

# single cotyledon (*sic*) Mutants of Pea and Their Significance in Understanding Plant Embryo Development

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**ABSTRACT** Angiosperms are divided into two distinct classes—the dicotyledons (dicots) and monocotyledons (monocots)—based in part on the number of cotyledons in mature embryos. In this paper, we describe single-cotyledon pea mutants, termed *sic* (single cotyledon), all of which show a degree of fusion between the cotyledons. The fusion in *sic1* is along the margin of one cotyledon and is less complete than in *sic2* embryos, but the effects of the mutations are additive in the double mutant. Occasionally *sic2* mutants will show fusion of the two cotyledons into one cylindrical embryo in which the shoot apex becomes surrounded by the cotyledons. Both *sic1* and *sic2* mutants produce fertile plants. In the *sic3* embryo, a single cotyledon is generated under the shoot apex that breaks the vascular connection between root and shoot, causing embryo lethality. The pattern of cotyledon development in all these mutants is identified by *in situ* mRNA hybridization and antibody labeling, using the storage protein vicilin as a cotyledon-specific marker. These patterns indicate that the joining of the cotyledons was due to zonal growth. The results indicate that there are genes in pea that influence the positioning and the morphology of the cotyledon. A model for cotyledon development in pea is proposed that is based on the regulation of the positioning of cell clusters by the *sic* genes. Dev. Genet. 25:11–22, 1999. © 1999 Wiley-Liss, Inc.

**Key words:** cotyledon; dicot; embryogenesis; monocot; mutants; pea; *Pisum sativum* L

## INTRODUCTION

Angiosperms have been classified into two classes, dicotyledons (dicots; Magnoliopsida) and monocotyledons (monocots; Liliopsida), based on the number of cotyledons in their mature seeds and other features such as the numbers of flower parts and pollen furrows, the leaf venation, the arrangement of the vascular bundles and the existence of secondary growth [Raven *et al.*, 1981]. Four types of mutants have been described in *Arabidopsis* and *Antirrhinum*, which alter the pattern or the number of cotyledons. The first type causes the deletion of the cotyledon, e.g., the *gurke* mutation in *Arabidopsis*, in which both cotyledon and shoot apex

are absent from the apical-basal axis [Mayer *et al.*, 1991]. The second type causes the fusion of the cotyledons, including *emb30/gnom*, *pin1-1*, and cup-shaped cotyledon (*cuc*) in *Arabidopsis* [Baus *et al.*, 1986; Mayer *et al.*, 1993; Liu *et al.*, 1993; Aida *et al.*, 1997] and *connata* in *Antirrhinum* [Stubbe, 1966], in which the cotyledon of some embryos exhibits a cylindrical or semicylindrical shape. Using T-DNA tagging, the *emb30/gnom* gene has been cloned [Shevell *et al.*, 1994]. Its predicted amino acid sequence is similar to that of a yeast protein, Sec7p, which is associated with Golgi and involved in the operation of vesicles in the secretory pathway. The *cuc* mutant is the result of the interaction between recessive alleles at two unlinked loci [Aida *et al.*, 1997]. The *CUC2* gene has been cloned and shows similarity to the NAM protein of *Petunia* [Souer *et al.*, 1996], which affects meristem development. The third type produces embryos with a variable number of cotyledons, as observed in the *pleiocotyledonea* (*pleic*) mutant of *Antirrhinum* [Stubbe, 1966], and the *pinoid* and *altered meristem program* (*amp*) mutants of *Arabidopsis*. Both *pinoid* and *amp* have one or more than two cotyledons; their phyllotaxy is changed as well [Bennett *et al.*, 1995; Chaudhury *et al.*, 1993]. Evidence suggests that PINOID plays a role in an auxin-related process. In *pleic*, removal of the supernumerary cotyledon reverts the mutant phyllotaxy to that of the wild type (WT), indicating that the arrangement

Contract grant sponsor: Biotechnology and Biological Sciences Research Council (BBSRC); Contract grant sponsor: Ministry of Agriculture, Fisheries and Food.

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Received 28 September 1998; Accepted 4 January 1999

of cotyledons could have a direct effect on the phyllotaxy of the leaves [Stubbe, 1966]. The fourth type of cotyledon mutant causes a homeotic change of cotyledons to leaves, represented by the *leafy cotyledon (lec)* mutant of *Arabidopsis* [Meinke, 1992]. The *LEC1* gene encodes a transcription factor homologue, the CCAAT box-binding factor HAP3 subunit [Lotan *et al.*, 1998]. In addition, mutants defective in other processes of pattern formation could also affect the number or morphology of the cotyledons. For example, the seedlings of the mutant *monopteros* (which has a deletion of the basal structure of the embryo including root and hypocotyl) also occasionally show a single cotyledon [Hardtke and Jürgens, 1998]. Nevertheless, none of the mutants identified so far produces a viable plant whose embryos show a complete transformation from two cotyledons to a single one [Meinke, 1995; Jürgens *et al.*, 1994].

After chemical mutagenesis [Wang *et al.*, 1990], several embryo mutants of pea have been identified that have cellular or morphological abnormalities [Johnson *et al.*, 1994; Liu *et al.*, 1996], including the formation of a single cotyledon. In this paper, three single-cotyledon mutants are described in detail. Two of these, *sic1* and *sic2*, are single recessive mutations that show a conversion from two to a single cotyledon; one, *sic3*, has a single cotyledon with an abnormal axis. A model is proposed to explain the formation of these mutant phenotypes and its relevance to monocot/dicot phylogeny is discussed.

## MATERIALS AND METHODS

### Plant Material

Mutants of *Pisum sativum* L. were obtained by chemical mutagenesis as described previously [Wang *et al.*, 1990]. The isolation numbers of the three cotyledon pattern mutants were E1137, E2391, and E4650; a preliminary description can be found in Johnson *et al.* [1994]. All material was grown in a glasshouse at 15°C/10°C (minimum day/night temperatures), with supplementary lighting as necessary to provide a 16-h photoperiod. Embryo developmental stages were designated according to Liu *et al.* [1995] after Marinov [1970]. Briefly, these are: stage 19, globular embryo; stage 20, late globular with shoot apex forming; stage 21, early heart-shaped, cotyledons being initiated; stage 22, heart-shaped, fresh weight ca.100 mg; stage 23 (embryo filling half the embryo sac) and 24 (embryo filling entire sac), main period of storage product accumulation, fresh weight up to 600 mg (for the BC1/RR parental line)[Hedley *et al.*, 1986].

### Histological Analysis

The procedures used for paraffin sections and L.R. White resin (London Resin Company, Hampshire, UK) sections have been described previously by Liu *et al.* [1995]. Wax sections of 8 µm were stained with Delafield's premixed haematoxylin for light microscopy

or 4,6-diamidino-2-phenylindole (DAPI) for fluorescence microscopy. Semithin resin sections were stained with 0.5% toluidine blue.

### In Situ mRNA Hybridization

The in situ hybridization method was based on that of Hauxwell *et al.* [1990] as modified by Welham *et al.* [1997], with the addition of 50% formamide to the post-RNase washes. Probes for the hybridization were generated by labeling the vicilin cDNA insert from pCD4 [Domoney and Casey, 1983], which was cloned into the transcription vector pGEM4-Z (Promega, Madison, WI), with digoxigenin-11-rUTP (DIG; Boehringer Mannheim, Lewes). Sense (control) labeling was carried out for all samples analyzed.

### Immunohistology

Wax-embedded sections, prepared as above, were blocked, incubated in primary antibody, secondary antibody (EXTRA-3, Sigma), stained using ExtrAvidin-peroxidase/Sigma Fast<sup>®</sup> kit as described elsewhere [Welham *et al.*, 1997], and mounted in Entallen. Vicilin antibody was a kind gift from Drs. R. Casey and C. Domoney, John Innes Centre.

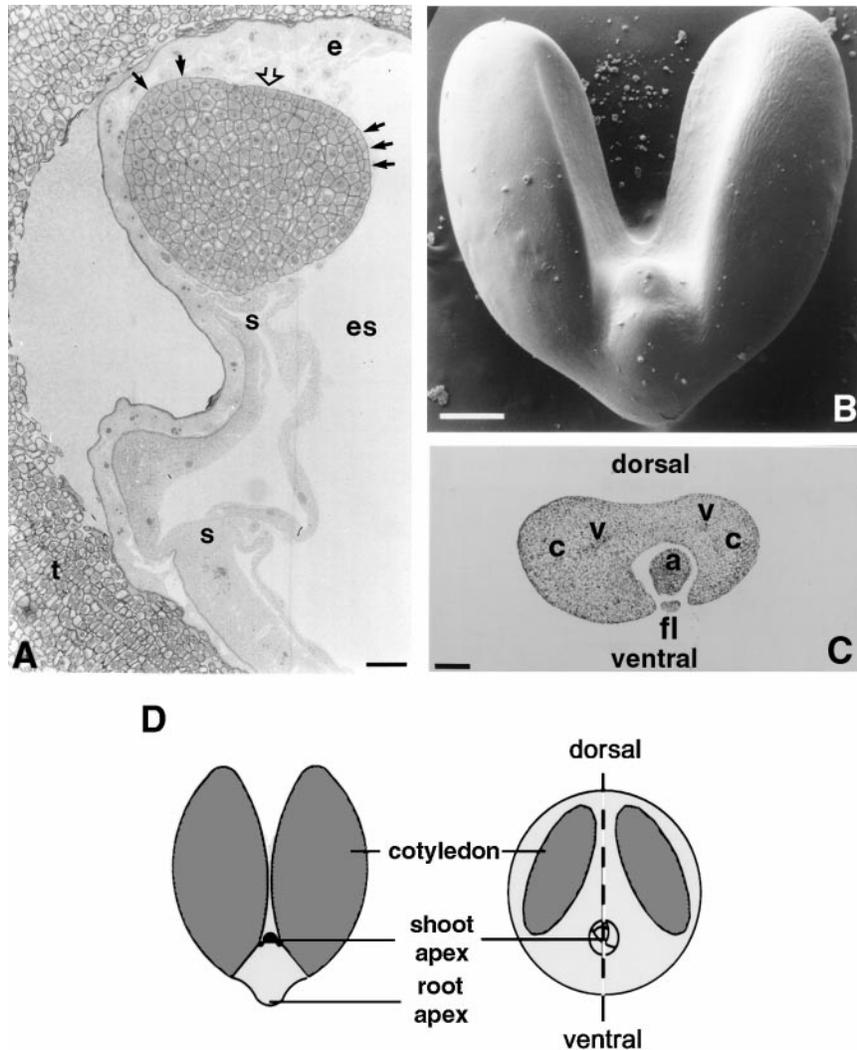
### Scanning Electron Microscopy

Pea embryos were dissected from ovules in a 5% glycerol solution, attached to stubs using double-sided tape and immediately transferred into a liquid nitrogen slush at -210°C. The frozen material was then transferred to the pre-chamber of a Hexland cryostage (at -195°C) attached to a CamScan Mark IV microscope. The temperature of the chamber was then raised to -80°C to remove the ice formed over the sample. After sublimation, the sample was withdrawn and sputter coated with gold at 2 mA for 5 min in an argon atmosphere. The coated sample was viewed at 16 kV and held at ca. -165°C.

## RESULTS

### Cotyledon Formation in the Wild-Type Embryo

During the development of the pea embryo, two cotyledons were initiated at stage 20 (Fig. 1A), after initiation of the shoot meristem. From the serial sections, it appeared that, close to the time of the cotyledon initiation, active periclinal cell divisions (Fig. 1A, arrows) occurred in the outermost cells at the regions in which cotyledons were to be generated, producing a single layer of protoderm cells, which, in turn, formed an epidermis covering the newly formed cotyledons. The whole embryo at this stage contained more than 1,000 cells. As soon as the cotyledon primordia formed, a single layer of protoderm was established, which covered the whole embryo. Thereafter, cell division in this layer occurred in an anticlinal manner only. The protoderm in pea was therefore formed after cotyledon initiation. The cotyledons appeared to be generated



**Fig. 1.** Cotyledon formation in wild-type pea embryos. **A:** Longitudinal section through a late globular stage (stage 20) embryo showing active periclinal divisions (*arrows*) in the outermost cell layer of the regions, where the cotyledons will be generated. The shoot apical meristem (*open arrow*) is just becoming apparent at the top of the embryo. Bar = 50  $\mu\text{m}$ . **B:** Scanning electron micrograph of a pea embryo at stage 22. Note that the position of the shoot apical dome to the fore of the embryo between the two cotyledons creates a single plane of symmetry. Note also that the lip to the rear of the apex means the apex can only be seen fully from one side. Bar = 200  $\mu\text{m}$ . **C:**

Transverse section of a pea embryo at stage 22 through the base of the cotyledons and the shoot apical dome. Note that the cotyledons are joined together along the dorsal side and the shoot apex is situated close to the ventral side. Bar = 250  $\mu\text{m}$ . **D:** Diagrammatic representation of the embryo (stage 22) from ventral side (*left*) and from above (*right*) to show the position of progenitor meristems the single plane of symmetry (*broken line*). a, apical meristem; c, cotyledon; e, endosperm; es, embryo sac; fl, first leaf; s, suspensor; t, testa; v, provascular bundle.

from two clusters of cells over the top and the lateral region of the globular embryo. The construction of an obvious root meristem was later than that of the shoot apex and the cotyledons and occurred once the two cotyledons were defined (stage 22; data not shown). During cotyledon growth, mitotic divisions were active in the region along the abaxial and distal region of the cotyledon. The cells in these regions were also smaller with a dense cytoplasm and a small vacuole, in contrast to the storage parenchyma.

The most appropriate time to analyze the embryo morphology of the pea mutants was found to be stage 22 (Fig. 1B), as all the embryonic domains had been generated, and no physical restraint had yet been applied to the developing embryo to alter its morphology. Such physical restraints were generated by the rapid growth of the embryo, especially the swift expansion of the cotyledons (after stage 23), and the limited space of the embryo sac, which caused bending of the axis and reorientation of the cotyledons. The WT pea

embryo has only a single plane of symmetry, in contrast to many dicots. To clarify this point, a dorsal-ventral concept from animal embryology was adopted to describe the morphology of the pea embryo. The ventral side was where the apical meristem was situated, which was close to the testa *in vivo*. The dorsal side was where the two cotyledons were situated, facing away from the adjacent testa. As a result, the shoot apex was only seen from the ventral side, and not from the dorsal side at stage 22, as shown by scanning electron microscopy (Fig. 1B, ventral view). Transverse sections of a young embryo through the shoot apex revealed that the two cotyledons were joined together along the dorsal side, and the first leaf was always formed at the ventral side (Fig. 1C). No procambium was seen in the joining region. A single plane of symmetry ran across the junction of these two cotyledons and across the shoot apex and the first leaf, as can be seen in Figure 1C and in the diagram, Figure 1D. Therefore, in the WT pea embryo, the cotyledons were formed from active cell divisions in two individual cell clusters, which were slightly closer to the dorsal side (Fig. 1D).

#### Mutant Isolation and Genetic Analysis

The three single-cotyledon mutants (designated E1137, E2391, and E4650) were isolated from a mutagenesis program by screening for cotyledon morphology of young embryos in immature seeds. The mutants were carried through a purification procedure by selected mutant-bearing plants from selfed heterozygotes for at least six generations. During the selection, each mutant gave segregation ratios consistent with being the product of single recessive genes [Johnson *et al.*, 1994]. Reciprocal crosses were therefore performed between the homozygous mutant plants of E1137 and E2391 as, in these lines, plants were viable. These two mutants were also backcrossed to the parental line. Segregation ratios of the F2 progeny supported the conclusion that they were single recessive genes. All F1 seeds from the intercrosses had a WT phenotype indicating 2 complementation groups. Hence at least two genes can mutate to produce single cotyledons in pea. F2 progeny from a cross between E1137 and E2391 were scored as developing embryos. The putative double mutant's phenotype was taken as that which bore different features to either E2391 (whether as a normal or as a cylindrical single cotyledon) or E1137. The phenotypes of embryos from segregating populations are described below. The ratios were 71:27:20:6 (WT:E2391:E1137:double mutant), with an expected value of 70:23:23:8. These data are consistent with E1137 and E2391 representing two independent loci ( $\chi^2 = 1.6$ ;  $P = 0.75-0.5$ ). These loci have been named *single cotyledon1* (*sic1*) and *single cotyledon2* (*sic2*), respectively. Identification of the putative double mutants necessitated their destruction and further genetic analysis to confirm their mutant identity was not possible.

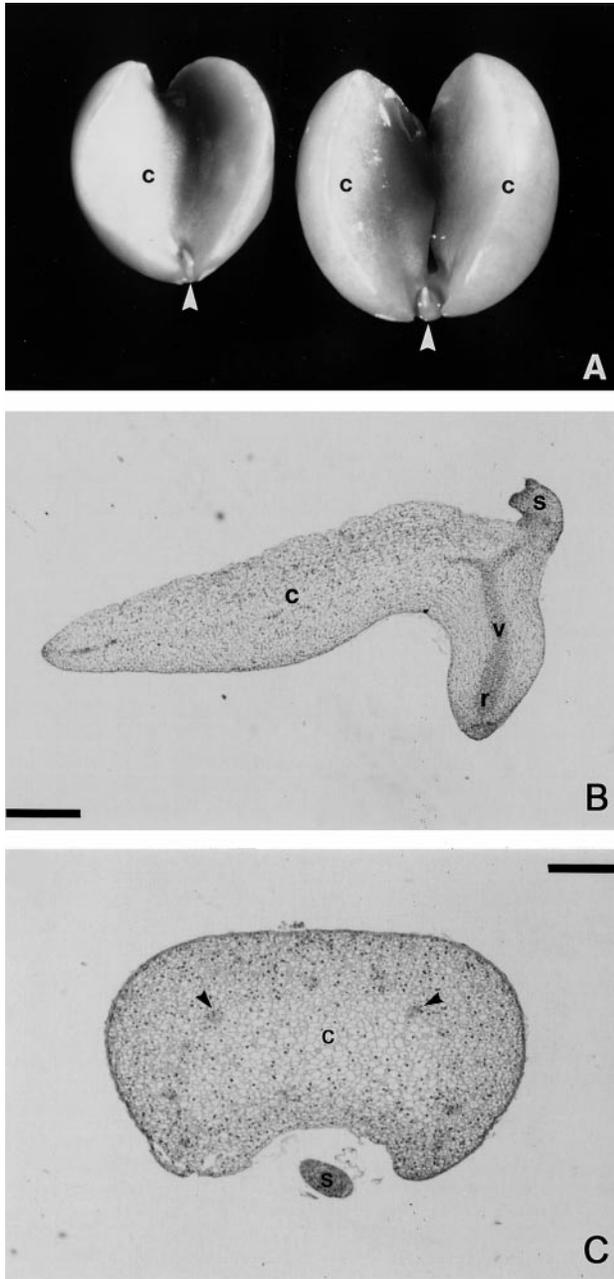
E4650 was embryo lethal and could only be crossed in a heterozygous state. Furthermore, the heterozygous plants were also of low fertility due, in part, to low pollen production. Progeny from crosses with either *sic1* or *sic2* were all of a WT phenotype (a 1:1 ratio of WT:mutant would be expected for alleles at the same locus), indicating that E4650 represents a third locus, which we have termed, therefore, *sic3*.

#### Cotyledon Formation in Mutants

**sic1.** The mutation at the *sic1* locus had the least effect on embryo development. Embryos of this mutant were similar to the WT except that they had a single cotyledon (Fig. 2A). The shoot apex (Fig. 2A, arrowheads) and root (Fig. 2B) were situated at the correct positions, as compared with that of the WT. A small depression was seen over the top of the single cotyledon. The growth rate of the mutant embryo was similar to that of the WT, producing mature seeds with a weight comparable to normal seeds. The dry seed encapsulating the mutant embryo could be distinguished from that of the WT, as there was a shallow depression in one side of the seed where the edge of the cotyledons met. In addition, the mutant seeds were not round, being thinner and longer than the WT. Although the morphology of the cotyledon and shape of the seeds had been changed in the mutant, the mature seeds could germinate normally to produce seedlings with a single cotyledon. There was no obvious difference in postembryonic development between the plants produced from the mutant seeds and those from the WT. All the embryos produced from such homozygous plants showed the mutant phenotype.

Longitudinal sections through 20 mg *sic1* embryos showed that they had normal organogenesis and tissue differentiation, like the WT, except that there was only one cotyledon connected to the axis (Fig. 2B). The shoot apex was located at a lateral position. The procambia (provascular bundles) were arranged properly between the shoot apex and the root meristem and branched to the cotyledon. Leaf primordia could be seen in the shoot apex. A transverse section through the mutant embryo along the shoot apex region showed that the provascular strands (Fig. 2C, the major pair indicated by arrowheads) were arranged in a half-circle within the single cotyledon.

**sic2.** The shape of most *sic2* mutant embryos was similar to that of the *sic1* embryo except for three differences: (1) the single cotyledon was narrower (Fig. 3A,B); (2) there was no notch at the top of the cotyledon (Fig. 3A); and (3) the mutant embryos occasionally possessed a single cylindrical cotyledon (Fig. 3C). In a similar way to the *sic1* embryo, the shoot apex was situated at the base of the cotyledon. Longitudinal sections through these mutant embryos showed that the tissue structure of the single-cotyledonary embryo was normal, with well-developed meristems and cotyledon (Fig. 3B). When mutant embryos of *sic2* showed a



**Fig. 2.** Phenotype of the *sic1* embryo. **A:** Ventral view of a *sic1* embryo (left) and the WT (right) at stage 23. Note that the cotyledons of the mutant are fused along their dorsal margins, with a depression at the top of the single cotyledon. *Arrowheads*, shoot apices. **B:** Longitudinal section through a mutant embryo, showing normal tissue differentiation, indicated by the correct positioning of the root, shoot, and provascular tissues. Bar = 500  $\mu$ m. **C:** Transverse section through a mutant embryo showing its cellular organization. The two main provascular strands are indicated by arrowheads. Bar = 250  $\mu$ m. Abbreviations as in Fig. 1, except: s, shoot apex; r, root apex.

complete fusion of their cotyledons at both the ventral and dorsal sides, the cotyledon displayed a cylinder-like structure (Fig. 3C), with the shoot apex inside and at

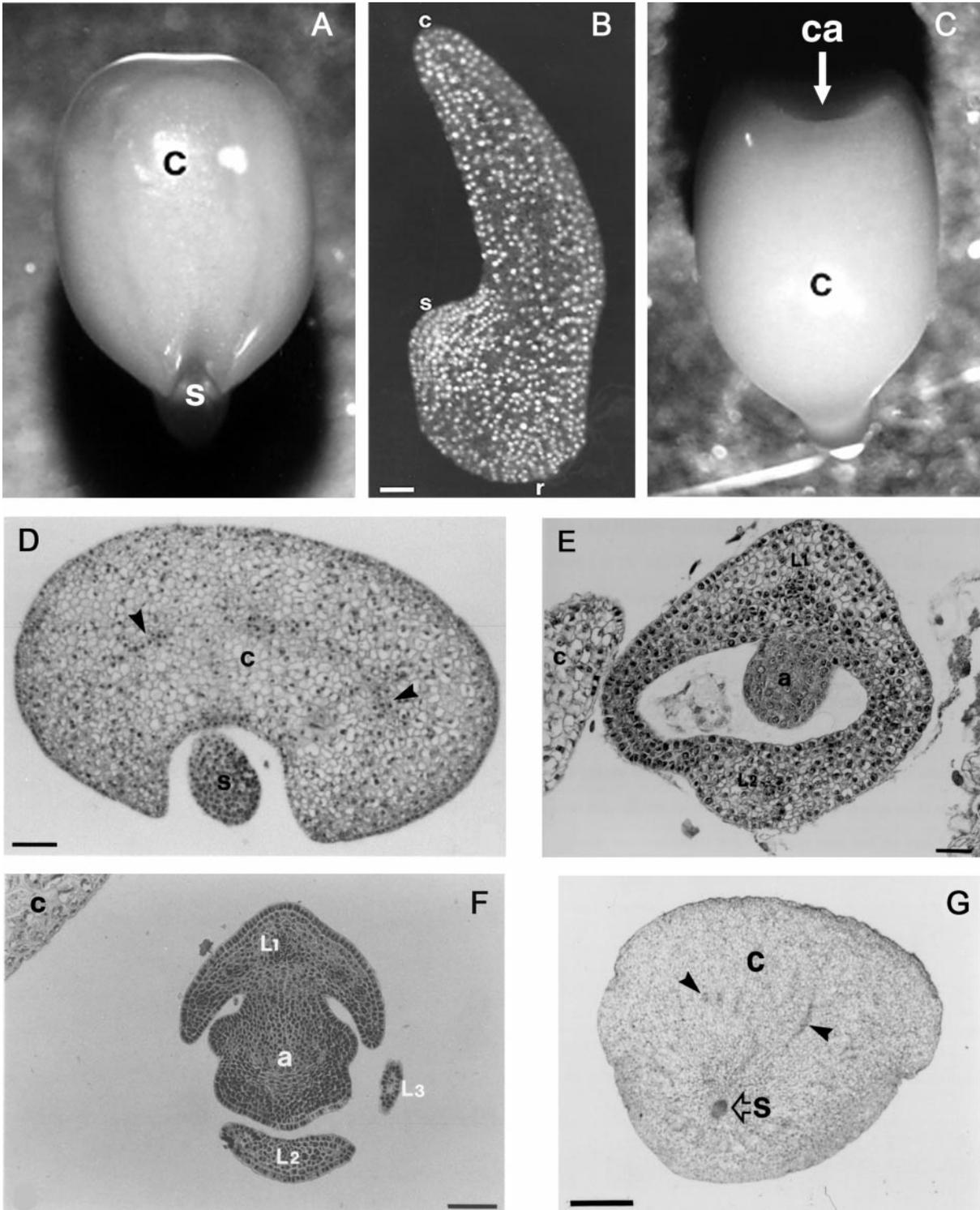
the bottom of the cylindrical cotyledon. The root had a normal WT morphology. Transverse sections through the noncylindrical single cotyledon near the shoot apex indicated that two major provascular bundles were situated in the cotyledon (Fig. 3D, arrowheads). Sections in the same plane through the shoot apex of a later stage embryo showed that there was a slight fusion at the base of the leaf primordium (Fig. 3E), although this had no effect on the morphology of the mature leaf. Such a fusion was not observed in the WT shoot apex, in which all leaf primordia emerged as isolated protrusions as soon as they were generated from the apical meristem (Fig. 3F).

Transverse sections through the cylindrical embryo showed that a shoot apex was present near the ventral side of the cylindrical cotyledon (Fig. 3G, open arrow). The cotyledon along the dorsal side (upper side in Fig. 3G) was much thicker than that along the ventral side (lower side, Fig. 3G). The shoot apex was more juvenile than that of the WT, with no leaf primordium present in the apex until stage 23. WT embryos at the same stage had at least two to three leaf primordia.

The dry seeds produced from the single-cotyledon embryos could be distinguished from the WT, because one side of the seed had a groove where the two edges of the cotyledon had failed to meet each other within the testa. These seeds germinated to form fertile plants. Seeds with fully cylindrical cotyledons (usually ca. 15%) germinated to produce roots. About one-third also produced shoots (Fig. 4A), but the cotyledons of the remainder remain intact in the soil for a long period. If the cotyledons of such embryos are cut back to where the shoot apex should be, a shoot emerged rapidly (within 30 min), indicating that these embryos also produced viable shoots whose growth is restricted physically (Fig. 4B,C). Close inspection of the cotyledon around the shoots in Figure 4A indicated that it had torn the adjacent tissue.

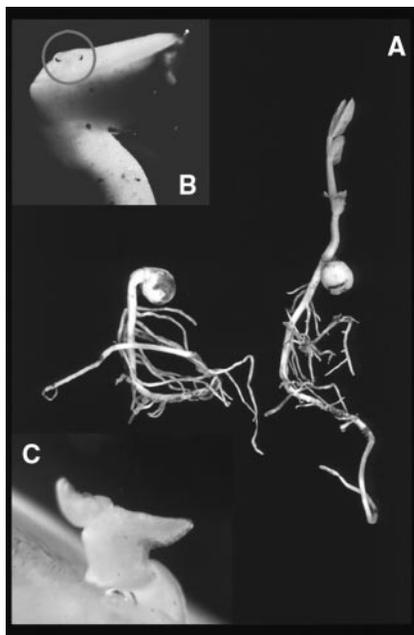
***sic1/sic2* double mutants.** As mentioned above, three morphological differences can be used to distinguish between the *sic1* and *sic2* cotyledon. In crosses between *sic1* and *sic2*, a third phenotype could be distinguished in which the notch was less pronounced and the cotyledon width intermediate (Fig. 5D). It was therefore considered that this phenotype represented the double mutant and the effects of the two mutations were additive, neither being epistatic to the other. In the cross between *sic1* and a *sic2* plant showing a high frequency of cylindrical cotyledons (ca. 50%), a fourth phenotype could be distinguished, which had neither a complete cylinder, nor a distinctive notch, although the latter was still present (Fig. 5I), again supporting the idea that the effects of these genes were additive. The segregation ratios for the F<sub>2</sub> population have been given in the section on genetic analysis.

***sic3*.** The mutant embryo of *sic3* had a sharply pointed root (instead of the more rounded root tip of the WT), a shoot apex, and a single and narrow cotyledon

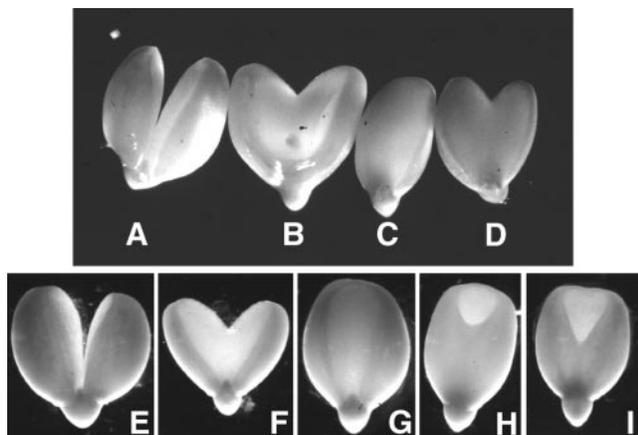


**Fig. 3.** Phenotype of the *sic2* embryo. **A:** Mutant embryo, illustrating the single cotyledon lacking a depression over the top. **B:** Longitudinal section through a mutant embryo stained with DAPI and observed using fluorescence microscopy. Note the well-formed root and shoot meristems with closely packed cells. Bar = 50  $\mu$ m. **C:** Mutant embryo with a cylinder-like cotyledon. Note that the shoot apex is hidden by the cotyledon and will be at the base of the cavity. **D:** Transverse section through a mutant embryo; *arrowheads*, main

provascular traces. The dorsal side of the embryo (*top*). Bar = 100  $\mu$ m. **E:** Transverse section through the shoot apex of the mutant, showing that the young leaves, L1 and L2 are linked. Bar = 50  $\mu$ m. **F:** Transverse section through the shoot apex of the WT, showing that the L1, L2, and L3 leaf primordia are separate. Bar = 100  $\mu$ m. **G:** Transverse section through a cylinder-like mutant embryo. *Open arrow*, shoot apex; *arrowheads*, provascular traces. Bar = 500  $\mu$ m. Abbreviations as in Fig. 2 plus *ca*, cavity.

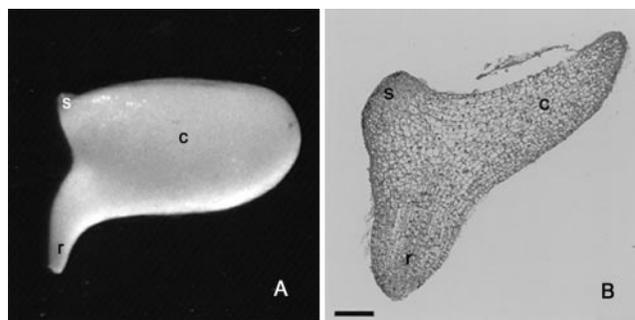


**Fig. 4.** Seedlings from *sic2* embryos with cylindrical cotyledons. **A:** Seedling with a cylindrical cotyledon (*left*) and one with cotyledons fused along a single edge (*right*). **B:** Higher power ( $\times 6.3$ ) of shoot (encircled) emerging from a dissected cotyledon, 2 h after cutting back. **C:** Magnified view ( $\times 32$ ) of the emerging shoot.



**Fig. 5.** Phenotypes of putative *sic1/sic2* double mutants. F2 segregation populations from two experiments, one (**A-D**) in the absence of the *sic2* cylindrical phenotype and one (**E-I**) in its presence. **A, E:** WT; **B, F:** *sic1*; **C, G, H:** *sic2*; **D, I:** putative double mutants.

(Fig. 6A). The cotyledon grew at right angles to the axis during early development, but its expansion at later stages "pushed" the shoot apex away from the root. The shoot apex, therefore, was situated at the top of the ventral side of the single cotyledon, whereas the root was situated more or less at the bottom of the cotyledon (Fig. 6A). The shoot apex looked less developed than that of the same-staged embryo in the WT. Leaf primordia could not be seen until a much later stage, as



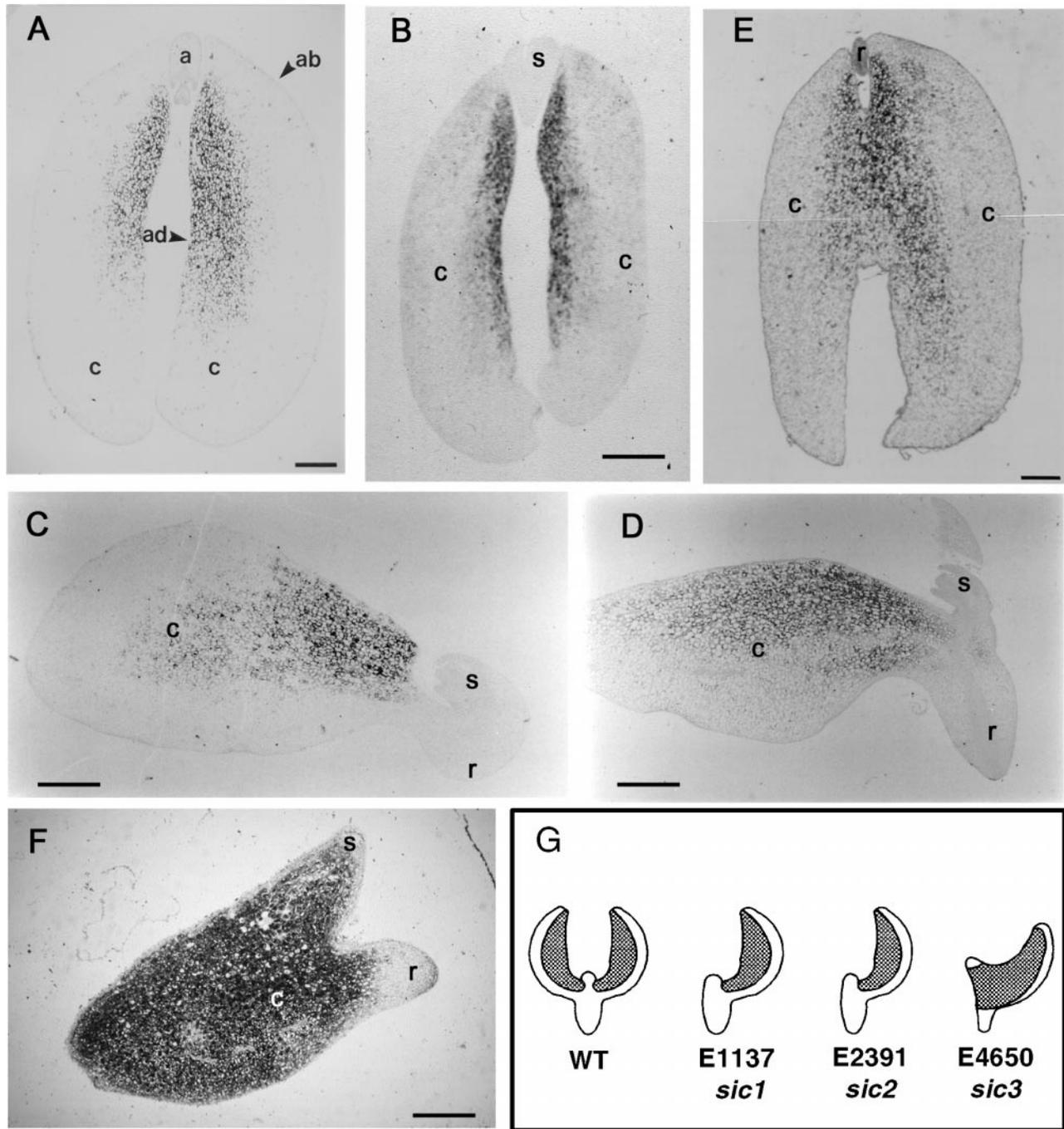
**Fig. 6.** Phenotypes *sic3* embryos. **A:** *sic3* embryo. Note the underdeveloped shoot apex(s) and sharply pointed root (r). **B:** Median section through a young *sic3* embryo. Note that no provascular traces can be seen between the root and shoot apices (cf. Fig. 2B). Bar = 100  $\mu$ m. Abbreviations as in Fig. 2.

compared to those of the corresponding WT embryos. In addition, the pointed root tip eventually turned brown. The mutant embryos survived to form mature seeds, although these seeds were smaller than those of the WT and they could not produce normal plants; the shoot apex and the root would grow, but only elongate slightly after germination. The embryonic roots sometimes elongated up to 2 cm, but thereafter the whole embryo became necrotic.

The mutant embryo was triangular in section, with the shoot apex, root apex and the single cotyledon sitting at each angle (Fig. 6B). Histological analysis showed that there was no proper vascular connection between the shoot apex and root (Fig. 6B). The cell arrangement in the region between the shoot and root apex (epicotyl and hypocotyl) was rather irregular, and more similar to that of the cotyledonary cells. A meristem and the tiers of cells in the pericycle and in the provascular bundle, typical of a normal root, could not be found in the root of this mutant.

#### Vicilin mRNA and Protein as a Cotyledon Marker

A major storage protein of pea, vicilin, was chosen as a marker for cotyledon parenchyma cells since its accumulation in such cells is well understood. Accumulation of mRNA starts quite early in those parenchyma cells undergoing expansion and whose nuclei are endoreduplicating. In the WT embryo, expression of vicilin mRNA was restricted to the cotyledons and was not present in the epidermis, the axis or in provascular areas. Expression occurred initially in the adaxial region of the cotyledon of the WT (Fig. 7A,C). The distribution of protein was essentially the same as its mRNA as exemplified in Figure 7B for the WT. In *sic1* and *sic2*, the distribution of mRNA was similar to that in the WT in that it was mainly in what would have been the adaxial surface of the cotyledons, closer to the axis (Fig. 7C). The region in which the cotyledons were joined in *sic1* and *sic2* clearly contained mRNA (Fig.



**Fig. 7.** Localization of vicilin mRNA and protein in WT and *sic* embryos, using in situ hybridizations and immunohistology. **A:** Longitudinal section through a WT embryo, showing the presence of mRNA toward the adaxial surface. Bar = 430  $\mu$ m. **B:** Section through the same embryo, showing the distribution of protein. Bar = 380  $\mu$ m. **C:** Transverse section of a WT embryo through the length of the adaxial region of one of the cotyledons and the edge of the axis, showing the distribution of mRNA. Bar = 140  $\mu$ m. **D:** Similar plane of section to C through a *sic1* embryo, but passing through the region in which the cotyledons are joined, showing the distribution of mRNA. Bar = 145

$\mu$ m. **E:** Longitudinal and tangential section from the root axis passing through the single cotyledon of *sic2*, showing mRNA distributed across the region in which the cotyledons are joined. Bar = 400  $\mu$ m. **F:** Transverse section through the length of a *sic3* embryo, indicating the presence of mRNA across most of the embryo. Bar = 175  $\mu$ m. **G:** Schematic representation of the pattern of vicilin gene expression in the embryos of the WT and *sic* mutants of pea. Shaded areas, regions in which mRNA corresponding to the pCD4 cDNA insert was detected in late heart-stage (20–30 mg fresh weight) embryos. Abbreviations as in Fig. 2, plus ad, adaxial surface; ab, abaxial surface.

7E). No vicilin mRNA was detected in the axis including the shoot apex, hypocotyl and root (Fig. 7A–E). By contrast, vicilin mRNA accumulation was seen in most regions of the *sic3* embryos, except the shoot apex, root tip, and the procambium (Fig. 7F). The accumulation of vicilin mRNA in the root approached the root tip, but was not in the root cap. No vicilin mRNA was found in the shoot and the root apices. The distribution of vicilin mRNA and protein in the WT and these three mutants at the late heart stage (20–30 mg fresh weight) is summarized in Figure 7G.

## DISCUSSION

### Cotyledon Mutants

Pattern formation in higher plants is brought about through a combination of programmed cell divisions, cell expansion and the interpretation of positional cues. There is no doubt that many genes are involved in these complex processes, which makes the study of pattern formation difficult. By mutagenesis, however, it is possible to alter such a program through the selection of pattern mutants. In this paper, mutants at three loci (*single cotyledon*, *sic*) are described in which cotyledon patterns have been changed. The term “single cotyledon” is used rather than monocotyledon, because the single cotyledon is believed to have been produced by additional growth between the normal cotyledonary meristems (see below). A similar situation was apparent for the *cup-shaped cotyledon* (*cuc*) mutant of *Arabidopsis* in which products of genes at unlinked loci (*cuc1* and *cuc2*) interacted to produce a single cup-shaped cotyledon [Aida *et al.*, 1997]. In this instance, however, the *cuc* mutant possessed no shoot apical meristem and was seedling lethal, in contrast to the situation in two of the three pea mutants described here where a fully developed shoot was generated that gave rise to a viable plant.

### Symmetry in the Pea Embryo

The existence of a dorsal-ventral difference may explain why several single cotyledon mutants have been identified in pea from such a relatively small screening project [Johnson *et al.*, 1994]. Stable transformation from a dicotyledonary embryo to a monocotyledonary embryo has only been reported once in the literature [Aida *et al.* 1997], even in the large screenings for embryo mutants in *Arabidopsis*, although some mutants occasionally produce embryos with a single cotyledon [Meinke, 1995; Jürgens *et al.*, 1994]. The single cotyledon in pea was generated by the fusion and/or expansion of the cell clusters along their dorsal sides. As stated by Johnson *et al.* [1994], it may not be correct to describe the cotyledons of these mutants as “fused,” because they appear to arise as a single structure in the early stages of embryo development. Such a developmental process is normally termed congenital

fusion rather than ontogenetic fusion [Cusick, 1966] because it is the result of zonal growth. The epidermal cells of the cotyledons would have to undergo a redifferentiation step for the two separate organs to grow into one so that they appear to be fused [Lolle *et al.*, 1992]. We have not observed this to be the case either by serial sectioning or by analyzing patterns of vicilin mRNA accumulation. The structure of the inner region of the single cotyledon also supports the view that zonal growth has occurred. The cotyledon in *sic* mutants appears to have been formed as a single integrated unit with normal tissue organization. The only difference was the occurrence of two major provascular strands in the single cotyledon, as opposed to one in each of the WT cotyledons (Figs. 2C, 3D). Thus, expansion of the two proliferation zones, rather than the loss of one zone, may be the cause of the single cotyledon and it is reasonable to believe that the size and the location of these meristematic clusters can directly influence the shape of the cotyledons and the whole morphology of the embryo.

A single plane of symmetry in the pea embryo, which was not distinguished by previous workers [Cooper, 1938; Reeve, 1948; Marinos, 1970], has been demonstrated in this investigation. The plane runs longitudinally through the axis and at a right angle to the vertical plane which passes through the two cotyledons (Fig. 1D). In most dicotyledonary embryos, e.g., *Brassica* and *Arabidopsis*, there are always two symmetry planes once the cotyledons have been formed, one is the same as that of the pea, the other is across the middle of the two cotyledons and the axis. The latter plane does not exist in pea, since the shoot apex is situated toward the ventral side, whereas the cotyledons are closer to the dorsal side. According to Reeve [1948], the growth of the cotyledons is initiated in a late globular embryo by two short and curving tiers of cells called rib-meristems. These rib-meristems could not be identified in the histological analysis conducted in this investigation. Nevertheless, active divisions were observed in clusters of cells at the top and lateral regions of the globular embryo, although they were not arranged in tiers (Fig. 1A). During cotyledon growth, cell divisions continued to occur in the abaxial and the distal regions of the cotyledon. The clusters of actively dividing cells should be situated over the top of the globular embryo, but slightly closer to the dorsal side, in contrast to the shoot apex which sits at the ventral side (Fig. 1D).

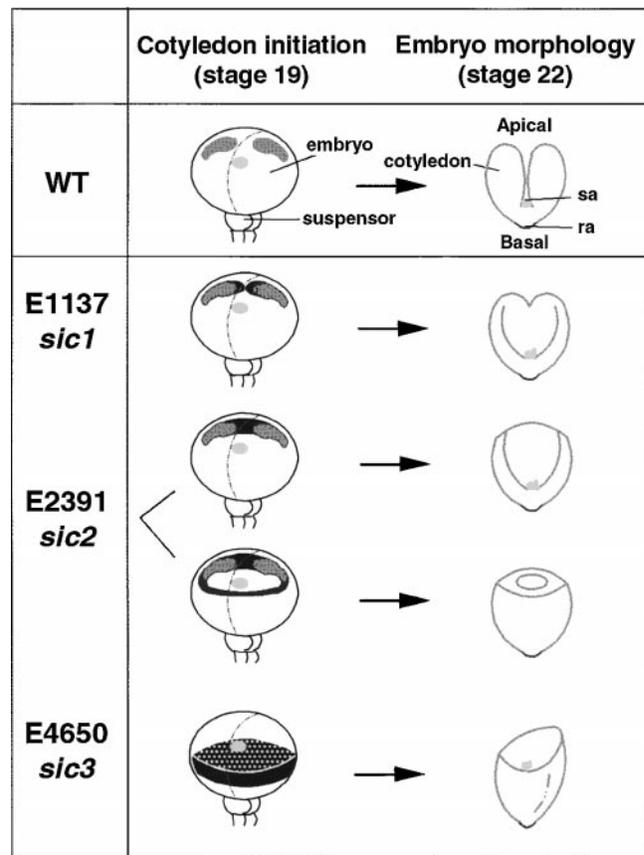
The expression of a gene encoding a vicilin, a major storage protein of pea, has been used as a marker of cotyledon development in this study. In pea, vicilin has been found to be present in the cotyledonary cells, once the DNA level reaches 5C or above [Corke *et al.*, 1987]. DNA endoreduplication generally occurs in the cotyledon of pea and other legumes at storage protein accumulation [Wang and Hedley, 1993]. The accumulation of vicilin mRNA was first detected in the inner region of the cotyledon near the shoot apex at stage 22. It then

spread to the outer region until all the parenchyma cells showed the presence of the mRNA [Hauxwell *et al.*, 1990, 1992]. Furthermore, accumulation did not occur in regions in which cell division was active. According to the *in situ* mRNA hybridizations and immunohistological analyses presented here, production of vicilin and its mRNA in the *sic1* and *sic2* mutants was initiated at an early stage in a similar region to that in the WT. The initiation of zonal growth in the dorsal region where the two cotyledons were joined would explain the extension of the region in the mutants and supports the conclusion that the altered cotyledon pattern in these two cotyledon mutants was caused by the alteration in the size and/or position of the actively dividing cell clusters. Similarly, a repositioning of the cells would account for the *sic3* phenotype. In this mutant, accumulation of mRNA is much greater than normal which may be related to the early developmental arrest of its embryo. Its cells may cease dividing earlier and prematurely age as occurs when embryos are cultured [Yang *et al.*, 1990]. Such cells accumulate more mRNA and protein than normal.

#### *Sic* Genes Define Cell Clusters That Form Cotyledons

Based on the results presented in this paper, a model of cotyledon formation in the WT and the four single cotyledon phenotypes is proposed (Fig. 8). The cotyledons in the WT are generated from two cell clusters at the late globular stage. Since the cotyledons are closer to the dorsal side and the shoot apex to the ventral side, the position of the cotyledon meristems and apical meristem also should be situated in this arrangement on top of the globular embryo. In *sic1* embryos, it appears that, instead of initiating two separate clusters as in the WT embryo, the two clusters *partially* come together along the dorsal side. Consequently, the cotyledons formed will be consolidated, but with a depression over the top of the cotyledon. Such an extension of cell proliferation activity could be achieved by the recruitment of a few additional cells into the original cluster at the time of cotyledon initiation. The mutant morphology of *sic2* may be created similarly, but by a complete coalescence of the clusters along the dorsal side. Thus only one cotyledon would be developed from such a single, crescent-shaped cluster. If extension of the cluster were to occur in both directions, dorsal and ventral, the cotyledon formed would exhibit a cylinder-like structure, as seen in some *sic2* embryos. The difference between the dorsal and ventral side should still be, and was, recognizable (Fig. 3G). Such an embryo with a cylinder-like cotyledon, therefore, would be different from those of *Brassica juncea* derived by the treatment of preembryos with auxin polar transport inhibitors, in which the embryos become radially symmetrical [Liu *et al.*, 1993], although the mechanism may be similar.

One can extrapolate this localised proliferation hypothesis to its extreme in the *sic3* embryo. Its cotyledon may be generated from cells that proliferate further



**Fig. 8.** Model of cotyledon formation in the WT and *sic* mutants of pea. In the WT, the cotyledons are initiated from small clusters of cells (mid-gray areas) near the dorsal side of the globular embryo, whereas the shoot apex (light gray) is initiated toward the ventral side. Initially (left column) in the *sic* mutants, the clusters are enlarged to different degrees and, in some instances, in different directions (dark gray). The resultant embryo morphologies at stage 22 are represented by the diagrams in the right-hand column. The diagrams are not to scale. ra, root apex; sa, shoot apex.

down and beneath the shoot apex (Fig. 8). Instead of two separate cell clusters as in the WT, the cluster in this mutant would be a single one under the apex, the latter still being located at the ventral side. The initiation of cotyledon formation under the shoot apex in this way (as confirmed by the cotyledon-specific marker) would split the vascular connection between the shoot and the root apex, causing embryo lethality. Such an explanation, though extreme, is plausible because no vascular connection between the shoot and the root apices was observed in serial sections of *sic3*. Hence the data from this mutant indicate that the vascular connection between the root and shoot apices is important for both embryonic and postembryonic development. The *Arabidopsis* mutant, *monopteros*, has no vascular connection between the shoot apical meristem and the root apex, although the vascular bundles are relatively normal in the cotyledons. The *monopteros* embryos,

regardless of whether they possess strong or weak alleles, are incapable of forming the proper root [Berleth and Jürgens, 1993], as was also observed in *sic3*. Nevertheless, roots can be induced on the mutant seedlings by auxin in tissue culture. The involvement of abnormal vascularization and auxin transport in the *monopteros* phenotype has been indicated by later studies [Przemeck *et al.*, 1996]. The *monopteros* gene has been cloned recently and has been shown to encode a transcription factor with possible binding activity to an auxin-inducible promoter [Hardtke and Berleth, 1998].

The function of the *Sic* genes could be to define the location and the size of the cell clusters that generate the cotyledons. They may act by limiting the territory of the clusters and any mutation in these genes, therefore, causes an alteration in the position of these clusters. In other words, the function of the WT genes could be to inhibit cell division and growth in those regions where organs are not initiated. By doing so, the position and shape of a new organ can be regulated precisely. The only gene with a similar function to *Sic* is *CUC2* which encodes a protein homologous to the *Petunia* NAM protein [Souer *et al.*, 1996]. Apart from cotyledon initiation, the *CUC2* gene is also involved in the development of shoot meristem [Aida *et al.*, 1997]. The *Sic* genes involved specifically in the formation and shaping of cotyledons, despite the fact that the latter are considered embryonic leaves, must be independent of the developmental pathway that regulates the formation of foliage leaves because none of the viable mutants showed alterations to their mature leaf morphology. Conversely, the leaf pattern mutants of pea described by previous workers [Marx, 1987] have no effect on the morphology of the cotyledons. It is unknown what kinds of signal molecules are involved in such regulation. According to Meinhardt's hypothesis, organ pattern formation might be the result of interactions between growth promoting and inhibiting substances in diffusion fields [Meinhardt, 1982]. One possible candidate signal molecule proposed by several workers is auxin because it can work in both a promoting and an inhibiting way depending on its concentration [Lyndon, 1994]. Treatment with auxin analogues and auxin transport inhibitors can cause the outgrowth of parts of the apical surface that would not normally do so [Soma, 1968; Liu *et al.*, 1993]. In monocots, it has also been found recently that auxin influences the change from radial symmetry to embryonic polarity [Fischer and Neuhaus, 1996]. Auxin might control the position, shape, and size of an organ through the adjustment of local surface wall extensibility [Lyndon, 1994]. Such a growth control mechanism might be a general mechanism for regulating organ pattern formation in higher plants.

In addition, the *sic2* mutant provides evidence for the role of physical forces in the shaping of the embryo. The growth of the cotyledons occurs within the boundaries of the seed coat. However, the shoot is normally held between the cotyledons until germination. In *sic2* em-

bryos with a cylindrical cotyledon, it appears that the shoot becomes trapped within the cotyledon. If it is sufficiently close to the surface, it can break through the cotyledon during germination. If it is not, it remains quiescent until the physical restriction of the mass of the cotyledon tissue is removed when it will then grow. The rapidity of this growth indicates that it is likely to be a turgor driven cell expansion event.

### Evolutionary Aspects

The results presented in this paper also give some indication of how monocots might have evolved from dicots. Our results agree with the hypothesis advanced by Sargent [1903] that monocots are derived phylogenetically from dicots through the fusion of the two cotyledons. She believed that the ancestral dicots might be similar to modern Nymphaeales, which show tendencies toward the fusion of the two cotyledons into one [Cronquist, 1981; Burger, 1981, 1996]. There have been arguments, however, that favor the hypothesis that the paired cotyledons of dicots are an early innovation in angiosperm evolution [Burger, 1981, 1996]. No evidence for suppression of one of the ancestral pair of cotyledons was found in any species by Haines and Lye [1979] despite earlier indications [cited in Foster and Gifford, 1974]. We have shown in this investigation that there are genes in pea which influence the positioning and the shaping of the cotyledon. One of the simplest explanations is that such an influence may be afforded via the inhibition of cell division in cell clusters which give rise to the cotyledons. Mutation of one of these genes would lead to a loss of functional inhibitor that would lead to the transformation of a two-cotyledon to a single-cotyledon embryo through additional growth along one or both margins of the cotyledons, without causing lethality. Such fusion of the cotyledons has also been observed in some species, e.g., *Nymphaea* and *Nuphar luteum*, which are closely related to monocots [Haines and Lye, 1975, 1979; Cronquist, 1981]. The embryo of *Nymphaea* resembles that of pea, showing a partial union along one margin of the cotyledons [Hains and Lye, 1975]. The full fusion of the cotyledon to a cylindrical structure in *sic2* also is quite similar to the cotyledonary tube found in many monocots [Burger, 1981].

There is no intention in this paper to indicate that pea is more closely related to monocots in an evolutionary sense, but rather that the single-cotyledon mutants of pea might repeat some of the events that occurred in the transition from the dicots to the monocots. Although a genetic approach has been used in the study of the evolution of flowers and inflorescences [Coen and Nugent, 1994], no such approach has been applied to embryogenesis. This work represents just one step toward understanding the monocot/dicot relationship. Further genetic and molecular studies of such mutants might provide more information on the phylogenetic relationships between these classes.

## ACKNOWLEDGMENTS

The authors thank Professors Ron Tyrl and David Meinke from Oklahoma State University; Cliff Hedley, Keith Roberts, and Rod Casey from the John Innes Centre for valuable comments; and Noel Ellis, of the John Innes Centre, for help with the genetic analysis. We also thank Kim Findlay for her assistance with the scanning electron microscopy.

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