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Disruption of morphogenesis and transformation of the suspensor in abnormal *sus*pensor mutants of *Arabidopsis*

Brian W. Schwartz[1], Edward C. Yeung[2] and David W. Meinke[1,*]

[1]Department of Botany, Oklahoma State University, Stillwater, OK 74078, USA
[2]Department of Biological Sciences, The University of Calgary, Calgary, Alberta T2N 1N4, Canada

*Author for correspondence

SUMMARY

The suspensor is the first differentiated structure produced during plant embryogenesis. In most angiosperms, the suspensor functions early in development to provide nutrients and growth regulators to the embryo proper. In *Arabidopsis*, the suspensor undergoes programmed cell death at the torpedo stage and is not present in mature seeds. We have identified at least 16 embryo-defective mutants of *Arabidopsis* that exhibit an enlarged suspensor phenotype at maturity. In this report, we focus on seven abnormal *sus*pensor mutants, which define three genetic loci (*sus1*, *sus2* and *sus3*). Recessive mutations at each of these loci disrupt morphogenesis in the embryo proper and consistently result in the formation of a large suspensor. Defects in the embryo proper appear by the globular stage of development; abnormalities in the suspensor are detected soon after at the heart stage. Storage protein and lipid bodies, which normally accumulate only in the embryo proper late in embryogenesis, are present in both the arrested embryo proper and enlarged suspensor. Therefore, cell differentiation in the embryo proper can proceed in the absence of normal morphogenesis, and the suspensor can be transformed into a structure with features normally restricted to the embryo proper. These observations are consistent with a model in which normal development of the embryo proper limits growth and differentiation of the suspensor. Altered development of the embryo proper in mutant seeds leads indirectly to proliferation of suspensor cells and expression of properties characteristic of the embryo proper. Ultimately, growth of the transformed suspensor is limited by the same genetic defect that disrupts development of the embryo proper. The availability of multiple alleles of *sus1* and *sus2*, including T-DNA tagged alleles of each, will facilitate the cloning of these essential genes and molecular analysis of interactions between the embryo proper and suspensor early in development.

Key words: suspensor, embryo proper, embryogenesis, morphogenesis, *sus* mutants, *Arabidopsis*

INTRODUCTION

Cell communication is essential to normal development of multicellular organisms. In higher plants, long-range communication between different tissues is accomplished primarily by hormones. Physiological studies on whole plants (Roberts and Hooley, 1988) and genetic analyses of mutants defective in hormone biosynthesis and response (Rothenberg and Ecker, 1993) have contributed greatly to our understanding of plant hormone action. Short-range interactions between adjacent cells have also been identified in plants and are currently being characterized at the molecular level. The *KNOTTED1* (*KN1*) gene of maize, for example, is expressed in the underlying corpus of shoot meristems but affects cell divisions and differentiation in the surrounding tunica layer (Hake, 1992). Recent evidence suggests that the KN1 protein itself could be the signal that moves from the corpus to cells of the tunica layer (Jackson et al., 1994). The nature of interactions between different parts of the early embryo are less well understood. Polar auxin transport is thought to be required for the transition from radial to bilateral symmetry at the heart stage of development (Liu et al., 1993), but the factors responsible for mediating the earliest developmental interactions in plant embryogenesis are not known. We propose that the suspensor and embryo proper comprise an attractive system for identifying molecular mechanisms that mediate developmental interactions during early embryogenesis.

In most flowering plants, the zygote divides asymmetrically to form a small, densely cytoplasmic terminal cell and a larger, highly vacuolated basal cell. The terminal cell undergoes many rounds of cell division and develops into the mature embryo proper (Raghavan, 1986; West and Harada, 1993). The basal cell undergoes more limited cell division and differentiates into the suspensor (Yeung and Meinke, 1993). The suspensor is the first differentiated structure produced by the developing embryo. Although the function of the suspensor was once thought to be limited to attachment of the embryo proper to maternal tissues, more recent evidence suggests that the suspensor plays an active role in supporting early development of the embryo proper by providing nutrients and growth regu-

lators (Corsi, 1972; Yeung and Sussex, 1979; Yeung, 1980; Ceccarelli et al., 1981; Piagessi et al., 1989).

Suspensor development and morphology vary widely among flowering plants (Lersten, 1983; Natesh and Rau, 1984). The suspensor may consist of a single cell or more than 100 cells, and it may be small or large relative to the embryo proper. Suspensors typically are filamentous, columnar, spherical, or irregular in shape, and the boundary between the suspensor and embryo proper can be distinct or diffuse. Following division of the zygote, the basal cell in *Arabidopsis* divides rapidly to form a fully differentiated suspensor by the globular stage of development (Mansfield and Briarty, 1991). This suspensor consists of an enlarged basal cell embedded in maternal tissues and a single file of 6-8 additional cells. The suspensor is a terminally differentiated structure that undergoes programmed cell death at the torpedo stage of development and is not present in the mature seed (Marsden and Meinke, 1985).

Several lines of evidence indicate that, in higher plants, normal development of the embryo proper restricts further growth of the suspensor (Haccius, 1963; Marsden and Meinke, 1985). For example, degeneration of the embryo proper following irradiation of young seeds often leads to abnormal growth of the suspensor (Devreux, 1963; Gerlach-Cruse, 1969; Akhundova et al., 1978). In *Arabidopsis* and maize, many embryo-defective mutants have been identified in which development is disrupted at an early stage. A significant number of these mutants exhibit unusually large suspensors late in development (Clark and Sheridan, 1991; Yeung and Meinke, 1993). The first abnormal suspensor mutant of *Arabidopsis* examined in detail arrested at the preglobular stage of development and exhibited an enlarged suspensor containing multiple columns of cells (Marsden and Meinke, 1985). As development progressed, mutant suspensor cells accumulated unusual starch grains and structures that resembled immature protein bodies. These results suggested that cells of the suspensor have an underlying developmental potential that is limited by normal development of the embryo proper. Developmental arrest or degeneration of the embryo proper at an early stage releases the normal inhibition of suspensor growth and allows further cell division and differentiation within the mutant suspensor. The molecular mechanisms that coordinate normal development of the embryo and accomplish communication between the embryo proper and suspensor remain to be identified.

In order to extend and refine the model of interaction between the developing embryo proper and suspensor, we have examined in detail the developmental anatomy of additional mutants of *Arabidopsis* that produce abnormally large suspensors. Many such mutants have been identified among a collection of over 250 embryonic-defective mutants isolated following EMS, X-ray and T-DNA insertional mutagenesis (Meinke, 1991, 1994). These mutants differ in the extent of seed and embryo pigmentation, the stage at which embryo development is arrested, and the morphology of the abnormal suspensor. The phenotype of mutant suspensors ranges along a continuum from subtle and occasional abnormalities to striking and consistent enlargement resulting from uncontrolled cell proliferation. In this report, we focus on seven abnormal *sus*pensor mutants defining three genetic loci (*sus1, sus2* and *sus3*). All of these recessive mutants consistently produce defective embryos with large suspensors. We demonstrate that in every case, morphological defects in the embryo proper precede visible defects in the suspensor. This is consistent with our model that abnormal growth of mutant suspensors is an indirect consequence of a defect in the embryo proper. Furthermore, we show that cell differentiation can proceed in the absence of morphogenesis in both the embryo proper and the abnormal suspensor. These results suggest that disruption of development in the embryo proper can lead to proliferation of suspensor cells and partial transformation of the suspensor into a structure resembling the mutant embryo proper.

MATERIALS AND METHODS

Mutant isolation and genetic characterization

The seven abnormal suspensor mutants of *Arabidopsis thaliana* described in this report were identified initially by the presence of defective seeds in siliques of heterozygous plants produced following either seed mutagenesis (Meinke, 1985, 1994; Franzmann et al., 1989; Meinke et al., 1989) or *Agrobacterium*-mediated seed transformation (Errampalli et al., 1991; Feldmann, 1991; Castle et al., 1993). Plants were grown in soil on a 16-hour/8-hour light/dark cycle as described previously (Heath et al., 1986; Franzmann et al., 1994). Heterozygous plants produced siliques with 25% defective seeds following self-pollination. Each mutant locus was mapped by crossing heterozygotes with tester lines carrying visible markers and scoring F_2 plants for both the embryonic mutation and visible markers (Patton et al., 1991; Castle et al., 1993; Franzmann et al., 1994). Complementation tests were performed between mutations causing similar phenotypes (*sus1-1* and *sus1-2*) and between mutations that mapped to a similar position (*sus1-3*; *sus2* alleles).

Nomarski microscopy of cleared seeds

Whole seeds in dissected siliques were soaked in Histochoice tissue fixative (Amresco, Solon, Ohio) for 15 to 30 minutes. Seeds were removed from siliques and cleared for 30 minutes to 16 hours in a drop of Hoyer's solution (7.5 g gum arabic, 100 g chloral hydrate, 5 ml glycerol in 30 ml water) on a microscope slide. Seeds at later stages of development required more time for clearing. Cleared seeds were examined using an Olympus BH-2 microscope equipped with Nomarski optics.

Light microscopy of sectioned embryos

Immature seeds were removed from dissected siliques, pierced with fine forceps and fixed in 2% formaldehyde, 2% glutaraldehyde in phosphate buffer (pH 6.8) for 24 hours at 4°C. Seeds were dehydrated with methyl cellosolve for 24 hours, followed by two changes of ethanol (24 hours each). Historesin embedding medium (Leica Canada, Toronto) was introduced gradually to 70%, followed by two changes of pure Historesin (Yeung and Law, 1987). Serial sections 2-3 μm thick were obtained using a Reichert-Jung 2040 rotary microtome with Ralph knives. Sections were stained with periodic acid-Schiff (PAS) reagent for total carbohydrate and counterstained with either Amido Black 10B or toluidine blue O (TBO) as described by Yeung (1984).

Electron microscopy of sectioned embryos

Seeds were punctured with fine forceps and fixed 2 hours in 2% glutaraldehyde, 0.1 M phosphate buffer on ice, followed by three washes in phosphate buffer. Seeds were postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin. 70-nm sections were prepared with a triangular glass knife, stained with uranyl acetate and lead citrate, and examined on a JEOL 100CX transmission electron microscope at the Electron Microscopy Center at Oklahoma State University.

Tissue culture of mutant embryos and seeds

Heterozygous siliques containing wild-type seeds at the cotyledon stage of development were surface sterilized by soaking first in 70% ethanol for 30 seconds and then in 20% Chlorox containing 0.1% Tween-20 for 5 to 10 minutes. After several rinses in sterile water, embryos were removed from mutant seeds with fine forceps and cultured on nutrient media containing the inorganic salts of Murashige and Skoog (1962) supplemented with B5 vitamins (Gamborg et al., 1968), 3% sucrose, 0.1% 2-[N-Morpholino]ethanesulfonic acid (pH 5.7), 0.8% Phytagar (BRL), naphthaleneacetic acid (0.1 μg/ml) and benzyladenine (1 μg/ml).

RESULTS

Isolation and genetic characterization of abnormal suspensor mutants

The seven abnormal suspensor mutants described here define three genetic loci, designated *sus1*, *sus2* and *sus3*. General features of these mutants are summarized in Table 1. Note that all three genes are located on chromosome 1. Mutant embryos typically consisted of a distorted embryo proper and an enlarged suspensor. Segregation ratios of defective seeds in heterozygous siliques were consistent with the presence of a single recessive mutation. Genetic and molecular evidence indicated that *sus1-1* and *sus2-1* were tagged with T-DNA (Castle et al., 1993).

Two lines (*sus2-2* and *sus2-3*) were not initially recognized as abnormal suspensor mutants because they exhibited subtle defects in suspensor morphology. These two mutations were identified as alleles of *sus2* only after they were mapped to the same position on chromosome 1 (Franzmann et al., 1994). Similarly, *sus1-3* was recognized as an allele of *sus1* only after mapping and complementation tests were performed. Duplicate alleles of embryo-defective mutations have been difficult to identify in *Arabidopsis* because there is a large number of target genes with essential functions during embryogenesis (Franzmann et al., 1994). As a consequence, mutants with similar phenotypes are often defective in different genes. The identification of multiple *sus1* and *sus2* alleles derived from a variety of mutagens has strengthened our interpretation of mutant phenotypes and will facilitate not only molecular isolation of these genes but also identification of important domains in their protein products.

Developmental profiles of abnormal suspensor mutants

Fig. 1 shows wild-type and mutant embryos from siliques at the globular, heart, torpedo and cotyledon stages of development. The globular stage embryo proper shown in Fig. 1A consists of 16 interior cells surrounded by a 16-cell protoderm. The interior cells include 8 isodiametric cells in the upper tier and 8 elongated cells in the lower tier. The suspensor contains a single file of 6-8 cells, including the hypophysis at the base of the embryo proper. Cotyledon primordia arise at the flanks of the upper tier to produce a heart-shaped embryo proper (Fig. 1B). At the same time, the hypophysis divides to form a region of cells from which the root apical meristem will arise. During the torpedo stage, the cotyledons and hypocotyl elongate and the suspensor begins to degenerate (Fig. 1C). The mature embryo proper (Fig. 1D) consists of two cotyledons adjacent to the hypocotyl.

Table 1. Summary of *sus* mutants in *Arabidopsis*

Mutant	*emb* designation*	Ecotype†	Mutagen	Tagging status‡	Map position§
sus1-1	*emb76-1*	WS	T-DNA	Yes	0.0
sus1-2	*emb76-2*	WS	T-DNA	No	0.0
sus1-3	*emb60*	Col.	X-Ray	No	0.0
sus2-1	*emb177*	WS	T-DNA	Yes	115.8
sus2-2	*emb14*	Col.	EMS	No	115.8
sus2-3	*emb33*	Col.	EMS	No	115.8
sus3	*emb158*	WS	T-DNA	No	86.2

*Locus name in previous publications (Franzmann et al., 1989; Meinke et al., 1989; Errampalli et al., 1991; Castle et al., 1993; Franzmann et al., 1994).
†Ecotypes: Wassilewskija (WS); Columbia (Col.).
‡Determined through genetic analysis as described by Errampalli et al. (1991) and Castle et al. (1993).
§Location (cM) on chromosome 1 of current map of embryo-defective mutations (Franzmann et al., 1994).

Elucidation of a gene's primary site and time of action is essential to understanding its role in embryogenesis. We examined the morphology of each of the *sus* mutants throughout development to determine the first visible manifestation of the mutation and to observe the progression of events leading to the terminal phenotype. The results of this analysis for *sus1-1*, *sus2-1* and *sus3* are shown in Fig. 1. Developmental stages of mutant embryos were determined by comparison to wild-type embryos within the same silique. Thus, 'torpedo stage' refers to a mutant embryo from a silique that contained wild-type embryos at the torpedo stage. The developmental profiles of *sus1-2* and *sus1-3* are nearly identical to that of *sus1-1*. Although the terminal phenotypes of *sus2-2* and *sus2-3* differ from that of *sus2-1*, the initial defects early in development are similar to those shown in Fig. 1.

The globular stage *sus1-1* embryo contains a morphologically normal suspensor and protoderm, but the embryo proper is elongated compared to the wild type (Fig. 1E). Elongation of the embryo proper results from abnormal cell divisions at the base of the embryo proper rather than from elongation of individual cells. The result is a tapered junction between the embryo proper and suspensor. Abnormal divisions in the suspensor first appear at the heart stage (Fig. 1F) and are elaborated during subsequent development (Fig. 1G,H). No root apical meristem is evident in the torpedo stage embryo proper (Fig. 1G). Cell divisions continue in both the embryo proper and suspensor through the cotyledon stage, resulting in an elongated embryo proper and a suspensor that often approaches the size of the embryo proper (Fig. 1H).

The first morphological defect in *sus2-1* embryos is the appearance of an irregular protoderm at the globular stage (Fig. 1I). The outer walls of protoderm cells are distended, producing an uneven surface on the mutant embryo proper. Also, the interior cells of the lower tier fail to undergo normal elongation. Abnormal cell divisions in the suspensor do not occur until the heart stage (Fig. 1J). These divisions usually occur predominantly in the lower half of the suspensor, away from the embryo proper, and spread outward along the length of the suspensor. However, the first abnormal divisions occasionally occur in cells adjacent to the embryo proper. Irregular cell divisions continue throughout the embryo proper and suspensor during the torpedo stage (Fig. 1K) to produce a

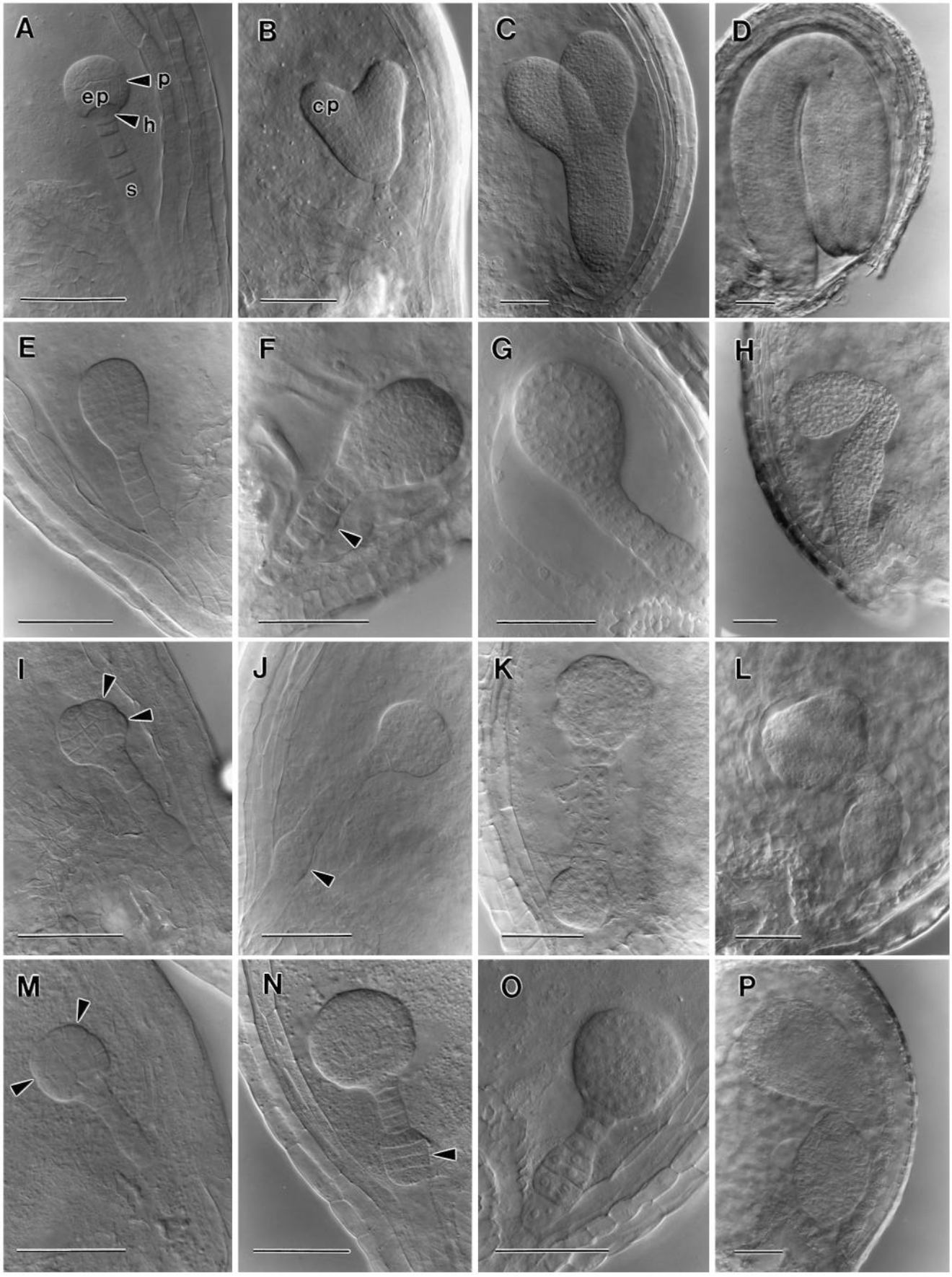
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Fig. 1. Development of wild-type and *sus* mutant embryos. A developmental profile is shown for wild type (A-D), *sus1-1* (E-H), *sus2-1* (I-L) and *sus3* (M-P). Each column depicts embryos from seeds of the same age. (A) Wild-type, globular embryo showing the embryo proper (ep) and suspensor (s). The protoderm (p) forms the outer edge of the embryo proper; the hypophysis (h) is located at the junction between the embryo proper and suspensor. (B) Wild-type, heart-stage embryo showing cotyledon primordia (cp). (C) Wild-type, torpedo-stage embryo showing elongating cotyledons and disintegrating suspensor. (D) Wild-type, cotyledon-stage embryo. (E) *sus1-1* globular-stage embryo. (F) *sus1-1* heart-stage embryo. (G) *sus1-1* torpedo-stage embryo. (H) *sus1-1* cotyledon-stage embryo. (I) *sus2-1* globular-stage embryo. (J) *sus2-1* heart-stage embryo. (K) *sus2-1* torpedo-stage embryo. (L) *sus2-1* cotyledon-stage embryo. (M) *sus3* globular-stage embryo. (N) *sus3* heart-stage embryo. (O) *sus3* torpedo-stage embryo. (P) *sus3* cotyledon-stage embryo. Arrows indicate abnormalities in the protoderm or suspensor. Scale bars, 50 μm.

mature embryo that consists of a large, distorted globular embryo proper and a massive suspensor (Fig. 1L).

The developmental profile of *sus3* embryos is similar to that of *sus2*. The earliest detectable abnormality is an irregular protoderm at the globular stage (Fig. 1M). Again, the interior cells in the lower tier fail to elongate. Abnormal cell divisions in the suspensor first appear at the heart stage, usually in cells near the base of the suspensor (Fig. 1N). Abnormal cell divisions in both the embryo proper and suspensor continue during the torpedo stage (Fig. 1O). The mature seed prior to desiccation contains an elongated embryo proper and a massive suspensor (Fig. 1P).

In summary, morphological defects in each of the mutants are detected first in the embryo proper at the globular stage of development. Abnormal cell divisions in the suspensor first appear at the heart stage. Morphological defects in the suspensor were never observed in any of the mutant embryos in the absence of visible defects in the embryo proper. These results are consistent with a model in which the primary effect of the *sus* mutations resides in the embryo proper. Therefore, abnormal development of the suspensor appears to be an indirect consequence of disrupted development in the embryo proper.

Cellular differentiation in the absence of morphogenesis in suspensor mutants

Cellular differentiation was monitored in wild-type and mutant seeds by the appearance of storage protein, lipid and starch in the embryo proper and suspensor. In wild-type embryos, these storage products are absent through the heart stage of development (Fig. 2A). Starch grains first appear in the embryo proper at the torpedo stage (Fig. 2B) and become prominent by the cotyledon stage (Fig. 2C). Starch grains then disappear, while storage proteins and lipids accumulate to high levels during maturation (Fig. 2D). Storage products are most abundant in cells of the epidermis and storage parenchyma (Fig. 2C,D) and are less abundant or absent in provascular cells (Fig. 2C). Storage products are not prominent in the wild-type suspensor at any stage of development.

Starch grains, protein bodies and lipid bodies are present in both the embryo proper and suspensor of *sus1-1* embryos (Fig. 3A-D). In contrast to wild type, these storage products accumulate throughout the embryo proper and suspensor (Fig. 3A). Lipid bodies are abundant in cells of the embryo proper (Fig. 3C) but are less common in the suspensor (Fig. 3D). Starch grains are more prominent in the mutant suspensor (Fig. 3D). Cells at the core of the mutant embryo proper degenerate during later stages of development (Fig. 3B). Electron microscopic examination of these cells revealed the complete absence of cytoplasm, indicating that the degeneration observed represents cell death and not simply vacuolation.

sus2-1 embryos contain starch grains, protein bodies and lipid bodies in both the embryo proper and suspensor (Fig. 3E-H). Storage products in *sus2-1* are restricted to the outer cell layers in the embryo proper (Fig. 3E), suggesting that cells of the mutant embryo proper correctly utilize positional information during storage tissue differentiation. Mutant embryos sometimes show a high degree of vacuolation in the embryo proper late in development (Fig. 3F). Unlike *sus1-1*, however, *sus2-1* embryos show vacuolation in cells at or near the margin

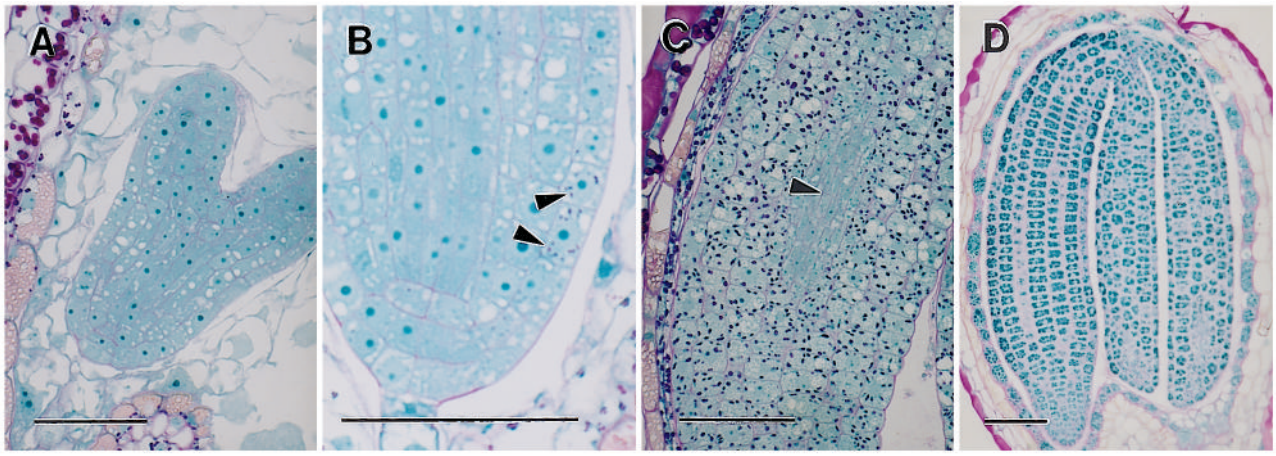

Fig. 2. Accumulation of storage products in wild-type embryos. (A) Heart-stage embryo. (B) Basal portion of the hypocotyl from a torpedo-stage embryo. Arrows indicate position of starch grains. (C) Hypocotyl of a curled-cotyledon stage embryo. Arrow indicates provascular tissue. (D) Cotyledon-stage embryo. All sections were stained with PAS for carbohydrate and counterstained with Amido Black 10B for protein. Starch grains stain purple; protein bodies stain dark blue. Scale bars, 50 μm.

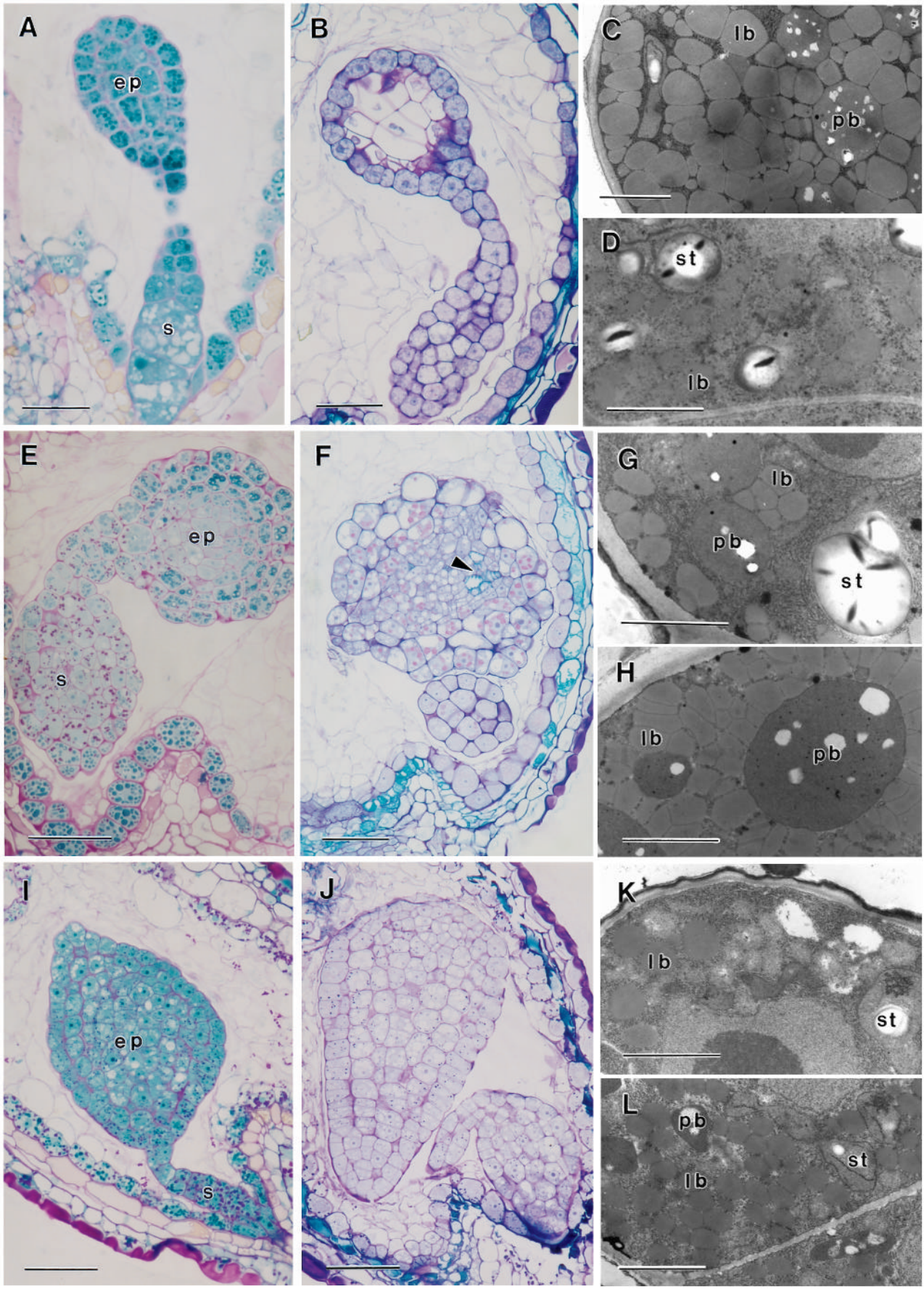
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Fig. 3. Accumulation of storage products in *sus* embryos. (A-D) *sus1-1*; (E-H) *sus2-1*; (I-L) *sus3*. (A,E,I) Light micrographs of sectioned embryos stained with PAS and counterstained with Amido Black 10B. Protein bodies stain dark blue and starch grains stain purple. (B,F,J) Light micrographs of sectioned embryos stained with PAS and counterstained with TBO. Arrow indicates differentiated xylem elements (F). (C,G,K) Electron micrographs of sections from the embryo proper. (D,H,L) Electron micrographs of sections from the suspensor. Embryo proper (ep), suspensor (s), lipid body (lb), protein body (pb), starch grain (st). Scale bars on light micrographs, 30 μm. Scale bars on electron micrographs, 2 μm.

of the embryo proper. Occasionally, cells in the interior of the embryo proper show signs of xylem differentiation (Fig. 3F).

Starch grains are prominent in both the embryo proper and suspensor of cotyledon stage *sus3* embryos (Fig. 3I). The presence of protein and lipid bodies in *sus3* embryos is more variable than in *sus1* or *sus2*. Many cotyledon stage embryos do not contain any protein bodies; others contain prominent protein bodies, particularly in cells near the base of the embryo proper (Fig. 3I). As in wild-type and *sus2* embryos, starch grains and protein bodies are restricted to the outer cell layers in the *sus3* embryo proper. Lipid bodies are present in both the embryo proper and suspensor (Fig. 3K,L), but they are not as abundant as in *sus1* or *sus2*.

Multiple alleles of *sus1* and *sus2*

We have identified three mutant alleles each of *sus1* and *sus2*. The development and terminal phenotypes of *sus1-2* and *sus1-3* are similar to those of *sus1-1* (Fig. 1E-H), suggesting that the allele strengths are the same. Although the developmental profiles of the *sus2* alleles are similar, differences in terminal phenotypes suggest differences in allele strengths. In mature seeds, *sus2-2* embryos consistently contain a small embryo proper with relatively few cells and a highly irregular protoderm (Fig. 4B). The cells are large and appear to be highly vacuolated. The embryo proper of *sus2-1* is larger and contains a more regular protoderm (Fig. 4A). The embryo proper contains more cells and the cells appear more densely cytoplasmic. The phenotype of *sus2-3* is more variable than that of the other two alleles, ranging from the severe defects typical of *sus2-2* to more normal development that can include formation of cotyledon primordia (Fig. 4C). Therefore, *sus2-2* appears to be the strongest allele, *sus2-1* is intermediate and *sus2-3* is the weakest allele.

The degree of suspensor abnormality is not strictly correlated with the severity of the embryo proper phenotype. Specifically, the intermediate allele, *sus2-1*, produces the largest and most abnormal suspensor (Figs 1L, 4A). Those *sus2-3* embryos that exhibit an intermediate embryo proper phenotype often contain a large suspensor, but embryos that develop more normally contain a normal suspensor that degrades by maturation (Fig. 4C). *sus2-2* produces a distorted suspensor that persists through maturation (Fig. 4B), but it contains only a few more cells than a normal suspensor at the globular stage. These results suggest that proliferation of suspensor cells in *sus2* mutants may be limited by the same genetic defect that limits development of the embryo proper. The most severe allele allows fewer cell divisions in both the embryo proper and abnormal suspensor.

Responses of mutant embryos in culture

Mutant embryos were removed from immature seeds and transferred to nutrient medium to determine their potential for growth and differentiation in culture. *sus1* embryos did not grow or differentiate after transfer to culture media. Apparently, these embryos are not able to overcome their defect even when grown in culture. The response of *sus2* embryos

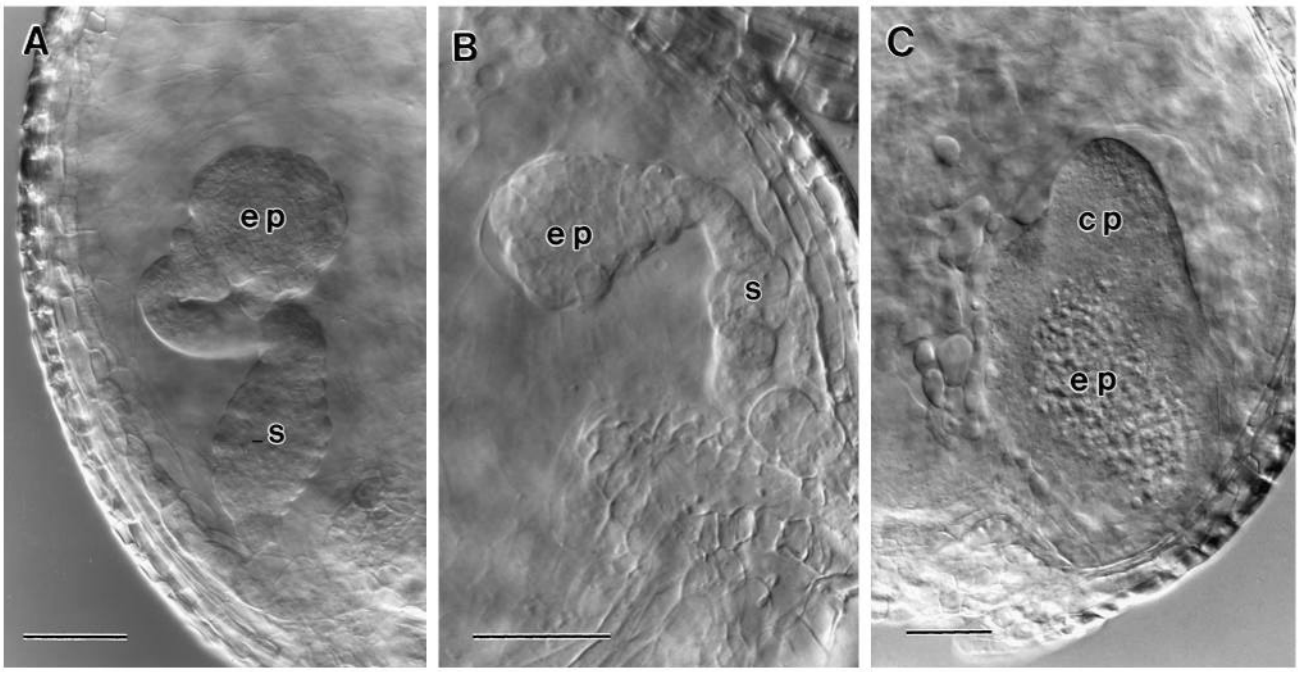


Fig. 4. Phenotypes produced by *sus2* mutant alleles. (A) *sus2-1*. (B) *sus2-2*. (C) *sus2-3*. All embryos are from seeds at the cotyledon stage of development. Embryo proper (ep), suspensor (s), cotyledon primordium (cp). Scale bars, 50 μm.

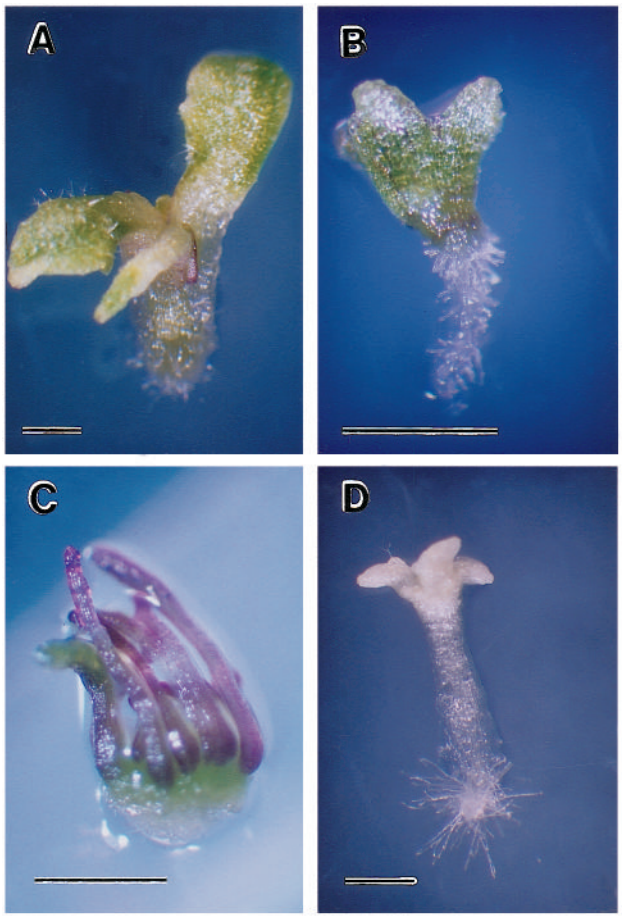


Fig. 5. Responses of mutant embryos in culture. (A) *sus2-1* embryo cultured for 10 days. (B) *sus2-1* embryo cultured for 10 days. (C) *sus2-3* embryo cultured for 10 days. (D) *sus3* embryo cultured for 15 days. Scale bars, 1 mm.

depended on the mutant allele. Culturing *sus2-1* embryos resulted in organogenesis or callus formation at a high frequency, up to 70% in some experiments. *sus2-1* embryos readily formed leafy structures (Fig. 5A) or root hairs, but only rarely were both a shoot and root formed (Fig. 5B). *sus2-3* responded similarly in culture, usually producing callus or leafy projections (Fig. 5C). Despite this high frequency of response, fertile plants were not obtained from any of these cultured plantlets. *sus2-1* and *sus2-3* embryos from older seeds that had begun to dehydrate did not respond at a high frequency in culture, suggesting that these embryos failed to establish desiccation tolerance. *sus2-2* embryos did not respond in culture, consistent with the proposal that *sus2-2* is a more severe allele.

Culturing immature *sus3* embryos led to both callus production and organogenesis. Embryos often produced leafy projections along with structures resembling the hypocotyl and root hairs (Fig. 5D). Root development was limited to production of root hairs at the base of the plantlet. None of the cultured plantlets were green, but some produced purple sectors on the leafy structures. No fertile plants were recovered from these plantlets, suggesting that either the culture conditions were inappropriate for complete embryo rescue or that the gene is required for normal vegetative development. As with *sus2* embryos, *sus3* embryos were sensitive to desiccation.

Isolated suspensors from immature *sus* embryos did not respond in culture. The suspensor is embedded in maternal tissues and is more difficult to remove from the seed than the embryo proper. Failure of mutant suspensors to respond in culture may therefore result from damage sustained during dissection. Alternatively, the different responses observed in culture for the mutant embryo proper and suspensor may indicate that embryogenic transformation of the suspensor is incomplete. In any case, failure of mutant suspensors to respond in culture is consistent with our view that abnormal growth of the suspensor in mutant seeds is not simply a direct consequence of cell-autonomous, uncontrolled proliferation of suspensor cells.

DISCUSSION

Abnormal suspensor mutants of *Arabidopsis* are an attractive system for studying the nature of interactions between different parts of the developing plant embryo. The most striking feature of these mutants is the presence of an enlarged suspensor in mature seeds prior to desiccation. The size of the abnormal suspensor in some cases equals that of the arrested embryo proper. Normal development of the suspensor in *Arabidopsis* involves the formation of a single column of 6-8 cells early in development, followed by programmed cell death prior to seed maturation. We have examined in detail the development of seven abnormal suspensor mutants of *Arabidopsis*, including three mutant alleles each of *sus1* and *sus2*. We find that in each mutant, the suspensor fails to undergo programmed cell death, proliferates inappropriately through renewed cell division and acquires features normally restricted to the embryo proper.

In all three abnormal suspensor mutants examined, morphological defects were detected first in the embryo proper at the globular stage of development. These defects included an abnormal protoderm (*sus2, sus3)* and disorganized cell divisions (*sus1, sus2* and *sus3*). Mutant embryos failed to undergo the transition from radial to bilateral symmetry during the heart stage. Despite this disruption of morphogenesis, mutant embryos accumulated starch, lipid and storage protein bodies during seed maturation. This result demonstrates that cellular differentiation is not strictly dependent on normal morphogenesis in plant development. This conclusion is consistent with observations made with other embryo-defective mutants analyzed in our laboratory (Patton and Meinke, 1990; B. Schwartz, D. Vernon and D. Meinke, unpublished observations). Furthermore, storage products in *sus2* and *sus3* were restricted to the outermost cell layers of the mutant embryo proper, indicating that these cells correctly interpreted their position in the embryo proper and differentiated as storage tissue rather than transmitting tissue. However, the persistence of starch grains in mutant embryos late in development suggests that *sus* mutations interfere with the temporal regulation of starch accumulation during embryogenesis.

Two of the abnormal suspensor mutants underwent differentiation events that are not normally associated with development of the embryo proper. In *sus1*, cells at the core of the embryo proper degenerated late in development. Cell death is

a normal feature of suspensor development and of vascular differentiation in adult plants, but it is not a normal feature of the embryo proper. In contrast to *sus1*, cells in the outermost layers of *sus2* embryos degenerated late in development. *sus2* embryos occasionally showed signs of precocious xylem differentiation in the innermost cell layers of the embryo proper, providing further evidence that cells of the mutant embryo proper can interpret positional cues accurately even in the absence of morphogenesis.

Morphological defects in the suspensor did not appear until the heart stage of development, and abnormal cell divisions in the suspensor were never detected in the absence of defects in the embryo proper. These observations support the earlier proposal that the primary defect in abnormal suspensor mutants resides in the embryo proper (Marsden and Meinke, 1985). We can now elaborate upon this model and propose that *SUS* genes normally act within the embryo proper and are required during the transition from the preglobular to the globular stage of development. Disruption of development in the embryo proper leads indirectly to proliferation of suspensor cells. Furthermore, our results demonstrate that cells of the abnormal suspensor not only proliferate when released from control by the embryo proper but also acquire characteristics normally restricted to cells of the embryo proper. These features include the appearance of storage protein, lipid and starch, products that normally accumulate in non-vascular tissues of the embryo proper late in embryogenesis. Therefore, the default developmental pathway of the suspensor is toward embryogenesis, and normal development requires inhibition of this default pathway. Presumably, transformation of the suspensor into a structure that resembles the embryo proper requires expression of developmental programs normally restricted to the embryo proper. Comparison of suspensor and embryo proper phenotypes among members of the *sus2* allelic series suggests that development of the transformed suspensor is limited to the same extent as development of the embryo proper.

A similar model was recently invoked to explain the phenotype of the *twin* (*twn*) mutant of *Arabidopsis*. Seeds of this mutant may contain two or more embryos capable of developing into mature plants upon germination. Vernon and Meinke (1994) showed that secondary embryos produced by this mutant were derived from the suspensor of the primary embryo. This mutant phenotype elegantly demonstrates that cells of the suspensor can duplicate the full spectrum of developmental programs characteristic of the embryo proper. The *TWIN* gene likely encodes either a product in the embryo proper required for sending the inhibitory signal to the suspensor or a product in the suspensor involved in receiving the inhibitory signal and maintaining suspensor cell identity.

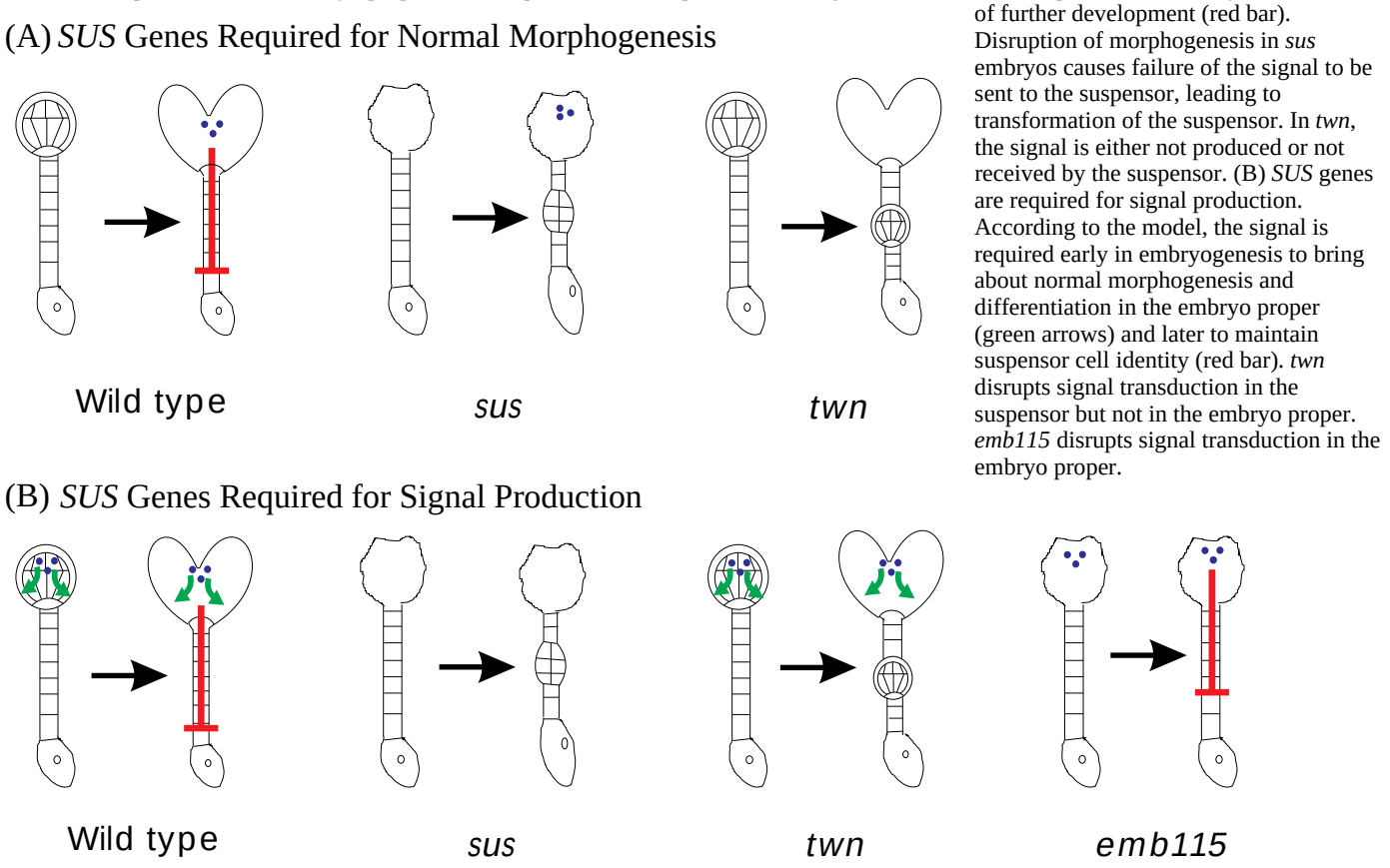
How can defects in development of the embryo proper lead to proliferation and transformation of the suspensor? One possible explanation is that the mutant embryo proper does not function as an efficient sink for nutrients and growth regulators transported through the suspensor. As a result, these substances might accumulate in the suspensor and induce cell proliferation and differentiation. This model can accommodate earlier observations that both destruction (Haccius, 1963; Akhundova et al., 1978) and arrest (Marsden and Meinke, 1985) of the embryo proper early in development can lead to suspensor cell proliferation. However, the model is not consistent with results reported here for *sus* mutants. In these mutants, the embryo proper apparently serves as an efficient sink at the time when abnormal suspensor cell proliferation first begins. The embryo proper continues to undergo cell division at that stage and even accumulates storage products later in development. We therefore favor an alternative model in which suspensor cell proliferation results from failure of the mutant embryo proper to send an inhibitory signal to the suspensor.

We offer two models to explain the role of *SUS* genes in development (Fig. 6). According to the first model, the *SUS* gene products are not involved directly in maintaining communication between the embryo proper and suspensor (Fig. 6A). Rather, *SUS* genes are required at the globular stage for normal morphogenesis. The *sus* mutations cause disruption of morphogenesis and failure of the embryo proper to convert from radial to bilateral symmetry. Such morphological defects in the embryo proper could block movement of a signal that requires cell polarity for transport. This model predicts a large number of target genes for *sus* mutations and explains the relative abundance of abnormal suspensor mutants in *Arabidopsis*. Mutations that interfere specifically with production or reception of the signal itself would be less frequent and would result in a relatively normal embryo proper and a transformed suspensor. The *twn* mutant might be defective in such a gene. One problem with this model is that it does not offer a simple explanation for other mutants, such as *emb115*, that have a relatively normal suspensor but an embryo proper with a pattern of abnormal development similar to that of the *sus* mutants (D. Vernon and D. Meinke, unpublished results). In order to explain the phenotype of such a mutant, the model would need to include a second mechanism of suspensor inhibition or signal transport that occasionally remains functional in the absence of normal morphogenesis in the embryo proper.

Alternatively, the *SUS* genes might be involved directly in the production of a signal responsible both for directing normal development of the embryo proper and maintaining suspensor cell identity (Fig. 6B). Differential responses in the embryo proper and suspensor could result from separate signal transduction pathways in the two tissues. According to this model, signal perception in the embryo proper is required for normal morphogenesis and differentiation along the longitudinal axis. Signal perception in the suspensor is required for maintenance of suspensor cell identity. Absence of the signal in *sus* mutants leads directly to disrupted morphogenesis in the embryo proper and transformation of the suspensor. This model is attractive because it explains the phenotypes of *sus* mutants as well as other mutants with related phenotypes. For example, mutations such as *twin* might disrupt signal transduction within the suspensor but not the embryo proper, allowing relatively normal development of the embryo proper in combination with embryogenic transformation of the suspensor. Conversely, mutations such as *emb115* might cause failure of signal transduction in the embryo proper but not the suspensor, resulting in disrupted morphogenesis in the embryo proper and production of a normal suspensor.

Regardless of the precise functions of *SUS* genes in growth and development, the abnormal suspensor phenotype illustrates the importance of establishing normal communication between the embryo proper and suspensor during early embryogenesis and the role of suppression of embryogenic pathways in maintaining differentiated cell states during plant

Fig. 6. Models of *SUS* gene action in embryogenesis. (A) *SUS* genes are required for normal morphogenesis. In wild-type embryos, a signal (blue dots) is produced in the embryo proper and transported to the suspensor, resulting in maintenance of suspensor cell identity and inhibition of further development (red bar). Disruption of morphogenesis in *sus* embryos causes failure of the signal to be sent to the suspensor, leading to transformation of the suspensor. In *twn*, the signal is either not produced or not received by the suspensor. (B) *SUS* genes are required for signal production. According to the model, the signal is required early in embryogenesis to bring about normal morphogenesis and differentiation in the embryo proper (green arrows) and later to maintain suspensor cell identity (red bar). *twn* disrupts signal transduction in the suspensor but not in the embryo proper. *emb115* disrupts signal transduction in the embryo proper.



development. The existence of T-DNA tagged alleles of *sus1* and *sus2* will facilitate the isolation of the corresponding genes and may lead to a better understanding of the molecular mechanisms of interaction between the embryo proper and suspensor early in development.

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