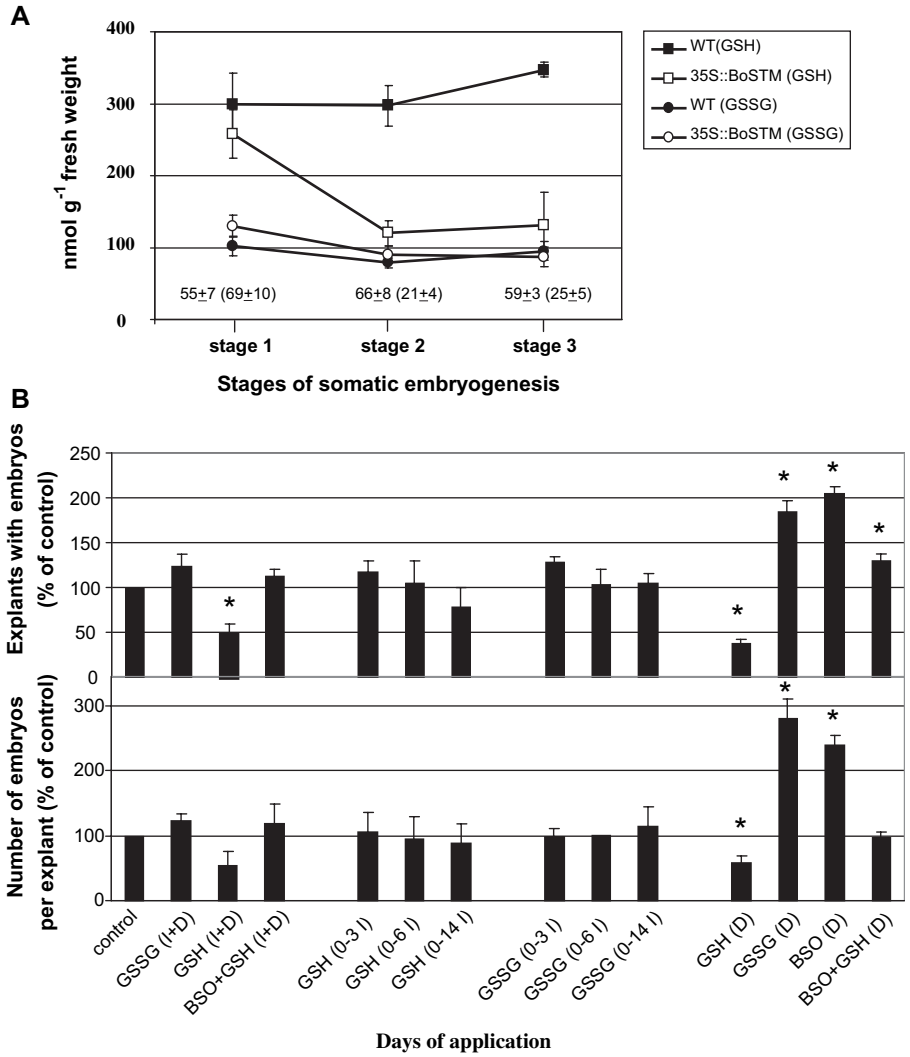

Fig. 9. Glutathione metabolism during *Arabidopsis* somatic embryogenesis. (A) Endogenous levels of reduced (GSH) and oxidized (GSSG) glutathione during different stages (0–3) of somatic embryogenesis in the WT line and the *35S::BoSTM* line 5. Numbers above the abscissa (values of the *35S::BoSTM* line in parentheses) indicate the activity of glutathione reductase (nmol g^{-1} fresh weight). Values ±SE are averages of at least three independent experiments. (B) Effects of exogenous applications of GSSG (1 mM), GSH (1 mM), and BSO (0.1 mM) on the number of explants with embryos and on the number of embryos per explant during somatic embryogenesis of the WT line. Compounds were added throughout the culture period (I+D) or on different days during induction (I) or development (D). Values ±SE (n=3) are expressed as percentages of the value in the untreated (control) tissue set at 100%. Asterisks above each bar indicate values which are significantly different from the control value ($P \leqslant 0.05$).

both *in vivo* and *in vitro* (Fig. 2) is very similar to that described for *At*ZLL during *Arabidopsis* embryogenesis (Moussian *et al.*, 1998). The participation of *At*ZLL in the formation of the primary embryonic SAM relies on the creation of positional cues for proper *STM* expression (Moussian *et al.*, 1998) and the enhancement of WUS function during the acquisition of stem cell fate (Tucker *et al.*, 2008). Involvement in SAM formation is a prerogative which is possibly retained by *Bn*ZLL-1 and *Bn*ZLL-2, as shown by the similarities shared between the *Arabidopsis* and *Brassica* genes

Based on sequence analyses, as well as on expression, localization, and phenotypic studies, the present results are consistent with the notion that *BnSTM*, *BnCLV1*, and *BnZLL-1* and *BnZLL-2* are the respective orthologues of *AtSTM*, *AtCLV1*, and *AtZLL*.

Ectopic expression of the Brassica *genes affects embryo production* in vitro

Somatic embryogenesis in *Arabidopsis* is a two-step process comprising an induction phase (in the presence of 2,4-D) in which the somatic cells of the explant acquire embryogenic competence and proliferate to produce a callus, and a development phase required for the growth of somatic embryos (Fig. 3A). The molecular mechanisms underlying the states of embryogenic competence acquisition and embryo development are poorly understood and it is still unclear what changes

somatic cells must undergo in order to become embryogenic. Over the past few years attempts have been made to identify genes with altered expression patterns during somatic embryogenesis (Lin *et al.*, 1996; Stasolla *et al.*, 2004), and to assess their function using molecular approaches (reviewed by Namasivayam, 2007). Although the effects of several loss-of-function mutants on somatic embryogenesis have also been described (Chaudhury *et al.*, 1993; Ogas *et al.*, 1999; Mordhorst *et al.*, 1998, 2002), there are only a few reports documenting changes in somatic embryogenic induction frequency as a result of ectopic gene expression (Hecht *et al.*, 2001; Stone *et al.*, 2001; Boutilier *et al.*, 2002; Zuo *et al.*, 2002). Furthermore among the genes tested in these studies only one, *WUS*, is related to SAM activity (Zuo *et al.*, 2002).

The present work shows that somatic embryo formation in *Arabidopsis* is affected by the introduction of *Brassica* (*Bn*, *Bo*, and *Br*) *STM* and *BnCLV1*, but not by *BnZLL-1* and *BnZLL-2* (Fig. 3). In *Arabidopsis*, *AtSTM* and *AtCLV1* have key roles in the maintenance of post-embryonic SAMs (Clark *et al.*, 1997; Schoof *et al.*, 2000), whereas *AtZLL* is only involved in the formation of a functional embryonic SAM without affecting its post-embryonic development (Moussian *et al.*, 1998; Lynn *et al.*, 1999). Although it is difficult to surmise function from gain-of-function phenotypes, the present overexpression experiments suggest that genetic factors regulating the maintenance of the SAM are also relevant for the generation of embryo-competent cells.

The STM-mediated redirection of cell fate is not a novel concept. *De novo* shoot meristems were produced in leaves of *Arabidopsis* plants overexpressing *KNAT1*, a member of the kn-1 like homeobox gene family which includes *STM* (Chuck *et al.*, 1996), and tobacco plants expressing the maize *STM* homologue *KNOTTED 1* (Sinha *et al.*, 1993). These observations are consistent with the role of STM in the formation and maintenance of the SAM. However, what is novel is the ability of STM to induce somatic embryos and not shoots in the present system (the identity of the embryos was verified by the presence of the bipolar apical meristems through histological analyses). It is postulated that a function of this gene when ectopically expressed in new domains is to encourage cells to dedifferentiate and acquire 'meristematic' identity. Depending on the physiological environment, such cells can then embark on an organogenic or embryonic developmental pathway. This flexibility in cell fate acquisition is common during *in vitro* development where positional cues are not as well defined as *in vivo*. The inductive effect of ectopic *STM* expression on *Arabidopsis* somatic embryogenesis is consistent with previous studies documenting an increased number of somatic embryos in spruce lines overexpressing *HBK3*, a class I KNOX homeobox gene, isolated from *Picea abies* (Norway spruce) (Belmonte *et al.*, 2007).

Another interesting point emerging from the present study is the different response in culture exhibited by the lines overexpressing *Brassica* (*Bo*, *Bn*, and *Br*) *STM* and *BnCLV1*. While the introduction of the *Brassica STM* encourages *Arabidopsis* somatic embryo formation, *BnCLV1* represses somatic embryogenesis (Fig. 3C). This competitive role of the *Brassica* genes *in vitro* is analogous to that fulfilled by *Arabidopsis AtSTM* and *AtCLV1 in vivo*, where *AtSTM* acts as a positive regulator of SAM activity by increasing the domain of meristematic cells (Lenhard *et al.*, 2002) and *AtCLV1* as a negative regulator which represses meristem proliferation and promotes cellular differentaition (Clark *et al.*, 1997). In line with this analogy it is therefore suggested that while *Brassica STM* encourages *Arabidopsis* somatic embryo formation by broadening the domain of embryogenic cells, *BnCLV1* limits the production of embryogenic cells by inducing differentiation. This notion is also supported by the signs of cellular differentiation (as estimated by the accumulation of storage products) observed in the SAMs of explants (bending-cotyledon embryos) overexpressing *BnCLV1* (Fig. 3B). The mechanisms underpinning the interaction between the *Brassica* STM and CLV1 in culture are elusive, although they might involve the participation of WUS (discussed in the next section), which is a signalling molecule component for the induction of somatic embryos (Chen *et al.*, 2009).

The altered expression of the *Brassica STM* gene also had profound effects on *B. napus* microspore-derived embryogenesis. A high yield of MDEs was observed in lines overexpressing *BnSTM*, whereas an opposite trend was noticed in lines with reduced *BnSTM* levels (Fig. 4). The phenotypic deviations (reduced embryo size and fused cotyledons) observed in many MDEs generated from lines down-regulating *BnSTM* (Fig. 5) confirm the notion that this gene is implicated in embryo development and meristem formation.

Overall these results clearly show that the overexpression of *Brassica STM* encourages somatic embryo formation in *Arabidopsis* and MDE production in *B. napus*. It is therefore suggested that the beneficial effect of this gene on *in vitro* embryogenesis is independent of the system utilized (direct embryogenesis from single microspores in *B. napus* or indirect somatic embryogenesis through a callus step formation in *Arabidopsis*) and the type of induction conditions (hormone-free medium in *B. napus* and auxin in *Arabidopsis*).

Genes and regulatory pathways altered by the ectopic expression of BoSTM *during* Arabidopsis *somatic embryogenesis*

Global changes in transcript levels during somatic embryogenesis in the WT line and in the *35S::BoSTM* line were analysed by microarray studies and validated by quantitative RT-PCR (Supplementary Fig. S6 at *JXB* online). Formation of embryos from *Arabidopsis* somatic cells requires a reprogramming in gene expression pattern, which is affected profoundly by the overexpression of *BoSTM*. In bending-cotyledon zygotic embryos (stage 0) the introduction of *BoSTM* resulted in the differential expression of 176 probes, some of which encoded cell structural components, hormone-responsive elements, stress-related enzymes, and transcription factors (data not shown). The most pronounced differences in expression levels occurred during the induction phase, demarcating the transition from stage 0 to stage 1. During the following development phase such

differences were attenuated, despite a large variation in function of the expressed probes (Table 1).

The induction phase

The induction phase was characterized by alterations in expression levels of several genes involved in hormone synthesis/response. The *BoSTM* activation of *adenylate isopentenyltransferase 7* (*IPT7*), a cytokinin biosynthesis gene, and *ARR5*, the primary cytokinin-responsive gene (Table 1), suggests that the role of this gene is to stimulate cytokinin synthesis and perception. A rapid elevation in the transcription of *IPT7* and *ARR5* was also observed in mature tissues of *Arabidopsis* plants ectopically expressing *AtSTM* and other *KNOTTED1* genes (Yanai *et al.*, 2006). Another role assigned to KNOTTED genes is to inhibit gibberellin synthesis through the repression of GA 20-oxidase (Sakamoto *et al.*, 2001). In the present system this regulation does not seem to occur as the expression of this gene increased in both WT and *35S::BoSTM* lines during the induction phase (Table 1).

Acquisition of embryogenic competence during *Arabidopsis* somatic embryogenesis relies on the ability of the tissue to respond to the exogenously supplied auxin (2,4-D). The altered expression of genes involved in auxin transport and signalling, together with the activation of several indole acetic acid (IAA)-induced elements (IAA1, 8, 9, and 28) during the induction phase (stage 0 versus 1) of the *35S::BoSTM* line (Table 1), suggest that the introduction of *BoSTM* modifies the response of the tissue to 2,4-D, possibly by making it more sensitive to this growth regulator. This is demonstrated by the sustained production of somatic embryos in *Arabidopsis* lines overexpressing *Brassica* (*Bo*, *Bn*, *or Br*) *STM* when exposed to reduced levels of 2,4-D which preclude embryogenesis in the WT line (Fig. 7). The requirement for 2,4-D is higher in the *35S::BnCLV1* lines, thus suggesting opposite effects of *Brassica STM* and *CLV1* on embryogenic competence acquisition and ultimately somatic embryo production. The mechanisms whereby auxin triggers embryonic competence are poorly understood (reviewed by Raghavan, 2004). Recent work (Su *et al.*, 2009) emphasized the relevance of the PIN-induced auxin gradient in the activation of *WUS* which, besides defining the organizing centre in the SAM *in vivo* (Mayer *et al.*, 1998), is associated with the production of totipotent embryogenic cells during *in vitro* morphogenesis (Chen *et al.*, 2009; Su *et al.*, 2009). A relationship between auxin requirement and *WUS* expression and localization is established in this study. The expression of *WUS* at the end of the induction period (stage 1) is higher in the *Bo*, *Br*, and *BnSTM* lines (Fig. 6A), which exhibit the lowest requirement for auxin, whereas it is repressed in the *BnCLV1* lines, which have the highest requirement for auxin. This trend was also confirmed by localization studies revealing an extended domain of *WUS* (which demarcates the formation of embryogenic cells) in lines overexpressing the *Brassica STM* gene and a limited *WUS* signal in the *35S::BnCLV1* line (Fig. 6B). A plausible regulatory mechanism explaining the increased expression of *WUS* in the *35S::BoSTM* line involves the kinase-associated protein phosphatase (KAPP), which is also activated during the induction phase of somatic embryogenesis (Table 1). Genetic studies have shown that during maintenance of the *Arabidopsis* SAM *in vivo*, KAPP activity attenuates the inhibitory effect of CLV signalling on the transcription of *WUS* by binding to the active CLV receptors (reviewed by Carles and Fletcher, 2003). This raises the intriguing possibility that mechanisms governing stem cell maintenance *in vivo* also regulate embryogenic cell formation *in vitro*. It must be said, however, that *KAPP* expression also increased in lines overexpressing *Brassica CLV1* (data not shown), possibly as a feedback mechanism to limit the ectopic activation of CLV signalling.

The activation of *WUS* during the induction phase of the *35S::BoSTM* line coincides with the increased expression of other elements playing a key role during the initiation of embryogenesis. These include transcription factors such as STM and BABY BOOM, the latter being responsible for triggering the conversion from vegetative to embryonic development (Boutilier *et al.*, 2002), as well as two ARGONAUTE members PINHEAD/ZWILLE and AGO 1 (Table 1), both involved in meristem formation (Moussian *et al.*, 1998).

Common embryogenesis-related programmes have been postulated to be under the control of genetic mechanisms, which often rely on the methylation state of the DNA regulated by auxin levels (Namasivayam 2007). Several studies on animal and plant systems showed that embryogenic cells or germline cells contain hypomethylated DNA (Monk *et al.*, 1987), and experimental hypomethylation of DNA of somatic cells induces a state of differentiation similar to that observed during the early phases of zygotic embryogenesis (Okkels, 1988). The expression of two key DNA methyltransferases is repressed in the *35S::BoSTM* line during the induction phase (stage 0–1, Table 1). One of these enzymes (At5g49160) has been identified as METHYLTRANSFERASE 1, which is responsible for maintaining CpG DNA methylation in *Arabidopsis* (Finnegan and Kovac, 2000). Investigations with isoschizomeric restriction endonucleases differentially sensitive to methylation of cytosine in CCGG sequences confirmed a lower DNA methylation state in the line overexpressing *BoSTM* at the end of the induction phase (Fig. 8A). This is in agreement with several independent studies suggesting an essential role for DNA hypomethylation in encouraging the acquisition of embryogenic competence of somatic cells (Lo Schiavo *et al.*, 1989; Munksgaard *et al.*, 1995). Pharmacological studies using azadC (Fig. 8B), a hypomethylating drug which inhibits METHYLTRANSFERASE 1 (Ghoshal *et al.*, 2005), confirm the requirement for DNA hypomethylation during the induction of *Arabidopsis* somatic embryogenesis.

The development phase

Compared with the induction phase, the number of differentially expressed probes during somatic embryo

development in WT and *35S::BoSTM* lines decreased, despite a significant variation in function (data not shown). Major differences in expression levels during the transition from stage 1 to stage 2 were observed for probes related to disease and defence processes, which included superoxide dismutase, enzymes involved in ascorbate metabolism, and two enzymes involved in glutathione metabolism (GSH2 and GR) (Table 1).

A key signal encouraging somatic embryo development involves a switch of the cellular glutathione pool [composed of reduced (GSH) and oxidized (GSSG) forms] towards an oxidized state, i.e. a low GSH/GSSG ratio. This concept has been demonstrated in several culture systems of gymnosperm and angiosperm species (Belmonte *et al.*, 2006, 2007). Somatic embryogenesis in spruce was favoured if the endogenous GSH/GSSG ratio was lowered through applications of either GSSG or BSO, a specific inhibitor of GSH *de novo* synthesis (Griffith and Meister, 1979). These conditions increased somatic embryo yield and quality (Belmonte *et al.*, 2007). Similar improvements were also described in *B. napus* microspore-derived embryogenesis where a low cellular GSH/GSSG ratio encouraged zygotic-like embryonic development (Belmonte *et al.*, 2006). Physiological and molecular studies have provided insights into the role of GSSG during *in vitro* embryogenesis (reviewed in Stasolla, 2010). The ectopic expression of *BoSTM* results in a drop in GSH levels during the first 3 d of somatic embryo development (stage 2) (Fig. 9A) possibly due to the transcriptional repression of GSH2, a biosynthetic enzyme catalysing the condensation of γ-glutamylcysteine and glycine to form GSH, and to a repression in transcription and activity of GR which recycles GSH from GSSG (Table 1, Fig. 9A). These changes, not observed in the WT line, would lower the GSH/GSSG ratio of the transformed line, thereby encouraging the development of somatic embryos. The beneficial effect of an oxidized glutathione environment was further demonstrated by the improvements in *Arabidopsis* somatic embryo production obtained in the WT line when GSSG or BSO, which lower the GSH/GSSG ratio (Belmonte *et al.*, 2007), was included in the development medium. This was in contrast to the application of GSH, which increases the endogenous GSH/GSSG ratio (Belmonte *et al.*, 2007) and represses the formation of explants able to form somatic embryos as well as the number of somatic embryos produced by each explant (Fig. 9B).

The relationship between *BoSTM* expression and glutathione redox state is difficult to define, although indirect evidence ties glutathione metabolism to the regulation of self-renewal and differentiation of stem cells in both plant and animal systems (Smith *et al.*, 2000; Jiang *et al.*, 2002).

Conclusion

This study demonstrates that *in vitro* embryogenesis, i.e. *Arabidopsis* somatic embryogenesis and *B. napus* microspore-derived embryogenesis, can be modulated by the overexpression of *Brassica* genes orthologous to the *Arabidopsis AtSTM*, *AtCLV1*, and *AtZLL*. The enhanced somatic embryogenesis observed in *Arabidopsis* lines overexpressing *Brassica STM* was associated with a lower requirement for the inductive signal 2,4-D, and a higher expression of *WUS* which demarcates the formation of embryogenic cells. This was in contrast to the *35S::BnCLV1* lines which, besides showing poor somatic embryo-forming capacity, had the highest requirement for exogenous 2,4-D and a reduced expression of *WUS*. The introduction of *BoSTM* (which showed the greatest improvement on *Arabidopsis* somatic embryogenesis among the other *Brassica STM* genes) was associated with the hypomethylation of the DNA during the induction phase of somatic embryogenesis, and with a switch of the glutathione pool towards an oxidized state during development. Such changes were critical for the successful production of somatic embryos. Altered expression of *Brassica STM* also had profound effects on *B. napus* microspore-derived embryogenesis. The number of MDEs increased in lines overexpressing *BnSTM* and significantly decreased in antisense lines down-regulating *BnSTM*. This suggests that the beneficial effect of the *Brassica STM* is independent of the embryogenic system utilized.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Identification of *Arabidopsis* lines ectopically expressing the *Brassica* genes.

Figure S2. Identification of *Brassica napus* lines transformed with *BnSTM* in the sense or antisense orientation.

Figure S3. Schematic representation of protein structure and amino acid sequence alignments of conserved domains of *Bn*STM, *Bn*CLV1, and *Bn*ZLL-1.

Figure S4. Phylogenetic trees of several class-1 KNOX proteins (A), CLAVATA 1-related proteins (B), and ZLL-like proteins (C).

Figure S5 Expression of selected probes showing a different behaviour between the WT and *35S::BoSTM* lines at stage 1 of *Arabidopsis* somatic embryogenesis.

Figure S6. Validation of microarray experiments by quantitative RT-PCR.

Table S1. Genes and primers used for validating the microarray results using quantitative RT-PCR.

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