# Stellingen

1. Cel-specifieke moleculaire merkers zijn essentieel voor de interpretatie van de fenotypische effecten van embryo mutaties.
   dit proefschrift.

2. Tijdens de *Arabidopsis* embryogenese worden patroonelementen eerst globaal en dan specifiek gedefinieerd.
   dit proefschrift.

3. Het Ac/Ds transposonsysteem leent zich, door het lage aantal transposon-inserties per plant, beter voor gebruik in gene / enhancer trap screens dan het En/I systeem.
   dit proefschrift; Aarts (1996), proefschrift LUW.

4. Het belang van het endosperm voor de voeding van de mens is duidelijker dan voor de voeding van het embryo.
   Lopes and Larkins (1993), Plant Cell 5, 1383-1399; West and Harada (1993), Plant Cell 5, 1361-1369; Meinke (1995), Annu. Rev. Plant Physiol. Plant Mol. Biol. 46, 369-394; Laux and Jürgens (1997), Plant Cell 9, 989-1000.

5. Ten gevolge van de fenotypische en functionele heterogeniteit is eenduidige definitie van een humane dendritische cel pas mogelijk nadat een unieke merker voor dit celtype gevonden is.
   Banchereau and Steinman (1998), Nature 392, 245-252; Hart (1997), Blood 9, 3245-3287; Peters *et al.* (1996), Immunol. Today 17, 273-278.

6. In ieder projectvoorstel zou een flinke post begroot moeten worden voor het schrijven van het volgende.

7. Het bedrijven van topsport en het uitvoeren van wetenschappelijk onderzoek kunnen synergetisch zijn.
   dit proefschrift.

8. De onderzoeksschool Experimentele Plantenwetenschappen (EPW) dient promovendi minstens één studiepunt te geven voor het succesvol invullen van het opleidings- en begeleidingsplan, en één voor het invullen van de formulieren ter verkrijging van het onderwijscertificaat.

**9.** Afgaande op het topsportbeleid van beide organisaties, zou de International Amateur Athletic Federation (I.A.A.F.) haar eerste A-tje over moeten dragen aan de Koninklijke Nederlandse Atletiek Unie (K.N.A.U.).

**10.** Zij die niet kunnen schrijven, schrijven handleidingen.

**11.** De modieuze borden 'Utrecht bereikbaar' langs Utrechts toegangswegen zijn een sterk staaltje van bestuurlijke ironie.

**12.** Het nieuwe ontwerp van de Wageningse markt is beslist geen eerbewijs aan het groene imago van de kennisstad.

**13.** Het verdedigen van een proefschrift op vrijdag de 13e tart het lot.

Stellingen behorende bij het proefschrift:
'Molecular markers in *Arabidopsis* embryos'
door Casper Vroemen, te verdedigen op 13 november 1998

# Molecular markers in *Arabidopsis* embryos

Promotoren : dr. A. van Kammen, emeritus hoogleraar in de moleculaire biologie
dr. S.C. de Vries, persoonlijk hoogleraar bij het laboratorium voor moleculaire biologie

Casper Willem Vroemen

# Molecular markers in *Arabidopsis* embryos

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C.M. Karssen,
in het openbaar te verdedigen
op vrijdag 13 november 1998
des namiddags te vier uur in de Aula.

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*"De kennis van de wetten waardoor mutaties optreden zal, naar het zich laat aanzien, er eens toe leiden dat men kunstmatig en willekeurig mutaties gaat opwekken en men op die manier geheel nieuwe eigenschappen bij planten en dieren zal laten ontstaan"*

Hugo de Vries, 'Die Mutationstheorie', 1901

# Contents


| | | |
|---|---|---|
| | Scope | 9 |
| **1** | Flowering Plant Embryogenesis | 11 |
| **2** | Pattern formation in the *Arabidopsis* embryo revealed by position-specific lipid transfer protein gene expression | 37 |
| **3** | Identification of genes expressed during *Arabidopsis thaliana* embryogenesis using enhancer trap and gene trap *Ds*-transposons | 51 |
| **4** | Molecular characterization of *Arabidopsis* enhancer trap and gene trap lines that exhibit *GUS* expression in embryos | 75 |
| **5** | Enhancer trap tagging of WET368, a regional marker for early *Arabidopsis* embryos | 103 |
| **6** | Summarizing discussion | 121 |
| | References | 129 |
| | Moleculaire merkers in *Arabidopsis* embryos: samenvatting | 145 |
| | Nawoord | 149 |
| | *Curriculum vitae* | 151 |
| | List of publications | 153 |
| | Appendix A: *Arabidopsis* mutants discussed in this thesis | 154 |

# Scope

In seed plants, sexual reproduction is initiated by pollen transfer from anther to stigma. One of the two sperm cells carried by the pollen grain fertilizes the egg cell in the flower's carpel, giving rise to a fertilized egg cell or zygote. The subsequent developmental process that represents the transition of the zygote to a multicellular seedling is termed zygotic embryogenesis. Zygotic embryos develop through a series of characteristic morphological stages, in dicots the globular, heart, torpedo, and bent-cotyledon stages. During this development, all distinct organs and tissues present in the seedling are arranged in their proper positions, a process called pattern formation. Along the apical-basal or longitudinal axis, the pattern consists of the shoot apical meristem, cotyledons (embryonic leaves), hypocotyl (embryonic stem) and radicle (embryonic root), including the root cap and root meristem. Along the radial axis, another pattern is apparent as a concentric arrangement of tissue types from outside to inside: the epidermis, ground tissue, and central vascular system. In the model plant *Arabidopsis* (wall cress), the sequence of cell divisions during zygotic embryogenesis is highly invariant, so that the origin of seedling organs and tissues appears traceable to specific cells in the early embryo. However, except for the early epidermal cell fate, no clonally transmitted lineages appear to be instrumental in pattern formation. Currently, numerous studies focus on the molecular events underlying plant embryo development. The current stage of this research area is discussed in chapter 1.

A widely followed approach to identify genes involved in pattern formation has been to screen for mutants with defects in the establishment of the embryo body plan. These genetic screens have yielded numerous embryo-defective mutants. However, a major difficulty that has emerged during these screens concerns the recognition and interpretation of informative phenotypes. Many different embryo-lethal mutants show quite similar phenotypes and the assessment of the precise effects of a mutation is often hampered by the inability to determine cell- or regional identity in the embryo mutant background. One way to partly circumvent these difficulties is to study the expression pattern of well defined molecular markers in embryo mutants. Markers reflecting cell- or regional identity or polarity in the developing embryo provide criteria other than morphology for the evaluation of the precise effects of an embryo mutation.

Chapter 2 describes the analysis of three embryo mutants using the *Arabidopsis thaliana* lipid transfer protein (*AtLTP1*) gene as a marker. In wild-type embryos, the *AtLTP1* gene is initially expressed in all epidermis cells, and later in the epidermal cells of the cotyledons and upper

hypocotyl, together representing the apical part of the embryo. Therefore, *AtLTP1* expression was used as tissue-layer specific marker for the epidermis to study the phenotypic defects in the *knolle* and *keule* mutants, both reported to have defects in the establishment of the epidermis. *AtLTP1* expression was used as marker for the apical part of the embryo to investigate effects of the *gnom* mutation on apical-basal embryo polarity.

Unfortunately, few other embryo marker genes are available to date, especially for the early stages of embryogenesis. This shortage of suitable molecular markers greatly hampers the recognition and interpretation of embryo phenotypes informative for the process of pattern formation. Therefore, we have performed an enhancer and gene trap insertional mutagenesis screen to identify *Arabidopsis* lines with *GUS* expression in embryos. This screen is described in chapter 3, and exploits two types of transposable *Ds* elements each carrying a *GUS* reporter gene that can respond to *cis*-acting transcriptional signals at the site of integration. The selected lines provide a set of markers that can be used to determine cell- or regional identity and polarity in *Arabidopsis* embryo mutants, and will allow the isolation of genes identified on the basis of their expression pattern in the *Arabidopsis* embryo.

Chapter 4 outlines the spectrum of *GUS* expression patterns observed in embryos during the screening of 431 enhancer trap and 373 gene trap lines. Four lines exhibiting remarkably early or localized *GUS* expression are described in more detail. Furthermore, electronic databases for the recording of screening data, and sequence analysis of genomic DNA flanking the transposon insertions in four enhancer trap and two gene trap lines are presented. Finally, the efficiency of enhancer and gene trap mutagenesis as a means of identifying genes that are important for embryo development is discussed.

Chapter 5 describes the identification of one specific enhancer trap line, WET368, that already shows uniform *GUS* expression in the 8-celled embryo. Later during embryogenesis, expression becomes restricted to a previously undefined region encompassing the shoot apical meristem and part of the cotyledon primordia. After germination, all aerial plant parts where meristems are or have been present are marked by WET368 *GUS* expression. Analysis of WET368 *GUS* expression in different mutants defective in the control of shoot meristem size or function provides an example of the way marker gene expression can extend morphological descriptions of mutant phenotypes.

Finally, a summarizing discussion of the research presented in this thesis is provided in chapter 6.

# 1

# Flowering Plant Embryogenesis

Casper W. Vroemen and Sacco C. de Vries

Modified from *Development - Genetics, epigenetics and environmental regulation* (Russo, E., Cove, D., Edgar, L., Jaenisch, R. and Salamini, F., eds.). Heidelberg: Springer-Verlag, in press.

## Introduction

An important area in plant developmental biology concerns the molecular basis of pattern formation, cell differentiation and organ development in the embryo. The majority of this work is being done in the model species *Arabidopsis thaliana*, and this plant species therefore features prominently in this chapter.

Although embryogenesis formally commences at fertilization, a brief introduction into the structure of female gametophyte or the embryo sac will be given first in order to describe the setting in which early embryogenesis occurs later. The embryo sac develops within the ovule, which is in turn found in the flower's carpel. The most common embryo sac form, usually called the polygonum-type embryo sac, is shown in Figure 1.1. It consists of seven cells: three antipodal cells at the chalazal pole, two synergids and one egg cell at the micropylar pole and one central cell with two polar nuclei in the center (reviewed by Reiser and Fischer, 1993). In some species, this is the final form of the embryo sac. In *Arabidopsis* however, the three antipodal cells degenerate and in maize they proliferate into as many as 100 cells in the mature embryo sac (Drews *et al.*, 1998). Sexual reproduction in seed plants is initiated when pollen is transferred from anther to stigma. Double fertilization of the egg cell and central cell by two sperm cells lead to the development of the zygotic embryo and the endosperm respectively. After fertilization, the zygote expands in a longitudinal direction. In *Arabidopsis*, the first division is asymmetric and results in an apical and a basal cell. The basal cell produces the suspensor and also contributes to the root meristem of the embryo, while from the apical cell the entire embryo except for part of the root meristem is formed (Figure 1.2).

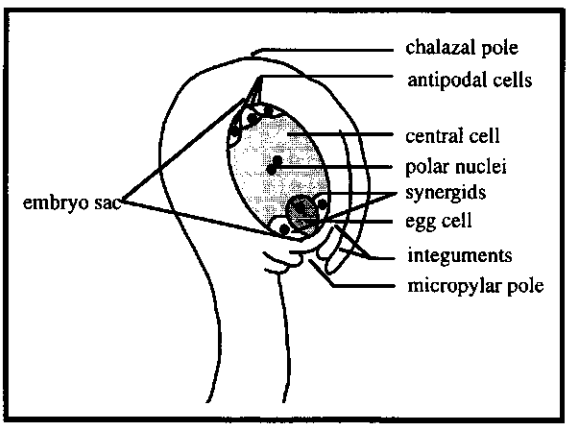


**Figure 1.1:** Schematic representation of an ovule with polygonum-type embryo sac. The orientation is such that the chalazal end is at the top and the micropylar end at the bottom of the drawing. From Mordhorst *et al.* (1997) with permission.

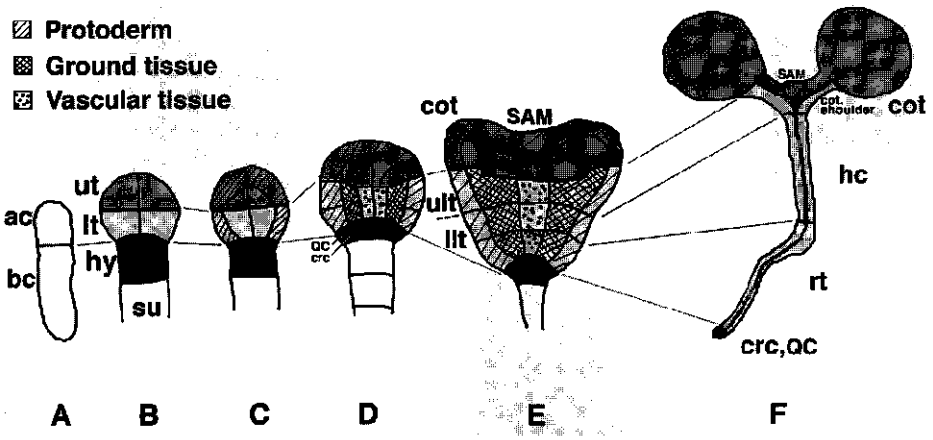


**Figure 1.2:** Pattern formation in the *Arabidopsis* embryo.

**A:** Two-cell stage. Asymmetric division of the zygote has yielded a small apical cell (ac) and a larger basal cell (bc).

**B:** Octant stage embryo. The apical cell has generated an embryo proper consisting of four upper tier (ut; grey) and four lower tier (lt; light grey) cells. The basal cell has given rise to the hypophyseal cell (hy; dark grey) and the suspensor (su; white).

**C:** Dermatogen stage embryo. Periclinal divisions have generated eight protoderm and eight inner cells.

**D:** Globular stage embryo. The inner cells of the lower tier have divided periclinally to yield ground tissue and vascular tissue. The hypophyseal cell has set off a lens-shaped cell that will give rise to the quiescent center (QC). The lower hypophyseal cell derivative will develop into the central root cap (crc).

**E:** Heart stage embryo. The apical domain, derived from the upper tier, has been partitioned into cotyledon (cot) primordia and shoot apical meristem (SAM). The central domain, derived from the lower tier, has been subdivided conceptually into the upper-lower (ult) and lower-lower (llt) tiers.

**F:** Seedling. The SAM and the largest part of the cotyledons are derived from the upper tier. The upper-lower tier has contributed to the cotyledon shoulders (cot. shoulder), while the lower-lower tier has formed the hypocotyl (hc), root (rt) and initials of the root meristem. The quiescent center and the central root cap are descendants of the hypophyseal cell.

Corresponding regions along the apical-basal axis of the developing embryo and seedling have corresponding grey scales. Tissue types along the radial axis are indicated by different fill patterns. Individual cells are shown in **A** to **C** and cell groups in **D** to **F**. Adapted from Laux and Jürgens (1997).

In contrast to animals, where the body pattern of the adult organism is complete after embryogenesis, most of the structures of adult higher plants are formed during postembryonic development from groups of proliferating cells, the root and shoot apical meristems (Kerstetter and Hake, 1997; Steeves and Sussex, 1989), both of which are formed during embryogenesis.

Studying pattern formation in the zygotic *Arabidopsis* embryo has the advantage that cell divisions are very regular, so that pattern elements can be traced back to their origins in the early embryo (Jürgens *et al.*, 1994a; Jürgens *et al.*, 1994b; Mansfield and Briarty, 1991; Scheres *et al.*, 1994). However, a regular pattern of early divisions is found in only a minority of plant species, and in certain *Arabidopsis* mutants and embryos of non-zygotic origin of this species (Mordhorst *et al.*, 1998; Wu *et al.*, 1992) there is very little regularity. Despite the variations in early divisions in such embryos, pattern formation appears normal and results in the correct positioning of all major pattern elements.

When compared to embryogenesis in other higher eukaryotes, plant embryogenesis is a unique process because it can be initiated not only from the zygote but also from other cells of the reproductive apparatus, including the gametes, and even from somatic cells. The plant zygote is therefore not unique in its property to develop into the entire multicellular organism. One of the challenges in plant embryogenesis is to unravel the molecular mechanisms that lead to the formation of a cell destined to form an embryo, whether the product of fertilization or of spontaneous or induced embryo development.

## Pattern formation, cell differentiation and organ development in the plant embryo

The plant embryo and the seedling derived from it after germination are arranged in a number of elements along an apical-basal or longitudinal axis, and along a radial or outside-to-inside axis (Figure 1.2). Along the apical-basal axis, the body pattern of a dicot embryo consists of the shoot apical meristem (SAM), cotyledons (embryonic leaves), hypocotyl (embryonic stem) and radicle (embryonic root), including the root cap and root meristem (RM). Along the radial axis, another pattern is apparent as a concentric arrangement of tissue types from outside to inside: the epidermis, ground tissue (cortex and endodermis), and central vascular system (pericycle, xylem and phloem) (Goldberg *et al.*, 1994; Jürgens, 1995; Jürgens *et al.*, 1994a; Jürgens *et al.*, 1994b; Mayer *et al.*, 1991; Vroemen *et al.*, 1996). The basic plant body pattern can be viewed as a superimposition of the apical-basal and radial patterns. In this section, recent insight obtained from molecular-genetic analysis in the events that generate this pattern in the *Arabidopsis* embryo will be described.

### *The apical-basal pattern*

In many plant species, the unfertilized egg cell as well as the zygote exhibit apical-basal polarity aligned with the chalazal-micropylar axis of the embryo sac (Figure 1.1). This is demonstrated by the

unequal distribution of cytoplasm and vacuoles (Mogensen and Suthar, 1979; Schulz and Jensen, 1968b). Before its first division, the *Arabidopsis* zygote elongates in the apical-basal direction. This elongation coincides with a re-orientation of microtubules to transverse cortical arrays (Webb and Gunning, 1991). The first division is an unequal transversal division, resulting in two cells of different developmental fates (Figure 1.2A). The basal daughter cell divides by a series of transversal divisions and finally gives rise to a filamentous suspensor consisting of 7 - 9 highly vacuolated cells (Figure 1.2B-E; Figure 1.3A-E; Mansfield and Briarty, 1991). The hypophyseal cell is the uppermost cell of the suspensor (Figure 1.2B-E; Figure 1.3A-E), and contributes to the embryo by forming part of the root, namely the central (columella) root cap and the quiescent center (Scheres *et al.*, 1994). The suspensor reaches maximum cell number by the globular stage. Subsequently, suspensor cell number decreases (Mansfield and Briarty, 1991; Mardsen and Meinke, 1985), until only a few remain at maturity.

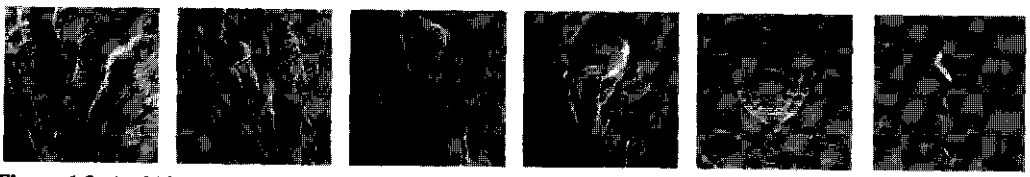

**Figure 1.3:** *Arabidopsis* embryogenesis. Differential interference contrast (**A** through **E**) and light (**E**) microscopic images of embryos and seedling at developmental stages as represented in Figure 1.2.

**A:** One-celled embryo proper. The suspensor already consists of approximately four cells.

**B:** Octant stage embryo.

**C:** Dermatogen stage embryo.

**D:** Globular stage embryo.

**E:** Heart stage embryo.

**F:** Seedling.

Scale of **F** is different from scale of **A** through **E**.

The apical daughter cell of the zygote undergoes two longitudinal divisions at right angles, followed by one transversal division (Jürgens and Mayer, 1994; Mansfield and Briarty, 1991). The latter plane of division divides the eight celled "embryo proper" (octant stage) into an upper and a lower tier (Figure 1.2B; Figure 1.3B). From the upper tier the shoot apical meristem and the main parts of the cotyledons are formed, while the lower tier contributes to the cotyledon shoulder, the entire hypocotyl, and part of the radicle (Scheres *et al.*, 1994); Figure 1.2C-F; Figure 1.3C-F). Until

the octant stage, there is a remarkable decrease in relative cell size (Mansfield and Briarty, 1991). As the octant embryo develops into a globular embryo of approximately 64 cells (Figure 1.2C, D; Figure 1.3C, D), the cells of the lower tier produce cell files along the apical-basal axis through oriented divisions and cell elongation, while most cells of the upper tier divide in more or less random orientations.

A dramatic change in embryo morphology occurs during the transition from the globular to the heart stage (Figure 1.2E; Figure 1.3E), as observed by the formation of juxtaposed cotyledon primordia at the apical side of the embryo (Jürgens and Mayer, 1994). At the heart stage the hypocotyl region also becomes visible due to further cell elongation in the lower tier, which is conceptually subdivided into an upper-lower tier and a lower-lower tier (Figure 1.2E) . At the same stage, the root meristem initials are defined from the most basal cells in the lower tier. With the completion of the apical-basal pattern in the form of cotyledons, hypocotyl and radicle the body plan of the seedling (Figure 1.2F; Figure 1.3F) is essentially complete in the heart stage embryo. The subsequent torpedo stage embryo is a result of cell elongation and expansion rather than continued division. The SAM can now for the first time be distinguished morphologically as a small group of cells between the bases of the cotyledons. The SAM therefore does not appear before the RM is nearly fully formed and functional (Barton and Poethig, 1993). During the transition from the torpedo to the final bent cotyledon stage, the cotyledons expand further and are finally folded backwards.

In conclusion, the subdivision of the octant embryo into upper tier, lower tier and hypophyseal cell is elaborated during subsequent stages of embryogenesis, to generate an ordered array of structures along the apical-basal axis. Through the regular pattern of cell divisions, and through fate map analysis (Scheres *et al.*, 1994), the origin of these structures can be traced back to progenitor cells in the early embryo with reasonable accuracy.

*Establishment of early apical-basal polarity*

In *Arabidopsis* the apical-basal axis of the embryo always forms in the same orientation relative to the surrounding embryo sac. This orientation can be dependent on the morphological polarity of the unfertilized egg cell, or imposed by the polar organization of the surrounding maternal tissues. Until now, only one gene, *SHORT INTEGUMENT1* (*SIN1*), has been identified that has a maternal effect on the apical-basal axis of the progeny upon mutation (Ray *et al.*, *1996*). Seedlings descending from *sin1* mutant mother plants show variable defects in the apical-basal axis. However, maize zygotes formed by *in vitro* fusion of isolated egg and sperm cells acquire polarity before undergoing an asymmetric division (Breton *et al.*, 1995). Egg cells of certain plant species appear apolar, or their polarity can be reversed upon fertilization (Johri, 1984), suggesting that no stable axis of polarity exists within the unfertilized egg cell, from which the polarity of the embryo is derived.

Suspensor cells can form secondary embryos if the primary embryo is aborted or arrested in development, such as in the *sus*, *twin*, and *raspberry* mutants (Schwartz *et al.*, 1994; Vernon and Meinke, 1994; Yadegari *et al.*, 1994; Yadegari and Goldberg, 1997). This suggests that the apical part of the embryo normally inhibits an 'embryo' fate in suspensor cells. In *twin2* (*twn2*) mutants, the development of the apical daughter cells of the zygote arrests after one or two zygotic divisions, and subsequently multiple embryos are formed from suspensor cells (Zhang and Somerville, 1997). In *twin1* (*twn1*) mutants, aberrant divisions occur in the embryo, and subsequently a secondary embryo is formed from the suspensor (Vernon and Meinke, 1994). Strikingly, the axis of polarity of the secondary embryo is either in the same, or in reverse orientation as that of the primary embryo. This raises the possibility that during normal embryo development, the position of the suspensor relative to the early embryo proper is instrumental in establishing the basal embryo pole. In this scenario, the basal pole of suspensor-derived *twin* embryos can form on either side, since the early secondary embryo is flanked by suspensor cells on both sides. Alternatively, the basal pole of suspensor-derived embryos may be established at random because in contrast to normal embryos, these embryos do not originate from a polarized zygote.

The acquisition of different cell fates after the first division of the zygote is reflected by the expression of the *ARABIDOPSIS THALIANA MERISTEM LAYER1* (*ATML1*) gene in the apical, but not in the basal cell (Lu *et al.*, 1996). This homeobox containing gene continues to be expressed in all derivatives of the apical cell until the octant stage. Mutations in the *Arabidopsis GNOM/EMB30* (*GN*) gene affect the asymmetric division of the zygote, resulting in an enlarged apical cell at the expense of the basal cell (Mayer *et al.*, 1993; Shevell *et al.*, 1994). While in *gnom* embryos the apical cell gives rise to an embryo proper that displays abnormal divisions, a shortened suspensor is formed from the basal cell. The *ARABIDOPSIS THALIANA LIPID TRANSFER PROTEIN1* (*ATLTP1*) gene, whose expression is normally restricted to the apical end of later stage embryos, and the *POLARIS* gene, that is normally only expressed in the "root pole", display variable and occasionally completely reversed expression along the apical-basal embryo axis (Topping and Lindsey, 1997; Vroemen *et al.*, 1996). The *GNOM* gene is zygotically required and the encoded protein shows sequence similarity to yeast guanine-nucleotide exchange factors involved in vesicle transport (Busch *et al.*, 1996; Peyroche *et al.*, 1996). This raises the possibility that directional vesicle transport is involved in stabilizing the apical-basal axis of the embryo, which would be reminiscent of the proposed axis stabilization mechanism in the brown algae *Fucus* (Kropf, 1997; Shaw and Quatrano, 1996).

Thus, the available evidence suggests that the formation of the apical-basal axis of the embryo is not yet fixed before fertilization, requires segregation of cell fates after the first division, but may only be completely stabilized later in embryo development. It has not been demonstrated unequivocally whether the maternal tissues surrounding the early embryo influence the orientation of the apical-basal axis, but the physical attachment of the embryo to the suspensor might be

instrumental in establishing the basal embryo pole.

*Establishment of embryonic domains along the apical-basal axis*

The different components that make up the final apical-basal pattern elements of the embryo do not originate simultaneously, but are established in steps. Thus, the establishment of the apical-basal pattern may be viewed conceptually as a series of partitioning events that sequester increasingly more specialized regions (West and Harada, 1993). The subdivision of the octant stage embryo into upper tier, lower tier and hypophyseal cell is elaborated during subsequent stages of embryogenesis. The upper tier gives rise to an apical domain, consisting of the shoot apical meristem and most of the cotyledons. The lower tier generates a central domain which comprises the "cotyledon shoulder", hypocotyl, root and the root meristem initials above the quiescent center. The remaining part of the root meristem, i.e. the quiescent center and the central root cap, is derived from the hypophyseal cell (Figure 1.2). It is evident that the three regions in the early globular stage embryo do not correspond precisely to the primordia of the different components of the apical-basal pattern in the later stage embryo. For example, the cotyledons are derived partly from the upper, and partly from the lower tier of the octant stage embryo, and the root meristem is composed of descendants from both the lower tier and the hypophyseal cell (Figure 1.2). Nevertheless, the significance of these three early regions for apical-basal patterning can be deduced from mutant embryonic phenotypes that affect one or more domains of the apical-basal pattern.

Mutations in the *GURKE/EMB22* (*GK*) gene affect the apical domain of the embryo (Torres-Ruiz *et al.*, 1996). The mutational defects are first seen in the derivatives of the upper tier in early heart-stage embryos, where abnormal or no divisions occur in the cotyledonary primordia. Later, the SAM and cotyledons are severely reduced or abolished, and in the most extreme manifestation of the phenotype, even the cotyledon shoulders and part of the hypocotyl, which are clonally derived from the lower tier, are deleted. This could mean that the cotyledons are initiated in the apical domain, which subsequently induces cells from the central domain to form the cotyledon shoulders and upper hypocotyl. Alternatively, *GK* may be functional in both apical and central domains of the embryo. *gk* mutants have a normal root and radial pattern.

Mutations in the *MONOPTEROS* (*MP*) gene cause deletion of the entire root and hypocotyl in the embryo and seedling (Berleth and Jürgens, 1993), and the *mp* mutant has thus been classified as an apical-basal pattern mutant affecting the central and basal regions of the embryo. The earliest deviation from wild-type is observed at the octant stage, when the *mp* embryo proper consists of four rather than two tiers of cells. Subsequently, cells of the central and basal domains, which are derived from the lower tier and the hypophyseal cell, divide abnormally. The inner cells of the central domain fail to produce elongated cell files that normally make up the vascular tissue of the hypocotyl and root.

At the same time, the basal cell that would normally become the hypophyseal cell divides like a suspensor cell to generate a central pile of cells continuous with the suspensor. The latter defect has been interpreted as an indirect consequence of the aberrations in the lower tier. In this view, the uppermost suspensor cell only becomes the hypophyseal cell after signalling from the lower tier cells. In an alternative view the *MP* product could be required in both the lower tier and the hypophyseal cell. The ability of *mp* seedlings to form adventitious roots was used to study the post-embryonic defects caused by the *mp* mutation. Although *mp* plants can make largely normal aerial structures, all organs contain differentiated, but insufficiently interconnected vascular strands, and polar auxin transport is reduced (Przemeck *et al.*, 1996). These observations led to the conclusion that the *MP* gene is primarily involved in "axialization", and the absence of *MP* gene activity would result in the formation of non-continuous cell files. The *MP* gene encodes a transcription factor also identified by others as a regulator of an early auxin-induced gene, possibly involved in relaying auxin signalling (Hardtke and Berleth, 1998). *MP* is expressed in all subepidermal cells of the globular embryo, and becomes gradually confined towards the sites of vascular differentiation during further embryonic and post-embryonic development. The *MP* gene is proposed to promote the formation of continuous cell files, that are required to relay axial information at the onset of the hypocotyl-root axis in the early embryo. Thus, basal embryonic patterning is either dependent on correct vascular differentiation, or both processes are directed by common apical-basal signalling.

Mutations in the *FACKEL* (*FK*) gene specifically delete the hypocotyl (Mayer *et al.*, 1991). In the globular stage *fk* embryo, the cells of the lower tier do not form the elongated vascular precursor cells of the future hypocotyl. *fk* mutants have, in contrast to *mp* mutants, a normal root. Since the root is derived from both the hypophyseal cell and the lower tier, this raises the possibility that the hypophyseal cells induce adjacent lower tier cells to become root meristem initials, which can then produce the root.

### *The radial pattern*

Periclinal divisions (perpendicular to the radial axis) of all cells of the octant stage embryo (Figure 1.2B; Figure 1.3B) lead to the dermatogen stage (Figure 1.2C; Figure 1.3C). The formation of an outer cell layer of epidermal precursor cells (protoderm) and an inner cell group, each consisting of eight cells, is the first visible sign of radial pattern formation. The protoderm will then be maintained by continued anticlinal (circumferential) divisions and develop into the epidermis of the entire embryo (Jürgens and Mayer, 1994; Mansfield and Briarty, 1991). The inner cells divide again and contribute in the lower tier to the innermost vascular tissue (procambium) and the parenchymal ground tissue. Together with the protoderm, three concentric tissue layers are thus established that make up the three radial pattern elements of the embryo. As such, the radial pattern is established in a preliminary form

when the embryo reaches the globular stage. At the late-globular stage, the procambium cells perform a periclinal division to generate the pericycle and the innermost vascular bundle. The ground tissue of the upper-lower tier forms an additional layer of ground tissue cells characteristic of the hypocotyl region. In the lower-lower tier, only one ground tissue layer is maintained, characteristic of the radicle. During the heart stage, the ground tissue of the hypocotyl and radicle splits into an inner layer of endodermis and an outer layer of cortex cells.

The progenitor cells of the epidermis, cortex-endodermis, and vascular tissue therefore become clonally distinct by the early globular stage of embryogenesis. At the late heart stage all tissue layers, from outside to inside the protoderm, one (in the radicle) or two (in the hypocotyl) cortex layers, the endodermis, pericycle, and vascular bundle, have been established. From then on, the tissue layers are extended by mitotic activity of the root meristem initials (Scheres *et al.*, 1995).

*Establishment of embryonic tissue types along the radial axis*

Like the components of the apical-basal pattern, the different tissue types that make up the radial pattern of the embryo are established in steps. The radial pattern is established in a preliminary form when the embryo reaches the early globular stage. At this stage, the protoderm, ground tissue and vascular tissue are present as three concentric tissue layers. Later during embryogenesis, the radial pattern is further elaborated in the central domain of the embryo, giving rise to a pericycle layer surrounding the vascular bundle, a double cortex layer in the hypocotyl, and separate cortex and endodermis layers in hypocotyl and radicle (see below under "Elaboration of the preliminary radial pattern in the central domain of the embryo").

Radial pattern formation is initiated when all eight cells of the octant stage embryo divide periclinally, yielding eight protoderm cells overlying eight inner cells. The *ATML1* gene is expressed in the apical daughter cell of the zygote and in all cells of the octant stage embryo (Lu *et al.*, 1996). Separation of the protoderm and inner cells coincides with restriction of *ATML1* expression to the protoderm. This raises the possibility that epidermal cell fate, as reflected by *ATML1* expression, is already established in the apical daughter cell of the zygote, and that the internal cells formed by periclinal divisions in the octant stage embryo represent the first non-epidermal cells. This idea was originally proposed for the determination of epidermal cell fate in *Citrus* embryos on the basis of the observation that the zygote and its apical daughter cells are already coated with a cuticular wax layer, which is a morphological marker for epidermal cells (Bruck and Walker, 1985a). Soon after inner cells are separated from protodermal cells, the latter start to express the *ARABIDOPSIS THALIANA LIPID TRANSFER PROTEIN1* (*ATLTP1*) gene.

In *knolle* mutants incomplete cell walls are formed, and the radial organization of the embryo is not established properly (Lukowitz *et al.*, 1996). In early *kn* embryos, inner cells cannot be

distinguished from the protodermal layer. This coincides with the lack of restriction of *ATLTP1* expression to the outer cell layer (Vroemen *et al.*, 1996). Apparently, the establishment and stable maintenance of cell fates along the radial axis requires the proper separation of tissue layers. The finding that fluorescent dye spreads within the hypocotyl epidermis of *Arabidopsis* seedlings, but not into the underlying ground tissue, demonstrates that these tissue layers are normally cytoplasmically isolated (Duckett *et al.*, 1994). In *kn* embryos, incomplete cell wall formation results in cytoplasmic connections between the outer and inner cell layers. As a result, hypothetical protoderm and inner cell fate determinants might not be segregated to the adjacent cell layers. More generally, the uncoupling of cytoplasmic connections may be instrumental in segregating different cell fates. Internal cells in *kn* embryos later discontinue *ATLTP1* expression and differentiate into vascular tissue. These changes may reflect the increased distance of the innermost cells from the outer cell layer. Alternatively, they could be the result of more complete cell wall formation, and thus an increased separation of tissue types. The *KN* gene is expressed in a "patchy" pattern of cells throughout the embryo from the octant stage onward, and encodes a syntaxin-like protein. Syntaxins are involved in vesicle trafficking during for example cell plate formation, which explains the observed incomplete cell walls in *kn* embryos (Lukowitz *et al.*, 1996).

*keule* (*keu*) mutant embryos have a protoderm layer consisting of bloated and irregularly arranged cells, while the ground and vascular tissues look normal. Detailed phenotypic analysis suggests that, like *KN*, *KEULE* is involved in cytokinesis (Assaad *et al.*, 1996). However, normal protoderm-specific *ATLTP1* expression in *keu* embryos suggests that the establishment of the radial pattern is unaffected by the *keu* mutation (Vroemen *et al.*, 1996).

In conclusion, only one mutant with an early embryonic defect in the establishment of the radial pattern has been identified so far. Most likely, no mutations have been identified yet in genes that convey specific information for radial pattern formation before the globular stage of embryogenesis. The *ATML1* gene may be such an instructive gene, but this awaits the phenotype upon inactivation of the gene.

*Elaboration of the preliminary radial pattern in the central domain of the embryo*

Several mutations in the radial pattern of the later stage embryo have been described (Benfey *et al.*, 1993; Scheres *et al.*, 1995). In *shortroot* (*shr*) and *scarecrow* (*scr*) mutants, the ground tissue layer does not segregate separate cortex and endodermis layers. *shr* embryos have a cortex layer, but fail to establish the endodermis. By contrast, the single cell layer in *scr* expresses both cortical and endodermal traits. Both phenotypes become apparent at the heart stage, when the periclinal division of the ground tissue cells that normally generates cortex and endodermis does not occur. Is the absence of a specific cell layer in *shr* and *scr* caused by defective cell fate specification in the ground tissue, or

by a defect in cell division that prevents the ground tissue cells from making the periclinal division necessary to generate two separate cell layers? To address this question, double mutants were made with the *fass* mutant (Scheres *et al.*, 1995). *fass* mutant embryos display an irregular sequence of cell divisions in the early embryo, which results in an increased number of cell layers along the radial axis (Torres-Ruiz and Jürgens, 1994; Traas *et al.*, 1995). *shr fass* double mutants have more ground tissue layers, but none of these display endodermis characteristics. These observations suggest that *SHR* specifies endodermal cell fate. By contrast, *scr fass* double mutants have one endodermal layer surrounded by multiple cortical layers. Apparently, SCR is required for the periclinal division in the ground meristem that leads to separate cortex and endodermis layers. The *SCR* gene encodes a novel putative transcriptional regulator (Di Laurenzio *et al.*, 1996), and is expressed in the ground tissue from the late heart stage onward. After the separation of ground tissue into cortex and endodermis, *SCR* expression continues in the endodermis. In the post-embryonic root, *SCR* is expressed in the cortex-endodermis initial and also in the endodermis (Di Laurenzio *et al.*, 1996; Malamy and Benfey, 1997). Therefore, SCR may not only be involved in regulating the division separating cortex and endodermis, but could also be involved in expressing endodermal attributes (Dolan, 1997).

Mutations in another gene, *WOODEN LEG* (*WOL*), result in a reduction of the number of vascular cells. Normally, vascular cells differentiate into xylem or phloem vessels late during embryogenesis. In *wol* mutant embryos, all vascular cells differentiate into xylem vessels, and no phloem is specified (Scheres *et al.*, 1995). An increased number of vascular cells in the *wol fass* double mutant restores phloem specification. This implies that *WOL*, like *SCR*, affects cell division rather than specifying a specific cell fate. It is noteworthy that the radial pattern mutations described here display the same defects in post-embryonically formed lateral roots (Scheres *et al.*, 1995), implying that mechanisms of pattern formation that operate in the embryo are also used in other developmental contexts.

The *scr* and *wol* phenotypes suggest that the number of cells or cell layers available is critical for the establishment of specific cell fates. The formation of xylem in *wol* mutants may consume all the available vascular cells, and as a result no phloem forms. The fact that xylem is formed, and not phloem, implies that xylem is normally specified before phloem. This "first-come-first-served" mechanism has also been suggested for the sequential allocation of cells to floral organ primordia (Laux *et al.*, 1996).

Taken together, the radial pattern of the embryo is established in steps, starting with the separation of inner cells from protodermal cells in the octant stage embryo. Several mutants have been identified that lack specific radial pattern elements in later stage embryos, and one of these (*shortroot*) seems primarily affected in specifying cell fate. A minimum number of cells seems to be required for the proper separation of all tissue-specific cell fates. The fact that the radial pattern is not elaborated in the same way in each of the regions along the apical-basal axis suggests that apical-basal positional cues influence the response of cells to radial patterning signals.

### *Establishment of the root and shoot meristems*

The root and shoot meristems are established at opposite ends of the apical-basal axis of the embryo. They are elements that are part of the apical-basal and the radial pattern, and for this reason are discussed here separately. While the shoot meristem originates entirely from the upper tier, the root meristem is derived from two clonally distinct regions, namely the lower tier and the hypophyseal cell (see Figure 1.2).

#### *The shoot apical meristem*

Although its precise origin cannot be deduced directly from histological data (Barton and Poethig, 1993; Irish and Sussex, 1992), the SAM becomes first recognizable at the torpedo stage of *Arabidopsis* embryogenesis as a small group of cytoplasmic dense cells between the bases of the cotyledons. The SAM is organized in three cell layers, L1, L2 and L3 (Satina *et al.*, 1940; Figure 1.4). The outermost L1 layer derives from the embryo protoderm cells that originate from the upper tier in the octant stage embryo. The L2 and innermost L3 layers derive from subepidermal cells located in the center of the apical region of the embryo. Strictly anticlinal divisions in L1 and L2 form two clonally distinct "tunica" layers, whereas the cells in L3 divide in various orientations to form the "corpus" (Barton and Poethig, 1993; Clark, 1997; McConnell and Barton, 1995).

Superimposed on the three horizontal cell layers, the SAM can be divided conceptually into three zones, the central zone (CZ), peripheral zone (PZ), and rib zone (RZ), although their

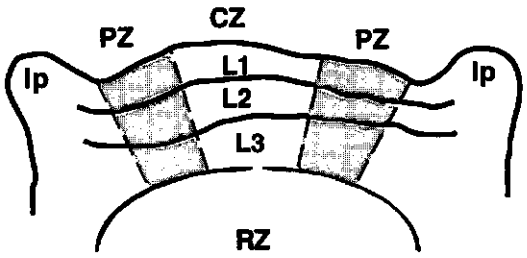


**Figure 1.4:** Schematic representation of the shoot apical meristem. The L1 and L2 tunica layers overly the L3 corpus. The zonation of the SAM includes a central zone (CZ) of undifferentiated stem cells, a peripheral zone (PZ) in which cells are incorporated into organ primordia, and a rib zone (RZ) that contributes to vascular tissue and interior stem structures. On each side of the meristem, a leaf primordium (lp) is indicated. The leaf primordia are formed late during *Arabidopsis* embryogenesis perpendicular to the cotyledons (not indicated; Laux and Jürgens, 1997).

morphology in the embryo is indistinct (Figure 1.4). The CZ consists of undifferentiated stem cells at the very center of the meristem. These produce daughter cells that adopt specific developmental fates as they enter the surrounding PZ, or the underlying RZ (Endrizzi *et al.*, 1996; Kerstetter and Hake, 1997; Steeves and Sussex, 1989). In the PZ, cells are incorporated into organ primordia, the first being two leaf primordia that are established perpendicular to the cotyledons before the embryo reaches maturity (Laux and Jürgens, 1997; Figure 1.4). Cells in the RZ contribute post-embryonically to the vasculature tissue and internal stem structures (Steeves and Sussex, 1989).

Mutations in the *SHOOT MERISTEMLESS* (*STM*) gene eliminate the entire SAM in embryos and seedlings (Barton and Poethig, 1993; Endrizzi *et al.*, 1996). The mutational defect becomes first apparent in mature embryos, when the characteristic bulge of cytoplasmic dense cells does not form between the bases of the cotyledons. Post-embryonically, adventitious shoots can form but terminate prematurely, likely to be due to a depletion of undifferentiated cells in the meristem center (Endrizzi *et al.*, 1996). STM activity is required to specify the meristematic nature of SAM cells during embryogenesis and thus to maintain a pool of undifferentiated cells in the center of the SAM. *STM* encodes a putative homeodomain transcription factor of the KNOTTED class (Long *et al.*, 1996), and is first expressed in one or two cells in the apical domain of the globular embryo, long before the visible presence of the SAM and the aberrant SAM morphology in *stm* mutant embryos (Long *et al.*, 1996). *STM* expression expands to include the entire histologically visible embryonic SAM, and during post-embryonic development, covers a central region of all shoot and floral meristems. It is unknown whether *STM* is sufficient for meristem formation, as is the case for its maize homologue *KNOTTED1* (Smith *et al.*, 1995). Ectopic expression of the *Arabidopsis KNOTTED*-like gene *KNAT1* (*KNOTTED ARABIDOPSIS THALIANA1*) is sufficient to induce ectopic shoot meristems on seedling leaves (Chuck *et al.*, 1996).

The *AINTEGUMENTA* (*ANT*) gene is expressed in two cell groups flanking the *STM* expressing cells in the apical domain of the globular stage embryo, and subsequently in the cotyledon primordia (Elliot *et al.*, 1996). The expression patterns of *STM* and *ANT* indicate that the apical domain of the globular embryo is partitioned into a central area destined to become the shoot meristem and a surrounding area from which the cotyledons develop.

Mutations in the *CLAVATA1* (*CLV1*) and *CLV3* genes result in an enlarged shoot meristem caused by accumulation of excessive numbers of undifferentiated cells in the CZ, a phenotype opposite to that of *stm* (Clark *et al.*, 1993; Clark *et al.*, 1995; Leyser and Furner, 1992; Weigel and Clark, 1996). The *clv* phenotypes become morphologically visible in the mature embryo. *clv1 clv3* double mutants suggest that the two corresponding genes act in the same pathway (Clark *et al.*, 1995). There are two possible models for the action of the *CLV* and *STM* genes. First, *CLV* genes may promote differentiation, and thus the transition of cells from the CZ into the PZ. Second, *CLV* genes may restrict the rate of cell division in the CZ, and thus the size of the CZ. In these models, *STM* would either limit differentiation, or promote cell division in the CZ. The *CLV1* gene encodes a

leucine-rich repeat transmembrane receptor kinase, and is expressed in the L3 layer of the meristem center, but in a region larger than the CZ. CLV1 may perceive positional information directed to cells expressing the receptor (Clark *et al.*, 1997). *clv* and *stm* mutants can dominantly suppress each others phenotypes, indicating that *CLV* and *STM* play opposite and possibly competitive roles in the regulation of meristem activity and size. Moreover, these data suggest that *CLV* and *STM* either both influence differentiation, or both affect cell division (Clark *et al.*, 1996)

An enlarged shoot apical meristem is found in mutant *altered meristem programming1/primordia timing* (*amp1/pt*) embryos. This phenotype is already apparent at the globular stage, when cells in the entire apical domain of the embryo acquire the dense cytoplasmic appearance typical of cells in the shoot meristem (Conway and Poethig, 1997). Unlike *clv*, the *amp1/pt* mutation has little effect on the post-embryonic shoot meristem. The highly pleiotropic effects of the *amp1/pt* mutation imply that *AMP1* function is not limited to the shoot apical meristem, but functions in many aspects of plant development.

*wuschel* (*wus*) mutants, like *stm* mutants, also lack a functional embryonic shoot meristem, which is first visible in the mature embryo. In contrast to *stm* shoot meristems, *wus* shoot meristems still contain central cells, but they are larger and more vacuolated than wild-type shoot meristem cells. *wus* seedlings have a flat rather than a convex shoot meristem, and no histological differences between cells of the central and peripheral zones are evident. *wus* seedlings repetitively initiate defective shoot meristems, that terminate prematurely during primordia initiation across the whole mutant shoot meristem, including the meristem center. Collectively, these observations suggest a role for WUS in specifying cell identity in meristem centers, rather than in the initiation of the shoot meristem (Endrizzi *et al.*, 1996; Laux *et al.*, 1996). This is in line with the epistasis of *STM* over *WUS* (Evans and Barton, 1997), so *STM* may act before *WUS*.

Mutations in the *ZWILLE* (*ZLL*) gene cause embryonic defects similar to those observed in *wus* mutant embryos (Endrizzi *et al.*, 1996). *zll* embryos and seedlings have a non-functional shoot meristem, whose cells are larger and more vacuolated than wild-type shoot meristem cells. However, post-embryonically formed adventitious shoot meristems can be initiated in *zll* mutants, as is also the case in *pinhead* (*pnh*) mutants (McConnell and Barton, 1995), in which the most extreme phenotype resembles *zll*. These observations correlate with a downregulation of *STM* expression in the *zll* meristem center of late stage embryos, suggesting that the cells in the CZ of the *zll* SAM have initiated differentiation. *STM* expression is normal during early embryonic stages and during post-embryonic development. Collectively, these data suggests that SAM initiation is not affected by the *zll* mutation, and that *ZLL* is specifically required to maintain meristematic cell fate in the CZ of the embryonic SAM. The *ZLL* gene encodes a novel protein, and is expressed in the SAM center of mature embryos, which correlates with the observed phenotypic defect. Surprisingly, *ZLL* expression was also observed in vascular cells, starting as early as the globular embryo stage (Moussian *et al.*, 1998). Although the significance of the *ZLL* expression in vascular cells is unclear, it could point towards a

transient requirement for signals from the vascular system for the partitioning of the embryonic SAM into a CZ of undifferentiated cells and a PZ of differentiating cells.

One other class of mutants has been identified that affects the formation of the SAM. Mutations in *CUP-SHAPED COTYLEDON* (*CUC*) genes result in embryos and seedlings without a SAM (Aida *et al.*, 1997). However, the most pronounced defect in *cuc* embryos seems to be an incomplete separation of the cotyledons. In *cuc* flowers, incomplete separation of sepals and stamens was observed. The mutant phenotype becomes apparent first in the heart stage embryo, when no distinct cotyledon primordia are visible, but is more pronounced during later stages of embryogenesis, when a collar-like structure forms at the apical end of the embryo. The SAM is completely lacking in *cuc* embryos, and post-embryonic adventitious SAM formation is partly inhibited by the *cuc* mutation. Whether the SAM and organ separation defects in *cuc* mutants are causally related is unclear. The *CUC* gene encodes a putative transcriptional activator and is homologous to the petunia *NO APICAL MERISTEM* (*NAM*) gene, that is expressed at boundaries of meristems and organ primordia, and mutation of which results in a similar phenotype as the *cuc* phenotype (Souer *et al.*, 1996).

The genetic and molecular data available so far show that a controlled balance between cell division and cell proliferation is required to maintain a functional SAM. The embryonic SAM seems to be established in at least two phases. First, cells in the apical domain of the globular embryo become specified towards SAM fate and initiate expression of specific genes such as *STM*. Second, a functional meristem is formed through partitioning of the SAM primordium into a central zone harboring undifferentiated cells, and peripheral and rib zones in which cells differentiate into organ primordia. It is thus far unknown whether the genes controlling this partitioning primarily regulate cell division, cell differentiation, or both. In any case, the *ZLL* expression pattern suggests that signalling from the vascular tissue may play a role.

*The root meristem*

The primary root meristem (RM) in the *Arabidopsis* embryo consists of two tiers of initial cells surrounding a group of four mitotically inactive cells, the quiescent center (QC; Dolan, 1998; Dolan *et al.*, 1993; Scheres *et al.*, 1994). The RM arises from two distinct groups of cells that are clonally separated at the first division of the zygote. The QC and the central root cap arise from the hypophyseal cell, that is, in turn, derived from the basal daughter cell of the zygote (Figure 1.2). The initials above the QC arise from the apical daughter cell of the zygote. The hypophyseal cell sets off a lens-shaped cell during the globular stage (Figure 1.2D; Figure 1.3D). The descendants of this cell will form the QC, while the lower hypophyseal cell derivative develops into the initials of the central root cap. Starting at the late heart stage, these initials will produce additional layers of central root cap

cells.

The initials above the QC display essentially the same radial organisation as the radicle: at the early heart stage, one layer each of epidermis, ground tissue, and pericycle surround a core of vascular precursor cells. At the late heart stage, the lowermost epidermal cell divides periclinally, giving rise to the lateral root cap initial and the epidermal initial. Further periclinal divisions of the lateral root cap initial produce additional layers of lateral root cap cells. Periclinal divisions in the daughters of the ground tissue initial give rise to the cortex and endodermis, turning the ground tissue initial into a "cortex-endodermis initial".

In conclusion, the RM is derived from two clonally distinct regions of the early embryo, and arises through a highly invariant sequence of cell divisions. The radial pattern of the RM is evident by the late heart stage of embryogenesis. Within the meristem, initial cells and their daughters perpetuate this pattern by ordered cell divisions.

The "hypophyseal cell group" mutants *hobbit* (*hbt*), *bombadil*, *gremlin*, and *orc* specifically affect the formation of the root meristem (Scheres *et al.*, 1996). The first recognizable defect in all these mutants is the aberrant development of the hypophyseal cell, indicating that the correct specification of the hypophyseal cell is essential for the establishment of a functional root meristem. The *hbt* defect becomes first apparent at the four-celled embryo stage, where atypical divisions occur in the uppermost suspensor cell, that normally forms the hypophyseal cell (Willemsen *et al.*, 1998). Subsequently, the cell-types normally derived from the hypophyseal cell, the quiescent center and the central root cap cells, are not specified. Furthermore, divisions of the lower-lower tier cells occupying the position of the root meristem initials in wild-type are strongly reduced or absent, and lateral root cap formation is disturbed. These defects can be explained by assuming that the *HBT* gene is involved primarily in the specification of the hypophyseal cell, which subsequently induces the adjacent lower-lower tier cells to form root meristem initials. This scenario has also been proposed on the basis of the *fackel* phenotype (see above under "Establishment of embryonic domains along the apical-basal axis"), and receives further support from the observation that laser ablation of a quiescent center cell at the seedling stage results in the differentiation of the normally undifferentiated adjacent initial cells (Dolan, 1998; van den Berg *et al.*, 1997). However, the current data cannot rule out the possibility that *HBT* is directly involved in the specification of all cell types of the root meristem, comprising the quiescent center, the central and lateral root cap, and the root meristem initials.

Based on the *monopteros*, *fackel* (see above under "Establishment of embryonic domains along the apical-basal axis) and *hobbit* mutant phenotypes, and the results from laser ablation studies (Dolan, 1998), a tentative model for the establishment of the root meristem includes two successive induction events across the clonal boundary between the lower tier and the hypophyseal derivatives. Initially, the lower tier cells induce the upper suspensor cell to develop into the hypophyseal cell, and later, the hypophyseal cell or its derivative, the quiescent center, induces the adjacent cells of the lower tier to form root meristem initials.

## Role of auxin in pattern formation and organ formation

The natural plant growth regulator indole 3-acetic acid (IAA), belonging to the auxins, is an important molecule affecting almost all aspects of plant growth and development. However, its role in embryo pattern formation has not been addressed until fairly recently. Except perhaps for the *Arabidopsis MONOPTEROS* gene product (Przemeck *et al.*, 1996), that is believed to be involved in the transduction of a polar signal (possibly auxin), and whose absence results in embryos and seedlings lacking a RM (see above under "Establishment of embryonic domains along the apical-basal axis"), embryo mutant analysis has not yet provided clues concerning the nature of the signals that are instrumental in setting up the embryo pattern. The question whether auxin gradients are instrumental in apical-basal pattern formation has been addressed by *in vitro* culturing of excised immature zygotic embryos. Inhibition of polar auxin transport in *in vitro* cultured globular zygotic embryos of Indian mustard (*Brassica juncea*), by application of 2,3,5-triiodobenzoic acid (TIBA) or 9-hydroxyfluorene-9-carboxylic acid (HFCA), led to the formation of a collar-like extension of fused cotyledons at the apical end of the embryo (Liu *et al.*, 1993). This fused cotyledon phenotype has similarities to the embryo phenotype observed in the *cuc* mutant (described above under "The shoot apical meristem"). The treated *Brassica* embryos formed shoot and root meristems (Liu *et al.*, 1993), suggesting that the specification of these pattern elements is either not dependent on polar auxin transport, or is completed before the globular stage. From further studies, employing the same system of immature *Brassica* zygotic embryo culture, Hadfi *et al.* (1998) concluded that auxin is not only involved in cotyledon formation, but also in establishing other elements of the apical-basal axis. Upon exposure of early globular embryos to appropriate concentrations of the natural auxin indole 3-acetic acid (IAA), ball- or egg-shaped embryos were formed that showed some normal cell differentiation and greening in the hypocotyl region, but no development of the shoot apical meristem. Inhibition of auxin transport in transition stage embryos resulted in different aberrations, most notably in collar-like cotyledons. In some embryos, more extreme abnormalities, such as axis duplication or axis broadening were observed (Hadfi *et al.*, 1998). In *in vitro* cultured globular wheat zygotic embryos, the addition of the polar auxin transport inhibitor TIBA influenced the position and development of the SAM while no RM was formed. The application of strong synthetic auxins prevented the development of bilaterally symmetric embryos. A model was proposed in which a non-homogenous distribution of auxin in the globular embryo is instrumental in mediating the transition between radial symmetry (as in the globular embryo) to bilateral symmetry (as in the transition stage embryo), finally leading to SAM formation (Fischer and Neuhaus, 1996). Later studies in the wheat system showed that application of NPA (*N*-1-naphthylphthalamic acid) and another polar transport inhibitor, 3,3',4',5,7-pentahydroxyflavone (quercetin) led to the formation of multiple shoot and root meristems, and in some cases even to a multiplication of embryonic axes, giving rise to zygotic polyembryos (Fischer *et al.*, 1997).

These results collectively suggest an important role of polar auxin transport in establishing at least certain elements of the apical-basal embryo pattern. From the available evidence, it is likely that the proposed auxin gradients are already established in the pre-globular embryo, preceding the establishment of some of the apical-basal pattern elements. Although intracellular levels of the synthetic auxin 2,4-D and the endogenous auxin indole-3-acetic acid (IAA) in entire cell clusters and embryos have been measured (Ivanova *et al.*, 1994; Michalczuk *et al.*, 1992; Ribnicky *et al.*, 1996), auxin gradients have not been determined so far in immature embryos. Whether auxin gradients are required for all aspects of apical-basal pattern formation in the embryo remains to be determined. The finding that some of the embryos treated with polar auxin transport inhibitors phenocopy certain *Arabidopsis* zygotic embryo mutants seems to support the idea that auxin gradients are instrumental in the establishment of the apical-basal embryo pattern. It is clearly a task of the immediate future to study auxin content and distribution in *Arabidopsis* zygotic embryo mutants.

## Flexibility in the initiation and in the early division pattern of embryogenesis

In plants, single cells other than the zygote are capable of setting course for an embryogenic pathway. This phenomenon occurs naturally in plants in the form of apomictic embryogenesis, commencing from a cell or cells of the female gametophyte or of the surrounding maternal somatic tissues (Koltunow, 1993). We assume that the mechanisms of embryo pattern formation by which plant embryos from non-zygotic origin develop are essentially the same as outlined above for the zygotic *Arabidopsis* embryo. However, there is no formal proof for this assumption yet.

From a comparison between zygotic and non-zygotic embryos of the same plant species, it is clear that, especially in the early stages, significant differences in cell division patterns exist (Mordhorst *et al.*, 1997). Evidence for this came from studies on microspore embryogenesis in rape seed, a close relative of *Arabidopsis*. Rape seed microspores at the unicellular stage can switch developmental fate from pollen development to embryogenesis. This process can be induced by applying heat shock to isolated microspores, and results in fully fertile plants (see Mordhorst *et al.* (1997)). The first division in microspore embryogenesis takes place inside the pollen coat or exine and is symmetric, in contrast to the asymmetric first division of the normal gametophytic pathway (Yeung *et al.*, 1996). While zygotic embryogenesis in rape seed follows the highly regular cell division pattern described for *Arabidopsis* (Yeung *et al.*, 1996), early divisions in embryogenic microspores appear to be random (Dolan, 1997; Malamy and Benfey, 1997; Telmer *et al.*, 1995; Yeung *et al.*, 1996). The resulting multicellular structure representing the early microspore embryo, while still developing inside the exine, exhibits an equal distribution of starch granules in all cells, implying that at that stage the embryo has not yet attained morphologically discernible polarity (Hause

*et al.*, 1994). After local rupture of the exine and release of the multicellular structure into the culture medium, starch granules disappear at the "broken side" (the future apical pole) and persist at the opposite side (the future root pole; Hause *et al.*, 1994; Yeung *et al.*, 1996). The multicellular structure released from the exine therefore appears to be 'self-organizing' in becoming a globular embryo. No typical suspensor nor a hypophyseal cell is observed (Yeung *et al.*, 1996), though the hypophyseal cell is considered to play a central role in the formation of the RM in zygotic embryogenesis (Scheres *et al.*, 1995). Based on these observations, one can conclude that it is apparently possible in microspore embryos to delay fixation of the apical-basal embryo axis until a multicellular stage is reached, and that essential functions attributed to certain cell types such as the hypophyseal cell are either not common to all types of embryogenesis, or can be taken over by other cells.

The most striking example of flexibility in plant embryogenesis remains the observation that *in vitro* somatic embryo development is possible in suspension cultures of carrot cells (Reinert, 1959). In carrot cell suspensions, it has been unequivocally demonstrated that single cells can develop into an embryo in completely synthetic media with only an auxin, usually the synthetic auxin 2,4-D, as growth regulator (Komamine *et al.* (1990) and Figure 1.5). Video analysis of many thousands of individual cells ("cell tracking") has shown that single embryo-forming cells are highly variable in morphology (Toonen *et al.*, 1994). In search of a molecular marker for single embryo-forming cells, Schmidt *et al* (1997) identified the *SOMATIC EMBRYO RECEPTOR-LIKE KINASE* (*SERK*) gene that is expressed in single embryo-forming suspension cells, as monitored by *SERK* promoter driven luciferase expression (Figure 1.5). The *SERK* gene is one of the very few genes described so far that appears to be expressed only in embryos and not in meristems or other parts of the adult plant. It is expressed during the few first cell divisions in the developing embryo and is turned off again at the globular embryo stage. The SERK protein encodes a transmembrane receptor kinase with some resemblance to the *Drosophila* Toll receptor kinase. The function of the SERK protein may be to

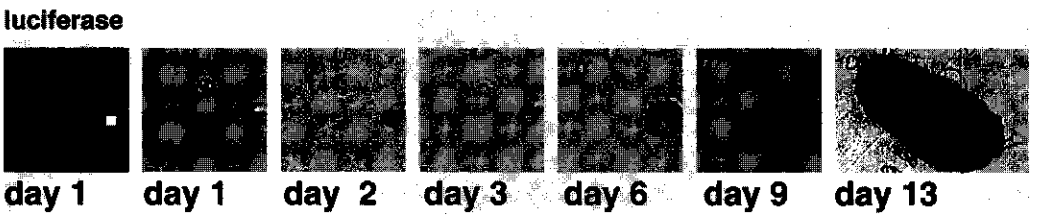


**Figure 1.5:** Luciferase expression under control of carrot *SERK* regulatory elements correlates with embryogenic competence of single cells or small cell clusters. Luciferase activity of immobilized cells from an embryogenic carrot cell culture was recorded at day 1, and subsequently the development of cells was recorded by video cell-tracking for a period of 13 days (Schmidt *et al.*, 1997).

transduce a signal that is instrumental in conferring an 'embryo-fate' to plant cells (Schmidt *et al.*, 1997).

The establishment of somatic embryogenesis in *Arabidopsis* (Mordhorst *et al.*, 1998) now allows to apply the molecular and genetic approaches used in the analysis of zygotic embryogenesis to the experimentally more accessible system of embryogenesis in cell culture. Embryogenic suspension cultures of *Arabidopsis* have been obtained by culturing immature zygotic embryos of the ecotype Columbia (Pillon *et al.*, 1996), and always arise from the SAM under *in vitro* conditions (Mordhorst *et al.*, 1998). Cell lines with a much higher embryogenic capacity *in vitro* were obtained from recessive mutants such as *primordia timing-1* (*pt-1*) and *clv*. These mutants were originally identified in genetic screens for SAM formation and altered flowering, respectively. Their most conspicuous embryo and seedling phenotype is a large SAM with a higher than normal number of dividing cells. While the *CLV1* gene encodes a Leucin Rich Repeat (LRR) type receptor kinase (Clark *et al.*, 1997), the *PT-1* gene has not yet been identified. A positive correlation between the size of the SAM in single and double mutants of *clv* and *pt* and the number of embryogenic cells that could be obtained under *in vitro* conditions suggests that the larger number of cycling SAM cells in the mutants may be the reason for the enhanced somatic embryogenesis phenotype (Mordhorst *et al.*, 1998).

Comparing early stages of zygotic and somatic embryo development in carrot (McWilliam *et al.*, 1974; Toonen *et al.*, 1994) and alfalfa (Dos Santos *et al.*, 1983) revealed that the early divisions in somatic embryos are less regular than those in their zygotic counterparts. Moreover, somatic embryos usually lack a suspensor (Fischer *et al.*, 1997; Toonen *et al.*, 1994; Xu and Bewley, 1992) and can develop via morphologically different cell clusters (Toonen *et al.*, 1994). In *Arabidopsis* somatic embryos, the number of cell files and the number of cells per file are higher and more variable than the highly regular numbers in zygotic embryos. Zygotic embryos formed on plants derived from somatic embryos display the normal regularity of the zygotic *Arabidopsis* embryo, indicating that the variability in cell numbers is associated with somatic embryogenesis (Mordhorst *et al.*, 1998).

Both microspore and somatic embryogenesis have frequently been used in screens for genes specifically expressed in embryos (e.g. Giroux and Pauls, 1996; Lin *et al.*, 1996; Schmidt *et al.*, 1997). Expression patterns of embryogenesis related mRNAs identified during these screens (Franz *et al.*, 1989; Osborne *et al.*, 1991; Sterk *et al.*, 1991; Wurtele *et al.*, 1993) turned out to be similar during somatic and zygotic embryo development, demonstrating that somatic and zygotic embryo development have common molecular characteristics.

In conclusion, non-zygotic embryos can develop from different types of plant cells, and can exhibit considerable morphological variability under *in vitro* conditions, while retaining common molecular characteristics. The fact that recessive mutations in two genetically unrelated genes, *PT* and *CLV*, result in highly increased embryogenic capacity *in vitro* may imply that embryogenic capacity is a trait that is suppressed in wild-type plants. The eventual number of mutants with increased embryogenic capacity and the identity of the corresponding genes will be important to understand the

concept of plant cell totipotency.

## Signalling molecules in somatic embryogenesis

In somatic embryogenesis, cells may switch from the somatic to the embryogenic state in a cell-autonomous way under the influence of exogenous growth regulators. Alternatively, they may depend on the perception of specific signals from neighboring cells. The identification of the *SERK* gene (see above under "Flexibility in the initiation and in the early division pattern in embryogenesis") suggests that the embryogenic transition process requires specific signalling events.

The beneficial effect of suspension cell cultures (Kranz *et al.*, 1991) or embryogenic microspore cultures on development of *in vitro* cultured zygotes (Holm *et al.*, 1994; Leduc *et al.*, 1996) proves the importance of cell-cell communication during initiation of embryogenesis. Similar effects have been observed in somatic embryogenesis after addition of medium conditioned by developing somatic embryos (De Vries *et al.*, 1988). By virtue of the accessibility of embryogenesis tissue culture systems, the compounds responsible for these beneficial effects have been purified from the conditioned medium and identified as proteins or proteoglycans that may be involved in cell-cell signalling events in early plant embryogenesis.

The carrot *EP3* endochitinase genes (Kragh *et al.*, 1996) encode proteins secreted into the culture medium that are able to rescue somatic embryo development in the temperature sensitive mutant *ts11* (De Jong *et al.*, 1992; De Jong *et al.*, 1995). The *EP3* genes are not expressed in somatic embryos, but in other cell types present in the suspension culture (Van Hengel *et al.*, 1998). Given the effect of the endochitinase proteins on somatic embryos these proteins may be part of a cell-cell communication system involved in somatic embryogenesis. Several recent studies indicate a role for certain arabinogalactan proteins (AGPs) in plant cell-cell communication. AGPs are 90-100 kD proteoglycans with a small protein core and consist of more than 90 % carbohydrate, predominantly in the form of arabinose and galactose, with minor amounts of other sugars. AGPs can promote embryogenesis in suspension cultures of carrot (Kreuger and Van Holst, 1993 and 1995; Toonen *et al.*, 1997) and *Pinus* (Egertsdotter and Von Arnold, 1995). Removal of a population of non-embryogenic single cells reduced embryogenesis but this negative effect could be counteracted by adding AGPs, suggesting that AGPs were the causative agent produced by the single cell population (Toonen *et al.*, 1997). AGPs react with the ß-glycosyl Yariv reagent (Fincher *et al.*, 1983; Kreuger and Van Holst, 1996). Binding of this reagent to cell wall AGPs of rose (*Rosa* sp.) suspension cells inhibited growth in a reversible fashion, probably due to suppression of the cell cycle, possibly in combination with prevention of cell expansion (Serpe and Nothnagel, 1994).

It has been proposed that carrot suspension cells decorated with the JIM8 AGP cell wall epitope are in a transition between the competent and embryogenic state (Pennell *et al.*, 1992). This

suggestion was based on the labelling of a subpopulation of single cells with the JIM8 antibody only in embryogenic cultures. Cell tracking of JIM8 labelled cell populations however failed to demonstrate a causal relationship between JIM8 labelling and embryo formation (Toonen *et al.*, 1996). Given the demonstrated promotive effects of certain AGPs, it is possible that the JIM8 decorated cells perform some accessory function in embryogenesis. Support for cell-cell signalling by a JIM8 epitope-containing molecule comes from a study where soluble molecules, possibly AGPs bearing the JIM8 epitope, are able to initiate somatic embryogenesis in suspension cells (McCabe *et al.*, 1997). This soluble JIM8 epitope is reminiscent of the one that labels a class of single cells in culture and may represent a component that is produced by one cell type and acts on another. These results suggest that AGPs may play a role in determining cell fate and regulating cell differentiation in plants (Chasan, 1994; Knox, 1995; Touraev *et al.*, 1996). The JIM8 epitope is found on suspensor cells and on the hypophyseal cell in the zygotic embryo, but not on descendants of the apical cell (Pennell *et al.*, 1991). Taken together, these findings point to signalling between suspensor and globular embryo, while suspension cells that have retained certain aspects of suspensor cells may take over the role of the suspensor cells *in vitro*.

If components of the conditioned medium of plant cell cultures such as chitinases and AGPs have a beneficial effect on somatic embryo formation, it is of interest to determine where such molecules are found during zygotic embryogenesis. Developing seeds have proven to be a rich source of AGPs able to promote embryogenic cell formation in tissue culture (Kreuger and Van Holst, 1993), while the carrot *EP3* chitinase genes appear to be expressed in the integuments of the seed coat and in the endosperm (Van Hengel *et al.*, 1998).

In conclusion, the available evidence from genetic and *in vitro* studies points to cell-cell communication between different domains in the early embryo, and also between the embryo and the surrounding endosperm and maternal tissues. These processes can now also be studied genetically through *Arabidopsis fertilization independent endosperm (fie)* and *fertilization independent seed (fis)* mutants, that show endosperm development without fertilization (Chaudhury *et al.*, 1997; Ohad *et al.*, 1996). While *fie* and *fis* mutants prove that endosperm development can take place without concurrent embryo development, no mutants have been identified so far in which embryo development takes place in the absence of endosperm. This finding may support the notion that embryo development is dependent on the surrounding endosperm.

## Outlook

It is evident that considerable progress is made towards understanding the initiation and maintenance of, for instance, the SAM. The genes that have been cloned based on meristem phenotype appear to be important regulators of the cellular differentiation state in the meristem. While extensive screens for early embryo mutants in *Arabidopsis* have been performed and novel screens are underway, one of

the emerging difficulties is the recognition of informative phenotypes. This problem appears less serious for SAM defectives than for mutations that affect polarity in the zygote or the two-celled stage. The inherent flexibility associated with embryogenesis in plants may greatly hamper identification of informative mutants based on morphology only. Additional efforts employing suitable marker genes are most likely of importance to make progress in this area. Unfortunately, very few genes are available that allow to detect early deviation from apical or basal cell fate. Precise molecular markers are necessary to determine cellular or regional identity in embryo mutants with phenotypes that are difficult to interpret. Enhancer and gene trap insertional mutagenesis screens have recently proven successful in identifying genes expressed during *Arabidopsis* embryogenesis (Vroemen *et al.*, 1998).

The discovery that interference with the polar auxin transport and thus endogenous auxin distribution creates embryo phenotypes reminiscent of genetically defined phenotypes appears to be a very promising lead to verify whether the classical plant growth regulator auxin is indeed a key molecule in embryo pattern formation.

The understanding of the mechanisms controlling the transition from a somatic or gametophytic cell into an embryogenic cell can be expected to help elucidate processes of early zygotic embryogenesis, as exemplified by studies on the *SERK* gene in both somatic and zygotic embryos. AGPs produced by non-embryogenic cells are candidate molecules that may perform an important role in conferring 'embryo identity' to plant cells. These effects of AGPs may reflect putative suspensor and endosperm functions in zygotic embryogenesis. Additional insight in the role of molecules such as AGPs in plant embryogenesis may be obtained by employing for instance reverse genetics approaches. Clearly, AGP synthesis and the precise modes of AGP action, including transport and recognition by target cells, are areas that need to be developed in the immediate future. *In vitro* embryogenesis could also provide clues on subjects such as the origin of polarity in seemingly unorganized clusters of embryogenic cells. Moreover, these systems should be amenable to sophisticated ways of experimental manipulation, such as cell ablation and *in vitro* complementation.

## Summary

Mutant screens have identified genes whose products are required for apical-basal and radial pattern formation in the embryo. Although not all of the genes identified by mutation have been cloned so far, and the precise mode of action of genes that have been cloned largely remains to be determined, the available evidence provides some conceptual insight into plant embryo development.

It appears that the acquisition of different cell fates during the establishment of the apical-basal embryo pattern is initially dependent on correct segregation of cell fates through specific and unequal

divisions. Mutations in genes such as *KNOLLE* and *GNOM* that affect the initial divisions of the embryo create severe embryo phenotypes that support this notion. The octant stage embryo is subdivided along the apical-basal axis into the upper and lower tiers, and the hypophyseal cell. Not all of the genes that are required to properly specify these regions are directly conferring regional identity, as demonstrated by for example the *MP* gene. Establishment of the different pattern elements along the apical-basal axis may require signalling between the three early embryonic regions. The *gk* phenotype suggests signalling between the upper and lower tiers, and the *mp*, *fk*, and *hbt* phenotypes a two-way signalling between the lower tier and hypophyseal cells.

Radial pattern formation starts when inner cells are separated from protoderm cells in the dermatogen stage embryo. Morphological and molecular observations suggest that protodermal cell fate is already established in the apical daughter cell of the zygote. Further elaboration of the radial pattern in the central domain of the embryo requires genes that specify cell fate, such as *SHR*, and genes, such as *SCR* and *WOL*, that affect cell division and are involved in the generation of sufficient concentric cell layers for the proper separation of all tissue-types.

The establishment of a functional SAM may require signals from the lower-tier derived vascular tissue, as suggested by *ZLL* expression, and signalling between the CZ and PZ, possibly involving *STM*, *CLV*, and *ZLL*.

The identification of LRR type receptor kinases as *SERK* and *CLV*, and transcriptional regulators such as *STM* appears to confirm the existence of elaborate systems of cell communication. The identified LRR receptors are of a type comparable to that found in animal cells, where they are commonly activated by peptide ligands. This may point towards the involvement of peptide-based growth factors (van de Sande *et al.*, 1996) in plant embryogenesis.

Positional information mediated through cell-cell communication appears of importance in making plant embryos. Evidence comes from mutant analysis and laser ablation studies (Dolan, 1998). The observation that the complete embryo pattern can be established *in vitro* without the precise sequence of oriented cell divisions characteristic of the zygotic *Arabidopsis* embryo also underscores the importance of positional information for the establishment of cell fates and pattern. This implies the existence of a flexible mechanism, independent of precise cell numbers and cell division patterns, to establish pattern. Polar auxin transport and unequal auxin distribution may be involved in embryo pattern formation or organ differentiation.

*In vitro* embryogenesis systems, in which non-zygotic embryos develop outside of the "normal" context of the seed, have provided evidence for the existence of cell-cell communication between non-embryogenic and embryogenic cells. AGPS have emerged in several systems as molecules that could be involved in or even responsible for the formation of embryogenic cells.

# 2

# Pattern formation in the *Arabidopsis* embryo revealed by position-specific lipid transfer protein gene expression

During *Arabidopsis* embryogenesis, the zygote divides asymmetrically in the future apical-basal axis; however, a radial axis is initiated only within the eight-celled embryo. Mutations in the *GNOM*, *KNOLLE*, and *KEULE* genes affect these processes: *gnom* zygotes tend to divide symmetrically; *knolle* embryos lack oriented cell divisions that initiate protoderm formation; and in *keule* embryos, an outer cell layer is present that consists of abnormally enlarged cells from early development. Pattern formation along the two axes is reflected by the position-specific expression of the *Arabidopsis* lipid transfer protein (*AtLTP1*) gene. In wild-type embryos, the *AtLTP1* gene is expressed in the protoderm and initially in all protodermal cells; later, *AtLTP1* expression is confined to the cotyledons and the upper end of the hypocotyl. Analysis of *AtLTP1* expression in *gnom*, *knolle*, and *keule* embryos showed that *gnom* embryos can also have no or reversed apical-basal polarity, whereas radial polarity is unaffected. *knolle* embryos initially lack but eventually form a radial pattern, and *keule* embryos are affected in protoderm cell morphology rather than in the establishment of the radial pattern.

Casper W. Vroemen, Sandra Langeveld, Ulrike Mayer[1], Gabriela Ripper[1], Gerd Jürgens[1], Ab van Kammen, and Sacco C. de Vries (1996) *Plant Cell* **8**, 783-791

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## Introduction

In flowering plants, the primary body plan of the seedling is laid down during embryogenesis (Steeves and Sussex, 1989). This body plan has been described as the superimposition of an apical-basal and a radial pattern (Mayer *et al.*, 1991). The apical-basal pattern visible in the seedling consists of distinct elements: two cotyledons, shoot meristem, hypocotyl, and root, including the root meristem. In *Arabidopsis*, the apical-basal polarity is already evident in the zygote, which elongates approximately threefold in the apical direction. An asymmetric division then generates a small apical cell from which all pattern elements are derived, except for part of the root, that is, the columella root cap and the quiescent center (Scheres *et al.*, 1994), and the suspensor, which are derived from the larger basal cell. Mutations resulting in a deletion of regions of the apical-basal pattern include *gurke, fackel, monopteros,* and *gnom* (Berleth and Jürgens, 1993; Mayer *et al.*, 1993; Mayer *et al.*, 1991) and *rootless, shoot meristemless,* and *topless* (Barton and Poethig, 1993).

In *gnom* embryos (Busch *et al.*, 1996; Mayer *et al.*, 1993; Mayer *et al.*, 1991), also called *emb30* embryos (Franzmann *et al.*, 1995; Shevell *et al.*, 1994), the zygote tends to divide symmetrically, producing an enlarged apical cell at the expense of the basal cell. *gnom* embryos have no root meristem and reduced or no cotyledons, and most *gnom* seedlings are cone shaped, retaining apical-basal polarity, although the pattern is severely compromised. Some *gnom* seedlings, however, are ball shaped, displaying no morphologically apparent apical-basal polarity (Mayer *et al.*, 1993). The radial pattern is arranged in three concentric layers of tissues: the outer protoderm, the inner mass of ground tissue, and the centrally located vascular bundles. This pattern is initiated within the first eight cells formed from the small apical cell (Jürgens *et al.*, 1991; Meinke, 1991). Each of these eight cells divides tangentially to give an outer epidermis precursor, or protodermal cell, and an inner cell. Repeated divisions of the inner cells generate the ground and vascular tissues. The protodermal cell layer expands by anticlinal cell divisions only and is thus maintained as a distinct cell layer.

Mutations in two genes, *KNOLLE* and *KEULE*, affect the radial pattern but also cause other major morphological defects; *knolle* and *keule* embryos and seedlings are strongly compressed in the apical-basal direction (Mayer *et al.*, 1991). In *knolle* embryos, the initial cell divisions are abnormal, so no inner cells can be clearly distinguished from an outer layer. At later stages, however, vascular tissue forms in the center of *knolle* embryos. *knolle* embryos and seedlings are round or tuber shaped. In *keule* embryos, a distinct outer cell layer is present, but it consists of abnormally enlarged cells, whereas the cells of both the ground tissue and the vasculature look normal. Thus, in this mutant, the shape rather than the initial formation of the protoderm cells seems to be affected (Mayer *et al.*, 1991).

The embryo protoderm is the precursor of the plant epidermis. During postembryonic development, the epidermis of aerial plant organs performs a number of functions essential for the stability of turgescent tissue. The most important function is to control water loss. In addition, the

epidermis provides mechanical and chemical defences against pathogens (Clark *et al.*, 1992). In the plant embryo, the protoderm may play a role in restriction of turgor-driven water uptake through the formation of a cuticular layer and also may act to protect the embryo from hydrolytic endosperm-degrading enzymes (Sterk *et al.*, 1991). Additional evidence for the importance of the protoderm for embryo development was found in somatic embryos of the temperature-sensitive carrot mutant line ts11. At the nonpermissive temperature, ts11 embryos arrest at the globular stage and have an aberrant, irregular protoderm with enlarged, vacuolated cells. At the permissive temperature or after rescue with a 32-kD endochitinase, ts11 embryos have a correctly formed protoderm (De Jong *et al.*, 1992; De Jong *et al.*, 1993). The protoderm of *Citrus jambhiri* embryos, once formed, could not be replaced by respecification of ground tissue after experimental removal (Bruck and Walker, 1985b), emphasizing the importance of protodermal differentiation for embryo development.

Sterk *et al.* (1991) identified the carrot *EP2* gene as a marker for the embryo protoderm in carrot. The *EP2* gene encodes a 10-kD lipid transfer protein (LTP) secreted into the medium of embryogenic carrot cell cultures. The *EP2* gene is expressed in protoderm cells of somatic and zygotic carrot embryos, starting at the early globular stage. The LTP is proposed to be involved in cuticle formation on the outer surface of protodermal cells (Sterk *et al.*, 1991). In arrested ts11 embryos, which have a morphologically aberrant protoderm, the *EP2* gene was found to exhibit either a uniform (De Jong *et al.*, 1993) or a diffuse subepidermal pattern of expression (Sterk *et al.*, 1991).

In this study, we show that in *Arabidopsis*, pattern formation along the apical-basal and radial axes is reflected in the position-specific expression of the *AtLTP1* lipid transfer protein gene (Thoma *et al.*, 1994), which is the *Arabidopsis* homolog of the carrot *EP2* gene (Sterk *et al.*, 1991). The *AtLTP1* gene is expressed in the protoderm soon after this cell layer is evident in the early globular embryo stage. *AtLTP1* expression was initially observed along the entire apical-basal embryo axis, but later became confined to the apical pole, including the cotyledons and the upper end of the hypocotyl. In a recent study, Yadegari *et al.* (1994) used *AtLTP1* expression to show that *raspberry* embryos, although they are morphologically arrested at the globular stage and have a grossly abnormal outer cell layer, initiate a protoderm-specific gene expression program in the outer cell layer of both the embryo proper and the suspensor. This indicates that in these mutant embryos, cell differentiation is uncoupled from morphogenesis. We have compared *AtLTP1* expression of wild-type embryos with that of *gnom*, *knolle*, and *keule* mutants. Our results suggest that embryo apical-basal polarity is still fully reversible in the zygote and that radial polarity is established in a centripetal fashion and employs more than one independent mechanism. Moreover, we emphasize the importance of using well-characterized tissue-specific markers in mutant embryo analysis.

## Results

### *Expression of an* AtLTP1 *promoter*-β-glucuronidase *gene fusion in wild-type and* gnom *embryos*

Embryos from transgenic plants homozygous for a fusion of a 1149-bp region of the *AtLTP1* promoter and a promoterless *β-glucuronidase* (*GUS*) gene (Thoma *et al.*, 1994) were analyzed histochemically for the presence of GUS activity. The results are presented in Figure 2.1. *GUS* expression was detected from the early globular stage, through the heart and torpedo stages, to the maturation stage (Figures 2.1A to 2.1C; data not shown). At the globular stage, *GUS* expression was uniform along the entire apical-basal axis of the embryo proper, including in the cells derived from the hypophyseal cell (Figure 2.1A). This staining pattern persisted in the heart-stage embryo (Figure 2.1B), whereas during the transition from the heart stage to the torpedo stage, *GUS* expression became confined to the apical pole of the embryo, including the cotyledons and the upper end of the hypocotyl (Figure 2.1C).

In maturation-stage embryos, *GUS* expression was most prominent in the cotyledons, especially in their tips, and less intense in the embryo hypocotyl (data not shown). No GUS staining was observed in the embryonic root of torpedo- and maturation-stage embryos. GUS staining was variable in the suspensor of globular- and heart-stage embryos (Figures 2.1A, 2.1B, and 2.1D), whereas after the heart stage, suspensor staining was never observed. The *AtLTP1* expression pattern in the mature embryo corresponds to the expression pattern observed in seedlings just after germination, at which time *AtLTP1* expression is highest in the tips of the cotyledons (Thoma *et al.*, 1994). In addition to embryo-specific GUS staining, intense GUS staining was evident in the developing seed coat (Figures 2.1D and 2.1H; data not shown). No GUS staining was observed in plants not containing the *AtLTP1* promoter-*GUS* fusion (data not shown).

We have used the gradual restriction of *AtLTP1-GUS* expression toward the apical end of the embryo to monitor changes in pattern formation caused by mutations in the *GNOM* gene (Mayer *et al.*, 1993). To localize *AtLTP1* promoter activity in *gnom* embryos, plants homozygous for the *AtLTP1-GUS* fusion were crossed to plants heterozygous for the *gnom* mutation. Histochemical GUS staining was performed with siliques of $F_1$ plants in which one-fourth of the embryos were mutant. Figures 2.1D to 2.1I show GUS-stained *gnom* embryos. Mutants clearly have a smooth surface due to the presence of a morphologically normal protoderm (Mayer *et al.*, 1993). In cone-shaped *gnom* embryos, the time course and a restriction of *GUS* expression to the apical end of the embryo were observed to be similar to those of wild-type embryos.

Temporal expression can be determined by comparing mutant and wild-type embryos in the same silique. In Figure 2.1E, which shows a cone-shaped *gnom* embryo of the same developmental

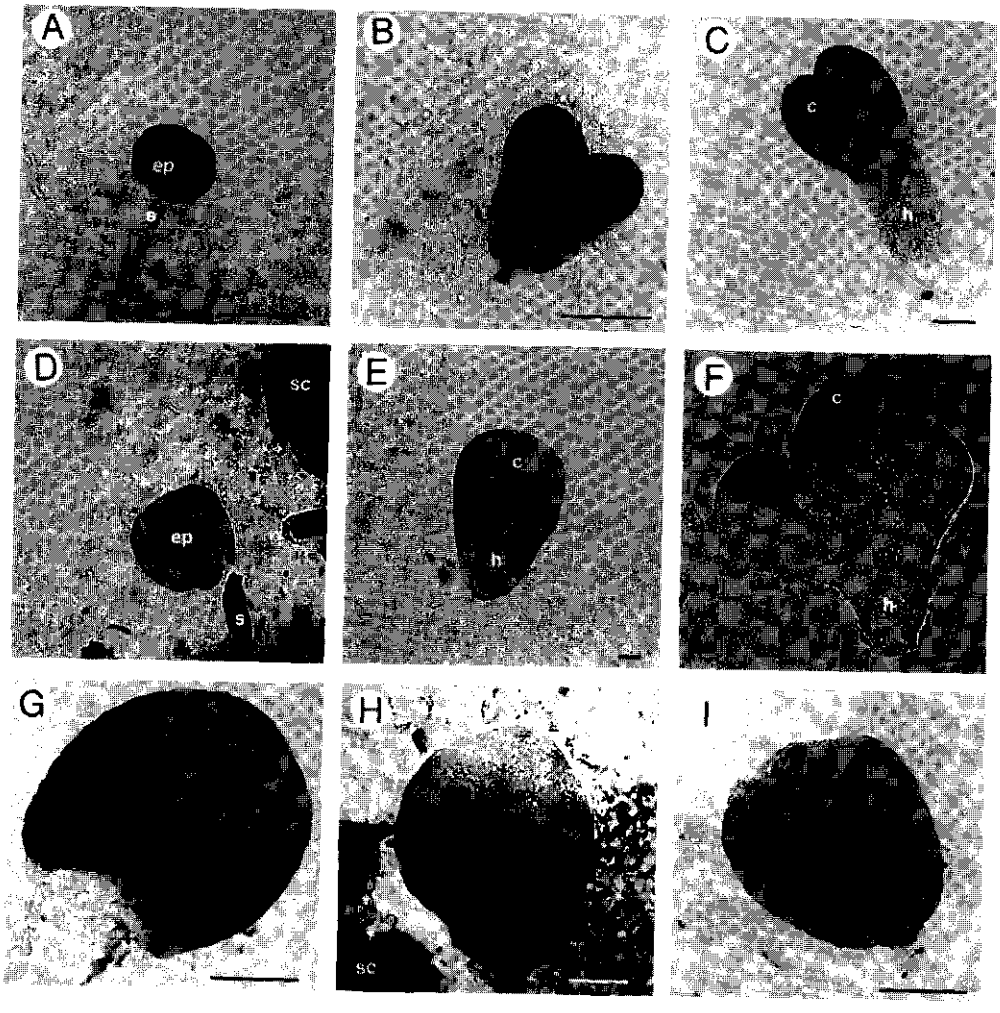


**Figure 2.1:** Histochemical localization of GUS activity in transgenic wild-type and *gnom* embryos containing an *AtLTP1* promoter-*GUS* fusion.

**A** to **C:** Developing wild-type embryos at the globular (**A**), heart (**B**), and torpedo (**C**) stages. Apical sides are oriented upward.

**D** to **I:** Developing *gnom* embryos from siliques in which wild-type embryos were at the globular (**D**), torpedo (**E**), and maturation (**F** to **I**) stages. Embryos in **E** and **F** are of the cone-shaped phenotype. Embryos in **G** to **I** are of the ball-shaped phenotype. Apical sides are oriented upward.

c, cotyledon; ep, embryo proper; er, embryonic root; h, hypocotyl; s, suspensor; sc, seed coat. Bars = 50 μm.

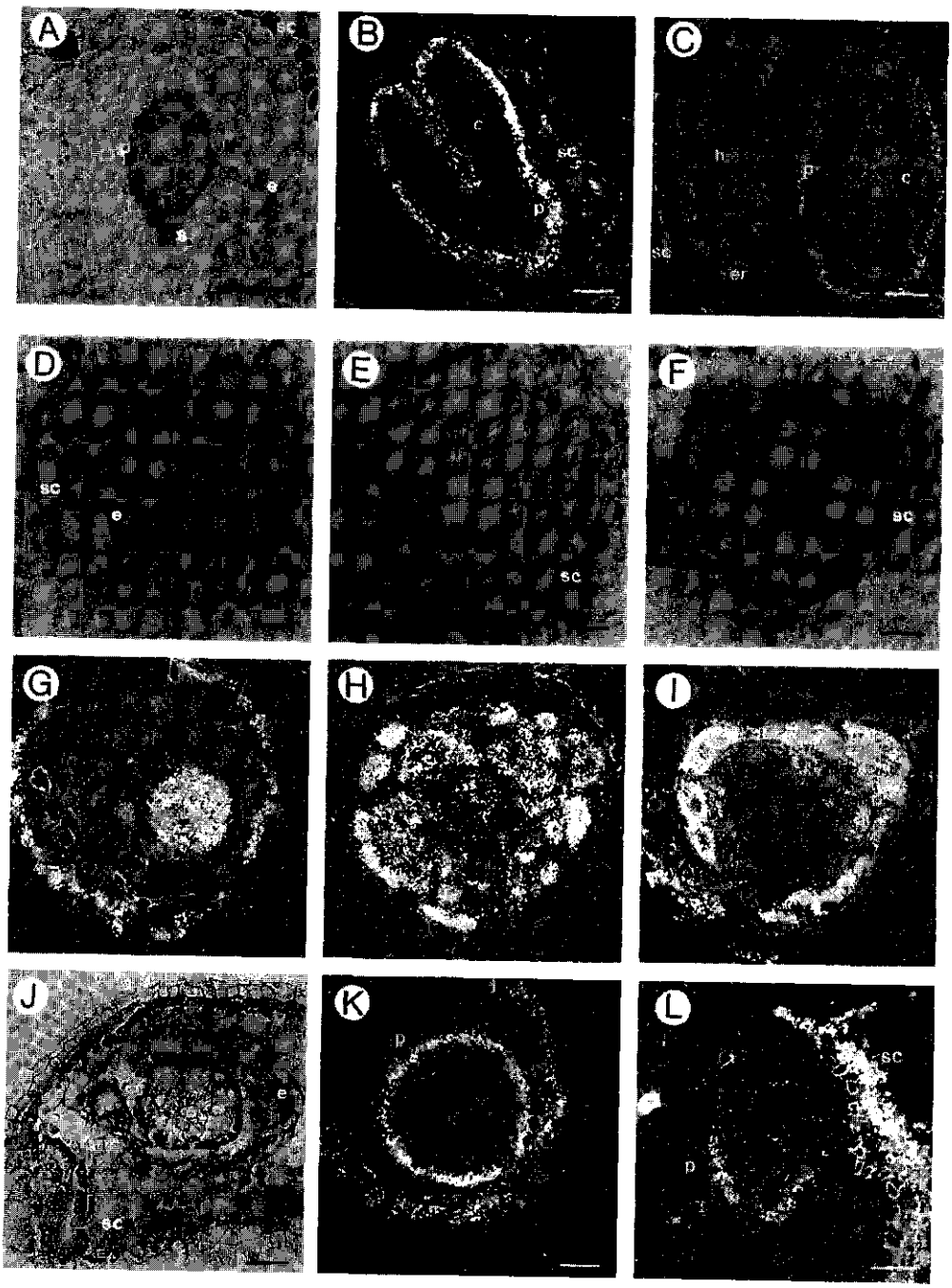


**Figure 2.2:** Localization of *AtLTP1* mRNA in wild-type, *knolle*, *keule*, and *gnom* embryos.

age as a wild-type torpedo-stage embryo, GUS staining is evident in the hypocotyl and in the fused cotyledons but is already reduced in the root. In cone-shaped *gnom* embryos from siliques that contain maturation-stage wild-type embryos, GUS staining is intense in the tips of the cotyledons, which are strongly reduced, sometimes fused, and aberrantly shaped (Figure 2.1F).

Among later stages of the ball-shaped *gnom* embryos, which represent the morphologically apparent loss of apical-basal polarity most dramatically, there are three distinct patterns of *GUS* expression. As in wild-type and cone-shaped *gnom* embryos, expression can be confined to the apical end of the embryo (Figure 2.1G), but it also can be completely reversed and restricted to the basal region of the embryo (Figure 2.1H; see also Figure 2.2L).

Finally, *GUS* expression can remain distributed uniformly (Figure 2.1I; see also Figure 2.2K). Of 51 ball-shaped *gnom* embryos, 18 displayed normal polarity, that is, apical staining; 21 displayed reversed polarity, that is, basal staining; and 12 were apolar, that is, they displayed uniform staining. Orientation in the 51 ball-shaped embryos was determined by the presence of the suspensor or part of the suspensor. Except for GUS staining, no other clear morphological change was found among the 51 ball-shaped embryos. GUS staining patterns in $F_2$ embryos derived from crosses of plants homozygous for the *AtLTP1-GUS* construct, and wild-type Landsberg *erecta* plants were equal to the GUS staining patterns in embryos from transgenic *AtLTP1-GUS* plants. These observations indicate that apical-basal polarity of the embryo is apparently still completely reversible in the zygote, because *GNOM* is a zygotic gene (Mayer *et al.*, 1993).

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Sections (7 μm thick) were hybridized with *AtLTP1* antisense RNA probes, which were labelled with either $^{35}$S-UTP (**B** to **I**, **K**, and **L**) or digoxygenin-UTP (**A** and **J**), as outlined in Matherials and Methods. Silver grains are visible as black dots in the bright-field images (**D** to **F**) and as bright white dots in dark-field images (**B**, **C**, **G** to **I**, **K**, and **L**). Digoxygenin labelling is visible as a blue-purple color (**A** and **J**). Staging of mutant embryos was performed according to the approximate corresponding developmental stages of wild-type embryos within the same silique.

**A** to **C:** Wild-type embryos. Stages shown are globular (**A**; ≈50 cells), bent cotyledon (**B**; oblique section not showing embryonic root), and maturation (**C**).

**D** to **I:** *knolle* embryos. Stages shown are globular (**D** and **G**), bent cotyledon (**E** and **H**), and maturation (**F** and **I**).

**J:** *keule* embryo (heart stage) deliberately overstained to show the absence of signal from inner cells.

**K:** Ball-shaped *gnom* embryo (bent cotyledon stage) with uniform protoderm-specific accumulation of *AtLTP1* mRNA.

**L:** Ball-shaped *gnom* embryo (bent cotyledon stage) with basal protoderm-specific accumulation of *AtLTP1* mRNA. Apical side is oriented upward.

c, cotyledon; e, endosperm; er, embryonic root; h, hypocotyl; p, protoderm; s, suspensor; sc, seed coat. Bars = 50 μm. Bars in **D** to **F** apply to **G** to **I**.

### *Cell-specific accumulation of* AtLTP1 *mRNA in wild-type,* knolle, keule, *and* gnom *embryos*

The spatial expression pattern of the *AtLTP1* gene was examined in more detail by *in situ* hybridization in sections of wild-type, *knolle*, *keule*, and *gnom* embryos at different stages. As shown in Figures 2.2A to 2.2C, *AtLTP1* transcripts accumulate exclusively in the protoderm of wild-type embryos from the globular stage (Figure 2.2A), the bent cotyledon stage (Figure 2.2B), and the maturation stage (Figure 2.2C). In the mature embryo (Figure 2.2C), *AtLTP1* expression is higher in the protoderm of the cotyledons than in the protoderm of the hypocotyl and is absent in the protoderm of the embryonic root. *AtLTP1* expression in wild-type embryos, as determined by *in situ* mRNA hybridization, is identical to the pattern of *AtLTP1* promoter activity, as determined by *AtLTP1* promoter-*GUS* expression studies.

In contrast to the protoderm-specific accumulation of *AtLTP1* transcripts in wild-type embryos, early *knolle* embryos show uniform *AtLTP1* mRNA accumulation in all cells (Figures 2.2D and 2.2G). At a developmental stage corresponding in time to the wild-type bent cotyledon stage, the *AtLTP1* mRNA level begins to be reduced in some cells in the center of *knolle* embryos (Figures 2.2E and 2.2H). At this moment, some vascular tissue appears in the center of the embryo (Mayer *et al.*, 1991). At the maturation stage, no *AtLTP1* mRNA was detected in most cells in the center of *knolle* embryos (Figures 2.2F and 2.2I). In the *knolle* embryos analyzed, *AtLTP1* mRNA was never confined completely to the outer cell layer at any stage of development.

To determine the specificity of the *knolle* defect, we also investigated *AtLTP1* expression in *keule* embryos. In these embryos, an outer cell layer is present, but it consists of abnormally enlarged cells from early development. The ground and vascular tissues in *keule* embryos are morphologically normal. Figure 2.2J shows that in *keule* embryos at a developmental stage comparable to the wild-type heart stage, *AtLTP1* mRNA is located specifically in the outer cell layer of the embryo proper. This indicates that *keule* embryos, although abnormal in protoderm cell morphology, have a spatially normal *AtLTP1* expression pattern. It also suggests that *AtLTP1* expression is not dependent on protodermal cell morphology.

Figures 2.2K and 2.2L show sections of ball-shaped *gnom* embryos at a developmental stage comparable to the wild-type bent cotyledon stage. *AtLTP1* mRNA is confined to the protoderm in both cone-shaped (data not shown) and ball-shaped (Figures 2.2K and 2.2L) *gnom* embryos. This indicates that the radial pattern, as exemplified by the protoderm-specific *AtLTP1* expression pattern, is unaffected in *gnom* embryos. Figure 2.2L shows a ball-shaped *gnom* embryo with basal protoderm-specific *AtLTP1* mRNA accumulation. Together with the uniform protoderm-specific mRNA accumulation (Figure 2.2K) and the apical protoderm-specific expression (data not shown) this confirms the observations made with the *AtLTP1-GUS* fusions (Figures 2.1G to 2.1I). In addition to the protoderm-specific signal, a signal is evident in the developing seed coat, also seen in

transgenic plants carrying the *AtLTP1* promoter-*GUS* fusion (see also Figures 2.1D and 2.1H). No signal above background was observed after hybridizing wild-type, *knolle*, *keule*, and *gnom* embryo sections with *AtLTP1* sense probes (data not shown).

## Discussion

### AtLTP1 *expression pattern in wild-type* Arabidopsis *embryos*

In this study, we examined the expression pattern of the *AtLTP1* gene during early *Arabidopsis* embryogenesis by *in situ* hybridization and by histochemical determination of *AtLTP1* promoter activity. Both gave essentially the same result, which was in contrast with a similar study of postembryonic development (Thoma *et al.*, 1994). By using digoxygenin-uridine 5'-triphosphate (UTP)-labelled *AtLTP1* probes for *in situ* hybridization and histochemical determination of *AtLTP1* promoter activity, we were able to detect *AtLTP1* gene expression in protodermal cells of embryos as early as the globular stage. By using *in situ* hybridization with $^{35}$S-UTP-labeled *AtLTP1* probes, we could not detect *AtLTP1* transcripts earlier than the torpedo stage. By using the same method, Yadegari *et al.* (1994) did not detect *AtLTP1* transcripts before the bent cotyledon stage. Thus, in our hands, the detection of *AtLTP1* mRNA with digoxygenin-UTP-labeled probes is more sensitive than with radiolabelled probes.

The *AtLTP1* expression pattern in *Arabidopsis* is temporally and spatially identical to that of the *EP2* gene in zygotic and somatic carrot embryos, although in carrot, this expression could be detected with $^{35}$S-labelled probes (Sterk *et al.*, 1991). *AtLTP1* expression in the protoderm of globular *Arabidopsis* embryos is consistent with the proposed role of the AtLTP1 protein in the assembly or deposition of cell wall or cuticular structural material (Sterk *et al.*, 1991; Thoma *et al.*, 1994), because globular-stage *Arabidopsis* (Rodkiewicz *et al.*, 1994), maize (Van Lammeren, 1986), *Capsella* (Rodkiewicz *et al.*, 1994; Schulz and Jensen, 1968a), and *Stellaria* (Rodkiewicz *et al.*, 1994) embryos are all reported to be covered with a cuticular layer. Although histochemical determination of *AtLTP1* promoter activity and *in situ* hybridization results matched perfectly in developing embryos, a discrepancy was seen in the developing seed coat. *In situ* hybridization showed the presence of *AtLTP1* transcripts in the seed coat of developing seeds containing embryos only up to the torpedo stage, whereas GUS staining was observed in seed coats of developing seeds containing embryos up to the maturation stage. Similar discrepancies were noted in a study of *AtLTP1* expression in postembryonic development (Thoma *et al.*, 1994). They could be due to a difference in stability between *AtLTP1* transcripts and the GUS protein, the result of additional, negative control elements not included in the *AtLTP1* promoter region used, or the result of promoter-

independent *GUS* expression (Uknes *et al.*, 1993).

From the combined results of *AtLTP1-GUS* expression and *in situ* hybridization in wild-type *Arabidopsis* embryos, we conclude that pattern formation in the *Arabidopsis* embryo is reflected by the position-specific expression of the *AtLTP1* gene: the *AtLTP1* gene is a marker for the protoderm from the globular stage to the maturation stage, and its expression is restricted to the apical end of the embryo after the heart stage. Thus, the temporal and spatial aspects of *AtLTP1* expression can be used to study cell identity and polarity in mutant embryos.

### AtLTP1 *expression pattern in* gnom, knolle, *and* keule *embryos: implications for pattern formation in the* Arabidopsis *embryo*

In embryos of all three mutants examined here, the temporal regulation of *AtLTP1* expression is similar to that observed in wild-type embryos. This agrees with observations of Yadegari *et al.* (1994) for the embryo mutant *raspberry*. The spatial pattern of *AtLTP1* expression is changed, however, when compared with the wild-type pattern in *gnom* and *knolle* (but not in *keule*) embryos. In *gnom* embryos, the apical-basal expression pattern is changed, whereas the radial expression pattern is as usual. Cloning of the *GNOM* gene (Busch *et al.*, 1996; Shevell *et al.*, 1994) has revealed that it encodes a protein that has similarity with the yeast SEC7 protein, which is involved in protein transport in the yeast secretory pathway. The significance of this sequence similarity for the role of the *GNOM* gene in apical-basal pattern formation remains unclear. In contrast, in *knolle* embryos, apical-basal *AtLTP1* expression is similar to that in wild-type embryos (Figure 2 and data not shown), but the radial distribution of *AtLTP1* mRNA is strikingly different. Interestingly, the *AtLTP1* expression pattern observed in *knolle* embryos shows remarkable similarities to the carrot *EP2* expression pattern of arrested embryos of the temperature-sensitive carrot mutant line ts11.

In arrested globular embryos of ts11, which, like *knolle* embryos, do not form a morphologically normal protoderm, *EP2* expression was found to be uniform or diffuse in subepidermal cells. In addition, the *EP2* gene was found to be expressed uniformly in proembryogenic masses of embryogenic carrot cell cultures (De Jong *et al.*, 1993; Sterk *et al.*, 1991). Recently, positional cloning of the *KNOLLE* gene (Lukowitz *et al.*, 1996) revealed that the predicted KNOLLE protein is similar to syntaxins, a family of proteins involved in vesicular trafficking. More detailed analysis of *knolle* embryos revealed many incomplete cell walls. The cell wall defects are variable and range from merely fragments of cross-walls to walls with small holes. These observations suggest that *knolle* embryos have groups of interconnected cells as a result of incomplete cytokinesis. Based on the finding that fluorescent dye taken up by hypocotyl cells of *Arabidopsis* seedlings readily spreads within the epidermis but not into the underlying ground tissue (Duckett *et al.*, 1994), one could envision that the failure of *knolle* embryos to establish a complete radial pattern

is the result of a continued connection between protodermal and internal cells. Such an interconnection of cells in the radial direction could prevent the initial cells in the *knolle* embryo from acquiring a nonepidermal cell fate.

In this scenario, the presence of *AtLTP1* mRNA in cells other than the outer cells, whether the result of a direct centripetal transport of *AtLTP1* mRNA or of expression of the *AtLTP1* gene in internal nuclei, illustrates the failure to specify internal cells with a fate different from that of the outer cells. Because the radial pattern defect of *knolle* embryos might be a consequence of a primary defect in cytokinesis, this implies that the *KNOLLE* gene does not convey specific information for radial patterning. In all scenarios, the exclusion of *AtLTP1* mRNA from the center of *knolle* embryos at later stages of development and the correct formation of provascular tissue (Mayer *et al.*, 1991) suggest that additional mechanisms not dependent on *KNOLLE* gene action are involved in radial patterning. In *keule* embryos, *AtLTP1* mRNA is confined to the grossly abnormal outer cell layer, as has also been seen in mutant *raspberry* embryos (Yadegari *et al.*, 1994), indicating that *AtLTP1* gene expression is not dependent on protodermal cell morphology.

In *gnom* embryos, the protoderm-specific accumulation of *AtLTP1* mRNA is unchanged, whereas the apical-basal *AtLTP1* expression pattern deviates from the usual. This finding supports the notion that the two body axes form independent of each other. *AtLTP1-GUS* expression is invariably confined to the apical end of wild-type advanced-stage embryos but variably distributed in *gnom* embryos of the same age, which may reflect an inherent variability of apical-basal polarity caused by the lack of *GNOM* activity in the zygote. Unfortunately, other molecular markers that would reveal polarity at an earlier stage of embryogenesis are not currently available. Nevertheless, the evidence presented here strongly suggests that the apical-basal polarity of the embryo is not fixed before fertilization, although the *Arabidopsis* egg cell is morphologically polar.

In other flowering plants, the egg cell appears apolar or polarity is reversed upon fertilization (Johri, 1984). Thus, apical-basal polarity of flowering plant embryos appears to be established within the zygote and fixed by its first division. Radial polarity presents a different case. Uniform *AtLTP1* expression observed in all cells of early *knolle* embryos suggests that in *knolle*, the inner cells retain protodermal character. *knolle* embryos therefore may not be defective in the specification of protodermal cells but instead fail to mark off the inner cells against the outer cell layer. This observation supports the notion that the epidermis may be a "ground state" (Bruck and Walker, 1985a) in plant embryogenesis that is associated with the presence of an outer cell wall partially inherited from the zygote.

It has been suggested that, based on the presence of a cuticle around *C. jambhiri* zygotes, the zygote develops an epidermal character perpetuated in all external cell derivatives of the zygote (Bruck and Walker, 1985a). Internal derivatives, finally giving rise to ground tissue and vascular bundles, would then diverge along a developmental route separate from their epidermal starting point. In this scenario, the walls of the inner cells may lack a wall-associated component of the zygote, and this

would be instrumental in determining radial polarity. Analogies for such a scenario exist in the Drosophila embryo, where the apical but not the basolateral cell membrane of blastoderm cells directly derives from the oocyte plasma membrane (Campos-Ortega and Hartenstein, 1985), and in the fate-determining ability of *Fucus* cell walls (Berger *et al.*, 1994).

The observation that *knolle* mutant embryos eventually show a reduction of the *AtLTP1* mRNA level internally and also form vascular tissue suggests that vascular differentiation, viewed as differentiation of the most internal radial pattern element, involves an additional mechanism that is not dependent on the initial ordered cell divisions in the early *Arabidopsis* embryo. This agrees with the observations that embryos of many plant species, such as cotton, grape, and *Datura* (Johri, 1984), as well as *Arabidopsis* mutant *fass* embryos (Torres-Ruiz and Jürgens, 1994) do not show an ordered pattern of early cell division yet develop complete body plans with all pattern elements present.

## Materials and methods

### *Plant strains and plant growth conditions*

The wild-type strain used was of the Landsberg *erecta* ecotype and was kindly provided by M. Koornneef (Department of Genetics, Wageningen Agricultural University). The mutants *knolle*, *keule*, and *gnom* are described by Mayer *et al.* (1991). Transgenic seed of the *Rschew* ecotype, carrying an *Arabidopsis thaliana* lipid transfer protein (*AtLTP1*) promoter-*β-glucuronidase* (*GUS*) fusion, was kindly provided by C. Somerville (Carnegie Institution, Stanford, CA) (for description, see Thoma *et al.* (1994))

Seeds were sown on wet filter paper (595 Rundfilter; Schleicher & Schuell, Inc., Keene, NH) in Petri dishes. The Petri dishes were stored at 4°C in the dark for at least 24 hr to break dormancy and then transferred to a room at 25°C with a 16-hr photoperiod (7 $W/m^2$). Seedlings were transferred to sterilized potting soil and grown in an air-conditioned greenhouse (at 18 to 23°C), with additional light during the winter (16-hr photoperiod; HP1-T, 400W lights; Philips, Eindhoven, the Netherlands).

Seeds from transgenic *AtLTP1-GUS* plants were surface-sterilized for 2 min in 70% ethanol, followed by three rinses of sterile distilled water. Seeds were placed on Murashige and Skoog medium (Murashige and Skoog, 1963) containing 1% sucrose and 50 mg/L kanamycin and germinated as described above. Kanamycin-resistant seedlings were transferred to sterilized potting soil and grown in a 25°C growth chamber at 80% humidity, with light cycles as described above.

### *Genetic crosses*

Plants heterozygous for *knolle, keule*, and *gnom*, respectively, were used as female parents; plants homozygous for the *AtLTP1-GUS* construct were used as males. Three flower buds of the plants to be used as female parent were emasculated by removing the anthers with forceps. These flowers were pollinated by touching the stigma with anthers from the male parent.

### *Histochemical localization of GUS activity*

Siliques were opened longitudinally and fixed in 0.3 % paraformaldehyde in 100 mM NaPi, pH 7.2, for 1 hr under vacuum. After washing in 100 mM NaPi, pH 7.2, they were immersed in the enzymatic reaction mixture containing 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide as catalysts in 100 mM NaPi, pH 7.2. The reaction was conducted overnight at 37°C in the dark (Jefferson *et al.*, 1987). After the reaction, ovules were mounted in 8:1:2 chloral hydrate-glycerol-water on a microscope slide with a cover slip and left for a period of 1 to 16 hr, depending on the stage. Embryos were removed from the ovules by applying pressure on the cover slip. Staining patterns were analyzed with an Optiphot-2 (Nikon Corp., Tokyo, Japan) using bright-field optics.

### In situ *mRNA hybridization*

*In situ* hybridization was performed essentially as described by Cox and Goldberg (1988). To facilitate handling, siliques or ovules were embedded in agarose before fixation and embedding in paraffin (Sterk *et al.*, 1991). RNA probes labelled with either $^{35}$S-UTP or digoxygenin-uridine 5'-triphosphate (UTP), were transcribed from the plasmid pJ5-3, which contains a cDNA of the *Arabidopsis AtLTP1* gene (kindly provided by C. Somerville (Thoma *et al.*, 1994)), or from the plasmid pAtEP2, which contains a 201-bp genomic insert from an *Arabidopsis LTP* gene, using either the T7 (sense controls) or the T3 (antisense) promoter. Hybridization was performed for 16 hr at 42°C.

For detection of $^{35}$S-UTP-labeled probes, slides were coated with LM-1 nuclear emulsion (Amersham), exposed for 3 weeks at 4°C, and developed (D19 developer; Kodak). Sections were stained with toluidine blue and photographed with an Optiphot-2 (Nikon), using bright- and dark-field optics. The more sensitive detection of digoxygenin-UTP-labeled probes was performed using a digoxygenin nucleic acid detection kit (Boehringer Mannheim), essentially according to the manufacturer's recommendations.

## Acknowledgements

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# 3

# Identification of genes expressed during *Arabidopsis thaliana* embryogenesis using enhancer trap and gene trap *Ds*-transposons

The technique of enhancer trap and gene trap mutagenesis has been exploited to identify new molecular markers for specific cell-types, tissues and regions in the *Arabidopsis thaliana* embryo and seedling. Screening of a population of 373 independent gene trap and 431 enhancer trap lines revealed that 25% of the gene trap insertions, and 81% of the enhancer trap insertions displayed *GUS* expression patterns in the embryo, seedling, silique, seed coat, or flower. A total of 39 lines expressed the *GUS* gene in the embryo. Except for one, all of these also displayed *GUS* expression at other stages of development. The insertion lines with specific *GUS* expression patterns in the embryo provide valuable markers for establishment of cell fate or position in embryo mutant backgrounds. Genomic DNA flanking the insertions was amplified by TAIL-PCR, and found to contain transcribed regions of a gene in all gene trap insertions, and in about a quarter of the enhancer trap insertions. Thus, enhancer trap and gene trap mutagenesis allow isolation of genes expressed during *Arabidopsis* embryogenesis based on expression pattern.

Casper W. Vroemen, Nicole Aarts, Paul M.J. In der Rieden, Ab van Kammen and Sacco C. de Vries (1998) In *Cellular integration of signalling pathways in plant development* (Lo Schiavo, F., Last, R. L., Morelli, G. and Raikhel, N. V., eds.). Berlin Heidelberg: Springer-Verlag, pp. 207-232.

## Introduction

A fundamental question in developmental biology concerns the molecular mechanisms underlying the establishment of polarity and body pattern. In plants, the stereotyped body organization of the seedling is laid down during embryogenesis, and may be viewed as the super-imposition of two patterns, one along the apical-basal or longitudinal axis, and one along the radial axis (Mayer *et al.*, 1991). *Arabidopsis* provides an excellent model system for the genetic dissection of pattern formation during embryogenesis, since, as in other crucifers, the cell division pattern is largely invariant. Also, numerous mutations affecting the body organization of the embryo have been described (reviewed by Mordhorst *et al.* (1997)). A serious problem in the analysis of embryo pattern formation is the shortage of molecular markers for specific cells and regions in the embryo (Jürgens, 1995). Such markers are important, because it is often difficult to establish cell-identity in mutant embryo backgrounds (Devic *et al.*, 1996; Vroemen *et al.*, 1996; Yadegari *et al.*, 1994). Molecular markers for specific cells or regions in the developing *Arabidopsis* embryo identified so far, include the *AtLTP1* gene, expressed in the embryo protoderm (Thoma *et al.*, 1994; Vroemen *et al.*, 1996; Yadegari *et al.*, 1994), the *SCARECROW* gene, expressed in the endodermal cell lineage (Di Laurenzio *et al.*, 1996), the *STM* gene (Long *et al.*, 1996) and the *CLV1* gene (Clark *et al.*, 1997), expressed in the presumptive shoot apical meristem, and the *ATML1* gene, which is expressed in all cells of the embryo proper until the eight-cell stage, in the protoderm from the sixteen cell-stage until the late heart-stage, and in the L1 layer of the shoot apical meristem in the mature embryo (Lu *et al.*, 1996). GUS markers for regions in the *Arabidopsis* embryo, such as the root tip (*POLARIS*), cotyledons and shoot and root apices (*EXORDIUM*), and root cap (*COLUMELLA*) have recently been used to investigate mechanisms involved in establishing polar organization in *Arabidopsis* embryos and seedlings (Topping *et al.*, 1994; Topping and Lindsey, 1997).

As part of a strategy to identify molecular markers for specific cell-types and regions in the *Arabidopsis* embryo, we have undertaken a gene / enhancer trap insertional mutagenesis screen, using the *Ac* / *Ds*-transposon based system described by Sundaresan *et al.* (1995). The gene and enhancer trap elements carry a *GUS* reporter gene that can respond to *cis*-acting transcriptional signals at the site of integration. A particularly useful aspect of this system is that it allows the identification of genes not only by mutant phenotype, but as well by their expression pattern. Many genes that have no visible phenotype upon disruption, because they are functionally redundant or their mutant phenotype is only visible under certain conditions and could be missed in screens for mutant phenotypes (Goebl and Petes, 1986), may be identified by expression pattern in gene trap and enhancer trap screens. Moreover, gene trap and enhancer trap mutagenesis can identify genes that are essential in both the development of the early embryo and later development. The function of such genes in later development can be obscured by an early lethal phenotype (Mlodzik *et al.*, 1990; Springer *et al.*, 1995).

Here we describe the use of gene and enhancer trapping to identify genomic sequences that are expressed or direct gene expression in the *Arabidopsis* embryo. The collection of insertions we have obtained provides a set of molecular markers for specific cell-types, tissues, organs and regions in the developing embryo, and can also be used to clone the corresponding genes.

### *Design of the gene and enhancer trap elements*

The two-element transposon system used in this study employs *Ac* starter lines, homozygous for an immobilized *Ac* element, that are crossed to one of two different *Ds* starter lines, *DsG* or *DsE*, homozygous for a non-autonomous gene trap or enhancer trap *Ds* transposon, respectively. This system was developed by Sundaresan *et al.* (1995). Figure 3.1 outlines schematically the *Ac*, *DsG*, and *DsE* T-DNA vectors and transposons. The *Ac* element contains a CaMV 35S promoter-*Ac* transposase fusion that causes high frequencies of *Ds* excision *in trans* (Swinburne *et al.*, 1992), and is "wings-clipped", meaning that it cannot transpose because it lacks one of the *Ac* termini.

The gene trap element *DsG* is designed to detect expression of a chromosomal gene when inserted within the transcribed region. For this purpose, the *DsG* element contains a promoterless *GUS* gene, whose expression relies on transcription from the tagged chromosomal gene (Friedrich and Soriano, 1991; Gossler *et al.*, 1989; Kerr *et al.*, 1989). Upstream of the *GUS* ATG startcodon, an intron of the *Arabidopsis GPA1* gene (Ma *et al.*, 1990) and a synthetic oligonucleotide containing two additional consensus splice acceptors have been fused, to provide for a splice acceptor in every reading frame. If *DsG* should insert into an intron, with the *GUS* gene in the same orientation as the tagged gene, as shown schematically in Figure 3.2A, splicing occurs from the splice donor of the chromosomal intron to the splice acceptors upstream of the *GUS* gene (Nussaume *et al.*, 1995). By contrast, if inserted in an exon in the correct orientation (Figure 3.2B), multiple splice donor sites, naturally existing at the 3' end of the *Ds* element and covering all possible reading frames (Wessler *et al.*, 1987), are exploited. The sequence between these splice donor sites and the splice acceptors just upstream of the *GUS* gene can be spliced out from the transcript, resulting in a fusion of the endogenous transcript and the *Ds* borne *GUS* transcript. The presence of three splice acceptor sequences, covering all three reading frames, in combination with multiple splice donors, also in each possible reading frame, ensures that for each possible reading frame the *DsG* element could insert into, at least one combination of splice donor and acceptor sequences generates an in-frame fusion between the endogenous and the *GUS* RNA (Nussaume *et al.*, 1995). As is clear from Figure 3.2C, no functional *GUS* fusion transcript is formed if the *DsG* element inserts into a gene in an orientation opposite to that of the tagged gene, which is expected to occur in half of the insertions.

With the *DsE* element, expression of the *GUS* reporter gene is dependent on *DsE* insertion near to chromosomal enhancer sequences. As shown in Figure 3.1, *DsE* contains a *GUS* gene fused

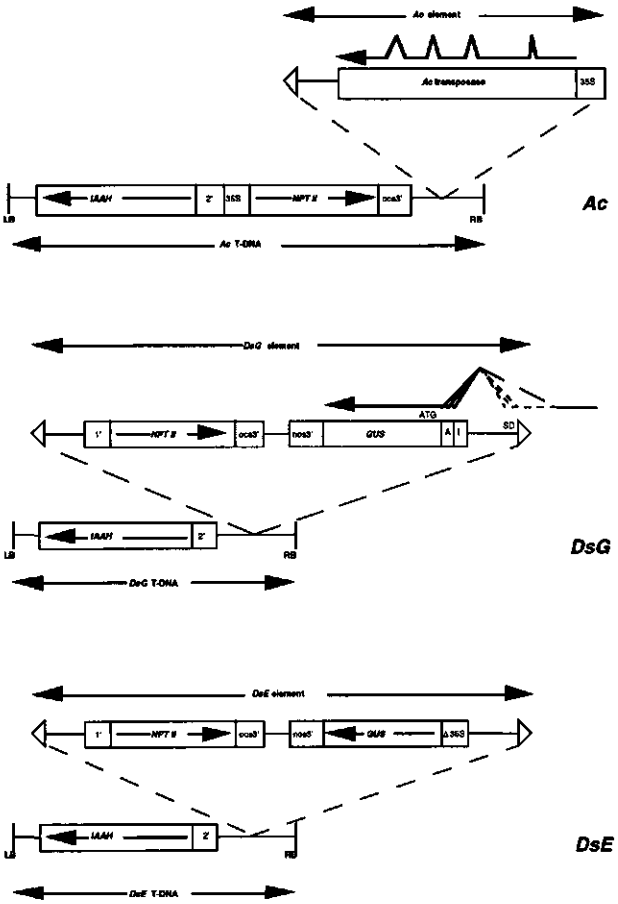


**Figure 3.1:** T-DNA vectors and transposon constructs used in this study.

***Ac*** : T-DNA carrying immobilized *Ac* element.

***DsG*** : T-DNA carrying gene trap element *DsG*.

***DsE*** : T-DNA carrying enhancer trap element *DsE*.

5' (drawn left) and 3' (drawn right) borders of the *Ds* element are represented as open triangles. (35S) CaMV 35S promoter; (*IAAH*) indole acetic acid hydrolase gene conferring sensitivity to NAM; (*NPTII*) neomycin phosphotransferase gene, conferring resistance to kanamycin; (1' and 2') 1' and 2' T-DNA promoter, respectively; (ocs3') octopine synthase terminator; (nos3') nopaline synthase terminator; (LB and RB) left border and right border sequences, respectively, of the T-DNA; (*GUS*) β-glucuronidase gene; (ATG) ATG-startcodon of *GUS* gene; (A) triple splice acceptor; (I) fourth intron of *Arabidopsis* G-protein gene *GPA1*; (SD) multiple splice donor sites at 3' end of *Ds*-element, covering all three reading frames; (Δ35S) -1 to -46 bp region of CaMV 35S promoter. Broken and dotted arrows in *DsG* represent splicing if insertion is into an intron or exon, respectively. This figure has been adapted from Sundaresan *et al.* (1995).

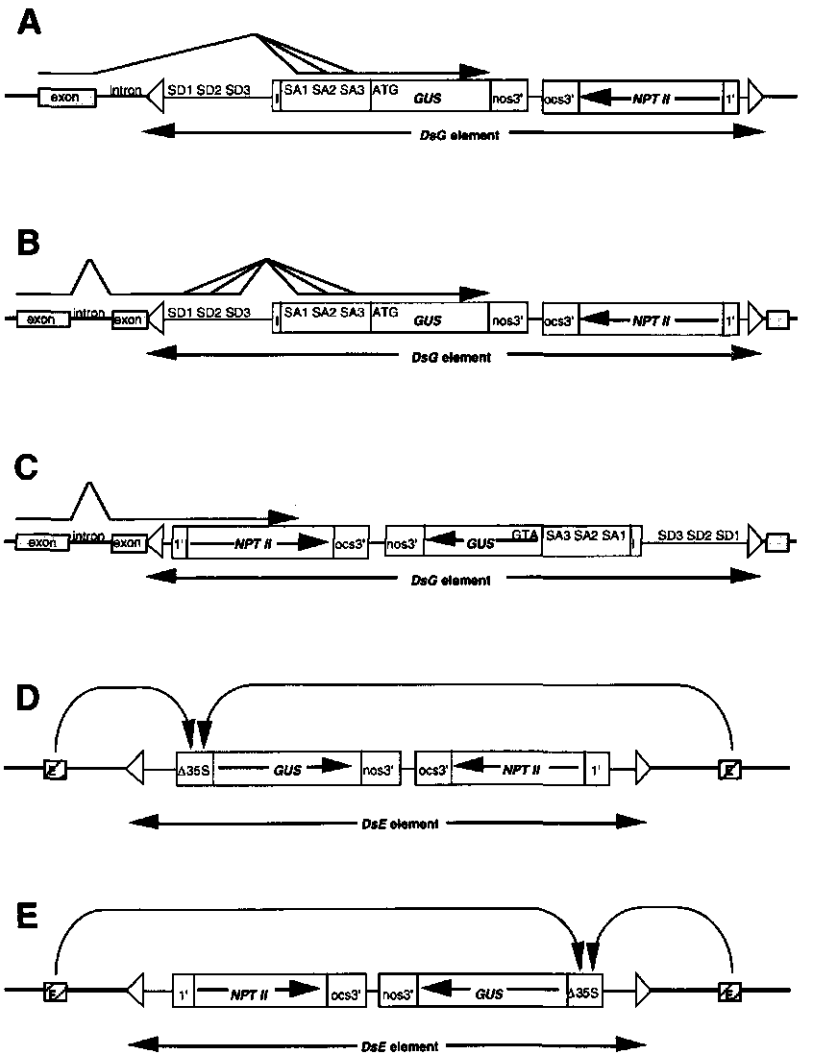


**Figure 3.2:** Different possibilities for chromosomal insertion of *DsG* and *DsE* elements.

**A:** *GUS* expression from *DsG* element after insertion into an intron.

**B:** *GUS* expression from *DsG* element after insertion into an exon.

**C:** no *GUS* expression from *DsG* element due to insertion in opposite orientation.

**D** and **E:** *GUS* expression from *DsE* element after insertion near a chromosomal enhancer, independent of orientation. (SD1, SD2, and SD3) splice donor sides at 3' end of *Ds* element, each in a different reading frame; (SA1, SA2, and SA3) splice acceptor sites, each in a different reading frame; (E) Chromosomal enhancer.

For further details see Figure 3.1. This figure has been adapted from Sundaresan *et al.* (1995).

to a minimal -1 to -46 bp CaMV 35S promoter, which is not active in the absence of enhancer sequences (Benfey *et al.*, 1989). When the *DsE* element inserts in the proximity of a chromosomal gene, within or outside of the coding region, *GUS* gene expression can be activated by a neighboring chromosomal enhancer (Bellen *et al.*, 1989; Bier *et al.*, 1989; Klimyuk *et al.*, 1995; O'Kane and Gehring, 1987; Sundaresan *et al.*, 1995; Wilson *et al.*, 1989). Since enhancers are known to act in an orientation-independent manner, insertions in either orientation may result in *GUS* expression (Figures 3.2D and 3.2E). In addition to the *GUS* reporter gene, both types of *Ds* elements carry a *NPTII* gene, which confers resistance to kanamycin as a selection marker.

### *Selection for plants carrying transposed* Ds *elements*

A limitation of the *Ac / Ds* transposable element system for transposon tagging is the preferential transposition to sites that are closely linked to the donor locus (Bancroft and Dean, 1993; Belzile and Yoder, 1992; Dooner and Belachew, 1989; Greenblatt, 1984; Jones *et al.*, 1990; Keller *et al.*, 1993; Osborne *et al.*, 1991). In the selection scheme used in this study, local transpositions are eliminated, because donor-T-DNA located enhancers could directly cause *GUS* expression in lines carrying a *Ds* insertion close to the donor T-DNA (Klimyuk *et al.*, 1995). Moreover, by selecting against local transpositions, a more or less random distribution of *Ds* insertions throughout the genome can be obtained.

Figure 3.3 outlines the selection scheme used to generate lines carrying a stable *Ds* insertion at a location unlinked, or loosely linked, to the donor T-DNA. These lines are referred to as transposants (Bellen *et al.*, 1989), and their selection is accomplished by selecting for the presence of the *Ds* element and, simultaneously, against both the *Ac* T-DNA and the *Ds* donor-T-DNA. Selection for the presence of the *Ds* element is possible due to the *NPTII* gene, conferring kanamycin resistance, that is located on both the *DsG* and *DsE* elements (Figure 3.1). Selection against the *Ac* and *Ds* donor-T-DNA is accomplished using the indole acetic acid hydrolase (*IAAH*) gene, present on the *Ac*-T-DNA, the *DsG* donor-T-DNA, and the *DsE* donor-T-DNA (Figure 3.1). The *IAAH* gene can be used as a counter selectable marker (Karlin-Neumann *et al.*, 1991), because it confers sensitivity to naphtalene acetamide (NAM), by converting it to the potent auxin naphtalene acetic acid (NAA), which causes severely stunted seedlings. Due to the selection against the presence of the *DsG* and *DsE* donor T-DNA, the recovery of transposed *Ds* elements depends on the recombination frequency between the *Ds* donor-T-DNA and the transposed *Ds* element. Only *Ds* elements that have transposed to locations unlinked or loosely linked to the donor-T-DNA are recovered.

Transposition of *Ds* elements is initiated by crossing *Ds* starter lines, homozygous for the *DsE* or *DsG* donor-T-DNA, to *Ac* starter lines, homozygous for the *Ac* T-DNA (Figure 3.3). In the resulting F1 plants, *Ds* transposition can occur under the influence of *Ac* transposase. After allowing

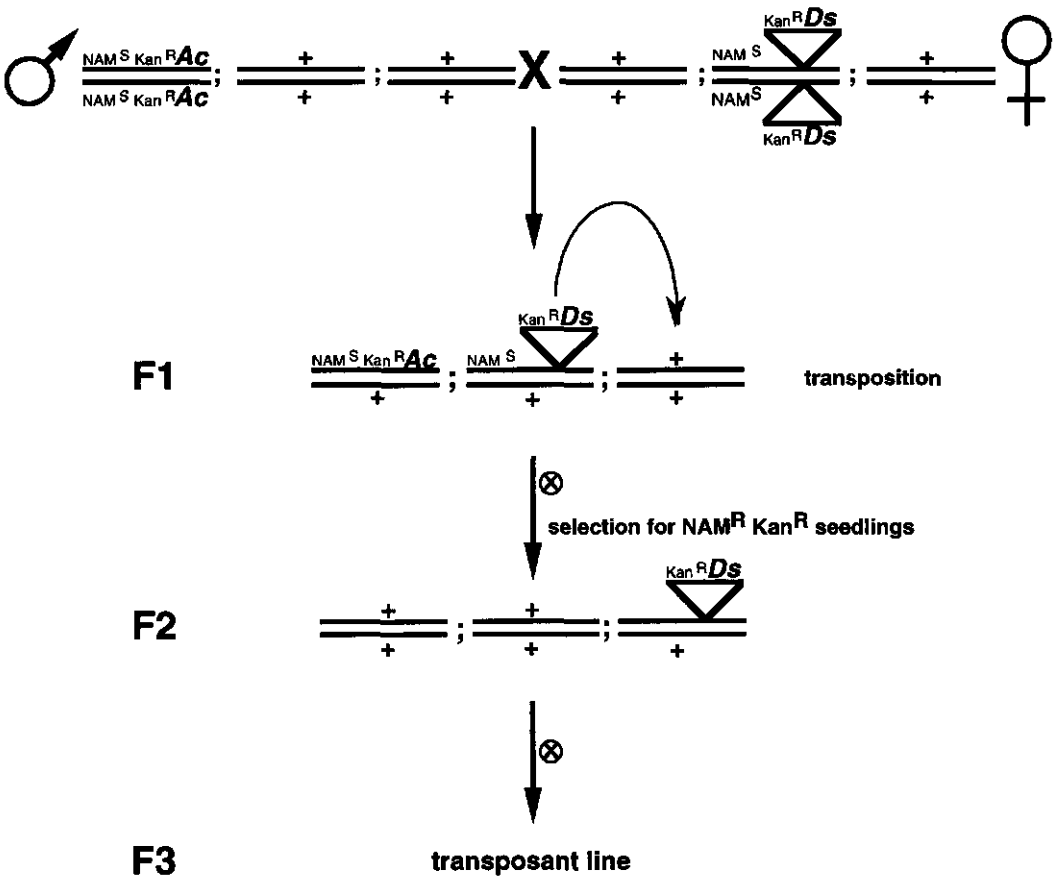


**Figure 3.3:** Generation of transposants, i.e. lines carrying transposed *Ds* elements by selection for stable, unlinked transposition events.
($NAM^S$) NAM sensitivity caused by *IAAH* gene; ($Kan^R$) kanamycin resistance caused by *NPTII* gene. Transposable element *Ds* is represented by a triangle. This figure has been adapted from Sundaresan *et al.* (1995).

the F1 plants to self-fertilize, the F2 seed families are germinated on plates containing kanamycin and NAM, and the double resistant seedlings are recovered as transposants. Transposant lines are maintained as F3 seed batches, obtained by self-fertilization of selected F2 plants. By selecting against *Ac*, the *Ds* element in selected transposant plants will not be able to re-transpose. Thus, the transposant lines obtained this way represent a collection of stable *DsE* and *DsG* insertion lines that serves as source for further screening and characterization.

# Results

### *Generation of independent gene and enhancer trap lines*

Gene trap and enhancer trap *Ds* elements were induced to transpose by crossing plants from *DsG* or *DsE* starter lines to plants from *Ac* starter lines. Table 3.1 outlines the different combinations of *Ac* and *DsG* or *DsE* starter lines used, and the numbers of independent F2 families that resulted from crosses of each combination. Each of two *Ac* starter lines were crossed to each of four *DsG* and each of four *DsE* starter lines, resulting in 2610 F1 plants heterozygous for *DsE* and *Ac*, and 1975 F1 plants heterozygous for *DsG* and *Ac*. Self-fertilization of the F1 plants and collection of F2 seed from each individual F1 plant yielded *DsG* and *DsE* F2 families. In the F2 generation, selection was performed by plating approximately 750-1000 F2 seeds on NAM-Kan plates, as described in Materials and Methods. Out of 1975 *DsG* F2 families, 373 independent gene-trap lines were established. Out of 2610 *DsE* F2 families, 431 independent enhancer-trap lines were recovered. Thus, 19% of the *DsG* carrying F1 plants (373/1975) and 17% of the *DsE* carrying F1 plants (431/2610) generated $NAM^RKan^R$ F2 progeny (transposants) in a frequency high enough to allow detection of at least one transposant per selection plate.

Over 80% of the F1 plants had no $NAM^RKan^R$ progeny, suggesting that in these plants there were either no *Ds* transpositions, only transpositions without re-integration, or only transpositions to sites closely linked to the donor locus, which are known to occur frequently in the *Ac-Ds* transposon system (Bancroft and Dean, 1993; Belzile and Yoder, 1992; Dooner and Belachew, 1989; Greenblatt, 1984; Jones *et al.*, 1990; Osborne *et al.*, 1991). F1 plants that did have $NAM^RKan^R$ progeny typically generated between 0.1% and 3% (i.e. 1-30 per 1000 F2 seedlings) $NAM^RKan^R$ F2 seedlings. In accordance with the results obtained by Sundaresan and co-workers (Sundaresan *et al.*, 1995) , the majority of F1 plants that did have $NAM^RKan^R$ progeny, yielded between 0.1% and 1% of $NAM^RKan^R$ F2 seedlings. It should be noted that, since only one-sixteenth (6.25%) of the progeny of an F1 plant is expected to be $NAM^R$, and the frequency of forward transposition, defined as the proportion of F2 plants in which the *Ds* element has excised from the donor locus, with the 35S-*Ac* transposase fusion used ranges from 5 - 50% in the F2 generation (Long *et al.*, 1993), a single transposition event is unlikely to result in more than 3% of $NAM^RKan^R$ progeny.

### *Molecular analysis of transposants*

To ascertain whether the NAM selection effectively selected against both the *Ac* T-DNA and the *Ds* T-DNA, and whether the kanamycin selection resulted in selection for inheritance of a *Ds* element, DNA

**Table 3.1:** Numbers of F2 families generated from different combinations of *Ac* and *DsE* or *DsG* starter lines, respectively, and numbers and frequencies of recovered transposant lines.

| starter lines | No. of F2 families generated | No. of transposant lines | % of F2 families yielding a transposant line |
|---|---|---|---|
| DsE1 x Ac1 | 477 | 107 | 22 |
| DsE1 x Ac2 | 584 | 65 | 11 |
| DsE2 x Ac1 | 225 | 38 | 17 |
| DsE2 x Ac2 | 455 | 55 | 12 |
| DsE3 x Ac1 | 70 | 39 | 56 |
| DsE3 x Ac2 | 657 | 93 | 14 |
| DsE6 x Ac1 | 60 | 13 | 22 |
| DsE6 x Ac2 | 82 | 21 | 26 |
| Total | 2610 | 431 | 17 |
| DsG1 x Ac1 | 519 | 103 | 20 |
| DsG1 x Ac2 | 409 | 82 | 20 |
| DsG6 x Ac1 | 95 | 32 | 34 |
| DsG6 x Ac2 | 349 | 20 | 6 |
| DsG7 x Ac1 | 123 | 38 | 31 |
| DsG7 x Ac2 | 419 | 89 | 21 |
| DsG8 x Ac1 | 12 | 5 | 42 |
| DsG8 x Ac2 | 49 | 4 | 8 |
| Total | 1975 | 373 | 19 |

from independent transposant seedlings was checked for the presence of the *IAAH* gene (which is located on both the *Ac* and *Ds* T-DNAs) and the *GUS* gene (which is located only on the *Ds* element). PCR using different sets of *IAAH* and *GUS* specific primers showed that all transposant lines contained the *GUS* gene, confirming inheritance of the *Ds* transposon, and not the *IAAH* gene, providing evidence for the absence of both *Ac* and *Ds* T-DNAs (data not shown). As expected, both the *GUS* gene and the *IAAH* gene were detected in *DsG* and *DsE* starter lines, whereas only the *IAAH* gene was detected in *Ac* starter lines. These results prove that the NAM-Kan selection procedure effectively selected for transposed *Ds* elements and against *Ac* and *Ds* T-DNAs. The selection against the *Ds* T-DNA results in selection against nearby re-insertion of the *Ds* element (Sundaresan *et al.*, 1995).

Figure 3.4 shows Southern blot analysis of DNA from 21 selected gene trap and enhancer trap transposant plants. Genomic DNA was digested with *Pst*I, which cuts once in the *NPTII* coding sequence within the *Ds* element. Probing with the entire *GUS* coding sequence showed that 20 of the transposants carried a single copy of the *Ds* element, whereas 1 transposant (WET115) contained 2 *Ds* elements inserted at different chromosomal locations. The different fragment lengths observed in the lanes in Figure 3.4 are indicative of the different chromosomal locations, and thus different flanking genomic sequences of *Ds* insertions in the selected transposants. A single transposed *DsG* or *DsE* element is essential for efficient screening of the transposant lines for specific *GUS* expression patterns, since more than one transposed *Ds* element, at different chromosomal locations, could result in overlapping or combined GUS expression patterns, under the control of regulatory regions at

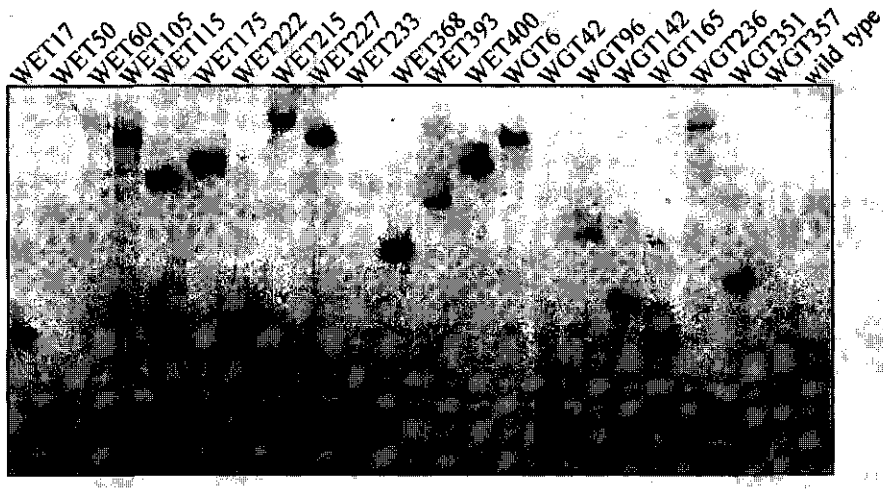


**Figure 3.4:** Southern blot of DNA from 21 selected gene trap and enhancer trap transposants.
The DNAs were digested with *Pst*I, and the probe used was the entire *GUS* coding sequence.

different sites in the genome. This would of course complicate interpretation of *GUS* expression patterns during the primary screen.

### *Screening transposants for* GUS *expression patterns*

All 373 gene trap (WGT) transposants and 431 enhancer trap (WET) transposants were examined for *GUS* expression patterns at various stages of plant development: seedling, flower, silique, developing seed and embryo. The results of the GUS staining data are summarized in Table 3.2. 27% of the WGT lines (100 out of 373) displayed *GUS* expression at some stage of the plant life cycle. The frequency of *GUS*-expressing WET lines amounted to 81% (317 out of 431), much higher than the frequency of *GUS* expressing WGT lines. In 58% of the *GUS* expressing WET lines (184 out of 317), GUS staining was only found in pollen grains. Due to the high frequency of this staining pattern in this and other screens employing a minimal 35S-promoter-*GUS* fusion for detection of enhancer action (Klimyuk *et al.*, 1995), and taking into account other reports on possible artefactual GUS staining in pollen (Klimyuk *et al.*, 1995; Mascarenhas and Hamilton, 1992; Uknes *et al.*, 1993), lines displaying this "pollen only" staining pattern are put in a separate class. It is unclear what the significance of the pollen GUS staining is. The fact that it occurs at much higher frequencies in enhancer trap lines than in gene trap lines (Table 3.2) suggests that it is not only the result of artefactual expression of the *GUS* gene independent of its genetic context, but that it can also be caused in some way by the minimal 35S promoter included in the enhancer trap *Ds* element. If the "pollen only" staining lines are excluded, the over-all GUS staining frequency of the WET lines (34%) is roughly similar to that of the WGT lines (25%). The lower staining frequency of the WGT

**Table 3.2:** Numbers and frequencies of *GUS* expressing WGT and WET lines.

| | WGT-lines | | WET-lines | |
|---|---|---|---|---|
| No. screened | 373 | | 431 | |
| No. showing *GUS* expression (%) | 100 | (27%) | 317 | (81%) |
| No. showing "pollen only" expression | 7 | | 184 | |
| No. other than "pollen only" (%) | 93 | (25%) | 133 | (34%) |

lines could be explained by both the orientation dependency of gene trap *Ds* insertions (see Figure 3.2), and the necessity of *DsG* insertion into the transcribed region of a gene, to be able to cause *GUS* expression.

Table 3.3 shows a summary of the frequencies of GUS staining patterns of WGT and WET lines found at different developmental stages and in different plant organs. Since our screen was primarily focused on detection of *GUS* expression in embryos and seedlings, GUS staining in other plant organs, such as the different flower organs, the silique and the seed coat, is put in a single category. The fact that "pollen only" staining occurs at high frequency among WET, but not among WGT lines, would complicate comparison of the staining frequencies in WGT and WET lines. Therefore, the class of "pollen only" staining lines is not included in Table 3.3. In a total of 39 lines, 12 WGT and 27 WET lines, *GUS* expression was detected in the embryo. The number of embryo-staining lines was rather low as compared to the total number of staining lines: only 13% of the GUS-positive WGT lines and 20% of the GUS-positive WET lines showed staining in the embryo. This corresponds to overall frequencies of 3-4% and 6-7% *GUS* expression in embryos, among gene and enhancer trap lines, respectively. Most of the lines that showed GUS staining in the embryo also had GUS staining in the seedling: 9 out of 12 for the embryo staining WGT lines, and 23 out of 27 for the WET lines. In some cases, the seedling staining pattern corresponded precisely to that in the embryo, whereas in other cases it was either completely different or resembled the embryo pattern only partially (data not shown). None of the WGT lines, and only one WET line showed embryo-specific *GUS* expression, taken as *GUS* expression only detectable in the embryo and not at any other stage of development or in any other plant organ. In this line, WET393, *GUS* expression is restricted to the suspensor (see Figure 3.5), which is *senso stricto* not even part of the embryo proper. In all other embryo staining lines, *GUS* expression was not restricted to the embryo, but also seen at other developmental stages, or in more than one organ or tissue. Among this class are also lines with a specific staining pattern in the embryo, that is perpetuated in the seedling. Other lines show, for example, GUS staining in the embryo and also in one or more flower organs. *GUS* expression in different organs or at different developmental stages could point to genes that are expressed in different developmental programs, or towards genes which are expressed in similar cell types or tissues in different plant organs (Sundaresan *et al.*, 1995). The frequency of lines that show *GUS* expression in seedlings is higher than the frequency of embryo staining lines. Among both the WGT and WET lines, more than half of the staining lines show *GUS* expression at the seedling stage (52% and 60%, respectively), and in approximately one third of these (17/48 and 24/80, respectively), *GUS* expression is restricted to the seedling stage. The majority of the GUS positive WGT and WET lines (77% for both) shows GUS staining somewhere in the flower, silique and / or seed coat. It should be noted that these include lines with staining patterns in, for example, a single flower organ, as well as lines with expression in different organs, and lines that are also expressed at the embryo and / or seedling stage.

**Table 3.3:** Summary of *GUS* expression in WGT and WET lines at different developmental stages.

| | WGT-lines | | WET-lines | |
|---|---|---|---|---|
| Total no. of *GUS* staining lines[a] | 93 | | 133 | |
| **Stain observed in:** | | | | |
| embryo (% of total staining lines[a]) | 12 | (13%) | 27 | (20%) |
| from which also in seedling | | 9 | | 23 |
| from which embryo-specific | | 0 | | 1 |
| seedling (% of total staining lines[a]) | 48 | (52%) | 80 | (60%) |
| from which seedling specific | | 17 | | 24 |
| flower / silique / seed coat (% of total staining lines[a]) | 72 | (77%) | 102 | (77%) |

[a]Lines with "pollen only" expression are not included.

Figure 3.5 shows an example of an enhancer trap line with a restricted *GUS* expression pattern, that was found in our screen for GUS staining patterns in the embryo. In this line, WET393, *GUS* expression is restricted to the basal cells of the suspensor from the pre-globular to the late heart stage (Figure 3.5 A-C). In later stage embryos, from the torpedo stage up to the mature embryo stage, *GUS* expression was seen in the entire suspensor, but not in the embryo proper (Figure 3.5 D-F).

### *Screening transposants for mutant phenotypes*

During our screen for *GUS* expression patterns in the embryo, we observed two WGT lines with morphological defects in the embryo (not shown). One of these lines segregated for embryos with strongly reduced cotyledons, and exhibited a good correlation between mutant phenotype and cotyledon specific *GUS* expression pattern. Our screening protocol for detection of *GUS* expression patterns in the developing embryo was optimised for detection of GUS staining in the embryo, and

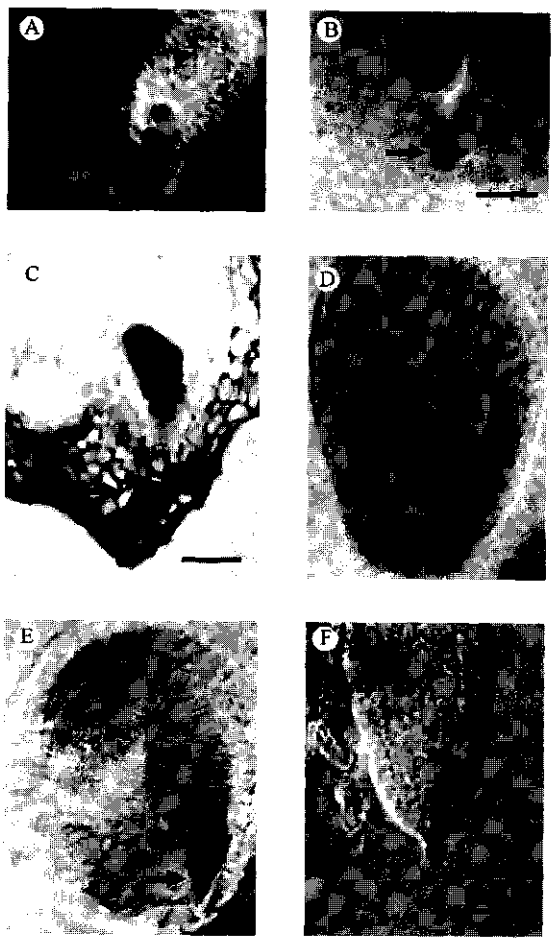


**Figure 3.5:** *GUS* expression pattern of enhancer trap line WET393.

**A:** developing seed with globular stage embryo.

**B:** transition stage embryo.

**C:** histological section of a developing seed with transition stage embryo.

**D:** developing seed with early torpedo stage embryo.

**E:** developing seed with bent cotyledon stage embryo.

**F:** basal region of bent cotyledon stage embryo after dissection out of the seed. The upper suspensor cell is still attached to the embryo proper.

Arrow points at GUS stained cells in the basal part of the suspensor in **A-C**, and in the entire suspensor, but not in the embryo proper, in **D-F**. Bar = 50 µm.

did not allow visualisation of subtle morphological aberrations in the embryo. This was caused by the fact that after the GUS staining reaction, clearing of the seed coat with Hoyers solution was much less efficient than without prior GUS staining. Therefore, it is likely that subtle phenotypic aberrations in the embryo were missed during our primary screen. A detailed screen of the GUS positive WGT and WET lines for phenotypically visible mutations in the developing embryo is currently being performed. In the case of gene trap lines, *DsG* insertion within the transcribed region of a gene is a prerequisite for *GUS* expression. Mutant embryo phenotypes caused by disruption of genes that are expressed in the embryo are, therefore, expected to occur at higher frequencies among embryo-staining WGT lines than among embryo-staining WET lines.

Morphologically visible phenotypes were more easily observed at developmental stages later than the embryo stage. Although not studied in detail, putative mutant phenotypes were observed in 26 out of 366 WGT lines (7%), and in 16 out of 400 WET lines (4%). Assuming an equal transposition behaviour for the *DsG* and *DsE* transposons, the frequency of insertion into a gene, possibly causing a mutant phenotype, is expected to be equal among gene trap lines and enhancer trap lines. Putative mutants included pigmentation mutants (chlorotic leaves), dwarfs, plants with retarded development, male steriles, plants with aberrant floral morphology, bushy plants and plants with aborted seeds. Detailed analysis, including verification of cosegregation of the mutant phenotype and the *Ds* element is in progress for some of the putative morphological mutants.

### *Amplification of genomic DNA flanking* DsG *and* DsE *insertions*

Genomic DNA flanking *DsG* and *DsE* insertions has been generated by TAIL-PCR (Liu *et al.*, 1995) for 27 of the 39 WGT and WET lines with *GUS* expression in the embryo. Typically, 50 bp to 1.5 kb of flanking DNA was obtained using this procedure. In our hands, the success rate of the TAIL-PCR procedure was on average 60%, meaning that in a successful experiment, flanking DNA was generated for approximately 60% of the lines. The sequence of flanking DNA from two gene trap lines showed that in both lines, the *DsG* element had inserted within the putative open reading frame of a gene. For three out of four enhancer trap lines from which flanking DNA was sequenced, no open reading frame could be detected in the sequence of the DNA flanking the *DsE* insertion. If an open reading frame is detected in the sequence of the flanking DNA, it may be possible to predict the function of the tagged gene from its sequence. This was the case for one of the two gene trap insertions of which flanking DNA was sequenced. Taken together, the TAIL-PCR procedure proved efficient in generating flanking DNA from WGT and WET insertions. In general, the generation of flanking DNA provides sequence information and molecular probes which can be used to determine the map position of the *Ds* insertions, and in library screening. For a selected number of lines, this work is currently in progress.

## Discussion

In this study, we have used the *Ac / Ds* based gene / enhancer trap system developed by Sundaresan and co-workers (Sundaresan *et al.*, 1995) to detect genes that exhibit position-, cell-type- or tissue-specific expression in the *Arabidopsis thaliana* embryo. The modification of insertional mutagens to contain a reporter gene overcomes the limitation of conventional insertional mutagens that only allow detection of genes that give a phenotype when disrupted. The reporter gene carried by a gene or enhancer trap element permits the detection of genes by expression pattern rather than by mutant phenotype only. Previously, Fedoroff and Smith (1993), Smith and Fedoroff (1995), and Klimyuk *et al.* (1995) have described the successful use of an enhancer trap *Ds* element for detection of plant enhancers. A serious limitation on the use of these elements as enhancer traps is imposed by the preferential transposition of *Ac / Ds* elements to closely linked sites. Enhancer sequences located on the *DsE* donor T-DNA could directly cause *GUS* expression in lines carrying a *DsE* insertion close to the donor T-DNA. Klimyuk *et al.* (1995) found that almost half of the lines that showed *GUS* expression revealed expression patterns similar or identical to those of the *DsE* starter lines they originated from. The parental GUS staining resulting from T-DNA located enhancers could partly or totally obscure novel GUS staining patterns conferred by endogenous enhancers. For this reason, and to obtain a more random distribution of *Ds* insertions across the genome, the mutagenesis scheme applied in this study includes a one-step plate selection against the *Ds* donor T-DNA, thus enriching for unlinked transpositions (Sundaresan *et al.*, 1995). At the same time, this procedure selects against lines carrying unexcised *Ds* elements, against the presence of the *Ac* T-DNA, and for re-insertion of the *Ds* element after excision from the donor T-DNA. This alleviates the need of large-scale PCR or Southern hybridization analyses, necessary in other systems (Klimyuk *et al.*, 1995) for selection of transposant lines. As an inevitable consequence of the relatively infrequent transposition of *Ds* elements to unlinked or loosely linked sites, only about one-fifth of the F1 plants yielded F2 progeny that survived our selection scheme: 19% for gene trap lines, and 17% in case of enhancer trap lines. These frequencies are somewhat lower than the frequency of plants with unique transposition events reported by Klimyuk *et al.* (1995), who obtained 87 of these plants starting from 314 F1 plants (28%). However, the mutagenesis scheme used in that study neither included selection against local transposition events, nor against the *Ac* T-DNA.

From the frequencies of recovered transposant lines resulting from crosses of each combination of starter lines (Table 3.1), it is apparent that particular combinations, such as for example *DsG6 Ac2*, and *DsG8 Ac2*, gave a very poor recovery of transposants. Such a poor recovery of $NAM^{R}Kan^{R}$ progeny could arise if the *Ac* and *Ds* T-DNA loci from two starter lines were linked in repulsion. In this case, F2 progeny would only survive NAM-Kan double selection if recombination would occur between the two loci. From recombinant inbred mapping (Lister and Dean, 1993), it is known that *DsG1* , *DsE1*, *DsE2*, and *DsE3* are not linked to either *Ac1* or *Ac2*

(Sundaresan *et al.*, 1995). For the other starter lines used in this study, such mapping data are currently unavailable. The fact that, in the case of the *DsG6 Ac2* and *DsG8 Ac2* combinations, only 14 transposant lines were recovered from nearly 400 F2 families, clearly points out the importance of testing each combination of *Ds* and *Ac* starter lines before proceeding with large scale mutagenesis. Determination of the map position of T-DNA donor loci might also help to select for more optimal combinations of *Ac* and *Ds* starter lines. In future large scale mutagenesis programs, the over-all yield of transposant lines might be improved by selecting combinations of starter lines based on the frequencies found in this study.

Molecular analysis of transposants obtained through the described selection procedure showed that 95% of the insertions generated by *Ds* transposition were single copies. This almost eliminates the possibility of detecting *GUS* expression patterns arising from more than one independent or multiple tandem insertions. This is in contrast to T-DNA promoter trap insertion methods (Kertbundit *et al.*, 1991; Koncz *et al.*, 1989; Topping *et al.*, 1994; Topping and Lindsey, 1995; Topping *et al.*, 1991), in which only 50-60% of the lines carried T-DNA at a single locus (Lindsey *et al.*, 1993). Moreover, these loci often contained rearranged or multiple tandem insertions of the T-DNA, which could influence reporter gene expression and complicate amplification of flanking DNA sequences. From the results of our screen, it is clear that the selection against local transpositions, and the fact that the vast majority of our lines has a single insert, greatly facilitates screening of transposant lines for *GUS* expression patterns.

### GUS *expression in gene / enhancer trap transposants*

The fraction of transposants that showed *GUS* expression at some stage of the plant life cycle was 27% for gene trap lines and 81% for enhancer trap lines. After correction for the very abundant class of "pollen only" expressing lines found among enhancer traps, these frequencies were 25% for gene traps, and 34% for enhancer traps. Sundaresan and co-workers obtained a similar frequency of *GUS* expressing gene trap lines (26%, Sundaresan *et al.*, 1995). Our frequency of 34% for *GUS* expression among enhancer trap lines is somewhat lower than the 48% reported by Sundaresan *et al.* (1995). This could be due to differences in the developmental stages at which transposants were assayed for *GUS* expression between the two different screens. Although not explicitly mentioned in their paper, Sundaresan and co-workers also found very frequent pollen staining among their collection of enhancer trap lines (R. Martienssen, pers. comm.).

Klimyuk *et al.* (1995) reported an over-all GUS staining frequency of 60% among a collection of enhancer trap lines. However, almost half of the *GUS* expressing lines revealed expression patterns similar or identical to the parental staining patterns, and besides those, the majority of the GUS-stained lines showed common staining patterns, such as pollen, stigma or stipule specific

staining. Only a limited number of lines (11%) showed unique expression patterns. It could be possible that, in some lines, the high level of parental GUS staining obscures weaker levels of *GUS* expression under the control of endogenous enhancers.

In our collection of WGT and WET lines, the frequency of GUS staining in embryos was relatively low as compared to the total frequency of GUS staining lines. The overall frequencies of *GUS* expression in embryos (3-4% among WGT lines and 6-7% among WET lines) compare well with the 3-4% frequency of *GUS* expression in embryos obtained with a T-DNA-based promoter trap approach, in which lines with T-DNA insertions at a single locus were studied (Topping *et al.*, 1994).

The overall frequencies found by us for *GUS* expression in seedlings were 13% and 19%, for WGT and WET lines, respectively. This is significantly higher than the frequencies of *GUS* expression in embryos. The low number of lines that show *GUS* expression in the embryo may reflect a relatively low number of genes expressed in the embryo, or may partly be caused by a low expression level of the *GUS* reporter gene in some lines, in combination with a low accessibility of the embryo for the GUS substrate. Surprisingly, only one line out of 39 lines in which GUS staining was observed in the embryo, showed *GUS* expression during embryogenesis only, and not during post-embryonic development. This enhancer trap line has restricted *GUS* expression in the basal cells of the suspensor, and during later stages of embryogenesis in all suspensor cells, indicating that in fact no real embryo-specific *GUS* expressing line was recovered. All other lines displaying *GUS* expression in the embryo also show GUS staining at other developmental stages, such as the seedling, flower, silique, or seed coat. These include lines with similar expression patterns in the embryo and the seedling, such as lines displaying hypocotyl or cotyledon specific staining in both embryo and seedling, and lines staining in tissues that exist in the embryo, seedling and mature plant, such as the shoot meristem and vascular tissue. In these lines, *GUS* expression seems to remain associated with a specific tissue or position in the plant throughout development. Thus, in our screen, far more tissue- and position-specific than embryo-specific expression patterns were detected. Examples of *Arabidopsis* genes exhibiting such tissue- or position-specific expression pattern include *AtLTP1* (Thoma *et al.*, 1994; Vroemen *et al.*, 1996), *ATML1* (Lu *et al.*, 1996) , *SCARECROW* (Di Laurenzio *et al.*, 1996), and *STM* (Long *et al.*, 1996).

The detection of lines with expression patterns in multiple organs was not surprising: such expression patterns could be explained by the occurrence of common cell types or activities, such as cell division (Springer *et al.*, 1995) or photosynthesis, in different organs, or by the repeated use of the same gene products in different developmental programs. Examples of the latter are commonly found in animal systems. For example, the components of the ras signalling pathway are involved in specifying cell fates during *Drosophila* embryogenesis, wing vein formation, eye development and oogenesis (e.g. reviewed by Ruohola-Baker *et al.* (1994)). So far, none of the genes identified in embryo mutant screens in plants, such as *Bio-1* (Schneider *et al.*, 1989), *EMB30/GNOM* (Busch *et al.*, 1996; Shevell *et al.*, 1994), *FUSCA-1* (Castle and Meinke, 1994), *PROLIFERA* (Springer *et al.*,

1995), *KNOLLE* (Lukowitz *et al.*, 1996), *STM* (Long *et al.*, 1996), *SCARECROW* (Di Laurenzio *et al.*, 1996), and *CLAVATA1* (Clark *et al.*, 1997), exhibit an expression pattern that is restricted to the embryo. A rare example of a gene expressed exclusively during early plant embryogenesis is the carrot *SERK* gene (Schmidt *et al.*, 1997), whose expression ceases after the early globular embryo stage.

### *Mutant phenotypes in gene / enhancer trap transposants*

The frequency of aberrant phenotypes observed among the transposant lines was 4 and 7% for WET and WGT lines, respectively. This is consistent with frequencies found in previous screens using *Ac / Ds* systems in *Arabidopsis* (2.1% (Bancroft *et al.*, 1993), 8% (Altmann *et al.*, 1995), 5% (Bhatt *et al.*, 1996)). This frequency is significantly lower than the frequency of transposant lines that show *GUS* expression. This can partly be attributed to the fact that the screening conditions applied were predominantly aimed at the detection of GUS staining and, in most cases, did not allow visualisation of very subtle phenotypic aberrations. Nevertheless, from our data it is clear that the frequency of mutants is low as compared to the frequency of expression patterns. This difference is not surprising for a number of reasons. First, many insertions, especially enhancer trap insertions, may result in *GUS* expression without gene disruption, if the insertion is upstream, downstream, or in a non-essential region of the coding region of the gene. In these cases, the generation of mutant alleles could be possible by inducing secondary transposition using *Ac*. Second, even in case of gene disruption, the resulting phenotype might be subtle, or only visible under non-standard growth conditions, and would therefore be missed in our screen. Finally, the tagged gene might be functionally redundant, so that even in case of a severe disruption of gene function, no phenotype arises. Together, these data imply that gene / enhancer trapping is particularly useful for the identification of genes that would be missed in genetic screens for mutant phenotypes.

### *Gene isolation*

Analysis of the genomic regions responsible for the observed *GUS* expression patterns has not been completed yet, but it is evident that TAIL-PCR amplification of genomic DNA flanking the *DsG* and *DsE* insertions is a straightforward method. In this context, an advantage of gene traps over enhancer traps is that in *GUS* expressing gene trap lines, the *DsG* element should be inserted within the coding region of the gene of which expression is visualised by the *GUS* reporter gene. This greatly facilitates isolation of the tagged gene, and sequence information may directly allow prediction of its function (Springer *et al.*, 1995). In fact, both gene trap insertions from which we sequenced flanking DNA

were inserted within the putative open reading frame of a gene, and for one of these, gene function could be predicted based on sequence homology to known genes. Although enhancer traps might also insert into the coding region of a gene, they do not rely on it for *GUS* expression. Flanking DNA sequences from four of our enhancer trap insertions revealed only one putative open reading frame. Little is known about the distance over which enhancers can act in plants. A large physical distance between an enhancer trap insertion and the gene(s) activated by the enhancer would seriously complicate gene isolation. An enhancer trap insertion conferring *GUS* expression in the root endodermis of *Arabidopsis* had inserted approximately 1 kb upstream of the *SCARECROW* gene, whose expression is also restricted to the root endodermis (Di Laurenzio *et al.*, 1996). This suggests that isolation of genes detected by enhancer trapping would be feasible using flanking DNA probes for genomic library screening, followed by expression analysis of the genes found to be close to the site of insertion.

### *Conclusions*

We have used gene trap and enhancer trap transposons to detect genes expressed during embryo development in *Arabidopsis*. Based on the data presented here, it appears that gene and enhancer trapping are particularly useful in the study of embryo development in different ways. First, the *GUS* expression patterns represent markers for specific cell-types, tissues, organs, and regions in the developing embryo. Such markers can be valuable for establishment of cell fate or position in embryo mutant backgrounds, and can supplement existing markers. Secondly, gene / enhancer trap insertions allow isolation of genes expressed during embryogenesis, without the requirement for a visible phenotype caused by gene disruption. This is particularly important if gene disruption does not cause a mutant phenotype, or if the mutant phenotype is embryo- or seedling-lethal. Thirdly, an advantage of gene / enhancer trapping over differential screening approaches is provided by the fact that gene / enhancer traps directly provide detailed information on the expression pattern of a gene, which can be an important criteria for selection of lines of interest. From our screen, enhancer traps appear to be more efficient in detecting expression patterns than gene traps. On the other hand, gene trap insertions only confer *GUS* expression if inserted into the transcribed region of a gene. This can greatly facilitate the cloning of the gene responsible for the observed *GUS* expression pattern, and may directly cause disruption of the tagged gene, possibly resulting in a mutant phenotype. By contrast, enhancer trap insertions that confer *GUS* expression might be outside of the coding region of the gene(s) activated by the enhancer. If so, gene disruption can only be achieved after remobilization of the *DsE* element, or by sequence based detection of a T-DNA or transposon insertion into the gene responsible for the observed *GUS* expression pattern. Taken together, the use of gene and enhancer trapping provides a powerful tool to dissect the molecular events involved in *Arabidopsis*

embryogenesis.

## Materials and Methods

### *Mobilization of* Ds *elements*

*DsG* elements were mobilized by crossing plants from *Ac* starter lines *Ac*1 and *Ac*2, both homozygous for the *Ac* T-DNA, to plants from *DsG* starter lines *DsG*1, *DsG*6, *DsG*7 and *DsG*8, all homozygous for the *DsG* T-DNA, in all possible pairwise combinations. In these crosses, *Ac* starter lines were used as the male and *Ds* starter lines as the female parental line. Likewise, *DsE* elements were mobilized by crossing plants from *Ac* starter lines *Ac*1 and *Ac*2 to plants from *DsE* starter lines *DsE*1, *DsE*2, *DsE*3 and *DsE*6, all homozygous for the *DsE* T-DNA, in all possible pairwise combinations. F1 seeds from the *Ac* x *DsG* and the *Ac* x *DsE* crosses were planted individually and the resulting F1 plants were allowed to self-fertilize. 1000-5000 seeds from each F1 plant were collected to establish independent F2-families.

### *NAM-Kan selection for transposants*

750-1000 (15-20 mg) F2 seeds from each F2 family were surface-sterilized by successive washes with 70% ethanol for 10 min, diluted bleach solution (containing 0.9% sodium hypo chlorite, and 0.1% Tween 20) for 10 min, and twice with sterile water. The seeds were then suspended in 5 ml of liquid MS-agar (containing 0.46% (w/v) MS salts (Duchefa, (Murashige and Skoog, 1963) adjusted to pH 5.7 with KOH, 1% sucrose and 0.7% agar (Difco)), and plated onto square 12 x 12 cm selection plates containing MS-agar supplemented with 50 µg / ml kanamycin sulphate (Duchefa), and 3.5 µM NAM (α-naphtalene-acetamide, Sigma). After 1-4 days at 4 °C, the plates were incubated for 4 days in a growth chamber at 25 °C with 16h light / 8h dark photoperiod. Transposant seedlings resistant to both NAM and kanamycin, recognizable by their green cotyledons, normal size and normal root development, were transferred to 60 mm round selection plates and further incubated to verify the double resistance. After reaching the second-leaf stage, transposants were transplanted to soil and allowed to self-fertilize. Flowers and siliques, that contained immature seeds, from these F2 plants were screened for *GUS*-expression. Mature seeds (the F3 generation) were harvested and stored as a transposant line, i.e. a gene trap (referred to as Wageningen Gene Trap lines WGT1 through WGT373) or enhancer trap line (Wageningen Enhancer Trap lines WET1 through WET431).

### *Histochemical localization of* GUS *expression*

For localization of *GUS* expression in seedlings, seeds from each gene trap and enhancer trap line were germinated in microtiter wells containing 400 µl of sterile water. After 5 days of incubation at 25 °C in the light, one volume of two times concentrated GUS staining solution was added, to make up final concentrations of 100 mM NaPi pH 7.2, 10 mM EDTA, 0.1% Triton X-100, 100 µg/ml chloramphenicol to inhibit bacterial growth, and 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid). The seedlings were vacuum infiltrated with GUS staining solution for 1 hour, and the reaction was allowed to proceed for up to 48 hours at 37 °C in the dark. After the reaction, seedlings were cleared through several washes in 70% alcohol at 37 °C. GUS staining patterns were viewed using a Nikon binocular (Nikon Corp., Tokyo, Japan).

Localization of *GUS* expression in flowers, developing seeds and embryos was performed either directly in the F2 plant generation (i.e. mature transposant plants after transplantation from selection plates to soil) or in the F3 generation. Prior to planting in soil, F3 seeds were germinated on MS agar plates containing 50 µg/ml kanamycin to select for individuals carrying the gene or enhancer trap transposon. Flowers were sampled from the plants and incubated in GUS staining solution as described above. Siliques with immature seeds covering all stages of embryo development (typically 3 - 5 siliques per line) were sampled from the plants, opened longitudinally, and incubated in GUS staining solution as described above. After the reaction, flowers and siliques (containing immature seeds) were cleared for a minimum of 16 hours in Hoyers solution (100 g chloral-hydrate, 2.5 g Arabic gum, 15 ml glycerol, 30 ml water). Flowers and immature seeds were mounted in Hoyers solution on a microscope slide. GUS staining patterns were viewed with a binocular and with a Nikon Optiphot-2 equipped with Normarski optics. If GUS staining was observed in embryos, the staining reaction was repeated in GUS staining solution containing 1.25 mM, or even 5 mM each of potassium ferrocyanide and potassium ferricyanide (Jefferson *et al.*, 1987), to minimize diffusion of the reaction intermediates and thereby improve the specificity of the localization of *GUS* expression in embryos. Very weak GUS staining, however, was in most cases only visible in the primary staining reaction, i.e. in the absence of ferricyanide and ferrocyanide.

### *Histological sections*

After the GUS staining reaction, immature seeds were transferred to FAA fixative (2% formaldehyde, 5% acetic acid, 65% ethanol). The fixative was vacuum infiltrated and the seeds were fixed for at least 3 days at 4 °C. After dehydration through an ethanol series, the seeds were infiltrated in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. In brief, subsequent changes of Technovit preparation solution (1 g hardener I, 2.5 ml PEG 400, 100 ml

Technovit 7100) of increasing concentrations in 96% ethanol (1:3, 1:1, 3:1) were done for one hour, followed by a one hour and an overnight incubation in 100% Technovit preparation solution. Seeds were embedded in Technovit embedding solution (1 ml hardener II, 15 ml Technovit preparation solution) and polymerization was allowed to proceed for one hour at 37 °C. Serial sections (3 µm thick) were cut with a Reichert-Jung microtome, transferred to microscope slides, stained with 0.01% Ruthenium Red (Sigma) for 1-10 min, and mounted in Euparal (Agar Scientific, Stansted, UK). Sections were analyzed with a Nikon Optiphot-2 using bright-field and dark-field optics.

### *Southern blot analysis*

Genomic DNA from individual transposant plants was isolated according to Bouchez (1996). 1-2 µg of genomic DNA was digested with *Pst*I, separated on a 1% agarose gel and blotted onto a Nitran Plus membrane (Schleicher & Schuell, Keen, NH, USA). Blotting and hybridization were performed according to the manufacturer's recommendations. A 2.2 kb [$\alpha^{32}$P-dATP] random prime labelled *GUS* fragment, covering the entire coding sequence, was used as probe. The blot was washed for 15 min with 2 x SSC, 0.1% SDS and for 15 min with 0.1 x SSC, 0.1% SDS at 65 °C (Sambrook *et al.*, 1989), before exposure to X-ray film (Amersham, 's Hertogenbosch, the Netherlands).

### *PCR-analysis*

For PCR detection of the *Ds* element, either a set of *GUS* primers was used, with sequences GUS-1, 5'-AGA CTG TAA CCA CGC GTC TG-3' and GUS-2, 5'-CCG ACA GCA GTT TCA TCA ATC-3', or a combination of a *GUS* specific primer and a primer specific for the 3' end of the *Ds* element, with sequences GUS-4, 5'-GCT CTA GAT CGG CGA ACT GAT CGT TAA AAC-3' and Ds3, 5'-TAT TTA ACT TGC GGG ACG GAA ACG AAA AC-3'. For detection of both the *Ac* and the *Ds* donor T-DNA, *IAAH* specific primers were used, with the following sequences: NAM3, 5'-CAT TCC CCA CCT TGA CGA ACT G-3' and NAM4, 5'-GGT CTG AAT CCG CTA ATC CA-3'. PCR conditions for all primer pairs were 5 min at 94°C, followed by 35 cycles of 94°C (1 min), 55°C (1 min 30 sec) and 72°C (1 min 30 sec). PCR products were separated on a 1% agarose gel. Transposant plants are expected to be positive for the *GUS* gene, and negative for the *IAAH* gene, whereas *Ds* starter lines should be positive for both. *Ac* starter lines should be negative for *GUS* and positive for *IAAH*.

### *TAIL-PCR*

Genomic DNA flanking *Ds* insertions was amplified by thermal asymmetric interlaced (TAIL) PCR, essentially as described by Liu *et al.* (1995). A set of three nested primers for the 5' end of *Ds*, Ds5-1, Ds5-2, and Ds5-3, was used in combination with one arbitrary primer, AD2 (Liu *et al.*, 1995), to amplify genomic DNA flanking the 5' end of either *DsG* or *DsE* insertions. The sequences of the primers are as follows: Ds5-1, 5'-CCG TTT ACC GTT TTG TAT ATC CCG-3'; Ds5-2 5'-CGT TCC GTT TTC GTT TTT TAC C-3'; Ds5-3, 5'-GGT CGG TAC GGA ATT CTC CC-3' and AD2, 5'-NGT CGA (G/C)(A/T)G ANA (A/T)GA A-3'. After three subsequent rounds of TAIL-PCR, the primary, secondary and tertiary reaction products were separated on a 3% agarose gel. In successful reactions, the tertiary reaction product should be 71 bp smaller than the secondary product. Typically, the reaction products of successful amplifications ranged in size from 200 to 1500 bp. Secondary and tertiary reaction products were either sequenced directly using Ds5-2 and Ds5-3 as sequencing primer, respectively, or cloned into the pGEM-T vector (Promega, Madison, WI, USA) and subsequently sequenced using T7 and SP6 sequencing primers.

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# 4

# Molecular characterization of *Arabidopsis* enhancer trap and gene trap lines that exhibit *GUS* expression in embryos

We identified 39 *Arabidopsis* lines that exhibit *GUS* expression in embryos by enhancer and gene trap insertional mutagenesis. These lines provide a source of molecular markers that define not only different cell types and tissues, but also mark previously unidentified regions in the developing embryo. *GUS* expression observed before the heart stage of embryogenesis is mostly uniform in all cells of the embryo, and in some lines early uniform expression becomes spatially restricted during later stages of embryo development. The expression pattern in five lines with early or localized *GUS* expression was analysed in detail. Electronic searchable databases were established to record screening data. Analysis of genomic DNA sequences flanking the transposon in two independent gene trap lines revealed putative open reading frames (ORFs) in both, and in one of these lines gene trap insertion had taken place into the 5' untranslated leader of an expansin gene. In the genomic DNA sequences flanking four independent enhancer trap insertions, only one putative ORF was identified. The corresponding enhancer trap line displays *GUS* expression and a mutant phenotype in the suspensor. Together, the selected lines provide a set of markers that can be used to determine cell- or regional identity and polarity in *Arabidopsis* embryo mutants, and will allow the isolation of genes identified on the basis of their expression pattern in the *Arabidopsis* embryo.

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## Introduction

The architecture of the adult plant is established in a preliminary form during embryogenesis. Many studies have been devoted to the elucidation of the molecular mechanisms underlying plant embryo development (reviewed by Vroemen and De Vries (1998)). The most widely followed approach has been to identify mutants with defects in the establishment of the embryo body plan, in some cases followed by the isolation of the mutated gene. These genetic approaches have yielded numerous embryo-defective mutants in *Arabidopsis* (Jürgens *et al.*, 1991; Mayer *et al.*, 1991; Meinke, 1991; Scheres *et al.*, 1995), maize (Sheridan and Clark, 1993), and rice (Hong *et al.*, 1995). However, a major difficulty that has emerged during genetic screens for embryo mutants defective in the establishment of the body plan concerns the recognition and interpretation of relevant phenotypes. Many embryo-lethal mutants show similar phenotypes (Feldmann, 1991; Meinke, 1991), and the assessment of the precise effects of a mutation is often hampered by the inability to establish cell- or regional identity in embryo mutants (Topping and Lindsey, 1997; Vroemen *et al.*, 1996; Yadegari *et al.*, 1994).

One way to partly circumvent these difficulties is to study the expression pattern of well defined molecular markers in embryo mutants. Markers reflecting cell- or regional identity or polarity in the developing embryo offer criteria other than morphology for the evaluation of the precise effects of an embryo mutation. Examples of this approach include the study of the *AtLTP1* expression pattern in mutant *knolle*, *keule*, *gnom* (Vroemen *et al.*, 1996), and *raspberry* (Yadegari *et al.*, 1994) embryos, and more recently, the expression analysis of the promoter trap markers *POLARIS*, *EXORDIUM* and *COLUMELLA* in mutant *hydra* and *emb30/gnom* embryos (Topping and Lindsey, 1997). Unfortunately, besides the ones mentioned above, few suitable embryo marker genes are available to date, especially for the early stages of embryogenesis. Therefore, we have performed an enhancer and gene trap insertional mutagenesis screen to identify *Arabidopsis* lines with *GUS* expression in embryos (Vroemen *et al.*, 1998). The *Ds* transposon based enhancer and gene trap elements used (Sundaresan *et al.*, 1995) carry a *GUS* reporter gene that can respond to *cis*-acting transcriptional signals at the site of integration. Apart from the generation of marker lines, an added benefit of enhancer and gene trap screens is that they allow the identification of genes that do not mutate to obvious phenotypes, for example due to functional redundancy or because the phenotypes are very subtle under the screening conditions employed. Subsequent gene isolation is facilitated by the transposon insertion in or close to the gene corresponding to the observed *GUS* expression pattern (Springer *et al.*, 1995).

Here, we describe the spectrum of *GUS* expression patterns observed during the screening of 431 enhancer trap and 373 gene trap lines. Furthermore, we present electronic searchable databases for the recording of screening data, and sequence analysis of genomic DNA flanking the transposon insertions in four enhancer trap and two gene trap lines. Finally, the efficiency of enhancer and gene

trap mutagenesis as a means of identifying genes that are important for embryo development is discussed.

## Results

### *Screening enhancer trap and gene trap lines for* GUS *expression in the embryo*

In order to identify *GUS* markers expressed in the *Arabidopsis* embryo, all enhancer and gene trap lines were examined for *GUS* expression in siliques containing seeds with (pre-)globular stage to mature embryos. Simultaneously, all lines were also assayed for *GUS* expression at other stages of the plant life cycle, such as the seedling and flower. A total of 39 lines, 27 WET lines and 12 WGT lines, exhibited *GUS* expression in the embryo. This corresponds to overall frequencies of 6% *GUS* expression in embryos among the WET lines, and 3% among the WGT lines (Vroemen *et al.*, 1998). Table 4.1 lists the results of a qualitative analysis of *GUS* expression patterns in plants of the 39 embryo-positive WET and WGT lines, and Figure 4.1 shows GUS staining in embryos and immature seeds of twenty of these lines. Since the aim of our screen was primarily the identification of *GUS* markers expressed in the embryo, Table 4.1 focuses mainly on *GUS* expression patterns in the embryo, seed and silique. GUS staining in pollen grains is mentioned in a separate column, since its significance in enhancer trap lines is unclear: 67% of all enhancer trap lines display GUS staining in pollen grains, compared to only 5% of all gene trap lines (Vroemen *et al.*, 1998). On the basis of their *GUS* expression pattern in developing embryos, the lines in Table 4.1 have been classified in four distinct classes. Class A represents lines in which *GUS* expression is initially uniform in the embryo, and becomes spatially restricted during later stages of embryogenesis. Examples include expression restricted to the hypocotyl and root (WET133, Figure 4.1), the vascular tissue of the hypocotyl (WET272, Figure 4.1), the shoot apical meristem and part of the cotyledon primordia (WET368, chapter 5), the shoot and root apices and the vasculature (WGT39, Figure 4.2), the shoot and root apices only (WGT142, Figure 4.1), the entire vascular tissue (WGT236, Figure 4.1), the cotyledons (WGT316, Figure 4.1), the root tip (WGT351, Figure 4.1), and to a region just above the root tip (WGT6, Figure 4.1). Class B harbors lines in which *GUS* expression marks a distinct embryonic region or tissue from the onset of expression onwards. Lines of this class display GUS staining in the internal embryonic tissues (WET42, Figure 4.1), the hypophyseal cell group (WET215, Figure 4.4), the shoot apex (WET233, Figure 4.5), the root and basal hypocotyl (WET45, Figure 4.1), the shoot apex and later the root apex (WET115, Figure 4.1), the cotyledon tips and root cap (WET6, Figure 4.1), a region encompassing the upper hypocotyl, the shoot apex and the bases of the cotyledons (WET16, Figure 4.1), the hypocotyl (WET17, Figure 4.1), the root

**Table 4.1:** Histochemical localization of GUS activity in plants of WET and WGT lines with GUS staining in the embryo or suspensor.

| | Line | Embryo | | | | | | seed coat/ endosperm | funicle attachment point | | | silique wall | sepal/petal abscission zone | flower | pollen grains |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (pre-) globular | heart | torpedo | mature | suspensor | seedling | | | funicle | placenta | | | | |
| A | WET133 | + | + | + | + | | + | + | | | | | | | |
| | WET272 | + | + | | + | + | + | + | | + | + | + | + | + | + |
| | WET368 | + | + | + | + | | + | + | + | | | + | | + | |
| | WGT39 | + | + | + | + | | + | + | + | + | + | + | + | + | |
| | WGT142 | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| | WGT236 | + | + | + | + | | + | | + | | | | | + | |
| | WGT316 | + | + | + | + | | + | + | + | | | + | + | + | + |
| | WGT351 | + | + | + | + | | + | + | + | + | | + | + | + | |
| | WGT357 | + | + | + | + | | + | + | + | + | + | + | + | + | + |
| | WGT6 | | | + | + | | + | | | + | | | | | |
| B | WET42 | + | + | + | + | | + | | + | + | + | | | + | + |
| | WET215 | | + | + | + | | + | | | | | | | | + |
| | WET233 | | + | + | + | | + | | | | | | | | |
| | WET45 | | | + | + | | | + | | | | | | | + |
| | WET115 | | | + | + | | + | + | | + | + | + | + | + | + |
| | WET6 | | | | + | + | + | + | + | + | + | + | + | + | + |
| | WET16 | | | | + | | | + | | + | + | | | + | |
| | WET17 | | | | + | | + | + | + | + | + | + | + | + | |
| | WET60 | | | | + | + | + | + | + | + | + | + | + | + | |
| | WGT320 | | | + | + | | + | | | | | | + | + | |
| | WET347 | | | | + | | | | | | | | | + | + |
| | WET393 | | | | | + | | | | | | | | | + |
| C | WET103 | + | + | + | + | + | + | + | + | + | + | | + | + | + |
| | WET175 | + | + | + | + | | + | + | | | + | + | + | + | + |
| | WET400 | + | + | + | + | | + | + | | + | + | + | | + | |
| | WET411 | + | + | + | + | + | n.d. | + | + | + | + | + | + | + | + |
| | WGT42 | +? | +? | +? | +? | | | + | | | | | | | |
| | WGT96 | +? | +? | +? | +? | | | + | | | | | | | |
| | WGT165 | + | + | + | + | | + | + | + | + | + | + | + | | |
| | WGT323 | +? | +? | +? | +? | | | + | | | | | | | |
| | WET24 | | + | + | + | | + | + | | | | + | | + | |
| | WET227 | | + | + | + | | + | + | | + | + | + | + | + | |
| | WET352 | | + | + | + | | + | + | + | + | + | + | + | + | + |
| | WET222 | | | + | + | | + | | | + | + | + | + | | |
| | WET322 | | | + | + | | + | | + | + | + | + | | + | + |
| | WET50 | | | | + | | + | + | + | + | + | + | + | + | + |
| | WET406 | | | | + | | n.d. | | | | | | + | + | |
| D | WET167 | + | + | | | + | + | + | + | | | | + | | + |
| | WET308 | + | + | + | | | + | + | + | + | | + | | + | |

+ , GUS activity detected; n.d., not determined; +?, GUS activity in embryo unsure due to GUS activity in seed coat.

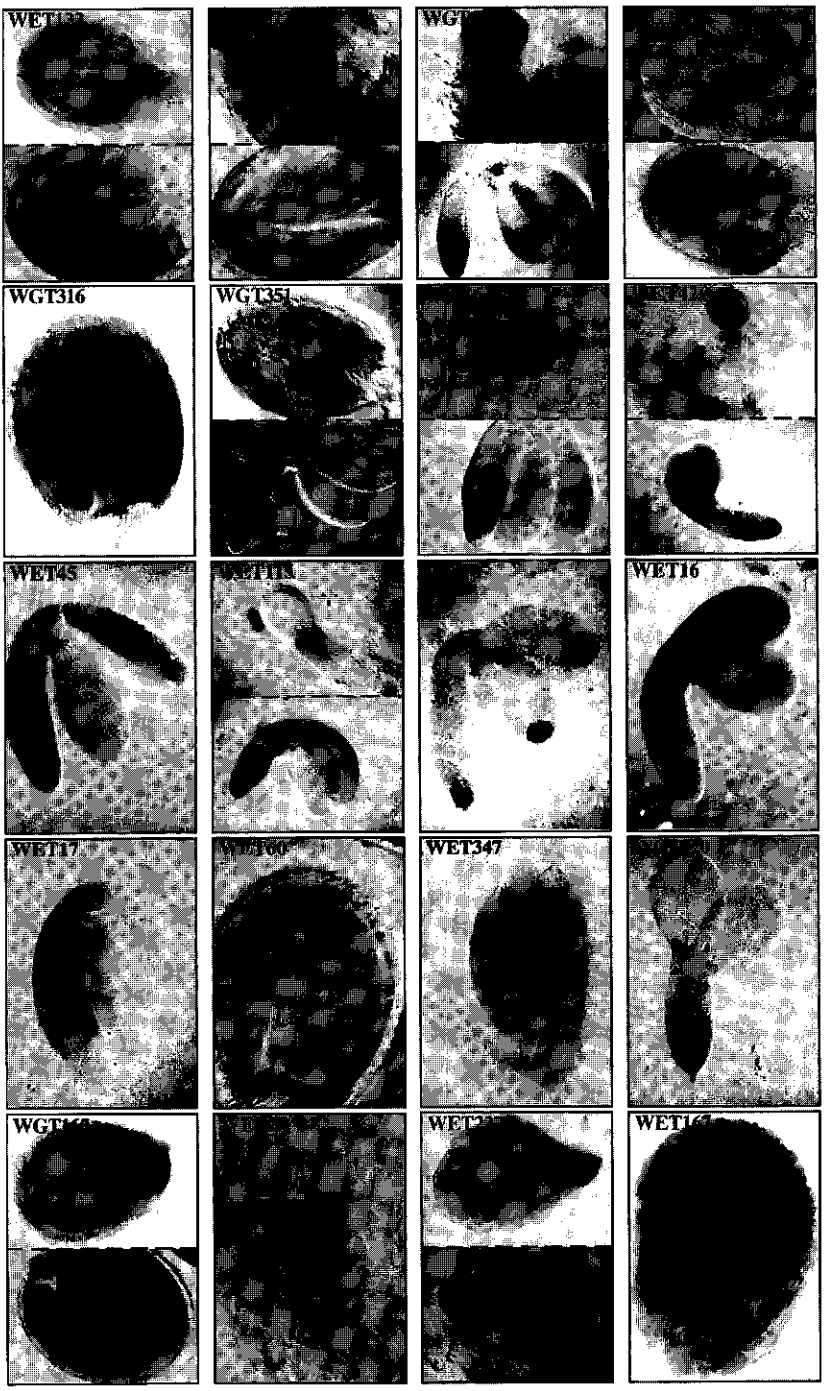



**Figure 4.1:** *GUS* expression patterns in seeds and embryos of twenty WET and WGT lines listed in Table 4.1.

tip (WET60, Figure 4.1), the cotyledons (WET347, Figure 4.1), a region just above the root tip (WGT320, Figure 4.1), and the suspensor (WET393, Figure 4.3). Class C lines exhibit uniform GUS staining in the embryo. In some lines, such as WGT165 (Figure 4.1), this uniform expression is already seen in pre-globular embryos, whereas in other lines, expression starts at a later stage (e.g. WET24 and WET227, Figure 4.1). Finally, class D consists of lines exhibiting early transient *GUS* expression, that disappears at later stages of embryo development. In the two class D lines, *GUS* expression is seen not only in the embryo, but instead, the entire developing seed, including seed coat, endosperm, and embryo show intense GUS staining. An example of such a line is WET167 (Figure 4.1), in which *GUS* expression in the embryo, endosperm and seed coat disappears after the heart stage of embryogenesis. Examination of *GUS* expression patterns in the subsequent generations showed that in all lines, the patterns of *GUS* expression were stably transmitted to the next generations, and were linked with the *Ds* elements (data not shown).

### GUS *expression patterns in WET393, WET215, WET233, WGT39*

On the basis of early or localized *GUS* expression observed during the primary screen, the class A line WGT39 and the class B lines WET393, WET215, and WET233 were chosen for a more detailed analysis of *GUS* expression patterns during the development of seeds, seedlings and mature plants. Class C lines exhibit uniform staining in the embryo, and both of the class D lines identified in our screen display uniform staining in the early embryo and the seed coat. Because our main interest was to identify lines exhibiting early and tissue-, cell-type- or region-specific *GUS* expression in developing embryos, no class C or D lines were subjected to more detailed analyses up to now.

WGT39 provides an example of a class A type of *GUS* expression pattern (see Table 4.1). Up to the globular stage, *GUS* expression is uniform in all cells of the embryo proper (Figure 4.2A).

**Figure 4.2:** *GUS* expression pattern in WGT39.

**A:** developing seed with globular stage embryo.

**B:** transition stage embryo.

**C:** early heart stage embryo.

**D:** early torpedo stage embryo.

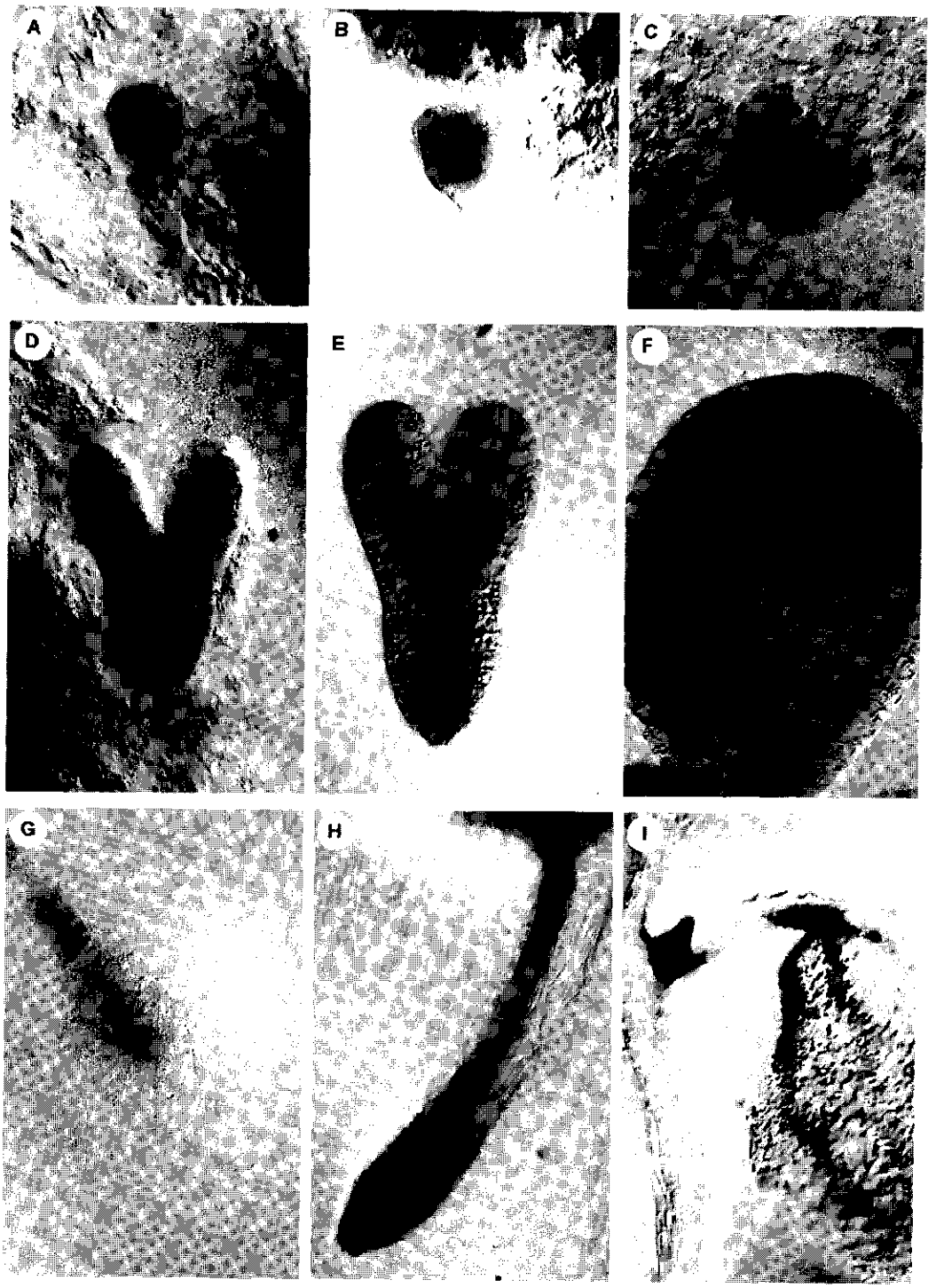
**E:** late torpedo stage embryo.

**F:** developing seed with bending cotyledon stage embryo.

**G:** root tip of seedling, 10 days post-germination.

**H:** lateral root of seedling, 10 days post-germination.

**I:** shoot apex of seedling, 5 days post-germination.


A
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Commencing at the transition stage, expression becomes gradually restricted to the internal cells of the embryo (Figure 4.2B and 4.2C), until it is only seen in the vascular tissue of the hypocotyl and cotyledons, and in the root apex at the early torpedo stage (Figure 4.2D). In late torpedo stage embryos, *GUS* expression becomes apparent in the shoot apex at the expense of the root apex expression (Figure 4.2E). During the final stages of embryogenesis, GUS staining remains associated with hypocotyl and cotyledonary vascular tissue and the shoot apex (Figure 4.2E and 4.2F). *GUS* expression in WGT39 seedlings is confined to the vascular tissue (Figure 4.2G, 4.2H, and 4.2I), the root meristem in early primary (data not shown) and lateral (Figure 4.2H) roots, and the shoot meristem (Figure 4.2I). GUS staining in primary and lateral roots initially covers the vascular tissue and most of the meristem (Figure 4.2H). Later during root development, *GUS* expression in the meristem decreases, until it is seen only in the root vasculature, where it abruptly ends in the cells adjacent to the quiescent center (Figure 4.2G). In mature WGT39 plants, *GUS* expression is observed in flowers and siliques, where it is most prominent in the vasculature, and in part of the seed coat (cf. Figure 4.2A, and data not shown).

GUS staining in WET393 plants is restricted to the embryo, meaning that no GUS staining is observed at any other stage of development or in any other plant organ (Vroemen *et al.*, 1998). However, in WET393 plants there is GUS staining in pollen, but this is considered to be artefactual (Klimyuk *et al.*, 1995; Mascarenhas and Hamilton, 1992; Uknes *et al.*, 1993; Vroemen *et al.*, 1998). At the globular embryo stage, *GUS* expression is observed in the entire suspensor including the hypophyseal cell (Figure 4.3A). This is the precise opposite of the expression pattern in early WET368 embryos, that show expression in all "apical" cells of the (pre-)globular embryo, but not in the hypophyseal cell and the suspensor (chapter 5). Beginning in the transition stage embryo, expression is gradually restricted to the lowermost cells of the suspensor (Figure 4.3B and 4.3C). During the torpedo (Figure 4.3D) and bent cotyledon (Figure 4.3E and 4.3F) stages, *GUS* expression is seen in the entire suspensor, but is completely absent from the embryo proper.

In heart stage embryos of WET215 plants, *GUS* expression is confined to all cells derived from the hypophysis, collectively designated as the hypophyseal cell group (Figure 4.4A). Figure 4.4B shows a section through the embryonic root of a torpedo stage WET215 embryo within the developing seed. Indigo blue crystals are present in all cells belonging to the hypophyseal cell group. At the bent-cotyledon stage (Figure 4.4C) GUS staining is most prominent in the hypophyseal cell group, while weaker GUS staining, most likely due to diffusion of reaction intermediates, was observed in a region just above the hypophyseal cells. In seedlings 5 days post-germination, expression is seen in the root cap, the hypocotyl and the base of the cotyledons (Figure 4.4D and 4.4E). Since GUS staining is much weaker in the hypocotyl and cotyledon bases as compared to the root cap, the seedling shown in Figure 4.4D and the seedling from which details are shown in Figures 4.4E, 4.4G and 4.4H were incubated in GUS staining solution for 48 hours. As a consequence, staining in the root cap was very intense and possibly not only restricted to cells

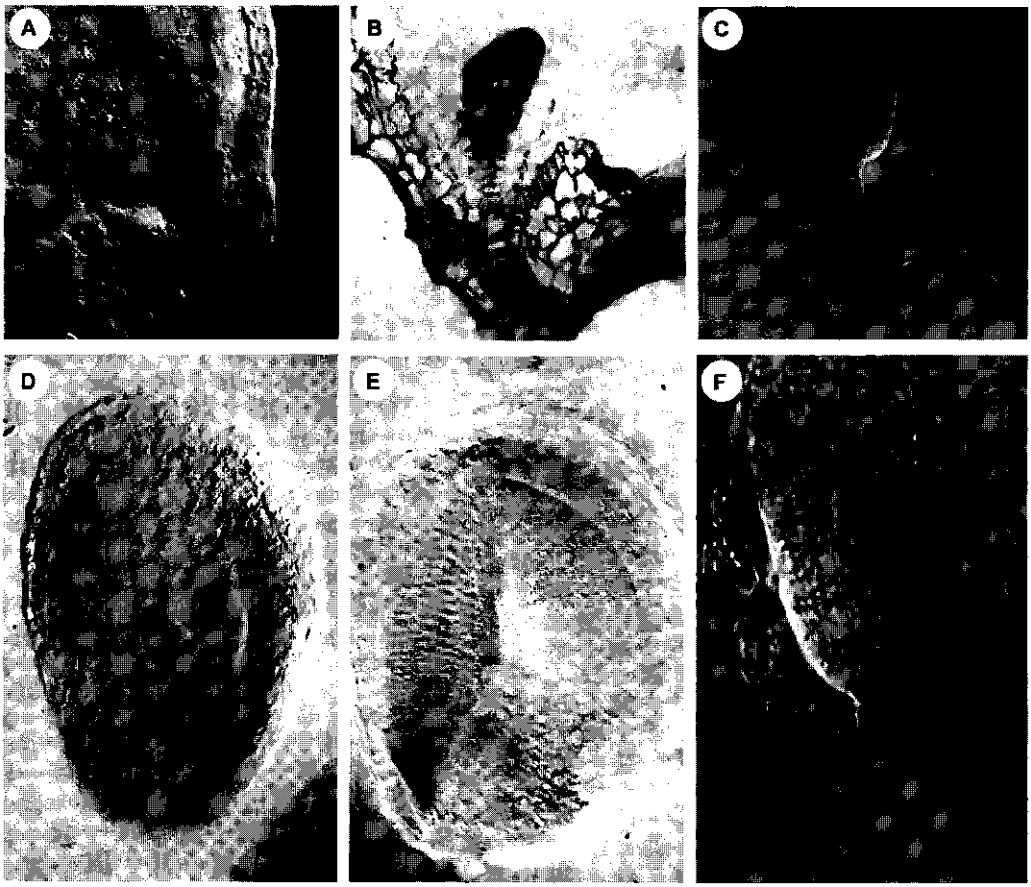



**Figure 4.3:** *GUS* expression pattern in WET393.

**A:** developing seed with globular stage embryo

**B:** section of developing seed with transition stage embryo

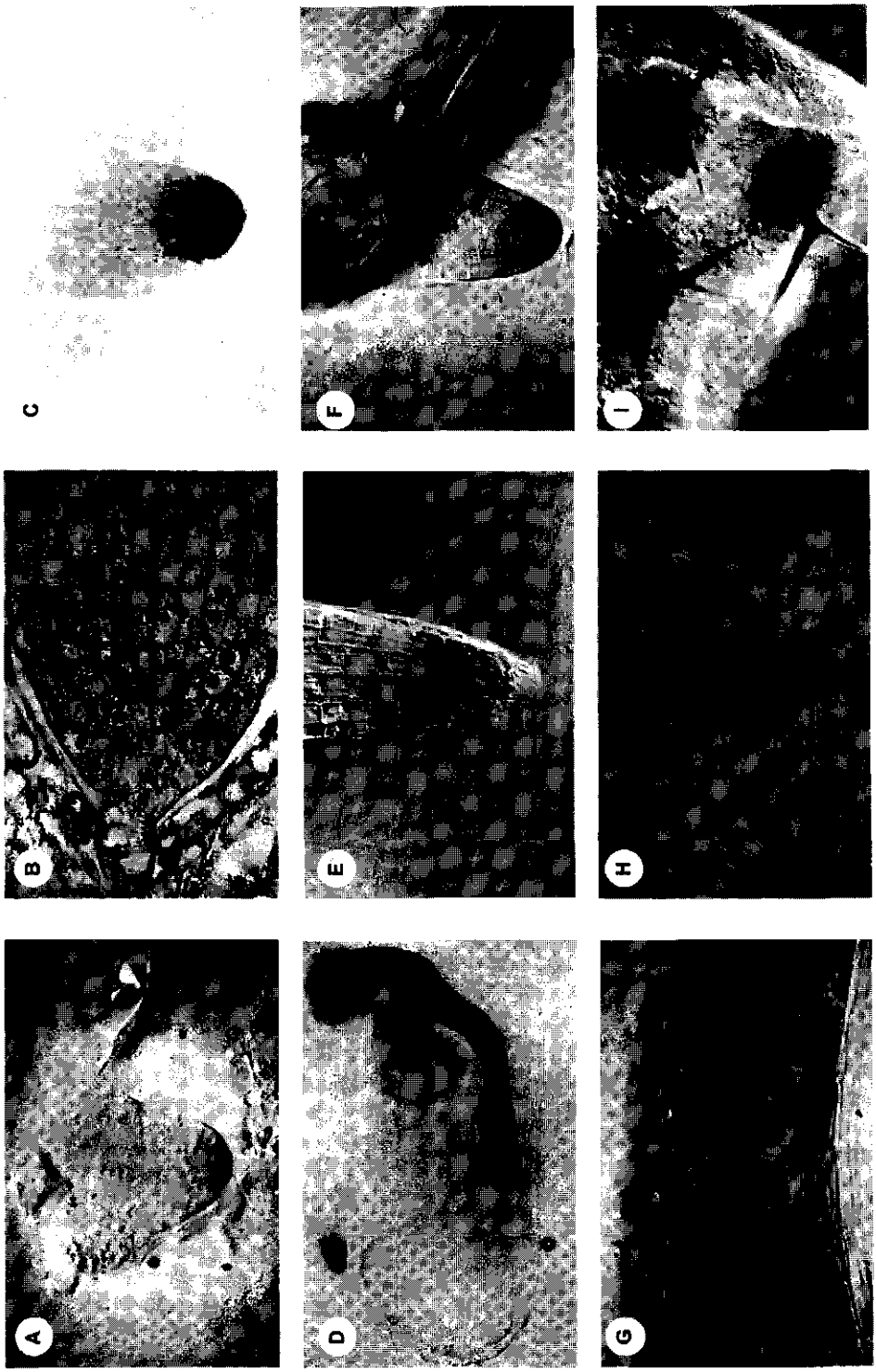
**C:** transition stage embryo

**D:** developing seed with early torpedo stage embryo

**E:** developing seed with bent cotyledon stage embryo

**F:** basal end of bent cotyledon stage embryo after dissection out of the seed. The upper suspensor cell is still attached to the embryo proper, and the staining reaction was performed under conditions that minimize diffusion of the reaction intermediates (see Materials and methods).

expressing the *GUS* gene. In order to precisely determine which cells of the root cap expressed *GUS*, WET215 seedlings 7 days post-germination were stained for 4 hours under staining conditions that minimize diffusion of reaction intermediates. These experiments indicated that *GUS* expression in the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columella root cap of primary (not shown) and lateral roots (Figure 4.4F) of WET215 seedlings is restricted to the upper two layers, which include columella root cap initials and their immediate daughters, and epidermal initials. Expression in the hypocotyl is restricted to the epidermis (Figure 4.4G). In the cotyledon bases, GUS staining is associated with the stomata (Figure 4.4H). In true leaves, strong GUS staining is seen in trichomes (Figure 4.4I).

In WET 233 embryos, *GUS* expression is confined to a region encompassing the shoot apical meristem (SAM) from the heart stage onwards (Figure 4.5A, 4.5B, and 4.5C). Unlike WET368 embryos, that display a similar *GUS* staining pattern at these stages of embryogenesis (chapter 5), WET233 embryos do not show *GUS* expression before the heart stage. Moreover, in contrast to WET368 seedlings (chapter 5), GUS staining in WET233 seedlings is not restricted to the SAM region, but is also seen in the cotyledons and upper hypocotyl (data not shown). No *GUS* expression is seen at developmental stages other than the embryo and seedling in WET233 plants.

### *Databases containing information on WET and WGT lines*

We established electronic searchable FileMaker Pro 2.0 (Claris™) databases based on a database format developed at Cold Spring Harbor Laboratory (R. Martienssen and V. Sundaresan). These databases allowed efficient recording of screening data, such as expression patterns and mutant phenotypes, of the WET and WGT lines, with emphasis on developing embryos. Two independent databases, one for WET lines and another, with identical set-up, for WGT lines, were established.

An outline of the WET database, that consists of three different layouts, is shown in Figure 4.6. The "list" layout (Figure 4.6, upper panel) represents a complete administration of all lines listed by number, and includes seed stock number of the parental F1 plant, genotype of the F1 parent (i.e.

**Figure 4.4:** *GUS* expression pattern in WET215.

**A:** heart stage embryo.

**B:** section of embryonic root of torpedo stage embryo in developing seed.

**C:** embryonic root of bent cotyledon stage embryo. To increase staining intensity, staining was performed for 3 days without inclusion of ferro / ferricyanides.

**D:** seedling, 5 days post-germination.

**E:** root tip of seedling, 5 days post-germination.

**F:** lateral root of seedling, 7 days post-germination.

**G:** hypocotyl of seedling, 5 days post-germination.

**H:** cotyledon of seedling, 5 days post-germination.

**I:** leaf of seedling, 7 days post-germination.

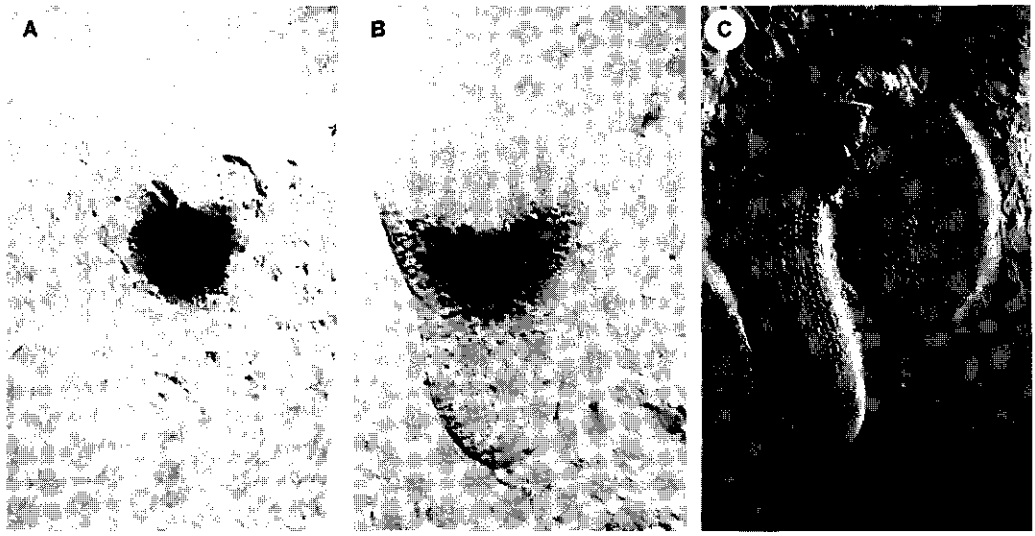



**Figure 4.5:** *GUS* expression pattern in WET233.

**A:** heart stage embryo.

**B:** torpedo stage embryo.

**C:** bent cotyledon stage embryo.

combination of *DsE* and *Ac* starter lines that were initially crossed to generate F1 seed), homo- or hemizygosity of the WET-line (i.e., the "pooled" F3 seed stock, see below and Materials and methods) for the *DsE* element, screening status at the seedling and embryo stage, and the last

**Figure 4.6:** Outline of the database containing information on all WET lines generated during our screen. An identical database exists for WGT lines. Upper ("**list**") layout contains genetic history, molecular data and screening status of each WET line. WET, Wageningen Enhancer Trap line; WAU, Wageningen Agricultural University seed stock; seedl., seedling; embr., embryo. "**Embryo**" layout contains observed *GUS* expression patterns and mutant phenotypes in embryos, seeds, siliques, flowers and other plant parts of each WET line screened. emb., embryonic; spor., sporophytic; cot., cotyledon; fun. att., funicle attachment point; absc. zone, sepal/petal abscission zone. "**Seedling**" layout contains observed *GUS* expression patterns and mutant phenotypes in seedlings of each WET line screened. vasc., vasculature; hypo. hypocotyl; lat., lateral; prim., primordia. Each layout contains buttons (dotted rectangles) to switch to either of the two other layouts of the same WET line. The "list" layout contains a "new" button to generate a new, empty record. "1" indicates weak, "2" intermediate, and "3" strong GUS staining. No entered number, or "0" indicates that no GUS staining was observed.

## List

| WET-line | F1 parent | F1 genotype | homo/hemi-zygous | screened seedl. | screened embr. | date | NEW |
|---|---|---|---|---|---|---|---|
| WET 213 | WAU 269-20 | DsE1/+ Ac2/+ | hemi | yes | yes | 24-6-98 | seedling |
| WET 214 | WAU 270-2 | DsE1/+ Ac1/+ | hemi | yes | yes | 24-6-98 | seedling |
| WET 215 | WAU 270-4 | DsE1/+ Ac1/+ | hemi | yes | yes | 24-6-98 | seedling |
| WET 216 | WAU 270-12 | DsE1/+ Ac1/+ | hemi | yes | yes | 24-6-98 | seedling |
| WET 217 | WAU 270-26 | DsE1/+ Ac1/+ | homo | yes | yes | 24-6-98 | seedling |

WET 215

## Embryo

embryo phenotype:

plant phenotype:

emb. segregation:

plant segregation:

Staining pattern

spor. stain (adult): 0
gametophytic stain: 2
embryo stain: 2
no stain:

notes: hypophyseal cell group

Expression patterns mimic pattern of morphological defects in *hobbit* mutant (Willemsen *et al.*, 1998 *Development* **125**, 521-531)

embryo tissue: protoderm, ground, vascular

| pre-globular | globular | heart | torpedo | bent cot. | non-embryo |
|---|---|---|---|---|---|
| suspensor | suspensor | suspensor | suspensor | suspensor | funiculus |
| uniform | uniform | uniform | uniform | uniform | placenta |
| upper tier | upper tier | cotyledons | cotyledons | cotyledons | silique |
| lower tier | lower tier | apex | cot. tips | cot. tips | carpel |
| hypophysis | hypophysis | hypocotyl | apex | apex | absc. zone |
| testa | testa | radicle | hypocotyl | hypocotyl | ovule |
| fun. att. | fun. att. | hypophysis 1 | radicle | radicle | pollen 2 |
| micropyle | micropyle | testa | hypophysis 2 | hypophysis 2 | other flower |
| | | fun. att. | testa | testa | elsewhere |
| | | micropyle | fun. att. | fun. att. | endosperm |
| | | | micropyle | micropyle | |

list seedling

## Seedling

WET 215

mutant phenotype:

segregation:

remarks: young: root + hypocotyl / older: 2 upper CRC layers in prim. and lat. root (after 4h stain), epidermis of hypocotyl (after >24 h stain), stomata in cotyledons, leaf trichomes. Looks like WET212.

Staining pattern

no stain:
seedling stain: 2
ubiquitous:

| cotyledons 1 | hypocotyl 1 | root 2 | tissue |
|---|---|---|---|
| cot. uniform | hypo. uniform 1 | root uniform | epidermis 1 |
| cotyledon tip | hypo. apical | root tip | ground tissue |
| cot. central | hypo. central | root cap 2 | vasculature |
| cot. base 1 | hypo. basal | root vasc. | |
| cot. vasc. | hypo. vasc. | root hairs | |
| cot. other | apex 1 | lat. root uniform | |
| | hypo other | lat. root prim. 2 | |
| leaf 2 | | lat. root tip 2 | |
| | root-hypo junction | lat. root vasc. | |
| | | root other | |

list

modification date of either of the three layouts of the WET-record. To the right of each record, two buttons allow access to the "seedling" and "embryo" layouts of the same WET-line. The "NEW" button adds a new empty record to the bottom of the list layout, to accommodate for newly generated WET lines. It should be noted that during the generation of WET lines, generally the seeds harvested from two NAM-kan resistant F2 plants were pooled to generate one single F3 seed batch, or WET-line (see Materials and Methods). 2/3 of the NAM-kan resistant F2 plants on a selection plate are expected to be hemizygous for the *Ds* transposon, and 1/3 homozygous. Only WET lines that were 100% kanamycin resistant were annotated "homozygous", and all WET lines segregating for kanamycin sensitivity as "hemizygous". Consequently, only 1/9 of all WET-lines are expected to be "homozygous". The number of 43 homozygous WET-lines (data not shown) obtained among the total population of 431 lines (10%) is well in line with this prediction.

The "Embryo" layout (Figure 4.6, middle panel) contains the results of the screening of each WET line for *GUS* expression patterns and mutant phenotypes in embryos, seeds, siliques, flowers and other parts of the adult plant. Since the aim of our screen was primarily the identification of *GUS* markers expressed in the embryo, *GUS* expression patterns in the embryo are documented in more detail than those in other plant organs. The embryo layout contains fields for mutant embryo and plant phenotypes and their segregation ratio, a "staining pattern" section that gives an overview of the stages at which *GUS* expression is observed, and an elaborate section which allows semi-quantitative description of the temporal and spatial *GUS* expression pattern. The latter section is subdivided in five columns, each representing a different stage of embryo and seed development, and a column listing extra-embryonic plant organs and tissues. The level of *GUS* expression is indicated in a numerical way, in which "1" indicates weak, "2" intermediate, and "3" strong *GUS* expression. No entered number, or "0" indicates that no GUS staining was observed. The section "embryo tissue" allows the recording of restrictions or differences in the level of *GUS* expression along the radial axis.

The "Seedling" layout (Figure 4.6, bottom panel) is similar to the "Embryo" layout, but concerns *GUS* expression patterns and mutant phenotypes at the seedling stage. The section harboring the observed *GUS* expression pattern consists of three columns for three different seedling elements (cotyledons, hypocotyl, and root) and a column listing the three main tissue types along the seedling's radial axis. The heading of the first three columns is followed by a numerical value that indicates the highest level of *GUS* expression in the corresponding seedling element.

The categorized and numerical fashion in which the *GUS* expression patterns are recorded in our databases enables searches for lines expressing *GUS* in any plant organ, tissue or region, or combinations thereof, that is defined in the "Embryo" or "Seedling" layouts. On the other hand, the spectrum of defined categories is not sufficient to describe all observed staining patterns in full detail. Therefore, both the "Embryo" and the "Seedling" layouts contain a field, named "notes" and "remarks", in which, where necessary, a more detailed description of the staining pattern and other

information can be recorded. Taken together, the combination of layouts in our databases provides a framework that first allows a detailed recording of screening data, and later permits easy identification of any set of lines of interest.

### *Genomic DNA sequences flanking* DsE *and* DsG *insertions*

*GUS* expression in gene trap lines relies on *DsG* insertion within the transcribed region of a gene. By contrast, *GUS* expression in enhancer trap lines can be activated from *DsE* elements inserted within or outside of the coding region of chromosomal genes. In order to determine whether the genomic sequences tagged by *DsG* and *DsE* insertions in GUS positive WGT and WET lines correspond to putative coding regions of chromosomal genes, DNA sequences flanking the 5' *Ds* ends in WET393, WET215, WET233, WET42, WGT39, and WGT236 were amplified by TAIL-PCR (Liu *et al.*, 1995; Vroemen *et al.*, 1998). Each product was cloned into a T/A cloning vector and sequenced. In all cases, the expected 5' *Ds* sequences were present, and only in line WGT236, *Ds* insertion had resulted in a 3 bp truncation of the 5' *Ds* end.

For WET393, a 645 bp PCR product was generated, that contained 532 bp of plant genomic DNA (Figure 4.7). Translation of the genomic DNA sequence into an amino acid sequence using the Sequence Navigator software (Applied Biosystems) revealed one putative open reading frame (ORF), spanning the entire 532 bp sequence. The predicted amino acid sequence contains four putative N-glycosylation sites, and is rich in serine and proline residues, reminiscent of certain cell wall proteins like extensins and hydroxyproline-rich glycoproteins. Furthermore, homopolymeric stretches of glutamine, serine, threonine, and proline residues account for 37% of the predicted amino acid residues (Figure 4.7). Domains rich in these residues have been shown to activate transcription (Johnson *et al.*, 1993), and are implicated to serve such role in the *Arabidopsis* SCARECROW protein (Di Laurenzio *et al.*, 1996). Thus, WET393 may encode a transcriptional regulator or a cell wall protein. It may also provide an example of an enhancer trap line in which the *DsE* element has inserted into the coding region of a transcribed gene. Further support for this possibility comes from the recent observation that developing embryos in WET393 plants display aberrant cell division patterns in the suspensor and hypophyseal cell group (data not shown). This mutant phenotype may be caused by gene disruption due to the *DsE* transposon insertion.

For WET215, a 514 bp PCR product containing 401 bp of genomic DNA was produced. The nucleotide sequence was found to be A/T rich, and no apparent ORF was identified, suggesting that the *DsE* element in WET215 is inserted outside of the coding region of chromosomal genes. Likewise, the 442 bp genomic flanking sequence from the 491 bp PCR product of WET42, and the 327 bp genomic flanking sequence from the 363 bp PCR product of WET233, did not contain clear putative ORFs.

```
1   GATAAGAGCTGTAACTCCTCCGGCGATTCCTCCGCCGTATCCGCCTCTGCAACGAGTAGT   60
      D  K  S  C  N  S  S  G  D  S  S  A  V  S  A  S  A  T  S  S

61  ACCGGTAACAATACAACGAACAGAGATCATTACCTGAGACAACTCAATAAGCTGTCTCAT  120
      T  G  N  N  T  T  N  R  D  H  Y  L  R  Q  L  N  K  L  S  H

121 AAGATATCAAAACCGACGAACTCTTCCTCCTCCGTCTCCGTCGCGAATCGTGAAATTGAT  180
      K  I  S  K  P  T  N  S  S  S  S  V  S  V  A  N  R  E  I  D

181 CTTCCACCTCCTCCACCGCTGCAAATCAATCAAGGGAATCTCCATCAACATCAACCTCCT  240
      L  P  P  P  P  P  L  Q  I  N  Q  G  N  L  H  Q  H  Q  P  P

241 GTTTACAATATCAACAAGAACGATTTCAGAGATGTTGTTCAGAAACTAACCGGTTCACCT  300
      V  Y  N  I  N  K  N  D  F  R  D  V  V  Q  K  L  T  G  S  P

301 GCACATGAACGGATCTCTGCTCCGCCGCAACAACCGATTCATCACCCTAAACCTCAACAG  360
      A  H  E  R  I  S  A  P  P  Q  Q  P  I  H  H  P  K  P  Q  Q

360 AGTTCGCGTCTACATAGGATCCGTCCTCCTCCTTTGGTTCACGTTATCAATCGTCCTCCT  420
      S  S  R  L  H  R  I  R  P  P  P  L  V  H  V  I  N  R  P  P

421 GGTTTGTTAAATGACGCACTTATCCCTCAAGGTTCTCATCACATGAATCAAAACTGGACC  480
      G  L  L  N  D  A  L  I  P  Q  G  S  H  H  M  N  Q  N  W  T

481 GGCGTTGGATTTAACCTTCGACCAACGGCGCCGCTTTCTTCTACACTCGACG   532
      G  V  G  F  N  L  R  P  T  A  P  L  S  S  T  L  D
```

**Figure 4.7:** Nucleotide and predicted amino acid sequence of genomic DNA flanking the *DsE* element in WET393. Putative N-glycosylation sites are boxed, and glutamine (Q), serine (S), threonine (T) and proline (P) residues (see text) underlined.

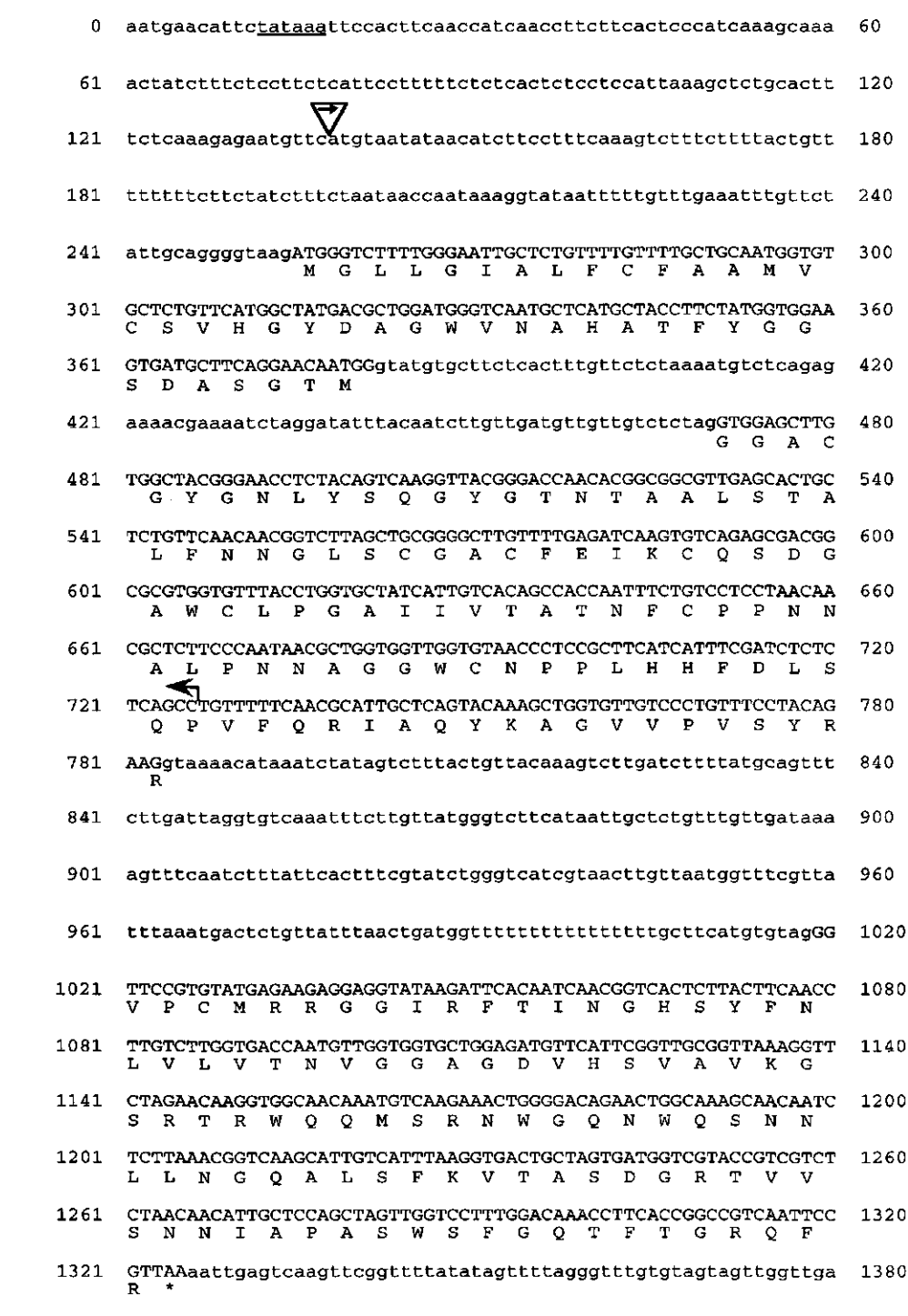
The 701 bp PCR product obtained from WGT39 contained 589 bp of genomic flanking DNA (Figure 4.8), that shows sequence similarity to expansin genes from *Arabidopsis* and other plant species. Expansins represent a highly conserved multigene family of proteins that mediate cell wall extension in plants (Shcherban *et al.*, 1995), and can have a role in mediating cell fate (Fleming *et al.*, 1997). The WGT39 flanking sequence was found to be most homologous on both the nucleotide and the amino acid level (Figure 4.9) to the *Arabidopsis thaliana AtEXP-1* cDNA (Shcherban *et al.*, 1995). Analysis of the nucleotide and amino acid sequence homologies revealed the presence of an 89 bp intron in the isolated flanking DNA. Further nucleotide sequence comparisons revealed an exact match of the WGT39 flanking DNA sequence with part of the genomic sequence of a BAC contig (Figure 4.8). Analysis of this genomic sequence indicated that the *DsG* element in WGT39 is located in the untranslated leader of a previously unidentified putative expansin gene, 117 bp upstream of the predicted ATG start codon (Figure 4.8). This implies that the observed GUS activity in WGT39 is the result of transcription of the *DsG* borne *GUS* gene under the control of the expansin promoter, followed by translation initiated from the ATG start codon of the *GUS* gene. Since *GUS* expression

does not rely on the formation of a translational fusion of the endogenous and the *GUS* gene products, the *DsG* element in WGT39 acts as a promoter trap rather than a gene trap. Thus, WGT39 represents an example of a *GUS* expressing gene trap line, in which the *DsG* element has inserted in the transcribed region, but outside of the coding region of a chromosomal gene.

For line WGT236, TAIL-PCR resulted in a 315 bp product, that contained 205 bp of genomic flanking DNA. The predicted amino acid sequence corresponding to the genomic flanking DNA revealed one putative ORF spanning the entire 205 bp. No significant similarity to known sequences was found on either the nucleotide or the amino acid level. Because gene trap lines are only expected to display *GUS* expression if the *DsG* element has inserted into the transcribed region of a chromosomal gene, this could mean that WGT236 carries a *DsG* insertion in an unknown gene. Alternatively, the *DsG* element in WGT236 could have inserted into an intron, a possibility that would be supported by the high (71%) A-T content of the flanking DNA (Bevan *et al.*, 1998). Collectively, sequence analysis of genomic DNA flanking *Ds* insertions in six lines displaying GUS staining in the embryo revealed one gene trap insertion into a gene with high sequence similarity to plant expansin genes, and one enhancer trap insertion into a putative gene with sequence motifs reminiscent of certain classes of cell wall proteins and transcriptional activators. Further molecular analysis will reveal whether the identified ORFs correspond to transcribed genes, and library screening will yield cDNA and genomic clones of genes whose expression pattern is mimicked by the GUS staining pattern in the corresponding gene or enhancer trap lines (Vroemen *et al.*, 1998).

## Discussion

Previously, we reported on the collection of 431 independent *Arabidopsis* enhancer trap lines and 373 independent gene trap lines, screened for *GUS* expression in the embryo and seedling. 27 WET lines and 12 WGT lines were found to exhibit *GUS* expression in the embryo (Table 4.1 and Figure 4.1), representing 6% of the WET lines, and 3% of the WGT lines (Vroemen *et al.*, 1998). The latter frequency is similar to the frequency of embryo *GUS* expression obtained with T-DNA based promoter traps (Topping *et al.*, 1994). Here, we show that the WET and WGT lines selected provide a source of molecular markers that define not only different cell types and tissues, but also mark different regions in the developing *Arabidopsis* embryo that were not identified as such in previous screens. As becomes immediately apparent from Table 4.1, only line WET393 displays *GUS* expression specific to the embryo, if we exclude the possibly artefactual pollen-staining observed as well (Vroemen *et al.*, 1998). The vast majority of all WET and WGT lines with GUS staining in the embryo also express *GUS* at other developmental stages. This includes lines in which *GUS* expression is associated with a specific tissue or region that exists in the embryo, seedling or mature plant, as exemplified by the shoot meristem region staining in WET368 (chapter 5). Such expression


0 aatgaacattctataaattccacttcaaccatcaaccttcttcactcccatcaaagcaaa 60
61 actatctttctccttctcattccttttttctctcactctcctccattaaagctctgcactt 120
121 tctcaaagagaatgttcatgtaatataacatcttcctttcaaagtctttctttttactgtt 180
181 ttttttcttctatctttctaataaccaataaaggtataatttttgtttgaaatttgttct 240
241 attgcaggggtaagATGGGTCTTTTGGGAATTGCTCTGTTTTGTTTTGCTGCAATGGTGT 300
M G L L G I A L F C F A A M V
301 GCTCTGTTCATGGCTATGACGCTGGATGGGTCAATGCTCATGCTACCTTCTATGGTGGAA 360
C S V H G Y D A G W V N A H A T F Y G G
361 GTGATGCTTCAGGAACAATGGgtatgtgcttctcactttgttctctaaaatgtctcagag 420
S D A S G T M
421 aaaacgaaaatctaggatatttacaatcttgttgatgttgttgtctctagGTGGAGCTTG 480
G G A C
481 TGGCTACGGGAACCTCTACAGTCAAGGTTACGGGACCAACACGGCGGCGTTGAGCACTGC 540
G Y G N L Y S Q G Y G T N T A A L S T A
541 TCTGTTCAACAACGGTCTTAGCTGCGGGGCTTGTTTTGAGATCAAGTGTCAGAGCGACGG 600
L F N N G L S C G A C F E I K C Q S D G
601 CGCGTGGTGTTTACCTGGTGCTATCATTGTCACAGCCACCAATTTCTGTCCTCCTAACAA 660
A W C L P G A I I V T A T N F C P P N N
661 CGCTCTTCCCAATAACGCTGGTGGTTGGTGTAACCCTCCGCTTCATCATTTCGATCTCTC 720
A L P N N A G G W C N P P L H H F D L S
721 TCAGCCTGTTTTTCAACGCATTGCTCAGTACAAAGCTGGTGTTGTCCCTGTTTCCTACAG 780
Q P V F Q R I A Q Y K A G V V P V S Y R
781 AAGgtaaaacataaatctatagtctttactgttacaaagtcttgatcttttatgcagttt 840
R
841 cttgattaggtgtcaaatttcttgttatgggtcttcataattgctctgtttgttgataaa 900
901 agtttcaatctttattcactttcgtatctgggtcatcgtaacttgttaatggtttcgtta 960
961 tttaaatgactctgttatttaactgatggtttttttttttttttgcttcatgtgtagGG 1020
1021 TTCCGTGTATGAGAAGAGGAGGTATAAGATTCACAATCAACGGTCACTCTTACTTCAACC 1080
V P C M R R G G I R F T I N G H S Y F N
1081 TTGTCTTGGTGACCAATGTTGGTGGTGCTGGAGATGTTCATTCGGTTGCGGTTAAAGGTT 1140
L V L V T N V G G A G D V H S V A V K G
1141 CTAGAACAAGGTGGCAACAAATGTCAAGAAACTGGGGACAGAACTGGCAAAGCAACAATC 1200
S R T R W Q Q M S R N W G Q N W Q S N N
1201 TCTTAAACGGTCAAGCATTGTCATTTAAGGTGACTGCTAGTGATGGTCGTACCGTCGTCT 1260
L L N G Q A L S F K V T A S D G R T V V
1261 CTAACAACATTGCTCCAGCTAGTTGGTCCTTTGGACAAACCTTCACCGGCCGTCAATTCC 1320
S N N I A P A S W S F G Q T F T G R Q F
1321 GTTAAaattgagtcaagttcggttttatatagttttagggtttgtgtagtagttggttga 1380
R *

patterns could represent genes encoding products involved repeatedly or continuously in developmental programs, such as meristem formation or maintenance, executed at different stages of development. Examples of such genes are commonly found in animals (e.g. reviewed by Ruohola-Baker *et al.* (1994)). In *Arabidopsis*, the *CLAVATA1* (Clark *et al.*, 1997), *SHOOT MERISTEMLESS* (Long *et al.*, 1996), and *SCARECROW* (Di Laurenzio *et al.*, 1996) genes provide examples of genes with putative regulatory functions during the development of both the embryo and the adult plant. Other lines exhibit more complex staining patterns throughout the plant life cycle. Such expression patterns could be associated with common cell types or activities, such as cell division (Springer *et al.*, 1995), in different plant organs or at different stages of plant development (Vroemen *et al.*, 1998).

***Molecular markers reveal polarity and the establishment of regions, cell- and tissue-types***

We have studied the expression patterns of lines WET368 (chapter 5), WET393, WET215, WET233 and WGT39 in more detail. WET368 and WET393 show complementary expression patterns in preglobular embryos: WET368 marks all cells descending from the apical, and WET393 all cells descending from the basal daughter cell of the zygote. As a consequence, these two expression patterns precisely reflect the segregation of cell fates along the apical-basal axis after the first zygotic division. All cells expressing WET368 at this stage have acquired embryo fate, whereas the cells marked by WET393 generate a file of cells of which all but the uppermost differentiate into the suspensor. The uppermost cell expressing WET393, the hypophyseal cell, will give rise to the quiescent center of the root meristem and de columella root cap. It has been proposed on the basis of the *monopteros* (Berleth and Jürgens, 1993), *hobbit* (Willemsen *et al.*, 1998), and *bodenlos* (Mayer and Jürgens, 1998) mutant phenotypes, that the hypophyseal cell only becomes part of the embryo proper after an inductive signalling event across the clonal boundary between the derivatives of the apical and basal daughter cells of the zygote (Mayer and Jürgens, 1998). The disappearance of WET393 *GUS* expression from the hypophyseal cell around the globular stage may coincide with its

**Figure 4.8:** Nucleotide sequence of genomic DNA flanking the *DsG* insertion in WGT39. The sequence represents part of a BAC contig (GenBank AC004138). The putative coding sequence of an expansin gene is in uppercase, and its predicted amino acid sequence is shown below the nucleotide sequence. The position of *DsG* insertion (between nucleotides 137 and 138) is indicated by a triangle, in which the direction of transcription of the *GUS* gene is marked by an arrow. A putative TATA box is underlined. The TAIL-PCR product generated from WGT39 DNA extended from the *DsG* element up to nucleotide 726 (marked by an arrow).

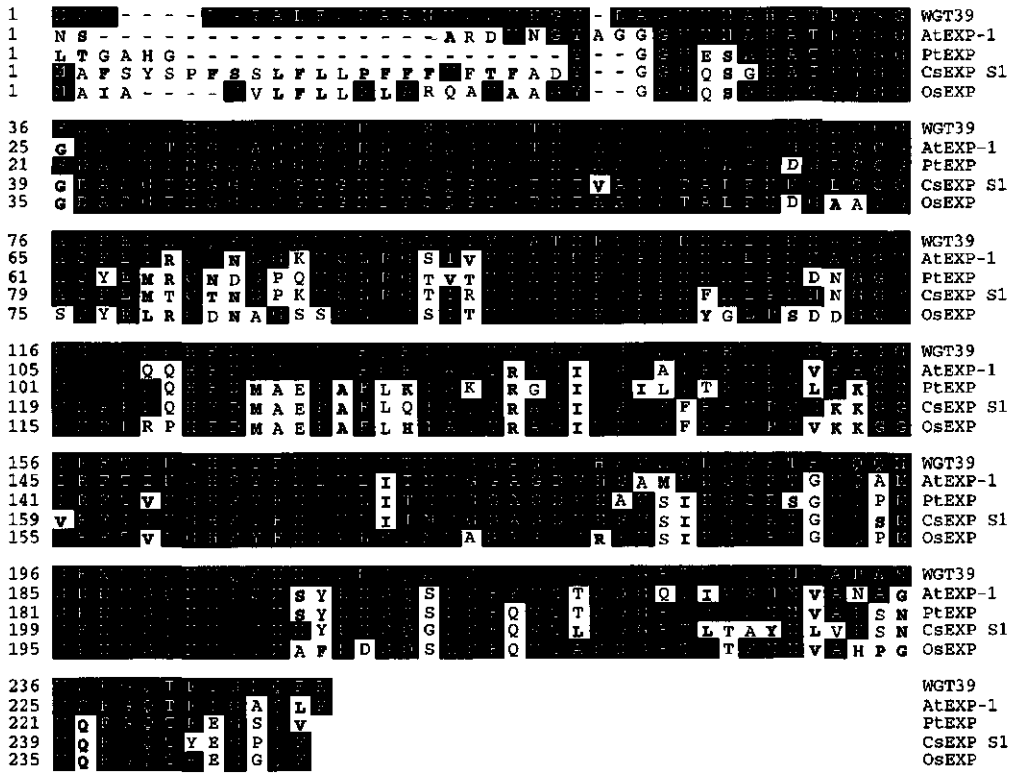



**Figure 4.9:** Comparison of the deduced amino acid sequence of the WGT39-expansin to expansin amino acid sequences of *Arabidopsis thaliana* (AtEXP-1, Accession Number gil1041702), *Pinus taeda* (PtEXP, Accession Number gil1778107), *Cucumis sativus* (expansin S1 precursor CsEXP S1, Accession Number gil1040875), and *Oryza sativa* (OsEXP, Accession Number gil1815681). Amino acid residues identical to the WGT39 residue are shaded; similar amino acids are indicated in bold face. Similar amino acids are grouped as follows: acidic (D, E); basic (H, K, R); hydrophobic (A, F, I, L, M, P, V, W); polar (C, G, N, Q, S, T, Y). Horizontal bars represent gaps introduced during alignment.

switch from suspensor to embryonic fate. The subsequent further restriction of *GUS* expression towards the basal cells of the suspensor indicates that suspensor cells express genes according to their relative positions, and reflects the existence of an apical-basal axis of polarity within the suspensor region. Thus, while initially marking all derivatives of the basal daughter cell of the zygote, WET393 expression is gradually restricted to the most basally located derivatives of the zygote during further embryo development. In this context, it is noteworthy that WET368 expression, while initially marking all derivatives of the apical daughter cell of the zygote, is gradually restricted to the SAM

region, which gives rise to the most apically located descendants of the zygote during post-embryonic development. In fact, mature plants display WET368 expression in all aerial meristems, and even in ovules, representing the last organ determined from the shoot meristem (see chapter 5). As such, WET393 and WET368 not only display opposite expression patterns in the early embryo, but also during later stages of embryo and plant development. The absence of WET393 expression after germination would in this scenario be the result of the absence of the suspensor, representing the most basally located descendants of the zygote, during post-embryonic development.

WET215 shows *GUS* expression in the hypophyseal cell group from the heart stage of embryogenesis onwards. In seedlings, columella root cap initials and their daughter cells, epidermal initials, hypocotyl epidermal cells, cotyledonary stomata and leaf trichomes show expression. This expression pattern coincides with the embryonic as well as the post-embryonic morphological aberrations in the *hobbit* (*hbt*) mutant (Willemsen *et al.*, 1998; V. Willemsen and B. Scheres, pers. comm.). Molecular mapping of the *DsE* insert in WET215 revealed that its map position is linked to, but does not coincide with the map position of *hbt* (V. Willemsen and B. Scheres, pers. comm.), and thus the WET215 expression pattern may reflect a component functioning in the same pathway as HOBBIT. The fact that cells marked by WET215 expression are either columella or epidermal cells implies that some intrinsic properties of epidermal cells are shared by columella root cap cells.

*GUS* expression in WET233 is similar to that in WET368 (chapter 5) from heart up to bent cotyledon stage embryos. Unlike WET368 expression, WET233 expression is not seen before the heart stage, and does not persist later than the seedling stage. Thus, while WET368 represents a marker for shoot meristem position throughout most of the plant life cycle, WET233 rather marks the shoot meristem region only in later stage embryos and seedlings, and not beyond the seedling stage. WET233 expression apparently reflects a gene function that is required in the SAM region in a more restricted developmental time frame than WET368.

In WGT39, *DsG* insertion has occurred just downstream of the promoter region of a previously unidentified putative expansin gene. WGT39 is initially expressed uniformly in the embryo proper, and becomes restricted to the vascular tissues and the root and shoot apices of later stage embryos and seedlings. Expansin proteins mediate cell wall extension in growing plant cells. Interestingly, the embryonic and post-embryonic WGT39 expression pattern has a remarkable, but not complete, similarity to the expression pattern of the *MONOPTEROS* (*MP*)gene, that encodes a transcription factor involved in embryo axis formation and vascular development (Hardtke and Berleth, 1998). The *MP* gene is expressed in embryonic and post-embryonic regions that undergo "axialization", meaning that continuous cell files are generated from previously isodiametric cells (Hardtke and Berleth, 1998). It seems plausible that changes in cell shape and size in axializing regions require cell wall extension, that, based on the WGT39 expression pattern, may be mediated by expansins.

### GUS *expression patterns in WET and WGT lines reflect different aspects of embryo development*

On the basis of the observed GUS staining pattern in developing embryos, we have classified the 39 embryo staining lines in four distinct classes. It should be noted that, due to the variety of observed staining patterns, such classification inevitably brings about difficulties in unequivocally assigning every single line to one of the predefined classes. As becomes clear from Table 4.1, the majority of lines in which *GUS* expression starts early during embryo development, meaning at or before the globular stage, falls into classes A and C. Thus, up to the globular stage, *GUS* expression is mostly uniform in the embryo proper. Only one line (WET42) with non-uniform expression in the embryo proper at the globular stage was identified. *GUS* expression in class C lines remains uniform at all stages of embryogenesis and might be associated with cellular processes that are common to all cell-types present in the embryo.

Two classes of lines display spatially restricted *GUS* expression at later stages of embryo development. In class A lines, the spatially restricted *GUS* expression is preceded by early uniform expression. Most class B lines do not show early, (pre)globular *GUS* expression, and *GUS* expression is restricted to a distinct embryonic region or tissue from its onset onwards. This indicates that the majority of lines exhibiting spatially restricted *GUS* expression in later stage embryos display either uniform (class A lines) or no (most class B lines) expression in (pre)globular embryos.

A tentative model for early plant embryogenesis includes first global and then more locally specified regions, that together generate the final body pattern by cellular interactions (Jürgens, 1995; chapter 5). The early uniform and subsequently more restricted expression patterns characteristic of class A lines seem to fit with the predictions made in this model for expression of genes involved in embryo patterning. The expression of early and uniformly expressed genes could gradually be repressed in certain regions, thereby giving rise to global, partly overlapping territories of gene expression. The superimposition of different gene expression patterns could subsequently allow the demarcation of more locally specified regions, each defined by the combined expression levels of a characteristic spectrum of genes. In this scenario, the distinct pattern elements and regions that finally make up the final body pattern would, once established, be marked by restricted *GUS* expression found in class B lines. An analogy for the demarcation of locally specified regions through gradual repression of early and uniformly expressed genes is found in *C. elegans*, where repeated localization of the PIE-1 protein to the totipotent germline blastomere correlates with a general repression of transcription within the germline (Mello *et al.*, 1996). The initially uniform and subsequently more restricted expression patterns of genes or enhancers such as those identified by class A lines could reinforce the notion that early acting patterning genes potentially mutate to early, not very informative embryo-lethal phenotypes (Meinke, 1991). The identification of such genes based on expression pattern appears to underscore the advantages of gene and enhancer trapping as one of the approaches

for unravelling the regulatory network governing embryo pattern formation. It is noteworthy that many of the regions marked by *GUS* expression in one or more of our gene or enhancer trap lines (Figure 4.1), do not correspond to regions previously identified by embryo morphology or histology, nor do they all consist of cells sharing a common clonal ancestry. An important implication of this would be that molecularly defined regions exist in the embryo, superimposed on morphologically or functionally recognizable regions. These molecularly defined regions could extend the description of the successive steps in the formation of a plant embryo (chapter 5).

### *Sequence analysis of genomic DNA flanking* DsE *and* DsG *elements: identified genes and insight into the molecular functioning of gene and enhancer traps*

Sequence analysis of genomic DNA flanking the *DsG* insertion in WGT39 revealed that in this line, the gene trap transposon has inserted into the 5' untranslated leader of an expansin gene. Consequently, the *DsG* element in WGT39 acts as a promoter trap, expressing *GUS* under the control of the expansin promoter just upstream of its insertion site. This demonstrates that the gene trap element we used not only functions if inserted into an intron or exon of the coding region of a transcribed gene (Sundaresan *et al.*, 1995), but also if inserted in the untranslated leader sequence. The genomic DNA sequence flanking the *DsE* element in WET393, and a putative mutant phenotype in WET393 embryos, collectively suggest the insertion of the enhancer trap element into the coding region of a gene, which might, based on the predicted amino acid sequence, encode a transcriptional activator or a cell wall protein. It is tempting to speculate that such a gene product is involved in the correct patterning of the suspensor or the hypophyseal cell group, or both. Both these cell groups descend from the basal zygotic daughter cell and initially show WET393 expression. In the flanking DNA of three other WET lines, no ORF was detected, nor was a mutant phenotype observed in plants of these lines, suggesting *DsE* insertion outside of the transcribed regions of chromosomal genes. This implies that *GUS* expression from the enhancer trap element used in this study can be activated after *DsE* insertion near a chromosomal enhancer, either within or outside of the transcribed region of a gene, as was the original purpose of the *DsE* element.

### *Efficiency of isolating genes important for embryo development by enhancer and gene trap transposon mutagenesis*

In this study, we have generated lines that exhibit different *GUS* expression patterns in the embryo. These lines represent 5% of the total number of lines screened, a percentage that roughly corresponds to the frequency of lines with GUS fusion activity in embryos obtained with T-DNA based promoter

traps (Topping *et al.*, 1994). Several other screens have been carried out, aimed at the isolation of genes transcribed in developing plant embryos. Most of these screens have focused on the isolation of "embryo enhanced" or "embryo specific" genes from carrot somatic embryos using a variety of differential screening approaches (Zimmerman, 1993). The frequencies of genes isolated on the basis of differential expression in somatic embryos or embryogenic cultures versus non embryogenic cultures, callus, or seedlings (Aleith and Richter, 1990; Choi *et al.*, 1987; Lin *et al.*, 1996; Sato *et al.*, 1995; Schmidt *et al.*, 1997; Wurtele *et al.*, 1993) vary widely, and are difficult to compare due to differences in screening criteria. Nonetheless, in all screens, the percentage of isolated clones was in the order of 0.01% to 0.1% of the total number of clones screened. Although this percentage is much lower than the percentage of enhancer and gene trap lines exhibiting *GUS* expression in embryos, it should be noted that each of the differential screens referred to above resulted in the isolation of 7 to 38 unique embryo enhanced or embryo specific clones, a number that is in the same order of magnitude as the number of embryo staining lines identified in our enhancer and gene trap screen. Taking into account the considerable effort needed to establish and screen populations of independent enhancer, gene or promoter trap lines (Sundaresan *et al.*, 1995; Topping *et al.*, 1994; Vroemen *et al.*, 1998), a direct comparison between the efficiency of both types of screens, purely based on the frequency of isolated lines or clones, can be misleading.

A more relevant evaluation of the results of different types of screens should take into account that attempts at cloning genes expressed in the embryo by differential screening were designed with the bias that genes that are important for embryo development should either not be expressed at all, or show greatly reduced expression in non-embryonic tissues. The recent cloning of genes with regulatory or putative regulatory functions during *Arabidopsis* embryogenesis, such as *SHOOT MERISTEMLESS* (Long *et al.*, 1996), *SCARECROW* (Di Laurenzio *et al.*, 1996), *CLAVATA1* (Clark *et al.*, 1997), *ZWILLE* (Moussian *et al.*, 1998), *MONOPTEROS* (Hardtke and Berleth, 1998), and *AtML1* (Lu *et al.*, 1996), and other genes that mutate to embryo phenotypes upon disruption, such as *KNOLLE* (Lukowitz *et al.*, 1996), *EMB30/GNOM* (Busch *et al.*, 1996; Shevell *et al.*, 1994), and *CUC2* (Aida *et al.*, 1997) has shown that the expression of none of these is restricted to the embryo. Therefore, any gene cloning scheme that involves differential or subtractive hybridization comparing embryo cDNA with cDNA from non-embryonic tissues will likely eliminate a substantial proportion of genes that are instructive in embryo development. So far, only few plant genes have been cloned that display embryo specific expression. Expression of the carrot *EMB-1* (Wurtele *et al.*, 1993) and the *Arabidopsis PEI1* (Li and Thomas, 1998) genes commences at the globular stage and continues up to the mature embryo stage. Expression of the carrot *SERK* gene (Schmidt *et al.*, 1997), that encodes a leucin-rich repeat containing receptor-like kinase that may be involved in an early embryo specific signal transduction cascade, starts as early as the one-cell stage, and continues no later than the globular stage. The *SERK* cDNA was isolated from a cold plaque screen, comparing mRNAs from embryogenic and non-embryogenic carrot single cell cultures

(Schmidt *et al.*, 1997). This indicates that, although embryo specific, the *SERK* mRNA was not abundant enough to allow detection by conventional differential screening approaches.

With the foregoing in mind, it may not be surprising that only one line exhibiting *GUS* expression restricted to embryogenesis was identified in our enhancer and gene trap screen. The results of different screens performed so far suggest that both enhancer, gene or promoter trapping and differential screening are suitable ways to identify genes important for embryo development. The bias of differential screening procedures against genes whose embryonic and non-embryonic expression levels do not significantly differ, seems to reduce the efficiency of these screens for the isolation of genes important for embryo development. On the other hand, the elimination of genes whose expression is not restricted to embryogenesis by differential screening or subtraction should provide access to the apparently rare class of genes with strictly embryo specific expression. Thus, if highly embryo specific processes underlie the acquisition of embryogenic potential and early embryo development, differential screening or subtraction approaches should allow the discrimination of the corresponding genes from genes involved in processes which are required for, but not unique to embryo development, such as for example *STM*, *CLV*, and many other genes identified in genetic screens on the basis of embryo morphology. Enhancer, gene or promoter trap screens offer the advantage of allowing selection of lines not only on the basis of *GUS* expression in the embryo, but more importantly, also based on its precise timing and spatial distribution in embryonic and non-embryonic tissues. The non-embryo specific expression pattern of recently cloned genes whose products are instructive in embryo development seems to underscore the importance of the latter criterion. Further molecular characterization of genomic regions tagged by our enhancer and gene trap elements will yield the identity of genes whose expression pattern is reflected by the observed GUS staining patterns. For gene trap lines this should be relatively straightforward, since *GUS* expression can only occur if the *DsG* element has inserted within the transcribed region of the corresponding gene, as appears the case for both gene trap lines from which flanking genomic DNA was sequenced. Analysis of genomic regions flanking our enhancer trap insertions should provide information on whether the observed *GUS* expression patterns in WET lines all precisely mimic the actual expression of a transcribed gene located close to the *DsE* insertion site.

## Materials and methods

### *Plant material*

A collection of 431 Wageningen Enhancer Trap (WET) and 373 Wageningen Gene Trap (WGT) lines was generated as previously described in detail (Vroemen *et al.*, 1998). WET plants contained

the enhancer trap transposable element *DsE* (Sundaresan *et al.*, 1995), that carries a β-glucuronidase (*GUS*) gene fused to a minimal 35S promoter, and a constitutively expressed neomycin phosphotransferase II (*NPTII*) gene conferring kanamycin resistance. WGT plants contained the gene trap transposable element *DsG*, that carries a promoterless *GUS* gene fused to a triple splice acceptor, and a constitutively expressed *NPTII* gene (Sundaresan *et al.*, 1995).

In order to establish lines carrying a stable enhancer or gene trap insertion at a locus unlinked or loosely linked to the original *DsE* or *DsG* T-DNA locus ("transposants" (Bellen *et al.*, 1989)), *DsE* and *DsG* elements were mobilized from their original T-DNAs by crossing *DsE* and *DsG* starter lines to *Ac* starter lines carrying an *Ac* transposase gene. Counter selection against both the *Ac* T-DNA and the original *DsE* or *DsG* T-DNA in the F2 generation of these crosses resulted in enhancer and gene trap transposants, of which the F3 seed (generally a pool of the seeds from two transposant plants) was harvested and stored as a WET or WGT line (Sundaresan *et al.*, 1995; Vroemen *et al.*, 1998). WET and WGT lines were maintained by selecting seeds on MS plates (Murashige and Skoog, 1963) containing 50 µg / ml kanamycin and bulking seeds from independent kanamycin resistant plants.

### *Histochemical localization of* GUS *expression*

For localization of *GUS* expression, tissues were vacuum-infiltrated with GUS staining solution (100 mM NaPi pH 7.2, 10 mM EDTA, 0.1% Triton X-100, 100 mg/ml chloramphenicol (to inhibit bacterial growth), and 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid)), and subsequently stained for up to 48 hours at 37 °C in the dark. Siliques with immature seeds covering all stages of embryo development (typically 3 - 5 siliques per line) were opened longitudinally prior to incubation in the staining solution. Stained tissues were cleared for a minimum of 16 hours in Hoyers solution (100 g chloral-hydrate, 2.5 g Arabic gum, 15 ml glycerol, 30 ml water). For the preparation of histological sections, plant tissues stained with X-Gluc were vacuum-infiltrated with 100 mM NaPi pH7.2, 4% formaldehyde, 4% DMSO and fixed for at least three days at 4°C. Fixed tissues were dehydrated through an ethanol series, and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions (Vroemen *et al.*, 1998). Serial sections 3 µm thick were cut with a Reichert-Jung microtome, transferred to microscope slides, stained with 0.01% Ruthenium Red (Sigma) for 1-10 min, and mounted in Euparal (Agar Scientific, Stansted, UK). GUS staining patterns were viewed using a binocular (Nikon Corp., Tokyo, Japan) and a Nikon Optiphot-2 microscope equipped with Normarski optics. GUS staining patterns were examined in more detail using GUS staining solutions containing equal amounts of potassium ferrocyanide and potassium ferricyanide in concentrations between 1.25 mM and 5 mM. These reaction conditions are reported to minimize diffusion of the reaction intermediates and thereby

improve the specificity of the localization of *GUS* expression (Jefferson *et al.*, 1987).

### *TAIL-PCR amplification of* DsE *and* DsG *flanking sequences*

Genomic DNA flanking *DsE* and *DsG* elements was amplified by thermal asymmetric interlaced (TAIL) PCR, essentially as described by Liu *et al.* (1995). Genomic DNA from individual WET and WGT plants was isolated according to Bouchez (1996), and analyzed by Southern blotting using a probe against the entire *GUS* coding sequence to determine the number of *Ds* inserts, as described previously (Vroemen *et al.*, 1998). For TAIL-PCR amplification, a set of three nested primers for the 5' end of *Ds*, Ds5-1, Ds5-2, and Ds5-3, was used in combination with one arbitrary primer, AD2 (Liu *et al.*, 1995), to amplify genomic DNA flanking the 5' end of either *DsE* or *DsG* elements. The sequences of the primers were as follows: Ds5-1, 5'-CCG TTT ACC GTT TTG TAT ATC CCG-3'; Ds5-2 5'-CGT TCC GTT TTC GTT TTT TAC C-3'; Ds5-3, 5'-GGT CGG TAC GGA ATT CTC CC-3' and AD2, 5'-NGT CGA (G/C)(A/T)G ANA (A/T)GA A-3'. Genomic DNA (approximately 10 ng) was used as substrate for the primary TAIL-PCR amplification, in the following 20 µl reaction mixture: 1 x PCR buffer (Promega, Southampton, UK), 0.2 mM (each) dNTPs, 0.2 µM Ds5-1, 3 µM AD2, and 0.05 u per µl *Taq* polymerase. PCR amplification was carried out in a GeneAmp PCR System 9600 (Perkin Elmer Cetus) using the following cycling conditions: denaturation 95°C 2 min; five "linear" cycles of 94°C 30 sec, 62 °C 1 min, 72°C 2.5 min; one "touchdown" cycle of 94°C 30 sec, 25°C 3 min, ramp to 72°C in 3 min, 72°C 3 min; 15 supercycles of 94°C 30 sec, 65°C 1 min, 72 °C 2.5 min, 94°C 30 sec, 65°C 1 min, 72°C 2.5 min, 94°C 30 sec, 44°C 1 min, 72°C 2.5 min; extension 72°C 5 min. The primary reaction products were diluted 50 times in sterile distilled water, and 1 µl was used as template for the secondary TAIL-PCR amplification, in the following 20 µl reaction mixture: 1 x PCR buffer, 0.2 mM (each) dNTPs, 0.2 µM Ds5-2, 2 µM AD2, 0.04 u per µl *Taq* polymerase. Cycling conditions for the secondary TAIL-PCR reaction consisted of the 15 supercycles applied in the primary reaction, with the two 65°C annealing steps performed at 64°C, followed by a 5 min 72°C extension. 1 µl of 50 times diluted secondary reaction products was used as template for the tertiary amplification. The reaction mixture was identical to that of the secondary amplification, with primer Ds5-3 instead of Ds5-2, and the cycling conditions were: 30 cycles of 94°C 30 sec, 44°C 1 min, 72°C 2.5 min, followed by a 5 min 72°C extension. The primary, secondary and tertiary reaction products were separated on a 3% agarose gel. In successful reactions, the tertiary reaction product was 71 bp smaller than the secondary product. Secondary and tertiary reaction products were purified using the PCR Purification Kit (Boehringer, Almere, the Netherlands), and either sequenced directly using Ds5-2 or Ds5-3 as sequencing primer, respectively, or cloned into the pGEM-T vector (Promega) according to the manufacturer's instructions, and sequenced using T7 and SP6 sequencing primers.

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# 5

# Enhancer trap tagging of WET368, a regional marker for early *Arabidopsis* embryos

In an enhancer trap screen for *GUS* markers expressed in the early *Arabidopsis* embryo, we identified line WET368 (for Wageningen Enhancer Trap line 368), that shows *GUS* expression commencing in the octant stage embryo. WET368 expression is initially uniform in all cells descending from the apical cell produced by the first division of the zygote. During later stages of embryogenesis, expression becomes restricted to a previously undefined region encompassing the shoot apical meristem (SAM) and part of the cotyledon primordia . After germination, WET368 expression remains associated with future, current and former positions of aerial meristems. Continued WET368 expression in the aberrant shoot apex regions of mutant *shoot meristemless*, *zwille*, *wuschel*, and *primordia timing* seedlings indicates that WET368 expression is independent of shoot meristem formation or activity, but is linked to the position of shoot meristem development. We have classified WET368 as a regional marker for apical meristem position, and propose that its expression is associated with positions in which cells can acquire shoot or floral meristem identity. The early WET368 expression implies that all embryo proper cells in the octant stage embryo, except the hypophyseal cell, can initially acquire SAM identity.

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## Introduction

Zygotic embryogenesis is the developmental process that represents the transition of a fertilized egg cell to a multicellular organism. During embryogenesis, different cell types, tissues and organs are arranged in a spatially defined pattern. In animals, pattern formation is essentially complete after embryogenesis, while most of the structures of adult higher plants are formed during postembryonic development from meristems (Kerstetter and Hake, 1997; Steeves and Sussex, 1989). The plant embryo develops into the seedling. The body plan of the seedling consists of shoot meristem, cotyledons, hypocotyl, and root including the root meristem arranged along the apical-basal axis of polarity, and epidermis, ground- and conductive tissue along the radial axis (Goldberg *et al.*, 1994; Jürgens, 1995; Jürgens *et al.*, 1994a; Jürgens *et al.*, 1994b; Mayer *et al.*, 1991; Vroemen *et al.*, 1996). In dicots, the body pattern of the seedling is established at the torpedo stage of embryogenesis. The origins of the apical-basal and radial pattern elements have been traced back to distinct cells or cell groups in the early embryo in *Arabidopsis* (Jürgens *et al.*, 1994a; Jürgens *et al.*, 1994b; Mansfield and Briarty, 1991; Scheres *et al.*, 1994). During *Arabidopsis* zygotic embryogenesis, the asymmetric division of the zygote yields two unequal daughter cells of distinct developmental fates. The smaller apical cell gives rise to most of the embryo proper, whereas the larger basal cell produces the suspensor and the hypophyseal cell, that produces the columella root cap and the quiescent center as part of the root meristem. The apical cell develops through a series of precise divisions through the 2-cell, quadrant, octant and dermatogen stages to reach the globular stage. Then, the embryo establishes bilateral symmetry as it passes through the transition, heart, torpedo and bent cotyledon stages until it becomes mature (Jürgens *et al.*, 1991; Scheres *et al.*, 1994). While the cell divisions during early *Arabidopsis* embryo development are highly regular, clonal and genetic analyses suggest that pattern formation largely depends on cell-cell communication and position-dependent cell fate specification (Irish, 1991; Laux and Jürgens, 1997; Scheres *et al.*, 1994).

During embryogenesis, two groups of stem cells are organized at opposite ends of the apical-basal axis, the root meristem and the shoot apical meristem (SAM; Steeves and Sussex, 1989). It is not known at what stage during embryo development cells adopt shoot meristem fate. One view considers that the top half of the globular embryo is the earliest form of shoot apical meristem, from which both the "embryonic leaves", or cotyledons, and the future vegetative meristem are derived (Kaplan, 1969; McConnell and Barton, 1995). An alternative view, based on histology, is that SAM initiation first occurs at the torpedo stage, between the base of the cotyledons (Barton and Poethig, 1993; Mansfield and Briarty, 1991). At this time point the L1, L2 and L3 layers of cells at the presumptive apex adopt division patterns that yield a tunica-corpus arrangement (Satina *et al.*, 1940). Divisions in the innermost L3 cell layer form the corpus, and anticlinal divisions in the L2 and uppermost L1 layer form two clonally distinct tunica layers (Barton and Poethig, 1993; Clark, 1997; McConnell and Barton, 1995). The L1 tunica layer derives from the embryo protoderm, reflected by

the expression patterns of the *AtLTP1* (Vroemen *et al.*, 1996) and *ATML1* (Lu *et al.*, 1996) genes. Superimposed on the three horizontal cell layers, the SAM can be divided conceptually into three zones, although their boundaries are often indistinct. The central zone (CZ) consists of undifferentiated stem cells at the very center of the meristem. These produce daughter cells that adopt specific developmental fates as they enter the surrounding peripheral zone (PZ), or flank meristem, or the underlying rib zone (RZ; Endrizzi *et al.*, 1996; Kerstetter and Hake, 1997; Steeves and Sussex, 1989). In the PZ, cells are incorporated into organ primordia, whereas cells in the RZ contribute to the vasculature and internal stem structures (Steeves and Sussex, 1989).

Genetic analyses have provided some insight into SAM organization and function. Mutations at the *CLAVATA1* (*CLV1*) and *CLV3* loci cause accumulation of excessive numbers of undifferentiated cells in the CZ, suggesting that *CLV* genes either promote the transition of cells into the PZ, or restrict the rate of cell division in the CZ (Clark, 1997; Clark *et al.*, 1993; Clark *et al.*, 1995; Leyser and Furner, 1992; Weigel and Clark, 1996). The *CLV1* gene encodes a leucine-rich repeat transmembrane receptor kinase, and is expressed in the L3 layer of the meristem center, but in a region larger than the CZ. CLV1 may perceive positional information directed to cells expressing the receptor (Clark *et al.*, 1997). Mutations in the *PRIMORDIA TIMING* (*PT*) gene result in a pleiotropic phenotype, including a broader embryonic and seedling SAM, polycotyly, and a higher number of rosette leaves than wild-type (Conway and Poethig, 1997; Mordhorst *et al.*, 1998). Mutations in the *SHOOT MERISTEMLESS* (*STM*) gene, that encodes a putative homeodomain transcription factor of the KNOTTED class (Long *et al.*, 1996), have effects opposite to those in *CLV* genes. A complete loss of *STM* function eliminates the entire SAM in embryos and seedlings (Barton and Poethig, 1993; Endrizzi *et al.*, 1996; Long *et al.*, 1996). Mutants with weak *stm* alleles retain a small number of cytoplasmic dense cells at the place were the wild-type SAM normally forms. Repetitively initiated shoot and floral meristems stop at the primordia stage, suggesting that the undifferentiated meristem cells are "used up". *STM* is first expressed in one or two cells in the apical hemisphere of the globular embryo, long before the visible presence of the SAM and the aberrant SAM morphology in mutants with strong *stm* alleles (Long *et al.*, 1996). *STM* expression expands to include the entire histologically visible embryonic SAM, and, during post-embryonic development, covers a central region of all shoot and floral meristems. These data indicate that STM is required to specify the meristematic nature of cells of the embryonic SAM, and to maintain a pool of undifferentiated cells in the center of the SAM.

Similar to *stm* mutants, *wuschel* (*wus-1*) mutant shoot and floral meristems terminate prematurely. In contrast to *stm* shoot meristems, embryonic and post-embryonic *wus-1* shoot meristems still contain central cells, but they are enlarged and non-functional. This suggests a role for WUS in specifying cell identity in meristem centers (Endrizzi *et al.*, 1996; Laux *et al.*, 1996). *wus-1* shoot meristems terminate in flat enlarged apices, or occasionally form a single terminal leaf at the apex. Mutations in the *ZWILLE* (*ZLL*) gene (Endrizzi *et al.*, 1996; Moussian *et al.*, 1998) result in

embryos and seedlings with non-functional shoot meristems, that are reduced in size and display a flat organization compared to wild-type shoot meristems. *zll* seedlings give rise to adventitious shoot meristems at the base of the cotyledons. These meristems give rise to shoots that eventually form fertile flowers. Thus, the *ZLL* gene seems to be involved in the specification of cell identity in the meristem center primarily during embryogenesis (Jürgens *et al.*, 1994b). Like *wuschel* seedlings, *zwille* seedlings either have terminated, flat apices, or form a single terminal leaf. Genetic analysis indicates that *WUS* and *ZLL* act downstream of *STM*. A model proposes that *STM* maintains a central pool of undifferentiated cells, and that *WUS* and *ZLL* are required for proper functioning of these cells (Endrizzi *et al.*, 1996).

So far, no mutants have been described that are instructive for the way SAM identity is established in cells of the early embryo, prior to the activity of STM. This could be due to a failure to recognize the relevant mutant phenotype. In addition, mutations in some developmentally important genes may not readily cause specific phenotypes due to functional redundancy (Aida *et al.*, 1997; Goebl and Petes, 1986) or very early lethality (Mlodzik *et al.*, 1990; Springer *et al.*, 1995), and may thus have been missed in extensive screens for embryo mutants (Castle *et al.*, 1993; Franzmann *et al.*, 1995; Jürgens *et al.*, 1991; Mayer *et al.*, 1991; Meinke, 1991). An approach to circumvent some of these problems would be the isolation of genes based on expression pattern, using gene or enhancer trapping (Devic *et al.*, 1995; Klimyuk *et al.*, 1995; Lindsey *et al.*, 1993; Sundaresan, 1996; Sundaresan *et al.*, 1995; Topping *et al.*, 1994; Topping and Lindsey, 1995; Topping *et al.*, 1991). We have established a collection of independent transgenic *Arabidopsis* lines containing gene and enhancer trap transposable elements and screened this collection for early embryo markers (Vroemen *et al.*, 1998). Here we describe Wageningen Enhancer Trap line WET368, that already shows *GUS* expression in the octant stage embryo, and becomes restricted to a region encompassing the shoot apical meristem and part of the cotyledon primordia at later stages of embryogenesis. After germination, WET368 *GUS* expression remains associated with all future, current and former positions were aerial meristems will, or can normally be formed. Based on its expression in wild-type and *stm*, *wus*, *zll*, and *pt* mutant embryos and seedlings, we propose that WET368 expression predicts which regions can acquire SAM identity and reflects SAM position. The early WET368 expression suggests that in the octant stage embryo, all embryo proper cells descending from the apical cell produced by the first division of the zygote, can initially acquire SAM identity.

## Results

### GUS *expression pattern of WET368 during embryogenesis*

In a screen for enhancer trap expression in *Arabidopsis* embryos (Vroemen *et al.*, 1998), line WET368 was selected because it showed *GUS* expression in a region encompassing the shoot apical meristem and part of the cotyledon primordia. Southern analysis with the *GUS* coding sequence as probe showed a single *DsE* element insertion, ensuring that the observed *GUS* expression pattern is directly correlated with the place of insertion (Vroemen *et al.*, 1998). A detailed analysis of the WET368 *GUS* expression pattern during embryogenesis was carried out using X-Gluc histochemistry. The WET368 enhancer trap confers uniform expression in all cells of the octant stage embryo, but not in the suspensor and the hypophyseal cell. Thus, at this stage, *GUS* expression is present in all 8 derivatives of the apical cell, but not in those of the basal cell of the two-celled embryo (Figure 5.1A). Starting in the transition stage embryo, the expression gradually shifts towards the apical end of the embryo proper, to a region encompassing the future shoot apical meristem (SAM) and part of the cotyledon primordia (Figure 5.1B). Figure 5.1C shows a section of a transition stage embryo, in which indigo blue crystals, that appear yellow or purple under dark field optics (Klimyuk *et al.*, 1995), are most abundant in the SAM region. From the early heart stage onwards, *GUS* expression becomes progressively restricted to the region encompassing the SAM and the base of the cotyledons, and becomes absent from the rest of the embryo proper (Figures 5.1D to 5.1F). Detailed analysis of serial sections through GUS stained WET368 embryos (Figure 5.1G) indicates that in the meristem itself, *GUS* expression is in L1, L2 and L3 and in both the central and the peripheral zone. In the base of the cotyledons, expression seems restricted to the epidermis. In mature embryos (Figure 5.1H) expression is mostly restricted to the SAM region. The dark field image of a GUS-stained, mature WET368 embryo (Figure 5.1I) shows that *GUS* expression is highest in the meristem itself, and decreases towards the periphery of the region. The same pattern of GUS activity was also observed under reaction conditions that prevent diffusion of the reaction intermediate through inclusion of high concentrations of the oxidative catalysts potassium ferri- and ferrocyanide (Jefferson *et al.*, 1987; Mascarenhas and Hamilton, 1992). We conclude that the expression conferred by the WET368 enhancer is initially linked to an embryonic region consisting of all descendants of the apical cell of the two-celled embryo, and later becomes restricted to a region encompassing the shoot meristem. Thus, although initially precisely reflecting the first division of the zygote, WET368 appears a regional marker rather than a marker reflecting a particular differentiation event.

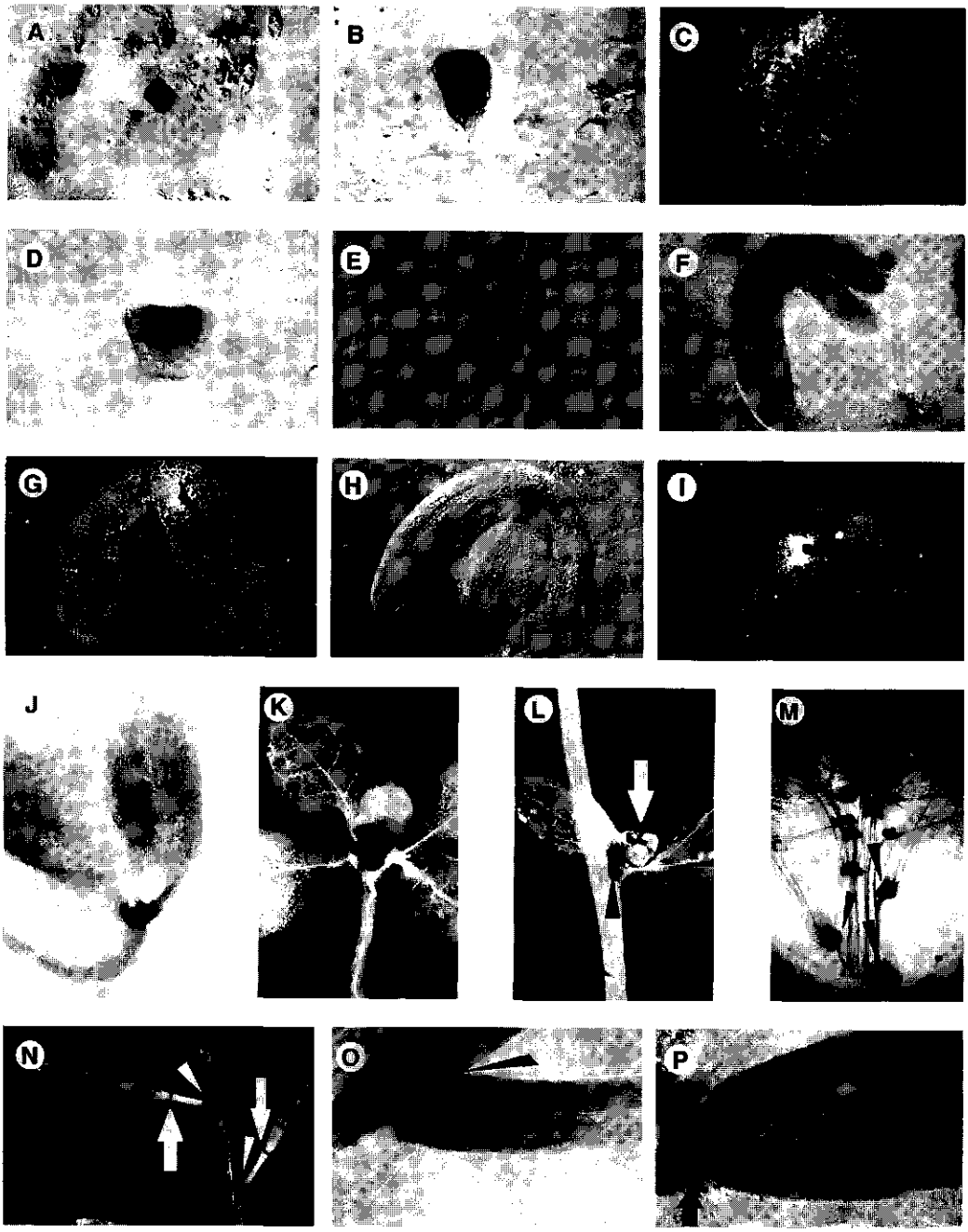


**Figure 5.1:** *GUS* expression patterns in WET368.

### GUS *expression pattern of WET368 during post-embryonic development*

After germination, *GUS* expression in WET368 remains associated with regions in which aerial meristems develop or have developed. In seedlings 5 days after germination (Figure 5.1J), GUS staining was essentially the same as in mature embryos: prominent in the shoot apical meristem, but also extending into the cotyledonary petioles. In 10 day old seedlings (Figure 5.1K), GUS staining extended into the petioles of the newly formed true leaves. In mature flowering plants, continued GUS activity was found in the axillary meristems, that form in the axils of cauline leaves (arrow in Figure 5.1L). These meristems are under normal circumstances not active, indicating that WET368 expression can precede axillary meristem activity. GUS activity was also observed in the axil of secondary inflorescences (arrowhead in Figure 5.1L) and in the axil of pedicels (arrowheads in Figures 5.1M, 5.1N, and 5.1O), with somewhat stronger staining adaxially than abaxially. These are the positions where the indeterminate secondary inflorescence meristem, and the determinate floral meristem, respectively, have branched from the main inflorescence stem. Finally, GUS activity was found in de petal-sepal abscission zone at the base of the carpel and silique (arrows in Figures 5.1N and 5.1P), and in ovules, developing seeds just after fertilization, carpels and silique walls (Figure 5.1P).

**A:** Octant stage embryo.

**B:** transition stage embryo.

**C:** Section of a transition stage embryo (dark field image).

**D:** early heart stage embryo.

**E:** late heart stage embryo.

**F:** bending cotyledon stage embryo.

**G:** section of a bending cotyledon stage embryo.

**H:** mature embryo.

**I:** mature embryo (dark field image).

**J:** seedling 5 days after germination.

**K:** seedling 10 days after germination.

**L:** axillary meristem in axil of cauline leaf, mature plant.

**M:** inflorescence, mature plant.

**N:** inflorescence stem with pedicels and siliques, mature plant.

**O:** axil at base of pedicel, mature plant.

**P:** base of silique just after fertilization, mature plant.

### GUS *expression pattern of WET368 in mutant backgrounds*

Because the WET368 enhancer confers *GUS* expression in a region encompassing the embryonic and seedling shoot apical meristem, its expression pattern was further investigated in different mutants defective in the control of shoot meristem size or function. Homozygous WET368 plants were crossed with plants carrying either the *shoot meristemless*, *wuschel*, *primordia timing*, or *zwille* mutation. Kanamycin resistant F2 progeny, homozygous for the mutation and containing the WET368 enhancer trap, were analyzed histochemically for the presence of GUS activity in 10 day-old seedlings. *stm-1* seedlings have strongly reduced WET368 expression when compared to wild-type seedlings of the same age (Figure 5.2A, cf. Figure 5.1K). Expression is localized to a small group of cells just above the point where the two cotyledonary vascular strands separate. Weak WET368 GUS staining was also seen in a small region just below the point where the partially fused petioles separate (data not shown).

*wus-1* seedlings have shoot meristems, that contain non-functional central cells, and terminate in flat enlarged apices or form a single terminal leaf at the apex. *wus-1* seedlings show almost wild-type levels of WET368 *GUS* expression (Figures 5.2B and 5.2C, cf. Figure 5.1K). Apparently, the central cells in *wus* seedlings show continued WET368 driven *GUS* expression. The region surrounding the *wus* SAM shows normal *GUS* expression. In case a terminal leaf is formed (Figure 5.2C), GUS staining marks the petiole of this leaf, just as the GUS staining in petioles of leaves in wild-type seedlings (cf. Figure 5.1K).

Seedlings homozygous for the *pt-1* mutation display strongly increased embryonic SAM size, and accordingly the region marked by the WET368 enhancer trap is increased (Figure 5.2D) when compared to wild-type seedlings of the same age (cf. Figure 5.1K). As in wild-type seedlings, the WET368 enhancer confers *GUS* expression in the petioles of the many leaves of the *pt* mutant seedling.

Like *wus* seedlings, *zll* seedlings either have terminated flat apices, albeit smaller in size, or form a single terminal leaf. As shown in Figure 5.2E, the region marked by the WET368 enhancer trap is reduced in *zll* seedlings with a small terminated apex when compared to wild-type seedlings of the same age (cf. Figure 5.1K). *zll* seedlings with terminal leaf show, like *wus* seedlings with terminal leaf, continued WET368 *GUS* expression in its petiole (Figure 5.2F). In contrast to *wus* plants, fertile secondary shoots are formed in mature *zll* plants. These shoots show only minor defects. Accordingly, the WET368 *GUS* expression in *zll* shoots is similar to that observed in wild-type shoots. GUS activity is present in axillary meristems in the axils of cauline leaves, in the axils of secondary inflorescences (Figure 5.2G, cf. Figure 5.1L), in the axil of pedicels, and in the abscission zone at the base of the silique (Figure 5.2H, cf. Figure 5.1N). Taken together, WET368 expression is strongly increased following the increased shoot meristem size in *pt*, and decreased in the reduced shoot meristematic region in *stm*. Continued GUS activity in the entire apices of *stm*, *zll*, and *wus*

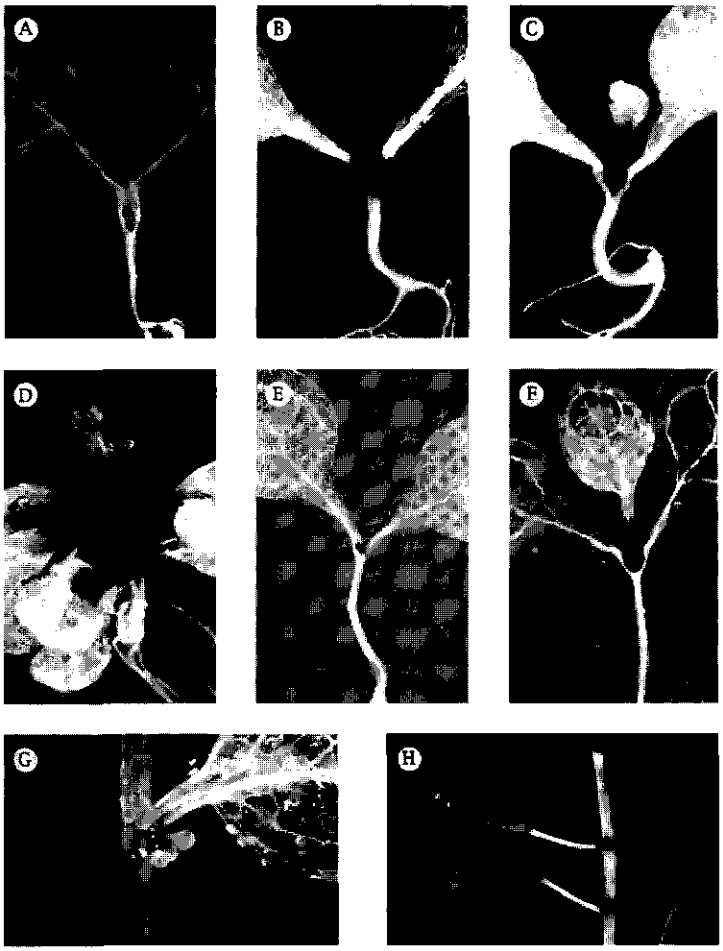


**Figure 5.2:** WET368 *GUS* expression patterns in *shoot meristemless*, *wuschel*, *primordia timing*, and *zwille* mutant backgrounds. All seedlings are 10 days after germination.

**A:** *stm-1* seedling.

**B:** *wus-1* seedling with terminated apex.

**C:** *wus-1* seedling with terminal leaf.

**D:** *pt-1* seedling.

**E:** *zll-3* seedling with terminated apex.

**F:** *zll-3* seedling with terminal leaf.

**G:** *zll-3* axillary meristem in axil of cauline leaf, mature plant.

**H:** *zll-3* inflorescence stem with pedicels and siliques, mature plant.

mutant seedlings indicates that WET368 expression is independent of meristem activity. This is in line with the onset of WET368 expression prior to the initiation of shoot meristem activity, and underlines the interpretation that WET368 represents a regional marker rather than a marker for active shoot meristematic cells.

## Discussion

### *WET368 is an early marker of embryo polarity*

The aim of our screen was to obtain molecular markers for early *Arabidopsis* embryos. The results presented in this study indicate that GUS staining in WET368 embryos was already seen at the octant stage. At this early developmental stage, WET368 marks all cells descending from the apical cell produced by the first division of the zygote, and none of the cells descending from the basal cell. Consequently, WET368 provides an early molecular marker for apical polarity in the *Arabidopsis* embryo. Few other markers are available for the early stages of *Arabidopsis* embryogenesis, the earliest being the *ATML1* gene (Lu *et al.*, 1996), which is expressed just after the first division of the zygote in the apical cell and its descendants, but not in the basal cell. Such markers can be important in evaluating regional identity and polarity in early embryo mutants defective in the establishment of apical-basal polarity, and mutants with early defects in the region encompassing the plane of the first division of the zygote. The observed gradual restriction in WET368 *GUS* expression towards a region encompassing the SAM and part of the cotyledon primordia during the transition stage identifies a region that has so far not been defined in morphological descriptions of *Arabidopsis* embryos. It also has not become apparent from phenotypes observed in extensive screens for embryo mutants. Histological sections of GUS stained embryos expressing the WET368 enhancer trap show it to be expressed in a group of cells in and around the shoot apex, that have in common their position in the apex. This implies that WET368 is not a marker for the shoot meristem itself, but rather represents a marker of cell position. The fact that WET368 expression is already apparent in the octant stage embryo in all derivatives of the apical, but not in those of the basal zygotic daughter cell, and subsequently gets restricted to a region at the embryo apex, classifies WET368 as a marker that reflects apical embryo polarity. Examples of polarity or cell-position markers expressed later during *Arabidopsis* embryo development include the apical position marker *AtLTP1* (Vroemen *et al.*, 1996), and the root tip position marker *POLARIS* (Topping and Lindsey, 1997).

Post-embryonically, *GUS* expression in WET368 remains associated with regions in which aerial meristems develop or have developed, i.e. in the seedling SAM region and at the base of petioles, and in axillary meristem regions of flowering plants. Noteworthy, GUS activity was also

seen in the axils of secondary inflorescences and pedicels. At this place, the indeterminate secondary inflorescence meristem and the determinate floral meristem, respectively, have branched from the main inflorescence stem. Consequently, GUS activity at these positions might be considered a remnant of previous secondary or floral meristem position. Whether the remaining GUS activity is the result of continued WET368 enhancer driven gene expression, or is due to high stability of the GUS protein is not known. The fact that GUS staining in the axils is somewhat stronger adaxially than abaxially would be in line with the decreasing staining intensity towards the periphery of the meristem region marked by WET368: the abaxial side of the secondary inflorescence or pedicel is further away from the original meristem center than is the adaxial side.

GUS activity in the petal / sepal abscission zone could also reflect its previous position at the base of the floral meristem region. However, the fact that many gene and enhancer trap lines (our unpublished results; Topping *et al.*, 1994), as well as promoter-*GUS* transgenes (Thoma *et al.*, 1994) display GUS activity in this zone could mean that this GUS staining is somehow artefactual. During flower and early silique development, continued GUS activity was seen in the ovule. Interestingly, the *ATML1* gene, that is expressed in the L1 layer of the vegetative SAM, inflorescence and floral meristems, and young floral organ primordia, also displays continued expression in the ovule (Lu *et al.*, 1996). This was postulated to indicate that the ovule retains some meristematic properties, thereby supporting the assumption of the possible phylogenetic origin of the ovule from the shoot (Herr, 1995; Lu *et al.*, 1996).

Taken together, WET368 expression initially precedes meristem formation, then accompanies the actual meristem, and finally remains behind after the meristem itself has moved more apically. Based on this expression pattern, it can be envisioned that WET368 expression demarcates regions of cells at positions in the plant where meristems can form. In this scenario, WET368 expression in a region would, independent of meristem activity, be a prerequisite for the acquisition of meristem identity by a subset of the cells in that region. Moreover, as a region marker that is independent of meristem activity (see below), WET368 remains expressed after meristem formation and even, in the case of floral and secondary inflorescence meristems, after the meristem itself has moved more apically. Therefore, we consider WET368 a marker of future, current and former positions of aerial meristems.

### *WET368 expression is independent of shoot meristem formation or activity*

To investigate the nature of the region marked by WET368 GUS staining in more detail, WET368 *GUS* expression was analyzed in *stm*, *wus*, *pt*, and *zll* mutants. Although no shoot meristematic cells are present in *stm-1* seedlings, continued WET368 *GUS* expression marked the shoot apical region in these seedlings. In *wus-1* and *zll-3* seedlings, the shoot meristematic cells are replaced by non-functional central cells, and the entire shoot apical region, including the non-functional central cells,

was marked by WET368 GUS staining. Finally, the WET368 marked region in *pt* mutant seedlings is enlarged compared to wild-type, as a result of the increased size of the functional shoot meristem. The implication of these observations, in combination with the observed early and regional expression pattern in wild-type, is that WET368 expression is independent of shoot meristem formation or activity, but is linked to the position of shoot meristem development. Consequently, we propose that WET368 represents a regional marker not only for embryonic, but also for post-embryonic SAM position. Since WET368 expression is both earlier and more global than that of the *STM* gene, of which the expression starts in one or two cells in the apical hemisphere of the globular embryo (Figure 5.3), WET368 expression may lead the way to a regulatory function upstream of *STM* in SAM formation.

### *WET368 expression and positional aspects of embryo development*

On the basis of its early and regional expression pattern, its persistent expression in later stages of development, and the fact that its expression precedes and is not dependent on *STM*, *WUS*, *ZLL*, and *PT* activity, we have classified WET368 as a regional marker for shoot apical meristem position. The region marked by WET368 *GUS* expression does not correspond to a region previously defined by embryo morphology or histology. An important implication of this would be that molecularly defined regions exist in the embryo, superimposed on morphologically or functionally recognizable regions. These molecularly defined regions could extend the description of the successive steps in the formation of a plant embryo. In this context, it is noteworthy that the early WET368 expression pattern in the globular embryo supports the view that SAM position is specified well before the meristem becomes histologically distinct at the torpedo stage of embryogenesis (Barton and Poethig, 1993; Endrizzi *et al.*, 1996; Kaplan, 1969).

The initially uniform and subsequently more restricted expression patterns of genes or enhancers such as *ATML1* (Lu *et al.*, 1996) and WET368 in the derivatives of the apical cell of the two-celled embryo could reinforce the notion that early acting patterning genes potentially mutate to early, not very informative embryo-lethal phenotypes (Meinke, 1991). If this were also the case for insertions into a gene or genes near the WET368 locus, it would be expected that such a gene or genes will have been missed in morphological screens for embryo pattern mutants. The identification of such genes based on expression pattern appears to underscore the advantages of gene / enhancer trapping as one of the approaches for unravelling the regulatory network governing embryo pattern formation. A tentative model for early plant embryogenesis suggests first global and then more locally specified regions, that generate the final body pattern by cellular interactions (Jürgens, 1995). How are such regions demarcated in the developing embryo? One possible mechanism that might account for the establishment of territories of differential gene expression in the embryo could involve gradual

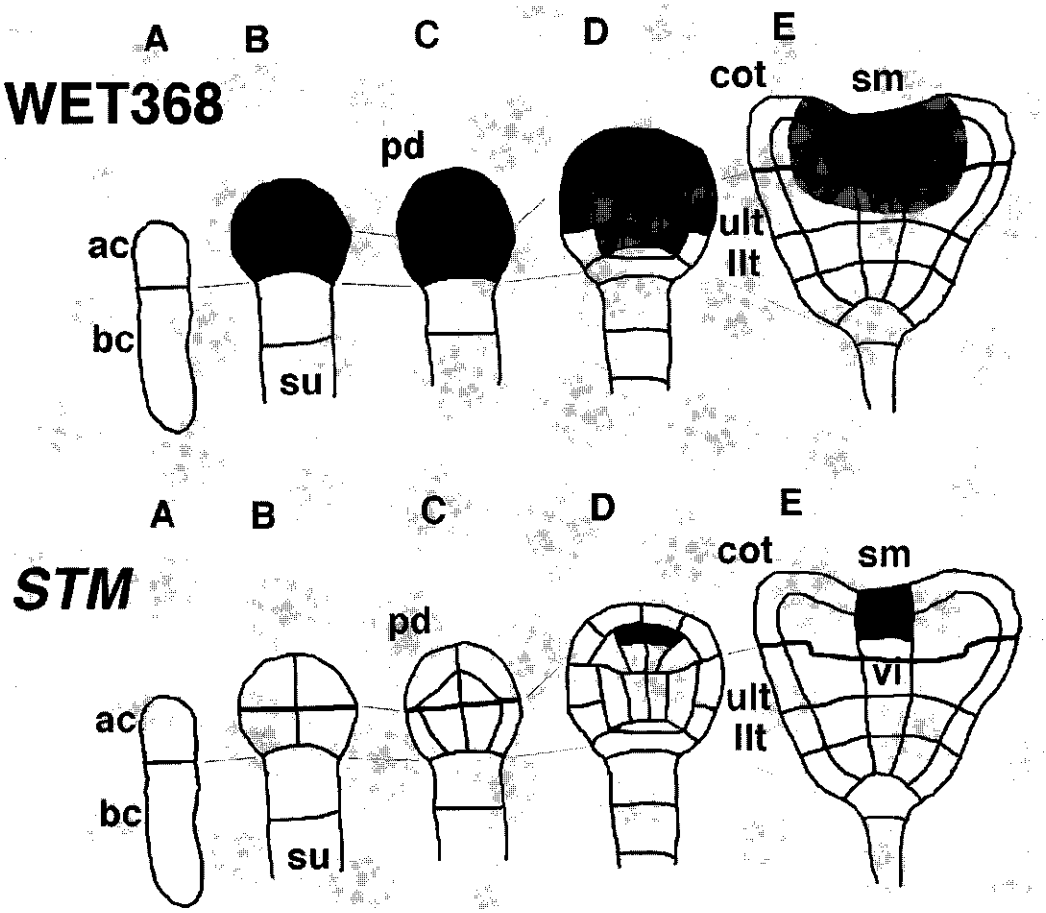


**Figure 5.3:** Schematic representation of WET368 and *STM* expression during early stages of *Arabidopsis* embryogenesis.

**A:** two-cell stage.

**B:** octant stage.

**C:** dermatogen stage.

**D:** globular stage.

**E:** heart stage.

ac, apical cell; bc, basal cell; su, suspensor; pd, protoderm; ult, upper lower tier; llt, lower lower tier; cot, cotyledon primordium; sm, shoot apical meristem; vi, vascular initials.

repression of early and uniformly expressed genes. An analogy for such a mechanism exists in *C. elegans*, where the PIE-1 protein is localized to the totipotent germline blastomere after each division in the early embryo. This localization correlates with a general repression of transcription within the germline, and thus PIE-1 may act as a localized general repressor that antagonizes the activity of a more broadly expressed set of transcriptional activators (Mello *et al.*, 1996). In plants, mechanisms exist that serve to continuously maintain a population of undifferentiated cells, as evidenced by the *clavata*, *shoot meristemless*, and *zwille* mutants. By analogy with PIE-1, WET368 could represent a broadly expressed transcriptional repressor that demarcates a region in which a population of undifferentiated cells is maintained. Noteworthy, our data show that WET368 confers *GUS* expression from the very early embryo, through the shoot meristem region, to the ovule, representing the last organ determined from the shoot meristem. Similar to the germ-line localization of PIE-1 in *C. elegans*, WET368 expression follows the region containing a population of undifferentiated cells throughout most of the diploid life cycle.

## Materials and methods

### *Plant material*

The enhancer trap transposon tagging screen performed in this study has been described in detail elsewhere (Vroemen *et al.*, 1998). Two types of starter lines (Sundaresan *et al.*, 1995), were used. *Ac* starter lines are homozygous for a single T-DNA insert containing a 35S promoter::*Ac* transposase gene (Scofield *et al.*, 1993) and a constitutive *indole-acetic acid hydrolase* gene, *IAAH*, driven by the 2' T-DNA promoter (Bancroft *et al.*, 1992). *DsE* starter lines are homozygous for a single T-DNA insert containing the *DsE* transposable element and another *IAAH* gene. Both starter lines were in the Landsberg *erecta* background.

A line heterozygous for the *shoot meristemless* (*stm-1*) mutation was obtained from Kathryn Barton (Barton and Poethig, 1993; Long *et al.*, 1996), and a homozygous *primordia timing* (*pt-1*) line (Conway and Poethig, 1997; Mordhorst *et al.*, 1998) from Igor Vizir (University of Nottingham, England). Crosses between enhancer trap line WET368 and mutant lines were performed using plants homozygous for the WET368 *DsE* element as one parental line, and plants heterozygous for *wus-1* (Laux *et al.*, 1996), or *stm*, or homozygous for *zll-3* (Endrizzi *et al.*, 1996; Moussian *et al.*, 1998) or *pt*, respectively, as the other parental line.

### *Mobilization of* DsE *elements*

*DsE* elements were mobilized by crossing plants from *Ac* starter lines *Ac*1 and *Ac*2, homozygous for the *Ac* T-DNA, to plants from *DsE* starter lines *DsE*1, *DsE*2, *DsE*3 and *DsE*6, all homozygous for the *DsE* T-DNA, in all possible pairwise combinations. Line WET368 was obtained from a cross between *Ac*2 and *DsE*3. F1 seeds from the *Ac* x *DsE* crosses were planted individually and the resulting F1 plants were allowed to self-fertilize. 1000-5000 seeds from each F1 plant were collected to establish independent F2-families.

### *NAM-Kan selection for transposants*

Lines carrying transposed *DsE* elements were selected among the F2 progeny seed by sowing on agar plates containing α-naphtalene acetamide (NAM) and kanamycin. 750-1000 (15-20 mg) F2 seeds from each F2 family were surface-sterilized by successive washes with 70% ethanol for 10 min, diluted bleach solution (containing 0.9% sodium hypo chlorite, and 0.1% Tween 20) for 10 min, and twice with sterile water. The seeds were then suspended in 5 ml of liquid MS-agar (containing 0.46% (w/v) MS salts (Duchefa; Murashige and Skoog, 1963) adjusted to pH 5.7 with KOH, 1% sucrose and 0.7% agar (Difco)), and plated onto square 12 x 12 cm selection plates containing MS-agar supplemented with 50 µg / ml kanamycin sulphate (Duchefa), and 3.5 µM NAM (Sigma). After 1-4 days at 4 °C, the plates were incubated for 4 days in a growth chamber at 25 °C with 16h light / 8h dark photoperiod. Seedlings carrying either T-DNA insert were stunted, because of the *IAAH* gene that conferred sensitivity to NAM (Karlin-Neumann *et al.*, 1991). Of the remaining seedlings, only those carrying a transposed *DsE* element survived on kanamycin, by virtue of a 1' T-DNA promoter::*NPTII* gene fusion carried within the *DsE* element. This selection scheme allowed the generation of "transposant" lines (Bellen *et al.*, 1989), carrying a stable *Ds* insertion at a location unlinked, or loosely linked, tot the *DsE* donor T-DNA. Transposant seedlings, resistant to both NAM and kanamycin, recognizable by their green cotyledons, normal size and normal root development, were transferred to 60 mm round selection plates and further incubated to verify the double resistance. After reaching the second-leaf stage, transposants were transplanted to soil and allowed to self-fertilize. Flowers and siliques, that contained immature seeds, from these F2 plants were screened for *GUS* expression. Mature seeds (the F3 generation) were harvested and stored as an enhancer trap line (Wageningen Enhancer Trap lines WET1 through WET431).

### *Histochemical localization of* GUS *expression*

For determination of *GUS* expression in seedlings, seeds from each enhancer trap line were germinated in microtiter wells containing 400 µl of sterile water. After 5 days of incubation at 25 °C in the light, one volume of two times concentrated GUS staining solution was added, to result in final concentrations of 100 mM NaPi pH 7.2, 10 mM EDTA, 0.1% Triton X-100, 100 mg/ml chloramphenicol (to inhibit bacterial growth), and 1 mg/ml X-Gluc (5-bromo-4-chloro-3-indolyl b-D-glucuronic acid). The seedlings were vacuum infiltrated with GUS staining solution for 1 hour, and the reaction was allowed to continue for up to 48 hours at 37 °C in the dark. Flowers and parts of the inflorescence were sampled from enhancer trap plants and incubated in GUS staining solution as described above. Siliques with immature seeds covering all stages of embryo development (typically 3 - 5 siliques per line) were opened longitudinally and incubated in GUS staining solution as described above. After the reaction, tissues were cleared for a minimum of 16 hours in Hoyers solution (100 g chloral-hydrate, 2.5 g Arabic gum, 15 ml glycerol, 30 ml water). GUS staining patterns were viewed with a binocular (Nikon Corp., Tokyo, Japan) and with a Nikon Optiphot-2 microscope equipped with Normarski optics. GUS staining patterns were examined in more detail using GUS staining solutions containing equal amounts of potassium ferrocyanide and potassium ferricyanide in concentrations between 1.25 mM and 5 mM. These reaction conditions are reported to minimize diffusion of the reaction intermediates and thereby improve the specificity of the localization of *GUS* expression (Jefferson *et al.*, 1987).

### *Histological sections*

After the GUS staining reaction, immature seeds were transferred to fixative (100 mM NaPi pH7.2, 4% formaldehyde, 4% DMSO). The fixative was vacuum infiltrated and the seeds were fixed for at least 3 days at 4 °C. After dehydration through an ethanol series, the seeds were infiltrated in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) according to the manufacturer's instructions. Briefly, subsequent changes of Technovit preparation solution (1 g hardener I, 2.5 ml PEG 400, 100 ml Technovit 7100) of increasing concentrations in 96% ethanol (1:3, 1:1, 3:1) were done for one hour, followed by a one hour and an overnight incubation in 100% Technovit preparation solution. Seeds were embedded in Technovit embedding solution (1 ml hardener II, 15 ml Technovit preparation solution) and polymerization was allowed to continue for one hour at 37 °C. Serial sections (3 mm thick) were cut with a Reichert-Jung microtome, transferred to microscope slides, stained with 0.01% Ruthenium Red (Sigma) for 1-10 min, and mounted in Euparal (Agar Scientific, Stansted, UK). Sections were analyzed with a Nikon Optiphot-2 using bright-field and dark-field optics.

***Southern blot analysis***

Genomic DNA from individual transposant plants was isolated according to Bouchez (1996). 1-2 µg of genomic DNA was digested with *Pst*I, separated on a 1% agarose gel and blotted onto a Nitran Plus membrane (Schleicher & Schuell, Keen, NH, USA). Blotting and hybridization were performed according to the manufacturer's recommendations. A 2.2 kb [$\alpha^{32}$P-dATP] random prime labelled *GUS* fragment, covering the entire coding sequence, was used as probe. The blot was washed for 15 min with 2 x SSC, 0.1% SDS and for 15 min with 0.1 x SSC, 0.1% SDS at 65 °C (Sambrook *et al.*, 1989) before exposure to X-ray film (Amersham, 's Hertogenbosch, the Netherlands).

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# 6

# Summarizing discussion

Sexual reproduction in seed plants starts with the fertilization of the egg cell by one of the sperm cells of the pollen grain. The resulting zygote develops through a series of characteristic stages into a multicellular seedling, in which organs and tissues are arranged in a specific spatial context, or pattern. An important area of plant developmental biology concerns the molecular mechanisms that underlie the acquisition of polarity and the establishment of the body pattern during embryogenesis. As reviewed in detail in chapter 1, the majority of the research in this area focuses on the model species *Arabidopsis thaliana*. One of the advantages of studying embryogenesis in *Arabidopsis* is provided by the highly invariant sequence of cell divisions, which allows the origin of pattern elements present in the seedling to be traced back to distinct cells or cell groups in the early embryo (Jürgens *et al.*, 1994a; Jürgens *et al.*, 1994b; Mansfield and Briarty, 1991; Scheres *et al.*, 1994). However, the rigid division pattern found in the *Arabidopsis* zygotic embryo might be misleading towards an understanding of the mechanisms underlying pattern formation. Numerous other plant species display a great plasticity in division patterns (Johri, 1984), yet develop complete body plans with all pattern elements correctly positioned. The same holds true for certain *Arabidopsis* mutants (Torres-Ruiz and Jürgens, 1994) and embryos of non-zygotic origin (Koltunow, 1993; Mordhorst *et al.*, 1998; Wu *et al.*, 1992). Therefore, the question arises what molecular clues are responsible for the correct specification and positioning of pattern elements during plant embryogenesis. A widely followed approach to identify genes involved in pattern formation has been to screen for mutants with defects in the establishment of the embryo body plan (reviewed in chapter 1). These genetic approaches have yielded numerous embryo-defective mutants (Jürgens *et al.*, 1991; Mayer *et al.*, 1991; Meinke, 1991; Scheres *et al.*, 1995). A major difficulty that has emerged during these screens concerns the recognition and interpretation of relevant phenotypes. Many embryo-lethal mutants show similar phenotypes (Feldmann, 1991; Meinke, 1991) and the assessment of the precise effects of a mutation is often hampered by the inability to establish cell- or regional identity in embryo mutants (Topping and Lindsey, 1997; Vroemen *et al.*, 1996; Yadegari *et al.*, 1994).

The subject of the research described in this thesis is the identification of genes expressed in the *Arabidopsis* embryo, and the use of such genes as molecular markers for the evaluation of the phenotypic effects of embryo mutations. The idea behind this approach is that molecular markers reflecting cell- or regional identity or polarity in the developing embryo can extend morphological observations in the interpretation of embryo mutant phenotypes. An example of a marker gene for developing *Arabidopsis* embryos is the *Arabidopsis thaliana* lipid transfer protein (*AtLTP1*) gene (Thoma *et al.*, 1994), which is the *Arabidopsis* homolog of the carrot *EP2* gene (Sterk *et al.*, 1991). In wild-type embryos, the *AtLTP1* gene appeared to be expressed in a radially restricted fashion: expression was seen initially in all protoderm cells, and not in the underlying ground and vascular tissues. Later, *AtLTP1* expression also became restricted along the apical-basal axis: expression became confined to the protodermal cells of the cotyledons and upper hypocotyl, together representing the apical part of the embryo (chapter 2). Therefore, pattern formation in the *Arabidopsis*

embryo is reflected by tissue- and position-specific *AtLTP1* expression along both the radial and the apical-basal axis. *AtLTP1* expression, measured by *in situ* mRNA hybridization and *AtLTP1-GUS* expression, was used as radial marker for the protoderm to study the phenotypic defects in the *knolle* and *keule* mutants, both reported to have defects in the establishment of the protoderm. In *knolle* mutants incomplete cell walls are formed as a result of disruption of a syntaxin gene involved in cytokinesis, and the radial organization of the embryo is not established properly (Lukowitz *et al.*, 1996). In early *knolle* embryos, inner cells cannot be distinguished from the protodermal layer. This coincided with uniform instead of protoderm-specific *AtLTP1* expression. Apparently, the establishment and stable maintenance of different cell fates along the radial axis requires the uncoupling of cytoplasmic connections and the proper separation of tissue layers. In *knolle* embryos, incomplete cell wall formation results in cytoplasmic connections between the outer and inner cell layers, such that hypothetical protoderm and inner cell fate determinants might not be segregated to the adjacent cell layers. The observation that this resulted in *AtLTP1* expression in outer and inner cells suggests that *knolle* embryos fail to specify inner cells, and that protoderm fate represents a "ground state" in embryogenesis (Bruck and Walker, 1985a), already present before the establishment of morphologically distinct outer and inner cells. This idea was originally proposed for the determination of epidermal cell fate in *Citrus* embryos on the basis of the observation that the zygote and its apical daughter cells are already coated with a cuticular wax layer, which is a morphological marker for epidermal cells (Bruck and Walker, 1985a). Later, it received support from the restriction of *ATML1* expression to the protoderm, after initial expression in the apical daughter cell of the zygote and all cells of the octant stage embryo (Lu *et al.*, 1996). Internal cells in *knolle* embryos later discontinued *AtLTP1* expression and differentiated into vascular tissue. These changes reflect the specification of internal cells with a fate different from that of the outer cells in later stage *knolle* embryos, possibly resulting from an increased distance of the innermost cells from the outer cell layer, or from more complete cell wall formation, and thus an increased separation of tissue layers. *keule* mutant embryos are, like *knolle* embryos, disturbed in cytokinesis. The protoderm layer of *keule* embryos consists of bloated and irregularly arranged cells, while the ground and vascular tissues look normal (Assaad *et al.*, 1996). Normal protoderm-specific *AtLTP1* expression in *keule* embryos suggests that the establishment of the radial pattern is unaffected by the *keule* mutation.

*AtLTP1* expression was used as marker for the apical part of the embryo to investigate effects of the *gnom* mutation (Mayer *et al.*, 1993) on apical-basal embryo polarity. Mutations in the *GNOM* gene affect the normally asymmetric division of the zygote, and the resulting enlarged apical daughter cell gives rise to an abnormal embryo proper. *AtLTP1* expression appeared variable and occasionally completely reversed along the apical-basal embryo axis. This finding was later substantiated by variable expression of the *POLARIS* gene, which is normally confined to the root pole of the embryo (Topping and Lindsey, 1997). Since *GNOM* is a zygotically required gene, the observed variability in apical-basal marker gene expression in *gnom* embryos suggests that apical-basal embryo polarity is

not yet established before fertilization, although the *Arabidopsis* egg cell is morphologically polar. In fact, in other flowering plants the egg cell appears apolar, or apical-basal polarity is reversed upon fertilization. These observations collectively suggest that apical-basal embryo polarity is established after fertilization, and that the apical-basal axis of polarity may only be fixed after the first division of the zygote.

The availability of other embryo marker genes, for example with expression patterns complementary to the *AtLTP1* expression pattern, would be useful to substantiate the results described in chapter 2. Because such markers were scarce, we performed an enhancer and gene trap insertional mutagenesis screen to identify *Arabidopsis* lines with *GUS* expression in embryos (chapter 3). The enhancer and gene trap system exploits two types of transposable *Ds* elements carrying a *GUS* reporter gene that can respond to *cis*-acting transcriptional signals at the site of integration. The enhancer trap element contains a minimal promoter, that only confers *GUS* expression if activated by a neighboring chromosomal enhancer, while the gene trap element carries a promoterless *GUS* gene whose expression is dependent on transcription from the tagged chromosomal gene. Over 800 enhancer and gene trap lines were generated using a novel selection scheme that only recovers lines with stable *Ds* insertions at chromosomal locations unlinked to the *Ds* donor-T-DNA. This selection has proven to yield stable lines, in which the *Ds* element does not re-transpose. Moreover, it rules out "background" GUS staining that is caused by T-DNA located enhancers, and is not related to transcriptional signals at the integration site. Such "background" staining has proven a serious problem in enhancer trap screens that do not include selection against the original transposon-bearing T-DNA (Aarts, 1996; Klimyuk *et al.*, 1995). 39 lines (27 enhancer and 12 gene traps) were found to exhibit GUS staining in embryos, including lines with *GUS* expression patterns that are confined to specific cell-types, tissues, organs, or regions of the developing embryo. The strategy of enhancer and gene trapping has thus proven successful towards the generation of molecular markers for the developing *Arabidopsis* embryo.

Chapter 4 outlines the spectrum of *GUS* expression patterns observed among all 39 lines with GUS staining in the embryo. Despite the wide variety of staining patterns, some general notions came out after classifying lines according to their *GUS* expression pattern. For example, in all but one line in which GUS staining was observed before, or at, the globular stage, it was uniform in the embryo proper. A prominent part of these early *GUS* expressing lines showed a gradual restriction of *GUS* expression during later stages of embryogenesis, as is described in detail for line WET368 in chapter 5. A tentative model for early plant embryogenesis suggests the demarcation of first global and then more locally specified regions, that generate the final body pattern by cellular interactions (Jürgens, 1995). The early uniform and subsequently more restricted expression patterns observed during our screen seem to fit with the predictions made in this model for expression of genes involved in embryo patterning. The expression of early and uniformly expressed genes could gradually be repressed in certain regions, thereby giving rise to global, partly overlapping territories of gene expression. The

superimposition of different gene expression patterns could subsequently allow the demarcation of more locally specified regions, each defined by the combined expression levels of a characteristic spectrum of genes. An analogy for the demarcation of locally specified regions through gradual repression of early and uniformly expressed genes is found in *C. elegans*, where repeated localization of the PIE-1 protein to the totipotent germline blastomere correlates with a general repression of transcription within the germline (Mello *et al.*, 1996).

If patterning genes also display early uniform and subsequently more restricted expression patterns, they could, as a result of an early and uniform requirement of their gene products, potentially mutate to early, not very informative embryo-lethal phenotypes (Meinke, 1991). Genes for which this is indeed the case may be missed in phenotypic screens for embryo mutants with pattern defects. The identification of such genes based on expression pattern appears to underscore the advantages of gene and enhancer trapping as one of the approaches for unravelling the regulatory network governing embryo pattern formation.

Line WET368, identified during the enhancer trap screen, exhibits *GUS* expression as early as the octant stage (chapter 5). Expression is initially uniform in all cells descending from the apical daughter cell of the zygote, and later becomes restricted to a previously unidentified region encompassing the SAM and part of the cotyledon primordia. Therefore, WET368 exemplifies an early uniform, and subsequently more restricted, expression pattern that would support the above described model postulating that locally specified regions in the developing embryo are preceded by earlier more global regions. WET368 expression is not limited to embryogenesis, since after germination it remains associated with all aerial regions were meristem formation will occur or has occurred. Intriguingly, WET368 expression initially precedes meristem formation, then accompanies the actual meristem, and finally remains behind after the meristem itself has moved more apically. Based on this expression pattern, it can be envisioned that WET368 expression demarcates regions of cells at positions in the plant where meristems can form. In this scenario, WET368 expression in a region would, independent of meristem activity, be a prerequisite for the acquisition of meristem identity by a subset of the cells in that region. Moreover, as a region marker that is independent of meristem activity (see below), WET368 remains expressed after meristem formation and even, in the case of floral and secondary inflorescence meristems, after the meristem itself has moved more apically.

The early embryonic expression pattern of WET368 identifies it as a molecular marker for early apical embryo polarity. Its subsequent gradual restriction to a region consisting of cells that have in common their position in or close to the SAM, but are not all part of the same cell lineage, classifies WET368 as a regional marker. Because the region marked by WET368 expression does not correspond to a region previously identified by morphology, histology, or function, molecularly defined regions can apparently extend existing descriptions of plant embryo development. WET368 continues to be expressed in the SAM region of *shoot meristemless*, *zwille*, *wuschel*, and *primordia timing* mutant seedlings, which are all defective in SAM size or function (Barton and Poethig, 1993;

Conway and Poethig, 1997; Endrizzi *et al.*, 1996; Laux *et al.*, 1996; Mordhorst *et al.*, 1998; Moussian *et al.*, 1998). This observation indicates that WET368 expression is not dependent on SAM formation or activity, and is consistent with the fact that WET368 expression precedes *STM* and *ZLL* expression and morphologically visible SAM formation (Long *et al.*, 1996; Moussian *et al.*, 1998). Instead, WET368 expression is linked to the normal position of SAM development, thereby supporting the classification of WET368 as a regional marker. At this stage, it is difficult to predict the molecular nature of the gene product encoded by a gene of which the expression is mimicked by WET368. In the above described scenario of gradual repression of gene expression, WET368 could represent a transcriptional repressor. However, scenarios in which WET368 represents part of a signalling pathway that functions in regional specification by activating gene expression, or by a combination of activating and repressing mechanisms, can currently not be ruled out. As exemplified by the spectrum of genes, such as *CLV*, *STM*, and *ZLL* , that are involved in shoot meristem specification or maintenance, the components of such pathways can vary widely in molecular identity (see chapter 5 and Appendix A).

We identified only one line in which *GUS* expression was limited to embryogenesis. In this line, WET393, *GUS* expression initially marks the entire suspensor, and subsequently becomes restricted to the most basal suspensor cells. Therefore, WET393 and WET368 represent complementary markers for basal and apical polarity, respectively, in the developing embryo. The fact that only one line with embryo-specific *GUS* expression was identified during our screen suggests that the vast majority of genes involved in embryo development is also involved in post-embryonic developmental programs. Indeed, expression of many recently identified genes with (putative) regulatory functions during *Arabidopsis* embryogenesis is not to restricted to embryogenesis (Aida *et al.*, 1997; Busch *et al.*, 1996; Clark *et al.*, 1997; Di Laurenzio *et al.*, 1996; Hardtke and Berleth, 1998; Long *et al.*, 1996; Lu *et al.*, 1996; Lukowitz *et al.*, 1996; Moussian *et al.*, 1998; Shevell *et al.*, 1994). Therefore, any gene cloning scheme that involves differential or subtractive hybridization comparing embryo cDNA with cDNA from non-embryonic tissues will likely eliminate a substantial proportion of genes that are instructive in embryo development. On the other hand, if highly embryo specific processes underlie the acquisition of embryogenic potential and early embryo development, differential screening or subtraction approaches should allow the discrimination of the corresponding genes from genes involved in processes which are required for, but not unique to embryo development, such as for example shoot meristem maintenance. The carrot *SERK* gene (Schmidt *et al.*, 1997), expressed during the few first cell divisions in the developing embryo and turned off again at the globular embryo stage, is one of the very few genes described so far that appears to be expressed only in embryos and not in meristems or other parts of the adult plant. Enhancer and gene trapping offer the advantage of allowing selection of lines not only on the basis of *GUS* expression in the embryo, but also based on its precise timing and spatial distribution in embryonic and non-embryonic tissues. The use of the same regulatory genes in embryonic and post-embryonic

developmental programs is not only found in plants, but has long been known in animal systems (Ruohola-Baker *et al.*, 1994), and seems to underscore the importance of the latter selection criterion.

Molecular characterization of genomic regions tagged by enhancer and gene trap elements is necessary to identify the actual genes whose expression pattern is reflected by the observed *GUS* staining patterns, and thus to demonstrate the suitability of enhancer and gene traps for the cloning of genes identified by expression pattern. For gene trap lines this is relatively straightforward, since *GUS* expression can only occur if the *DsG* element has inserted within the transcribed region of the corresponding gene, as has been shown for the *PROLIFERA* gene (Springer *et al.*, 1995). Analysis of genomic DNA sequences flanking the *DsG* transposon in two of our gene trap lines revealed putative open reading frames in both, and one of these appeared to represent a novel expansin gene. By contrast, *GUS* expression from an enhancer trap does not require *DsE* insertion within the open reading frame of a transcribed gene, and among four enhancer trap lines, only one displayed a putative open reading frame in the genomic DNA directly flanking the insertion. Further molecular analysis should reveal whether the observed *GUS* expression patterns in enhancer trap lines all mimic the expression of actually transcribed genes located close to the *DsE* insertion site.

## Epilogue

The research described in this thesis has given ample support for the value of molecular markers for the recognition and interpretation of mutant phenotypes, relevant to the acquisition of polarity and the establishment of the body pattern during *Arabidopsis* embryogenesis. The employed enhancer and gene trap mutagenesis system has proven successful towards the isolation of *GUS* markers for distinct cell- or tissue-types and regions in the developing embryo. These markers can not only be used for the phenotypic analysis of embryo mutants, but can also refine the existing descriptions of plant embryogenesis by demarcating novel regions that have not been identified previously by morphology, histology or function. Besides generating markers, molecular analysis has shown that enhancer and gene traps also allow the isolation of genes identified on the basis of their expression pattern. In both ways, the established collection of enhancer and gene trap lines may contribute to a more comprehensive understanding of the molecular events underlying plant embryogenesis.

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# Moleculaire merkers in *Arabidopsis* embryo's: samenvatting

Bij hogere planten begint de geslachtelijke voortplanting als stuifmeelkorrels afkomstig uit de meeldraden terechtkomen op de stempel van een bloem. Een van de twee zaadcellen uit een stuifmeelkorrel bevrucht daarna een eicel in de stamper, waardoor een onrijp zaadje met daarin een bevruchte eicel, of zygote ontstaat. Tijdens het proces dat zygotische embryogenese genoemd wordt, deelt de zygote vele malen zodat uiteindelijk na de zaadkieming een veelcellig kiemplantje ontstaat. Zygotische embryo's ontwikkelen zich via een reeks ontwikkelingsstadia, die aangeduid worden met de vorm van het embryo. In twee-zaadlobbigen zijn dit het globulaire, hart, torpedo en gebogen-cotyl stadium. Tijdens de embryo ontwikkeling worden alle verschillende organen en weefsels waaruit een zaailing is opgebouwd aangelegd en op de juiste manier gerangschikt. Dit proces heet patroonvorming. Het patroon bevat langs de lengte-, of apicaal-basale as, van boven naar beneden gezien de volgende elementen: het scheutmeristeem, de cotylen (kiemblaadjes of zaadlobben), de hypocotyl (embryo-stengel) en de wortel met het wortelmutsje en het wortelmeristeem. Loodrecht daarop is langs de radiale as een tweede patroon te onderscheiden, dat van buiten naar binnen gezien, bestaat uit een aantal elkaar omringende weefsels: de epidermis (opperhuid), het grondweefsel of cortex, en de vaatbundel. In de modelplant *Arabidopsis* (zandraket) is het verloop van de celdelingen tijdens de zygotische embryogenese heel constant, waardoor precies bekend is van welke cellen in het jonge embryo de verschillende zaailing-organen en weefsels afstammen. Veel minder is er bekend over de processen die de ontwikkeling van het embryo sturen en ervoor zorgen dat alle cellen uiteindelijk de juiste identiteit krijgen en de verschillende organen en weefsels op de juiste plaats aangelegd worden. Daarom proberen tegenwoordig veel ontwikkelingsbiologen de moleculaire mechanismen die hieraan ten grondslag liggen te ontrafelen. Hoofdstuk 1 van dit proefschrift geeft een uitgebreid overzicht van de huidige stand van zaken binnen dit onderzoeksveld.

Een veel gevolgde methode om genen te vinden die betrokken zijn bij de patroonvorming bestaat uit het willekeurig aanbrengen van mutaties in het erfelijk materiaal van planten, om vervolgens te zoeken naar mutanten die afwijkingen vertonen in de aanleg van het patroon tijdens de embryogenese. Zulke genetische benaderingen hebben talrijke mutanten opgeleverd met fenotypisch afwijkende embryo's. Het blijkt echter een groot probleem om embryo fenotypes die relevant zijn voor de patroonvorming te herkennen en correct te interpreteren. De fenotypes van veel embryo-lethale mutaties lijken sterk op elkaar, waardoor het vaak moeilijk is echte patroonmutanten te onderscheiden van mutanten die een afwijking hebben in een meer algemeen proces, zoals

bijvoorbeeld het celmetabolisme. Bovendien wordt het vaststellen van de precieze gevolgen van een mutatie vaak bemoeilijkt doordat het niet mogelijk is de identiteit van cellen en regio's in het mutante embryo te bepalen. Een strategie om deze problemen gedeeltelijk te omzeilen is de bestudering van het expressiepatroon van goed gedefinieerde moleculaire merkergenen in mutante embryo's. Zulke merkergenen komen in normale "wild-type" embryo's tot expressie in een specifiek celtype of in een bepaalde regio van cellen en kunnen daardoor dienen als moleculaire merkers die de identiteit van cellen en celregio's, of de polariteit in het ontwikkelende embryo aangeven. Naast bestudering van de morfologie, of vorm, van een mutant embryo worden door merkergenen dus extra criteria geboden om de precieze gevolgen van een embryo-mutatie vast te stellen.

Hoofdstuk 2 van dit proefschrift beschrijft de analyse van drie embryo-mutanten met behulp van het "*Arabidopsis thaliana* lipide transport eiwit" (*AtLTP1*) gen als merker. In wild-type embryo's komt het *AtLTP1* gen aanvankelijk tot expressie in alle epidermiscellen en later tijdens de embryo ontwikkeling alleen in de epidermiscellen van de cotylen en het bovenste gedeelte van de hypocotyl, dus in de epidermiscellen van het bovenste of "apicale" deel van het embryo. *AtLTP1* expressie werd gebruikt als weefsel-laag specifieke merker voor de epidermis bij de bestudering van de fenotypes van de *knolle* en de *keule* mutanten. Van deze mutanten was beschreven dat ze afwijkingen vertoonden in de aanleg van de epidermis. In *knolle* embryo's is geen morfologisch onderscheid zichtbaar tussen epidermis en onderliggende cellen en de vorming van celwanden tussen de cellen in het embryo is niet compleet, waardoor cellen die normaal van elkaar gescheiden zijn met elkaar in verbinding staan. In *knolle* embryo's blijkt het *AtLTP1* gen aanvankelijk in alle cellen tot expressie te komen in plaats van alleen in de buitenste cellaag, hetgeen suggereert dat deze mutant niet primair gestoord is in het aanleggen van de epidermis, maar van de onderliggende cellen. Blijkbaar is een complete celwand, en dus een volledige scheiding van de verschillende cellagen in het embryo een vereiste voor de aanleg van verschillende celtypen langs de radiale embryo-as. In *keule* embryo's is wel een afzonderlijke buitenste cellaag te onderscheiden, maar de cellen ervan zien er abnormaal gezwollen uit. De vraag rees dus of de buitenste cellaag in *keule* embryo's wel epidermis identiteit heeft. *AtLTP1* expressie in *keule* embryo's is, net zoals in wild-type embryo's, alleen te zien in de buitenste cellaag, waaruit geconcludeerd kan worden dat *keule* embryo's wel een normale epidermis aanleggen, alleen zien de epidermiscellen er anders uit dan in wild-type embryo's. Omdat het *AtLTP1* gen tijdens de latere embryo ontwikkeling alleen tot expressie komt in de epidermiscellen in het apicale deel van het embryo, werd *AtLTP1* expressie gebruikt als apicale merker bij de bestudering van het effect van de *gnom* mutatie op de apicaal-basale polariteit van het embryo. Tijdens de ontwikkeling van *gnom* embryo's vindt geen normale aanleg plaats van de organen langs de apicaal-basale, of lengteas. *AtLTP1* expressie in *gnom* embryo's blijkt lang niet altijd beperkt tot het apicale deel van het embryo, en was soms zelfs alleen zichtbaar in het basale deel. Dit suggereert dat de apicaal-basale polariteit van *gnom* embryo's variabel en soms zelfs helemaal omgedraaid is. Omdat de *gnom* mutatie pas effect heeft na de bevruchting geeft dit tevens aan dat de apicaal-basale polariteit van het *Arabidopsis* embryo nog niet volledig vastligt voor de bevruchting.

Helaas waren er, vooral voor vroege stadia in de embryo ontwikkeling, weinig andere merkergenen beschikbaar. Dit tekort aan geschikte moleculaire merkers staat de herkenning en interpretatie van embryo fenotypes die informatief kunnen zijn voor het proces van patroonvorming danig in de weg. Daarom is tijdens het in dit proefschrift beschreven onderzoek gezocht naar meer genen die tot expressie komen in het *Arabidopsis* embryo. Met behulp van de techniek van "gene / enhancer trap insertie mutagenese" is gezocht naar *Arabidopsis* plantenlijnen die het *GUS* merkergen in embryo's tot expressie brengen. Cellen waarin dit *GUS* merkergen tot expressie komt kunnen door middel van een blauwkleuringsreactie zichtbaar gemaakt worden. Het gene / enhancer trap systeem en de manier waarop het door ons gebuikt is, is uitvoerig beschreven in hoofdstuk 3 van dit proefschrift. Het systeem maakt gebruik van twee verschillende typen transposons (stukken DNA die zich via excisie en insertie van de ene plaats binnen het celkern-DNA van de plant naar de andere kunnen verplaatsen): een gene trap transposon en een enhancer trap transposon. Deze transposons bevatten een *GUS* merkergen dat in specifieke cellen tot expressie kan komen, onder invloed van in die cellen aanwezige regulerende signalen. Zo zullen in bijvoorbeeld scheutmeristeemcellen signalen aanwezig zijn die genen met een functie in het meristeem tot expressie laten komen. Vangt het *GUS* merkergen in een bepaalde plantenlijn zo'n signaal op dan kan het in het scheutmeristeem tot expressie komen, en in de kleurreactie een blauw scheutmeristeem opleveren. Met behulp van dit systeem is een uitgebreide set van *Arabidopsis* plantenlijnen gevonden, die elk het *GUS* merkergen op een specifieke plaats in het embryo tot expressie brengen. Deze set plantenlijnen levert een collectie moleculaire merkers op die gebruikt kan worden om in mutante embryo's cel- of regio-identiteit, of polariteit vast te stellen, op een vergelijkbare manier als eerder is gedaan met het *AtLTP1* gen. Ook kan met behulp van moleculair biologische technieken het plantengen uit het celkern-DNA geïsoleerd worden dat in de buurt van de transposon-insertie ligt, en op dezelfde manier tot expressie komt als het *GUS* reportergen.

Hoofdstuk 4 van dit proefschrift geeft een overzicht over het spectrum aan *GUS* expressiepatronen dat gevonden is in embryo's na het doorzoeken van 431 enhancer trap en 373 gene trap plantenlijnen. Vier lijnen waarin het *GUS* merkergen heel vroeg of op een heel precieze plaats tijdens de embryo ontwikkeling tot expressie komt worden meer gedetailleerd beschreven. Verder worden elektronische databanken gepresenteerd die ontwikkeld zijn om gegevens van alle verschillende gene en enhancer trap plantenlijnen op te slaan en eenvoudig te kunnen doorzoeken. Van vier enhancer trap en twee gene trap lijnen is het celkern-DNA waarin het transposon zich bevindt geanalyseerd. In één geval werd een gen gevonden waarvan de functie bekend is, en in twee andere mogelijk een onbekend gen. Aan het einde van hoofdstuk 4 wordt de efficiëntie van het gene / enhancer trap systeem voor het isoleren van genen die een rol spelen bij de embryo ontwikkeling besproken. Hierbij lijkt voornamelijk de mogelijkheid om genen op te sporen aan de hand van hun expressiepatroon in het embryo, zichtbaar gemaakt door middel van de expressie van het *GUS* merkergen, een voordeel van het gene / enhancer trap systeem.

In hoofdstuk 5 van dit proefschrift wordt een van de gevonden enhancer trap lijnen, genaamd

WET368, besproken. In de WET368 plantenlijn wordt al in het 8-cellig embryo expressie van het *GUS* merkergen waargenomen. Wat later tijdens de ontwikkeling van het embryo wordt alleen nog expressie waargenomen in een niet eerder beschreven regio van cellen, die het scheutmeristeem en een deel van de kiemblaadjes omvat. Na de zaadkieming worden alle gebieden in de plant waar scheut- of bloemmeristemen aanwezig zijn of waren gekenmerkt door *GUS* expressie. Het zou kunnen dat door middel van WET368 expressie regio's in de plant aangelegd worden waarbinnen bepaalde cellen zich tot meristeemcellen kunnen ontwikkelen. De analyse van WET368 *GUS* expressie in een serie mutanten met afwijkingen in de grootte of functie van het scheutmeristeem laat zien hoe merkergen-expressie de morfologische beschrijving van mutante fenotypes kan aanvullen.

Aan het eind van dit proefschrift, in hoofdstuk 6, worden de belangrijkste resultaten van het beschreven onderzoek samengevat en bediscussieerd.

# Nawoord

Hoewel dit nawoord voor velen waarschijnlijk het meest begrijpelijke deel van dit proefschrift is, was het misschien wel het moeilijkste deel om te schrijven. Is het bij het schrijven van een wetenschappelijk stuk tekst al lastig de juiste afbakening te vinden, bij het schrijven van een dankwoord is dit welhaast onmogelijk. Vandaar dat ik op deze plaats wil beginnen met een ieder die op wat voor manier dan ook een bijdrage heeft geleverd aan de totstandkoming van dit proefschrift te bedanken. Zonder degenen die er na het lezen van dit nawoord achter komen dat ze niet genoemd zijn tekort te willen doen, wil ik een aantal mensen bij naam bedanken.

Allereerst mijn begeleider en groepsleider Sacco de Vries. Sacco, het is een eer je eerste AIO te zijn, die jou promotor mag noemen in plaats van co-promotor! Onze discussies, je brede inzicht en je vele contacten die ertoe bijdroegen dat mijn werkterrein zich verder dan Wageningen uitstrekte, hebben mij de afgelopen jaren enorm gemotiveerd voor het doen van onderzoek. Daarnaast is je interesse in zaken die verder gingen dan onderzoek voor mij van grote waarde geweest. Ab van Kammen, mijn andere en lange tijd enige promotor. Leek je in het begin van mijn promotie iemand die ergens op de achtergrond de grote lijnen bewaakte, tijdens het schrijven van publicaties en dit proefschrift bleek dat je bijdragen en onze gesprekken voor mij zeer nuttig en fascinerend waren. Als ik dacht dat een publicatie echt af was, bleken er na het ontcijferen van je opmerkingen altijd weer wat nieuwe gezichtspunten te zijn.

De collega's van de embryo-groep, zoals zij heet sinds ik aan *Arabidopsis* ging werken, hebben ieder een eigen bijdrage geleverd aan mijn promotie-onderzoek en bovenal aan een goede werksfeer. Dank aan mijn collega-AIOs Marcel, Arjon en Paul, de laatste eerst nog als student, en alle anderen die voor korte of lange tijd het embryo-lab bevolkten, met name Marijke, Ed, Andreas, Valérie, Theo, Ellen, Kim, Flavia en Arina. Een speciaal woord van dank gaat uit naar de doctoraal-studenten die als afstudeeronderzoek een bijdrage geleverd hebben aan het in dit proefschrift beschreven onderzoek. Eddy van de Honing, Sandra Langeveld, Han Gerrits, Nicole Aarts en Paul in der Rieden, behalve jullie resultaten herinner ik mij vooral dat het gewoon erg leuk was met jullie samen te werken. Het doet mij goed jullie nog in het onderzoeksveld te zien werken. Verder bedank ik de andere "Molbi-mensen" voor hun praktische en soms puur sociale bijdragen, die de tijd op Moleculaire Biologie zo leuk maakten.

In de categorie "buiten de vakgroep" ben ik dank verschuldigd aan Gerd Jürgens en Ulrike Mayer voor de jarenlange prettige samenwerking, en aan Rob Martienssen, Venkatesan Sundaresan, Patricia Springer en Ueli Grossniklaus voor een mooie tijd op Cold Spring Harbor en het bieden van

de mogelijkheid te participeren in het gene / enhancer trap project. Voorts ben ik Bertrand Dubreucq en Harald Wolkenfelt erkentelijk voor hun onmisbare en leerzame hulp bij het screenen, en Maarten Koornneef voor zijn waardevolle adviezen en het op eigen initiatief markeren van mogelijk mutante planten in de *Arabidopsis* kas.

Ook mijn nawoord wordt afgesloten met "het thuisfront", omdat dit wellicht de belangrijkste, maar in elk geval ook de minst concrete steunpilaar gevormd heeft tijdens het promotie-onderzoek. Op deze plaats wil ik dan ook mijn vrienden bedanken voor hun steun, interesse, en de mooie momenten in de afgelopen jaren; mijn lieve ouders Paula en Bert, en mijn broers Jeroen en Simon, omdat ze er altijd voor me zijn en zullen zijn; Loes en Henk, omdat zij de laatste jaren zo merkbaar dichtbij waren. En tenslotte mijn liefste Annemarie, jouw steun tijdens mijn promotie is niet met woorden te beschrijven; ik ben blij dat een heel klein stukje ervan voor iedereen in een oogopslag zichtbaar is!

# *Curriculum vitae*

Casper Willem Vroemen werd geboren op 11 mei 1969 te Delft. In 1987 behaalde hij het VWO diploma aan het Mencia de Mendoza Lyceum te Breda en begon hij met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. In oktober 1988 slaagde hij met lof voor het propaedeutisch examen en zette hij de studie voort in de chemisch-biologische oriëntatie. In november 1992 werd het ingenieursdiploma behaald, met afstudeervak Moleculaire Genetica van Industriële Micro-organismen aan de Landbouwuniversiteit (Dr. J. Visser en Dr. L. de Graaff), en stages Levensmiddelenchemie bij Gist-brocades, Seclin, Frankrijk (Dr. C. Grassin) en Moleculaire Genetica bij het Department of Nematology van de University of California at Riverside, Verenigde Staten van Amerika (Dr. D. Bird). Tijdens laatstgenoemde stage maakte hij deel uit van de atletiekploeg van de universiteit. Van januari 1993 tot juli 1997 was hij als assistent in opleiding (AIO) verbonden aan het laboratorium voor Moleculaire Biologie van de Landbouwuniversiteit Wageningen, waar hij onder leiding van Prof. Dr. S.C. de Vries en Prof. Dr. A. van Kammen het in dit proefschrift beschreven promotie-onderzoek uitvoerde. Als onderdeel van dit onderzoek werd in de periode maart tot en met juni 1994 een werkbezoek gebracht aan de laboratoria van Dr. R. Martienssen en Dr. V. Sundaresan, op het Cold Spring Harbor Laboratory in de staat New York van de Verenigde Staten van Amerika. Het promotie-onderzoek combineerde hij door middel van een deeltijdaanstelling met het bedrijven van atletiek op nationaal en internationaal niveau, waarbij midden-lange afstand lopen en 3 kilometer steeple chase zijn voornaamste disciplines waren. Vanaf januari 1998 is hij werkzaam als onderzoeksmedewerker (post-doc) op het laboratorium voor Moleculaire Biologie van de Landbouwuniversiteit Wageningen, in dienst van de Nederlandse organisatie voor Wetenschappelijk Onderzoek (NWO).

# List of publications

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**Vroemen, C.W., Aarts, N., In der Rieden, P.M.J., Van Kammen, A. and De Vries, S.C.** (1998) Identification of genes expressed during *Arabidopsis thaliana* embryogenesis using enhancer trap and gene trap *Ds*-transposons. In *Cellular integration of signalling pathways in plant development* (Lo Schiavo, F., Last, R. L., Morelli, G. and Raikhel, N. V., eds.). Berlin Heidelberg: Springer-Verlag, pp. 207-232.

**Vroemen, C.W. and De Vries, S.C.** (1998) Flowering Plant Embryogenesis. In *Development-Genetics, epigenetics and environmental regulation* (Russo, E., Cove, D., Edgar, L., Jaenisch, R. and Salamini, F., eds.). Heidelberg: Springer-Verlag, in press.

# Appendix A: *Arabidopsis* mutants discussed in this thesis

**(in order of appearance)**

| Mutant | embryo phenotype | proposed embryonic function of mutated gene | gene encodes | reference |
|---|---|---|---|---|
| *knolle* (*kn*) | incomplete cell walls; radial tissue organization defects | cytokinesis | syntaxin | Lukowitz *et al.*, 1996 |
| *keule* (*keu*) | incomplete cell walls; radial tissue organization defects | cytokinesis | | Assaad *et al.*, 1996 |
| *gnom/emb30* (*gn*) | variable apical-basal polarity | asymmetric cell division | guanine nucleotide exchange factor ? | Shevell *et al.*, 1994 Busch *et al.*. 1996 |
| *short integument* (*sin1*) | defects in apical-basal (and radial) axis; no cotyledons | apical-basal polarization | | Ray *et al.*, 1996 |
| *abnormal suspensor* (*sus1, sus2, sus3*) | embryo formation from suspensor; arrest of primary embryo | suppression of embryo-fate in suspensor | spliceosome assembly factor (*SUS2*) | Schwartz *et al.*,1994 |
| *twin* (*twn1, twn2*) | embryo formation from suspensor; *twn2*: arrest of primary embryo | suppression of embryo-fate in suspensor | valyl-tRNA synthetase valRS (*TWN2*) | Vernon and Meinke, 1994; Zhang and Somerville, 1997 |
| *raspberry* (*1,2*) | embryo formation from suspensor; arrest of primary embryo | embryo morphogenesis | | Yadegari *et al.*, 1994 |
| *gurke/emb22* (*gk*) | no / reduced SAM and cotyledons | specification of upper tier | | Torres-Ruiz *et al.*, 1996 |
| *monopteros* (*mp*) | no root and hypocotyl; non-continuous vascular strands | axialization | transcription factor | Hardtke and Berleth, 1998 |
| *shortroot* (*shr*) | no endodermis | specification of endodermal cell fate | | Scheres *et al.*, 1995 |
| *scarecrow* (*scr*) | no separate cortex and endodermis | periclinal division in ground tissue | bZIP-transcription factor | Di Laurenzio *et al.*, 1996 |
| *fass/tonneau* (*ton*) | irregular early cell divisions; increased number of cell layers along radial axis | directional cell expansion; cell division plane allignment | | Torres-Ruiz and Jürgens, 1994; Traas *et al.*, 1995 |
| *wooden leg* (*wol*) | no phloem | regulation of cell division in vascular tissue | | Scheres *et al.*, 1995 |
| *shoot meristemless* (*stm*) | no shoot meristem | SAM specification and maintenance | homeodomain transcription factor | Long *et al.*, 1996 |
| *aintegumenta* (*ant*) | | (cotyledon-) primordia initiation | AP2-domain transcription factor | Elliot *et al.*, 1996 |
| *clavata* (*clv1, clv2, clv3*) | enlarged SAM | restriction of SAM size | leucin-rich repeat receptor kinase (*CLV1*); leucin-rich repeat transmembrane protein (*CLV2*); small protein (*CLV3*) | Clark *et al.*, 1997; Jeong *et al.*, 1998; Fletcher *et al.*, 1998 |
| *altered meristem programming/ primordia timing* (*amp1/pt*) | supernumerary structures; enlarged SAM (pleiotropic) | | | Conway and Poethig, 1997; Mordhorst *et al.*, 1998 |
| *wuschel* (*wus*) | no functional shoot meristem | specification of cell identity in SAM center | | Laux *et al.*, 1996 |

| | | | | |
|---|---|---|---|---|
| *zwille/pinhead* (*zll/pnh*) | no functional shoot meristem | specification of cell identity in SAM center | protein of unknown identity | Moussian *et al.*, 1998; McConnell and Barton, 1995 |
| *cup-shaped cotyledon* (*cuc1, cuc2*) | fused cotyledons; no SAM ( *cuc1 cuc2* double mutant) | organ separation | putative transcriptional activator (*CUC2*) | Aida *et al.*, 1997 |
| *hobbit* (*hbt*) | misspecification of hypophyseal cell; no RM activity | specification of hypophyseal cell or RM | | Willemsen *et al.*, 1998 |
| *bombadil* (*bbl*) | misspecification of hypophyseal cell; no RM activity | specification of hypophyseal cell or RM | | Scheres *et al.*, 1996 |
| *gremlin* | misspecification of hypophyseal cell; no or limited RM activity | specification of hypophyseal cell or RM | | Scheres *et al.*, 1996 |
| *orc* | misspecification of hypophyseal cell; no or limited RM activity | specification of hypophyseal cell or RM | | Scheres *et al.*, 1996 |
| *fertilization independent endosperm/seed* (*fie/fis*) | endosperm / seed development without fertilization | linkeage of endosperm / seed development to fertilization | transcription factor (*FIS2*) | Ohad *et al.*, 1996; Chaudhury *et al.*, 1997; Luo *et al.*, 1998 |
| *rootless* (*rtl*) | no RM;sometimes no SAM (pleiotropic) | | | Barton and Poethig, 1993 |
| *topless* | no SAM and cotyledons | specification of upper tier | | Barton and Poethig, 1993 |
| *bio1* | biotin auxotrophy; embryo *lethality* | biotin synthesis | 7,8 diamino-pelargonic *acid aminotransferase* | Patton *et al.*, 1996 |
| *fusca* (*fus*) | abnormal anthocyanin accumulation | signal transduction | hydrophylic protein (*FUS6*) | Castle and Meinke, 1994 |
| *prolifera* (*prl*) | embryo lethality | cell proliferation; initiation of DNA replication | yeast MCM2-3-5 like protein | Springer *et al.*, 1995 |
| *hydra* (*hyd*) | abnormal cell size and shape; morphological defects | cell expansion | | Topping and Lindsey, 1997 |
| *bodenlos* (*bdl*) | misspecification of hypophyseal cell | lower tier-hypophyseal cell signalling | | Mayer *et al.*, 1998 |

**Genes expressed in *Arabidopsis* embryos identified by approaches other then mutant screens**

| | | | | |
|---|---|---|---|---|
| *ARABIDOPSIS THALIANA LIPID TRANSFER PROTEIN* (*ATLTP1*) | | cuticle formation on epidermis | lipid transfer protein | Thoma *et al.*, 1994; Vroemen *et al.*, 1996 |
| *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*ATML1*) | | establishment of apical-basal and radial embryo polarity; epidermal cell fate specification | homeodomain transcription factor | Lu *et al.*, 1996 |
| *POLARIS* | | root pole specification | 37 amino acid peptide | Topping and Lindsey, 1997; Lindsey *et al.*, 1998 |
| *WAGENINGEN GENE TRAP 39* (*WGT39*) | | cell wall extension | expansin | this thesis, chapter 4 |
| *PEI-1* | arrest at heart stage (in antisense plants) | specification of apical domain | transcription factor | Li and Thomas, 1998 |