THE UNIVERSITY OF CALGARY

Physiological Control of Shoot Apical Meristem Formation in *Brassica napus* cv Topas

by

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A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

Calgary, Alberta

April, 1999

Abstract

This thesis examines how shoot apical meristem (SAM) formation is controlled physiologically in microspore-derived (MD) and cultured zygotic embryos of *Brassica napus*. Auxin levels were shown to play an important role during early embryogenesis. Globular-stage MD and zygotic embryos, in the presence of the auxin transport inhibitor, tri-iodobenzoic acid (TIBA), were unable to exhibit bilateral symmetry. In the presence of TIBA only one fused cotyledon was initiated. TIBA interfered with the formation and function of the SAM. In MD embryos, the SAM assumed a more axially elongated shape and was composed of less cells compared to the control. The opposite occurred in the treated zygotic embryos as the SAM expanded laterally with a reduced number of cell layers. The difference in the pattern of reaction suggests that SAM formation in MD and zygotic embryos is different. The TIBA treated MD and zygotic embryos also exhibited a dramatic decline in the ability to produce shoots post-embryonically.

The change in the shape and size of the cells in the TIBA treated embryos was accompanied by a change in the orientation of the cortical microtubules of the cells. This change prevented the cells in the SAM from maintaining their normal cell division patterns and accounts for the reduced ability to produce shoots. As TIBA has been shown to inhibit the polar transport of auxin, these observations suggest that TIBA treatment leads to an increase of auxin in the apical half of the embryo. The change in auxin distribution pattern associated with TIBA treatment is detrimental to the formation of the SAM and its subsequent ability to function. In addition to auxin, another physiological agent, abscisic acid (ABA) was shown to greatly improve the characteristics of the SAM and the resulting shoots in MD embryos.

These observations suggest that the physiological environment during the early stages of embryogenesis plays a critical role in the formation and function of the SAM. However, SAM formation is a long and dynamic process that may last for the entire length of embryogenesis and requires the interaction of many physiological agents and genes.

Acknowledgments

During the course of this work there are many people that have helped me along the way - academically and personally. I have learned many new things about science and myself from them that I will never forget.

Firstly, I am grateful to my supervisor, Dr. Ed Yeung, who opened up his lab to me and gave me an opportunity to continue my education by doing what I love to do. In addition to teaching me all about the wonderful world of plant development, Dr. Yeung has also taught me how to appreciate science and life from all different angles. His variety of supervisory techniques, ranging from eliciting terror to serving tea, always brought out the best in me. I also appreciate all the encouragement support and advice that I received from Dr. Thorpe who has been like a second supervisor to me.

I also appreciate all the work of Ken Girard and Bonny Smith and for always finding room for canola. Thanks to Dr. A. Ferrie (PBI, Saskatoon) for taking the time to show me the MD embryo system and troubleshooting with me. Thanks also to Dr. Dirk Hays and Dr. Larry Holbrook for their advice on the MD embryo system.

I also wish to thank the many dear friends that I have made as a result of doing this work. Thanks to my growth chamber for acting up and killing all my plants, otherwise I would not have had that first beer with Alison. Thank you for always cheering me up and being there to celebrate the good times. Thanks to Simon and Jess for being excellent friends and always being poised to go for Chinese food at any give moment in time. Thanks also to Claudio for working like a 'crazy person', making me feel guilty, and making me work even harder. A special thanks also to Sindy B. and Natalia L. for always listening. I am also indebted to Cindy P. for all those chocolate bar breaks - I know I would not have made it through without all that sugar. Finally, thanks to Sarita C., Lisa H., Steve Z., Rick K., Pete M., and Scott R. for making the past two and a half years more interesting.

Finally, I wish to acknowledge the unwavering support of the Ramesar and Fortner households. Thank you for always believing in me and encouraging me. My heart is brimming with gratitude for my husband, Lee, for always being there.

This work is dedicated, with all my heart, to my beloved Lee,
who always restores my faith, instills me with confidence,
and sees only the best in me.

This would not have been without you.

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Abbreviations

ABA	Absisic acid
BSA	Bovine serum albumin
DAC	Days after the initiation of culture
IAA	Indole-3-acetic acid
MD	Microspore-derived
MS	Murashige and Skoog
NPA	*N*-(1-naphthyl) phthalamic acid
PAS	Periodic acid schiff's
PBS	Phosphate buffered saline
PCIB	2-(p-chlorophenoxy)-2-methylpropionic acid
RAM	Root apical meristem
SAM	Shoot apical meristem
TIBA	Tri-iodobenzoic acid
TBO	Toluidine blue O

CHAPTER ONE

General Introduction

One of the unique characteristics of plants is the maintenance of designated regions where cell division can occur throughout the life span of the plant. These areas, known as meristems, are responsible for being the ultimate source of all cells in a plant. Therefore they are considered essential to the survival of the plant as 'meristems make the plant' (Sussex, 1989). The most important of these meristems are the apical meristems that are formed during embryogenesis. The shoot apical meristem (SAM) and the root apical meristem (RAM) are then responsible for producing all of the aerial and subterranean parts of the plant respectively. It is therefore imperative that these generating centres are formed properly during embryogenesis or they may compromise the development of the plant. It is also not surprising that there have been many studies focused on understanding how these areas form and function.

Organization of the SAM

The SAM is the distal-most portion of the shoot and consists of a small number of cells that have unique characteristics. The cells of this region are characteristically densely cytoplasmic, have large nuclei, and very little vacuolation. It has been suggested that this region may be analogous to the stem cells that exist in animal systems. In keeping with this classification, the cells of SAM have the ability to proliferate, replace themselves, give rise to a variety of cell types, and to regenerate the meristem itself if damaged (Steeves and Sussex, 1989).

The cells of the SAM are basically organized into two groups: the initials that are the ultimate source of the entire plant, and the cells that are the progenitors of the tissues and the lateral organs. The spatial organization of the two groups of cells has been described using two models: the concept of layers and cytohistological zonation (Fig 1.1).

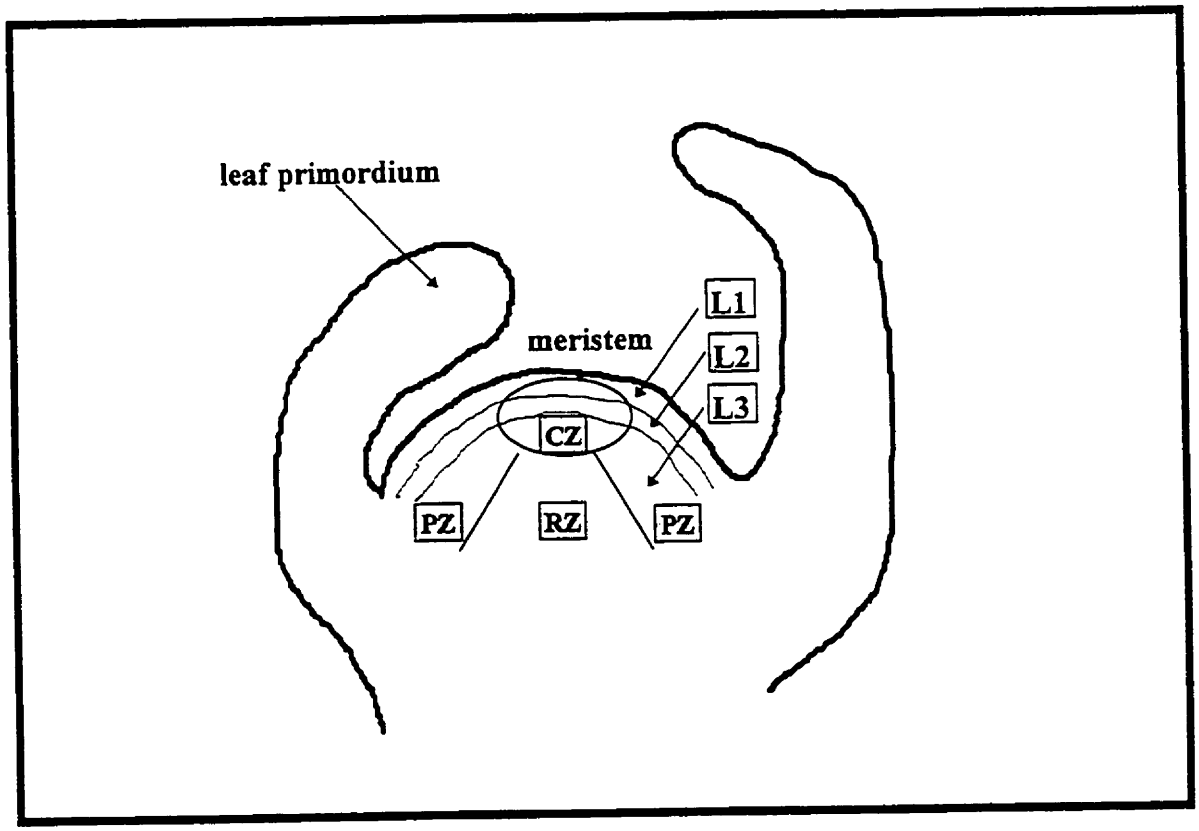
The concept of layers is based on the work of early chimera studies which showed that all the tissues in the mature shoot are derived from three distinct layers of the SAM (Satina et al., 1940; Steeves and Sussex, 1989). Cells in the outermost layer of the SAM, L1, are restricted to dividing in an anticlinal direction and give rise to the epidermis of the shoot. The subepidermal layer, referred to as L2, also divides in a predominantly anticlinal direction or periclinal direction where organ initiation is taking place. This layer will give rise to the palisade layer and lower spongy mesophyll in the leaf (Poethig, 1989). The L1 and L2 layers have also been referred to as the tunica, but only if all the cells are restricted to dividing in the anticlinal direction. In the third layer from the surface, L3, the cells divide in an anticlinal and periclinal plane and give rise to the cells in the interior of the mature organs. Similarly, this region where there is an irregular arrangement of cells has been referred to as the corpus of the meristem.

In contrast to this layer model, the regions within the SAM have also been defined in terms of the cytohistological characteristics of the cells (Steeves and Sussex, 1989). The central zone, located at the distal end of the SAM, is made up of slowly dividing cells and are the initials for the other zones. These cells in this zone are usually larger, have more prominent nuclei, and are more vacuolated than the surrounding cells. The cells of the peripheral zone surround the central zone and function mainly in giving rise to lateral organs. Finally, the rib zone is found at the base of the meristem. It is a transition zone between the undifferentiated cells of the meristem proper and the differentiated cells. The cells in this zone give rise to the tissues in the pith of the stem. However, it has also been suggested that it may act as an organizing centre of the shoot that transmits signals to and from the meristem (Sachs, 1991; Medford, 1992).

It should be noted that while these two models may appear distinct, there is considerable overlap of the regions referred to in each of the models, as cells from each zone contain cells from all three layers. The disparity lies mainly in defining the depth of the SAM that is functionally significant. Chimera studies indicate that three layers are sufficient for a fully functional SAM. However, it is apparent from the zonation and microsurgery studies that the functional meristem can be defined to a greater depth (Steeves and Sussex, 1989).

Figure 1.1:

Diagram of a dicotyledon shoot apical meristem showing the two different models used to describe the organization of the cells. In the layer model, indicated by the unshaded boxes, the L1 layer is the outermost layer and where the cells divide in a predominantly anticlinal fashion; the cells of the L2, or second layer, are able to divide in a periclinal or anticlinal direction; the cells in the L3 can divide in any plane. In the cytohistological zonation model, indicated by the shaded boxes, the central zone (CZ) essentially serves as the initial cells; organ initiation takes place from the peripheral zone (PZ); and the rib zone (RZ) gives rise to the pith of the stem. The diagram also indicates that there is an overlap in the two models. Diagram modified and redrawn from Laufs et al. (1998).


leaf primordium
meristem
L1
L2
L3
CZ
PZ
RZ
PZ

Pattern of the SAM formation during embryogenesis

In many dicotyledons, including the members of the Brassicaceae, early embryogenesis is marked by a precise and invariable set of cell divisions. This regularity of cell divisions has made it possible to trace the development of the zygote to the mature embryo.

After fertilization, the zygote, which is elongated, undergoes an anticlinal asymmetric division that gives rise to a larger basal cell and a small apical cell (Fig 1.2). The basal cell will eventually give rise to the suspensor and depending on the species, it can also contribute to the columella initials of the root apical meristem, while the apical cell will give rise to the rest of the embryo. The cell wall that is found between the apical cell and basal cell has been termed the 'O boundary'. The position of the 'O boundary' can be followed all through the development of the embryo and in essence indicates the boundary between all the derivatives of the apical cell and basal cell. The apical cell undergoes two vertical divisions at right angles to each other to give rise to a 4-celled embryo. Each of these cells, in turn, undergo a transverse division to give rise to an eight-celled embryo. The protoderm is established when each of these 8 cells undergoes a periclinal division (Fig 1.2: 5). The four inner cells in the upper layer of the 16 celled embryo then undergo a round of divisions that lead to the formation of the epiphysis, the precursors of the SAM. There are two models describing the method of origin of the epiphysis that are based on the plane of cell division (Swamy and Krishnamurthy, 1977). After the division of the four inner cells in the upper layer of the proembryo the cells can either be aligned in a single tier (Fig 1.3: Model 1) or in a two-tiered configuration (Fig 1.3: Model 2). The difference between the two models can be distinguished by the plane of cell division. In the former type, the division is oblique and outwardly curved leading to a single tier of cells. In the latter, the division is transverse and thus leads to two tiers of cells. Within either group of eight cells, however, the cells of the future SAM are evident (stippled cells in Fig 1.3), and this region is therefore often referred to as the embryonic shoot apex. Therefore, even at this very early stage in development, the future

Figure 1.2:

Diagram showing the early division patterns found in the embryo proper during embryogenesis of *Brassica napus*. **1:** The zygote, which is formed after fertilization is elongated. **2:** The first division of the zygote is an asymmetric, transverse division that forms the smaller apical cell (A) and a larger basal cell (B). The 'O boundary' (O) is formed by the cell wall found between the apical and basal cells. Following the development in the proembryo:- **3:** Subsequently 2 vertical divisions at right angles to each other occur in the apical cell and lead to the formation of the quadrant stage. During this time the basal cell also is dividing and the hypophysis (*h*) is formed. Divisions in the hypophysis will eventually lead to the formation of the RAM. **4:** Each of the 4 cells of the embryo proper then undergoes a transverse division to give rise to the an octant stage embryo. **5:** The octant cells then divide periclinally to give rise to the cells of the future protoderm (*p*). These cells then divide anticlinally (**6**). **7:** A globular stage embryo. Each of the cells of the octant embryo undergo more-or-less vertical division. The cells in the upper part will form the future SAM (shaded cells: epiphyseal cells) and cotyledons of the embryo while the cells in the lower part will form the future procambium (*pr*) and ground meristem (*g*) of the embryo. *B. napus* follows the pattern in Model 1. Modified and redrawn from Tykarska (1976).

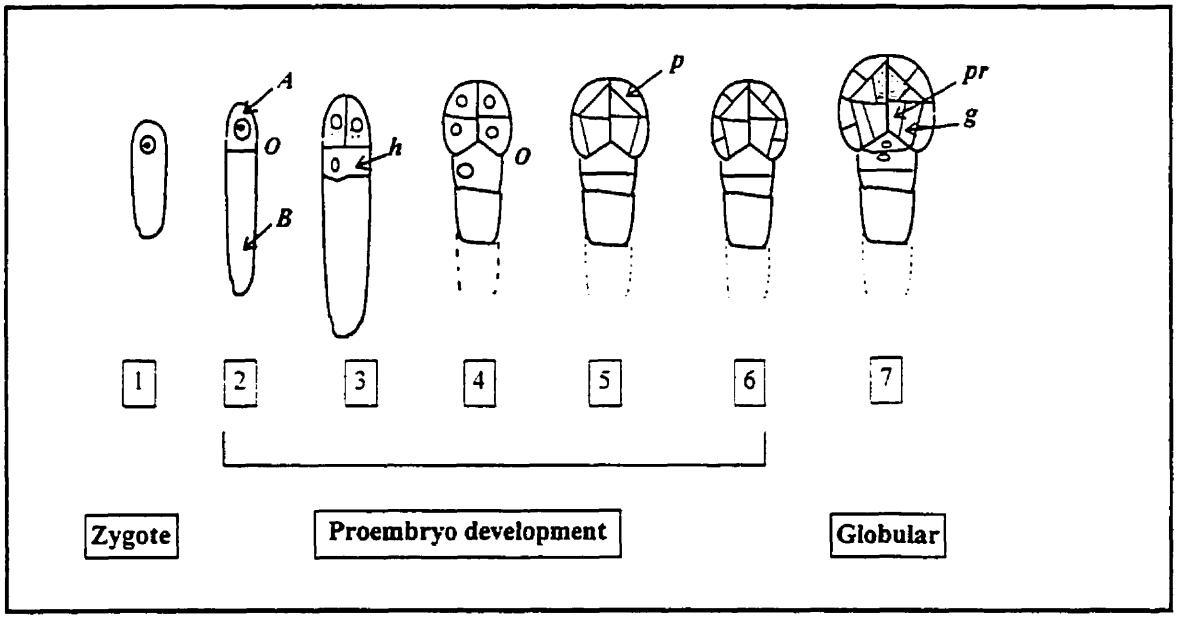
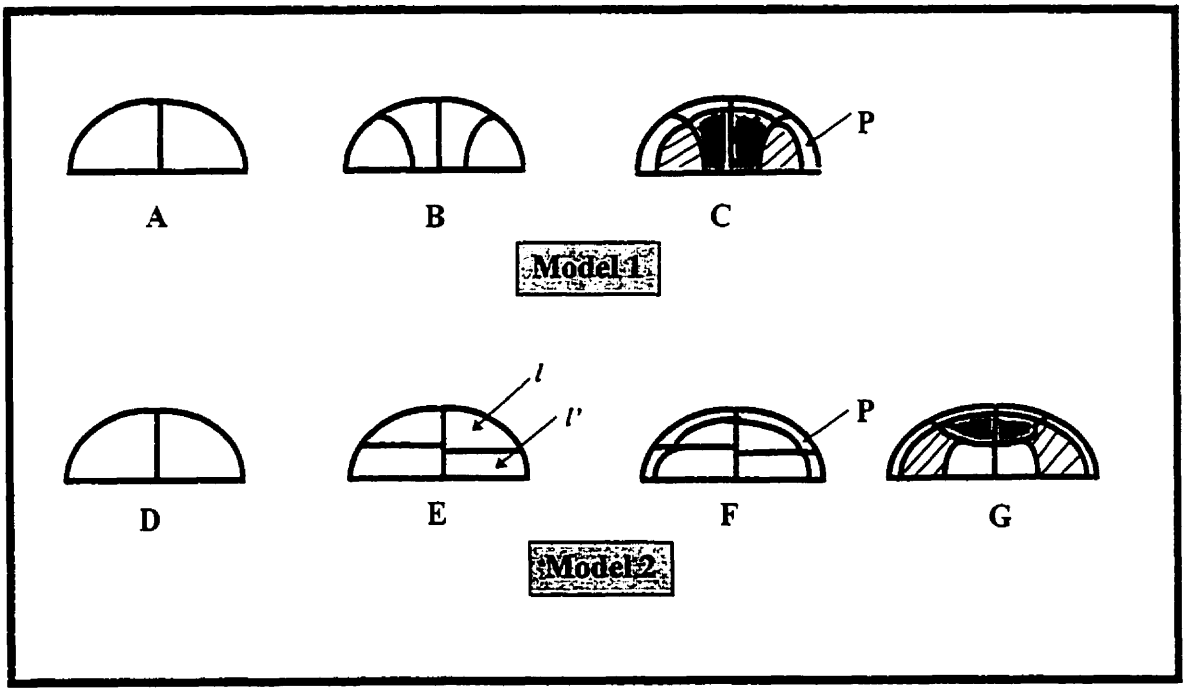

A
O
B
h
O
p
pr
g
1
2
3
4
5
6
7
Zygote
Proembryo development
Globular

Figure 1.3:

Diagram showing the difference in the two models used to describe the origin of the epiphysis in dicotyledons viewed in a longitudinal section. In Model 1: the apical cell of the zygote divides vertically (A); the third round of cell division, which is oblique leads to the configuration in (B); once the protoderm (P) begins to differentiate, the epiphyseal cells are aligned a single tier (C) The two inner cells that are stippled represent the epiphyseal cells that will be incorporated into the SAM, the two outer cells with diagonal lines will divide to form the cotyledons In Model 2: the free hemispherical cell of the zygote divides vertically (D), as in model 1; the third cell division, however, is transverse (E) which leads to two layers (*l* and *l'*); once the protoderm (P) differentiates, the epiphyseal cells can be found in two tiers (F). G: After another cell division and re-arrangement of the cells of *l'*, the two cells that are stippled will go on to form the SAM and the cells with the diagonal lines will form the cotyledons. Diagram modified and re-drawn from Swamy and Krishnamurthy (1977).


P
A
B
C
Model 1
l
l'
P
D
E
F
G
Model 2

SAM can be distinguished. In *Brassica*, Tykarska (1976), has shown that the pattern of epiphyseal formation follows the path similar to Model 1 (cf. Fig 1.2, 7).

Throughout development to the heart-stage, the cells of the epiphysis rarely divide, and if they do, they divide in an anticlinal plane (Swamy and Krishnamurthy, 1977). These cells remain relatively quiescent and go on to form the central mother cells of the mature SAM. It should be noted, however, that an epiphysis may not be evident in all systems. For example, in developing microspore-derived embryos of *B. napus*, the SAM develops from a group of cells that are not well organized and cannot be termed the epiphyseal cells (Yeung et al., 1996). Relying on the presence or absence of an epiphysis, determined by histological evidence, has often led to confusion concerning the timing of the formation of the SAM. The mature form of the SAM is only evident in late heart-stage embryos (Yeung et al., 1996) or fully formed embryos (Barton and Poethig, 1993). During this time, the epiphyseal region widens due to cell division. Concomitantly, there is a change from a random distribution of cell divisions to a concentration of mitoses around the quiescent regions (Krishnamurthy, 1994).

Physiological Control of SAM Formation

What signals the epiphysis, or for that matter, the hypophysis to form at a specific stage? Both of these future generating regions become organized and recognizable simultaneously, and the cells within them are also distinctive from the surrounding cells (Krishnamurthy, 1994). Even at this very early stage, the cells exhibit relatively larger nuclei and distinctive staining reactions (Swamy and Krisnamurthy, 1977; Krishnamurthy, 1994). It has been suggested that polarized gradients play an important role in this early differentiation. Embryo-like structures developed *in vitro* from callus, that show no inherent polarity, do not exhibit any distinctive epiphysis or hypophysis, or any subsequent tissue differentiation (Krishnamurthy, 1994). These results indicate that the environment surrounding the embryo plays an important part in events occurring within the embryo and also that the formation of the two polar centres is necessary for subsequent tissue differentiation.

Changes that occur within the embryo, in particular symmetry changes, are often associated or correlated with changes in the endosperm (Krishnamurthy, 1994). The differentiation of the two polar centres corresponds with the initiation of wall formation in the nuclear endosperm. Any delay in the endosperm development also leads to changes or delays in embryo development. Therefore, it is evident that the physiological and/or physical environment may be an important factor(s) in meristem differentiation during embryogenesis. However, the exact nature of the signals that are involved in this process *in vivo* have been difficult to study primarily because of the inaccessibility of the developing embryo.

However, numerous *in vitro* studies dating back to the classic work of Skoog and Miller (1957), have clearly shown the importance of the plant growth regulators (PGRs) in meristem formation. More importantly, these studies also suggest that there is an interaction of the various PGRs, and in particular auxin and cytokinins, in determining whether these meristems give rise to shoots or roots. Goldsworthy and Rathore (1985) observed that shoot formation in tobacco callus was significantly increased when a weak electrical current was administered between the callus and the medium in the presence of exogenous indole-3-acetic acid (IAA). The current was thought to enhance the uptake of IAA and effect a polar transport of auxin through the tissue. Furthermore, the alignment of several adjacent cells in culture may increase the chance, under the appropriate conditions, for meristems to form. Brawley et al. (1984) and Rathore et al.(1988) showed that, at the late globular stage, there is an inward current that is centred on the apical pole and an outward current greatest at the developing radicle end. This current was attributed to the basipetal polar transport of auxin that is initiated at this time in development.

There have been other studies that have demonstrated the important role of auxin during embryogenesis. In another study using somatic embryos of *Daucus carota* (carrot), Schiavone and Cooke (1987) noted that exogenous auxins or auxin transport inhibitors blocked the transition from globular to heart-stage embryos. Instead, they became enlarged globular and oblong embryos. It is during this transition that the meristems become distinct and most of the body-plan is laid down. Therefore, it is conceivable that the polar transport of auxin may be involved in the differentiation of the

apical meristems. Fry and Wangermann (1976) suggested that if cytokinins are uniformly distributed in the globular stage embryo, then the initiation of the polar transport of auxin would lead to a high cytokinin/auxin ratio at the future shoot apex and a low cytokinin/auxin ratio at the future root apex. These differing ratios would then influence the type of meristem would form and would be consistent with the results from the early *in vitro* studies of Skoog and Miller (1957).

It is evident, therefore, that auxin, and more specifically the polar transport of auxin, plays a critical role in meristem formation. How is it that auxin may regulate this process? It has been demonstrated that the direction of auxin flow influences the arrangement of cortical microtubules and therefore the orientation of the cell axis (Warren Wilson and Warren Wilson, 1993). In the SAM of angiosperms, the cortical microtubules are arranged anticlinally in the tunica layers (L1 and L2) which permits predominantly anticlinal divisions (Sakaguchi et al., 1988). In the corpus (L3), the microtubules are arranged randomly and is reflected in the random nature of the cell divisions in this region. These observations then support the layer concept of meristem organization. In addition, the arrangement of the microtubules can be related to the amount of polarity within the region (Warren Wilson and Warren Wilson, 1993). For example, in meristematic cells (as those found in the SAM), although there are high levels of auxin, there is little polar transport but rather diffusion occurring. Therefore, the microtubules are randomly oriented (in the corpus) and the cells divide in random planes. In the derivatives of the meristem, where the auxin movement is more polarized, the microtubules are arranged predominantly transverse to the direction of the auxin flow and this results in longitudinal expansion.

Genetic Control of SAM Formation

More recent investigations into the nature of the SAM formation have taken a more genetic and molecular approach and have led to further insights. There have been many mutants described that are defective in the apical-basal patterning (see Meinke, 1995). Several mutants that lack a SAM have also been identified in a variety of species

including, for example, *Arabidopsis* (Barton and Poethig, 1993) and petunia (Souer et al., 1996). The many corresponding genes may therefore interact during meristem development. These genes can be broadly characterized as either affecting SAM initiation, maintenance, or both.

To date there are several genes whose expression has been shown to be correlated with the formation of the SAM. Analysis of the expression of these genes have confirmed the early cell division patterns that SAM formation occurs very early on in embryogenesis. The *WUSCHEL* (*WUS*) gene, identified in *Arabidopsis*, has been shown to play a role in regulating the fate of the cells destined to become the SAM (Laux et al., 1996; Mayer et al., 1998). In the *wus* mutants, a normal wild-type SAM never developed, and upon germination the SAM was observed to broaden considerably (Laux et al., 1996). The mutant SAM was not able to maintain the cytohistological zones characteristic of the wild type SAM as periclinal divisions were observed in the subepidermal layer of the CZ. Further analysis indicated that the *WUS* gene encoded a homeodomain protein (Mayer et al., 1998). The *WUS* gene was shown to be expressed as early as the 16 cell stage of embryogenesis. It was first expressed in the inner four cells in the upper layer of the proembryo. Once these cells divided to form the epiphysis and the precursors of the cotyledons, the *WUS* gene was expressed only in the cells of the epiphysis (Mayer et al., 1998). Throughout the rest of embryogenesis, *WUS* expression was confined to the centre of the developing SAM.

Another important gene, *KNOTTED1 (KN1)*, was isolated and cloned from maize (Smith et al., 1995). The sequencing of the cDNA revealed that it encoded a homeodomain, an evolutionary conserved DNA-binding protein. These homeodomain proteins act as transcription factors and regulate the expression of a host of other specific target genes. In *Drosophila*, in which the homeobox was first identified, the expression pattern of these regulatory molecules are established very early on in embryo development (Lincoln et al., 1994). It was later determined, that the *Arabidopsis SHOOT MERISTEMLESS* (*STM*) gene also encodes a *KN*-like protein (Long et al., 1996).

Mutant embryos, homozygous for *STM-1* gene, are normal in all aspects except that they fail to develop a SAM during embryogenesis. The cells that would normally

divide to give rise to the SAM failed to do so. The cells that occupied the normal position of the SAM were indistinguishable from the surrounding cells, and particularly resembled the cells from the cotyledons (Barton and Poethig, 1993). These cells were very parenchyma-like and accumulated starch. *STM* gene transcript was shown to accumulate at an very early stage during the globular stage in the one to two cells that will go on to form the SAM (Long et al., 1996). As development proceeds, the transcript is restricted to those cells forming the SAM. This defined localization of the STM transcript within the developing embryo is an indication that the gene expression is sensitive to positional information starting at the globular stage (Evans and Barton, 1997). This restricted gene expression is also an indication that the cells that will give rise to the SAM are uniquely characterized from a very early (globular) stage in embryogenesis although the SAM does not become histologically distinct at a late (bending cotyledon) stage in embryogenesis. Therefore, the *STM* gene expression can serve as an objective marker for SAM initiation.

Further analysis of weak *stm* allele mutants showed that the cells in the centre of the 'meristem', which in the wild-type would remain undifferentiated, are incorporated into ectopic primordia and become differentiated (Endrizzi et al., 1996). The depletion of the central cells of the meristem leads to meristem termination. It was proposed that the *STM* gene therefore is not only required for meristem initiation, but also its maintenance. It may function, therefore, to suppress differentiation in the central region by positively regulating the inhibitors of differentiation or it may be a positive regulator of cell proliferation in the meristem centre (Endrizzi et al., 1996). This may tie in to the suggestion made by Hake et al. (1995), that *KN1* and related genes may be affecting the ratio of PGRs, either auxin or cytokinin, in the plant. Transgenic plants overexpressing the *KN1* gene often resemble transgenic plants overexpressing cytokinin biosynthetic gene in that they both show ectopic production of shoots on mature leaves (from Hake et al., 1995). *STM* may promote SAM formation by transcriptionally or translationally regulating other genes (Evans and Barton, 1997). It will now be important to determine what are the target genes for these homeobox proteins in order to complete the picture.

Two other genes similar to *STM* implicated to be involved in embryonic SAM formation are the *PNH* (*PINHEAD*) gene and the recently described *CUC* (*CUP-SHAPED COTYLEDON*) gene both from *Arabidopsis*. Although a detailed analysis of the embryo development of the *pnh* mutant was not performed, the authors were able to show that a functional SAM does not form in these mutants (McConnell and Barton, 1995). In the seedlings of the mutants, the SAM terminated in a single determinate organ ranging from a slender pinlike structure with radial symmetry to a single terminal leaf. The most extreme variation is that a 'flat meristem' develops with no obvious leaf primordia. These observations suggest that the wild-type PINHEAD gene product is necessary for the formation of the SAM. McConnell and Barton (1995) surmise that *PNH* and *STM* work in concert to give rise to a mature, functional SAM. The *STM* gene product may be required to establish a 'meristem primordium' which would include the basic tunica/corpus organization. Both the *PNH* and *STM* gene products may then be necessary for a functional SAM to form. The *cuc* mutant embryos also lack a distinguishable SAM by the end of embryogenesis (Aida et al., 1997). The cells in the location of the SAM were very vacuolated and contained small nuclei similar to the cells surrounding them. In addition, however, these mutants also failed to have two separated cotyledons and instead have a ring of cotyledonary tissue indicating a loss of bilateral symmetry. It is interesting to note that in these mutants where there is a loss of bilateral symmetry that there also seems to be a disruption in the SAM formation during embryogenesis.

It is therefore evident that SAM formation occurs very early on in embryogenesis with a precise cell division pattern and is accompanied by unique gene expression pattern. However, the process of SAM formation is a dynamic process requiring the action and interaction of many gene products and requires the entire period of embryogenesis for the it to be completed.

The *Brassica napus* microspore-derived embryo system: a model system for the study of plant development

The study of plant embryogenesis has often been hindered primarily because of the inaccessibility of the developing embryo. As it is buried deep within the maternal tissue, experimental manipulation and observation is difficult (Goldberg et al., 1994). The realization that somatic embryogenesis, the development of embryos from somatic cells, can occur *in vitro* under controlled conditions led to the opportunity to study and detail the early events of embryogenesis. The somatic culture system is an important tool that provides an excellent opportunity to study embryonic processes, as it allows for experimental manipulation which is not always possible with zygotic embryos.

The successful production of haploid embryos derived from anthers of *D. innoxia* were first reported by Guha and Maheshwari (1964). Subsequently, the ability to produce haploid embryos was reported to be feasible from a wide variety of species including many *Brassica* species (Palmer et al., 1996). However, as was noted for other species, despite the abundance of microspores within the anther that could give rise to these haploid embryos, the frequency of embryogenesis was very low (Lichter, 1982; Palmer et al., 1996). It was suggested that the walls of the anther imposed a constraint on embryo development. Therefore, methods were developed to isolate and culture only the microspores from the anthers (Lichter, 1982). Consequently, this technique has been applied successfully to many *Brassica* species.

There have been numerous investigations and studies that have used microspore-derived (MD) embryos of *Brassica* spp. One of the main purposes behind all of this research resides in the fact that the *Brassicas* include some very important oilseed and vegetable crops, such as canola (*B. napus*), broccoli, cauliflower, cabbages, and kales (different varieties of *B. olearacea*). The production of haploid embryos present an opportunity to quickly develop new breeding lines and new cultivars. However, there are drawbacks that always have to be considered. Some of the factors that influence MD embryogenesis include the genotype of the donor plant, the growth conditions of the donor plants, the stage of microspore development, the culture conditions and

environment. Due to the potential agronomic use there have been and continue to be countless studies performed in an attempt to improve the efficiency of the MD embryo system (Chuong and Bevesdorf, 1985; Pechan and Keller, 1988; Telmer et al., 1992; Ilic-Grubor et al., 1997). It is evident, from all of these studies, as Palmer et al. (1996) discuss in detail, the improvement and optimization of this system is a continuous process.

The MD embryo system of *Brassica napus* is also a very important research tool, in that it allows for experimental manipulation and better understanding of what occurs during embryogenesis. MD embryos are able to proceed through all the developmental stages (i.e. globular, heart, torpedo and cotyledonary) that are observed in zygotic embryos Several studies have attempted to define the events and changes that occur during MD embryogenesis. In this technique, microspores at the late-uninucleate to early binucleate stage of development are isolated from the anthers and maintained in a defined medium. One of the most fundamental steps during the process is an initial heat shock when the microspores are subjected to a high temperature (32 ° C) for a period of 72 hours. It is believed that it is during this time that the normal developmental pathway of the microspore is switched off and the pathway to develop into an embryo is turned on (Zaki and Dickinson, 1990; Telmer et al., 1993). After the heat-shock treatment, microspores which would normally undergo an asymmetrical division underwent more symmetrical divisions which is the first step towards an embryogenic pathway. This indicates the importance of the arrangement of the microtubules and the plant cytoskeleton from very early on in the induction process. Telmer et al. (1993) also showed that the high temperature disrupted the cell wall structure of the microspores. They suggest that the heat shock may disrupt normal cellular protein synthesis to allow for the synthesis of heat shock proteins. During this time, pollen development is halted and the activation of processes leading to the embryo formation can then occur.

In addition to these studies that have sought to understand the early processes involved in the induction of the embryogenic pathway in microspores, there have also been many studies that have characterized how the actual developing embryos may be similar to or different from their zygotic counterparts. Yeung et al. (1996) made a histological comparison of the MD and zygotic embryogenic systems. The pattern of cell

division during the early stages of embryogenesis was not very well defined in the MD embryos as they were in the zygotic embryos. In addition, they also found that the origin of the apical meristems were not as clearly marked as it was in the zygotic embryos. Upon prolonged culture, the quality of the MD embryos were observed to degenerate suggesting that the culture conditions were not optimal. Hays (1996) also observed differences in the physiology of the two systems which may account for some of the degradation that occurs in the MD embryos.

However, despite these differences, the MD embryo system of *B. napus* can still be deemed an attractive system to use for the study of embryogenesis as there are relatively few differences from the zygotic counterparts. Some of the advantages of using such a system, once optimized, are: relative ease of the isolation and culture of the microspores, production of large numbers of embryos at any given time, ease of maintenance of the embryos, ease of experimental manipulation and observation particularly because the system is made up from a uniform population of single cells, and relative convenience for potential mutagenesis and *in vitro* selection of desirable homozygous lines.

Objectives of this research

Although one of the most fundamental processes to occur during plant development is the formation of the shoot apical meristem, our understanding of the physiological control of its formation and function during embryogenesis is limited. The MD embryo system of *B. napus* presents an opportunity to experimentally examine the different physiological controls of shoot apical meristem development.

Recently, Liu et al. (1993) developed a system that would allow immature (from as early as globular stage) zygotic embryos to be cultured *in vitro*. These cultured embryos have a similar development to their *in vivo* counterparts. This system allows for the experimental manipulation of different physiological agents to determine their effect on embryogenesis. The MD embryo and *in vitro* systems of *B. napus* will allow for the comparison of how the process of SAM formation is physiologically regulated in a somatic and zygotic systems.

One of the primary objectives of this project is to determine how auxins may be involved in the development of the SAM. In order to do this, the levels of auxins within developing zygotic and somatic embryos will be altered using an auxin transport inhibitor, tri-iodobenzoic acid (TIBA). TIBA acts to inhibit the polar transport of auxin by preventing the auxin from binding to an efflux carrier that transports the auxin out of the cell. Therefore, this results in an accumulation of auxin in the cell (Depta et al., 1983; Lomax et al., 1995). A histological approach will be used to analyze the effects of the altered auxin levels on SAM development. The effect of auxin levels on the ability of the SAM to function will be assessed by using the conversion frequency of the treated embryos. The ability of an embryo to convert, or produce vegetative leaves, from the SAM can be used as an assay of the meristem to function efficiently. Secondly, the mechanism through which auxin may be influencing SAM development will be investigated.

In summary, the main objectives of this work are to:

I Examine the role of auxin levels in SAM formation and function in MD embryos

II A) Examine the role of auxin levels in SAM formation and function in *in vitro* zygotic embryos

B) Compare the effects of altered auxin levels in MD and *in vitro* zygotic embryos

III Examine a mechanism through which auxin levels may influence SAM formation

IV Determine if there are any physiological influences on SAM maturation

CHAPTER TWO

Materials and Methods

The microspore-derived embryo system

Plant growth conditions

Brassica napus cv Topas 4086 seeds were obtained from the Plant Biotechnology Institute in Saskatoon. Plants were grown in growth chambers under 16 hour day conditions at 20°C day/15°C night until the crown of the plants were formed and buds were just visible (approximately 3-4 weeks). The plants were then maintained at 15°C day/10°C night for the remainder of the experimental period. The plants were fertilized every two weeks with a commercial fertilizer (Peters® 20-10-20). The terminal as well as the lateral racemes were used for microspore isolation

Microspore isolation

Microspores were isolated according to the methods of Ferrie and Keller (1995). Approximately 600 buds were processed in each preparation. The buds were picked while the plants were in the dark phase of the photoperiod and placed on ice. Buds 3-4 mm in length were selected and divided into 6 groups with 100 buds each. Dividing the buds into these groups facilitated a greater amount of microspores to be collected. Each group of buds was placed in a metal basket and surface sterilized in 30% Javex® solution for 15 minutes then rinsed twice in sterile distilled water for 5 minutes each. Each group of buds was then placed into a sterile 200ml beaker with 5 ml of half-strength modified B5 medium with 13% sucrose (Table 1). The buds were then crushed in this medium and the mixture was filtered through 44 μm nylon screencloth into a sterile beaker. The

beaker that the buds were crushed in was rinsed with additional 5 ml of B5-13 medium and this was also passed through the screencloth. The filtrate was then put into a sterile 50 ml centrifuge tube and centrifuged at 150 *g* for 5 minutes. The supernatant was discarded and the resulting pellet was re-suspended in 5 ml of half-strength B5-13 medium and centrifuged two more times. The last pellet was suspended in full-strength NLN medium with 13% sucrose (Table 2.1).

Microspore culture conditions

The microspores were plated out in 100 × 15 mm petri plates at a density of 1 flower bud to 1mL of NLN-13 with a total of 10 ml of the microspore solution in each petri plate. The petri plates were sealed with Parafilm®. The plates were incubated in a growth chamber, in the dark at 32°C for 72 hours and the transferred to a dark chamber at 25°C. For the purpose of consistency in experimentation, the day after heat shock was considered to be Day 0. The microspores were subcultured on Day 2 with a 3X dilution of cell density. The final density of microspores was approximately 10^5 microspores per ml. The plates were continued to be maintained at 25°C until Day 10 when they were transferred to a shaker at 70 rpm in the dark for the remainder of the experimental time period (up to Day 30).

Table 2.1: Composition of media used for the culture of MD embryos of *Brassica napus*

Half-strength B5-13*		Full-strength NLN-13*	
Macroelements	**mg l^{-1}**	**Macroelements**	**mg l^{-1}**
KNO_3	1500	KNO_3	125
$(NH_4)_2SO_4$	75	$MgSO_4.7H_2O$	125
$MgSO_4.7H_2O$	250	$NaH_2PO_4.H_2O$	125
$CaCl_2.2H_2O$	75	$Ca(NO_3)_2.4H_2O$	500
$NaH_2PO_4.H_2O$	75		
Sequestrene	14	**Sequestrene**	40
Micronutrients		**Micronutrients**	
KI	0.38	KI	0.83
H_3BO_3	1.50	H_3BO_3	10.00
$MnSO_4.H_20$	6.60	$MnSO_4.H_20$	25.00
$ZnSO_4.7H_2O$	1.00	$ZnSO_4.7H_2O$	10.00
$Na_2MoO_4.2H_2O$	0.13	$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.01	$CuSO_4.5H_2O$	0.025
$CoCl.6H_2O$	0.01	$CoCl.6H_2O$	0.025
Vitamins and amino acids		**Vitamins and amino acids**	
Thiamin.HCl	5.00	Thiamin.HCl	0.50
Nicotinic acid	0.50	Nicotinic acid	5.00
Pyrodoxine.HCl	0.50	Pyrodoxine.HCl	0.50
Inositol	50.00	Glycine	2.00
		Biotin	0.05
		Folic acid	0.50
		Glutamine	800
		Glutathione	30
		Inositol	100
		Serine	100
Sucrose	130g	**Sucrose**	130g
pH	5.8	**pH**	5.8

*Both media were filter sterilized using 1L disposable Nalgene filter units into sterile glass bottles and maintained at 4°C.

The *in vitro* zygotic embryo system

Plant growth conditions

Plants of *Brassica napus* cv Topas were grown in a greenhouse-mix soil in a growth chamber maintained at 25 ° C days and 16 ° C nights with a 16 hour photoperiod. The plants started to flower approximately 6-7 weeks after the initiation of germination. Flowers were hand-pollinated and tagged on the day of anthesis.

Culture of zygotic embryos

Developing siliques were picked and surface-sterilized in 30% (v/v) Javex® with 1% (v/v) Tween for 15 minutes and subsequently washed three times with sterile water. The embryos were at the globular stage of development at approximately 6 days after pollination and at the heart stage at approximately 8 days after pollination. Within one silique the ovules could contain embryos at various stage of development but these could be easily differentiated under the dissecting microscope. Ovules containing globular and heart stage embryos were cut transversely using a sterile razor blade to expose the developing embryo, and placed on a modified enriched maturation medium (Table 2.2; Liu et al., 1993a) either with or without treatment (10 μM TIBA). A stock solution of TIBA was prepared and filter sterilized (for details see Experimental Methods below). The appropriate amount was added to the medium after it had been autoclaved and before being poured into the petri plates.

Table 2.2: Composition of maturation medium for culture of zygotic embryos of *Brassica napus* (Liu et al., 1993a)

Macroelements	mg l^{-1}
NH_4NO_3	200
KNO_3	1500
$CaCl_2.5H_2O$	850
$MgSO_4.7H_2O$	400
$KH_2PO_4.2H_2O$	79
$Na_2EDTA.2H_2O$	33
$FeSO4.7H_2O$	28
Microelements	
KI	0.75
H_3BO_3	3.00
$MnSO_4.H_20$	13.20
$ZnSO_4.7H_2O$	2.00
$NaMoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl.6H_2O$	0.025
Sugar Mixture	
Sucrose	40 g
Glucose	20 g
Fructose	100
Ribose	100
Xylose	100
Mannose	100
Rhamnose	100
Cellobiose	100
Sorbitol	100
Mannitol	100

Organic Acids	mg l^{-1}
Sodium pyruvate	20
Citric acid	40
Malic acid	40
Fumaric acid	40
Vitamins and amino acids	
Inositol	500
Glutamine	200
Thiamin.HCl	1
Nicotinic acid	0.1
Pyrodoxine.HCl	0.1
d-Biotin	0.01
Organic Supplements	
Coconut water*	100 ml l^{-1}
Casein hydrolysate	100
Agar	10g

* Sterile coconut water was obtained from Sigma (C-5915)

Zygotic embryo culture conditions

The petri plates containing approximately 20-25 embryos were placed in the dark for 2 days and then under 16 hour photoperiod for a further 12 days. At the end of the maturation period, the embryos were transferred to a conversion medium (half-strength MS medium with 2% sucrose and 0.8% agar) and maintained under similar conditions. At various times during the maturation the embryos were observed to determine the number of embryos with fused cotyledons. Pictures were taken under the dissecting microscope with Kodak Kodacolor ASA 200 film. The embryos were also fixed and processed at different stages during the maturation or conversion process for histological observations as outlined below.

Experimental Methods

Auxin Effects

To test the effects of altering the levels of auxin on embryogenesis, the polar auxin transport inhibitor, 2,3,5-triiodobenzoic acid (TIBA, sodium salt: Sigma T-7267) in the concentrations of 1μM, 2μM, 5μM, and 10 μM were added to culture plates on day 5, 10, 15, 20 and 25. A stock solution of TIBA was prepared in water, filter sterilized and maintained at 4°C. The inhibitor were then added to the culture plates on the appropriate day.

To assess whether the effects of TIBA were due to its ability to inhibit the polar transport of auxin, an auxin antagonist, 2-(p-chlorophenoxy)-2-methylpropionic acid (PCIB, Sigma C-7142), was added to the TIBA treated embryos on days 5 and 10. A stock solution of PCIB was prepared by first dissolving it in anhydrous ethyl alcohol and then filter sterilizing the solution. PCIB was then added in the concentrations of 2, 5 and 10 μM at the same time that the TIBA (2 μM) was added to the cultures.

Exogenous indole-3-acetic acid (IAA, Sigma I-2886) was also added to the developing MD embryos at various times during their development similar to the TIBA treatments. A stock solution of IAA was prepared by first dissolving it into anhydrous

ethyl alcohol and then filter sterilizing the solution. Appropriate amounts were added to the culture plates to give final concentrations of either 1, 2, 5 or 10 μM.

Observations were made at intervals of 5 days throughout the experimental time period (to Day 30). At these times, the number of MD embryos with fused cotyledons was counted under a dissecting microscope and recorded in each of three different petri plates. The embryos were then fixed and processed as outlined below. Each experiment was repeated a minimum of three times, with embryos in at least 3 different petri dishes counted. In the graphs drawn, the bars represent the mean and the error bars represent the standard deviation around the mean.

Abscisic Acid Effects

Similarly, to determine the effects of abscisic acid (ABA) on embryogenesis, ABA (± cis, trans-ABA, Sigma A-1012) was added to the culture plates in the concentrations of 2μM, 5μM, 10μM, and 15μM on days 5, 10, 15, 20, and 25. A stock solution of ABA was prepared by first dissolving the ABA in anhydrous ethyl alcohol. Each experiment was repeated a minimum of three times with at least 3 replicate petri dishes for each concentration.

Conversion Frequencies

To assess the effects of the treatments (TIBA and ABA) on the ability of the MD embryos to produce a normal plantlet, the conversion frequency of control and treated embryos was tested. The conversion frequency was determined by the production of both roots and shoots upon germination. Control and treated embryos that were 15, 20, 25 and 30 days old were transferred to a germination medium (half-strength MS with 2% sucrose, 0.9% agar and 0.1% activated charcoal). The embryos were placed on the medium such that the root end was inserted into the medium and the embryo was vertical. Excess liquid medium was removed from the embryos by using sterile filter paper to absorb the liquid. The conversion frequency, the number of embryos that possessed

leaves divided by the total number of embryos plated, was then assessed 3 weeks after the transfer to the germination medium. The shoot apex of these plantlets were fixed and processed as outlined below. Conversion frequency experiments were repeated a minimum of 3 times independently with a minimum of 30 embryos for each transfer day and each time of treatment in each experiment. In the graphs drawn, the bars represent the means and the error bars represent the standard deviation around the mean.

Fixation and Processing of Samples

In vitro zygotic and MD embryos were fixed at various times during the experimental periods in 2.5% glutaraldehyde and 1.6% paraformaldehyde buffered with 0.05M phosphate buffer, pH 6.8, for 24 hours at 4 ° C.

The samples were then dehydrated in methylcellosolve (BDH Chemicals) for 24 hours, followed by two changes of 100% ethanol for 24 hours each at 4 ° C. The embryos were infiltrated gradually (3:1, 1:1, and 1:3 100% ethanol:Technovit® 7100, 24 hrs each) with Technovit® 7100 (Kluzer and Co. Gmbh, Bereich Technik, D6393 Wehrheim, Germany), followed by two changes of pure Technovit® 7100. The tissue was then embedded according to Yeung and Law (1987).

Median longitudinal serial sections, 3 μm thick, were cut using a Ralph knife on a Reichert-Jung 2040 Autocut rotary microtome. Sections were stained according to Yeung (1984) with Periodic Acid Schiff's (PAS) reagent for total carbohydrate and counter-stained with either 0.05% (w/v)Toluidine Blue O (TBO) in benzoate buffer for general histology or 1% (w/v) amido black 10B in 7% acetic acid for protein. The sections were viewed under a Leitz photomicroscope. A minimum of 30 sections for each treatment and each time point were observed. Photographs of sections were taken using Kodak Technical Pan ASA 80 black and white film. All photographs presented are oriented such that the cotyledons (and the apex) of the embryo are towards the top of the photographs.

Immunolocalization of α-Tubulin

Fixation and Processing

At various intervals during the development of control and TIBA treated zygotic and MD embryos, samples were fixed and processed for the immunolocalization of α-tubulin similar to the methods of Baluška et al. (1992). Prior to fixation, the embryos were trimmed to allow for better positioning during the embedding process. This also facilitated the fixation process. The embryos were fixed in freshly prepared 4% paraformaldehyde in MT-stabilizing buffer (MTSB: 50mM PIPES buffer, 50mM $MgSO_4$, 50mM EGTA with 10% (v/v)dimethyl sulphoxide (DMSO) and 1% Triton-X) for 3 hours at room temperature. The paraformaldehyde was dissolved into the MTSB by gently heating the solution in the fume hood. Following 2 rinses with MTSB for 30 minutes each, the vials containing the samples were placed on a slowly rotating rotor. The samples were dehydrated in a graded ethanol series to 90% ethanol at 4 ° C. This was followed by two changes of 100% ethanol at room temperature for 30 minutes each. The samples were then infiltrated at 37 ° C with mixtures of 2:1, 1:1 and 1:2 of 100% ethanol: polyester wax (BDH) overnight, followed by two changes of pure wax. The samples were then embedded by allowing the wax to solidify at room temperature.

Median longitudinal sections were cut at 10μm on a rotary microtome using steel knives and mounted in water on slides coated with the Vectabond ™ Reagent (Vector Laboratories, California). The slides were then allowed to thoroughly dry overnight and then stored in slide boxes.

Immunolocalization

The tissues were dewaxed in three changes of absolute ethanol for 10 minutes each. The slides were removed from the ethanol and air dried for 15 minutes at room temperature to allow the sections to adhere to the slides as per Richardson and Dym (1994). The tissues were then rehydrated in 90% and 70% ethanol for 10 minutes each. After a brief rinse in phosphate buffered saline (PBS), the sections were blocked with 4%

bovine serum albumin (BSA) in PBS to reduce any non-specific binding. The sections were then incubated in mouse monoclonal antibody (Sigma T-9026; 1:200 dilution in 1% BSA in PBS) raised against chick brain α-tubulin for 60 minutes at 37 ° C. Control slides were incubated in mouse IgG (Sigma, I-5381) at the same dilution as the primary antibody. The slides were rinsed and washed in PBS for 10 minutes and then incubated with fluorescein isothiocyanate- (FITC-)conjugated anti-mouse IgG raised in goat (Sigma, F-8771) diluted 1:64 in PBS for 60 minutes at 37 ° C. The sections were rinsed and washed with PBS for 10 minutes and then mounted in an anti-fade mountant citifluor (Marivac Ltd., Halifax, Nova Scotia). The fluorescence was observed on a photomicroscope equipped with epifluorescence and FITC filters. The immunolocalization experiments were repeated 3 times each for MD and zygotic embryos. Photographs were recorded with Tri-X Pan ASA 400 black and white film. All photographs are oriented such that the cotyledons and the apex of the embryo are towards the top of the photograph.

CHAPTER THREE

The effects of altered auxin levels on developing microspore-derived embryos

Introduction

It has long been recognized that the phytohormones play a crucial role during plant development. Auxins play a key role in a variety of plant processes including, most importantly, the regulation of cell growth and differentiation. They have also been implicated in playing a role in a number of morphogenic processes, including induction of vascular differentiation, stimulation of cambial activity, control of apical dominance, promotion of root initiation, senescence and abscission (Goldsmith, 1977; Warren Wilson and Warren Wilson, 1993). In addition, the polar transport of auxin has been associated with polar development as well as differentiation and growth phenomena, for example: apical dominance, vascular development and tropisms (Lomax et al., 1995). In shoot tissues, auxin transport has been shown to be basipetal such that auxin moves from the apical to the basal region. However, the polarity of transport in roots has not been firmly established (Lomax et al., 1995).

Polarity in the embryo is determined morphologically by the establishment of the root-shoot axis and it has been suggested that the polar transport of auxin that is initiated during embryogenesis is responsible for physiological polarity. There has been relatively little work on the physiological processes that control embryogenesis primarily due to the limited access of the developing zygotic embryo. Therefore, much of the information that is available on the role of the polar transport auxin has come from somatic embryos and other *in vitro* systems. Using somatic embryos of *Daucus carota*, Brawley et al. (1984) were able to detect electrical currents around the developing embryos. They found that the patterns of current either entering or leaving the embryos remained the same during development and suggested that this bioelectric current along the axis of the embryo may be involved in the establishment of morphological polarity. First, an inward current is

centred on the apical pole, but then moves and intensifies at sites where cotyledon differentiation is occurring. The outward current is established at the elongation zone of the radicle. Brawley et al. (1984) were also able to determine that only changes in H^+ affected the density of the currents significantly, indicating that the main part of this current could be carried by protons. Furthermore, exogenous application of indole-3-acetic acid (IAA) diminished the currents altogether. The flow of auxin could be responsible for this bioelectric current (Rathore et al., 1988; Warren Wilson and Warren Wilson, 1993).

Several studies that disrupt the polar transport of auxin provide additional evidence that auxins play a significant role during early embryogenesis. Inclusion of indole-3-acetic acid (IAA), a natural auxin, in the culture medium of developing carrot embryos resulted in the embryos reverting to undifferentiated callus (Schiavone and Cooke, 1987). It has been suggested that the exogenous, symmetrical application of IAA reduced the internal polarized transport and prevented further development. Schiavone and Cooke (1987) also demonstrated that application of 2,3,5-triiodobenzoic acid (TIBA) and *N*-(1-naphthyl) phthalamic acid (NPA), inhibitors of the polar transport of auxin, to carrot cultures prevented the polarized axial growth associated with the late globular stage. Instead, large globular and oblong embryos were formed. This indicated to the authors that the embryos acquired the ability to transport auxin at this stage in development and the subsequent accumulation of auxin in the basal region resulted in the elongation of the axis. In addition, they also found that cotyledon formation was absolutely dependent on the polar transport. The initiation of the polar transport of auxin at this stage correlates well also with anatomical observations. It is at the late globular-heart stage transition that differentiation of the internal tissue begin to occur and particularly the cells in the centre of the embryo elongate to form the procambium (cf. Yeung et al., 1996). This ties in with auxin's role in cell elongation and differentiation.

Recently, Liu et al. (1993b) demonstrated that the polar transport of auxin is also essential in the early *in vitro* zygotic embryogenesis of *Brassica juncea*. Unlike carrot somatic embryos, these embryos were able to undergo elongation even when treated with TIBA at the globular stage. However, the authors also found that auxin transport

inhibitors interfered with cotyledon formation. One fused trumpet-shaped cotyledon was initiated at the late globular stage indicating that these embryos failed to develop bilateral symmetry. Furthermore, within the ring of cotyledonary tissue, there were six vascular bundles compared to the two found in control embryos. Hadfi et al. (1998) elaborated on this system and also showed that IAA and TIBA can also prevent the embryos from elongating in high enough concentrations. These disruptions in embryonic pattern formation are not limited to the dicots as Fisher and Neuhaus (1996) also observed that *in vitro* zygotic embryos of *Triticum aestivum* L. (wheat) failed to exhibit bilateral symmetry if they were treated with TIBA at the globular stage. It is clear from these studies that the polar transport of auxin exerts its effects very early on in development. However, these studies were important in mainly observing the morphological effects of disrupting auxin levels during embryogenesis. They did not undertake to observe the histological effects of altering auxin transport or to determine if the disruption in polar transport affected another important early developmental process - the formation of the SAM. In fact, Liu et al. (1993b) observed that TIBA-treated embryos showed a delay in shoot formation upon initiation of germination. This further suggests that the SAM is altered upon TIBA treatment.

Therefore, this chapter examines the role that auxin levels play in the development of the SAM during MD embryogenesis of *Brassica napus*. The MD embryo system offers the advantage of having a large number of embryos that can be used for experimental manipulation in a relatively short space of time.

Results

The effect of TIBA on the morphology of developing MD embryos

During the normal development of MD embryos, they were observed to undergo similar developmental stages as their zygotic counterparts (Fig 3.1). At 10 days old, the majority of the embryos were at the globular stage of embryogenesis (Fig 3.1 A). However, there were also some embryos that were at the heart stage of development (Fig 3.1 B) as the embryos started to elongate and two separate cotyledons emerged. These embryos continued to develop and were considered to be at the torpedo stage of development by day 15 (Fig 3.1C). At this stage, the hypocotyl region had elongated considerably, however, the cotyledons were not very well developed. As the embryos continued to mature, the cotyledons increased in size and the embryo increased in overall length (Fig 3.1 D). The embryos were considered to be completely mature by day 30 (Fig 3.1 E). Embryos with fused cotyledons were not ever observed in the more than 2200 untreated embryos observed (Fig 3.2).

The morphology of the TIBA treated embryos depended on the timing of TIBA treatment. If TIBA was added to the culture medium at Day 5, the pre-globular stage, a high percentage (74% of a total 1887) of embryos developed one fused cotyledon instead of two separate cotyledons found in the control embryos (Fig 3.2, Fig 3.3A, B). Similarly, if TIBA was added to the culture medium when the embryos were at the globular stage of development (Day 10), more than 48% (of a total of 1692) of the embryos developed one fused cotyledon (Fig 3.2, Fig 3.3C). These 'trumpet-shaped' embryos were evident as early as about Day 12 when normally the two cotyledons would start to emerge on the control embryos. If TIBA was added to the culture medium after the globular stage (i.e. at Day 15, 20 or 25), the embryos had morphologies comparable to the control embryos with two separate cotyledons (Fig 3.2, Fig 3.2 D-F). These observations were reproducible with all the concentrations of TIBA tested (1, 2, 5, 10, 20 μM). However, the concentration of 2 and 5 μM produced the most consistent results and concentrations higher than these had deleterious effects on the developing embryos.

Figure 3.1:

Morphology of untreated MD embryos of *Brassica napus* at various times during the culture period. A: a globular stage embryo predominantly found at Day 10. B: a heart stage embryo also usually found at Day 10. C: a torpedo stage embryo found at Day 15. D: an embryo considered to be at the mid-cotyledonary stage found in the cultures at Day 20 - 25. E: a mature embryo at the late-cotyledonary stage at Day 30. Note that from the heart stage onwards two separate cotyledons are visible. Scale bar = 1.5mm

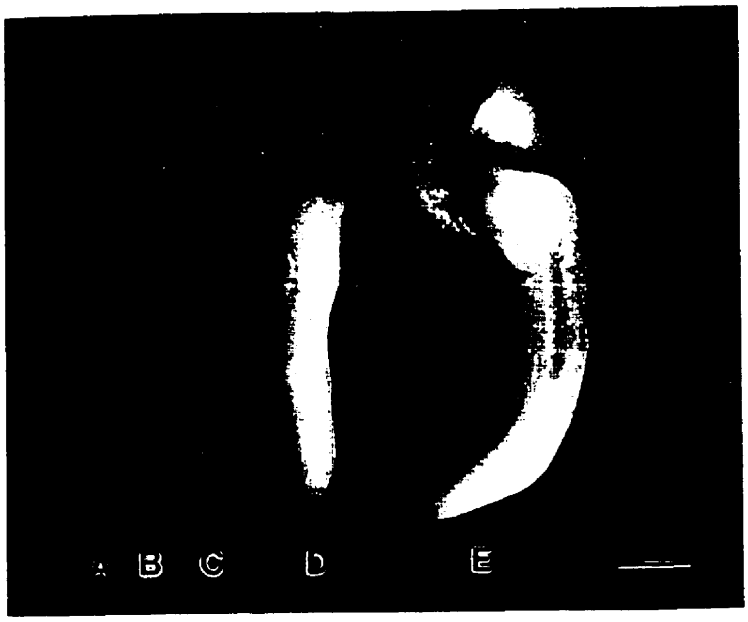

A B C D E

Figure 3.2:

Response of MD embryos of *B. napus* treated with TIBA at various times. The percent of embryos with fused cotyledons was scored at the end of the experimental time period of 30 days. The bars represent the mean percent of fused cotyledons present in that treatment. Error bars represent the standard deviation around the plotted average.

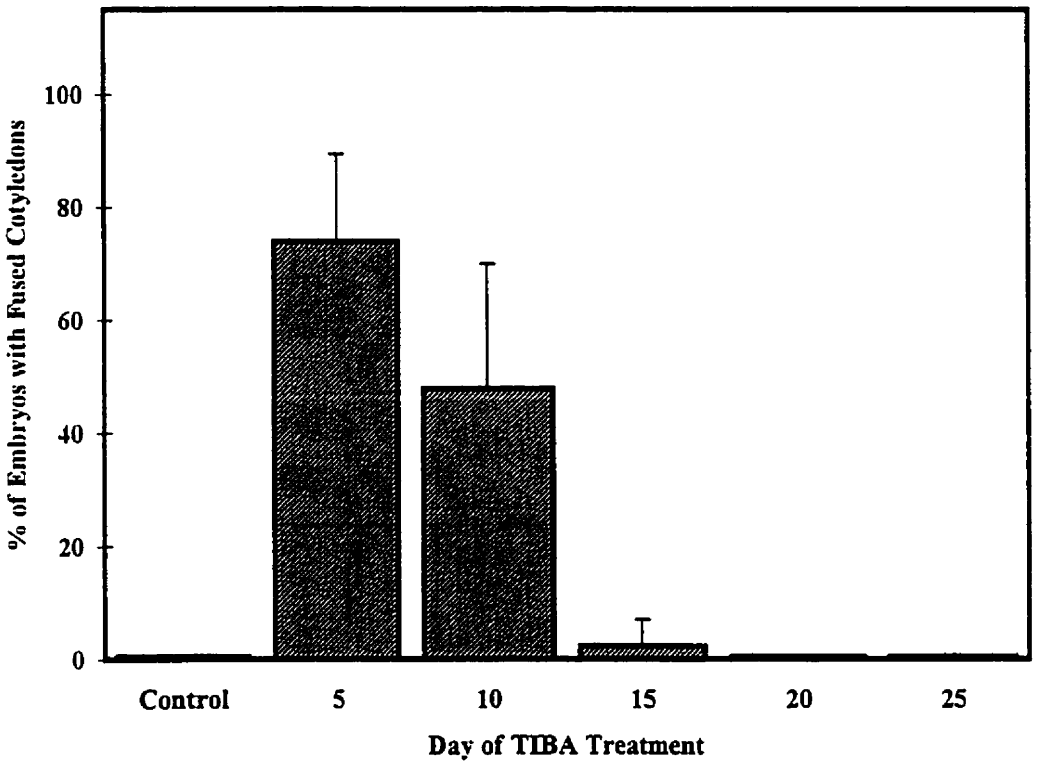
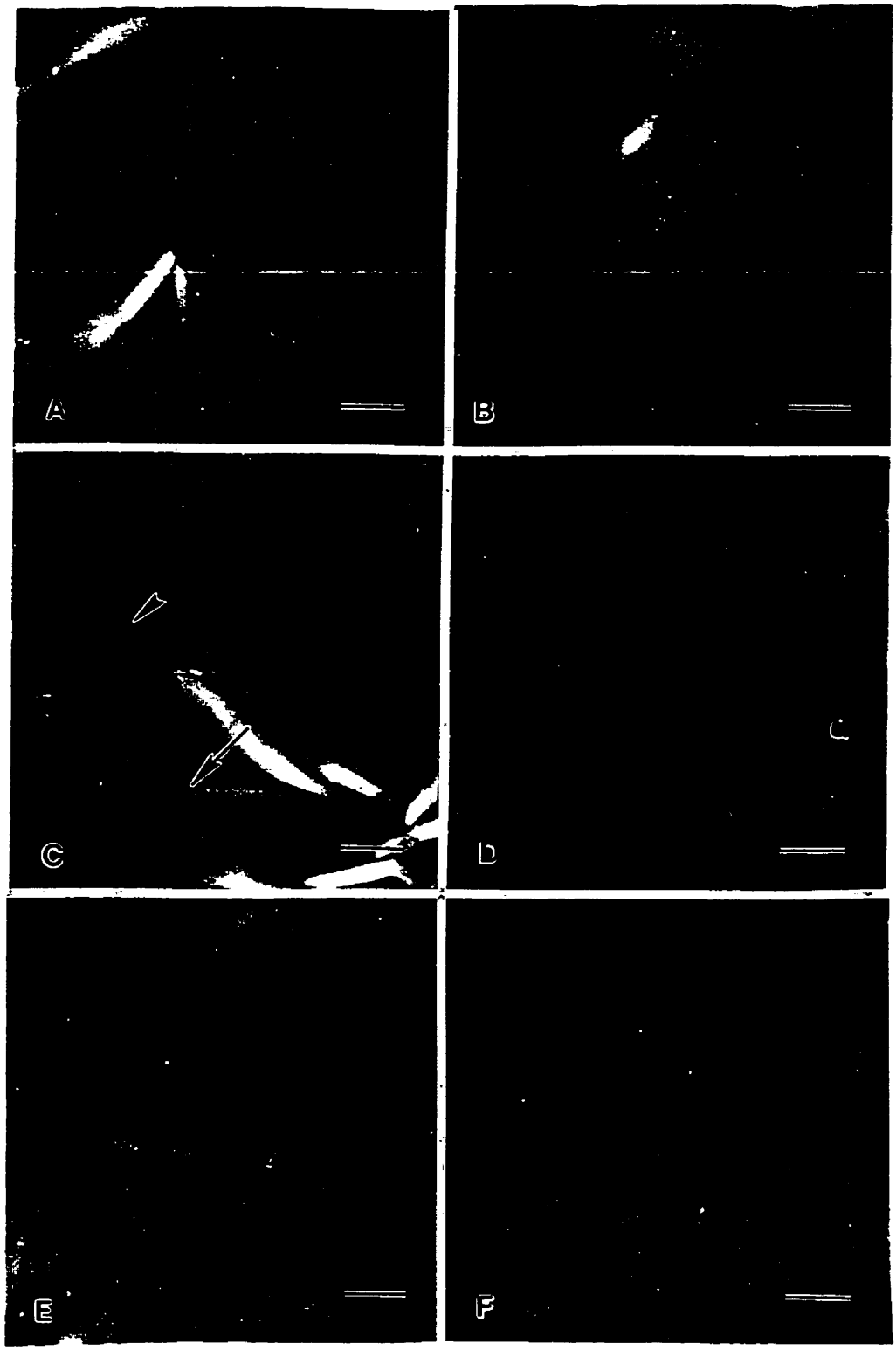

% of Embryos with Fused Cotyledons
100
80
60
40
20
0
Control
5
10
15
20
25
Day of TIBA Treatment

Figure 3.3:

Morphology of MD embryos of *B. napus* at Day 30. A: Untreated embryos all with two separate cotyledons. B-F: Embryos treated with 2 μM TIBA. B: Embryos that were treated at Day 5 all have one fused cotyledon. C: Some embryos treated at Day 10 have one fused cotyledons (➔) while some have two separate cotyledons (➢). D: Embryos treated at Day 15 all have two separate cotyledons. E: Embryos treated at Day 20 all have two separate cotyledons. F: Embryos treated at Day 25 all have two separate cotyledons. All scale bars = 2.0mm


A
B
C
D
E
F

Consequently the results reported and pictures presented are those of embryos treated with 2 μM TIBA.

To determine whether the effects of TIBA on the morphology of the developing embryos is due to its ability to block the polar transport of auxin and not because of a pharmacological side effect, an auxin antagonist, PCIB, was added at the time of TIBA treatment. Figure 3.4 indicates that if PCIB was added to the medium at the same time that TIBA was added at Days 5 and 10, then the number of embryos with fused cotyledons was reduced by approximately 60%. This was observed when PCIB was added in the concentrations of 2, 5 or 10 μM. However, 2 μM PCIB produced the most consistent results. Embryos with fused cotyledons were not ever observed if they were treated with PCIB alone (Fig 3.4). Embryos treated with PCIB and TIBA appeared normal with two separate cotyledons similar to control embryos (Fig 3.5 A, C). Furthermore, embryos treated with PCIB alone also appeared normal (Fig 3.5 B).

The effects of TIBA treatment on SAM development

To determine if TIBA treatment had any effect on the formation of the SAM in MD embryos, the histology of the treated embryos was examined and compared to the control embryos. By Day 15, the SAM of the control embryos was well developed and defined as a group of tightly packed isodiametric cells located in the apical notch between the two emerging cotyledons (Fig 3.6 A). These cells were cytoplasmically dense with very little vacuolation and the nuclei were quite prominent. The pattern of observations of embryos treated at day 5 or day 10 with TIBA were similar, therefore only the sequence of results of those treated at day 5 are reported. In embryos that were treated with TIBA (2μM) on day 5 and sampled on day 15, it was clear that the SAM was altered by this time. The SAM was less broad in comparison to the SAM of the control embryo (Fig 3.6B). There were less cells that composed the SAM and some of the characteristics of these cells were changed. The cells were still fairly dense but there was evidence of vacuolation beginning at the apical half of the cells in the tunica layer of the SAM.

Figure 3.4:

Response MD embryos of *B. napus* treated with TIBA and PCIB at various times. The control embryos were treated with TIBA or PCIB at Day 5. The percent of embryos with one fused cotyledon was assessed at Day 30. The concentrations were 2 μM TIBA and 5 μM PCIB. The bars represent the mean percent of embryos with one fused cotyledon present in that treatment. Error bars represent the standard deviation around the plotted mean.

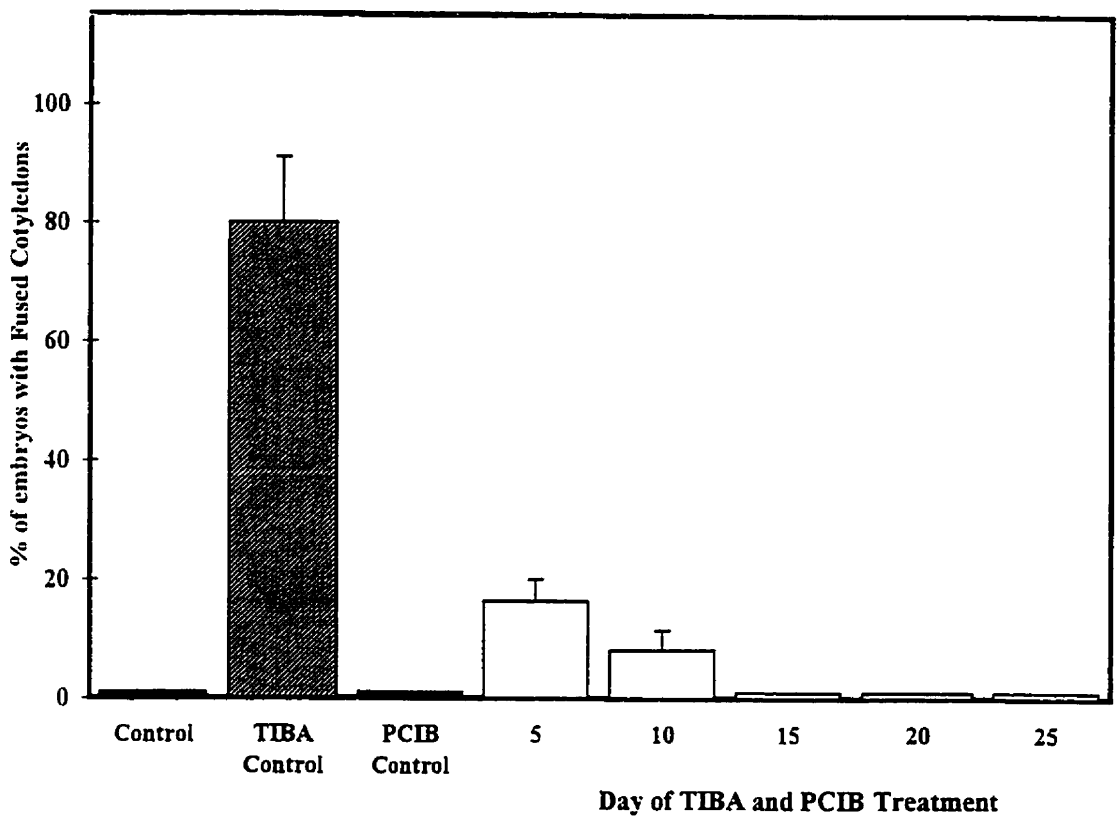

% of embryos with Fused Cotyledons
100
80
60
40
20
0
Control
TIBA Control
PCIB Control
5
10
15
20
25
Day of TIBA and PCIB Treatment

Figure 3.5:

Morphology of MD embryos of *B napus* at Day 15. A: Untreated embryos all with two separate cotyledons. B: Embryos treated with 5 μM PCIB at Day 5 all have two separate cotyledons. C: If 5μM PCIB is added to the embryos at the same time that 2μM TIBA is at Day 5, then all the embryos have two separate cotyledons. All scale bars = 2.0mm

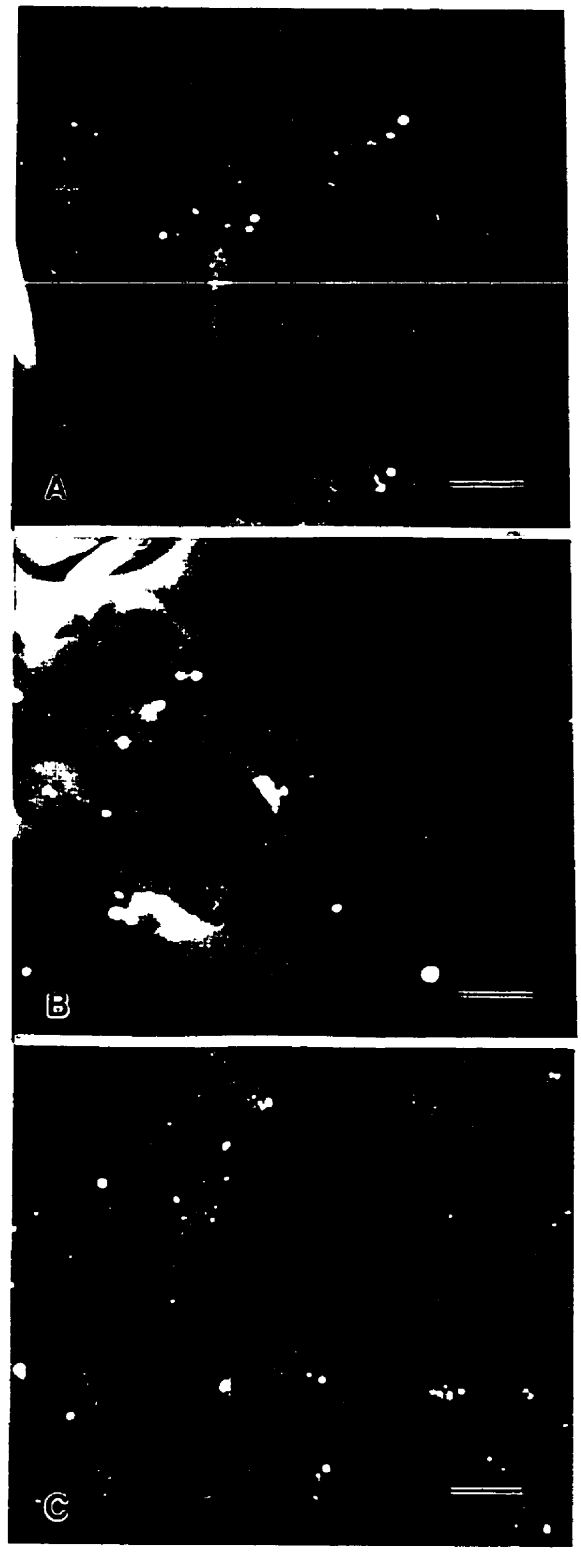
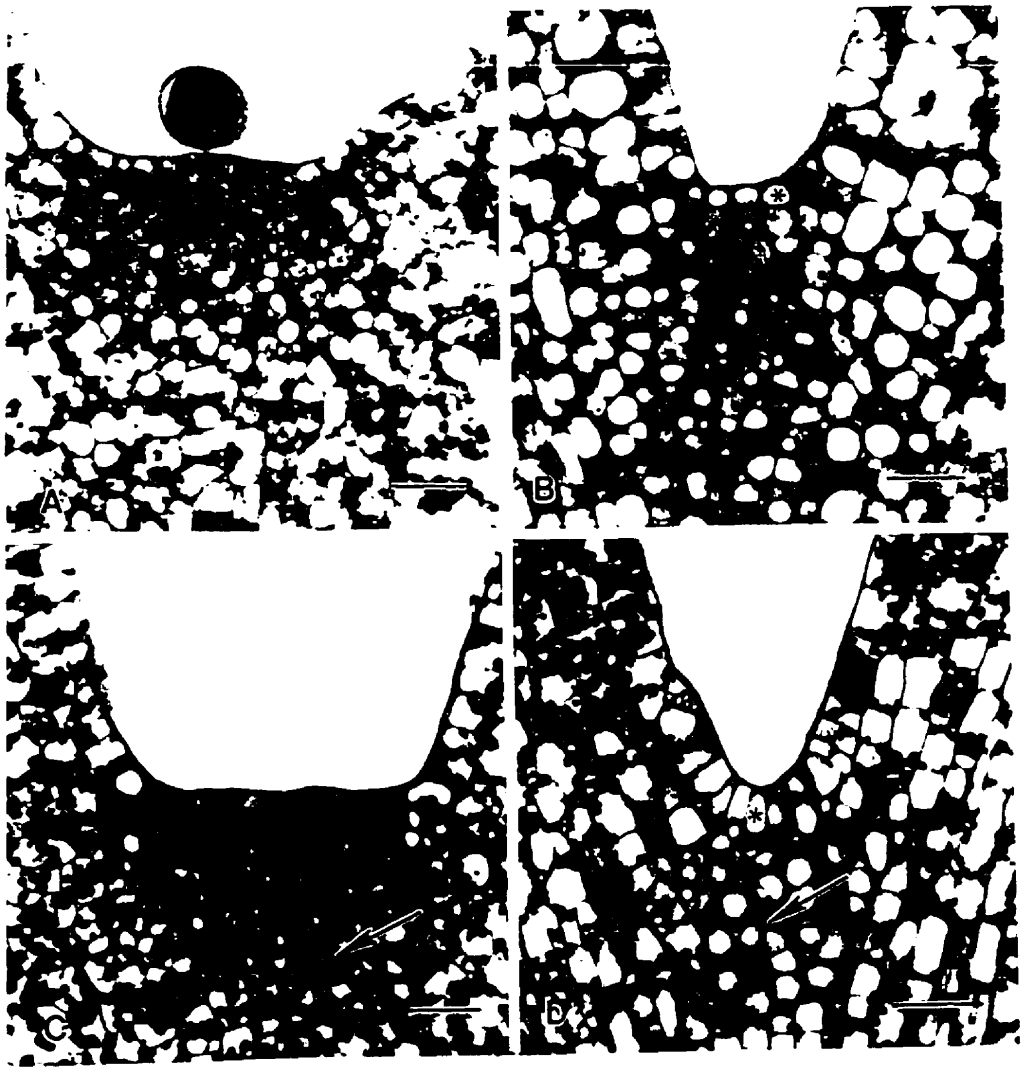

A
B
C

Figure 3.6:

Histology of the SAM of control and TIBA-treated MD embryos. Embryos were treated with TIBA at Day 5. A and B: embryos sampled at day 15. A: The SAM of this control embryo is very well organized with many cells in the width and depth. B: The SAM of this TIBA treated embryo is much reduced in width. Vacuolation is evident in the apical half of the cells in the tunica (*). C and D: embryos sampled at day 20. C: The SAM of a control embryo continues to maintain its overall shape and size but starch is beginning to accumulate in the peripheral cells of the corpus (➔). D: Vacuolation is now evident in all cells of the SAM of this TIBA treated embryo (*) and starch is also beginning to encroach into the cells of the corpus (➔). All scale bars = 20μm.


A
B
C
D

These tunica layer cells also appeared to be more axially elongated and lost their isodiametric appearance.

By day 20 the SAM of the control embryos continued to maintain its overall shape, size and appearance. However, there was some evidence of vacuolation beginning to occur in some of the cells (Fig 3.6 C). Vacuolation continued to increase in the cells of the SAM of the TIBA treated embryos and was observed in the cells of the corpus region (Fig 3.6 D). These cells began to appear more elongated, similar to the cells of the tunica layer. The overall area occupied by the SAM decreased as starch began to encroach in the cells on the periphery of the SAM.

Generally the SAM of the control embryos began to further deteriorate by day 25. Starch was observed to begin accumulating in these cells and there was an increase in vacuolation in the area (Fig 3.7 A). However, the cells continued to maintain there overall isodiametric shape and the limits of the SAM were still recognizable. By this time, all the cells of the TIBA-treated embryos had accumulated starch (Fig 3.7 B). The SAM was extremely reduced in size and in some cases, no more than six cells could be designated as belonging to the SAM. The rest of the cells became starch-filled parenchyma-like cells that resembled the cells of the cotyledons

The cells of the tunica layer of the SAM in the control embryos were extremely vacuolated by day 30 and starch filled most of the cells on the periphery of the corpus (Fig 3.7 C). All the cells of the SAM of TIBA treated embryos had some evidence of starch (Fig 3.7 D). The cells continued to be axially elongated, and heavily vacuolated. The cells in the area surrounding the SAM appeared to accumulate more starch than that observed in the control embryos.

The effect of TIBA on the SAM seemed to be less dramatic if the embryos were treated on day 15, 20, or 25 (Fig 3.8 B-D). The overall appearance of the SAM of these treated embryos was very similar to their control counterparts (Fig 3.8 A). In particular, the size and shape of the cells as well as the entire SAM were very similar to the control embryos and their developmental pathway was the same when compared to control.

Figure 3.7:

Histology of the SAM of control and TIBA-treated MD embryos. Embryos were treated with TIBA at Day 5. A and B: embryos sampled at Day 25. A: The SAM of a control embryo. Vacuolation is evident in many cells of the SAM (*) and starch is continuing to encroach into the cells (➔). The cells, however, still remain fairly cytoplasmic. B: The cells in the SAM of the TIBA treated embryo continue to appear elongated with a high amount of vacuolation (*) and starch accumulation (➔)

C and D: Embryos sampled at Day 30. C: All the cells in the SAM of this control embryo show starch accumulation (➔) and vacuolation (*). Similarly the cells of the SAM in the TIBA treated embryo (D) also show starch accumulation and a high degree of vacuolation. All scale bars = 20 μm

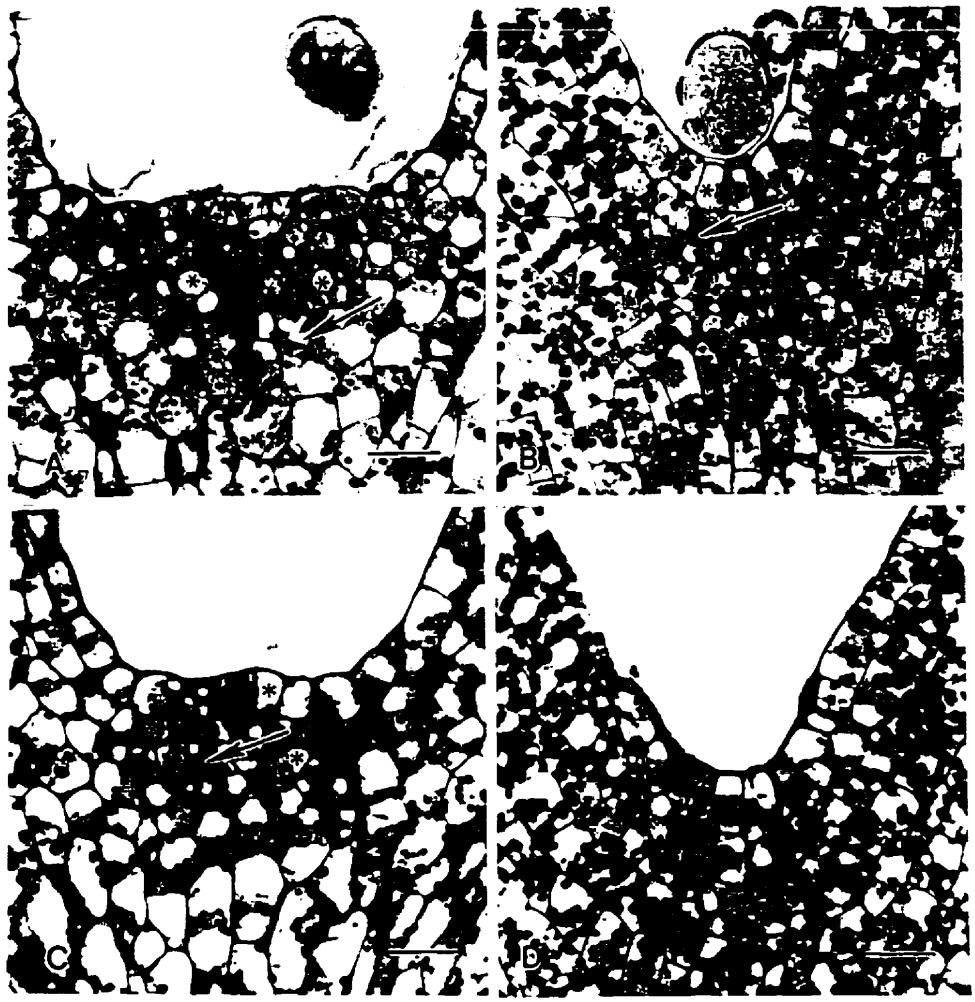
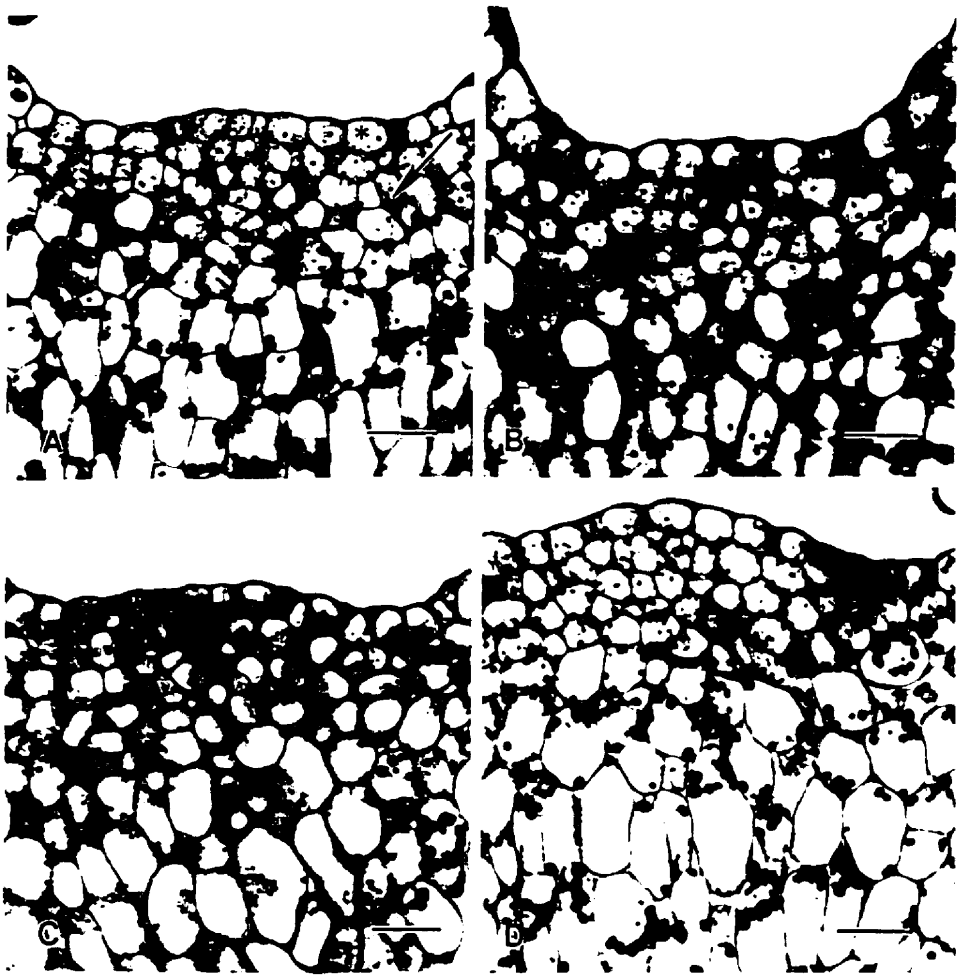

A
B
C
D

Figure 3.8:

Histology of the SAM of control and TIBA-treated MD embryos sampled at Day 30. A: The cells of the SAM of a control embryo, for comparison, show some vacuolation (*) and starch accumulation (➔). Embryos that were treated with TIBA at Day 15 (B), Day 20 (C), or Day 25 (D) have SAMs with comparable characteristics to the control embryo. All scale bars = 20μm


A
B
C
D

The effects of TIBA on the conversion frequency of developing MD embryos

To assess the normality and functionality of the embryos during various stages of development, they were transferred to a conversion medium and their ability to produce normal shoots and roots was determined. The embryos were transferred to the conversion medium at days 15, 20, 25 and 30. After 2-3 weeks, the number of embryos that had converted was divided by the total number of embryos to determine the conversion frequency. The conversion frequency of the control embryos ranged from 48% to 69% at different stages (Figs 3.9-3.12). However, there were no significant differences in the conversion frequencies at these different stages. Embryos that were transferred to the conversion medium at Days 15 and 20 produced the best plantlets often with one to two leaves already formed by the end of the experimental time period (Fig 3.13 A, C). However, at the end of the experiment, only a very small leaf was observed to be protruding from between the cotyledons of the embryos that were transferred at Day 25 and 30 (Fig 3.14 A, C). Embryos that did not convert usually turned a yellow colour and died.

Embryos that were treated with TIBA were plated out on the conversion medium at different days after their treatment. Their ability to convert generally depended on when they were treated with TIBA. Similar to the control embryos, within two days of transfer to the medium, the embryos turned completely green and the root began to elongate. The embryos that were treated with TIBA at Days 5 and 10 that had fused cotyledons had extremely low conversion frequencies (less than 10%) compared to the control regardless of when they were transferred to the germination medium (Fig 3.9-3.12, Fig 3.13 B, D, Fig 3.14 B, D). In those that were considered to have converted, there was a small rounded protrusion visible within the trumpet-shaped cotyledon. However, this protrusion was not ever observed to expand or resemble a normal leaf. The embryos that were treated with TIBA on Day 15 and later had conversion frequencies comparable to their respective controls (Fig 3.10-12).

The SAM of the control embryos that did convert increased in size and the cells maintained their overall cytoplasmic density and isodiametric shape (e.g. Fig 3.15 A, B).

Figure 3.9:

Conversion frequency of MD embryos transferred to the conversion medium at Day 15. The conversion frequency was assessed 3 weeks after the transfer. Embryos that were treated with TIBA on DAY 5 or Day 10 had conversion frequencies significantly lower than that of the control embryos. The bars represent the mean conversion frequency and the error bars represent the standard deviation around the plotted mean.

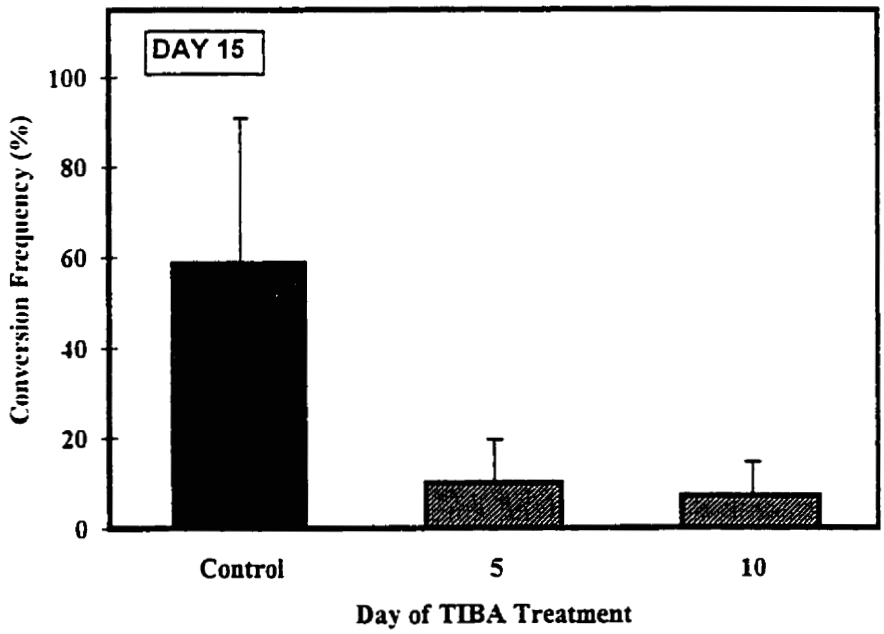

DAY 15
100
80
60
40
20
0
Conversion Frequency (%)
Control
5
10
Day of TIBA Treatment

Figure 3.10:

Conversion frequency of MD embryos transferred to the conversion medium at Day 20. The conversion frequency was assessed 3 weeks after the transfer. Embryos that were treated with TIBA on DAY 5 or Day 10 had conversion frequencies significantly lower than that of the control embryos. However, embryos that were treated on Day 15 had a conversion frequency comparable to the control. The bars represent the mean conversion frequency and the error bars represent the standard deviation around the plotted mean.

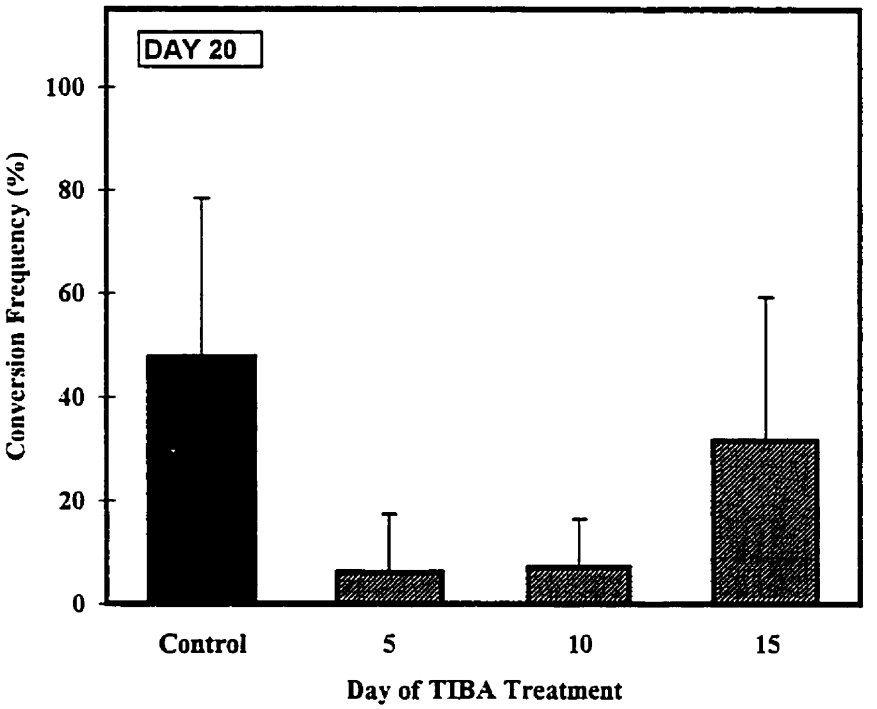

DAY 20
100
80
60
40
20
0
Conversion Frequency (%)
Control
5
10
15
Day of TIBA Treatment

Figure 3.11:

Conversion frequency of MD embryos transferred to the conversion medium at Day 25. The conversion frequency was assessed 3 weeks after the transfer. Embryos that were treated with TIBA on DAY 5 or Day 10 had conversion frequencies significantly lower than that of the control embryos. However, embryos that were treated on Day 15 or Day 20 had conversion frequencies comparable to the control. The bars represent the mean conversion frequency and the error bars represent the standard deviation around the plotted mean.

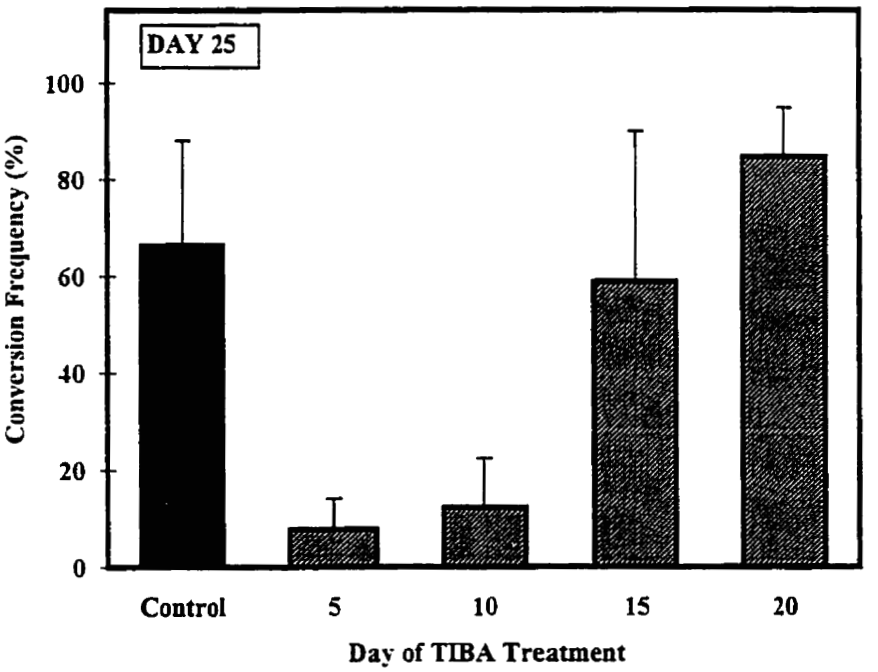

DAY 25
Conversion Frequency (%)
100
80
60
40
20
0
Control
5
10
15
20
Day of TIBA Treatment

Figure 3.12:

Conversion frequency of MD embryos transferred to the conversion medium at Day 30. The conversion frequency was assessed 3 weeks after the transfer. Embryos that were treated with TIBA on DAY 5 or Day 10 had conversion frequencies significantly lower than that of the control embryos. However, embryos that were treated on Day 15, 20 or 25 had conversion frequencies comparable to the control. The bars represent the mean conversion frequency and the error bars represent the standard deviation around the plotted mean.

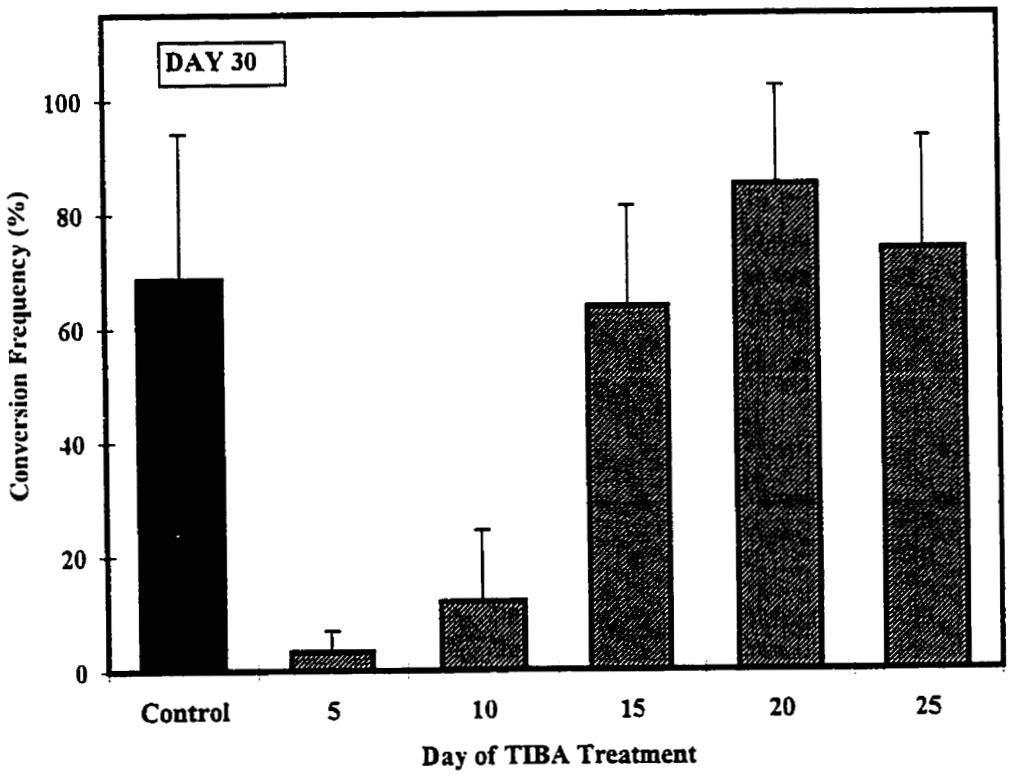

DAY 30
Conversion Frequency (%)
100
80
60
40
20
0
Control
5
10
15
20
25
Day of TIBA Treatment

Figure 3.13:

Morphology of MD embryos three weeks after the transfer to the conversion medium. Embryos were treated at Day 5. A and B: These embryos were transferred at Day 15. A: There are two leaves (➔) emerging from within the cotyledons of the control embryo. At this time the TIBA-treated embryo (B) has greened and expanded but does not show an signs of organogenesis.
C and D: These embryos were transferred to the conversion medium at Day 20. C: The control embryo shows signs of leaf formation (➔) but the TIBA treated embryo (D) does not. All scale bars = 1mm

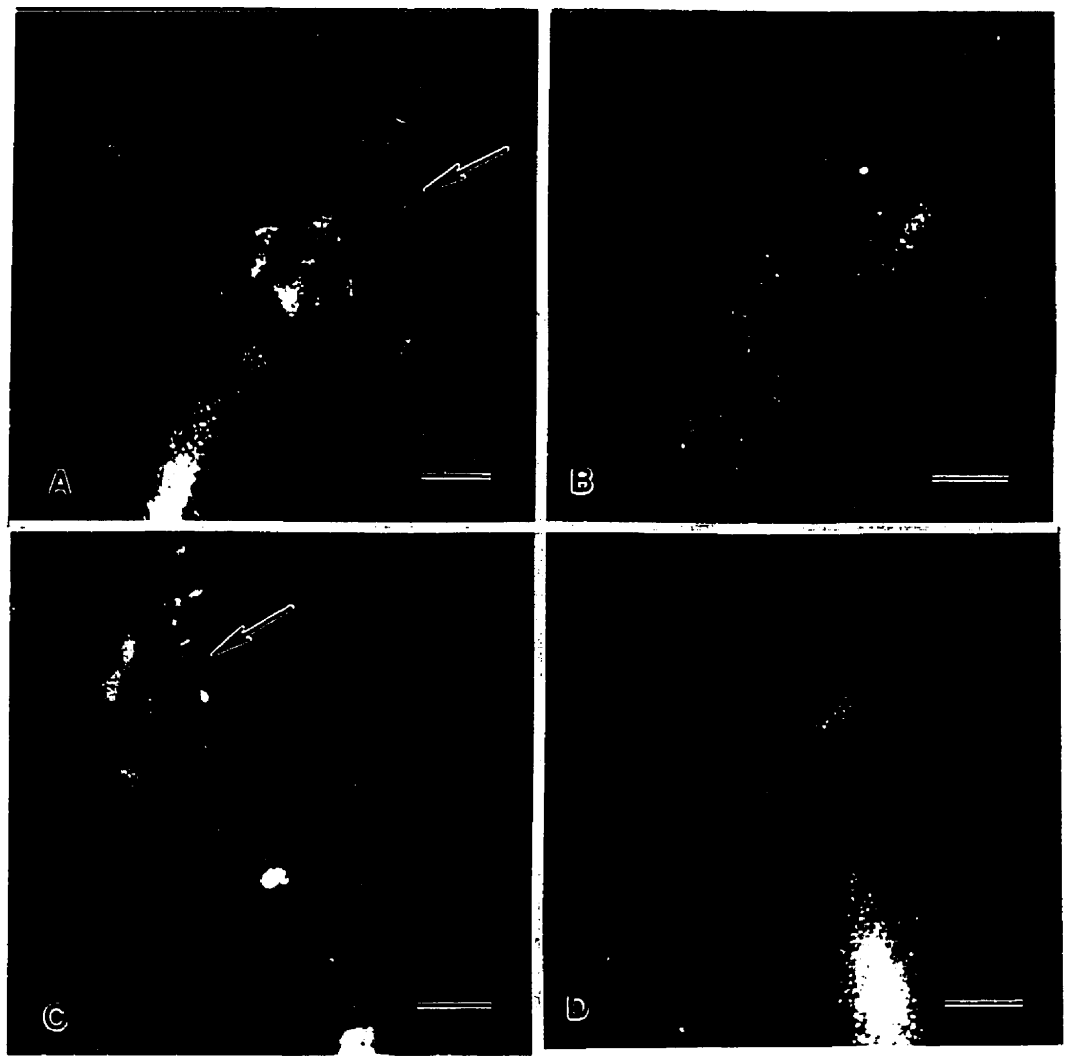
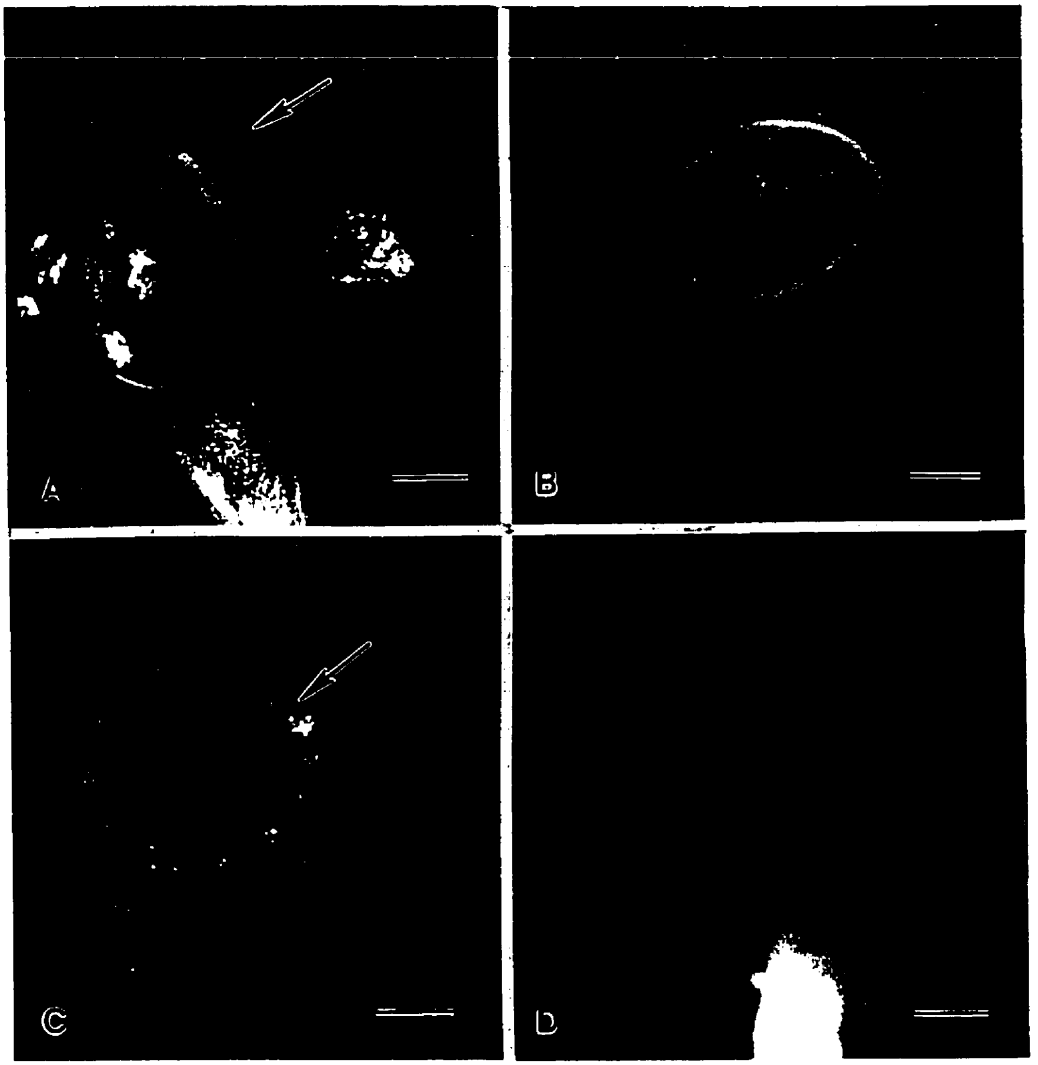

A
B
C
D

Figure 3.14:

Morphology of MD embryos three weeks after the transfer to the conversion medium. Embryos were treated at Day 5. A and B: These embryos were transferred at Day 25. A: This embryo is able to produce stem (➔) and leaves while the TIBA treated embryo (B) was not. C and D: These embryos were transferred at Day 30. C: A small leaf protrusion (➔) is present on this control embryo while there is no evidence of leaf formation on the TIBA treated (D) embryo. All scale bars = 1mm.


A
B
C
D

In the TIBA treated embryos that were transferred to the germination medium on Day 15 in which no 'leaf' protrusion was observed, the overall shape of the SAM remained distorted. The SAM appeared concave compared the convex shape observed in the control (cf. Fig 3.15 A and C). There appeared to be an increase in the number of cells in the SAM but these cells were not as organized as that seen in the control. The densely cytoplasmic cells extended up into the cotyledon. This unorganized pattern was also observed in other TIBA treated embryos that were transferred to the germination medium at later days (e.g. Day 30, Fig 3.15D). The SAMs of the TIBA treated embryos in which a 'leaf' protrusion was observed also exhibited very disorganized pattern of organization and cell division pattern (Fig 3.15 E, F). In some cases anticlinal divisions were observed in the tunica layer (Fig 3.15 E). It appeared that the 'leaf' was produced abnormally from cells that had extended up into the cotyledon region.

The effects of exogenous auxin on MD embryo development

Exogenous indole-3-acetic acid (IAA) was added in the concentrations of 1, 2, 5 and 10 μM. The higher concentration of 5 and 10 μM appeared to be toxic to the microspores as the number of embryos that developed were greatly reduced and those embryos that did develop were very distorted. The most effective concentration was 1 μM. Similar to the treatments with TIBA, IAA caused an alteration in embryo morphology and this depended on the timing of IAA treatment. Approximately 50% of the embryos that were treated with IAA at Day 5 had fused cotyledons (Fig 3.16 and Fig 3.17 A, B). This percentage declined if the embryos were treated at Day 10 (Fig 3.16 and Fig 3.17 C). If the embryos were treated at day 15 or later, 100% of the embryos had normal cotyledon morphology (Fig 3.16 and Fig 3.17 D).

The effect of IAA on SAM development was similar to that observed with TIBA. If the embryos were treated at an early stage (Day 5 or 10) in development, the SAM was less broad (Fig 3.18 A) than compared to that found in control embryos (Fig 3.6 A). The characteristics of the cells were also similar to those found in the TIBA-treated embryos. By Day 30 these cells also became highly vacuolated and there was an increase in starch

in these cells (Fig 3.18 B). Similar to the observations made with TIBA treated embryos, if IAA was added to the medium when the embryos were 15, 20 or 25 days old, the SAM appeared comparable to the control embryos (cf. Fig 3.18 C and Fig 3.7 C).

Comparable to the results obtained with TIBA treated embryos, the conversion frequency of the IAA treated embryos depended on the timing of IAA treatment.

Figure 3.15:

Histology of the SAM of MD embryos that have been on the conversion medium for three weeks. A, C, E: transferred at Day 15; A: The SAM of this control embryo is very well organized with densely packed cells. C: The SAM of this non-converted TIBA treated embryo was very disorganized with many cells characteristic of the meristem extending up the side of the cotyledon. The cells remained quite elongated. E: SAM of a 'converted' TIBA treated embryo that shows very unusual cell division patterns. Anticlinal divisions (➢) are evident in the tunica layer. B, D, F: transferred to the conversion medium on Day 30. B: SAM of a control embryo again showing good organization. D: SAM of a 'non-converted' TIBA treated embryo showing very unorganized cell division patterns. F: SAM of a 'converted' TIBA treated embryo that again shows a very disorganized pattern of cell division. All scale bars = 20μm.

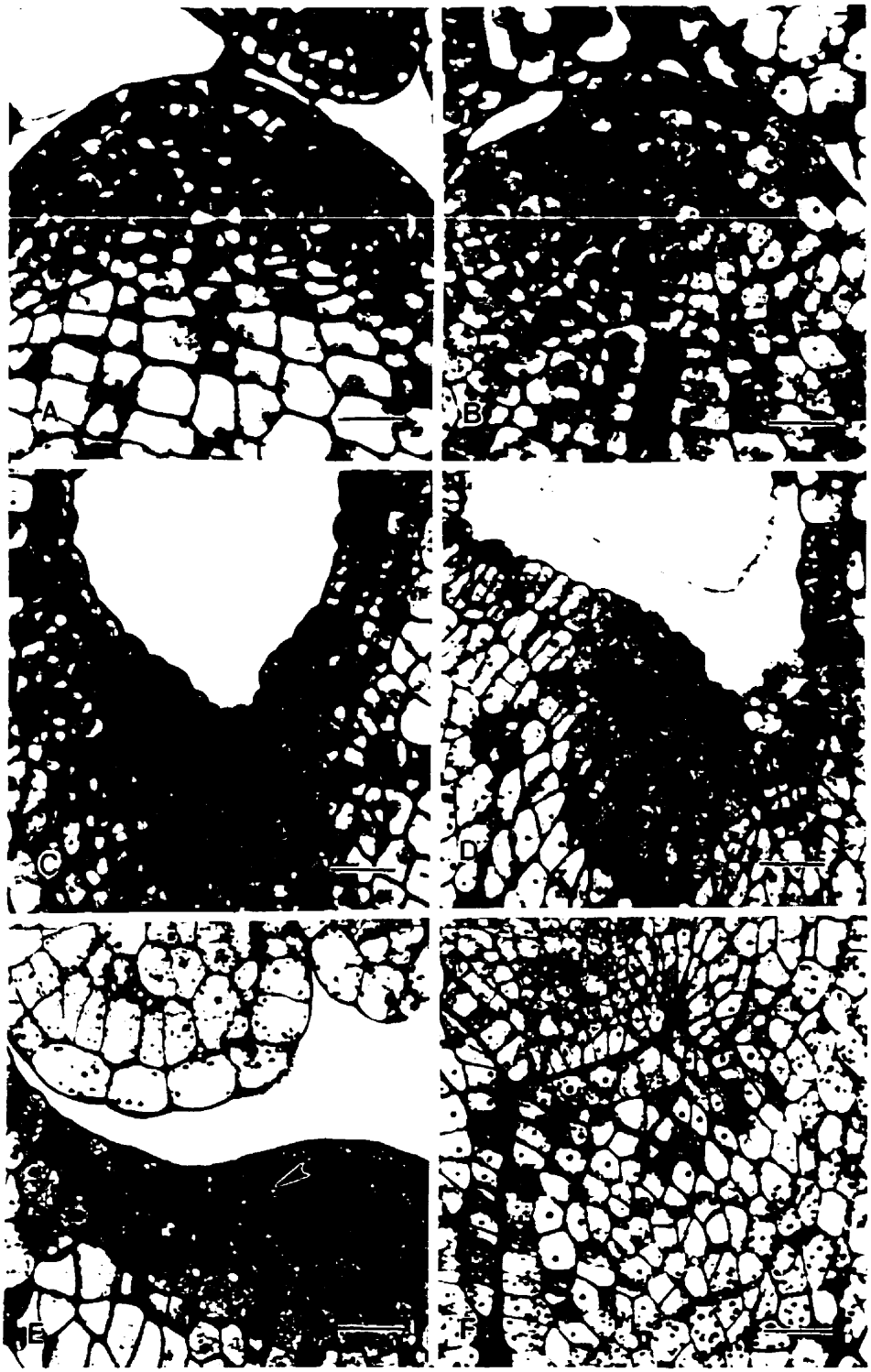

A
B
C
D
E
F

Figure 3.16:

Response of MD embryos to treatment with IAA at different times during the culture period. The bars represent the mean percent of embryos with fused cotyledons and the error bars represent the standard deviation around the plotted mean.

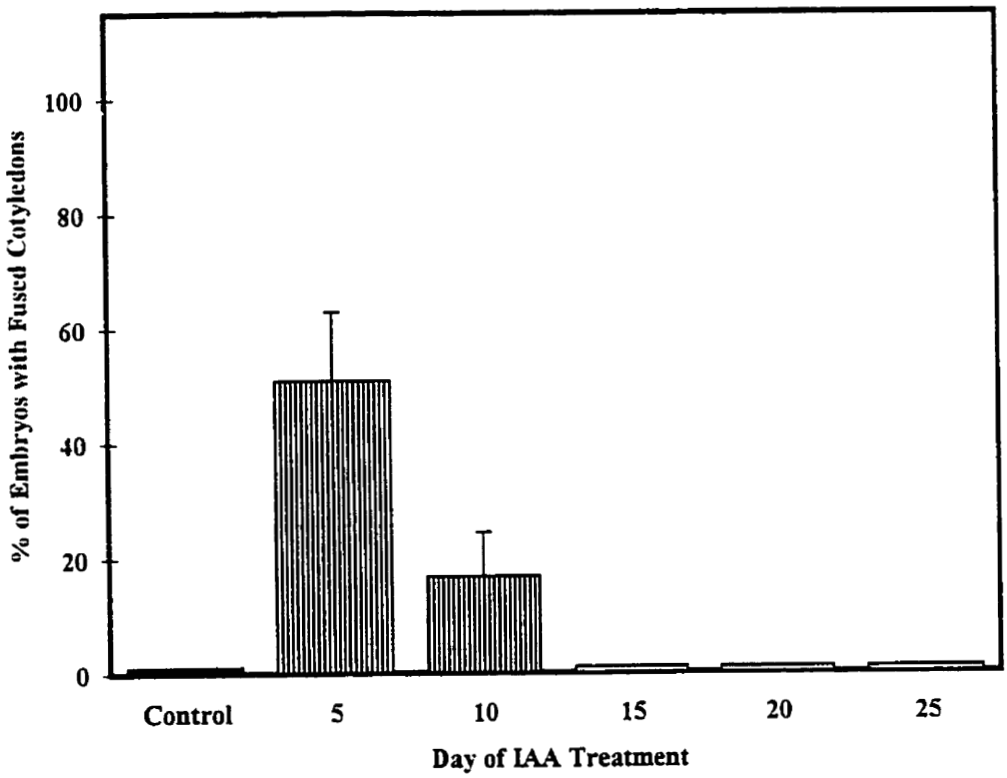

% of Embryos with Fused Cotyledons
100
80
60
40
20
0
Control
5
10
15
20
25
Day of IAA Treatment

Figure 3.17:

Morphology of MD embryos treated with IAA at different times. The embryos were sampled at Day 30. A: Control embryos have two separate cotyledons. Embryos treated at Day 5 (B) or Day 10 (C) have one fused cotyledon. Embryos treated at Day 15 (D) are similar to the control with two separate cotyledons. All scale bars = 1.5mm.

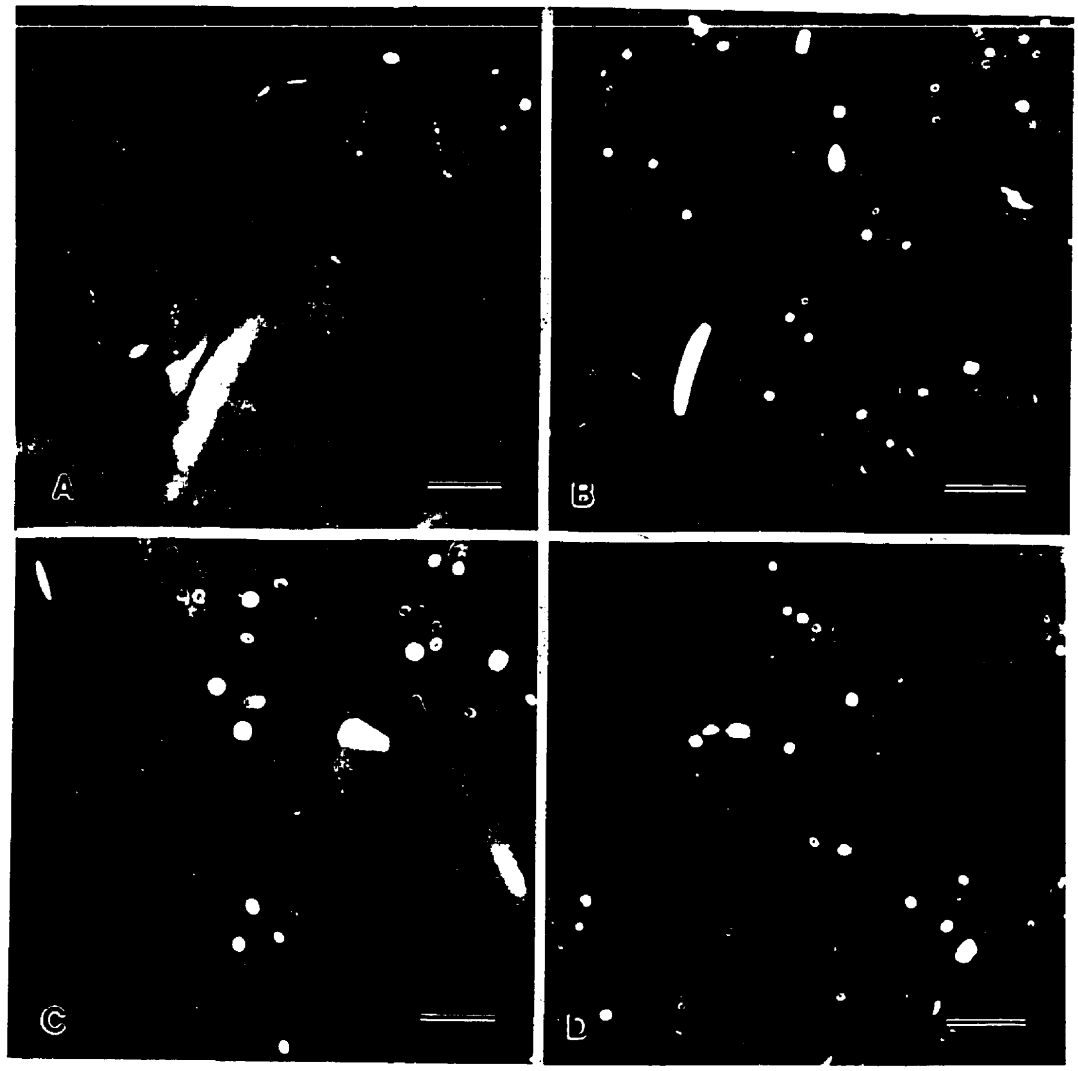
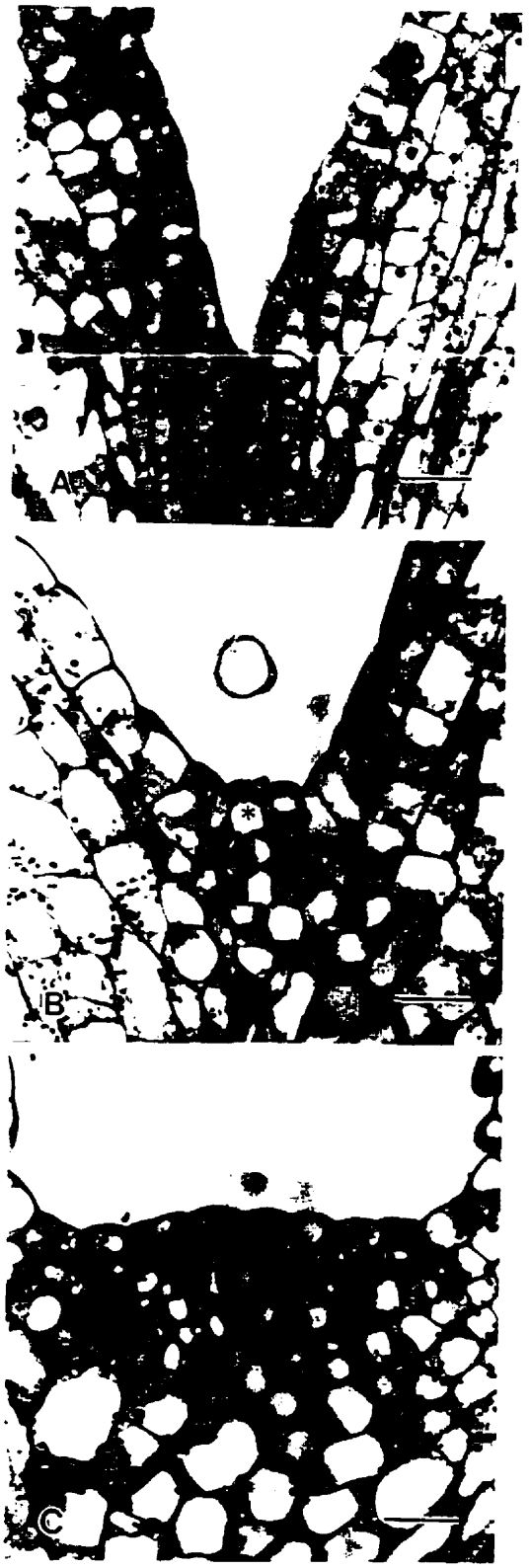

A
B
C
D

Figure 3.18:

Histology of SAMs of MD embryos treated with IAA. A: This embryo was treated with IAA on Day 5 and sampled on Day 15. The cells of the SAM appear very elongated. B: This embryo was treated with IAA at Day 5 and sampled at Day 30. The cells of the SAM continue to be elongated but also show increased vacuolation (*) and starch is beginning to accumulate in these cells. C: This embryo was treated with IAA at Day 15 and the SAM appears comparable to that of a control embryo (cf. Fig 3.7C). All scale bars = 20 μm.


A
B
C

Discussion

Embryogenesis of the MD embryos was affected by an alteration in auxin levels. Furthermore, the results also indicate that SAM formation and subsequent function are affected in MD embryos.

Auxin levels affect embryo morphology

Upon TIBA treatment, the morphology of the embryos was altered. When the embryos were treated at the pre-globular or globular stage, the embryos were not able to exhibit bilateral symmetry. Instead of the normal two cotyledons that would be formed, one, fused, trumpet-shape cotyledon emerged. These results were also reported in several other systems. Liu et al. (1993b) and later Hadfi et al. (1998) also observed that *in vitro* zygotic embryos of *Brassica juncea* treated with TIBA at the globular stage continued to maintain their radial symmetry throughout their development. Choi et al. (1997) also demonstrated that somatic embryos of ginseng developing in the presence of TIBA had 'jar-shaped' cotyledons. The loss of the ability to make the transition from radial to bilateral symmetry was also reported in the *Daucus carota* somatic embryo system (Schiavone and Cooke, 1987). However, these embryos were not able to develop cotyledons but remained at the globular stage and merely enlarged in size. Furthermore, an alteration in symmetry is not limited to dicots as Fisher and Neuhaus (1996) also reported that TIBA also affected the overall symmetry of the monocot wheat. It is also interesting to note that the ability of TIBA to induce various anomalous growth patterns was recognized from very early on. Wardlaw (1952) observed that when TIBA was placed on the growing apices of tomato plants, a 'cup-like rim' developed and became elevated on the rapidly dividing stem. The author also reports other observations that TIBA can cause the 'connation of leaves and formation of tubular structures'.

The importance of auxin for the establishment of bilateral symmetry is also supported by work with mutants. The *pin-formed* mutant of *Arabidopsis* has been shown to have extremely reduced polar transport of auxin activity (Okada et al., 1991). A survey

of the embryos of heterozygous *pin* mutants revealed that approximately 20% of them had abnormal cotyledon morphologies (Liu et al., 1993b). The embryos of the *cup-shaped* mutant of *Arabidopsis* also have fused cotyledons (Aida et al., 1997) and may be an indication that the auxin transport system has been altered as a consequence of the mutation.

The symmetry of the embryos was not affected if they were treated with TIBA after the globular stage. It has been suggested that it is at the globular stage that the basipetal polar transport of auxin is established and is necessary for the transition in symmetry to occur (Schiavone and Cooke, 1987; Liu et al., 1993b). Liu et al. (1993b) suggested two models that could account for the formation of fused cotyledon in the presence of an auxin transport inhibitor. In the first model, auxin is synthesized in the shoot primordium and transported to the cotyledon forming regions where it would stimulate the growth of the cotyledons. In the other model, auxin is synthesized in a ring of cells outside of the shoot primordium and then transported to the cotyledon forming regions. In either model, the presence of an auxin transport inhibitor would lead to a local increase in auxin and the formation of a ring of cotyledonary tissue as opposed to two separate cotyledons. From his observations, Wardlaw (1952) also suggested that in the mature plant, TIBA prevented the 'embryonic cells' from dividing but allowed the cells on the periphery to continue dividing. The results presented here provide further evidence that the stages leading up to and including the globular stage of embryogenesis are the most critical for the embryo to establish its proper symmetry. A disruption in the polar transport of auxin would lead to uniform build up of auxin in the apical half of the embryo and result in a ring of cotyledonary tissue being formed. This would lend more support for the second model proposed by Liu et al. (1993b). Interestingly, as noted by Hadfi et al. (1998), although the polar transport of auxin was inhibited, the embryos were still able to undergo hypocotyl elongation. The authors suggest that hypocotyl elongation may be a separate process from the activities at the apex.

In addition, it is noteworthy that if the gradient of auxin along the embryos was altered then the symmetry was also altered. Exogenous application of IAA also resulted in the formation of fused cotyledons. However, this reaction was not as dramatic as that

with TIBA. This may be due to the possibility that the polar transport of auxin was not totally inhibited but the exogenous IAA disrupted the overall gradient of auxin in the embryo. Consequently, radial symmetry could not be maintained. Hadfi et al. (1998) also observed that zygotic embryos of *B. juncea* did not exhibit bilateral symmetry under very high concentrations (10-40 μM) of IAA. Indeed at these concentrations, most of the embryos were not able to elongate and simply remained in a 'ball/egg' shape. Schiavone and Cooke (1987) also observed similar results when carrot somatic embryos developed in an exogenous auxin environment. Therefore, the polar transport of auxin is necessary to establish a gradient of auxin along the axis of the embryo and for bilateral symmetry to occur.

The ability of TIBA as well as exogenous IAA to disrupt proper embryogenesis and lead to the formation of the trumpet-shaped embryos provides an indication that the internal auxin levels have been disturbed. Therefore, as Liu et al., (1993b) discuss, although there is little information about the site of synthesis of auxin, it can be speculated that auxin is synthesized in the apical half of the embryo and then transported towards the future radicle. The TIBA treatment leads to an increase in auxin in the apical half of the embryo.

Auxin levels influence SAM formation

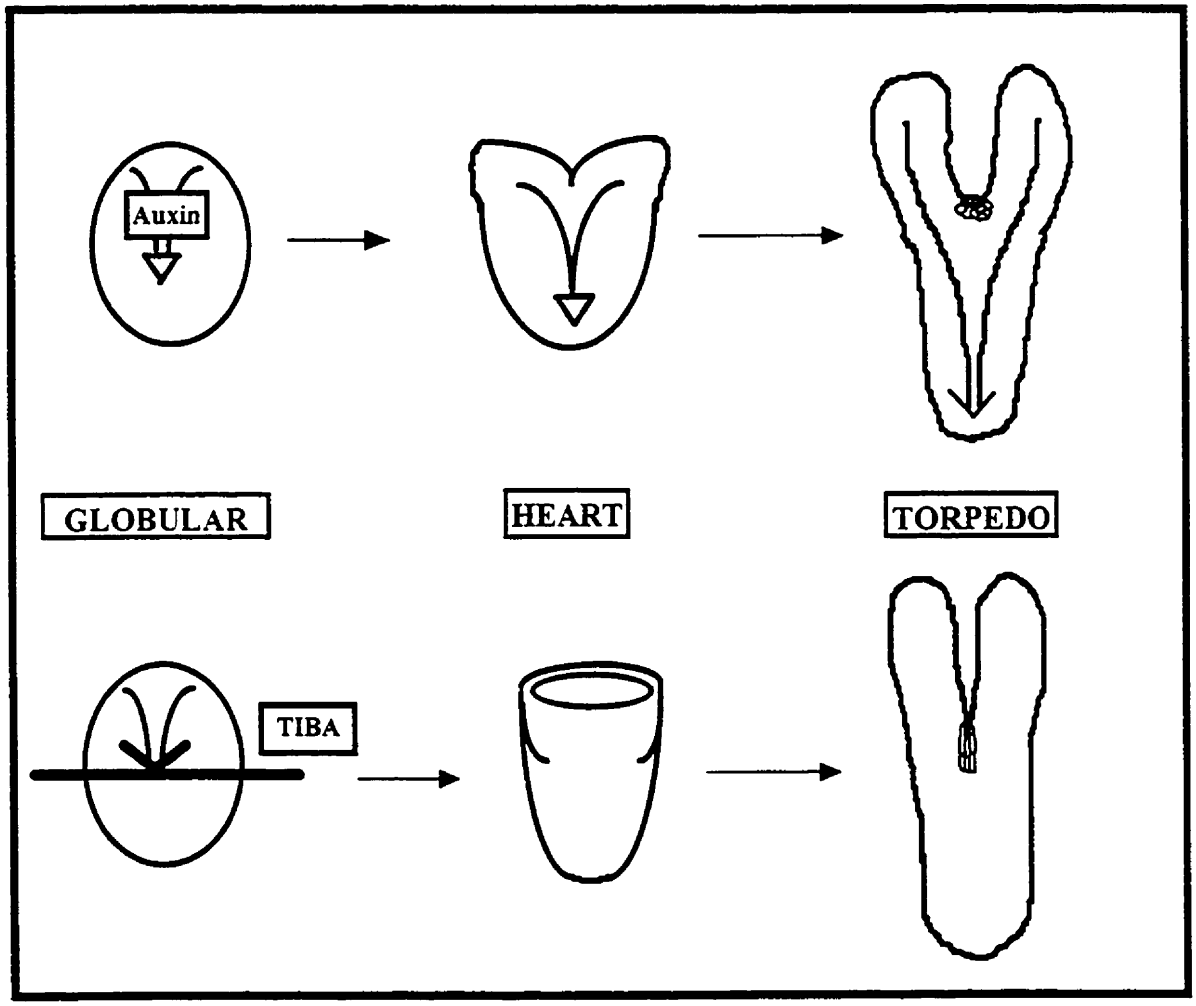
In many of the previous studies that have examined the effects of altered auxin levels on embryogenesis, there has been little attention paid to the effects on the shoot apical meristem. Liu et al. (1993) noted that the TIBA treated embryos with fused cotyledons exhibited a delay in shoot production when compared to the control. This is an indication that the SAM had been affected in some respect. Therefore, this study was undertaken to determine if altered auxin levels had any effect on SAM formation or function. From the results, it is evident that if the embryos were treated with TIBA prior to and including the globular stage, the SAM was altered. However, if the embryos were treated after the globular stage, the SAM was not affected. These observations again point to the importance of the processes and activities that are occurring at the globular

stage of embryogenesis. If the polar transport of auxin is initiated at this stage in embryogenesis, then treatment with TIBA would lead to an accumulation of auxin in the apical half of the embryo (Liu et al., 1993b). As a consequence, this would lead to an increase in auxin in the apical notch. From a histological examination, this increase in auxin prevents the SAM from developing fully. In normal development, from the heart stage onwards until approximately day 20, the SAM usually increases in size by mitotic division of cells in the region (Yeung et al., 1996). However, in the TIBA treated embryos, the cells SAM appeared to be limited in the amount of divisions that could occur. With prolonged treatment in TIBA, the SAM was not able to increase in size and most of the cells of the SAM gradually became differentiated into starch filled parenchyma cells. The general profile of these cells resembled that of the procambial cells. For example, they appeared to be axially elongated, have a high accumulation of starch and extremely vacuolated.

The cells of the developing SAM were sensitive to TIBA treatment for a long period of time. Until the embryos were at the heart stage of embryogenesis or later the fate of the cells that were to become the SAM was not fixed. Once this critical stage had passed the cells were able to maintain their meristematic identity and were not altered by the change in the auxin levels. These results suggest that, during embryogenesis, a lower concentration of auxin in the apical notch is necessary for normal development of the SAM to occur. As the polar transport of auxin appears to be initiated at the globular stage, the auxin would flow from the initiating cotyledons in a basipetal direction through the central cylinder and to the future radical (Fig 3.19). Essentially, this correlates with the timing of the differentiation of the procambial strand. Sachs (1991) referred to this process as 'canalization' where a specialized path for auxin flow leads directly to the formation of axially elongated cell files that transport the auxin. As the auxin follows this path, it could create a pocket of lower auxin concentration between the developing cotyledons. This lower auxin concentration may allow for the cells in this area to respond differently from the surrounding cells and adopt a different fate, i.e. a meristematic fate. However, with the TIBA treatment and a higher concentration of auxin in this region, this may

Figure 3.19:

A model of how the flow of auxin and TIBA may be influencing the morphology of the embryo and development of the SAM. In normal development (the upper sequence), the polar transport of auxin is initiated at the globular stage of embryogenesis. This coincides with histodifferentiation as well as the transition to the heart stage. The basipetal flow of auxin from the developing cotyledons may create a pocket in the apical notch where the auxin concentration will be lower than the surrounding area. This would allow for the normal development of the SAM to occur and for bilateral symmetry to be maintained throughout the rest of development. The lower sequence depicts development under the influence of TIBA. TIBA blocks the basipetal flow of auxin at the globular stage. This results in an increase in the auxin levels in the apical half of the embryo and the formation of one fused cotyledon. The increase of auxin in the apical notch disturbs the normal development of the SAM and leads to a narrow, elongate shaped SAM. The cells of the SAM also become elongated, vacuolated and starch-filled.


Auxin
GLOBULAR
HEART
TORPEDO
TIBA

account for the SAM cells adopting an appearance resembling the cells of the procambium.

Auxin levels influence SAM function

Another indication of the importance of the events occurring at the globular stage of embryogenesis is the observation that the embryos treated with TIBA during this stage were unable to produce shoots. The TIBA-treated embryos had extremely low conversion frequencies when compared to the control embryos. However, the conversion frequencies of the embryos treated after this critical stage were comparable to their respective controls. Liu et al. (1993b) observed that there was a delay in shoot emergence from the embryos with fused cotyledons. However, they did not report the percentage of embryos that were able to produce shoots in comparison to the control. The authors also reported that the shoots emerged from the base of the fused cotyledon and essentially split the trumpet open. However, this was never observed in the present investigation. From the histological observations, the SAMs of the TIBA treated embryos were not able to maintain a normal organization. The cells were not able to maintain their normal cell division patterns. The main part of the corpus appeared very disorganized, and this is the region that is most associated with organogenesis. Therefore, this would account for the low conversion frequency that was observed. During normal development, a lower concentration of auxin in the apical notch is not only necessary for proper formation of the SAM but also for proper functioning of the SAM upon the initiation of germination.

CHAPTER FOUR

The effect of altered auxin levels on SAM formation and function in *in vitro* zygotic embryos

Introduction

Embryogenesis, in plants, is marked by a set of characteristic events that establishes the basic pattern of the future plant. One of the most fundamental events to occur is the formation of the two apical meristems. The proper formation of these generating centres is critical for subsequent normal development to occur. The shoot apical meristem (SAM) consists of a uniquely characterized group of cells that will eventually generate all the aerial parts of the plant. There have been numerous studies that have detailed the structural organization and function of the mature apical meristem in a variety of species ranging from the lower vascular plants through to the angiosperms. These studies have led to further understanding of how the regions of the meristem function post-embryonically to produce lateral organs and how these different regions within the meristem may be integrated to form a functioning whole. Despite this wealth of information, relatively little is known about how this unique group of cells is formed, maintains its unique identity, and what are the underlying physiological and molecular processes controlling the precise pattern of formation exhibited during embryogenesis.

In the previous chapter, it was demonstrated that TIBA affected the development and function of the SAM of MD embryos. However, as discussed by Yeung (1995) there is evidence to suggest that somatic embryos often differ from their zygotic counterparts. Yeung et al. (1996) as well as Hays (1996) have previously demonstrated that *Brassica* MD embryos do, in fact differ from zygotic embryos in their developmental pathways and physiology. In zygotic embryos, particularly of the Brassicaceae, the pattern of cell division is very precise and extremely predictable (Tykarska, 1976; Yeung et al., 1996). During the early stages of embryogenesis, the pattern leading to the formation of the epiphyseal cells is well documented. In the MD embryos, Yeung et al. (1996) noted that

the early division patterns were very varied and it was not until the embryo assumed an oblong shape that the cells of the future SAM were discernible. Furthermore, there was no distinct pattern of division or accompanying epiphysis that gave rise to this group of cells. Swamy and Krishnamurthy (1977) also emphasize that these epiphyseal cells are different from the surrounding cells in their staining pattern which may indicate a difference in physiology. Therefore, one of the key features that seem to separate the developmental pattern of zygotic and somatic embryos is the regularity of the early cell division patterns.

While the MD embryo system can offer many advantages, there are also some drawbacks to the protocol. Although there can be hundreds of embryos developing within one petri plate, there can also be sub-populations of embryos at different stages of development. Therefore, when the treatments are added to the petri plates, embryos at different stages of meristem formation are being treated and being categorized together. A system with all the embryos synchronously progressing through the different stages of development would be ideal.

As mentioned previously, the processes occurring during zygotic embryogenesis are often difficult to study because of the inaccessibility of the embryo deep within the maternal tissue. However, Liu et al. (1993a) recently developed a moderately successful protocol for the *in vitro* culture of young zygotic embryos. This system provides an excellent opportunity to investigate some of the physiological controls of embryogenesis in a system that is as close as possible to being *in vivo*. It also provides the additional advantage of being able to pick specific stages of embryogenesis that are of interest. Therefore, this chapter focuses on whether the polar transport of auxin is also associated with the proper formation of the shoot apical meristem during embryogenesis and its subsequent function in a zygotic system. The effect of an auxin polar transport inhibitor, TIBA, on SAM formation during zygotic embryogenesis will be analyzed.

Results

The effect of TIBA on the morphology of in vitro zygotic embryos of B. napus

Embryos were at the globular stage of embryogenesis at 7 days post-anthesis (Fig 4.1A). These embryos were colour-less at the initiation of culture. Embryos were observed to exhibit a higher survival rate if the ovules were buried in the medium. By 24 hours after the plates with the globular-stage embryos were placed in the light, they began to turn green. By 4 days after culture (DAC), globular stage embryos were at the torpedo stage of development with two cotyledons clearly visible in the untreated embryos (Fig 4.2 A). Of the more than 500 globular stage embryos treated with TIBA, 66% did not develop two separate cotyledons (Fig 4.3). Instead, a ring of cotyledonary tissue developed by 4 DAC, indicating a continuation of radial symmetry (Fig 4.2B). The control embryos were not ever observed to have a fused cotyledon. Both control and treated embryos maintained their respective symmetries and continued to increase in size until 14 DAC, the end of the maturation period (Fig 4.2 C and D).

Embryos that were cultured at the heart stage were green at the onset of culturing (Fig 4.1). By 4 DAC both control and treated embryos were at the cotyledonary stage (Fig 4.4 A and B). At the end of the maturation period, 14 DAC, both control and treated embryos had developed to the late cotyledonary stage and were considered mature (Fig 4.4 C and D). The embryos were never observed to have one fused cotyledon at any time point during the maturation process (Fig 4.3).

Figure 4.1

Morphology of zygotic embryos at the initiation of culture (D0). A: A globular stage embryo approximately 7 days post anthesis. B: A heart stage embryo approximately 9 days post anthesis. All scale bars = 0.5 mm.

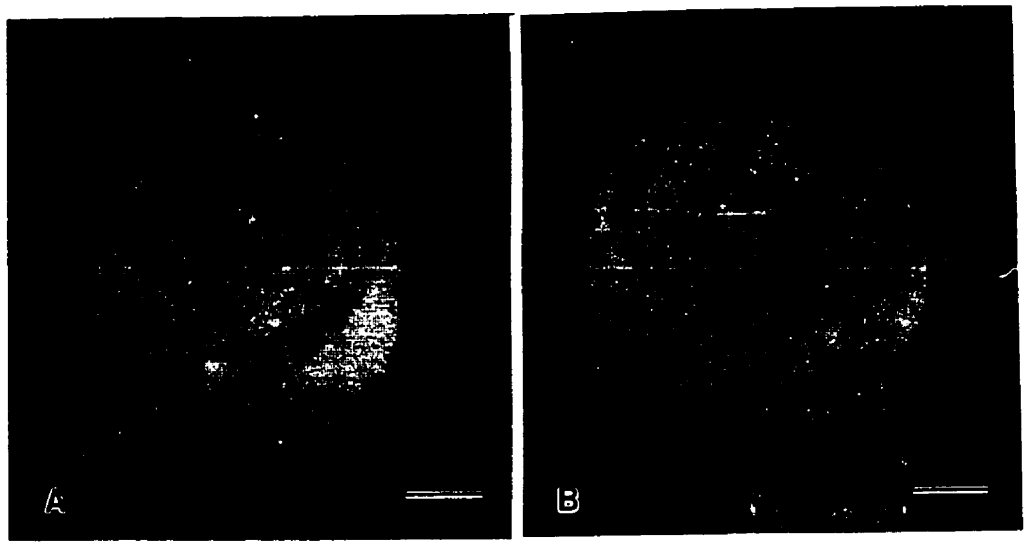

A
B

Figure 4.2:

Development of globular stage zygotic embryos at different stages throughout the maturation process. A and B: embryos are 4 days old. A: A control embryo, still in the ovule, with two distinctly separate cotyledons; B: An embryo that was grown in the presence of TIBA. It has one fused cotyledon (➔). C and D: embryos are 14 days old, at the end of the maturation period. C: A control embryo with two separate cotyledons; D: An embryo that was grown in the presence of TIBA. It still maintains its one fused cotyledon (➔). All scale bars = 0.5mm.

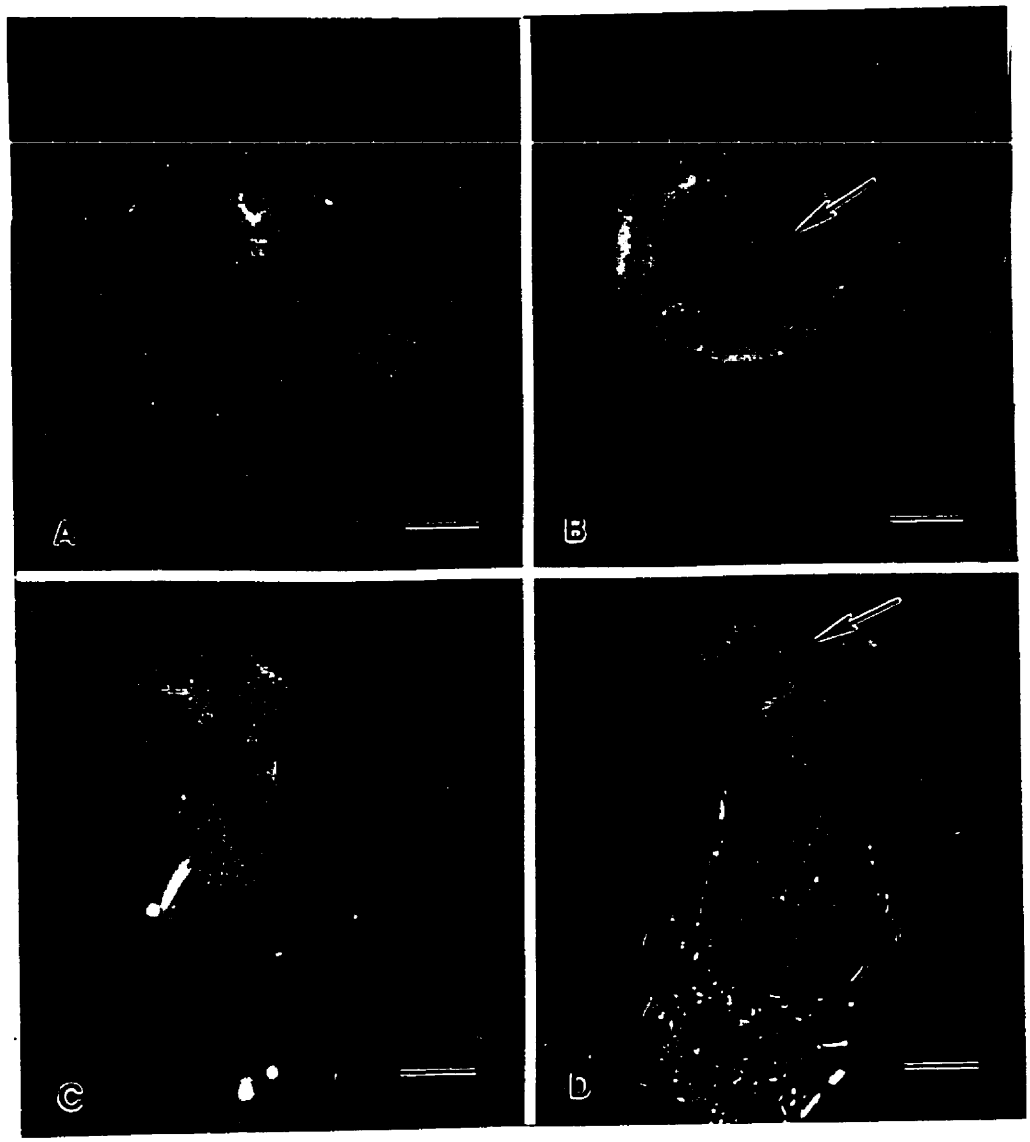

A
B
C
D

Figure 4.3:

Response of zygotic embryos to TIBA treatment. Embryos were grown in TIBA-containing medium either from the globular or heart stage. The percent of embryos with one fused cotyledon was assessed and compared to the untreated embryos. The bars represent the mean, and the error bars are the standard deviation around the mean.

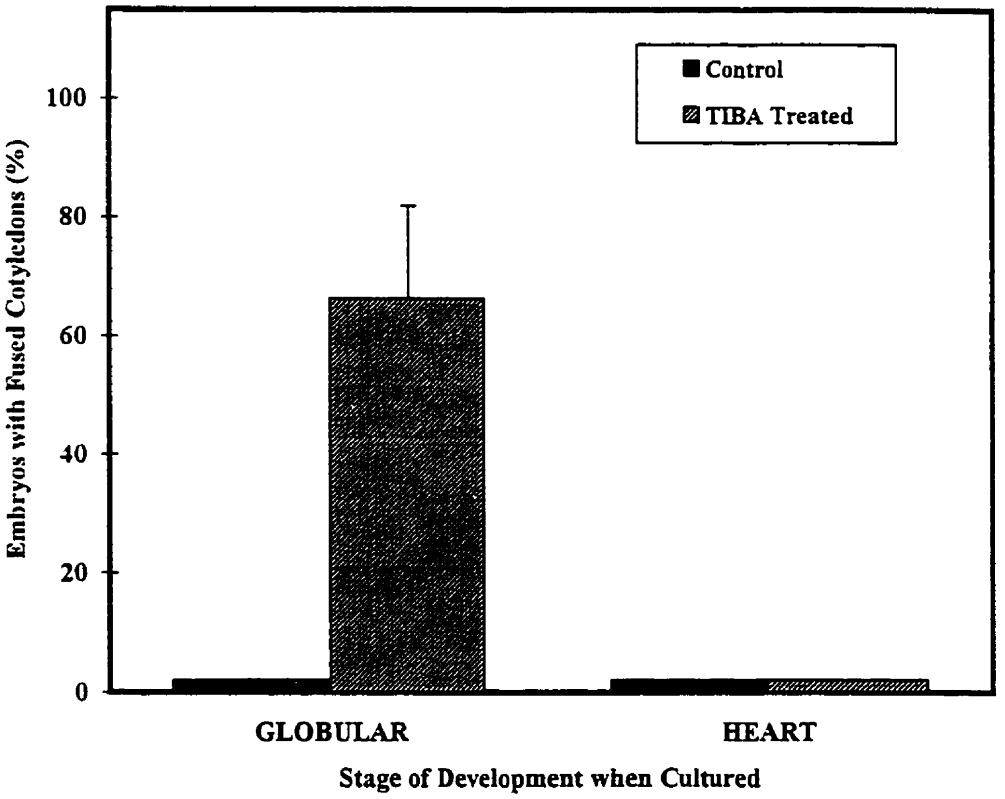
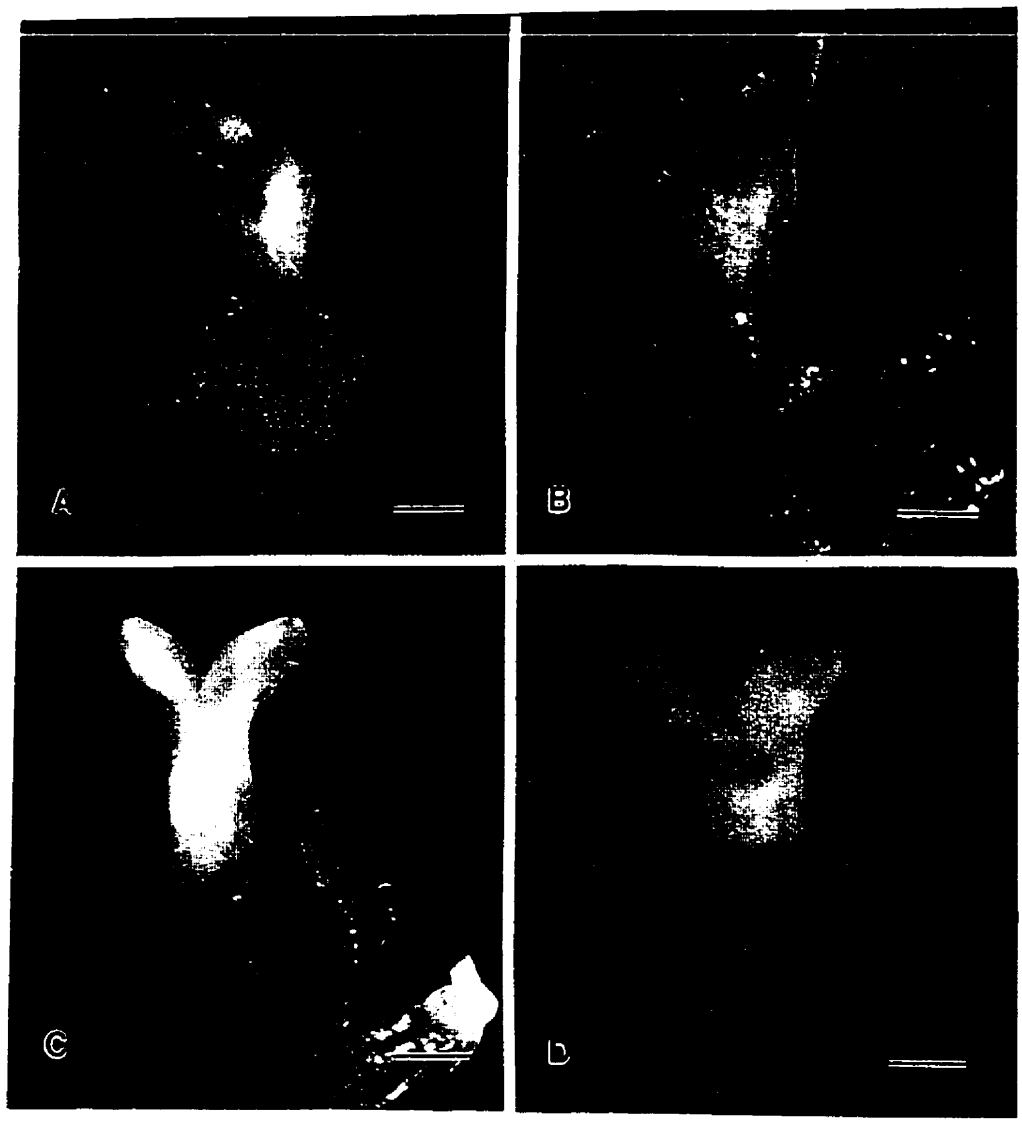

Control
TIBA Treated
Embryos with Fused Cotyledons (%)
100
80
60
40
20
0
GLOBULAR
HEART
Stage of Development when Cultured

Figure 4.4:

Development of heart stage zygotic embryos at different stages throughout the maturation process. A and B: embryos are 4 days old. The control embryo (A) and the embryo grown in TIBA (B) both have two separate cotyledons. C and D: embryos are 14 days old. Again, the control embryo (C) and the embryo grown in TIBA (D) maintain two separate cotyledons. All scale bars = 0.5 mm.


A
B
C
D

The effect of TIBA on the development of the SAM of in vitro zygotic embryos

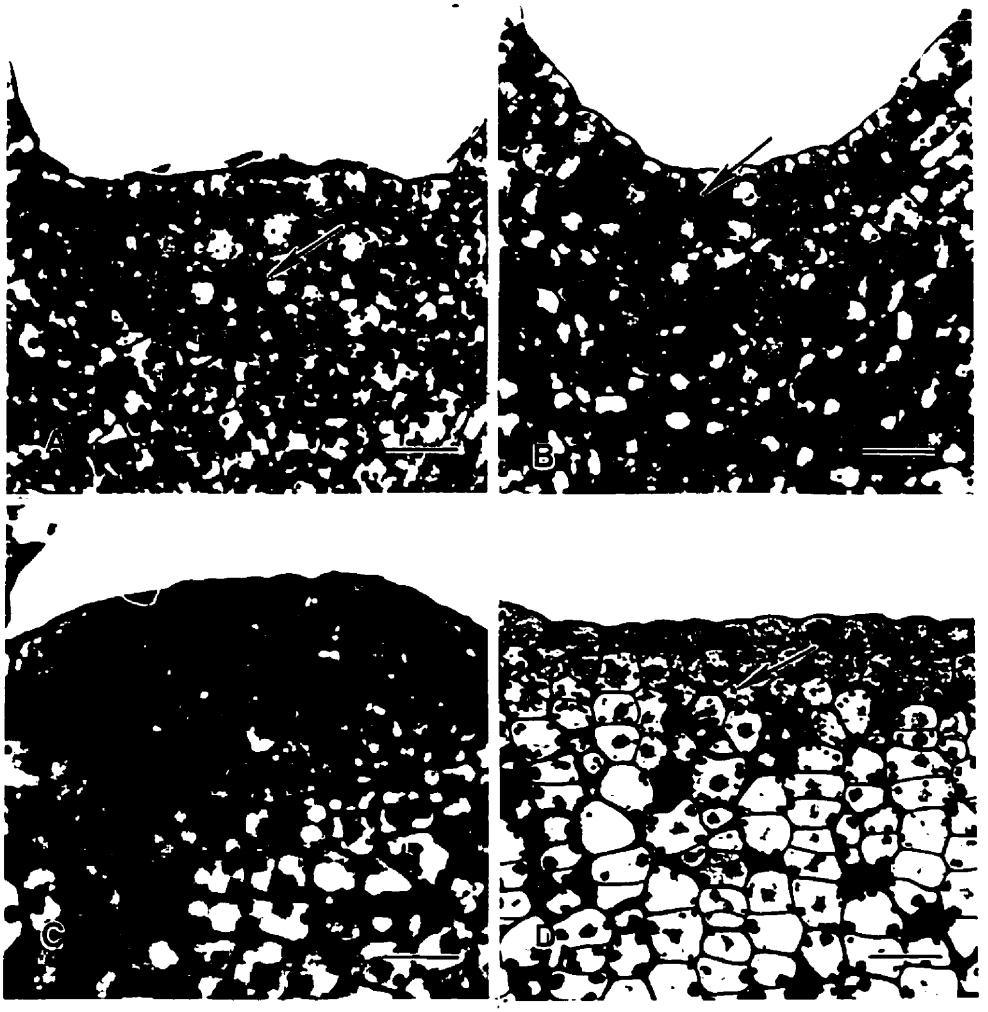
Cultured at globular stage

The SAM consisted of a tightly packed group of cells in the apical notch by 4 DAC (Fig 4.5A). The cells were fairly cytoplasmic with very few vacuoles. These cells were isodiametric in shape with very large nuclei. However, interestingly, at this stage in development there was a high amount of starch accumulation in the cells of the SAM (Fig 4.5A). The SAM of the TIBA treated embryos had very similar characteristics to the control embryos (Fig 4.5B).

By 8 DAC, differences in the SAM of the control and treated embryos were becoming evident. The SAM of the control embryos continued to increase in size, i.e. width and depth and assumed a more dome-shaped appearance. There was a decrease in the accumulation of starch in the region. The cells continued to maintain their isodiametric shape and remain densely cytoplasmic with large nuclei. These characteristics of the SAM were maintained until the end of the maturation period at 14 DAC (Fig 4.5C). It was also at about 8 days in culture, when the SAM of the TIBA treated embryos exhibited characteristics maintained until the end of the culture period (Fig 4.5D). The SAM of the TIBA treated embryos appeared to be flattened with an increase in width due to an increase in the number of cells. However, it appeared to be limited to approximately only 2 cell layers in depth. These cells were small and isodiametric but continued to show a high degree of starch accumulation and vacuolation when compared to the control. The cells of the other layers also became enlarged, starch filled and increasingly vacuolated. This trend was maintained to the end of the maturation period at 14 DAC.

Figure 4.5:

Development of the SAM in zygotic embryos cultured at the globular stage. A and B: These embryos were maintained in culture for 4 days. A: A SAM of a control embryo. The cells show a high amount of starch accumulation (➔). B: A SAM of an embryo grown in TIBA. The characteristics of the cells are similar to the control embryo. These cells also have a high degree of starch accumulation (➔). C and D: These embryos were maintained in culture for 14 days. C: The SAM of a control embryo. The cells show less starch accumulation and continue to be densely cytoplasmic. Note that the SAM can be considered to be 5-6 layers of cells deep. D: A SAM of an embryo grown in TIBA containing medium. The SAM appears to consist of one to two cell layers. These cells continue to exhibit a high degree of starch accumulation (➔). All scale bars = 20 μm.


A
B
C
D

Cultured at heart stage

After 4 days in culture, the SAMs of both control (Fig 4.6A) and TIBA-treated (Fig 4.6B) embryos consisted of a group of compactly arranged isodiametric cells. All the cells were densely cytoplasmic, and had very few vacuoles or starch. At 14 DAC, the end of the maturation period, the SAMs of both control and treated embryos had increased in size and appeared as a dome-shaped mound (Fig 4.6 C and D). The characteristics of the cells of the SAM in both embryos were extremely similar.

The effect of TIBA on the function of SAM

Embryos cultured at the globular stage

To test the ability of the SAM to function, i.e. to produce shoots, control and TIBA treated embryos were placed on a conversion medium at the end of the maturation period. By 4 days after being placed on the conversion medium leaves were visible on 63% of the control embryos that were cultured at the globular-stage (Fig 4.7). The SAM of these control embryos appeared as a well-organized convex dome with very cytoplasmic cells (Fig 4.8A). Leaf formation was not evident on the TIBA-treated globular embryos at this time (Fig 4.7). The SAM, however, continued to increase in width and the cells maintained their starch-filled appearance (Fig 4.8B).

The conversion frequency of the control embryos increased to 93% after 8 days of being on the germination medium while a very low percentage (less than 15%) of the TIBA treated embryos had a small protrusion within the trumpet shaped cotyledon (Fig 4.7). This protrusion, however, never expanded or took on the form of a normal leaf.

By day 12, the conversion frequency of the control embryos was 100% (Fig 4.7). The plantlets that were formed had produced 2 to 3 leaves that were clearly visible (Fig 4.9A). The SAM continued to maintain its overall shape and characteristics (Fig 4.8C). The cells were very densely cytoplasmic with very little starch accumulation and prominent nuclei. The conversion frequency of the TIBA-treated embryos never increased significantly (Fig 4.7). Most of the embryos simply expanded in size and there was no evidence of leaf formation in the enlarged fused cotyledon (Fig 4.9B). By this

time, the SAM had doubled in width, however, the depth of the SAM remained limited to 2 cell layers (Fig 4.8D).

Embryos cultured at the heart stage

The conversion frequencies of both the control and TIBA-treated embryos were 100% after being 4 days in the conversion medium. By 8 days in the conversion medium, both treated and untreated embryos had produced several leaves (Fig 4.9 C and D). The SAMs of both control and treated plantlets were similar. They appeared well organized with densely cytoplasmic cells with very little starch and few vacuoles (Fig 4.8E and F).

Figure 4.6:

Development of the SAM in zygotic embryos cultured at the heart stage. A and B: These embryos were 4 days old when sampled. A: A SAM of a control embryo. B: A SAM of an embryo grown in TIBA-containing medium. C and D: these embryos were 14 days old when sampled. C: A SAM of a control embryo. B: A SAM of a TIBA treated embryo. Note that the SAMs of the TIBA treated embryos appear very similar to those of the control embryos. All scale bars = 20 μm.

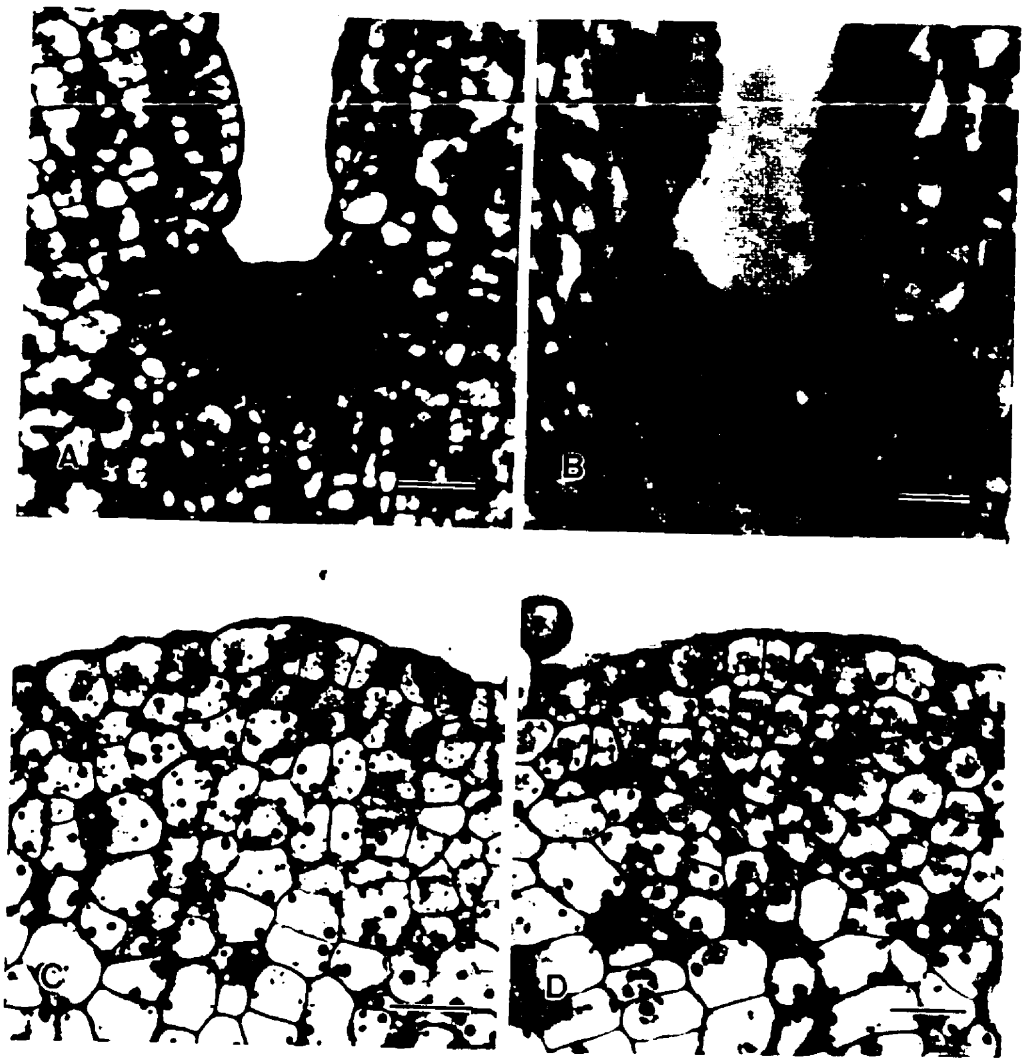

A
B
C
D

Figure 4.7:

Conversion frequencies of zygotic embryos cultured at the globular stage. At the end of the maturation process, control and TIBA treated embryos were transferred to a conversion medium. The conversion frequency was assessed at various times throughout the culture period. The bars represent the average conversion frequency and the error bars are the standard deviation around the plotted mean.

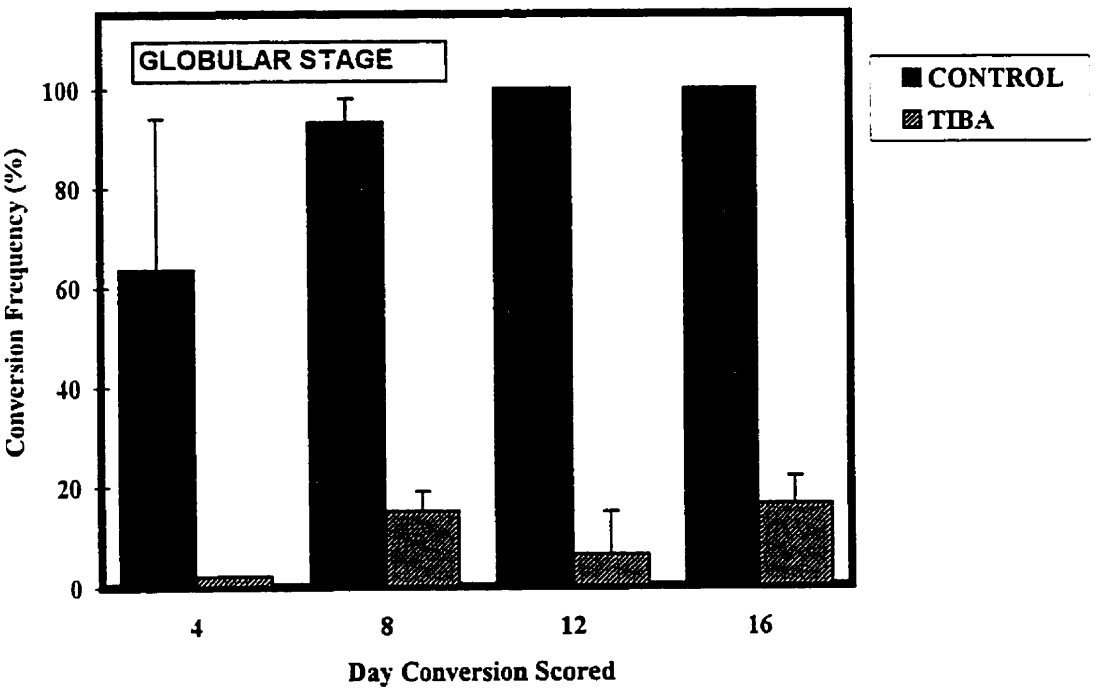

GLOBULAR STAGE
CONTROL
TIBA
Conversion Frequency (%)
100
80
60
40
20
0
4
8
12
16
Day Conversion Scored

Figure 4.8:

SAMs of zygotic embryos at various times during conversion. The embryos were allowed to mature and then transferred to a conversion medium. A and B: These were initially globular-stage embryos that were maintained on the conversion medium for 4 days. A: A SAM of a control embryo. B: A SAM of an embryo that was grown in TIBA-containing medium. C and D: These were globular-stage embryos that were maintained on the conversion medium for 12 days. C: A SAM of a control embryo. D: A SAM of an embryo that was grown in TIBA containing medium. E and F: These were initially heart stage embryos that were maintained in the conversion medium for 8 days. E: SAM of a control embryo. F: SAM of a TIBA-treated embryo. All scale bars = 20 μm.

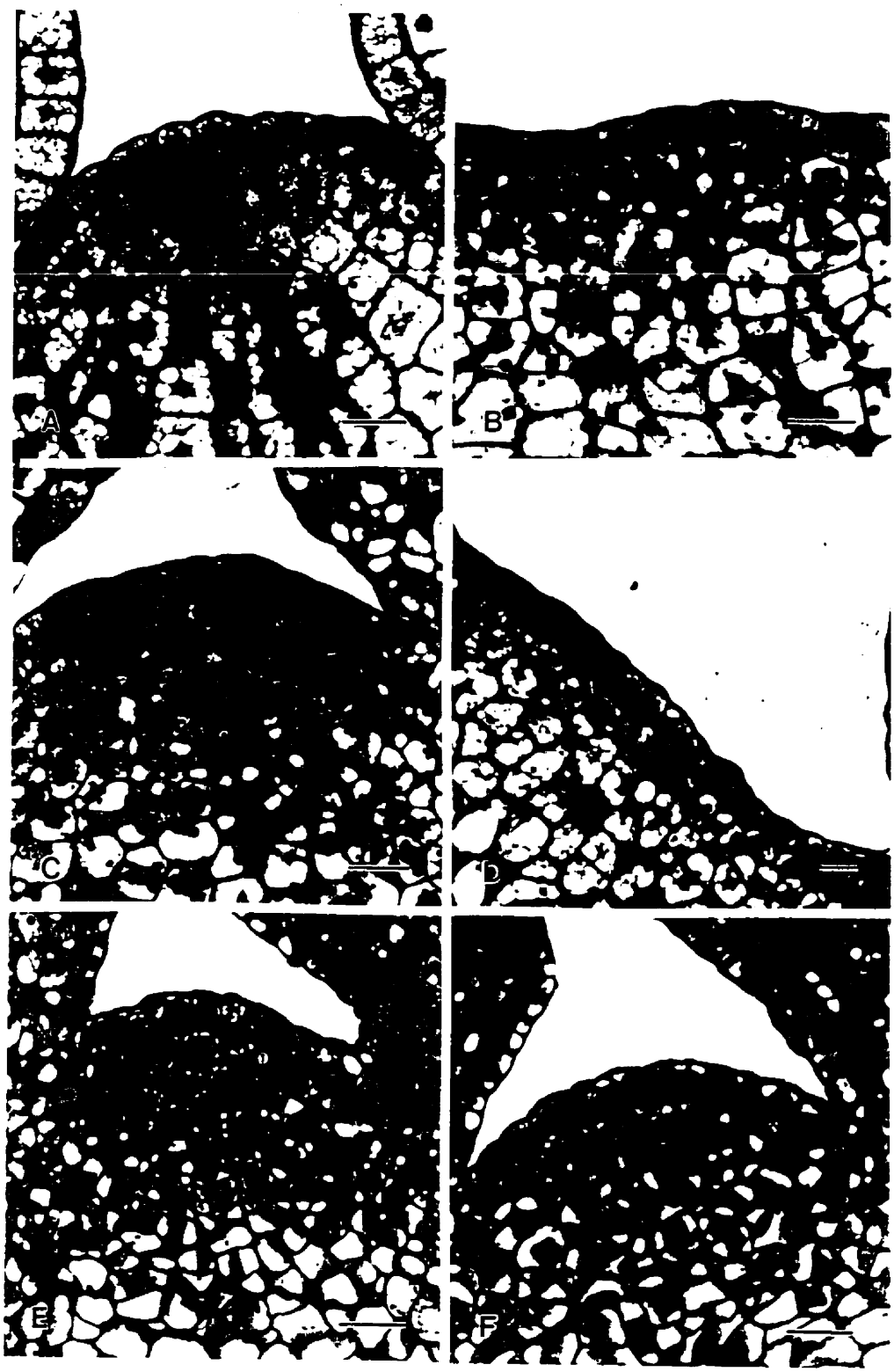
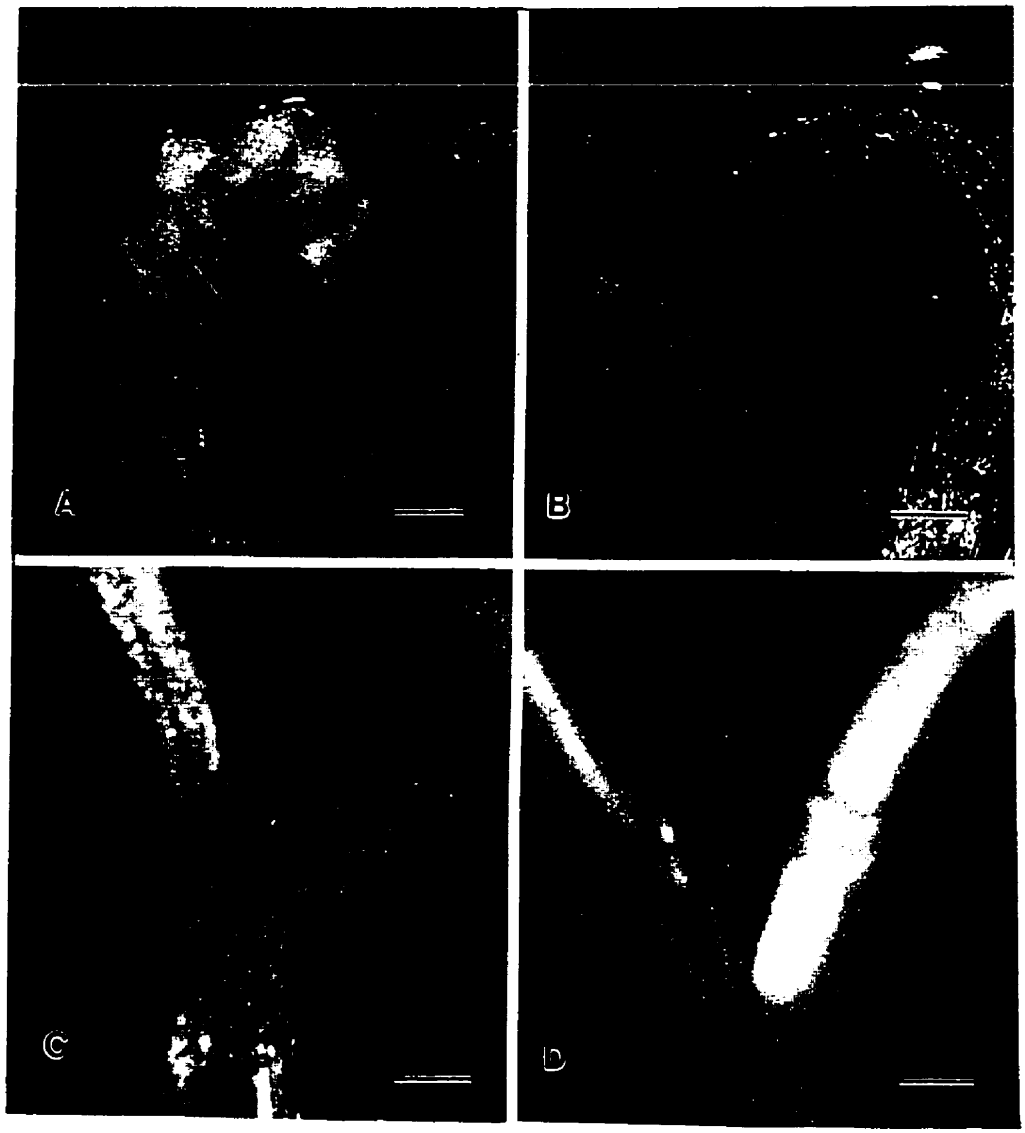

A
B
C
D
E
F

Figure 4.9:

Morphology of embryos 12 days after the initiation of conversion. A and B: Embryos were initially at the globular stage. A: A control embryo shows the formation of 2 leaves. B: A TIBA-treated embryo does not show any sign of leaf formation. C and D: Embryos were initially at the heart stage. Both the control embryo (C) and the TIBA-treated embryo (D) have formed leaves. All scale bars = 0.5 mm


A
B
C
D

Discussion

From our observations, it is evident that the formation of the SAM during embryogenesis is affected by the disruption of the polar transport of auxin. The earliest progenitors of the SAM are the cells of the epiphysis that are distinguishable at the globular stage (Swamy and Krishnamurthy, 1977). These cells are distinct from the surrounding cells usually by being more isodiametric, cytoplasmically dense and having large nuclei. Recently, Mayer et al. (1998) have shown that these cells uniquely express the *WUS* gene prior to when the SAM becomes morphologically distinguishable. The authors suggested that *WUS* may act to maintain the meristematic nature of the cells that it is expressed in. Long et al. (1996) previously showed that at the globular stage, when the embryo comprises of about 100 cells, the expression of the *STM* gene, implicated in playing a role in the maintenance of the SAM, is localized to the cells that will be incorporated into the SAM proper. The results presented here also indicate that at this early stage of SAM formation, these cells may be responding differently to an auxin gradient. Liu et al. (1993b) suggested that TIBA may act to prevent the polar transport of auxin to the basal half of the embryo. As discussed in Chapter 3 (Fig 3.19), this would the lead to an increase in auxin levels in the apical half of the embryo including the apical notch. This can account for the formation of the ring of cotyledonary tissue being produced as opposed to two separate cotyledons. Liu et al. (1993b) also noted that the SAM of TIBA treated embryos simply appeared more 'juvenile' than the control. However, the present study has shown that the SAM is altered when treated with TIBA.

The effect of auxin levels is stage specific

TIBA did not have any adverse effects on the overall morphology or the SAM of the embryos that were treated at the heart stage of development. These results suggest that the stages leading up to and including the globular stage are very crucial to the proper development of the plant. It appears that after this 'sensitive' stage has passed, the fate of the cells of the embryo seems more determined and is not affected by external influences.

Under the influence of TIBA, the build-up of auxin in the apical notch prevents the SAM from forming properly. The SAM of the treated embryos increased in width i.e. there was an increase in the number of cells in the tunica layers of the SAM. Hadfi et al. (1998) also noted that NPA, another auxin transport inhibitor caused alterations in the SAM of zygotic embryos of *B. juncea*. The authors observed that the SAM of the embryos were concave and also that they appeared to be broadened. However, the authors did not indicate if all the layers of the SAM increased in width or describe the cytology of the cells of the SAM. In the present study, the number of cells in the corpus of the SAM seemed to be drastically reduced so that the SAM appeared very flat and broad. This suggests that the increasing auxin levels in this region may have prevented the cells of the corpus from dividing and maintaining a reservoir of cells. Instead, these cells lost their meristematic identity and accumulated starch. However, the cells of the tunica were able to continue dividing perhaps in an attempt to replace the cells of the corpus. The cells of the tunica are usually restricted to dividing anticlinally and therefore, this would result in the increased width of the SAM in the TIBA treated embryos.

There is a great deal of evidence from somatic as well as zygotic systems that suggest that the polar transport of auxin is initiated during the globular stage of embryogenesis. It has been proposed that auxin synthesis starts in the apical half of the embryo and more specifically in the developing cotyledons. The auxin is then transported towards the centre of the embryo and towards the future radicle. In a normal embryo the basipetal transport of auxin could lead to a 'pocket' in the apical notch where the concentration of auxin is lower than in the surrounding cells or where the cells can respond differently to auxin. This would be consistent with the fact that the procambial strand that develops at this time is strictly limited to a predetermined position. However, upon TIBA treatment, a build up of auxin in the apical notch would affect the cells in this region and lead to a disruption in the normal SAM formation pattern. The cells of the SAM would not be usually subjected to such high levels of auxin. It is also important to note that TIBA did not have such a dramatic effect on SAM formation when the embryos were treated at the heart stage of development. This suggests that, by the heart stage of embryogenesis, the cells that will form the SAM are developmentally determined and

their physiology is not easily perturbed. Therefore, there may be a small window of time (during the globular stage) when these cells require the proper auxin level to initiate the program that will allow them to develop into meristematic cells.

Auxin levels affect the ability of the SAM in zygotic embryos to function

The ability of the SAM to function and produce shoots was also affected by TIBA treatment during the globular stage. The SAM of the treated embryos continued to expand laterally and interestingly resembled the SAM of the *wus* mutants (Laux et al., 1996). In these mutants, the meristem also assume a very broad and shallow appearance and suggested to the authors that *WUS* may be involved in the maintenance of the meristematic identity of the SAM. It is possible that there may be some interaction between the level of auxin and *WUS* activity that would infer a unique identity to the cells of the SAM.

The treated embryos exhibited a substantial decline in the ability to form shoots compared to the control embryos. This further supports the previous observation that events occurring during the globular stage of embryogenesis are not only critical for the proper development of the SAM but also its subsequent activity. Furthermore, our results show that if the embryos are treated at a later stage of development, this does not alter the ability of the embryos to produce normal shoots. Liu et al. (1993b) also noted that TIBA treated globular stage embryos exhibited a delay in shoot formation. The authors observed that emerging shoots eventually split the elongated trumpet shaped cotyledon in half. However, that was not ever observed in the present investigation. Although a leaf-like protrusion was observed in the centre of the trumpet, it never expanded or took on the form of a true leaf similar to the observations made by Hadfi et al. (1998). These authors noted that in zygotic embryos treated with IAA and NPA, the leaves that were produced were not ever able to grow out of the collar cotyledon. In the majority of the TIBA treated embryos, the SAM simply continued to increase in width with no evidence of organogenesis occurring. As mentioned earlier, the SAM can be divided into functional layers or regions. In the TIBA-treated embryos the ability to produce leaves

was probably reduced primarily because the layers (L2 and L3) that are involved in organ formation were most affected.

The effects of TIBA on SAM development differed in the MD and zygotic embryo systems

There are many differences in the way that TIBA affected the SAM of MD and zygotic embryos. One of the primary differences is the resulting shape of the SAM and the cells within the SAM. It is not very clear why there would be such a dramatic difference in the effect of TIBA. However, there may be a few reasons that could explain such differences in the reaction.

First, the two experimental systems may not be comparable. In the MD embryo system, the developing embryos were exposed to the treatment from the initial stages of development, i.e. prior to the formation of the proembryo. The embryos were submersed in a liquid medium that would facilitate the exposure of all the cells of the embryo to the treatment. TIBA would be able to exert its effects from very early on and the cells at the apical half of the embryo would be exposed to higher levels of auxin for an increased amount of time. In the zygotic embryo system, the embryos were placed on to a solid medium. Therefore, the TIBA was not surrounding the entire embryo and all the cells of the embryo were not exposed to the treatment simultaneously. The TIBA may have had to be absorbed by the embryo first before inhibiting the polar transport of auxin. This would account for the SAM (of zygotic embryos) of the treated embryos appearing very similar to the control embryos shortly (4 DAC) after TIBA treatment. It was only after prolonged exposure to TIBA, and presumably time for TIBA to block the transport, were any effects noticeable.

Second, the culturing of very young globular stage zygotic embryos is a very difficult process. The system developed by Liu et al. (1993a) used in this study is the only recent one that has had a relative amount of success. However, the youngest embryos that could be cultured were at the globular stage as any earlier stages usually die *in vitro*. Even at this very early stage in development, there is a high degree of

organization. In *Arabidopsis*, which is genetically very similar to *Brassica* species, it has been shown that the few cells that could be identified as the precursors of the SAM, show distinctive gene expression at the globular stage (Long et al., 1996; Mayer et al., 1998). Furthermore, Tykarska (1976, 1979) have shown that the precursors to all the internal tissues of the plant are already established by the globular stage. If these cells are unique by this early stage of development, then perhaps TIBA is not able to dramatically alter their development into the SAM. Once the specific genes are activated, these cells may be able to maintain their unique identity to a certain degree. In the MD embryo system, TIBA was present before the embryos were being formed. Therefore, it may have prevented the appropriate genes from being activated and the cells were not able to maintain their meristematic identity. To make the zygotic system more comparable to the MD system, it would be ideal if the zygote, formed just after fertilization, could be cultured *in vitro* and allowed to develop in the specific treatment.

CHAPTER FIVE

The Influence of Altered Auxin Levels on Microtubule Orientation in the SAM

Introduction

Plants are sessile organisms. As a consequence they are only able to respond to environmental stimuli by changes in their growth and development. Furthermore, plant cells are immobile because of the rigid cell walls. Therefore, an adaptive response often requires that there are changes in the shape and size of the cells and this is translated to changes at the organismal level. Cell shape is primarily controlled either by cell division or the direction of cell expansion. These in turn, are controlled by the cytoskeleton of the cell. Therefore, signals that influence the cytoskeleton indirectly affect the overall morphology of the plant.

The cytoskeleton of eukaryotic cells is a network of filaments that is found in the cytoplasm. The main components of the cytoskeleton are the microtubules (MTs) and microfilaments which can be differentiated by the size of the filaments, and several related proteins. The cytoskeleton plays a vital role in several key processes in plants including cell division and expansion, organelle distribution and positioning, cytoplasmic streaming, as well as cell differentiation. There are several unique microtubular arrays found in plants that are not found in animal cells (reviewed in Cyr, 1994; Nick, 1998). It is this component of the cytoskeleton, and in particular the cortical MTs, that appear to be the most involved in cell division and expansion. The MT cytoskeleton is very dynamic and its configuration depends on the developmental stage of the cell. The different MT arrays that could be found in plants are the interphase cortical array, the pre-prophase band, the spindle apparatus, and the phragmoplast. In a cell that is in interphase, the cortical MTs are usually aligned parallel to each other and are oriented perpendicular to the long axis of the cell. As the cell enters into division, this cortical array is reorganized

into the preprophase band (PPB) that surrounds the nucleus. It is the PPB that always predicts the future site of the cell wall. As the cell continues through the division cycle, the PPB breaks down and gives rise to the spindle array. This is the only array that plant cells have in common with animal cells. Finally, a new array, the phragmoplast, forms to essentially create the new cell plate. Similarly, the cortical MTs are intimately connected with any subsequent expansion of the cell that may occur as the orientation of the MTs are always closely connected to the orientation of the newly deposited cell wall material. Therefore, because of the dynamic nature of the MTs in plants and their close connection to the eventual shape and size of the cell, it has been suggested that the main role of the cortical MTs is to intercept external and internal signals that influence cells, effect any changes and ultimately alter the developmental pathway of the plant (Nick, 1998).

A cursory survey of the cells in a plant reveals that the size and shape of a cell changes over its life. Meristematic cells are usually comparatively small and isodiametric in shape. However, the derivatives of these cells, upon differentiation, usually enlarge and often assume a more cylindrical shape such as the cells found in the hypocotyls or the internodes. This change in the shape of the cells is often mirrored by changes in the cortical microtubules. Cells of the SAM and RAM often have MTs with distinct orientations. Sakaguchi et al. (1988, 1990) demonstrated that in shoot apices of several angiosperms, the MTs were oriented in a longitudinal direction in the tunica layer and randomly in the corpus. This correlates with the planes of cell division that are predominantly found in these two areas of the SAM. For example, the longitudinal orientation of the MTs in the tunica would allow for anticlinal divisions to occur. However, the random orientation of the MTs in the corpus allows the cells to maintain their isodiametric shape and also to divide in any plane of direction. Baluska et al (1992) found that the cells of the root apical meristem, which are also isodiametric in shape, exhibit a random orientation of cortical MTs. However, in more mature tissues, as the cells differentiate, the MTs assume a more organized arrangement. The cortical MTs are arranged in a more transverse direction which would be parallel to the predominant plane of cell division (Baluska et al., 1992). This transverse arrangement of the MTs is also a reflection of the transverse arrangement of the cellulose microfibrils that acts as a

reinforcement mechanism to maintain the longitudinal growth axis in these cells (Hardham, 1982).

Cyr (1994) brings attention to the fact that the manner in which cortical MTs and cellulose microfibrils interact could be likened to any other signal transduction pathway. In a signal transduction pathway there is always a sensor, the transduction machinery, and an effector event. The alignment of the cellulose microfibrils can be considered the effector event that allows the cell to respond to changes in the environment. The microtubular cytoskeleton is the transduction machinery that serves to interpret the signal. However, the signal can be one of many factors and is still an area of speculation. It has been suggested that the phytohormones, which are involved in many developmental responses, and are also associated with changes in cell size and shape, could act as signals to provide spatial information to orient the MTs and affect the behaviour of a cell.

There is a great deal of evidence to show that auxin may be one of the primary candidates that could influence MT orientation. Auxin has been shown to cause cells to elongate in a direction that is parallel to the flow of the auxin (Czaja, 1935). Several investigations have shown that the development and orientation of tracheary elements is influenced by the direction of auxin flow and the predominantly transverse banding of the secondary wall in these cells are always arranged perpendicular to the flow of auxin (Sachs, 1991; Warren Wilson and Warren Wilson, 1993). This arrangement also reflects the transverse arrangement of the cortical MTs. Several studies using shoots and coleoptiles have shown that auxin does cause a re-alignment of the MTs in a direction that is perpendicular to the flow of auxin (Nick et al., 1990). Furthermore, bioelectric currents, that could be associated with the polar transport of auxin, have been shown to affect the alignment of the MTs. The cortical MTs were always oriented perpendicular to the flow of the current (Blackman and Overall, 1995).

From the results of the previous two chapters it is evident that an increased level of auxin in the apical region affects the overall shape of the cells in the SAM. Therefore, it is reasonable to surmise that the cortical MTs would also be affected. This chapter utilizes a quick and simple technique to determine whether the TIBA treatments also resulted in an alteration of the cortical MT arrangement in the cells of the SAM.

Results

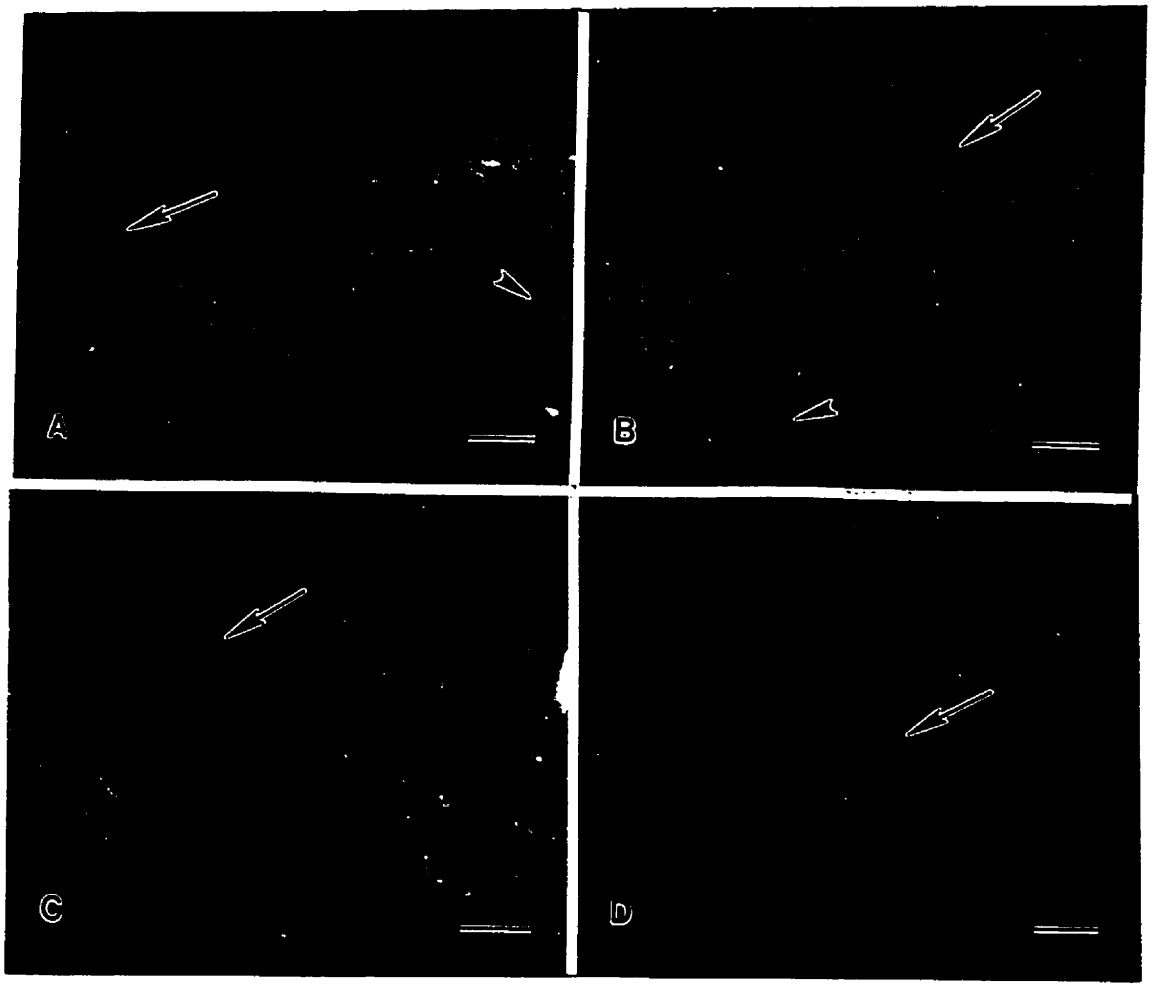
The effect of TIBA on MT orientation in the SAM of MD embryos

At day 11, the SAM of control MD embryos was well organized. There were two main patterns of MT orientation that were observed at this time. In the tunica layer, the cells had a predominantly longitudinal arrangement of MTs (Fig 5.1A, arrow) and they were positioned very close together. In the corpus region, most of the cells were observed to have a random orientation of MTs (Fig 5.1A, arrowhead). This pattern of MT arrangement persisted throughout most of the development until about day 30 when the MTs in the tunica layer took on a more random arrangement (Fig 5.1C, arrow).

From previous histological sections of TIBA treated embryos, the cells of the tunica layer were quite elongated by day 11. However, these cells exhibited a pattern of MT arrangement that was similar to the control embryos with the tunica cells having longitudinal arrangement (Fig 5.1B, arrow) and the corpus cells having a random orientation (Fig 5.1B, arrowhead). The only difference observed was that there appeared to be less MTs found in these cells as the MTs were situated further apart. This pattern changed drastically by Day 30, when all the cells of the SAM exhibited a transverse alignment of MTs (Fig 5.1D, arrow).

Figure 5.1:

Arrangement of cortical MTs in the SAM of MD embryos at various stages of development. A and B: These embryos were 15 days old. A: SAM of a control embryo. The MTs are arranged in a longitudinal direction in the cells of the tunica (➞), and randomly in the corpus (≻). B: A SAM of an embryos that was treated with TIBA on Day 5. Similar to the control, the MTs are arranged longitudinal in the tunica (➞), and randomly in the corpus (≻). C and D: These embryos were sampled at Day 30. C: SAM of a control embryo. The MTs in the tunica appear more random. D: SAM of an embryo that was treated with TIBA at Day 5. The MTs are now arranged transversely in the tunica and corpus (➞). All scale bars = 60 μm.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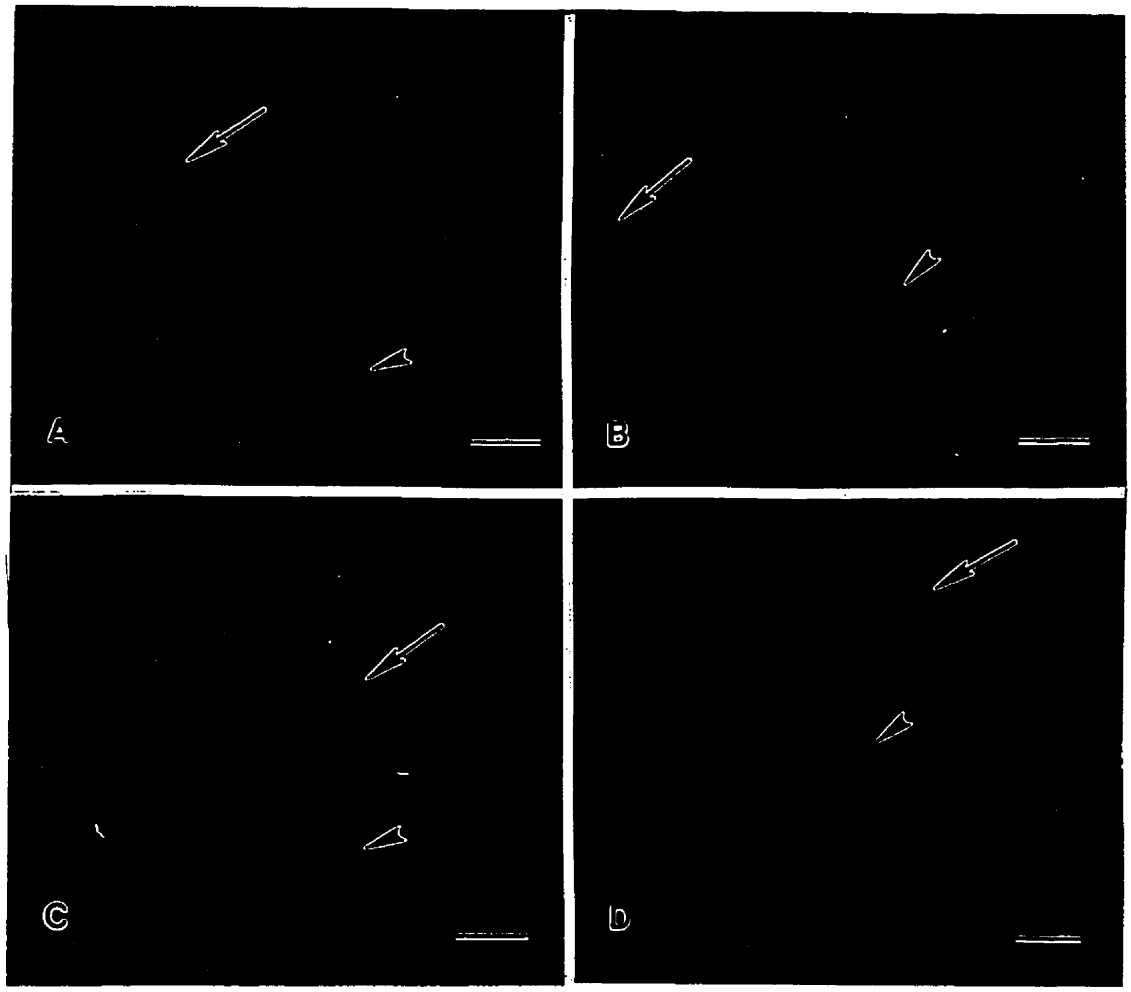
The effect of TIBA on MT orientation in the SAM of zygotic embryos

By 5 DAC (Days after Culture), the cells of the SAM of control zygotic embryos exhibited a pattern similar to that of the MD embryos. The cells of the tunica layer had MTs arranged in a longitudinal orientation (Fig 5.2A, arrow) while the cells of the corpus had MTs arranged in a random fashion (Fig 5.2A, arrowhead). This general pattern was maintained until 14 DAC when the embryo was considered to be mature (Fig 5.2C).

The globular stage embryos that were maintained in TIBA containing medium had a similar pattern of MT arrangement to the control embryos (Fig 5.2B). During the maturation period, the longitudinal arrangement of MTs was also observed very frequently in the second layer of cells of the SAM (Fig 5.2D, arrowhead). The other cells continued to maintain a random arrangement of MTs. This pattern was maintained until the end of the maturation period.

Figure 5.2:

Arrangement of the cortical MTs in the SAM of *in vitro* zygotic embryos at different stages during the maturation process. A and B: These embryos were 5 DAC. In the SAM of both the control (A) and the TIBA-treated (B) embryo, the MTs in the tunica are arranged longitudinally (➞) and the in the corpus they are arranged randomly (➢). C and D: These embryos were 14DAC. Similarly, in the older control embryo (C), the MTs were arranged longitudinally in the tunica (➞) and randomly in the corpus (➢). In the TIBA treated embryos (D), the cells in the tunica continued to maintain MTs arranged longitudinally (➞), but there was a greater number of cells in the corpus that had a longitudinal arrangement of MTs (➢). All scale bars = 60 μm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Discussion

The arrangement of the cortical MTs has been shown to be intimately involved in determining the shape and size of a cell. However, the physiological controls that influence the orientation of the MTs has not been conclusively elucidated. There have been many suggestions that the phytohormones may act as signals to change the orientation of the MTs (Warren Wilson and Warren Wilson, 1993). Auxin, in particular, can be considered as a prime candidate for affecting MT orientation as it is involved in many physiological processes that are accompanied by a change in cell shape and size.

MT arrangement in the SAM

The specific arrangements of MTs found in the tunica and corpus regions of both the MD and zygotic embryos are characteristic of the SAM organization and have been reported in other systems. Sakaguchi et al. (1988, 1990) performed a series of studies to determine the arrangement of the MTs in mature SAMs of various species. Most notably the authors found that, in the tunica, the MTs were arranged longitudinally, and in the corpus the MTs had a random orientation. This is the same pattern, observed in this study, in the developing SAM of young embryos. The specific arrangement of the MTs in the tunica would allow for cell division as well as expansion in an anticlinal manner. The cellulose microfibrils of these cells were shown to be oriented in a similar fashion (Sakaguchi et al., 1988). This preferred direction of growth may be a reflection of the function of this layer in the formation of the epidermis of the mature part of the plant. The random arrangement of the MTs in the corpus cells would allow for cell division in any plane and isotropic cell expansion. This arrangement may also be a reflection of the function of this region in forming the main internal tissues of the developing plant and having the ability to divide in any plane would therefore be important prerequisite.

TIBA treatment alters the MT pattern in the SAM

The embryos that were treated with TIBA had SAMs that were altered in the overall shape and cell characteristics. In the MD embryos, the SAM assumed a more elongated shape and the cells also gradually became more elongated. Although the pattern of MT arrangement was initially similar to that of the control embryos, the MTs in all the cells of the SAM eventually became oriented in a transverse direction. As discussed previously, the TIBA treatment most likely resulted in an increase in auxin in the apical region. This would have resulted in an axial elongation of the cells in this area which is consistent with the ability of the auxin to cause an elongation of cells. This shift from the normal direction of expansion in these cells was accompanied in a shift in the orientation of the MTs of these cells. MTs and cellulose microfibrils in cells in maize coleoptiles have been observed to re-orient to a predominantly transverse direction after exogenous auxin application (Nick et al., 1992). The microtubular pattern found in the tunica and corpus regions that is characteristic for SAMs of angiosperms was destroyed by TIBA treatment. Therefore, the cells could not maintain the ability to divide in specific planes and this may account for the extremely low conversion frequencies observed previously. From these observations, it is reasonable to suggest that a lower level of auxin is required for the cells of the SAM to maintain specific MT orientations that are necessary for the function of the meristem.

In the zygotic embryos, the TIBA treatment had a different effect on the SAM. The bulk of the corpus of the SAM was reduced as the cells became starch-filled and parenchyma-like. However, the tunica layer, which appeared to be extended to two layers, continued to expand laterally. The cells in the first layer of the SAM continued to maintain MTs in a longitudinal orientation in TIBA treatment. However, there were an increase in the number of cells in the second layer of the SAM with longitudinally oriented MTs compared to the control. This increase in the number of cells with longitudinally arranged MTs would result in an increase in the number of anticlinal cell divisions and account for the increase in the width of the meristem observed. A reduction in the number of cells with randomly oriented MTs in the corpus could also account for

the low conversion frequency of the TIBA treated embryos. The concentration of auxin in the apical region may not be as high as that found in the TIBA treated MD embryos, therefore, the cells reacted differently. An observation that is common to both systems is that any deviation from the normal pattern of MT arrangement is an indication that the cells have been perturbed and are not likely to function normally. The longitudinal arrangement of the MTs in the tunica and the random arrangement in the corpus can therefore be considered as a marker for normal SAM development and function.

The influence of the polar transport of auxin on MT orientation in the SAM

During the life of a cell, its specific MT orientation could be correlated to its influence by the polar transport of auxin. A meristematic cell of the SAM, although located near the source of auxin, may not necessarily be directly involved in the polar transport pathway. As discussed in previous chapters, the polar transport of auxin may create a 'pocket' in the apical notch where the auxin concentration in lower that the surrounding area. In this 'pocket', there would not be an active polar transport of auxin. This would account for the random arrangement of MTs and cellulose microfibrils observed in the corpus. The derivatives of the SAM have a higher level of auxin and the polar transport of auxin is active in these cells. The MTs are arranged perpendicular to the flow of auxin and the cells expand in a predominantly axial direction which results in an elongated cell. Overall, the polar transport of auxin could then serve to provide positional information to the cells in the developing embryo, allow for them to adopt the appropriate MT orientation, and follow through with the appropriate plane of cell division and direction of cell expansion.

The mechanism by which auxin could be influencing MT orientation is still an area of speculation. However, it has been suggested that auxin could interact with the second messenger, calcium, that has been shown to affect MT stability. Furthermore, the basipetal transport of auxin has also been linked to the acropetal transport of calcium (Dela Fuente and Leopold, 1973) and a lowered concentration of Ca^{2+} reduces the active transport (Allan and Rubery, 1991). Cyr (1991) demonstrated that Ca^{2+}, coupled with an

effector protein, calmodulin, can affect the stability of microtubules *in situ*. Further investigation of this interaction determined that calmodulin is able to affect the stability of MTs depending on the local concentration of Ca^{2+} (Fisher et al., 1996). At low Ca^{2+} concentrations, the MTs are stabilized by calmodulin. However, once the Ca^{2+} concentrations pass a threshold level, calmodulin dissociates from the MTs which can be destabilized. Therefore, it is possible that in the SAM, where there is no active polar transport of auxin, the Ca^{2+} levels would be lower and the cortical MTs would be stabilized by calmodulin. However, in the TIBA treated embryos where there is an increase in the concentration of auxin in the SAM, the Ca^{2+} levels would be increased, and calmodulin would act to de-stabilize the MTs.

CHAPTER SIX

Physiological influences on SAM maturation

Introduction

Somatic embryogenesis is the process by which an embryo can develop from a cell other than a gamete of the product of a gametic fusion (Merkle et al., 1995). The ability to bring this process into an *in vitro* system has provided an excellent opportunity to develop propagation protocols that are useful to the agriculture, horticulture and forestry industry, but it has also been a tool that can be used to further the understanding of the process of embryogenesis in plants. Thorpe (1993) reported that there are more than 150 species reported to be able to be able to undergo somatic embryogenesis *in vitro*.

In many of the systems studied, the process through which embryos develop from somatic cells is very similar to that of zygotic embryos. For example, the embryos can be observed to proceed through the same morphological transitions from globular, heart, torpedo to cotyledon stages as observed in zygotic embryos. However, it should be noted that in the culture environment, there are many more aberrant morphologies that are observed. These include abnormal cotyledon morphology, multiple cotyledons, axis duplication (see Yeung, 1995). Furthermore, while the somatic embryos may have similar internal organization to the zygotic embryos, the histodifferentiation pathways may also be quite different in the two systems (Yeung, 1995; Yeung et al., 1996). One other area that is quite different between the two systems is that several *in vitro* studies have demonstrated that the somatic embryos are unable to continue the post-embryonic development efficiently as compared to the zygotic embryos (Nickle and Yeung, 1993; 1994; Yeung et al., 1996).

It is quite clear that *in vitro* somatic embryogenesis occurs in an artificial system and this may account for the many differences that are observed between somatic and zygotic embryos. The culture environment does not simulate exactly the conditions

found *in ovulo* (Carman, 1989) and many of the physiological signals that originate from the maternal tissue will undoubtedly be absent. During seed development there appears to be a concert of physiological events occurring with the rise and fall of various hormones throughout the process (Rock and Quatrano, 1995)

Nickle and Yeung (1993; 1994) were able to demonstrate that the low conversion frequency (production of shoots and roots) of somatic embryos of *Daucus carota* was due to a deterioration of the SAM during embryogenesis. The low conversion frequency was improved, however, by the addition of abscisic acid (ABA) into the medium. ABA was found to improve the integrity of the SAM and therefore allowed for an increased conversion frequency. However, it is important to note that ABA was able to cause the greatest improvement in the conversion frequency of embryos treated during the globular stage.

ABA has been shown to play a role in the maturation processes of embryo development. Concomitantly, ABA levels have been shown to peak during this time (Rock and Quatrano, 1995). It has been suggested that ABA may play a general role in suppressing precocious germination of the embryo while it is still developing. Perhaps at this time it may also act to trigger some maturation events in the SAM that would make it more stable. *In vitro* somatic embryos develop in a different environment from their zygotic counterparts. This, in part, accounts for the differences observed in the somatic embryo development. Yeung et al. (1996) showed that the conversion frequency of microspore-derived canola embryos was highest at the late torpedo and early cotyledon stage but rapidly declined with older embryos. The decline in the conversion frequency was also attributed to the deterioration of the shoot apical meristem. This suggests that there is a phase of embryogenesis when, although the meristem has formed, it is not functional. A period of 'maturation' has to occur before it can become active. Recent work has shown that the levels of several hormones are significantly lower in microspore-derived somatic embryos as compared to zygotic embryos of canola. Hays (1996) found that, on average, abscisic acid (ABA) levels were 10 fold lower in the somatic embryos.

In Chapter 3, it was observed that the conversion frequency of the MD embryos was always relatively low. In comparison, in Chapter 4, the conversion frequency of the

zygotic embryos was always 100%. Therefore, this chapter considers the role that ABA may play in restoring the conversion frequency of the MD embryos to that of zygotic embryos.

Results

The effect of ABA on the morphology of MD embryos

ABA was added to the medium of the developing MD embryos on Days 5, 10, 15, 20 and 25. The embryos that were treated at Day 5, 10 and 15 and allowed to develop in the ABA-containing medium remained relatively small during their maturation (Fig 6.1). However, the embryos that were treated later in development (i.e. Days 20, 25) resembled the control in terms of their size (Fig 6.1). Embryos that were treated with ABA regardless of the concentration or the timing of treatment exhibited bilateral symmetry. This observation was consistent for the different concentrations (2, 5, 10 and 15 μM) of ABA tested. However, 5 μM ABA gave the most consistent results, therefore, only pictures and results using this concentration are reported.

The effect of ABA on the conversion frequency of MD embryos

Control and ABA-treated embryos were transferred to the conversion medium at Days 15, 20, 25 and 30. The embryos were allowed to grow for a period of 3 weeks before the conversion frequency was assessed. Figure 6.2 is a summary of the conversion frequencies of the control and ABA treated embryos that were transferred to the conversion medium at different times throughout the culture period. From these graphs, it is evident that ABA treatment improved the conversion frequency regardless of time of treatment or how old the embryos were. However, it is important to note that the amount of time that the embryos were exposed to ABA also had an effect on the conversion frequency. The greatest improvement in conversion was observed when the embryos were treated at Day 5 and 10 and allowed to develop in ABA until they were 25 or 30 days old. At these times the conversion frequency was 100% compared to a conversion frequency of about 50% to 60% in the control. This indicates that the embryos had to be exposed to ABA for a minimum of 25 to 30 days for any significant improvement to be made.

Figure 6.1:

Morphology of MD embryos at Day 30. A: A control embryo. B: an embryo treated with ABA at Day 5. C: An embryos treated with ABA at Day 10. D: An embryo treated with ABA at Day 15. E: An embryo treated with ABA at Day 20. F: An embryo treated with ABA at Day 25. Scale bar = 1.5 mm.

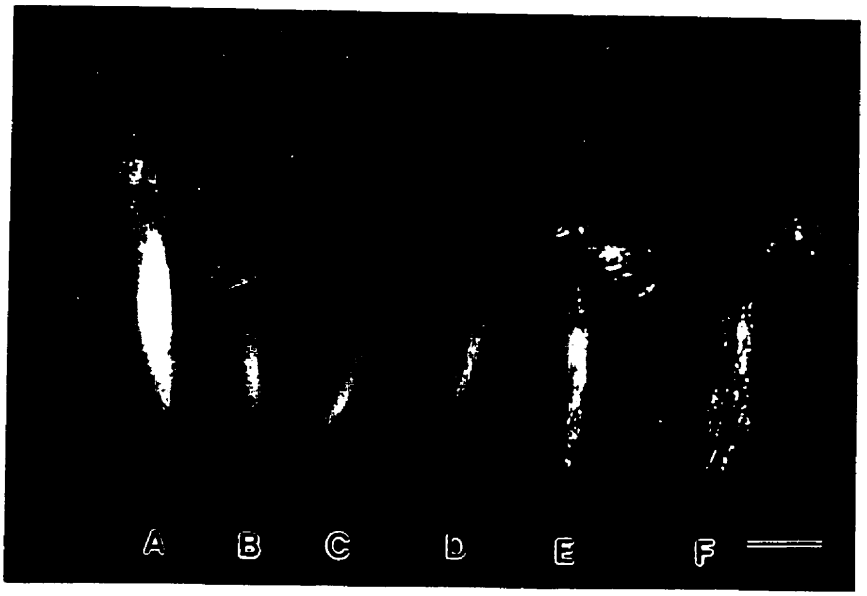
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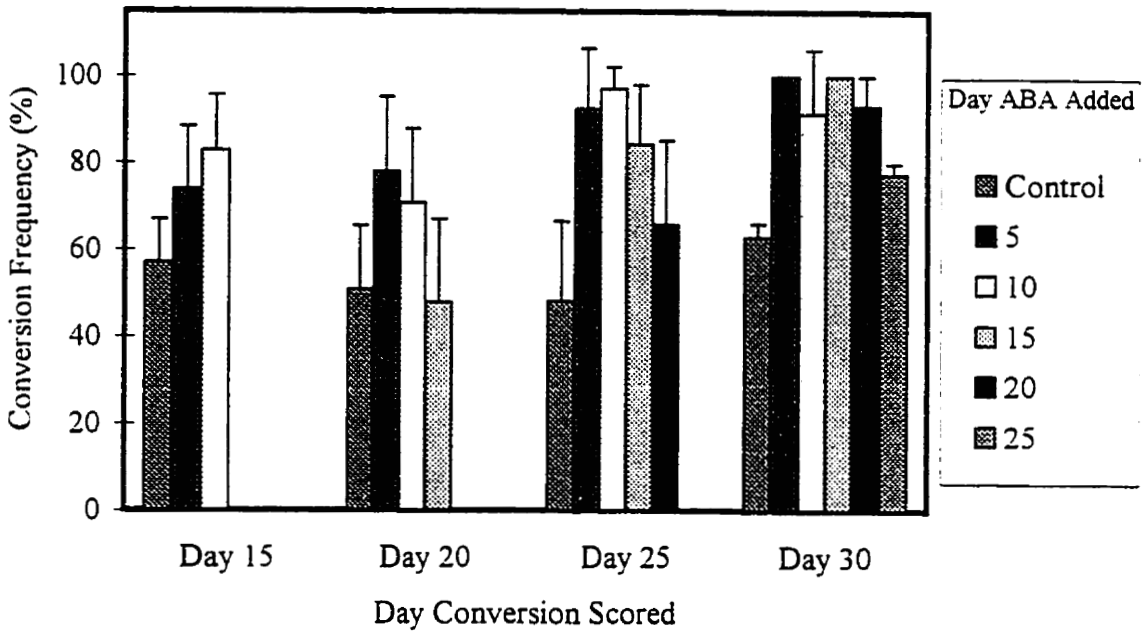
Figure 6.2:

Conversion frequencies of control and ABA-treated MD embryos at different stages throughout maturation. The embryos were transferred to the conversion medium and the conversion frequency was scored approximately 3 weeks after the transfer. Bars represent the mean conversion frequency and the error bars represent the standard deviation around the plotted mean.


Conversion Frequency (%)
100
80
60
40
20
0
Day 15
Day 20
Day 25
Day 30
Day Conversion Scored
Day ABA Added
Control
5
10
15
20
25

However, if ABA was added to the medium for only 5 days prior to transfer to the initiation of conversion, the conversion was not significantly improved over that of the control.

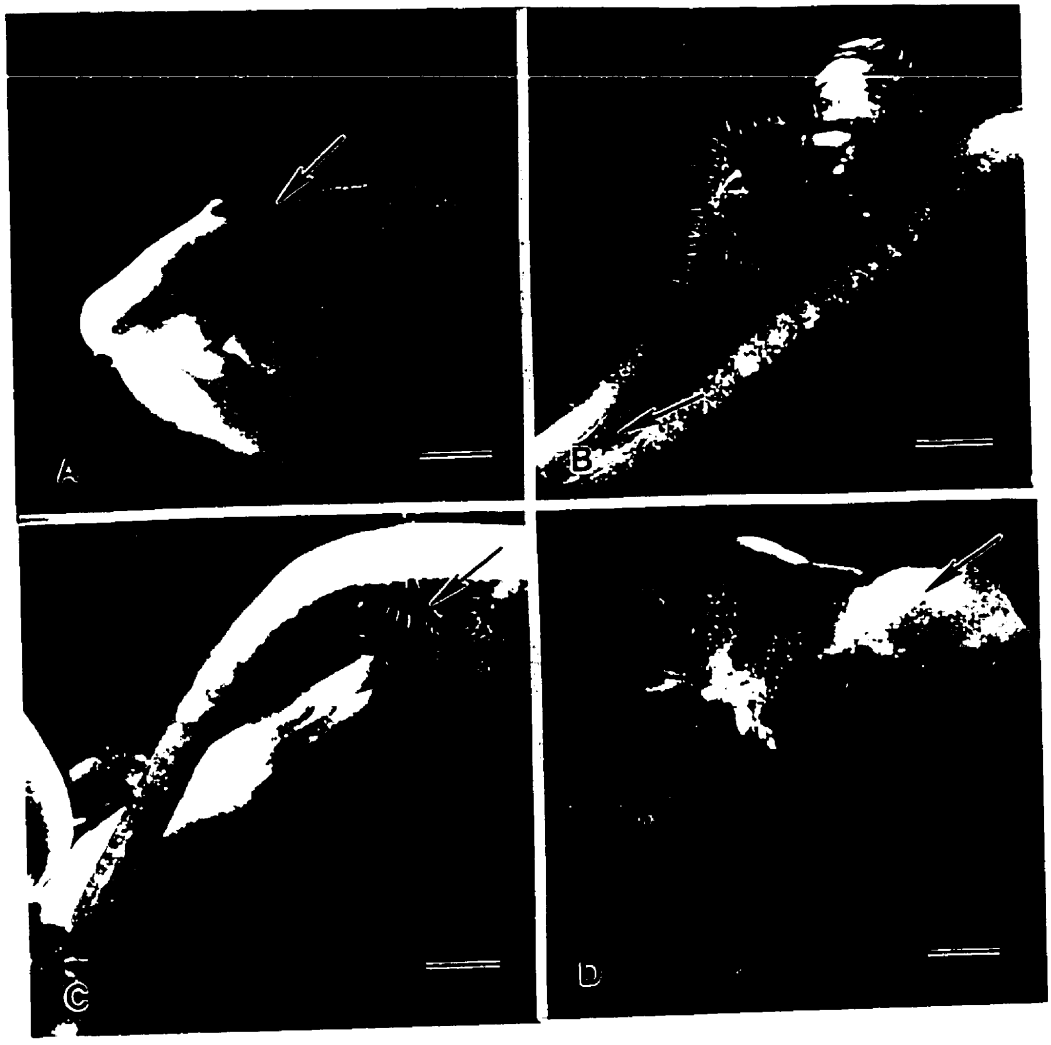
Furthermore, the general quality of the resultant plantlets also depended on the amount of time that the embryos were exposed to ABA. At the time that the conversion was scored, most of the control and ABA-treated embryos had produced one small leaf that was just emerging from between the cotyledons (cf. Fig 6.3A). There were also usually secondary embryos that were associated with the plantlet and callus forming at the hypocotyl-root junction. However, the embryos that were treated at Day 5 or Day 10 and allowed to develop in ABA until Day 25 before the initiation of conversion, had produced 3-4 leaves (Fig 6.3 B and C). These plantlets did not have any secondary embryos or callus associated with them. If the embryos were treated at Day 15 or later and similarly transferred to the conversion medium on Day 25 (Fig 6.3D), the resultant plantlet resembled that of the control.

The effect of ABA on the conversion frequency of TIBA-treated MD embryos

Observing that ABA markedly improved the conversion of the control embryos, ABA was added with TIBA to the developing MD embryos to determine if this would also improve the conversion frequency of the TIBA treated embryos. The ABA/TIBA-treated embryos still developed one, fused cotyledon. The trumpet-shaped embryos were transferred to the conversion medium at various times throughout their development. However, the conversion frequency was not improved significantly.

Figure 6.3:

Morphology of MD embryos transferred to the conversion medium at Day 25. The conversion frequency was scored 3 weeks after the transfer. A: A control embryo showing a leaf just emerging from between the cotyledons (➡). B: An embryo that was treated with ABA on Day 5. There are 2 well developed leaves and a third just emerging (➡). Similarly the embryo that was treated with ABA on Day 10 (C) also has well developed leaves (➡). The embryo that had been treated with ABA on Day 15 (D) resembles the control embryo with just one leaf (➡) emerging. All scale bars = 1.5 mm.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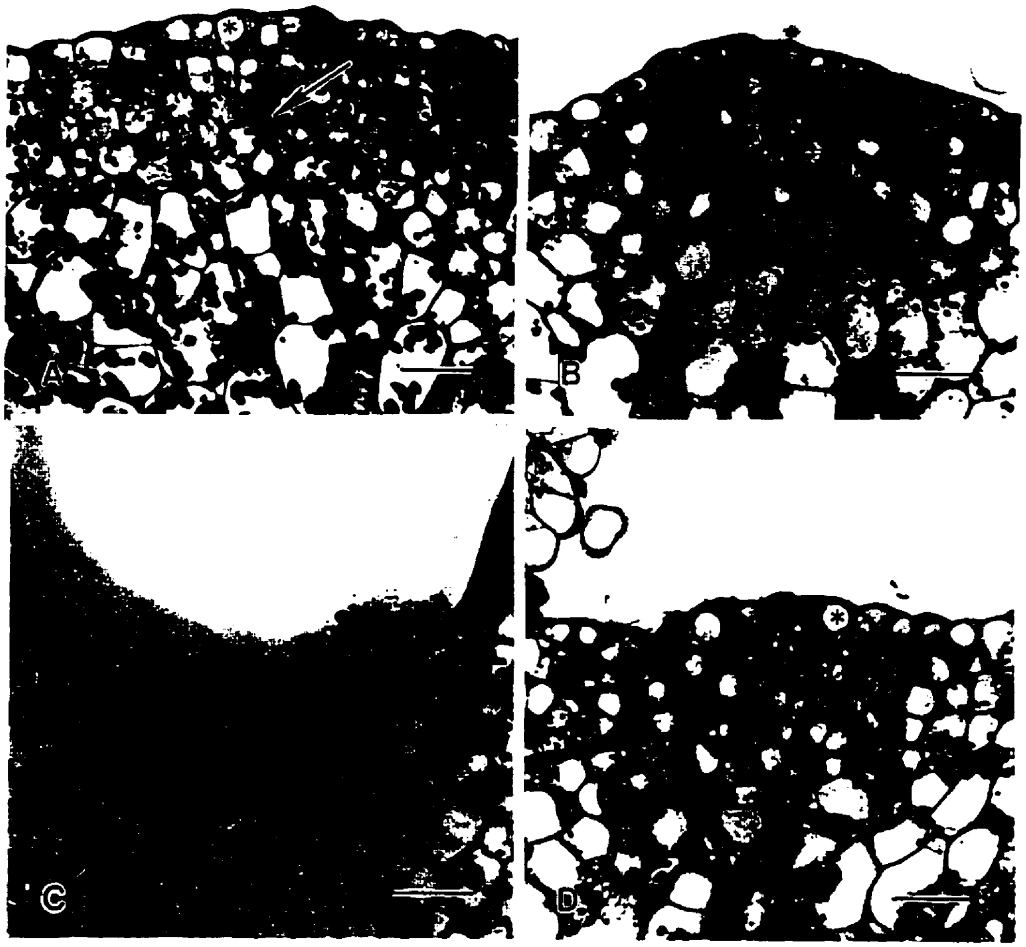
The effect of ABA on the SAM of MD embryos

Noting the improvement in the conversion frequency of ABA-treated embryos, an examination of the histology of the SAM was performed. Embryos that were treated early in development and allowed to develop in ABA for a short length of time had SAMs similar to those of the control embryos. However, the embryos that were treated early and allowed to develop for the entire length of their maturation period showed significant improvements in the quality of the meristem. Figure 6.4 shows an example of SAMs of embryos that were sampled at Day 25. The SAM of the control embryos at this point in development was usually heavily vacuolated and showed a high degree of starch accumulation (Fig 6.4A). In embryos that were treated with ABA at Day 5 (Fig 6.4 B) or Day 10 (Fig 6.4 C), the SAM appeared very well organized. The cells were able to maintain their cytoplasmic density and there was very few starch grains or vacuole present in the SAM. This was in contrast to the SAM of embryos that were treated at Day 15 or later in development (Fig 6.4 D). The cells in these SAMs were comparable to that of the control embryos with highly vacuolated, starch-filled cells.

Figure 6.4:

Histology of control and ABA-treated MD embryos at Day 25. A: SAM of a control embryo. Note the vacuolation (*) and encroachment of starch (➞) in the cells of the SAM. The SAM of an embryo that was treated with ABA at Day 5 (B) or Day 10 (C) is well organized into a dome-shaped mound and the cells are densely cytoplasmic with very little starch deposition. The SAM of an embryo treated with ABA on Day 15 (D) resembles that of the control with the cells being highly vacuolated (*). All scale bars = 20 μm.


A
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C
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Discussion

ABA influences the morphology of MD embryos

The results obtained in this study indicate that ABA plays a role in the maturation process of MD embryos of *B. napus*. MD embryos are often larger in size than their zygotic counterparts which is perhaps due to the fact that the ovule is not present to restrict the size. From the morphological observations, embryos that were treated with ABA early in development were reduced in size and their cotyledons were never observed to be as expanded as those of the control embryos. This reduction in size was also noted by Pomeroy et al. (1994) who further reported that the dry weight of the ABA treated embryos was drastically reduced. Similarly, Nickle (1993) also observed that upon ABA treatment, somatic embryos of *Daucus carota*, did not continue to grow or develop past the torpedo stage. This suggested that that the embryos were perhaps undergoing a 'dormancy' period. It is possible that ABA may act to slow the maturation of the MD embryos.

In a study that compared the physiologies of zygotic and MD embryos, Hays (1996) documented many differences between the zygotic and MD embryo system. One significant finding was that the concentration of ABA in the MD embryos was 100 times less than that found in zygotic embryos. In zygotic embryos, ABA has been shown to be important in the process of zygotic seed maturation by preventing precocious germination and stimulating the synthesis and deposition of a range of proteins (Rock and Quatrano, 1995). Considering the results from this investigation, a relatively high level of ABA may be needed to normalize the development of the MD embryos and allow them to be more zygotic-like. High levels of ABA may promote some maturation programs or suppress the germination program in zygotic embryos that are not stimulated in the MD embryos. The expanded cotyledons and larger size found in the MD embryos may be a reflection of germinative processes that are not suppressed because the ABA levels are not high enough. Indeed, Hause et al. (1994), using other characteristics have likened MD embryos to germinating zygotic embryos.

ABA preserves the qualities of the SAM in MD embryos

ABA was observed to maintain the overall integrity of the cells of the SAM in the MD embryos. However, it is important to note that ABA was only able to influence the quality of the SAM if it was present at the very early stages of development, i.e. the preglobular or globular stage. Furthermore, the beneficial effects of the ABA were not apparent unless the embryos were allowed to develop in the ABA for a minimum of 20 to 25 days. In a study that documented the levels of ABA throughout development, Hays (1996) reported that zygotic embryos exhibited a biphasic increase over time. The first peak, that occurred very early in development, was from the surrounding maternal tissue. The second peak, due to an embryonic source, occurred later in development. The first external source of ABA is obviously not present in the MD embryo system. However, it may be necessary for complete maturation of the MD embryo to occur. Hays (1996) also found that the MD embryos showed a biphasic increase in ABA levels but these levels may not be as high enough to initiate the necessary maturation processes. Nickle (1993) also noted that the globular stages of *D. carota* were the most responsive to ABA treatment. Therefore, an early external source of ABA as well as high level of ABA may play a role in the maintaining the quality of the SAM of MD embryos.

ABA improves the conversion frequency of MD embryos

The improvement of the SAMs in ABA-treated embryos is also reflected in an increase in the conversion frequency of the embryos and in the quality of the plantlets produced. ABA was observed to increase the conversion frequency by approximately 50% in some cases. The ability of ABA to improve the conversion frequency has also been noted by Nickle and Yeung (1993, 1994). Similar to this previous study, ABA appears to promote the retention of densely cytoplasmic cells in the apical notch that maintain a meristematic identity and are able to initiate organogenesis. ABA also seems to prevent these cells from becoming excessively vacuolated or accumulating any starch.

The greatest improvement in the conversion frequency and best plantlet quality was observed only if the embryos were exposed to ABA for a minimum of 25 days. If the embryos were exposed to ABA for less time, they were able to produce 1-2 leaves but then growth stopped. The SAM of embryos that were not exposed to ABA for a very long time were vacuolated and had some starch accumulation. It is possible that while some of these cells were able to partake in organogenesis, the meristem may have exhausted its resources and been unable to continue making leaves. The requirement for a prolonged exposure to ABA suggests that there may be a subtle process of meristem maturation that is extended throughout the entire period of embryogenesis. It is also an indication that a shoot apical meristem is not necessarily efficiently functional even though it is morphologically recognizable.

It is also important to note that ABA treatment was not able to reverse the effects of TIBA or even improve the conversion frequency of the TIBA-treated embryos. This is an indication of the importance of auxin during the formative stages of the SAM. It appears that a lowered concentration of auxin is absolutely necessary for the proper formation of the SAM and ABA is not able to exert its influences if the cells have already been affected by TIBA. This result sheds additional light on the physiological regulation of SAM formation *in planta*. Initially a low concentration of auxin is necessary for the cells of the SAM to develop their unique characteristics (shape, MT orientation, and division planes). However, during this time a higher level of ABA is also needed to maintain the cytoplasmic qualities of the cells of the SAM perhaps by delaying gene expression associated with the germination process. Previous studies have shown that ABA treated MD embryos appeared more zygotic like in their ability to initiate storage protein and lipid synthesis that is associated with embryo maturation (Maquoi et al., 1993; Pomeroy et al., 1994). Therefore, it is evident that the process of SAM formation and maturation is complex and requires the presence of many physiological factors.

CHAPTER SEVEN

Summary and Future Studies

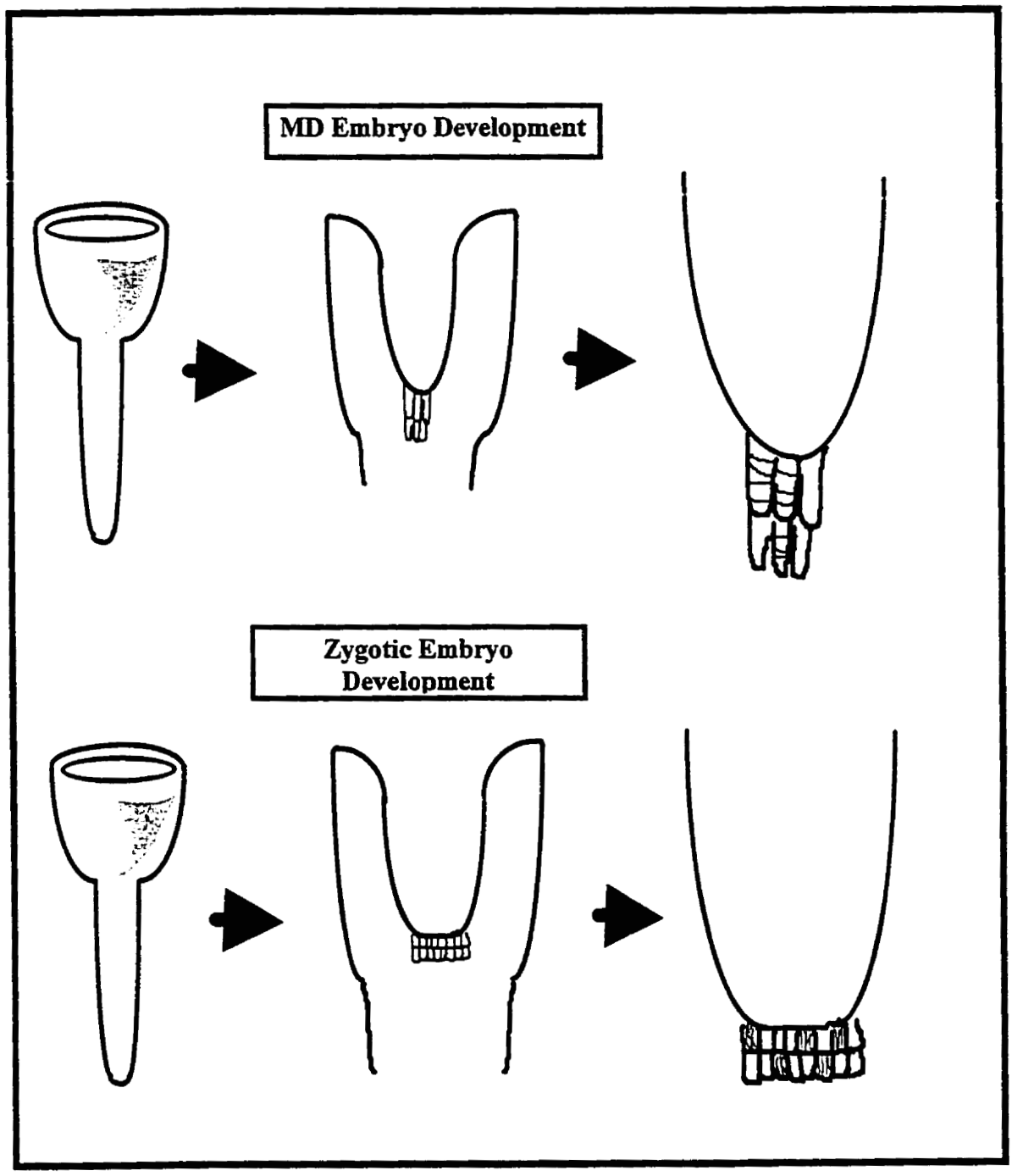
One of the most fundamental processes to occur during plant embryogenesis is the formation of the shoot and root apical meristems that are responsible for essentially creating the main body of the plant. It is not surprising that there have been many studies that have characterized the pattern of formation of these meristems during the embryogenesis of a variety of species. However, it is only recently that there has been any insight into the physiological control of these unique areas in the plant. The purpose of this thesis was to investigate the role of physiological agents in the formation and function of the shoot apical meristem in *Brassica napus*.

B. napus was chosen as a model system because of previous work performed using this specimen. The early cell division patterns that occur during embryogenesis have been well documented and have been shown to be extremely stable and precise. The well-established system of producing microspore-derived embryos offers the advantage of an easily manipulated experimental system. Furthermore, the recently developed *in vitro* method of culturing young zygotic embryos provides the opportunity to compare the results observed in the MD system to those in a zygotic system.

The results from this thesis provide an indication of the important role of auxin during the development of the SAM during embryogenesis. In both the zygotic and MD systems, an inhibition of the polar transport of auxin during the early stages of embryogenesis had dramatic effects on the characteristics of the SAM. In both systems, the auxin polar transport inhibitor, TIBA, was found to interfere with SAM formation only if the embryos were treated prior to the globular stage. In the MD system (Chapter 3) TIBA, caused the SAM to be narrower and it assumed a more elongated shape (Fig 7.1). There were less cells that comprised the SAM and the cells themselves were larger, and more axially elongated. In the zygotic embryo system (Chapter 4) the opposite seem to occur. The SAM of the TIBA treated embryos appeared transversely expanded and shallower.

Figure 7.1:

Summary diagram of the effect of TIBA treatment on MD and zygotic embryos. In MD embryo development (the upper panel), TIBA treatment resulted in the formation of embryos with one, fused cotyledon. The SAM of these embryos appeared elongated with less cells in the SAM. The cells themselves were also axially elongated in shape. The MTs in these cells appeared to be arranged transversely to the long axis of the cell. In zygotic embryos, the TIBA treatment also caused the embryos to develop one, fused cotyledon. However, the SAM of these embryos appeared more broad and less shallow. The MT were primarily arranged parallel to the long axis of the cell in the layers of the SAM that remained.


MD Embryo Development
Zygotic Embryo Development

There was an increase in the number of cells in the width of the SAM, however, there was a decrease in the number of cell layers that were found in the SAM (Fig 7.1).

These results may suggest that there are fundamental differences in the process of SAM formation in zygotic and MD embryos. In zygotic embryos, previous studies have shown that, there is a very precise pattern of cell division that leads to the formation of the SAM (Tykarska, 1976; Yeung et al., 1996). The progenitors of the SAM, the epiphyseal cells, can be distinguished from as early as the octant stage of the globular-stage embryo. These cells have distinct histological characteristics as well as unique gene expression patterns. These characteristics may be an indication that they are able to respond to the polar transport of auxin, that is initiated some time during the globular stage, and adopt a different fate from the surrounding cells - a meristematic fate. It is possible that the unique genes expressed in these cells may be involved with allowing the cells to lower the auxin concentration in the area by degradation or conjugation. Alternatively, the gene expression may be associated with altering the sensitivity of these cells to auxin by changing the number of receptors available. In previous studies it has been suggested that auxin is synthesized, at the globular stage, at the sites of the future cotyledons and transported basipetally to the future radicle (Schiavone and Cooke, 1987; Liu et al., 1993b). If there is a flood of auxin through the embryo, then the SAM will not develop properly. The cells destined to become the SAM may have the ability to react differently to auxin.

The pattern of results obtained reflect some of these concepts. In the zygotic embryos, only embryos at the globular stage and older were cultured due to the limitations of the protocol. However, it is clear that even at this early stage, that the cells of the epiphysis are already distinct. Perhaps the cells were 'fixed' in their fate such that the disturbances in the auxin levels were not able to disrupt their characteristics dramatically. However, it appears that the fate of all the cells in the SAM may not be set at the same time. Only two cell layers of the SAM were able to maintain their meristematic characteristics while the other layers became starch-filled parenchyma cells.

The differences observed in the characteristics of the SAM of the TIBA treated MD embryos could be accounted for by the differences in the manner in which the SAM

is formed. Yeung et al. (1996) noted that distinct epiphyseal cells are not ever observed in the MD embryos. Perhaps the gene expression patterns in the cells that will form the SAM in MD embryos are different from those found in the epiphyseal cells in the zygotic embryos. It is possible that these cells in the MD embryos were compromised in their ability to react to the increased auxin concentration that may have occurred in the apical notch in TIBA treated embryos. This could account for the dramatically different pattern observed in the TIBA treated zygotic and MD embryos. For example, in the zygotic embryos, the cells in the apical notch may have retained their ability to lower the concentration of auxin which allowed the cells to continue to divide and maintain their overall characteristics. However, in the MD embryos, if the cells in the apical notch were not able to reduce the auxin build-up due to TIBA, then this would have resulted in the characteristics observed.

In both the MD and zygotic embryo systems, the ability of the SAM to function was impaired by the TIBA treatment at the globular stage. If the embryos were treated later in development then this did not affect the ability of the SAM to produce leaves. This is another important indication that the physiological events that are occurring during the early parts of embryogenesis really lay the foundation for the SAM to function efficiently later on in development.

In addition to the noted effect that TIBA had on the formation of the SAM, these results also raise other interesting issues of when the cells of the SAM become determined or fixed in their fate and what factors are involved in promoting the stability of the meristem. These questions have long been a concern of many investigators (see Wareing, 1979; McDaniel, 1984). Wareing (1979) considered whether the cells of the apical meristems may be inherently determined to adopt a meristematic fate or if they came under the influence of some physiological agent that then allowed them to adopt a unique identity. The results from this study would suggest that the cells that will eventually be a part of the SAM are not inherently determined. If they were, then their fate could not be altered, however, the TIBA treatment was observed to affect their ability to maintain a meristematic identity. The fate of the epiphyseal cells are then not determined from the time of their initiation. However, once these cells have divided and

the embryo is at the heart stage, it appears that the cells have become more stable in their identity and perhaps determined. The polar transport of auxin could be the signal that provides positional information to the epiphyseal cells that allows them to react differently from the surrounding cells. McDaniel (1984) suggests that auxin may have been involved in meristem initiation and stabilization during the early stages of meristem evolution. Other mechanisms for meristem stabilization may have evolved later, however, the role of auxin in the formation of the meristem was maintained.

Chapter 5 presented evidence that auxin may be influencing the cytoskeleton of the cells of the SAM. The cells of the SAM may be able to maintain their overall shape and division patterns because of the levels of auxin found in the region. Although the pattern of MT orientation differed in the TIBA treated zygotic and MD embryos (Fig 7.1), it is clear that an increase in auxin concentration did cause an alteration in the pattern. Furthermore, it also suggests that any deviation from the normal pattern of MT orientation does not allow for the pattern of cell divisions necessary for organogenesis to occur.

In Chapter 6, ABA was shown to greatly improve and maintain the characteristics of the SAM in MD embryos. This is an indication of the complexity of the physiological processes occurring during the early stages of embryogenesis *in planta* that has not been replicated *in vitro* at this point in time. It is evident that there are more than one hormone that influence SAM development during embryogenesis. Although ABA was shown to be necessary during the early stage of MD embryo development, its beneficial effects were not noticeable until the embryos were almost mature.

All of these results taken together suggest that before the SAM can be morphologically identified there is a group of cells, at the 16 cell stage of development, that are destined to become the future SAM. This group of cells is unique from the surrounding cells. The physiological environment affecting this group of cells is extremely important for the subsequent proper development and function of the SAM.

To summarize the important points that have arisen from this work:

- The cells of the SAM attain a unique identity from as early as the 16 cell stage of embryogenesis. The epiphyseal cells, however, are not fixed in their identity. Once this critical stage has been passed, the cells are stable in their identity.
- A lower level of auxin during the critical stages of formation is required for these cells to maintain their unique identity throughout embryogenesis.
- The pattern of SAM formation differs in zygotic and MD embryos. The lack of epiphyseal cells in the MD embryos suggest that this early cell division pattern is important in stable SAM formation. This precise cell division pattern may be associated with unique gene expression.
- The physiological events that occur during the early stage of embryogenesis also play an important role in the subsequent post-embryogenic function of the SAM.
- The process of SAM formation, maturation and function is a complex event that involves many physiological agents and occurs throughout the entire duration of embryogenesis.

The results from this thesis provide the framework for many future investigations:

- It would be a great asset if a system could be developed that would allow for the culture of zygotic embryos prior to the globular stage. It will be important to determine if auxin levels are altered prior to the formation of the epiphyseal cells, if the response will be similar to that observed in the MD embryos.
- It may also be useful to make a careful histological examination of the SAM formation in some of the developmental mutants of *Arabidopsis* that have been shown to inherently have a lowered polar transport of auxin.
- Examining the gene expression patterns of some of the genes unique to the SAM, for example *STM* or *WUS*, in control and TIBA-treated embryos may also provide insight into how auxin levels may be affecting SAM formation.
- It has been well established that the ratio of auxin to cytokinin influences whether a shoot or root producing meristem forms *in vitro*. Knowing what the effects of an increased level of auxin on SAM formation are, it is possible to examine if increasing the level of cytokinins can reduce these effects.
- Using these two systems, the possibility that auxin may be influencing the cytoskeleton of the cells of the SAM through the Ca^{2+}/calmodulin system can be investigated by observing whether the TIBA treatment has any effect on the pattern of Ca^{2+} or calmodulin distribution

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