

**Molecular Characterization of Several *Brassica* Shoot Apical Meristem Genes
and the Effect of Their Altered Expression during *in vitro* Morphogenesis.**

BY

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A Thesis

Submitted to the Faculty of Graduate Studies in Partial
Fulfilment of the Requirements
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THE UNIVERSITY OF MANITOBA
FACULTY OF GRADUATE STUDIES

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This thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements for the degree of ***Doctor of Philosophy*** in **Plant Science**.

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ABSTRACT

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Molecular characterization of several *Brassica* shoot apical meristem genes and the effect of their altered expression during *in vitro* morphogenesis.

Supervisor: Dr. Stasolla

A common event during *in vitro* morphogenesis (either embryogenesis or shoot organogenesis) is the ability of somatic cells within the explants to de-differentiate and acquire “meristematic identity”. The developmental program of such meristematic cells can then be re-routed to form shoots or embryos depending on the imposed culture environment. The objective of this research is to investigate how the altered expression of *Brassica* genes regulating meristematic activity *in vivo* affects *in vitro* morphogenesis. It is predicted that ectopic expression of positive regulators of the shoot apical meristem, *SHOOT MERISTEMLESS* (*STM*) and *ZWILLE* (*ZLL*) which increase the pool of meristematic cells within the apical meristem, has a beneficial effect on somatic embryogenesis and shoot organogenesis. Conversely the over-expression of *CLAVATA1* (*CLV1*), a negative regulator which depletes the pool of meristematic cells, should inhibit both processes. Over-expression of the *Brassica STM* in *Arabidopsis* enhanced the production of somatic embryos and shoots *in vitro* possibly by reducing the requirement of the tissue for exogenous auxin, which is the inductive signal for the production of embryogenic and organogenic cells. This was also accompanied by profound alterations in gene expression patterns affecting components of DNA methylation and glutathione metabolism, which are beneficial for embryo formation. The introduction of *STM* also enhanced *Arabidopsis* shoot organogenesis through profound transcriptional changes in cytokinin signalling. While the ectopic expression of the *Brassica CLV1* inhibited both somatic embryogenesis and shoot organogenesis, the expression of *ZLL* had no effects on the production of somatic embryos but encouraged the formation of shoots. Taken together these results suggest the existence of similar genetic mechanisms regulating the formation of meristem cells *in vivo* and embryogenic/organogenic cells *in vitro*.

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ABBREVIATIONS

2,4 D, 2,4-dichlorophenoxyacetic acid

ABA, Absciscic acid

AGO, ARGONAUTE gene family

ARRs, Arabidopsis response regulators

At, *Arabidopsis thaliana*

BA, *N*⁶-benzyladenine

BBM1, BABY BOOM1

PCIB, *p*-chlorophenoxyisobutyric acid

Bn, *Brassica napus*

Bo, *Brassica oleracea*

Br, *Brassica rapa*

BSO, buthionine sulfoximine

CIM, callus induction medium

CLV1, CLAVATA 1

CLV2, CLAVATA2

CLV3, CLAVATA3

CZ, central zone of SAM

DR5, direct repeat 5

GA3, gibberellic acid 3

GAs, gibberellins

GSH, reduced glutathione

GSSG, oxidized glutathione

HAP3, Heme-Activated Proteins 3 subunit

IAA, indole acetic acid

IBA, indole-3-butyric acid

KAPP, a kinase protein phosphatase

KDa, kilo Dalton

LEA, late embryogenesis abundant proteins

LEC1, LEAFY COTYLEDON 1

LEC2, LEAFY COTYLEDON 2

LRR, leucine rich repeat protein

MAPK, mitogen-activated proteins

MDEs, microspore derived embryos

MS medium, Murashige and Skoog medium

NAA, naphthaleneacetic acid

NADP⁺, nicotinamide adenine dinucleotides phosphate oxidized

NADPH, nicotinamide adenine dinucleotides phosphate reduced

PAS, periodic acid-Schiff's reaction

PINs, auxin efflux carrier proteins

PZ, peripheral zone of SAM

RISC, RNA-induced silencing complex

ROP, Rho-GTPase

RZ, rib zone of SAM

SAM, shoot apical meristem

SE, somatic embryogenesis

SERK, SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE

SIM, shoot induction medium

STM, SHOOT MERISTEMLESS

TBO, Toluidine blue O

TCS, the two component output sensor

TDZ, thidiazuron

TIBA, 2, 4, 6-triiodobenzoic acid

WUS, WUSCHEL

YUC, flavin-containing monooxygenase encoding enzyme

ZLL, ZWILLE

EDAC, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride

BPS, phosphate buffer

BSA, bovine serum albumin

B5 medium, Gamborg B5 medium

WT, wild type

FORWARD

This thesis has been written in manuscript style. A general introduction and literature review precedes the four manuscripts which are the integral part of the thesis. Each manuscript contains an introduction, materials and methods, results, and discussion. A general summary, prospective plans for future studies, a list of references cited, and appendices follow the manuscripts.

CHAPTER 1

1.1. INTRODUCTION

Unlike animals, higher plants are characterized by continuous growth throughout their life cycle in order to generate new organs and tissues. This unique feature is due to the presence of defined groups of cells at the poles of the plant body called meristems. The word meristem derives from the Greek “merizein” which means “to divide” (Evans and Barton, 1997). According to their position within the plant body, meristems can be classified into apical and lateral. Apical meristems are located at the apices of the plants and they contribute cells for the apical-basal pattern of growth, whereas lateral meristems produce cells required for radial growth. The uppermost meristem, which is called the shoot apical meristem (SAM), is responsible for producing the above-ground tissues and organs of the plant. The SAM is formed during the globular stage of embryogenesis and occupies a position between the two incipient cotyledons (Lenhard and Laux, 1999), where it remains inactive until germination. Once the SAM is activated post-embryonically, it contributes cells for the formation of the vegetative tissues and it is therefore referred to as “vegetative SAM”. As reviewed by (Steeves and Sussex, 1989), the morphology and activity of the SAM vary among species and also during development within the same species. In conifers the SAM is generally narrow and conical, while in some monocot plants it is more elongated. In dicot plants it is usually dome-shaped and characterized by cells with different structural features (discussed later). Changes in SAM structure and architecture are also observed during development. A classical example is related to the alterations in SAM size and shape during plastochron, which is defined as the time gap separating the formation of two successive

primordial (Nougarède and Rembur, 1985). Upon the perception of developmental and environmental cues, the vegetative SAM can produce reproductive organs, i.e. flowers. These changes mark the transition from a vegetative SAM to a reproductive or floral SAM (Bernier et al., 1993). Physiological and genetic studies accompanying these changes reveal complex mechanisms of meristem regulation (Traas and Vernoux, 2002).

In *Arabidopsis*, four genes contribute to the regulation of the SAM: *SHOOT MERISTEMLESS* (*STM*), *CLAVATA1* (*CLV1*), *ZWILLE* (*ZLL*), and *WUSCHEL* (*WUS*). *SHOOT MERISTEMLESS* (*STM*), which encodes a knotted-like homeobox protein, was first isolated by (Barton and Poethig, 1993). While a mutation of this gene results in the failure to establish and maintain a functional SAM (Long et al., 1996), ectopic expression of *STM* induces the formation of meristems from vegetative tissue, i.e. leaves (Sinha et al., 1993). These results, together with additional studies (Lenhard et al., 2002), demonstrate the requirement of *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.

Another important player in the SAM regulation is *WUS*, a homeobox gene which confers “stem cell” identity (Dodsworth, 2009). *WUSCHEL* expression is confined to a small group of cells within the SAM which is referred to as “stem cell niche” or “organizing center” (Mayer et al., 1998). The function of *WUS* is to maintain the stem cells within the organizing center in an undifferentiated state. This concept is exemplified by studies on *wus* mutant plants which exhibit a differentiation of the meristematic cells (Laux et al., 1996). The expression of *WUS* is tightly regulated by *CLV1*, a transmembrane receptor serine/threonine kinase with a leucine rich repeat (LRR) (Clark

et al., 1997). The role of CLV1 is to promote the differentiation of meristematic cells by repressing the expression of *WUS* through a complex signaling model involving other CLV members (Dodsworth, 2009). In this model, CLV3 (a ligand produced by the apical layer of the SAM) binds to the CLV1/CLV2 receptor kinase complex which through the activation of downstream signaling components represses *WUS* expression (Dodsworth, 2009). *clv1* mutant lines showed enlarged meristems as a result of the accumulation of stem cells in the central zone leading to stem fasciation, i.e. abnormal broadening, flattening, and clumping of the stem (Schoof et al., 2000). Thus, in contrast to *WUS* which keeps the stem cells in an undifferentiated state, CLV1 promotes the acquisition of an organ-specific cell fate (Clark et al., 1996).

ZWILLE is also required for the formation of the embryonic SAM but, unlike *STM*, it is not needed for its post-embryonic (organogenic) growth (McConnell and Barton, 1995; Moussian et al., 1998; Lynn et al., 1999). Molecular studies revealed that *ZLL* belongs to the *ARGONAUTE* (*AGO*) gene family. Several studies have showed that *AGO* members participate in the formation of an RNA-induced silencing complex in animals and plants (Cerutti et al., 2000). Two functions of *ZLL* are to define the proper expression of *STM* (Moussian et al., 1998) and to potentiate the *WUS* domain during embryogenesis (Tucker et al., 2008).

Based on the above information, a model has been proposed suggesting that *STM* and *ZLL* are positive regulators of the SAM by promoting the accumulation of undifferentiated cells within the apical pole, while CLV1 is a negative regulator which promotes the differentiation of meristematic cells (Dodsworth, 2009). In this model the activity of the SAM is regulated by a feed-back mechanism between the CLV complex

(CLV1, 2, and 3) and WUS, with STM and ZLL affecting WUS expression (Fig. 1.1). This elaborated interaction would ensure a proper balance between cell division and differentiation within the SAM.

The acquisition of meristematic cell identity is considered a fundamental aspect of plant tissue culture. Since the concept of totipotency, i.e. the ability of a single cell to regenerate the whole plant, was introduced, tissue culture has represented a very useful tool to study morphogenesis *in vitro*. Morphogenesis is a biological process in which tissues, organs, and the entire body of an organism are elaborated as a result of specific environmental and developmental stimuli. In culture, there are two morphogenic pathways responsible for the regeneration of the whole plant (Phillips, 2004). The first pathway is embryogenesis while the second is organogenesis. Both pathways can be executed directly, without an intervening callus phase, or indirectly via the formation of callus. Compared to organogenesis which produces only unipolar organs (shoots or roots), *in vitro* embryogenesis generates bipolar structures (embryos). The latter process is therefore the most desirable propagation method as it guarantees the simultaneous formation of the apical poles. *In vitro* embryos can be generated through two processes: somatic embryogenesis or androgenesis. Somatic embryogenesis refers to the production of embryos from somatic cells (cells other than gametes). The first description of the process was reported by Levine (1947). Since then, huge efforts have been exerted to optimize the culture conditions for several species (Thorpe and Stasolla, 2001).

The *Arabidopsis* somatic embryogenesis system has been recently used as a model to investigate the molecular regulation of embryo development (Mordhorst et al., 2002).

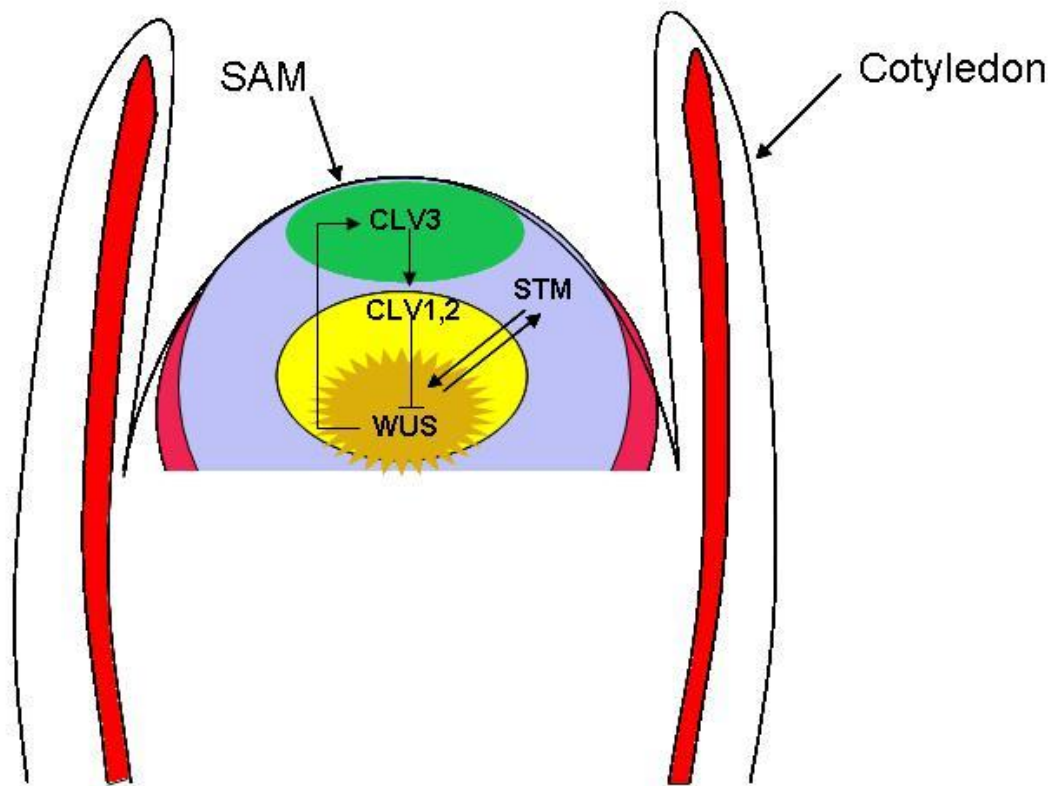


Figure 1.1: Molecular regulation of the *Arabidopsis* shoot apical meristem (SAM) through the interaction of *CLAVATA* (*CLV*), *WUSCHEL* (*WUS*), *SHOOT MERISTEMLESS* (*STM*), and *ZWILLE* (*ZLL*). *CLAVATA3* (*CLV3*) expression is restricted to the apical layers [layer1 (L1) and layer2 (L2)] of the central zone (green color), while *CLAVATA1* and 2 (*CLV1* and 2) can only be detected in layer 3 (L3) of the central zone and rib zone (yellow color). *WUS* is expressed in few central cells of the "organizing center" (orange color). *STM* and *ZLL* are expressed within the whole SAM region. *CLV* and *WUS* regulate themselves through a feed back mechanism. *STM* affects *WUS* expression, while *ZLL* (red color) potentiates *WUS* expression and ensures the proper localization of *STM*.

Arabidopsis somatic embryos can be either induced from immature zygotic (seed) embryos (Ikeda-Iwai et al., 2002; Mordhorst et al., 2002; Bassuner et al., 2007) or mature cells (O'Neill and Mathias, 1993; Luo and Koop, 1997). In the former protocol somatic embryogenesis is induced using bent-cotyledon zygotic embryos as explants on an auxin-containing medium. In this medium, the explants proliferate forming a callus in which the cells acquire embryogenic competence. Embryo development is achieved on a development medium devoid of growth regulators.

Besides somatic embryogenesis, *in vitro* embryos can also be induced from either male or female gametophytes. When male gametophytes (immature pollen) are used, the process is referred to as androgenesis. Androgenesis has been applied to many plant species and implemented in many breeding programs because the endogenous genetic variation can be captured through the recovery of diploid homozygous plants once the chromosome number has been doubled (Yao et al., 1997). 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 and proteins characterizing the initial transition of gametophytic cells into embryogenic cells (Joosen et al., 2007; Malik et al., 2007; Tsuwamoto et al., 2007), and the subsequent development of the microspore-derived embryos MDEs (Stasolla et al., 2008).

Shoot organogenesis, the *de novo* development of shoots from somatic cells in culture, was first reported by White (1939). In *Arabidopsis* shoot organogenesis can be

induced using many explants such as roots, hypocotyls, and cotyledons (Morris and Altmann, 1994). In recent studies roots have become the preferred choice of explants (Akama et al., 1992). Optimized protocols involving manipulations of exogenous auxins and cytokinins have been designed (Zhao et al., 2002).

A common event during *in vitro* morphogenesis (either embryogenesis or shoot organogenesis) is the ability of somatic cells within the explants to re-differentiate and acquire a “meristematic identity”. The developmental program of such meristematic cells can then be re-routed to form shoots or embryos depending on the imposed culture environment. To date there is very limited information on how this de-differentiation step is executed and how the fate of the cells can be switched to different programs. The objective of this research is to investigate how the altered expression of *Brassica* genes, fulfilling different functions during the formation and maintenance of the meristematic cells within the SAM affects *in vitro* morphogenesis. The hypothesis to be tested is that genes which are positive regulators of the SAM (*STM* and *ZLL*) and increase the pool of undifferentiated meristematic cells should have a beneficial effect on shoot organogenesis and somatic embryogenesis. Conversely negative regulators of the SAM, such as *CLV1* which depletes the pool of meristematic cells, should exercise negative effects on *in vitro* morphogenesis.

1.2. LITERATURE REVIEW

As indicated in the previous section, apical meristems, including the shoot apical meristem (SAM) are formed early during embryogenesis and contribute cells to support post-embryonic growth. The meristematic cells harbored within the SAM play a crucial role in providing derivatives which differentiate and mature into different cell types. Proper balance between cell division and differentiation is therefore critical for ensuring “homeostasis” within the SAM (Shani et al., 2006). Formation of meristematic cells plays also an important role during *in vitro* morphogenesis. A key step for tissue or organ formation involves the de-differentiation of somatic cells which first acquire a “meristematic fate” and then initiate specific developmental programs. This review will provide background information on the regulation of the SAM and methods to regenerate organs in culture through embryogenesis and organogenesis.

1.2.1. The shoot apical meristem (SAM)

The architecture of the SAM has been the subject of many structural investigations, especially in early days, prior to the development of molecular and genetic techniques. These “classical” studies have revealed the presence of domains with the SAM characterized by distinct cell division patterns and cytophysiological features. It has been only in the last two decades that the genetic network regulating the activity of the SAM has been investigated. Several reviews are currently available (Traas and Vernoux, 2002; Gross-Hardt and Laux, 2003; Carles and Fletcher, 2003; Shani et al., 2006; Tahir and Stasolla, 2006; Veit, 2006; Tucker and Laux, 2007; Ioio et al., 2008; Veit, 2009).

1.2.1.1. Structural organization of the SAM

Steeves and Sussex (1989) reviewed two main models describing the structure of the SAM in angiosperm species. In the first model (Fig. 1. 2 A), the SAM is divided into layers. The surface region, i.e. the tunica, consists of two layers (denoted as L1, and L2) characterized by anticlinal divisions contributing cells to each individual layer. The underlying corpus region (often referred to as layer 3 or L3) is an arrangement of cells that divide in all planes, anticlinally and periclinally. Each layer is responsible for contributing cells to specific tissues. In *Arabidopsis Thaliana* L1 contributes cells to the epidermis of the shoot, leaves and flowers; L2 provides cells to the ground tissue and germ cells; while the corpus layer produces the vascular tissue and the most internal tissues of leaves and flowers (Fletcher, 2002; Carles and Fletcher, 2003). Szymkowiak and Sussex (1996), however, showed that this arrangement is not fixed. Changes in the planes of cell division are often observed during development and in response to changing environmental conditions.

The second model describing the structure of the shoot meristem is based on “cytophysiological zonations” (reviewed by Steeves and Sussex 1989). Cytochemical studies of the SAM revealed three distinct zones: a central zone (CZ), a peripheral zone (PZ), and a rib zone (RZ) (Fig.1.2 B). Cells within each zone are characterized by different staining patterns, degrees of vacuolation, and unique planes of divisions (Steeves and Sussex, 1989). The PZ contains cells that will be included in organ primordia while the RZ includes cells which are incorporated in the ground tissue. The CZ, which occupies the apical region of the meristem, is the source of cells for the RZ and PZ. Cells within the CZ (which are often referred to as “stem cells”) stain faintly

with standard histological stains, have a higher degree of vacuolation, and a lower rate of cell division (Steeves and Sussex, 1989). Maintenance of the CZ is crucial for proper meristem function as the stem cells produce initials which replace cells “consumed” during the formation of primordia. As cells of the PZ and RZ differentiate and mature into specific cell types they are incorporated into new organs. These cells are soon replaced by a new subset of cells provided by the CZ, which in turn replenishes itself through new rounds of cell divisions (Lenhard and Laux, 2003). Through this mechanism, the SAM maintains itself as a stable entity in which the rate of differentiation equals the rate of cell division. However these cytophysiological patterns of the SAM are no longer recognizable during time of dormancy when cells enter a quiescent phase (Steeves and Sussex, 1989).

1.2.1.2. Genetic regulation of the SAM

The molecular regulation underpinning the establishment of the SAM and its post-embryonic maintenance has been outlined in many reviews (Vernoux et al., 2000; Fletcher, 2002; Golz, 2006; Adrian et al., 2009; Barton, 2009). The activity of the SAM is governed by specific key genes that regulate, organize and maintain its layering and zonation pattern. Integrated expression and interaction of these genes through feed back mechanisms ensure the proper balance between stem cell renewal and differentiation. These concepts have been demonstrated through mutant analyses which have revealed the existence of a subset of genes crucial for proper SAM formation and maintenance. Such genes are collectively referred to as SAM marker genes (reviewed by Gross-Hardt and Luax, 2003; Tahir and Stasolla, 2006).

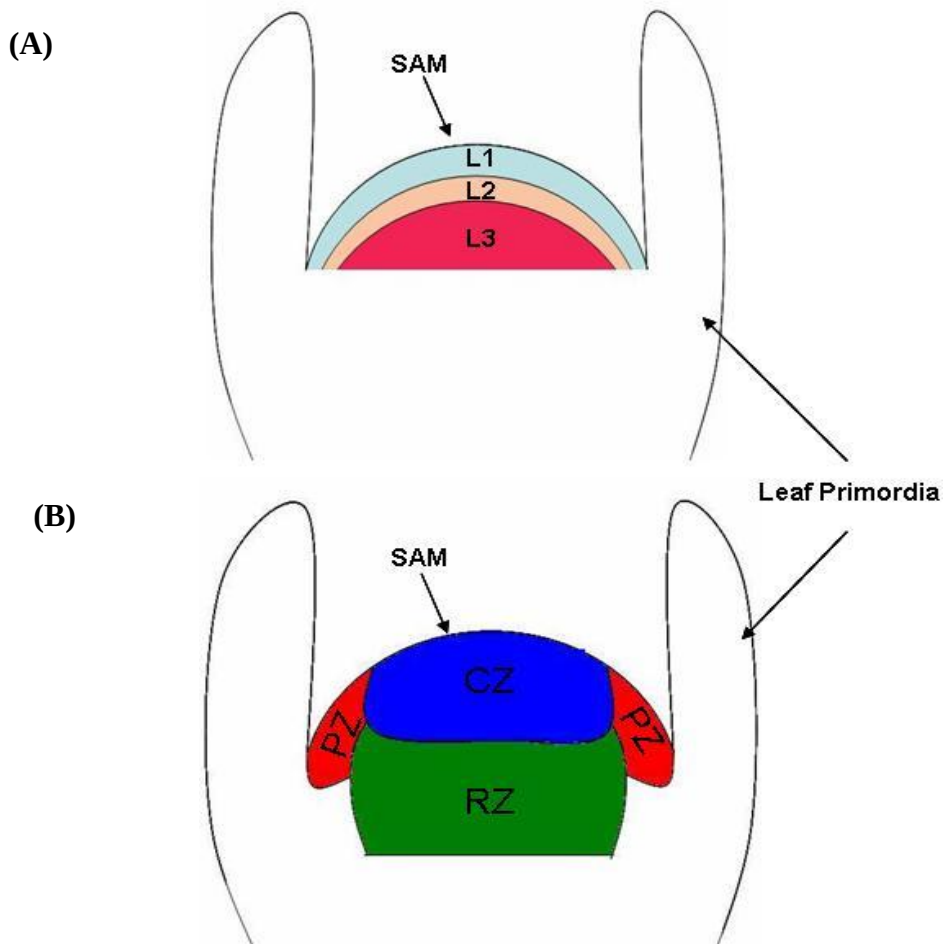


Figure 1.2: A schematic diagram of the structural organization of shoot apical meristem (SAM). **(A)** Organization of the SAM in layers. The epidermal (L1) and sub-epidermal (L2) layers characterized by anticlinal cell divisions form the tunica. Cells subtending L2 form the corpus (L3). Both anticlinal and periclinal divisions occur in the corpus. **(B)** Cytophysiological zonation of the SAM. The central zone (CZ) acts as a reservoir of cells that replenishes the differentiating cells of the peripheral (PZ) and rib (RZ) zones, consumed during the formation of lateral organs.

Four SAM marker genes described in this review are *SHOOT MERISTEMLESS (STM)*, *CLAVATA 1 (CLV1)*, *ZWILLE (ZLL)*, and *WUSCHEL (WUS)*. Despite their undisputed role in the regulation of SAM (Fig. 1.1) evidence on their mode of action has only been documented recently (Barton, 2009). Several aspects related to their specific functions are still unknown.

1.2.1.2.1. *SHOOT MERISTEMLESS (STM)*

The *Arabidopsis STM* is a homeobox gene which encodes a member of the class-1 KNOX homeodomain containing proteins (Long et al., 1996; Janosevic and Budimir, 2006). Members of this class of proteins are generally expressed in the SAM where they fulfill key roles in the regulation of the meristematic cells (Cole et al., 2006). The function of this gene appears to be conserved, as demonstrated by the existence of many orthologs in other species including maize (Kerstetter et al., 1994), rice (Matsuoka et al., 1993), tomato (Hareven et al., 1996), and tobacco (Tamaoki et al., 1997).

Unique features of STM, also found in other members of the class-1 KNOX sub-family of transcription factors are the two domains KNOX1 and KNOX2; the former with a suggested repressive effect on the transcription of target genes and the latter involved in dimer formation and transactivation (Sakamoto et al., 2001a). Additional conserved regions are the ELK domain which has been proposed to contain a nuclear localization signal and the Homeodomain involved in DNA binding (Schofield and Murray, 2006). The homeodomain belongs to the three amino acid loop extension (TALE) class of homeodomains, and interacts with DNA-targets (Bellaoui et al., 2001).

The previous studies revealed that transcripts of *AtSTM* are first detected in a few cells of globular stage embryos (Sharma and Fletcher, 2002). As the embryos develop further the expression domain of the gene expands to the peripheral zone of the SAM and encompasses all the meristematic cells between the two emerging cotyledons. During post-embryonic growth, *STM* expression is detected in both vegetative and floral meristems, although minute differences in localization can be detected (Long et al., 1996). *Arabidopsis stm* plants fail to develop a functional SAM indicating that this gene is a fundamental player for the establishment of the embryonic meristem and its post-embryonic maintenance (Long et al., 1996). The processes of SAM formation and maintenance are regulated by the overlapping function of several homeobox genes (Sentoku et al., 1999). It was reported that two rice homeobox genes, *OSH1* and *OSH15*, are co-expressed in the SAM of embryos where they exercise complementary roles (Sato et al., 1998). Genetic and molecular analyses revealed that *STM* is required in the central region of the SAM to inhibit differentiation and maintain a pool of undifferentiated cells (Long and Barton, 1998). A possible mode of action of *STM* is to repress the expression of MYB-related genes, such as *ASYMETRIC LEAVES 1 (AS1)* which are required to initiate the genetic programs leading to organ (leaf) formation (Lenhard et al., 2002).

Functional studies further revealed severe abnormalities in leaf morphology following over-expression of *KNOX* genes, including *STM* (Sinha et al., 1993; Chuck et al., 1996; Hareven et al., 1996; Kessler et al., 2002; Hay and Tsiantis, 2006; Liu et al., 2008). These observations suggest that besides regulating SAM activity, *KNOX* proteins might play a critical role during morphogenesis (Lincoln et al., 1994). It is likely that *KNOX* proteins keep the leave marginal blastozones, i.e. a group of cells competent for

organogenesis located at the leaf margin, in an active state. This activation is responsible for the changes in leaf morphology observed in plants over-expressing *KNOX* genes (Barth et al., 2009).

The developmental roles of *KNOX* proteins are tightly linked to various phytohormones. It is known that cytokinins delay tissue maturation, and their levels increase in *KNOX* over-expressing plants (Shani et al., 2006). Frugis et al. (2001) found that the introduction of *KNAT1*, a class 1-*KNOX* gene, in lettuce results in an increase in the endogenous cytokinin levels indicating that the function of this gene may be mediated by cytokinins (Barth et al., 2009). Additional information is available on the relationship between *KNOX* genes and gibberellins. Tissue differentiation and maturation are often promoted by gibberellins (GAs). *KNOX* genes suppress GA biosynthesis, and the degree of abnormalities in leaf structure seen in *KNOX* over-expressing plants can be reduced by exogenous applications of GAs (Tanaka-Ueguchi et al., 1998; Hay et al., 2002). Comprehensive information on the roles of plant growth regulators on the SAM will be presented in the next sections.

1.2.1.2.2. CLAVATA1 (CLV1)

CLAVATA1 (CLV1) is a receptor-like kinase containing the 21 tandem leucine-rich repeats (LRRs) domain with a single pass transmembrane domain, and the cytoplasmic serine/threonine kinase domain (Clark et al., 1997). CLV1 is an important component of an integral signaling pathway regulating the differentiation of stem cells within the SAM. In this pathway CLV1 binds to CLV2 to form a CLV1/CLV2 receptor complex to which CLV3, a small polypeptide containing a CLV3/ESR-related domain,

binds. This interaction triggers a MAPK cascade which represses the expression domain of WUS (Brand et al., 2002; Lenhard and Laux, 2003; Song et al., 2006) (Fig.1.3). The related phosphatases POLTERGEIST and POLTERGEIST LIKE 1 (PLL1) are downstream regulators which ensure the proper expression of WUS in the organizing center of the SAM. Both POL and PLL1 work downstream of CLV1 (Song et al., 2006). Since the identification of the first CLV genes, several studies have tried to establish their relation. (Brand et al., 2002) demonstrated that CLV1 works downstream of CLV3 because the phenotype observed in plants over-expressing CLV3 is exceedingly dependant on the presence of CLV1. Conclusive evidence supporting this idea was provided by *in vitro* assays showing that the CLV3/CLE peptide is able to bind to CLV1 (Ogawa et al., 2008). The function of CLV might not only be restricted to the SAM, but also to the root apical meristem. Exogenous applications of the *in vitro* translated C-terminus CLV3/ESR domain to the root apex were able to alter the fate of root meristematic cells (Fiers et al., 2005) indicating that CLV1 works redundantly with other genes to regulate meristem development.

As indicated previously, the role of CLV signaling is antagonistic to that exercised by STM, with the former promoting the differentiation of the meristematic cells and the latter ensuring the maintenance of an undifferentiated pool of cells within the SAM. This notion was demonstrated by several genetic studies showing a strong interaction among CLV 1 and 3 and STM. The *stm1* and *stm2* phenotype is partially suppressed by *clv1* and *clv3* mutations (Clark et al., 1996). The same work showed that embryos with functional SAMs are produced by *clv1 stm* double mutant lines. Furthermore CLV3 expression levels increased in *stm* plants indicating a plausible

involvement of STM in the formation of the CLV3/CLV1/CLV2 protein complex (Brand et al., 2002). These results clearly indicate that STM and CLV1 have antagonistic roles with STM maintaining stem cells in an undifferentiated state (by preventing meristematic cells from adopting an organ-specific cell fate), and CLV1 limiting the expansion of the undifferentiated stem cell population in the SAM (by promoting differentiation).

1.2.1.2.3. ZWILLE (ZLL)

ZWILLE belongs to the ARGONAUTE (AGO) family of genes and it is also known as either PINHEAD or AGO10 (Tucker et al., 2008). In *Arabidopsis*, ZLL contains three different domains, PIWI, PAZ, and DUF. The PIWI domain, also referred to as the ARGONAUTE-like domain, is the main component of an RNA-induced silencing complex (RISC). The PIWI domain is located within the C-terminal region of AGO and consists of two sub-domains, one of which binds to RNAs and the other is the catalytic site for silencing (Song and Joshua-Tor, 2006). The PAZ domain is an integral part of the RNA-induced silencing complex (RISC) with an endonuclease activity that plays a key role in the RNA interference pathway. (Ma et al., 2004) recognized that PAZ functions as a nucleic acid binding domain, with a strong preference for single-stranded nucleic acids (RNA or DNA) or RNA duplexes with single-stranded 3' overhangs. It has been suggested that the PAZ domain provides a unique mode for the recognition of the two 3'-terminal nucleotides in single-stranded nucleic acids and removes the 3' OH group.

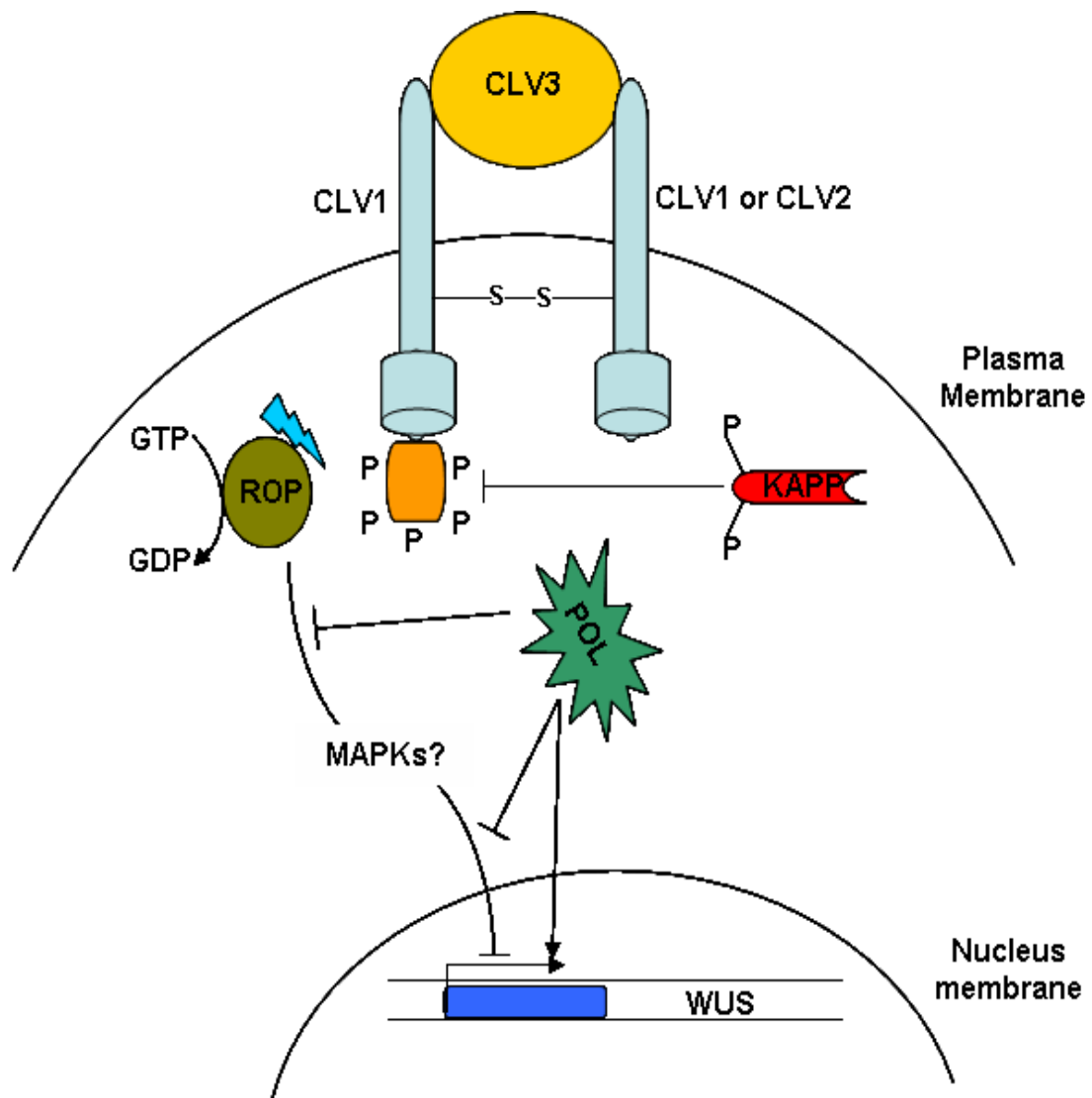


Figure 1.3: Schematic representation of CLV-WUS feedback regulation of the SAM. In this pathway, CLV1 and CLV2 form homo-or-heterodimers through the formation of a disulfide bond between conserved cysteine pairs. The binding of CLV3 (released from the apical layer L1 of the SAM) induces the phosphorylation and activation of the CLV1 and/or CLV2 receptor (located in the L2-L3 layers). This activation results in the assembly of the 450 KDa complex which also includes a protein phosphatase (KAPP), and a Rho-GTPase (Werner et al.). KAPP de-phosphorylates the CLV1/CLV2 receptor complex and acts as a negative regulator of the CLV signaling pathway. ROP, a positive regulator, transmits the signal of the CLV complex downstream, and through the activation of mitogen-activated proteins (MAPKs), represses WUS expression. POL functions within the SAM as either a direct suppressor of CLV1 signaling or a positive regulator of WUS.

The PAZ may recognize the characteristic 3' overhangs in siRNAs within RISC and other complexes (Hall, 2005). The DUF is a short domain located upstream the PAZ and its biological function is still unknown.

In situ studies indicate that ZLL is initially expressed in all cells of young globular embryos. Upon further development the expression of this gene becomes localized to the vascular tissue, the SAM, and the adaxial side of the cotyledons (Lynn et al., 1999; Tucker et al., 2008). Genetic studies in *Arabidopsis* showed that homozygous *zll* plants have a variable SAM size. In extreme cases no SAMs are formed, whereas in other instances enlarged SAMs are produced (Moussian et al., 1998). These meristems are not functional and abort during post-embryonic development. However adventitious meristems can form from the aborted SAM and ensure the post-embryonic growth of the mutants (Lynn et al., 1999). The observation that the SAMs in *zll* mutants cannot be rescued by *WUS* indicates that the two genes do not have antagonistic functions (Moussian et al., 2003). However the rescue of SAMs in the *zll clv* double mutant homozygous lines suggests that CLV and ZLL counteract each other's function in the regulation of the shoot meristem (Moussian et al., 2003). Further genetic studies revealed that like STM, ZLL is required for the formation of the embryonic SAM but, unlike STM, it is not needed for its post-embryonic (organogenic) growth (McConnell and Barton, 1995; Lynn et al., 1999).

The mechanisms whereby ZLL exercises its functions in the SAM are not known. Recent work indicates that a very strong signal of ZLL is detected in the vascular tissue, while a weak signal is noticed in the shoot meristem (Tucker et al., 2008). In the same study it was shown that the introduction of ZLL driven by the promoter of AtHB8, a gene

expressed in the SAM, did not rescue the SAM of *zll* plants. The authors proposed the existence of a signal produced by the vascular tissue (where *ZLL* is mostly expressed) which migrates to the apical pole where it regulates the development of the SAM.

1.2.1.2.4. WUSCHEL (WUS)

WUS is a homeobox gene which encodes a transcription factor involved in the establishment and maintenance of the “organizing center” of the SAM (Mayer et al., 1998) (Fig. 1.1 and 1.3). Mutations of this gene result in a depletion of the central zone (containing the stem cells) followed by a reduced production of leaf primordia and the subsequent abortion of the SAM. This phenotype is the result of a loss of “pluripotency” of the meristematic cells (Leibfried et al., 2005). Molecular studies have revealed that the specification of stem cells in the shoot meristem is regulated by *WUS* through a feed back mechanism interaction with *CLV* signaling (Bhalla and Singh, 2006). The nature of this interaction, depicted in Figs. 1.1 and 1.3, is critical for the proper maintenance of the SAM as it ensures a balance between the formation of stem cells and the rate of differentiation. Perturbation of this regulatory process often results in meristem abortion.

During embryogenesis the expression of *WUS* is first visible in the inner apical cells of 16-cell embryos. Upon further growth, the expression of this gene becomes localized in the sub-apical cells demarking the organizing center of the SAM (Mayer et al., 1998). A diagram summarizing the expression patterns of *WUS*, as well as that of the other meristem marker genes, is shown in Fig. 1.4.

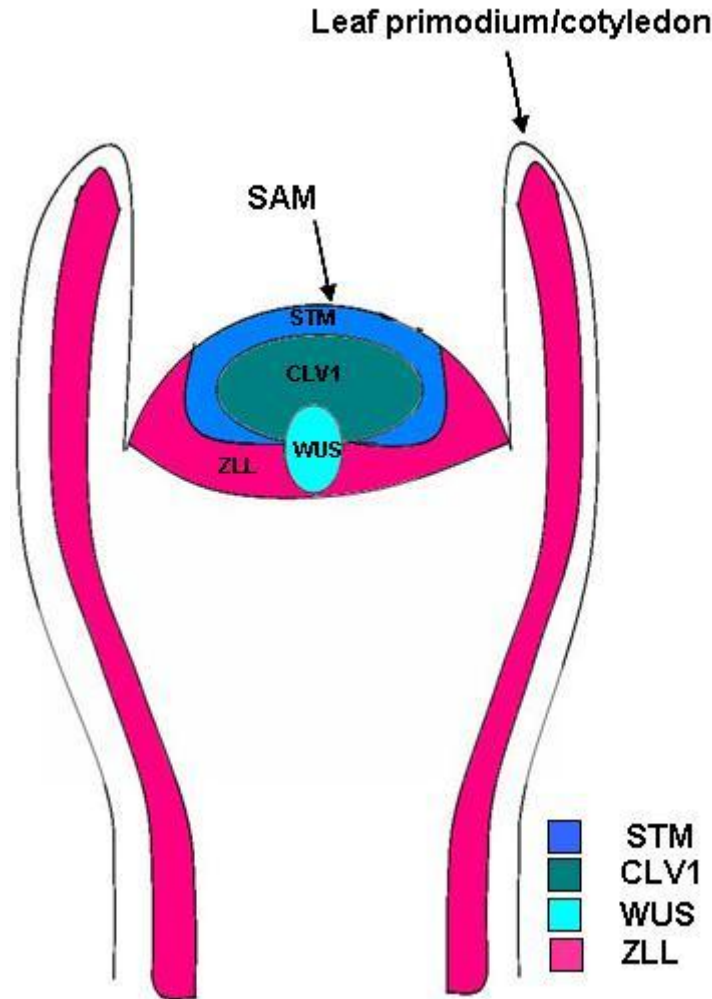


Figure 1.4: A diagram of the shoot apical meristem showing the expression domains of the SAM-regulatory genes. STM mRNAs (blue color) are detected in L1, L2, and L3 of the central zone, but not in the peripheral zone. CLV1 (as well as CLV2) is expressed in the central region of the SAM and includes cells of L2 and L3 (green color). The WUS domain demarks the "organizing center" of the SAM (turquoise color). ZLL transcripts are localized in the vascular tissue and the SAM (red color).

1.2.13. Hormonal regulation of the SAM

Recent evidence suggests that the activity of the SAM might be under the control of plant growth regulators, which occupy specific domains within the apical pole (Hedden and Thomas, 2006). The CZ of the SAM is characterized by high levels of cytokinins, required to confer “stem” identity to the meristematic cells, while the PZ is enriched in auxins and gibberellins which have been implicated in the formation of lateral primordia (Fig. 1.5) (Veit, 2009). The involvement of each plant growth regulator during the maintenance of the SAM will be discussed in the next sections.

1.2.13.1. Auxins

The word “auxins” derives from the Greek *auxein* which means to grow, to increase or to enlarge (Baluska et al., 2002). This definition is very appropriate, given the role of this class of plant growth regulators in eliciting many morphogenic responses in plants (Teale et al., 2006). Expression studies using the auxin-induced promoter DR5 indicate that auxin is present throughout the SAM, but with highest levels in the PZ (Veit, 2009). Besides containing lower endogenous auxin levels, cells within the CZ are also insensitive to this growth regulator. This concept is exemplified by the observations that exogenous auxins induce the formation of leaf primordia in the PZ, but not in the CZ (Reinhardt, 2005).

Unlike the CZ, the PZ is sensitive to auxins which act as a cue for the formation of leaf primordia. Molecular studies uncovered the essential roles of auxins during SAM development, especially in relation to primordium initiation and phyllotaxy (Reinhardt et

al., 2000). Galinha et al. (2009) proposed that auxins might suppress genes conferring meristematic identity, thereby committing cells to differentiate into lateral organs. This notion is consistent with the auxin maxima observed at the future sites of primordial growth (de Reuille et al., 2006). Asymmetric distribution of auxin within the SAM is mediated by PIN1 which has been shown to transport auxin towards the incipient leaf primordia (Reinhardt et al., 2003). These observations have also been supported by computer simulations describing the distribution of auxin transporters and auxin levels in the apical pole (Benková et al., 2003; Reinhardt et al., 2003; de Reuille et al., 2006).

Heisler et al. (2005) suggested that the heterogeneous distribution of auxin in the SAM might be responsible for the proper localization pattern of *STM*. Expression of this gene is found in domains characterized by low auxin levels, whereas no expression is observed in domains with high auxin concentrations (*STM* expression is lost during the initiation of the leaf primordium). This notion is further supported by Scanlon (2003) who showed that expression of members of the class-1 *KNOX* gene family (which also include *STM*) is altered in maize seedlings treated with auxin transport inhibitors, which also affect the sites of leaf initiation. In an effort to explain the relationship between auxin and *KNOX1* genes, Shani et al. (2006) proposed a feed back mechanisms operating within the SAM and ensuring a balance between cell division and differentiation. It must be kept in mind that besides *STM*, auxin levels affect the expression of other genes participating in the maintenance of the SAM, including *CUPSHAPED COTYLEDON-1* and -2 (*CUC 1* and -2), which are expressed at the boundary between the SAM and the incipient leaf primordium (Laufs et al., 2004). Detailed information on the role of auxin and auxin transporters on leaf formation and phyllotaxy is available in (Reinhardt, 2005).

1.2.1.3.2. Cytokinins

The inductive effects of cytokinins on shoot formation have long been recognized (Skoog and Miller, 1957), indicating that the formation of the SAM depends on the level of cellular cytokinins. Several pharmacological and molecular experiments using *in vitro* and *in vivo* systems have shown the crucial roles played by this class of plant growth regulators during the formation and maintenance of meristems (Higuchi et al., 2004; Werner et al., 2008). The over-expression of cytokinin oxidase, a cytokinin degrading enzyme, results in a reduction in the size of the SAM (Werner et al., 2003) suggesting the involvement of cytokinins in the maintenance of the meristematic cells. To further investigate this notion, Müller and Sheen (2008) introduced a novel cytokinin reporter system: the two component output sensor (TCS). The TCS is composed of a concatemericized B-TYPE ARR binding motif fused to a minimal 35S promoter and a luciferase or GFP reporter. The TCS-transformed *Arabidopsis* plants showed a very strong signal in cells of the CZ specifying the *WUS* expression domain (Gordon et al., 2009). These results indicate that high levels of cytokinins are found in *WUS*-expressing cells within the organizing center. Therefore it can be suggested that cytokinin maxima are required for the acquisition of “stem” identity in the CZ of the SAM. This concept was further demonstrated by the enlarged organizing center in meristems of plants over-expressing cytokinin biosynthetic genes (Sinha et al., 1993; Giulini et al., 2004; Leibfried et al., 2005; Jasinski et al., 2005; Barton, 2009). An opposite phenotype was noticed in rice plants with reduced expression of *LOG*, a gene encoding a factor responsible for the activation of endogenous cytokinins (Kurakawa et al., 2007).

1.2.1.3.3. Gibberellins

Morphogenesis and leaf initiation in the SAM are also affected by gibberellins (GAs). In the *Arabidopsis* SAM, high levels of GAs were observed in regions characterized by auxin maxima, i.e. young leaf primordia, suggesting that both hormones are involved in cellular differentiation and maturation processes (Shani et al., 2006). Within the CZ of the SAM, however, the endogenous levels of GA are maintained low, possibly due to the increased activity of catabolic enzymes and/or decreased activity of GA biosynthetic enzymes (Bolduc and Hake, 2009). Jasinski et al. (2005) further proposed that KNOX proteins are directly required to lower cellular GA levels in the CZ of the SAM. Functional studies indicated that KN1, a class 1 KNOX protein, represses GA 20-oxidase, a GA biosynthetic enzyme involved in the oxidation and elimination of carbon-20 which is required to produce the biologically active C₁₉-GAs (Sakamoto et al., 2001b; Chen et al., 2004). The expression level of this enzyme is significantly decreased in leaves over-expressing *STM* and *BREVIPEDICELLUS* indicating that KNOX proteins regulate GA levels through the down-regulation of genes encoding GA biosynthetic enzymes (Hay et al., 2002). Recent work Kessler et al. (2006) further suggested that KNOX protein can also affect GA degrading enzymes by altering their expression. One of such enzymes, GA2-oxidase-1, is up-regulated in *Kn1-N* mutants of maize (Kessler et al., 2006). Collectively the above evidence suggests the existence of fine regulatory mechanisms responsible for lowering GA levels in the central regions of the SAM which involve the participation of key developmental elements, i.e. the KNOX proteins.

1.2.1.3.4. Ethylene

No much information is currently available on the effects of ethylene on SAM formation and maintenance *in vivo*. (Hamant et al., 2002) observed that plants with a mutated *CONSTITUTIVE TRIPLE RESPONSE 1* gene, a negative regulator of the ethylene response pathway in *Arabidopsis*, exhibited a disorganized SAM and reduced *KNAT2* expression levels. Although without any conclusive evidence, this study demonstrates the involvement of ethylene signaling during meristem function. *In vitro* studies showed that high levels of ethylene disrupt the architecture of the SAM and induce the formation of intercellular spaces which compromise the functionality of the meristematic cells resulting in meristem abortion at germination (Belmonte et al., 2006).

1.2.1.3.5. Absciscic acid (ABA)

The role of ABA in the regulation of the SAM is still not completely clear. Prolonged culture of *Brassica* microspore derived embryos (MDEs) results in structural alterations of the SAM, including the formation of intercellular spaces which disrupt the integrity of the apical pole leading to meristem abortion. These abnormalities can be overcome if ABA is included in the culture medium (Belmonte et al., 2006; Stasolla, 2009). ABA-treated MDEs produce zygotic-like SAMs and are able to regenerate plants at high frequency, denoting enhanced meristem functionality.

Indirect evidence on the role of ABA in the regulation of meristematic cells is provided by studies conducted on the root apical meristem. (Rosado et al., 2006) observed that *TETRATRICOPEPTIDE-REPEAT THIOREDOXIN-LIKE 1 (TTL1)*, an ABA responsive gene, affects the sensitivity of *Arabidopsis* root cells to ABA and

osmotic stress. *ttl1* mutant plants show disorganized roots indicating that TTL1 is required for root meristem regulation. Further evidence on the role of ABA during meristem maintenance comes from studies conducted on *LATD*, a *Medicago* gene involved in ABA signaling (Benková and Hejácíko, 2009). The severe structural abnormalities observed in the root meristems of *latd* lines were abolished by exogenous applications of ABA. Given the increasing amount of evidence suggesting the existence of similar regulatory mechanisms governing the shoot and the root apical meristems (Wong et al., 2009), it is suggested that ABA might also have a critical role during SAM development.

1.2.2. *In vitro* regeneration of plants

Over the past years, plant tissue culture has been used not only for some practical applications, such as clonal propagation, germplasm culture, plant modification and improvement, but also as a system to study plant morphogenesis (Hicks, 1994). Plant regeneration *in vitro* can be achieved through two distinct pathways: embryogenesis (via somatic or gametophytic) and organogenesis (via root or shoot formation) (Phillips, 2004). Both pathways can occur directly, without an intervening callus phase, or indirectly with the generation of a callus from which embryos or organs originate. This variation reflects the great developmental flexibility of *in vitro* culture systems.

The mechanisms whereby plant regeneration occurs during embryogenesis and organogenesis are quite complex and somehow similar among the two systems. As a first step, cells within the explants must have the potential to regenerate embryos or organs. Secondly, cells must be competent to respond to exogenous signals. Thirdly, competent

cells must be induced by specific signals (often plant growth regulators) and become “committed” to initiate the embryogenic/organogenic pathway. Commitment to initiate a morphogenetic pathway requires a de-differentiation step, in which mature cells of the explants lose their specific fate and acquire a meristematic condition (Sugiyama, 1999). Formation of meristematic cells on the surface of the explant (in the case of a direct regeneration pathway) or within the callus (in the case of an indirect regeneration pathway) can be localized or extended to large areas. Meristematic cells can then initiate a new developmental fate and regenerate embryos or organs (shoots or roots) depending on the culture conditions provided.

Initial cytological differences between cells committed to form organs (pluripotent cells) or embryos (totipotent cells) have been described. As reviewed by (Verdeil et al., 2007), pluripotent organogenic cells are characterized by a high nucleus/cytoplasm ratio, one or multiple nucleoli, nuclei of spherical shape, the absence of amyloplasts, the presence of many plasmodesmata, a higher heterochromatin versus euchromatin content, and a thin primary cell wall. On the other hand, totipotent embryogenic cells are distinguished by nuclei with irregular shape, a single large nucleolus, amyloplasts which are adjacent to the nucleus, a lower heterochromatin versus euchromatin content, a modified cell wall with callose deposits, and very few or no plasmodesmata.

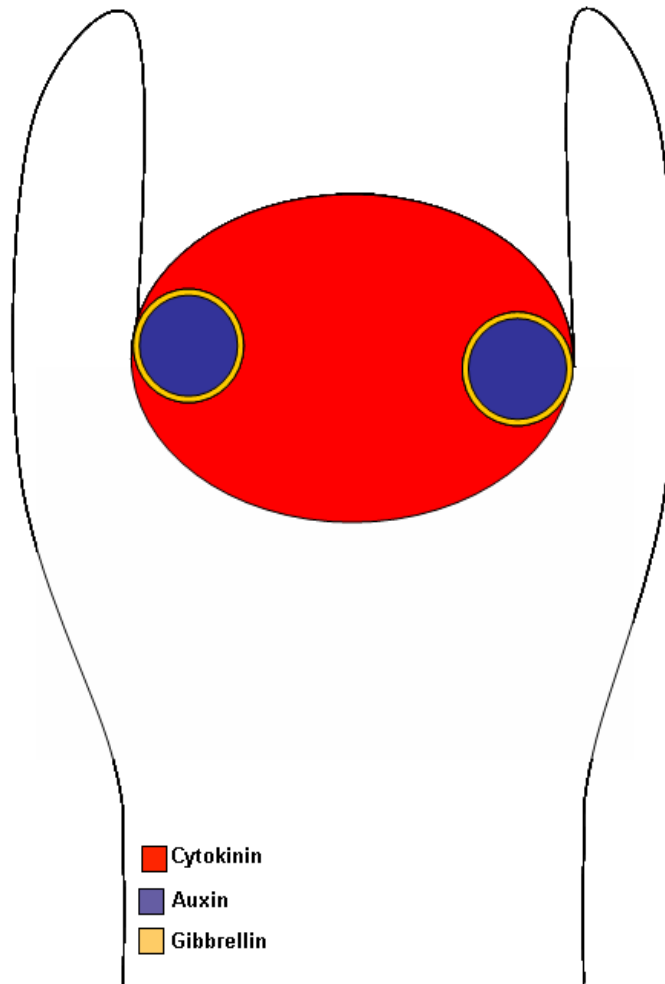


Figure 1.5: Distribution of plant growth regulators within the shoot apical meristem. Domains of hormone maxima are shown by different colors.

1.2.2.1. Embryogenesis

Double fertilization in higher plants marks the formation of the sporophyte and results in the establishment of a single-celled zygote and a founder cell of the endosperm. The subsequent development of the zygote is referred to as embryogenesis during which the zygote undergoes a series of complex morphological events. Embryogenesis can also occur without fertilization through the generation of asexual embryos, also referred to as apomictic embryos, which are formed from maternal tissue or the unfertilized egg cell (Nogler, 1984). Formation of asexual embryos can also be achieved in culture through two processes: gametophytic and somatic embryogenesis. Gametophytic embryogenesis is the generation of haploid embryos from the male or female gametophyte. In general male gametophytic embryogenesis (also referred to as androgenesis) is the preferred method and involves the utilization of microspores and pollen grains (Raghavan, 2000; Maraschin et al., 2005). Somatic embryogenesis, the second method to obtain embryos in culture, is initiated from somatic cells, i.e. cells other than gametes (Bhojwani and Razdan, 1996; Raghavan, 2000).

In dicotyledonary species the developmental sequence of embryogenesis, denoted by the shape of the embryo body as globular, heart, and torpedo, has been stereotyped and investigated (Willemsen and Scheres, 2004). These stages are also observed during *in vitro* embryogenesis, thereby revealing the existence of common regulatory pathways guiding cell patterning and tissue differentiation *in vivo* and *in vitro*. This observation is very important because it allows the use of culture embryos to investigate physiological and molecular events occurring in seed embryos, which are very difficult to study. Seed embryos are in fact embedded in the maternal tissue and very difficult to dissect. The

main advantages of using *in vitro* systems is that (1) a large number of embryos can be obtained in a short period of time and (2) in the majority of systems embryo development is synchronized, which permits the harvesting of specific stages. However, there are some limitations in using the *in vitro* system to study embryogenesis; these include the lack of dormancy of the embryos, somaclonal variation, and the affect that a specific genotype might have on the process.

During my studies, two *in vitro* techniques have been utilized to generate embryos in culture: androgenesis (microspore-derived embryogenesis) in *Brassica napus* and somatic embryogenesis in *Arabidopsis*. Both systems have been used in many previous studies (O'Neill and Mathias, 1993; Huang and Yeoman, 1995; Yeung et al., 1996; Barinova et al., 2004; Belles-Boix et al., 2006; Chan and Pauls, 2007; Malik et al., 2007; Malik et al., 2008), and they differ in that *Brassica* androgenesis is a direct system (without a callus phase), whereas somatic embryo development in *Arabidopsis* requires a callus step.

A key event during androgenesis in *B. napus* is the re-differentiation step in which the genetic program of the microspores is redirected towards the embryogenic pathway. This transition is induced by several stress treatments, including elevated temperatures (usually 32° C, Keller and Armstrong (1979); Custers et al. (1994), ethanol (Pechan and Keller, 1989), gamma irradiation (Pechan and Keller, 1989), colchicine (Zhao et al., 1996), sucrose starvation (Touraev et al., 1996), low temperatures (Sunderland et al., 1974; Kasha et al., 1995) and changes in medium pH (Barinova et al., 2004). The first sign of de-differentiation of the microspores, as reviewed by (Telmer et al., 1992), involves changes in cytoskeletal organization. Simmonds and Keller (1999) observed that the pre-prophase band (composed by arrays of microtubules) tends to localize in the

middle region of induced microspores. This positioning ensures the symmetric cell division of the microspore, which demarks the completion of the inductive events and the acquisition of the embryogenic program (Yeung, 2002). This is in contrast to the gametophytic pathway which is started with an asymmetric mitotic division of the microspore. Of the two daughter cells originating from the microspore one is committed to form the suspensor of the embryo, while the other, referred to as “the proembryogenic cell” will generate the embryo proper (Fig. 1.6 A). Through a series of anticlinal and periclinal divisions the proembryogenic cell gives rise to a cluster of cells demarking the globular stage of embryogenesis. Growth of the globular embryo results in the production of the first visible tissue, the protoderm, which is the precursor of the epidermis. After about fifteen days in culture the globular embryos develop further into heart-shaped embryos characterized by the formation of incipient cotyledons. It is during this stage that the apical meristems become visible. A detailed description of the histodifferentiation process can be found in (Yeung et al., 1996).

Unlike *Brassica* androgenesis, *Arabidopsis* somatic embryogenesis is an indirect system involving a callus step (Mordhorst et al., 1997). Somatic embryos can be produced from mature tissue (O'Neill and Mathias, 1993; Huang and Yeoman, 1995) or zygotic embryos (Mordhorst et al., 1998). In the latter case bent-cotyledon embryos are placed on an induction medium containing the auxin 2,4-D. Under these conditions embryogenic tissue develops from the adaxial side of the cotyledons. Embryo development is then encouraged in a medium devoid of plant growth regulators (Fig. 1.6 B).

1.2.2.1.1. Physiological aspects of *in vitro* embryogenesis

As reviewed by Thorpe and Stasolla (2001), formation of *in vitro* embryos is usually accompanied by profound physiological and metabolic changes, which include fluctuations in amino acid, polyamine, DNA, RNA, and protein synthesis. (Malabadi and Van Staden, 2005) observed stage-specific alterations in amino acid biosynthesis and a significant improvement in the number of *Pinus patula* somatic embryos when an amino acid mixture was added to the culture medium. This notion was also confirmed by Malabadi and van Staden (2005) who observed an increased rate of cell division and enhanced embryonic yield in carrot suspension cultures supplemented with α -alanine. A similar result was documented by Skokut et al. (1985) in alfalfa culture systems. Polyamines, small aliphatic amines, are also necessary for the completion of the embryogenic program. (Yadav and Rajam, 1998) found that eggplant leaf disc explants from the apical part of the donor plant are more embryogenic than explants located at the base of the plant. These results correlated to the endogenous levels of polyamines, which were higher in the upper regions of the donor plant. Further studies Mengoli et al. (1989) documented higher levels of putrescine and spermidine in developing carrot somatic embryos. Overall these findings suggest a requirement of polyamines during embryogenesis. This notion was further confirmed by the inhibitory effects of methylglyoxal bis, an inhibitor of the polyamine precursor S-adenosylmethionine, on carrot somatic embryogenesis (Minocha et al., 1991), and by several studies showing an improvement in embryogenic output in systems supplemented with exogenous polyamines. Biosynthesis of polyamines also affects the level of S-adenosylmethionine, a key metabolite involved in several other processes. S-adenosylmethionine can be

converted to S-adenosylhomocysteine with the production of methyl groups leading to DNA methylation (Brown, 1989). Changes in DNA methylation levels have profound effects on the initiation of embryogenesis in both animal and plant systems (Leljak-Levani et al., 2004), and will be discussed in the next chapter. Based on the above information it is apparent that polyamine levels play a direct and an indirect role during *in vitro* embryogenesis.

Changes in the rate of DNA, RNA, and protein biosynthesis are also observed during specific stages of embryo development. Extensive work (Ashihara et al., 1981; Stasolla et al., 2001; 2002; 2003) revealed that DNA and RNA metabolism is affected by fluctuations in purine and pyrimidine synthesis and degradation. Using different culture systems these studies demonstrated that production of nucleotides, through the salvage of nucleobases, is required especially during the induction phase of embryogenesis. During the subsequent phases of development such changes attenuate.

One of the key events during *in vitro* embryogenesis is the formation of the SAM which is initiated in late globular-stage embryos and completed in heart-stage embryos (Yeung et al., 1996).

Differently from zygotic embryogenesis, the formation of *in vitro* SAMs occurs in a sub-optimal environment and this is reflected by the structural abnormalities noticed in the apical region of the embryos (Stasolla, 2009). The most apparent structural deviation consists in the formation of intercellular spaces, which disrupt the architecture of the apical pole, and the differentiation of the meristematic cells which accumulate storage products and form large vacuoles.

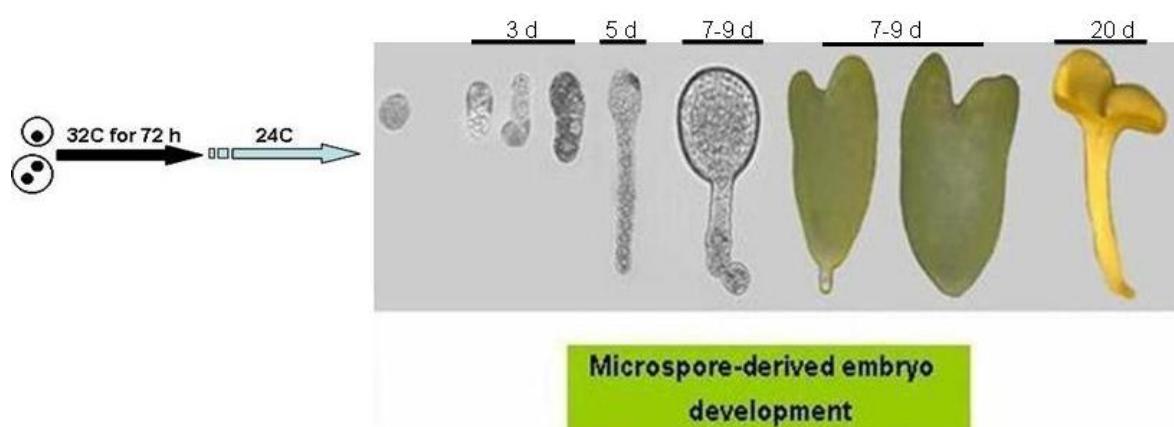
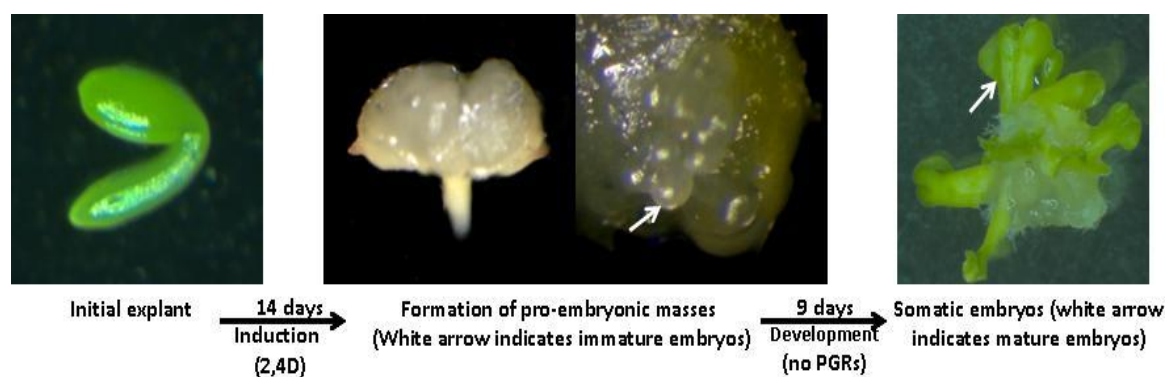
A**B**

Figure 1.6. Schematic representation of microspore-derived embryogenesis in *Brassica napus* (A) and somatic embryogenesis in *Arabidopsis* (B). *In vivo* pollen development in *B. napus* starts with the formation of haploid uninucleate and/ or early binucleate microspores. These microspores undergo microgametogenesis and further develop into pollen grains. The gametophytic development is arrested by exposing the microspores to 32 °C for 72 hours. The initial stressed explants (immature pollens) are transferred to 24°C in order to develop microspore-derived embryos. The number above each developmental stage of MDEs indicates the days in culture. *Arabidopsis* somatic embryogenesis is initiated by culturing bent-cotyledon embryos on an induction medium enriched with the auxin 2,4-D. After two weeks a callus forms from the adaxial side of the cotyledons. Production of embryos, originating from the surface of the callus occurs on a development medium devoid of plant growth regulators (PGRs).

These events compromise the functionality of the SAM and preclude the ability of the embryos to germinate and regenerate viable plants (Belmonte et al., 2006; Stasolla, 2009).

Improvements of culture conditions in an effort to enhance the quality of the SAMs have been documented in many studies, and include applications of ABA (Hays, 1996), osmoticum agents such as polyethylene glycol (Ilic -Grubor et al., 1998), and alterations in the glutathione redox state (Belmonte et al., 2005; Belmonte et al., 2006). Changes in the glutathione redox state, which is regulated by the contribution of the reduced (GSH) or oxidized form (GSSG) of glutathione, have profound effects on embryo development and meristem formation. Belmonte et al. (2005) showed that an experimental shift of the glutathione pool towards an oxidized state (low GSH/GSH+GSSG ratio), affected by applications of buthionine sulfoximine, an inhibitor of glutathione *de novo* synthesis, encourages the production of embryos with zygotic-like meristems. Such meristems have a dome-shaped appearance, lack intercellular spaces, and are characterized by a large cluster of central cells which are highly meristematic.

The ability to experimentally manipulate the architecture of the SAM *in vitro* is very important for gaining insights into the physiological requirements which ensure the establishment of a functional meristem. This is demonstrated by the work conducted by (Ramesar-Fortner and Yeung, 2001) who showed that applications of 2,4,6-triiodobenzoic acid (TIBA), an inhibitor of the polar transport of auxin affect the bilateral symmetry of *Brassica* MDEs and prevent the formation of a functional SAM.

Changes in glutathione metabolism and applications of TIBA in culture will be used in this thesis to affect the architecture of the SAM in *B. napus* MDEs.

1.2.2.1.2. Hormone regulation of *in vitro* embryogenesis

Plant hormones play crucial roles during embryogenesis (Friml et al., 2003). As indicated in the previous section, a key element for the initiation of the embryogenic process is represented by the inductive signals which trigger competent cells to acquire embryogenic potential. Plant hormones and especially auxins are directly involved in this step (Pasternak et al., 2002). This is also demonstrated *in vivo* where increasing auxin levels are observed during the initial phases of carrot fertilization (Ribnicky et al., 2002).

1.2.2.1.2.1. Auxins

The use of auxin to induce somatic cells to produce embryos is very common. Nomura and Komamine (1985) were able to encourage embryogenic competence in carrot somatic cells with applications of 2,4-D. This synthetic auxin is currently utilized to induce somatic embryos in many angiosperm and gymnosperm species (Feher et al., 2003). Applications of exogenous 2,4-D might act either directly, or indirectly, by increasing the endogenous auxin levels. Rajasekaran et al. (1987) documented elevated levels of the naturally occurring auxin, indole 3-acetic acid (IAA), upon exogenous applications of 2,4-D. This notion was consistent with the observation that cellular IAA increases during the initial phases of somatic embryogenesis (Charrière et al., 1999; Pasternak et al., 2002).

The roles exercised by auxin during the induction step are elusive. Speculations argued for its action as a “stress signal” (Feher et al., 2003), and its involvement in DNA methylation (Phillips et al., 1994) and ABA synthesis (Woeste et al., 1999). Alterations in DNA methylation are often observed when auxins, including 1-naphthaleneacetic acid

(NAA), 2,4-D, and IAA are applied to the culture medium (Phillips et al., 1994; von Aderkas and Bonga, 2000). Changes in DNA methylation patterns have been associated to the induction of embryogenic competence in both animal and plant systems (Morrish and Vasil, 1989). While the acquisition of embryogenic competence requires auxins, embryo development often occurs in an auxin-free environment. This concept has been demonstrated in many species (Feher et al., 2003).

Preliminary work also suggests that a possible role of 2,4-D is to increase the biosynthesis of ABA, as revealed by the higher levels of 9-cis-epoxycarotenoid dehydrogenase, the enzyme which catalyses the first step of abscisic acid biosynthesis from carotenoids in plants exposed to auxins (Woeste et al., 1999). It is well known that ABA is a stress hormone *in planta*, and its applications induce somatic embryogenesis in several systems (Nishiwaki et al., 2000; Senger et al., 2001). During the induction phase of carrot somatic embryogenesis, the expression level of *ABI3*, an ABA signaling gene, increases after four days of 2,4-D treatments and this is followed by a rise in cellular ABA (Shiota et al., 1998). Increasing levels of endogenous ABA are indeed required to promote embryo development both *in vivo* (Ackerson, 1984) and *in vitro* (Garcia-Martin et al., 2005).

1.2.2.1.2.2. Cytokinins

Besides auxins, cytokinins are also involved during the initial phases of embryogenesis (Sagare et al., 2000). In some plants such as *Dendrobium*, applications of thidiazuron (TDZ) in the induction medium promote direct somatic embryo production from leaf tip explants (Chung et al., 2005). This finding was supported by Kuo et al.

(2005) who found clusters of directly-induced somatic embryos originating from leaf segments of *Phalaenopsis* cultured in the presence of benzyl adenine (BA) and TDZ (Canhoto and Cruz, 1994). In the same study it was shown that the number of somatic embryos produced was proportional to the amount of cytokinins added in the medium. In many plant species cytokinins and auxins are added together to induce the acquisition of embryogenic potential (Guohua, 1998; Panaia et al., 2004; Chung et al., 2005).

1.2.2.1.2.3. Absciscic acid

Absciscic acid (ABA) is an important plant growth regulator for zygotic and somatic embryogenesis (Jimenez, 2005). Fluctuations in ABA levels and sensitivity are observed during zygotic embryo development. In both gymnosperms and angiosperms, cellular ABA increases during the early phases of embryogenesis, reaching a maximum level at the globular stage of development before declining sharply during the subsequent phases (Rajasekaran et al., 1982; Kong and Von Aderkas, 2007). This decline is also accompanied by a reduction in ABA sensitivity (Finkelstein et al., 1985). These fluctuations were also described during *in vitro* embryogenesis (Kamada and Harada, 1981), suggesting a key role of ABA during the middle stages of embryonic growth. Pharmacological studies showed that applications of glyphosate and fluridone, inhibitors of ABA synthesis, to the culture medium of *Pennisetum purpureum* inhibited somatic embryo development. This inhibitory effect was however reversed if ABA was applied together with the inhibitors (Rajasekaran et al., 1987).

Absciscic acid is important for several developmental processes, including the accumulation of storage products and late embryogenesis abundant (LEA) proteins

(reviewed by Hundertmark and Hinch, 2008) and for preventing precocious germination, as illustrated by several ABA insensitive mutants (Koornneef et al., 1989). A further role associated to ABA is related to its ability to regulate direct or indirect embryo conversion. During the early phases of germination, cultured embryos can regenerate plants directly, i.e. by converting into seedlings, or indirectly, through the formation of secondary somatic embryos. In bermuda grass, Li and Qu (2002) found that removal of ABA from the culture medium induced the formation of secondary somatic embryos. An opposite result was observed by (Ogata et al., 2005), who reported that ABA applications to carrot embryogenic cultures strongly promoted the formation of secondary and tertiary somatic embryos. This discrepancy in results clearly suggests that the effect of ABA is species dependent.

1.2.2.1.3. Genetic aspects of *in vitro* embryogenesis

In vitro embryogenesis is an attractive system for studying the molecular bases of plant embryo development (Lin et al., 1996; Singla et al., 2007; Malik et al., 2007; 2008; Karami et al., 2009; Karami and Saidi, 2009; Karami et al., 2009). As discussed above, embryogenesis is triggered by a switch in cell fate allowing somatic cells to acquire embryogenic potential. This transition, critical for the process, is underpinned by elaborated molecular mechanisms requiring the participation of several genes. Over the past few years efforts have been directed to isolate and characterize these genes and today several candidates have been identified. These include *SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE (SERK)*, *LEAFY COTYLEDON 1 and 2 (LEC1 and 2)*, *PICKLE (PKL)*, *BABY BMOOM (BBM1)*, and *WUSCHEL (WUS)*.

SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE (SERK) was originally isolated from embryogenic cells of carrot suspension cultures (Schmidt et al., 1997). Localization studies indicate that *SERK* is expressed in zygotic embryos up to the globular stage of development (Salaj et al., 2008). Ectopic expression of the *Arabidopsis AtSERK*, orthologous to the carrot *SERK*, significantly improved the efficiency of somatic embryogenesis in *Arabidopsis* (Hecht et al., 2001). Expression analyses in *Poa pratensis* showed that *PpSERK* is involved in the production of apomictic embryos (Albertini et al., 2005). According to the previous information, it appears that *SERK* may induce the embryogenic pathway in several systems, both *in vivo* and *in vitro*. Hu et al. (2005) further demonstrated that the *Oryza sativa OsSERK*, was significantly induced by pathogenic stress. This observation suggests that the expression of this gene is activated as a result of imposed stresses, which play a key role during the induction of *in vitro* embryogenesis (Feher et al., 2003).

Another candidate gene involved in the acquisition of embryogenic competence is *LEAFY COTYLEDON1 (LEC1)*, which encodes a transcription factor related to the Heme-Activated Proteins 3 (HAP3) subunit of the CCAAT (Lotan et al., 1998). *lec* mutant zygotic embryos show an abnormal morphology in the trichomes of the cotyledons. These embryos fail to acquire desiccation tolerance and do not accumulate late embryogenic abundant (LEA) proteins (Vicent et al., 2000). Over-expression of *AtLEC1* in *Arabidopsis* induced embryo-like structures from vegetative tissues indicating that this gene is involved in the initiation of the embryogenic program (Lotan et al., 1998). As for *SERK*, the existence of *LEC1* orthologs in other plants suggests a redundant role of this gene across species (Kwong et al., 2003).

During *in vitro* embryogenesis *LEC 1* is only expressed in embryogenic lines, but not in their non-embryogenic counterparts (Karami et al., 2009). A related member of the LEC family is *LEC2*, which was also shown to regulate many aspects of embryogenesis (Stone et al., 2008). *LEC2* encodes a transcription factor with a B3 DNA binding domain. Loss-of-function mutations in *LEC2* influence the specification of cotyledon identity, maintenance of embryonic cell fate, and desiccation acquisition (Meinke et al., 1994). Karami et al. (2009) reported that in *lec2* plants direct (without a callus phase) somatic embryogenesis is completely compromised, whereas indirect (with a callus phase) somatic embryogenesis was retained, despite a fewer number of embryos produced. Based on these observations the authors speculated that the function of *LEC2* during *in vitro* embryogenesis is restricted to direct systems. Recently, Stone et al. (2008) found that ectopic expression of *LEC2* increases the expression of *YUC2* and *YUC4*, key enzymes in the triptophan-dependant auxin biosynthetic pathway, resulting in increased synthesis of auxin (Cheng et al., 2006). These studies suggest an intimate interaction between *LEC2* and auxin which is the inductive signal for the acquisition of embryogenic competence (discussed above).

A third candidate gene involved during the initial phases of embryogenesis is *PICKLE (PKL)*, which encodes a chromatin remodeling factor ubiquitously expressed in *Arabidopsis* (Eshed et al., 1999). The observation that a mutation of this gene induces indirect somatic embryogenesis from root explants. Ogas et al. (1997) suggests that *PKL* represses the initial stages of embryo development. This repressive effect might be exercised through the down-regulation of *LEC* genes, as suggested by Ogas et al. (1997) who observed high *LEC* expression levels in *pkl* plants. Recent evidence also suggests a

possible interaction between PKL and plant growth regulators, especially gibberellins, which are able to rescue the *pkl* phenotype (Rider et al., 2004).

The *BABY BOOM1* (*BBM1*) gene, identified from canola microspore- derived embryos encodes an AP2/ERF transcriptional factor (Boutilier et al., 2002). Ectopic expression of *this gene* in canola seedlings results in the production of somatic embryos from the leaf margins, while its over-expression in *Arabidopsis* leads to the formation of cotyledon-like structures (Boutilier et al., 2002). These phenotypic characteristics were also associated to other changes in leaf and flower morphology, neoplastic growth, and hormone-free regeneration of explants. *BBM1* expression promotes organogenesis and embryogenesis without applications of exogenous hormones emphasizing that *BBM1* may stimulate the production of hormones and/or increase the sensitivity of cells to growth regulators (Boutilier et al., 2002).

The last gene affecting embryogenic potential is *WUSCHEL* (*WUS*), a homeobox gene which encodes a transcription factor required for the establishment and maintenance of the “organizing center” of the SAM (Mayer et al., 1998). Zuo et al. (2002) reported that *WUS* promotes the transition from vegetative to embryonic development in several tissues and organs without hormonal applications. Over-expression of this gene triggers the formation of direct somatic embryos from somatic cells, bypassing the requirement of auxin. As for *BBM1*, these characteristics suggest that *WUS* might interfere with auxin synthesis/signaling in the acquisition of embryogenic competence.

Besides the identification of the genes mentioned above, which are able to induce or repress *in vitro* embryogenesis through their altered expression, several studies have focused on the identification of genes differentially expressed during embryo

development. Using subtractive hybridization followed by differential screening Tsuwamoto et al., (2007) isolated and identified 136 non redundant ESTs expressed during the induction phase of *Brassica napus* MDEs. Of these ESTs, 14 were identified as androgenesis-related and were classified into two groups. Group A contained 10 genes repressed in the microspores by heat-shock, whereas group B included 4 genes whose expression peaked in globular embryos before declining during the subsequent stages of development (Tsuwamoto et al., 2007). One of the genes identified in this study encoded a glutathione S- transferase which has been reported to participate in the acquisition of embryogenic competence, possibly by removing reactive oxygen species during the early mitotic divisions. Another group of genes expressed during the initial phases of *B. napus* microspore-derived embryogenesis encode Rop GTPases (Chan and Pauls, 2007). The authors suggested that Rop GTPases affect embryogenesis by acting as specific cues needed for the histodifferentiation process.

Recent studies using sequencing techniques further contributed to the identification of differentially expressed elements during specific stages of embryogenesis. Malik et al. (2007) identified 16 *B. napus* “embryogenesis-marker” genes. These genes were classified into three distinct groups: (1) embryo specific genes (*FUS3*, *LEC1*, *LEC2*, *UP1*, and *UP2*), (2) embryos expressed genes (*NAPIN*, *BBM1*, *WOX9*, *AB13*, and *ATS1*), and (3) sporophyte expressed genes (*LRR1*, *CP1*, *CYP78A*, *WOX2*, and *CYP81F*). A similar approach was also employed by Joosen et al. (2007) who identified transcript/protein pairs induced during the microspore-embryo transition in *B. napus*.

Analyses of genes expressed during the late phases of MDE development, i.e. globular-heart-cotyledonary stage, were conducted by (Stasolla et al., 2008). Overall these studies indicate that embryogenesis is a complex process requiring the activation of several primary and secondary metabolic pathways including lipids, amino acids, and carbohydrates (reviewed by Pauls et al., 2006). Perturbation of these pathways often results in the abolishment of the embryogenic process.

1.2.2.2. Organogenesis

Organogenesis refers to the process whereby unipolar organs, i.e. shoots or roots, are generated from somatic cells. The process is initiated by the formation of primordia, which through a series of defined events form a well developed shoot or root system (Steeves and Sussex, 1989). The formation of one organ (shoot or root) must be followed by the regeneration of the complementary organ in order to obtain bipolar plants characterized by two apical meristems. In the case of shoot organogenesis, developed shoots are transferred onto a rooting medium which encourages the formation of viable roots. *In vitro* organogenesis was first observed by White (1939) who obtained shoots from tobacco explants and Nobecourt (1939) who induced roots from carrot callus. Since then many other species have been propagated through organogenesis (Thorpe and Stasolla, 2001). As also documented for embryogenesis, *in vitro* shoot organogenesis depends on the application of phytohormones, mainly auxins and cytokinins (as exogenous signals), and the ability of the explants to respond to these exogenous signals (Zhao et al., 2002).

Several structural changes are observed during the induction of shoot organogenesis and the subsequent development of shoots. The first cytological “marker” of the process is a random cell division within the explants occurring after a few days in culture. As for somatic embryogenesis, sustained cell division can generate an organ directly or indirectly, through an intervening callus phase. In the case of direct organogenesis dividing cells are able to organize themselves into apolar meristemoids (Fig. 1.7 A), which are characteristic clusters of isodiametric, densely cytoplasmic cells containing a large number of organelles (Thorpe, 1980). Such meristemoids further develop into primordia (Fig. 1.7 B), displaying a higher degree of cellular organization, and eventually fully functional shoots. In *Arabidopsis*, which has become a good model system (Zhao et al., 2002), shoots can be generated from root/hypocotyl explants through an intervening callus phase (Valvekens et al., 1988). Explants are first preincubated on an auxin-containing callus induction medium (CIM) and then transferred onto a cytokinin-containing shoot induction medium (SIM) where shoots form (Fig. 1.7 C).

As reviewed by Sugiyama (1999) three phases are recognized during *in vitro* organogenesis: a competence acquisition step, a canalization phase, and morphogenesis. The competence acquisition step in *Arabidopsis* is triggered by auxins present in the CIM which induce some cells to de-differentiate. Several studies (Gallois et al., 2002); (Zhao et al., 2002) showed that this step requires the activation of several genes, including *WUS* (discussed in the next chapters). The second phase is the “canalization” step, in which some de-differentiated cells initiate a shoot-specific developmental pathway. Sauer et al. (2006) demonstrated that auxins also play an important role during the canalization step. A sub-cellular reorganization of the auxin-efflux PIN transporters produces an auxin

gradient within the explant, which is perceived as a positional signal by some cells. This signal is suggested to be responsible for the acquisition of the shoot-specific fate. The final phase is morphogenesis in which meristemoids form shoots in response to the cytokinins in the SIM (Sugiyama, 1999). The developmental stages of *Arabidopsis* shoot organogenesis from root explants are shown in Fig. 1.7 C.

1.2.2.2.1. Physiological aspects of *in vitro* organogenesis

Physiological studies on the events leading to the formation of organs in culture are very few. This is mainly due to the fact that the initial phases of organogenesis involve the activation of sub-apical cells which cannot be separated from the remaining “inactive” or unresponsive cells of the explant. Studies on carbohydrate metabolism indicate that heavy accumulation of starch, resulting from the activation of starch biosynthetic enzymes precedes the formation of meristemoids (Thorpe and Murashige, 1968). Starch might serve as a source of energy for the dividing cells. This is also confirmed by the higher respiratory rate and increasing levels of the glycolytic and pentose phosphate pathways observed in explants cultured under shoot-forming conditions, compared to those cultured in a non-shoot-forming environment (Thorpe and Meier, 1972). These observations clearly indicate that reactivation of cell division followed by meristemoid formation is an energy-expensive process requiring profound changes in cellular metabolism. Such changes are also accompanied by an increased production of reducing power (NADPH), possibly as a result of an active pentose phosphate pathway and malate metabolism (reviewed by Thorpe, 1980) observed a rapid utilization of NADPH (compared to NADP^+) in shoot forming tissue and argued that

changes in the NADPH/ NADP⁺ ratio represent a key metabolic switch for the organogenic process.

The increasing levels of carbohydrate production are not only required as a “food source” but are also needed to create a low osmoticum environment. This has been demonstrated by the replacement of a portion of exogenously supplied sucrose with mannitol (Brown et al., 1979) and by measurements of water potential (Brown and Thorpe, 1980). In the latter study it was shown that compared to the non-shoot forming counterpart, shoot-forming tissue has the ability to adjust its water status by maintaining a lower osmotic potential. As suggested by Thorpe (1983) these adjustments are the result of several metabolic fluctuations including the accumulation of prolines, malate, and free sugars. The increased metabolic rate observed during meristemoid formation also correlated to the enhanced incorporation and utilization of several radio-labeled intermediates of primary metabolism: glucose and acetate (Beaudoin-Eagan and Thorpe, 1985). These studies also showed the requirement of carbon skeletons for biosynthetic processes. Increasing levels of aromatic aminoacids were the results of higher activities of several enzymes involved in the shikimate pathway (Beaudoin-Eagan and Thorpe, 1984).

Besides carbohydrates, nitrogen utilization is also affected during the initial phases of shoot organogenesis. Hardy and Thorpe (1990) documented higher levels of total nitrogen, as well as increasing levels of nitrogen assimilation in proteins due to the higher activities of nitrite and nitrate reductase. Fluctuations in the biosynthesis and utilization of polyamines were also observed (Kumar and Thorpe, 1989).

Like any morphogenic process, organogenesis is directly affected by the levels of plant growth regulators supplemented in the culture medium. In particular auxins and cytokinins play a key role during organ initiation and development. Overall, a high auxin/cytokinin ratio favors the formation of roots whereas a low ratio induces the formation of shoots (Murashige and Skoog, 1962).

It is well known that high levels of auxin induce callus formation in some systems and roots in others (Mendoza and Kaeppler, 2002). As discussed previously, auxin might act as an inductive signal for the acquisition of organogenic competence, possibly by interacting with other culture components. Studies in soybean culture systems show that morphogenesis is indeed induced by an intimate interaction between auxin and carbohydrates (Lazzeri et al., 1988). The effects of auxin depend on the concentration applied and also on the type of auxin utilized (Paek and Hahn, 2000). For example, inclusions of IAA and auxin indole-3-butyric acid (IBA) in the culture medium encourage root formation in *Eustoma grandiflorum* while NAA induces the production of shoots (Paek and Hahn, 2000). Similarly, Sunandakumari et al. (2005) observed that while the combined application of BA and NAA induces shoot formation in *Euphorbia nivulia*, 2,4-D and BA promote somatic embryo formation. Shetty et al. (1992) observed that the endogenous level of BA could be increased by applications of ABA, which promoted direct shoot organogenesis in cucumber. This observation confirms the notion that *in vitro* morphogenesis is regulated by the interaction of different growth regulators.

Together with auxins, cytokinins also affect organogenesis by encouraging the formation of shoots. Jiang et al. (2005) reported that addition of 1 μ M TDZ in *Arnebia euchroma* (a medicinal plant) significantly increased the number of regenerated shoots

from cotyledon and hypocotyl explants. On the same line, applications of BAP increased the number of regenerated shoots in lentils (Omran et al., 2008). The inductive effects of cytokinins during shoot organogenesis are well characterized and this class of growth regulators is routinely used to propagate angiosperm and gymnosperm species in culture (Thorpe, 1982).

While auxins and cytokinins have a promotive effect on organogenesis, gibberellins (GAs) and ethylene exercise a repressive role. Early studies Murashige (1961) showed that applications of GA3 inhibit shoot formation in tobacco by lowering the respiratory rate, which is a key requirement for meristemoid initiation (Thorpe and Meier, 1972). However such inhibitory effects are only exercised during the early phase of organogenesis. Once meristemoids are formed, inclusions of GA3 in the medium encourage the formation of shoots (Jarret et al., 1981). Similarly to GAs, the accumulation of ethylene inhibits shoot formation in *Arabidopsis* (Tsuchisaka and Theologis, 2004). This was also demonstrated by Sethi et al. (1990) who documented the inhibition of shoot organogenesis in *Brassica oleracea* when *S*-adenosylmethionine and 1-aminocyclopropane-1-carboxylate (precursors of ethylene biosynthesis) were added in the medium. This inhibitory effect is species dependent, as in a few instances ethylene has been reported to induce shoot formation (Economou, 1990).

1.2.2.2.2. Genetic components of organogenesis

Like *in vitro* embryogenesis, organogenesis is characterized by major structural events which are governed by the activation or repression of several genes. However, due to the limited number of studies, which are mostly descriptive, this molecular

regulation is poorly understood. Phillips (2004) showed that during the initial phases of cellular de-differentiation and organogenic potential acquisition several genes are expressed and include SRD1, SRD2, SRD3, and CYCD3. While SRD2 is involved in the acquisition of root organogenic potential, SRD3 has been associated to the acquisition of shoot fate (Sugiyama, 1999). Microarray work conducted by Che et al. (2002) during *Arabidopsis* organogenesis indicated that 2-3% of the genes are differentially expressed during these initial phases. These genes include members of auxin signaling, thus confirming the role of this growth regulator during the de-differentiation step. A heavy change in gene expression pattern during the initial stages of *Populus* organogenesis was also observed by (Bao et al., 2009). In this study it was found that several F-box genes, as well as many members of auxin and cytokinin signaling were induced during the formation of callus. A similar result was described by (Su et al., 2007) in rice.

Following the acquisition of organogenic competence, the cells within the explants become committed to form organs. In the case of *Arabidopsis* shoot organogenesis from root explants, this step is characterized by the expression of several SAM-related genes including *STM*, *CLV1*, *CUC1* and *2* (Cary et al., 2002; Tahir and Stasolla, 2006). *CUP-SHAPED COTYLEDONS1* and *2* are expressed first followed by *WUS*, *STM* and *CLV1*.

Independent work (Cary et al., 2002; Gallois et al., 2002; Zhao et al., 2002) revealed that the expression of *WUS* is associated to the proliferation of un-differentiated cells, whereas *STM* is expressed once the meristemoids are fully formed. While the precise function of *CUC 1* and *2* during shoot organogenesis is unclear, transformation experiments suggest that they play a key role during the process. Ectopic expression of

either *CUC 1* or *CUC2* enhances shoot organogenesis in *Arabidopsis* roots only in the presence of cytokinins, suggesting that these genes cannot bypass the hormonal requirements (Daimon et al., 2003).

Aida et al. (1999) proposed that a function of the *CUC* genes during shoot organogenesis is to ensure the proper expression of *STM* once the meristemoids are formed. Additional studies revealed the importance of transcription factors during shoot initiation. Shoot formation in *Arabidopsis* was significantly enhanced by over-expressing *ENHANCER OF SHOOT DEVELOPMENT 1 (ESR1)*, a putative transcription factor with an AP2/EREBP domain (Banno et al., 2001).

The determination of the developmental fate during shoot organogenesis is controlled by the cytokinin signaling pathway (Phillips, 2004). Dissection of this pathway has identified several components, including the histidine protein kinases (AHKs), the putative cytokinin receptors, and histidine phosphotransfer proteins (AHPs) which transfer the signal from AHKs to nuclear response regulators (ARRs). In *Arabidopsis* two types of ARRs have been identified: A- and B-types, which induce different morphogenic responses. An overall up-regulation of the cytokinin signaling, elicited by the over-expression of the receptor, has been shown to induce shoot formation (Kakimoto, 1996). Changes in the expression of ARRs were also investigated. While ectopic over-expression of *ARR2*, a B-type response regulator in *Arabidopsis*, significantly increased shoot induction from root explants (Hwang and Sheen, 2001), the up-regulation of A-type ARRs decreased the shoot-forming ability (Hirose et al., 2007).

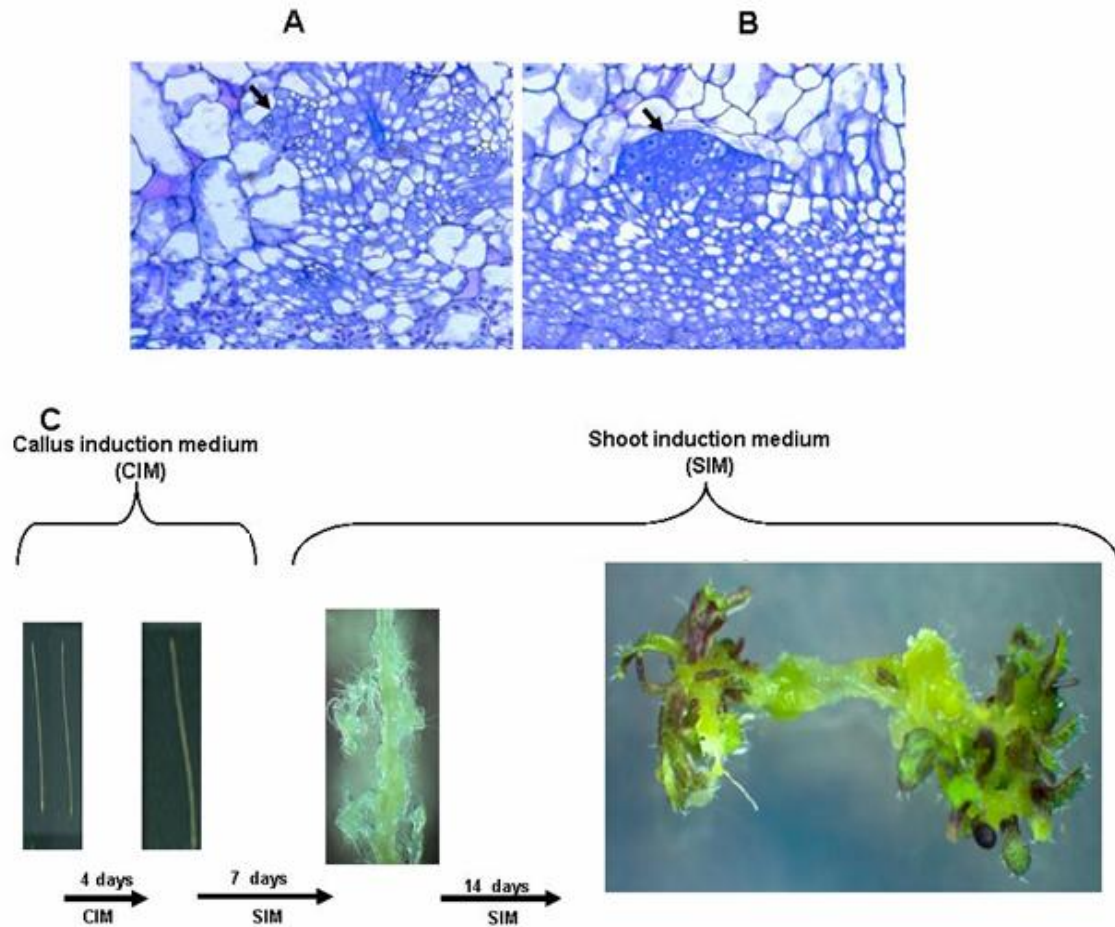


Fig. 1.7: *Arabidopsis* shoot organogenesis. Reactivation of cell division leads to the formation of meristemoids (**A**), which then develop into primordia (**B**). Formation of shoots (**C**) is initiated by culturing roots on a callus induction medium (CIM) for 4 days. Roots are then transferred onto a shoot induction medium (SIM) where callus and shoot formation is encouraged. Meristemoids and primordia are induced during the first week in shoot induction medium.

The late phases of shoot organogenesis involve the formation of functional shoots which exhibit a similar architecture to the SAMs produced *in vivo* and discussed previously.

1.2. Objectives of this study

Acquisition of organogenic or embryogenic potential in cultured cells is a key step during *in vitro* morphogenesis. As discussed above, competent cells within the explants must be able to respond to inductive signals, usually provided by specific culture addenda, and de-differentiate into meristematic cells. Such cells are then “canalized” into specific developmental pathways leading to the formation of organs or embryos, depending on the imposed culture conditions. Formation and maintenance of meristematic cells within the explants are therefore critical for ensuring successful plant regeneration.

The aim of this thesis is to investigate if altered expression of genes involved in the establishment and maintenance of meristematic cells within the SAM *in vivo* also affects the formation of meristematic cells *in vitro* and ultimately the production of organs and embryos.

My hypothesis is that positive regulators of SAM activity, i.e. *STM* and *ZLL*, which increase the domain of meristematic or undifferentiated cells in the apical meristem, have a positive effect on *in vitro* morphogenesis. Conversely, negative regulators of the SAM, such as *CLV1* which represses meristem proliferation, have an opposite effect. This hypothesis will be tested by ectopically expressing *Brassica* genes, orthologous to the *Arabidopsis* *STM*, *CLV1*, and *ZLL* in *Arabidopsis* (used as a “proof of

concept” system due to the amenable transformation procedure) in order to examine their effects on *in vitro* morphogenesis (embryogenesis and organogenesis). Gene(s) favoring the embryogenic process will be subsequently over-expressed or down-regulated via sense or antisense transformation in *B. napus* to verify if their function is retained during microspore-derived embryogenesis. Research conducted in this thesis is presented in several sections (chapters):

Chapter 2: This chapter deals with the isolation and characterization of the *Brassica* genes and the effects of their ectopic expression on *Arabidopsis* somatic embryogenesis and *Brassica* microspore-derived embryogenesis. The effects of the *Brassica STM* on global changes in transcript levels during embryo development are further assessed using microarray techniques.

Chapter 3: Investigations on the relationship between the inductive signal auxin (2,4-D) and the *Brassica STM* during *Arabidopsis* somatic embryogenesis are presented.

Chapter 4: The effects of the *Brassica* genes on *Arabidopsis* shoot organogenesis are studied. Molecular analyses will also define the relationship between the *Brassica* genes and cytokinin signaling.

Chapter 5: While the previous chapters deal with the effects of the *Brassica* genes during *in vitro* morphogenesis, this chapter illustrates the effects of ectopic expression of these genes during *in vivo* post-embryonic development.

**CHAPTER 2: MODULATION OF EMBRYO-FORMING CAPACITY IN
CUTURE THROUGH THE EXPRESSION OF *BRASSICA* GENES INVOLVED
IN THE REGULATION OF THE SHOOT APICAL MERISTEM**

2.1. ABSTRACT

Somatic embryogenesis in *Arabidopsis* is achieved by culturing bent-cotyledon embryos on a 2,4-D containing induction medium for 14 days followed by a transfer onto a hormone-free development medium. Several genes orthologous to the *Arabidopsis* *SHOOT MERISTEMLESS* (*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 over-expressing *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 over-expressing the *Brassica* *STM* was associated to a lower requirement for the inductive signal 2,4-D, and a higher expression of *WUSCHEL* which demarks 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 WT line and a *BoSTM* over-expressing 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 the *Brassica STM* also had profound effects on *B. napus* microspore-derived embryogenesis. The yield of microspore-derived embryos increased in lines over-expressing *BnSTM* and significantly decreased in antisense lines down-regulating *BnSTM*.

2.2. 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 *SHOOT MERISTEMLESS* (*STM*), *ZWILLE* (*ZLL*), and *CLAVATA 1* (*CLV1*). *SHOOT MERISTEMLESS* 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 of *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. 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 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 *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 on 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 to 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 towards *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 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; Mordhorst et al., 2002), very few deal with over-expression experiments (Zuo et al., 2002). The aim of the present work is to characterize

Brassica genes orthologous to the *Arabidopsis* *STM*, *ZLL*, and *CLV1*, and to investigate the effects of their ectopic expression on embryogenesis *in vitro*. Our results indicate that (1) somatic embryogenesis in *Arabidopsis* is affected by the introduction of the *Brassica* *STM* and *CLV1*, but not by the *Brassica* *ZLL*; (2) over-expressions of *BnSTM* and *BnCLV1* have antagonistic effects on *Arabidopsis* somatic embryo formation with *BnSTM* having a promotive role and *BnCLV1* a repressing role; (3) the increased *Arabidopsis* somatic embryo production in tissue ectopically expressing the *Brassica* *STM* entails profound changes in the expression of genes involved in different functions, and (4) besides improving *Arabidopsis* somatic embryogenesis (an indirect embryogenic system requiring a callus phase), the introduction of the *Brassica* *STM* also facilitates microspore-derived embryogenesis in *Brassica napus* (a direct embryogenic system without an intervening callus phase).

2.3. MATERIALS AND METHODS

2.3.1. Culture treatments

Production of *Brassica napus* cv Topas DH4079 microspore-derived embryos (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, and subsequently

transferred into dark at 24 C°. Fully developed microspore-derived embryos were observed after 25 days in culture (Belmonte et al., 2006).

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

2.3.2. Isolation of *BnSTM*, *BnCLV1*, *BnZLL-1*, and *BnZLL-2* and expression studies (*BnZLL1* and *BnZLL2* were isolated by Dr. Tahir and Mr. Khamiss)

Total RNA extracted from *Brassica napus* MDEs after 25 days in culture was utilized for cDNA synthesis using SuperScriptTM II Reverse Transcriptase kit (Invitrogen, Carisbad, CA). PCR-based amplification of full length *BnSTM* cDNA (Appendix 1, GU480584) was obtained using primers (5'-ATGGAAAAGTGGTTCCAACA-3' and 5'-ATCCGGGACAATGCTTTGA-3') designed from the sequence of *Brassica oleracea* *BoSTM* (AF193813) identified by Teo et al. (2001). *BnZLL-1* (EU329719) and *BnZLL-2* (Appendix 2, GU731230) were isolated using primers (5'-ATGCCGATTAGGCAAATGAA-3' and 5'-GAGTCATGTTTTACTGCTAA-3') based on the sequence of *Arabidopsis* *ZLL* (NM 123748). *B. napus* *BnCLV1* (Appendix 3, GU480585) was isolated by 5' and 3' RACE [First Choice RLM-RACE kit (Ambion, USA)] using a partial *CLV1* sequence (AY283519) from the same species. RACE was utilized to confirm the 3' and 5' ends of the cDNAs.

Expression studies of *AtSTM*, *AtCLV1*, and *AtZLL* in different tissue of 25 day old *Arabidopsis* plants and *BnSTM*, *BnCLV1*, *BnZLL-1* and *-2* in 40 day old *Brassica* plants were performed by semiquantitative RT-PCR. Briefly, total RNA was extracted using QIAGEN RNeasy[®] Plant mini Kit (Cat. # 74904) and used to generate cDNA with Invitrogen SuperScript[®] II Reverse Transcriptase (Cat. # 19064-002). For RT-PCR studies in *Brassica* the following primers were used: *BnSTM* (5'-TGATGGTCCGATGTGTCCTA-3' and 5'-GCACCAGAGGAAGGAGAACA-3'), *BnCLV1* (5'-CGGCGGGGACTATTCCTA-3' and 5'-AAGGAAGTGTCTGAAGACTAGG-3'), *BnZLL-1* (5'-CGACGACGACGCCGCTCAGCTCAGA-3' and 5'-CAGACTCTTTGTATAGTCTCACTA-3'), *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'-CTTAAATACCTCTCTTTTCGGTGGA-3' and 5'-ATCTCTATGCAAGATCAATGGTGA-3'), *AtZLL* (5'-CTGGTAAACGGGCAGATTGT-3' and 5'-TTTTTCTTTCCCGTTCTCGTGATAC-3'). As an internal control polyubiquitin10 (NM_178968) was amplified using the primers 5'-GATCTTTGCCGGAACAATTGGAGGATGGT-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). The fold change in the expression level was

calculated for each gene by normalizing its amplification intensity against that of UBQ10 or Actin.

2.3.3. 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). In brief, the plant tissues were fixed with 4% (W/V) freshly prepared paraformaldehyde in PBS (pH 7.4), then vacuum infiltrated for 15 min, and incubated on a rotary shaker for 3-h at room temperature. The samples were dehydrated in an ethanol series for 45 min at 4 °C and left over night in 100% ethanol. Xylene was added to the samples 3X with increasing concentrations. A few paraffin pellets were subsequently added to the samples and incubated overnight at 42 °C. Xylene was then replaced by molten paraffin at 60 °C. The blocks were made after 8 changes in paraffin. The paraffin-embedded plant tissues were sectioned at a thickness of 10 µm using disposable blades in a Leica (RM2145) microtome. The sections were deparaffinized by two changes of xylenes and re-hydrated.

Probe preparation was performed according to the following. The full length *BnSTM*, *BnZLL* and *BnCLV1* cDNA were amplified from the previously prepared clones using M13 forward and reverse primers. The T7 and SP6 activation sites (See pGEM®-Teasy Vector genetic map), were then used for *in vitro* transcription using digoxigenin-11-UTP, (Roche Applied Science) as described in the DIG-RNA labeling kit. Sense and antisense probes were hydrolyzed for the appropriate time at 60 °C in the presence of 60 mM Na₂CO₃ and 40 mM NaHCO₃ and stored at -80 °C until use.

Pre-hybridization and tissue treatment were done exactly as ascribed by Canton et al. (1999). The hybridization and color development procedures were conducted according to Tahir et al. (2006).

For histological examination, the tissue was fixed with 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.05 M phosphate buffer, pH 6.9, dehydrated with methyl cellosolve followed by two changes of absolute ethanol, and infiltrated and embedded in Histo-resin (Leica Canada, Toronto) according to Yeung (1999). Serial sectioning (3 µm) was performed by a Reichert-Jung 2040 Autocut rotary microtome. These sections were stained with periodic acid-Schiff (PAS) reaction for total carbohydrates, and counterstained with toluidine blue O (TBO) for general histological organization.

2.3.4. Transformation studies and *in vitro* regeneration

Arabidopsis transformation experiments were carried out using Gateway technology (Invitrogen, 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).

Full length *BoSTM*, *BrSTM*, *BnSTM*, *BnCLV1*, and *BnZLL-1* and *Bn-ZLL-2* cDNAs were inserted in the Gateway entry clone pDONR™221 (Invitrogen, USA) 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 *Arabidopsis* plants via standard *Agrobacterium*-

mediated transfection techniques (Weigel and Glazebrook, 2002). Many transformed plants were generated and the transcript level of the transgenes was measured by quantitative RT-PCR.

Brassica transformation with *BnSTM* in sense or antisense orientation was performed as described by Bhalla and Singh (2008) with some modifications. Briefly, surface sterilized seeds were germinated for 5 days on $\frac{1}{2}$ 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 2mg/L BA) for 4 days, and then incubated with the same *Agrobacterium* strain used for *Arabidopsis* transformation, harboring the pK2GW7 (for sense transformation) or pK2WG7 (for antisense transformation) 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 $\frac{1}{2}$ MS basal medium with 1% sucrose and 50 mg/l kanamycin and the resulting F1 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 F1 plants were cultured to produce haploid MDEs which were germinated on $\frac{1}{2}$ MS medium supplemented with 1% sucrose and 70 mg/l 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 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.

2.3.5. Microarray experiment

2.3.5.1. Microarray development: experimental design

Differences in global changes in transcript levels during somatic embryogenesis of wild type (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 our 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 three and nine days in development medium (stage 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 vs. 35S::*BoSTM* at stage 0, 1, 2, and 3), i.e. direct comparison analysis. A swapped-dye experiment (stage 1 vs. common reference) was also conducted to verify the effect of the dye on the hybridization outcome (Stasolla et al., 2008).

2.3.5.2. 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 was 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 in (<http://ag.arizona.edu/microarray/methods.html>). Briefly, after a pre-hybridization treatment in a buffer containing 5xSSC, 0.1%SDS, and 0.1 mg/ml BSA at 37°C for 1h, the slides were washed three times in 0.1xSSC at 22°C for 5 min and once in water at 22°C for 30 sec. The slides were dried by centrifugation at 1,000 x g for 2 min and stored until hybridization. The slides were then hybridized using 200 ng of Cy5-labeled target and 350 ng of Cy3-labeled target added to warm (42°C) hybridization solution containing 2% formamide, 5xSSC, 0.1%SDS, and 0.1mg/ml salmon sperm DNA. Hybridization was carried out for 16h at 42°C in a hybridization oven. Post-hybridization washes were performed by immersing the slides in a solution composed of 2xSSC, 0.1%SDS for 15 min at 37°C, followed by two washes in 1xSSC for 2 min at 22°C, and three washes in

0.1xSSC for 2 min at 22°C. The slides were dried by centrifugation and fluorescence levels of the Cy dyes were determined using a Axon GenePix 4000B laser scanner (MDS Analytical Technologies) with GenePix® Pro. Software Version 6.

2.3.5.3. Microarray data analysis (Performed by Dr. Gulden)

Differences in gene expression were determined using a mixed model approach developed by (Wolfinger et al., 2001) and used in our 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 vs. 35S::*BoSTM*), the data were analyzed as a one-way treatment structure. This reduced the impact of missing data points on the key comparisons that were required. Data were analyzed within each gene considering treatment as the fixed effect and array as the random effect. Within each analysis, ‘apriori’ expression differences were examined between 10 pairs of treatments. Differences were evaluated with four direct comparisons of WT vs. transformed tissue at each time point (WT vs. 35S::*BoSTM* at stage 0, 1, 2, and 3), and changes in gene expression over time were evaluated with three comparisons among successive time points (stage 0 vs. 1, 1 vs. 2, and 2 vs. 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 ≤ 0.01 and an absolute difference in expression ratios higher than 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).

2.3.5.4. Validation of microarray data

Microarray data were validated by quantitative real-time RT-PCR according to Stasolla et al. (2003) for several selected genes (Appendix 4, and 5) that showed differential expression patterns during embryo development. The relative level of gene expression was analyzed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001) using actin (AY139999) as a reference.

2.3.6. Glutathione metabolism

Measurements of glutathione content, and glutathione reductase activity were performed according to Zhang and Kirkham (1996). Briefly, 0.2 g the explants were

homogenized in 2ml cold 5 % meta-phosphoric acid. The homogenate was centrifuged at 22000g for 15 min at 4 C° and the supernatant was collected for glutathione, and glutathione reductase measurements. One ml of the supernatant was neutralized by 1.5 ml of 0.5 M phosphate buffer (pH 7.5), then 50 µl H₂O was added. This sample was used to assay the total glutathione (GSH + GSSG). Another 1 ml of the supernatant was neutralized by 1.5 ml of 0.5 M phosphate buffer (pH 7.5), then 50 µl 2-vinylpyridine was added to mask GSH, and the contents of the tube were mixed until an emulsion formed. The tube was then incubated for 60 min at room temperature. This sample was used for GSSG estimation. GSH was assayed as the difference between total glutathione and GSSG. Glutathione content was measured in a 3 ml reaction mixture containing 0.2 mM NADPH, 100 mM phosphate buffer (pH 7.5), 5 mM EDTA, 0.6 mM DTNB, and 3 units of glutathione reductase (GR). The reaction was started by adding 0.1 ml of extract sample previously obtained. The reaction rate was monitored by measuring the change in absorbance at 412 nm for 1 min. A standard curve was developed based on GSH in the range 0-50 µM/ml.

For GR assay, 0.3 g of tissue was homogenized in 4 ml ice-cold 25 mM phosphate buffer (pH 7.5) containing 1% PVP and 0.2mM EDTA. The homogenate was filtered first and centrifuged at 18000g. The supernatant was collected and used for determination of GR activity. The activity of GR was measured at 340 nm for 1 min. once 1mM GSSG was added to the reaction mixture [1mM EDTA, 2mM NADPH, 0.2mM dehydroascorbic acid, 50 mM Na-phosphate buffer (pH 7.8), and 0.5 ml of crude extract]. Glutathione applications were exactly performed as described by Belmonte et al. (2006).

2.3.7. Methylation assays

DNA methylation was estimated using the Coupled Restriction Enzyme Digestion and Random Amplification (CRED-RA), as reported by Leljak-Levani et al. (2004). Briefly, the DNA extracted from WT and 35S::*BoSTM* lines at stage 1 of somatic embryogenesis was digested with *HpaII* and *MspI* endonucleases (which cut the sequence 5'-C/CGG-3' with different sensitivity to cytosine methylation; *MspI* cuts if the inner C is methylated, whereas *HpaII* cannot cleave in the presence of methyl groups), and then used as a template, in a PCR reaction 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 (primer5), and 5-CAACCGGTCT-3 (primer 6). The PCR reactions were preformed in a total volume 25 µl using: 1 µl (50 ng) digested DNA, 1 µl 10nM primer, 0.5 µl 10 nM MgCl₂, 11.25 µl nuclease free H₂O, and 11.25 µl 2XBioMix master mix (GE Healthcare UK Limited, Amersham, UK). The PCR conditions and measurements of the methylated bands were carried out exactly as described by Cai et al. (1996) and Leljak-Levani et al. (2004).

2.3.8. Statistical analysis

Tukey's post-hoc test for multiple variance (Zar, 1974) was used to compare differences between treatments and control.

2.4. RESULTS

2.4.1. Isolation and characterization of *Brassica napus* *BnSTM*, *BnCLV1*, *BnZLL-1*, and *B-ZLL2*

Using the available nucleotide sequence of STM, CLV1 and ZLL from *Arabidopsis* and other *Brassica* species, full length *Brassica napus* *BnSTM*, *BnCLV1*, and *BnZLL-1* and -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* orthologs (91% for *BnSTM*, 88% for *BnCLV1*, and 96% for *BnZLL-1* and -2) and with all the conserved domains. These include the KNOX1, KNOX2, ELK, and a Homeodomain for *BnSTM*, a serine/threonine kinase domain for *BnCLV1*, and the PIWI and PAZ domains for *BnZLL-1* and -2 (Fig. 2.1). Phylogenetic analyses further confirm the similarities of the *Brassica* genes with their *Arabidopsis* orthologs (Fig. 2.2).

The relative expression of the *Brassica napus* genes in *B. napus* plants was measured in different tissues and compared to the expression pattern of the *Arabidopsis* orthologs measured in *Arabidopsis* plants (Fig. 2.3). 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* orthologs were also expressed (Fig. 2.3). Both *BnSTM* and *BnCLV1* were expressed in the SAM of *B. napus* zygotic and MDEs (Fig. 2.4). The combined signal of *BnZLL-1* and -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 embryos. A similar localization pattern to that

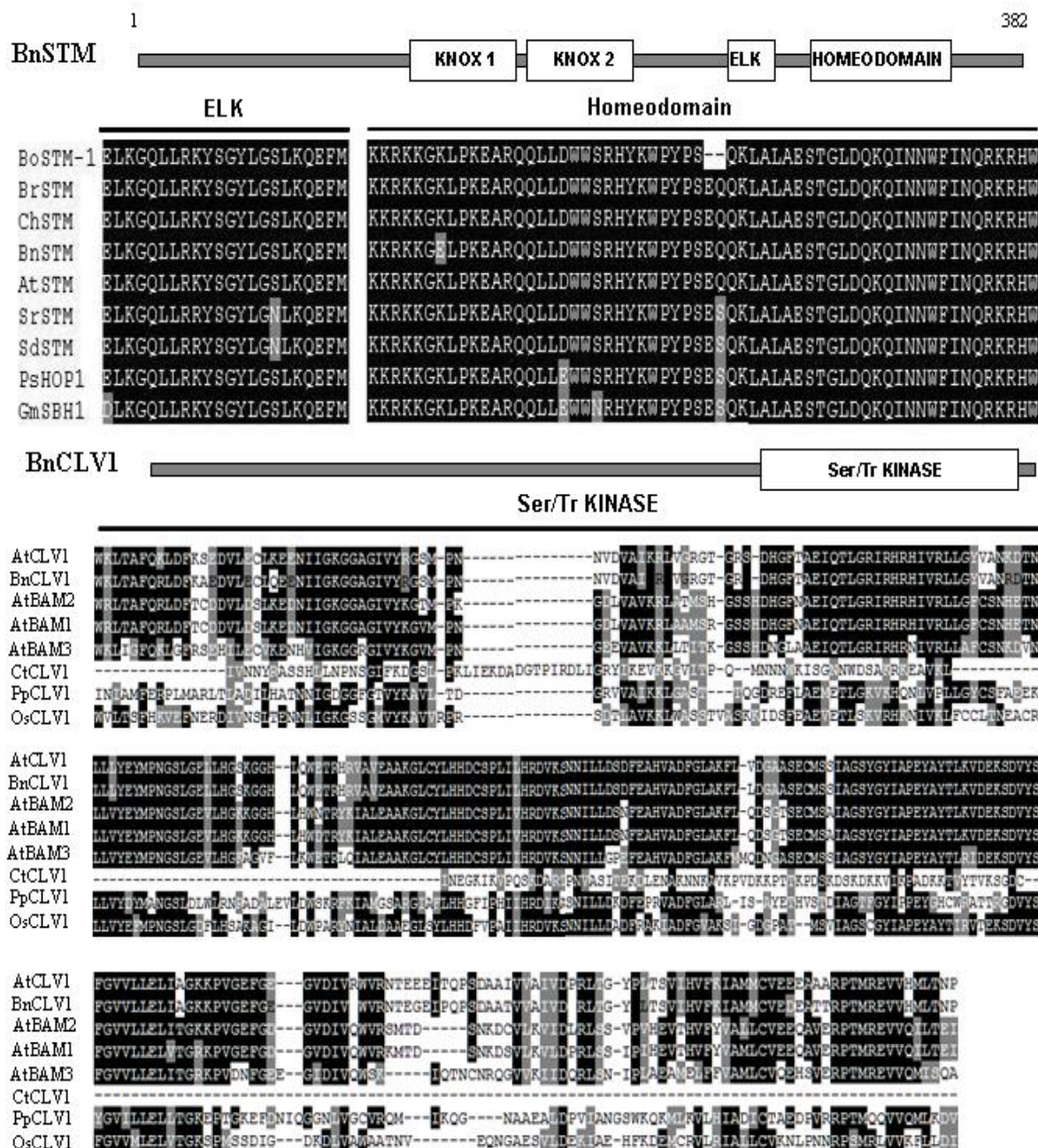
observed for zygotic embryos was also observed in MDEs (Fig. 2.4). The expression domains of the *Brassica* genes in *B. napus* zygotic and microspore-derived embryos coincided perfectly with those described for the *Arabidopsis* orthologs, which are also expressed in the SAM of *Arabidopsis* embryos (reviewed by (Dodsworth, 2009).

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

Given the very low efficiency of *Brassica 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 *Brassica 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 (Fig. 2.5) and utilized to produce somatic embryos by first culturing bent-cotyledon zygotic embryos on a 2,4-D enriched induction medium for 14 days and then transferring them on a hormone-free development medium (Fig. 2.6 A).

The number of explants (bent-cotyledon zygotic embryos) producing somatic embryos doubled in three *35S::BnSTM* lines (2-4) analysed (Fig. 2.6 B). This prompted us to ascertain whether these results could be replicated, and possibly further improved, by over-expressing the *STM* orthologs from *B. oleracea* (AF193813) and *B. rapa* (GU480585) (denoted as *BoSTM* and *BrSTM* respectively), which are the progenitor species of *B. napus*.



C

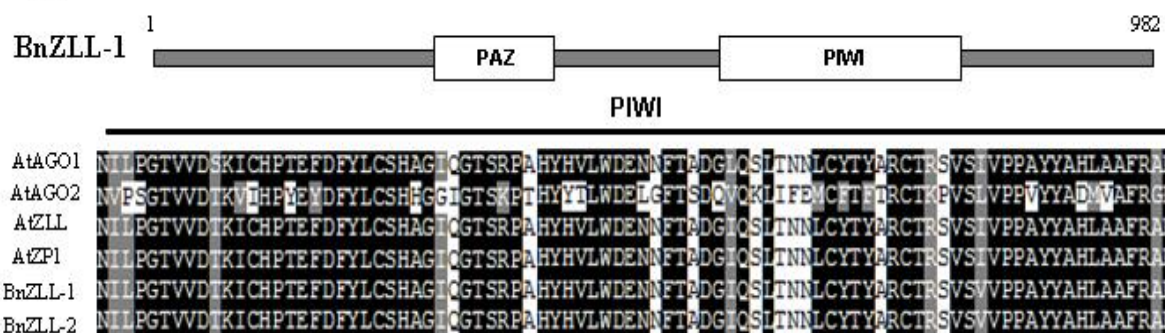


Figure 2.1. Schematic representation of protein structure and amino acid sequence alignments of conserved domains of *BnSTM*, *BnCLV1*, and *BnZLL-1*. High levels of identity with domains of related proteins of other species are shown for the ELK and homeodomain regions of *BnSTM* **(A)**, for the serine/threonine kinase domain of *BnCLV1* **(B)**, and for the PIWI domain of *BnZLL-1* **(C)**. The structure and the PIWI domain of *BnZLL-2* is identical to those shown for *BnZLL-1*. *Brassica oleracea* *BoSTM* (AAM89270); *Brassica rapa* *BrSTM* (ADD64788); *Cardamine hirsute* *ChSTM* (ABF59514); *Brassica napus* *BnSTM* (ADD64787); *Arabidopsis Thaliana* *AtSTM* (NP_176426); *Streptocarpus rexii* *SrSTM* (AAW33774); *Streptocarpus dunnii* *SdSTM* (AAW33773); *Pisum sativum* *PsHOP1* (AAC33008); *Glycine max* *GmSBH1* (AAA20882); *Arabidopsis Thaliana* *AtCLV1* (NP_177710); *Brassica napu* *BnCLV1* (ADD64789); *Arabidopsis Thaliana* *AtBAM2* (NP_190536); *Arabidopsis Thaliana* *AtBAM1* (NP_201371); *Arabidopsis Thaliana* *AtBAM3* (NP_193760); *Clostridium tetani* *CtCLV1* (NP_782560); *Physcomitrella patens* *PpCLV1* (XP001763746); *Oryza sativa* *OsCLV1* (NP_001062883); *Arabidopsis Thaliana* *AtAGO1* (NP_175274); *Arabidopsis Thaliana* *AtAGO2* (NP_174413); *Arabidopsis Thaliana* *AtZLL* (NP_199194); *Arabidopsis Thaliana* *AtZP1* (NP_199194); *Brassica napus* *BnZLL1* (ABY52943); *Brassica napus* *BnZLL2* (ADE06082). Multiple alignment analyses were conducted using T-Coffee/ClustalW2 (<http://www.ebi.ac.uk/Tools/t-coffee/>)

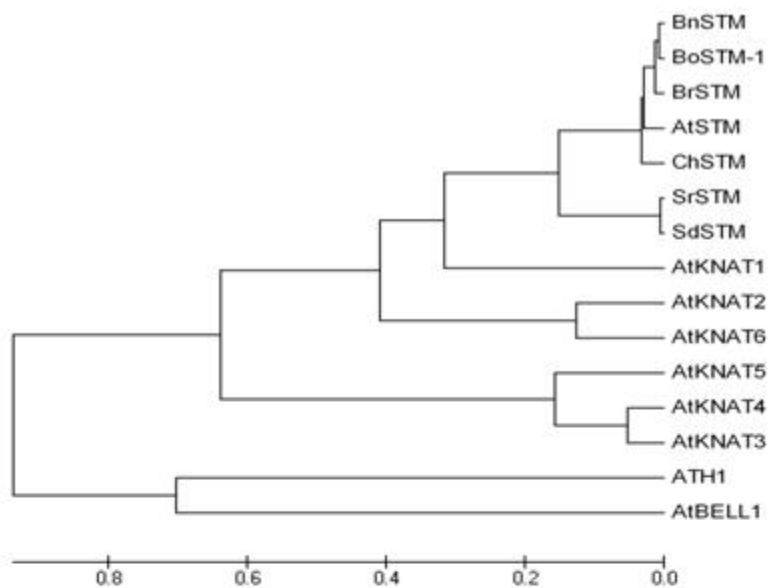
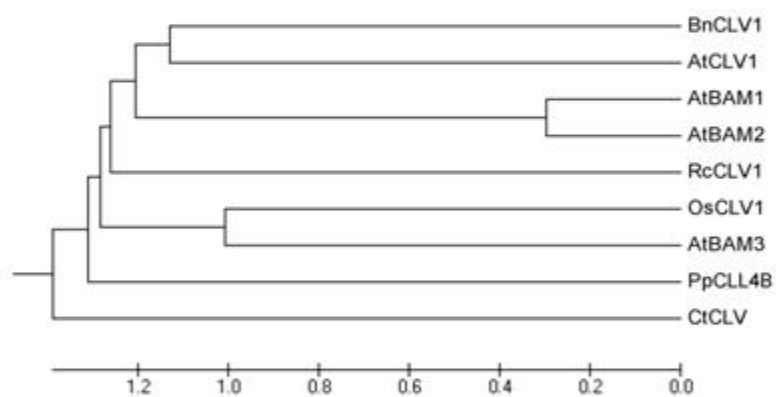
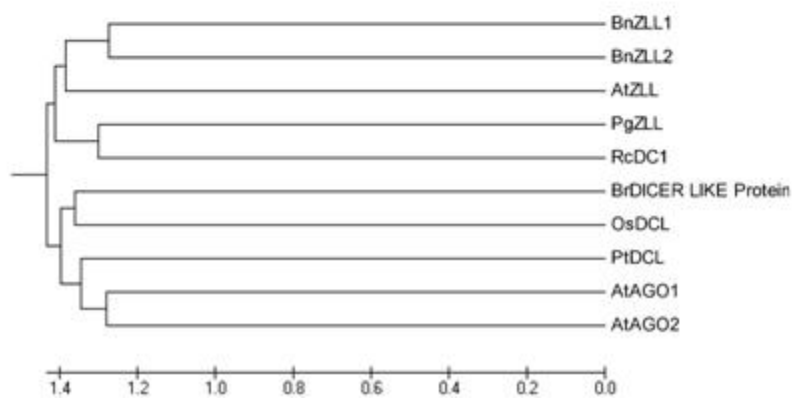
A**B****C**

Figure 2.2. Phylogenetic trees of several class-1 KNOX proteins **(A)**, CLAVATA 1-related proteins **(B)**, and ZLL-like proteins **(C)**. Phylogenetic relationship was based on the analyses of amino acid sequences of full length proteins. *Brassica napus* BnSTM (ADD64787); *Brassica oleracea* BoSTM-1 (AAM89270); *Brassica rapa* BrSTM (ADD64788); *Arabidopsis Thaliana* AtSTM (NP_176426); *Cardamine hirsute* ChSTM (ABF59514); *Streptocarpus rexii* SrSTM (AAW33774); *Streptocarpus dunnii* SdSTM (AAW33773); *Arabidopsis Thaliana* Homeobox protein knotted-1-like1 AtKNAT1 (NP_192555); *Arabidopsis Thaliana* Homeobox protein knotted-1-like2 AtKNAT2 (NP_177208); *Arabidopsis Thaliana* Homeobox protein knotted-1-like 6 AtKNAT6 (NP_173752); *Arabidopsis Thaliana* Homeobox protein knotted-1-like5 AtKNAT5 (NP_194932); *Arabidopsis Thaliana* Homeobox protein KONTTED-1 Like 4 (KNAT4) (CAC03454); *Arabidopsis Thaliana* Homeobox protein knotted-1-like3 AtKNAT3 (NP_001031938); *Arabidopsis Thaliana* Homeobox1 AtH1 (NP_195024); *Arabidopsis Thaliana* BEL1-like homeodomain 1 AtBELL1 (AAK43836); *Brassica napus* BnCLV1 (ADD64789); *Arabidopsis Thaliana* CLAVATA1 protein AtCLV1 (NP_177710); *Arabidopsis Thaliana* BARELY ANY MERISTEM1 protein AtBAM1 (NP_201371); *Arabidopsis Thaliana* BARELY ANY MERISTEM2 protein AtBAM2 (NP_190536); *Ricinus communis* CLV1 receptor protein kinase RcCLV1 (XP_002517850); *Oryza sativa* (Japonica) CLV1 receptor kinase-like protein OsCLV1 (BAD23458); *Arabidopsis Thaliana* BARELY ANY MERISTEM3 protein AtBAM3 (NP_193760); *Physcomitrella patens* CLAVATA1-like receptor S/T protein kinase protein PpCLL4B (XP_001763746); *Clostridium tetani* CLV1 receptor kinase protein CtCLV1 (NP_782560); *Brassica napus* ZWILLE 1 protein BnZLL1 (ABY52943); *Brassica napus* ZWILLE 2 protein BnZLL2 (ADE06082); *Arabidopsis Thaliana* ZWILLE protein AtZLL (CAA11429); *Picea glauca* ZWILLE protein PgZLL (AAY67884); *Ricinus communis* DICER1 protein RcDC1 (XP_002515097); *Brassica rapa* BrDICER-like protein (ACE60552); *Oryza sativa* DICER LIKE protein OsDCL (ABB20894); *Populus trichocarpa* DICER LIKE protein PtDCL (XP_002308384); *Arabidopsis Thaliana* ARGONAUTE1 protein AtAGO1 (ACB30738); *Arabidopsis Thaliana* ARGONAUTE2 protein AtAGO2 (ACB30762). The phylogenetic trees were constructed with the UPGEMA method using MEGA 4.0.2 software (Tamura et al., 2007).

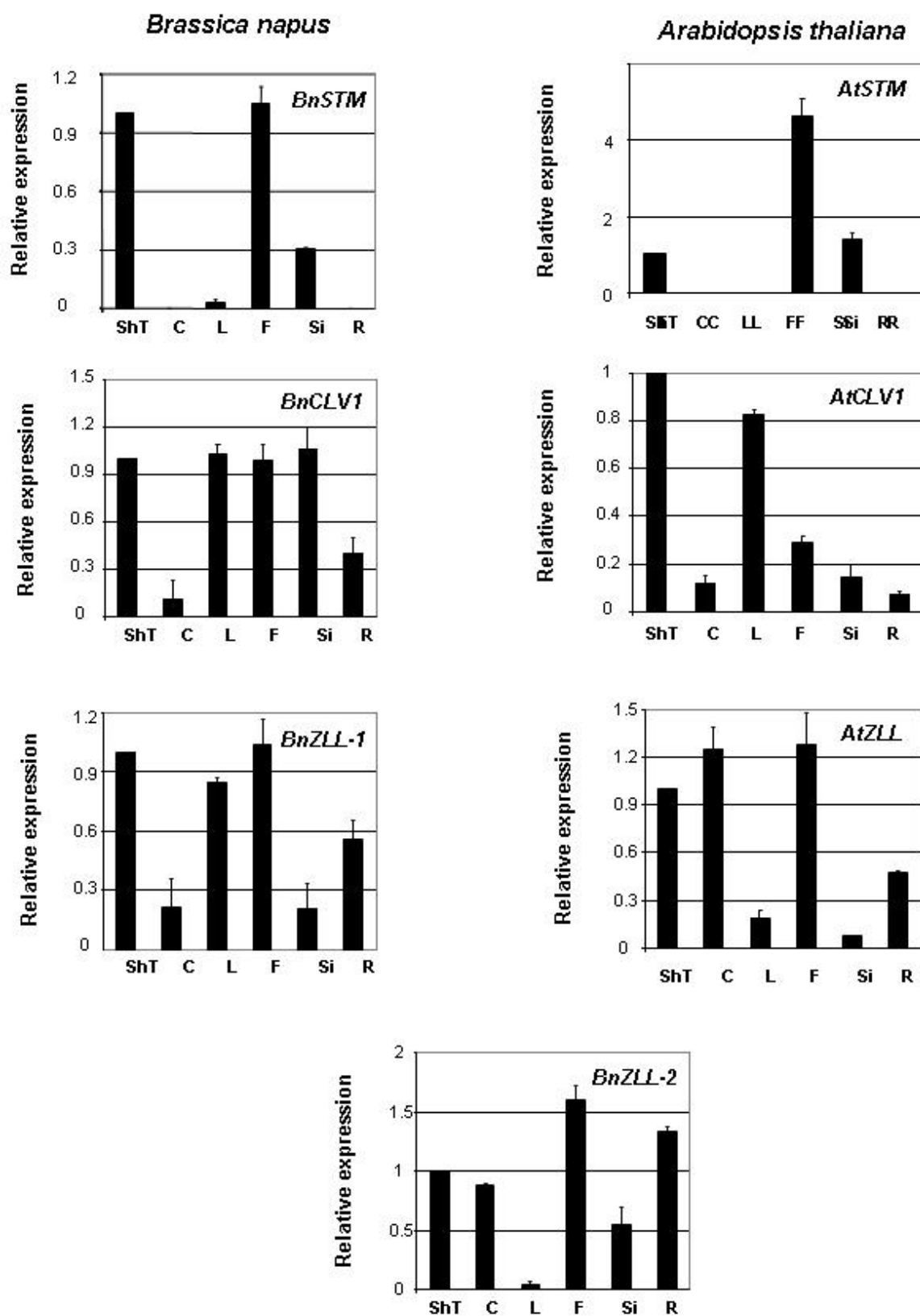


Figure 2.3. Expression studies by semiquantitative RT-PCR of *BnSTM*, *BnCLV1*, *BnZLL-1* and -2 in *B. napus* tissues and *AtSTM*, *AtCLV1* and *AtZLL* in *Arabidopsis* tissues. Shoot tips (ShT), cotyledons (C), leaves (L), flowers (F), siliques (Si), and roots (R) harvested from *Brassica napus* (40 day old plants, with the exception of C harvested from 10 day old plants) and *Arabidopsis* (28 day old plants with the exception of C harvested from 10 day old plants). Values $\pm$ SE (n=3) were normalized to the expression level in ST set at 1.

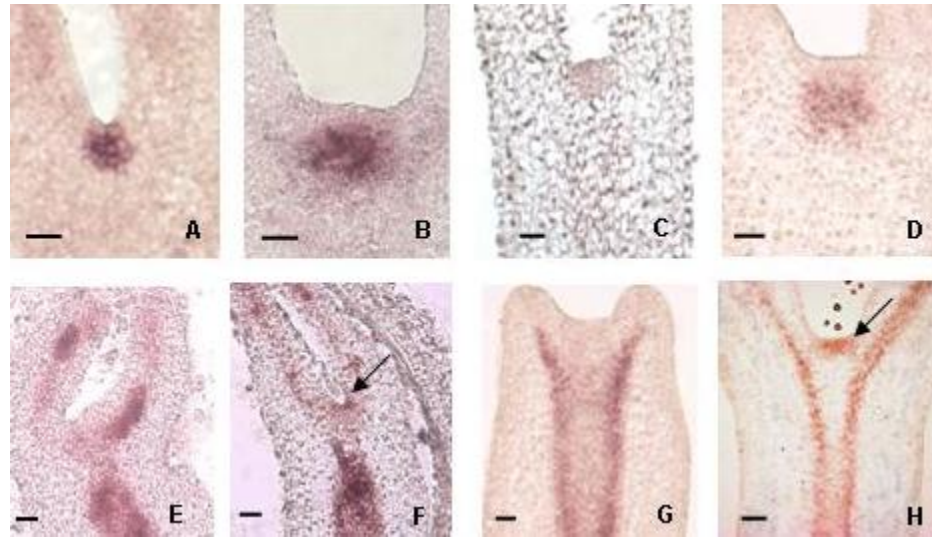


Figure 2.4. RNA-in situ hybridization of *BnSTM*, *BnCLV1*, *BnZLL-1* and -2 in *Brassica napus* zygotic embryos at the late cotyledonary stage (**A**, **C**, and **F**) or at the middle cotyledonary stage (**E**) of development, and in microspore-derived embryos (MDEs) cultured for 15 days (**G**) or 25 days (**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 *BnZLL-1* and -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 day old MDE (**G**), and then extended to the apical regions (arrows) of late cotyledonary zygotic embryos (**F**) and 25 day old MDEs (**H**). All scale bars = 40 μm .

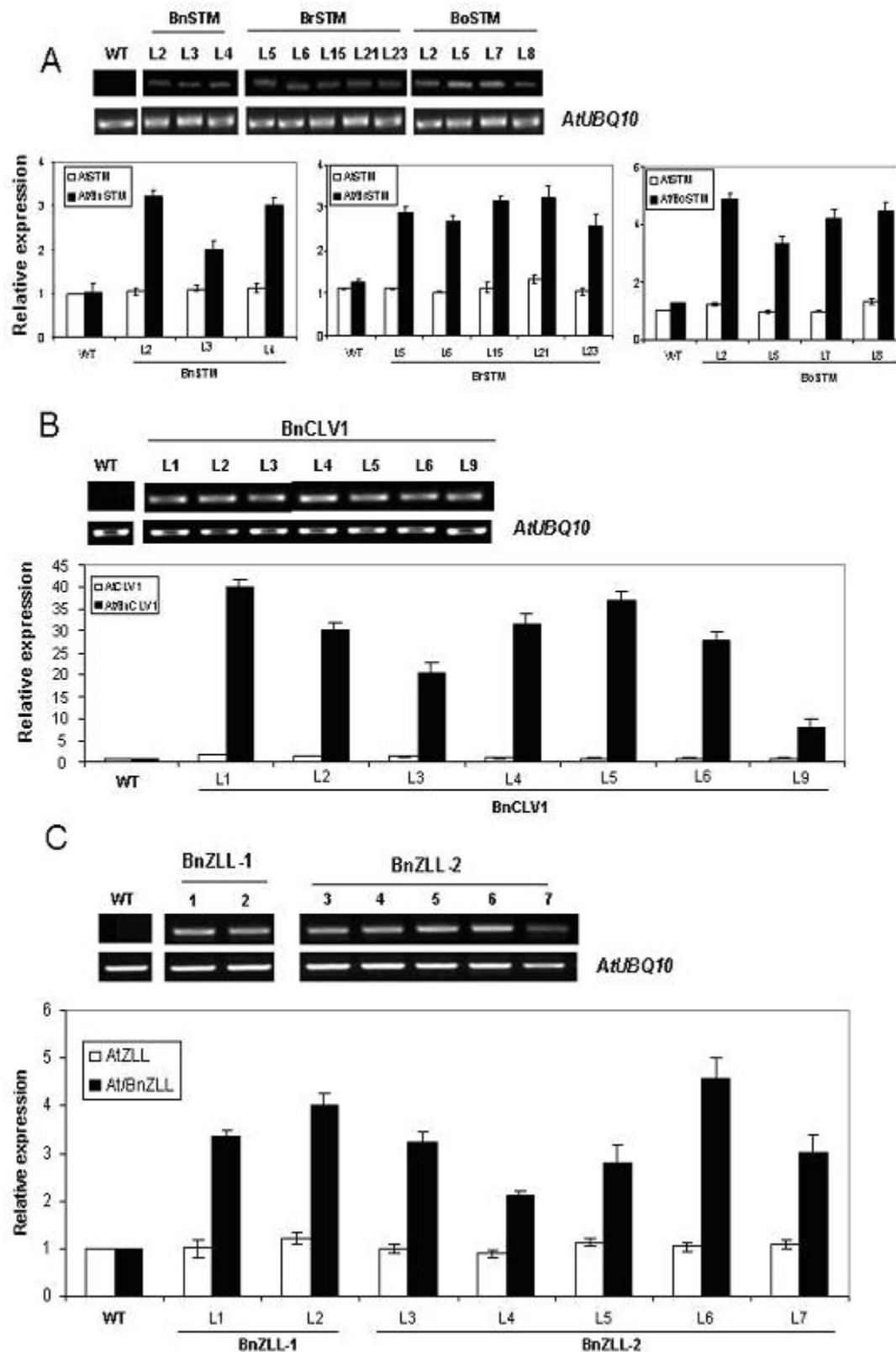


Figure 2.5. Identification of *Arabidopsis* lines ectopically expressing the *Brassica* genes. Expression studies by quantitative RT-PCR were conducted using 10 day old seedlings. **(A)** Identification of lines expressing *Brassica napus* *BnSTM* (line 2, 3, and 4), *B. rapa* *BrSTM* (line 5, 6, 15, 21, and 23) and *B. oleracea* *BoSTM* (line 2, 5, 7, and 8). Top panel: Insertion of the transgene in the genomic DNA was verified by PCR using a forward 35S promoter primer (5'-TGGACCCCCACCCACGAG-3') and a reverse gene specific primer common for the three genes (5'-GCACCAGAGGAAGGAGAACA-3'). Ubiquitin (*AtUB10*) primers (5'-GATCTTTGCCGAAAACAATTGGAGGATGGT-3' and 5'-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3') were used to assess the quality of the DNA for PCR. Bottom panels: the level of endogenous *AtSTM* (5'-TAGATCAAAAAAGCTTTA-3' and 5'-CATTACTTCATGGATAATGTCTTGGG-3') and endogenous + exogenous, i.e. *AtSMT* + the relative *Brassica STM* (5'-TGATGGTCCGATGTGTCCTA-3' and 5'-GCACCAGAGGAAGGAGAACA-3') measured by quantitative qRT-PCR. **(B)** Identification of 7 lines ectopically expressing *BnCLV1* (line 1, 2, 3, 4, 5, 6, and 9) using the same approach as in (A). *BnCLV1* specific primers used for the amplification of DNA: (5'-TCTCCTTCAACGACCTCTCG-3' and 5'-CTGGTGAAGATAACACAGTCCTTTTCG-3'), and for the qRT-PCR analysis: *AtCLV1* (5'-GTAAAATTTCTCTATTACAAATGATAA-3' and 5'-AAGATGAGTCTTCAAAAGTCTCATCGC-3') and endogenous + exogenous, i.e. *AtCLV1* + *Brassica CLV1* (5'-TCTCCTTCAACGACCTCTCG-3' and 5'-AGACGATCCCCGCTCCGCCTTTG-3'). **(C)** Identification of lines over-expressing *BnZLL-1* (line 1 and 2) or *BnZLL-2* (line 3, 4, 5, 6, and 7) using the same approach as in (A). Specific *BnZLL* primers used for the amplification of DNA (5'-AACTCCAACCTTGCGCCGAGA-3' and 5'-TTTTCTTTCCCATTCATGATAC-3'), and for the qRT-PCR analysis: *AtZLL* (5'-CTTCTCTAGCGTCACTCTTCTTCTT-3' and 5'-TCACCGGAGAAGGAGAAGGAGGA-3'), and endogenous + exogenous (*AtZLL+BnZLL-1*) or -2(*BnZLL-1*, 5'-GACGACGACGGCGGCAGCTCAGAGCC-3' and 5'-CAGACTCTTTGTATAGTCTCACTA-3'; *BnZLL-2*, 5'-ACTCCGAGCCAAGCTTCTTCAC-3' and 5'-CAGACTCTTTGTATAGTCTCACTA-3'). Values $\pm$ SE ($n=3$) were compared to the expression level of endogenous *At* gene in WT plants set at 1. The relative level of gene expression was analyzed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001).

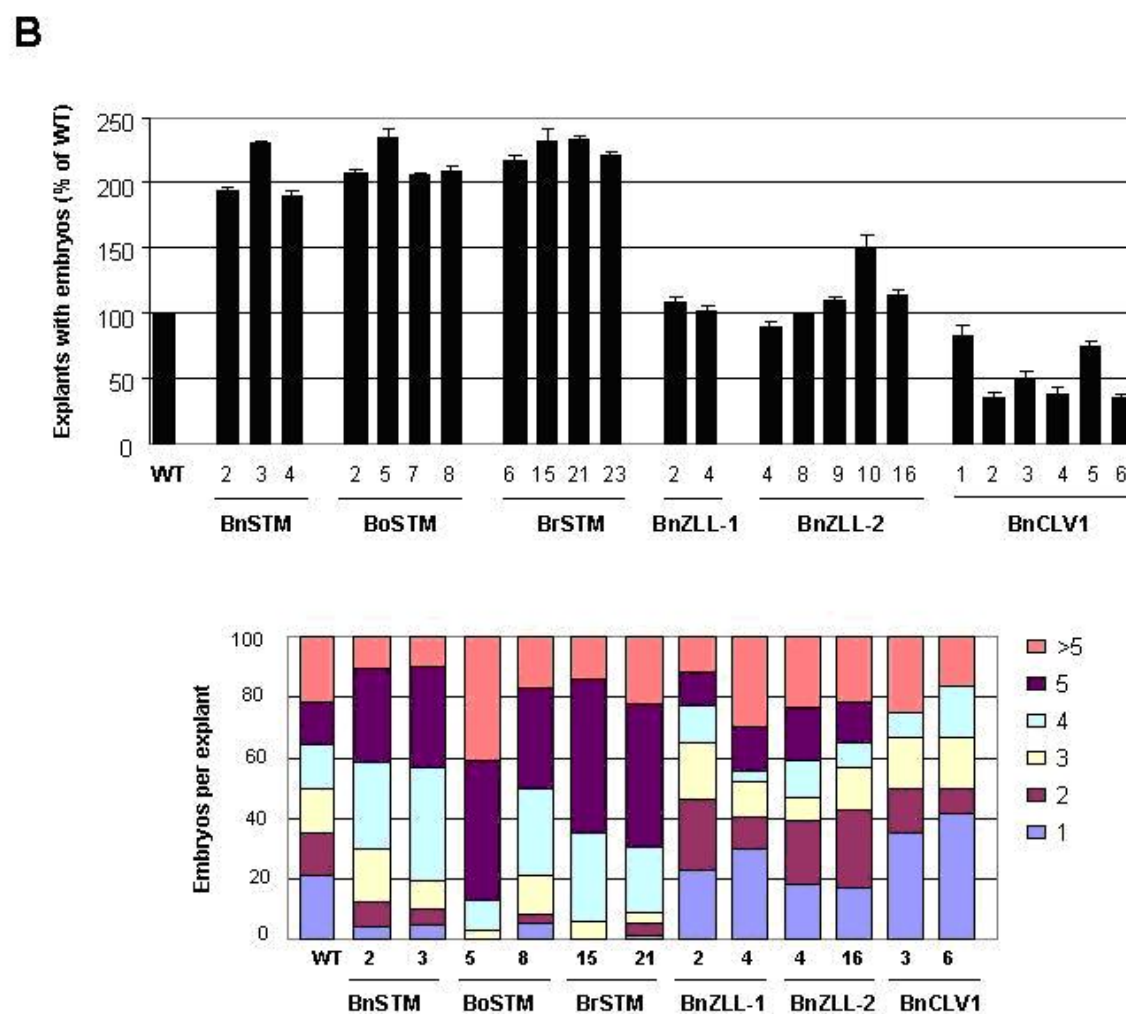
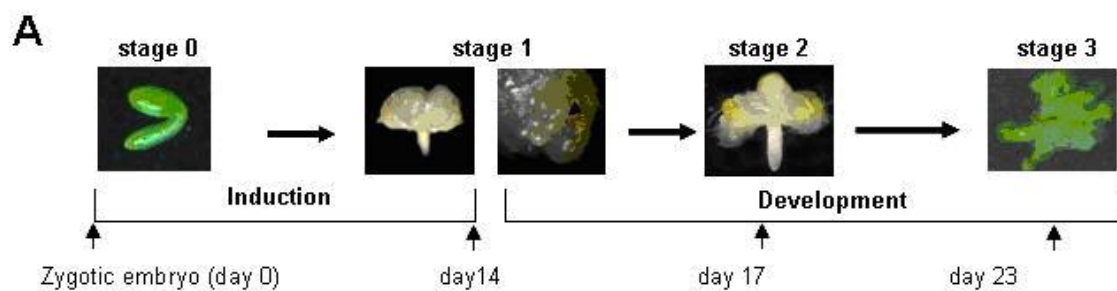


Figure 2.6. (A) Schematic representation of the somatic embryogenic process in *Arabidopsis* and stages, with relative time points, used for the microarray experiment. Bent-cotyledon zygotic embryos were cultured on induction medium for 14 days and then transfer on development medium. Embryogenic cells 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)** 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 $\pm$ SE are averages of at least three independent experiments. WT total explants in each replicate were 12. The average number of somatic embryos that were produced by WT was 2.35 somatic embryos/explant. The number of somatic embryos was counted at day 23 of the culture.

The two cDNAs were isolated using a PCR-based approach followed by RACE. Protein sequence alignment revealed a high degree of identity between *BnSTM* and the two *Brassica* orthologs (97% for *BrSTM*, and 99% for *BoSTM*). Ectopic expression of both *BoSTM* and *BrSTM* (Fig. 2.5) increased the response of the explants to the culture conditions (Fig. 2.6B).

The number of embryo-forming explants in the *35S::BnZLL-1* and 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. 2.6 B).

Arabidopsis somatic embryo composition was further examined in all transformed plants and results of representative lines are shown in Figure 2.6B. The number of embryos produced by each responsive explant increased in all lines over-expressing the *Brassica* (*Bo*, *Bn*, and *Br*) *STM*, especially in line 5 (*35S::BoSTM*) in which almost 90% of the responsive explants generated 5 or more embryos. No appreciable differences in embryo composition were observed between the *35S::BnZLL-1* and -2 lines and the WT counterpart, whereas the number of explants producing only one somatic embryo almost doubled in all *35S::BnCLV1*.

2.4.3. Effects of *BnSTM* over-expression or down-regulation on *B. napus* microspore-derived embryogenesis

To evaluate if the beneficial effects of *Brassica STM* over-expression were only restricted to embryogenic systems with an intervening callus phase, such as *Arabidopsis* somatic embryogenesis, we induced microspore-derived embryogenesis in *Brassica*

napus lines over-expressing or down-regulating *BnSTM* (Fig. 2.7). In this system microspores develop directly into embryos (microspore-derived embryos, MDEs) without the formation of callus. Differentiation of microspores into MDEs increased significantly in sense lines over-expressing *BnSTM*, whereas an inhibition in embryo forming capacity was observed in antisense lines with reduced *BnSTM* levels (Fig. 2.8). The down-regulation of *BnSTM* resulted in profound morphological abnormalities (Fig. 2.9). 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 over-expressing *BnSTM* (Fig. 2.9).

2.4.4. 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* over-expressing line showing the most pronounced enhancement of somatic embryogenesis, i.e. *35S::BoSTM* (line 5). This comparison would allow us to identify genes responsive to the introduction of the *Brassica STM* and important to the formation of somatic embryos.

In the WT line, 885 probes were differentially expressed (fold change > 4 and $p \leq 0.01$) 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 probe being differentially expressed during the first two stage comparisons (1334 between stage 0 and 1 and 1032 between stage 1 and 2).

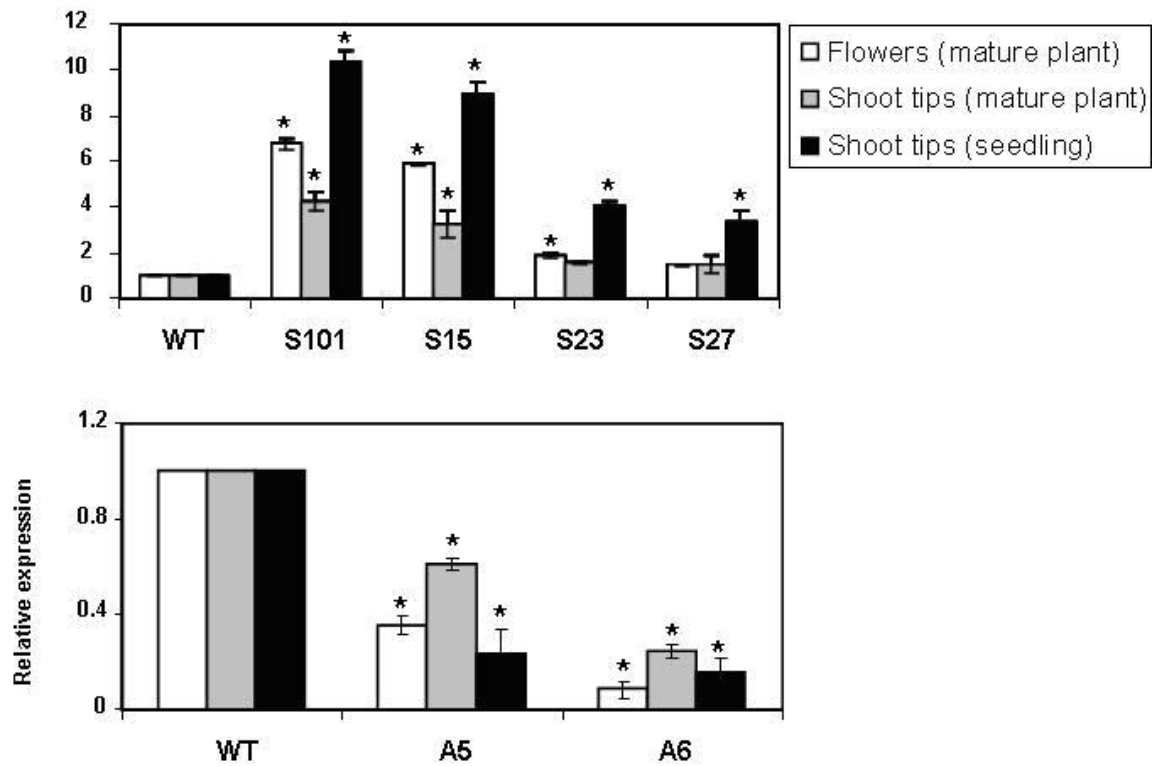


Figure 2.7. Identification of *Brassica napus* lines transformed with *BnSTM* in sense (S, line 101, 15, 23, and 27) or antisense (A, line 5 and 6) orientation. The expression level was measured in different organs of seedlings (15 day old) or mature (40 day old) plants. For mature plants fully open flowers and 3 cm long siliques were used. Expression studies were conducted by quantitative RT-PCR using the following primers (5'-TGATGGTCCGATGTGTCCTA-3' and 5'-GCACCAGAGGAAGGAGAACA-3'). Actin (5'-TAAAGTATCCGATTGAGCATGGTAT-3' and 5'-GACATTAAAGAGAAGCTTGCTTACG-3') was used as the internal control. Values $\pm$ SE, which are means of three independent experiments, were compared to the expression of the endogenous *BnSTM* in the respective WT organ. The relative level of gene expression was analyzed with the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (2001).

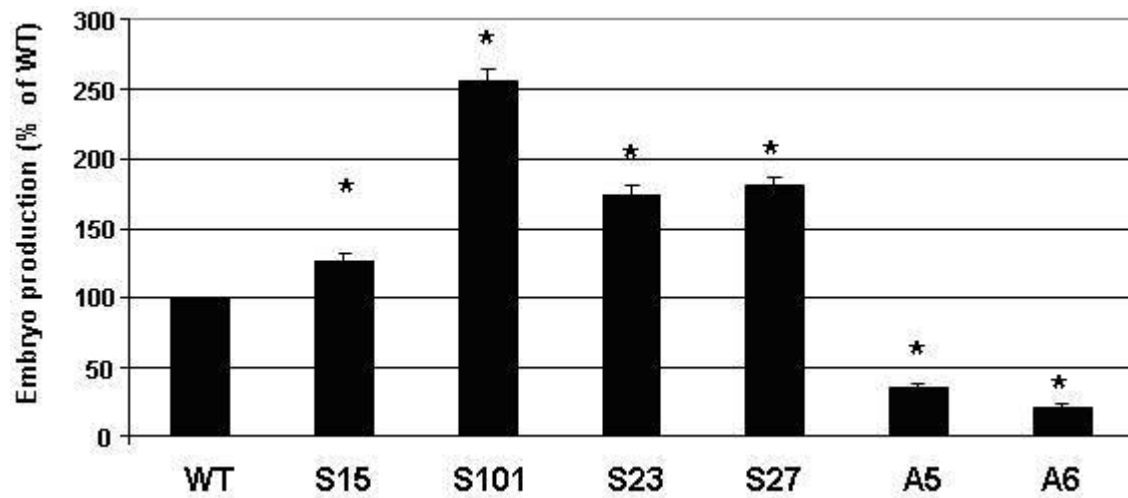


Figure 2.8. Production of microspore-derived embryos (MDEs) from *Brassica napus* plants with altered BnSTM expression. Antisense lines (A, line 5 and 6) with lower BnSTM expression or sense line (S, line 15, 101, 23, and 27) with increased BnSTM expression were utilized to generate MDEs. For each experiment 40,000 microspores were plated and embryo number was counted after 25 days in culture. Values $\pm$ SE (n=3) are expressed as a percentage of WT. Stars above each bar indicate values which are significantly different from the WT value ($P \leq 0.05$).

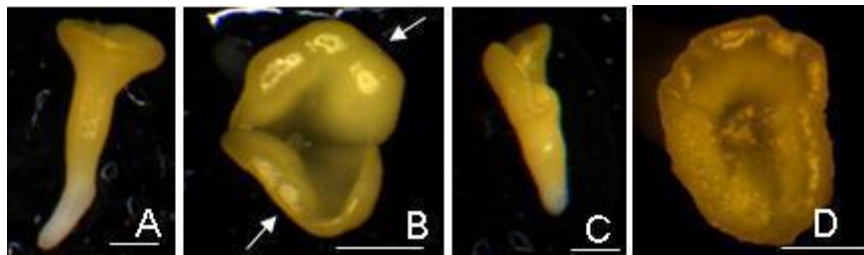


Figure 2.9. Morphology of microspore-derived embryos (MDEs) produced by *B. napus* lines with altered *BnSTM* expression. Microspore-derived embryos were collected after 25 days in culture. MDEs obtained from the WT line **(A)** were characterized by two distinct cotyledons (arrows) **(B)**. MDEs produced 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 over-expressing *BnSTM*. All scale bars = 0.3 mm.

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 2.1. Many probes implicated in hormone synthesis and signaling showed different behavior, whereas probes involved in meristem function (*STM*, *WUSCHEL*, *PINHEAD/ZWILLE*, *AGO1*, and *FIDDLEHEAD*) were induced only in the *35S::BoSTM* line between stage 0 and 1. Differences between WT and *BoSTM* over-expressing lines were also observed for glutathione reductase (GR) and glutathione synthase (GSH2), both repressed in the transformed line during the first days in development (stage 1 vs. 2; Table 2.1). Two probes encoding DNA (cytosine-5)-methyltransferases were also repressed in the *35S::BoSTM* line during the induction phase (stage 0 vs. 1; Table 2.1).

A direct comparison analysis was also performed to identify differentially expressed probes between *35S::BoSTM* and WT 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 TCA cycle were upregulated in the *35S::BoSTM* line (Fig. 2.10A). 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 *WUSCHEL* and *REVOLUTA* (HB 6 and 7) (Fig. 2.10B). Different profiles were detected for probes clustered in the categories of disease and defense (D&D), metabolism (M), and hormonal control. A probe (M 11) encoding for S-adenosylmethionine synthase was significantly upregulated in the *35S::BoSTM* line (Fig. 2.10C).

Table 2.1. List of selected genes showing a differential expression between progressive stages of somatic embryo development in a comparison of WT and 35S::BoSTM *Arabidopsis* lines.

Stages	WT			35S::BoSTM		
	0-1	1-2	2-3	0-1	1-2	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 (homolog 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 Beta-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	-	-

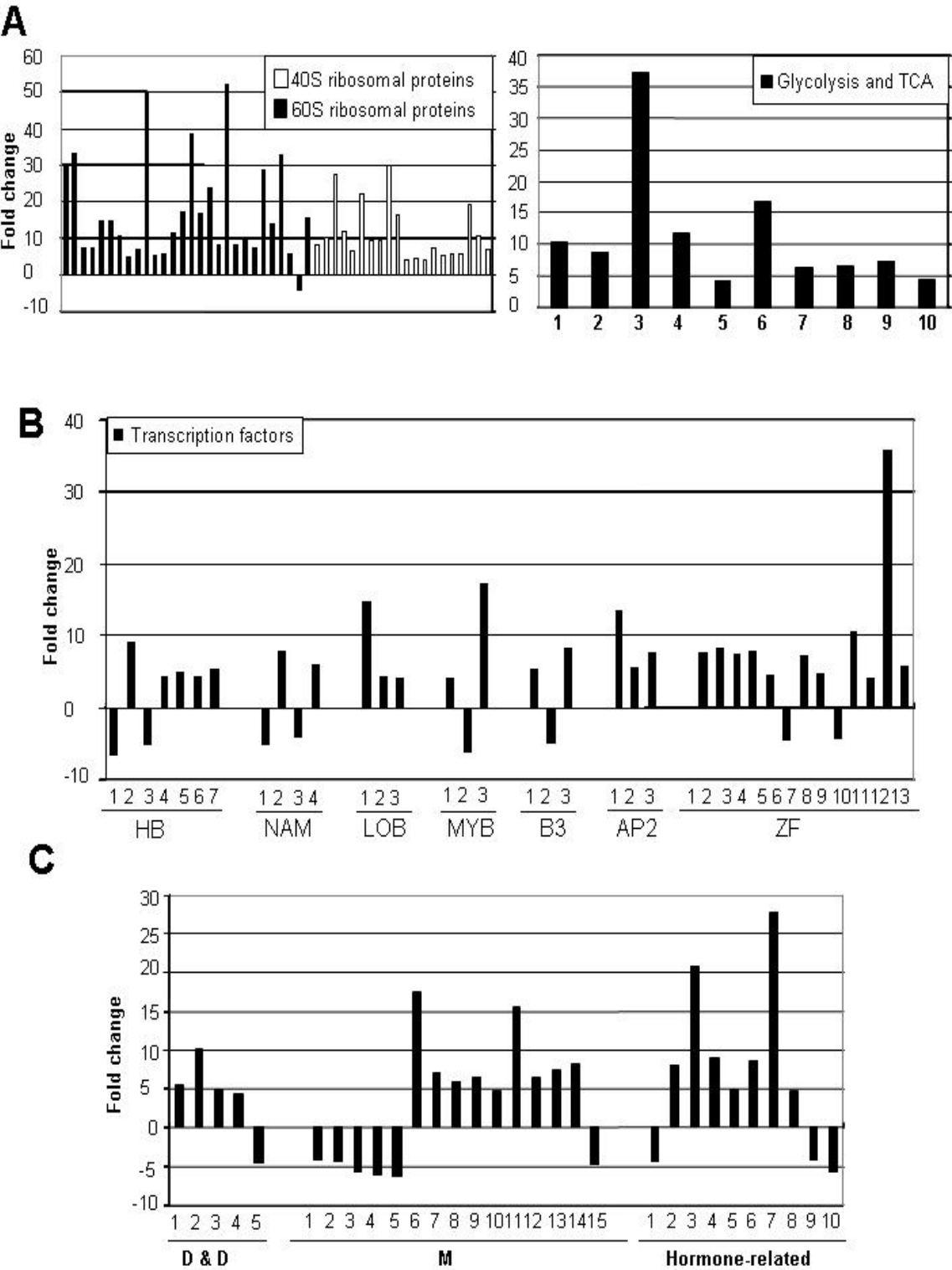


Figure 2.10. Expression of selected probes showing a different behaviour between the WT and 35S::BoSTM lines at stage 1 of *Arabidopsis* somatic embryogenesis. Up-regulation or down-regulation in 35S::BoSTM embryos (compared to WT embryos) is indicated by positive or negative values respectively. **(A)** Expression profiles of probes encoding ribosomal proteins and enzymes involved in glycolysis and TCA cycle. 1, Aconitase hydratase (At2g0510); 2, Malate dehydrogenase (At1g04410); 3, Malate dehydrogenase (At2g22780); 4, Pyruvate dehydrogenase (At5g50850); 5, Pyruvate kinase (At5g52920); 6, Phosphoglycerate mutase (At4g38370); 7, Fructose biphosphate aldolase (At2g01140); 8, Fructose biphosphate aldolase (At3g52930); 9, Phosphofructokinase (At1g12000); 10, triosephosphate isomerise (At2g21170). **(B)** Expression behavior of probes encoding transcription factors. Homeodomain-containing (HB) proteins: 1 KNAT5 (At4g32040); 2 Homeobox-leucine zipper (At1g52150); 3, BEL1-like homeodomain protein (At1g19700); 4, HAT14 (At5g06710); 5, HB9 At1g30490; 6, WUSCHEL (At2g17950); 7, REVOLUTA (At5g60690). NAM-containing probes: 1, RD26 (At4g27410); 2, NAM –similar to TIP (At4g35580); 3, similar to NAC2 (At3g10500); 4, similar to NAC2 (At3g10490). Lateral Organ Boundary (LOB) class: 1, LBD12 (At2g30130); 2, LBD16 (At2g42430); 3, LBD41 (At3g02550). Myb-containing (MYB) probes: 1, myb transcription factor (At2g38300); 2, MYB32 (At4g34990); 3, MYB91 (At4g32010). B3-containing probes: 1, B3 family protein (At5g1800); 2, B3 family protein (At4g30080); 3, low similarity to FUSCA (At4g32010). AP2-containing probes: 1, RAP 2.2. (At3g14230); 2 and 3, AP2-containing proteins (At4g23750 and At1g36060). Putative transcription factors with zinc finger (ZF) motives: 1, (At2g24790); 2 (At1g75540); 3, (At3g10810); 4, (At5g01960); 5,

(At3g19910); 6, (At5g01960); 7, (At2g19810); 8, (At3g02830); 9, (At4g36620); 10, (At4g32890); 11, (At3g54810); 12, (At5g25830); 13, (At4g24470). **(C)** Expression of probes involved in disease and defence (D&D), Metabolism (M), and Hormone-related responses. D&D: 1, Superoxide dismutase [Cu-Zn] (At1g08830); 2, Superoxide dismutase [Fe] (At5g23310); 3, Superoxide dismutase [Mn] (At3g10920); 4, Ascorbate peroxidase 1 (APX1) (At1g07890); 5, L-ascorbate peroxidase (APX) (At4g08390). Metabolism (M): 1, Sucrose-phosphatase 2 (SPP2) (At3g52340); 2, Sucrose synthase (At5g49190); 3, Mannitol dehydrogenase (At4g39330); 4, Galactose dehydrogenase (At4g33670); 5, Ribose-5-phosphate isomerase-related (At5g44520); 6, Glutamate synthase (At5g53460); 7, Glutamine synthetase (GS1) (At3g17820); 8, Glutamine synthetase, putative (At1g48470); 9, Glutamine synthetase, putative (At5g37600); 10, Glutathione synthetase (GSH2) (At5g27380); 11, S-adenosylmethionine synthetase (At1g02500); 12, S-adenosylmethionine-sterol-C-methyltransferase (At1g20330); 13, Nitrate reductase (At1g 77760); 14, Nitrate reductase 2 (At1g37130); 15, Nitrate-responsive NPOI protein (At5g40645). Hormone-related probes: 1, 1-Aminocyclopropane 1-carboxylate synthase (At4g37770); 2, Gibberellin-responsive modulator (At3g03450); 3, Gibberellin-regulated family protein (At5g14920); 4, Auxin-responsive GH3 protein (At5g54510); 5, Auxin-responsive protein (At3g23050); 6, Auxin-responsive protein (At2g22670); 7, Auxin-responsive protein (At5g65670); 8, Ethylene responsive element binding factor (ERF3) (At1g50640); 9, Ethylene responsive transcriptional co-activator (At3g24500); 10, Brassinosteroid signaling positive regulator (BZR1) (At1g75080).

2.4.5. *WUSCHEL (WUS)* expression and localization during *Arabidopsis* somatic embryogenesis

Expression of *WUS* was measured by semiquantitative RT-PCR at stage 1 of *Arabidopsis* somatic embryogenesis in the WT line as well as in lines over-expressing *BoSTM*, *BnSTM*, *BrSTM*, and *BnCLV1* (Fig. 2.11). Compared to the WT line, *WUS* expression increased in all lines over-expressing the *Brassica STM* (confirming our microarray data, Table 2.1 and Fig. 2.10 B) whereas it decreased in the two *BnCLV1* lines (Fig. 2.11 A). In order to assess if these alterations in expression levels were associated to 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. 2.11 B₁) and to the meristemoids (Fig. 2.11 B₂). *WUS* was expressed in the meristematic cells which stained blue with toluidine blue (TBO) (Fig. 2.11 B_{3,4}). In the *35S::BoSTM* line (line 5) the localization of *WUS* was extended to all apical and sub-apical cells of the callus (Fig 2.11 B₅). Compared to the subtending parenchyma cells, the *WUS* expressing cells were smaller and highly cytoplasmic (Fig. 2.11 B₆). 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. 2.11 B_{7,8}). 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. 2.11B₉), although in many instances no expression level was detected (Fig.2.11 B₁₀).

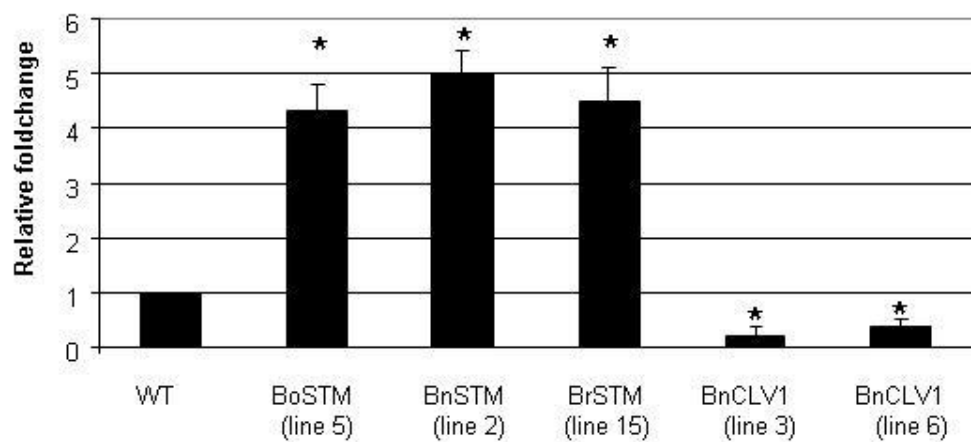
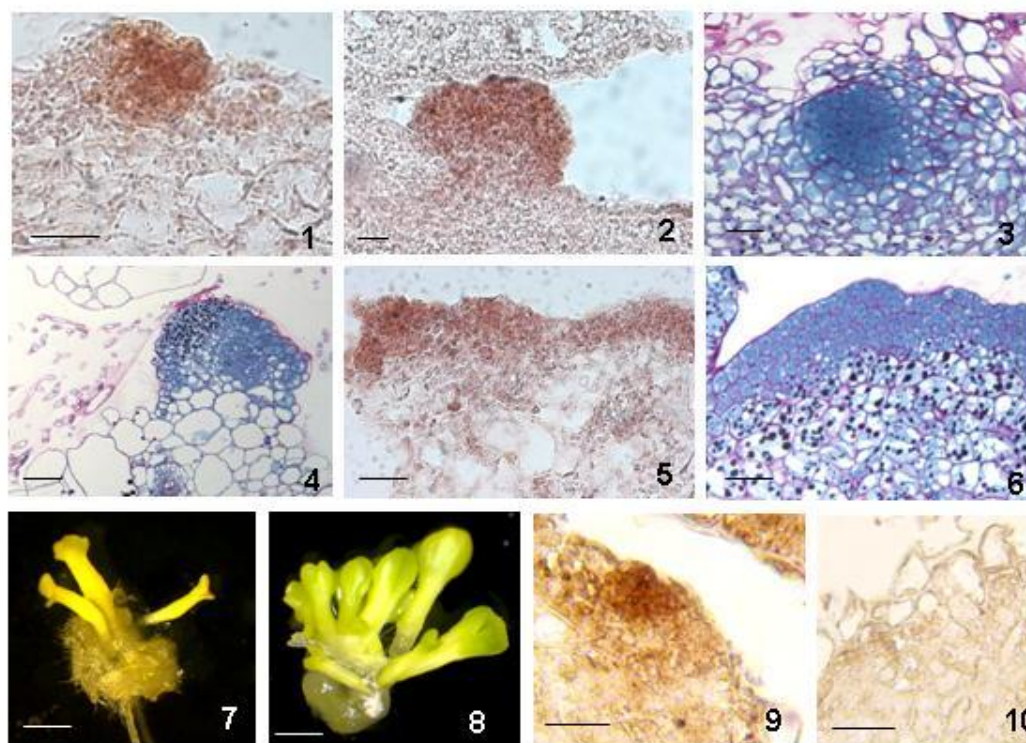
A**B**

Figure 2.11. *WUSCHEL* (*WUS*) expression (measured by semiquantitative RT-PCR) and localization (by DIG-labeled RNA in-situ hybridization) at the end of the induction period (stage 1) of *Arabidopsis* somatic embryogenesis in the WT line and in lines over-expressing *BoSTM* (line 5), *BnSTM* (line 2), *BrSTM* (line 15), and *BnCLV1* (line 3 and 6). Values $\pm$ SE are means of three independent experiments and are normalized to the value of the WT line set at 1. Stars indicate values which are significantly different from the WT value ($P \leq 0.05$) **(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 demarked the formation of embryogenic cells which stained blue with toluidine blue (TBO) **(B_{3,4})**. In the line over-expressing the *BoSTM*, the *WUSCHEL* domain was extended to the surface of the cotyledons **(B₅)** corresponding to apical and sub-apical layers occupied by embryogenic cells **(B₆)**. The extent of the *WUS* domain at stage 1 of somatic embryogenesis correlated to 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 line over-expressing the *Brassica CLV1*, *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 μ m (1-6 and 9, 10) and 1 mm (7, 8).

2.4.6. 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. 2.6A) of *Arabidopsis* somatic embryogenesis was evaluated in the WT line and lines ectopically expressing BoSTM (line 5), BnSTM (lines 2), BrSTM (line 15) and BnCLV1 (lines 3 and 6). Somatic embryo production in the WT line decreased to 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. 2.12). 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 to 3.5 μM (Fig. 2.12).

2.4.7. 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 2.1) of *Arabidopsis* somatic embryogenesis affected the pattern of DNA methylation, we performed a coupled restriction enzyme digestion and random amplification (CRED-RA) in the WT line and the 35S::*BoSTM* line 5 at stage 1 of somatic embryogenesis. As shown in Fig. 2.13A, the degree of methylation of cytidine residues was lower in the transformed line.

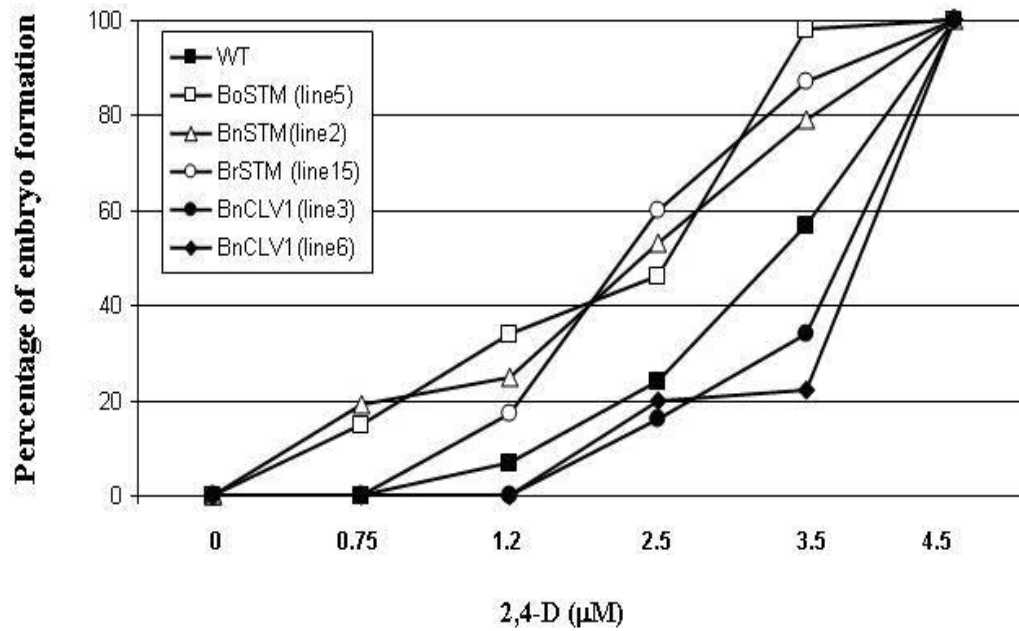


Figure 2.12. Auxin requirement for the induction of *Arabidopsis* somatic embryogenesis. The WT line, as well lines ectopically expressing *BoSTM* (line 5), *BnSTM* (line 2), *BrSTM* (line 15) and *BnCLV1* (lines 3 and 6) were cultured on the induction medium with different levels of 2,4-D. Values ($n=3$) are presented as a percentage of the embryo production occurring at the optimal 2,4-D level (4.5 μM).

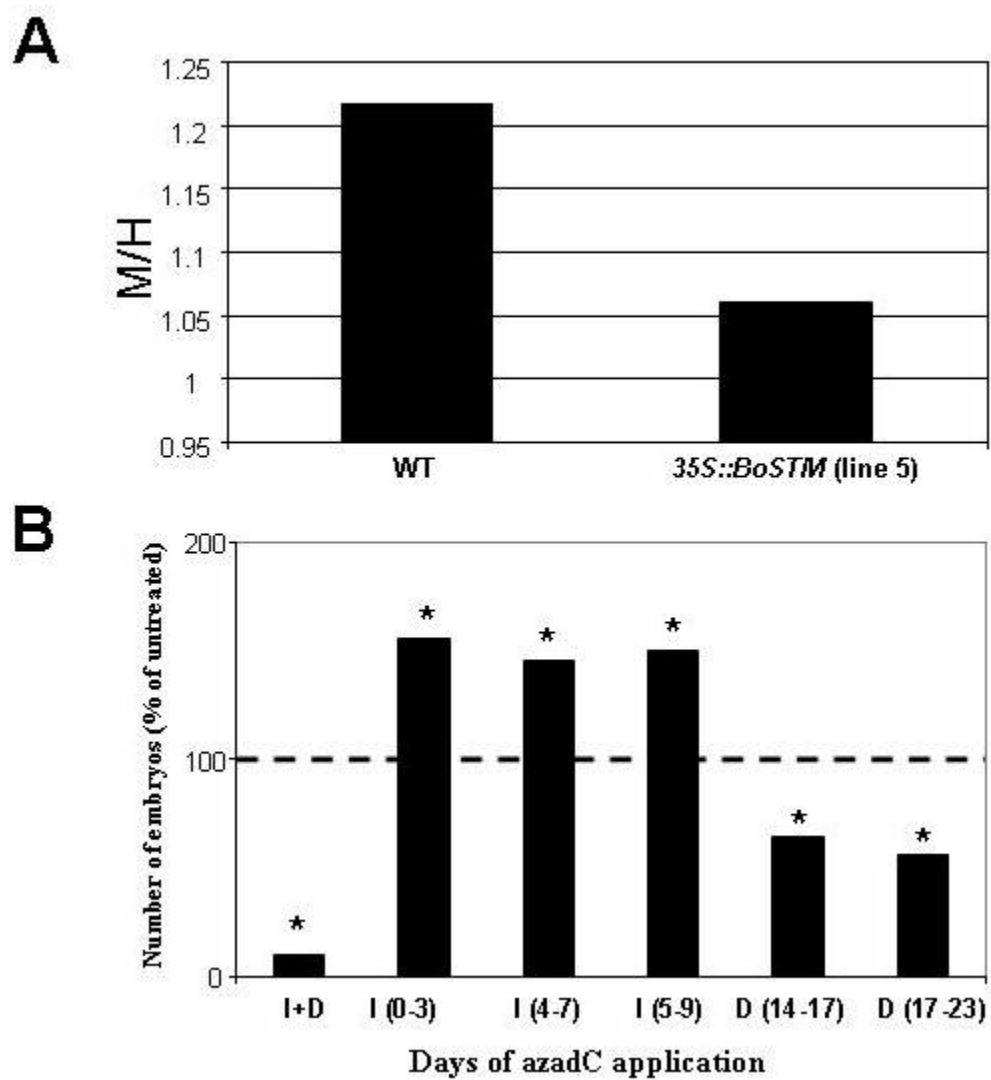


Figure 2.13. (A) DNA methylation analysis in the WT line and in the 35S::*BoSTM* line 5 at stage 1 of *Arabidopsis* somatic embryogenesis. Ratios of consistent amplified bands following digestion with *MspI* (M) and *HpaII* (H). DNA was extracted at stage 1, digested with *HpaII* and *MspI* endonucleases (which cut the sequence 5'-C/CGG-3' with different sensitivity to cytosine methylation; *MspI* cuts if the inner C is methylated, whereas *HpaII* cannot cleave in the presence of methyl groups), and then used as a template in a PCR reaction 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 at different days in the induction (I) or development (D) medium. 5-azadeoxycytidine was also added through the culture period (I+D). Values (n=3) are expressed as percentages of the value of untreated tissue (set at 100%). Stars indicate values which are significantly different from the untreated value ($P \leq 0.05$) time.

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 favored the production of WT somatic embryos. An opposite trend was observed if azadC was added in the development medium (Fig. 2.13B).

Transcriptional studies showed that during the initial days in the development medium (stage 1-2) glutathione reductase (GR) and glutathione synthase (GSH2) are repressed in the *35S::BoSTM* line (Table 2.1). This observation prompted us to investigate the role of glutathione metabolism during *Arabidopsis* somatic embryogenesis. Compared to the WT line, the endogenous level of reduced glutathione (GSH) in the *35S::BoSTM* line 5 decreased sharply within the first three days in the development medium (from stage 1 to stage 2) and remained low during the following days in culture (Fig. 2.14A). This profile correlated with the reduced activity of glutathione reductase (GR) in the *35S::BoSTM* line after stage 2. No differences in the endogenous content of oxidized glutathione (GSSG) were observed between lines during somatic embryogenic (Fig. 2.14A).

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 oxidized environment (low GSH/GSSG ratio) favoring somatic embryo development and a reduced environment (high GSH/GSSG) having an 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

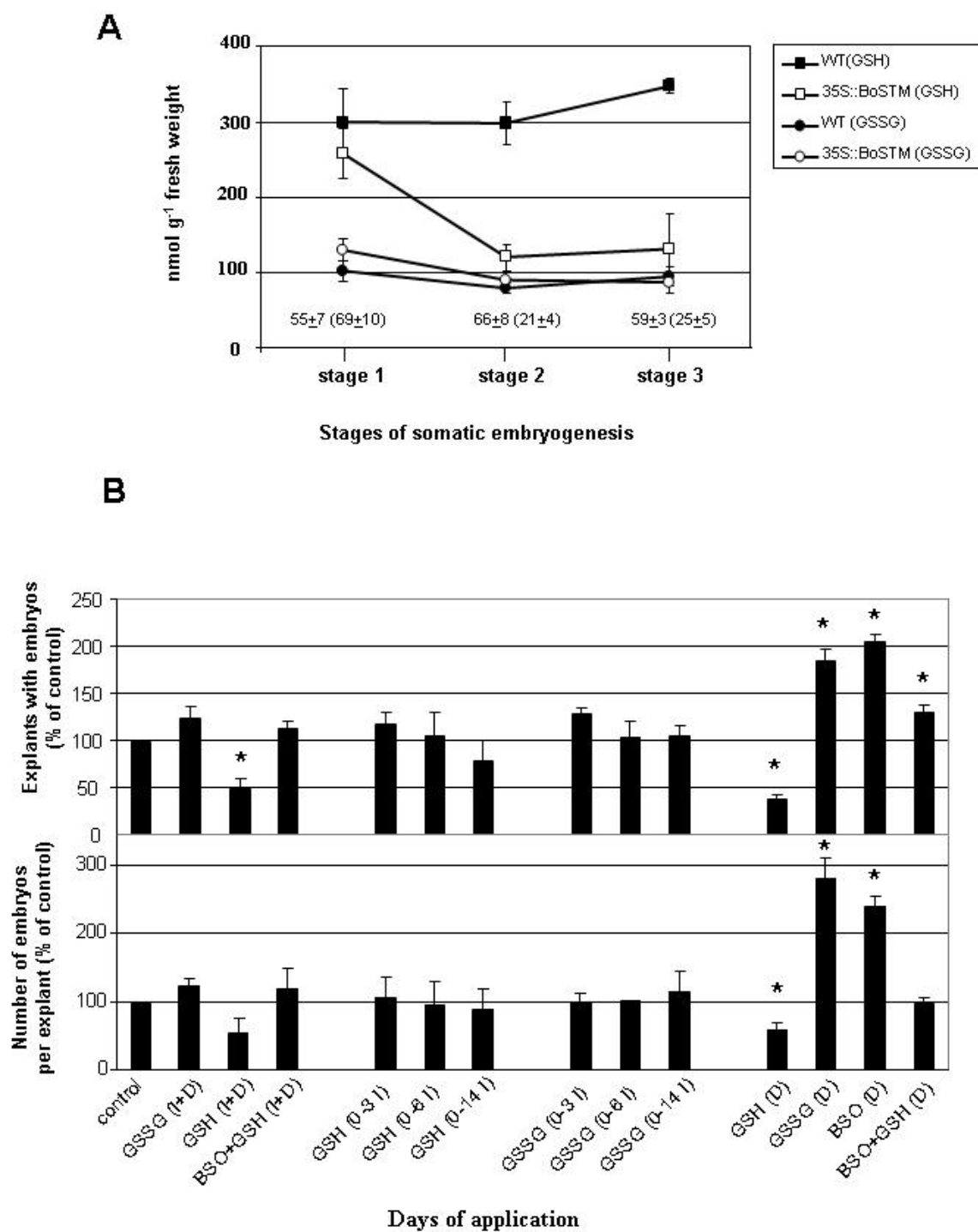


Figure 2.14. Glutathione metabolism during *Arabidopsis* somatic embryogenesis. **(A)** Endogenous levels of reduced (GSH) and oxidized (GSSG) glutathione during different stages (0-3) of somatic embryogenesis in the WT line and the *35S::BoSTM* line 5. Numbers above abscissa (values of the *35S::BoSTM* line in brackets) indicate the activity of glutathione reductase (nmol g^{-1} fresh weight). Values $\pm$ SE are averages of at least three independent experiments. **(B)** Effects of exogenous applications of GSSG (1mM), GSH (1mM), 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 at different days during induction (I) or development (D). Values $\pm$ SE (n=3) are expressed as percentages of the value in the untreated (control) tissue set at 100%. Stars above each bar indicate values which are significantly different from the control value ($P \leq 0.05$).

reduced or oxidized glutathione environment, (Belmonte et al., 2006) or BSO, 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. 2.14B). The inhibitory effect of GSH was reversed by the 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. 2.14B).

2.5. DISCUSSION

2.5.1. Characterization of *B. napus* shoot apical meristem genes

The putative orthologs of the *Arabidopsis* *STM*, *CLV1*, and *ZLL* were isolated from *Brassica napus* tissue and denoted as *BnSTM*, *BnCLV1*, and *BnZLL-1* and *-2*. The *Brassica* genes displayed high levels of sequence identity with their respective *Arabidopsis* orthologs with which they share all the conserved domains (Fig. 2.1 and 2.2). Unique features of *BnSTM* 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. 2.4), 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 *AtSTM*, *BnSTM* is involved in the establishment and maintenance of a functional SAM. The *B. napus* *BnCLV1* contains all the signatures of its respective *Arabidopsis* ortholog *AtCLV1* receptor kinase, including the conserved C-terminal serine/threonine kinase domain (Fig. 2.1). Similarities among the two genes (*BnCLV1* and *AtCLV1*) were also observed in their localization patterns

during embryogenesis (Fig. 2.4 and Clark et al., 1997) and expression during post-embryonic development (Fig. 2.3). The termination of the SAMs due to differentiation of the meristematic cells observed in *Arabidopsis* seedling over-expressing *BnCLV1* (data not shown), suggests that *BnCLV1* regulates SAM activity through mechanisms analogous to those ascribed to *AtCLV1*, which limits the accumulation of undifferentiated or uncommitted cells and promotes differentiation (reviewed by Dodsworth, 2009).

The two *BnZLL* genes, denoted as *BnZLL-1* and -2 share a high degree of amino acid sequence identity (96%) with *AtZLL* and also contain two conserved regions, the PIWI and PAZ domains, which are unique features of the AGO members, including *AtZLL* (Cerutti et al., 2000). Additional similarities shared by *BnZLL-1* and 2 and the *Arabidopsis AtZLL* include the two distinct expression domains (vascular tissue and SAM) during embryogenesis. The localization of *BnZLL-1* and 2 in the vascular tissue of immature embryos and in the SAM of fully developed cotyledonary embryos both *in vivo* and *in vitro* (Fig. 2.4) is very similar to that described for *AtZLL* during *Arabidopsis* embryogenesis (Moussian et al., 1998). The participation of *AtZLL* 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 *BnZLL-1* and -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, our results are consistent with the notion that *BnSTM*, *BnCLV1*, and *BnZLL-1* and -2 are the respective orthologs of *AtSTM*, *AtCLV1*, and *AtZLL*.

2.5.2. 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. 2.6A). 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; Mordhorst et al., 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, *WUSCHEL*, 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 -2 (Fig. 2.6). 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, our over-expression 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 over-expressing *KNAT1*, a member of the *kn-1* like homeobox gene family which include *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 our system (the identity of the embryos was verified by the presence of the bipolar apical meristems through histological analyses). We postulate 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 into 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 our previous studies documenting an increased number of somatic embryos in spruce lines over- expressing *HBK3*, a class I KNOX homeobox gene, isolated from *Picea abies* (Norway spruce) (Belmonte et al., 2007).

Another interesting point emerging from our study is the different response in culture exhibited by the lines over-expressing the *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. 2.6B). This competitive role of the *Brassica* genes *in vitro* is analogous to that fulfilled by the *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 differentiation (Clark et al., 1997). In line with this analogy it is therefore suggested that while the *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 (bent-cotyledon embryos) over-expressing *BnCLV1* (data not shown). 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 component for the induction of somatic embryos (Kurdyukov et al., 2009).

The altered expression of the *Brassica* *STM* also had profound effects on *B. napus* microspore-derived embryogenesis. A high yield of MDEs was observed in lines over-expressing *BnSTM* whereas an opposite trend was noticed in lines with reduced *BnSTM* levels (Fig. 2.8). The phenotypic deviations (reduced embryo size and fused cotyledons) observed in many MDEs generated from lines down-regulating *BnSTM* (Fig. 2.9) confirm the notion that this gene is implicated in embryo development and meristem formation.

Overall these results clearly show that the over-expression of the *Brassica* *STM* encourages somatic embryo formation in *Arabidopsis* and MDEs production in *B. napus*.

It is therefore suggested that the beneficial effect of this gene on *in vitro* embryogenesis is independent from 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*).

2.5.3. 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 (Appendix 4 and 5). Formation of embryos from *Arabidopsis* somatic cells requires a reprogramming in gene expression pattern, which is affected profoundly by the over-expression of *BoSTM*. In bent-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. The most pronounced differences in expression levels occurred during the induction phase, demarking 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 2.1).

2.5.3.1. 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 2.1) suggests that a 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., 2005). Another role assigned to *KNOTTED* genes is to inhibit gibberellin synthesis through the repression of GA 20 oxidase (Sakamoto et al., 2001b). In our 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 2.1).

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 IAA-induced elements (*IAA1*, 8, 9, 28) during the induction phase (stage 0 vs. 1) of the *35S::BoSTM* line (Table 2.1), 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 over-expressing the *Brassica* (*Bo*, *Bn*, or *Br*) *STM* when exposed to reduced levels of 2,4-D which preclude embryogenesis in the WT line (Fig. 2.12). The requirement for 2,4-D is higher in the *35S::BnCLV1* lines, thus suggesting opposite effects of the *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 organising center in the SAM *in vivo* (Mayer et al., 1998), is associated with the production of totipotent embryogenic cells during *in vitro* morphogenesis (Kurdyukov 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. 2.11A), which exhibit the lowest requirement for auxin (Fig. 2.12), 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 demarks the formation of embryogenic cells) in lines over-expressing the *Brassica STM* and a limited *WUS* signal in the *35S::BnCLV1* line (Fig. 2.11B). 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 2.1). 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 over-expressing the *Brassica CLV1* (data not shown), possibly as a feed-back 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, which is 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 2.1), both involved in meristem formation (Moussian et al., 1998).

Common embryogenesis-related programs 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 germ line 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 2.1). 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 over-expressing *BoSTM* at the end of the induction phase (Fig. 2.13A). 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 azadeoxycytidine (Fig. 2.13B), a hypomethylating drug which inhibits METHYLTRANSFERASE 1 (Ghoshal et al., 2005), confirm the

requirement of DNA hypomethylation during the induction of *Arabidopsis* somatic embryogenesis.

2.5.3.2. The development phase

Compared to 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. Major differences in expression levels during the transition from stage 1 to stage 2 were observed for probes related to disease and defense processes, which included superoxide dismutase, enzymes involved in ascorbate metabolism, and two enzymes involved in glutathione metabolism (GSH2 and GR) (Table 2.1).

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. 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 favored if the endogenous GSH/GSSG ratio was lowered either through applications of GSSG or DL-buthionine-[S,R]-sulfoximine (BSO), a specific inhibitor of the 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 on the role of GSSG during *in vitro* embryogenesis reviewed in Stasolla (2009).

The ectopic expression of *BoSTM* results in a drop in GSH levels during the first three days of somatic embryo development (stage 2) (Fig. 2.14A) possibly due to the transcriptional repression of glutathione synthase (GSH2), a biosynthetic enzyme catalyzing the condensation of γ -glutamylcysteine and glycine to form GSH, and to a repression in transcription and activity of glutathione reductase (GR) which recycles GSH from GSSG (Table 2.1, Fig. 2.14A). 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), were included in the development medium. This was in contrast to the application of GSH, which increases the endogenous GSH/GSSG (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. 2.14B).

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., 2003).

In Conclusion, this study demonstrates that *in vitro* embryogenesis, i.e. *Arabidopsis* somatic embryogenesis and *B. napus* microspore-derived embryogenesis can be modulated by the over-expression of *Brassica* genes orthologous to the *Arabidopsis* *AtSTM*, *AtCLV1*, and *AtZLL*. The enhanced somatic embryogenesis observed in

Arabidopsis lines over-expressing the *Brassica STM* was associated to a lower requirement for the inductive signal 2,4-D, and a higher expression of *WUSCHEL* which demarks 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 to the hypomethylation of the DNA during the induction phase of somatic embryogenesis, and to 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 the *Brassica STM* also had profound effects on *B. napus* microspore-derived embryogenesis. The number of microspore-derived embryos increased in lines over-expressing *BnSTM* and significantly decreased in antisense lines down-regulating *BnSTM*. This suggests that the beneficial effect of the *Brassica STM* is independent from the embryogenic system utilized.

**CHAPTER 3: OVER-EXPRESSION OF THE *BRASSICA STM* ATTENUATES
THE DELETERIOUS EFFECTS OF THE AUXIN TRANSPORT INHIBITOR
TIBA ON SOMATIC EMBRYO NUMBER AND MORPHOLOGY**

3.1 ABSTRACT

The auxin transport inhibitor 2, 3, 5-tri-iodobenzoic acid (TIBA) is a useful compound for investigating the role of auxin flow during plant development. In the WT line, applications of TIBA during the induction phase of *Arabidopsis* somatic embryogenesis inhibit embryo development and induce the differentiation of the meristematic cells of the SAM leading to the fusion of the cotyledons. These abnormalities were associated to changes in the expression levels of auxin transporter genes (*PINs*) and endogenous distribution of IAA. Treatments of TIBA caused a rapid accumulation of IAA within the epidermal and cortical root cells of the explants (bent-cotyledon zygotic embryos), as well as in the apical and sub-apical cells of the callus generated by the cotyledons of the explants. Within the callus only a few number of cells acquired “meristematic characteristics” and this was linked to the repression of genes involved in embryogenic cell fate acquisition, such as *WUSCHEL* (*WUS*), *LEAFY COTYLEDON 1* and 2. All these deleterious effects were attenuated when TIBA was added to lines over-expressing the *STM* isolated from *Brassica oleracea* (*Bo*), *B. napus* (*Bn*), and *B. rapa* (*Br*). Of interest, TIBA-treated explants of the *Brassica STM* over-expressing lines were able to produce a large number of embryogenic cells and somatic embryos which exhibited a normal morphology with two distinct cotyledons. A proposed reason for this behaviour was the ability of the transformed tissue to retain a normal distribution of auxin in the presence of

TIBA. Proper localization of auxin might be required for the normal expression of several genes necessary for the acquisition of embryogenic competence and formation of somatic embryos.

3.2. INTRODUCTION

Somatic embryogenesis exemplifies plant plasticity in that somatic cells are induced to dedifferentiate and initiate an embryogenic program (Feher et al., 2003). This intriguing process is executed under appropriate culture conditions, and requires specific inductive signals triggering the embryogenic transition from somatic cells. In many species the use of auxins, such as IAA (Ahmed et al., 1996), NAA and 2,4-D (Baker and Wetzstein, 1994), dicamba (Eapen and George, 1993), and picloram (Ozias-Akins et al., 1992) is a routine to induce somatic embryogenesis. In *Arabidopsis* bent-cotyledon zygotic embryos cultured with 2,4-D can be used as explants to induce somatic embryos reviewed by (Raghavan, 2004). Despite this extensive utilization, the role played by auxins during the initial phases of somatic embryo development remains elusive. According to Su et al. (2009) the generation of auxin gradients within the explants might be required to demark the localization of *WUSCHEL* (*WUS*), which is associated to the production of totipotent cells during *in vitro* morphogenesis (Kurdyukov et al., 2009; Su et al., 2009). Expression of *WUS* might be sufficient to induce somatic embryos. Zuo et al. (2002) showed that the over-expression of *PLANT GROWTH ACTIVATOR*, a *WUS*-related gene promotes somatic embryogenesis from vegetative tissue in a hormone-independent manner.

Many developmental and physiological processes promoted by auxin are mediated by its directional transport within the plant body, regulated by influx proteins of the AUX/LAX family and the efflux carrier proteins of the PIN-FORMED (PIN) family, as well as the multidrug resistance (MDR)-like ABC transporter proteins (Noh et al., 2003). Four members of the PIN protein family are expressed during zygotic embryogenesis in *Arabidopsis*: PIN1, 3, 4, and 7, where they contribute to the accumulation of auxin in specific domains (reviewed by Friml et al., 2003; Chen and Chang, 2005). In pre-globular embryos auxin is channelled from the suspensor to the embryo proper by PIN7. At the globular stage of embryogenesis a re-localization of PIN1, 4, and 7 contributes to the formation of an apical-basal flow of auxin which specifies the root meristem (Friml et al., 2003). Thomas and Jiménez (2006) demonstrated that a precise redistribution of PIN proteins also occurs during *in vitro* embryogenesis where it is required to increase the endogenous levels of auxin in specific domains of the explants acquiring embryogenic competence. High levels of endogenous auxin have been associated to improved somatic embryogenesis in several systems including alfalfa (Ivanova et al., 1994); *Pennisetum purpureum* (Rajasekaran et al., 1987); *Dactylis glomerata* (Wenck et al., 1988), and wheat (Kopertekh and Butenko, 1995). The relevance of the polar transport/distribution of auxin during plant development, including embryogenesis, has been demonstrated by the use of auxin transport inhibitors, such as 2,3,5-triiodobenzoic acid (TIBA) which competes with the auxin binding site on the efflux carriers (Gaspar et al., 2003). Independent studies revealed that applications of TIBA preclude the polarized axial growth of globular stage carrot somatic embryos (Schiavone and Cooke, 1987) and affect the bilateral symmetry of *Brassica juncea* zygotic embryos which develop one single

trumpet-shaped cotyledon (Liu et al., 1993). This effect was also replicated in cultured embryos of *Brassica napus* (Ramesar-Fortner and Yeung, 2001). A decrease in somatic embryo number and structural abnormalities were also observed when TIBA was added in those embryogenic systems requiring exogenous 2,4-D (Choi et al., 2001; Ramarosandratana and Van Staden, 2004) suggesting that perturbations of auxin flow in an auxin-enriched environment are also deleterious for embryo growth. To date no information is available of the effect of this inhibitor during the induction phase of *Arabidopsis* somatic embryogenesis.

Previous studies (Chapter 2) demonstrated that somatic embryo production in *Arabidopsis* is encouraged by the ectopic expression of *Brassica* genes orthologous to the *Arabidopsis* *SHOOT MERISTEMLESS (STM)*. The independent introduction of *Brassica napus*, *B. oleracea*, and *B. rapa STM* (denoted as *Bn*, *Bo*, and *BrSTM*) doubled the percentage of explants, i.e. bent-cotyledon embryos, able to form somatic embryos and enhanced the number of embryos produced by each explant. Gene expression studies and hormone sensitivity analyses revealed that the introduction of the *Brassica STM* modifies the response of the tissue to 2,4-D, possibly by making it more sensitive to this plant growth regulator. This notion was demonstrated by the sustained production of somatic embryos produced by the *Brassica STM* over-expressing lines when exposed to reduced levels of 2,4-D which preclude embryogenesis in the WT line. As an extension of that previous work, the current study examines if the polar transport of auxin is required for the induction of *Arabidopsis* somatic embryos and whether the over-expression of *BoSTM*, *BnSTM*, and *BrSTM* can overcome the detrimental effects of TIBA applications.

3.3. MATERIALS AND METHODS

3.3.1. Plant Materials

Generation of *Arabidopsis* plants and induction of somatic embryogenesis were conducted as described in Chapter 2. For this study three transformed lines were used: *35S::BnSTM* (line 2), *35S::BoSTM* (line 5), and *35S::BrSTM* (line 6). Characterization of these lines was reported in Chapter 2. Applications of TIBA were performed at different days during the induction period. Embryo production was measured after 9 days on a TIBA-free development medium.

3.3.2. Histological analyses

Structural analyses were conducted following the procedure described in Chapter 2.

3.3.3. Expression studies

Measurements of transcript levels of genes involved in embryo development [*WUSCHEL* (*WUS*), *CLAVATA3* (*CLV3*), *CUP-SHAPED COTYLEDON1* (*CUC1*), *FUSCA3* (*FUS3*), *LEAFY COTYLEDON1* and 2 (*LEC 1* and *LEC2*)], and auxin flow (*PIN1*, 2, 3, 4, and 7) were performed by quantitative qRT-PCR as indicated in Chapter 2. A list of primers utilized for the experiment is compiled in Appendix 6.

3.3.4. Auxin localization

Immunolocalization of endogenous IAA was carried out as described by (Thomas et al., 2002) with some minor modifications. Plant material was first pre-fixed in freshly

prepared 4% aqueous 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDAC; Sigma) 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 ethanol series, embedded in paraplast, sectioned (10 µm), and deparaffinised in xylene. The sections were incubated in blocking solution [10 mM PBS pH 7, 0.1% Tween 20, 1.5% glycine, and 5% bovine serum albumin (BSA)] at room temperature for 1 hour. 150 µl of monoclonal primary IAA-antibodies (1 mg/ml, Sigma) 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 150 µM 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 [anti-mouse IgG alkaline phosphates conjugate (1 mg/ml), Promega, USA], following the manufacturer instruction overnight in a high humidity chamber, washed 2 times in 10 mM PBS containing 150 µM NaCl, 0.1 % Tween 20, and 0.8 % BSA for 10 minutes, and then incubated in water for 15 min to remove excess of secondary antibodies. Samples were stained using 250 µl Western blue (Promega) for 40 minutes.

3.3.5. Statistical analysis

Statistical analysis was preformed as described in Chapter 2.

3.4. RESULTS

3.4.1. Effects of TIBA applications on somatic embryos produced by the WT line

In order to find the optimal level of TIBA several concentrations of this inhibitor were added to the induction medium of the WT line (Fig. 3. 1A). The number of somatic embryos produced by the WT line decreased with increasing levels of TIBA and no somatic embryos were observed when TIBA was applied at concentrations higher than 2 μ M. The highest level of TIBA producing a substantial number of somatic embryos was 1.5 μ M which resulted in a 27% of embryo formation compared to control (untreated) conditions.

The effects of 1.5 μ M TIBA at different days during the induction period were also assessed (Fig. 3.1B). The most pronounced decline in WT somatic embryo formation was measured when 1.5 μ M TIBA was added to the induction medium after day 3 (day 3-14). This treatment, which will be used for all the subsequent experiments, resulted in a 10% production of somatic embryos compared to the control (100%).

3.4.2. Over-expression of *STM* attenuates the inhibitory effects of TIBA on embryo formation

In the absence of TIBA (control), somatic embryo formation was enhanced in all transformed (35S::*BnSTM*, 35S::*BoSTM*, and 35S::*BrSTM*) lines (Fig. 3.2A). A severe repression of somatic embryogenesis was observed when TIBA was added to the WT line. A considerable number of somatic embryos (close to control conditions of the WT line) was still produced when TIBA was added to the lines over-expressing the *Brassica STM*.

The number of somatic embryos in the WT line showing fused cotyledons increased to more than 25% in the presence of TIBA (Fig. 3.2B). In the transformed lines cotyledon fusion was never observed, not even when TIBA was added to the medium. To determine if TIBA treatments affected the formation of the shoot apical meristem (SAM), histological analyses were conducted on fully developed somatic embryos. Control somatic embryos produced by the WT line had normal shoot apical meristems (SAMs), characterized by a few layers of highly cytoplasmic cells (Fig. 3.2C₁). Applications of TIBA disrupted the architecture of these SAMs by inducing differentiation of the meristematic cells, as estimated by the accumulation of storage products in the apical and sub-apical cells (Fig. 3.2C₂). Several SAMs of TIBA-treated embryos were smaller in size and the cotyledons often fused at their base resulting in the typical “trumpet” shaped-phenotype (Fig. 3.2C₃). In the absence of TIBA, somatic embryos of lines over-expressing the *Brassica STM* displayed enlarged SAMs (Fig. 3.2C₄) and retained a dome-shaped, functional meristem even when TIBA was applied to the culture medium (Fig. 3.2C₅).

3.4.3. Expression analyses of genes involved in somatic embryo production

The expression levels of several genes affecting the induction of somatic embryo development were examined in the middle (day 7) and at the end (day 14) of the induction period in the WT line and 35S::*BoSTM* line. This transformed line was used since it produced the highest number of embryos in the absence or presence of TIBA (Fig. 3.2A). At day 7 the expression levels of *WUSCHEL* (*WUS*), *LEAFY COTYLEDON*

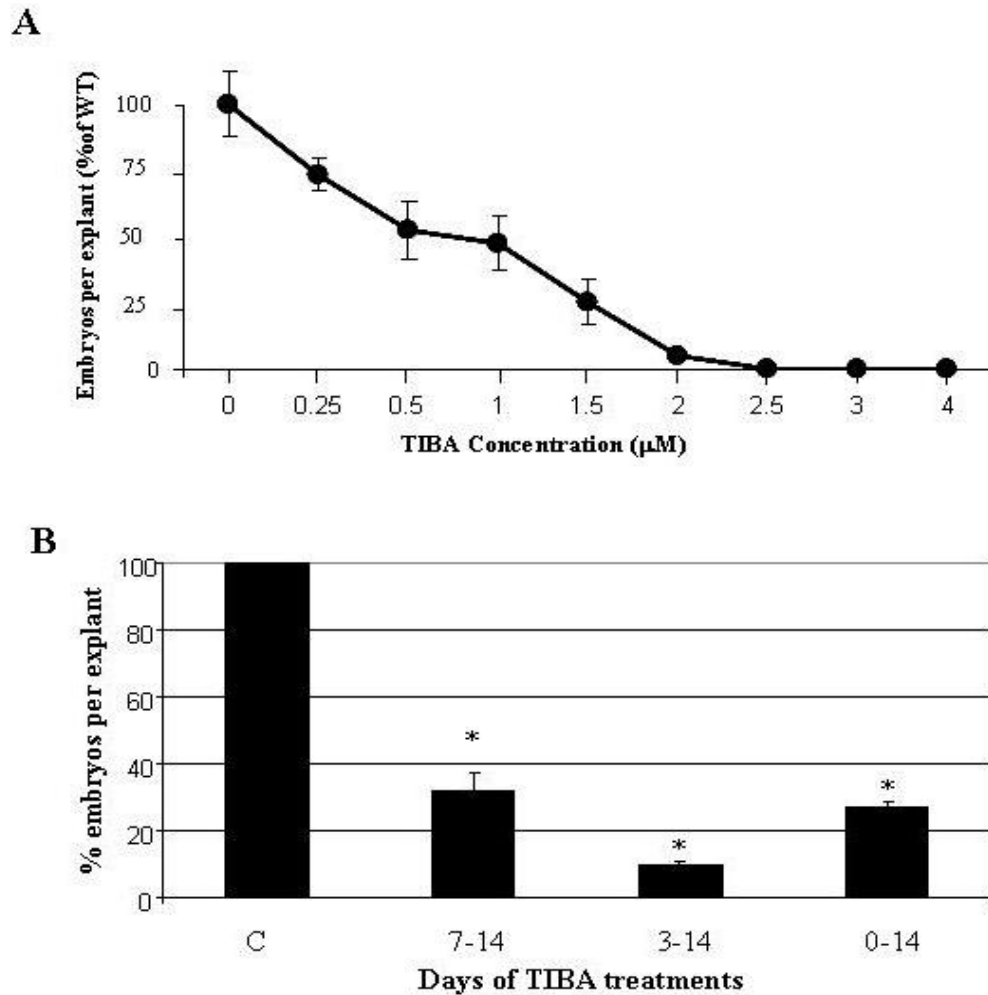


Figure 3.1. (A) Effects of increasing concentrations of 2,3,5-triodobenzoic acid (TIBA) on somatic embryos produced by the WT line. TIBA was added throughout the induction period. Values are means $\pm$ SE of at least three independent experiments. (B) Effects of applications of 1.5 μM TIBA on somatic embryo formation. TIBA was added at different days during the induction period. Values are means $\pm$ SE of at least three independent experiments. * indicate statistically significant values ($P \leq 0.01$) compared to untreated (control, C) tissue. WT total explants in each replicate were 12. The average number of somatic embryos that were produced by WT was 2.51 somatic embryos/explant. The number of somatic embryos was counted at day 23 of culture.

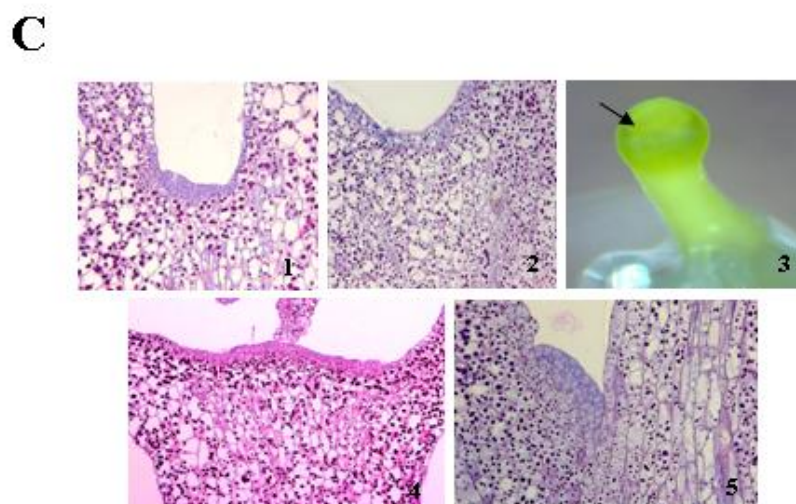
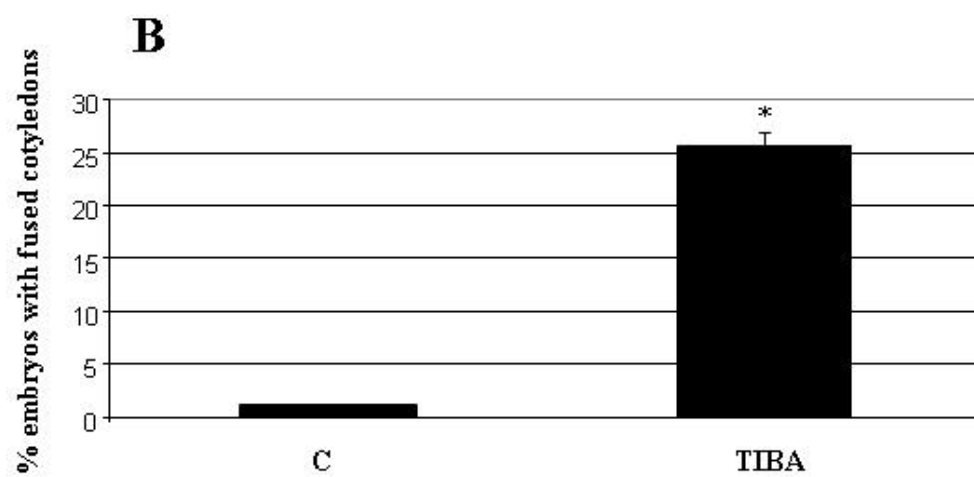
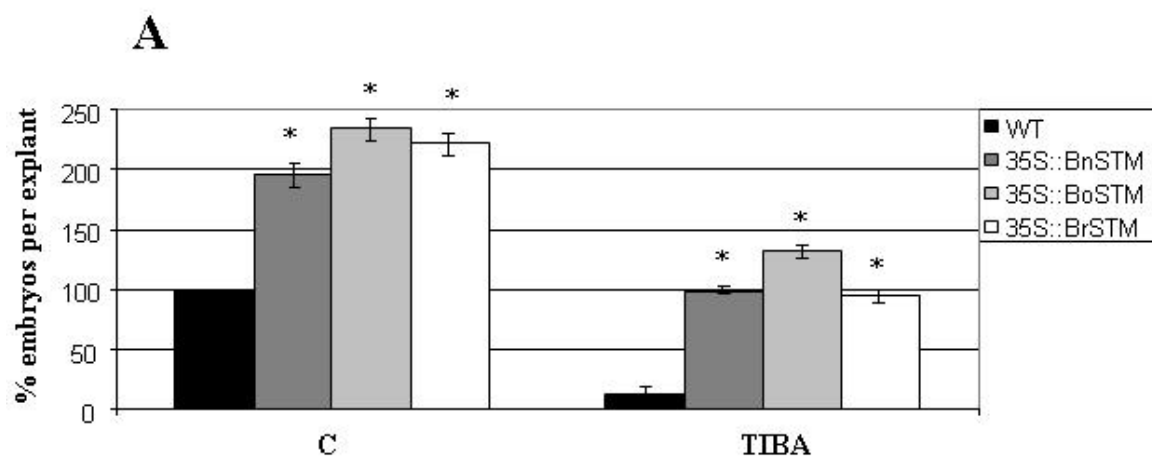


Figure 3.2. (A) Percentage of somatic embryo production in the WT lines and lines over-expressing the *Brassica napus* *BnSTM*, *Brassica oleracea* *BoSTM*, and *Brassica rapa* *BrSTM* cultured in the absence (control, C) and presence of TIBA. Values are means $\pm$ SE of at least three independent experiments. Values are normalized to the WT (control) value set at 100%. * indicate statistically significant values ($P \leq 0.01$) compared to WT.

(B) Percentage of WT somatic embryos with fused cotyledons in the absence (control, C) and presence of TIBA. Values are means $\pm$ SE of at least three independent experiments.

(C) Structure of the shoot apical meristem (SAM) of WT somatic embryos cultured in the absence **(1)** or presence **(2)** of TIBA. Morphology of “trumpet”-shaped WT embryos grown in the presence of TIBA. Architecture of the SAM in *35S::BoSTM* embryos growth in the absence **(4)** or presence **(5)** of TIBA. WT total explants in each replicate were 12. The average number of somatic embryos that were produced by WT was 2.51 somatic embryos/explant. The number of somatic embryos was counted at day 23 of culture.

1 and 2 (*LEC 1* and 2), *CUP-SHAPED COTYLEDON 1* (*CUC1*) and *FUSCA3* (*FUS3*) were highest in the transformed line regardless of the treatment (Fig. 3.3).

A different pattern was observed for *CLAVATA 3* (*CLV3*), in which the highest expression level was measured in the WT line. At the end of the induction period (day 14), the expression levels of *WUS*, *CUC1*, and *FUS3* were higher in the *35S::BoSTM* line, compared to the respective treatment applied to the WT line.

3.4.4. Expression analyses of auxin carrier proteins

Inclusions of TIBA in the culture medium affected the expression of several auxin carriers. The transcript levels of *PIN1*, 3, and 7 were generally always higher in the transformed line (cultured in the presence or absence of TIBA) compared to the respective WT treatment (Fig. 3.4). An opposite trend was observed for *PIN2* which showed its highest expression in the WT line treated with TIBA at any day during the induction period. The highest transcript levels of *PIN4* were measured in the *35S::BoSTM* line cultured in the absence of TIBA (Fig. 3.4).

3.4.5. Immunohistochemical localization of IAA and microscopy

In zygotic *Arabidopsis* embryos IAA was evenly distributed with a stronger signal in the SAM (Fig. 3.5A). The specificity of the signal was demonstrated by performing the immunolocalization procedure in the absence of the primary antibody (Fig. 3.5B). After 7 days in the induction medium the dissected zygotic embryos (explants) elongated and callus formation was demarked by the swelling of the adaxial regions of the cotyledons.

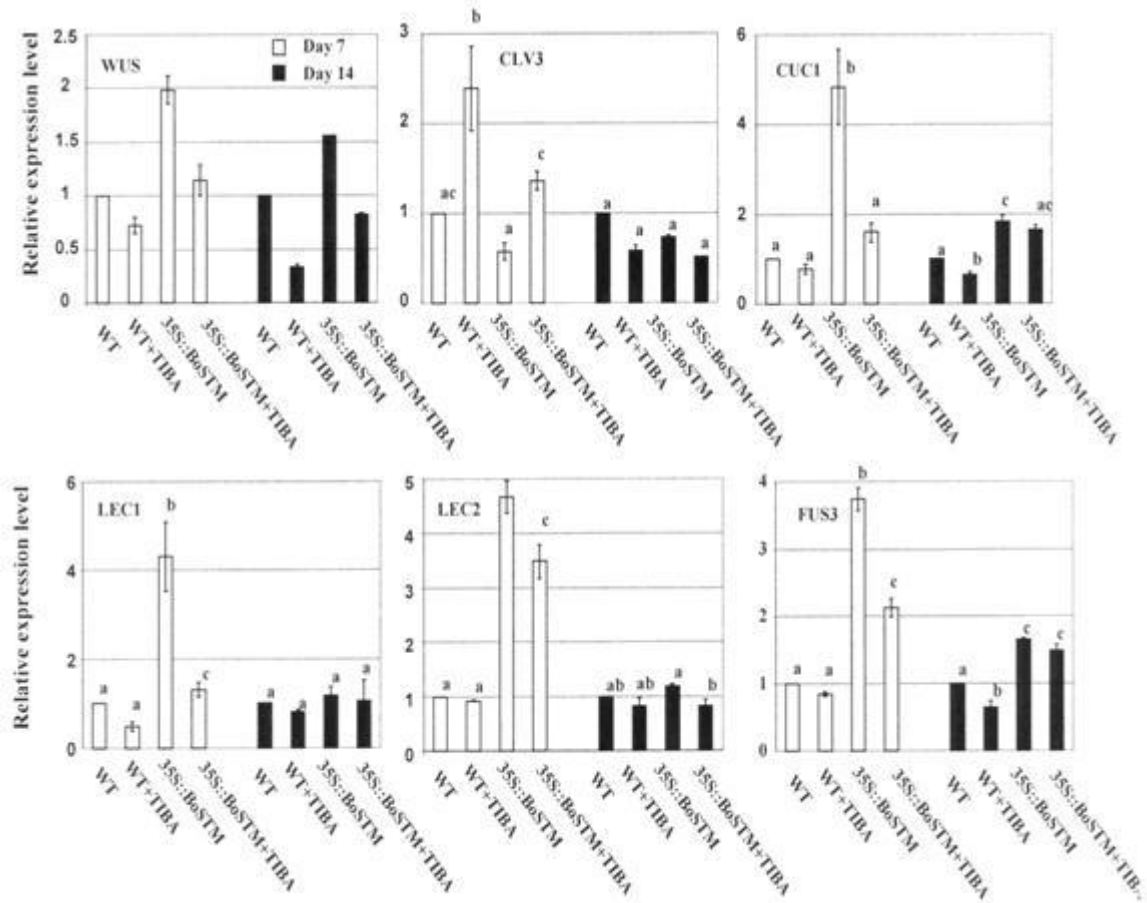


Figure 3.3. Relative expression measured by qRT-PCR of *WUSCHEL* (*WUS*), *CLAVATA 3* (*CLV3*), *CUP-SHAPED COTYLEDON 1* (*CUC1*), *LEAFY COTYLEDON 1* and *2* (*LEC1* and *2*), and *FUSCA3* (*FUS3*), at day 7 (open bars) and day 14 (close bars) during the induction period of *Arabidopsis* somatic embryogenesis. Graphs include data of WT and 35S::BoSTM tissue cultured in the absence or presence of TIBA. Values are means $\pm$ SE of at least three independent experiments and are normalized to the WT value (set at 1) of the respective day in culture. Statistically significant values ($P \leq 0.01$) are indicated by the letters above the bars.

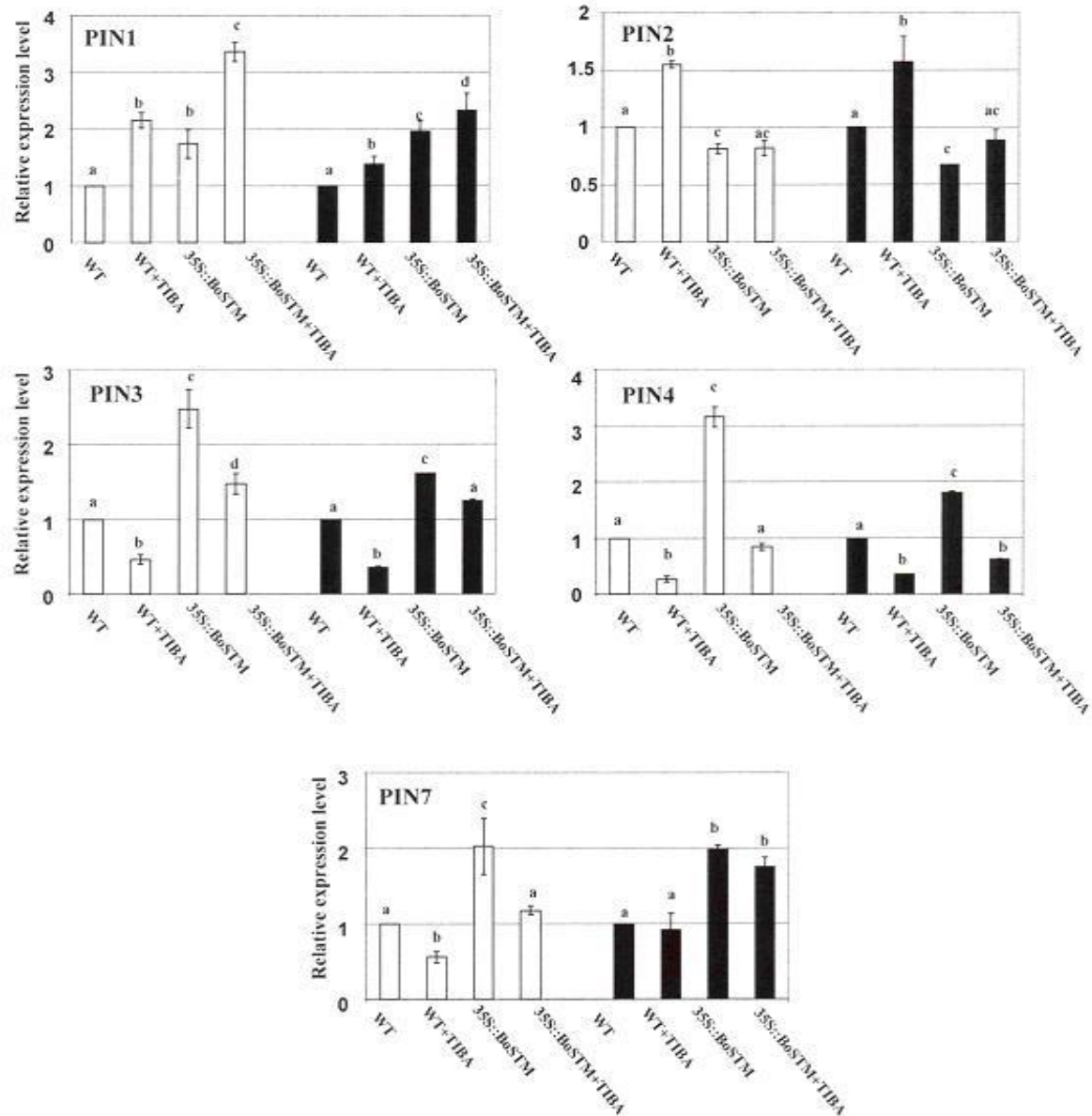


Figure 3.4. Relative expression measured by qRT-PCR of *PIN1*, 2, 3, 4, and 7, at day 7 (open bars) and day 14 (close bars) during the induction period of *Arabidopsis* somatic embryogenesis. Graphs include data of WT and 35S::BoSTM tissue cultured in the absence or presence of TIBA. Values are means $\pm$ SE of at least three independent experiments and are normalized to the WT value (set at 1) of the respective day in culture. Statistically significant values ($P \leq 0.01$) are indicated by the letters above the bars.

In the upper section of the explants produced by the WT line, IAA was mainly localized in the vascular tissue (arrow), and within the cells occupying the apical pole (arrowhead, Fig. 3.5C). Within the root, auxin accumulated in the root tip (*) and a heavy signal was also observed in the vascular tissue, mainly xylem cells (arrow) and in the cortical cells adjacent to the apical region (arrowheads) (Fig. 3.5D). Differences in auxin localization patterns among lines and treatments were observed mainly in the epidermal and cortical cells. Compared to control (untreated) WT line (Fig. 3.5E), applications of TIBA increased auxin accumulation within the epidermal and cortical cells (Fig. 3.5F). This was in contrast to explants of the *35S::BoSTM* line which exhibited a reduced accumulation of auxin in the absence or presence of TIBA (Fig. 3.5G).

At the end of the induction period (day 14) an intensive proliferation of callus produced by the cotyledons was observed. Compared to the WT line cultured under control conditions (Fig. 5.3H), inclusions of TIBA in the induction medium resulted in a heavy accumulation of IAA in the apical and subapical cells of the callus (Fig. 3.5I). Lower auxin levels were detected in the callus of the *35S::BoSTM* line cultured in the absence (Fig. 3.5J) or presence (Fig. 3.5K) of TIBA. The specificity of the signal was demonstrated by the omission of primary antibodies (Fig. 3.5L).

A differential distribution of embryogenic cells, characterized by the presence of a densely stained cytoplasm, was observed among lines and treatments. Within the callus of the WT line, embryogenic cells were organized in distinct domains (arrows, Fig. 3.5M). Addition of TIBA to the culture medium resulted in a reduced number of cytoplasmic cells and a concomitant increased number of differentiated cells which had large vacuoles and accumulated storage products, mainly starch (arrows, Fig. 3.5N).

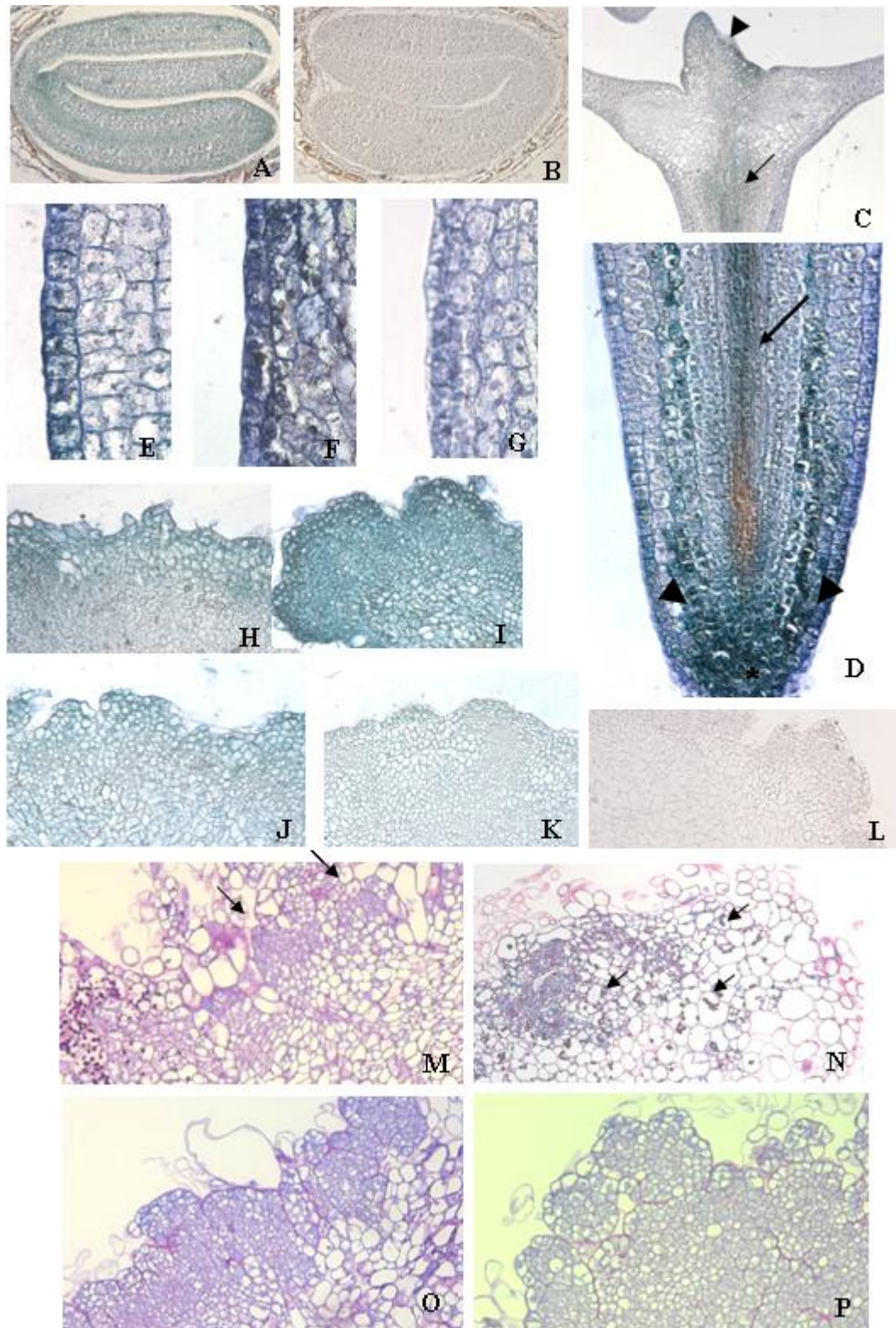


Figure 3.5. Auxin localization and microscopy. **(A)** Immunolocalization of IAA in zygotic *Arabidopsis* embryos. **(B)** Immunolocalization performed in the absence of the primary antibody. **(C)** Distribution of IAA in the upper portion of WT zygotic embryos cultured for 7 days on the induction medium. The signal was mainly localized in the vascular tissue (arrows) and apical pole (arrowhead). **(D)** Root of WT zygotic embryos cultured for 7 days on the induction medium showing accumulation of IAA in the root meristem (*), vascular tissue (arrow), and in the cortical regions adjacent to the apex (arrowheads). Epidermal and sub-epidermal cells of roots of WT embryos **(E)**, WT embryos treated with TIBA **(F)**, and 35S::*BoSTM* embryos **(G)**. An identical IAA localization pattern to that described in **(G)** was also observed in 35S::*BoSTM* embryos treated with TIBA. Auxin localization in the callus originated after 14 days in culture from the cotyledons of WT explants **(H)**, WT explants treated with TIBA **(I)**, 35S::*BoSTM* embryos **(J)**, and 35S::*BoSTM* explants treated with TIBA **(K)**. The specificity of the signal was verified by omitting the primary antibody **(L)**. Structural organization of the densely cytoplasmic embryogenic cells (arrows) formed by the WT explants **(M)**, WT explants treated with TIBA showing accumulation of storage products (arrows) **(N)**, 35S::*BoSTM* explants **(O)**, and 35S::*BoSTM* explants treated with TIBA **(P)**.

A large number of embryogenic cells covered the surface of the callus produced by the 35S::BoSTM line (Fig. 3.5O) and this arrangement was also retained in the presence of TIBA (Fig. 3.5P).

3.5. DISCUSSION

Manipulations of the culture conditions through the inclusion of compounds affecting the polar transport of auxin have been extensively used to investigate the role of auxin during *in vitro* morphogenesis (Choi et al., 2001; Ramesar-Fortner and Yeung, 2006; Hakman et al., 2009). One of the most commonly used auxin transport inhibitor is TIBA, which blocks the directional auxin flow (Friml and Palme, 2002). Independent studies revealed that applications of TIBA inhibit somatic embryogenesis in a variety of species such as sweet potato (Chen et al., 2004), carrot (Nissen and Minocha, 1993), geranium (Hutchinson et al., 1996), ginseng (Choi et al., 1997) and spruce (Hakman et al., 2009). In particular, the inhibitory effect of this compound was also retained in embryogenic systems requiring exogenous auxin (Schiavone and Cooke, 1987), suggesting that the polar transport of auxin is still necessary to provide positional cues for proper cellular differentiation and tissue patterning even in an auxin-enriched environment. This is not surprising given the involvement of auxin flow in a variety of morphogenic events (Leyser, 2005).

The present study shows that the polar transport of auxin plays an important role during the induction phase of somatic embryogenesis and that embryo formation is compromised by inclusions of TIBA. Applications of this inhibitor in the WT line reduced embryo production in a dosage-dependent manner and this effect was partially

attenuated by the introduction of *BoSTM*, *BnSTM*, and *BrSTM*. One hypothesis supported by this observation is that the polar transport of auxin is required for the specification of embryogenic cells, and that lines over-expressing the *Brassica STM* are less susceptible to perturbations of auxin flow and therefore able to retain a larger number of embryo-forming cells in the presence of TIBA. This was tested by analysing the expression levels of genes involved in meristem activity (*WUS*, *CLV3*, and *CUC1*), given the involvement of meristem genes in specifying embryogenic cell fate (discussed in Chapter 2), and genes with an established role in embryogenic cell formation (*LEC1*, 2 and *FUS3*) (Gaj et al., 2005).

Regulation of meristem activity *in vivo* is under the control of the *WUS/CLV* signalling, with the former involved in the maintenance of a reservoir of undetermined cells in the organizing center of the SAM (Mayer et al., 1998) and the latter repressing the expression of *WUS* through feed back mechanisms initiated by the interaction of *CLV3* with the receptor kinase *CLV1* and *CLV2* (reviewed by Dodsworth, 2009). While mutations of *WUS* result in determined SAMs (Lenhard et al., 2002), 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 (Schoof et al., 2000). Genetic work shows that the role of *CLV1* is to promote the differentiation of the meristematic cells thereby reducing the pool of the undifferentiated stem cells (Clark et al., 1996). As outlined in Chapter 2, this competitive regulation might also operate *in vivo*, with *WUS* promoting embryogenic cell formation and *CLV* having an opposite effect.

The activation of *CLV3* observed at day 7 in the WT line treated with TIBA might exercise a repressive effect on *WUS* expression, which declines during the following days in culture, i.e. day 14 (Fig. 3.3). Reduced *WUS* expression would inhibit embryogenic cell formation and promote cellular differentiation within the embryogenic tissue, as estimated by the accumulation of storage products (Fig. 3.5N). These changes do not occur in the *35S::BoSTM* line treated with TIBA, which compared to the WT counterpart has higher levels of *WUS* transcripts and retains a larger number of embryogenic cells (Fig. 3.5P). The antagonistic effect of these two genes (*WUS* and *CLV1*) and their reciprocal regulation was also documented during *Medicago truncatula* somatic embryogenesis (Kurdyukov et al., 2009).

The retention of embryo-forming capacity in transformed lines cultured with TIBA also correlates with the increased expression levels of *LEC1*, 2 and *FUS3*, during the first days of the induction period. Besides their established role during zygotic embryogenesis, these genes are required for the establishment of embryogenic cells, as a reduction of somatic embryogenic potential was observed in *lec* and *fus Arabidopsis* plants (Gaj et al., 2005). *LEC* genes play key roles in many aspects of plant embryogenesis and their involvement during zygotic embryogenesis has been investigated in details (Harada, 2001). Post-embryonic over-expression of *LEC1* and 2 is sufficient to confer embryogenic competence to somatic cells (Stone et al., 2001). In the same line, the expression of the carrot *LEC1* is restricted to embryogenic cells and early embryos, but it is excluded from non-embryogenic cells (Yazawa et al., 2004). The expression analyses presented in this study suggest that during the initial phases of

induction the high levels of *LEC1*, 2 and *FUS* retained by the *35S::BoSTM* line treated with TIBA might be sufficient for the acquisition of embryogenic potential.

Independent studies suggest that several regulators of embryogenic competence acquisition including *WUS*, *LECs* and *FUS3* are regulated by auxin (Gaj et al., 2005; Kurdyukov et al., 2009). In plants the direction of auxin flow is determined by the distribution of PIN proteins (Friml et al., 2003). Despite the functional redundancy during auxin transport, these proteins are involved in unrelated developmental events ranging from vascular tissue differentiation to gravitropism and embryo development (Vieten et al., 2005). However studies on their expression patterns, coupled with auxin localization analyses have been utilized successfully to estimate auxin movement and distribution (Friml et al., 2003). During the induction of *Arabidopsis* somatic embryos two major differences in auxin distribution are apparent in the explants of the WT line cultured with TIBA; the first along the outer cells, i.e. epidermis and cortex, of the root and the second within the callus arising from the cotyledons. Auxin flux in the root occurs through a PIN1-mediated basipetal movement in the stele, followed by a fine tuning mechanisms whereby auxin is accumulated in the root meristem and columella (which functions as the gravity perception of the root, Feraru and Friml 2008) by PIN4, and moved laterally by PIN3 and 7 before being refluxed back to the apex through epidermal and cortical cells via PIN2 (Blilou et al., 2005). In WT roots the accumulation of auxin in the stele with a maximum in the root cap (Fig. 3.5D) agrees with this model. In explants of the WT line cultured in the presence of TIBA the high expression level of *PIN2* and the pronounced accumulation of auxin in the epidermal and cortical cells (Fig. 3.5F) suggest an enhanced upward movement of auxin. This is in contrast to the explants of the *35S::BoSTM* line

(with or without TIBA) which show reduced IAA levels in the epidermal cells (Fig. 3.5G) and lower *PIN2* expression. Furthermore, the high expression of *PIN1* and *PIN3* observed in these explants is suggestive of an enhanced basipetal flow of auxin. Another variation in auxin distribution pattern is visible within the callus produced by the explants, with more auxin being accumulated in the WT line treated with TIBA (possibly due to the *PIN2*-increase in upward flow). Precise gradients of auxin distribution occurring within the embryogenic masses are necessary for the proper expression of *WUS* (Su et al., 2009), and the establishment of such gradients might be compromised in WT tissue treated with TIBA. In particular, addition of TIBA to the *35S::BoSTM* explants does not seem to affect the pattern of auxin localization, despite fluctuations in the expression patterns of *PINs*. This discrepancy can be explained by the functional redundancy of *PIN* proteins and their ability to cross-regulate each others and readjust variations in auxin flow due to physiological perturbations (Vieten et al., 2005). The normal auxin distribution observed in TIBA-treated *35S::BoSTM* explants might be the reason for their retained ability to generate embryogenic cells and somatic embryos.

As observed in other systems, including spruce (Hakman et al., 2009), *Brassica juncea* (Liu et al., 1993), and *B. napus* (Ramesar-Fortner and Yeung, 2006) applications of TIBA affect the bilateral symmetry of several WT embryos which display a trumpet-like phenotype, due to abnormalities in the meristematic regions (Fig. 3.2B and C). This phenotype is consistent with a malfunction of *PIN* carriers as fused cotyledons are also visible in *pin* mutant embryos (Furutani et al., 2004). Aida et al. (2002) suggested that *PINs* regulate cotyledon formation through the proper expression of *CUC1*, which demarks the boundaries of the incipient primordia (Fleming, 2005). Fusion of cotyledons

was not observed in the somatic embryos produced by the 35S::*BoSTM* line which form functional meristems even in the presence of TIBA. This observation is difficult to explain but it might also be related to the ability of these embryos to retain a normal distribution of auxin and /or to the high levels of *CUC1*.

In conclusions, this study shows that the introduction of *Brassica STM* in *Arabidopsis* attenuates the inhibitory effect of TIBA on somatic embryogenesis and allows the explants to produce embryogenic cells and normal embryos even in the presence of this inhibitor, which compromises the formation of somatic embryos in the WT line. A proposed reason for this behavior is the ability of the transformed tissue to retain a normal distribution of auxin despite fluctuations in the expression levels of auxin efflux carriers. Proper localization of auxin would be required for the expression of several genes responsible for embryogenic cell acquisition and embryo formation.

**CHAPTER 4: SHOOT FORMATION FROM *ARABIDOPSIS* ROOTS IS
DIFFERENTIALY AFFECTED BY THE OVER-EXPRESSION OF THE
BRASSICA GENES.**

4.1. ABSTRACT

Shoot organogenesis refers to the biological process whereby unipolar organs, i.e. shoots, are generated from somatic cells. In *Arabidopsis* shoot production can be induced by culturing roots on a 2,4-D enriched callus induction medium (CIM), followed by a transfer onto a shoot induction medium (SIM) containing the cytokinin 6-(δ,δ -dimethylallylamino-purine (2iP). The ectopic expression of *SHOOT MERISTEMLESS* (*STM*) isolated from *Brassica napus* (*Bn*), *B. oleracea* (*Bo*), and *B. rapa* (*Br*), as well as *BnZWILLE* (*BnZLL*) encouraged shoot organogenesis from *Arabidopsis* roots. Conversely, the ectopic expression of *BnCLAVATA1* (*BnCLV1*) repressed shoot organogenesis. The STM-stimulation of shoot production was associated to an increased sensitivity of the explants to auxin in the CIM and a transcriptional activation of the cytokinin signal transduction in the SIM, as shown by the up-regulation of the cytokinin receptors *AHK4* and *CKI1* as well as many type-B *ARRs* (known activators of the cytokinin response). This was in contrasts to the line over-expressing *BnCLV1* which exhibited reduced auxin sensitivity in the CIM and a repression of cytokinin signaling in the SIM, as revealed by the down-regulation of the cytokinin receptors *AHK3* and 4, and the induction of type-A *ARRs* (repressors of the cytokinin-mediated signal). The marginal increase in shoot forming ability observed in *BnZLL* over-expressors was associated to a small increase in auxin sensitivity in the CIM, but not to changes in

cytokinin response. Overall these results suggest that the over-expression of genes involved in meristem function has contrasting effects on the number of shoots produced *in vitro*.

4.2. INTRODUCTION

Shoot organogenesis refers to that process whereby *de-novo* shoots are initiated from somatic cells of the explants. Shoot formation in various species can be classified as direct, if it occurs without an intervening callus phase, or indirect, with a callus step. Examples of both have been documented by (Hicks, 1994; Bhojwani and Razdan, 1996). Morphological, physiological, and genetic characterizations of shoot organogenesis have identified distinct phases: competence acquisition, canalization, and morphogenesis (reviewed by Sugiyama, 1999). In the first phase, cells within the explant acquire the ability, i.e. competence, to respond to induction signals. These competent cells are then “canalized” into the shoot developmental program which culminates to “morphogenesis”, that is the formation of shoots. In *Arabidopsis* viable shoots can be produced by several explants, including petals and sepal (Tanaka, 2001), hypocotyls (Campos-Cuevas et al., 2008) and roots (Valvekens et al., 1988). In the latter case young roots are induced to form shoots by a procedure consisting of a preculture onto an auxin-containing medium (callus induction medium, CIM), followed by a transfer onto a cytokinin-containing medium (shoot induction medium, SIM) (Valvekens et al., 1988; Che et al., 2002). Utilization of this efficient system has been very fruitful in examining events related to competence acquisition (Che et al., 2007) and the requirement of specific factors needed for proper shoot formation (Ozawa et al., 1998; Che et al., 2002; Lee et al., 2007).

Judicious selection of hormones is a prerequisite for successful production of shoots *in vitro*. Competence acquisition requires auxins, which are known to activate three distinct classes of genes, the Aux/IAA, GH3, and SAUR (Abel and Theologis, 1996; Berleth et al., 2004). Microarray studies conducted by Che et al. (2002) revealed the existence of several members of the Aux/IAA family which are up-regulated in the auxin-containing CIM and considered to be possible candidates for the acquisition of competence during shoot organogenesis. Initiation of shoot formation in the SIM is encouraged by applications of cytokinins and relies on profound changes in cytokinin perception and signaling (Che et al., 2002). Genetic dissection of the cytokinin pathway has identified several components, including sensor histidine kinases (AHKs), histidine phosphotransmitters (AHPs) and response regulators (ARRs). The *Arabidopsis* genome contains more than 30 ARRs which are grouped into two distinct types based on defined structural features. While the A-type ARRs contain a receiver domain and a very short C-terminal extension, B-Type ARRs are characterized by a longer C-terminal extension which contains regions acting as transcription regulators (Sakai et al., 2000). Genetic evidence indicates that type B ARRs are transcription activators of cytokinin-induced genes, as well as type A ARRs which act as feed-back repressors of the initial cytokinin transduction pathway (Hwang and Sheen, 2001). Over-expression of several B-type ARRs has been shown to induce shoot formation *in vitro* in the absence of exogenous cytokinin (Hwang and Sheen, 2001; Osakabe et al., 2002), thereby confirming the key role played by these regulators during *in vitro* organogenesis.

Induction of shoots involves considerable genetic re-programming, the magnitude of which has been estimated by several microarray experiments (Che et al., 2002;

Yanghuan et al., 2009). Ectopic expression studies have also been employed to analyze the molecular mechanisms underpinning the process. These include the over-expression of *WUSCHEL*, *WOX5*, *CALRETICULUM 1B*, and *BABYBOOM1* (Rashid et al., 2007; Jin et al., 2007; Srinivasan et al., 2007; Passarinho et al., 2008; Rashid and Kyo, 2008; Rashid and Kyo, 2010), with no information related to the introduction of other shoot meristem genes such as *SHOOT MERISTEMLESS (STM)*, *CLAVATA 1 (CLV1)* and *ZWILLE (ZLL)*, which play a key role in the formation and maintenance of the apical pole.

In Chapter 2, it was shown that the introduction of the *Brassica* orthologs of the *Arabidopsis STM*, *CLV1*, and *ZLL* have different effects on the generation of embryogenic cells and somatic embryos. While the ectopic expression of *STM* encouraged the formation of cells with embryonic potential, over-expression of *BnCLV1* repressed the production of somatic embryos. No effects were observed by over-expressing *BnZLL-1* and -2. In the same study, it was shown that the beneficial effect of the *Brassica STM* was possibly mediated by an increased sensitivity to the inductive signal, i.e. auxin, and a higher expression of *WUS*, which is a key component for embryogenic competence acquisition.

The objective of this work is to investigate if (1) the ectopic expression of the *Brassica STM*, *CLV1*, *ZLL-1* and -2 also influences shoot organogenesis from *Arabidopsis* root explants, and (2) the introduction of these genes is associated to transcriptional changes in auxin and cytokinin signaling.

4.3. MATERIALS AND METHODS

4.3.1. Plant material and transformation

Generation and characterization of *Arabidopsis* plants transformed with *STM* isolated from *Brassica oleracea* (BoSTM), *Brassica napus* (BnSTM), *Brassica rapa* (BrSTM), *BnCLV1*, *BnZLL-1* and -2 were described in the previous Chapters (2-3).

4.3.2. Shoot organogenesis

Shoot organogenesis from *Arabidopsis* root explants was induced exactly as described by Valvekens et al. (1988). Briefly, seeds were sterilized in 10% bleach for 10 min, rinsed 3 times in sterile water, and germinated on ½ MS solid medium for 7 days. The germinated seeds were then transferred on ½ MS liquid medium and incubated in the dark for 7 days on a shaker (150 rpm) at 27°C. The roots were harvested, cut in small (0.5 cm) segments, and placed on callus induction medium (CIM) containing 2,4-D (0.5 mg/L). After 4 days, roots and the newly formed callus were transferred on shoot induction medium (SIM) supplemented with 5 mg of the cytokinin 6-(δ,δ-dimethylallylamino)-purine (2iP) for 3 weeks after which shoots were counted.

Analyses of auxin and cytokinin requirements were performed by changing the levels of 2,4-D or 2iP in the CIM and SIM respectively and assessing the number of shoots formed.

4.3.3. Gene expression studies

Expression studies of genes involved in auxin and cytokinin signaling were performed by quantitative qRT-PCR exactly as described in Chapter 2 using the primers

listed in Appendix 7. The selection of genes was based on the work of Che et al. (2002) who identified 8 auxin responsive genes (IAA1, 5, 8, 9, 11, 19, 20 and 29) which were up-regulated in the CIM and several genes involved in cytokinin signaling which showed distinct expression profiles during the organogenic process. These studies were performed using the following representative lines: WT, *BoSTM* (L7), *BnSTM* (L1), *BrSTM* (L23), *BnCLV1* (L3), *BnZLL-1* (L1) and *BnZLL-2* (L5).

4.3.4. RNA in situ hybridization studies

Localization of *WUS* in the root explants was conducted as outlined in Chapter 2

4.3.5. Statistical analysis

This was done as outlined in Chapter 2.

4.4. RESULTS

4.4.1. Shoot formation is altered by the ectopic expression of the *Brassica* genes

The ectopic expression of the *Brassica* genes affected the number of shoots originating from *Arabidopsis* root explants (Fig. 4.1A). The introduction of *Brassica STM* (*BoSTM*, *BnSTM*, and *BrSTM*) favored the organogenic process with the most pronounced improvements observed in tissue expressing the *B. napus STM*, which showed a 400% increase in shoot formation. Unlike WT tissue, where the shoots emerged along the root surface as separate entities, clusters of shoots were often visible in *35S::BnSTM* explants (Fig. 4.1B). A similar increase in shoot organogenesis, although to a lower extent, was also obtained in *BnZLL-1* and -2 over-expressors. All five

35S::*BnCLV1* lines analyzed in this study exhibited a reduced ability to produce shoots in culture (Fig. 4.1A). No apparent alterations in shoot morphology were observed among the transformed lines.

The different response in culture was also investigated by following the expression and localization patterns of *WUSCHEL* (*WUS*), which is a reliable marker of shoot formation both *in vivo* (Mayer et al., 1998) and *in vitro* (Gordon et al., 2007). In the WT line *WUS* transcripts were induced in the cytokinin-rich SIM reaching a maximum level at the end of the culture period (Fig. 4.2A). The introduction of *Bo*, *Br*, and *BnSTM* resulted in higher levels of *WUS* mRNAs in the CIM and a rapid activation of this gene once the tissue was transferred on the SIM. The ectopic expression of *BnZLL-1* and -2 did not affect *WUS* expression whereas a decline in *WUS* transcript levels were measured in *BnCLV1* over-expressors cultured on the SIM (Fig. 4.2A). Compared to the WT line, where *WUS* was localized in the apical and a few sub-apical cells of the explants (Fig. 4.2B₁), the localization of this gene in the lines over-expressing *BnSTM* was extended to a larger domain (Fig. 4.2B₂). In the 35S::*BnCLV1* line only a few cells of the explant expressed *WUS* (Fig. 4.2B₃). No differences in *WUS* localization patterns were observed between WT and *BnZLL-1* and -2 lines.

4.4.2. Auxin and cytokinin requirements for shoot formation

The requirement of the tissue for exogenously supplied auxin was examined by culturing the root explants on a CIM with different levels of 2,4-D.

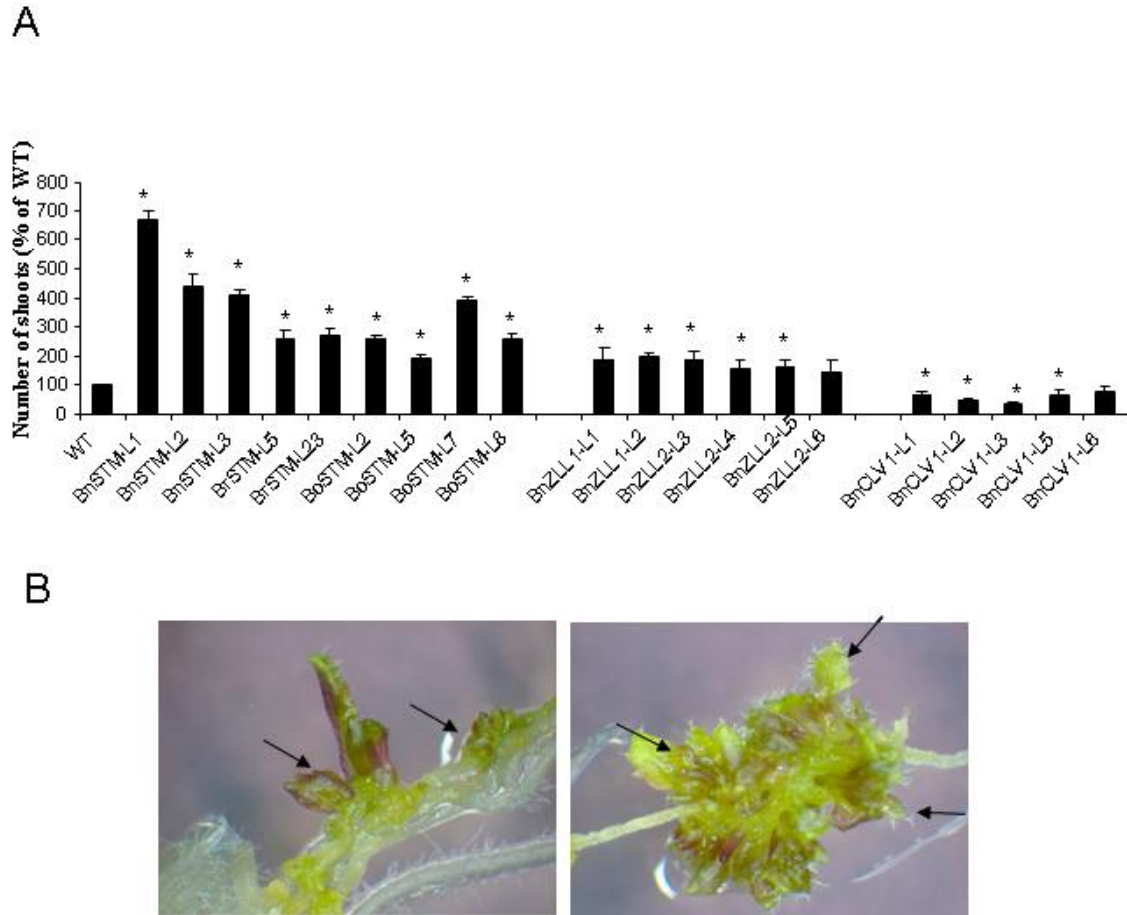


Figure 4.1. (A) Effects of the ectopic expression of *BnSTM*, *BoSTM*, *BrSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* on the number of shoots produced by *Arabidopsis* root explants. Values (n=3) are expressed as a percentage of control. Statistically significant values ($P \leq 0.01$) are indicated by the letters above the bars. **(B)** Compared to WT explants (left panel) in which shoots (arrows) formed individually along the surface of the root, the introduction of *STM* (right panel) resulted in an increased number of shoots which clustered together (arrows). The number of shoots per centimeter of root was counted at day 25 of culture. The length of root explants used in each replicate was about 25 cm. WT formed 3.4 shoots/cm explant.

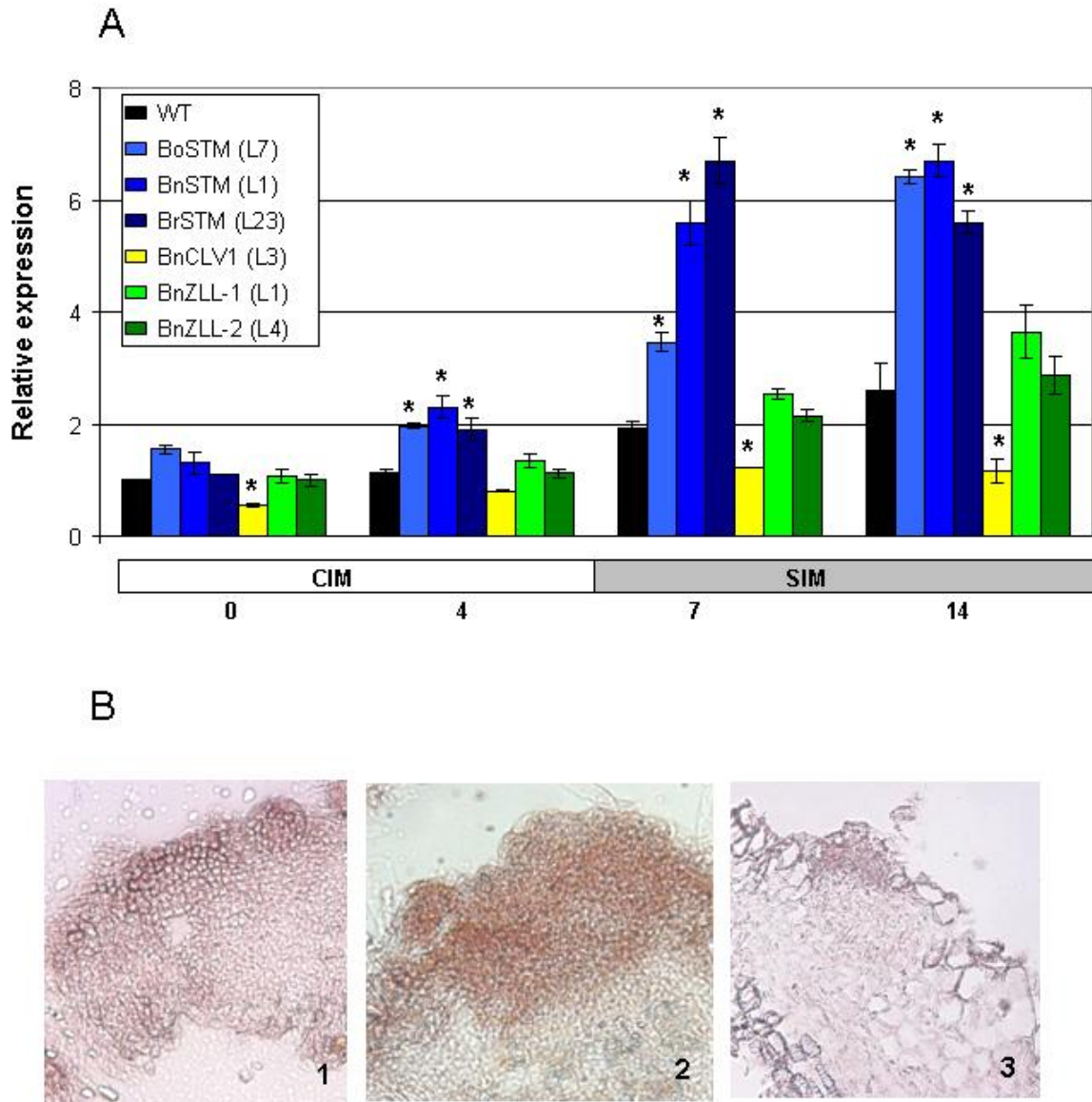


Figure 4.2. (A) Effects of the ectopic expression of *BnSTM*, *BoSTM*, *BrSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* on the expression of *WUSCHEL* during culture on the callus induction medium (CIM) and shoot induction medium (SIM). Values $\pm$ SE ($n=3$) are shown relative to the WT value at day 0 set at 1. Statistically significant values ($P \leq 0.01$) are indicated by the * above the bars. **(B)** Localization of *WUSCHEL* transcripts by RNA in situ hybridization at the end of the callus induction period in the WT line (**B₁**) and in lines over-expressing *BnSTM* (**B₂**) and *BnCLV1* (**B₃**).

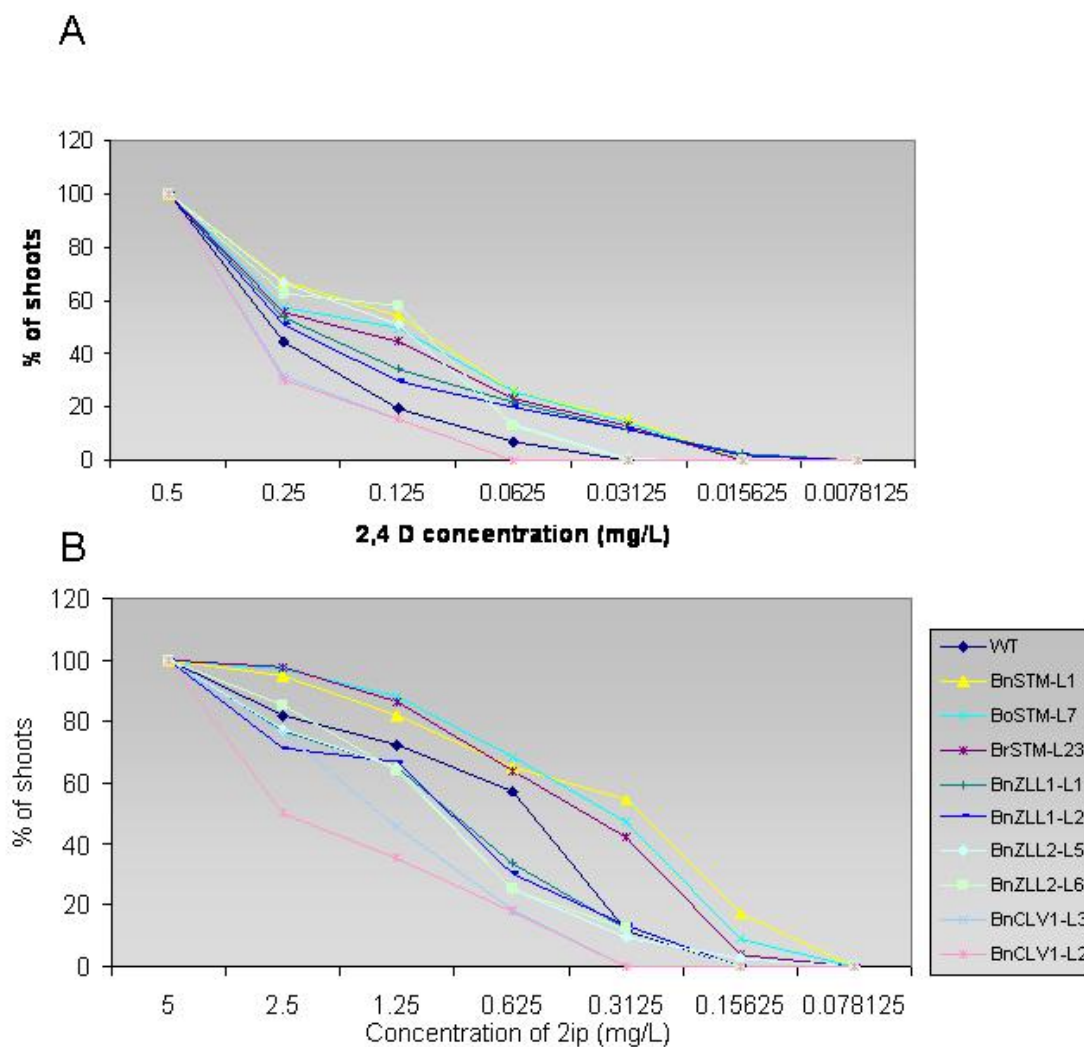


Figure 4.3. Effects of different levels of the auxin 2,4-D in the callus induction medium **(A)** and the cytokinin 2iP in the shoot induction medium **(B)** on the percentage of shoots produced from *Arabidopsis* roots.

In WT tissue, shoot formation was halved when the concentration of 2,4-D was reduced to 0.25 mg/L (Fig. 4.3A). A more severe reduction of this growth regulator significantly compromised shoot organogenesis as no shoots were observed at a 2,4-D concentration lower than 0.06 mg/L. Overall all lines over-expressing the *Brassica* (*Bo*, *Bn*, and *Br*) *STM* were more sensitive to auxin. About 50% of shoots (compared to 20% in the WT tissue) were still produced when the level of 2,4-D was lowered to 0.12 mg/L. Shoot production was still obtained at those auxin levels which precluded morphogenesis in the WT line. A similar response to that described for the *STM* over-expressors was also observed in the *35S::ZLL-1* and *-2* lines. The introduction of *BnCLV1* decreased sensitivity to 2,4-D as no shoots were formed when the concentration of this growth regulator was lower than 0.12 mg/L (Fig. 4.3A).

A decline in shoot formation in the WT tissue was observed when the level of 2iP in the SIM was reduced below the optimal concentration (Fig. 4.3B). A similar response (with the exception of 0.6 mg/L 2iP) was also obtained in lines over-expressing *BnZLL-1* and *-2*. *STM* over-expressors were still able to produce a substantial number of shoots with low cytokinin levels (below 0.6 mg/L 2iP), whereas an opposite profile characterized the *35S::BnCLV1* lines. In the two lines analyzed (*35S::BnCLV1* line 2 and 3) the production of shoots was severely reduced at a 2iP concentration of 0.6 mg/L (Fig. 4.3B).

4.4.3. Auxin response during shoot organogenesis

The expression levels of several *Aux/IAA* genes which were identified as being involved in the auxin response during shoot formation *in vitro*, and strictly related to the

presence of 2,4-D in the CIM (Che et al., 2002) were measured at different days in culture. As shown in Fig. 4.4, all genes (*IAA1*, 5, 8, 9, 11, 19, 20 and 29) were activated in WT tissue cultured on the auxin-rich CIM, and then repressed during the subsequent transfer onto the SIM. Overall four different expression patterns were observed (Fig. 4.4 and 4.8). Some genes (*IAA 5 and 9*) displayed similar expression profiles among lines. The expression of others (*IAA 8, 11, and 19*) was induced in the CIM especially in lines over-expressing the *Brassica STM* and *ZLL* (although the expression of *IAA 19* in the *35S::BnZLL-2* line was not statistically different than that of WT tissue), whereas it remained low in the line over-expressing *BnCLV1*.

Different expression profiles were observed for *IAA 20* which was induced only in the *BnZLL* over-expressors, and *IAA29* which was induced in lines over-expressing the *Brassica STM* (Fig. 4.4).

4.4.4. Cytokinin response during shoot organogenesis

Since cytokinins play a key role during the initiation of shoot formation the expression patterns of several genes encoding putative cytokinin receptors (*AHK3*, 4, and *CKI1*), type-A response elements (*ARR 4, 5, 7, 15, and 16*), and type-B response elements (*ARR1, 2, 10, 12, and 13*), previously described in relation to shoot organogenesis (Che et al., 2002) were measured. Of the three cytokinin receptors, only *AHK4* and *CKI1* showed a pronounced activation in the SIM (Fig. 4.5). Compared to WT, both genes were up-regulated in lines ectopically expressing the *Brassica STM*, with *CKI1* peaking at day 7 and *AHK4* at day 14 in the SIM. Lower levels of *AHK3*, *CKI1* and *AHK4* were generally detected in the *35S::BnCLV1* line (Fig. 4.5).

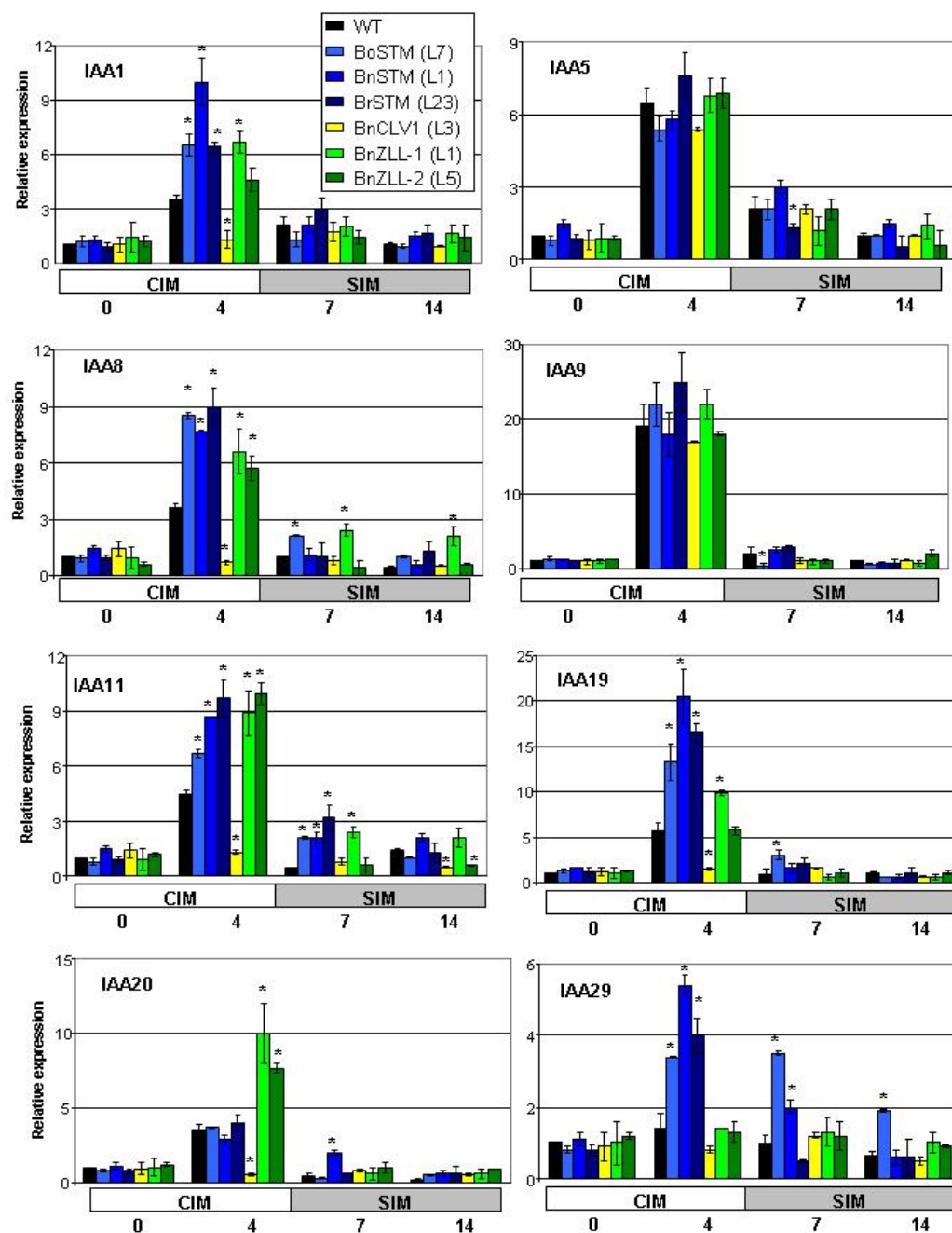


Figure 4.4. Effects of the ectopic expression of *BnSTM*, *BoSTM*, *BrSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* on the expression of several members of the *Aux/IAA* family in the callus induction medium (CIM) and shoot induction medium (SIM). Values $\pm$ SE ($n=3$) are shown relative to the WT value at day 0 set at 1. Statistically significant values ($P \leq 0.01$) within each day in culture are indicated by * above the bars.

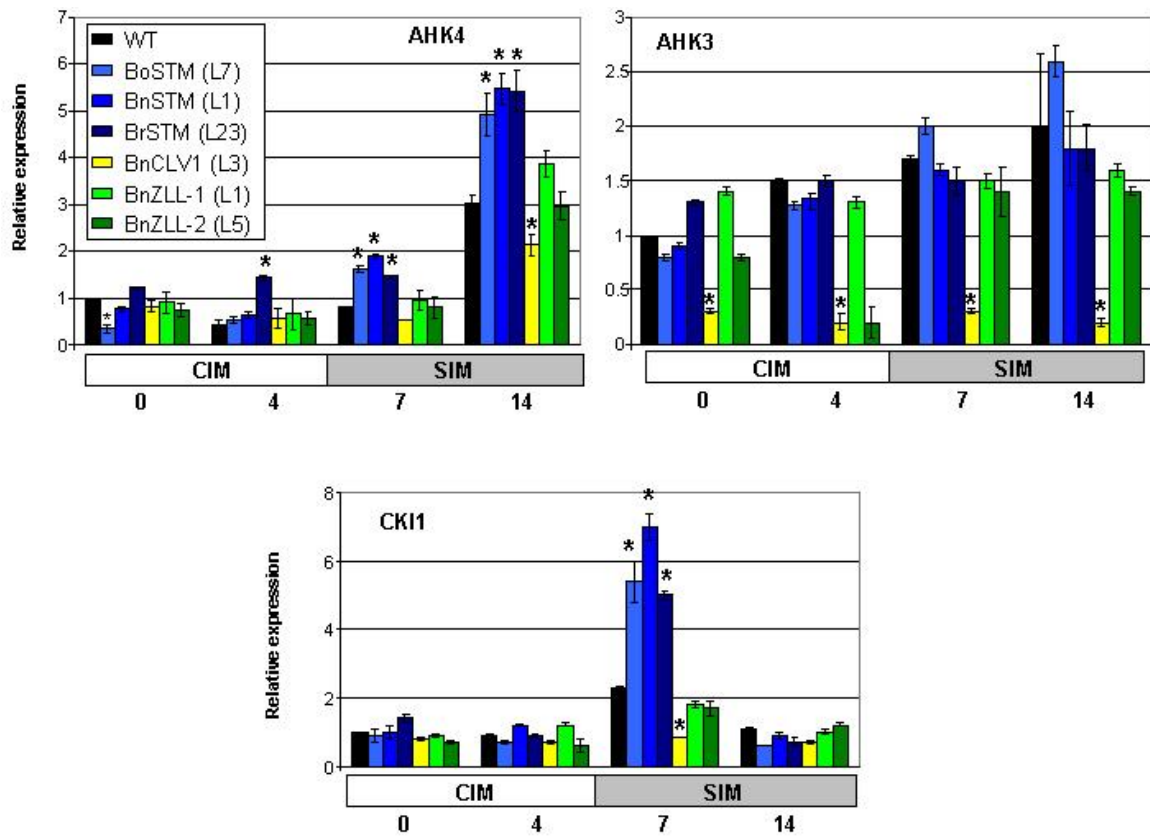


Figure 4.5. Effects of the ectopic expression of *BnSTM*, *BoSTM*, *BrSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* on the expression of three histidine kinase genes involved in cytokinin perception in the callus induction medium (CIM) and shoot induction medium (SIM). Values $\pm$ SE (n=3) are shown relative to the WT value at day 0 set at 1. Statistically significant values ($P \leq 0.01$) within each day in culture are indicated by * above the bars.

Among type-A *ARRs* the expression profiles of *ARR 4* and *16* did not show any pronounced fluctuation throughout the culture period (CIM+SIM) within each line (Fig. 4.6). Higher levels of *ARR 4* transcripts were measured in the *35S::BnCLV1* line. The remaining *ARRs* (*ARR5*, *7*, and *15*) were induced in the SIM. Compared to WT, the introduction of the *Brassica STM* repressed the expression of the three genes, whereas an opposite trend was obtained in the line over-expressing *BnCLV1*. No major differences in the expression profiles of *ARR5*, *7*, and *15* were observed between the WT and the *335S::ZLL-1* and *-2* lines (Fig. 4.6).

The expression levels of several type-B *ARRs* were also measured during shoot organogenesis. Within each line, the expression profile of *ARR13* did not show profound changes during the culture period (Fig. 4.7). An overall induction of *ARR1*, *2*, *10*, and *12* occurred in the SIM, especially in the lines over-expressing the *Brassica STM*. The lowest transcript levels of *ARR2*, *10* and *12* were observed in the *35S::BnCLV1* line.

A simplified diagram summarizing the expression patterns of the genes investigated in this study is shown in Fig. 4.8.

4.5. DISCUSSION

The developmental events culminating in the formation of shoots *in vitro* are exemplified in *Arabidopsis* through a two step process. Root explants are first cultured on CIM for 4 days and subsequently transferred on SIM for 14 days. As shown by Cary et al. (2002), explants acquire competence on the CIM and commitment to form shoots on the SIM. At the end of the culture period, fully developed shoots emerge from the root surface (Fig. 4.2).

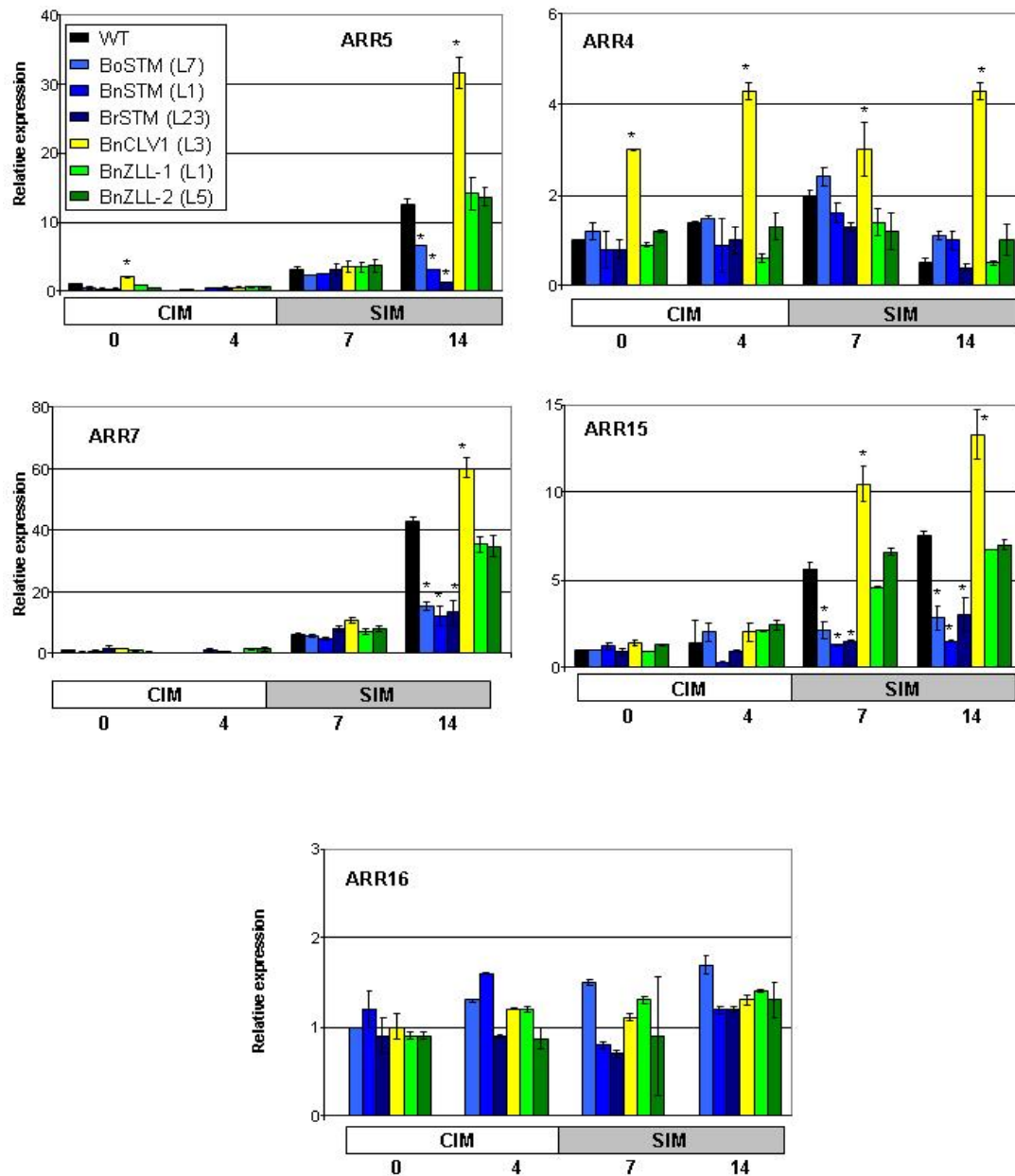


Figure 4.6. Effects of the ectopic expression of *BnSTM*, *BoSTM*, *BrSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* on the expression of several type-A *Arabidopsis* response regulators (ARRs) in the callus induction medium (CIM) and shoot induction medium (SIM). Values $\pm$ SE ($n=3$) are shown relative to the WT value at day 0 set at 1. Statistically significant values ($P \leq 0.01$) within each day in culture are indicated by * above the bars.

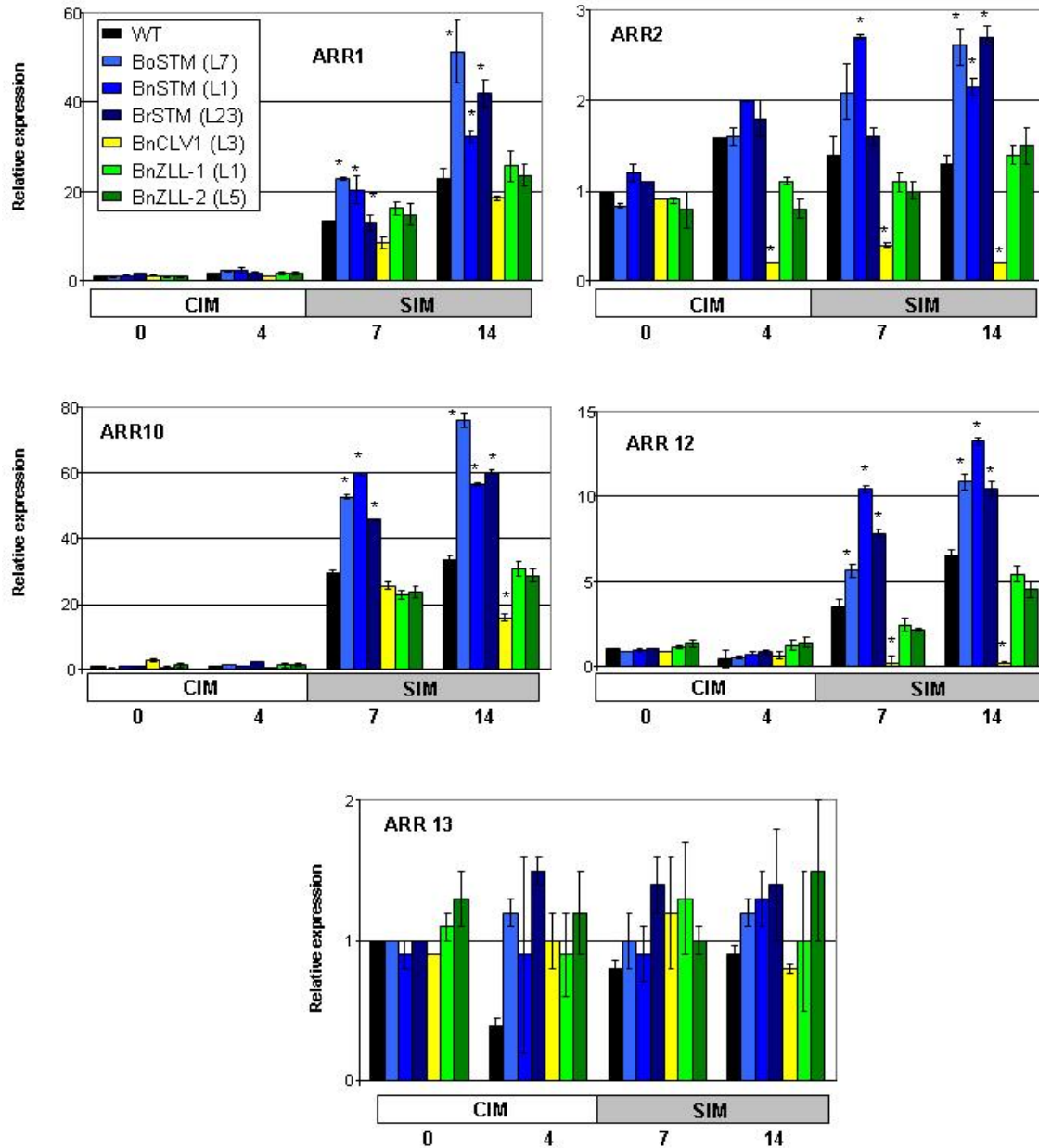


Figure 4.7. Effects of the ectopic expression of *BnSTM*, *BoSTM*, *BrSTM*, *BnCLV1*, *BnZLL-1* and *BnZLL-2* on the expression of several type-B *Arabidopsis* response regulators (ARRs) in the callus induction medium (CIM) and shoot induction medium (SIM). Values $\pm$ SE ($n=3$) are shown relative to the WT value at day 0 set at 1. Statistically significant values ($P \leq 0.01$) within each day in culture are indicated by * above the bars.

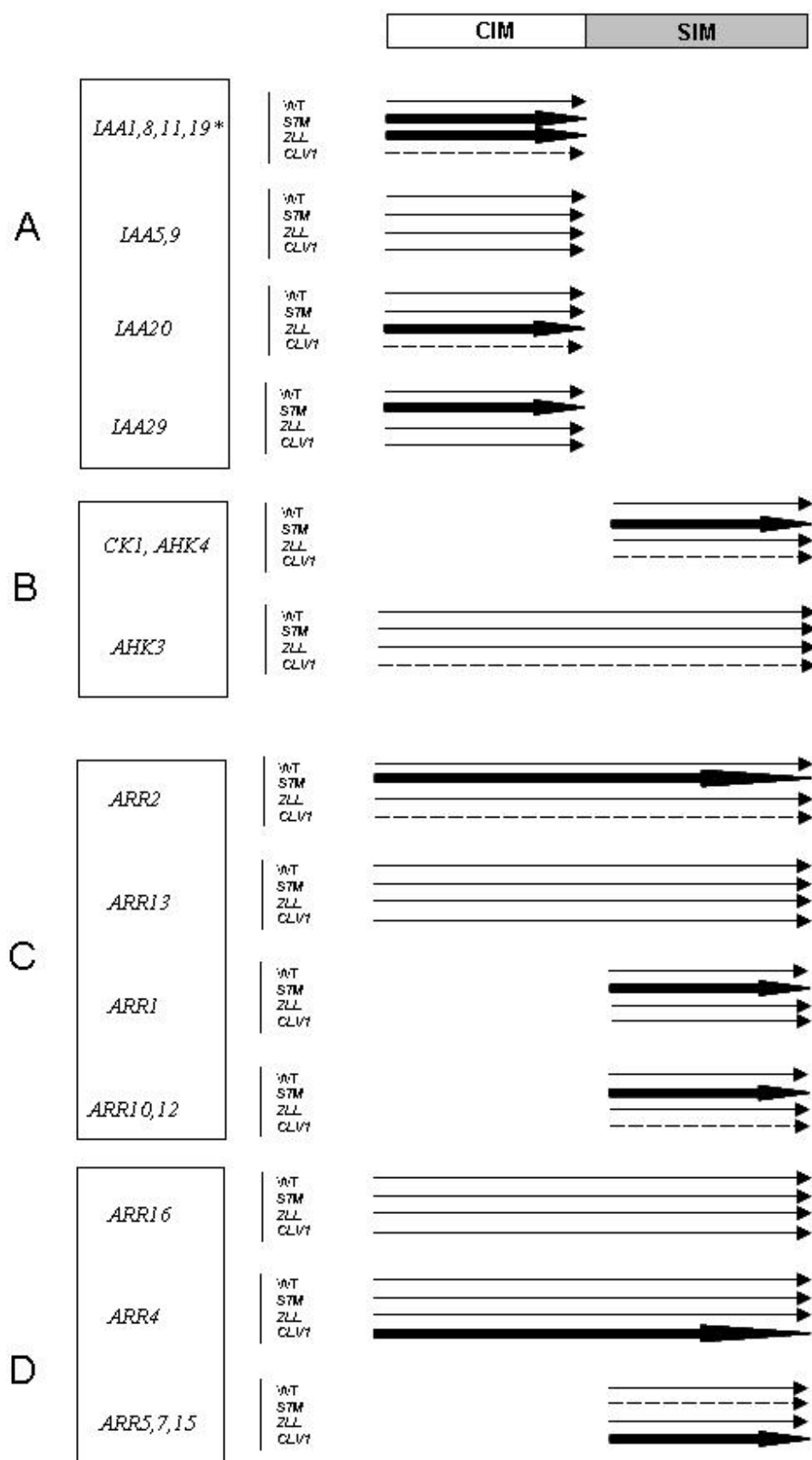


Figure 4.8. Schematic representation of changes in profiles of several *Aux/IAA* members **(A)**, putative cytokinin receptors **(B)**, type-B *ARRs* **(C)** and type-A *ARRs* **(D)** during organogenesis in tissue transformed with the *Brassica* genes. Values of *BnZLL-1* and -2 are combined to facilitate the interpretation of the results. CIM, callus induction medium; SIM, shoot induction medium. Up-regulation or down-regulation compared to WT is indicated by large or broken arrows respectively. * response observed only in *35S::BnZLL-1* explants.

In previous studies (Chapter 2) it has been reported that *Brassica* genes (*BnSTM*, *BnCLV1*, *BnZLL-1* and -2), orthologous to the *Arabidopsis* *STM*, *CLV1*, and *ZLL* involved in the formation and maintenance of the SAM (Barton and Poethig, 1993; Clark et al., 1997; Moussian et al., 1998), have different effects on the generation of embryogenic cells and somatic embryos. While the ectopic expression of the *Brassica* *STM* encourages the acquisition of embryogenic fate and embryo development, *BnCLV1* has an opposite effect (Chapter 2). The function exercised by these two genes during *in vitro* morphogenesis seems to be retained during organogenesis as shoot formation is enhanced in all lines over-expressing the *Brassica* *STM* and inhibited in the *35S::BnCLV1* lines (Fig. 4.1A). The *STM*-mediated redirection of cell fate is not a new concept since ectopic shoot meristems from vegetative tissue were observed in *Arabidopsis* plants over-expressing members of the *kn-1* like homeobox gene family which include *STM* (Chuck et al., 1996), and tobacco plants ectopically expressing the maize *STM* (Sinha et al., 1993). The *BnCLV1*-inhibition of shoot formation is however a novel finding which suggests that these two genes (*STM* and *CLV1*) fulfilling opposite functions in the SAM *in vivo* (Brand et al., 2002) may also have a competitive roles during shoot initiation *in vitro*. What is also relevant is the improvement of the organogenic process observed in the *35S::BnZLL-1* and -2 lines.

4.5.1. The auxin response

The opposite behavior in culture of the different lines might be related to their requirements and response to exogenously applied auxin. Lines able to produce many shoots, i.e. *STM* and *ZLL* over-expressors, were more sensitive to 2,4-D, whereas those

with reduced shoot formation, i.e. *CLV1* over-expressors, were less sensitive to applications of this growth regulator (Fig. 4.3A). These responses correlated with different expression profiles of Aux/IAA genes, which together with the GH3 gene family and the SAUR gene family, participate in the auxin response (Abel and Theologis, 1996). The Aux/IAA genes encode small proteins that interact with each other and with auxin-response factors (ARFs), thereby modulating their activity and ultimately initiating auxin signaling (reviewed by (Berleth et al., 2004). Due to their quick induction (within minutes) by exogenously supplied auxin, members of the *Aux/IAA* family are often referred to as “early auxin-induced” genes (Abel and Theologis, 1996). All the *Aux/IAA* genes measured in the present study have been suggested to play an important role during shoot organogenesis due to their transcriptional activation in the auxin-rich CIM (Che et al., 2002). Of interest, the improved shoot-forming capacity observed in lines over-expressing the *Brassica STM* and *ZLL* is associated to higher expression levels of several *Aux/IAA* (*IAA1*, 8, 11, 19, and 20) compared to the WT (Fig. 4.4 and 4.8). Opposite expression profiles were measured in the *BnCLV1* line (Fig. 4.4) exhibiting reduced shoot formation. It is therefore apparent that the different behavior of the transformed lines may be a consequence of their altered responsiveness to 2,4-D, which confers competence to the shoot-induction signal (cytokinin).

All *STM* over-expressing *Arabidopsis* lines, showing improved organogenesis and increased sensitivity to auxin, exhibited higher expression level of *WUS*, which is localized in extended domains of the root explants. These characteristics were also associated to the enhanced somatic embryo-forming capacity observed in lines ectopically expressing the *Brassica STM* (Chapter 2). In that context it was discussed that

the expression of *WUS*, which is involved in the formation and maintenance of the organizing center in the SAM (Mayer et al., 1998), is regulated by the PIN-induced auxin gradient within the explants and demarks the formation of embryogenic cells in culture (Kurdyukov et al., 2009; Su et al., 2009). It is therefore suggested that the introduction of the *Brassica STM* encourages the formation of embryogenic cells during somatic embryogenesis and organogenic cells during shoot organogenesis through similar *WUS*-mediated mechanisms. However the fact that *WUS* expression is unaltered in the *35S:BnZLL-1* and *-2* lines, which showed a similar auxin response and shoot forming ability to those of the *STM* over-expressors, indicates the existence of more complex regulatory mechanisms during shoot organogenesis.

4.5.2. The cytokinin response

While auxin is required for the acquisition of organogenic competence, cytokinin is the inductive signal which re-directs cells within the explants towards a “shoot fate” pathway. Cytokinin receptors in *Arabidopsis* are hybrid kinases similar to the two-component systems containing an extracellular cytokinin-binding domain and two cytoplasmic regions: the transmitter and receiver domains (To and Kieber, 2008). While *AHK3* and *4* exhibit these features, *CKI1* lacks the receiver domain (Hwang et al., 2002) and its original function as a receptor (Kakimoto, 1996) is being questioned (Che et al., 2002). This gene was however included in this study since its over-expression was sufficient to induce shoot formation in culture in a cytokinin-independent manner (Kakimoto, 1996). The expression of *CKI1* peaks at day 7 in the SIM and this is consistent with other studies (Che et al., 2002) relating this gene to shoot commitment (a

developmental phase in which shoots are produced even in the absence of exogenous cytokinin) which also occurs around the same day (Kakimoto, 1996). At day 7 (SIM) the upregulation of this gene in the *STM* over-expressing lines and its repression in the *35S::BnCLV1* line compared to WT tissue correlate to the shoot forming-abilities of the explants, and suggest the existence of differences in cytokinin perception among lines. A similar trend but at the end of the culture period (day 14) was also observed for *AHK4* (also referred to as *WOODENLEG 1* and *CYTOKININ RESPONSE 1*), which is the first characterized cytokinin receptor able to confer cytokinin responsiveness in yeast (Yamada et al., 2001), and critical for shoot formation *in vitro* (Inoue et al., 2001). The developmental up-regulation of *CKI1* and *AHK4* (the former at day 7 and the latter at day 14 in SIM) indicated that precise transcriptional changes in cytokinin perception must occur during shoot organogenesis. A failure to execute such changes, as observed in the line over-expressing *BnCLV1* may compromise the response in culture. The other receptor *AHK3* (Suzuki et al., 2001) does not show any transcriptional change during shoot organogenesis.

Cytokinin signaling relies on the induction of type-A response regulators (ARRs), feedback repressors which reduce cytokinin response, and type-B ARRs, activators of cytokinin-induced genes (Imamura et al., 1998; Hwang et al., 2002). While the reduced shoot forming-ability of the *35S::BnCLV1* line is linked to the up-regulation of type-A ARRs, the improved shoot organogenesis of *STM* over-expressors is associated to the induction of type-B ARRs. The introduction of *BnCLV1* increases the expression of *ARR 4*, *5*, *7* and *15*, well characterized inhibitors of the cytokinin response (To et al., 2004; To et al., 2007). In *arr4* and *5* mutants both root elongation and lateral root formation were

more sensitive to cytokinin inhibition and these effects were reversed by the introduction of the WT, functional gene (To et al., 2004). In the same study it was also demonstrated that both mutants were able to produce shoots in culture at low levels of cytokinin. Altered cytokinin response was also observed in plants over-expressing *ARR 7* and *15*. Using genome-wide expression profiling, Lee et al. (2007) demonstrated that the expression of many early cytokinin-induced genes involved in several biological processes was repressed in *ARR7* over-expressors. A similar conclusion was reached by Kiba et al. (2003) who observed a reduced sensitivity to exogenous cytokinin in root elongation assays and *in vitro* shoot formation in *ARR15* over-expressors. During shoot organogenesis *ARR5* expression increases in those undifferentiated cells of the callus destined to produce shoots but it decreases rapidly during shoot initiation (Che et al., 2002). The high expression of *ARR5* at the end of the culture period in the line over-expressing *BnCLV1* suggests that cells within the explants are able to de-differentiate but fail to organize a shoot meristem. The involvement of type-A ARRs during shoot organogenesis was also demonstrated *in vivo* where formation and maintenance of the SAM require the WUS-repression of several type-A ARRs (*ARR5*, *7*, and *15*). This regulation might also operate *in vitro* as shown by the negative correlation between the expression of *WUS* and several type-A ARRs in lines over-expressing the *Brassica STM* and *CLV1* (Fig. 4.2 and 4.8).

Many type-B ARRs, genetic activators of cytokinin signaling (To and Kieber, 2008) are promoted by the introduction of the *Brassica STM* and repressed in the *BnCLV1* line. These include *ARR1* and *2* which have been demonstrated to activate cytokinin responses in the absence of exogenous cytokinin when over-expressed in

protoplasts (Sakai et al., 2001), and *ARR10* and *12* which confer sensitivity to cytokinin and are required for normal shoot growth (Argyros et al., 2008). A direct involvement of these ARRs during shoot organogenesis has also been demonstrated. (Hwang and Sheen, 2001) showed that elevated *ARR2* expression is sufficient to promote shoot formation *in vitro* in the absence of cytokinin (Hwang and Sheen, 2001). Abnormal shoots, characterized by reduced expression of *CYCD3;1*, a cytokinin induced D-type cyclin (Riou-Khamlichi et al., 1999), were observed in *arr-1 arr10 arr12* triple mutants (Argyros et al., 2008). From these observations, it appears that the differential expression of these ARRs between lines over-expressing the *Brassica STM* and *CLV1* may account for the differences in shoot-forming capacity (Fig. 4.1).

In conclusion, shoot formation was encouraged by the introduction of the *Brassica STM* and *ZLL* and inhibited by the introduction of *BnCLV1*. The *STM*-stimulation of shoot production was associated to an increased sensitivity of the explants to auxin in the CIM and a transcriptional activation of the cytokinin signal transduction in the SIM, as shown by the up-regulation of the cytokinin receptors *AHK4* and *CKI1* as well as many type-B ARRs (known activators of the cytokinin response). This was in contrast to the line over-expressing *BnCLV1* which exhibited reduced auxin sensitivity in the CIM and a repression of cytokinin signaling in the SIM, as revealed by the down-regulation of the cytokinin receptors *AHK3* and *4*, and the induction of type-A ARRs (repressors of the cytokinin-mediated signal). The marginal increase in shoot forming ability observed in *ZLL* over-expressors was associated to a small increase in auxin sensitivity in the CIM, but not to changes in cytokinin response.

CHAPTER 5: FURTHER CHARACTERIZATION OF THE *BRASSICA* GENES DURING MICROSPORE-DERIVED EMBRYOGENESIS IN *B. NAPUS* AND POST EMBRYONIC DEVELOPMENT IN *ARABIDOPSIS*.

5.1. ABSTRACT

Plant morphology is mainly controlled by the activity of the shoot apical meristem (SAM). This work investigates the localization patterns of the *Brassica* genes, *BnSTM*, *BnCLV1*, *BnZLL-1* and -2, during microspore-derived embryogenesis in *Brassica napus* and the effects of their over-expression during *Arabidopsis* development. RNA-in situ studies indicate that all genes are expressed within the SAM of the developing MDEs and their localization domains are altered by treatments which affect SAM architecture and functionality. These alterations were observed early during embryogenesis prior to any sign of SAM formation, thereby suggesting that these genes can be used as markers to estimate meristem quality in culture. Their ectopic expression causes profound changes in the phenotype of *Arabidopsis* plants. The introduction of *STM* isolated from *Brassica oleracea* (*Bo*), *B. rapa* (*Br*) and *B. napus* (*Bn*) increased the size of the embryonic SAM, resulting in faster meristem activation at germination possibly through the expression of other SAM-regulatory genes, induced the formation of ectopic meristems and produced leaves with altered morphology. This was in contrast to lines over-expressing the *Brassica CVL1* which produced embryos with smaller SAMs and with a delayed activation of the shoot at germination. A premature termination of the apical meristem was also observed in these lines. No apparent morphological differences were noticed during development of *BnZLL* over-expressing lines. These phenotypic changes are

discussed in relation to the different hormone profiles measured in the transformed lines.

5.2. INTRODUCTION

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; 2008; 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 over 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. Like 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) (reviewed by (Yeung and Stasolla, 2000). Reduced post-embryonic growth was observed in those MDEs which develop abnormalities within the apical pole, including vacuolation and differentiation of meristematic cells (Belmonte et al., 2006). 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 GSH synthesis (Griffith and Meister, 1979), induced the formation of functional “zygotic-like” meristems able to reactivate at high frequency at germination (Belmonte et al., 2006). On the contrary, complete loss of SAM functionality was observed when 2,3,5-triiodobenzoic acid (TIBA), an auxin transport inhibitor, was added to the medium (Ramesar-Fortner and Yeung, 2006). These studies clearly show the relevance of the *Brassica in vitro* system to study meristem

formation and maintenance, although they do not provide solid evidence on how these alterations correlate to the activity of SAM “marker genes”. Therefore, the characterization of SAM regulatory genes in *Brassica* is critical to expand our knowledge on meristem function.

The *Arabidopsis* shoot apical meristem (SAM) is regulated by a set of key genes, including *SHOOT MERISTEMLESS (STM)*, *CLAVATA 1 (CLV1)*, *WUSCHEL (WUS)*, and *ZWILLE (ZLL)*, which establish and maintain the characteristic layering and zonation patterns of the apical pole (reviewed by Gross-Hardt and Laux, 2003; Tahir and Stasolla, 2006). The *Arabidopsis* STM is a member of the class-1 KNOX homeodomain containing proteins (Long et al., 1996; Janosevic and Budimir, 2006) required for the formation and maintenance of the SAM by retaining a pool of undifferentiated cells at the apical pole (Barton and Poethig, 1993). 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 (Endrizzi et al., 1996). Proper expression of *STM* within the SAM is ensured by *ZLL* (Moussian et al., 1998), a member of the ARGONAUTE (AGO) family, involved in the formation of RNA-induced silencing complexes in both animal and plants (Cerutti et al., 2000). 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 (McConnell and Barton, 1995; Moussian et al., 1998; Lynn et al., 1999). Proper maintenance of SAM activity also relies on the feedback mechanism of the WUS-CLV signaling. *WUSCHEL* is a homeobox gene encoding a transcription factor which regulates the “organizing

center” of the SAM by maintaining a reservoir of undetermined cells (Mayer et al., 1998). The expression of *WUS* is repressed by the CLV signaling upon the interaction of CLV3 with the receptor kinase complex (CLV1 and CLV2, reviewed by Dodsworth, 2009). A mutation of any *CLV* members, including *CLV1*, disrupts the balance between cell division and differentiation within the SAM resulting in larger meristems and abnormal expression pattern of *WUS* (Schoof et al., 2000). According to Clark et al. (1996) the role of CLV1 is to promote the differentiation of the meristematic cells thereby reducing the pool of undifferentiated stem cells.

The molecular regulation of the SAM is mediated by the activity of plant growth regulators. Independent studies show that KNOX proteins, which include STM, affect the metabolism of cytokinins and gibberellins (GAs, reviewed by Veit, 2009). 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 isopentenyltransferase genes (Yanai et al., 2005). By contrast the levels of GA in the central zone of the SAM are maintained low by the suppression exercised by KNOX members on GA20 oxidase (Hay et al., 2002). High GA levels are observed in the peripheral region of the SAM demarking the initiation of lateral organs (see reviewed by Veit, 2009). No information is currently available on the regulation of plant growth regulators by other genes participating in SAM formation and maintenance.

The objective of this study is to further characterize the previously isolated *Brassica* genes (Chapter 2), orthologous to the *Arabidopsis* *STM*, *CLV1*, and *ZLL*. This was achieved by assessing (1) their expression and localization patterns during normal and abnormal MDE development in *B. napus*, (2) the effects of their ectopic

expression on embryonic and post-embryonic development in *Arabidopsis*, and (3) their relationship with plant growth regulators.

5.3. MATERIALS AND METHODS

5.3.1. Plant material and treatments

Growth conditions of *Brassica napus* (Topas DH4079) plants and production of MDEs were described in Belmonte et al. (2006). For microspore-derived embryogenesis days in culture were counted from the imposition of the heat shock treatment. Alterations in embryo and SAM structure were induced by following previous protocols utilizing 0.1 mM DL-buthionine sulfoximine (BSO, Belmonte et al., 2006), 2 μ M 2,3,5-triiodobenzoic acid (TIBA), and 2 μ M 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB) (Ramesar-Fortner and Yeung, 2006).

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 as described in Chapter 2. The lines used in this study were *BnSTM* (L5, 8, and 23); *BrSTM* (L5, 15, 21), *BoSTM* (L5, 7, 11, and 33), *BnCLV1* (L2, L3, and L9), *BnZLL-1* (L2 and 16), and *BnZLL-2* (L8 and 16) (Appendix. 8).

The reactivation of the shoot apical meristem was estimated by counting the number of 5 days old *Arabidopsis* seedlings which displayed newly formed primordia. The seeds were germinated on ½ MS medium containing 1% sucrose.

5.3.2. Expression studies

Expression studies in *Brassica* and *Arabidopsis* were carried out using semi-quantitative RT-PCR. Primers and number of cycles are listed in Appendix 9. The

transcript levels of *BnSTM*, *BnCLV1*, *BnZLL-1* and 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 and cotyledons) 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.

5.3.3. RNA-in situ hybridization and light microscopy

Both techniques were performed as described in Chapter 2.

5.3.4. Hormone treatments and analyses

The effects of hormone applications on the expression of the *Brassica* genes were evaluated exactly as described by (Liu et al., 2008). Ten day-old *Brassica napus* seedlings grown on vermiculite were collected and their roots were immersed in solutions containing different levels of plant hormones [indole acetic-acid (IAA, 30 µM), abscisic acid (ABA, 100 µM), benzylamine purine (BAP, 100 µM), and gibberellin-3 (GA3, 100 µM)]. The shoot of the seedlings, without the cotyledons, was harvested at different times (0, 1, 3, and 6h) for RNA extraction.

For hormone analyses, 3 week-old shoots from wild type *Arabidopsis* plants and plants over-expressing *BoSTM* (line 5) and *BnCLV1* (line 2) were collected and lyophilized. The plant hormone analysis was performed at the National Research Council of Canada-Plant Biotechnology Institute, Saskatoon, Canada (<http://www.nrc-cnrc.gc.ca/eng/facilities/pbi/plant-hormone.html>) 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).

Sensitivity to ABA and 2,4-D was assayed by performing germination and root growth tests as outlined in (Weigel and Glazebrook, 2002).

5.3.5. Immunolocalization of IAA

This was performed using the procedure described in Chapter 3

5.3.6. Statistical analysis

Statistical analysis was performed as described in Chapter 2.

4.5. RESULTS

4.5.1. Expression and localization of the *Brassica napus* genes during normal and abnormal development of MDEs.

To establish a functional relationship between the four *Brassica* genes (*BnSTM*, *BnCLV1*, *BnZLL-1* and -2) and the development of the shoot meristem we investigated their expression and localization patterns in *B. napus* MDEs in which the SAM structure was experimentally altered through manipulations of the culture medium (Belmonte et al., 2006; Ramesar-Fortner and Yeung, 2006). 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 (Belmonte et al., 2006). 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 (Appendix 10). TIBA-treated embryos developed non functional SAMs and exhibited a characteristic fusion of the cotyledons at their base (Ramesar-Fortner and Yeung,

2006). These abnormalities were reverted if 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB), an auxin antagonist, was added with TIBA (Ramesar-Fortner and Yeung, 2006). The expression levels of all *Brassica* genes were repressed in MDEs developed in the presence of TIBA (Fig. 5. 1), 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 (Fig. 5. 1).

In situ hybridization studies revealed that the four *B. napus* genes were 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 “zygotie-like” expression, which was restricted to the central region of the apical pole (Fig. 5. 2A₁₋₄).

In other embryos transcripts of this gene could be detected in unusual domains, such as the peripheral region of the apical pole (Fig. 5. 2A₅₋₇), or in separate pockets of cells (Fig. 5. 2A₈₋₁₁). This observation is consistent with the notion that the SAMs of untreated MDEs develop under sub-optimal conditions (Belmonte et al., 2006). 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 (Fig. 5. 2A₁₂₋₁₄). In the presence of TIBA, *BnSTM* expression was restricted to the apical pole in immature embryos (Fig. 5. 2A₁₅₋₁₆) but was lost in cotyledonary embryos with an abnormal SAM (Fig. 5. 2A₁₇). Both structural and expression abnormalities induced by TIBA were reversed in TIBA+PCIB treated MDEs (Fig. 5. 2A₁₈₋₁₉). No signal was detected with a sense probe (Fig. 5. 2A₂₀).

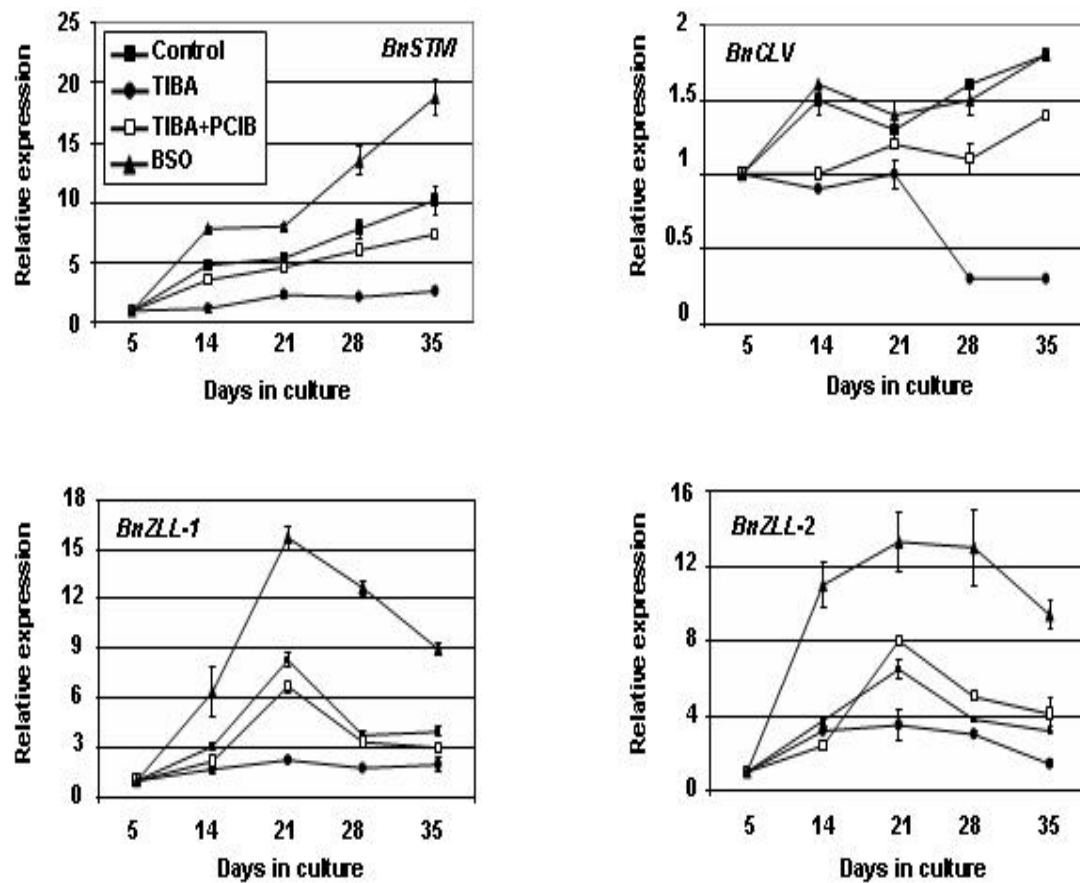


Figure 5.1. Expression profiles of *BnSTM*, *BnCLV1*, *BnZLL-1* and -2 during *B. napus* MDEs cultured in the presence of 2,3,5-triiodobenzoic acid (TIBA), 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB), and DL-buthionine sulfoximine (BSO). The compounds were added after 5 days in culture. Values $\pm$ SE are means of at least 3 independent experiments.

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 (Fig. 5. 2B₁₋₃). In the presence of BSO a larger group of sub-apical cells expressed *BnCLV1* in globular embryos (Fig. 5. 2B₄). The expression in the sub-apical domain was also retained in fully developed BSO-treated MDEs (Fig. 5. 2B₅). TIBA reduced the expression domain of *BnCLV1* in globular embryos, and to a certain extent in cotyledonary embryos (Fig. 5. 2B₆₋₇). Embryos treated with TIBA+PCIB exhibited a control-like expression pattern (data not shown), whereas no signal was observed in tissue hybridized with sense probe (Fig. 5. 2B₈).

The combined expression of *BnZLL-1* and -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 (Fig. 5. 2C₁₋₅). Within the SAM the signal was often delocalized to the peripheral region (Fig. 5. 2C₄). Inclusions of BSO enlarged the localization domain of *BnZLL-1* and -2 throughout the SAM (Fig. 5. 2C₆), whereas no signal was observed in the SAMs of TIBA-treated embryos (Fig. 5. 2C₇). The signal was recovered if PCIB was applied with TIBA (Fig. 5. 2C₈). Specificity of the hybridization was demonstrated using a sense riboprobe (Fig. 5. 2C₉).

5.4.2. Phenotypic characterization of *Arabidopsis* plants ectopically expressing the *Brassica* genes

Given the difficulties to transform *Brassica* plants, the effects of the *Brassica* genes were investigated in *Arabidopsis*. During zygotic embryo development the ectopic expression of the *Brassica CLV1* resulted in the formation of small SAMs whereas an opposite effect was observed with the introduction of *STM* (Fig. 5.3A).

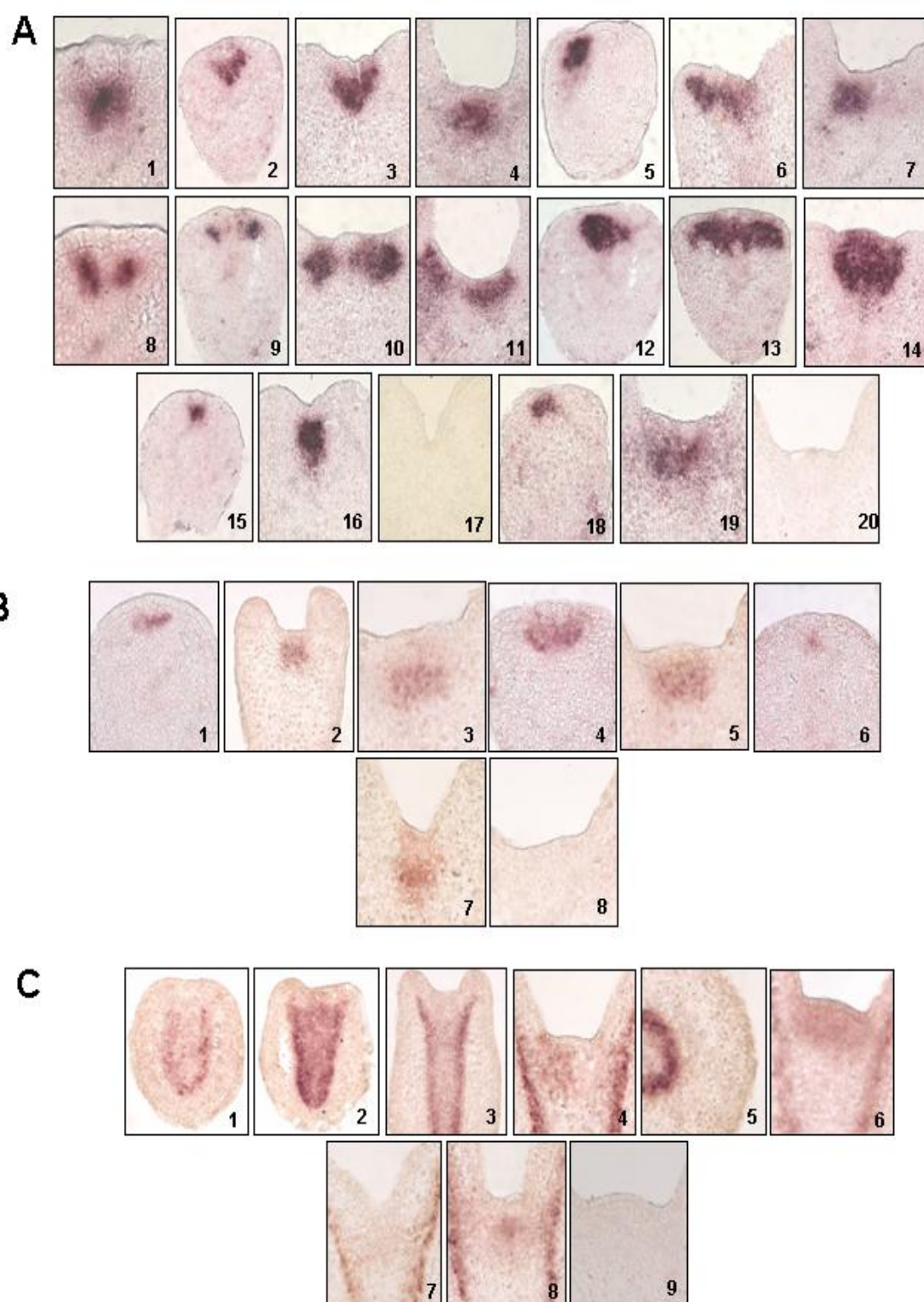


Figure 5.2. RNA-in situ hybridization studies of *BnSTM*, *BnCLV1*, *BnZLL-1* and -2 during *B. napus* microspore-derived embryogenesis. **(A)** Expression pattern of *BnSTM* during *B. napus* MDE development. Signal for *BnSTM* was first observed in small clusters of sub-apical cells in pre-globular embryos (1) and retained in the apical pole of globular (2), early cotyledonary (3) and cotyledonary (4) embryos. In some instances *BnSTM* expression was delocalized to the side of the apical pole throughout development (5-7), or in non-adjacent clusters of cells (8-11). In the presence of BSO, *BnSTM* expression expanded in globular (12), heart-shaped (13), and cotyledonary (14) embryos. The signal of *BnSTM* was reduced in immature embryos treated with TIBA (15, 16) and completely lost in fully developed embryos (17). These alterations were recovered in immature (18) and cotyledonary (19) embryos when PCIB was added along with TIBA. No signal was observed with the sense probe (20). **(B)** Localization pattern of *BnCLV1* in untreated MDEs at the globular (1), early cotyledonary (2) and cotyledonary (3) stage of development, in BSO-treated MDEs (4, 5), and in TIBA-treated embryos (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 (8). **(C)** Expression of *BnZLL-1* and -2 in longitudinal sections of globular (1), early cotyledonary (2), middle cotyledonary (3) and cotyledonary (4) untreated MDEs, as well as in cross sections of cotyledonary embryos (5). *BnZLL-1* and -2 localization in cotyledonary MDEs treated with BSO (6), TIBA (7), and TIBA+PCIB (8). No signal was observed with sense probes (9).

Compared to WT, the SAM of the *Brassica STM* over-expressing embryos was dome-shaped and composed of a larger cluster of meristematic cells (Fig. 5. 3A). No effects on meristem size were noticed in plants over-expressing the *Brassica ZLL-1* and -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* (Fig. 5.3B). This faster meristem reactivation was also observed by over-expressing the *STM* orthologs from *B. oleracea* (AF193813) and *B. rapa* (GU480585) (denoted as *BoSTM* and *BrSTM* respectively), which are the progenitor species of *B. napus* and share a high degree of amino acid sequence identity with *BnSTM* (97% for *BrSTM*, and 99% for *BoSTM*).

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 visible in *BoSTM* plants. In lines with the highest *BoSTM* expression, such as line 33 (Appendix 8), ectopic shoots (Fig. 5.3C₁) and small lobed leaflets (Fig. 5.3C₂) emerged from the adaxial 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 serrate leaves (Fig. 5. 3C₃), larger inflorescences characterized by more flowers (Fig. 5.3C₄) and curved siliques (Fig. 5.3C₆). Increased number of siliques and flowers, as well as reduced plant height were also observed in all *STM* over-expressors (Table 5. 1). A reduction in hypocotyl elongation and termination of the SAM (Fig. 5.3C₇) were observed in lines with higher expression levels of *BnCLV1*.

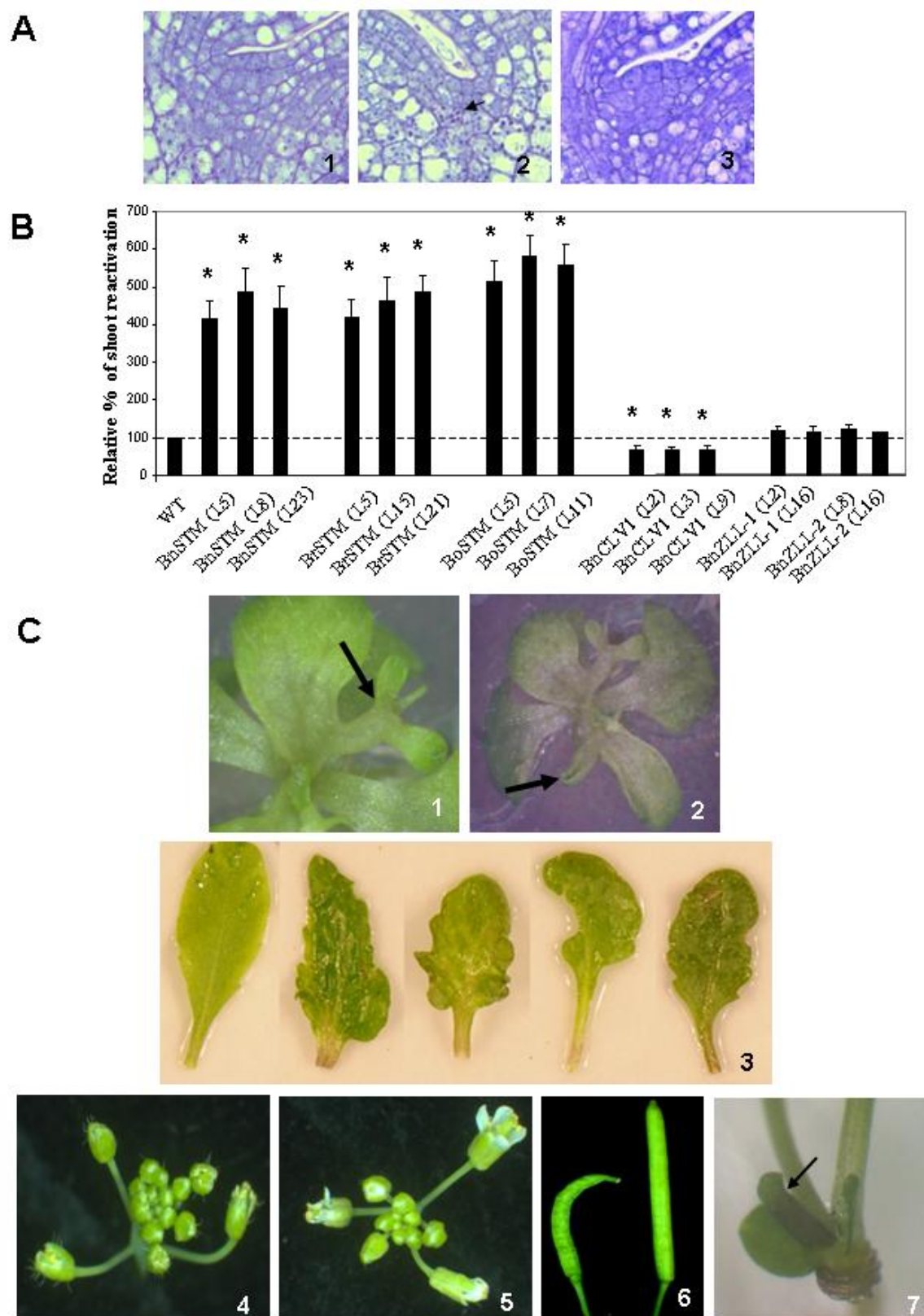


Figure 5.3. Phenotypic characterization of *Arabidopsis* lines ectopically expressing the *Brassica STM*, *CLV1*, *ZLL-1* and *-2*. **(A)** Structure of the shoot apical meristem (SAM) in WT cotyledonary embryos (1) and embryos expressing the *Brassica CLV1* (2) characterized by accumulation of storage products (arrow) and *STM* (3). The SAM of *BnZLL-1* and *-2* over-expressing embryos was similar to that of WT embryos (data not shown). **(B)** Number of transformed *Arabidopsis* seedlings producing leaf primordia after 5 days in germination. Values are means of at least 3 independent experiments $\pm$ SE. Stars indicate values which are significantly different from the WT value ($P \leq 0.01$). **(C)** Production of ectopic meristems (1) or lobed leaves (2) from high *STM* over-expressors. The introduction of the *Brassica STM* also resulted in the production of abnormal leaves (WT leaf on the left side) (3), larger inflorescences (4) compared to WT plants (5), and curved siliques (6). Termination of the SAM with a determined stem-like structure (arrow) was often observed in lines ectopically expressing *BnCLV1* (7).

No major morphological abnormalities were observed in *Arabidopsis* lines ectopically expressing *BnZLL-1* and -2, the only exception being a reduced number of branches (Table 5.2).

5.4.3. Expression analysis of SAM-related genes in the transformed lines

The expression level of several SAM-related genes was measured in the shoot of transformed *Arabidopsis* seedlings after 1 week in germination. The introduction of *Brassica STM* activated the expression of *KNAT6*, *WUSCHEL (WUS)* and *CUPSHAPED COTYLEDON-1 (CUC 1)*, whereas an opposite trend was observed in the *BnCLV1* over-expressing line (Fig. 5. 4). A less pronounced increase in expression of the three genes was also measured in lines ectopically expressing *BnZLL-1* and 2. 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* (Fig. 5. 4).

5.4.4. Effects of hormone treatments on the expression of the *Brassica* genes

The endogenous levels of *BnSTM*, *BnCLV1*, *BnZLL-1* and -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. An opposite expression profile was measured in the presence of ABA and GA3 (Fig. 5.5). The expression level of *BnCLV1* increased in both control seedlings and seedlings treated with BAP and IAA. The transcript levels of this gene increased in the presence of ABA and decreased with GA3 applications. Hormone treatments did not affect the expression of *BnZLL-1* and -2.

Table 5.1. Phenotypic characterization of lines over-expressing the *Brassica napus* (Bn), *B. rapa* (Br), and *B. oleracea* (Bo) *STM*. Values, expressed as a percentage (control values set at 100%) are average $\pm$ SE of at least three independent experiments. H. Plant Height; B. Branches; S, Siliques; CL. Cauline leaves; RL. Rosette leaves; F. Flowers. * Indicates values which are significantly different from the WT value ($P \leq 0.01$).

	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)	109.3 (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)	178.8* (± 13.2)	168.7* (± 15.6)	103.3 (± 8.9)	87.2 (± 10.3)	124.1 (± 14.6)

Table 5.2. Phenotypic characterization of lines over-expressing the *Brassica napus* *CLV1*, *ZLL-1* and -2. Values, expressed as a percentage (control values set at 100%) are average $\pm$ SE of at least three independent experiments. H. Plant Height; B. Branches; S, Siliques; CL. Cauline Leaves; RL. Rosette Leaves; F. Flowers. * Indicates values which are significantly different from the WT value ($P \leq 0.01$).

	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	77.6 (± 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)

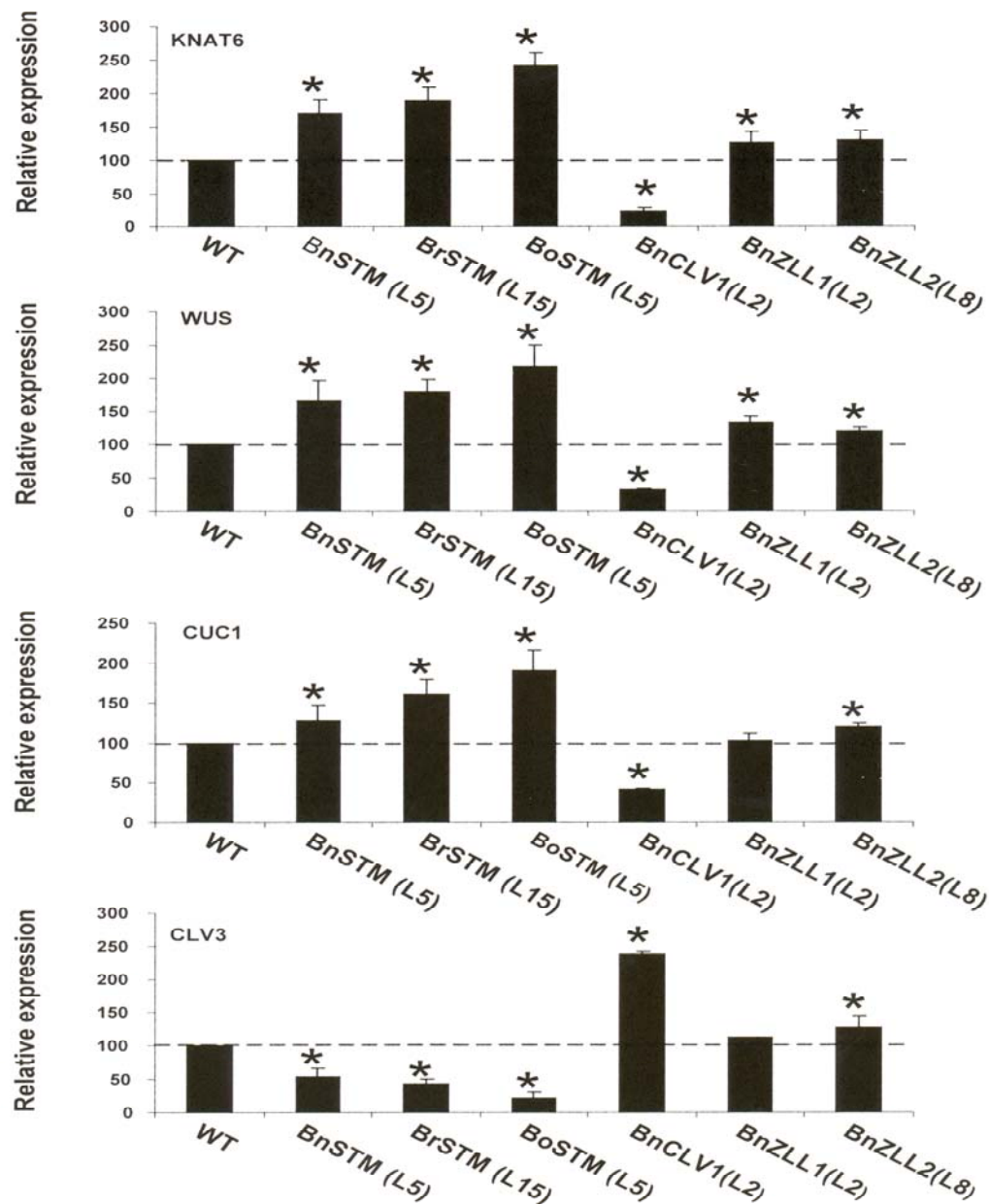


Figure 5.4. Expression levels measured by semi-quantitative RT-PCR of the meristem-related genes *KNAT6*, *WUS*, *CUC1*, and *CLV3* in the transformed *Arabidopsis* plants after 1 week in germination. Values $\pm$ SE are means of at least three independent experiments. Stars indicate values which are significantly different from the WT value ($P \leq 0.01$). The values of the transformed lines were normalized to the value of WT plants set at 100.

5.4.5. Endogenous hormone analysis of the transformed *Arabidopsis* plants

For hormone analysis three *Arabidopsis* lines were selected: WT, as well as *BoSTM* (L5) and *BnCLV1* (L2), as they exhibited the most severe phenotypes among the fertile 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). Lower levels of t-ZR and isopentenyladenine (2iP) were measured in the shoots of the *35S::BnCLV1* line (Fig. 5. 6A). Zeatin and dehydrozeatin were below detectable levels in all the lines analysed.

Profile of auxin metabolites showed a significantly increased level of indole-3-acetic acid (IAA) in the *BoSTM* line. No differences in the levels of the other metabolites, i.e. IAA-alanine (IAA-Ala), IAA-aspartic acid (IAA-Asp), and IAA-glutamic acid (IAA-Glu) were detected among lines (Fig. 5. 6B).

Compared to the other lines analysed, the *BoSTM* line had lower endogenous levels of abscisic acid (ABA), dehydrophaseic acid (DPA), ABA-glucose ester (ABA-GE), and phaseic acid (PA). No statistically significant differences were detected among lines for 7'-hydroxy-ABA (7'-OH-ABA) (Fig. 5. 6C). Neo-phaseic acid was below detectable levels.

5.4.6. Hormone sensitivity assays

Sensitivity to ABA was assayed using a seed germination test. A similar inhibition profile with increasing concentrations of ABA was observed between WT seeds and seeds obtained from *35S::BnCLV1*, *BnZLL-1* and -2 lines (Fig. 5.7A).

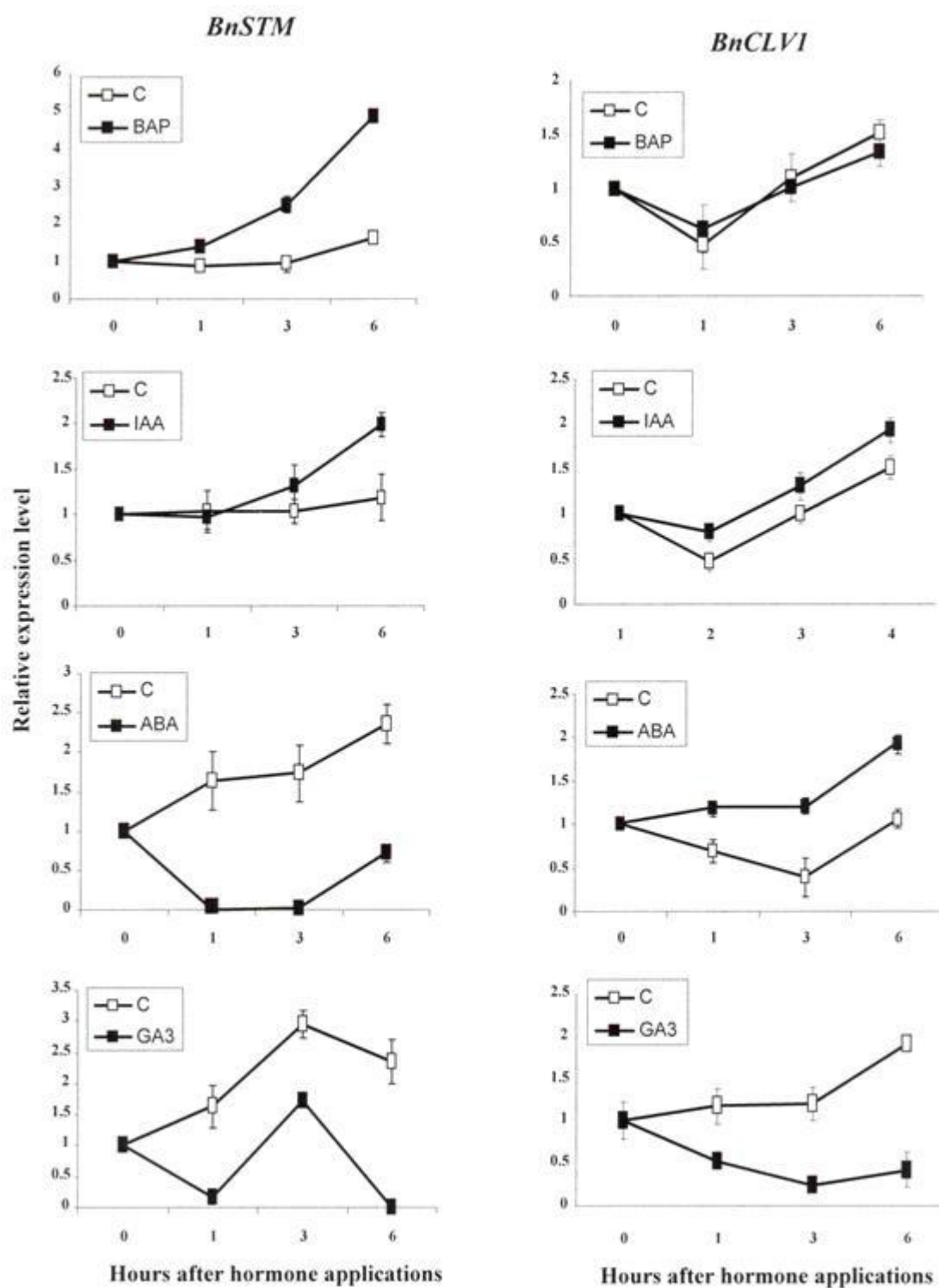


Figure 5.5. Effects of hormone treatments on the expression level of *BnSTM* and *BnCLV1* measured by semi-quantitative RT-PCR. Hormones [indole acetic-acid (IAA, 30 μ M), abscisic acid (ABA, 100 μ M), benzylamine purine (BAP, 100 μ M), and gibberellin-3 (GA3, 100 μ M)] were applied to 10-day old *Brassica napus* plants. The shoots, without the cotyledons, were harvested at different times (0, 1, 3, and 6h) after incubation and utilized for expression studies. Values $\pm$ SE are means of at least three independent experiments.

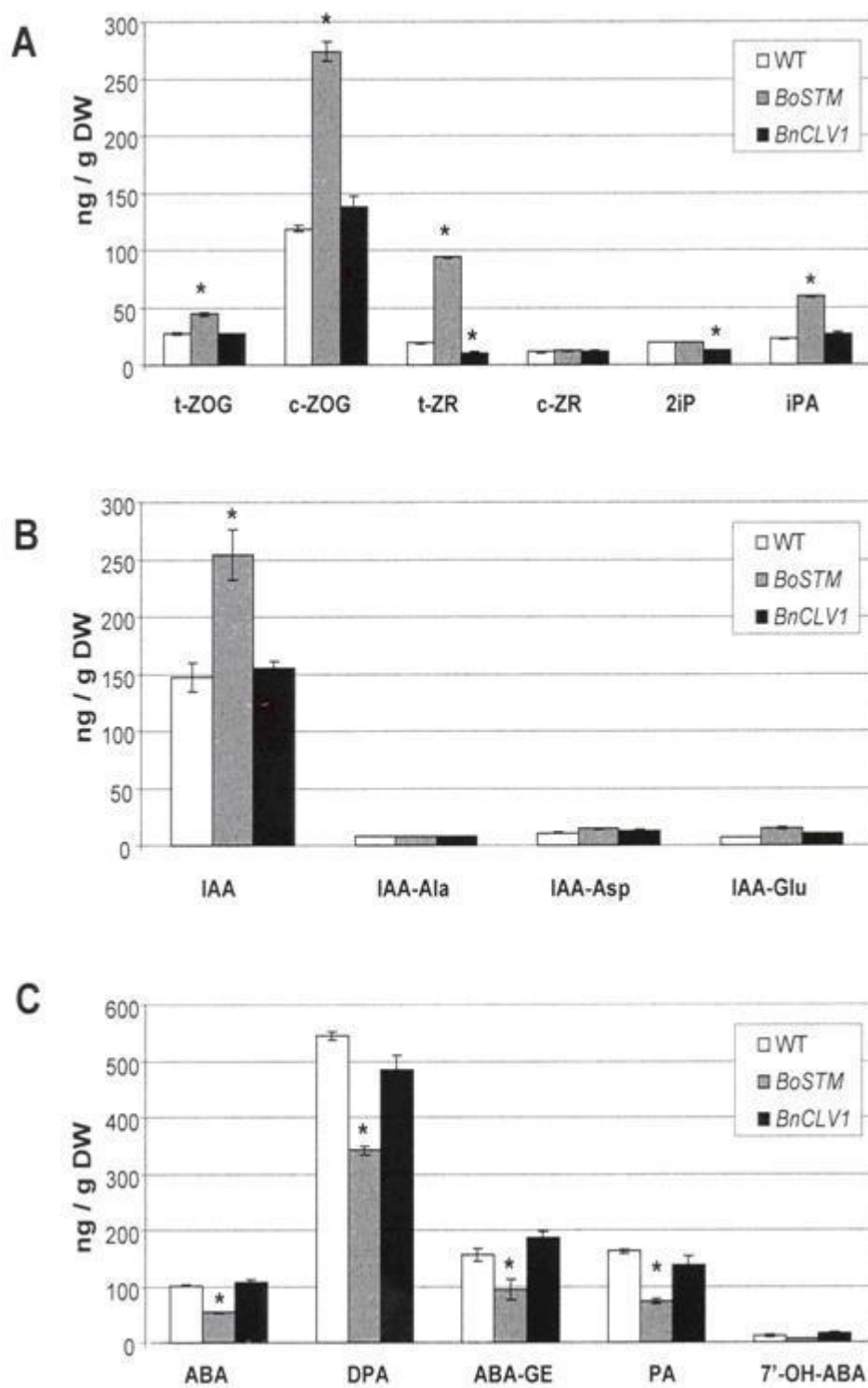


Figure 5.6. Hormone profiles of cytokinin **(A)**, auxin **(B)**, and abscisic acid **(C)** metabolites in 3 week old *Arabidopsis* shoots. tZOG, trans-zeatin-O-glucoside; cZOG, cis-zeatin-O-glucoside; tZR trans-zeatin riboside; cZR, cis-zeatin riboside; 2iP, isopentenyl adenine; iPA, isopentenyl adenosine; IAA, indole 3-acetic acid; IAA-Ala, indole 3-acetic acid alanine; IAA-Asp, indole 3-acetic acid aspartate; IAA-Glu, indole 3-acetic acid glutamic acid; ABA, abscisic acid; DPA, dehydrophaseic acid; ABA-GE, abscisic acid glucose ester; PA, phaseic acid; 7'-OH-ABA, 7'-hydroxyl abscisic acid. Values $\pm$ SE are means of at least three independent analyses. Stars indicate values which are significantly different from the WT value ($P \leq 0.01$).

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, 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 (Fig. 5.7B).

5.5. DISCUSSION

5.5.1. 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 have functional meristems able to reactivate upon transfer onto germination medium. This percentage can be increased to almost 80% when BSO is added to the culture medium (Belmonte et al., 2006). Applications of TIBA, which inhibits the polar transport of auxin, reduced meristem functionality by 90% (Ramesar-Fortner and Yeung, 2006). This detrimental effect is not due to the toxicity of the compound as the combined inclusion of TIBA + PCIB (an auxin antagonist reversing the effect of TIBA) rescues meristem functionality to control levels (Ramesar-Fortner and Yeung, 2006).

Using this approach it is suggested that *BnSTM*, *BnCLV1*, and *BnZLL-1* and *-2* might be 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

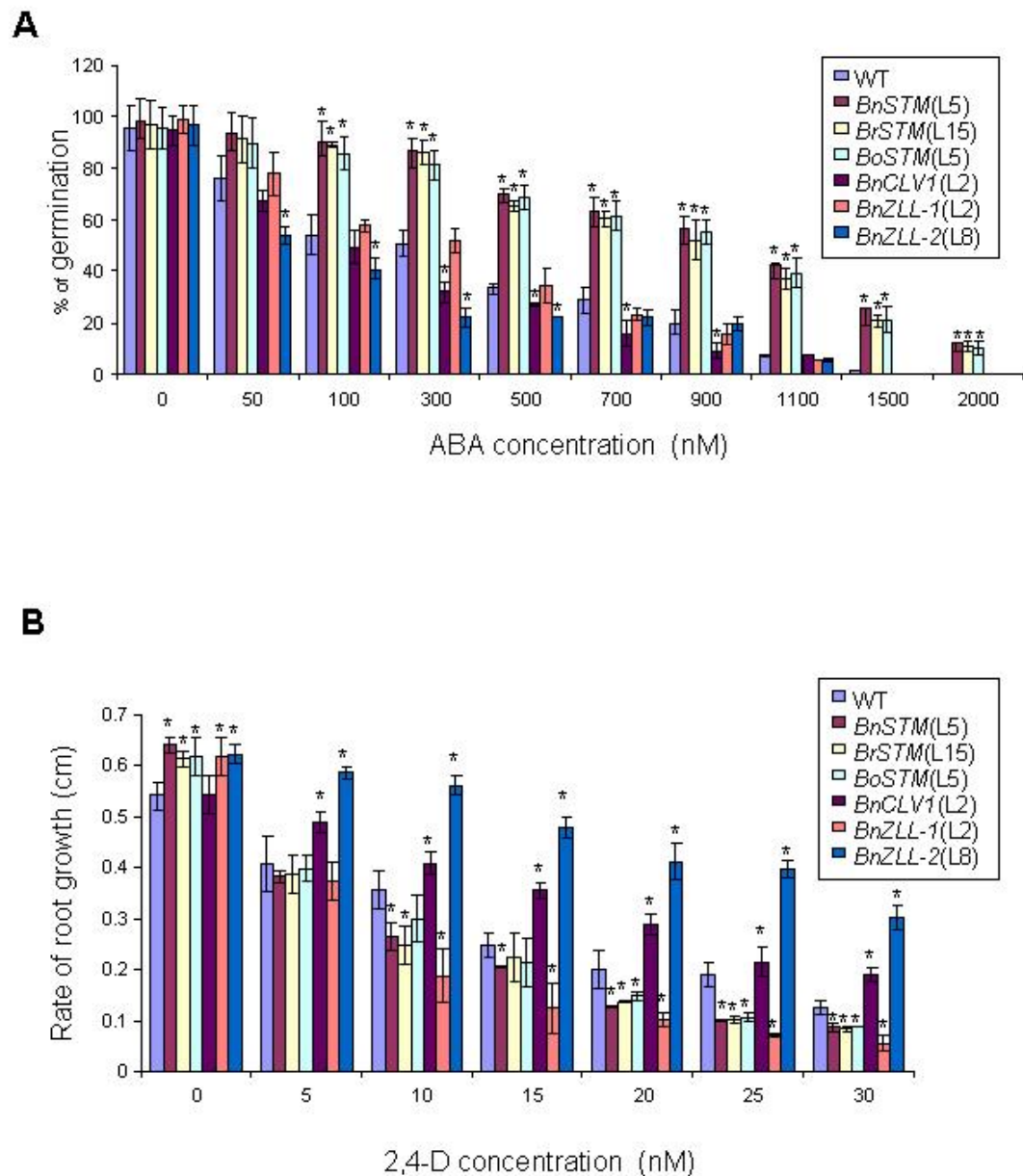


Figure 5.7. Hormone sensitivity assays performed on the transformed lines. Effects of increasing ABA levels on the percentage of seed germination **(A)** and 2,4-D on root growth **(B)**. Values $\pm$ SE are means of at least three independent analyses. Stars indicate values which are significantly different from the WT value ($P \leq 0.01$).

extended to the entire apical pole of the embryos (Fig. 5. 2A₁₋₄), as also observed in zygotic embryos (Chapter 2). The inability of many (about 60%) untreated *B. napus* MDEs to convert, Belmonte et al. (2006) correlates with the early (prior to the formation of a meristem) miss-expression of *BnSTM* either in the peripheral region (Fig. 5. 2A₅₋₇), or in two separate domains of the SAM (Fig. 5. 2A₈₋₁₁). A deviation in expression pattern in control MDEs was also observed for *BnZLL-1* and -2 which are first expressed in the vascular tissue and later in the SAM (Fig. 5. 2C). However in many MDEs the expression domain of *BnZLL-1* and -2 in the SAM is restricted to the peripheral zone (Fig. 5. 2C₄).

The BSO-improvement of meristem functionality correlates with the increased transcript levels and localization patterns of *BnSTM*, *BnZLL-1* and -2 (Fig. 5. 1 and 5. 2). Of interest, the enlarged *BnSTM* signal in the apical pole of MDEs cultured with BSO (Fig 2A_{12,13}) became apparent before any visible SAM, thereby suggesting that the *BnSTM* localization pattern in early embryos can be used to predict the quality of the meristem.

The expression of all *Brassica* genes is compromised in non-functional (TIBA-treated) MDEs (Fig. 5. 1). The SAMs of these embryos do not express *BnSTM*, *BnZLL-1* and -2, while the signal of *BnCLV1* is reduced (Fig. 5. 2). 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 (Appendix 10). In the shoot meristem of *Arabidopsis* high levels of auxins, generally associated to the sites of primordium formation, induce cell differentiation and reduce the expression of KNOX genes, including STM (reviewed by Veit, 2009).

Based on the information presented above it emerges 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 produced *in vitro*.

5.5.2. Phenotypic characterization of *Arabidopsis* plants ectopically expressing the *Brassica* genes

Ectopic expression of the *Brassica STM* and *CLV1* influences the rate of SAM reactivation at germination, with the former accelerating meristem conversion and the latter having a retarding effect (Fig. 5. 3B). These results can be possibly attributed to the different organization of the embryonic SAMs observed in the transformed line (Fig. 5. 3A). The role of these two genes during SAM maintenance is well established (Clark et al., 1996), 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 *CLV1* repressing the function of *STM* (Clark et al., 1996), it can be argued 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. 5. 3A₂) and the termination of the SAM a few days after germination (Fig. 5. 3C₇) support this idea.

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. 5.4). *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 to abolish the residual meristematic activity of weak *stm-2* plants (Belles-Boix et al., 2006). 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 (Hibara et al., 2003). A third gene induced by the introduction of the *Brassica STM* is *WUSCHEL* (*WUS*), which encodes a WOX-class homeodomain transcription factor (Mayer et al., 1998) required for maintaining the meristematic cells of the central zone in an “undetermined” 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 (Laux et al., 1996). These three regulators of meristem activity are repressed in the *BnCLV1* lines (Fig. 5. 4) 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, Fig. 5. 3C₇) and depletion of the stem cells through the repression of *WUS* (Fig. 5. 4 and Schoof et al., 2000). This is in contrast to the *STM* over-expressors characterized by lower *CLV3* expression (Fig. 5. 4).

In the *STM* over-expressing lines, the up-regulation of several genes involved in SAM activity also correlates with the formation of ectopic shoots, abnormal leaf morphology, and increased production of reproductive organs (Fig. 5.3 and Table 5.1). The *STM*-induction of ectopic meristems from leaf tissue was also reported in several systems, including *Arabidopsis*, tobacco, and soybean (Sinha et al., 1993; Chuck et al., 1996; Liu et al., 2008). Similarly, the abnormalities in leaf morphology observed in our study (Fig. 5.3C₃) are consistent with the over-expression of other *KNOX-1* genes (Lincoln et al., 1994; Liu et al., 2008; Barth et al., 2009; Ikezaki et al.,

2010) and can be attributed to meristematic growth originating from the margins of the leaves. A novel trait linked to the introduction of *STM* is the larger inflorescence size, resulting in a higher number of flowers and siliques (Fig. 5.3 and Table 5.1). This phenotype can also be the result of the increased meristematic activity encouraging floral meristem formation.

The lack of a distinct phenotype in *BnZLL* lines (Table 5.2) agrees with other studies using the *Arabidopsis ZLL* (McConnell and Barton, 1995; Moussian et al., 1998; Lynn et al., 1999), and it suggests that *BnZLL*, like its *Arabidopsis* ortholog, is only required during the establishment of the embryonic meristem, but not for its post-embryonic development.

5.5.3. Interaction between the *Brassica* genes and plant growth regulators

Increasing evidence demonstrates a close relationship between the meristem-related genes and plant growth regulators (reviewed by Veit, 2009). However, only a few studies are available regarding a possible hormone regulation on the transcription of *STM*, *CLV1* and *ZLL* (Rupp et al., 1999; Liu et al., 2008; di Giacomo et al., 2008). This work shows a link between *STM* expression and cytokinins. While applications of BA activate *BnSTM* (Fig. 5.5), over-expression of the *Brassica STM* induces the accumulation of zeatin-type cytokinins, i.e. c- and t-ZOG, as well as tZR, and iP-type cytokinins, i.e. iPA (Fig. 5.6). High levels of these classes of cytokinins were observed during growth of Douglas-fir buds (Kong et al., 2008). As also reported in previous studies (Kong et al., 2008), the concentrations of free zeatin and dehydrozeatin were below detectable levels, implying a fast turnover to ZR. The *STM*-mediated increase in the levels of cytokinins 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 (reviewed by Kamada-Nobusada and Sakakibara, 2009). As demonstrated previously (Chapter 2), the introduction of *BoSTM* in *Arabidopsis* tissue induces the transcription of adenylate isopentenyltransferase 7, in agreement with other studies over-expressing several *KNOTTED1* genes (Yanai et al., 2005). The 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 (Fig. 5.3), and the production of ectopic meristems (Fig. 5.3C). While the role of cytokinins for meristem formation has long been recognized (Steeves and Sussex, 1989), it was the work of Lindsay et al. (2006) which documented that applications of cytokinins to the *Arabidopsis* apical pole induced an enlarged SAM. Yanai et al. (2005) further demonstrated that expression of cytokinin biosynthetic genes driven by the *STM* promoter partially rescued the abnormal meristem of *stm* plants. Other phenotypes observed in plants over-expressing *STM*, such as the formation of curved siliques and the increased number of flowers can result from high cytokinins levels (Lindsay et al., 2006).

The poor architecture of the *35S::CLV1* SAMs in developing embryos and their termination during post-embryonic growth may be caused by the low endogenous levels of tZR, and 2iP (Fig. 5. 6), the latter having one of the highest biological activities among all cytokinins (Kamada-Nobusada and Sakakibara, 2009). Recent work (Gordon et al., 2009) shows that sustained cytokinin levels in the SAM induce the expression of *WUS*, which is required for the maintenance of “stem cell niche” or “organizing center” (Mayer et al., 1998). These conditions might not be met in lines over-expressing *BnCLV1* which have low cytokinins levels and reduced *WUS* expression.

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). Quantification of several GAs by HPLC-ES-MS/MS, i.e. GA1, 3, 4, 8, 9, 19, 20, 24, 29, 44, and 53) revealed no differences between WT and transformed plants. However, a localized effect of SAM-related genes on GAs cannot be excluded given the large size of the tissue analyzed.

The high levels of free IAA (Fig. 5. 6) and the increased sensitivity to supplied 2,4-D (Fig. 5. 7) exhibited by lines over-expressing the *Brassica STM* suggest a possible role of auxin in the *STM* phenotype. As discussed in the Chapter 2, 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 a maximum of auxin ensured by its basipetal transport (Sarkar et al., 2007). The lobed leaves and the formation of ectopic meristems observed in lines over-expressing the *Brassica STM* can be a direct consequence of high auxin levels which maintain the cells within the blastozone in a proliferative state. Cell proliferation from the leaf margin was also induced by the ectopic expression of *KNOX* genes (Barth et al., 2009).

Compared to the 35S::*CLV1* line, the biosynthesis of ABA, its degradation to PA and DPA via the 8'hydroxylation pathway, as well as its conjugation to sugars to form ABA-glucose ester (ABA-GE) were lower in plants over-expressing *STM*. These plants also exhibited a lower sensitivity to ABA. 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. Jacquemard et al. (1995) showed that elevated ABA concentration increases the time of DNA replication in the meristematic cells of *Sinapis alba* and delays their proliferation during post-embryonic growth.

In conclusion, this work shows that the expression levels and localization patterns of the *Brassica STM*, *CLV1*, *ZLL-1* and *-2* correlate with the quality of the SAM established during *in vitro* embryo development in *B. napus*, and can be utilized to estimate meristem functionality. The ectopic expression of these genes in *Arabidopsis* 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.

CHAPTER 6: SUMMARY AND CONCLUSION

The acquisition of morphogenic (embryogenic and organogenic) competence *in vitro* is an important and essential step in which some cells within the explants respond to an inductive signal which allows them to de-differentiate, acquire meristematic identity, and initiate a new developmental program. In order to explore this transition this thesis investigates how the ectopic expression of *Brassica* genes fulfilling different functions in the establishment and maintenance of the SAM *in vivo* affects morphogenic competence *in vitro*. The hypothesis tested is that the over-expression of positive regulators of SAM activity (*STM* and *ZLL*), which increase the pool of undifferentiated cells, has positive effects on morphogenesis, whereas the over-expression of negative regulators, i.e. *CLV1*, which promotes the differentiation of meristematic cells, has opposite effects. This study was carried out using *Brassica* genes (instead of *Arabidopsis* genes), as they will be utilized for future studies on meristem function during *B. napus* microspore-derived embryogenesis, a preferred *in vitro* embryogenic system compared to *Arabidopsis* somatic embryogenesis.

In the first Chapter the full length cDNAs of the *Brassica* genes were isolated and their deduced protein sequence shared high degree of similarities with their *Arabidopsis* homologs (91% for *BnSTM*, 88% for *BnCLV1*, and 96% for *BnZLL-1* and -2). Expression and localization studies suggested that the *Brassica* genes are orthologous to the *Arabidopsis STM*, *CLV1* and *ZLL*, and their ectopic expression had profound effects on *Arabidopsis* somatic embryogenesis. While *BnZLL-1* and -2 did not influence the yield of somatic embryos, *BnCLV1* repressed *in vitro* embryogenesis. A different effect was observed for *BnSTM*, which enhanced both the number of explants able to produce somatic embryos and the number of somatic

embryos per explants. These results were also replicated and partially improved by the introduction of *STM* isolated from *Brassica oleracea* and *rapa*, which are the progenitor species of *B. napus*. The *STM*-improvement of embryogenesis was also observed during microspore-derived embryogenesis in *Brassica napus*; the number of MDEs increased in *BnSTM* over-expressors whereas it decreased in *BnSTM* antisense lines. These results suggest that the effects of the *Brassica STM* are independent from the nature of the embryogenic system utilized, i.e. indirect (with an intervening callus phase, such as in the case of *Arabidopsis*) or direct (without a callus step, such as in the case of *B. napus*). The introduction of the *Brassica STM* improved *Arabidopsis* somatic embryogenesis possibly by lowering the requirement of the tissue for auxin, which is the inductive signal, and by promoting the expression of *WUSCHEL*, which is associated to the production of totipotent embryogenic cells during *in vitro* morphogenesis (Kurdyukov et al., 2009; Su et al., 2009). Additional effects related to the introduction of the *Brassica STM* include changes in the DNA methylation status and alterations in glutathione metabolism, both of which encourage embryogenesis.

Besides altering the requirement for auxin, the introduction of *STM* also affects the transport of auxin within the explants, as demonstrated by the experiments performed using TIBA (Chapter 3). Auxin immunolocalization studies and expression analyses of auxin transporters indicate differences in auxin distribution during the initial phase of *Arabidopsis* somatic embryogenesis, especially during the formation of callus. Applications of TIBA in the WT line increased the accumulation of endogenous IAA in the epidermal/cortical cells of the explants and on the surface of the callus. This increase in auxin levels, not observed when TIBA was added to lines over-expressing the *Brassica STM*, might cause a miss-expression of *WUS*, the expression of which is dependent upon precise changes in auxin gradients.

As presented in Chapter 4, the introduction of the *Brassica* genes also had profound effects on shoot organogenesis from *Arabidopsis* roots, with *STM* and *ZLL* enhancing the number of shoots produced in culture and *CLV1* repressing the process. As observed for somatic embryogenesis, these different responses are associated to a different requirement for auxin (which is needed for inducing cells of the explants to become competent and respond to the shoot-promoting cytokinin). Lines able to produce many shoots, i.e. *Brassica STM* and *ZLL* over-expressors required less 2,4-D whereas those with reduced shoot formation ability, i.e. *BnCLV1*, required more 2,4-D. These differences in auxin requirement were also related to the expression and localization pattern of *WUS*, which demarks the sites of organogenic cells. The expression of this gene was induced by the introduction of *STM* and repressed by that of *CLV1*.

The opposite organogenic response in culture observed in lines over-expressing the *Brassica STM* and *CLV1* correlated to changes in transcript levels of many genes involved in cytokinin perception and signaling. Of interest, the ectopic expression of *STM* activated two cytokinin receptors (*AHK4* and *CKI1*) and many type-B *ARRs* which are known activators of cytokinin signaling. This was in contrast to *CLV1* which induced the expression of several type-A *ARRs* (repressors of cytokinin signaling) and the repression of type-B *ARRs*.

A correlation between the *Brassica* genes and plant growth regulators was established in Chapter 5, which further characterized the function of these genes during *Arabidopsis* development. Besides demonstrating that the *Brassica STM*, *CLV1*, and *ZLL-1* and *-2* can be used as markers for SAM quality during MDE development in *B. napus*, experiments in that Chapter show that their over-expression in *Arabidopsis* caused profound phenotypic deviations during embryonic and post-

embryonic development. Some of the phenotypic alterations were associated to changes in endogenous hormone levels. Specifically the ectopic expression of *BoSTM* increased the levels of several biologically active cytokinins and IAA, whereas it reduced the levels of many ABA metabolites. Low concentrations of several cytokinins were observed in a line over-expressing *BnCLV1*.

In conclusion, the original hypothesis was confirmed. The ectopic expression of positive regulators of the SAM (*STM* and *ZLL*) encourages *in vitro* morphogenesis, whereas the over-expression of *CLV1*, a negative regulator, represses both embryogenesis and organogenesis. The opposite behavior in culture observed between the *STM* and *CLV1* over-expressor lines suggests a competitive role of the two genes *in vitro* analogous to that observed *in vivo* during the maintenance of the meristematic cells within the SAM. This raises the intriguing possibility that mechanisms governing stem cell maintenance *in vivo* also regulate embryogenic/organogenic cells *in vitro* (Fig. 6.1)

The introduction of *STM* reduces the requirement of the *Arabidopsis* explants (bent-cotyledon zygotic embryos for somatic embryogenesis or roots for shoot organogenesis) for auxin, which is the inductive signal. This might be due to either an increased sensitivity to 2,4-D and/or a higher endogenous auxin level. Proper levels/distributions of auxin would increase *WUS* expression which is known to specify embryogenic/organogenic cell fate. While embryogenic cells can develop into somatic embryos without plant growth regulators, shoot formation from organogenic cells requires cytokinins. This step is also affected by *STM*, which induces the transcription of cytokinin receptors and type-B ARR, while repressing type A-ARRs (Fig. 6.1). Opposite events would occur in lines over-expressing *CLV1*, leading to the preclusion of somatic embryogenesis and shoot organogenesis.

The introduction of *ZLL* only affects organogenesis, possibly by reducing the requirement of auxin in a similar fashion to that described for *STM*.

These findings provide a solid framework for further studies which will assess the function of these genes during *Brassica* MDE development (*B. napus* lines expressing *BnCLV1*, *ZLL-1* and -2 in sense and antisense orientation have been established, but they are not ready for analysis) in order to define similarities and differences between an indirect embryogenic system (*Arabidopsis* somatic embryogenesis) and a direct one (*B. napus* microspore-derived embryogenesis).

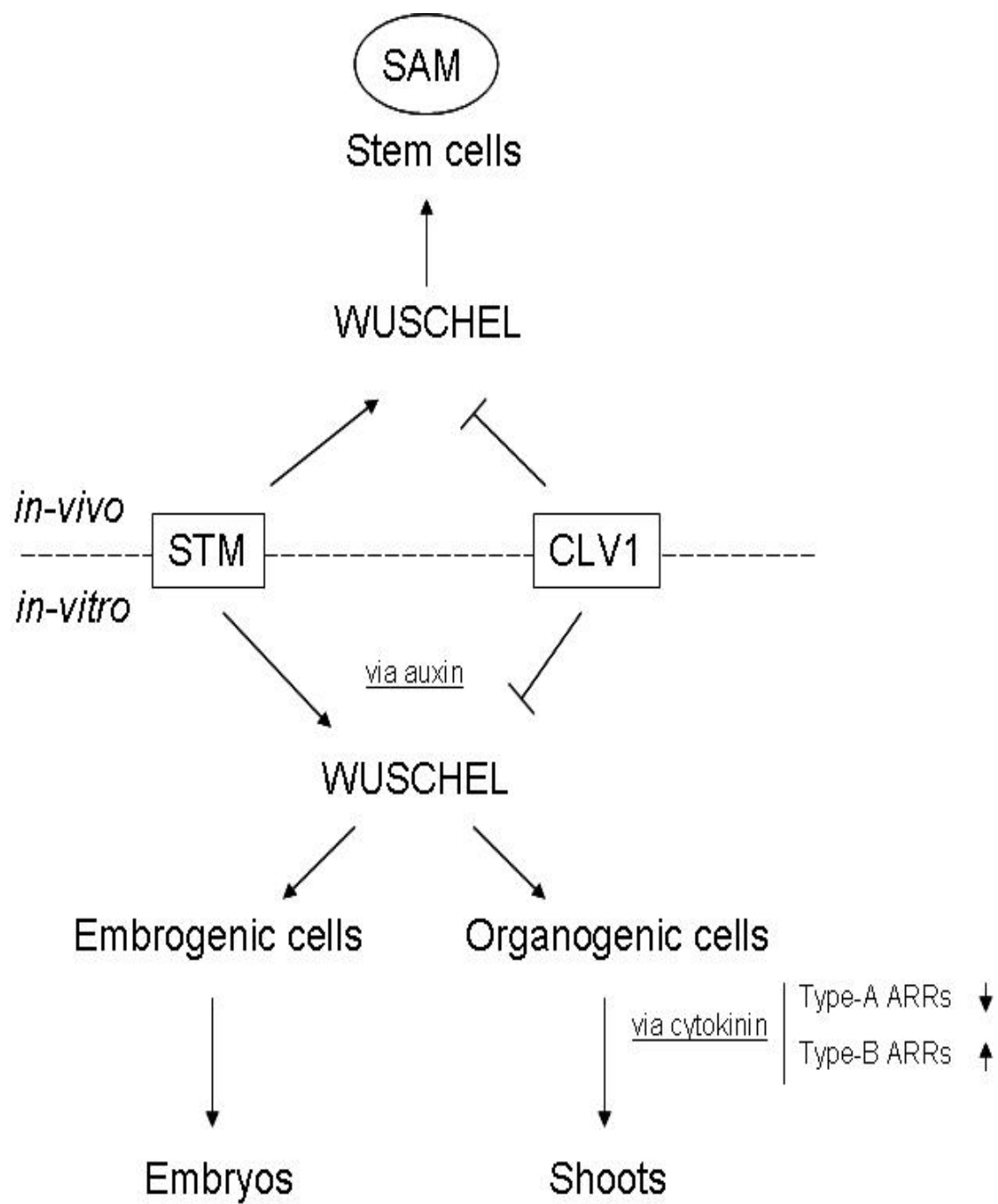


Figure 6.1. Schematic model outlining possible analogous functions of *STM* and *CLV1* *in vivo* and *in vitro*. During *in vivo* development *STM* maintains the *WUS*-expressing cells, required for the specification of the stem cells, in an undifferentiated state by preventing meristematic cells from adopting an organ-specific cell fate. This is in contrast to the role of *CLV1* which suppresses *WUS* expression and promotes differentiation of the stem cells (Clark et al., 1996). A similar regulation might occur *in vitro* with *STM* inducing *WUS* expression, possibly through modulations in auxin levels/distribution/sensitivity and *CLV1* having an opposite effect. *WUS* is required for the specification of embryogenic and organogenic cells. During organogenesis the *STM*-transcriptional activation of components involved in cytokinin perception and response (type-B responsive regulators ARR_s) would ensure shoot formation. These events are precluded by *CLV1*, which represses type-B ARR_s and activates type-A ARR_s (repressors of cytokinin signaling).

APPENDICES

Appendix 1 (Chapter 2):

Sequence analysis of *BnSTM*

ATGGAAGTGGTTCCAACAGCACTTCTTGTCGAATGGCTTTTGCCGGGGATAATAGTGATGGTCCGATGT
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TTTGTAGTAATGGACGCAACACATCCTCACCATTACTTTATGGACAATGTCATGGGAAATCCTTTCCCA
TTGATCACATCTCCTCGACCATGCTTTGA

Deduced protein sequence of *BnSTM*

MESGSNSTSCPMFAGDNSDGPMSMMMMMPVITSHQQHHGHDDQQHQQHQQHDDGYAYQSHHQHSSLLFL
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CQKVGAPPEVQARLEETCSSAAAAAASMGPTGSLGEDPLDQFMEAYCEMLVKYEQELSKPFKEAMVFLQ
HVECQFKSLSLSSPSSFSGYGEAAIERNNNGSSEEEVDMNNEFVDPQAEDRELKGQLLRKYSGLGSLKQ
EFMKRRKKGELPKEARQQLLDWWSRHYKWPYPSEQQLALAEESTGLDQKQINNWFINQRKRHWKPSMDQ
FVMDATHPHHYFMDNVMGNPFIDHISSTML

Sequence analysis of *BrSTM*

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GTCCTATGATGATGATGATGATGCCCCGCATAACATCACATCAACAACATCATGGTCATGATCAACAACA
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GGCAGAAGATTGGGAGCTTAAAGGACAGCTCTTGCAGCAAGTACAGTGGTTATTTAGGCAGTCTGAAGCAA
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Deduced protein sequence of *BrSTM*

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FVMDATHPHHYFMDNVMGNPFIDHISSTML

Appendix 2 (Chapter 2):**Sequence analysis of *BnZLL2***

ATGCCGATTAGGC AAATGAAAGATAGCTCAGAGACTCATTTACTACCAAACCCAAGCATCTCCCCAA
AGCCGTGCAAAACGCTAAAGCCCCTCCTACTCCGAGCCAAGCTTCTTCACCTTCTCCTCCGTCCAAGA
ACCGTAGCCGGAGGAGAAACCGCGGTGGTGC AAAATCAGACCAAGGAGATGTCTGTATGAGGCCTAGC
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CTAA

Deduced protein sequence of *BnZLL2*

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GDVCMRPSSRPRKPPQNAAPVAAVSGTEIVAVNHQMGMVVRGSSKNSNFAPRPGFGQLG
TKCIVKANHFADLPKDL SHYDVTITPEVSSKSVNRAIIAELVRLYKESELGSRLPAYD
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NFSRSVQENVARGF CNELGQMCEVSGMEFNPEPVIPIYSARPDQVEKALKHYHTAMDKT
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APPENDICES

ARCTRSVSVVPPAYYAHAAFRARFYMEPEIMQDNGSPGKKNTKTTTVGDHGVVGGGVKP
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Appendix 3 (Chapter 2):**Sequence analysis of *BnCLV1***

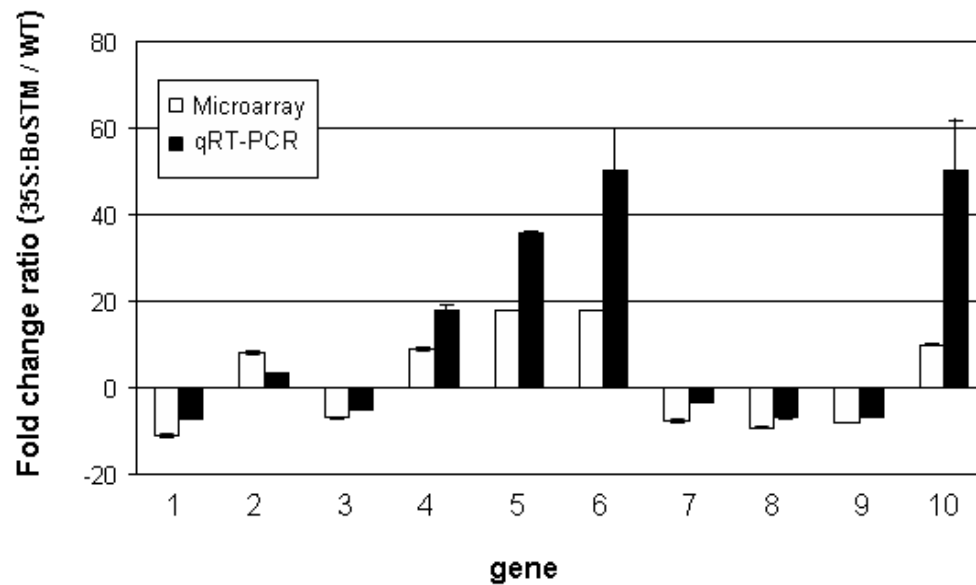
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Deduced protein sequence of *BnCLV1*

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VRWVRNTEGEIPQPSDAATVVAIVDQRLTGYPLTSVIHVFKIAMMCVEDEATTRPTMREVVHMLTNPPKS
VTNLIAF

Appendix 4 (Chapter 2): Genes and primers used for validating the microarray results

Gene	ID	Forward primer	Reverse primer
Cell division protein	AT1g78770	AAAGCTGTGCGGTGGTTTGAGAAG	TTGACCACAGTTGGTCCCAGGAT
Oxidoreductase	AT4g10500	GCGACCAAATGCAGGTCATAAGCA	AGTCGGAATCGATAACCGCTCGTT
Leucin-rich repeat	AT4g29240	TTGTATTCCCGGAGGAATTCGCA	TCCAACACTTATCGCAGCACAAAGC
Short chain dehydrogenase	AT3g12800	TATTCTTGTTAACGCCGCTGCTGG	GCTGCGTGACACATGTTGAATGTTCC
S-adenosylmethionine synthase	AT1g02500	AGGGCACATGTTTGTTATGCCAC	TTAGCCAGGCGCAAGTACCATTCT
Ketol-acid reductoisomerase	AT3g58610	AGACTGCATACAGCGCATCCTTCT	ACCGGCTAAGACAACACTTCGGAT
Peroxidase	At4g26010	GCGCAGACATTGTAACCTTGCCA	ATGCGCTAACCGGAATTGTTGGTC
Zinc finger (C3HC4) protein	At5g58580	ACATCGCCGGAGAAGACGAAGAAA	AAACCTCCGAGTGGGAGAGAAGGTAT
DNA Helicase	AT1g60930	GAGGATATTCTCGCAGAGGGTGTT	CGGCCCTTIGATCTGTTACCTTCA
Male sterility-MS5	AT5g4885	TGGGAGAATGGAGGAGCAAGTTGA	CGGCCGATAAGTACTTGGCTTGTT

Appendix 5 (Chapter 2): Validation of microarray experiments.

The expression level of ten genes was compared using the microarray data and quantitative qRT-PCR analyses. Values $\pm$ SD are expressed as foldchange ratios (35S::*BoSTM* / WT). Measurements were performed at the following stages of embryogenesis: stage 0 (gene 1 and 2); stage 1 (genes 3-6); stage 2 (genes 7, 8); stage 3 (genes 9, 10). List of genes used: 1, (At4g10500); 2, (At1g78770); 3, (At4g29240); 4, (At3g12800); 5, (At1g02500); 6, (At3g58610); 7, (At4g26010); 8, (At5g58580); 9, (At5g4885); and 10 (At1g60930). See Appendix 1 for sets of primers. Values for qRT-PCR measurements are means of three independent experiments.

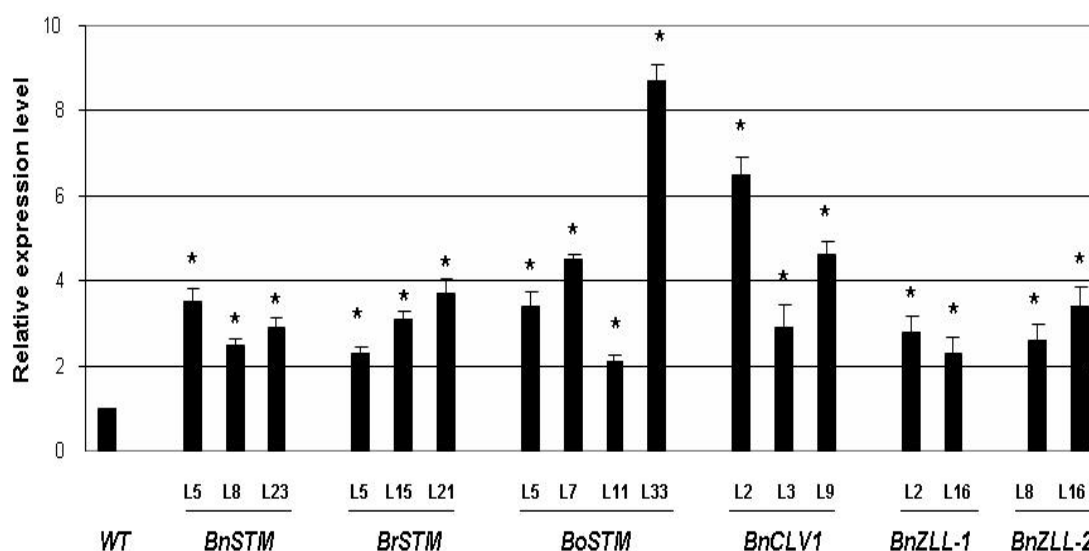
Appendix 6 (Chapter 3): List of primers used in chapter 3 for qRT-PCR:

Gene	ID	Primers (5' → 3')	
<i>WUS</i>	AT2G17950	F	ACCAACAGCCGATCAGATCCAGAA
		R	TGGGTGATGAAGATGGTGTGGTCA
<i>CLV3</i>	AT2G27250	F	CGTTCAAGGACTTTCCAACCGCAA
		R	TTGTTTCTTGGCTGTCTTGGTGGG
<i>CUC1</i>	AT3G15170	F	TCTGGATTTGGGATCATCGGGACA
		R	ACCGCATGGCGATCAGAGAGTAAA
<i>FUS3</i>	AT3G26790	F	GGCACTTGAATGCAAGGAAGGGAT
		R	AAGTCACCTAGCTGCAGACCATGA
<i>LEC1</i>	AT1G21970	F	AAATGGTATTGGGTTTCACGGCCC
		R	ACTGGATTTCATCTTGACCCGACGA
<i>LEC2</i>	AT1G28300	F	ACAATCGCTCGCACTTCACAACAG
		R	TTTGCGTAACCGGGATCTGAGGAT
<i>PIN1</i>	AT1G73590	F	ATCTCCGAGCAGTTTCCAGA
		R	CAGCTTCTCCAGGACCAAAG
<i>PIN2</i>	AT5G57090	F	CTTCTTTGGCAGGCGTTTAGCC
		R	TGATGCTCTGAAGGACCACGATCT
<i>PIN3</i>	AT1G70940	F	AGGTCCAACCTCCTCGACCTT
		R	TCCAGACGAACATGTGAAGC
<i>PIN4</i>	AT2G01420	F	ACTTTTCTCTCTTAACGGTTTCTTT
		R	TTGGAACCAAAAACCAAAGG
<i>PIN7</i>	AT1G23080	F	TTCACTCGCTCCGGTAGTCT
		R	GCTTACCATCGTCACCGATT

Appendix 7 (Chapter 4): List of primers used in chapter 4 for qRT-PCR:

Gene	ID	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
IAA1	AT4G14560	AGGACACAGAGCTTCGTTTGGGAT	CGTTGTTCTTGCCTTGTGTGTC
IAA5	AT1G15580	TCCGCTCTGCAAATTCTGTTCGG	TCTCCAGCAAGCATCCAATCTCCA
IAA8	AT2G22670	GGTGATTGGATGCTTGTGGCGAT	ACTGCACCTGGAGCTAAGCCAATA
IAA9	AT5G65670	TGGATCTAATGGAGCTGCTGGGAA	TCTCCACGGAACATCTCCAACAA
IAA11	AT4G28640	AGGGAATGAGCTTGAGCTTGGTCT	AGCTGTCCTCTTGATCCCAGCAAT
IAA19	AT3G15540	TTCATCTGGTGGTGACGCTGAGAA	TAAGGCACACCATCCATGCTCACT
IAA20	AT2G46990	ATGGCTACCGCGACTTGATCAGAA	TGCGTACGTTAGCACGTGACTCTT
IAA29	AT4G32280	TTCACCTTCCAAGGGAAAGAGGGT	TGGTCCGATTTGAACGCCTATCCT
AHK4	AT2G01830	AGGATCACCCGCAACTCTCAA	TGACCACTCTCTGCACAAACCACT
AHK3	AT1G27320	TGCAAAGTCACAGTTCCTTGCCAC	ACTTGCCTGTGCGGTCTTAACATA
CKI1	AT1G47430	AAGTTTCGGGCGTTTCTCTGAGAT	TGCACGCATATGCATCTCTCTCCT
ARR4	AT1G10470	TGGAATAATTCTGGGATGCCGTGT	AGGCGCGAGAGATTAAAGGGACAT
ARR5	AT3G48100	TGATCGGAAGTTCATCGAGCGTT	TCCCAGGCATAGAGTAATCCGTCA
ARR7	AT1G19050	GTTGCATGTTCTTGCCGTCGATGA	ACTGCAAAGCCCTAGTTCCACTCT
ARR15	AT1G74890	CCGGAGTTACATGTTCTTGCCGTT	TCCCCTCTCAACAGTCGTCCTT
ARR16	AT2G40670	CAGTTCAGGAGGTTCTTGTTTCGTC	GCGCATTCTCTGCTGTTGTCCTT
ARR1	AT3G16857	GCAGCCGATGTTATCAAACCGCAT	TCACGGGAAGTTCTGGTTGGAAGT
ARR2	AT4G16110	AAAGAGTGGCGGAGACAGTGAATT	GCCTTCCTGTTTGAGAAATGCGCT
ARR10	AT4G31920	TTGACGGCGATGACTATGGAGCAA	TATTGGCAGCGCTGAAGCAAAGTC
ARR12	AT2G25180	ACGGATGCTATGGCTCTGTTGAGT	ACAAATCTCCCTGGCTCTGTTCCCT
ARR13	AT2G27070	ATATAACCCTCAGCCACCTGCCAA	ATGTTACGAAGGTCCAGTCACCA

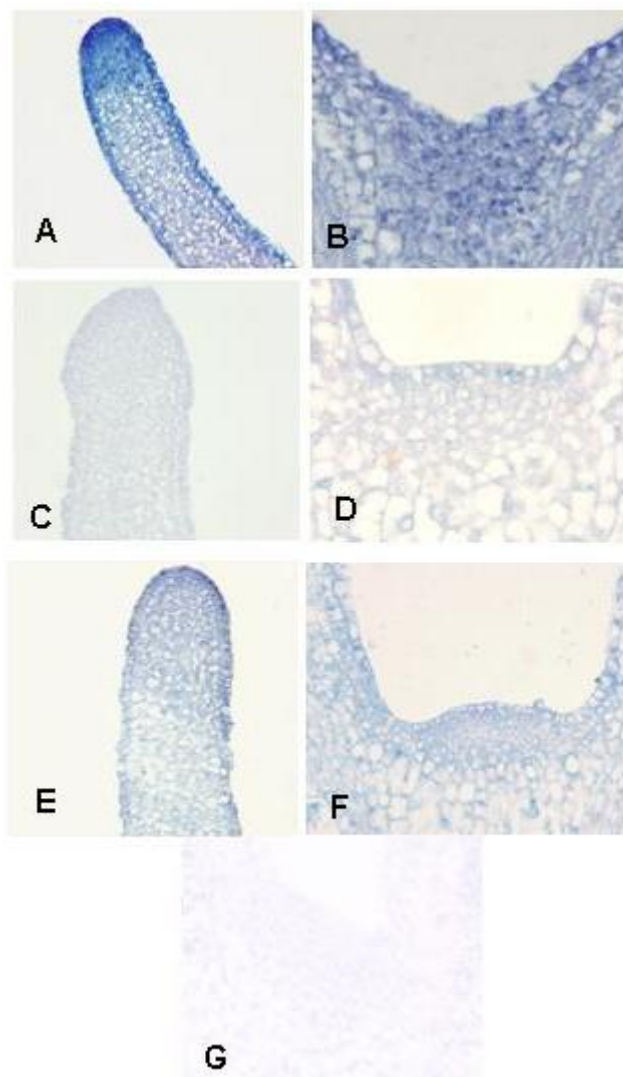
Appendix 8 (Chapter5): Characterization of *Arabidopsis* lines transformed with the *Brassica STM*, *CLV1*, *ZLL-1* and -2.



Expression level by quantitative qRT-PCR of *Arabidopsis* lines (10 day old seedlings) transformed with the *Brassica oleracea* (*Bo*), *B. rapa* (*Br*), and *B. napus* (*Bn*) *STM*, *BnCLV1*, and *BnZLL-1* and -2. Values $\pm$ SE are means of at least three independent analyses. Stars indicate values which are significantly different from the WT value ($P \leq 0.01$) set at 1. The primers utilized for these analyses recognize both the native *Arabidopsis* gene and the *Brassica* transgene (see Fig. 2.4. for details on primers). No differences in the expression levels of the native genes (*AtSTM*, *AtCLV1* and *AtZLL*) were observed between the WT line and the transformed lines.

Appendix 9 (Chapter 5): Primer sets, product size and number of cycles utilized to measure the expression levels of *Brassica* and *Arabidopsis* genes by semi-quantitative PCR.

Gene	Forward primers	Reverse Primers	Cycles
<u>Brassica</u>			
STM	5-TGGGACCCACAGGTTCTTTA-3	5-CCAGTTGATTCCGCTAGTGC-3	27
CLV1	5-TCTCCTTCAACGACCTCTCG-3	5-TGGTGAAGATAACACAGTCCTTTCCG-3	23
ZLL1	5-GACGACGACGGCGGCA GCTCAGAGCC-3	5-CAGACTCTTTGTATAGTCTCACTA-3	24
ZLL2	5-ACTCCGAGCCAAGCTTCTTCAC-3	5-CAGACTCTTTGTATAGTCTCACTA-3	26
Actin	5-TAAAGTATCCGATTGAGCATGGTAT-3	5-CGTAGGCAAGCTTCTCTTTAATGTC-3	22
<u>Arabidopsis</u>			
KNAT6	5-AGCGGTTTCATATTATTCTTCTTCTTCA-3	5-AAAAAGAGGTTATTTTATTCACCG-3	27
CUC1	5-TTCTTCTTGTGCCGACAATG-3	5-TCAGAGAGTAAACGGCCACA-3	25
WUS	5-CCAGCAAGTTGTTTCTTGC-3	5-CATCATAGAGATAAACGGTTGTCA-3	27
CLV3	5-TGGATTGGAAGAGTTTCTGC-3	5-GAAAATCATGAGATATAATAGTGC-3	29
UBQ10	5-GATCTTTGCGGAAAAACAATTGGAGGATGG-3	5-CGACTTGTCTTAGAAAAGAAAGAGATAACAGG-3	21

Appendix 10 (Chapter 5):

Immunolocalization of IAA in 25 day old *Brassica napus* microspore-derived embryos. Micrographs show cotyledons (**A**, **C**, and **E**) and shoot apical meristems (**B**, **D**, and **F**) of embryos cultured in the presence (**A**, **B**) or absence (**C**, **D**) of 2 μ M 2,3,5-triodobenzoic acid (TIBA). Embryos were also growth in the presence of TIBA plus 2 μ M 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB) (**E**,**F**). Specificity the signal was demonstrated by the omission of primary antibodies (**G**).

REFERENCES CITED

- Abel S., Theologis A. (1996) Early genes and auxin action. *Plant Physiology* 111:9-18.
- Ackerson R. (1984) Regulation of soybean embryogenesis by abscisic acid. *Journal of Experimental Botany* 35:403.
- Adrian J., Torti S., Turck F. (2009) From decision to commitment: the molecular memory of flowering. *Molecular plant* 2:628.
- Ahmed R., Dutta Gupta S., De D. (1996) Somatic embryogenesis and plant regeneration from leaf derived callus of winged bean [*Psophocarpus tetragonolobus* (L.) DC.]. *Plant Cell Reports* 15:531-535.
- Aida M., Ishida T., Tasaka M. (1999) Shoot apical meristem and cotyledon formation during *Arabidopsis* embryogenesis: interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. *Development* 126:1563.
- Aida M., Vernoux T., Furutani M., Traas J., Tasaka M. (2002) Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the *Arabidopsis* embryo. *Development* 129:3965.
- Akama K., Shiraishi H., Ohta S., Nakamura K., Okada K., Shimura Y. (1992) Efficient transformation of *Arabidopsis Thaliana*: comparison of the efficiencies with various organs, plant ecotypes and *Agrobacterium* strains. *Plant Cell Reports* 12:7-11.
- Albertini E., Marconi G., Reale L., Barcaccia G., Porceddu A., Ferranti F., Falcinelli M. (2005) SERK and APOSTART. Candidate genes for apomixis in *Poa pratensis*. *Plant Physiology* 138:2185.

- Argyros R., Mathews D., Chiang Y., Palmer C., Thibault D., Etheridge N., Argyros D., Mason M., Kieber J., Schaller G. (2008) Type B response regulators of *Arabidopsis* play key roles in cytokinin signaling and plant development. *The Plant Cell* 20:2102.
- Ashihara H., Fujimura T., Komamine A. (1981) Pyrimidine nucleotide biosynthesis during somatic embryogenesis in a carrot cell suspension culture. *Z. Pflanzenphysiol* 104:129-137.
- Baker C., Wetzstein H. (1994) Influence of auxin type and concentration on peanut somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* 36:361-368.
- Baluska F., Hlavacka A., Samaj J., Palme K., Robinson D., Match T., McCurdy D., Menzel D., Volkmann D. (2002) F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. *Plant Physiology* 130:422.
- Banno H., Ikeda Y., Niu Q., Chua N. (2001) Overexpression of *Arabidopsis* ESR1 induces initiation of shoot regeneration. *The Plant Cell* 13:2609.
- Bao Y., Dharmawardhana P., Mockler T., Strauss S. (2009) Genome scale transcriptome analysis of shoot organogenesis in *Populus*. *BMC Plant Biology* 9:132.
- Barinova I., Clement C., Martiny L., Baillieul F., Soukupova H., Heberle-Bors E., Touraev A. (2004) Regulation of developmental pathways in cultured microspores of tobacco and snapdragon by medium pH. *Planta* 219:141-146.

- Barth S., Geier T., Eimert K., Watillon B., Sangwan R., Gleissberg S. (2009) KNOX overexpression in transgenic *Kohleria* (Gesneriaceae) prolongs the activity of proximal leaf blastozones and drastically alters segment fate. *Planta* 230:1081-1091.
- Barton M. (2009) Twenty years on: The inner workings of the shoot apical meristem, a developmental dynamo. *Developmental Biology*.
- Barton M., Poethig R. (1993) Formation of the shoot apical meristem in *Arabidopsis Thaliana*: an analysis of development in the wild type and in the shoot meristemless mutant. *Development* 119:823.
- Bassuner B., Lam R., Lukowitz W., Yeung E. (2007) Auxin and root initiation in somatic embryos of *Arabidopsis*. *Plant Cell Reports* 26:1-11.
- Beaudoin-Eagan L., Thorpe T. (1985) Tyrosine and phenylalanine ammonia lyase activities during shoot initiation in tobacco callus cultures. *Plant Physiology* 78:438.
- Bellaoui M., Pidkowich M., Samach A., Kushalappa K., Kohalmi S., Modrusan Z., Crosby W., Haughn G. (2001) The *Arabidopsis* BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. *The Plant Cell* 13:2455.
- Belles-Boix E., Hamant O., Witiak S., Morin H., Traas J., Pautot V. (2006) KNAT6: an *Arabidopsis* homeobox gene involved in meristem activity and organ separation. *The Plant Cell* 18:1900.
- Belmonte M., Tahir M., Schroeder D., Stasolla C. (2007) Overexpression of HBK3, a class I KNOX homeobox gene, improves the development of Norway spruce (*Picea abies*) somatic embryos. *Journal of Experimental Botany* 58:2851.

- Belmonte M., Donald G., Reid D., Yeung E., Stasolla C. (2005) Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*). *Journal of Experimental Botany* 56:2355.
- Belmonte M., Ambrose S., Ross A., Abrams S., Stasolla C. (2006) Improved development of microspore-derived embryo cultures of *Brassica napus* cv Topaz following changes in glutathione metabolism. *Physiologia Plantarum* 127:690-700.
- Benjamini Y., Hochberg Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B (Methodological)* 57:289-300.
- Benková E., Hejácíko J. (2009) Hormone interactions at the root apical meristem. *Plant Molecular Biology* 69:383-396.
- Benková E., Michniewicz M., Sauer M., Teichmann T., Seifertová D., Jürgens G., Friml J. (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* 115:591-602.
- Berleth T., Krogan N., Scarpella E. (2004) Auxin signals--turning genes on and turning cells around. *Current opinion in plant biology* 7:553-563.
- Bernier G., Havelange A., Houssa C., Petitjean A., Lejeune P. (1993) Physiological signals that induce flowering. *The Plant Cell* 5:147-1.
- Bhalla P., Singh M. (2006) Molecular control of stem cell maintenance in shoot apical meristem. *Plant Cell Reports* 25:249-256.
- Bhalla P., Singh M. (2008) *Agrobacterium*-mediated transformation of *Brassica napus* and *Brassica oleracea*. *Nature Protocols* 3:181-189.

REFERENCES

- Bhojwani S., Razdan M. (1996) Plant tissue culture: theory and practice Elsevier Science Ltd.
- Blilou I., Wildwater M.V., Paponov I. (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* 433:39-44.
- Bolduc N., Hake S. (2009) The maize transcription factor KNOTTED1 directly regulates the gibberellin catabolism gene *ga2ox1*. *The Plant Cell Online* 21:1647.
- Boutilier K., Fiers M., Liu C., Geest A. (2005) Biochemical and molecular aspects of haploid embryogenesis. *Haploids in crop improvement II*:73-95.
- Boutilier K., Offringa R., Sharma V., Kieft H., Ouellet T., Zhang L., Hattori J., Liu C., van Lammeren A., Miki B. (2002) Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryonic growth. *The Plant Cell Online* 14:1737.
- Brand U., Grunewald M., Hobe M., Simon R. (2002) Regulation of CLV3 expression by two homeobox genes in *Arabidopsis*. *Plant Physiology* 129:565.
- Brown D., Thorpe T. (1980) Changes in water potential and its components during shoot formation in tobacco callus. *Physiologia Plantarum* 49:83-87.
- Brown D., Leung D., Thorpe T. (1979) Osmotic requirement for shoot formation in tobacco callus. *Physiologia Plantarum* 46:36-41.
- Brown P. (1989) DNA methylation in plants and its role in tissue culture. *Genome* 31:717-729.
- Cai Q., Guy C., Moore G. (1996) Detection of cytosine methylation and mapping of a gene influencing cytosine methylation in the genome of Citrus. *Genome* 39:235-242.

- Campos-Cuevas J., Pelagio-Flores R., Raya-González J., Méndez-Bravo A., Ortiz-Castro R., López-Bucio J. (2008) Tissue culture of *Arabidopsis Thaliana* explants reveals a stimulatory effect of alkamides on adventitious root formation and nitric oxide accumulation. *Plant Science* 174:165-173.
- Canhoto J., Cruz G. (1994) Improvement of somatic embryogenesis in *Feijoa sellowiana* berg (Myrtaceae) by manipulation of culture media composition. *In Vitro Cellular & Developmental Biology-Plant* 30:21-25.
- Canton F., Suárez M., Jose-Estanyol M., Canovas F. (1999) Expression analysis of a cytosolic glutamine synthetase gene in cotyledons of Scots pine seedlings: developmental, light regulation and spatial distribution of specific transcripts. *Plant Molecular Biology* 40:623-634.
- Carles C., Fletcher J. (2003) Shoot apical meristem maintenance: the art of a dynamic balance. *Trends in plant science* 8:394-401.
- Cary A., Che P., Howell S. (2002) Developmental events and shoot apical meristem gene expression patterns during shoot development in *Arabidopsis Thaliana*. *The Plant Journal* 32:867-877.
- Cerutti L., Mian N., Bateman A. (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends in biochemical sciences* 25:481.
- Chan J., Pauls P. (2007) *Brassica napus* Rop GTPases and their expression in microspore cultures. *Planta* 225:469-484.

- Charrière F., Sotta B., Miginiac É., Hahne G. (1999) Induction of adventitious shoots or somatic embryos on in vitro cultured zygotic embryos of *Helianthus annuus*: Variation of endogenous hormone levels. *Plant Physiology et Biochemistry* 37:751-757.
- Chaudhury A., Letham S., Craig S., Dennis E. (1993) amp1-a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. *The Plant Journal* 4:907-916.
- Che P., Lall S., Howell S. (2007) Developmental steps in acquiring competence for shoot development in *Arabidopsis* tissue culture. *Planta* 226:1183-1194.
- Che P., Gingerich D., Lall S., Howell S. (2002) Global and hormone-induced gene expression changes during shoot development in *Arabidopsis*. *The Plant Cell* 14:2771.
- Chen H., Banerjee A., Hannapel D. (2004) The tandem complex of BEL and KNOX partners is required for transcriptional repression of *ga20ox1*. *The Plant Journal* 38:276-284.
- Chen J., Chang W. (2005) TIBA affects the induction of direct somatic embryogenesis from leaf explants of *Oncidium*. *Plant Cell, Tissue and Organ Culture* 79:315-320.
- Cheng Y., Dai X., Zhao Y. (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. *Genes & development* 20:1790.
- Chiwocha S., Abrams S., Ambrose S., Cutler A., Loewen M., Ross A., Kermode A. (2003) A method for profiling classes of plant hormones and their metabolites using

liquid chromatography-electrospray ionization tandem mass spectrometry: an analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. The Plant Journal 35:405-417.

Choi Y., Katsumi M., Sano H. (2001) Triiodobenzoic acid, an auxin polar transport inhibitor, suppresses somatic embryo formation and postembryonic shoot/root development in *Eleutherococcus senticosus*. Plant Science 160:1183-1190.

Choi Y., Kim H., Soh W., Yang D. (1997) Developmental and structural aspects of somatic embryos formed on medium containing 2, 3, 5-triiodobenzoic acid. Plant Cell Reports 16:738-744.

Chuck G., Lincoln C., Hake S. (1996) KNAT1 induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. The Plant Cell 8:1277.

Chung H., Chen J., Chang W. (2005) Cytokinins induce direct somatic embryogenesis of *Dendrobium chiengmai* pink and subsequent plant regeneration. In Vitro Cellular & Developmental Biology-Plant 41:765-769.

Clark S., Williams R., Meyerowitz E. (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 89:575-585.

Clark S., Jacobsen S., Levin J., Meyerowitz E. (1996) The CLAVATA and SHOOT MERISTEMLESS loci competitively regulate meristem activity in *Arabidopsis*. Development 122:1567.

Cole M., Nolte C., Werr W. (2006) Nuclear import of the transcription factor SHOOT MERISTEMLESS depends on heterodimerization with BLH proteins expressed in

discrete sub-domains of the shoot apical meristem of *Arabidopsis Thaliana*. Nucleic acids research 34:1281.

Custers J., Cordewener J., Nölle Y., Dons H., Van Lockeren Campagne M. (1994) Temperature controls both gametophytic and sporophytic development in microspore cultures of *Brassica napus*. Plant Cell Reports 13:267-271.

Daimon Y., Takabe K., Tasaka M. (2003) The CUP-SHAPED COTYLEDON genes promote adventitious shoot formation on calli. Plant and Cell Physiology 44:113.

de Reuille P., Bohn-Courseau I., Ljung K., Morin H., Carraro N., Godin C., Traas J. (2006) Computer simulations reveal properties of the cell-cell signaling network at the shoot apex in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America 103:1627.

di Giacomo E., Sestili F., Iannelli M., Testone G., Mariotti D., Frugis G. (2008) Characterization of KNOX genes in *Medicago truncatula*. Plant Molecular Biology 67:135-150.

Dodsworth S. (2009) A diverse and intricate signalling network regulates stem cell fate in the shoot apical meristem. Developmental Biology 336:1-9.

Eapen S., George L. (1993) Somatic embryogenesis in peanut: influence of growth regulators and sugars. Plant Cell, Tissue and Organ Culture 35:151-156.

Economou A. (1990) Ethylene and shoot formation in vitro. In Vitro Culture, XXIII IHC 300:35-44.

Endrizzi K., Moussian B., Haecker A., Levin J., Laux T. (1996) The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in

Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE. *The Plant Journal* 10:967-979.

Eshed Y., Baum S., Bowman J. (1999) Distinct mechanisms promote polarity establishment in carpels of *Arabidopsis*. *Cell* 99:199-209.

Feher A., Pasternak T., Dudits D. (2003) Transition of somatic plant cells to an embryogenic state. *Plant Cell, Tissue and Organ Culture* 74:201-228.

Feraru E., Friml J. (2008) PIN polar targeting. *Plant Physiology* 147:1553.

Fiers M., Golemiec E., Xu J., Van Der Geest L., Heidstra R., Stiekema W., Liu C. (2005) The 14-amino acid CLV3, CLE19, and CLE40 peptides trigger consumption of the root meristem in *Arabidopsis* through a CLAVATA2-dependent pathway. *The Plant Cell* 17:2542.

Finkelstein R., Tenbarge K., Shumway J., Crouch M. (1985) Role of ABA in maturation of rapeseed embryos. *Plant Physiology* 78:630.

Finnegan E., Kovac K. (2000) Plant DNA methyltransferases. *Plant Molecular Biology* 43:189-201.

Fleming A. (2005) Formation of primordia and phyllotaxy. *Current opinion in plant biology* 8:53-58.

Fletcher J. (2002) Coordination of cell proliferation and cell fate decisions in the angiosperm shoot apical meristem. *BioEssays* 24:27-37.

Friml J., Palme K. (2002) Polar auxin transport—old questions and new concepts? *Plant Molecular Biology* 49:273-284.

- Friml J., Vieten A., Sauer M., Weijers D., Schwarz H., Hamann T., Offringa R., Jurgens G. (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. *Nature* 426:147-153.
- Frugis G., Giannino D., Mele G., Nicolodi C., Chiappetta A., Bitonti M., Innocenti A., Dewitte W., Van Onckelen H., Mariotti D. (2001) Overexpression of KNAT1 in lettuce shifts leaf determinate growth to a shoot-like indeterminate growth associated with an accumulation of isopentenyl-type cytokinins. *Plant Physiology* 126:1370.
- Furutani M., Vernoux T., Traas J., Kato T., Tasaka M., Aida M. (2004) PINFORMED1 and PINOID regulate boundary formation and cotyledon development in *Arabidopsis* embryogenesis. *Development* 131:5021.
- Gaj M., Zhang S., Harada J., Lemaux P. (2005) Leafy cotyledon genes are essential for induction of somatic embryogenesis of *Arabidopsis*. *Planta* 222:977-988.
- Galinha C., Bilsborough G., Tsiantis M. (2009) Hormonal input in plant meristems: A balancing act, Elsevier. pp. 1149-1156.
- Gallois J., Woodward C., Reddy G., Sablowski R. (2002) Combined SHOOT MERISTEMLESS and WUSCHEL trigger ectopic organogenesis in *Arabidopsis*. *Development* 129:3207.
- Garcia-Martin G., Manzanera J., Gonzalez-Benito M. (2005) Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos. *Plant Cell, Tissue and Organ Culture* 80:171-177.

- Gaspar T., Kevers C., Faivre-Rampant O., Crèvecoeur M., Penel C., Greppin H., Dommes J. (2003) Changing concepts in plant hormone action. *In Vitro Cellular & Developmental Biology-Plant* 39:85-106.
- Ghoshal K., Datta J., Majumder S., Bai S., Kutay H., Motiwala T., Jacob S. (2005) 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Molecular and cellular biology* 25:4727.
- Giulini A., Wang J., Jackson D. (2004) Control of phyllotaxy by the cytokinin-inducible response regulator homologue ABPHYL1. *Nature* 430:1031-1034.
- Golz J. (2006) Signalling between the shoot apical meristem and developing lateral organs. *Plant Molecular Biology* 60:889-903.
- Gordon S., Chickarmane V., Ohno C., Meyerowitz E. (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. *Proceedings of the National Academy of Sciences of the United States of America* 106:16529.
- Gordon S., Heisler M., Reddy G., Ohno C., Das P., Meyerowitz E. (2007) Pattern formation during de novo assembly of the *Arabidopsis* shoot meristem. *Development* 134:3539.
- Griffith O., Meister A. (1979) Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (S-n-butyl homocysteine sulfoximine). *Journal of Biological Chemistry* 254:7558.

- Gross-Hardt R., Laux T. (2003) Stem cell regulation in the shoot meristem. *Journal of Cell Science* 116:1659.
- Guohua M. (1998) Effects of cytokinins and auxins on cassava shoot organogenesis and somatic embryogenesis from somatic embryo explants. *Plant Cell, Tissue and Organ Culture* 54:1-7.
- Hakman I., Hallberg H., Palovaara J. (2009) The polar auxin transport inhibitor NPA impairs embryo morphology and increases the expression of an auxin efflux facilitator protein PIN during *Picea abies* somatic embryo development. *Tree physiology*.
- Hall T. (2005) Structure and function of argonaute proteins. *Structure* 13:1403-1408.
- Hamant O., Nogue F., Belles-Boix E., Jublot D., Grandjean O., Traas J., Pautot V. (2002) The KNAT2 homeodomain protein interacts with ethylene and cytokinin signaling. *Plant Physiology* 130:657.
- Harada J. (2001) Role of *Arabidopsis* LEAFY COTYLEDON genes in seed development. *Journal of Plant Physiology* 158:405-409.
- Hardy E., Thorpe T. (1990) Nitrate assimilation in shoot-forming tobacco callus cultures. *In Vitro Cellular & Developmental Biology-Plant* 26:525-530.
- Hareven D., Gutfinger T., Parnis A., Eshed Y., Lifschitz E. (1996) The making of a compound leaf: genetic manipulation of leaf architecture in tomato. *Cell* 84:735-744.
- Hay A., Tsiantis M. (2006) The genetic basis for differences in leaf form between *Arabidopsis Thaliana* and its wild relative *Cardamine hirsuta*. *Nature genetics* 38:942-947.

Hay A., Kaur H., Phillips A., Hedden P., Hake S., Tsiantis M. (2002) The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. *Current Biology* 12:1557-1565.

Hays D. (1996) The role of hormones in *Brassica napus* embryo development, PhD thesis. University of Calgary, Calgary, Alberta, Canada.

Hecht V., Vielle-Calzada J., Hartog M., Schmidt E., Boutilier K., Grossniklaus U., De Vries S. (2001) The *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiology* 127:803.

Hedden P., Thomas S. (2006) Plant hormone signaling Wiley-Blackwell.

Heisler M., Ohno C., Das P., Sieber P., Reddy G., Long J., Meyerowitz E. (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. *Current Biology* 15:1899-1911.

Hibara K., Takada S., Tasaka M. (2003) CUC1 gene activates the expression of SAM-related genes to induce adventitious shoot formation. *The Plant Journal* 36:687-696.

Hicks G. (1994) Shoot induction and organogenesis *in vitro*: a developmental perspective. *In Vitro Cellular & Developmental Biology-Plant* 30:10-15.

Higuchi M., Pischke M., Mähönen A., Miyawaki K., Hashimoto Y., Seki M., Kobayashi M., Shinozaki K., Kato T., Tabata S. (2004) In planta functions of the *Arabidopsis* cytokinin receptor family. *Proceedings of the National Academy of Sciences of the United States of America* 101:8821.

- Hirose N., Makita N., Kojima M., Kamada-Nobusada T., Sakakibara H. (2007) Overexpression of a type-A response regulator alters rice morphology and cytokinin metabolism. *Plant and Cell Physiology* 48:523.
- Hu H., Xiong L., Yang Y. (2005) Rice SERK1 gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. *Planta* 222:107-117.
- Huang B., Yeoman M. (1995) Somatic embryogenesis in *Arabidopsis Thaliana* L. *Biotechnology in Agriculture and Forestry*:371-371.
- Hundertmark M., Hinch D. (2008) LEA (Late Embryogenesis Abundant) proteins and their encoding genes in *Arabidopsis Thaliana*. *BMC genomics* 9:118.
- Hutchinson M., Murch S., Saxena P. (1996) Morphoregulatory role of TDZ: evidence of the involvement of endogenous auxin in TDZ-induced somatic embryogenesis of Geranium (*Pelargonium X hortorum* Bailey). *Journal of Plant Physiology* 149:573-579.
- Hwang I., Sheen J. (2001) Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* 413:383-389.
- Hwang I., Chen H., Sheen J. (2002) Two-component signal transduction pathways in *Arabidopsis*. *Plant Physiology* 129:500.
- Ikeda-Iwai M., Satoh S., Kamada H. (2002) Establishment of a reproducible tissue culture system for the induction of *Arabidopsis* somatic embryos. *Journal of Experimental Botany* 53:1575.

- Ikezaki M., Kojima M., Sakakibara H., Kojima S., Ueno Y., Machida C., Machida Y. (2010) Genetic networks regulated by ASYMMETRIC LEAVES1 (AS1) and AS2 in leaf development in *Arabidopsis Thaliana*: KNOX genes control five morphological events. *The Plant Journal* 61:70-82.
- Ilic -Grubor K., Attree S., Fowke L. (1998) Induction of microspore-derived embryos of *Brassica napus* L. with polyethylene glycol (PEG) as osmoticum in a low sucrose medium. *Plant Cell Reports* 17:329-333.
- Imamura A., Hanaki N., Umeda H., Nakamura A., Suzuki T., Ueguchi C., Mizuno T. (1998) Response regulators implicated in His-to-Asp phosphotransfer signaling in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* 95:2691.
- Inoue T., Higuchi M., Hashimoto Y., Seki M., Kobayashi M., Kato T., Tabata S., Shinozaki K., Kakimoto T. (2001) Identification of CRE1 as a cytokinin receptor from *Arabidopsis*. *Nature* 409:1060-1063.
- Ioio R., Linhares F., Sabatini S. (2008) Emerging role of cytokinin as a regulator of cellular differentiation. *Current opinion in plant biology* 11:23-27.
- Ivanova A., Velcheva M., Denchev P., Atanassov A., Onckelen H. (1994) Endogenous hormone levels during direct somatic embryogenesis in *Medicago falcata*. *Physiologia Plantarum* 92:85-89.
- Jacqmard A., Houssa C., Bernier G. (1995) Absciscic acid antagonizes the effect of cytokinin on DNA-replication origins. *Journal of Experimental Botany* 46:663.

- Janosevic D., Budimir S. (2006) Shoot apical meristem structure and STM expression in has mutant of *Arabidopsis Thaliana*. *Biologia Plantarum* 50:193-197.
- Jarret R., Hasegawa P., Bressan R. (1981) Gibberellic acid regulation of adventitious shoot formation from tuber discs of potato. *In Vitro Cellular & Developmental Biology-Plant* 17:825-830.
- Jasinski S., Piazza P., Craft J., Hay A., Woolley L., Rieu I., Phillips A., Hedden P., Tsiantis M. (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. *Current Biology* 15:1560-1565.
- Jiang B., Yang Y., Guo Y., Guo Z., Chen Y. (2005) Thidiazuron-induced in vitro shoot organogenesis of the medicinal plant *Arnebia euchroma* (Royle) Johnst. *In Vitro Cellular & Developmental Biology-Plant* 41:677-681.
- Jiang K., Meng Y., Feldman L. (2003) Quiescent center formation in maize roots is associated with an auxin-regulated oxidizing environment. *Development* 130:1429.
- Jimenez V. (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. *Plant Growth Regulation* 47:91-110.
- Jin Z., Ryu S., Hong J., Song C., Lim C., Choi Y., Lee K., Chung W., Lee S., Lim C. (2007) Over-expression of Chinese cabbage calreticulin 1b, BrCRT1b, induces initiation of organogenesis in *Arabidopsis*. *Korean Journal of Genetics* 29:409.
- Joosen R., Cordewener J., Supena E., Vorst O., Lammers M., Maliepaard C., Zeilmaker T., Miki B., America T., Custers J. (2007) Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of *Brassica napus* microspore-derived embryo development. *Plant Physiology*.

- Kakimoto T. (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. *Science* 274:982.
- Kamada-Nobusada T., Sakakibara H. (2009) Molecular basis for cytokinin biosynthesis. *Phytochemistry* 70:444-449.
- Kamada H., Harada H. (1981) Changes in the endogenous level and effects of abscisic acid during somatic embryogenesis of *Daucus carota* L. *Plant and Cell Physiology* 22:1423.
- Karami O., Saidi A. (2009) The molecular basis for stress-induced acquisition of somatic embryogenesis. *Molecular Biology Reports*:1-15.
- Karami O., Aghavaishi B., Mahmoudi Pour A. (2009) Molecular aspects of somatic-to-embryogenic transition in plants. *Journal of Chemical Biology* 2:177-190.
- Karimi M., Inzé D., Depicker A. (2002) GATEWAY (TM) vectors for *Agrobacterium*-mediated plant transformation. *Trends in plant science* 7:193-195.
- Kasha K., Yao Q., Simion E., Oro R., Hu T. (1995) Production and application of doubled haploids in crops, IAEA.
- Keller W., Armstrong K. (1979) Stimulation of embryogenesis and haploid production in *Brassica campestris* anther cultures by elevated temperature treatments. *TAG Theoretical and Applied Genetics* 55:65-67.
- Kerstetter R., Vollbrecht E., Lowe B., Veit B., Yamaguchi J., Hake S. (1994) Sequence analysis and expression patterns divide the maize knotted1-like homeobox genes into two classes. *The Plant Cell* 6:1877.

Kessler S., Seiki S., Sinha N. (2002) Xcl1 causes delayed oblique periclinal cell divisions in developing maize leaves, leading to cellular differentiation by lineage instead of position. *Development* 129:1859.

Kessler S., Townsley B., Sinha N. (2006) L1 division and differentiation patterns influence shoot apical meristem maintenance. *Plant Physiology* 141:1349.

Kiba T., Yamada H., Sato S., Kato T., Tabata S., Yamashino T., Mizuno T. (2003) The type-A response regulator, ARR15, acts as a negative regulator in the cytokinin-mediated signal transduction in *Arabidopsis Thaliana*. *Plant and Cell Physiology* 44:868.

Kong L., Von Aderkas P. (2007) Genotype effects on ABA consumption and somatic embryo maturation in interior spruce (*Picea glauca* x *engelmanni*). *Journal of Experimental Botany* 58:1525.

Kong L., Abrams S., Owen S., Graham H., Von Aderkas P. (2008) Phytohormones and their metabolites during long shoot development in Douglas-fir following cone induction by gibberellin injection. *Tree physiology* 28:1357.

Koornneef M., Hanhart C., Hilhorst H., Karssen C. (1989) In vivo inhibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis Thaliana*. *Plant Physiology* 90:463.

Kopertekh L., Butenko R. (1995) Naturally occurring phytohormones in wheat explants as related to wheat morphogenesis in vitro. *Russian Journal of Plant Physiology* 42:488-491.

- Kumar P., Thorpe T. (1989) Putrescine metabolism in excised cotyledons of *Pinus radiata* cultured in vitro. *Physiologia Plantarum* 76:521-526.
- Kuo H., Chen J., Chang W. (2005) Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. *In Vitro Cellular & Developmental Biology-Plant* 41:453-456.
- Kurakawa T., Ueda N., Maekawa M., Kobayashi K., Kojima M., Nagato Y., Sakakibara H., Kyojuka J. (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445:652-655.
- Kurdyukov S., Kereszt A., Gresshoff P., Rose R. (2009) The association of homeobox gene expression with stem cell formation and morphogenesis in cultured *Medicago truncatula*. *Planta* 230:827-840.
- Kwong R., Bui A., Lee H., Kwong L., Fischer R., Goldberg R., Harada J. (2003) LEAFY COTYLEDON1-LIKE defines a class of regulators essential for embryo development. *The Plant Cell* 15:5.
- Laufs P., Peaucelle A., Morin H., Traas J. (2004) MicroRNA regulation of the CUC genes is required for boundary size control in *Arabidopsis* meristems. *Development* 131:4311.
- Laux T., Mayer K., Berger J., Jurgens G. (1996) The WUSCHEL gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* 122:87.
- Lazzeri P., Hildebrand D., Sunega J., Williams E., Collins G. (1988) Soybean somatic embryogenesis: interactions between sucrose and auxin. *Plant Cell Reports* 7:517-520.

REFERENCES

- Lee D., Park J., Ku S., Ha Y., Kim S., Kim M., Oh M., Kim J. (2007) Genome-wide expression profiling of *ARABIDOPSIS* RESPONSE REGULATOR 7 (ARR7) overexpression in cytokinin response. *Molecular Genetics and Genomics* 277:115-137.
- Leibfried A., To J., Busch W., Stehling S., Kehle A., Demar M., Kieber J., Lohmann J. (2005) WUSCHEL controls meristem function by direct regulation of cytokinin-inducible response regulators. *Nature* 438:1172-1175.
- Leljak-Levani D., Bauer N., Mihaljevi S., Jelaska S. (2004) Changes in DNA methylation during somatic embryogenesis in *Cucurbita pepo* L. *Plant Cell Reports* 23:120-127.
- Lenhard M., Laux T. (1999) Shoot meristem formation and maintenance. *Current opinion in plant biology* 2:44-50.
- Lenhard M., Laux T. (2003) Stem cell homeostasis in the *Arabidopsis* shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. *Development* 130:3163.
- Lenhard M., Jurgens G., Laux T. (2002) The WUSCHEL and SHOOTMERISTEMLESS genes fulfil complementary roles in *Arabidopsis* shoot meristem regulation. *Development* 129:3195.
- Leyser O. (2005) Auxin distribution and plant pattern formation: how many angels can dance on the point of PIN? *Cell* 121:819-822.

- Li L., Qu R. (2002) *In vitro* somatic embryogenesis in turf-type bermudagrass: roles of abscisic acid and gibberellic acid, and occurrence of secondary somatic embryogenesis. *Plant Breeding* 121:155-158.
- Lichter R. (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. *Z. Pflanzenphysiol* 105:427-434.
- Lin X., Hwang G., Zimmerman J. (1996) Isolation and characterization of a diverse set of genes from carrot somatic embryos. *Plant Physiology* 112:1365.
- Lincoln C., Long J., Yamaguchi J., Serikawa K., Hake S. (1994) A knotted1-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *The Plant Cell* 6:1859.
- Lindsay D., Sawhney V., Bonham-Smith P. (2006) Cytokinin-induced changes in CLAVATA1 and WUSCHEL expression temporally coincide with altered floral development in *Arabidopsis*. *Plant Science* 170:1111-1117.
- Liu C., Xu Z., Chua N. (1993) Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. *The Plant Cell* 5:621.
- Liu J., Ha D., Xie Z., Wang C., Wang H., Zhang W., Zhang J., Chen S. (2008) Ectopic expression of soybean GmKNT1 in *Arabidopsis* results in altered leaf morphology and flower identity. *Journal of Genetics and Genomics* 35:441-449.
- Livak K., Schmittgen T. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2- $^{-\Delta\Delta CT}$ method. *Methods* 25:402-408.

- Lo Schiavo F., Pitto L., Giuliano G., Torti G., Nuti-Ronchi V., Marazziti D., Vergara R., Orselli S., Terzi M. (1989) DNA methylation of embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. *TAG Theoretical and Applied Genetics* 77:325-331.
- Long J., Barton M. (1998) The development of apical embryonic pattern in *Arabidopsis*. *Development* 125:3027.
- Long J., Moan E., Medford J., Barton M. (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. *Nature*.
- Lotan T., Ohto M., Yee K., West M., Lo R., Kwong R., Yamagishi K., Fischer R., Goldberg R., Harada J. (1998) *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell* 93:1195-1205.
- Lund R. (1975) Tables for an approximate test for outliers in linear models. *Technometrics* 17:473-476.
- Luo Y., Koop H. (1997) Somatic embryogenesis in cultured immature zygotic embryos and leaf protoplasts of *Arabidopsis Thaliana* ecotypes. *Planta* 202:387-396.
- Lynn K., Fernandez A., Aida M., Sedbrook J., Tasaka M., Masson P., Barton M. (1999) The PINHEAD/ZWILLE gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the ARGONAUTE1 gene. *Development* 126:469.
- Ma J., Ye K., Patel D. (2004) Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* 429:318-322.

- Malabadi R., Van Staden J. (2005) Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. In Vitro Cellular & Developmental Biology-Plant 41:181-186.
- Malik M., Wang F., Dirpaul J., Zhou N., Polowick P., Ferrie A., Krochko J. (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiology 144:134.
- Malik M., Wang F., Dirpaul J., Zhou N., Hammerlindl J., Keller W., Abrams S., Ferrie A., Krochko J. (2008) Isolation of an embryogenic line from non-embryogenic *Brassica napus* cv. Westar through microspore embryogenesis. Journal of Experimental Botany 59:2857.
- Maraschin S., De Priester W., Spaink H., Wang M. (2005) Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. Journal of Experimental Botany 56:1711.
- Matsuoka M., Ichikawa H., Saito A., Tada Y., Fujimura T., Kano-Murakami Y. (1993) Expression of a rice homeobox gene causes altered morphology of transgenic plants. The Plant Cell 5:1039.
- Mayer K., Schoof H., Haecker A., Lenhard M., Jürgens G., Laux T. (1998) Role of WUSCHEL in regulating stem cell fate in the *Arabidopsis* shoot meristem. Cell 95:805-815.
- McConnell J., Barton M. (1995) Effect of mutations in the PINHEAD gene of *Arabidopsis* on the formation of shoot apical meristems. Developmental Genetics 16:358-366.

Meinke D., Franzmann L., Nickle T., Yeung E. (1994) Leafy cotyledon mutants of *Arabidopsis*. The Plant Cell 6:1049.

Mendoza M., Kaeppler H. (2002) Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). In Vitro Cellular & Developmental Biology-Plant 38:39-45.

Mengoli M., Bagni N., Luccarini G., NUTI R. (1989) *Daucus carota* cell cultures: polyamines and effect of polyamine biosynthesis inhibitors in the preembryogenic phase and different embryo stages. Journal of Plant Physiology 134:389-394.

Minocha S., Papa N., Khan A., Samuelsen A. (1991) Polyamines and somatic embryogenesis in carrot. III. Effects of methylglyoxal bis (guanyldrazone). Plant and Cell Physiology 32:395.

Monk M., Boubelik M., Lehnert S. (1987) Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. Development 99:371.

Mordhorst A., Toonen M., De Vries S. (1997) Plant embryogenesis. Critical Reviews in Plant Sciences 16:535-576.

Mordhorst A., Hartog M., El Tamer M., Laux T., de Vries S. (2002) Somatic embryogenesis from *Arabidopsis* shoot apical meristem mutants. Planta 214:829-836.

Mordhorst A., Voerman K., Hartog M., Meijer E., van Went J., Koornneef M., de Vries S. (1998) Somatic embryogenesis in *Arabidopsis Thaliana* is facilitated by mutations in genes repressing meristematic cell divisions. Genetics 149:549.

- Morris P., Altmann T. (1994) Tissue culture and transformation. *Arabidopsis*:173–222.
- Morrish F., Vasil I. (1989) DNA methylation and embryogenic competence in leaves and callus of napiergrass (*Pennisetum purpureum* Schum.). *Plant Physiology* 90:37.
- Moussian B., Haecker A., Laux T. (2003) ZWILLE buffers meristem stability in *Arabidopsis Thaliana*. *Development genes and evolution* 213:534-540.
- Moussian B., Schoof H., Haecker A., Jürgens G., Laux T. (1998) Role of the ZWILLE gene in the regulation of central shoot meristem cell fate during *Arabidopsis* embryogenesis. *The EMBO journal* 17:1799-1809.
- Müller B., Sheen J. (2008) Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. *Nature* 453:1094-1097.
- Munksgaard D., Mattsson O., Okkels F. (1995) Somatic embryo development in carrot is associated with an increase in levels of S adenosylmethionine, S adenosylhomocysteine and DNA methylation. *Physiologia Plantarum* 93:5-10.
- Murashige T. (1961) Suppression of shoot formation in cultured tobacco cells by gibberellic acid. *Science* 134:280.
- Murashige T., Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15:473-97.
- Namasivayam P. (2007) Acquisition of embryogenic competence during somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* 90:1-8.

- Nishiwaki M., Fujino K., Koda Y., Masuda K., Kikuta Y. (2000) Somatic embryogenesis induced by the simple application of abscisic acid to carrot (*Daucus carota* L.) seedlings in culture. *Planta* 211:756-759.
- Nissen P., Minocha S. (1993) Inhibition by 2, 4-D of somatic embryogenesis in carrot as explored by its reversal by difluoromethylornithine. *Physiologia Plantarum* 89:673-680.
- Nogler G. (1984) Gametophytic apomixis. *Embryology of angiosperms*. Springer-Verlag: Berlin, etc:475-518.
- Noh B., Bandyopadhyay A., Peer W., Spalding E., Murphy A. (2003) Enhanced gravi- and phototropism in plant *mdr* mutants mislocalizing the auxin efflux protein PIN1. *Nature* 423:999-1002.
- Nomura K., Komamine A. (1985) Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. *Plant Physiology* 79:988.
- Nougarède A., Rembur J. (1985) Le point végétatif en tant que modèle pour l'étude du cycle cellulaire et de ses points de contrôle. *Bulletin de la Société Française de Botanique* 132:9-34.
- O'Neill C., Mathias R. (1993) Regeneration of plants from protoplasts of *Arabidopsis Thaliana* L. cv. Columbia (C24), via direct embryogenesis. *Journal of Experimental Botany* 44:1579.
- Ogas J., Cheng J., Sung Z., Somerville C. (1997) Cellular differentiation regulated by gibberellin in the *Arabidopsis Thaliana* pickle mutant. *Science* 277:91.

- Ogas J., Kaufmann S., Henderson J., Somerville C. (1999) PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America 96:13839.
- Ogata Y., Iizuka M., Nakayama D., Ikeda M., Kamada H., Koshiba T. (2005) Possible involvement of abscisic acid in the induction of secondary somatic embryogenesis on seed-coat-derived carrot somatic embryos. Planta 221:417-423.
- Ogawa M., Shinohara H., Sakagami Y., Matsubayashi Y. (2008) *Arabidopsis* CLV3 peptide directly binds CLV1 ectodomain. Science 319:294.
- Okkels F. (1988) A theory explaining formation of somatic embryogenic cells by auxin induced inhibition of DNA methylation. Physiologia plantarum 73.
- Omran V., Bagheri A., Moshtaghi N. (2008) Direct in vitro regeneration of lentil (*Lens culinaris* Medik.). Pak. J. Biol. Sci 11:2237-2242.
- Osakabe Y., Miyata S., Urao T., Seki M., Shinozaki K., Yamaguchi-Shinozaki K. (2002) Overexpression of *Arabidopsis* response regulators, ARR4/ATRR1/IBC7 and ARR8/ATRR3, alters cytokinin responses differentially in the shoot and in callus formation. Biochemical and biophysical research communications 293:806-815.
- Ozawa S., Yasutani I., Fukuda H., Komamine A., Sugiyama M. (1998) Organogenic responses in tissue culture of *srd* mutants of *Arabidopsis Thaliana*. Development 125:135.
- Ozias-Akins P., Anderson W., Holbrook C. (1992) Somatic embryogenesis in *Arachis hypogaea* L.: genotype comparison. Plant Science 83:103-111.

REFERENCES

- Paek K., Hahn E. (2000) Cytokinins, auxins and activated charcoal affect organogenesis and anatomical characteristics of shoot-tip cultures of *Lisianthus* [*Eustoma grandiflorum* (Raf.) Shinn]. *In Vitro Cellular & Developmental Biology-Plant* 36:128-132.
- Panaia M., Senaratna T., Dixon K., Sivasithamparam K. (2004) The role of cytokinins and thidiazuron in the stimulation of somatic embryogenesis in key members of the Restionaceae. *Australian Journal of Botany* 52:257-266.
- Passarinho P., Ketelaar T., Xing M., van Arkel J., Maliepaard C., Hendriks M., Joosen R., Lammers M., Herdies L., den Boer B. (2008) BABY BOOM target genes provide diverse entry points into cell proliferation and cell growth pathways. *Plant Molecular Biology* 68:225-237.
- Pasternak T., Prinsen E., Ayaydin F., Miskolczi P., Potters G., Asard H., Van Onckelen H., Dudits D., Feher A. (2002) The role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. *Plant Physiology* 129:1807.
- Pauls K., Chan J., Woronuk G., Schulze D., Brazolot J. (2006) When microspores decide to become embryos-cellular and molecular changes. *Canadian Journal of Botany* 84:668-678.
- Pechan P., Keller W. (1989) Induction of microspore embryogenesis in *Brassica napus* L. by gamma irradiation and ethanol stress. *In Vitro Cellular & Developmental Biology* 25:1073-1074.
- Phillips G. (2004) *In vitro* morphogenesis in plants-recent advances. *In Vitro Cellular & Developmental Biology-Plant* 40:342-345.

Phillips R., Kaeppler S., Olhoft P. (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. *Proceedings of the National Academy of Sciences of the United States of America* 91:5222.

Raghavan V. (2000) *Developmental biology of flowering plants* New York, USA.: Springer-Verlag 354 pp.

Raghavan V. (2004) Role of 2, 4-dichlorophenoxyacetic acid (2, 4-D) in somatic embryogenesis on cultured zygotic embryos of *Arabidopsis*: cell expansion, cell cycling, and morphogenesis during continuous exposure of embryos to 2, 4-D. *American journal of botany* 91:1743.

Rajasekaran K., Vine J., Mullins M. (1982) Dormancy in somatic embryos and seeds of *Vitis*: changes in endogenous abscisic acid during embryogeny and germination. *Planta* 154:139-144.

Rajasekaran K., Hein M., Vasil I. (1987) Endogenous abscisic acid and indole-3-acetic acid and somatic embryogenesis in cultured leaf explants of *Pennisetum purpureum* Schum.: Effects *in vivo* and *in vitro* of Glyphosate, Fluridone, and Paclobutrazol. *Plant Physiology* 84:47.

Ramarosandratana A., Van Staden J. (2004) Effects of auxins and 2, 3, 5-triiodobenzoic acid on somatic embryo initiation from Norway spruce zygotic embryos (*Picea abies*). *Plant Cell, Tissue and Organ Culture* 79:105-107.

Ramesar-Fortner N., Yeung E. (2001) Tri-iodobenzoic acid affects shoot apical meristem formation and function in zygotic embryos of *Brassica napus* cv. Topas. *Canadian Journal of Botany* 79:265-273.

- Ramesar-Fortner N., Yeung E. (2006) Physiological influences in the development and function of the shoot apical meristem of microspore-derived embryos of *Brassica napus* 'Topas'. *Botany* 84:371-383.
- Rashid S., Kyo M. (2008) *In vivo* functional assay of WUS ortholog in tobacco, ISHS. pp. 161-166.
- Rashid S., Kyo M. (2010) Ectopic expression of ARR1 DDK in tobacco: alteration of cell fate in root tip region and shoot organogenesis in cultured segments. *Plant Biotechnology Reports* 4:53-59.
- Rashid S., Yamaji N., Kyo M. (2007) Shoot formation from root tip region: a developmental alteration by WUS in transgenic tobacco. *Plant Cell Reports* 26:1449-1455.
- Reinhardt D. (2005) Phyllotaxis--a new chapter in an old tale about beauty and magic numbers. *Current opinion in plant biology* 8:487-493.
- Reinhardt D., Mandel T., Kuhlemeier C. (2000) Auxin regulates the initiation and radial position of plant lateral organs. *The Plant Cell* 12:507.
- Reinhardt D., Pia Stieger T., Kurt Baltensperger M., Jan Traas J. (2003) Regulation of phyllotaxis by polar auxin transport. *Nature* 426:255-260.
- Ribnicky D., Cohen J., Hu W., Cooke T. (2002) An auxin surge following fertilization in carrots: a mechanism for regulating plant totipotency. *Planta* 214:505-509.
- Rider S., Hemm M., Hostetler H., Li H., Chapple C., Ogas J. (2004) Metabolic profiling of the *Arabidopsis* pkl mutant reveals selective derepression of embryonic traits. *Planta* 219:489-499.

- Riou-Khamlichi C., Huntley R., Jacqmard A., Murray J. (1999) Cytokinin activation of *Arabidopsis* cell division through a D-type cyclin. *Science* 283:1541.
- Rosado A., Schapire A., Bressan R., Harfouche A., Hasegawa P., Valpuesta V., Botella M. (2006) The *Arabidopsis* tetratricopeptide repeat-containing protein TTL1 is required for osmotic stress responses and abscisic acid sensitivity. *Plant Physiology* 142:1113.
- Rupp H., Frank M., Werner T., Strnad M., Schmülling T. (1999) Increased steady state mRNA levels of the STM and KNAT1 homeobox genes in cytokinin overproducing *Arabidopsis Thaliana* indicate a role for cytokinins in the shoot apical meristem. *The Plant Journal* 18:557-563.
- Saama P., Patel O., Bettgowda A., Ireland J., Smith G. (2006) Novel algorithm for transcriptome analysis. *Physiological genomics* 28:62.
- Sagare A., Lee Y., Lin T., Chen C., Tsay H. (2000) Cytokinin-induced somatic embryogenesis and plant regeneration in *Corydalis yanhusuo* (Fumariaceae)--a medicinal plant. *Plant Science* 160:139-147.
- Sakai H., Aoyama T., Oka A. (2000) *Arabidopsis* ARR1 and ARR2 response regulators operate as transcriptional activators. *The Plant Journal* 24:703-711.
- Sakai H., Honma T., Aoyama T., Sato S., Kato T., Tabata S., Oka A. (2001) ARR1, a transcription factor for genes immediately responsive to cytokinins. *Science's STKE* 294:1519.
- Sakamoto T., Kamiya N., Ueguchi-Tanaka M., Iwahori S., Matsuoka M. (2001a) KNOX homeodomain protein directly suppresses the expression of a gibberellin

biosynthetic gene in the tobacco shoot apical meristem. *Genes & development* 15:581.

Sakamoto T., Kobayashi M., Itoh H., Tagiri A., Kayano T., Tanaka H., Iwahori S., Matsuoka M. (2001b) Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. *Plant Physiology* 125:1508.

Salaj J., von Recklinghausen I., Hecht V., de Vries S., Schel J., van Lammeren A. (2008) AtSERK1 expression precedes and coincides with early somatic embryogenesis in *Arabidopsis Thaliana*. *Plant Physiology and Biochemistry* 46:709-714.

Sano H., Kamada I., Youssefian S., Wabiko H. (1989) Correlation between DNA undermethylation and dwarfism in maize. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1009:35-38.

Sarkar A., Luijten M., Miyashima S., Lenhard M., Hashimoto T., Nakajima K., Scheres B., Heidstra R., Laux T. (2007) Conserved factors regulate signalling in *Arabidopsis Thaliana* shoot and root stem cell organizers. *Nature* 446:811-814.

Sato Y., Sentoku N., Nagato Y., Matsuoka M. (1998) Isolation and characterization of a rice homeobox gene, OSH15. *Plant Molecular Biology* 38:983-997.

Sauer M., Balla J., Luschnig C., Wi niewska J., Reinöhl V., Friml J., Benková E. (2006) Canalization of auxin flow by Aux/IAA-ARF-dependent feedback regulation of PIN polarity. *Genes & development* 20:2902.

REFERENCES

Scanlon M. (2003) The polar auxin transport inhibitor N-1-naphthylphthalamic acid disrupts leaf initiation, KNOX protein regulation, and formation of leaf margins in maize. *Plant Physiology* 133:597.

Schiavone F., Cooke T. (1987) Unusual patterns of somatic embryogenesis in the domesticated carrot: developmental effects of exogenous auxins and auxin transport inhibitors. *Cell differentiation* 21:53-62.

Schmidt E., Guzzo F., Toonen M., De Vries S. (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. *Development* 124:2049.

Schoof H., Lenhard M., Haecker A., Mayer K., Jürgens G., Laux T. (2000) The stem cell population of *Arabidopsis* shoot meristems is maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 100:635-644.

Scofield S., Murray J. (2006) KNOX gene function in plant stem cell niches. *Plant Molecular Biology* 60:929-946.

Senger S., Mock H., Conrad U., Manteuffel R. (2001) Immunomodulation of ABA function affects early events in somatic embryo development. *Plant Cell Reports* 20:112-120.

Sentoku N., Sato Y., Kurata N., Ito Y., Kitano H., Matsuoka M. (1999) Regional expression of the rice KN1-type homeobox gene family during embryo, shoot, and flower development. *The Plant Cell* 11:1651.

- Sethi U., Basu A., Guha-Mukherjee S. (1990) Control of cell proliferation and differentiation by modulators of ethylene biosynthesis and action in *Brassica* hypocotyl explants. *Plant Science* 69:225-229.
- Shani E., Yanai O., Ori N. (2006) The role of hormones in shoot apical meristem function. *Current opinion in plant biology* 9:484-489.
- Sharma V., Fletcher J. (2002) Maintenance of shoot and floral meristem cell proliferation and fate. *Plant Physiology* 129:31.
- Shetty K., Shetty G., Nakazaki Y., Yoshioka K., Asano Y., Oosawa K. (1992) Stimulation of benzyladenine-induced *in vitro* shoot organogenesis in *Cucumis melo* L. by proline, salicylic acid and aspirin. *Plant Science* 84:193-199.
- Shiota H., Satoh R., Watabe K., Harada H., Kamada H. (1998) C-ABI3, the carrot homologue of the *Arabidopsis* ABI3, is expressed during both zygotic and somatic embryogenesis and functions in the regulation of embryo-specific ABA-inducible genes. *Plant and Cell Physiology* 39:1184.
- Simmonds D., Keller W. (1999) Significance of preprophase bands of microtubules in the induction of microspore embryogenesis of *Brassica napus*. *Planta* 208:383-391.
- Singla B., Tyagi A., Khurana J., Khurana P. (2007) Analysis of expression profile of selected genes expressed during auxin-induced somatic embryogenesis in leaf base system of wheat (*Triticum aestivum*) and their possible interactions. *Plant Molecular Biology* 65:677-692.

Sinha N., Williams R., Hake S. (1993) Overexpression of the maize homeobox gene, KNOTTED-1, causes a switch from determinate to indeterminate cell fates. *Genes & development* 7:787.

Skokut T., Manchester J., Schaefer J. (1985) Regeneration in alfalfa tissue culture: stimulation of somatic embryo production by amino acids and N-15 NMR determination of nitrogen utilization. *Plant Physiology* 79:579.

Skoog F., Miller C. (1957) Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology* 11:118-131.

Smith J., Ladi E., Mayer-Pröschel M., Noble M. (2000) Redox state is a central modulator of the balance between self-renewal and differentiation in a dividing glial precursor cell. *Proceedings of the National Academy of Sciences of the United States of America* 97:10032.

Song J., Joshua-Tor L. (2006) Argonaute and RNA--getting into the groove. *Current opinion in structural biology* 16:5-11.

Song S., Lee M., Clark S. (2006) POL and PLL1 phosphatases are CLAVATA1 signaling intermediates required for *Arabidopsis* shoot and floral stem cells. *Development* 133:4691.

Srinivasan C., Liu Z., Heidmann I., Supena E., Fukuoka H., Joosen R., Lambalk J., Angenent G., Scorza R., Custers J. (2007) Heterologous expression of the BABY BOOM AP2/ERF transcription factor enhances the regeneration capacity of tobacco (*Nicotiana tabacum* L.). *Planta* 225:341-351.

Stasolla C. (2009) Glutathione redox regulation of in vitro embryogenesis. *Plant Physiology and Biochemistry*.

Stasolla C., Kong L., Yeung E., Thorpe T. (2002) Maturation of somatic embryos in conifers: morphogenesis, physiology, biochemistry, and molecular biology. *In Vitro Cellular & Developmental Biology-Plant* 38:93-105.

Stasolla C., Loukanina N., Ashihara H., Yeung E., Thorpe T. (2001) Changes in pyrimidine nucleotide biosynthesis during germination of white spruce (*Picea glauca*) somatic embryos. *In Vitro Cellular & Developmental Biology-Plant* 37:285-292.

Stasolla C., Van Zyl L., Egertsdotter U., Craig D., Liu W., Sederoff R. (2003) The effects of polyethylene glycol on gene expression of developing white spruce somatic embryos. *Plant Physiology* 131:49.

Stasolla C., Bozhkov P., Chu T., van Zyl L., Egertsdotter U., Suarez M., Craig D., Wolfinger R., Von Arnold S., Sederoff R. (2004) Variation in transcript abundance during somatic embryogenesis in gymnosperms. *Tree physiology* 24:1073.

Stasolla C., Belmonte M., Tahir M., Elhiti M., Khamiss K., Joosen R., Maliepaard C., Sharpe A., Gjetvaj B., Boutilier K. (2008) Buthionine sulfoximine (BSO)-mediated improvement in cultured embryo quality in vitro entails changes in ascorbate metabolism, meristem development and embryo maturation. *Planta* 228:255-272.

Steeves T., Sussex I. (1989) *Patterns in plant development* Cambridge Univ Pr.

Stone S., Kwong L., Yee K., Pelletier J., Lepiniec L., Fischer R., Goldberg R., Harada J. (2001) *LEAFY COTYLEDON2* encodes a B3 domain transcription factor that

induces embryo development. Proceedings of the National Academy of Sciences of the United States of America 98:11806.

Stone S., Braybrook S., Paula S., Kwong L., Meuser J., Pelletier J., Hsieh T., Fischer R., Goldberg R., Harada J. (2008) *Arabidopsis* LEAFY COTYLEDON2 induces maturation traits and auxin activity: Implications for somatic embryogenesis. Proceedings of the National Academy of Sciences of the United States of America 105:3151.

Su N., He K., Jiao Y., Chen C., Zhou J., Li L., Bai S., Li X., Deng X. (2007) Distinct reorganization of the genome transcription associates with organogenesis of somatic embryo, shoots, and roots in rice. Plant Molecular Biology 63:337-349.

Su Y., Zhao X., Liu Y., Zhang C., O'Neill S., Zhang X. (2009) Auxin-induced WUS expression is essential for embryonic stem cell renewal during somatic embryogenesis in *Arabidopsis*. The Plant Journal 59:448-460.

Sugiyama M. (1999) Organogenesis in vitro. Current opinion in plant biology 2:61-64.

Sunandakumari C., Zhang C., Martin K., Slater A., Madhusoodanan P. (2005) Effect of auxins on indirect in vitro morphogenesis and expression of gusA transgene in a lectinaceous medicinal plant, *Euphorbia nivulia* Buch.-Ham. In Vitro Cellular & Developmental Biology-Plant 41:695-699.

Sunderland N., Dunwell J., Street H. (1974) Plant tissue and cell culture. Anther and Pollen Culture:223-265.

REFERENCES

- Suzuki T., Miwa K., Ishikawa K., Yamada H., Aiba H., Mizuno T. (2001) The *Arabidopsis* sensor His-kinase, AHK4, can respond to cytokinins. *Plant and Cell Physiology* 42:107.
- Szymkowiak E., Sussex I. (1996) What chimeras can tell us about development. *Annual. Review of Plant Molecular Biology* 47:351-376.
- Tahir M., Stasolla C. (2006) Shoot apical development during in vitro embryogenesis. *Canadian Journal of Botany* 84:1650-1659.
- Tahir M., Law D., Stasolla C. (2006) Molecular characterization of PgAGO, a novel conifer gene of the ARGONAUTE family expressed in apical cells and required for somatic embryo development in spruce. *Tree physiology* 26:1257.
- Tamaoki M., Kusaba S., Kano-Murakami Y., Matsuoka M. (1997) Ectopic expression of a tobacco homeobox gene, NTH15, dramatically alters leaf morphology and hormone levels in transgenic tobacco. *Plant and Cell Physiology* 38:917.
- Tamura K., Dudley J., Nei M., Kumar S. (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular biology and evolution* 24:1596.
- Tanaka-Ueguchi M., Itoh H., Oyama N., Koshioka M., Matsuoka M. (1998) Over-expression of a tobacco homeobox gene, NTH15, decreases the expression of a gibberellin biosynthetic gene encoding GA 20-oxidase. *The Plant Journal* 15:391-400.
- Tanaka S. (2001) Formation of callus and redifferentiation of shoots from *Arabidopsis Thaliana* petal and sepal explants. *Memoirs of Osaka Kyoiku University. Ser. 3. Natural Science and Applied Science* 49:277-283.

- Teale W., Paponov I., Palme K. (2006) Auxin in action: signalling, transport and the control of plant growth and development. *Nature Reviews Molecular Cell Biology* 7:847-859.
- Telmer C., Simmonds D., Newcomb W. (1992) Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. *Physiologia Plantarum* 84:417-424.
- Teo W., Kumar P., Goh C., Swarup S. (2001) The expression of *Brostm*, a KNOTTED1-like gene, marks the cell type and timing of *in vitro* shoot induction in *Brassica oleracea*. *Plant Molecular Biology* 46:567-580.
- Thomas C., Jiménez V. (2006) Mode of action of plant hormones and plant growth regulators during induction of somatic embryogenesis: molecular aspects. *Somatic Embryogenesis*:157-175.
- Thomas C., Bronner R., Molinier J., Prinsen E., van Onckelen H., Hahne G. (2002) Immuno-cytochemical localization of indole-3-acetic acid during induction of somatic embryogenesis in cultured sunflower embryos. *Planta* 215:577-583.
- Thorpe T. (1980) Organogenesis in vitro: structural, physiological and biochemical aspects. *Perspectives in Plant Cell and Tissue Culture*:71-111.
- Thorpe T. (1982) Callus organization and *de novo* formation of shoots, roots and embryos *in vitro*. *Techniques and Applications of Plant Cell and Tissue Culture to Agriculture and Industry* (D. Tomes, BE Ellis, PM Harney, KJ Kasha and R. L. Peterson, eds.). University of Guelph, Ontario:115-138.
- Thorpe T. (1983) Morphogenesis and regeneration in tissue culture. pp. 258-302.

- Thorpe T., Murashige T. (1968) Starch accumulation in shoot-forming tobacco callus cultures. *Science* 160:421-422.
- Thorpe T., Meier D. (1972) Starch metabolism, respiration, and shoot formation in tobacco callus cultures. *Physiologia Plantarum* 27:365-369.
- Thorpe T., Stasolla C. (2001) Somatic embryogenesis. Current trends in the embryology of angiosperms:35–64.
- To J., Kieber J. (2008) Cytokinin signaling: two-components and more. *Trends in plant science* 13:85-92.
- To J., Deruere J., Maxwell B., Morris V., Hutchison C., Ferreira F., Schaller G., Kieber J. (2007) Cytokinin regulates type-A *Arabidopsis* response regulator activity and protein stability via two-component phosphorelay. *The Plant Cell* 19:3901.
- To J., Haberer G., Ferreira F., Deruere J., Mason M., Schaller G., Alonso J., Ecker J., Kieber J. (2004) Type-A *Arabidopsis* response regulators are partially redundant negative regulators of cytokinin signaling. *The Plant Cell* 16:658.
- Touraev A., Pfosser M., Vicente O., Heberle-Bors E. (1996) Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/pollen embryogenesis. *Planta* 200:144-152.
- Traas J., Vernoux T. (2002) The shoot apical meristem: the dynamics of a stable structure. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences* 357:737.

- Tsuchisaka A., Theologis A. (2004) Unique and overlapping expression patterns among the *Arabidopsis* 1-amino-cyclopropane-1-carboxylate synthase gene family members. *Plant Physiology* 136:2982.
- Tsuwamoto R., Fukuoka H., Takahata Y. (2007) Identification and characterization of genes expressed in early embryogenesis from microspores of *Brassica napus*. *Planta* 225:641-652.
- Tucker M., Laux T. (2007) Connecting the paths in plant stem cell regulation. *Trends in cell biology* 17:403-410.
- Tucker M., Hinze A., Tucker E., Takada S., Jurgens G., Laux T. (2008) Vascular signalling mediated by ZWILLE potentiates WUSCHEL function during shoot meristem stem cell development in the *Arabidopsis* embryo. *Development* 135:2839.
- Valvekens D., Montagu M., Lijsebettens M. (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis Thaliana* root explants by using kanamycin selection. *Proceedings of the National Academy of Sciences of the United States of America* 85:5536.
- Veit B. (2006) Stem cell signalling networks in plants. *Plant Molecular Biology* 60:793-810.
- Veit B. (2009) Hormone mediated regulation of the shoot apical meristem. *Plant Molecular Biology* 69:397-408.
- Verdeil J., Alemanno L., Niemenak N., Tranbarger T. (2007) Pluripotent versus totipotent plant stem cells: dependence versus autonomy? *Trends in plant science* 12:245-252.

Vernoux T., Autran D., Traas J. (2000) Developmental control of cell division patterns in the shoot apex. *Plant Molecular Biology* 43:569-581.

Vicient C., Bies-Etheve N., Delseny M. (2000) Changes in gene expression in the leafy cotyledon1 (*lec1*) and *fusca3* (*fus3*) mutants of *Arabidopsis Thaliana* L. *Journal of Experimental Botany* 51:995.

Vieten A., Vanneste S., Wisniewska J., Benkova E., Benjamins R., Beeckman T., Luschnig C., Friml J. (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* 132:4521.

von Aderkas P., Bonga J. (2000) Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. *Tree physiology* 20:921.

Weigel D., Glazebrook J. (2002) *Arabidopsis: a laboratory manual* Cold Spring Harbor Laboratory Pr.

Wenck A., Conger B., Trigiano R., Sams C. (1988) Inhibition of somatic embryogenesis in orchardgrass by endogenous cytokinins. *Plant Physiology* 88:990.

Werner T., Motyka V., Laucou V., Smets R., Van Onckelen H., Schmulling T. (2003) Cytokinin-deficient transgenic *Arabidopsis* plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *The Plant Cell* 15:2532.

Werner T., Holst K., Pors Y., Guivarc'h A., Mustroph A., Chriqui D., Grimm B., Schmulling T. (2008) Cytokinin deficiency causes distinct changes of sink and source parameters in tobacco shoots and roots. *Journal of Experimental Botany* 59:2659.

- Willemsen V., Scheres B. (2004) Mechanisms of pattern formation in plant embryogenesis. *Annual Review of Genetics* 38:587-614
- Woeste K., Ye C., Kieber J. (1999) Two *Arabidopsis* mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. *Plant Physiology* 119:521.
- Wolfinger R., Gibson G., Wolfinger E., Bennett L., Hamadeh H., Bushel P., Afshari C., Paules R. (2001) Assessing gene significance from cDNA microarray expression data via mixed models. *Journal of Computational Biology* 8:625-637.
- Wong C., Singh M., Bhalla P. (2009) Molecular processes underlying the floral transition in the soybean shoot apical meristem. *The Plant Journal* 57:832-845.
- Yadav J., Rajam M. (1998) Temporal regulation of somatic embryogenesis by adjusting cellular polyamine content in eggplant. *Plant Physiology* 116:617.
- Yamada H., Suzuki T., Terada K., Takei K., Ishikawa K., Miwa K., Yamashino T., Mizuno T. (2001) The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant and Cell Physiology* 42:1017.
- Yanai O., Shani E., Dolezal K., Tarkowski P., Sablowski R., Sandberg G., Samach A., Ori N. (2005) *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. *Current Biology* 15:1566-1571.
- Yanghuan B., Palitha D., Todd M., Steven S. (2009) Genome scale transcriptome analysis of shoot organogenesis in *Populus*. *BMC Plant Biology* 9.

- Yao Q., Simion E., William M., Krochko J., Kasha K. (1997) Biolistic transformation of haploid isolated microspores of barley (*Hordeum vulgare* L.). *Genome* 40:570-581.
- Yazawa K., Takahata K., Kamada H. (2004) Isolation of the gene encoding carrot leafy cotyledon1 and expression analysis during somatic and zygotic embryogenesis. *Plant Physiology and Biochemistry* 42:215-223.
- Yeung E. (1999) The use of histology in the study of plant tissue culture systems—some practical comments. *In Vitro Cellular & Developmental Biology-Plant* 35:137-143.
- Yeung E. (2002) The canola microspore-derived embryo as a model system to study developmental processes in plants. *Journal of Plant Biology* 45:119-133.
- Yeung E., Stasolla C. (2000) Somatic embryogenesis—apical meristems and embryo conversion. *Korean J. Plant Tiss. Cult* 27:299-307.
- Yeung E., Rahman M., Thorpe T. (1996) Comparative development of zygotic and microspore-derived embryos in *Brassica napus* L. cv Topas. I. Histodifferentiation. *International Journal of Plant Sciences* 157:27-39.
- Zar J. (1974) *Biostatistical analysis* Prentice-Hall Englewood Cliffs, NJ.
- Zhang J., Kirkham M. (1996) Antioxidant responses to drought in sunflower and sorghum seedlings. *New Phytologist* 132:361-373.
- Zhao J., Simmonds D., Newcomb W. (1996) Induction of embryogenesis with colchicine instead of heat in microspores of *Brassica napus* L. cv. Topas. *Planta* 198:433-439.

REFERENCES

Zhao Q., Fisher R., Auer C. (2002) Developmental phases and STM expression during *Arabidopsis* shoot organogenesis. *Plant Growth Regulation* 37:223-231.

Zuo J., Niu Q., Frugis G., Chua N. (2002) The WUSCHEL gene promotes vegetative-to-embryonic transition in *Arabidopsis*. *The Plant Journal* 30:349-359.