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Genetic analysis of pattern formation in the *Arabidopsis* embryo

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Summary

Virtually nothing is known about the mechanisms that generate the basic body pattern in plant embryogenesis. As a first step towards the analysis of pattern formation, we have isolated and begun to characterise putative pattern mutants in the flowering plant, *Arabidopsis thaliana*. A large-scale screen for morphologically abnormal seedling mutants yielded about 250 lines for further study, and genetic evidence suggests saturation of the genome for this kind of mutation. The phenotypes of putative pattern mutants fall into distinct categories, classes and groups, which may reflect specific aspects of embryonic pattern formation. Mutant seedling phenotypes result from abnormal development in the early embryo. The implications of our findings are discussed with regard to the prospects for a mechanistic understanding of pattern formation in the plant embryo.

Key words: *Arabidopsis*, embryo, pattern formation, mutants.

Introduction

Survival and successful reproduction of multicellular organisms depend on the precise spatial arrangement of differentiated cell types, tissues and organs. The developmental process which brings about the body organisation has been called pattern formation. This term essentially means that cells of a seemingly homogeneous population adopt different developmental fates according to their relative positions.

The basic features of the body pattern are established during embryogenesis. This is obvious in those animals whose postembryonic development merely involves growth and sexual maturation. The same principle, however, holds true for holometabolous insects like *Drosophila* in which the adult fly radically differs from the juvenile larva. Postembryonic development of *Drosophila* uses as reference the pattern laid down in embryogenesis (Simcox *et al.* 1989). A similar reasoning may be applied to flowering plants. The young seedling, which results from pattern formation in the embryo, looks very different from the mature plant. The seedling contains two meristems, the shoot and the root meristems, located at opposite poles of the main body axis. Despite the fact that the mature plant is almost exclusively derived from the two meristems, the overall organisation of the plant body appears to be conditioned by the pattern generated in the embryo. Two lines of evidence support this notion. (1) The apical–basal polarity of the embryo is stably maintained during postembryonic development. (2) The leaves, which are produced by the shoot meristem during vegetative growth, are normally arranged in a specific manner called phyllotaxis. If the number of cotyledons (seedling leaves) is increased from two to three, there is a corresponding change in the phyllotaxis.

Pattern formation in the embryo has been intensively studied in the fruit fly, *Drosophila melanogaster*. A combination of genetic analysis, experimental embryology and molecular studies has led to remarkable insights into the mechanisms that generate the body pattern in the *Drosophila* embryo (Nüsslein-Volhard *et al.* 1987; Ingham, 1988). Three lessons can be learned from the analysis of pattern formation in *Drosophila*. (1) Relatively few genes specifically contribute to pattern formation in the embryo. (2) If any one of these genes is inactive due to mutation, embryonic development is not arrested but the embryo forms an incomplete or abnormal body pattern. (3) The pattern forms in steps, proceeding from global to local events. This procedure reflects an underlying hierarchy of interacting genes, most of which code for regulatory molecules including transcription factors and components of signal transduction. Whether the *Drosophila* mode of pattern formation is unique or represents common principles can only be determined when pattern formation in other organisms has been studied to a comparable level.

Plants differ in their life strategy from animals. Comparing the plant mode with the *Drosophila* mode of pattern formation may therefore be the most critical test for common principles in biological pattern formation. Unfortunately, virtually nothing is known about pattern formation in the plant embryo. Exper-

imental manipulation of plant embryogenesis *in situ* is very difficult, if not impossible, as the embryo develops inside a shell, the seed coat, which in turn is enclosed in the fruit. Similarly, a direct molecular approach to pattern formation in the embryo faces the problem of what molecules to look for and how to assess their biological roles. The only approach not afflicted with these difficulties is a genetic analysis of pattern formation which would recognise relevant genes by their mutant phenotypes.

The genetic analysis of pattern formation is confined to a few species in which mutants can easily be isolated and characterised. This limitation is not critical in the study of plant development because flowering plants are so closely related to one another that findings with any one species are likely to apply to all the others. The wall cress *Arabidopsis thaliana*, a weed of the crucifer family, is particularly well suited for a genetic analysis of pattern formation in the embryo. Advantageous features include the small size of the nuclear genome, the small size of the mature plant, the short generation time, the selfing of hermaphroditic flowers and the number of progeny per flower (Rédei, 1970; Meyerowitz, 1987). All these features combine to make large-scale screens for developmental mutants feasible and in principle to enable molecular cloning of genes solely defined by mutant phenotype and map position.

Here we outline a genetic approach to the analysis of pattern formation in the *Arabidopsis* embryo which involves a large-scale screen for putative pattern mutants. We discuss the strategy used, the implications of our findings and the prospects for a mechanistic understanding of pattern formation in the plant embryo.

Results

Descriptive embryology

Embryogenesis in *Arabidopsis* closely resembles the text-book type of embryogenesis in the dicotyledonous flowering plants (Vandendries, 1909; Meinke and Sussex, 1979*a*). Embryogenesis in *Arabidopsis* is faster, taking about 12 days, and produces a smaller seedling compared to many other plants. In addition, the mature embryo fills up the seed almost completely and there is no starchy endosperm, as for instance in maize, which would require sectioning of the seed for anatomical studies of the embryo. Instead, *Arabidopsis* embryos can readily be analysed in simple whole-mount preparations of entire seeds.

The zygote is a small polarised cell which divides asymmetrically in the future apical–basal axis of the embryo. The smaller apical daughter cell gives rise to the embryo except for the root which derives from the larger basal daughter cell. The basal cell also produces the extraembryonic suspensor which supplies the young embryo with nutrients from the mother plant. The apical cell undergoes 2 or 3 'cleavage' divisions before further cell divisions increase the size of the incipient embryo. The growing embryo undergoes shape changes which result from both local cell division and oriented cell elongation as the cell wall makes cell movements impossible.

Stages of plant embryogenesis have been named after the shapes the embryo attains successively. The designations most commonly used are octant, globular, heart and torpedo (Fig. 1). We have introduced a new stage called 'triangular' which indicates the change of symmetry in early embryogenesis (Fig. 1).

The octant stage is precisely defined by the number of cells derived from the apical cell. Periclinal cell divisions delaminate 8 outer epidermal precursor cells from 8 inner cells, marking the beginning of the globular stage. Regional differences can be observed in the late globular embryo which is still very small, measuring only 40 μm in diameter. The incipient root primordium becomes distinct at the basal end of the embryo, and just above the root primordium a centrally located group of 8 narrow cells forms which will give rise to the procambium (vascular primordium) of the hypocotyl.

Cell elongation in the vascular primordium stretches the embryo in the apical–basal axis and, at the same time, cell divisions spread out the apical surface of the embryo. Since the basal surface of the embryo is constrained by the narrow root primordium, these cell activities result in a triangular shape of the embryo. The triangular stage is very short-lived as cell divisions in the apical region rapidly enlarge the laterally located primordia of the cotyledons so that the embryo becomes heart-shaped. The hypocotyl also expands by cell division and cell elongation during the heart stage, and the root primordium is completed at the basal end of the embryo.

As the cotyledons extend further in the apical direction, the heart stage is succeeded by the torpedo stage. Eventually, the growing cotyledons bend over and come to lie adjacent to the hypocotyl in the mature embryo. When the ripe seed dries, the mature embryo enters a stage of dormancy in which it can persist until favourable conditions induce the germination of the seed and the onset of postembryonic development.

Pattern of the seedling

The seedling is the result of plant embryogenesis much as the larva is the result of embryogenesis in *Drosophila*. However, the body pattern of a plant seedling is simple compared with the complex pattern of the *Drosophila* larva.

The main body axis of the seedling is the apical–basal axis. Along this axis, we can distinguish 4 pattern elements from top to bottom: the epicotyl including the shoot meristem, the cotyledons, the hypocotyl, and the root including the root meristem (Fig. 2). The upper end of the hypocotyl is marked by a ring of anthocyan pigmentation just below the site at which the cotyledons are inserted into the axis. The apical–basal pattern elements share the same few tissues which are arranged in radial layers: the outer epidermis, the ground tissue, and the central vascular system. Although these layers may be regarded as elements of a radial pattern

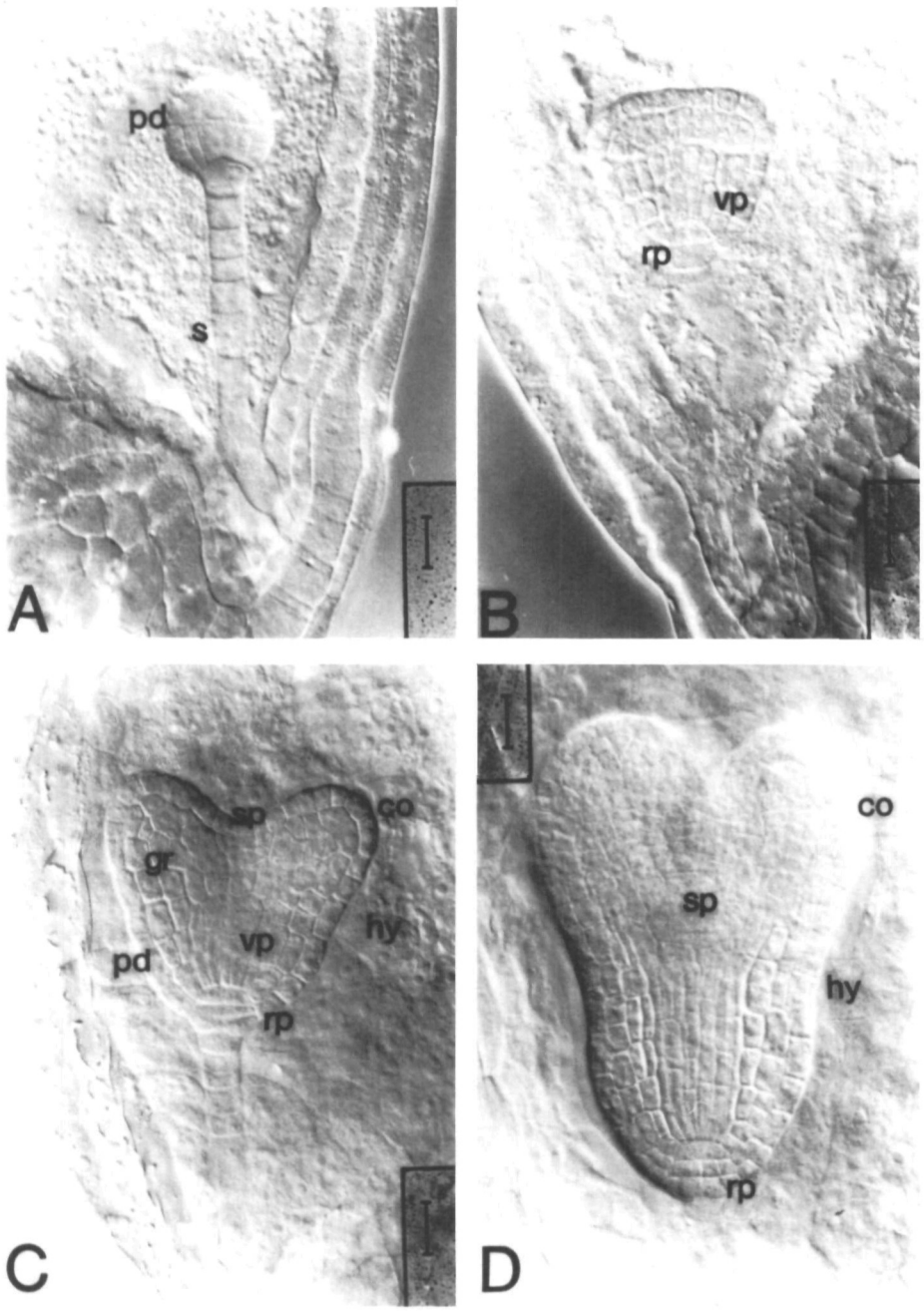

Fig. 1. Stages of embryogenesis in *Arabidopsis*. (A) globular, (B) triangular, (C) heart, (D) torpedo. The root primordium becomes distinct in slightly older globular embryos than the one shown in (A). co, cotyledonary primordium; gr, primordium of ground tissue; hy, primordium of hypocotyl; pd, protoderm (primordium of epidermis); rp, root primordium; s, suspensor; sp, shoot primordium; vp, procambium (primordium of vascular tissue of hypocotyl). Nomarski optics, scale bar 16 μm, apical pole up.

perpendicular to the apical–basal axis, we will not consider this aspect in the present discussion. The seedling pattern is very simple indeed, which may facilitate the analysis of pattern formation in the plant embryo.

Origin of pattern elements in the embryo

The apical–basal pattern elements of the seedling can be traced back to the heart-shaped embryo when their primordia become distinct. Embryogenesis beyond the heart stage merely involves growth of primordia and cell differentiation, whereas pattern formation must have occurred earlier (Fig. 2). The heart stage is reached after 30% of embryogenesis and during this period the embryo has grown relatively little. If one takes into account that the heart shape results from cell activities that are themselves responses to pattern formation, no more than 100 cells are present in the young embryo when the pattern is generated. This small size of the young embryo certainly does not exceed the dimension of a cell population in which stable concentration gradients of diffusible morphogens can be established (Crick, 1970; Driever and Nüsslein-Volhard, 1988).

It is not clear precisely when in embryogenesis the basic pattern of the plant body is generated. The apical–basal polarity of the embryo derives from the polarity of the unfertilised egg cell. The progenitor of the root primordium becomes distinct in the globular embryo. Similarly, the future vascular cells of the

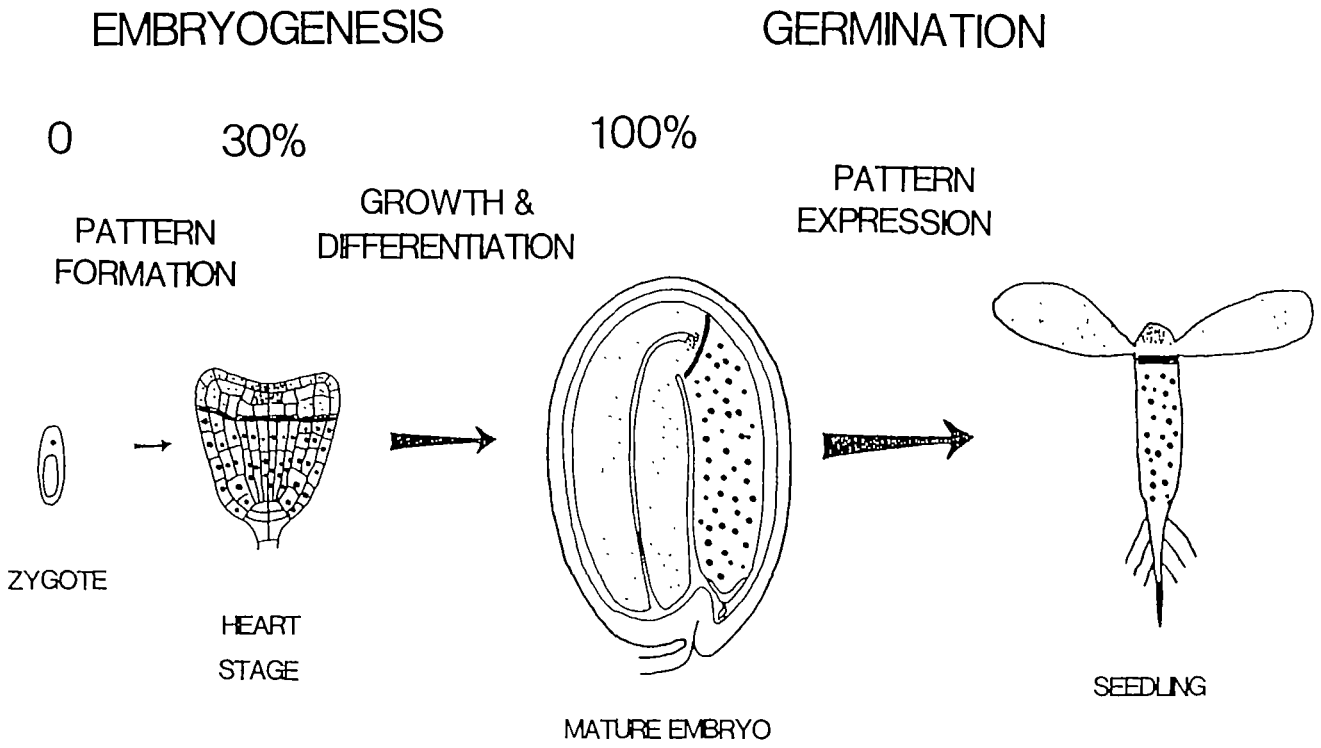

Fig. 2. Pattern formation in the embryo. The pattern elements of the seedling and their corresponding primordia in the embryo are shaded in the same manner.

hypocotyl elongate before the transition from globular to triangular stage. Thus, mutations affecting pattern formation in the plant embryo should show clear deviations from normal development not later than the heart stage.

Screening for putative pattern mutants: rationale

A genetic dissection of pattern formation in the plant embryo seeks answers to the following questions. (1) Can the process of pattern formation be broken down into distinct events, which would be reflected in the phenotypes of pattern mutants? (2) What is the genetic complexity of the process that generates the morphologically simple pattern of the seedling? (3) What molecular mechanisms are responsible for producing the stereotyped pattern?

The first step in the genetic analysis involves the identification of most, if not all, relevant genes by their mutant phenotypes, which requires a large-scale screen for pattern mutants. This approach poses a number of problems. The most critical problem concerns the phenotypic criteria by which to recognise pattern mutants, and related to that, the developmental stage at which mutant phenotypes are to be scored.

The plant embryo lives on its own rather than being supplied with large amounts of substances produced by the mother plant. This condition of life implies that mutational inactivation of genes coding for rather general cell functions including the metabolic machinery, protein synthesis and the like, are fatal to the embryo. For instance, biotin auxotrophy causes embryonic lethality (Schneider *et al.* 1989). We therefore anticipated that embryonic lethality is not a valid criterion by which to recognise pattern mutants. Furthermore, we took the view that genes critical for pattern formation constitute a separate class of genes. Putative pattern mutants might therefore be expected to complete embryogenesis but fail to form the normal seedling pattern. We were encouraged in this view by the results obtained in *Drosophila* where pattern mutants do not arrest embryonic development, no matter how grossly abnormal the mutant pattern may be (e.g. Nüsslein-Volhard *et al.* 1987). The application of *Drosophila* concepts to problems of plant development may be debatable. However, before we started to search for pattern mutants systematically, we found a very interesting pattern abnormality which seems to support our notion (Fig. 3). We call this phenotype *doppelwurzel* (double-root) although this term does not describe the pattern abnormality precisely. The top end of the pattern, including the shoot meristem, is deleted and replaced by the remaining pattern elements in mirror-image orientation. The plane of symmetry runs through the cotyledons which are followed by hypocotyl and root on both sides. Despite gross rearrangement of the pattern, the seedling looks well differentiated. Thus, abnormal pattern formation need not interfere with the completion of embryogenesis.

Screening for pattern mutants at the seedling stage offers the advantage of selecting against embryonic-lethal mutants. However, 'true' pattern mutants would still have to be distinguished from other mutants also

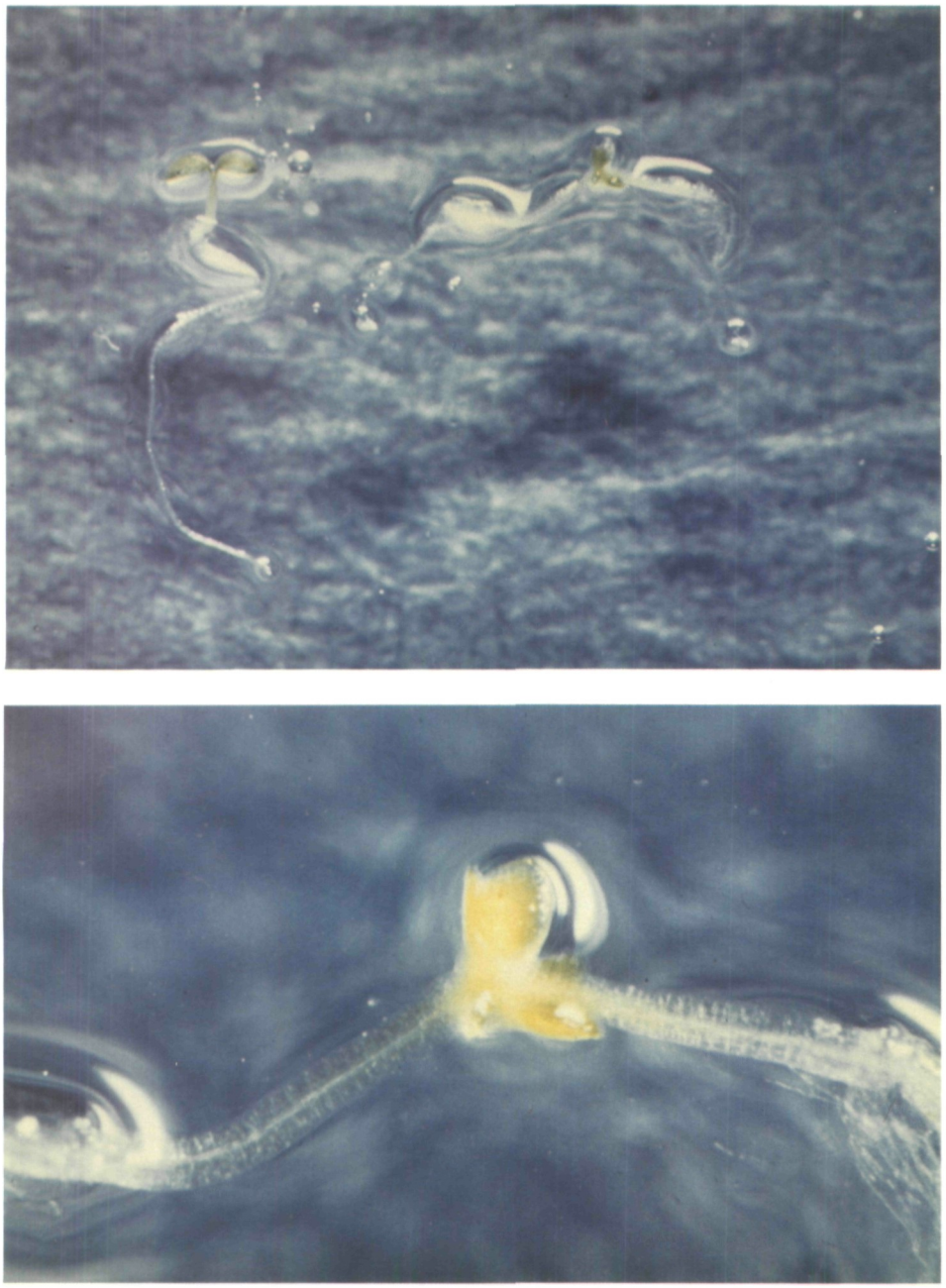

Fig. 3. *doppelwurzel* phenotype. The upper panel shows a wild-type seedling at the left and a *doppelwurzel* seedling at the right. Lower panel: *doppelwurzel* seedling at higher magnification. The plane of mirror-image symmetry runs through the fused cotyledons which are flanked by hypocotyl on both sides. Note the vascular strands in the two hypocotyls.

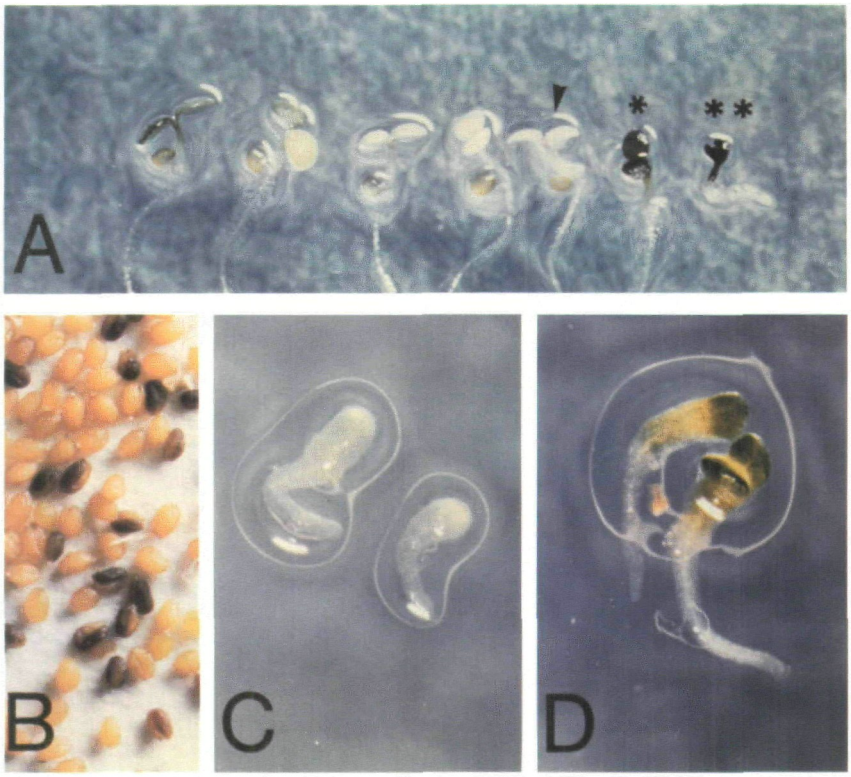

Fig. 5. Mutant seedling phenotypes. (A) Examples of pigmentation mutants as classified by the colour of their cotyledons. From left to right: wild type, yellow-green, pale green, light yellow, albino (arrow-head), 2 different *fusca* mutants of different colour intensities (asterisks). (B) Two-coloured seed phenotype of *fusca* mutants. Wild-type seeds are yellow-brown. (C), (D) Two examples of morphologically abnormal seedling mutants ('quappe').

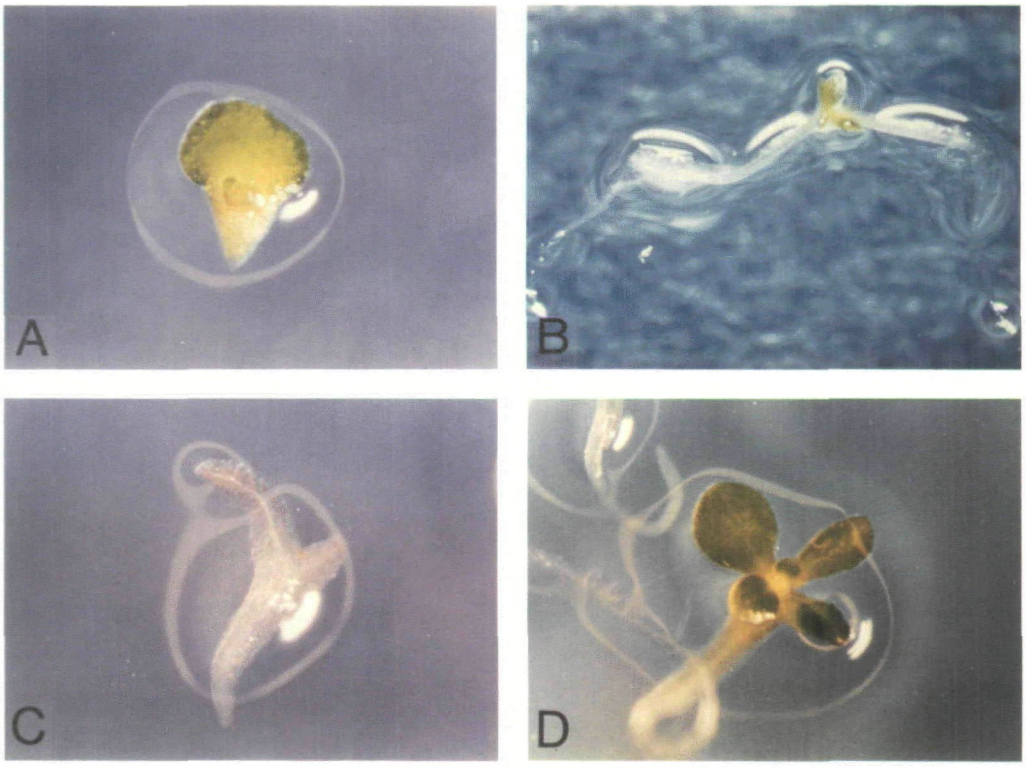

Fig. 6. Putative pattern mutants. The seedling phenotypes shown exemplify the four different phenotype categories (see text). (A) *gnom* (deletion); (B) *doppelwurzel* (deletion/duplication); (C) *toro* (transformation); (D) *häuptling* (multiplication).

producing morphologically abnormal seedlings. There is no *a priori* criterion by which pattern mutants can reliably be recognised at the seedling stage even if some phenotypes might show more specific pattern alterations than do others. Putative pattern mutants would later have to pass a more rigorous test. One requirement is that a pattern mutant should deviate from wild-type at the time when the pattern is generated in the embryo.

Following the paradigm of the *Drosophila* analysis, we reasoned that it would be necessary to identify all relevant genes in the *Arabidopsis* genome if we want to aim at a mechanistic understanding of pattern formation. This aim poses the problem of how many mutagenised lines are to be screened in the search for pattern mutants.

In terms of statistics, at least 5 alleles per gene have to be isolated on average to achieve saturation. According to the Poisson formula, the proportion of genes not represented by mutant alleles would then be negligible, i.e. well below 1%. In planning the saturation screen, we had to make assumptions about the number of essential genes in the *Arabidopsis* genome and about the efficiency of mutagenesis, as these two parameters are relevant for estimating the scale of the screen necessary for saturation:

$$\text{number of lines required} = 5 \times \frac{\text{number of essential genes}}{\text{efficiency of mutagenesis}}.$$

Essential genes include all the genes of an organism that are required for survival and successful reproduction. Since there are no valid estimates of the total number of essential genes in the *Arabidopsis* genome, we reckoned that both *Caenorhabditis elegans* and *Drosophila melanogaster*, which have similar genome sizes, do not differ grossly in genetic complexity from *Arabidopsis*. Based on this comparison, we assumed that *Arabidopsis* may have about 5000 essential genes. Although essential genes of *Arabidopsis* may mutate not only to embryonic lethality, but also to seedling lethality, sterility or haplo-lethality, we concentrated on embryonic-lethal mutations for practical reasons. Embryonic lethality is easy to score, and this class of essential genes is large enough to represent average genes. To be on the safe side, we therefore intended to screen a number of lines corresponding to 25000 embryonic lethal mutations divided by the efficiency of mutagenesis. The latter parameter depends on the dose of the mutagen used. We chose the chemical ethylmethylsulfonate (EMS) because it predominantly causes point mutations which alter the activity of individual genes. The mutant phenotype would result from a single gene change. Our previous experience with EMS mutagenesis suggested that about one-half of the lines derived from seeds treated with 0.3% EMS for 8 h would carry at least one embryonic-lethal mutation. This value translates into a mean value of m=0.7 embryonic-lethal mutations per treated genome. Thus, at least 35000 mutagenised lines would have to be screened for putative pattern mutants in order to achieve statistical saturation.

Screening for putative pattern mutants: procedure

Our screening procedure is outlined in Fig. 4. Mutagenesis of seeds is developmentally analogous to mutagenesis of mature animal embryos producing germ-line clones. In *Arabidopsis*, there are only two cells in the shoot meristem of the mature embryo that give rise to the germ cells of the plant (Müller, 1965; Li and Redei, 1969). If one of these diploid precursor cells suffers a mutational event, this cell would produce a clone of heterozygous descendants which, on average, populate the reproductive tissue in every other flower. A necessary requirement of our screening procedure is the fact that *Arabidopsis* flowers are hermaphroditic, containing both female and male reproductive organs, and that each flower normally self-fertilises. Thus, about one-quarter of the progeny of an individual heterozygous flower are homozygous in the F_1 generation derived from the mutagenised seed. The flowers outside the mutant sector do not produce the same mutant progeny. We analysed only one or two fruits per plant and harvested the seeds from individual fruits separately. If the same phenotype appeared in the F_1 generation from the two fruits of the same plant, this occurrence was regarded as one mutant. In this way, we made sure that any mutants that later turned out to affect the same gene were indeed independent alleles. We scored the phenotypes of F_1 seedlings and, when we found distinct abnormalities, we grew their normal-looking sibs to maturity. Two-thirds of the sibs were expected to be heterozygous for the putative mutation. Seeds were then harvested from individual F_1 plants and checked for the segregation of the same abnormal seedling phenotypes that had first appeared in the previous generation. Upon confirmation of the phenotype, lines were established for further analysis of the mutants.

Yield of large-scale screen: quantitative aspect

A total of 44000 lines were tested (Table 1). These lines carried approximately 25000 embryonic-lethal mutations. Thus, our screen for putative pattern mutants probably fulfilled the statistical requirement for saturation if the genes involved in pattern formation mutate

Table 1. *Yield of mutants*

Type of mutation	Number*	%†
Embryonic-lethal mutations	25000‡	100
Abnormal-seedling mutations	5000	20
A. abnormal pigmentation	2500	10
albino-type mutations§	800	3
fusca-type mutations§	80	0.3
B. abnormal morphology	2500	10
putative pattern mutations§	250	1

44000 lines were screened.

* all numbers given are approximations.

† the number of embryonic-lethal mutations (57% of the lines) was taken as 100% to indicate relative mutation frequencies.

‡ calculated from the number of lines without embryonic-lethal mutations using the Poisson formula to account for more than one hit per line.

§ distinct phenotypes.

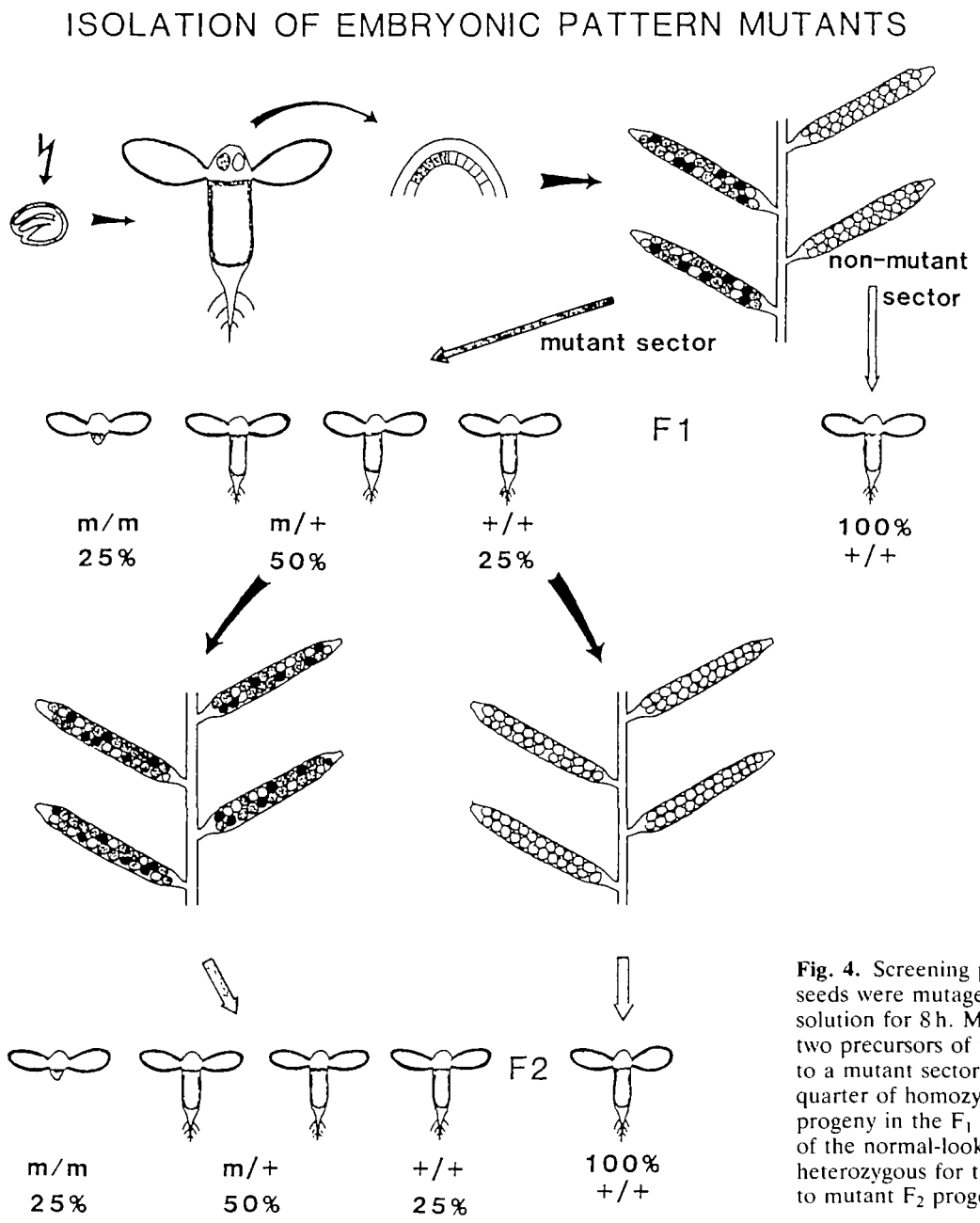

Fig. 4. Screening procedure. Wild-type seeds were mutagenised in 0.3% EMS solution for 8 h. Mutation in one of the two precursors of the germ cells gives rise to a mutant sector which produces one quarter of homozygous mutant (m/m) progeny in the F_1 generation. Two thirds of the normal-looking sibs are heterozygous for the mutation, giving rise to mutant F_2 progeny. For details see text.

like average genes. The embryonic-lethal mutations were not analysed in detail. We noted that embryonic development was arrested at various stages, depending on the line. Our observations agree with previous reports on embryonic-lethal mutants (Müller, 1963; Meinke and Sussex, 1979*b*; Meinke, 1985).

About 5000 lines segregated for seedling phenotypes which included abnormal pigmentation or abnormal morphology or both. Morphologically normal but abnormally pigmented seedlings were segregated in about 2500 lines. A spectrum of pigmentation defects was observed ranging from pure white to light yellow to yellow-green to purplish-green (Fig. 5). The white or light yellow seedlings often turned purple when exposed to light. Of all these pigmentation phenotypes, only two were considered distinct in that the phenotype was easy to score and did not change as the seedlings grew older. About 800 lines segregated for *albino* seedlings, which lacked any of the yellow and green pigments found in wild-type seedlings and appeared white or had various shades of purple colour (Fig. 5). The *albino* seedlings do not form any true leaves but stay small and eventually die.

About 80 lines segregated for the other distinct pigmentation phenotype called *fusca* which was first described in *Arabidopsis* by Müller (1963). The *fusca* mutants can be recognised by unscheduled anthocyan production late in embryogenesis which gives rise to two-coloured seeds, half yellow-brown and half dark brown (Fig. 5). The dark brown colour results from the superposition of two colours, the purple colour of the anthocyan accumulated in the cotyledons of the mutant

embryo and the yellow-brown colour of normal seeds. Upon germination, the seedling normally turns green as a result of chloroplast differentiation. In the *fusca* mutants, this process is not affected but the abnormal anthocyan accumulation makes the seedling appear purplish-green (Fig. 5). Subsequent development is impaired in *fusca* mutants for reasons not well understood. It is not the excessive production of anthocyan *per se* which eventually causes developmental arrest, as lack of anthocyan does not suppress the lethality of *fusca* mutants (our unpublished observation).

About 2500 lines segregated for morphologically abnormal seedlings. In about 90% of these lines, the mutant seedlings clearly differed from wild-type seedlings and yet, their defects appeared to be rather unspecific. Our generic term is 'quappe' phenotype, which implies that the mutant seedling is less well shaped than the wild-type seedling (Fig. 5). 'Quappe' seedlings have reduced or otherwise abnormally shaped cotyledons which sometimes are also less well pigmented. The root is also often shortened or less differentiated than normal. In brief, the 'quappe' phenotype appears to be a syndrome caused by some more general defect which may affect late embryogenesis but does not interfere with germination. However, we cannot at present rule out the possibility that among these mutants with seemingly unspecific pattern defects, there might be some with very interesting primary defects.

About 250 lines segregated for specific morphological abnormalities to be described later. These phenotypes may include pattern defects resulting from mutations in genes which are critical for pattern formation in the embryo. We therefore classify these lines as putative pattern mutants and have begun to study them carefully.

In our screen, embryonic-lethal mutations were about 100 times more frequent than putative pattern mutations. If we assume equal mutabilities for these two gene classes, only 1% of the essential genes in *Arabidopsis* appear to be critical for pattern formation in the embryo. This value may be a slight overestimate as there are other essential genes that mutate to sterility or haplolethality rather than embryonic lethality. Thus, *Arabidopsis* seems to spend only a small proportion of its genetic information on the formation of the basic body pattern in the embryo.

Phenotypic classification of putative pattern mutants

Having identified a large number of putative pattern mutants, we attempted to classify the mutants on the basis of their pattern abnormalities at the seedling stage. This was to serve two purposes. First, we wanted to see if we could establish distinct subdivisions of mutant phenotypes that might reveal different aspects of the pattern-forming process. Another purpose of our attempt at classifying phenotypes was to reduce the labour usually necessary to define genes by complementation analysis. If we could subdivide our collection of mutants on the basis of distinct phenotypes, we would have to testcross fewer phenotypically related mutants. This strategy relies on the validity of the assumption that genes mutate to distinct phenotypes.

In our attempt to classify the putative pattern mutants, we started by establishing rather broad categories based on the kinds of pattern alteration (Table 2). The seedling pattern can be altered by deletion, duplication, transformation or multiplication of pattern elements. Examples of mutant phenotypes representing the four categories are shown in Fig. 6. Which part of the pattern is affected in a mutant seedling, depends on the kind of pattern alteration. Whereas only one class of phenotype is observed in each of the categories of pattern duplication, transformation and multiplication, different pattern elements can be deleted. According to the position of the affected elements, four different classes of pattern deletions can be distinguished: apical, basal, apical and basal, and central (Table 2). The classification of the pattern deletions can be taken one step further by grouping together those mutants that look phenotypically similar within each class. This refined system of classification resulted in 15 different phenotype groups each containing at least five members.

Our system of phenotype classification is still preliminary and coarse-grained. Despite its shortcomings, this scheme is being used as a reference for the genetic analysis of putative pattern mutants. As the genetic and phenotypic analysis of mutants progresses, certain modifications of the scheme may become necessary, e.g. some phenotype groups may have to be

Table 2. *Classification of pattern defects observed in putative pattern mutants*

Phenotype category*	Phenotype class†	Phenotype group‡
Deletion	apical and basal	*fass, gnom, keule, knopf, tulpe*
	apical only	*gurke, knolle, laterne*
	basal only	*möve, monopteros, wurzellos*
	central	*fackel*
Deletion/duplication	apical deletion and basal duplication	*doppelwurzel*
Transformation	of cotyledons into shoots	*toro*
Multiplication	of cotyledons	*häuptling*

* kind of pattern defect.
† position of pattern defect.
‡ specific phenotype.

assigned to different phenotype classes. We believe, however, that the general structure of our classification of putative pattern mutants will prove to be consistent.

Genetic characterisation of putative pattern mutants

We have begun to define genes by complementation tests among mutants within phenotype groups. So far we have concentrated on mutants in the class of pattern deletions, and in the few cases tested the phenotype groups turned out to be good criteria for predicting allelism. Some mutants with unrelated phenotypes have also been tested and found to complement one another whereas phenotypically similar mutants can indeed result from mutations in the same gene. At present, four putative patterning genes with 5 to 10 alleles each have been identified. Three of these genes, *gnom*, *knopf* and *monopteros*, appear to mutate to alleles with nearly identical phenotypes whereas one of these genes, *fass*, is represented by alleles of varying strengths (U. Mayer, manuscript in preparation; R. A. Torres Ruiz and T. Berleth, unpublished observations). These observations suggest that thresholds of gene activity determine the phenotypic effect in the former group while residual gene activity is quantitatively reflected by the phenotype in the latter.

Our preliminary complementation data support the notion that we have saturated the *Arabidopsis* genome for purative pattern mutants. However, patterning genes appear to represent a small subgroup of the essential genes in the *Arabidopsis* genome and may differ in mutability from average genes. If we could independently assess the degree of saturation achieved in our screen, we would be able to estimate the number of essential genes mutating to embryonic lethality and use this figure to determine the proportion of putative patterning genes. For this purpose, we chose the *fusca* mutants which do not affect the seedling pattern and thus might represent average genes in the *Arabidopsis* genome. The advantage of the *fusca* mutants is that they constitute a homogeneous class of mutant phenotype, although the primary defect may be different in different mutants. The number of *fusca* mutants is large enough to be representative, and, on the other hand, small enough to be completely analysed by complementation tests without too much effort. We used the two-coloured seed phenotype as the criterion for non-complementation. In order to minimise the labour, we started out by crossing 13 *fusca* mutants in all possible pair-wise combinations. This established eight initial complementation groups which were subsequently used for testing additional mutants. So far, we have defined nine genes with three alleles on average. The total number of *fusca* genes may be close to one dozen each with six or seven alleles. Thus, we have probably identified all the genes mutating to those phenotypes for which we have saved the mutants. This conclusion appears to hold true for putative pattern mutants and others alike.

We have begun to map putative patterning genes as defined by complementation analysis as well as a few single mutants that have not been tested for complementation. This is being done for two reasons. First, we wish to see if the genetic lesion causing the abnormal pattern can actually be localised to a specific position on the genetic map (Koornneef *et al.* 1983, 1987). If this is the case, the pattern alteration would very likely result from the mutational inactivation of a single gene. Second, RFLP mapping is to provide a starting point for molecular cloning of patterning genes (Chang *et al.* 1988; Nam *et al.* 1989). Our preliminary observations on ten putative patterning genes indicate that the mutant phenotype is the result of a defined genetic lesion and that the map positions are randomly distributed in the genome.

Early mutant phenotypes: testing the rationale

Our screen was based on the assumption that the mutant seedling pattern reflects alterations in the process of pattern formation in the early embryo (Fig. 2). To test the validity of this assumption, we have begun to study early embryogenesis in putative pattern mutants. In the mutants so far tested, the seedling phenotype is closely correlated with a specific deviation from normal development in the early embryo. In the following, we discuss some developmental features of pattern mutants as exemplified by mutant alleles of the *gnom* gene.

The *gnom* gene is not required for cell differentiation. The mutant seedling contains cell types derived from the three tissue layers of the embryo, i.e. epidermis, ground tissue and vascular tissue (Fig. 7). The abnormally shaped seedling is covered by an epidermal cell layer which includes the characteristic guard cells of stomata normally found in the cotyledons. The presence of guard cells in *gnom* seedlings suggests that the apical region corresponds to the cotyledonary pattern element of wild type. Tracheary elements derived from the vascular tissue are also present in *gnom* seedlings. However, these elements are not interconnected to form strands as in wild type but are either arranged in clusters or even occur as single, isolated cells. Thus, differentiated cells of both hypocotyl and cotyledons are found at the correct relative positions in the apical–basal axis. By contrast, cells with the specific features of root elements are lacking.

The *gnom* embryos can reliably be recognised at the heart stage by their abnormal shape (Fig. 7). Several factors contribute to the deviation from wild type. There are no cells corresponding to the root primordium of wild type identified by their characteristic shapes and by their location next to the filamentous suspensor. In addition, there is no central block of elongated cells that normally constitute the vascular primordium, and the presumptive cotyledonary regions are less distinct than in wild type.

The *gnom* gene meets the criteria for patterning genes. First, it is required during the pattern-forming phase of embryogenesis. Second, lack of *gnom* gene activity does not block subsequent development but rather eliminates part of the seedling pattern.

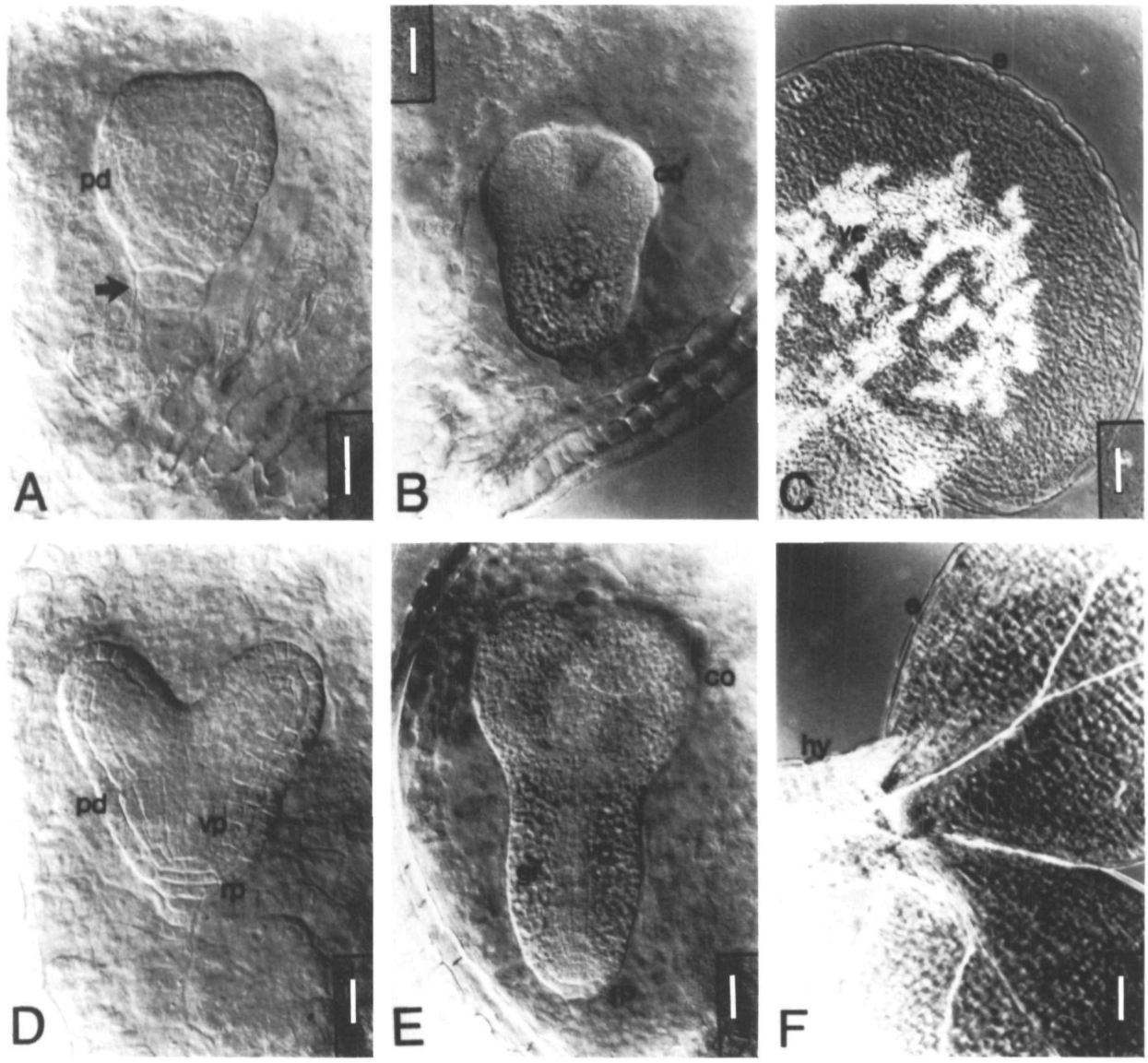

Fig. 7. Embryogenesis of the *gnom* mutant. (A,D) heart, (B,E) torpedo, (C,F) seedling; (A–C) *gnom*, (D–F) wild type. Note group of differently shaped cells (arrow in A) in place of root primordium in *gnom* heart-stage embryo. co, cotyledonary primordium in (E), cotyledon of seedling in (F); co′, rudimentary cotyledonary primordium of *gnom* mutant; e, epidermis; gr, ground tissue; pd, primordium of epidermis; hy, hypocotyl; rp, root primordium; vc, vascular cell (arrowhead); vp, primordium of vascular tissue; vs, vascular strand. Nomarski optics, scale bar 16 μm in (A) and (D), 25 μm in (B) and (E), 50 μm in (C) and (F), apical pole up.

Discussion

Starting from scratch with the genetic analysis of pattern formation in the plant embryo poses both theoretical and practical problems. With regard to the practical problems, we have worked out a selection scheme that enables the isolation of putative pattern mutants in *Arabidopsis*. Subsequent characterisation of these mutants has shown that pre-selection at the seedling stage does enrich for mutant alleles of genes directing pattern formation in the embryo. We believe that further genetic and phenotypic studies will single out the patterning genes from among our collection of putative pattern mutants. However, if we aim to understand pattern formation in the embryo as a gene-directed process, we have to identify all the relevant components by mutant alleles. Although the large scale of our screen was certainly sufficient for statistical saturation of the genome, our screening procedure may have selected against specific types of pattern mutants.

Is the search for pattern mutants by phenotypic criteria comprehensive?

Our approach was based on the assumption that pattern formation in the embryo is directed by a separate class of genes not involved in common cell processes. This assumption, which cannot at present be tested, is indirectly supported by the following evidence. Several embryonic-lethal mutations have been shown to affect

the competitiveness of the growing pollen tube resulting in fewer mutant embryos than expected from random selfing (Müller, 1963; Meinke, 1982; Meinke and Baus, 1985). This feature suggests that the gene in question is required for general cell processes common to both the vegetative pollen tube cell and dividing cells of the embryo. By contrast, the mutants that we believe identify patterning genes in the embryo generally do not show reduced transmission in the male gametophyte (our unpublished observation). Thus, the embryonic patterning genes appear to constitute a different class.

Although we have been able to isolate pattern mutants by their seedling phenotype, it is conceivable that mutations in other patterning genes may alter early embryogenesis so drastically as to cause developmental arrest. Mutants of this kind would have been missed in our screen. We have checked about 100 embryonic-lethal mutants for pattern abnormalities in the embryo and have not found any that would qualify as a pattern mutant. Although this result means that it is unlikely that there are many genes of this kind, it does not rule out their existence.

Embryonic patterning genes might also be required for analogous processes at other stages of the life cycle, especially in the haploid gametophyte. If a patterning gene performed the same role in both embryogenesis and the development of the gametophyte from which the embryo derives, this would prevent the formation of mutant zygotes and hence this gene could not be identified by its mutant phenotype at the seedling stage. The present techniques of genetic analysis do not enable the recovery of this type of mutant in *Arabidopsis*.

The apical–basal polarity of the unfertilised egg cell raises the problem that some genes directing embryonic pattern formation might be active exclusively in the female gametophyte. Along the same lines, there is also the possibility that premeiotic gene activity directly influences pattern formation in the embryo, which would show up as maternal-effect mutations. No mutants of this kind have been described in *Arabidopsis* or any other plant species.

Finally, we may have introduced a systematic bias ourselves by the phenotypic criteria on which we picked the 'putative pattern mutants' from among a much larger number of lines segregating for morphologically abnormal seedlings. Some patterning genes may mutate to inconspicuous phenotypes, and their mutant alleles would have been missed. However, we consider it unlikely that a patterning gene exclusively mutates to inconspicuous phenotypes. The situation is different for patterning genes whose activity translates quantitatively into pattern. If weak alleles were difficult to recognise phenotypically, this would still leave one or more strong alleles which we would have classified as putative pattern mutants. By the same token, patterning genes whose strong alleles arrest embryonic development would be identified by weaker alleles with interesting seedling phenotypes. In both instances, fewer than average alleles would be recovered. However, with an average allele frequency of five, such genes should still be represented in our collection of putative pattern mutants.

Genetic complexity of pattern formation in the plant embryo

Our collection of putative pattern mutants probably includes mutant alleles of most, if not all, embryonic patterning genes mutating to distinct seedling phenotypes. The ongoing complementation analysis is to yield the actual number of genes represented by mutant alleles as well as the distribution of allele frequencies. Our preliminary data suggest that at least 15 and probably less than 50 zygotically active genes direct pattern formation in the *Arabidopsis* embryo. The lower estimate derives from the number of distinct phenotype groups, the upper estimate reflects the proportion of putative pattern mutants relative to the embryonic-lethal mutations. However, the total number of genes mutating to embryonic lethality is not known. If we use the proportion of *fusca* mutants isolated in the same screen, we arrive at a slightly lower estimate. The *fusca* mutants that most likely represent one dozen genes correspond to approximately 0.3 % of the embryonic-lethal hits, which would lead to an estimated total number of 4000 essential genes required for embryogenesis and hence about 40 embryonic patterning genes. Whatever the actual number may be, pattern formation in the *Arabidopsis* embryo appears to be directed by a fairly small number of essential genes.

The significance of our estimate can be assessed by comparison with the genetic complexity of embryonic pattern formation in *Drosophila*, the only other organism for which data are available from comparable large-scale screens. Zygotically active genes mutating to pattern abnormalities in the cuticle of the larva represent about 3 % of the essential genes (Nüsslein-Volhard *et al.* 1984; Jürgens *et al.* 1984; Wieschaus *et al.* 1984). In *Drosophila*, there are also maternally active genes that contribute to the formation of the basic body pattern, and these amount to about 1 % of the essential genes (reviewed by Anderson, 1989; Nüsslein-Volhard and Roth, 1989). A more direct comparison would only involve pattern formation in the longitudinal body axes, the apical–basal axis of *Arabidopsis* and the anterior–posterior axis of *Drosophila*. About 1 % of the essential genes direct segmentation, including both partitioning and diversification, in the *Drosophila* embryo (reviewed by Akam, 1987). This figure is slightly higher than our estimate of the number of genes directing the analogous process in *Arabidopsis*. It seems as if *Arabidopsis* makes its simple body pattern with almost as many genes as *Drosophila* needs for its elaborate body segmentation.

Classification of mutant phenotypes and the structure of the pattern-forming process

The pattern abnormalities at the seedling stage probably reflect perturbations of the pattern-forming process in the early embryo. A consistent classification of

mutant phenotypes may therefore reveal distinct events of pattern formation. This phenotype taxonomy was successfully applied to the segmentation genes of *Drosophila* which were initially ranked at different levels in the process on the basis of their mutant phenotypes and later shown to act in a hierarchical order (Nüsslein-Volhard and Wieschaus, 1980; Ingham, 1988).

We have sorted the putative pattern mutants of *Arabidopsis* by phenotypic criteria. The resultant system of classification involves three major subdivisions. Categories, classes and groups represent kinds of pattern change, positions of pattern defect and specific phenotypes, respectively. In the following, we discuss how our classification system might be related to the structure of the pattern-forming process in the embryo.

The four different categories (deletion/duplication, transformation, multiplication and deletion) represent the kinds of pattern change observed in putative pattern mutants. In terms of pattern formation, the categories may represent a heterogeneous assemblage of diverse processes. The deletion/duplication is reminiscent of the *bicaudal* phenotype in *Drosophila* which was taken as evidence for the graded distribution of some positional information in the anterior–posterior axis of the *Drosophila* embryo (Nüsslein-Volhard, 1977). This analogy raises the possibility that pattern formation in apical–basal axis of the plant embryo has a formally similar basis. It is worthy of note that we observed only one class of deletion/duplication phenotype in which basal pattern elements replaced apical pattern elements in mirror-image symmetry. The lack of the reciprocal phenotype, i.e. basal elements replaced by apical ones, may suggest that the root pole is 'epistatic' to the shoot pole.

Two other categories also include single phenotype classes. The transformation phenotype affects the leaf-like cotyledons which are replaced by shoot-like structures. By analogy to the homeotic phenotypes in *Drosophila* which change segmental identity without affecting the formation of segments (e.g. Lewis, 1978), we may infer from the transformation of cotyledons that lateral appendages are formed but their normal identity is not established. This phenotype may also be regarded as atavistic if leaves evolved from shoot-like structures as is commonly believed. Regardless of the evolutionary origin of leaves, the homeotic transformation of leaves into shoots suggests that shoot is the 'ground state' in plant development.

The multiplication phenotype also affects the cotyledons, increasing their number from two to four or more. It is not clear at present whether this alteration specifically affects the cotyledons. Alternatively, the region of the apical–basal pattern giving rise to the cotyledons might be expanded at the expense of the shoot meristem. In the former case, the two categories of transformation and multiplication would both reflect events related to the elaboration of the pattern rather than to the earlier partitioning of the apical–basal axis. In the latter case, the lack of the most apical pattern element and the concomitant expansion of the remaining pattern would suggest that a failure of partitioning causes the multiplication phenotype.

The category of pattern deletions can be subdivided into different classes according to the position of the defect in the apical–basal axis. These phenotype classes suggest that pattern formation in the embryo involves partitioning of the apical–basal axis into distinct regions, apical, central and basal. How the class of apical and basal deletion fits in with this scheme is not at all obvious. One might argue that only one of the deletions is the primary defect which then causes the other deletion. This seems to be the case with *gnom* which appears to affect the basal region of the pattern primarily. Alternatively, the two deletions might result from independent requirements for the same gene at the two opposite ends of the embryo. More detailed studies of mutant embryos are necessary to resolve this problem.

Our preliminary classification of pattern abnormalities hints at a mosaic mode of pattern formation. With the exception of the deletion/duplication phenotype, different parts of the pattern can be affected independently of other parts. This may mean that each patterning gene is required for one specific aspect of the process but is dispensible for other aspects.

The idea that different parts of the pattern are largely specified independently can be tested in combinations of deletion mutants which affect different parts of the pattern. On the assumption of independent development, double mutants should phenotypically correspond to the sum of their components. In any case, the study of double mutant phenotypes should also reveal functional relationships between patterning genes and thus facilitate the analysis of pattern formation as a process of interacting genes. For instance, if one or the other mutant phenotype were epistatic the two genes would be said to act at different hierarchical levels in the process. If an entirely novel phenotype resulted from the combination, the two genes most likely interact synergistically.

Prospects for a mechanistic understanding of pattern formation in the plant embryo

Compared to *Drosophila*, the analysis of pattern formation in the plant embryo is just beginning. However, there are reasons to believe that pattern formation in the *Arabidopsis* embryo may be understood at a comparable level in the near future. The seedling pattern is simple, and the number of genes directing pattern formation is small. Our collection of pattern mutants provides raw material for further genetic analysis. Mutant phenotypes will be studied carefully in order to assign each gene a particular role in the process of pattern formation, and the analysis of double mutants will outline the logical structure of the pattern-forming process.

A mechanistic understanding of pattern formation in the plant embryo requires a molecular analysis of the genes involved and their products. Once a patterning gene has been cloned, genetic hypothesis about its role in the process can be tested at the molecular level. Of

special interest to us are answers to the following questions. (1) How does the patterning gene act, i.e. what is the molecular nature of its gene product? (2) How is the expression of the patterning gene regulated, i.e. does the spatial distribution of its mRNA correspond to the spatial requirement of gene function as inferred from the mutant phenotype? (3) How does the patterning gene fit in with the genetic network directing pattern formation in the embryo, i.e. is the pattern of gene expression changed in embryos of other pattern mutants as predicted from the analysis of double mutant phenotypes?

Molecular cloning of genes solely defined by mutant phenotype and map position can be achieved in *Arabidopsis* in two ways, by gene tagging (Feldmann *et al.* 1989) or by RFLP mapping and chromosomal walking (Chang *et al.* 1988; Nam *et al.* 1989). The latter approach is feasible in *Arabidopsis* because the nuclear genome is small and largely devoid of interspersed repetitive sequences (Pruitt and Meyerowitz, 1986). By bringing together the genetic analysis and the techniques of molecular biology, we may soon be able to study pattern formation in the plant embryo at the molecular level.

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