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The role of the *monopteros* gene in organising the basal body region of the *Arabidopsis* embryo

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SUMMARY

The *monopteros* (*mp*) gene contributes to apical-basal pattern formation in the *Arabidopsis* embryo. *mp* mutant seedlings lack basal body structures such as hypocotyl, radicle and root meristem, and this pattern deletion has been traced back to alterations in the octant-stage embryo. Cells of the embryo proper and the uppermost cell of the suspensor fail to establish division patterns that would normally generate the basal body structures. The resulting absence of a morphological axis seems to be responsible for another phenotypic trait of *mp* seedlings, variable positioning of cotyledons. This relationship is suggested by weak *mp* seedling phenotypes in which the presence of a short hypocotyl is correlated with normal arrangement of cotyledons. Root formation has been induced in *mp* seedlings grown in tissue culture. This result supports the notion that the *mp* gene is required for organising the basal body region, rather than for making the root, in the developing embryo.

Key words: *Arabidopsis*, embryo, pattern formation, *monopteros* gene

INTRODUCTION

In flowering plants, the body organisation is generated by two distinct processes. Embryonic pattern formation lays down the primary organisation of the plant body, as represented by the seedling, including the meristems of the shoot and the root. The meristems then take over to produce the adult plant during postembryonic development (Steeves and Sussex, 1989). The primary body organisation of the seedling has been viewed as a superimposition of two patterns: an apical-basal pattern along the main body axis of polarity and a radial pattern perpendicular to the axis (Jürgens et al., 1991; Mayer et al., 1991). Each pattern consists of an array of morphologically distinct elements. Shoot meristem, cotyledon(s), hypocotyl, radicle (embryonic root) and root meristem are elements of the apical-basal pattern while the three major tissue types, such as epidermis, ground tissue and vascular tissue, are concentrically arranged to give the radial pattern. With the exception of the shoot meristem, the primordia of pattern elements are morphologically recognisable in the heart-shaped embryo.

How the basic body pattern is generated in the plant embryo is unknown. One way to analyse this process is a genetic approach: mutants with relevant phenotypes are isolated and characterised in order to identify genes specifically involved in embryonic pattern formation. This approach has been highly successful in favourable animal species such as *Drosophila* and *Caenorhabditis*, providing a basis for subsequent analysis of underlying molecular mechanisms (reviews by Ingham, 1988, and Schnabel, 1991). Developmental mutants resulting in various types of embryo defects have also been described in several plant species (reviews by Meinke, 1991, and Sheridan, 1988; Clark and Sheridan, 1991). The prevalent class of mutations arrest embryogenesis, and it is thus not clear from the mutant phenotypes whether embryonic pattern formation or other developmental processes are affected. A complementary approach was taken in *Arabidopsis thaliana* by focusing on the seedling phenotypes of embryonic mutants that did complete embryogenesis (Jürgens et al., 1991). A set of putative pattern genes were thus identified by mutations that affect different aspects of the seedling organisation (Mayer et al., 1991).

Four genes, including the *monopteros* (*mp*) gene, appear to be involved in apical-basal pattern formation. Mutations in the *mp* gene eliminate the basal pattern elements, hypocotyl, radicle and root meristem. Here, we present a detailed analysis of *mp* mutant embryos and seedlings, including tissue-culture experiments in which mutant seedlings were assayed for the ability to form roots. Our results suggest that the *mp* gene is primarily required in the early embryo for organising the development of the basal body region.

MATERIALS AND METHODS

Plant growth conditions, plant strains and genetic crosses

Plants were grown as previously described (Mayer et al., 1993).

The wild-type strain used was the *Landsberg erecta* (*Ler*) ecotype. For mapping the *mp* gene, we used the marker strain W100 (*an ap-1*; *er py*; *hy-2 gl-1*; *bp cer-2*; *ms-1 tt-3*) kindly provided by M. Koornneef, Agricultural University, Wageningen, The Netherlands (for description of mutants, see Koornneef et al., 1983; Koornneef, 1990). The *monopteros* (*mp*) mutants were isolated on the basis of their seedling phenotypes following EMS mutagenesis of *Ler* seeds (Mayer et al., 1991). Genetic crosses were done as previously described (Mayer et al., 1993). In complementation tests, seedling progeny (at least 60) from 3 or more cross-pollinated flowers were phenotyped on agar plates.

Recombination mapping

The *mp* gene was mapped against 9 marker mutations in the W100 strain. mp^{U21} heterozygous plants were crossed with homozygous W100 marker plants and F_1 plants heterozygous for *mp* were identified by the phenotypes of their progeny. Individual F_2 plants were phenotyped for the recessive markers and analysed for the production of *mp* mutant seedlings. The recombination frequency (RF) between *mp* and each marker was determined as $RF=x/(2-x)$, with x representing the proportion of *mp* heterozygous plants among all F_2 plants homozygous for this marker. Map distances were calculated from the recombination frequencies using the mapping function. For 8 markers, the x values were close to 66% (58-71%), indicating free segregation whereas only 33% (11/33) of the F_2 plants homozygous for *an* were also heterozygous for *mp*. From this x value, a recombination frequency (RF) of 20% and a map distance of 25.5 cM between *an* and *mp* were calculated.

Phenotypic analysis

For the analysis of seedling phenotypes, seeds were plated on medium containing 0.3% agar and 50 μg/ml ampicillin, stored for several days at 4°C in the dark to break dormancy and then germinated in the light (constant illumination, 24°C). Seedling phenotypes were usually analysed about 7 days after germination, using an Olympus stereoscope and an Olympus Ti-4 camera attached to it.

Whole-mount preparations were used for microscopic analysis of embryo and seedling phenotypes. Embryos or 7-day old seedlings were fixed for 1-4 hours in ethanol/acetic acid (6:1) at room temperature. After several washes in 100% ethanol and finally in 70% ethanol, embryos or seedlings were mounted in a mixture of chloralhydrate/glycerol/water (8:1:2) and cleared for about 1 hour at room temperature. Whole-mount preparations could be stored at room temperature for at least several weeks. Whole-mount preparations were used to detect specific deviations of *mp* mutants from wild-type embryo development, by analysing the progeny of *mp* heterozygous and wild-type plants from the same lines. Large numbers of wild-type and mutant embryos were thus compared in order to assess the specificity of defects before embryos were analysed in histological sections.

For histological analysis of embryos, ovules were collected from siliques of appropriate ages, washed in PBT (50 mM sodium phosphate pH 7.2, 0.15 % Tween 20) and fixed in a mixture of 4% paraformaldehyde, 0.25% glutaraldehyde in 50 mM sodium phosphate (pH 7.2) at 4°C for 1 or 2 days. Fixed ovules were processed for embedding in Spurr's resin (Spurr, 1969). Ovules were dehydrated in a series of increasing concentrations of acetone. After several washes in 100% acetone, Spurr's embedding medium was added in increasing concentrations over a period of 4 hours and finally exchanged several times against fresh embedding medium to remove all traces of acetone. After hardening (overnight at 60°C), sections, 4.5 μm thick, were cut with a Reichert-Jung microtome, placed onto polylysine-coated microscope slides, stained with toluidine-blue and mounted in Spurr´s medium. Photographs of sections were taken with a Zeiss Axiophot microscope-camera, using an Agfa-Pan film (25 ASA).

Tissue culture

Seeds from mutant lines were surface-sterilised in 5% calcium hypochloride/0.15% Tween 20 for 8 minutes, washed at least 4 times in water and plated on medium containing 0.3% agar and 50 μg/ml ampicillin. After germination, the seedlings were kept on these plates for 7 days (constant illumination, 24°C) to allow for the completion of cell expansion. Mutant seedlings were manipulated with foreceps in either of two ways, depending on the allele under investigation.

Mutant seedlings with weak alleles were wounded immediately below the cotyledons; bisection was attempted but not always achieved. The apical part, including the shoot meristem and the cotyledons, was placed on fresh medium containing 0.3% agar, 50 μg/ml ampicillin and half-strength Murashige-Skoog basal medium (Murashige and Skoog, 1962), supplemented with 0.5% sucrose and 3 μg/ml indole butyric acid (IBA). The plates were sealed with parafilm and kept under constant illumination at 24°C for several weeks.

Mutant seedlings with strong *mp* alleles were dissected and pieces of tissue (mostly cotyledon, occasionally including meristematic tissue) were cultured on a medium of similar composition as above, but supplemented with 0.2 μg/ml kinetin, in addition to 0.3 μg/ml IBA. After three weeks, explants were transferred to fresh medium containing 0.3 μg/ml IBA as the only hormone and scored for root development at weekly intervals.

RESULTS

Genetic analysis of *monopteros* mutants

A large-scale screen for putative pattern mutants in *Arabidopsis* yielded a group of mutants with very similar seedling phenotypes: the basal pattern elements, root and hypocotyl, were deleted, and the number and relative position of the cotyledons were variably altered (Fig. 1A). This group of mutants was named *monopteros* (*mp*) after the frequent occurrence of only a single cotyledon in mutant seedlings, and complementation analysis established one gene with 11 mutant alleles (Mayer et al., 1991). Two additional mutants with similar phenotypes, which had initially shown aberrant segregation ratios, also proved to be *mp* alleles. All mutant alleles were tested in reciprocal crosses with each of three reference alleles ($mp^{TÜ399}$, mp^{T1} and mp^{U21}), and no complementation was observed. Upon outcrossing to wild-type, normal segregation ratios close to 25% were observed for all 13 alleles. Thus, the *monopteros* phenotype appears to result from recessive mutations in a single nuclear gene that is normally active in the embryo but has no apparent function in the gametophytes. The *mp* gene was mapped to the first chromosome, approximately 25 cM from the *angustifolia (an)* gene (see Materials and methods). This initial mapping result was confirmed by localising the *mp* gene on RFLP maps as a first step towards molecular cloning (Chang et al., 1988; Nam et al., 1989; C. Hardtke and T.B., unpublished data).

Two groups of *mp* alleles were distinguished by morphological criteria: most notably, one group of 3 alleles (mp^{G92}, mp^{U348} and mp^{T366}) occasionally produced mutant seedlings with a short hypocotyl whereas this was never observed for the other 10 alleles, which were therefore classified as strong (for details, see later). Trans-heterozygotes of weak and strong alleles showed typical features of the weak phenotype, suggesting that the weak alleles have residual *mp*

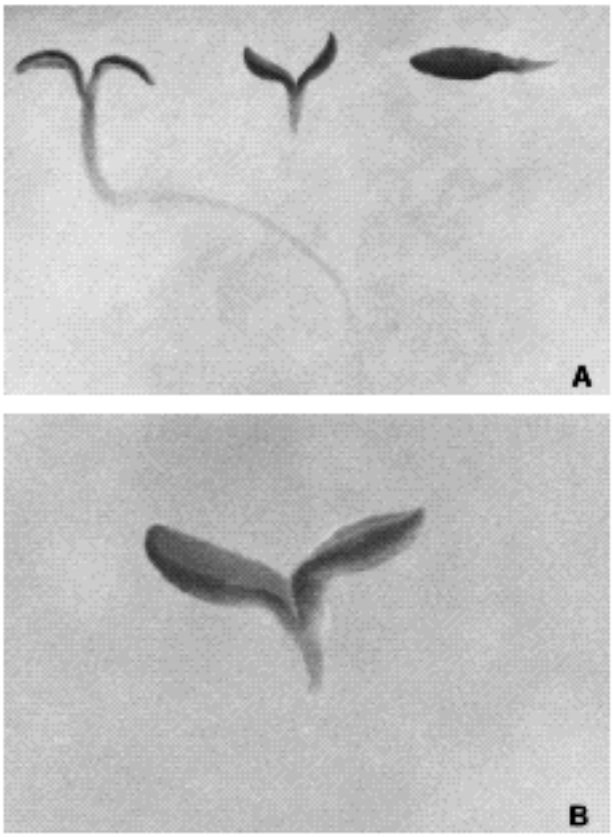

Fig. 1. *monopteros* mutant seedling phenotypes (allele mp^{T370}). (A) Wild-type (left) and two mutant seedlings (centre, right) lacking basal structures and also representing the extremes of the variable apical phenotype. (B) Completely symmetric *mp* mutant seedling. Unless otherwise noted, descriptions in the text refer to this phenotype.

gene activity. We will first describe the seedling phenotype of strong alleles which very likely results from complete inactivation of the *mp* gene.

The seedling phenotype of strong *mp* alleles

All strong *mp* alleles produce essentially the same seedling phenotype: basal structures are deleted while the arrangement of cotyledons may be normal or variably altered (Fig. 1A). The *mp* gene thus seems to be absolutely required for the formation of basal structures but not for the spatial arrangement of cotyledons (see later). Disregarding the apical variability in this section, we compare organ differentiation in mutant and wild-type seedlings.

The *mp* seedling phenotype has been described as a specific type of apical-basal pattern deletion in that apical structures such as shoot meristem and cotyledons are present whereas basal structures such as hypocotyl, radicle and root meristem are missing (Mayer et al., 1991). *mp* seedlings end basally in a conical structure we have named 'basal peg' which is attached to the cotyledons (Fig. 1B). We have analysed the 'basal peg' in order to determine whether this structure is unique to *mp* seedlings or whether it corresponds to one or another basal element of the wild-type seedling. The 'basal peg' does not show any root-specific morphological features, such as root hairs, root cap etc., nor does it show the internal differentiation that characterises the hypocotyl (Fig. 2A-D). The wild-type hypocotyl has a concentric arrangement of different cell types, and this radial pattern is invariant along the cylindrical hypocotyl (Fig. 2A,C). By contrast, the 'basal peg' contains large cells without any morphological distinctions (Fig. 2B,D). In addition, the 'basal peg' is variably oriented relative to the cotyledons, in contrast to the fixed position of the hypocotyl (see later; Figs 3, 4). Thus, the 'basal peg' may be regarded as a unique structure of *mp* seedlings that develops by default (see section on embryogenesis and Discussion).

In contrast to the deletion of basal pattern elements, the apical structures such as cotyledons and shoot meristem are present in *mp* seedlings and well differentiated. The cotyledons of *mp* seedlings consist of the tissue types that are found in wild-type cotyledons: epidermis, ground tissue (mesophyll) and vascular tissue. The epidermal layer is composed of tightly connected flat cells, which are typically irregular in shape, and interspersed guard cells of stomata. This organisation, including the average spacing of stomata, is indistinguishable between wild-type and *mp* cotyledons in both the upper and lower epidermal layers (Fig. 2H,M). No significant differences were observed in the mesophyll layers of *mp* cotyledons as compared to wild-type (Fig. 2I,L). The cells of the palisade mesophyll are similar in shape and arrangement, and only a single layer of palisade cells was found in cross-sections of both, wild-type and mutant cotyledons (data not shown). Although *mp* cotyledons may contain supernumerary layers of spongy mesophyll, this feature seems to be rather unspecific as similar defects were also observed in wild-type cotyledons of unusual curvature. By contrast, two specific deviations from the wild-type arrangement were noted for the vascular tissue in the cotyledons of *mp* seedlings. First, tracheid cells appear incompletely interconnected although they form long strands (Fig. 2K). Preliminary data suggest that these strands nonetheless function sufficiently to support considerable postembryonic growth (T. B., unpublished data). Second, the vascular system is reduced to a centrally located strand in *mp* cotyledons whereas in wild-type, the central strand is interconnected with two half-circles extending into the blade (Fig. 2E,F). The expression of this vascular phenotype depends on the *mp* allele studied (see later).

The other apical pattern element, the shoot meristem, is normal in *mp* mutant seedlings. Little, rapidly growing bulges of small cells, which were observed at germination (Fig. 2G), subsequently formed functional primary leaves (data not shown). The number and time of appearance of the primary leaves was often normal. However, if the cotyledons were abnormally arranged the development of the primary leaves was also affected. Similar effects have been observed for other *Arabidopsis* seedlings with only a single cotyledon (T. B., unpublished observation).

In summary, the strong seedling phenotype indicates that the *mp* gene activity is absolutely required for the formation of basal pattern elements such as hypocotyl, radicle and root meristem. By contrast, the two apical pattern elements, shoot meristem and cotyledons, can develop without *mp* gene activity except that the vascular system of the cotyledons appears to be reduced (see Discussion).

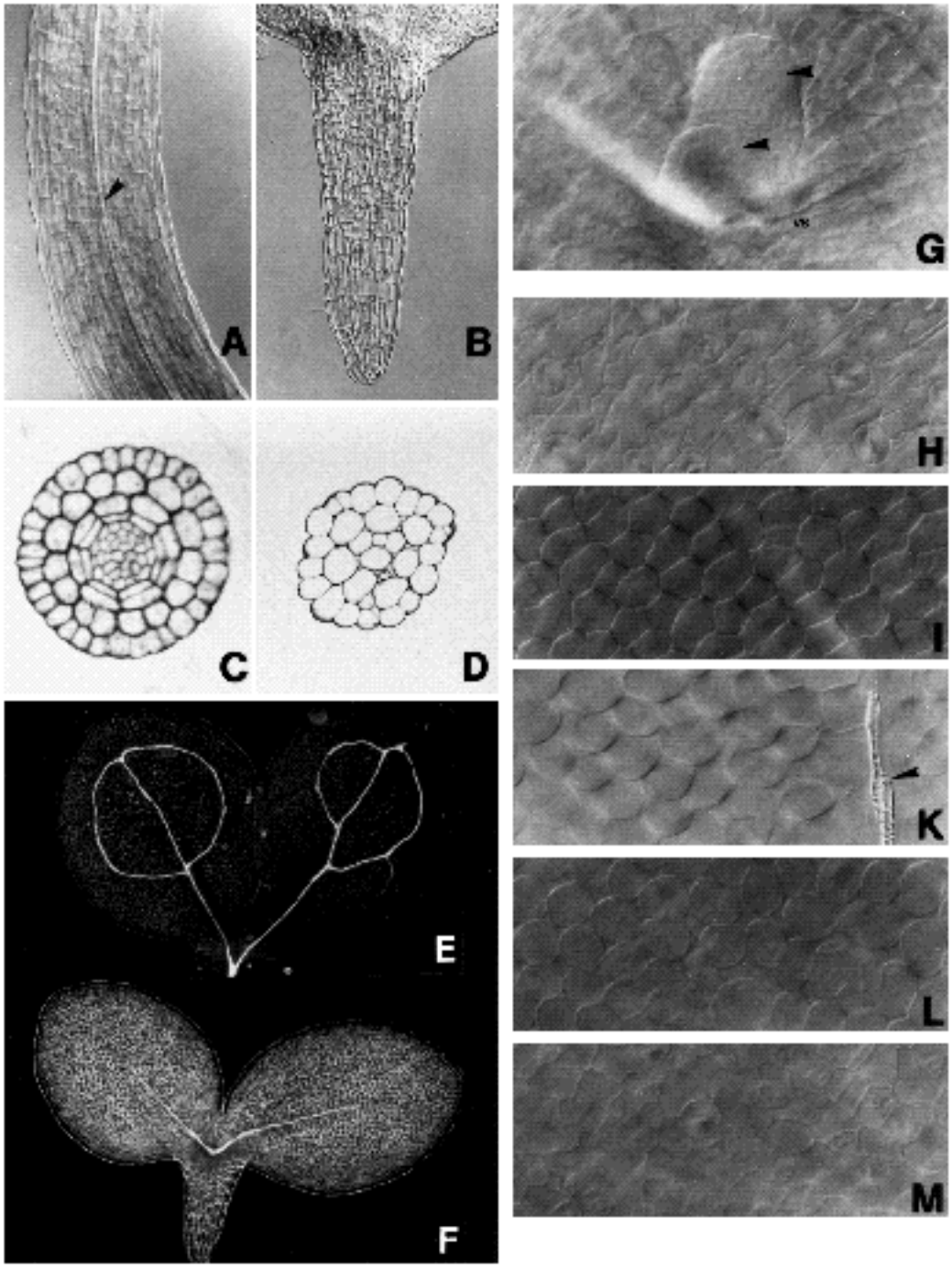

Fig. 2. Differentiation in strong *mp* mutants. (A-D) Basal body structures: part of wild-type hypocotyl with centrally located xylem strands (arrowhead; A) and complete 'basal peg' (allele mp^{U21}; B), shown at same magnification. Whole-mount preparations, Nomarski optics. Cross section to show radial differentiation of wild-type hypocotyl (C) and mutant 'basal peg' (allele mp^{U21}; D) in nearly mature embryos. (E,F) Internal seedling structures: the vascular system of the cotyledons is reticulated in wild-type (E) but reduced to the central strand in *mp* mutant (allele mp^{T313b}; F). Whole-mount preparations, dark-field optics. (G) Shoot meristem in *mp* mutant seedling (allele mp^{U242}) shortly after germination. The arrowheads mark the primordia of the primary leaves. A single vascular strand (vs) extends from below the shoot meristem into the right cotyledon and is horizontally connected to the other one extending to the left (not visible; compare with F). Whole-mount preparation, Nomarski optics. (H-M) Optical sections through cotyledon of *mp* mutant seedling (allele mp^{T370}), focusing on the upper (H) and lower (M) epidermis, the palisade (I) and the spongy mesophyll (L). Tracheid cells are improperly aligned (K; arrowhead). Whole-mount preparation, Nomarski optics.

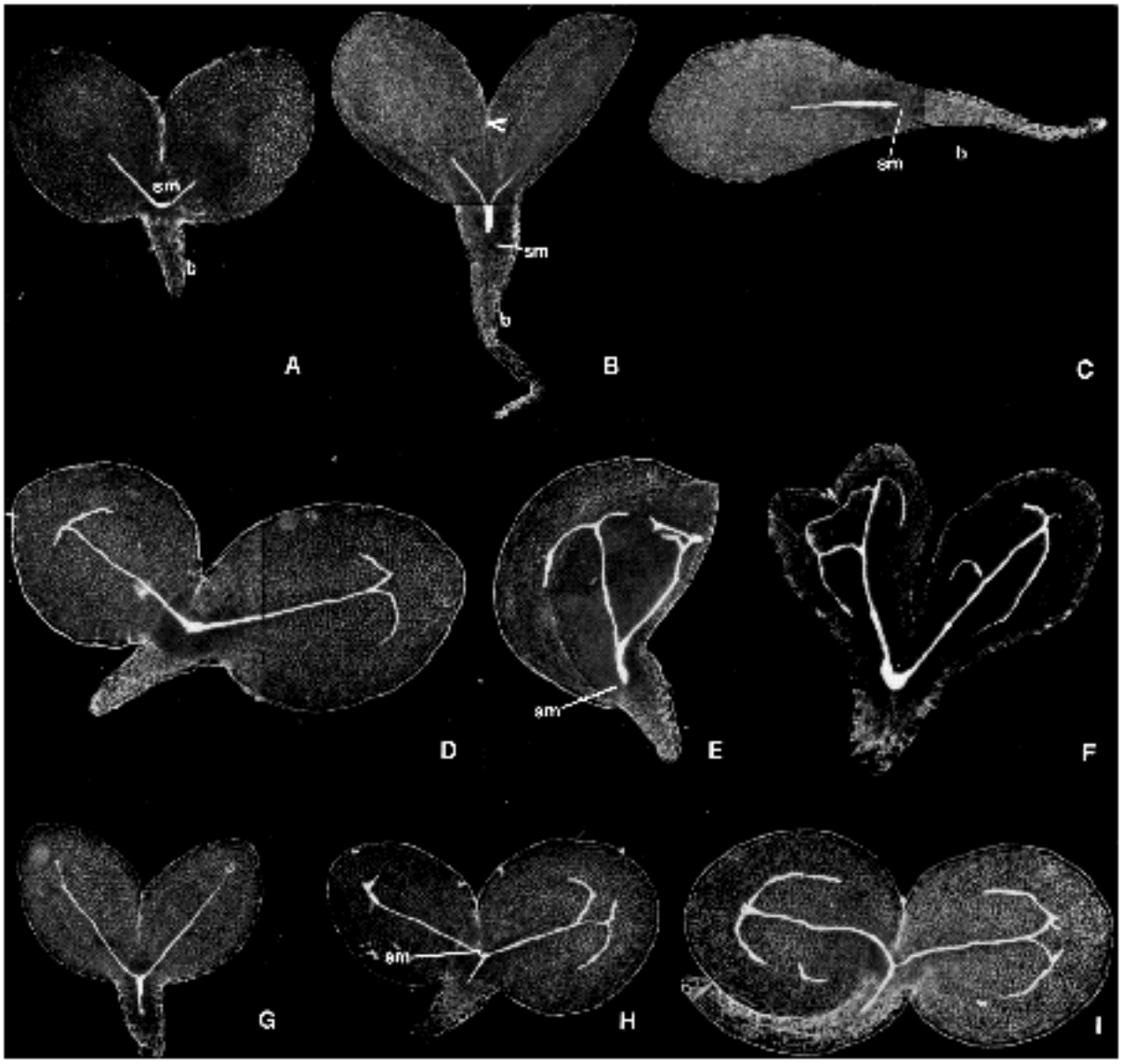

Fig. 3. Vascular-system phenotypes of *mp* mutant seedlings. (A-C) The only phenotypes produced by strong alleles: A and C represent class 'straight' of Table 1, B class 'fused'. The arrowhead in B marks the point of cotyledon fusion. Note that the cotyledonary vascular strands in B terminate in a common strand at the base of the fused cotyledon. This was verified in each case by focusing on the adjacent epidermis. (D-I) Phenotypes exclusively produced by weak alleles: D and E represent class 'branched' of Table 1; F, class 'circles'. Note closed circles (arrowhead) of vascular strands in F. (G-I) Seedlings with hypocotyl. Note that a short vascular strand extends from the attachment point of the cotyledons into the hypocotyl, as visible in cross section (see text; compare with B). These mutants were classified as 'hypocotyl' in Table 1, regardless of cotyledon phenotype. The position of the shoot meristem (sm) and the 'basal peg' (b) is indicated, where necessary to facilitate orientation. Whole-mount preparations, dark-field optics.

Vascular-system phenotypes reveal differences in allele strength

The strong seedling phenotype as described above is characteristic for ten of the thirteen *mp* alleles while the remaining three alleles (mp^{G92}, mp^{U348} and mp^{T366}) produce additional, weaker seedling phenotypes. As the most distinguishing feature, a reduced hypocotyl can occur in seedlings with weak *mp* alleles (Fig. 3G-I). The hypocotyl is generally much shorter than in wild-type seedlings, although hypocotyls of intermediate length were also found. When analysed in cross-sections, such longer hypocotyls displayed the concentric layers of radial pattern elements as found in wild-type seedlings (data not shown). In order to quantitate differences between seedling phenotypes produced by strong and weak *mp* alleles, we determined the frequency at which a vascular strand extended basally from the cotyledon(s) for 10 mutant alleles. No such vascular strand was found in mutant seedlings with the 8 strong alleles, in contrast to about 5% of the mutant seedlings with the 2 weak alleles (Fig. 3G-I; Table 1).

Weak and strong *mp* alleles can also be distinguished by the extent to which the vascular system reticulates in the cotyledons of mutant seedlings. In seedlings with strong alleles, just one vascular strand runs along the midline of the

Table 1. Vascular-system phenotypes of *mp* mutant seedlings

mp allele	No. of seedlings	NVS.* %	Straight %	Fused %	Branched %	Circles %	Hypocotyl %
R34	141	0	74	26	0	0	0
S4	162	0	80	20	0	0	0
T313b	87	1	83	16	0	0	0
T370	246	4	85	11	0	0	0
TÜ399	112	2	71	27	0	0	0
U21	74	0	76	24	0	0	0
U252	293	0	81	19	0	0	0
U368	81	2	89	9	0	0	0
G92	199	0	40	6	43	6	5
U348	107	2	34	20	37	1	6

*No vascular strands.

The other phenotypic classes are shown in Fig. 3. Each seedling was grouped into only one of the classes according to hierarchically ordered criteria: from "hypocotyl", regardless of the cotyledon phenotype, to "circles" of vascular strands, to "branched" vascular strands in the cotyledons. Seedlings with a single vascular strand in each cotyledon were classified as "fused" rather than "straight" if the basal ends of the vascular strands from the two fused cotyledons were also fused. The double line separates 2 weak from 8 strong *mp* alleles.

cotyledon(s) in the vast majority of mutant seedlings (Figs 2F, 3A-C; Table 1). By contrast, the vascularisation of cotyledons was more elaborate in 40-50% of mutant seedlings with the weak alleles: the vascular strands were often branched, and even half-circles of vascular strands like those in wild-type seedlings were observed (Fig. 3D-I; Table 1). Separate phenotypic classes such as 'branched' and 'circles' were established to indicate different degrees of branching although in reality the vascular phenotypes merge. There was no marked correlation between the presence of a hypocotyl and the extent of vascularisation in the cotyledons. For example, the mutant seedling shown in Fig. 3I has an almost complete hypocotyl and yet the vascular system reticulates poorly in the cotyledons.

The vascular-system phenotypes seem to reflect differences between *mp* alleles rather than effects of unknown second-site modifiers. This conclusion is based on two lines of evidence. First, different sub-lines carrying the same allele produced essentially the same frequency distribution of vascular phenotypes. Second, sub-lines of *mp* alleles that were established after repeated out-crossing to wild-type, also showed vascular phenotypes not markedly different from those of the original lines. It is thus likely that the weaker seedling phenotypes are due to residual *mp* gene activity.

The stability of the apical pattern is allele-specific

In wild-type seedlings, the two cotyledons are stably positioned, forming a straight line. Exceptions to this rule are extremely rare and may occur only once in several hundred seedlings. By contrast, *mp* seedlings often have abnormally positioned cotyledons, with the angle between the cotyledons ranging from less than 180° to almost zero (Fig. 4). Thus, although all apical structures can be formed in the absence of *mp* gene activity, the stability of their spatial arrangement is reduced in mutant seedlings. In the extreme case, a *mp* seedling essentially consists of nothing but a single cotyledon (Fig. 4E,F).

In order to quantitate the degree of apical stability in different *mp* alleles, we classified mutant seedlings with respect to the number and position of the cotyledons. Regardless of other seedling features, three major classes were distinguished: 'two cotyledons', which refers to the wild-type arrangement (Fig. 4A,B), 'single cotyledon' (Fig. 4E,F), and 'intermediate' phenotypes between the two extremes, which includes partially fused cotyledons (Fig. 4C,D). In the 8 strong *mp* alleles analysed, one-quarter to one-half of the mutant seedlings showed the wild-type arrangement while 30-50% of the mutant seedlings fell into the single-cotyledon class (Table 2). By contrast, two weak alleles predominantly resulted in mutant seedlings with cotyledons positioned in a straight line while less than one-fifth of the mutant seedlings represented the single-cotyledon class (Table 2). In order to assess the statistical significance of the result, we determined the phenotypic variability between different sub-lines carrying the same *mp* allele and compared the mean values for different alleles. As shown in Fig. 5, the single-cotyledon class of seedling phenotypes was reduced for the same two *mp* alleles that had been classified as weak by the criterion of producing mutant seedlings with a short hypocotyl (see Table 1). In order to determine whether the two phenotypic traits are correlated, mutant seedlings were pre-selected for the occurrence of a short hypocotyl, and 95% (35 of 37) of these seedlings also showed the wild-type arrangement of cotyledons. This result suggests that the hypocotyl may stabilise the spatial arrangement of cotyledons (see Discussion).

Table 2. Apical pattern phenotypes of *mp* mutant seedlings

mp allele	No. of seedlings	Two cotyledons %	Fused cotyledons %	Single cotyledon %
R34	73	49	6	45
S4	183	44	12	44
T313b	97	31	17	52
T370	277	40	19	41
TÜ399	131	25	21	54
U21	165	29	19	52
U252	421	47	15	38
U368	109	44	26	30
G92	249	59	23	18
U348	117	68	17	15

The phenotypic classes are shown in Fig. 4: "two cotyledons" (A,B), "fused cotyledons" (C,D), "single cotyledon" (E,F). The double line separates 2 weak from 8 strong *mp* alleles.

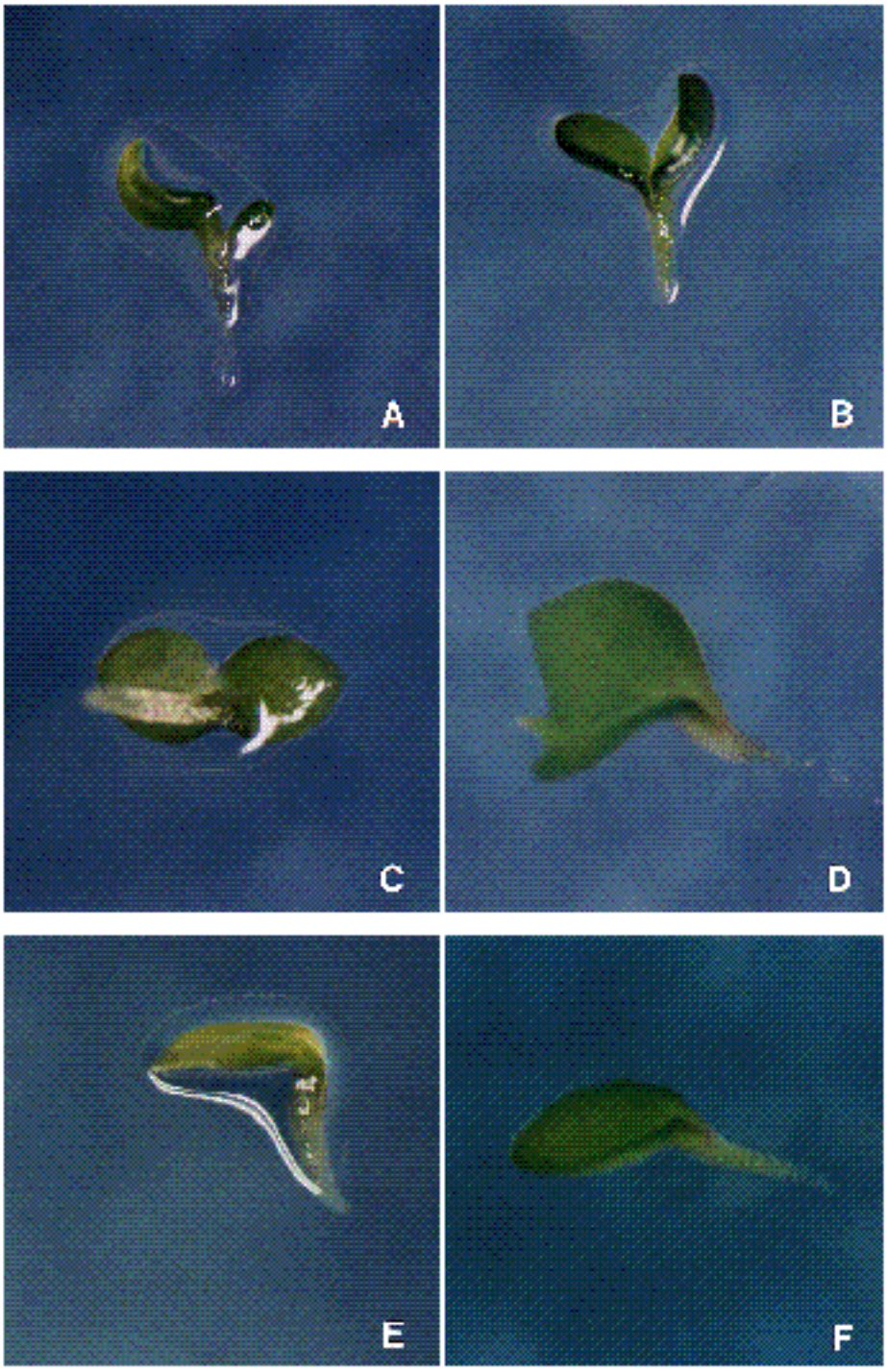

Fig. 4. Spatial arrangement of cotyledons in *mp* mutant seedlings. (A, B) Normal position of the cotyledons (class 'two cotyledons' in Table 2) which can differ in size. (C, D) Intermediate types of cotyledon fusions (class 'intermediate' in Table 2), ranging from angles slightly less than 180°between the cotyledon axes (C) to almost completely fused coteledons (D). Note that this class also includes weaker types of cotyledon fusions than the class 'fused' in Table 1. (E, F) Seedlings with a single cotyledon (class 'single cotyledon' in Table 2) which can be oriented differently relative to the 'basal peg'. Mutant alleles: mp^{T1} (A-C, E, F); mp^{T370} (D).

Development of *monopteros* mutant embryos

The *mp* seedling phenotype had previously been correlated with pattern changes in the heart-shaped embryo (Mayer et al., 1991). We have now studied the development of mutant embryos from early stages in order to determine how the mutant seedling phenotype evolves from primary alterations in the embryo. In the following, we will compare *mp* and wild-type embryos at four stages of embryogenesis: octant, triangular/early-heart, late-heart and mid-torpedo stages. For the sake of clarity, we will describe the development of symmetric mutant embryos first and the variable features later.

At the octant stage, the wild-type embryo shows a highly reproducible cell arrangement: two tiers of 4 cells each make up the embryo proper, which is derived from the apical daughter cell of the zygote. 6-9 cells, which are the descendants of the basal daughter cell of the zygote, form a filamentous suspensor (Fig. 6A). This cell arrangement is altered in *mp* mutant embryos: four, rather than two, embryonic cell tiers are attached to a suspensor that consists

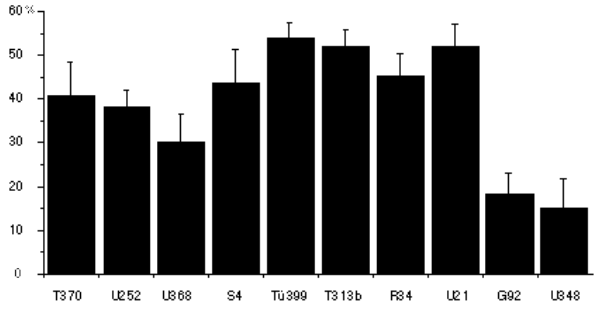

Fig. 5. Allele-specific proportions of *mp* mutant seedlings with single cotyledon. The numbers (from Table 2) are cumulative values for several sub-lines of each mutant allele. The proportion of single-cotyledon seedlings was separately determined among 30-45 mutant seedlings for each sub-line, which was used for calculating the standard deviation for each allele (indicated by the bar in each column).

of the same number of cells as in wild-type (Fig. 6E). The cells of the supernumerary tiers show the intense staining normally characteristic of the embryo proper as opposed to the suspensor cells. The origin of those cells has not been determined (see Discussion). The tiers are often not aligned in parallel, with cell walls running obliquely across an abnormally shaped embryo proper.

Due to the lack of cell migration, different parts of the octant-stage embryo have been correlated with the production of specific elements of the seedling body in *Arabidop - sis* and other crucifers (Vandendries, 1909; Mansfield and Briarty, 1991; Jürgens and Mayer, 1993; *Brassica napus*: Tykarska, 1976, 1979; *Capsella bursa pastoris*: Schulz and Jensen, 1968). The cells of the upper tier (*ut*) will give rise to the shoot meristem and the cotyledons, the derivatives of the lower tier (*lt*) will produce the hypocotyl, the embryonic root (radicle) and the upper part of the root meristem while the uppermost suspensor cell becomes the hypophysis which, by a precise pattern of cell divisions, will generate the remainder of the root meristem. Following tangential divisions that give off an outer layer of epidermal precursor cells, the *ut* and *lt* cells begin to differ in shape and patterns of cell division. The *ut* cells maintain their isodiametric shape and do not show any preferential direction of division. By contrast, the *lt* cells elongate in the apical-basal axis and divide in a highly ordered pattern so as to produce precisely oriented cell files (Fig. 6B). These files extend from the basal end of the embryo where the hypophysis has produced a lens-shaped upper daughter cell which eventually gives rise to the centre of the root meristem (Jürgens and Mayer, 1993). The oriented cell divisions of the *lt* derivatives result in continual growth of the primordia of hypocotyl and radicle, and by the mid-heart stage a morphological axis is well differentiated with respect to the three tissue primordia: the outer epidermis, the ground tissue and the centrally located vascular tissue (Fig. 6C). From the triangular stage on, *ut* derivatives at two specific sites show increased mitotic activity which leads to the formation and subsequent growth of the cotyledonary primordia (Fig. 6B-D).

Two features that characteristically distinguish *mp* from wild-type embryos can most clearly be recognised at the triangular/early-heart stage (compare Fig. 6F with 6B). First, all inner cells of *mp* embryos behave as if they were *ut* derivatives: they are isodiametric in shape and do not produce the cell files that are characteristic of *lt* derivatives (compare Figs 6F,K,L with 6B). Second, the uppermost suspensor cell does not undergo the highly stereotyped division pattern of the wild-type hypophysis. Instead, a 'central pile' of two to four cells is visible at the basal end of the embryo proper and appears to be contiguous to the suspensor (Fig. 6G,K,L).

Past the triangular stage, the shape of the *mp* mutant embryo gradually approaches the shape of the mutant seedling, due to the outgrowth of the cotyledonary primordia and the relative narrowing-down of the basal part of the embryo (Fig. 6G,H). Internally, the heart-shaped *mp* embryo lacks the cell files of hypocotyl and radicle which normally extend from the incipient root primordium to the base of the cotyledonary primordia (compare Fig. 6G with 6C). The vascular primordium, which in the wild type consists of about 6 files of narrow cells, is absent, and a 'central pile' of fairly large, isodiametric cells protrudes from the upper end of the suspensor into the embryo (Fig. 6G,H). This 'central pile', which is often observed from the triangular stage on (not visible in Fig. 6F; see Fig. 6K,L), extends to the base of the cotyledonary primordia by the mid-torpedo stage, being about eight cells high (Fig. 6H). The origin of the 'central pile' could not definitively be established although its position and growth from the upper end of the suspensor suggest that it may have been produced by abnormal divisions of the uppermost suspensor cell.

The mutant torpedo-stage embryo shows normal growth and morphological differentiation in the cotyledonary primordia as compared to wild-type embryos of the same stage (compare Fig. 6H with 6D). In addition to the epidermis, derived from the outer layer of the early embryo, strands of narrow cells were found in the basal region of the cotyledonary primordia, and these cells seem to correspond to the primordia of the vascular strands. By contrast, wild-type and mutant embryos are markedly different in the basal region (Fig. 6D,H): the wild-type hypocotyl is enlarged and radially differentiated whereas the basal part of *mp* mutant embryo contains relatively few indistinct cells, indicating that little growth has occurred between the late-heart and mid-torpedo stages (Fig. 6G,H). The basal part of the mutant embryo gives rise to the 'basal peg'.

A considerable proportion of *mp* mutant embryos do not undergo the symmetric development described above. At the early-heart stage, *mp* mutant embryos often show differential outgrowth of the two cotyledonary primordia (Fig. 6K) or seem to form only a single cotyledon (Fig. 6L). Distortion of symmetry was often observed in even younger *mp* mutant embryos, presumably due to oblique cell divisions in the basal part of the embryo (Fig. 6I). This early asymmetric development may be responsible for the variable arrangement of cotyledons (see Discussion).

In summary, *mp* mutant embryos appear to lack the information that is required for the specification of the lower tier of the octant-stage embryo and the adjacent hypophysis, resulting in abnormal patterns of cell division. Other processes such as the formation of the suspensor, the segregation of epidermal precursor cells or the outgrowth of cotyledonary primordia are apparently not affected.

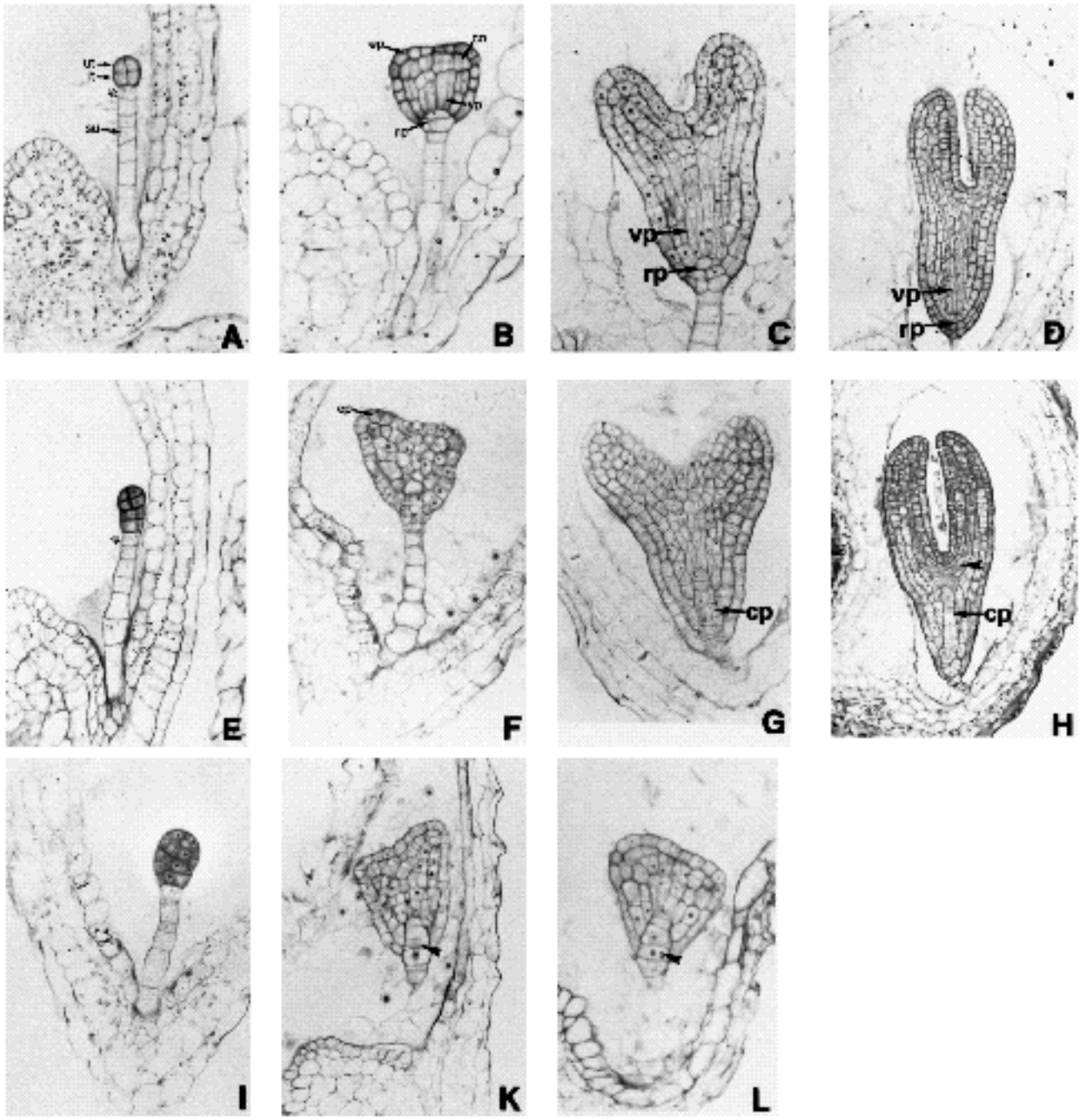

Fig. 6. Wild-type (A-D) and *mp* mutant (E-H) embryogenesis. (A,E) Octant stage: the cells of the embryo proper stain darkly and the size of the suspensor (su) is similar in A and E. The asterisk marks the uppermost suspensor cell. *ut*, *lt*, upper and lower cell tiers that give rise to apical and basal seedling structures (see text). (B,F) Triangular/early-heart stage: the wild-type embryo (B) shows the primordia of all major pattern elements: (ep) epidermis, (co) cotyledon primordium, (vp) vascular primordium, (rp) root primordium. Note the uniform shape of the inner cells in the mutant (F) embryo. (C,G) Late-heart stage: the wild-type root primordium (rp) is a characteristic assemblage of twelve cells (six are visible in this section; C). Note that in the mutant embryo (G), the 'central pile' (cp) is contiguous to the suspensor. (D,H) Mid-torpedo stage: the vascular system develops in the cotyledon primordia of the mutant embryo (arrowhead, H). Note the considerable growth and differentiation of hypocotyl and radicle in wild-type (compare D with C), in contrast to the basal part of the *mp* mutant (compare H with G). (I-L) Asymmetric development of *mp* mutant embryos at mid-globular (I) and triangular/early-heart (K, L) stages. Embryo in K shows cotyledon primordia of different sizes, while the embryo in L seems to produce only a single cotyledon. Note the contiguity of the suspensor to the 'central pile' in the *mp* mutant (arrowheads in K, L). Suspensor runs out of the plane of section. Mutant alleles: mp^{R34} (E-H), mp^{T313b} (I, K), mp^{U21} (L). Embryos shown in A,E; B,F; C,G and D,H correspond to stages 6, 11/12, 14 and 16 of Jürgens and Mayer (1993). For each stage, wild-type and mutant embryos are shown at the same magnification. Staging of mutant embryos is approximate. Histological sections.

Root formation by *mp* mutant tissue in culture

All *mp* mutant embryos, regardless of the allele studied, are incapable of forming the root and the root meristem. Mutant seedlings with the 10 strong alleles also invariably lack the hypocotyl, while 3 alleles classified as weak allow for the occasional formation of a short hypocotyl. Although these seedling phenotypes argue for the *mp* gene product organising the basal body region rather than being specifically involved in making the root, we directly tested whether root formation can be induced in *mp* mutant tissue by appropriate culture conditions. Due to the different seedling phenotypes of weak and strong alleles, two different procedures were applied.

Mutant seedlings with two of the weak alleles (mp^{G92} and mp^{U348}) that had developed a short hypocotyl were subjected to a modification of the treatment described by Weiland and Müller (1972; see Materials and methods). Briefly, mutant seedlings were wounded at the level of the hypocotyl and cultured on an auxin-containing medium. Roots emerged from the wounded seedlings in 21 of 28 cases (75%) for allele mp^{G92} and in 7 of 17 cases (41%) for allele mp^{U348} (Fig. 7A,B). For comparison, bisected wild-

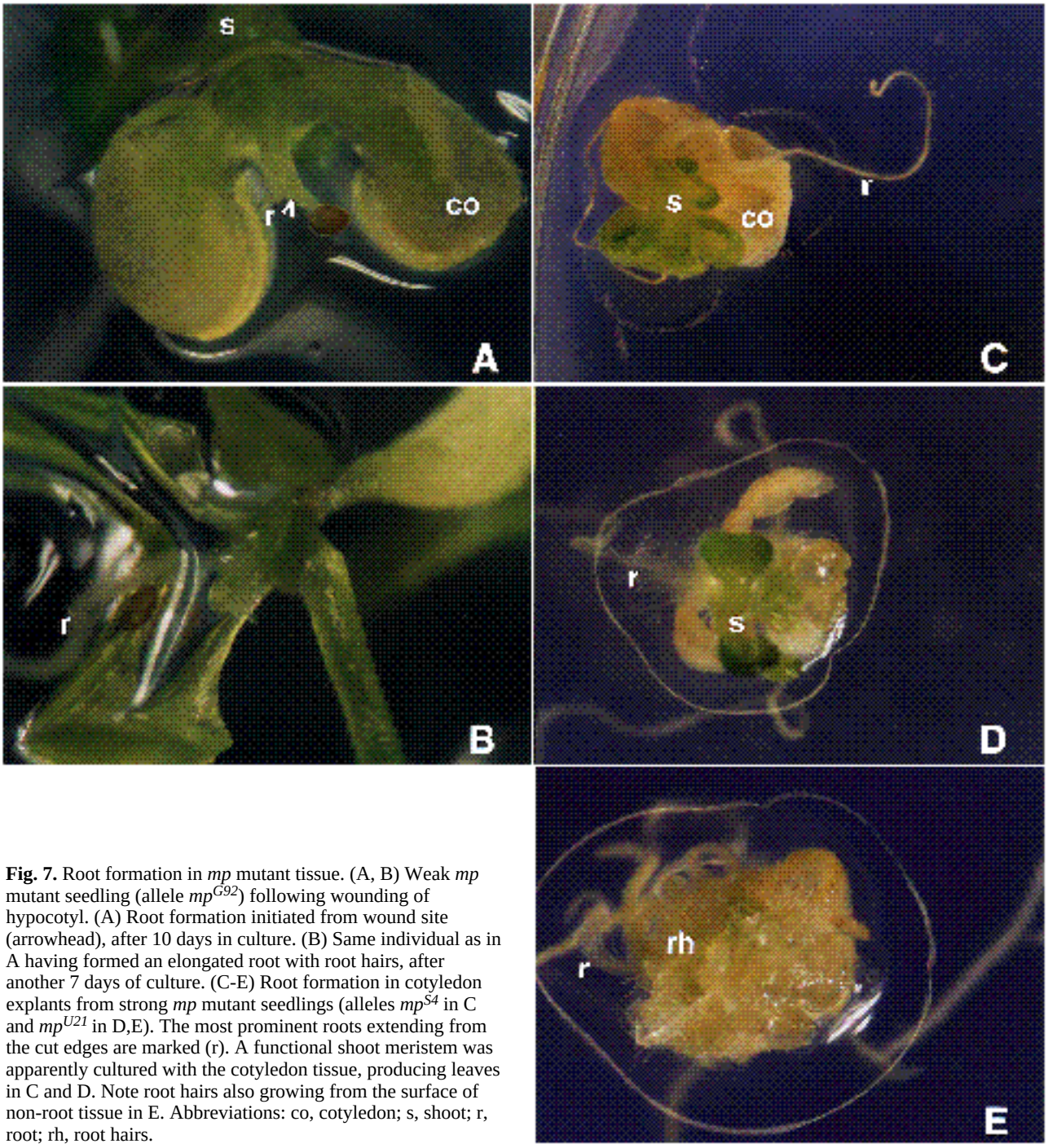

Fig. 7. Root formation in *mp* mutant tissue. (A, B) Weak *mp* mutant seedling (allele mp^{G92}) following wounding of hypocotyl. (A) Root formation initiated from wound site (arrowhead), after 10 days in culture. (B) Same individual as in A having formed an elongated root with root hairs, after another 7 days of culture. (C-E) Root formation in cotyledon explants from strong *mp* mutant seedlings (alleles mp^{S4} in C and mp^{U21} in D,E). The most prominent roots extending from the cut edges are marked (r). A functional shoot meristem was apparently cultured with the cotyledon tissue, producing leaves in C and D. Note root hairs also growing from the surface of non-root tissue in E. Abbreviations: co, cotyledon; s, shoot; r, root; rh, root hairs.

type seedlings formed roots in 45 of 47 cases (96%). Thus, root formation can be induced in wounded seedlings that lack root and root meristem because of reduced *mp* gene activity in embryogenesis.

A more stringent test was performed with tissue from *mp* seedlings that were mutant for two presumed null alleles (mp^{S4} and mp^{U21}). Pieces of tissue were dissected from mutant seedlings and cultured as described in Materials and methods. The tissue pieces were mostly of cotyledonary origin although a functional shoot meristem was sometimes included, and explants thus developed into plantlets or looked callus-like after four to six weeks in culture (Fig. 7C-E). The appearance of the cultured tissue had no marked effect on root formation. Roots were observed in 14% (4 of 29) of the explants from allele mp^{S4} and in 24% (8 of 33) of the cases for allele mp^{U21}. Thus, root formation can occur in mutant tissue that presumably lacks functional *mp* gene product.

DISCUSSION

Apical-basal pattern formation in the flowering plant embryo generates a reproducible sequence of seedling structures, including the meristems of the shoot and the root at opposite ends of the axis. The isolation of putative pattern mutants in *Arabidopsis* has led to the identification of a gene, *monopteros* (*mp*), that appears to be specifically required for the formation of basal body structures such as hypocotyl, radicle and root meristem (Mayer et al., 1991). We have now explored the role the *mp* gene may play in embryonic pattern formation. The findings indicate that the *mp* gene is primarily required for organising the basal region of the developing embryo from early on. The variable arrangement of cotyledons, as seen in mutant seedlings, seems to result indirectly from the lack of organised growth in the basal region.

Developmental basis for the deletion of basal pattern elements in *mp* seedlings

The *mp* seedling phenotype has been traced back to the early embryo in order to obtain clues as to the developmental process affected. *mp* mutant embryos show abnormalities as early as the octant stage. In contrast to the expectation that a basal deletion phenotype derives from a mutant embryo with fewer cells than wild-type, the *mp* embryo proper contains more cells than normal. However, these cells are not functionally equivalent to the lower tier of the wild-type embryo proper, as judged by the behaviour of their descendants.

The basal body structures that are deleted in *mp* seedlings normally derive from specific regions of the octant-stage embryo: the lower tier of the embryo proper and the uppermost suspensor cell (Schulz and Jensen, 1968; Tykarska, 1976, 1979; Mansfield and Briarty, 1991; Jürgens and Mayer, 1993). From the early-globular stage on, the descendants of those cells show specific patterns of cell division and cell elongation. *mp* mutant embryos seem to lack instructions for this organised, directional growth of the basal region. The descendants of the lower tier(s) show a random cell-division pattern, thus resembling the upper-tier descendants. Similarly, the uppermost suspensor cell does not establish the precisely ordered pattern of cell division that characterises the wild-type hypophysis. Since the 'central pile' of cells appears in place of the hypophyseal derivatives, it seems likely to us that the 'central pile' derives from the uppermost suspensor cell which, in this view, would continue to divide horizontally, as normal suspensor cells do. As this 'central pile' extends into the growing embryo, it is gradually ensheathed by adjacent cells derived from the mutant embryo proper, and this assemblage of cells later gives rise to the 'basal peg'. Thus, the position of the early defect correlates well with the basal deletion of the seedling, and this correlation holds throughout embryogenesis.

mp gene activity is essential for directional growth in the basal region of the embryo which eventually gives rise to the root. By contrast, *mp* mutations do not interfere with the regenerative pathway of root formation that can be induced experimentally in various tissues (Smith and Murashige, 1970; Feldman, 1976; Feldman and Torrey, 1976; Schiavone and Racusen, 1991). Thus, the embryonic pathway of root formation seems to involve specific events that occur exclusively in the basal region of the embryo. As this region develops from two adjacent cell groups of different origin, the lower tier of the embryo proper and the hypophysis, organising the growth pattern may require their interaction. To this effect, the *mp* gene may be active in both cell groups. Alternatively, only one of the two may autonomously require *mp* gene product, with the other depending on instructions from the former. For example, the uppermost suspensor cell may normally become the hypophysis in response to some signal from the embryo proper, and the *mp* mutant embryo proper may fail to produce that signal. Such a scenerio would explain why the primary role of the *mp* gene is confined to embryogenesis. The embryo-specific requirement distinguishes the *mp* gene from another gene involved in apical-basal pattern formation, the *gnom* gene, which has recently been shown to be necessary for root formation in the embryo as well as in tissue culture (Mayer et al., 1993).

Origin of apical defects in *mp* mutant seedlings

The *mp* gene is not absolutely required for proper spatial arrangement of cotyledons as indicated by the large proportion of strong *mp* mutant seedlings showing normal cotyledon arrangement (see Table 2). In the absence of *mp* gene activity, the developmental process generating the arrangement of cotyledons seems to be labile, suggesting that a different *mp*-dependent process normally ensures that the cotyledonary primordia are established in their proper places. Our data as well as other observations make it very likely that the body axis plays this stabilising role.

The spatial arrangement of cotyledons is variable in *mp* mutant seedlings lacking a hypocotyl. Conversely, the cotyledons are properly spaced in mutant seedlings that have a hypocotyl, however short. This correlation also holds for other mutants. For example, mutations in other genes that result in seedlings with a short hypocotyl and no roots, do not affect the spacing of cotyledons (T. B., unpublished observation). By contrast, mutations in the *fackel* gene, which specifically affect the hypocotyl, result in abnormal

arrangement of cotyledons (Mayer et al., 1991). Similarly, *fass* mutant seedlings have an abnormally wide hypocotyl, and this phenotype can be associated with supernumerary cotyledons (Mayer et al., 1991; R. A. Torres Ruiz and G. J., unpublished data). In these cases, the primary lesion seems to be in the seedling axis rather than in the cotyledons themselves.

Cotyledonary primordia become recognisable in the apical region of the early-heart stage embryo: two buttresses are symmetrically positioned with respect to the body axis which is formed by the derivatives of the lower tier (Schulz and Jensen, 1968; Tykarska, 1976, 1979; Mansfield and Briarty, 1991; Jürgens and Mayer, 1993). In early *mp* mutant embryos, an incipient body axis does not form as indicated by isodiametric cell shape and random cell-division patterns in the basal region. This failure frequently results in asymmetric shapes of globular embryos, and the cotyledonary primordia are thus often abnormally positioned at the early-heart stage. In this way, the primary defect of *mp* mutant embryos, i.e. lack of organised basal growth, seems to result in the variable apical defect.

How the symmetric positioning of cotyledonary primordia is achieved in the wild-type embryo is not known. By analogy to the spacing of leaf primordia in which inhibitory interactions have been considered to play a role (Steeves and Sussex, 1989, and references therein), one might speculate that positioning of cotyledonary primordia also involves interaction. This idea receives support from mutations that specifically alter number or spacing of cotyledons, without obviously affecting the seedling axis (Jürgens et al., 1991, and unpublished observation). Mutually inhibitory influences have the inherent tendency to enhance initial asymmetries such as those seen in early *mp* mutant embryos, which would explain the large proportion of *mp* mutant seedlings with only a single cotyledon. This view implies a labile balance between the early cotyledonary primordia: proper spacing of cotyledons would thus be favoured if the primordia were initiated nearly simultaneously and symmetrically about the body axis. In *mp* mutant embryos, however, a body axis does not form, resulting in variable initiation of cotyledonary primordia whose fate would then be determined by their mutually inhibitory interaction.

mp mutant seedlings showed another apical defect, incomplete vascularisation of cotyledons, and this defect was strictly dependent on *mp* gene activity, in contrast to the spatial arrangement of cotyledons. All strong *mp* mutant seedling phenotypes invariably showed this trait while weaker seedling phenotypes tended towards normality. In individual *mp* seedlings of the latter class, there was no clear correlation between the basal pattern deletion and this apical defect, and their relationship, if any, is thus not obvious. One might consider the possibility that the *mp* gene product is required for two seemingly unrelated processes in embryogenesis. Such a redeployment of a developmental gene would not be without precedence as this has amply been documented for early-acting segmentation genes in *Drosophila* (Ingham, 1988).

Implications for apical-basal pattern formation

From the analysis of mutant phenotypes, we previously proposed that the apical-basal axis may initially be partitioned into regions (Mayer et al., 1991). Such regions may be established in the octant-stage embryo, with different cell groups giving rise to distinct parts of the apical-basal pattern. This notion seems to be supported by the early phenotype of *mp* mutant embryos: the defects are restricted to the basal cells of the embryo proper and the uppermost suspensor cell, whereas the apical cells of the embryo proper are not affected. The *monopteros* gene may thus be viewed as a region-specific patterning gene that directs development of contiguous cells originally defined by position in the octant-stage embryo. Within its domain of action, more *mp* gene activity seems to be required for the production of very basal structures, as the residual gene activity of weak alleles occasionally generates a short hypocotyl but not a root. This spatial difference may reflect different growth dynamics, influenced by continual *mp* gene activity.

A good candidate for a gene involved in early partitioning is *gnom*, as indicated by two observations (Mayer et al., 1993). First, the *gnom* gene is already required for the asymmetric division of the zygote. Second, *gnom* is epistatic to *mp*, indicating that *gnom* gene activity is a prerequisite for *mp* gene action. Genes like *gnom* may act transiently to confine the action of region-specific genes to their proper domains. Genes like *mp* may subsequently remain active for longer periods of time, directing the development of specific body regions. Such a mode of pattern formation would provide enough flexibility to account for diverse growth patterns as seen in early embryos from different flowering plant species (Rutishauser, 1969; Johri, 1984).

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