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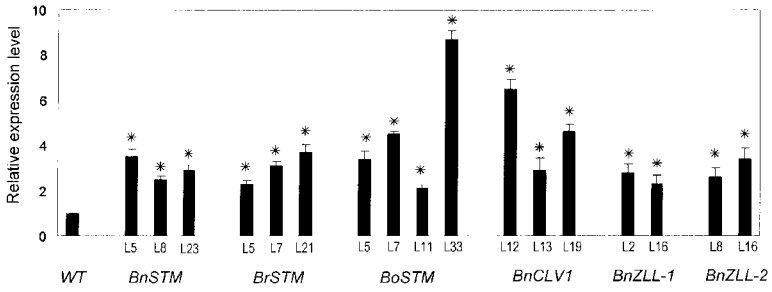

Figure 1

(57) Abstract: A method for increasing seed yields in agronomic plants comprising modulating expression of STM genes in the plants thereby increasing the production of reproductive organs and yield components. A method for increasing seed yields in an agronomic crop plant comprising transforming a selected plant cell from the plant by incorporating therein an expression vector comprising a STM gene.

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TITLE: INCREASED PRODUCTION OF REPRODUCTIVE ORGANS AND YIELD COMPONENTS IN PLANTS

TECHNICAL FIELD

The present invention relates to methods for increasing grain yields in seed-producing agronomic crops by modulating therein expression of *STM* genes.

BACKGROUND

Over the past few years *Brassica napus* androgenesis, i.e. the formation of embryos from microspores, has become a model system to study plant embryogenesis and meristem formation (Yeung, 2002; Boutilier *et al.*, 2005; Joosen *et al.*, 2007; Malik *et al.*, 2007; Stasolla *et al.*, 2008). This system is very suitable for developmental studies since a large number of synchronized embryos can be produced without an intervening callus phase during a short period of time. Furthermore, the genetic similarities between *Brassica* and *Arabidopsis* can be exploited to isolate and characterize *Brassica* genes involved in embryogenesis. As in any other *in vitro* embryogenic system, the establishment of a functional shoot apical meristem (SAM) is a prerogative for the successful regeneration of *Brassica* microspore-derived embryos (MDEs). Reduced post-embryonic growth is commonly observed in those MDEs which develop abnormalities within the apical pole, including vacuolation and differentiation of meristematic cells. Experimental manipulations of culture conditions have been used to alter the structure of the SAM. Applications of DL-buthionine sulfoximine (BSO), a specific inhibitor of glutathione synthesis (Griffith et al., 1979, *Potent and specific inhibition of glutathione synthesis by gluthionine sulfoximine (s-n-Butyl Homocysteine Sulfoximine)*, J. Biochem. Chem. 254:7558-7560), induced the formation of functional "zygotic-like" meristems able to reactivate at high frequency at germination (Belmonte *et al.*, 2006, *Improved development of microspore derived embryo cultures of Brassica napus cv Topaz following changes in glutathione metabolism*, Physiol. Plant. 127:690-700). On the contrary, complete loss of SAM functionality was observed when 2,3,5-triiodobenzoic acid (TIBA), an auxin flow inhibitor, was added to the medium. (Ramesar-Fortner et al., 2006, *Physiological influences in the development and function of the shoot apical*

meristem of microspore-derived embryos of Brassica napus cv. Topas. Can. J. Bot. 84:371-383).

The *Arabidopsis* shoot apical meristem (SAM) is regulated by a set of key genes, including *SHOOTMERISTEMLESS (STM), CLAVATA 1 (CLV1), WUSCHEL (WUS),* and *ZWILLE (ZLL),* which establish and maintain the characteristic layering and zonation patterns of the apical pole. The *Arabidopsis* STM is a member of the class-1 KNOX homeodomain containing proteins required for the formation and maintenance of the SAM by retaining a pool of undifferentiated cells at the apical pole. Structural abnormalities, including the terminal differentiation of the apical cells, are observed in the shoot meristems of *stm* plants which are not able to reactivate and grow post-embryonically. Proper expression of *STM* within the SAM is ensured by ZLL, a member of the ARGONAUTE (AGO) family, which is involved in the formation of RNA-induced silencing complexes in both animal and plants. Unlike STM which is needed for the development of the embryonic SAM and its post-embryonic activity, ZLL is only implicated in the formation of the SAM during embryogenesis. Genetic studies showed that *zll* embryos fail to develop proper meristems. However, adventitious SAMs originating between the cotyledons ensure post-embryonic development. Proper maintenance of SAM activity also relies on the feedback mechanism of the WUS-CLV signalling. *WUSCHEL* is a homeobox gene encoding a transcription factor which regulates the "organizing center" of the SAM by maintaining a reservoir of undetermined cells. The expression of *WUS* is repressed by the CLV signalling upon the interaction of CLV3 with the receptor kinase complex. A mutation of any *CLV* members, including *CLV1,* disrupts the balance between cell division and differentiation within the SAM resulting in enlarged meristems and atypical expansion of the *WUS* domain. It appears the role of CLV1 is to promote the differentiation of the meristematic cells thereby reducing the pool of the undifferentiated stem cell population.

The molecular regulation of SAM is mediated by the activity of plant growth regulators. Numerous studies show that KNOX proteins, which include STM, affect the metabolism of cytokinins and gibberellins. Increasing levels of cytokinins, which are linked to the maintenance of undifferentiated cells in the central domain of the SAM are attributed to the KNOX-activation of isopentenyltransferse genes. By

contrast, the levels of GAs in the central zone of the SAM are maintained low by the suppression exercised by KNOX members on GA20 oxidase. High GA levels are observed in the peripheral region of the SAM demarking the initiation of lateral organs. No information is currently available on the regulation of plant growth regulators by other genes that may participate in SAM formation and maintenance.

SUMMARY

This disclosure provides methods for increasing grain yields in agronomic crop plants. The methods pertain to manipulating selected agronomic crop plants to produce therein over-expression of *STM* genes. Some exemplary methods pertain to transformation of selected agronomic crop plants by incorporating therein expression vectors comprising *STM* genes.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will be described in conjunction with reference to the following drawings, in which:

Figure 1 is a chart showing expression levels by 10-day-old *Arabidopsis* seedlings transformed with the *Brassica oleracea STM* gene *(BoSTM)*, *B. rapa STM* gene *(BrSTM)*, *B. napus STM* gene *(BnSTM)*, *B. napus CLV1* gene (*BnCLV1*, *B. napus ZLL*-1 gene (*BnZLL-1*), and *B. napus ZLL*-2 gene (*BnZLL-2*). Values $\pm$ SE are means of three independent analyses. Stars indicate values which are significantly different from the WT value ($P \leq 0.05$) set at 1;

Figures 2A – 2G are micrographs showing immunolocalization of IAA in 25-day-old *Brassica napus* microspore-derived embryos. The micrographs show cotyledons (2A, 2C, 2E) and shoot apical meristems (2B, 2D, 2F) of embryos cultured in the presence (2A, 2B) or absence (2C, 2D) of 2mM 2,3,5-triodobenzoic acid (TIBA). Embryos were also growth in the presence of TIBA plus 2μM 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB) (2E, 2F). Specificity of staining was demonstrated by the omission of primary antibodies (2G);

Figure 3 is a chart showing the expression profiles of (A) *BnSTM,* (B) *BnCLV1,* (C) *BnZLL-1*, and (D) *BnZLL-2* in *B. napus* microspore-derived embryos

cultured in the presence of 2,3,5-triiodobenzoic acid (TIBA), 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB), and DL-buthionine sulfoximine (BSO);

Figures 4(1) – 4(20) are micrographs illustrating the expression patterns of *BnSTM* during development of *B. napus* microspore-derived embryos. The signal for *BnSTM* was first observed in a small clusters of sub-apical cells in pre-globular embryos 4(1) and retained in the apical pole of globular 4(2), early cotyledonary 4(3) and cotyledonary 4(4) embryos. In some instances *BnSTM* expression was delocalized to the side of the apical pole throughout development 4(5-7), or in non-adjacent clusters of cells 4(8-11). In the presence of BSO, *BnSTM* expression expanded in globular 4(12), heart-shaped 4(13), and cotyledonary 4(14) embryos. The signal of *BnSTM* was reduced in immature embryos treated with TIBA 4(15, 16) and completely lost in fully developed embryos 4(17). These alterations were recovered in immature 4(18) and cotyledonary 4(19) embryos when PCIB was added along with TIBA. No signal was observed with the sense probe 4(20);

Figures 5(1) – 5(8) are micrographs illustrating the localization patterns of *BnCLV1* in untreated microspore-derived embryos at the globular 5(1), early cotyledonary 5(2) and cotyledonary 5(3) stage of development, in BSO-treated MDEs 5(4, 5), and in TIBA-treated embryos 5(6-7). A localization signal similar to that observed in untreated embryos was also detected in MDEs cultured with TIBA+PCIB (data not shown). No signal was visible with the sense probe 5(8);

Figures 6(1) – 6(9) are micrographs illustrating the expression of *BnZLL-1* and *BnZLL-2* in longitudinal sections of globular 6(1), early cotyledonary 6(2), middle cotyledonary 6(3) and cotyledonary 6(4) untreated MDEs, as well as in cross sections of cotyledonary embryos 6(5). *BnZLL-1* and *BnZLL-2* localization in cotyledonary MDEs treated with BSO 6(6), TIBA 6(7), and TIBA+PCIB 6(8). No signal was observed with sense probes 6(9);

Figures 7(1) – 7(3) are micrographs illustrating phenotypic characterization of *Arabidopsis* lines ectopically expressing the *Brassica STM, CLV1, ZLL-1* and *ZLL-2*: Fig. 7(1) shows the structure of the shoot apical meristem (SAM) in WT cotyledonary embryos, Fig. 7(2) shows the structure of SAM in embryos expressing the *Brassica CLV1*, and Fig. 7(3) shows the structure of SAM in embryos expressing the *Brassica*

STM (3). The meristematic cells of *35S::BnCLV1* embryos accumulated storage products (2, arrow);

Figure 8 is a chart showing the number of transformed *Arabidopsis* seedlings producing leaf primordia 5 days after germination;

Figures 9(1) – 9(7) are micrographs showing production of ectopic meristems 9(1, arrow) or lobed leaves 9(2, arrow) from high *STM* over-expressors. The introduction of the *Brassica STM* also resulted in the production of abnormal leaves 9(3, with WT leaf on the left side), larger inflorescences 9(4) compared to WT plants 9(5), and curved siliques 9(6). Termination of the SAM with a determined stem-like structure (arrow), was often observed in lines ectopically expressing *BnCLV1* 9(7);

Figures 10(A) – 10(D) are charts showing expression levels measured by semi-quantitative RT-PCR of the meristem-related genes: 109(A) *KNAT6*, 10(B) *WUS*, 10(C) *CUC1*, and 10(D) *CLV3* in the transformed *Arabidopsis* plants after 1 week of germination. The values of the transformed lines were normalized to the value of WT plants set at 100;

Figures 11(A) – 11(D) are charts showing the effects of hormone treatments on the expression level of *BnSTM* as measured by semi-quantitative RT-PCR in shoots from 10-day old *Brassica napus* plants, harvested at different times after application of the hormone treatments. Fig 11(A) shows the effects of benzylamine purine (BAP, 100 μM), Fig. 10(B) shows the effects of indole acetic-acid (IAA, 30 μM), Fig. 11(C) shows the effects of abscisic acid (ABA, 100 μM), and Fig. 11(D) shows the effects of gibberellin-3 (GA3, 100 μM). Figures 11(E) – 11(H) are charts showing the effects of hormone treatments on the expression level of B*nCLV1*, wherein Fig. 11(E) shows the effects of BAP (100 μM), Fig. 11(F) shows the effects of IAA (30 μM), Fig. 11(G) shows the effects of ABA (100 μM), and Fig. 11(G) shows the effects of GA3 (100 μM); and

Figure 12(A) is a chart showing the effects of increasing ABA levels on the percentage of seed germination, Figure 12(B) is a chart showing the effects of increasing 2,4-D levels on root growth.

DETAILED DESCRIPTION

The present invention relates to methods for increasing seed yields of agronomic crops wherein the numbers of reproductive organs produced per plant are significantly increased in comparison to parental lines.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. In order that the invention herein described may be fully understood, the following terms and definitions are provided herein.

As used herein, the term "synthetic DNA" means DNA sequences that have been prepared entirely or at least partially by chemical means. Synthetic DNA sequences may be used, for example, for modifying native DNA sequences in terms of codon usage and expression efficiency.

The word "comprise" or variations such as "comprises" or "comprising" will be understood to imply the inclusion of a stated integer or groups of integers but not the exclusion of any other integer or group of integers.

As used herein, the word "complexed" means attached together by one or more linkages.

The term "a cell" includes a single cell as well as a plurality or population of cells.

The term "about" or "approximately" means within 20%, preferably within 10%, and more preferably within 5% of a given value or range.

The term "nucleic acid" refers to a polymeric compound comprised of covalently linked subunits called nucleotides. Nucleic acid includes polyribonucleic acid (RNA) and polydeoxyribonucleic acid (DNA), both of which may be single-stranded or double-stranded. DNA includes cDNA, genomic DNA, synthetic DNA, and semisynthetic DNA.

The term "gene" refers to an assembly of nucleotides that encode a polypeptide, and includes cDNA and genomic DNA nucleic acids.

The term "recombinant DNA molecule" refers to a DNA molecule that has undergone a molecular biological manipulation.

The term "vector" refers to any means for the transfer of a nucleic acid into a host cell. A vector may be a replicon to which another DNA segment may be attached so as to bring about the replication of the attached segment. A "replicon" is any genetic element (e.g., plasmid, phage, cosmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*, i.e., capable of replication under its own control. The term "vector" includes plasmids, DNA-protein complexes, and biopolymers. In addition to a nucleic acid, a vector may also contain one or more regulatory regions, and/or selectable markers useful in selecting, measuring, and monitoring nucleic acid transfer results (transfer to which tissues, duration of expression, etc.).

The term "cloning vector" refers to a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment. Cloning vectors may be capable of replication in one cell type, and expression in another ("shuttle vector").

A cell has been "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. A cell has been "transformed" by exogenous or heterologous DNA when the transfected DNA effects a phenotypic change. The transforming DNA can be integrated (covalently linked) into chromosomal DNA making up the genome of the cell.

The term "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules"), or any phosphoester anologs thereof, such as phosphorothioates and thioesters, in either single stranded form, or a double-stranded helix. Double stranded DNA—DNA, DNA-RNA and RNA—RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms.

Modification of a genetic and/or chemical nature is understood to mean any mutation, substitution, deletion, addition and/or modification of one or more residues. Such derivatives may be generated for various purposes, such as in particular that of enhancing its production levels, that of increasing and/or modifying its activity, or that of conferring new pharmacokinetic and/or biological properties on it. Among the derivatives resulting from an addition, there may be mentioned, for example, the chimeric nucleic acid sequences comprising an additional heterologous part linked to one end, for example of the hybrid construct type consisting of a cDNA with which one or more introns would be associated.

Likewise, for the purposes of the invention, the claimed nucleic acids may comprise promoter, activating or regulatory sequences, and the like.

The term "promoter sequence" refers to a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3′ direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3′ terminus by the transcription initiation site and extends upstream (5′ direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background.

A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if the coding sequence contains introns) and translated into the protein encoded by the coding sequence.

The term "homologous" in all its grammatical forms and spelling variations refers to the relationship between proteins that possess a "common evolutionary origin," including homologous proteins from different species. Such proteins (and their encoding genes) have sequence homology, as reflected by their high degree of sequence similarity. This homology is greater than about 75%, greater than about 80%, greater than about 85%. In some cases the homology will be greater than about 90% to 95% or 98%.

"Amino acid sequence homology" is understood to include both amino acid sequence identity and similarity. Homologous sequences share identical and/or similar amino acid residues, where similar residues are conservative substitutions for, or

"allowed point mutations" of, corresponding amino acid residues in an aligned reference sequence. Thus, a candidate polypeptide sequence that shares 70% amino acid homology with a reference sequence is one in which any 70% of the aligned residues are either identical to, or are conservative substitutions of, the corresponding residues in a reference sequence.

The term "polypeptide" refers to a polymeric compound comprised of covalently linked amino acid residues. Amino acids are classified into seven groups on the basis of the side chain R: (1) aliphatic side chains, (2) side chains containing a hydroxylic (OH) group, (3) side chains containing sulfur atoms, (4) side chains containing an acidic or amide group, (5) side chains containing a basic group, (6) side chains containing an aromatic ring, and (7) proline, an imino acid in which the side chain is fused to the amino group. A polypeptide of the invention preferably comprises at least about 14 amino acids.

The term "protein" refers to a polypeptide which plays a structural or functional role in a living cell.

The term "corresponding to" is used herein to refer to similar or homologous sequences, whether the exact position is identical or different from the molecule to which the similarity or homology is measured. A nucleic acid or amino acid sequence alignment may include spaces. Thus, the term "corresponding to" refers to the sequence similarity, and not the numbering of the amino acid residues or nucleotide bases.

The term "derivative" refers to a product comprising, for example, modifications at the level of the primary structure, such as deletions of one or more residues, substitutions of one or more residues, and/or modifications at the level of one or more residues. The number of residues affected by the modifications may be, for example, from 1, 2 or 3 to 10, 20, or 30 residues. The term derivative also comprises the molecules comprising additional internal or terminal parts, of a peptide nature or otherwise. They may be in particular active parts, markers, amino acids, such as methionine at position −1. The term derivative also comprises the molecules comprising modifications at the level of the tertiary structure (N-terminal end, and the like). The term derivative also comprises sequences homologous to the sequence

considered, derived from other cellular sources, and in particular from cells of human origin, or from other organisms, and possessing activity of the same type or of substantially similar type. Such homologous sequences may be obtained by hybridization experiments. The hybridizations may be performed based on nucleic acid libraries, using, as probe, the native sequence or a fragment thereof, under conventional stringency conditions or preferably under high stringency conditions.

An exemplary embodiment of the present invention pertains to methods for increasing the numbers of reproductive organs produced per plant of a selected seed-producing crop wherein expression of the SHOOT-MERISTEMLESS (i.e., *STM*) gene is manipulated in a manner such that the *STM* gene is over-expressed in comparison to non-manipulated plants.

We have surprisingly found that genetically transformed plants having incorporated therein *STM* genes under control of a 35S promotor, produce significantly more numbers of reproductive organs per plant than do the parent un-transformed plants. A suitable source for *STM* genes for transforming seed-producing agronomic crop plants is *Brassica napus*. The *STM* gene obtained from *B. napus* is referred to herein as the BnSTM gene. The sequence of the *BnSTM* gene is given in SEQ ID NO: 1.

Grain-producing agronomic crop plants transformed with an STM gene produce significantly greater numbers of reproductive organs and yield components exemplified by: flowers per plant, siliques per plant, branches per plant, pods per plant, seeds per pods, seeds per plant.

An exemplary embodiment of the present invention relates to a method for increasing the yields of oil-seed crops by manipulating therein the expression of *STM* genes so that the *STM* genes are over-expressed thereby increasing flowering and the production of yield components. An aspect pertains to transforming oil-seed crops by incorporating therein a *STM* gene exemplified by the *BnSTM* gene. Suitable oil-seed crops are exemplified by canola, mustard, flax, sunflower, camelina and the like.

SEQ ID NO: 1.

1 atggaaagtg gttccaacag cacttcttgt ccaatggctt ttgccgggga taatagtgat
61 ggtccgatgt gttctatgat gatgatgatg atgcccgtca taacatcaca tcaacaacat
121 catggtcatg atcaacaaca tcaacatcaa caacaacatg atggttatgc atatcagtca
181 caccaccaac atagtagcct ccttttttctt caatcactaa ctcctccgtc tcaagaagcg
241 aagaacaaag ttagatcttc ttgttctcct tcctctggtg ctcctgctta ttctttcatg
301 gagatcaatc accaaaacga actcctcgca ggaggactca atccctgttc ttcagcctct
361 gtcaaggcca aaatcatggg tcatcctcac taccaccgcc tcttgctcac ctatgtcaat
421 tgccagaagg tgggagctcc accggaagtg caggcgaggc tggaagaaac atgctcgtct
481 gcggctgccg ccgcagcgtc gatgggaccc acaggttctt taggtgaaga tccagggctt
541 gatcagttca tggaagcgta ctgtgaaatg ctcgttaagt acgagcaaga actctctaaa
601 ccttttaaag aagctatggt cttccttcaa cacgtcgagt gtcaattcaa atccctctct
661 ctctcctcgc cgtcctcctt ctctggttat ggagaggcag ctattgagag aaacaacaat
721 gggtcatctg aggaagaagt cgatatgaac aatgaatttg tagatccgca ggcagaagat
781 agggagctta aaggacagct cttgcgcaag tacagtggtt acttaggcag tctgaagcaa
841 gagttcatga agaagaggaa gaaaggagag cttcctaaag aagctcgcca gcaactactt
901 gactggtgga gccgacacta caaatggcct tacccttcgg agcagcaaaa gctagcacta
961 gcggaatcaa ctgggctgga ccagaaacag ataaacaact ggttcataaa ccagaggaaa
1021 aggcattgga aaccgtcgga ggatatgcag tttgtagtaa tggacgcaac acatcctcac
1081 cattacttta tggacaatgt catgggaaat cctttcccca ttgatcacat ctcctcgacc
1141 atgctttga

An exemplary embodiment of the present invention relates to a method for increasing the yields of pulse crops by manipulating therein the expression of *STM* genes so that the *STM* genes are over-expressed thereby increasing flowering and the production of yield components. An aspect pertains to transforming pulse crops by incorporating therein a *STM* gene exemplified by the *BnSTM* gene. Suitable pulse crops are exemplified by soybeans, field peas, lentils, chickpeas, dry beans, faba beans and the like.

An exemplary embodiment of the present invention relates to a method for increasing the yields of cereal crops by manipulating therein the expression of *STM* genes so that the *STM* genes are over-expressed thereby increasing flowering and the production of yield components. An aspect pertains to transforming cereal crops by

incorporating therein a *STM* gene exemplified by the *BnSTM* gene. Suitable cereal crops are exemplified by wheat, winter wheat, malting barley, feed barley, food barley, triticale, oats, and the like.

An exemplary embodiment of the present invention relates to a method for increasing the yields of maize crops by manipulating therein the expression of *STM* genes so that the *STM* genes are over-expressed thereby increasing flowering and the production of yield components. An aspect pertains to transforming maize crops by incorporating therein a STM gene exemplified by the *BnSTM* gene.

EXAMPLES

Example 1: Expression and localization of the *Brassica napus* genes during normal and abnormal embryo development

To establish a functional relationship between the four *Brassica* genes i.e., (i) *BnSTM*, (ii) *BnCLV1*, (iii) *BnZLL-1*, and (iv) *BnZLL-2*, and their effects on the development of shoot apical meristems, their expression and localization patterns in *B. napus* MDEs in which the SAM structure was experimentally altered through manipulations of the culture medium as disclosed herein.

Plant material and treatments:

Greenhouse culture conditions for growing and maintaining *Brassica napus* (Topas DH4079) plants and for the production of microspore-derived embryos (MDEs) from the plants followed the methods disclosed by Belmonte et al. (2006, *Improved development of microspore derived embryo cultures of Brassica napus cv Topaz following changes in glutathione metabolism*, Physiol. Plant. 127:690-700). *Brassica napus* cv Topaz seeds were grown at 25° C day/16° C night temperature with a 16-h photoperiod until the first flower buds appeared (approximately 5 weeks). Plants were then transferred to 12° C day/7° C night temperature cold treatment where they remained for bud collection. Flower buds (2–3 mm long) were collected from cold-treated plants and the microspores were isolated *and* suspended in NLN medium with 13% sucrose (pH 5.8). The microspores were then subjected to a heat shock

treatment of 32° C for 72 h, and then transferred onto a gyratory shaker and allowed to develop in the NLN medium up to 35 days under dark conditions.

For microspore-derived embryogenesis, "days in culture" were counted from the imposition of the heat shock treatment. Alterations in MDE and SAM structures were induced by following the methods taught by: (1) Belmonte et al. for 0.1 mM DL-buthionine sulfoximine (BSO), and (2) by Ramesar-Fortner et al. (2006, *Physiological influences in the development and function of the shoot apical meristem of microspore-derived embryos of Brassica napus cv. Topas*. Can. J. Bot. 84:371-383) for 2μM 2,3,5-triiodobenzoic acid (TIBA), and 2μM 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB). *B. napus* cv Topaz seeds were grown at 25° C day/16° C night temperature with a 16-h photoperiod until the first flower buds appeared (approximately 5 weeks). Plants were then transferred to 12° C day/7° C night temperature cold treatment where they remained for bud collection. Flower buds (2–3 mm long) were collected from cold-treated plants and the microspores were isolated *and* suspended in NLN medium with 13% sucrose (pH 5.8). The microspores were then subjected to a heat shock treatment of 32° C for 72 h, and then transferred onto a gyratory shaker and allowed to develop in the NLN medium up to 35 days under dark conditions. Microspore-derived embryos were harvested at day 5, 14, 21, 28, and 35 for gene expression and transcript localization studies.

Transgenic *Arabidopsis* plants ectopically expressing the *Brassica napus BnSTM* (GU480584), *B. rapa BrSTM* (GU480585), *B. oleracea BoSTM* (AF193813), *BnCLV1* (GU480585), as well as *BnZLL-1* (EU329719) and *BnZLL-2* (GU731230) were generated following the methods disclosed by Elhiti et al. (2010, *Modulation of embryo-forming capacity in culture through the expression of Brassica genes involved in the regulation of the shoot apical meristem*. J. Exp. Bot. 61:4069-4085). Full-length cDNAs were inserted in the Gateway entry clone vector pDONR221 (Invitrogen Canada Inc., Burlington, ON, Canada) and then transferred into the pK2GW7 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) once the plants started flowering. All plants were grown at 22° C under long day conditions (16 h light and 8 h dark). Seeds of transformed plants were

screened on 1× MS medium supplemented with 1% sucrose and kanamycin (50 µg ml−1).

The lines used in this study were *BnSTM* (L5, 8, and 23); *BrSTM* (L5, 15, 21), *BoSTM* (L5, 7, 11, and 33), *BnCLV1* (L12, L13, and L19), *BnZLL-1* (L2 and 16), and *BnZLL-2* (L8 and 16) (Figure 1).

The reactivation of the embryonic shoot apical meristem in transformed lines was estimated by germinating the *Arabidopsis* seeds on ½ MS medium containing 1% sucrose, and counting the number of 5-day-old seedlings which displayed newly formed leaf primordia.

Expression studies:

Expression studies in *Brassica* and *Arabidopsis* were carried out using semi-quantitative reverse transcriptase (RT)-PCR. Primers and number of cycles are listed in Table 1. The transcript levels of *BnSTM, BnCLV1, BnZLL-1* and *BnZLL-2* were measured during *Brassica* MDE development. The expression levels of *KNAT6, WUS, CUC1*, and *CLV3* were measured in the aerial part (seedling without roots) of 1-week old *Arabidopsis* plants germinated on ½ MS medium containing 1% sucrose. actin and ubiquitin were used as internal controls for *Brassica* and *Arabidopsis* respectively (Table 1).

Histological studies were performed exactly as described by Yeung (1999). Tissue was fixed in 2.5% glutaraldehyde and 1.6% paraformaldehyde buffered with 0.05M phosphate buffer pH6.9, dehydrated with methylcellosolve, followed by two changes of absolute alcohol, and then infiltrated and embedded in Historesin. Sectioning was carried out with glass knives on a Reichert-Jung 2040 Autocut microtome. Serial longitudinal sections were cut at a thickness of 3 µm. The sections were stained with 0.05% toluidine blue O in benzoate buffer, pH 4.4 and observed under a compound microscope.

Table 1: Primer sets and numbers of cycles used to measure the expression levels of *Brassica* and *Arabidopsis* genes by semi-quantitative PCT.

Brassica genes		Forward primers	Cycles
STM	SEQ ID NO:2	5-TGGGACCCACAGGTTCTTTA-3	27
CLV1	SEQ ID NO:4	5-TCTCCTTCAACGACCTCTCG-3	23
ZLL1	SEQ ID NO:6	5-GACGACGACGGCGGCAGCTCAGAGCC-3	24
ZLL2	SEQ ID NO:8	5-ACTCCGACCAAGCTTCTTCAC-3	26
Actin	SEQ ID NO:10	5-TAAAGTATCCGATTGAGCATGGTAT-3	22
Brassica genes		Reverse primers	
STM	SEQ ID NO:3	5-CCAGTTGATTCCGCTAGTGC-3	
CLV1	SEQ ID NO:5	5-TGGTGAAGATAACACAGTCCTTTCG-3	
ZLL1	SEQ ID NO:7	5-CAGACTCTTTGTATAGTCTCACTA-3	
ZLL2	SEQ ID NO:9	5-CAGACTCTTTGTATAGTCTCACTA-3	
Actin	SEQ ID NO:11	5-CGTAGGCAAGCTTCTCTTTAATGTC-3	
Arabidopsis genes		Forward primers	
KNAT6	SEQ ID NO:12	5-AGCGGTTTCATATTATTCTTCTTCTTCA-3	27
CUC1	SEQ ID NO:14	5-TTCTTCTTGTGCCGACAATG-3	25
WUS	SEQ ID NO:16	5-CCAGCAAGTTGTTTTCTTGC-3	27
CLV3	SEQ ID NO:18	5-TGGATTCGAAGAGTTTTCTGC-3	29
UBQ10	SEQ ID NO:20	5-GATCTTTGCGGAAAACAATTGGAGGATGG-3	21
Arabidopsis genes		Reverse primers	
KNAT6	SEQ ID NO:13	5-AAAAAGAGGTTATTTTTATTCACCG-3	
CUC1	SEQ ID NO:15	5-TCAGAGAGTAAACGGCCACA-3	
WUS	SEQ ID NO:17	5-CATCATAGAGATAAACGGTTGTCA-3	
CLV3	SEQ ID NO:19	5-GAAAATCATGAGATATAATAGTGC-3	
UBQ10	SEQ ID NO:21	5-CGACTTGCATTAGAAAGAAAGAGATAACAGG-3	

Hormone treatments and hormone analyses

The effects of hormone applications on the expression of *Brassica* genes were evaluated exactly as described by Liu *et al.* (2008). Briefly, ten day-old *Brassica napus* seedlings grown on vermiculite were collected and their roots were immersed in solutions containing difference levels of plant hormones: IAA (30 μM), ABA (100 μM), BAP (100 μM), and GA3 (100 μM). Shoots of the seedlings, without the cotyledons, were harvested for RNA extraction at 0, 1, 3, and 6h after hormone treatment. Ten day-old *Brassica napus* seedlings grown on vermiculite were collected and their roots were immersed in solutions containing difference levels of plant hormones: IAA (30 μM), ABA (100 μM), BAP (100 μM), and GA3 (100 μM). Shoots of the seedlings, without the cotyledons, were harvested for RNA extraction at 0, 1, 3, and 6h after hormone treatment.

For hormonal analyses, 3 week-old shoots from *Arabidopsis* wild type plants and plants over-expressing *BoSTM* (line 5) and *BnCLV1* (line 12) were collected and lyophilized. The plant hormone analysis was performed at the National Research Council of Canada (Plant Biotechnology Institute, Saskatoon, SK, CA) by high performance liquid chromatography electrospray tandem mass spectrometry (HPLC-ES-MS/MS) using deuterated internal standards, as described in Chiwocha *et al.* (2003) and Kong *et al.* (2008). Sensitivities to ABA and 2,4-D were assayed by performing germination and root growth tests as outlined in Weigel and Glazebrook (2002). For ABA sensitivity assays, seeds were germinated on ½ strength MS medium supplemented with 1% sucrose and different levels of ABA. The percentage of germinating seeds was scored at different days in culture using a dissecting microspore. For 2,4-D sensitivity assays, seeds were germinated on ½ strength MS medium supplemented 1% sucrose. Established seedlings were then transferred to a similar medium supplemented with different levels of 2,4D, and root growth was measured over time.

Immunolocalization of IAA

Fully developed MDEs were first pre-fixed in freshly prepared 4% aqueous 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDAC; Sigma-

Aldrich Canada Ltd., Oakville, ON, CA) at 4°C for 2h, and then post-fixed in FAA (10% formalin, 5% acetic acid, and 50% ethanol) overnight at 4° C. The fixed tissue was dehydrated in a ethanol series, embedded in paraplast, sectioned (10 µm), and deparaffinised in xylene. The sections were incubated in blocking solution ([10 mM phosphate buffer pH 7 (PBS), 0.1% Tween 20, 1.5% glycine, and 5% BSA) at room temperature for 1 hour. 150 µl of monoclonal primary IAA-antibodies (1 mg/ml, Sigma-Aldrich Canada Ltd., Oakville, ON, CA) diluted 1:200 in 10 mM PBS containing 0.8% BSA were applied to the sections and incubated in a high humidity chamber for 4 hours at room temperature. The slides were washed first in 10 mM PBS containing 0.88 g/L NaCl, 0.1 % Tween 20, and 0.8 % BSA for 5 minutes, and then in 10 mM PBS with 0.8% BSA for 5 minutes in order to remove any excess of Tween 20. The slides were incubated in 200 µl secondary antibodies i.e., 1 mg/ml anti-mouse IgG alkaline phosphates conjugate (Promega Corp., Madison, WI, USA). following the manufacturer's instruction overnight in a high humidity chamber, washed 2 times in 10 mM PBS containing 0.88 g/L NaCl, 0.1 % Tween 20, and 0.8 % BSA for 10 minutes, and then incubated in water for 15 min to remove the excess of secondary antibodies. Samples were stained using 250 µl Western blue (Promega Corp., Madison, WI, USA) for 40 minutes.

Pharmacological studies

The SAM in MDEs was enhanced by inclusions of DL-buthionine sulfoximine (BSO), which produced better organized SAMs with a zygotic-like appearance. These meristems regenerated shoots at high frequency denoting an improved functionality Formation of the SAM was compromised in MDEs cultured with 2,3,5-triiodobenzoic acid (TIBA). This compound blocks the basipetal flow of auxin resulting in an accumulation of auxin at the tip of the cotyledons and at the SAM (Figure 2). TIBA-treated embryos developed non-functional SAMs and exhibited a characteristic fusion of the cotyledons at their base. These abnormalities were reverted if 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB), an auxin antagonist, was added with TIBA. The expression levels of all *Brassica* genes were repressed in MDEs developed in the presence of TIBA (Figures 3A-3D), whereas BSO increased the transcript levels of *BnSTM*, *BnZLL-1* and *BnZLL-2*. Intermediate levels of expression were detected in control embryos and embryos cultured with TIBA+PCIB. The

similar expression profiles observed for *BnZLL-1* and *2* suggest that these two genes might have a similar function during microspore-derived embryogenesis (Figures 3A-3D).

In situ hybridization studies revealed that the four *B. napus* genes are expressed in the SAM during *in vitro* embryogenesis. In untreated MDEs, three distinct *BnSTM* localization patterns could be detected. In some MDEs, *BnSTM* exhibited a "zygotic-like" expression, which was restricted to the central region of the apical pole (Figures 2(1) – 2(4)). In other embryos, transcripts of this gene could be detected in unusual domains, such as the peripheral region of the apical pole (Figures 4(5) – 4(7)), or in separate pockets of cells (Figures 4(8) – 4(11)). In the improved SAMs of BSO-treated MDEs, the localization domain of *BnSTM* was always central and encompassed a larger group of cells, especially in heart-shaped embryos (Figures 4(12) – 4(14)). In the presence of TIBA, *BnSTM* expression was restricted to the apical pole in immature embryos (Figures 4(15) – 4(16)) but was lost in cotyledonary embryos with an abnormal SAM (Figure 4(17)). Both structural and expression abnormalities induced by TIBA were reversed in TIBA+PCIB treated MDEs (Figures 4(18) – 4(19)). No signal was detected with a sense probe (Figure 4(20)).

Expression of *BnCLV1* was first visible in the sub-apical cells of globular MDEs and then extended to a larger domain in middle and late cotyledonary embryos (Figure 5(1) – 5(3)). In the presence of BSO, a larger group of sub-apical cells expressed *BnCLV1* in globular embryos (Figure 5(4)). The expression in the sub-apical domain was also retained in fully developed BSO-treated MDEs (Figure 5(5)). TIBA reduced the expression domain of *BnCLV1* in globular embryos, and to a certain extent in cotyledonary embryos (Figure 5(6) – 5(7)). Embryos treated with TIBA+PCIB exhibited a control-like expression pattern (image not shown) whereas no signal was observed in tissue hybridized with sense probe (Figure 5(8)).

The combined expression of *BnZLL-1* and *BnZLL-2* (the high degree of similarity in the sequences of the two cDNAs did not allow for the design of specific probes) was first detected in the vascular tissue of developing MDEs, and then extended to the SAM of cotyledonary embryos (Figure 6(1) – 6(5)). Within the SAM the signal was often delocalized to the peripheral region (Figure 6(4)). Inclusions of BSO enlarged the localization domain of *BnZLL-1* and *BnZLL-2* throughout the SAM

(Figure 6(6)), whereas no signal was observed in the SAMs of TIBA-treated embryos (Figure 6(7)). The signal was recovered if PCIB was applied with TIBA (Figure 6(8)). Specificity of the hybridization was demonstrated using a sense riboprobe (Figure 6(9)).

Phenotypic characterization of *Arabidopsis* plants ectopically expressing *Brassica* genes

Given the very low efficiency of *Brassica napus* Topas (DH4079 embryogenic line) transformation, the effects of ectopic expression of the four *Brassica* genes during development were analysed in *Arabidopsis*. During zygotic embryo development the ectopic expression of the *Brassica napus BnCLV1* resulted in the formation of small SAMs (which also accumulated storage products), whereas an opposite effect was observed with the introduction of *STM*. Compared to WT, the SAM of the *BnSTM* over-expressing embryos was dome-shaped and often composed of a larger cluster of meristematic cells (Figures 7(1) – 7(3)). No effects on meristem size or shape were noticed in plants over-expressing the *BnZLL-1* and *BnZLL-2*, which showed a WT phenotype. These structural differences resulted in alterations in the rate of meristem reactivation. Overall SAM reactivation at germination was delayed by the introduction of *BnCLV1* and accelerated by the over-expression of *BnSTM* (Figure 8).

Similar phenotypic deviations from WT were observed among all the *Brassica STM* over-expressing lines (*35S::BnSTM, BoSTM,* and *BrSTM*), although the most severe phenotypes were obtained in *BoSTM* plants. In lines with the highest *BoSTM* expression, such as line 33 (Figure 1) ectopic shoots (Figure 9(1)) and small lobed leaflets (Figure 9(2)) emerged from the adaxal side of the rosette leaves. These lines were sterile and eliminated form further characterization. The remaining lines with lower levels of *STM* expression produced small lobed and serrated leaves (Figure 9(3)), larger inflorescences characterized by more flowers (Figures 9(4) – 97(5)) and curved siliques (Figure 9(6)). Increased number of siliques and flowers, as well as reduced plant height were observed in almost all *STM* over-expressors (Table 2).

Table 2: Phenotypic characterization of lines over-expressing the *Brassica napus* (*Bn*), *B. rapa* (*Br*), and *B. oleracea* (*Bo*) *STM*.

	WT	BnSTM L5	BnSTM L8	BnSTM L23	BrSTM L5	BrSTM L15	BrSTM L21	BoSTM L5	BoSTM L7	BoSTM L11
H	100	81.1 (±4.6)	80.76 (±8.4)	86.59 (±5.5)	83.1 (±7.3)	91.6 (±5.4)	86.4 (±7.2)	74.6 (±9.8)	70.7 (±5.9)	80.1 (±7.1)
B	100	110 (±10.3)	116.1 (±10.5)	106 (±8.9)	122.6 (±10.9)	119.3 (±14.3)	130.5 (±9.2)	98.4 (±6.7)	103.6 (±9.6)	105.6 (±10.2)
S	100	149.27 (±10.3)	158.16 (±10.2)	159.72 (±9.7)	161.3 (±11.9)	159.7 (±11.6)	160.1 (±13.7)	110.3 (±9.8)	97.6 (±10.2)	119.3 (9.7)
CL	100	98.27 (±8.2)	102 (±7.6)	96.5 (±6.3)	97.6 (±12.3)	109 (±8.9)	115.3 (±7.9)	72.3 (±6.2)	69.4 (±7.8)	68.7 (±6.8)
RL	100	100.5 (±12.2)	80.75 (±9.8)	90.79 (±9.7)	113.4 (±10.2)	98.2 (±7.9)	93.7 (±6.9)	109.6 (±13.2)	117 (±10.2)	98.9 (±14.2)
F	100	163.79 (±12.2)	161.49 (±16.3)	175.1 (±14.2)	169.3 (±7.9)	178.6 (±13.2)	168.7 (±15.6)	103.3 (±8.9)	87.2 (±10.3)	124.1 (±4.6)

H: plant height

B: branches

S: siliques

CL: cauline leaves

RL: rosette leaves

F: flowers

A reduction in hypocotyl elongation and termination of the SAM (Fig. 9(7)) were observed in lines with higher expression levels of *BnCLV1*. Overall, the introduction of *BnCLV1* resulted in a reduction in the number of siliques and cauline leaves (Table 3). No major morphological differences were observed in *Arabidopsis* lines ectopically expressing *BnZLL-1* and *BnZLL-2*, the only exception being a reduced number of branches (Table 3).

Table 3: Phenotypic characterization of lines over-expressing the *Brassica napus* CLV1, ZLL-1 and ZLL-2 genes, expressed as a percentage of controls.

	WT	BnCLV1 L1	BnCLV1 L3	BnCLV1 L9	BnZLL-1 L2	BnZLL-1 L16	BnZLL-2 L8	BnZLL-2 L16
H	100	109.5 (±10.9)	104.7 (±9.8)	113.61 (±14.6)	79.6 (±8.3)	85. 8 (±6.3)	90.4 (±8.9)	91.3 (±10.3)
B	100	107.7 (±18.3)	105.1 (±9.3)	143.7 (±10.7)	76.8 (±13.8)	52.5 (±8.8)	65.4 ±(11.3)	67.4 (±4.5)
S	100	87.9 (±11.3)	85.8 (±6.9)	78.7 (±7.8)	92.2 (±12.3)	91.9 (±8.1)	96.3 (±7.5)	90.5 (±6.3)
CL	100	67.6 (±10.1)	78.2 (±9.7)	90.5 (±8.9)	97.4 (±11.6)	104.1 (±16.3)	99.5 (±13.6)	104.8 (±15.5)
RL	100	86.8 (±8.9)	86.8 (±8.3)	100.5 (±9.1)	105.7 (±13.6)	98.8 (±8.9)	100.8 (±7.9)	99.6 (±9.3)
F	100	92.1 (±13.5)	83.7 (±8.4)	87.8 (±10.9)	98.5 (±11.3)	98.8 (±7.1)	95.5 (±12.3)	90.7 (±9.3)

H: plant height

B: branches

S: siliques

CL: cauline leaves

RL: rosette leaves

F: flowers

Expression analysis of SAM-related genes in the transformed lines

The expression level of several SAM-related genes was measured in the shoot of some transformed *Arabidopsis* seedlings after 1 week of germination. The introduction of *Brassica STM* activated the expression of *KNAT6*, *WUSCHEL (WUS)* and *CUPSHAPED COTYLEDON-1 (CUC 1)* (Figures 10A – 10C) whereas an opposite trend was observed in the *BnCLV1* over-expressing line (Figure 10D). A less pronounced increase in expression of the three genes was also obtained in lines ectopically expressing *BnZLL-1* and *BnZLL-2* (Figures 10A – 10C). The expression of *CLAVATA 3* (*CLV3*) was repressed by the introduction of the *Brassica STM*, whereas it increased in lines over-expressing *BnCLV1* and *BnZLL-2*.

Effects of hormone treatments on the expression of the Brassica genes

The endogenous levels of *BnSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* were measured in *Brassica* seedlings treated with different hormones. Compared to control (untreated tissue), applications of the cytokinin BAP and the auxin IAA increased the level of *BnSTM* after a few hours (Figures 11A – 11B). An opposite expression profile was measured in the presence of ABA and GA3 (Figures 11C – 11D).

The expression level of *BnCLV1* increased in both control seedlings and seedlings treated with BAP and IAA (Figures 11E – 11F). The transcript levels of this gene were induced by ABA (Figure 11G) and repressed by GA3 (Figure 11H) applications.

Hormone treatments did not affect the expression of *BnZLL-1* and *BnZLL-2*.

Endogenous hormone analysis of the transformed Arabidopsis plants

For hormone analysis three *Arabidopsis* lines were selected: WT, *BoSTM* (L5), and *BnCLV1* (L12); with the last two exhibiting the most severe phenotypes among all the transformed lines. No *ZLL* over-expressing lines were selected as they did not show any major phenotypic deviation from WT plants. Compared to WT, the introduction of the *Brassica STM* increased the endogenous levels of the cytokinins trans-zeatin-O-glucoside (t-ZOG), cis-zeatin-O-glucoside (c-ZOG), trans-zeatin riboside (t-ZR), and isopentenyladenosine (iPA). Undetectable or low levels of cytokinins were measured in the shoots of the *35S::BnCLV1* line (Table 4). Zeatin and dehydrozeatin were below detectable levels in all the lines analysed.

Profile of auxin metabolites showed a significant increased level of indole-3-acetic acid (IAA) in the 35S::*BoSTM* line. IAA-alanine (IAA-Ala) was not detected in the three lines whereas very low levels of IAA-aspartic acid (IAA-Asp) and IAA-glutamic acid (IAA-Glu) were measured.

Compared to the other lines analysed, the line over-expressing the *Brassica STM* had lower endogenous levels of abscisic acid (ABA), dehydrophaseic acid (DPA), ABA-glucose ester (ABA-GE), and phaseic acid (PA). The highest

concentration of DPA was measured in the *35S::BnCLV1* line. 7'-hydroxy-ABA (7'-OH-ABA) was below detectable levels (Table 4).

Table 4: Hormone profiles of *Arabidopsis* shoots ectopically expressing *Brassica oleracea SHOOTMERISTEMLESS* (BoSTM) gene and *Brassica napus CLAVATA1* (BnCLV1) gene.7

Cytokinins	tZOG	cZOG	t-ZR	c-ZR	2iP	iPA
WT	28±1	119±2	9±1	11±0	20±1	23±1
BoSTM	45±4*	274±9*	94±1*	12±1	20±0	60±1*
BnCLV1	ND	ND	<	5±0*	<	10±1 *

Auxins	IAA	IAA-Ala	IAA-Asp	IAA-Glu
WT	147±13	ND	11±1	ND
BoSTM	254±22*	ND	14±0	14±1*
BnCLV1	138±17	ND	<	ND

ABA metabolism	ABA	DPA	ABA-GE	PA	7'OH-ABA
WT	102±2	545±7	157±12	162±4	ND
BoSTM	55±1*	342±8*	95±18*	75±5*	ND
BnCLV1	109±2	745±62 *	83±2*	152±13	ND

tZOG: trans-zeatin-O-glucoside
cZOG: cis-zeatin-O-glucoside
tZR: trans-zeatin riboside
cZR: cis-zeatin riboside
2iP: isopentenyl adenonine
iPA: isopentenyl adenosine
IAA: indole 3-acetic acid
ABA: abscisic acid
IAA-Ala: indole 3-acetic acid alanineDPA:
DPA: dehydrophaseic acid
IAA-Asp: indole 3-acetic acid aspartate
ABA-GE:abscisic acid glucose ester
IAA-Glu: indole 3-acetic acid glutamic acid
PA: phaseic acid
7'-OH-ABA: 7'-hydroxyl abscisic acid
< : indicates that the signals were below the limit for quanitification
ND : not detected

Hormone sensitivity assays

Sensitivity to ABA was assayed using a seed germination test. A similar inhibition profile with increasing concentrations of ABA was observed for WT seeds, as well as seeds obtained from *35S::BnCLV1, BnZLL-1* and *-2* lines (Figure 12A). Seeds over-expressing the *Brassica STM* showed a reduced sensitivity to the inhibitory effect of ABA and a significant percentage of germination was still measured at those ABA levels precluding germination in WT seeds.

Root growth assays were performed to test the tissue sensitivity to 2,4-D. Compared to WT, the rate of root growth was generally higher in the *35S::BnCLV1* and *BnZLL-2* lines. The introduction of the *Brassica STM* increased sensitivity to high levels of 2,4-D, as estimated by the reduced root growth (Figure 12B).

The *Brassica* genes are reliable markers of SAM functionality

Meristem functionality in *B. napus* MDEs, estimated by the ability of the SAMs to convert, i.e. produce viable shoots at germination, can be easily altered in culture. In our system, about 40% of the MDEs produce functional meristems able to reactivate upon transfer onto germination medium. It appears that *BnSTM*, *BnCLV1*, and *BnZLL-1* and *BnZLL-2* are implicated in meristem function during MDE development. In control (untreated) MDEs, *BnSTM* signal is first activated in a pocket of sub-apical cells in globular-stage embryos where it demarks the future site of the SAM and later extended to the entire apical pole of the embryos (Figs. 4(1)-4(4)). The inability of many (about 60%) untreated *B. napus* MDEs to convert correlates with the early (prior to the formation of a meristem) miss-expression of *BnSTM* either in the peripheral region (Figs. 4(5)-4(7)), or in two separate domains of the SAM (Figs. 4*8)-4(11)). A deviation in expression pattern in control MDEs was also observed for *BnZLL-1* and *BnZLL-2* which are first expressed in the vascular tissue and later in the SAM (Fig. 6). In many MDEs the expression domain of *BnZLL-1* and *BnZLL-2* in the SAM is restricted to the peripheral zone (Figure 6(4)).

The BSO-improvement of meristem functionality correlates to the increased transcript levels and enlarged localization patterns of *BnSTM, BnZLL-1 andBnZLL -2*

(Figures 4 and 6). Of interest, the enlarged *BnSTM* signal in the apical pole of MDEs cultured with BSO (Figs. 4(12)-4(13)) became apparent before any visible SAM, thereby suggesting that the *BnSTM* localization pattern in early embryos can be used to estimate the quality of the meristem.

The expression of all *Brassica* genes is compromised in non-functional (TIBA-treated) MDEs (Figure 3). The SAMs of these embryos do not express *BnSTM*, *BnZLL-1* and *-2*, while the signal of *BnCLV1* is reduced. Poor meristem function and repression of gene expression can be attributed to the TIBA-induced accumulation of IAA within the apical regions of the MDEs, especially in the SAM (Figure 2).

Based on this information, it appears that the *Brassica* genes might be implicated in meristem functionality and can be utilized as ``SAM-markers` to estimate the quality of the shoot meristems of cultured embyros.

Phenotypic characterization of Arabidopsis ectopically expressing the Brassica genes

Ectopic expression of the *Brassica STM* and *CLV1* influence the rate of SAM reactivation at germination, with the former accelerating meristem conversion and the latter having an opposite effect (Fig. 8). These results can be possibly attributed to the different organization of the embryonic SAMs observed in the transformed line (Fig. 7). The role of these two genes during SAM maintenance is well established but their influence on meristem reactivation has not been documented. If the two *Brassica* genes fulfill similar roles to those assigned to their *Arabidopsis* orthologs, with *STM* increasing the domain of meristematic cells and *CLV*1 repressing the function of *STM*, then it follows that the rate of meristem reactivation relies on the pool of undifferentiated cells present within the apical pole of the embryos. The accumulation of storage products, a sign of cellular differentiation, in the meristematic cells of *35S::CLV1* embryos (Fig. 7(2)) and the termination of the SAM a few days after germination (Fig. 9(7)) support this observation.

The STM-acceleration of shoot meristem conversion at germination may be due to the early activation of several genes involved in SAM activity, such as *KNAT6*, *WUS*, and *CUC1* (Fig. 10). *KNAT 6*, a *KNOTTED*-like gene, is a downstream

effector of the STM pathway required for the promotion of meristematic proliferation. Inactivation of *KNAT6* has been shown by others to abolish the residual meristematic activity of weak *stm-2* plants. An additional function of this gene is to regulate lateral organ separation, in an integrated pathway including CUC1, which delimits the boundaries of primordia produced in the peripheral zone of the SAM. Fusions of lateral organs often occur in *cuc1* mutants. A third gene induced by the introduction of the *Brassica STM* is *WUSCHEL (WUS),* which encodes a WOX-class homeodomain transcription factor required for maintaining the meristematic cells of the central zone in an "indeterminate" state. Mutations of *WUS* result in the differentiation and incorporation of the cells occupying the central domain of the SAM into leaf primordia, leading to the abortion of the meristem. These three regulators of meristem activity are repressed in *BnCLV1* lines (Fig. 10) characterized by a delayed conversion of the SAM at germination. The up-regulation of *CLV3* in plants ectopically expressing *BnCLV1* further suggests that the CLAVATA pathway (requiring both CLV1 and CLV3) is highly operative. This would result in the promotion of cellular differentiation (leading to the termination of the SAM, Figs. 7(2)) and depletion of the stem cells through the repression of *WUS* (Fig. 10). This is in contrast to the *STM* over-expressors characterized by lower *CLV3* expression (Fig. 10).

The up-regulation of several genes involved in SAM activity in *Arabidopsis* lines ectopically expressing the *Brassica STM* also correlates with the formation of adventitious shoots, abnormal leaf morphology, and increased production of reproductive organs (Fig. 9 and Table 2). The abnormalities in leaf morphopogy observed in our study (Fig. 9) are consistent with the over-expression of other *KNOX-1* genes observed in other systems and can be attributed to meristematic growth originating from the margins of the leaves.

A novel trait linked to the introduction of S*TM* is the enlarged inflorescence size, resulting in a higher number of flowers and siliques (Fig. 9 and Table 2). This phenotype can also be the result of the increased meristematic activity encouraging floral meristem formation.

Interaction between the Brassica genes and plant growth regulators

The studies disclosed herein show a positive correlation between *STM* expression and cytokinins. While applications of BAP activate *BnSTM* (Fig. 11), over-expression of the *Brassica STM* induces the accumulation of both zeatin-type cytokinins, i.e. c- and t-ZOG, as well as tZR, and iP-type cytokinins, i.e. iPA (Table 4). The concentrations of free zeatin and dehydrozeatin were below detectable levels, implying a fast turnover to ZR. The STM-mediated increase in cytokinins levels might be exercised through the activation of adenylate isopentenyltransferases, which are the first enzymes involved in the biosynthesis of cytokinins in both the methylerythritol phosphate and mevalonate pathways. The introduction of *BoSTM* in *Arabidopsis* tissue induces the transcription of adenylate isopentenyltransferase 7. This intimate interaction between STM and cytokinins, which was not observed for CLV1, might explain some of the phenotypic deviations noticed in the transformed plants, including the formation of larger SAMs (Figure 5A), and the production of ectopic meristems (Fig. 9).

The poor architecture of the *35S::CLV1* SAMs in developing embryos and their termination during post-embryonic growth may be caused by the extremely low endogenous levels of cytokinins (Table 4). The lines over-expressing *BnCLV1* which, besides having low cytokinin levels, may also have reduced WUS expression (Fig. 10 and Table 4).

The GA3 repression on the transcription of *STM* and *CLV1* suggests an interaction between SAM-related genes and GAs. Several studies have shown that proper SAM functions require low levels of GAs and this condition can be attributed to *STM* (and other *KNOX1* genes) suppressing the transcription of GA20 oxidase (Hay *et al.,* 2002). All the GAs measured by HPLC-ES-MS/MS, i.e. GA1, 3, 4, 7, 8, 9, 19, 20, 24, 29, 34, 44, 51 and 53 were below detectable levels among the three lines. However, a localized effect of SAM-related genes on GAs cannot be excluded given the large size of the tissue analysed.

The high levels of free IAA (Table 4) and the increased sensitivity to supplied 2,4-D (Fig. 8) exhibited by lines over-expressing the *Brassica STM* suggests a

possible role of auxin in the *STM* phenotype. During in vitro morphogenesis, auxin might be required for the acquisition of embryogenic fate through the induction of *WUS*. It is not clear whether a similar interaction occurs in vivo with auxin conferring stem fate to the cells of the organizing center through the activation of *WUS*. This intriguing possibility cannot be excluded given the positive correlation between high IAA content and elevated *WUS* expression observed in this study, and the mechanistic parallels between the organizing center in the SAM and the quiescent center in the root meristem. The root quiescent center is specified by the expression of *WUS*-like genes and this specification requires an auxin maximum ensured by its basipetal transport. The lobed leaves and the formation of ectopic meristems observed in the lines over-expressing the *Brassica STM* (Fig. 9) can be a direct consequence of high auxin levels which maintain the cells within the blastozone of the leaves in a proliferative state.

Compared to the *35S::CLV1* line, the biosynthesis of ABA, as well as its degradation to PA and DPA via the 8'hydroxylation pathway were lower in plants over-expressing *STM* (Table 4). These plants also exhibited a lower sensitivity to ABA (Fig. 8). The lack of available information relating STM to ABA does not allow any speculation on these results; however, the low endogenous levels and sensitivity to ABA might be responsible for the quick reactivation of the apical meristems at germination.

Example 2: Over-expression of the BnSTM gene in *Brassica napus*

Brassica transformation with BnSTM in sense orientation was performed following the method taught by Bhalla and Singh (2008, *Agrobacterium mediated transformation of Brassica napus and Brassica oleraceae*. Nature Protocols 3:181-189) with some modifications. Briefly, surface sterilized seeds were germinated for 5 days on ½ 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 2mg/L BA) for 4 days, and then incubated with the same *Agrobacterium* strain used for *Arabidopsis* transformation, harboring the pK2GW7 vector for 2 min. Following a 4 day co-cultivation period, the explants were plated on shoot induction medium (MS-B5 supplemented with 5mg/l AgNO3, 2mg/l BA, 3% sucrose, 20 mg/l timentin and 50 mg/l kanamycin) for 8 weeks. The emerging shoots were first

transferred on elongation medium (MS-B5 supplemented with 3% sucrose, 0.1 mg/l GA3, and 1 mg/l BA) for 4 weeks and then placed on rooting medium (MS-B5 supplemented with 1% sucrose, 2 mg/l NAA) for 4 weeks. Rooted shoots were transferred onto soil to generate fully mature plants (F0). Seeds from F0 plants were germinated on ½ MS basal medium with 1% sucrose and 50 mg/l kanamicin and the resulting F1 generation was screened for the presence of the transgene by RT-PCR using a forward 35S promoter primer (SEQ ID NO:22; 5'-TGGACCCCCACCCACGAG-3') and a reverse gene specific primer (SEQ ID NO:23; 5'-GCACCAGAGGAAGGAGAACA-3'). Microspores harvested from the positive F1 plants were cultured to produce haploid MDEs which were germinated on ½ MS medium supplemented with 1% sucrose and 70 mg/l kanamycin for 4 weeks. Thehaploid 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 qRT-PCR, selected plants were used as a source of seeds to generate the next (F2) generation. The expression level of BnSTM in these plants was measured by quantitative RT-PCR.

The transformed *B. napus* plants over-expressing *BnSTM* produced 20% more siliques than were produced by the wild type control plants.

In conclusion, this work shows that the expression levels and localization patterns of the *Brassica STM*, *CLV1*, and ZLL correlate with the quality of the SAM established during embryo development, and can be utilized to estimate meristem functionality. The ectopic expression of these genes induces profound phenotypic changes during both embryonic and post-embryonic development. While some of these changes are similar to those produced by the ectopic expression of their respective *Arabidopsis* orthologs, others are unique and might be the consequence of profound alterations in endogenous hormone levels, especially cytokinins, auxins, and ABA.

Claims:

1. A method for increasing seed yields in an agronomic crop, comprising:

modulating an expression of a *STM* gene in plants comprising the agronomic crop whereby the numbers of reproductive organs produced per modulated plant are significantly increased in comparison to a non-modulated plant.

2. A method for increasing seed yields in an agronomic crop, comprising:

transforming a selected plant cell from a plant from the agronomic crop, by incorporating therein an expression vector comprising a *STM* gene.

3. The method according to claim 2, wherein the *STM* gene is a *Brassica napus STM* gene.

4. The method according to claim 3, wherein the *STM* gene comprises a nucleotide sequence set forth in SEQ ID NO: 1, or comprises a nucleotide sequence that exhibits from about 80% to 100% identity with SEQ ID NO: 1.

5. A plant cell produced according to the method of any of claims 1 to 3.

6. The method according to claim 1, wherein the agronomic crop is selected from a group consisting of oil-seed crops, pulse crops, cereal crops, and maize.

7. The method according to claim 6, wherein the oil-seed crop is selected from a group consisting of canola, mustard, flax, sunflower, and camelina.

8. The method according to claim 6, wherein the pulse crop is selected from a group consisting of soybeans, field peas, lentils, chickpeas, dry beans, and faba beans.

9. The method according to claim 6, wherein the cereal crop is selected from a group consisting of wheat, winter wheat, malting barley, feed barley, food barley, triticale, and oats.

10. Use of a *STM* gene to transform an agronomic crop for increasing seed yield therein.

11. Use according to claim 10, wherein the *STM* gene is a *Brassica napus STM* gene.

12. Use according to claim 11, wherein the *STM* gene comprises a nucleotide sequence set forth in SEQ ID NO: 1, or comprises a nucleotide sequence that exhibits from about 80% to 100% identity with SEQ ID NO: 1.

13. Use according to claim 10, wherein the agronomic crop is selected from a group consisting of oil-seed crops, pulse crops, cereal crops, and maize.

14. Use according to claim 13, wherein the oil-seed crop is selected from a group consisting of canola, mustard, flax, sunflower, and camelina.

15. Use according to claim 13, wherein the pulse crop is selected from a group consisting of soybeans, field peas, lentils, chickpeas, dry beans, and faba beans.

16. Use according to claim 13, wherein the cereal crop is selected from a group consisting of wheat, winter wheat, malting barley, feed barley, food barley, triticale, and oats.

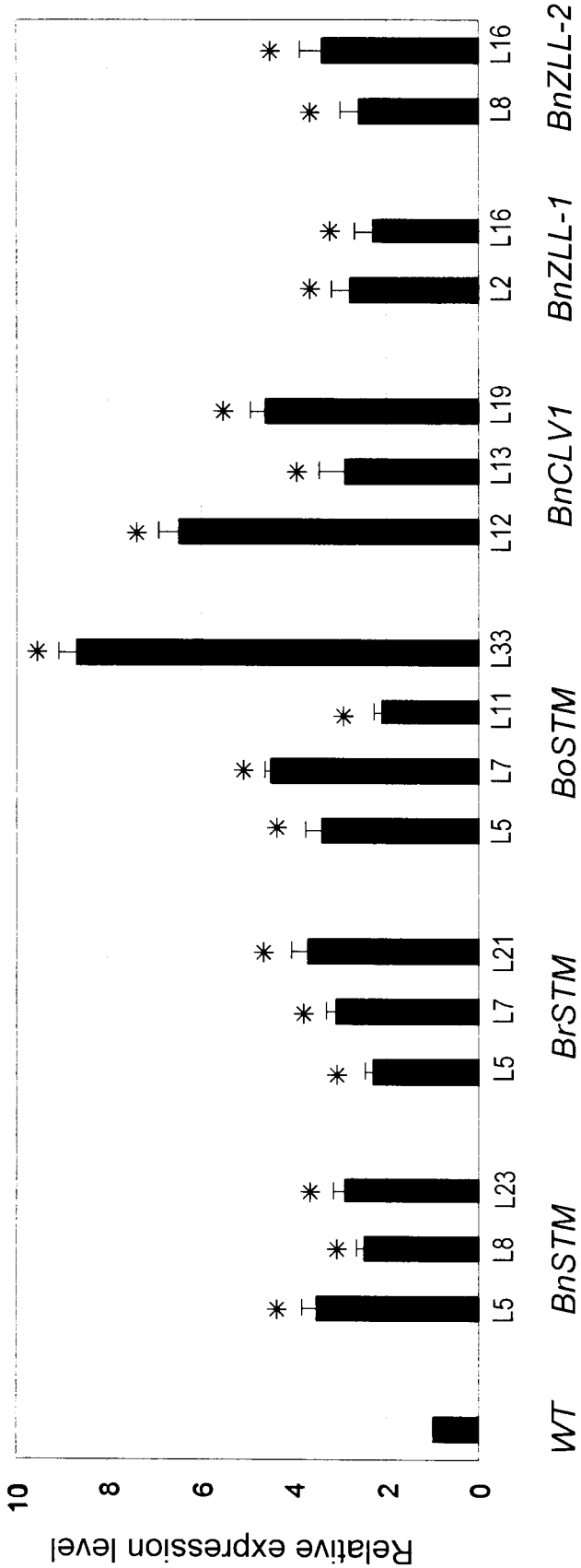

Relative expression level
0
2
4
6
8
10
WT
BnSTM
L5
L8
L23
BrSTM
L5
L7
L21
BoSTM
L5
L7
L11
L33
BnCLV1
L12
L13
L19
BnZLL-1
L2
L16
BnZLL-2
L8
L16

Figure 1

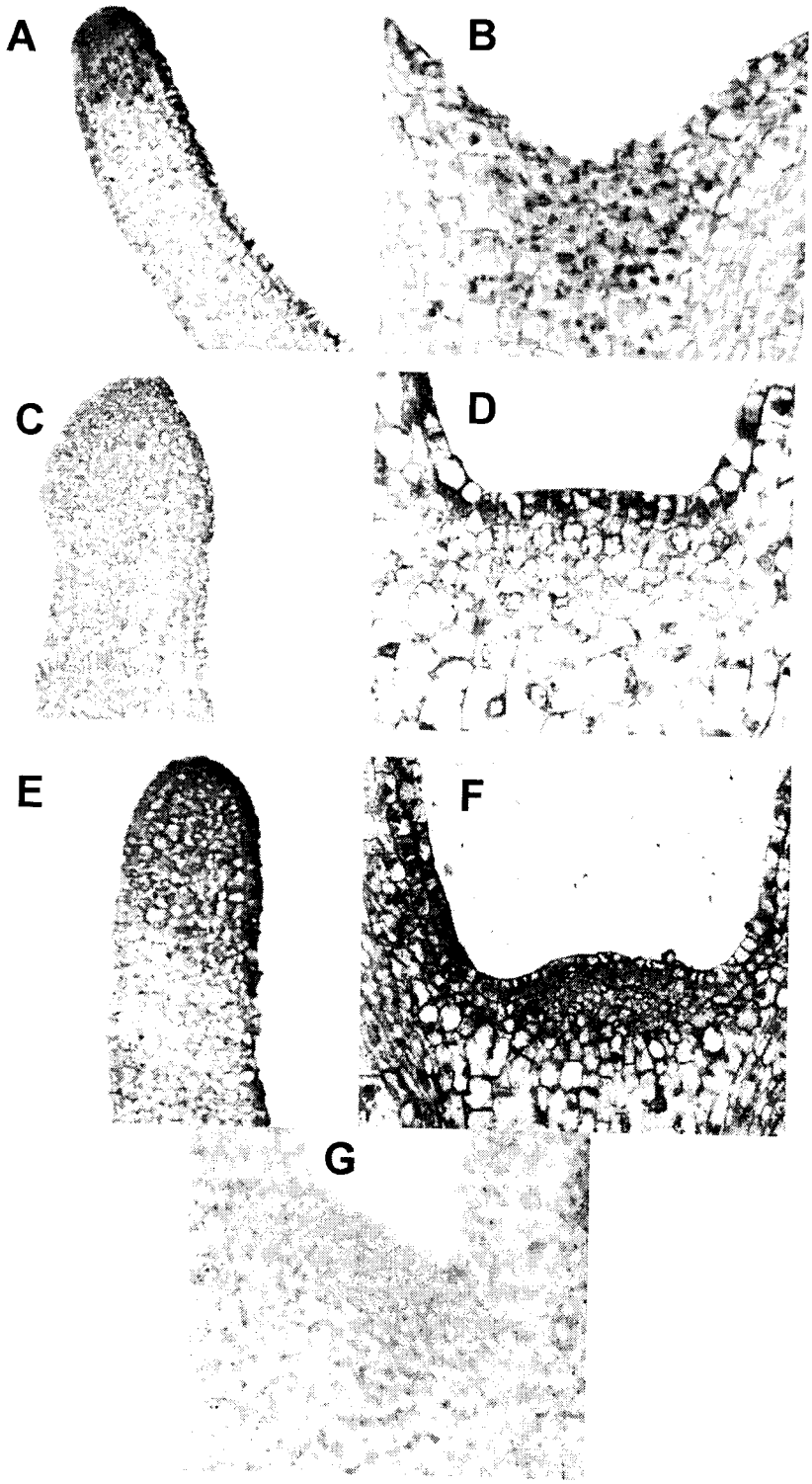


Figure 2

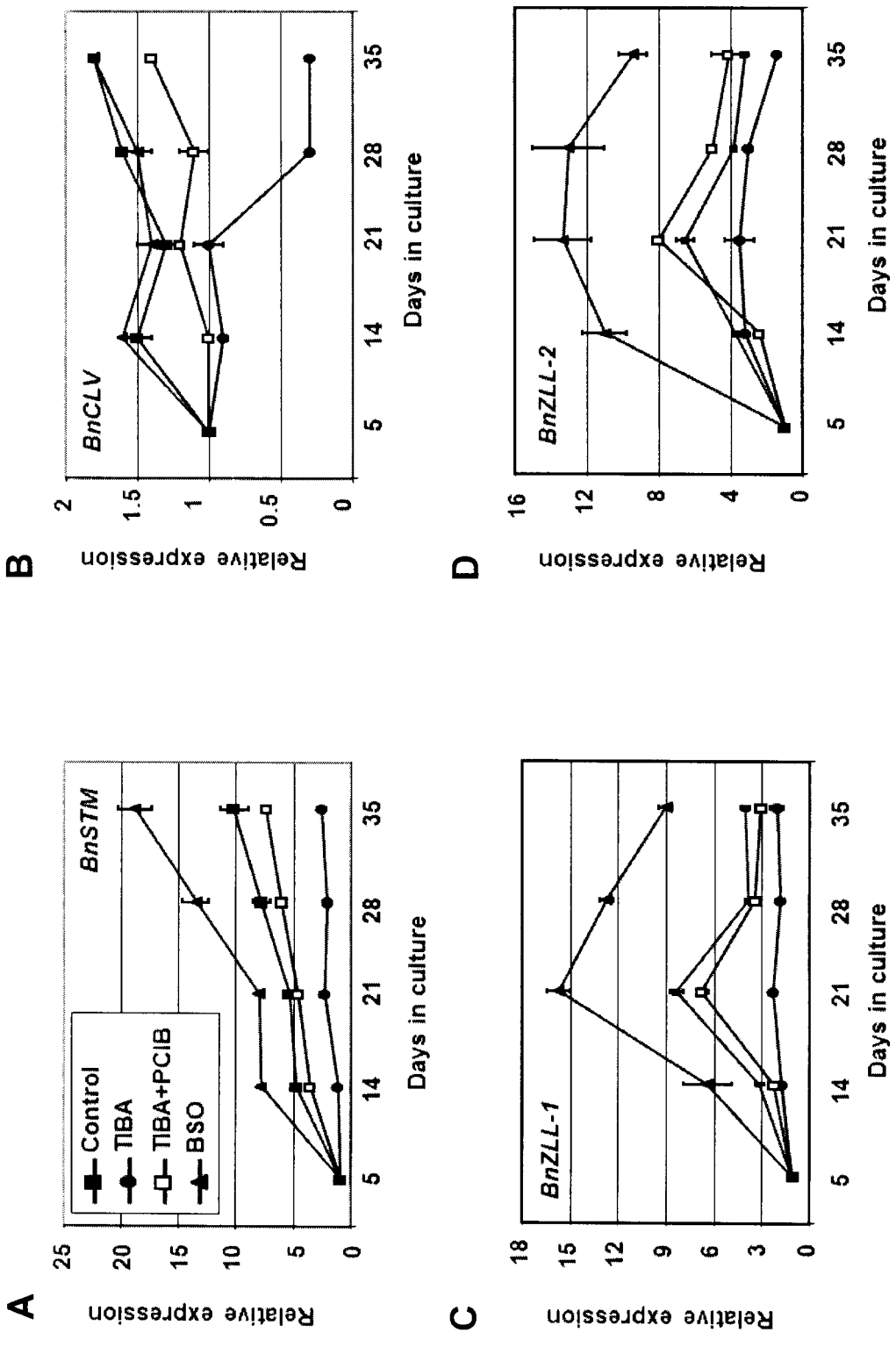
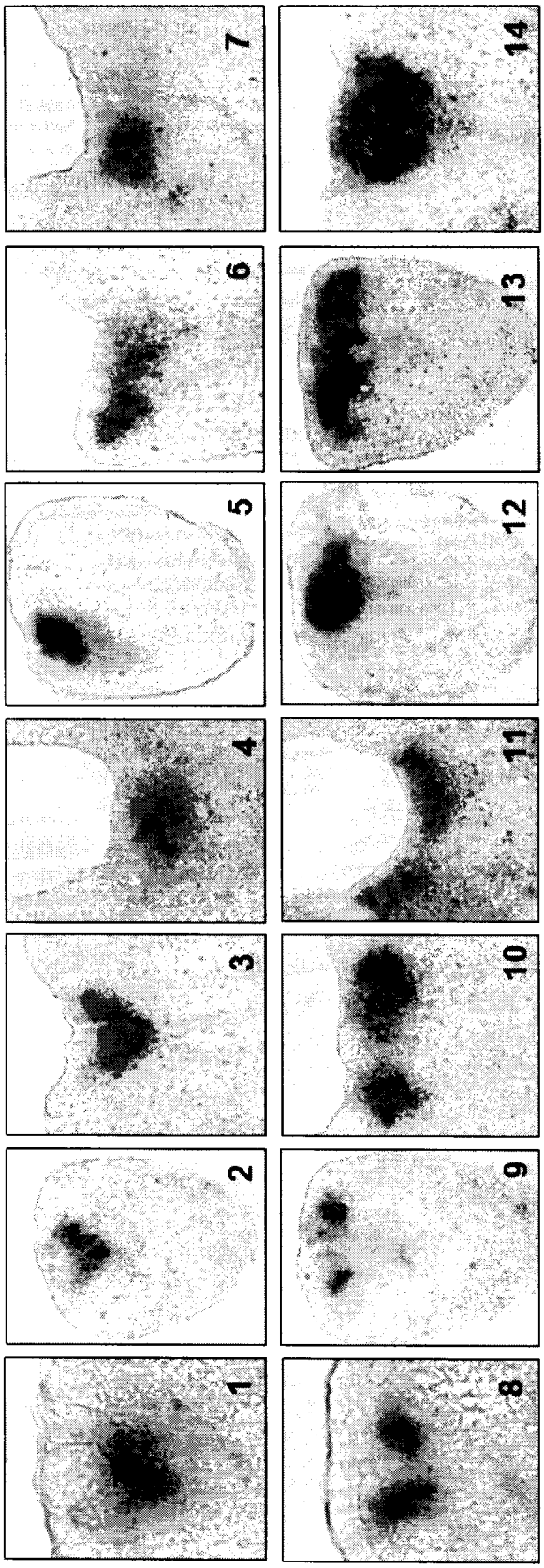

A
BnSTM
Control
TIBA
TIBA+PCIB
BSO
Relative expression
Days in culture
B
BnCLV
Relative expression
Days in culture
C
BnZLL-1
Relative expression
Days in culture
D
BnZLL-2
Relative expression
Days in culture

Figure 3



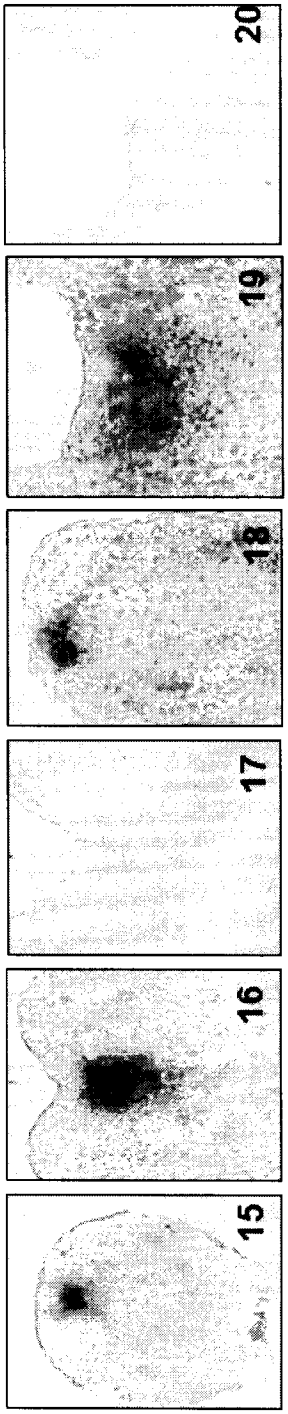


Figure 4

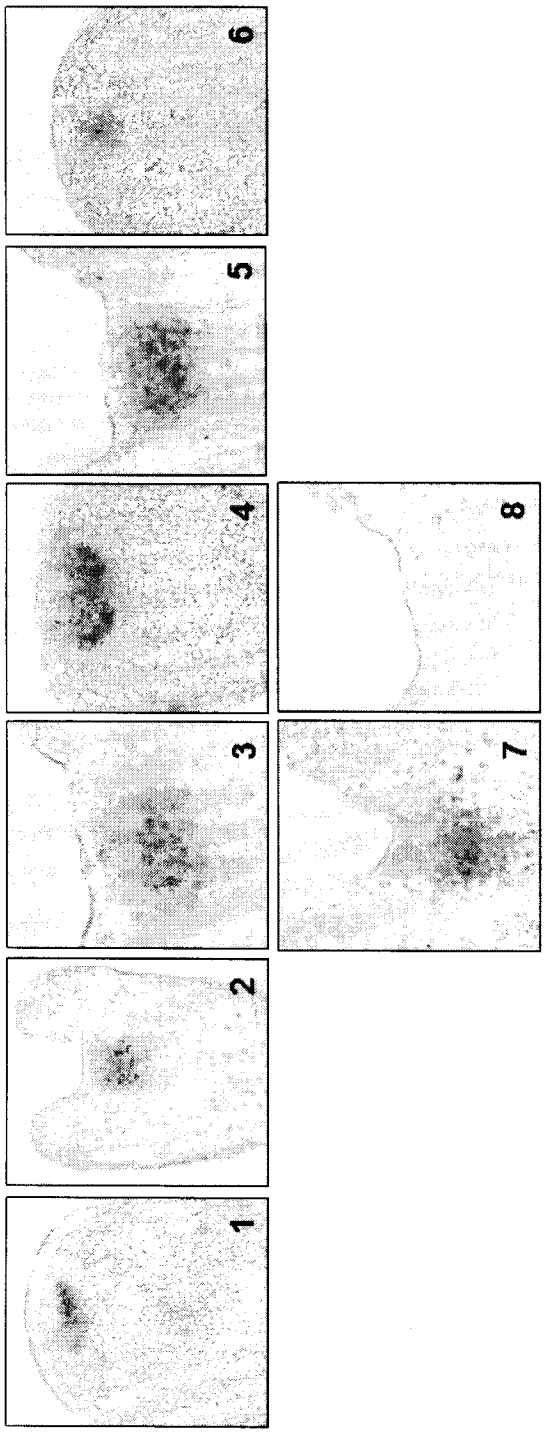


Figure 5

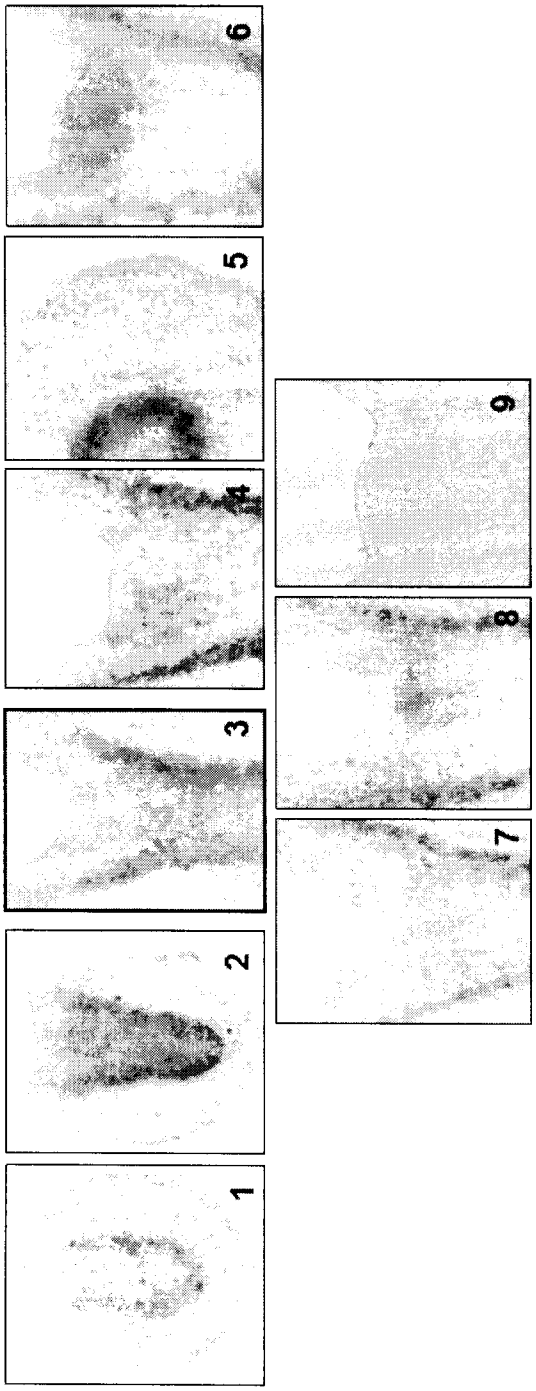


Figure 6

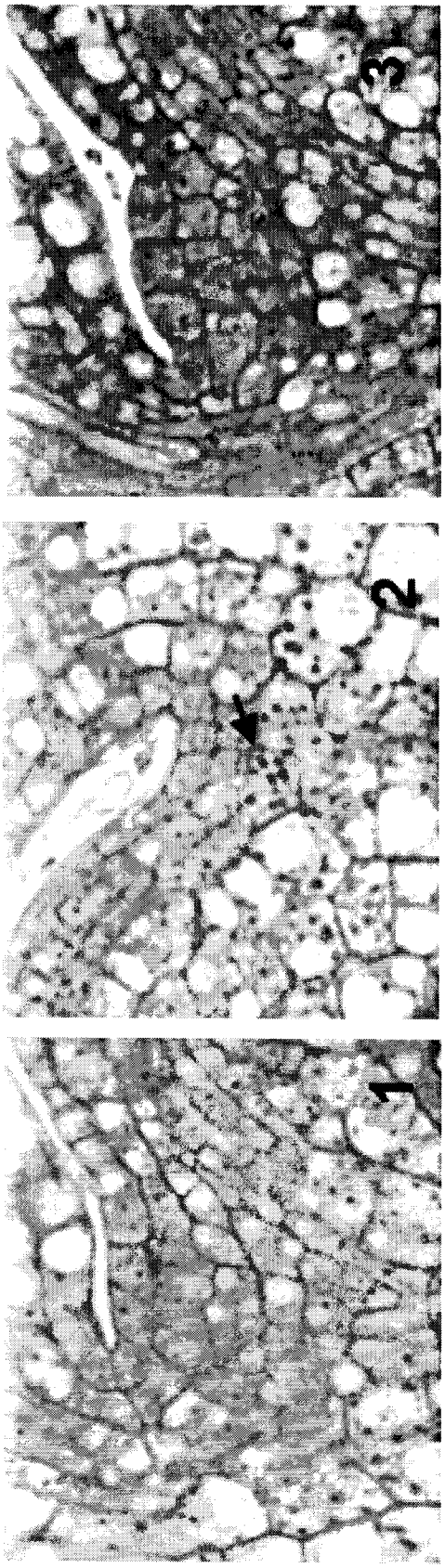


Figure 7

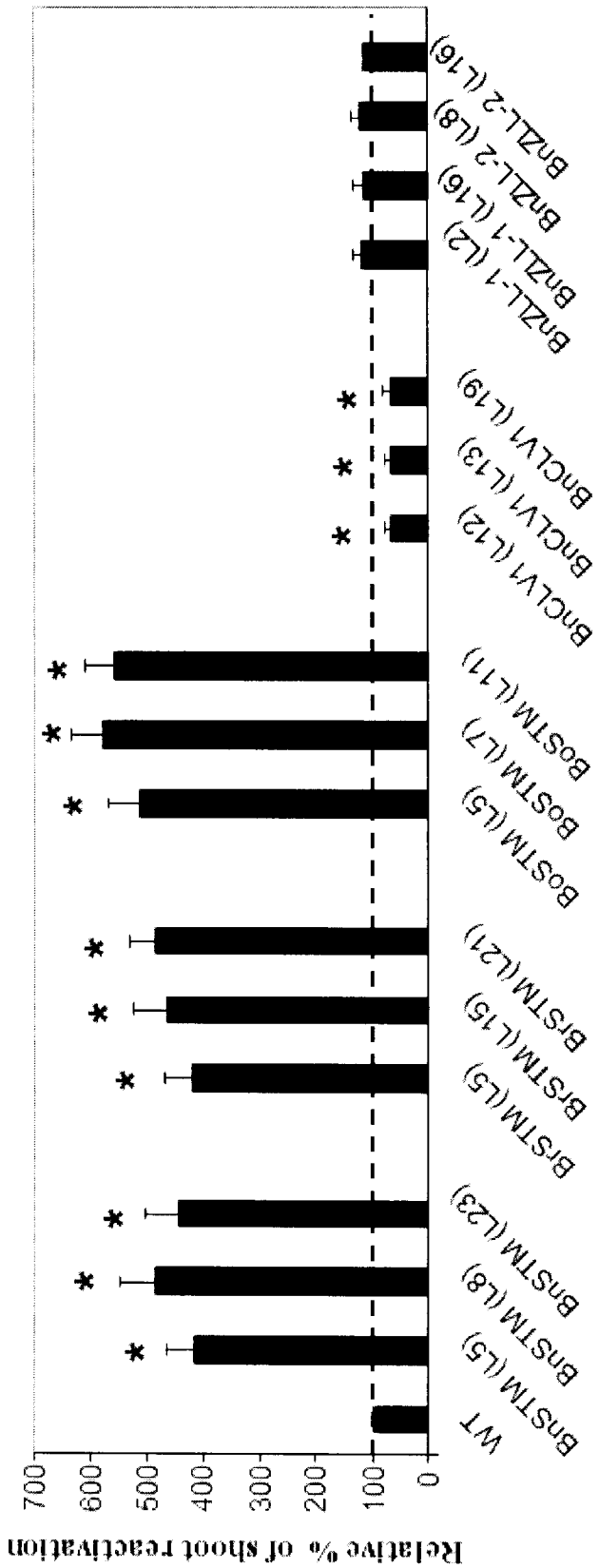

Relative % of shoot reactivation
0
100
200
300
400
500
600
700
WT
BnSTM (L5)
BnSTM (L8)
BnSTM (L23)
BrSTM (L5)
BrSTM (L15)
BrSTM (L21)
BoSTM (L5)
BoSTM (L7)
BoSTM (L11)
BnCLV1 (L12)
BnCLV1 (L13)
BnCLV1 (L19)
BnZL-1 (L2)
BnZL-1 (L16)
BnZL-2 (L8)
BnZL-2 (L16)

Figure 8

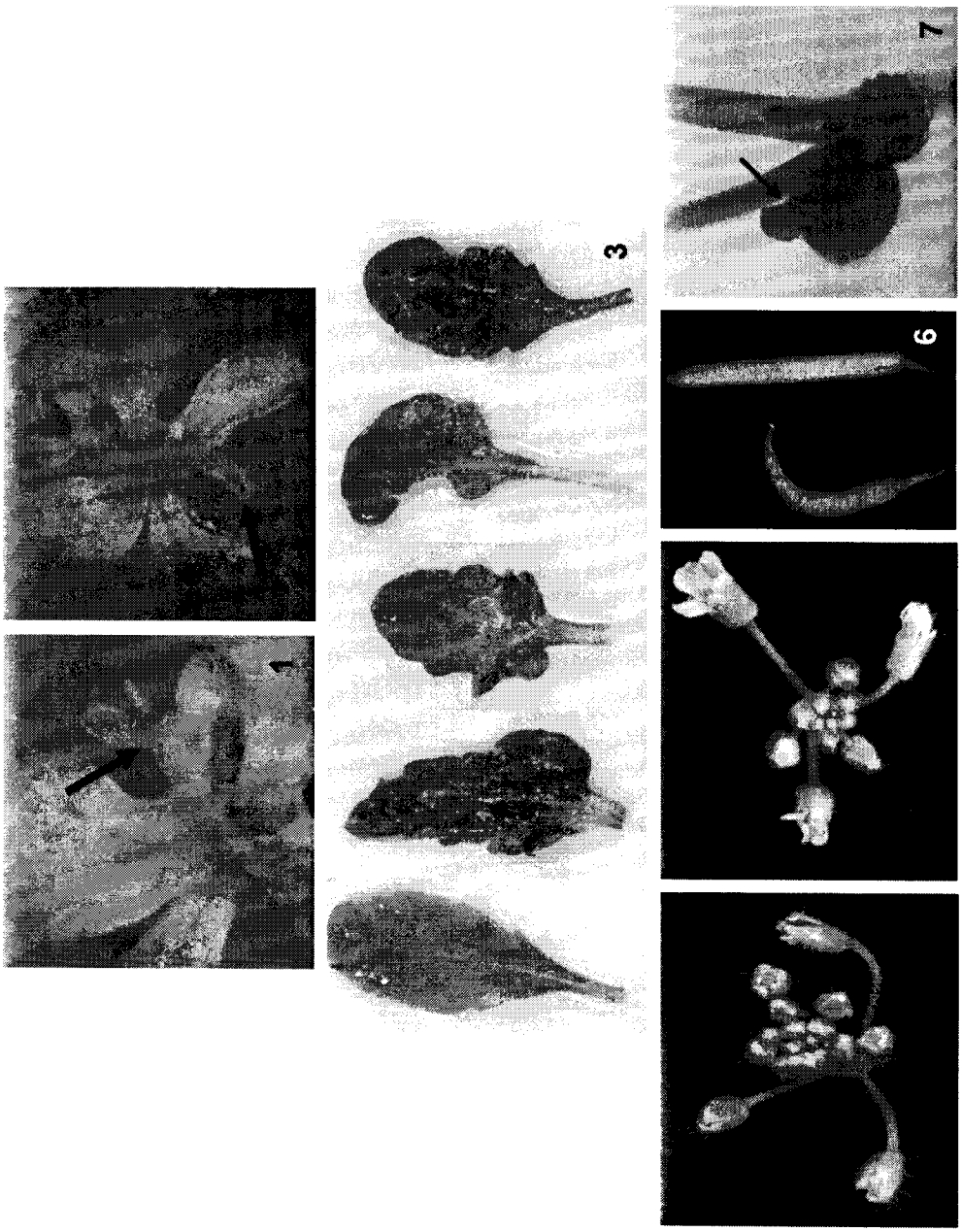


Figure 9

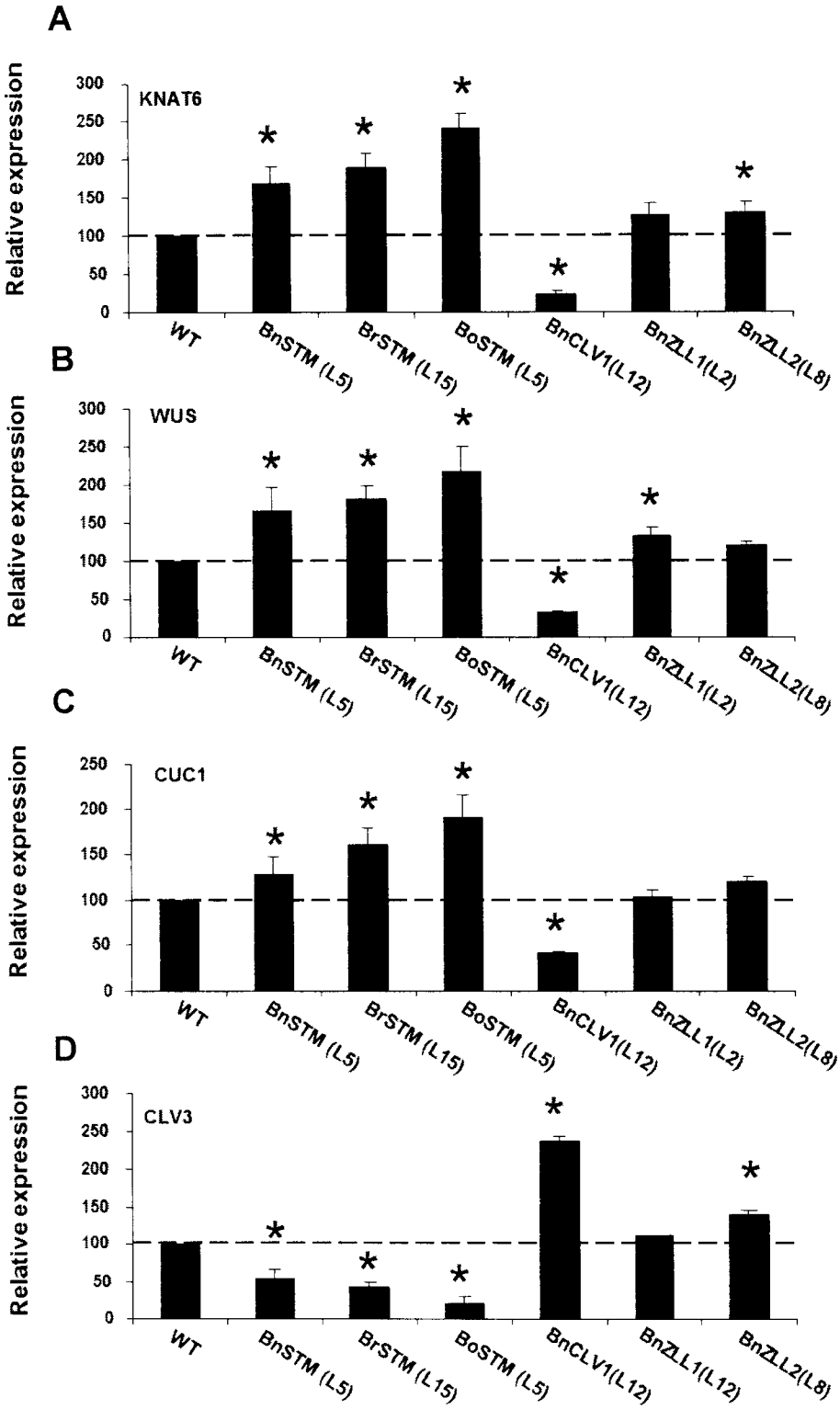

A
KNAT6
Relative expression
300
250
200
150
100
50
0
WT
BnSTM (L5)
BrSTM (L15)
BoSTM (L5)
BnCLV1(L12)
BnZLL1(L2)
BnZLL2(L8)
B
WUS
Relative expression
C
CUC1
Relative expression
D
CLV3
Relative expression
BnZLL1(L12)

Figure 10

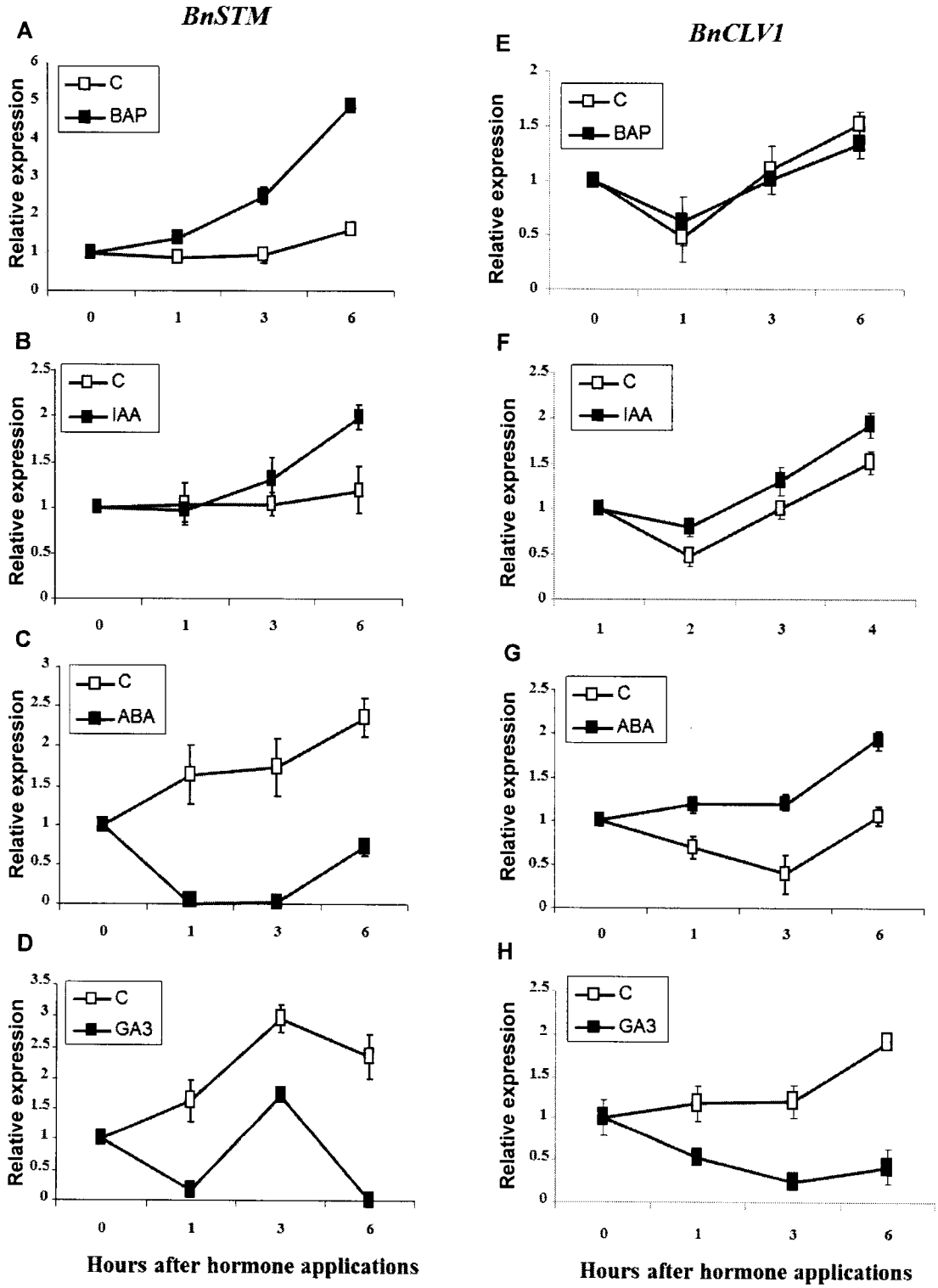

BnSTM
BnCLV1
A
B
C
D
E
F
G
H
C
BAP
IAA
ABA
GA3
Relative expression
Hours after hormone applications

Figure 11

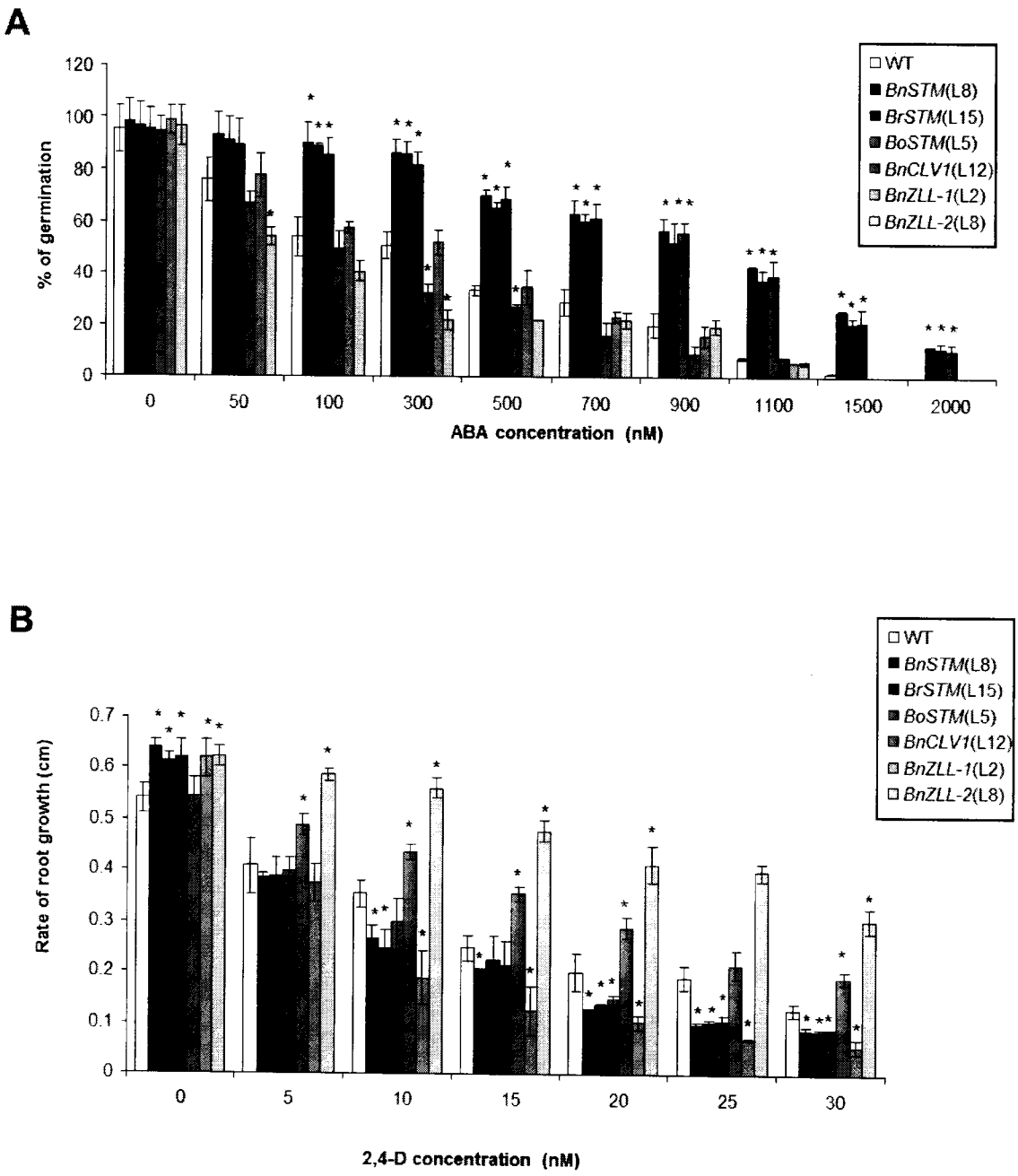

A
% of germination
120
100
80
60
40
20
0
0
50
100
300
500
700
900
1100
1500
2000
ABA concentration (nM)
WT
BnSTM(L8)
BrSTM(L15)
BoSTM(L5)
BnCLV1(L12)
BnZLL-1(L2)
BnZLL-2(L8)
B
Rate of root growth (cm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
2,4-D concentration (nM)
WT
BnSTM(L8)
BrSTM(L15)
BoSTM(L5)
BnCLV1(L12)
BnZLL-1(L2)
BnZLL-2(L8)

Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000164

A. CLASSIFICATION OF SUBJECT MATTER

IPC: ***C12N 15/00*** (2006.01) , ***A01H 5/00*** (2006.01) , ***A01N 65/08*** (2009.01) , ***A01P 21/00*** (2006.01) , ***C12N 15/29*** (2006.01) , ***C12N 5/10*** (2006.01) ***C12N 15/82*** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: ***C12N 15/00*** (2006.01) , ***A01H 5/00*** (2006.01) , ***A01N 65/08*** (2009.01) , ***A01P 21/00*** (2006.01) , ***C12N 15/29*** (2006.01) , ***C12N 5/10*** (2006.01) ***C12N 15/82*** (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Scopus, TotalPatent, Agricola, Canadian Patent Database, GenomeQuest. Keywords: shoot meristemless, STM, seed, yield, Brassica, transformed plant

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ELHITI, MOHAMED ABDELSAMAD. Molecular characterization of several *Brassica* shoot apical meristem genes and the effect of their altered expression during in vitro morphogenesis. Doctoral Thesis. University of Manitoba, 16 August 2010 (16-08-2010). Retrieved from the Internet: http://hdl.handle.net/1993/4053 *chapters 2 and 5*	1-16
A	ELHITI, MOHAMED et al. "Modulation of embryo-forming capacity in culture through the expression of *Brassica* genes involved in the regulation of the shoot apical meristem". Journal of Experimental Botany. 20 August 2010 (20-08-2010), 61(14):4069-85. ISSN: 0022-0957 *whole document*	1-16
A	SCOFIELD, SIMON et al. "The *KNOX* gene *SHOOT MERISTEMLESS* is required for the development of reproductive meristematic tissues in Arabidopsis". The Plant Journal. June 2007 (06-2007), 50(5): 767-81. ISSN: 0960-7412 *whole document*	1-16

[ ] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 2012 (25-05-2012)	01 June 2012 (01-06-2012)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Mary Murphy (819) 994-4066

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2012/000164

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [ ] in the international application as filed

   [X] together with the international application in electronic form

   [ ] subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments :