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Mutations in the *FASS* gene uncouple pattern formation and morphogenesis in *Arabidopsis* development

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SUMMARY

The pattern of cell division is very regular in *Arabidopsis* embryogenesis, enabling seedling structures to be traced back to groups of cells in the early embryo. Recessive mutations in the *FASS* gene alter the pattern of cell division from the zygote, without interfering with embryonic pattern formation: although no primordia of seedling structures can be recognised by morphological criteria at the early-heart stage, all elements of the body pattern are differentiated in the seedling. *fass* seedlings are strongly compressed in the apical-basal axis and enlarged circumferentially, notably in the hypocotyl. Depending on the width of the hypocotyl, *fass* seedlings may have up to three supernumerary cotyledons. *fass* mutants can develop into tiny adult plants with all parts, including floral organs, strongly compressed in their longitudinal axis. At the cellular level, *fass* mutations affect cell elongation and orientation of cell walls but do not interfere with cell polarity as evidenced by the unequal division of the zygote. The results suggest that the *FASS* gene is required for morphogenesis, i.e. oriented cell divisions and position-dependent cell shape changes generating body shape, but not for cell polarity which seems essential for pattern formation.

Key words: fass, embryo, *Arabidopsis*, morphogenesis, pattern formation, polarity, shape, elongation

INTRODUCTION

Embryogenesis generates the primary plant body of the seedling which is fairly uniform among flowering plants (Steeves and Sussex, 1989). How this body organisation is established in the plant embryo has become a focus of genetic analysis, using *Arabidopsis* as a model (Jürgens et al., 1991). A large-scale screen for mutations affecting the seedling body organisation identified genes that appear to play specific roles in one of three different aspects: apical-basal pattern, radial pattern or shape (Mayer et al., 1991). Mutations in those genes were shown to alter embryogenesis before or at the heart stage when the primordia of seedling structures become morphologically recognisable.

In *Arabidopsis* and other crucifers, the embryo grows by oriented cell divisions such that the seedling structures can be traced back to groups of cells in the early embryo (Schulz and Jensen, 1968; Tykarska, 1976, 1979; Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). Such regularity of cell division pattern is not common to all flowering plant embryos (Natesh and Rau, 1984), and thus it is not obvious how it relates to embryonic pattern formation. However, recent studies of embryonic pattern mutants in *Arabidopsis* have provided evidence that oriented cell divisions reflect processes underlying pattern formation. Mutations in the *GNOM* gene render the normally asymmetric division of the zygote nearly symmetric, resulting in an enlarged apical daughter cell which also divides abnormally; subsequent cell divisions are highly irregular, yielding a seedling with a grossly perturbed apical-basal pattern (Mayer et al., 1993). Mutations in the *MONOPTEROS* gene alter the cell division pattern of the basal region of the embryo from the early-globular stage, producing seedlings without hypocotyl and root (Berleth and Jürgens, 1993).

Mutations in the *FASS* (*FS*) gene were shown to drastically change the shape of the seedling without altering its body pattern; furthermore, *fs* embryos did not display the characteristic cell shapes or cell arrangements by which the primordia of seedling structures are normally recognised at the heart stage (Mayer et al., 1991). These observations raised the possibility that the seedling body is generated by two distinct processes in the *Arabidopsis* embryo, pattern formation and morphogenesis, and that the *FS* gene might only be involved in the latter but not the former; the term 'morphogenesis' is used to cover cell activities, such as oriented cell divisions and cell shape changes, that generate body shape (for definition of the terms see e.g. Slack, 1991). To test this idea, we have carried out a detailed genetic and developmental analysis, and the results indicate that the *FS* gene is continuously required for the elaboration of cell shape and for correct orientation of cell walls

from very early stages of embryogenesis to the adult stage. Thus, mutations in the *FS* gene uncouple pattern formation and morphogenesis. Since cell polarity appears not to be affected in *fs* embryos, we propose that polarity plays a pivotal role in pattern formation.

MATERIALS AND METHODS

Plant strains

The wild-type strain used was the Landsberg *erecta* (*Ler*) ecotype. The *fass* (*fs*) mutants were isolated on the basis of their seedling phenotypes following EMS mutagenesis of *Ler* seeds (Mayer et al., 1991). Mapping and complementation experiments involved the marker strains W100 (*an ap1; er py; hy2 gl1; bp cer2; ms1 tt3*), W134 (*ttg ga3*) and *ttg yi*, which were kindly provided by M. Koornneef, Agricultural University, Wageningen, The Netherlands (for description of mutants, see Koornneef et al., 1983, 1987; Koornneef and Hanhart, 1983).

Plant growth conditions and genetic crosses

Plants were grown as previously described (Mayer et al., 1993). Flowers of plants to be used as female parents were emasculated with foreceps and cross-pollinated on one of the following 3 days. The anthers of male parents were either used directly after removal from flowers or after storage at 4°C for up to 10 days.

Complementation tests

All lines had been isogenised by selfing at least four times. Plants to be used as parents were tested for *fs* heterozygosity by phenotypic analysis of embryos from selfed flowers. Younger flowers from heterozygous plants were then test-crossed. Each complementation test was repeated at least once. In total, more than 50, and often up to 400, F_1 seedlings from each combination were phenotyped on agar plates.

Recombination mapping

Pollen from *fs* heterozygous plants was used to pollinate flowers of homozygous W100 and *ttg yi* plants. F_1 plants were progeny-phenotyped for *fs* heterozygosity. The F_2 progeny were scored for W100 marker phenotypes and for segregation of *fs* seedlings (F_3 generation). Recombination frequencies (RF) were determined as $RF = \mathbf{x}/(2\text{-}\mathbf{x})$ where **x** is the proportion of *fs* heterozygous plants among all F_2 plants showing the same marker phenotype. Map distances were calculated from RF using Haldane´s mapping function.

Analysis of seedling phenotypes

Analysis of seedling phenotypes was carried out as described in Mayer et al. (1993). Dark-grown seedlings were obtained by the following modification: the Petri dishes were wrapped in aluminium foil, stored at 4°C for 4 days and incubated at 25°C for up to 2 weeks. Two batches of (strong) *fs* seedlings, grown on soil, were sprayed with 10^{-5} M gibberellin (90% GA_3; Sigma) every 5 days during a period of 3 weeks.

Length measurements of seedlings

For quantitative comparisons, conditions were kept as similar as possible: seeds from parental lines and F_1 seeds of trans-heterozygous combinations were stored in paper bags for at least 3 weeks, sown on agar plates, vernalised at 4°C for at least 5 days and transferred to 25°C under constant light. Due to the large number of crosses (and resulting F_1 seedlings), the data were collected from three series of experiments as indicated by different symbols in Fig. 2.

Length and width of seedlings were measured after germination on agar plates using an Olympus VM stereomicroscope equipped with an eye-piece scale bar (10 mm, 100 divisions). The seedlings were examined at 40× magnification (one division corresponding to 0.025 mm). The length of the seedling was measured from the tip of the basal end to the cotyledonary node, i.e. where the cotyledons are inserted into the axis. The width of the seedling was measured across the cotyledonary node. The mean values of length measurements fell into three different groups, which were designated ‘strong’, ‘intermediate’ and ‘weak’ (see Fig. 2). Trans-heterozygous combinations were classified as ‘intermediate’ if their standard deviation values (taken as s_{n-1}) did not overlap with the mean value of their parents. All combinations, except one, fulfilled this criterion and were classified accordingly. The exceptional case was the trans-heterozygote from the cross $fs^{3\text{-}515} \times fs^{U93\text{-}11}$ (field 55 in Table 1); classification as ‘intermediate’ was retained since the standard deviation overlapped only slightly with the mean value of one parent.

Whole-mount preparations

In most cases embryos or seedlings were directly mounted in gum arabic/chloral hydrate/glycerol (30 g/200 g/20 g in 50ml). Whole mounts were used to study the phenotypic effects of *fs* mutations during early and late development by comparing large numbers of wild-type and mutant embryos. Several strong and weak *fs* alleles were analysed with a Zeiss Axiophot using phase-contrast or Nomarski (DIC) optics. Photographs were taken using an Agfa Pan 25 film.

For DAPI staining, young leaves were fixed in 3.7% formaldehyde in PBT buffer for 4 hours (room temperature) or overnight (4°C), washed 3× with PBT and stained with DAPI (1 μg/ml) in PBT buffer for 1 hour. Excess stain was removed by washing 3× with PBT. The leaves were mounted in glycerol and analysed with the Zeiss Axiophot equipped with an epifluorescence lamp (filter no. 487901, UV-H365).

Histological analysis

Ovules were removed from staged siliques, transferred to fixative (3.7% formaldehyde, 5% acetic acid, 50% ethanol, 0.01% Triton X-100) and fixed overnight at 4°C. Fixed ovules were processed for embedding in Spurr’s resin (Spurr, 1969). Ovules were washed twice for 20 minutes in 50% ethanol and then dehydrated in a series of increasing concentrations of ethanol (60%, 70%, 80%, 90% and twice in 100% for 20 minutes each). After the final 100% ethanol treatment Spurr’s embedding medium was added in increasing concentrations (5:1, 2:1, 1:1, 1:3 of ethanol : Spurr’s; each treatment for at least 30 minutes). Finally, two changes of Spurr´s embedding medium were applied to remove remaining traces of ethanol. The embedding medium was hardened by incubating overnight at 60°C. Sections 3-5 μm thick were made with a Reichert-Jung microtome, placed onto poly-L-lysine coated microscope slides, stained with toluidine-blue and mounted in Spurr’s medium. Photographs were taken with a Zeiss Axiophot microscope-camera using Agfa-Pan (25 ASA), Kodak Ektachrome (64 ASA) or Kodak Gold II (100 ASA) films.

RESULTS

Genetic characterisation of *fass* mutants

Twelve EMS-induced mutants were independently isolated on the basis of their characteristic seedling phenotype (Mayer et al., 1991). The seedlings from nine mutant lines were extremely compressed in the apical-basal axis and enlarged in diameter as compared to wild-type seedlings (Fig. 1A). The three other mutants showed a similar but less drastic seedling phenotype (see Fig. 1B). Crosses of two of the mutants with the remaining mutants established a single complementation group which was named *FASS* (*FS*) after the barrel shape of mutant seedlings.

Subsequent complementation analysis of all possible pair-

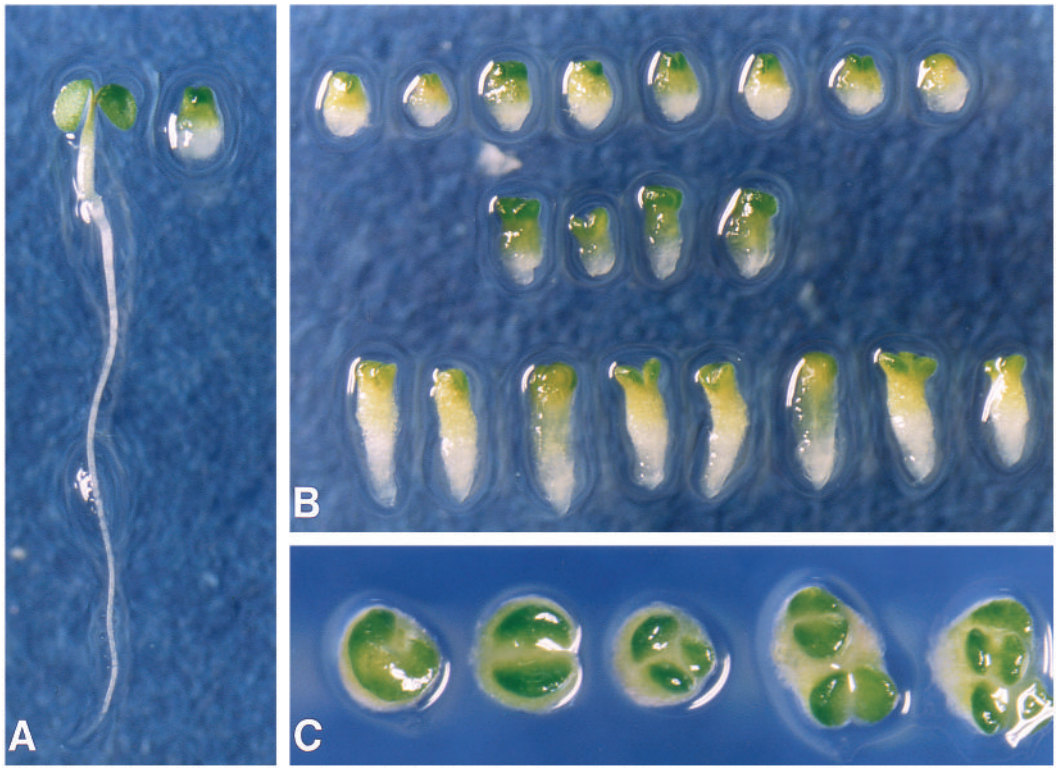

Fig. 1. (A) Seedling phenotype of the strong allele *fass*$^{R226\text{-}32}$ (right) showing extreme compression of all body elements; wild-type on the left. Colours indicate orientation in the mutant: the dark green at the apical end represents the cotyledons, the light-green region corresponds to the hypocotyl ending in a broad, white root end (9X). (B) Comparison of homozygous strong (*fs*$^{R226\text{-}32}$/*fs*$^{R226\text{-}32}$), trans-heterozygous (*fs*$^{R226\text{-}32}$/*fs*$^{U93\text{-}11}$) and homozygous weak (*fs*$^{U93\text{-}11}$/*fs*$^{U93\text{-}11}$) *fass* seedlings. (C) Cotyledon number variation of *fass* seedlings (allele *fs* $^{R2\text{-}3}$, from left to right, one to five cotyledons). Note the π and pairwise arrangement in seedlings with three or more cotyledons respectively.

wise combinations yielded three classes of phenotype among the F_1 trans-heterozygous seedlings: strong, weak and intermediate (Fig. 1B, Table 1). The strong or weak phenotypes resulted from crossing any two strong or weak alleles, respectively. Intermediate phenotypes were only observed in crosses of any strong with any weak allele. Although visual inspection of seedlings enabled classification of mutant phenotypes, there was some variability within each class so we quantitated the phenotypes by measuring the lengths of mutant seedlings (Fig. 2). Care was taken to compare F_1 trans-heterozygous seedlings with mutant seedlings from their parental lines under identical conditions (for details, see Material and Methods). The nine strong alleles and all their trans-heterozygous combinations produced very short seedlings (0.5-0.75 mm long), which showed little variability in length. The three weak alleles and their trans-heterozygous combinations yielded *fs* seedlings that were more variable in length, ranging from 1.25 mm to 2 mm. Trans-heterozygous combinations of strong and weak alleles gave seedlings of intermediate length, ranging from 0.75 to 1.25 mm. There was almost no overlap between the intermediate and the other two phenotypes, suggesting that the former reflected a distinct residual level of gene activity.

The *FS* gene was mapped against morphological markers, using the W100 and *ttg yi* strains (for details, see Material and Methods). For most markers, the recombination frequencies were close to 50%, suggesting independent segregation. By contrast, only 9% (11/124) of all *ttg* plants tested produced *fs* seedlings, which corresponds to a genetic distance of about 5 cM between the two genes. In the same region of chromosome 5 lies *GA3*, a gene required for the biosynthesis of gibberellic acid, which mutates to produce a dwarf plant phenotype (Koornneef et al., 1983). We tested for the possibility that *ga3* and *fs* might be mutations in the same gene by crossing *fs*/+ with *ga3* plants. All their F_1 progeny were normal. To exclude the possibility that *ga3* and *fs* might be complementing alleles, lines were established from F_1 plants that produced both *fs* seedling and *ga3* plants, and normal-looking plants of the next generation were progeny-tested. Some F_2 plants were recombinant since they did not produce *fs* seeds. In addition, *fs* seedlings did not respond to gibberellic acid whereas *ga3* plantlets do, by elongating their axis. Thus, the two phenotypes are caused by mutations in different, though closely linked, genes.

The *fs* seedling phenotype

Although *fs* seedlings have grossly abnormal shapes, they do not lack any of the pattern elements that characterise normal seedlings, and the overall body pattern is normal (Fig. 3). In whole-mount preparations of wild-type seedlings, the following elements of the apical-basal pattern can be recognised by specific features: shoot meristem, cotyledons, hypocotyl, root and root meristem (Fig. 3A). The cotyledons have circles of vascular strands which are bisected by a central strand. The root-hypocotyl boundary is marked by an abrupt change in birefringence and by the presence of root hairs below the boundary. The root meristem consists of compact cells which appear bright in dark-field optics. By these criteria, the same apical-basal pattern elements are present in *fs* seedlings although extremely compressed in the strong phenotype and less so in the weak phenotype (Fig. 3B,C,E). Specifically, the vascular ramifications in the cotyledons are essentially as in wild-type, indicating that the cotyledons are distally complete. Root hairs are present in *fs* at the same relative position as in wild type (Fig. 3B). Of the radial pattern elements, two appear

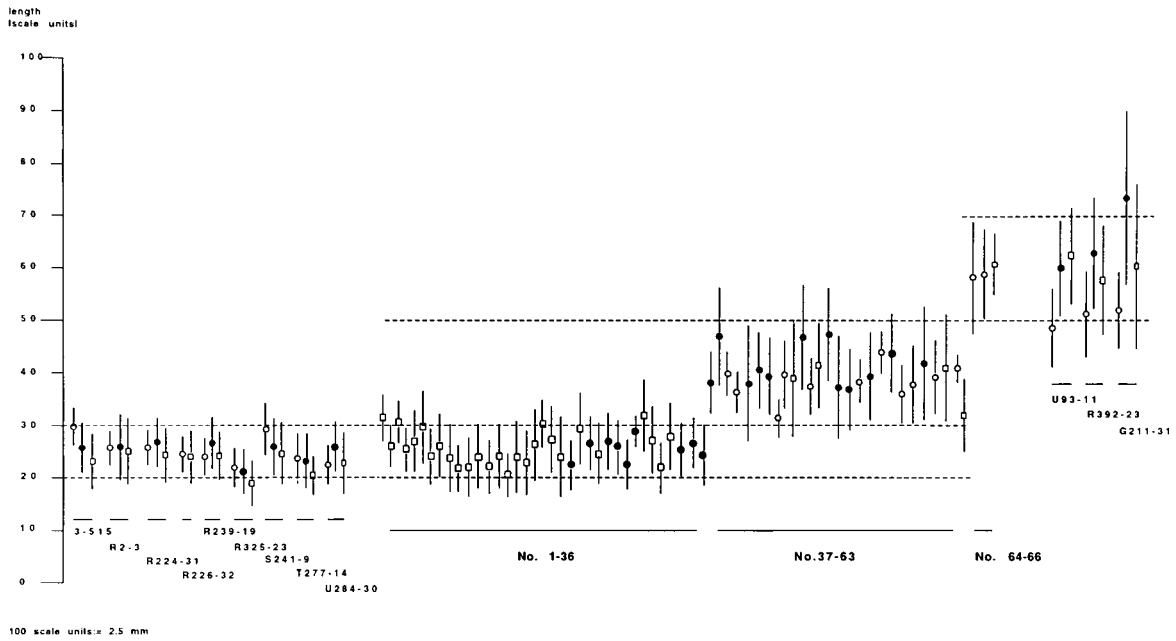

Fig. 2. Length measurements of *fass* homozygous and trans-heterozygous seedlings resulting from the crosses shown in Table 1. Data are given for measurements taken from three series of crosses. The corresponding mean values with standard deviations, s_{n-1}, are indicated by three different symbols: open circle for the first, filled circle for the second and open square for the third series (for details see Materials and Methods and text). Values for homozygous mutant seedlings, from selfing of weak or strong alleles, are grouped by alleles (horizontal bars) as indicated. Data for trans-heterozygous *fass* seedlings are grouped by combinations (see Table 1): strong/strong (No. 1-36), strong/weak (No. 37-63) and weak/weak (No. 64-66) trans-heterozygotes. Dotted lines serve as orientation on the scale.

Table 1. Complementation matrix of *fass* mutant

	3-515	R2-3	R224-31	R226-32	R239-19	R325-23	S241-9	T277-14	U284-30	G211-31	R392-23	U93-11
3-515	**X**	S 1 –/9	S 2 22/–	S 4 –/23	S 7 16/19	S 11 28/–	S 16 –/24	S 22 –/18	S 29 27/–	IN 37 20/–	IN 46 20/18	IN 55 29/–
R2-3		**X**	S 3 54/–	S 5 16/–	S 8 18/20	S 12 21/–	S 17 28/24	S 23 23/–	S 30 15/–	IN 38 23/–	IN 47 19/–	IN 56 25/–
R224-31			**X**	S 6 19/31	S 9 22/36	S 13 25/–	S 18 –/23	S 24 –/26	S 31 4/21	IN 39 28/–	IN 48 24/–	IN 57 30/–
R226-32				**X**	S 10 –/20	S 14 19/24	S 19 –/22	S 25 24/–	S 32 –/21	IN 40 22/–	IN 49 –/17	IN 58 24/19
R239-19					**X**	S 15 –/19	S 20 –/15	S 26 –/19	S 33 17/–	IN 41 29/–	IN 50 28/–	IN 59 21/–
R325-23						**X**	S 21 –/15	S 27 –/25	S 34 22/–	IN 42 14/–	IN 51 29/–	IN 60 –/24
S241-9							**X**	S 28 –/17	S 35 22/–	IN 43 31/–	IN 52 26/–	IN 61 –/20
T277-14								**X**	S 36 16/–	IN 44 24/–	IN 53 –/24	IN 62 –/18
U284-30									**X**	IN 45 27/8	IN 54 –/24	IN 63 17/20
G211-31										**X**	W 64 25/31	W 65 30/–
R392-23											**X**	W 66 –/26
U93-11												**X**

Parental alleles are given on the left and at the top. Strong and weak alleles are separated by double lines. For easy reference in the text every combination is given a number in the upper right corner of the respective field (total 66 fields). The phenotypes of F_1 seedlings were classified for each combination as strong (S), intermediate (IN) or weak (W) (based on length measurements, see text and Fig. 2). Numbers indicate percentages of *fass* seedlings from each cross. Slashes separate the results of reciprocal crosses (ca. 20% of the cases). Values to the left or right of the slash refer to the female parent shown on the left or at the top, respectively.

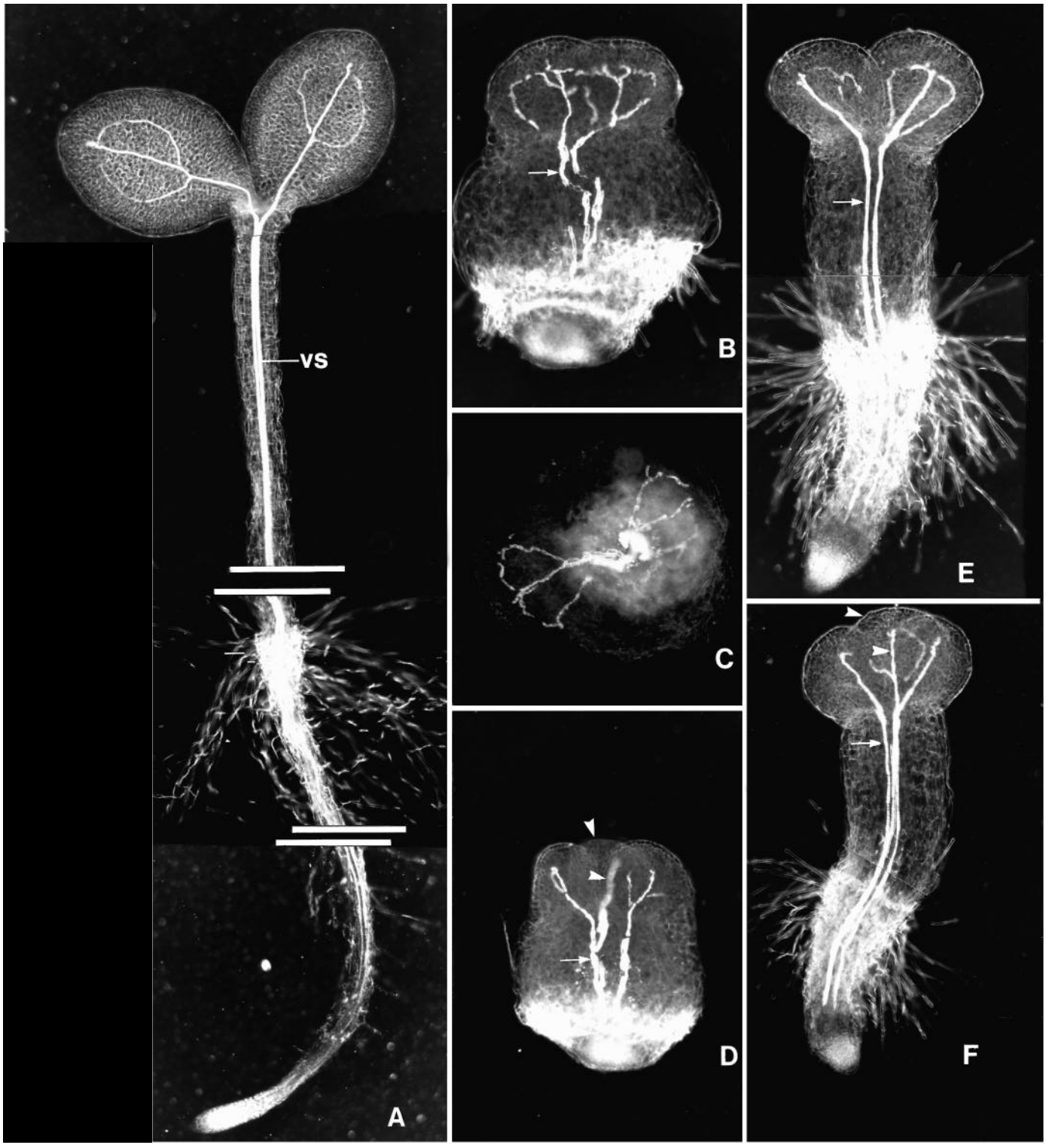

Fig. 3. Whole-mount preparations of wild-type and *fs* seedlings showing internal structures. (A) Wild-type with a simply reticularised vascular system in the cotyledons. The vascular strands run tightly together through the center of the hypocotyl into the root. The upper end of the root is marked by root hairs and a more intense birefringence, which is also seen in the apical and especially the basal end (root meristem zone). The wild-type is not shown in full length (interruptions indicated). (B-F) *fs* seedlings (same stage and same magnification as the wild type). The main features of the wild type are also visible in *fs* seedlings (note the epidermal layer in the cotyledons). Vascular strands are clearly separated from each other in the hypocotyl and the root (small arrows). (B-D) Strong *fs* phenotype. Dicotyledoneous seedlings in B (side view) and C (top view): vascular system is sometimes interrupted. (D) *fs* seedling with three cotyledons: arrowheads point to the supernumerary vascular strand and cotyledon respectively. (E,F) Weak *fs* phenotypes: compare with B and D. Dark-field optics.

bright in dark-field optics: the epidermal layer and the centrally located, closely spaced vascular strands (Fig. 3A). In *fs* seedlings, the epidermal layer is best seen in the cotyledons (Fig. 3B,E). The two vascular strands running the length of the axis are separated in both weak and strong phenotypes; in the latter, the vascular strands are abnormally shaped and often interrupted (Fig. 3B,C). Some mutant seedlings have three cotyledons (see below); in these cases, there are also three, rather than two, vascular strands in the hypocotyl (Fig. 3D,F).

Histological sections of strong *fs* seedling phenotypes revealed regional differences in cell size and cell shape (Fig. 4A). The hypocotyl and the embryonic root lack the regular arrangement of cell files found in the wild type and show irregularly enlarged cells both in the epidermis and in the ground tissue. Narrow cells in the centre may represent the vascular tissue (Fig. 4B). Near the lower end of the seedling axis, large surface cells form bulges, which may correspond to incipient root hairs (Fig. 4C). The enlarged root pole is covered by layers of compressed cells, presumably the central portion of the root cap, which overlie the small, densely cytoplasmic cells of the root meristem; no quiescent centre can be recognised by anatomical criteria (Fig. 4D). The cotyledons consist, by and large, of normal-sized cells. The epidermal layer is clearly recognisable, and stomatal guard cells are well-differentiated (Fig. 4E,F). Narrow cells in the centre seem to correspond to the vascular cells found in wild-type cotyledons (Fig. 4F). The shoot meristem is represented by a group of small, densely cytoplasmic cells bulging between the bases of the cotyledons (Fig. 4G). Thus, the various tissues and organs are present in *fs* seedlings, although cell shapes and arrangements are highly abnormal.

The abnormal shapes and arrangements of cells are probably responsible for the compression of the seedling axis. We tested whether this defect could be reversed or mitigated by growing *fs* seedlings in the dark since light is known to have an inhibitory effect on stem elongation. We compared the seedling phenotypes of the three weak and two strong alleles in the light and in the dark, by measuring their lengths and their widths (Table 2). In the strong alleles, the seedlings were almost as wide as long in the light and they elongated very little in the dark. By contrast, the weak-phenotype seedlings elongated markedly in the dark although much less than did wild-type seedlings. Among the weak alleles, $fs^{U93\text{-}11}$ was less responsive than the other two and gave broader seedlings both in the light and in the dark, suggesting that this allele is slightly stronger (see also below).

Abnormal cotyledon numbers in *fs* seedlings

Mutant seedlings, in spite of their altered shape, essentially showed the overall body pattern seen in wild-type seedlings,

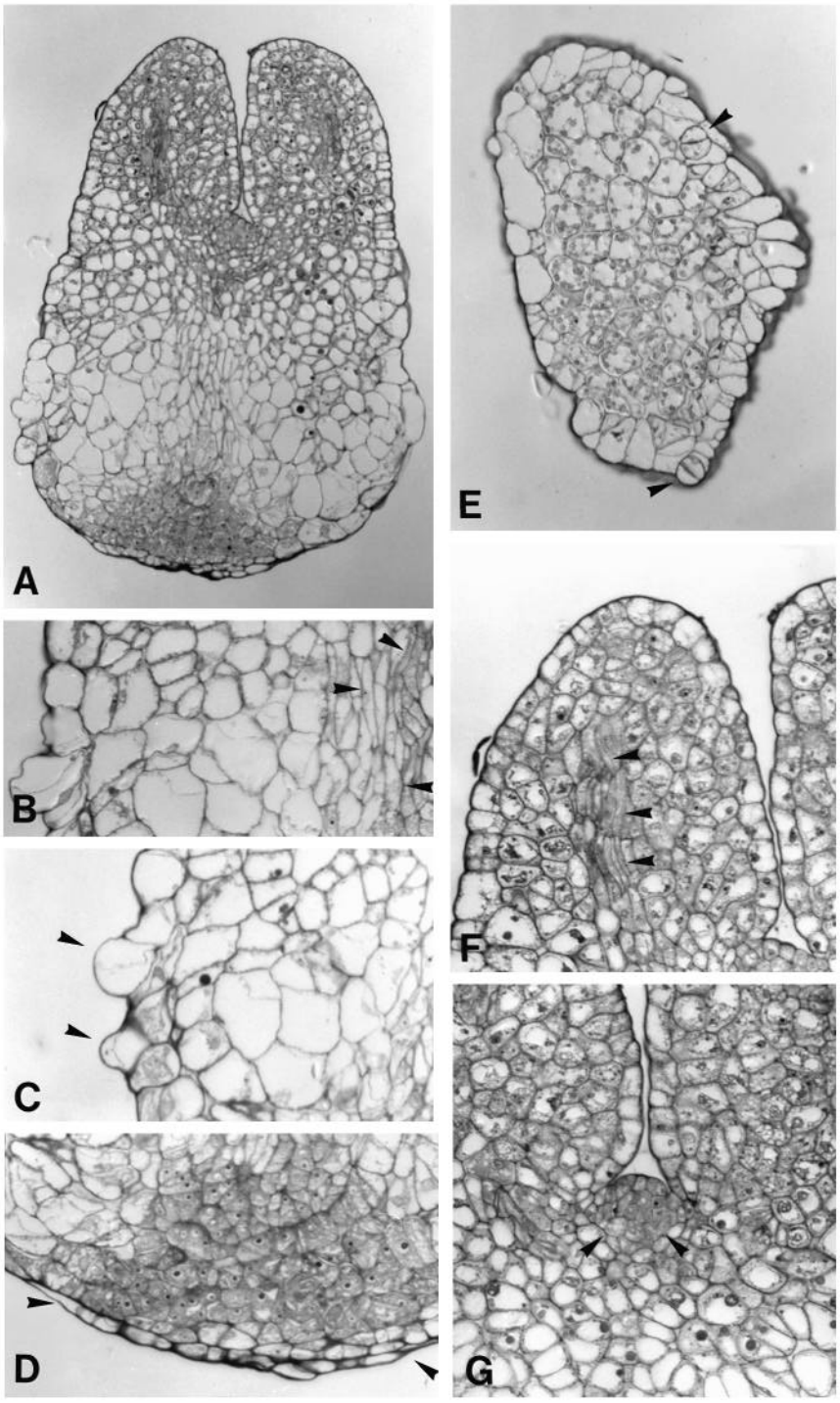

Fig. 4. Histological sections of young *fs* seedlings. (A) Longitudinal section. (B-G) Higher magnification: sections of different seedling regions, specific features marked by arrowheads (not all taken from the same section as in A). (B) Hypocotyl: elongated cells (vascular precursors) in the center. (C) Upper root region: putative root hair cells. (D) Root end: root cap (darkly stained), cell walls abutting a group of densely cytoplasmic cells, which include the root meristem. (E) Cotyledon: stomatal cells (tangential section). (F) Cotyledon: elongated cells (including vascular precursors). (G) Shoot apical meristem zone.

Table 2. Width and length measurements of *fass* seedlings in the light and the dark*

Allele		Width of *fass* seedlings Light(l) (scale units†)	Dark(d) (scale units†)	l/d	Length of *fass*-seedlings Light (l) (scale units†)	Dark (d) (scale units†)	l/d
R224-31		24.0±4.4	23.1±3.6	1.04	31.8±5.3	36.7±9.0	0.87
R226-32		25.2±2.9	23.8±3.4	1.06	28.0±5.0	32.4±6.0	0.86
G211-31		17.2±1.8	15.4±1.1	1.12	60.5±5.6	120.2±21.6	0.50
R392-23		16.8±1.7	14.0±1.6	1.20	62.3±6.8	118.5±23.1	0.53
U93-11		20.8±2.6	17.8±1.6	1.17	64.9±9.4	98.5±17.3	0.66
	Hypocotyl				68.2±7.1	367.6±64.0	0.19
Wt	Root				410.4±46.0	210.4±40.4	1.95
	Total	7.8±0.9	6.9±0.7	1.13	478.6±53.1	578.0±104.4	0.83

*Taken 5 days after germination.
†100 scale units=2.5 mm.
The double line separates strong *fass* alleles, weak *fass* alleles and wild type (wt) respectively.

Table 3. Number of cotyledons in *fass* plants

Allele	No. of seedlings scored	*fs* seedlings (%)	Monocot.* %	Dicot.* %	Tricot.* %	π-arrangement† (%)	Tetracot.* %
3-515	1826	410 (22.5)	<1	88	11	(58)	<1
R2-3	1885	413 (21.9)	<2	81	16	(57)	<2‡
R224-21	1867	438 (23.5)	<1	82	17	(73)	<1
R226-32	2194	491 (22.4)	<1	89	10	(49)	n.d.
R239-19	1643	366 (22.3)	<1	83	15	(73)	<2
R325-23	1742	437 (25.1)	<2	82	16	(61)	<1
S241-9	1457	327 (22.4)	<1	80	19	(61)	<1
T277-14	1924	397 (20.6)	<2	84	13	(62)	<2
U284-30	1823	466 (25.6)	<2	84	14	(66)	<1
G211-31	2622	524 (20.0)	1	93	6	(59)	n.d.
R392-23	2607	603 (23.1)	<2	90	8	(38)	<1
U93-11	2912	791 (27.2)	<1	84	15	(61)	<1

*Refers to *fass* seedlings.
†% of *fass* seedlings with three cotyledons, two arranged as a pair and the third positioned at 90° relative to the pair (see text and Fig. 1C).
‡Also rare pentacotyledonous seedlings observed (see Fig. 1C).
The double line separates strong and week alleles.

except for one recurrent deviation. Regardless of the *fs* allele, cotyledon number was abnormal in a large proportion of mutant seedlings (Table 3). The vast majority of exceptional seedlings had three cotyledons, some had only one cotyledon and others had four or even five cotyledons. Supernumerary cotyledons correlated well with the widths of mutant seedlings. The strong alleles produced 10-19% mutant seedlings with three cotyledons as compared to 15% for the weak allele $fs^{U93\text{-}11}$ and 6-8% for the other two alleles (Table 3). The allele $fs^{U93\text{-}11}$ also caused a larger seedling width than the other weak alleles did, suggesting that the increased number of cotyledons is a secondary defect (see Table 2).

The supernumerary cotyledons were arranged in specific ways (Fig. 1C). In tricotyledonous seedlings, two different configurations were observed. Either all three cotyledons were spaced evenly, forming a triangle; or, more often, two cotyledons facing each other abutted the third at a right angle, thus forming a π-shaped arrangement. Nearly all seedlings with four cotyledons (and those with five) had the cotyledons grouped in pairs which, in turn, were variably positioned relative to each other.

Development of *fs* embryos

It had been shown previously that *fs* embryos exhibit abnormal arrangements of cell walls at the heart stage (Mayer et al., 1991). We analysed, in histological sections, *fs* embryos from very early stages in order to determine when their development starts to deviate from normal and how the extreme compression of the seedling axis comes about. We compared mutant with wild-type embryos of approximately the same stages (Fig. 5). Since the cellular features that are used for staging wild-type embryos (Jürgens and Mayer, 1994) are lacking in *fs* embryos, we tentatively identified equivalent stages by size, approximate cell numbers and the corresponding developmental stage of the endosperm (Mansfield and Briarty, 1990). To facilitate the description of *fs* embryogenesis, we will first briefly describe wild-type embryo development (for details, see Mansfield and Briarty, 1991; Jürgens and Mayer, 1994).

Very early development of wild-type embryos is characterised by a sequence of precise cell divisions, resulting in the octant-stage embryo (Fig. 5A,C). The zygote divides asymmetrically to give a small apical and a large basal cell. These two cells and their progeny differ in staining intensity: the apical cell, which is densely cytoplasmic, stains more darkly, while the basal cell, which has a large vacuole stains more lightly (Mansfield and Briarty, 1991). The apical cell undergoes three rounds of cell division, two vertical and one horizontal, to generate the proembryo of the octant stage, which is

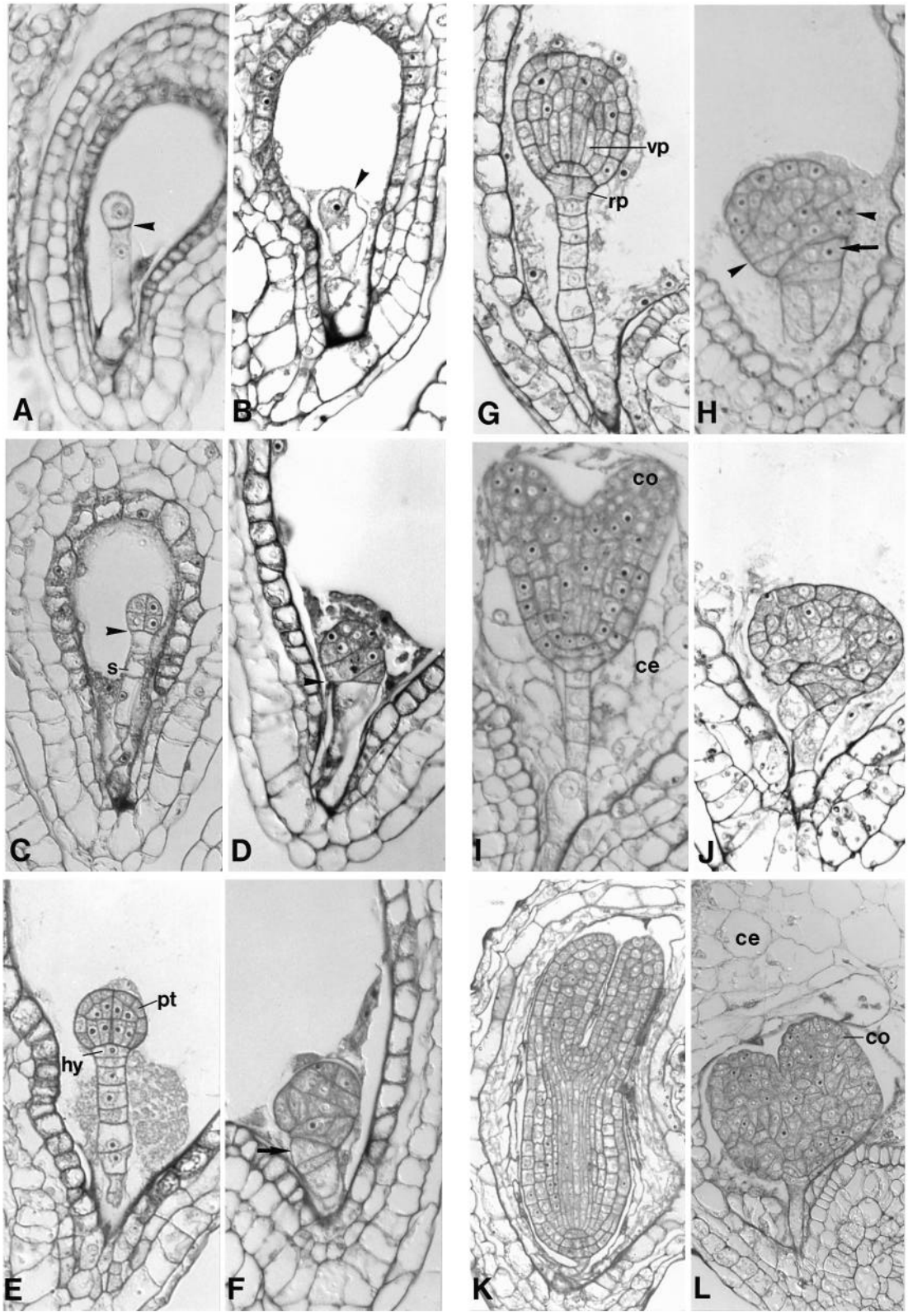

Fig. 5. Embryo development of *fass* in comparison to wild type (comparable stages, wild-type: A, C, E, G, I, K, *fs*: B, D, F, H, J, L). All sections same magnification except K and L (approx. half the magnification of A-J). (A,B) One cell stage: the apical cell is densely cytoplasmic and darkly stained. The first cell wall (arrowhead) is horizontal in A and oblique in B (note the nuclei with nucleoli; approx. 400×). (C,D) Octant cell stage: the cell wall separating suspensor (s) and proembryo is horizontal (arrowheads, note highly vacuolated suspensor cells). (E,F) Early globular stage: the epidermis primordium (protoderm, pt) and the hypophysis (hy) are recognisable in E but not in F. A cell at the equivalent position of the hypophysis is marked in F. (G,H) Late globular stage: root primordium (rp) and vascular primordium (vp, elongated cells). Irregularly arranged cells (arrow) in H may correspond to the rp. Irregular surface layer in H (note cells extending into the embryo, arrowheads). (I,J) Heart stage: cotyledon primordia (co) and cellular endosperm (ce) visible in I; the latter has been probably damaged in J. (K,L) Torpedo stage: cotyledon primordia (co) are distinguishable in *fs*. Note also cellular endosperm (ce).

composed of an upper and a lower tier, each of four cells. The basal cell produces, by repeated horizontal divisions, a file of 7-9 cells of which all but the uppermost one form the suspensor (Fig. 5E,G). The uppermost derivative of the basal cell, which also stains lightly, becomes the hypophysis which joins the proembryo. Region-specific changes transform the octant stage into a globular embryo. Initially, tangential cell divisions produce the primordium of the epidermis (protoderm) as an

outer cell layer (Fig 5E). Slightly later, the innermost cells can be recognised as the primordium of the vascular tissue (Fig. 5G). Oriented cell divisions of the lower-tier derivatives result in elongation of the embryonic axis, and the hypophysis generates, by a series of highly regular divisions, the incipient root primordium. Within a short period of time, the symmetry of the developing embryo changes from radial to bilateral. Rapid cell divisions in the apical region of the late-globular embryo initiate the two cotyledonary primordia, giving the embryo a heart shape (Fig. 5G,I). At the basal pole, the derivatives of the hypophysis are now arranged in three cell layers (Fig. 5I). The late-heart stage embryo displays the primordia of the main tissues and structures of the seedling. The endosperm, which has been nuclear, cellularises during this stage (Fig. 5I). During the torpedo stages, the embryo mainly grows by elongation of both the cotyledons and the hypocotyl which now consist of several layers of cell files (Fig. 5K). Bending of the cotyledons, which eventually reach the level of the root pole, and enlargement of the shoot meristem and the root primordium complete the growth of the embryo.

Very early embryos from *fs*/+ plants were inspected for deviation from the regular pattern of cell division (Fig. 5B,D). As in the wild type, *fs* zygotes divided asymmetrically, giving two daughter cells of different sizes and staining intensity. Occasionally, the cell wall separating the apical from the basal daughter cell was oriented obliquely, instead of perpendicular to the axis, suggesting that the *FS* gene normally acts before the division of the zygote (compare Fig. 5A and B). However, the majority of mutant embryos could not be recognised by the abnormal orientation of the first cell wall, neither at the one-cell stage nor at later stages when other features enabled recognition of *fs* embryos. The apical cell also tended to divide irregularly, and by the octant-stage, *fs* embryos looked abnormal. The basal cell often produced fewer derivatives which were separated by horizontal or oblique cell walls (Fig. 5B,D). The shorter suspensor frequently failed to push the developing embryo into the cavity of the embryo sac.

In *fs* embryos, the octant cells did not produce a distinct outer cell layer (Fig. 5F), and the innermost cells of the mid-globular embryo did not show the morphological features of the vascular primordium (Fig. 5H). The derivatives of the 'lower tier' divided more or less at random, and the positional equivalent of the hypophysis produced a cell group that, by morphological criteria, did not resemble the incipient root primordium (Fig. 5H). The suspensor cells, which were recognisable by their light staining, had irregularly oriented cell walls (Fig. 5H). The growing embryo merely increased in size but did not change its shape and thus, by the time the wild-type embryo had turned heart-shaped, the mutant embryo resembled an oversized globular embryo.

The continuing irregular cell divisions of *fs* embryos made the cells at the surface form a sloppy layer; although discernable as a layer, the surface cells varied in size and shape (Fig. 5J). The internal cells as well remained disorganised with respect to cell wall orientation. The cells in the centre did not show morphological features of vascular precursors except that in some sections, they appeared narrower than the surrounding cells of the putative ground tissue. From the late-heart stage, bulges representing cotyledonary primordia developed from the apical surface of the embryo (Fig. 5L). The cells between their bases as well as cells at the basal pole of the embryo exhibited features of meristematic cells: they were small, rich in cytoplasm and less vacuolated than other cells. Some heart-stage embryos had a basally located cell group that was marked off against the main cell mass. This cell group may have been derived from the hypophysis (Fig. 5F,H); by size and vacuolation, these cells resembled suspensor cells. In contrast to the wild type where the derivatives of the hypophysis are arranged in a highly regular manner, the basal cell group of *fs* embryos showed variable cell wall orientations, sometimes horizontal, in other cases oblique or vertical (Fig. 5F,H,J). This variability made it difficult to recognise the basal cell group unambiguously as the incipient root primordium. There were fewer suspensor cells than in wild-type and they differed from embryonic cells by staining lightly (Fig. 5H,J,L). The endosperm of developing *fs* seeds was normal. Nuclear endosperm was associated with the embryo until the heart-stage and then replaced by cellular endosperm (compare Fig. 5J with L). Continuing irregular cell divisions resulted in a mature embryo that was extremely compressed in the apical-basal axis but considerably enlarged radially. Although the cotyledons did not bend, the embryo filled up the entire seed at maturity.

Postembryonic development of *fs* mutants

Mutant *fs* seedlings, though short and thick, were not lethal and developed into adult plants that were extremely dwarfed, even in comparison to *ga3* plants (Fig. 6A). Weak *fs* alleles completed postembryonic development in a regular manner whereas strong alleles rarely did. Initially, a thick root emerged from the root pole of the seedling, and the shoot meristem produced primary leaves which appeared in the region between the stunted cotyledons (Fig. 6B). Subsequent activity of the shoot meristem generated organs that were compressed in their proximal-distal axis. The extent to which organ shape was altered reflected the relative strength of the *fs* allele. For example, the leaf blade seemed to be immediately attached to the stem in strong alleles while a thick petiole was recognisable in weak alleles (compare Fig. 6C with G,H). In some *fs* plants, different leaf arrangements were observed, which may be correlated with the different spatial relations of the cotyledons described above (Fig. 6G,H).

The *fs* phenotype was also expressed at the cellular level, which was most clearly seen in the leaf epidermis. Epidermal cells, which are characteristically interdigitated in wild type, appeared roundish in *fs* plants (compare Fig. 6K with L). Furthermore, leaf trichomes, which normally have three branches positioned in a very specific way (Fig. 6I; Hülskamp et al., 1994) were abolished in strong *fs* alleles; weak alleles were associated with unbranched trichomes (Fig. 6J).

The flowers of adult *fs* plants showed the normal number and position of organs (compare Fig. 6D with E). However, flower organs were compressed in their proximal-distal axis (Fig. 6F). This effect was particularly pronounced for stamens and pistils, and the pistils contained thickened ovules. Regardless of the allele strength, the *fs* plants examined did not set seed. These results suggest that the *FS* gene is required during post-embryonic development in a similar manner as in embryogenesis.

DISCUSSION

The analysis of pattern formation in the *Arabidopsis* embryo

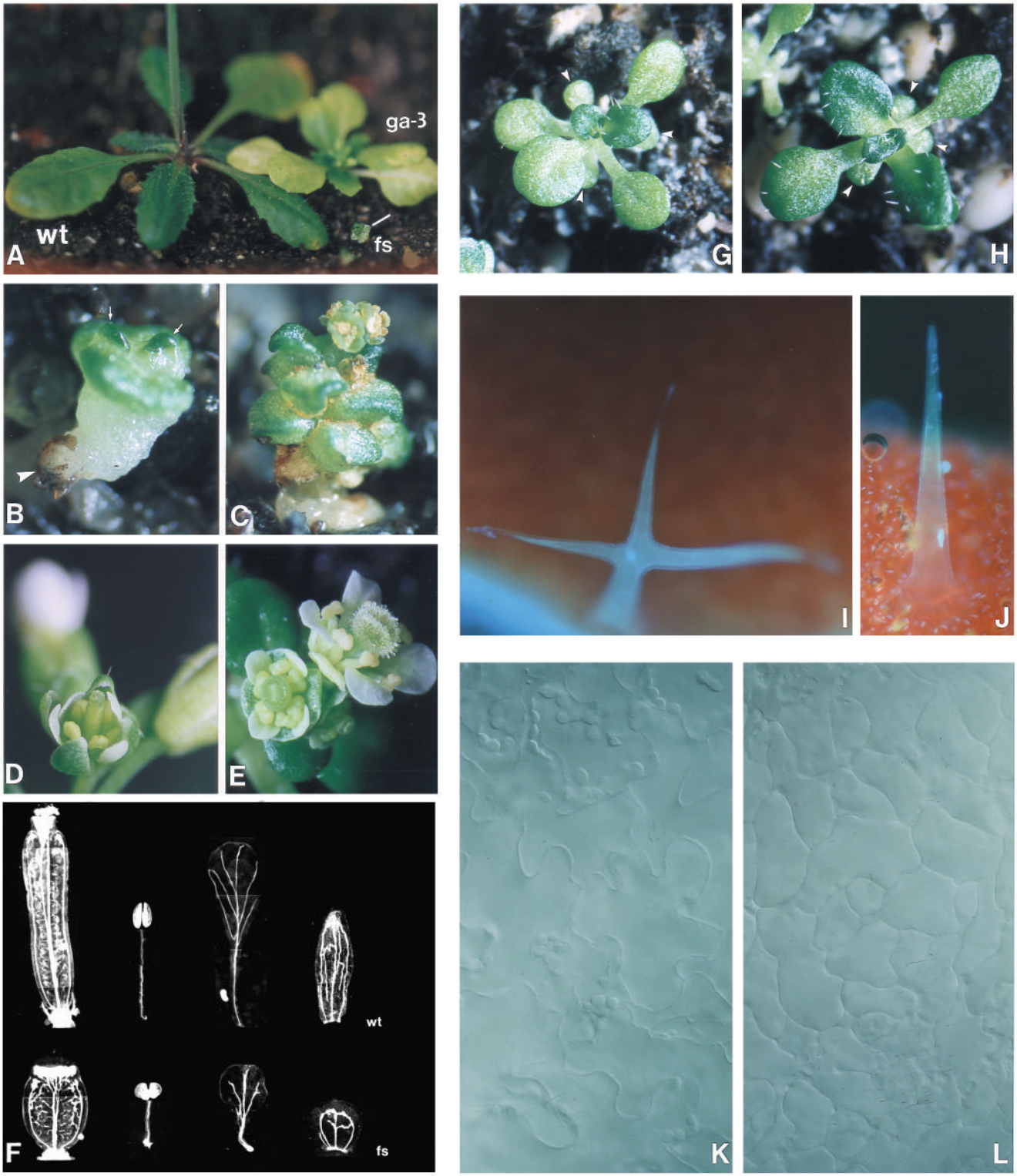

Fig. 6. Postembryonic development of *fs*. (A) Comparison of adult wild-type (wt), dwarf mutant (*ga-3*) and *fs* plants (*fs*, marked by a white line). (B) Strong-phenotype seedling with emerging root (arrowhead) and primary leaves (arrows). (C) Adult plant with flowers (strong allele). (D,E) Comparison of wild-type (D) and *fs* flowers (E, weak allele). (F) Comparison of wild-type (upper row) and *fs* (lower row) flower organs (from left to right: pistil, stamen, petal and sepal). (G,H) Tricotyledoneous *fs* plants (weak allele); G: cotyledons evenly spaced (arrowheads), and three staggered primary leaves; H: cotyledons irregularly spaced, two primary leaves. (I,J) Leaf trichome: wild type (I), *fs* trichome (J; weak allele); DAPI-stain (note the densely stained nuclei). (K,L) Epidermal cell layer: wild-type (K) and *fs* (L; weak allele).

is facilitated by the regularity of cell divisions that characterise the early stages of embryogenesis (Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). The primordia of seedling structures which become recognisable in the heart-stage embryo can thus be traced back to cell groups in the very early embryo. Oriented cell divisions and changes in cell shape are associated with the formation of seedling primordia as well as with their elaboration during later stages of embryogenesis. These cell activities are also responsible for generating seedling body shape and may thus be covered by the term 'morphogenesis'. Although in wild-type development, pattern formation and morphogenesis are intimately coupled, mutations in the *FASS* gene affect morphogenesis but not pattern formation, suggesting that the regularity of cell division is not instrumental in pattern formation in the *Arabidopsis* embryo. In the following, we address two questions: (1) what is the primary effect of the *FASS* gene in development, and (2) what can be inferred from the *fass* phenotype about pattern formation in the *Arabidopsis* embryo?

The primary effect of the *FASS* gene in development

Mutations in the *FS* gene cause a strong compression of the longitudinal axes of the plant body and its organs throughout development. By phenotypic criteria, the mutant alleles form two distinct classes, strong and weak. Any trans-heterozygous combination of weak with strong alleles gave a novel, intermediate, phenotype which did not overlap the other two classes. This result suggests that the strong phenotype reflects the lack of function of the gene and that the intermediate phenotype results from half the residual gene activity that is associated with the weak alleles. A likely interpretation is that the *FS* gene normally acts in a quantitative manner, with the amount of functional product directly determining the biological effect. If the expression of the *fs* phenotype reflects residual gene activity, why did we not isolate alleles that would produce intermediate phenotypes on their own? The answer might be that the weak *fs* alleles represent a particular class of mutations which make less product, rather than less active product. This idea is consistent with the lack of partial complementation between weak alleles, which stands in contrast to the interaction between specific classes of *gnom* alleles (Mayer et al., 1993).

That the strong alleles probably lack *FS* gene activity is also supported by other observations. All strong alleles show essentially the same extreme phenotype which varies little. Furthermore, the *fs* alleles were isolated in a mutagenesis screen that achieved saturation by statistical criteria and the number of *fs* alleles was above the average allele frequency (Jürgens et al., 1991; Mayer et al., 1991). We therefore consider it unlikely that stronger *fs* alleles might mutate to embryonic lethality and would thus not have been found. In summary, the strong alleles probably lack gene activity but direct evidence will only come from the molecular analysis of the *FS* gene.

The *FS* gene is probably required throughout development and in all cell types. Lack of active *FS* gene product causes different cellular phenotypes, depending on the tissue as well as the developmental stage. In the *fs* embryo, cell walls are oriented abnormally, and continuing cell divisions result in grossly abnormal cell shapes and arrangements. At the seedling stage, the postmitotic cells of the hypocotyl do not elongate properly, even if challenged by the absence of light. In the leaf, the epidermal cell layer does not acquire the characteristically interdigitated pattern of lobed cells, and the trichome cells, which normally undergo extensive morphogenesis (Hülskamp et al., 1994), fail to grow out of the leaf surface in plants with strong alleles or fail to branch if *FS* activity is reduced. All these cellular processes, orderly division, elongation and morphogenesis, require specific changes in the organisation of the cell. Such changes may be brought about by the reorientation of the cytoskeletal microtubules and the cellulose microfibrils in the cell wall (for review see Staiger and Lloyd, 1991). For example, the arrangement of microtubules changes during elongation of the *Arabidopsis* zygote before the asymmetric division occurs (Webb and Gunning, 1991), and similar observations have been made on elongating cells of the pea hypocotyl (Duckett and Lloyd, 1994). Cell shape is also influenced by light and plant hormones such as auxin, ABA and GA_3 which alter the orientation of cortical microtubules (Seagull, 1989; Sachs, 1991; Sakiyama-Sogo and Shibaoka, 1993). The strong *fs* phenotype is not markedly influenced by light or by GA_3, suggesting that the primary defect resides within the cells. This is supported by the rare occurrence of twin embryos of which one was mutant and the other was wild-type (our unpublished observations). Considering the variety of abnormal cell activities in *fs* mutants, it is tempting to speculate that the primary function of the *FS* gene product may be required for the proper organisation of the cell, either for its maintenance or for mediating changes in response to spatial cues.

Pattern formation and morphogenesis in embryogenesis

Unlike many other plant species (Johri et al., 1992), *Arabidopsis* and other crucifers display a regular pattern of cell division in early embryogenesis (Schulz and Jensen, 1968; Tykarska, 1976, 1979; Mansfield and Briarty, 1991; Jürgens and Mayer, 1994). This regularity enabled primordia of seedling structures to be recognised from early embryogenesis and facilitated the identification of precursors for the meristems of the root and the shoot (Dolan et al., 1993; Barton and Poethig, 1993). Moreover, the mutant seedling phenotypes of two genes involved in pattern formation in the *Arabidopsis* embryo, *GNOM* and *MONOPTEROS*, were shown to correlate topographically with abnormal patterns of cell division in the early embryo (Mayer et al., 1993; Berleth and Jürgens, 1993). These results supported the view that oriented cell divisions are associated with pattern formation in the *Arabidopsis* embryo. The *fs* phenotype, however, suggests that oriented cell divisions are not instrumental for pattern formation: although the primordia of seedling structures cannot be recognised by morphological criteria at the heart stage, the body pattern of the seedling is essentially normal (we interpret the occurrence of supernumerary cotyledons as a secondary effect which is caused by an enlarged hypocotyl). Thus, *fs* embryos mimic the situation found in plant species such as cotton: the pattern of cell division in the embryo is not regular and yet the seedling has the same body pattern as in crucifers (Pollock and Jensen, 1964). It is therefore appropriate to view the regularity of cell division in the *Arabidopsis* embryo as a special case: cells at specific positions respond to pattern formation by changing cell shape or by undergoing oriented cell division. In this view, morphogenesis would be the consequence of pattern formation.

The *fs* phenotype indicates that pattern formation is not dependent on morphogenesis. Are the two processes independent of each other or is morphogenesis a consequence of pattern formation? The additive phenotype of the *fass gnom* double mutant suggests that the two processes are independent (Mayer, 1993), which raises the question of how pattern formation and morphogenesis are normally integrated to produce the wild-type seedling.

In order to identify factors influencing pattern formation in the *Arabidopsis* embryo, one might thus ask what is not changed in *fs* embryos. Although the division of the zygote produces a variably oriented cell wall and subsequent divisions of both its daughter cells can be abnormal, cell polarity is not affected since the apical and the basal cell differ in staining intensity as they do in wild-type. This is consistent with the zygote being polarised in most plant species (Rutishauser, 1969; Natesh and Rau, 1984). The *fs* seedling itself demonstrates that polarity is maintained throughout development since it represents a polarised structure. Thus, cell polarity as set up in the zygote might be essential for cells to acquire information about their relative positions which may underlie pattern formation. In some plant species, cell fate is then presaged by very regular patterns of cell division and cell arrangements, if the *FS* gene (or its homologue) is functional. In essence, the *fs* phenotype suggests that pattern formation and morphogenesis are two distinct processes in establishing the body organisation of the seedling.

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