EMBRYO-LETHAL MUTANTS OF ARABIDOPSIS THALIANA: RESPONSE OF MUTANT EMBRYOS IN CULTURE AND ANALYSIS OF THE DISTRIBUTION OF ABORTED SEEDS IN HETEROZYGOUS SILIQUES

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CHAPTER I

INTRODUCTION

Arabidopsis thaliana (L.) Heynh. is a small, weed-like plant in the Brassicaceae (Cruciferae) that is widely distributed throughout the northern hemisphere (Figure 1). This inconspicuous member of the mustard family has been used extensively as a research tool in many different areas of plant biology (Rédei, 1970, 1975a,b). The widespread interest in Arabidopsis can be attributed to a number of desirable characteristics, a few of which include: (1) the size of the plant is small, allowing populations of thousands to be grown under laboratory conditions; (2) the life cycle is completed in 5 to 6 weeks enabling many generations to be grown in a short period of time; (3) each plant has the potential to produce more than 10,000 seeds; (4) the chromosome number is low (n=5); and (5) a wide range of mutants can easily be isolated following seed mutagenesis (Rédei, 1975b).

The earliest studies with A. thaliana were begun over 40 years ago and focused primarily on the areas of physiological and biochemical genetics in an attempt to uncover the basic features of plant growth and development (Laibach, 1943, 1951; Kugler, 1947). Numerous studies describing the genetics and basic biology of this plant have also been

Figure 1. Drawing of Arabidopsis thaliana (L.) Heynh. Mature plant showing basal rosette, main stem with terminal inflorescence and lateral branches (A); epidermal hairs on the surface of leaves (B); flower at the time of pollination (C); petal (D); stamens (E, F); compound pistil (G); mature silique splitting along both sides of the central septum to reveal 2 rows of seeds (H); and mature seed (I). Drawing from Ross-Craig (1948).


C
×8
D
×8
E
×8
F
×8
G
×12
B
×12
A
×1
H
×4
I
×24

reported in various review articles (Rédei 1970, 1975a,b; Müller, 1961).

More recent studies with A. thaliana have expanded to many different areas of plant biology. Physiological and biochemical studies are still being pursued but from a different approach than that of the early researchers. Valuable information, for example, has been obtained from studies on the physiological factors affecting seed germination (Baskin and Baskin, 1983) and seed dormancy (Cone and Spruit, 1983), the ultrastructure of cellular organelles in the root cap (Olsen et al., 1984), and the biochemical analysis of metabolites produced following azide mutagenesis (Rosichan et al., 1983).

A. thaliana has also played an important role in many plant tissue culture projects (Negrutiu et al., 1975). Factors influencing plant regeneration from in vitro cultures of A. thaliana have been examined (Negrutiu and Jacobs, 1978a,b), and this information has enabled subsequent studies upon somatic embryogenesis (Huang and Yeoman, 1983) and in vitro organogenesis (Huang and Yeoman, 1984). Anther culture of this plant has been reported to produce haploid callus and plants (Gresshoff and Doy, 1972) as well as dihaploid shoots (Scholl and Amos, 1980). In addition, protoplasts of A. thaliana have been fused with those of Brassica campestris to generate a somatic-cell hybrid, Arabidobrassica (Gleba and Hoffmann, 1980). Such intergeneric hybrids provide interesting material for genetic and biochemical analyses.

Agrobacterium tumefaciens has been used as a vector for genetic engineering of plants through the incorporation of foreign DNA into the genome of plant cells (Schell et al., 1983; Horsch et al., 1984). A. thaliana is an ideal host for such transformation studies because it has an extremely small haploid genome (7×10^7 nucleotide pairs) and contains few repetitive DNA sequences (Leutwiler et al., 1984). Not only have crown gall tumors produced from A. tumefaciens been induced on A. thaliana (Aerts et al., 1979), but subsequent studies have demonstrated that stable transmission of the crown gall DNA from infected A. thaliana plants to seed progeny is possible (Pavingerova et al., 1983).

A wide variety of mutants of A. thaliana has been generated for detailed studies (Table I). Mutants unable to assimilate nitrogen have been isolated by selecting for plants that are resistant to high concentrations of chlorate (Oostindiër-Braaksma and Feenstra, 1973). This technique has allowed rapid isolation of nitrogen reductase-deficient mutants for detailed mutant characterization (Braaksma and Feenstra, 1982a), nitrogen uptake studies (Doddema, et al., 1978), and the analysis of the nitrate reductase complex in revertants (Braaksma and Feenstra, 1982b).

A. thaliana mutants have also been used in numerous plant hormone studies. Recent reports have described mutants that are less sensitive to exogenous ABA than wild-type lines (Koornneef et al., 1984), have reduced levels of ABA (Koornneef et al., 1982b), are unable to germinate and develop normally unless giberellin is applied (Koornneef and

TABLE I

A REPRESENTATION OF A FEW OF THE MUTANTS OF ARABIDOPSIS THALIANA

Mutant	Method of Induction	Reference
GA sensitive	EMS, X-rays or fast neutrons	Koornneef and van der Veen, 1980
Nitrate-reductase deficient	EMS or NG	Braaksma and Feenstra, 1982a
ABA-insensitive	EMS	Koornneef et al., 1984
ABA-deficient	EMS or fast neutrons	Koornneef et al., 1982b
Thiamine auxotrophs	EMS	Li and Rédei, 1969
Photosynthesis and photorespiration	EMS	Somerville and Ogren, 1979, 1980, 1981, 1983; Somerville et al., 1982;
Auxin-resistant	EMS	Maher and Martindale, 1980
Chloroplast-lipid deficient	EMS	Browse et al., 1985
Chlorophyll-deficient	EMS	Müller, 1963
Leaf shape	EMS or X-rays	Rédei and Hirono, 1964
Fused flower	EMS or chemical	MeKelvie, 1962
Long hypocotyl	EMS, X-rays or fast neutrons	Koornneef et al., 1980
Trichome-deficient	EMS or X-rays	Rédei and Hirono, 1964

TABLE I (CONTINUED)

Distorted trichomes	EMS, X-rays of fast neutrons	Feenstra, 1978; Koornneef et al., 1982a
Embryo-lethal	EMS or X-rays	Müller, 1963; Meinke, 1985

NG = N-methyl-N-nitro-N-nitrosoguanidine

EMS = Ethylmethane sulfonate

van der Veen, 1980), and have both an increased resistance to 2,4-D and an altered geotropic response (Maher and Martindale, 1980; Mirza et al., 1984).

Research involving mutants has also been helpful in determining factors regulating a number of major biochemical pathways. Mutants of A. thaliana with a decreased activity of ribulose 1, 5 bisphosphate (RuBP) carboxylase (Somerville et al., 1982), a defective chloroplast dicarboxylate transporter (Somerville and Ogren, 1983), a deficiency in phosphoglycolate phosphatase (Somerville and Ogren, 1979), an inactive leaf glutamate synthetase (Somerville and Ogren, 1980), and reduced serine trans hydroxyl methylase activity (Somerville and Ogren, 1981) have been used to study both photosynthesis and photorespiration in plants. In addition, analysis of a mutant of A. thaliana lacking a chloroplast-specific lipid has proven to be a useful tool for investigating the relationship between fatty acyl composition and membrane function (Browse et al., 1985).

Mutants in which development is altered or severely impaired have also been reported. Mutants of A. thaliana with fused flowers (McKelvie, 1962), distorted trichomes (Feenstra, 1978; Koornneef et al., 1982a), altered hypocotyls (Koornneef et al., 1980), and abnormal leaf shapes (Rédei and Hirono, 1964) have been analyzed in various developmental studies. In addition, embryo-lethal mutants of A. thaliana have been isolated and characterized through a variety of experimental techniques (Müller 1963; Meinke, 1979b, 1985;

Meinke et al., 1985). These mutants provide a promising system for the study of plant embryo development because the defect interrupts development at various stages. Embryo-lethal mutants are the focus of this project and will be discussed in greater detail in the following paragraphs.

Embryo-lethal mutants, which are blocked at some stage during embryogenesis, are unable to develop into normal, mature plants. In higher plants, these mutants have been described extensively in corn (Jones, 1920; Mangelsdorf, 1926; Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980, 1982), carrot (Breton and Sung, 1982) and A. thaliana (Müller, 1963; Meinke and Sussex, 1979a,b; Meinke, 1982, 1985; Meinke et al., 1985). Corn and Arabidopsis mutants are maintained as heterozygotes because homozygotes are usually unable to germinate in vivo. Ethyl methanesulfonate (EMS) or irradiation with X-rays are the two methods that are most commonly used to induce these mutants.

Jones (1920) was the first to report lethal factors that disrupted seed development in corn. Many of these factors, which were shown to be the result of Mendelian inherited genes, were termed defective seed and were designated "de". Subsequent studies have described defective seed mutants of corn in more detail. Mangelsdorf (1926), for example, performed extensive studies on 14 defective seed mutants isolated in various genetic backgrounds. Analysis of defective and normal seeds revealed that each mutation was inherited as a Mendelian recessive. Complementation tests between the different heterozygous plants indicated that two

of the mutants (de-5 and de-11) were defective in the same gene (i.e. allelic). The weights of both mature defective and normal kernels were then compared. The average weight of mutant kernels ranged from 2% of normal kernel weight in de-14 to 59% in de-2 (Table II). The percent germination of mature mutant kernels was also compared with that of the normal kernels at the same weight (Table II). From these results Mangelsdorf was able to conclude that something other than the decreased weight of the mutant kernels was affecting the germination rate.

In later experiments, Lowe and Nelson (1946) analyzed the defective seed mutant termed miniature seed. Although kernels carrying this mutation are only 20% of the weight of normal seeds from the same ear, they are able to produce plants that do not differ significantly in size from normal plants. Light microscopic studies of sections of defective seeds revealed that at nine days after pollination, the chalazal cells connecting the vascular tissue to the ovary wall of the caryopsis began to break down. By fourteen days after pollination, growth of the embryo and endosperm had ceased completely. These results suggested that the small size of the miniature seed at maturity was a result of the disruption of nutrient flow to the growing embryo and endosperm.

Brink and Cooper (1947) analyzed the effect of the de-17 allele on development of the corn caryopsis. Kernels homozygous for this allele are similar to the miniature seed

TABLE II

KERNEL DEVELOPMENT AND GERMINATION FREQUENCIES IN 14 DEFECTIVE SEED MUTANTS OF CORN STUDIED BY MANGELSDORF (1926)

Mutant	Weight of Mutant Kernels*	Germination of Mutant Kernels	Expected Germination**
de-2	59%	45%	94%
de-1	50%	46%	91%
de-4	37%	4%	75%
de-6	34%	12%	72%
de-3	30%	54%	66%
de-7	19%	20%	49%
de-5	18%	11%	47%
de-8	15%	0%	42%
de-10	14%	0%	39%
de-11	7%	1%	22%
de-9	6%	0%	17%
de-13	5%	0%	12%
de-12	4%	0%	10%
de-14	2%	0%	4%

*Expressed as average percentage of normal weight reached by mutant kernels at maturity.

**Germination frequency observed when normal kernels of the same weight were removed from an immature ear, dried, and planted.

kernels in that the mature mutant seeds are small (i.e. 75% the weight of normal seeds), while the resulting homozygous plants are phenotypically normal. Light microscopic studies indicated that the basal cells of the endosperm began to collapse after 10 days. This collapse of cells reduced the absorption and transfer of nutrients to the seed, causing the reduced seed size.

B-A translocation have been used extensively in corn mutants to study embryo-endosperm interactions (Robertson, 1952; Neuffer and Sheridan, 1980; Sheridan and Neuffer, 1980, 1982). B chromosomes are accessory chromosomes found in some plant species that produce very little phenotypic effect. In corn, these chromosomes undergo non-disjunction during the second post-meiotic mitosis of pollen formation to produce one sperm cell lacking B chromosomes and one sperm cell containing two B chromosomes. Through irradiation of pollen grains containing B chromosomes, it is possible to produce B-A translocations such that a portion of a normal A chromosome is exchanged with a portion of a B chromosome. When B-A translocations are crossed with heterozygous individuals, corn kernels in which the genotype of the embryo and endosperm differ can be obtained.

Neuffer and Sheridan (1980) successfully isolated 194 defective kernel mutants of corn following EMS pollen mutagenesis. To determine the effect of a normal embryo on development of a mutant endosperm and vice versa, 19 of these mutants were crossed with B-A translocations to construct a kernel with a normal embryo and a mutant endosperm. The

reciprocal genotype was also constructed. In a majority of the 19 mutants, a normal endosperm was unable to help the mutant embryo proceed through normal stages of development. These results suggested that the mutation was affecting both endosperm and embryo development. Sheridan and Neuffer (1980) also examined the growth in culture of 102 defective kernel mutants. Eighty-one of these mutants produced shoots and roots on at least one medium, while 16 produced more tissue on the basal, and 23 exhibited better growth on the enriched medium. Ten of the 23 were recognized as possible auxotrophs. One of the ten was positively identified as a proline-requiring auxotroph that was allelic to the pro-1 (proline-requiring) seedling lethal previously described by Gavazzi et al. (1975).

In their most recent report, Sheridan and Neuffer (1982) studied 14 defective kernel mutants in which the embryos were unable to form leaf primordia at maturity (Figure 2). The endosperm-embryo interaction of 11 of these mutants was examined by using B-A translocations. In all cases, a normal endosperm was not able to recover a mutant embryo, but a normal embryo was able to rescue a mutant endosperm (Table III). The embryos in the later case formed primordia and germinated. Although the endosperm genotype may impair growth in some cases, it is the genotype of the embryo that determined the extent of embryonic development in this group of mutants.

Figure 2. Stages of developmental arrest of 17 defective kernel mutants of corn and the corresponding stages of immature normal embryos. The bottom row represents normal embryo development from the proembryo to stage 2 as described by Abbe and Stein (1954). The lines indicate the stage of development in which the corresponding mutant kernels arrest. Four mutants allelic to E792 are not illustrated. With the exceptions of E1311C, E1399 and E1429A, the mutant kernels are unable to form leaf primordia. Figure taken from Sheridan and Neuffer (1982).

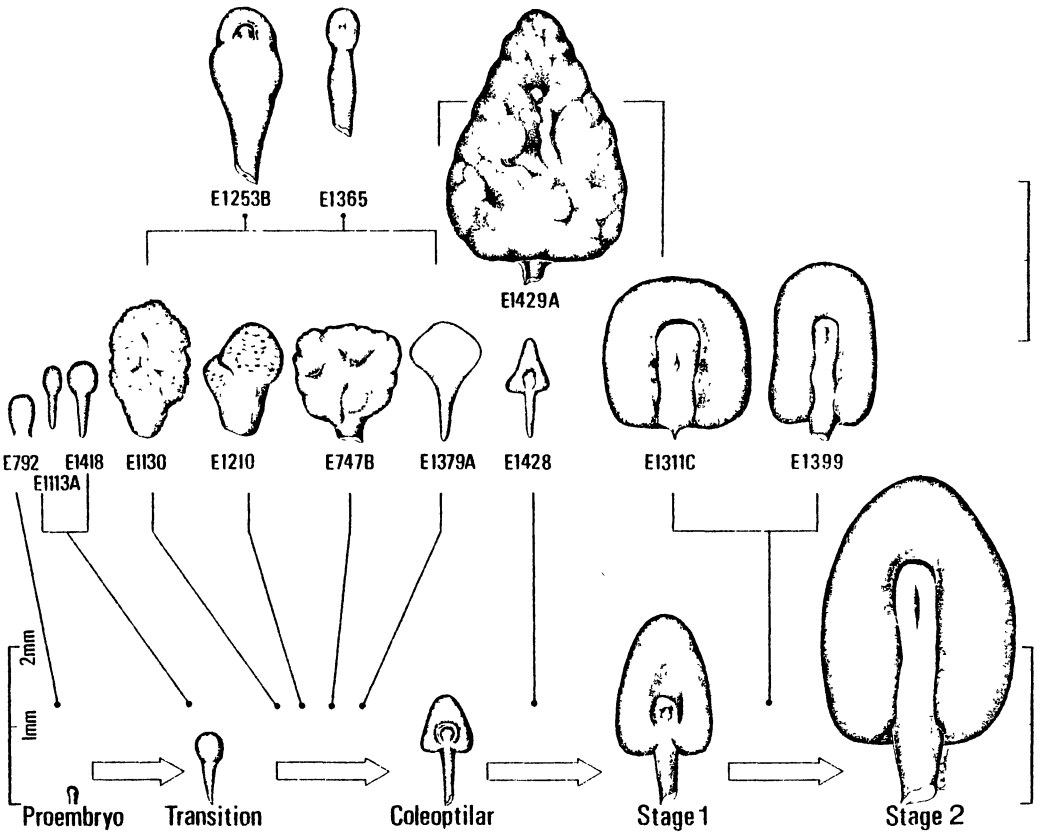

E1253B
E1365
E1429A
E792
E1418
E1113A
E1130
E1210
E747B
E1379A
E1428
E1311C
E1399
2mm
1mm
Proembryo
Transition
Coleoptilar
Stage 1
Stage 2

TABLE III

RESPONSE OF SEVEN DEFECTIVE KERNEL MUTANTS IN WHICH THE GENOTYPES OF THE EMBRYO AND ENDOSPERM DIFFER (ADAPTED FROM SHERIDAN AND NEUFFER, 1982)*

	Embryo and Seedling Response	
Mutants	Normal endosperm Mutant embryo	Mutant endosperm Normal embryo
E792	defective embryo--lethal	slow but normal plant
1113A	defective embryo--lethal	seedling--lethal
1130	defective embryo--lethal	normal plant
1365	defective embryo--lethal	normal plant
1379A	defective embryo--lethal	seedling--lethal
1409	defective embryo--lethal	normal plant
1429A	defective embryo--lethal	seedling--weak

* Refer to Figure 2 for the stages of development reached by the mutant embryos

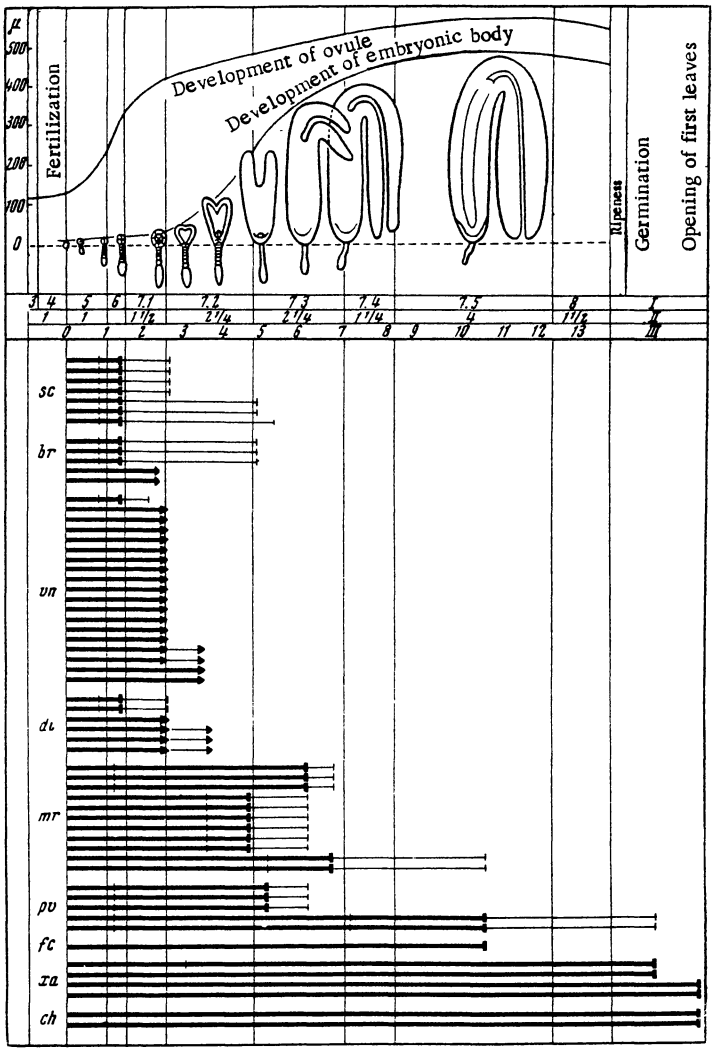
Regeneration of plants from cells growing *in vitro* has been described in many plant species (Evans et al., 1981). Cultivated carrot was one of the first plants in which this process was reported to occur via somatic embryogenesis (Steward, 1958; Reinert, 1959). Since that time, numerous studies have analyzed the carrot cell culture system in detail (Halperin and Wetherell, 1964; Evans et al., 1981; Ammirato, 1983).

The carrot system, which is capable of producing many embryos in culture, has proven to be a valuable system for studying the control of embryogenesis (Sung et al., 1979, Sung, 1979). In a recent report, Breton and Sung (1982) described a number of temperature-sensitive carrot variants (ts-emb$^-$) impaired in early embryogenesis. The enrichment procedure used to select these variants involved filtering the cultured cells of a haploid line of domestic carrot, *Daucus carota* variety Juwarot, removing the clumps of cells and embryos greater than 100 um in diameter and plating the remaining fraction of cells on a callus-inducing medium at 24°C. The resulting colonies were tested for their ability to produce callus and form embryos at 24°C as well as 32°C. The variants (i.e. those cell lines that can grow at one temperature but not the other) were then arranged into three classes according to their phenotype. The first class was unable to produce any type of growth at all on an embryonic medium. The second class was capable of producing callus on the same medium but could not form embryos. The third class,

however, formed embryos that could not exceed the globular stage of development on this medium. The phenotype of the majority of these variants was stable in culture and was maintained through plant regeneration. Characterization of these mutants may be useful in determining the fundamental mechanisms regulating growth and differentiation in carrots.

Embryo-lethal mutants of A. thaliana are readily obtained by subjecting wild-type seeds to various physical and chemical agents (Rédei, 1970). While various researchers have analyzed the effect of specific mutagens on seeds of A. thaliana (Usmanov and Müller, 1970; Usmanov and Sokhibnazarov, 1974), the most detailed study in this area was reported by Müller (1963), who recovered over 3,000 recessive lethal mutants of A. thaliana by subjecting approximately 20,000 seeds to X-irradiation. From these mutants, 60 randomly-selected mutant lines were chosen for further studies. The normal and aborted embryos of each mutant line were examined under a dissecting scope to determine the stage in which developmental arrest of the mutant embryos had occured. It was then possible to arrange the 60 mutant lines into 6 different classes of embryo-lethal mutants and 3 different classes of seedling mutants (Figure 3). The number of aborted and normal seeds were also recorded in order to calculate the segregation ratio for each mutant line. The majority of these mutants segregated as Mendelian recessives. However, further studies with this group of mutants has not been possible because the seed stocks were not maintained.

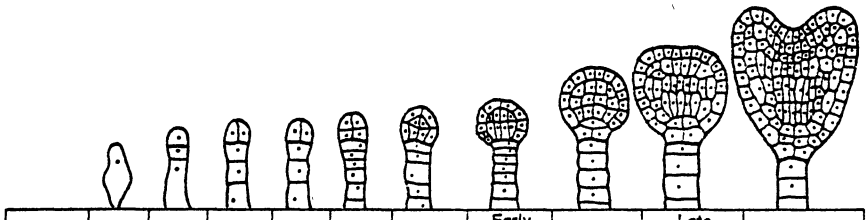
Figure 3. Stages of developmental arrest for 60 embryo-lethal mutants of Arabidopsis thaliana studied by Müller (1963). The normal stages of embryo development are listed across the top of the figure. The letters along the left side of the figure represent abbreviations for the 9 different classes of mutants. Each horizontal line represents a single mutant with the thick bar indicating normal development of the embryo and the thin bar representing the lethal phase. The lines ending in arrows indicate that the corresponding embryos exhibited abnormal development. (Figure from Usmanov and Müller, 1970).


μ
500
400
300
200
100
0
Fertilization
Development of ovule
Development of embryonic body
Ripeness
Germination
Opening of first leaves
sc
br
vn
di
mr
pv
fc
xa
ch

Recently, new groups of embryo-lethal mutants have been reported (Meinke and Sussex, 1979b; Meinke, 1985) enabling further studies on this system. Meinke and Sussex (1979b) isolated a number of embryo-lethal mutants of A. thaliana by subjecting wild-type seeds to EMS. Six of these lines, designated 123B, 79A, 124D, 87A, 71E and 50B, were characterized in detail. Segregation ratios of approximately 25% aborted seeds to 75% phenotypically normal seeds were obtained for each line by screening heterozygous siliques. Examination of the mutant embryos under a dissecting microscope revealed that the lethal phase in each mutant line occurred at a very early stage of embryogenesis (Figure 4). Pairwise crosses were performed between mutant lines to test for allelism. Results from these crosses indicated that all six of the mutants were defective in different genes. Finally, the segregation ratios of heterozygous plants grown at 18°C, 25°C and 32°C were analyzed. There was no significant difference in these ratios for the three temperatures, indicating that the mutant lines were not temperature sensitive.

The distribution of aborted seeds in heterozygous siliques for each mutant line was analyzed in a later publication (Meinke, 1982). Two of the six mutants, 79A and 124D, were observed to have a non-random distribution of aborted seeds. In 79A, 59.9% of the aborted seeds were found in the top half of the silique, while in 124D, 61.5% of the aborted seeds were found in the top half. The corresponding

Figure 4. Stages of developmental arrest for 6 embryo-lethal mutants of Arabidopsis thaliana studied by Meinke and Sussex (1979b). The normal stages of embryo development are illustrated across the top of the figure. The solid bar represents the stage in which developmental arrest of the corresponding mutant embryo occurs. Developmental arrest of the embryo proper in mutant 50B (*) is followed by abnormal growth of the suspensor. Figure from Meinke (1982).



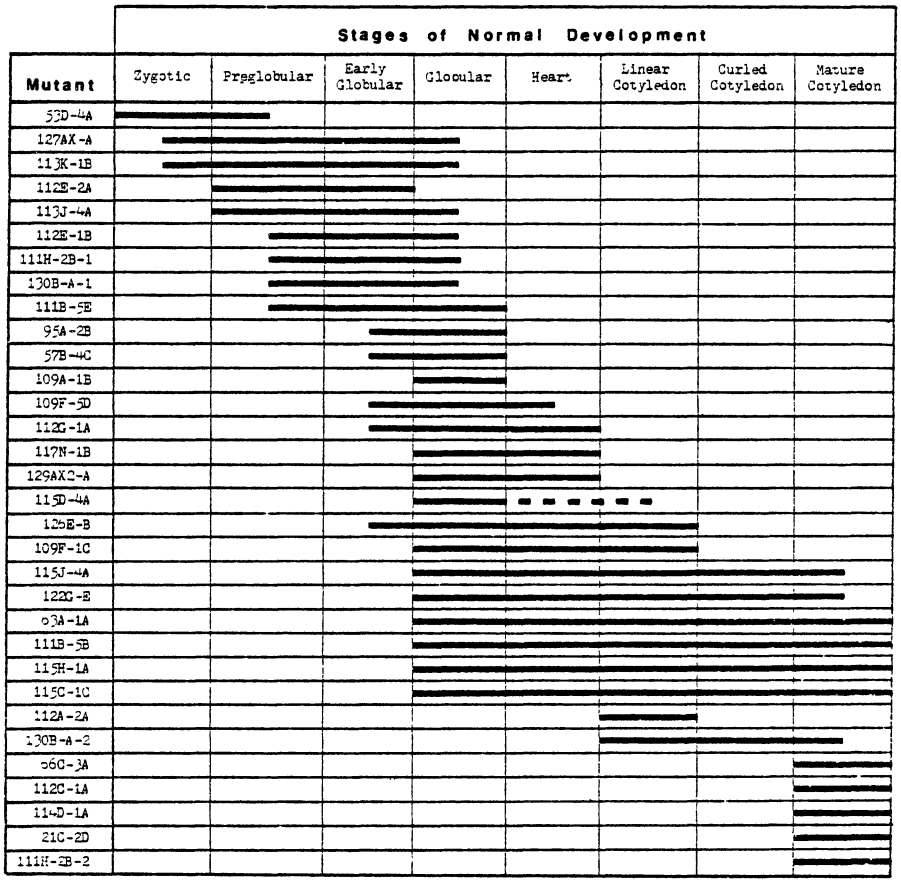
Mutant	Zygote	1 cell	2 cell	4 cell	8 cell	16 cell	Early Globular	Globular	Late Globular	Heart
87A									■	■
123B								■	■	
79A				■	■	■	■			
50B			■	■	■	■*				
71E		■	■	■	■	■				
124D		■	■	■	■	■				

Chi-square values of 48.5 and 71.3 proved to be highly significant. These results suggested that the mutant gene in these two lines may be expressed during gametogenesis as well as embryogenesis.

Mutant 50B was also of special interest because it was the only one of the six mutant lines in which the arrested embryo did not resemble a normal embryo (Meinke and Sussex, 1979b). Marsden and Meinke (1985) prepared 12 aborted seeds of mutant 50B for light and electron microscopy. Examination of sectioned material revealed that the embryo proper arrested at a preglobular stage of development. The suspensor, however, was extremely large (e.g. 15 to 150 cells). Wild-type suspensors from seeds at the same stage of development consisted of only 6-8 cells. These results suggested that the the mutant gene was inhibiting further development of the embryo proper but not the suspensor. Since the suspensor of the arrested embryo was able to continue growing, this mutant may provide evidence that the growth of the suspensor is normally inhibited by subsequent growth of the embryo proper (Marsden and Meinke, 1985).

In a recent publication, Meinke (1985) reported the isolation of 32 new embryo-lethal mutants of A. thaliana following EMS seed mutagenesis (Figure 5). These mutants were characterized as described previously. In contrast to the original 6 mutants previously described, these new mutant lines represented a large range of lethal phases (i.e. zygotic to mature cotyledon). In addition, the aborted seeds and arrested embryos of the 32 mutant lines differed greatly

Figure 5. Stages of developmental arrest for 32 embryo-lethal mutants of Arabidopsis thaliana reported by Meinke (1985). The solid bar represents the lethal phase of each mutant. Boxes only partially covered with a solid bar indicate that the mutant embryo is only occassionally observed to arrest at the corresponding stage of development. The dashed line drawn for mutant 115D-4A indicates that the exact stage of developmental arrest can not be determined from the appearance of the mutant embryos. Figure from Meinke (1985).


Stages of Normal Development
Mutant
Zygotic
Preglobular
Early Globular
Glocular
Heart
Linear Cotyledon
Curled Cotyledon
Mature Cotyledon
55D-4A
127AX-A
113K-1B
112E-2A
113J-4A
112E-1B
111H-2B-1
130B-A-1
111B-5E
95A-2B
57B-4C
109A-1B
109F-5D
112G-1A
117N-1B
129AX2-A
115D-4A
126E-B
109F-1C
115J-4A
122C-E
93A-1A
111B-5B
115H-1A
115C-1C
112A-2A
130B-A-2
96C-3A
112C-1A
114D-1A
21C-2D
111H-2B-2

in color, size and appearance. Interestingly, eight of these new mutants showed a non-random distribution of aborted seeds. This high frequency reinforces the hypothesis that many genes that are required for the completion of embryo development are also expressed prior to fertilization.

In this report I will describe several different approaches that I have used to analyze embryo-lethal mutants of A. thaliana isolated previously following EMS seed mutagenesis (Meinke, 1985). The initial characterization of five recessive embryo-lethal mutants with characteristic lethal phases is presented in Chapter 2. The phenotypes of the aborted seeds and arrested embryos differ greatly among the mutant lines. Three of the five mutants show a non-random distribution of aborted seeds.

In Chapter 3, the response in culture of aborted seeds and arrested embryos from the different mutant lines is described and compared with that of wild-type seeds and embryos. Some mutants lines produce significant amounts of callus on both basal and enriched media, while others only produce callus on an enriched medium. Interestingly, those lines in which the mutant embryos arrest at an early stage of development generally do not produce significant amounts of callus.

A detailed analysis of mutant 122G-E will be described in Chapter 4. This mutant is particularly interesting because it grows much better in vitro on an enriched 2,4-D medium than it does on a basal 2-4-D medium. In addition,

homozygous mutant plants develop on an enriched NAA medium but not on a basal NAA medium. An attempt was made to determine which component of the enriched medium enhanced growth, but the results were inconclusive. The homozygous plants generated in culture were transplanted into pots with vermiculite and grown under laboratory conditions. These plants appeared normal through the time of flowering, but subsequent growth resulted in pale, abnormal flowers and a premature cessation of flowering. No seed set was observed in this group of plants. To determine whether the abnormal flowering was also an effect of the same mutant allele that was blocking embryo development, homozygous mutant plants were subcultured into deep dish containers with enriched NAA media and into deep dish containers with basal NAA media. Although no seed set was observed in either case, the plants grown on the enriched NAA medium appeared to grow more normally than those on the basal NAA medium. These results suggest that the mutant gene is expressed not only during embryogenesis but during flower formation as well.

The results of controlled crosses between wild-type plants and heterozygous plants with a non-random distribution of aborted seeds will be described in Chapter 5. These crosses were performed to determine if a non-random distribution of aborted seeds in heterozygous siliques is a result of expression of the mutant gene on the male side prior to fertilization. Mutant 126E-B was chosen for these crosses because it showed an extremely non-random distribution of aborted seeds along the length of heterozygous

siliques. Results from these crosses showed that when the wild-type plant was the male parent, the heterozygous seeds in the resulting F-1 siliques were distributed randomly, but when the heterozygous mutant plant was the male parent, the heterozygous seeds were distributed non-randomly. These results clearly indicated that the mutant allele interrupting embryogenesis also disrupted gametogenesis on the male side and not the female side.

CHAPTER II

CHARACTERIZATION OF FIVE EMBRYO-LETHAL MUTANTS

Introduction

Five embryo-lethal mutants of A. thaliana that differed significantly in lethal phase and appearance of aborted seeds and arrested embryos were selected from a larger group of mutants isolated following EMS seed mutagenesis (Meinke, 1985). In order to perform the detailed studies on these mutant lines that will be presented in subsequent chapters, it was first necessary to examine the aborted seeds and arrested embryos to accurately characterize the lethal phase, phenotype and segregation ratio of each mutant. In this chapter, I will summarize the data accumulated from these studies. Although I performed the majority of the work presented for these five mutant lines, other laboratory members should be acknowledged for contributing to the screening project described in this section.

Materials and Methods

Maintenance of Plants

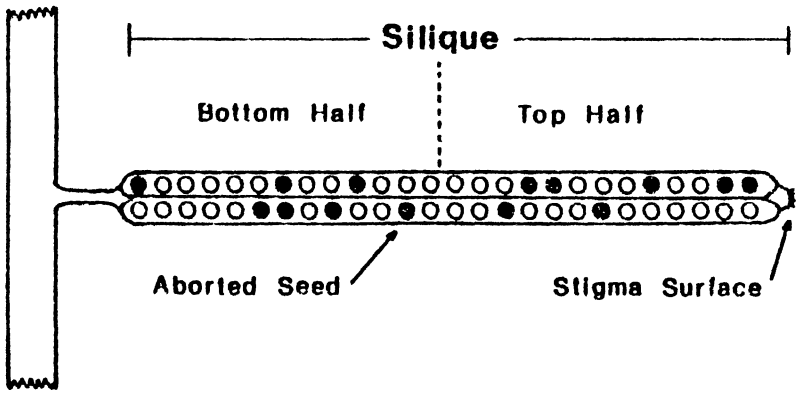
The five mutant lines were maintained as described previously (Meinke and Sussex, 1979a). Phenotypically normal seeds from heterozygous plants of Arabidopsis thaliana (L.)

Heynh. strain "Columbia" were used to maintain the mutant lines because homozygous mutant seeds were unable to germinate *in vivo*. Mature seeds were suspended in water and then transferred with Pasteur pipets to 3" pots filled with a 12:3:1 mixture of coarse vermiculite, potting soil and sand. The soil surface was misted periodically to prevent the seeds from drying out. After the seeds had germinated (usually within 3 to 5 days), the soil around the plants was top-watered with either distilled water or a dilute fertilizer solution. Care was taken to insure that the cotyledons did not get wet. After two pairs of true leaves were formed, the plants were bottom-watered regularly with a solution containing 4 g/gal of 7-6-19 All Purpose Hyponex and 0.25 g/gal of 15-16-17 Peat Lite Special. The plants were maintained at 24°C $\pm$ 2°C under light benches that provided 16 hour/8 hour light/dark cycles. Proper illumination was obtained with cool white high-output fluorescent lights.

Characterization of Mutant Lines

Heterozygous plants were identified by the presence of two phenotypically different classes of seeds in the mature siliques: normal and aborted (Figure 6). Green, mature siliques from these plants were then harvested, split along both sides of the central septum with forceps, and observed under a dissecting microscope. The color and size of the aborted seeds and the arrested embryos were noted. The lethal phases of the arrested embryos were also recorded.

Figure 6. Heterozygous silique of Arabidopsis thaliana (L.) Heynh. Two phenotypically different classes of seeds are present: 75% are normal (homozygous and heterozygous wild) and 25% are aborted (homozygous mutant). Adapted from Meinke (1982).


Silique
Bottom Half
Top Half
Aborted Seed
Stigma Surface

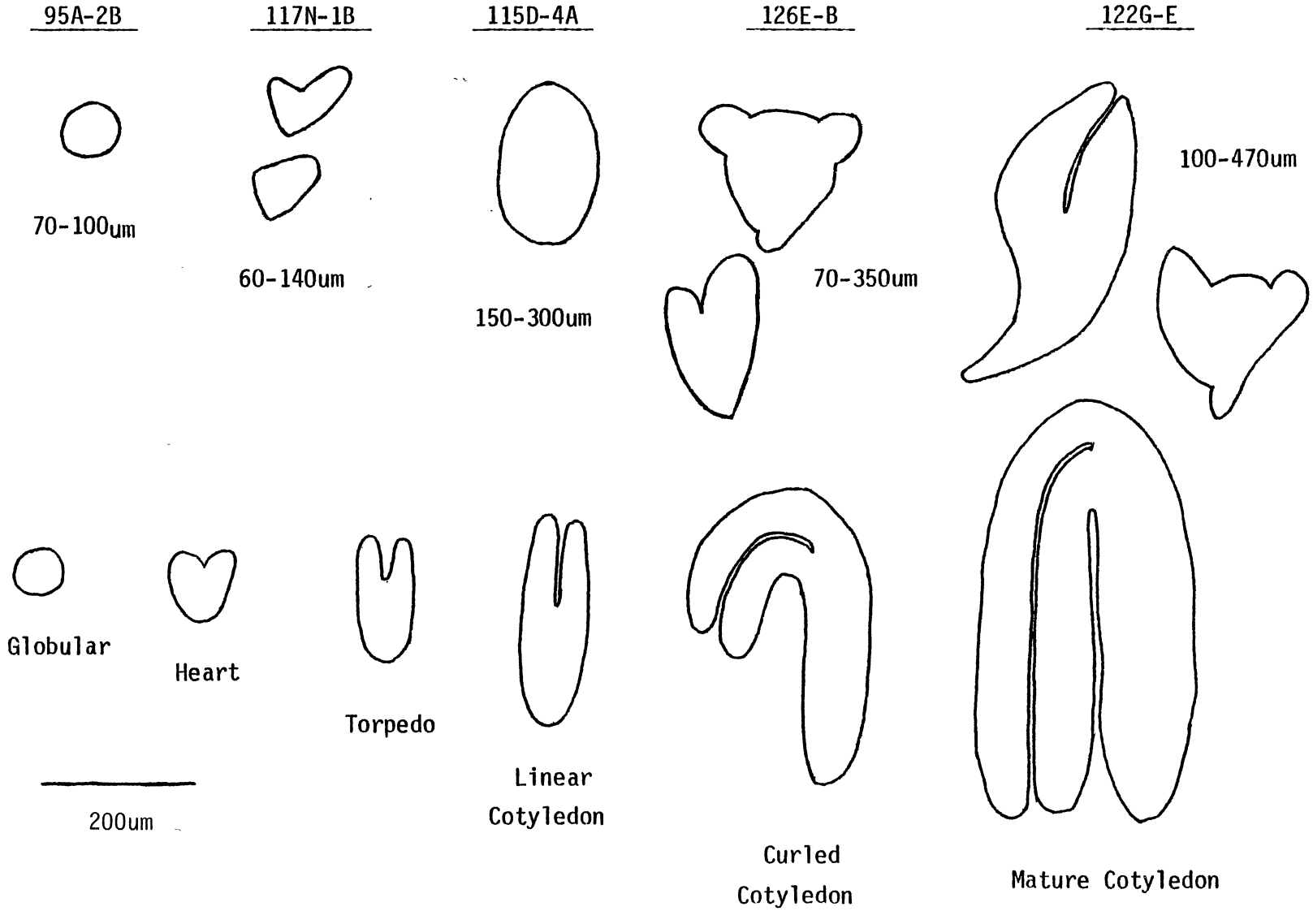
The exact position of each aborted seed along the length of the silique was recorded by drawing a silique on graph paper, designating one box for each seed, and placing symbols in the boxes corresponding to aborted seeds. The total number of normal and aborted seeds was recorded to determine the segregation ratio. The silique was then divided in half perpendicular to the central septum (Figure 6), and the total number of aborted seeds in each half was calculated. This information was obtained from a minimum of 20 siliques, each containing 40 to 75 seeds. Siliques from heterozygous plants grown at 18°C were screened in the same manner. In addition, each mutant was tested for temperature sensitivity by growing heterozygous plants first at 24°C $\pm$ 2°C, screening the earliest mature siliques formed to assure the presence of two phenotypically different classes of seeds, and then transferring the heterozygous plants to a growth chamber at 18°C. The first 20 siliques formed at the lower temperature were screened and the percent aborted seeds was calculated.

Results

Appearance of Arrested Embryos

Phenotypically normal and abnormal arrested embryos were observed in heterozygous siliques from the five mutant lines (Figure 7). The mutant embryos of 95A-2B resembled wild-type embryos at the same stage of development. The majority of the arrested embryos observed in mutants 117B-1B, 126E-B and 122G-E were abnormal in appearance, but occassionally,

Figure 7. Appearance of mature, arrested embryos from five embryo-lethal mutants of Arabidopsis thaliana and the corresponding stages of normal embryo development. The normal stages of embryo development are shown on the bottom row of the figure. The arrested embryos from each mutant line and the corresponding size range of each are listed across the top. Refer to Table IV for the range of colors exhibited by each arrested embryo. See Figure 10 for a greater variety of arrested embryos from mutant 122G-E.


95A-2B
117N-1B
115D-4A
126E-B
122G-E
70-100um
60-140um
150-300um
70-350um
100-470um
Globular
Heart
Torpedo
Linear Cotyledon
Curled Cotyledon
Mature Cotyledon
200um

phenotypically normal arrested embryos were observed. The mutant embryos of 115D-4A, in contrast, exhibited an extremely abnormal pattern of development. This line is often referred to as the "green blimp" because the embryos are usually green, elongated, and do not have a distinct hypocotyl or cotyledons.

Lethal Phase of the Arrested Embryos

The five mutant lines varied considerably in stage of developmental arrest (Figure 8). The mutant embryos of 95A-2B and 117N-1B always arrested over a very narrow range of developmental stages. In these lines, the defect appeared to be in a gene that is essential for the completion of a certain stage of plant embryo development. In lines 126E-B and 122G-E, the mutant embryos characteristically arrested over several stages of development. There are various interpretations for these mutants. One possible explanation is that the mutation is "leaky" such that the function of the defective gene is not totally eliminated but is simply altered. A reduction of gene product could account for a broad lethal phase with numerous phenotypes. Another explanation suggests that the defect does not immediately cause arrest of the mutant embryo but allows some degree of normal development. In mutant 122G-E, for example, it was observed that aborted embryos from immature heterozygous siliques generally arrested at a younger stage of development than did aborted embryos from mature heterozygous siliques,

Figure 8. Stages of developmental arrest for five embryo-lethal mutants of Arabidopsis thaliana. Each solid bar represents the stage in which the corresponding mutant embryo arrests. Stages that are only reached occassionally by the mutant embryo are partially covered with a solid line. The dashed line in mutant 115D-4A indicates that the lethal phase cannot accurately be determined based on the appearance of the mutant embryo.

STAGES OF NORMAL DEVELOPMENT								
Mutant	Zygotic	Preglobular	Early Globular	Globular	Heart	Linear Cotyledon	Curled Cotyledon	Mature Cotyledon
95A-2B			———	———				
117N-1B				———	———			
115D-4A				———	- - - - -	- - -		
126E-B			———	———	———	———		
122G-E				———	———	———	———	———

again suggesting that expression of the mutant gene did not immediately cause embryonic lethality. Finally, the defect could be disrupting endosperm development which would indirectly affect the pattern of embryogenesis. Mutant 115D-4A, however, showed such an abnormal pattern of embryo development that the exact lethal phase could not be determined based on the appearance of the arrested embryos.

Colors of Aborted Seeds and Arrested Embryos

The range of colors expressed by aborted seeds and arrested embryos of each mutant line also varied (Table IV). Aborted seeds and arrested embryos of mutant 117N-1B were consistently white. In mutant 95A-2B, the arrested embryos were usually white and only occassionally very pale yellow-green, whereas the aborted seeds were usually very pale yellow-green and only occassionally white. The aborted seeds and arrested embryos of mutant 115D-4A were different shades of pale green with the arrested embryos generally darker green than the aborted seeds. Mutant 126E-B produced aborted seeds and arrested embryos that ranged from white to pale green. The arrested embryos, however, often appeared to have pale green cotyledons and white hypocotyls. In mutant 122G-E, the arrested embryos also ranged from white to pale green, while the aborted seeds were always very pale yellow-green or pale green. In addition, some of the aborted seeds and arrested embryos of mutants 115D-4A, 126E-B and 122G-E were observed to turn a darker green upon drying.

TABLE IV

COLOR OF ABORTED SEEDS AND ARRESTED EMBRYOS
OF FIVE EMBRYO-LETHAL MUTANTS OF
ARABIDOPSIS THALIANA

Mutant	Seed Color	Embryo Color
95A-2B	wh**, vpyg	wh, vpyg**
117N-1B	wh	wh
115D-4A*	vpyg, pg, g**	vpyg, pg, g
126E-B*	wh, vpyg, pg	wh, vpyg, pg**
122G-E*	vpyg, pg	wh, vpyg, pg

wh = white or cream

vpyg = very pale yellow-green

pg = pale green

g = green

* Embryos turn darker upon drying.

** Color is only observed occassionally.

Segregation Ratios

The segregation ratios of aborted seeds found in heterozygous siliques following self-pollination are presented in Table V. Mutants segregating for a single recessive gene were expected to have approximately 25% aborted seeds in heterozygous siliques. Segregation ratios for most of the lines examined in this study differed significantly from this expected value. For example, significantly less than 25% aborted seeds were found in mutant 126E-B at 18°C and mutants 115D-4A and 95A-2B at both temperatures. The segregation ratio for mutant 117N-1B, in contrast, was slightly greater than 25%. The presence of aborted seeds in heterozygous siliques grown at both temperatures indicated that the five mutant lines were not able to restore the function of the defective gene at a lower temperature. The disruption of expected segregation ratios observed in mutants 95A-2B, 115D-4A and 126E-B appeared to be caused by an effect of the mutant allele on pollen-tube growth.

Distribution of Aborted Seeds

The distribution of aborted seeds in heterozygous siliques from the five mutant lines is presented in Table VI. In mutants 117N-1B and 122G-E, approximately equal numbers of aborted seeds were found in the top and bottom halves of the heterozygous siliques produced at both 24°C and 18°C. In mutants 115D-4A and 126E-B at both temperatures and mutant

TABLE V

SEGREGATION RATIOS FOR EMBRYO-LETHAL MUTANTS OF ARABIDOPSIS THALIANA GROWN AT 24° C AND 18° C

Mutant	Temperature	Seeds Screened	% Aborted Seeds[a]	Chi-square
95A-2B	24° C	3,683	23.1	6.01*
	18° C	3,439	21.5	22.43**
117N-1B	24° C	2,791	26.7	4.36*
	18° C	520	21.7	2.79
115D-4A	24° C	2,662	18.7	53.76**
	18° C	2,013	19.0	38.63**
126E-B	24° C	2,232	25.2	0.03
	18° C	2,042	12.2	177.92***
122G-E	24° C	2,526	26.5	3.05
	18° C	989	24.4	0.18

* Significantly different from 25.0% at P = 0.05; ** at P = 0.01; *** at P = 0.001.

[a] Mature heterozygous siliques should contain approximately 25% aborted seeds.

TABLE VI

DISTRIBUTION OF ABORTED SEEDS IN HETEROZYGOUS SILIQUES FROM FIVE MUTANT LINES OF ARABIDOPSIS THALIANA

Mutant	Temperature	Total Seeds	% Aborted Seeds[a]	% Top Half	Chi-square
95A-2B	24° C	2,290	24.5	40.6	19.62***
	18° C	3,206	22.0	51.6	0.63
117N-1B	24° C	1,075	25.9	49.3	0.03
	18° C	520	21.7	55.0	0.90
115D-4A	24° C	1,896	18.8	57.9	8.50**
	18° C	1,836	19.1	61.5	18.23***
126E-B	24° C	2,056	23.8	65.9	49.03***
	18° C	2,029	12.3	84.0	114.24***
122G-E	24° C	2,084	26.7	50.2	<0.01
	18° C	910	24.8	53.5	1.00

** Significantly different from 50.0% at P = 0.01; *** at P = 0.001.

[a] Percentage of total aborted seeds located in the top half of the silique. If aborted seeds are distributed randomly within the silique, this number should be approximately 50%.

95A-2B at 24° C, the distribution of aborted seeds was non-random. In mutants 115D-4A and 126E-B the percent of aborted seeds in the top half was much greater than the percent in the bottom half of the silique. This difference was extremely significant at the lower temperature. In mutant 95A-2B, however, there were significantly more aborted seeds in the bottom half of the silique. This effect was only observed at the higher temperature.

Discussion

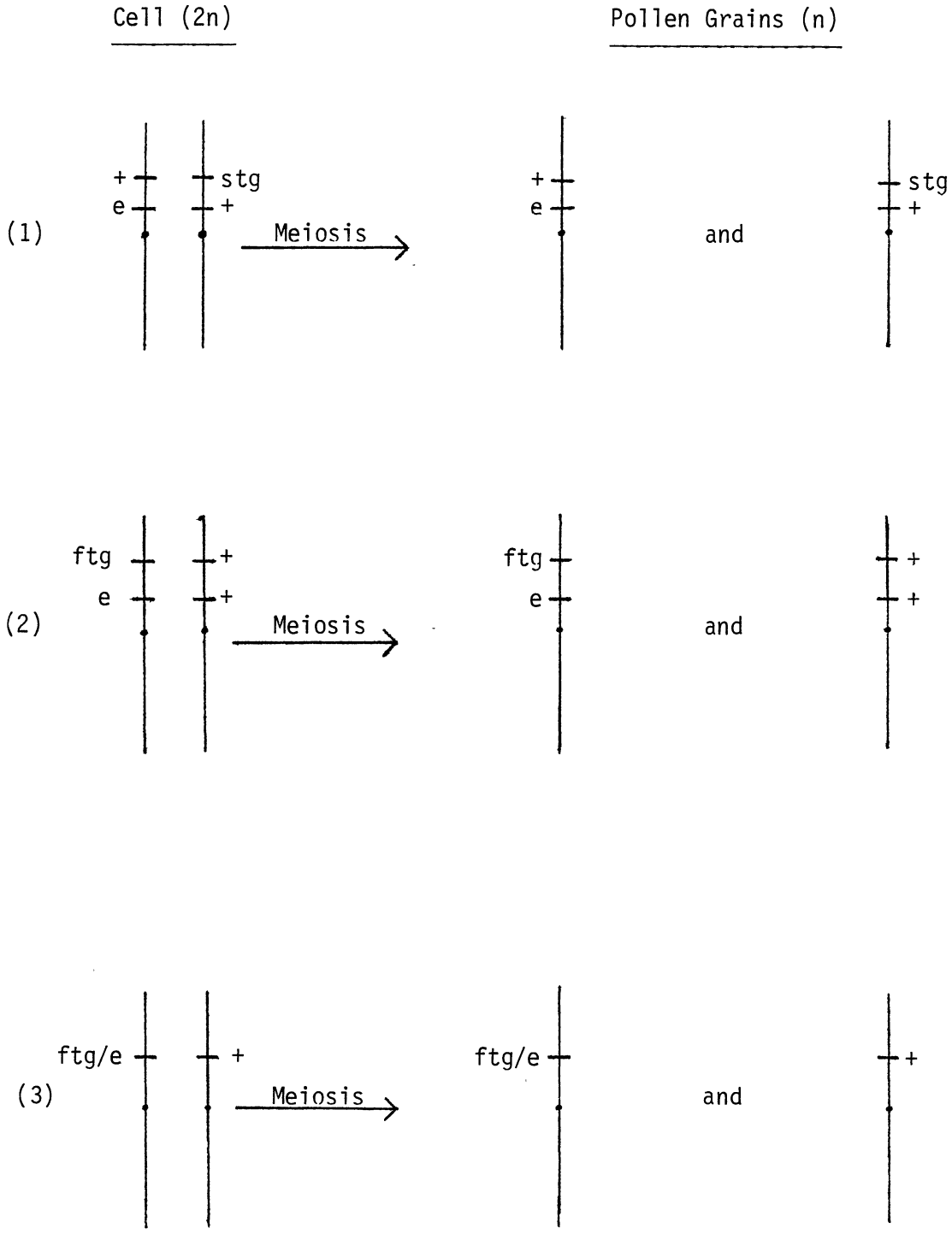
Although all five mutant lines do not contain precisely 25% aborted seeds following self pollination, they are all considered to be segregating as simple Mendelian recessive lethals. The increased number of aborted seeds present in heterozygous siliques from mutant 117N-1B is only slightly significant, indicated by the Chi-square value of 4.36. In this line, it is possible that the increased segregation ratio is a result of an effect of the mutant allele on pollen development or pollen-tube growth. Those pollen grains carrying the mutant allele may be competitively more advantageous than wild-type pollen grains. However, if this were the case, a non-random distribution of aborted seeds with significantly more aborted seeds located in the bottom of the silique would be expected. The random distribution of aborted seeds observed in mutant 117N-1B heterozygous siliques grown at both temperatures suggests that this explanation is incorrect. Another possible explanation is that two very closely linked genes that produce the same

lethal phase may be affected. A third possibility is that the siliques screened may not have been an accurate representation of the population. If this explanation were true, screening more siliques would be expected to lower the segregation ratio to 25%. Finally, there exists the possibility that some other unrecognized factor may be acting in this mutant line.

The deviation from 25% aborted seeds in mutants 95A-2B, 115D-4A and 126E-B may be explained by gametophytic expression of the mutant genes (Meinke, 1982). In mutants 115D-4A and 126E-B, the mutant genes appear to be causing embryo lethality as well as decreasing the rate of pollen-tube growth, particularly at the lower temperature. The wild-type pollen tubes are therefore more competitive and more readily fertilize ovules at the base of the silique. This possibility would also produce a non-random distribution of aborted seeds such that there are significantly more aborted seeds in the top half of the silique. Table VI indicates that this outcome is exactly what is observed in these two mutant lines. In addition, crosses involving mutant 126E-B determined that the mutant allele is indeed affecting pollen development or pollen-tube growth such that those pollen grains carrying the mutant allele are less likely to fertilize ovules at the base of the silique. Results from these crosses will be discussed in greater detail in Chapter V.

The decreased segregation ratio observed in heterozygous siliques of mutant 95A-2B cannot be explained with the same logic presented for mutants 115D-4A and 126E-B, because the aborted seeds of 95A-2B are distributed randomly when heterozygous siliques are grown at 18°C and non-randomly such that there is a greater number of aborted seeds at the base of the silique when heterozygous siliques are grown at 24°C. Three possible explanations for the prevalence of aborted seeds in the bottom half of the silique in this mutant is presented in Figure 9. One possible explanation is that two very closely linked genes are being affected; one causes embryo lethality while the other decreases pollen-tube growth. The defective alleles, however, must be located in trans (i.e. on opposite homologues) instead of cis (i.e. on the same homologue). The pollen grains produced would either contain (1) the wild-type pollen-tube allele and the embryo-lethal allele, which would cause normal pollen-tube growth and embryo-lethality; or (2) the defective pollen-tube allele and the normal embryo development allele, which would cause decreased pollen-tube growth but normal embryo development. Since the two genes are closely linked, the combination of both embryo lethality and decreased pollen-tube growth would rarely occur. A second possible explanation is that two very closely linked genes are being affected; one causes embryo-lethality while the other one causes increased pollen-tube growth. However, these two defective genes are located cis instead of trans. Two types of pollen grains would be expected, one carrying the alleles for normal pollen-tube

Figure 9. Three possible explanations for the prevalence of aborted seeds in the bottom half of 95A-2B heterozygous siliques. In the first situation, two very closely linked genes located in trans are being affected. One causes embryo lethality (e) while the other causes slow pollen-tube growth (stg). In the second, two closely linked genes on the same allele are being affected. One causes fast pollen-tube growth (ftg), while the other causes embryo-lethality. In the third, one gene locus is affected. This mutant gene causes both fast pollen-tube growth and embryo lethality.


Cell (2n)
Pollen Grains (n)
(1)
+
e
stg
+
Meiosis
and
stg
+
(2)
ftg
e
+
+
Meiosis
and
+
+
(3)
ftg/e
+
Meiosis
and
+

growth and normal embryo development while the other carries the alleles for increased pollen-tube growth and embryo lethality. Only rarely would crossing-over occur to produce pollen grains with only one altered allele. A third possible explanation suggests that only one gene locus is affected. This defective gene causes embryo lethality and increases pollen-tube growth. Again, two types of pollen grains are expected; a wild-type pollen grain which causes normal pollen-tube growth and normal embryo development; and a mutant pollen grain which causes increased pollen-tube growth and embryo lethality. However, all three possible explanations must somehow be influenced by the temperature, because a random instead of a non-random distribution of aborted seeds is observed in heterozygous siliques grown at 18°C. If any one of the three explanations were acting the expected segregation ratio would be significantly greater than 25%. This result is definitely not what is observed in mutant 95A-2B. What is actually happening in this mutant to cause these unexpected results cannot be explained from the data obtained in this project. In future experiments, it would be interesting to see what would happen to the segregation ratio and distribution of aborted seeds if heterozygous plants were grown at higher temperatures.

The five mutant lines described in this chapter were chosen for detailed study because the arrested embryos differed significantly in lethal phase. Another approach that can be taken to further characterize the lethal

condition of these mutant lines is to observe the *in vitro* response of the aborted seeds and arrested embryos. Mutants 115D-4A, 126E-B and 122G-E appear to be the most promising for this type of study because the the mutant embryos arrest at later stages of embryogenesis and are therefore more likely to be rescued.

The approach I chose to further study these mutants was to (1) analyze gametophytic expression of mutant 126E-B; and (2) observe the *in vitro* response of aborted seeds and arrested embryos from the five mutant lines. Results from these projects will be discussed in the following chapters.

CHAPTER III

RESPONSE IN VITRO OF FIVE EMBRYO-LETHAL MUTANTS OF ARABIDOPSIS THALIANA

Introduction

Tissue culture has been used to investigate the factors regulating numerous biochemcal, physiological and genetic pathways in higher plant species. For example, the *in vitro* response of embryo-lethal mutants of corn (Neuffer and Sheridan, 1980) and carrot variants (Breton and Sung, 1982) has been studied in an attempt to analyze the factors controlling plant embryo development. Although the growth of *Arabidopsis* cells in culture has been described previously (Negrutiu et al., 1975; Negrutiu and Jacobs, 1978a,b; Huang and Yeoman 1983, 1984) the response in culture of embryo-lethal mutants has not been reported.

In this chapter, the growth response of aborted seeds and arrested embryos from five embryo-lethal mutants of *A. thaliana* cultured on basal and enriched callus media will be described. This approach was taken to recover homozygous mutant plants for additional genetic analyses, generate callus for biochemical studies, and identify potential auxotrophic mutants that are unable to grow in the absence of a specific amino acid, nucleoside or vitamin.

Materials and Methods

Maintenance of Plants

The five embryo-lethal mutants of Arabidopsis thaliana (L.) Heynh. strain "Columbia" analyzed in this study were isolated following EMS seed mutagenesis (Meinke, 1985). Homozygous and heterozygous wild-type plants were identified and maintained as described in Chapter I.

Preparation of Media

Four different media were prepared: BC2D1/2K, BC2N1/2K, EC2D1/2K, and EC2N1/2K. The basal callus (BC) medium consisted of the inorganic salts described by Murashige and Skoog (1962), 3% (w/v) glucose, 0.8% (w/v) purified Difco agar, 0.55 mM inositol, 5 uM thiamine hydrochloride, 0.5 mg/l kinetin and 2 mg/l of either 2,4-D (BC2D1/2K) or NAA (BC2N1/2K). Additional modified media containing 5 mg/l NAA and 1 mg/l kinetin (BC5N1K) or 10 mg/l NAA and 0.5 mg/l kinetin (BC10N1/2K) were later prepared to further characterize the in vitro growth of mutant 115D-4A. To prepare basal callus (BC) media, the components were mixed (except the agar) and the pH was adjusted to 5.8 with NaOH. The media were then transferred to a flask and the agar was added. These flasks were sealed with cotton and foil and then autoclaved for 20 minutes at 122°C and 18 psi. After the medium had cooled sufficiently but had not begun to harden, it was transferred to a laminar flow hood where

approximately 10 ml was poured into sterile 60 mm x 15 mm plastic petri plates. The plates were then allowed to cool before they were transferred to plastic containers where they were stored at room temperature until needed.

The enriched callus (EC) media were prepared as described for the basal callus media except that inositol and thiamine were not added. In addition, the organic supplements listed in Table VII were sterilized with a 0.2 um Gelman filter and then added to the flask after the components had been autoclaved and allowed to cool to approximately 35°C. The media were then poured and stored as described above.

Culturing Procedure

The pedicel of mature green siliques harvested from normal and heterozygous wild-type plants was dipped in hot paraffin to seal the cut end. These siliques were transferred to a laminar flow hood and surface sterilized in 95% ethanol for 10 seconds and then in a solution of 20% Clorox with a small amount of FL-70 detergent for 4 minutes. The siliques were then placed in a sterile beaker and rinsed with sterile distilled water three times. One at a time, the siliques were transferred to an autoclaved slide placed under a Wild stereomicroscope and opened along both sides of the central septum with sterile forceps. The selected seed or dissected embryo was then transferred to the appropriate culture medium. The seeds were oriented such that the funiculus was in contact with the agar. Seeds and embryos

TABLE VII

ORGANIC SUPPLEMENTS OF ENRICHED CALLUS (EC) MEDIA

Components
0.2 mM each of 20 L-amino acids*
0.1 mM each of 5 nucleosides**
0.5 uM each of 7 vitamins***
50.0 uM choline chloride
0.5 mM each of DL-malic and citric acids

* Ala, Arg-HCl, Asn, Asp, Cys, Glu, Gln, Gly, His-HCl, Ile, Leu, Lys-HCl, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val

** adenosine, cytidine, guanosine, thymidine, uridine

***p-aminobenzoic acid, biotin, folic acid, nicotinamide, Ca-pantothenate, pyridoxine HCl, riboflavin-5'-phosphate

that appeared to be buried in the agar were lifted to the surface to allow proper gas exchange. The exterior portion of each plastic petri plate was wiped with 70% ethanol and the edges sealed with 2 strips of parafilm. The cultured agar plates were then placed under light benches that provided 16 hour/8 hour light/dark cycles at 24°C.

Results

In Vitro Response of Wild-Type Seeds and Embryos

As a control, it was first necessary to determine the response of mature wild-type seeds and embryos cultured on the prepared media. These results are described in Table VIII. Intact seeds and isolated embryos formed extensive pale, friable callus equally well when cultured on either a basal or enriched medium containing 2,4-D and kinetin. This callus, when subcultured to fresh media, continued to grow. Extensive green nodular callus with numerous roots formed when isolated seeds and embryos were cultured on basal media containing NAA. This callus was also able to continue growing when subcultured. Relatively little callus was observed from seeds and isolated embryos cultured on enriched media with NAA. Instead, the embryos often germinated. If callus generated on the basal medium containing NAA was subcultured onto the enriched medium, continued callus growth with numerous roots was observed. These results indicate that the prepared media were able to support the growth and proliferation of wild-type tissue in culture.

TABLE VIII

RESPONSE OF MATURE WILD-TYPE SEEDS AND EMBRYOS IN VITRO

Media	Response of Seeds and Embryos
BC2D1/2K	Extensive, pale friable callus
BC2N1/2K	Extensive, green nodular callus; roots form
EC2D1/2K	Extensive, pale friable callus
EC2N1/2K	No callus; often germinate

In Vitro Response of Mutant 95A-2B

Aborted seeds from mutant 95A-2B were unable to produce significant growth *in vitro* (Table IX). Although a slight amount of cell division occurred in the mutant embryo when aborted seeds were cultured on basal media, this response was extremely rare and occurred only after only 6 to 7 weeks *in vitro*. A limited amount of cell division may have occurred in the aborted seeds cultured on the enriched media but was undetected because it was not large enough to crack the seed coat.

In Vitro Response of Mutant 117N-1B

The growth response of aborted seeds from mutant 117N-1B is listed in Table X. This response was observed after two months in culture. The white callus produced by aborted seeds from mutant 117N-1B cultured on basal media ranged from seed size (i.e. approximately 600 um) to four times that size. No continued growth was observed even when the callus was transferred to fresh media. When the aborted seeds were cultured on the enriched medium with NAA, the tissue produced just barely broke the seed coat. Upon subculturing, no further growth was observed even when the seed coat was removed. These results indicate that only a limited amount of cell division in the arrested embryo was possible on this medium. The aborted seeds did produce slightly more pale yellow, friable callus when cultured on the enriched medium

TABLE IX

RESPONSE OF MUTANT 95A-2B IN VITRO

Medium	Number of Seeds Cultured	Percent of Seeds Responded	Size of Tissue after 6 weeks in Culture*	Appearance of Tissue
BC2D1/2K	76	1	2X seed size	vpyg callus
BC2N1/2K	73	1	seed size	cream callus
EC2D1/2K	72	0	no callus formed	no callus formed
EC2N1/2K	77	0	" " "	" " "

* Mature seeds are approximately 600 um long.

vpyg = very pale yellow-green

TABLE X

RESPONSE OF MUTANT 117N-1B IN VITRO

Medium	Number of Seeds Cultured	Percent of Seeds Responded	Size of Tissue after 8 Weeks in Culture*	Appearance of Tissue
BC2D1/2K	99	43	4X seed size	white callus
BC2N1/2K	98	51	2X seed size	white callus
EC2D1/2K	87	51	8X seed size**	pale yellow, friable callus
EC2N1/2K	86	20	seed size	white callus

* Mature seeds are approximately 600 um long.

** Tissue grows only slightly when subcultured.

with 2,4-D than on the basal media. However, when this callus was subcultured onto fresh media, only a limited amount of continued growth occurred after a long time in culture.

In Vitro Response of Mutant 115D-4A

The combined response of aborted seeds and arrested embryos from mutant 115D-4A is summarized in Table XI. The aborted seeds responded much better than the isolated embryos. The aborted seeds cultured on basal media did occassionally break the seed coat to produce white callus ranging from seed size to two times that size, but no further growth was observed even when this tissue was transferred to new media. The response of the aborted seeds was slightly better on the enriched media. In this case, the arrested embryos occassionally appeared to produce a cream-colored elongated hypocotyl ranging from seed size to four times that size. However, continued growth was not observed when this tissue was transferred to fresh media. Attempts to stimulate further growth of mutant 115D-4A were performed by culturing aborted seeds and arrested embryos on the modified basal media, BC5N1K and BC10N1/2K. The responses on these media were the same as the responses produced on the regular basal callus medium. No attempt was made to culture aborted seeds on enriched callus media with higher levels of NAA.

TABLE XI

RESPONSE OF MUTANT 115D-4A IN VITRO

Medium	# of Seeds and Embryos Cultured	% of Seeds and Embryos Responded	Size of Tissue after 4 Weeks in Culture*	Appearance of Tissue
BC2D1/2K	73	15	2X seed size	cream callus
BC2N1/2K	72	11	3X seed size	cream callus
EC2D1/2K	100	20	4X seed size	cream callus[a]
EC2N1/2K	84	15	3X seed size	cream callus[a]

* Mature seeds are approximately 600 um long.

[a] Resembles an elongated hypocotyl.

In Vitro Response of Mutant 126E-B

The combined response of aborted seeds and arrested embryos for mutant 126E-B is listed in Table XII. The best reponse in this line was obtained from mutant embryos that arrested at a late stage of development. Extensive pale, friable callus was produced by aborted seeds and arrested embryos cultured on basal and enriched media containing 2,4-D. This callus continued to divide and proliferate when subcultured to fresh media. A significant amount of callus was also produced on the basal medium containing NAA. This callus appeared pale when it was first formed but as it continued to grow it became pale green and produced numerous root hairs. This tissue was also able to continue growing on fresh media. Callus was not observed on the enriched media containing NAA. Instead, the seeds appeared to germinate but were unable to continue growing on fresh media. However, green nodular callus produced from one aborted seed cultured on the basal NAA medium and then transferred to the enriched NAA medium was observed to produce two plantlets with numerous leaves. An attempt was made to transplant these plants into pots with vermiculite, but the plants did not survive. The plants appeared on the enriched NAA medium after approximately one month in culture. Although it is possible that the callus developed from the accidental culture of a wild-type seed, the slow response *in vitro* suggests that the tissue was produced from aborted seeds.

TABLE XII

RESPONSE OF MUTANT 126E-B IN VITRO

Medium	# of Seeds and Embryos Cultured	% of Seeds and Embryos Responded	Size of Tissue after 4 Weeks in Culture*	Appearance of Tissue
BC2D1/2K	103	31	5X seed size***	cream, friable callus
BC2N1/2K	111	46	3X seed size***	cream to pale green callus with root hairs
EC2D1/2K	104	36	15X seed size***	cream, friable callus
EC2N1/2K	127	49	3X seed size	appeared to germinate

* Mature seeds are approximately 600 um long.

*** Tissue continues to grow when subcultured.

In Vitro Response of Mutant 122G-E

Mutant 122G-E produced a very interesting response in culture (Table XIII). Aborted seeds and arrested embryos cultured on the enriched 2,4-D medium produced extensive amounts of pale, friable callus that continued to grow when subcultured. However, only a slight amount of callus (i.e. seed size to 1.5 times that size) was observed on the basal 2,4-D medium, and this callus did not continue to grow when subcultured to new media. Aborted seeds and arrested embryos cultured on the basal medium with NAA produced only a slight amount (i.e. 2 times seed size) of very pale yellow-green callus. Again, continued growth was not observed when the callus was subcultured. However, aborted seeds and arrested embryos cultured on the enriched medium with NAA developed into phenotypically normal plantlets (see Chapter IV).

Discussion

Mutant embryos arrested at a globular stage of development were not cultured as isolated embryos because even wild-type embryos cultured at this early stage of development fail to respond consistently *in vitro* (Raghavan, 1976). Although wild-type seeds cultured at early stages of development complete embryogenesis *in vitro*, those mutant lines in which the embryos arrest prior to the heart stage of development generally did not produce significant amounts of callus when aborted seeds were placed *in vitro*. Aborted seeds of mutant 95A-2B, for example, consistently contained

TABLE XIII

RESPONSE OF MUTANT 122G-E IN VITRO

Medium	# of Seeds and Embryos Cultured	% of Seeds and Embryos Responded	Size of Tissue after 3 Weeks in Culture*	Appearance of Tissue
BC2D1/2K	397	71	2X seed size	cream, friable callus
BC2N1/2K	214	72	2X seed size	vpyg callus
EC2D1/2K	334	60	15X seed size***	cream, friable callus
EC2N1/2K	1,293	41	no callus formed	green plants formed

* Mature seeds are approximately 600 um long.

*** Tissue continues to grow when subcultured.

vpyg = very pale yellow-green

embryos that arrested at a globular stage of development. These seeds, when cultured on basal media, were only rarely able to produce a small amount of callus after 6 to 7 weeks in culture. At first it would appear that the culture conditions are not optimal for in vitro growth of aborted seeds containing globular embryos. However, when wild-type seeds with embryos at a globular stage of development were cultured on this same medium, the embryos normally completed embryogenesis (Meinke, 1979). There was no sign that the arrested embryos of mutant 95A-2B advanced to a later stage of embryo development. These results clearly suggest that the lethal mutation in 95A-2B is blocking callus formation as well as the completion of embryogenesis.

Mutant 117N-1B also arrests at a very early stage of embryogenesis (i.e. globular to heart), but is able to produce more callus in vitro than mutant 95A-2B. Slightly more callus was observed when aborted seeds were cultured on the enriched 2,4-D medium than on the basal 2,4-D medium. However, this mutant does not appear to be amenable for further studies because the difference in response of aborted seeds on basal and enriched callus media is only slight; the rate of growth observed even on the enriched medium is very slow; and finally, the amount of callus produced on the enriched 2,4-D medium cannot be maintained in vitro. It is possible that further callus growth in mutant 117N-1B is inhibited by the accumulation of some secondary metabolite produced during early stages of growth in vitro.

The response in culture for aborted seeds and arrested embryos of mutant 115D-4A appears to be low when compared to the response of other mutants that arrest at similar stages of embryogenesis. This value is misleading, because both aborted seeds and isolated embryos were cultured to produce this value, but only the aborted seeds were observed to respond. Whether the poor response of the isolated embryos was a result of external damage during culturing, improper gas exchange on the surface of the agar or reduced nutrient uptake has not been established. As observed for mutant 117N-1B, the response of aborted seeds on the enriched media is slightly better than the response on basal media. From these results, it appears that the enriched callus media may be better than the basal callus media for the growth of arrested embryos. Occassionally, the tissue produced by aborted seeds from mutant 115D-4A cultured on the enriched callus media resembled an elongated hypocotyl. Attempts to stimulate further growth by culturing aborted seeds on modified media (BC5N1K and BC1ON1/2K) failed to produce positive results. These results suggest that something other than a missing nutrient is inhibiting growth in mutant 115D-4A.

Mutants 126E-B and 122G-E both produce interesting results *in vitro*. The best response from both mutants occurs if arrested embryos at later stages of development are used. Aborted seeds and arrested embryos from mutant 126E-B respond as wild-type seeds and embryos do, except that continued

growth of aborted seeds on the enriched NAA medium does not occur. It is possible that the defect in mutant 126E-B destroys the function of a gene that codes for a product that plays a minor or secondary role in development. Alternately, mutant 126E-B could be an example of a leaky mutant. In this case, the defect would alter the function of a gene such that only a reduced amount of the gene product is available. In either case, the mutation would allow a certain amount of normal growth. The response of aborted seeds and arrested embryos from mutant 122G-E on the basal and enriched media suggests that this line may be an example of an auxotrophic mutant. Detailed studies upon this mutant will be discussed in the following chapter.

Sheridan and Neuffer (1980) reported the response of 102 defecive kernel corn mutants cultured on basal and enriched media. These studies were performed to search for auxotrophs and at the same time accurately characterize the lethal phase of the arrested embryos and determine the morphological effect of the mutation. Results from these studies indicated that embryos from 21 mutants enlarged only slightly or failed to grow at all on any of the media tested; 81 produced shoots and roots on at least 1 medium; and 23 grew better on an enriched medium than basal. However, the approach taken by Sheridan and Neuffer (1980) differed significantly from the approach taken in this study. The media used by Sheridan and Neuffer were designed to rescue the defective kernels rather than induce callus growth. In addition, 84 of the 102 mutants chosen were able to form at least one leaf primordium

before being developmentally blocked, indicating that the majority of the mutants cultured were not actually embryo-lethals. However, Sheridan and Neuffer found that only one of the mutants that arrested during embryogenesis was able to form a single root when cultured on the basal medium. This mutant, E792, arrested during a transition stage of development (Figure 2). These results also suggest that those mutant lines that arrest during young stages of embryo development generally do not produce significant growth *in vitro*.

The question that cannot be answered with the data obtained from these experiments is what causes some aborted seeds from a given mutant to respond *in vitro* while other remain inactive. In future experiments, it may be possible to improve the growth response of these mutant lines by modifying the concentrations of the amino acids, nucleosides and vitamins present in the enriched media, adding different combinations of plant hormones to the media, and altering the temperature and lighting conditions under which the cultured seeds and embryos are grown.

CHAPTER IV

GROWTH AND DEVELOPMENT OF 122G-E HOMOZYGOUS MUTANT PLANTS IN CULTURE

Introduction

Mutant 122G-E appears to be segregating for a single Mendelian recessive allele that causes embryonic arrest between the globular and mature cotyledon stages of development. Aborted seeds from this mutant range in color from very pale yellow-green to pale green. Mutant embryos are white or very pale green and are usually abnormal in appearance. Aborted seeds and arrested embryos produce a very interesting response in culture. Only a slight amount of callus that is unable to grow on fresh media is produced when aborted seeds and arrested embryos are cultured on basal 2,4-D or basal NAA media. In contrast, aborted seeds and arrested embryos produce extensive callus when cultured on an enriched 2,4-D medium, and homozygous mutant plants when cultured on an enriched NAA medium. This differential response of mutant embryos on basal and enriched callus media suggested that mutant 122G-E might be an auxotroph and an interesting mutant to analyze in detailed studies. This chapter will describe various experiments that have been performed to further characterize mutant 122G-E.

Materials and Methods

Length and Stage of Development of Arrested Embryos

Heterozygous wild-type plants of Arabidopsis thaliana (L.) Heynh. were identified and maintained as described in Chapter II. Green siliques with wild-type seeds ranging from a mature to linear cotyledon stage of development were harvested from these plants such that the oldest siliques were harvested first and the youngest were harvested last. These siliques were then placed in labelled vials arranged in decreasing stage of maturity and were observed one at a time under a Wild stereomicroscope starting with the oldest silique. Each silique was opened along both sides of the central septum with forceps. The stage of development of the wild-type embryos from each silique was determined by dissecting wild-type seeds and observing the corresponding embryos. Each aborted seed was also dissected to observe the mutant embryo. The stage of developmental arrest and color of the mutant embryos were noted and then the size of each was determined with a stage micrometer.

Preparation of Media

Modifications of the enriched callus (EC) media described in Chapter III were prepared to determine which component of the media enhanced growth of mutant 122G-E aborted seeds and arrested embryos. These media, designated EC2D1/2K-a, EC2D1/2K-b, EC2D1/2K-c, EC2N1/2K-a, EC2N1/2K-b,

and EC2N1/2K-c, are described in Table XIV. The modified media were identical to either EC2D1/2K or EC2N1/2K except that one component of the organic supplements was absent in each of the modified media. Those media with the subscript "a" did not contain amino acids; those with the subscript "b" did not contain nucleosides or the organic acids, malic acid and citric acid; and those media with the subscript "c" did not contain vitamins. In addition, modifications of the basal callus media were prepared. These modified media, designated BC2D1/2K+a, BC2D1/2K+b, BC2D1/2K+c, and BC2D1/2K+gln, are summarized in Table XV. These media were prepared as described for the basal 2,4-D medium in Chapter III, except that BC2D1/2K+a contained amino acids; BC2D1/2K+b contained nucleosides; BC2D1/2K+c contained vitamins; and BC2D1/2K+gln contained glutamine. These organic supplements were filter sterilized in a 0.2 um Gelman filter and added to the autoclaved basal components just prior to pouring the plates.

Liquid media were also prepared as added nutrients for plants that were produced in vitro and transplanted to pots. These media were prepared as described in Chapter III except that agar was not added. In addition, glucose was not added to those media designated "-g".

Culturing Procedure

Heterozygous green, mature siliques were surface sterilized as described in Chapter III. The aborted seeds and arrested embryos were cultured on one half of the agar

TABLE XIV

COMPONENTS OF MODIFIED ENRICHED CALLUS MEDIA

Medium	Basal Medium Components*	Inositol and Thiamine	2,4-D	NAA	Amino acids	Organic Acids and Nucleosides	Vitamins
EC2D1/2K	X	---	X	---	X	X	X
EC2D1/2K-a	X	---	X	---	---	X	X
EC2D1/2K-b	X	X	X	---	X	---	X
EC2D1/2K-c	X	---	X	---	X	X	---
EC2N1/2K	X	---	---	X	X	X	X
EC2N1/2K-a	X	---	---	X	---	X	X
EC2N1/2K-b	X	X	---	X	X	---	X
EC2N1/2K-c	X	---	---	X	X	X	---

X = present in medium; --- = not present in medium

a = amino acids; b = organic acids and nucleosides; c = vitamins

* Inositol, thiamine and organic acids are not included.

TABLE XV

COMPONENTS OF MODIFIED BASAL CALLUS MEDIA

Medium	Basal Medium Components	2,4-D	Amino Acids	Nucleosides	Vitamins	Glutamine
Basal	X	---	---	---	---	---
BC2D1/2K	X	X	---	---	---	---
BC2D1/2K+a	X	X	X	---	---	---
BC2D1/2K+b	X	X	---	X	---	---
BC2D1/2K+c	X	X	---	---	X	---
BC2D1/2K+gln	X	X	---	---	---	X

X = present in medium

--- = not present in medium

a = amino acids; b = nucleosides; c = vitamins

plates, and as an internal control, phenotypically normal seeds and embryos from the same silique were cultured on the other half. Callus produced from aborted seeds and arrested embryos cultured on the basal and enriched callus media were subcultured directly to fresh media in the laminar flow hood with sterile forceps. Leaves produced *in vitro* from homozygous mutant plants and phenotypically normal plants were also subcultured directly onto basal 2,4-D and enriched 2,4-D callus media.

Transplanting Technique for Plants Produced In Vitro

Plants produced *in vitro* were removed from the agar plates with sterile forceps after two pairs of true leaves had formed, and the roots were carefully rinsed with distilled water to remove any remaining clumps of agar. The plants were then quickly planted in 3" pots containing standard soil mixture (12:3:1 coarse vermiculite, potting soil and sand) that had previously been allowed to soak in distilled water for 10 minutes. Care was taken to handle the plants only by the leaves. The pots were placed in 1000 ml Pyrex beakers that were partially sealed with Saran wrap and then transferred to light benches providing 16 hour/8 hour light dark cycles at 24°C. The plants were top-watered every day for one week with distilled water. During this time, the Saran wrap was slowly unrolled from the mouth of the beaker. During the second week, the plants were watered with a dilute fertilizer solution, and the Saran wrap was completely

removed. At the beginning of the third week, the pots were removed from the beaker, placed in tubs, and top-watered with full strength fertilizer solution.

Techniques to Produce Siliques with Seed Set

In an attempt to induce seed set in 122G-E homozygous mutant plants, a selected group of plants produced *in vitro* was transferred to pots with the standard soil mixture and watered every other day during the time of bolting with either 20 ml of EC2N1/2K (-g) or 20 ml of basal medium (-g). As a control, wild-type plants produced *in vitro* were also transferred to pots and watered in the same way. Homozygous mutant plants produced *in vitro* were also aseptically subcultured to deep dish containers containing 50 ml of either BC2N1/2K, EC2N1/2K, basal medium, or basal medium (-g). A wild-type plant was included in each container as a control. These experiments did not appear to be successful because both the homozygous mutant and wild-type plants failed to set seed. A different approach involved subcultured a selected group of homozygous mutant plants along with wild-type controls to sterile deep dish containers containing either the standard soil mixture or a 1:1 mixture of the standard soil mixture and perlite, and watering these plants with 35 ml of either basal medium (-g), basal medium, or enriched NAA medium (-g).

Results

Phenotypes of Arrested Embryos

Mutant embryos of 122G-E arrest over a very broad range of developmental stages (Figure 8). The arrested embryos occassionally resemble phenotypically normal embryos at the same stage of development, but the majority are abnormal in appearance (Figure 10). The approximate percent of arrested embryos observed at each stage of development was determined for 254 aborted seeds from mutant 122G-E. The results summarized in Table XVI indicated that an irregular torpedo stage of development was most frequently observed. In addition, significantly more mutant embryos were observed to have irregular phenotypes than normal phenotypes.

Lengths of Arrested Embryos

Arrested embryos from three classes of siliques that differed in stage of development of the phenotypically normal embryos were measured because arrested embryos dissected from aborted seeds in older siliques appeared to be significantly larger than arrested embryos from aborted seeds in younger siliques. The lengths of arrested embryos from the three classes of siliques are presented in Table XVII. There appears to be no significant difference between the size reached by the majority of the mutant embryos from siliques that contain wild-type embryos at a late mature stage of development as compared to siliques that contain wild-type

Figure 10. Examples of arrested embryos from mutant 122G-E.

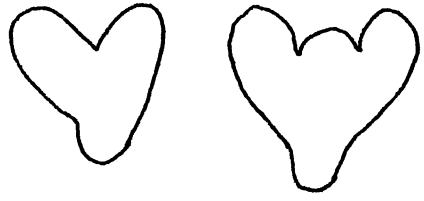
Globular

Irregular Globular

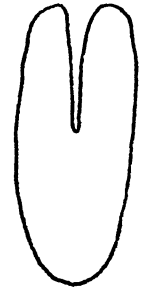
Heart

Irregular Heart

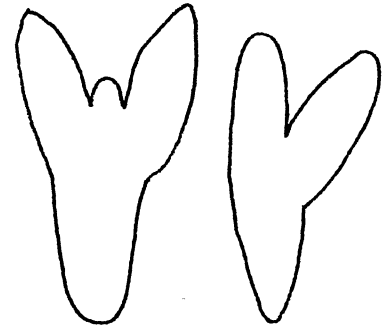
Torpedo



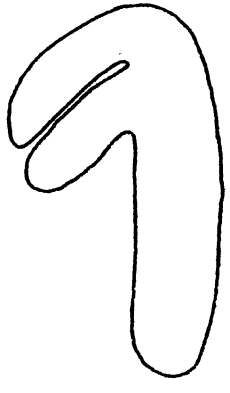
Irregular Torpedo



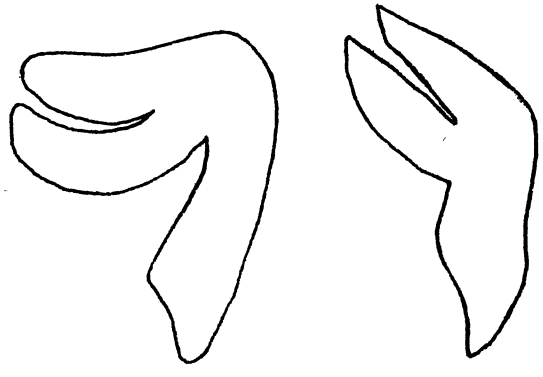
Linear



Irregular Linear



Curled Cotyledon



Irregular Curled Cotyledon

Indistinguishable

TABLE XVI

STAGES OF EMBRYO DEVELOPMENTAL ARREST OBSERVED IN 254 ABORTED SEEDS OF MUTANT 122G-E*

Stage of developmental arrest	Number of arrested embryos observed	Approximate percent of arrested embryos observed
globular	3	1.2
irregular globular	23	9.1
heart	1	0.4
irregular heart	26	10.3
torpedo	10	3.8
irregular torpedo	91	35.7
linear cotyledon	18	7.1
irregular linear cotyledon	26	10.3
early curled cotyledon	11	4.3
irregular early curled cotyledon	15	5.9
mature	1	0.4
indistinguishable	3	1.2
no embryo found	26	10.3

* See Figure 10 for the appearance of the mutant embryos arrested at each stage of development.

TABLE XVII

LENGTHS OF 122G-E ARRESTED EMBRYOS IN HETEROZYGOUS SILIQUES THAT DIFFER IN STAGE OF DEVELOPMENT OF THE PHENOTYPICALLY NORMAL EMBRYOS

Stage of Development of Normal Embryos	Number (Percent) of Arrested Embryos			
	100 - 190 um	200 - 290 um	300 - 390 um	> 400 um
Linear to Curled	27 (40)	29 (43)	9 (13)	3 (4)
Early Mature	14 (13)	35 (31)	48 (44)	15 (14)
Late Mature	4 (6)	10 (14)	37 (52)	20 (28)

embryos at an early mature stage of development. In both cases, the largest class of mutant embryos observed ranged from 300 um to 390 um in length. However, the second largest class of mutant embryos found in siliques that contained wild-type embryos at a late mature stage of development was greater than 400 um in length, while the second largest class of mutant embryos found in siliques that contained wild-type embryos at an early mature stage of development was only 200 um to 290 um in length. The largest class of mutant embryos observed in siliques that contained wild-type embryos at a linear to a curled cotyledon stage of development ranged from 200 um to 290 um in length, while the second largest class of arrested embryos from this group of siliques ranged from 100 um to 190 um. Rarely were arrested embryos greater than 400 um observed in siliques which contained wild-type embryos at a linear to curled cotyledon stage of development. These results indicated that 122G-E arrested embryos found in older siliques were clearly larger than those found in younger siliques.

Stage of Development of the Arrested Embryos

The number and percent of mutant embryos that arrested at a specific stage of development were also determined for the three classes of siliques that differed in stage of development of the wild-type embryos (Table XVIII). Interestingly, the most common stage of arrest for the mutant embryos from all three classes of siliques occurred at a torpedo to linear stage of development, while the second most

TABLE XVIII

DEVELOPMENTAL STAGES OF 122G-E ARRESTED EMBRYOS REMOVED FROM SILIQUES AT DIFFERENT STAGES OF DEVELOPMENT

Stage of Development of Normal Embryos	Number (Percent) of Arrested Embryos at Each Stage of Development		
	Globular to Heart	Torpedo to Linear	Curled Cotyledon to Mature
Linear to Curled	14 (20)	48 (71)	6 (9)
Early Mature	20 (18)	82 (71)	13 (11)
Late Mature	12 (17)	48 (68)	11 (15)

common stage of embryo arrest occurred between a globular to a heart stage of development. These results suggested that the presence of larger arrested embryos in older siliques of mutant 122G-E was not caused by a difference in the stage of developmental arrest of mutant embryos but by continued growth of the mutant embryos after embryonic arrest occurred.

Response in Culture

The response of 122G-E mutant embryos and callus cultured on basal and enriched media is presented in Table XIX. As described previously, mutant embryos produced only a slight amount of callus on basal 2,4-D and basal NAA media. In contrast, mutant embryos cultured on enriched 2,4-D media produced an extensive amount of callus that continued to grow when subcultured to either basal 2,4-D or enriched 2,4-D media. On the enriched NAA medium, mutant embryos developed into plants. The response of 122G-E mutant and wild-type seeds and embryos cultured on the modified enriched callus media is presented in Figure 11 and Table XIX. There was no significant difference in response observed for aborted seeds and isolated embryos cultured on BC2D1/2K+gln and BC2D1/2K. On both media, a slight amount of callus was produced by aborted seeds and arrested embryos. These results suggested that the enhanced growth of mutant embryos on the enriched media was not a result of a reduced nitrogen deficiency. There was also no significant difference between the amount of callus growth observed on the modified enriched 2,4-D

TABLE XIX

RESPONSE OF MUTANT 122G-E IN CULTURE

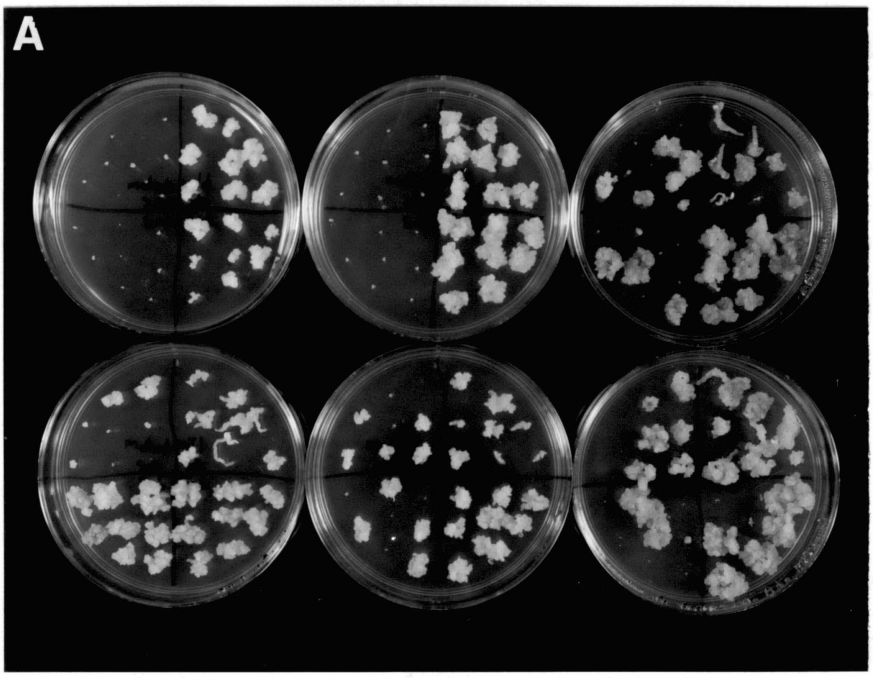
Explant	Nutrient Medium	Response in Culture
Mutant embryo	Basal 2,4-D	Slight callus forms
" "	Basal NAA	" " "
Mutant embryo	Enriched 2,4-D	Extensive callus forms
" "	Enriched NAA	Green plantlets form
Mutant callus	Basal 2,4-D	Continued callus growth
" "	Enriched 2,4-D	" " "
Mutant embryo	BC2D1/2K+a	Slight callus forms
" "	BC2D1/2K+b	" " "
" "	BC2D1/2K+c	" " "
Mutant embryo	EC2D1/2K-a	Extensive callus forms
" "	EC2D1/2K-b	" " "
" "	EC2D1/2K-c	" " "
Mutant embryo	EC2N1/2K-a	Green plantlets form
" "	EC2N1/2K-b	" " "
" "	EC2N1/2K-c	" " "

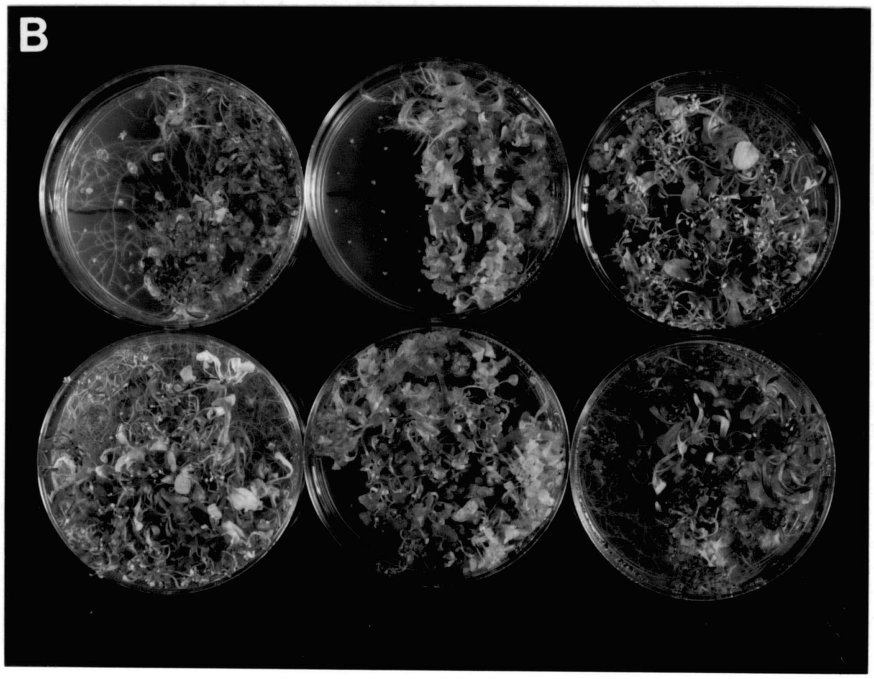
a = amino acids

b = nucleosides and organic acids (malic and citric acids)

c = vitamins

Figure 11. Response of 122G-E mutant and wild-type seeds and embryos on modified enriched callus media. In each plate, aborted seeds are cultured on the top left quadrant; isolated arrested embryos are on the bottom left quadrant; wild-type seeds are on the top right quadrant; and isolated wild-type embryos are on the bottom right quadrant. The media used in Fig. A moving clockwise starting with the upper left corner are BC2D1/2K, BC2D1/2K+gln, EC2D1/2K, EC2D1/2K-c, EC2D1/2K-b, and EC2D1/2K-a. The media used in Fig. B moving clockwise starting with the upper left corner are BC2N1/2K, BC2N1/2K+gln, EC2N1/2K, EC2N1/2K-c, EC2N1/2K-b, and EC2N1/2K-a.

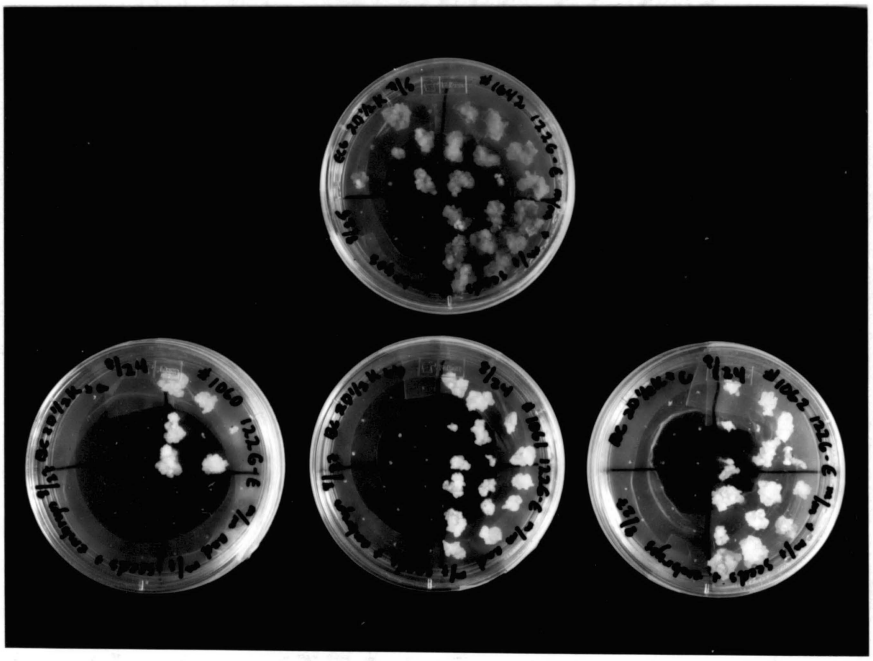

A


B

callus media (EC2D1/K-a, EC2D1/2K-b, and EC2D1/2K-c) as compared to the amount of callus growth observed on the original enriched 2,4-D callus medium (EC2D1/2K). Similar results were observed for mutant and wild-type seeds and embryos cultured on the modified enriched NAA media. Homozygous mutant plants were able to form equally well from aborted seeds and arrested embryos cultured on EC2N1/2K, EC2N1/2K-a, EC2N1/2K-b, and EC2N1/2K-c, but homozygous mutant plants were not observed on BC2N1/2K or BC2N1/2K+gln. After several weeks *in vitro*, however, 122G-E mutant seeds and embryos were observed to become slightly larger and significantly greener on BC2N1/2K when wild-type seeds and embryos cultured on the same plate produced plants with roots that extended across the bottom of the petri plate (Figure 11). This same response was observed again when roots produced by wild-type seeds cultured on BC2N1/2K surrounded aborted seeds of mutant 122G-E. This suggests that the wild-type tissue may produce a diffusible substance that is utilized by the aborted seeds to enhance cell division.

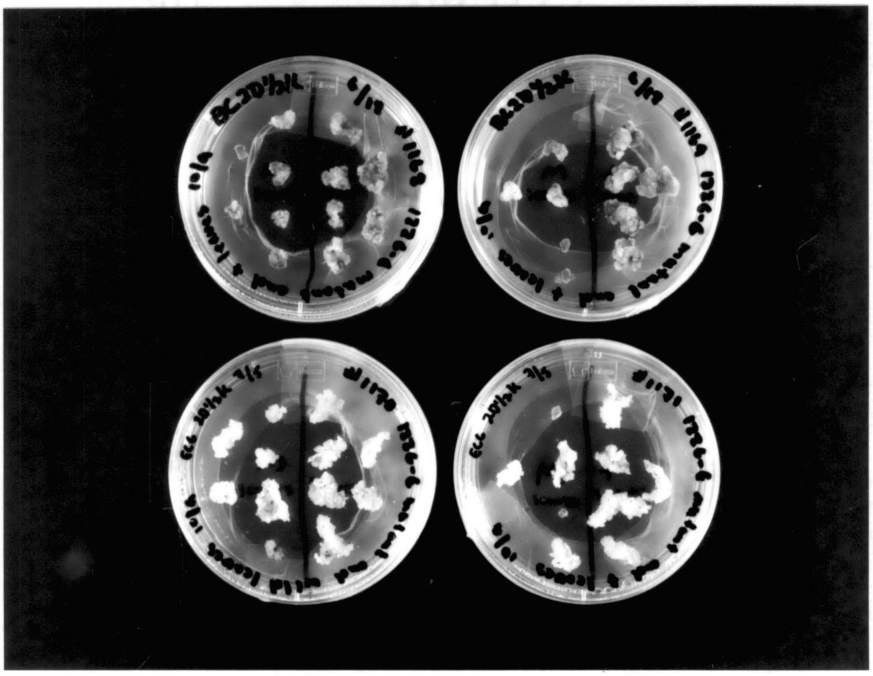
The response of 122G-E mutant and wild-type seeds and embryos on modified basal callus media is presented in Figure 12 and Table XIX. Aborted seeds and arrested embryos were unable to produce significantly more callus on the modified basal 2,4-D media (BC2D1/2K+a, BC2D1/2K+b, and BC2D1/2K+c) than on the original basal 2,4-D medium. However, wild-type seeds and embryos cultured on the same modified basal 2,4-D media were able to produce significant amounts of callus.

Figure 12. Response of 122G-E mutant and wild-type seeds and embryos on modified basal callus media. In each plate, aborted seeds are cultured on the top left quadrant; isolated arrested embryos are on the bottom left quadrant; wild-type seeds are on the top right quadrant; and isolated wild-type embryos are on the bottom right quadrant. The media used starting with the top petri plate and moving clockwise are EC2D1/2K, BC2D1/2K+c, BC2D1/2K+b, and BC2D1/2K+a.



Pale, friable callus produced from aborted seeds and arrested embryos on EC2D1/2K was subcultured numerous times to different media. Although continued callus growth occurred on all four original media, the appearance of the callus often changed after several subcultures to different media. When the enriched 2,4-D callus was subcultured to BC2D1/2K, the callus remained pale and friable, but the growth rate was slightly slower than the growth rate of callus produced and maintained on the enriched 2,4-D media. Callus initiated on an enriched 2,4-D medium and then subcultured to an enriched NAA medium became dark green and nodular and produced many root hairs. Occassionally, the individual pieces of callus were observed to produce several shoot primordia, indicating that undifferentiated cells were capable of producing shoots in vitro. Enriched 2,4-D callus subcultured to enriched NAA media and then subcultured twice to basal NAA media became pale green and remained friable. Root hairs were visible on some calli, but there were no obvious signs of shoot morphogenesis. Leaves from 122G-E homozygous mutant and wild-type plants produced in vitro were also observed to form pale, friable callus when subcultured on BC2D1/2K and EC2D1/2K (Figure 13). However, slightly faster growth of the callus was observed on EC2D1/2K than on BC2D1/2K. Continued growth of enriched 2,4-D callus on basal and enriched 2,4-D media suggested that the mutant gene was not expressed during callus proliferation. Similarly, callus production by leaves from homozygous mutant plants indicated

Figure 13. Response of 122G-E homozygous mutant leaves and wild-type leaves produced *in vitro* and subcultured to BC2D1/2K (top 2 plates) and EC2D1/2K (bottom 2 plates). In each plate, the mutant leaves are cultured on the left half and the wild-type leaves on the right.



that the mutant gene did not block cell division in undifferentiated cells.

Homozygous 122G-E mutant plants developed more readily from isolated arrested embryos cultured on an enriched NAA medium than aborted seeds on the same medium. The majority of these mutant seedlings were green and phenotypically normal (Figure 14). However, not all 122G-E arrested embryos were able to develop into plants. Those embryos that arrested at later stages of development more readily produced plants *in vitro*. In addition, plants were only observed to develop from arrested embryos that came from heterozygous siliques produced at 24°C. Heterozygous plants grown at 18°C produced smaller arrested embryos that were unable to develop into plants when placed on an enriched NAA medium. Homozygous mutant plants left in culture for a long period of time developed an abundance of leaves and relatively few roots.

Response of Plants In Vivo

Watered with Fertilizer Solution. Plants at an early stage of development (i.e. two pairs of true leaves) were easily transferred to pots with the standard soil mixture. The appearance of the 122G-E homozygous mutant plants transferred to pots, watered with the standard fertilizer solution, and allowed to grow several weeks without added nutrients is presented in Figure 15. These plants were occassionally smaller than wild-type plants but were definitely green and appeared normal until the time of

Figure 14. 122G-E homozygous mutant plantlets produced from arrested embryos cultured on EC2N1/2K. One of the arrested embryos in the top picture did not develop into a plant.

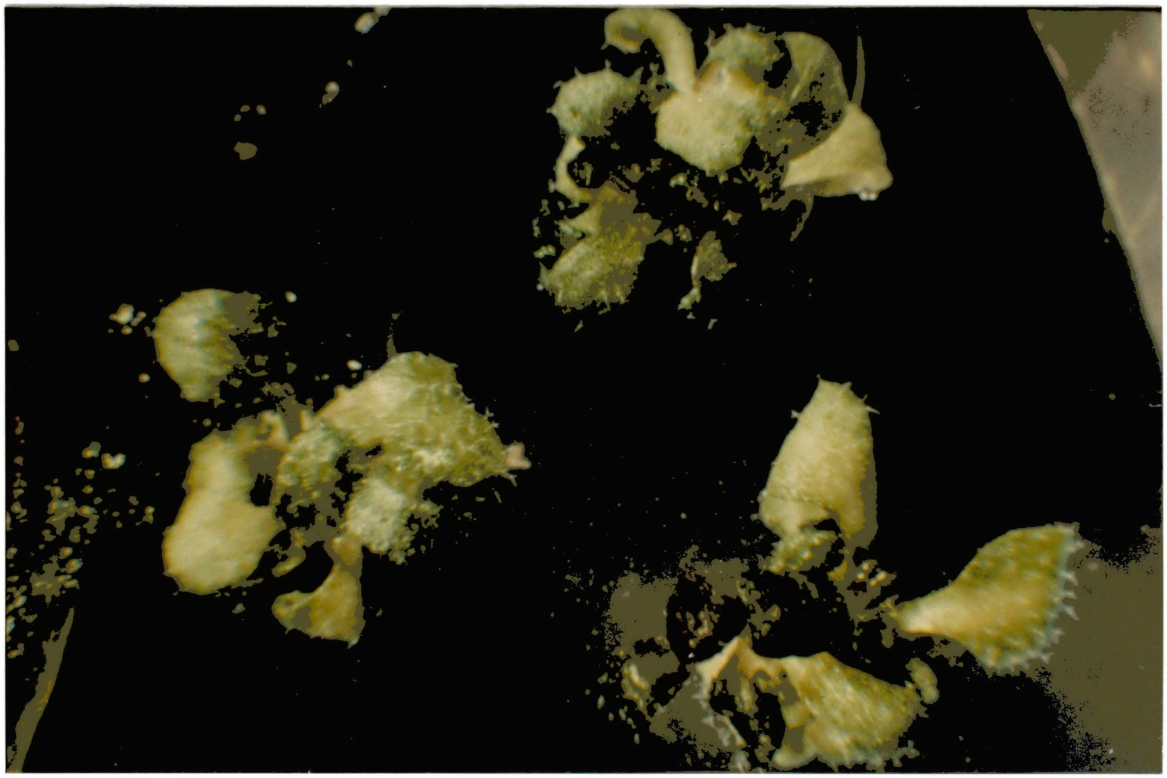


Figure 15. Nine 122G-E homozygous mutant plants that have been transferred to pots containing 12:3:1 coarse vermiculite, potting soil and sand without added nutrients. The pot on the left contains the youngest plants which have been growing for two weeks. These plants are green and just beginning to bolt. The pot in the center contains older plants which have been growing for one month. These plants are bolting and starting to turn pale. The pot on the right contains the oldest plants which have been growing for one month and 4 days. These plants are pale and have very abnormal flowers.

bolting. Just prior to flowering, however, the cauline leaves proximal to the inflorescence became very pale. The buds produced were a variety of shapes and sizes, but almost all were white or very pale green. The most normal-looking buds were found on very young inflorescences that had not yet begun to turn pale. One fairly normal bud contained 4 normal sepals, 6 normal dried stamens, 3 white dried petals, and a white silique that was slightly larger than the stamens. After the plants turned pale, bolting ceased (i.e. the main axis was approximately 3.0 cm to 5.5 cm tall) and no further buds were produced. Two very young buds on these homozygous mutant plants were very abnormal in appearance and were found to contain two sepals, both of which were pale green, but one was slightly larger and curled over the smaller one; a silique reduced to one-fourth of the normal size; three small, beige stamens one-third the size of the bud; one filament without an anther; and two very small, dried petals one-third the size of the bud. This abnormal response was observed in every homozygous mutant plant transferred to pots and watered with fertilizer solution. The wild-type controls, however, flowered normally and set seed. These results suggested that the same mutant allele blocking embryo development was also disrupting flower formation.

Watered with Liquid Media. The response of homozygous mutant plants and wild-type plants produced *in vitro*, transplanted to pots with soil, and watered with defined liquid media is summarized in Table XX. The wild-type plants

TABLE XX

RESPONSE OF 122G-E HOMOZYGOUS MUTANT PLANTS AND WILD-TYPE PLANTS PRODUCED IN VITRO, TRANSPLANTED TO SOIL, AND WATERED WITH LIQUID MEDIA

Liquid Medium Added to Pots*	Number of Mutant Plants Observed	Response of Mutant Plants	Response of Wild-type Plants
BC1N1/2K	6	Grew slightly but became pale at bolting; No normal flowers or seed set observed;	Not tested
EC1N1/2K	17	" " "	Slightly smaller than normal but bolted, flowered and produced siliques with seeds;
Basal	6	" " "	" " "

* Media did not contain glucose.

watered with EC2N1/K (-g) or basal medium (-g) were able to bolt, flower, and set seed normally. The response of wild-type plants watered with BC2N1/2K (-g) was not tested. The homozygous mutant plants watered with BC2N1/2K (-g), EC2N1/2K (-g) or basal medium (-g) appeared to grow normally at first. The homozygous mutant plants bolted but consistently became pale at the onset of flowering and eventually died. No normal flowers or mature siliques were observed. However, microbial contamination was present on the soil surface of all pots. If the same mutant allele blocking embryogenesis was also affecting flower formation, the addition of liquid enriched NAA medium to the pots containing homozygous mutant plants should be able to enhance normal flower development. This response was not observed probably because of competition between the plants and the microorganisms, or the concentration of the essential nutrient(s) was not sufficiently high to support growth of whole plants.

Response of Plants In Vitro

On Sterile Soil Mixtures with Added Liquid Media. To eliminate possible competition between the homozygous mutant plants and microbial contaminants, the homozygous mutant and wild-type plants produced *in vitro* were subcultured aseptically to various sterilized soil mixtures and watered with sterile liquid media. Results from these experiments are described in Table XXI. Wild-type plants grown on both

TABLE XXI

RESPONSE OF 122G-E HOMOZYGOUS MUTANT PLANTS AND WILD-TYPE PLANTS ON STERILIZED SOIL MIXTURES IN DEEP DISH CONTAINERS AND WATERED WITH STERILE LIQUID MEDIA

Soil	Liquid Medium	Number of Mutant Plants Subcultured	Response of Mutant Plants	Response of Mutant Plants
A	Basal-g	2	Grew slightly but became pale and did not flower or set seed	Bolted and flowered but did not set seed
B	Basal-g	2	" " "	" " "
A	Basal	2	" " "	" " "
B	Basal	2	" " "	" " "
A	EC2N1/2K-g	2	Appeared normal; bolted and flowered but did not set seed	" " "
B	EC2N1/2K-g	2	" " "	" " "

A = standard soil mix (12:3:1 coarse vermiculite, potting soil and sand)

B = 1:1 mixture of standard soil mix and perlite

g = glucose

types of soil mixtures and watered with basal medium, basal medium (-g) or EC2N1/2K (-g) appeared to grow normally. The majority of the wild-type plants were observed to bolt and flower but were unable to set seed. The homozygous mutant plants watered with the same liquid medium generally appeared to grow slightly better on the soil mixture containing the standard soil mixture than on the 1:1 standard soil and perlite mixture. Those homozygous mutant plants watered with the basal medium (-g) were usually able to begin bolting but consistently produced pale, abnormal buds around the time of flowering. The cauline leaves proximal to the buds were also observed to become pale. Similar results were observed for homozygous mutant plants watered with basal medium, except that these plants appeared to be slightly greener. No phenotypically normal flowers or siliques with seed set were produced by homozygous mutant plants watered with either basal media. The homozygous mutant plants watered with EC2N1/2K (-g), in contrast, were able to bolt and produce normal-looking flowers, but no seed set was observed. Occassionally, a few of the cauline leaves appeared slightly pale but the response was not extensive. Some component of the enriched NAA medium appeared to be allowing homozygous mutant plants to form phenotypically normal flowers. These results suggest that the mutant allele disrupting embryogenesis is also blocking flower formation.

On Defined Agar Media. The response of plants produced in vitro and subcultured aseptically to deep dishes

containing defined agar media is summarized in Table XXII. Wild-type plants subcultured to BC2N1/2K and BC2N1/2K+gln media formed normal rosettes but were never observed to bolt. Instead, these plants produced a slight amount of pale green callus with many small root hairs. Wild-type plants on basal medium (-g) also appeared normal at first but were unable to bolt or flower. However, wild-type plants on basal medium and EC2N1/2K appeared normal, bolted and flowered but did not set seed. Homozygous mutant plants subcultured to either BC2N1/2K or BC2N1/2K+gln produced significant amounts of callus with numerous root hairs. Homozygous mutant plants subcultured to basal medium (-g) appeared normal at first but consistently became pale and died before bolting. Homozygous mutant plants on basal medium usually bolted but became pale before flower formation and were unable to produce siliques with seed set. However, the majority of the homozygous mutant plants cultured on EC2N1/2K appeared phenotypically normal, bolted and flowered but did not set seed. In an attempt to provide more space for flowering, homozygous mutant plants subcultured to deep dish containers containing EC2N1/2K were placed in autoclaved, 1 gallon mayonnaise jars. The top of the deep dish container was removed and the mayonnaise jar were then sealed with parafilm. The homozygous mutant plants in these jars flowered profusely but were still unable to produce siliques with seeds. These results further support the conclusion that the mutant allele blocking embryogenesis is also disrupting flower formation.

TABLE XXII

RESPONSE OF 122G-E HOMOZYGOUS MUTANT PLANTS AND WILD-TYPE PLANTS TRANSFERRED ASEPTICALLY TO DEEP DISH CONTAINERS WITH DEFINED AGAR MEDIA

Medium	Number of Mutant Plants Subcultured	Response of Mutant Plants	Response of Wild-type Plants
BC2N1/2K	5	Grew slightly; extensive pale green callus produced; no bolting or seed set observed	Grew slightly; slight pale green callus produced; no bolting or seed set observed
BC2N1/2K+gln	4	" " "	" " "
Basal-g	6	Appeared normal 2 weeks after subculture, then became pale; no bolting or seed set observed	Appeared normal 2 to 3 weeks after subculture; no bolting or seed set observed
Basal	6	Appeared normal at first, occassionally bolted but became pale before flowers formed; no seed set observed	Most produced normal-looking plants that bolted and flowered but did not set seed
EC2N1/2K	14	Most produced normal-looking plants that bolted and flowered but did not set seed	" " "

gln = glutamine; g = glucose

Discussion

Auxotrophic mutants unable to grow in the absence of a required nutrient have been described extensively in fungi (Cove, 1976; Kevec and Peberdy, 1984) and bacteria (Li et al., 1967) and to a lesser extent in liverworts (Schieder, 1976) and mosses (Ashton and Cove, 1977). Relatively few auxotrophic mutants have been well characterized in higher plants (Flick, 1983). Those auxotrophic mutants that have been identified in higher plants have been useful in various studies to elucidate biochemical pathways (Müller and Grafe, 1978) and select somatic hybrids by genetic complementation (Pental et al., 1984). Landridge and Brock (1961) reported a spontaneous, single gene mutant of tomato that was unable to produce normal-looking plants in the absence of thiamine. However, the cotyledons of these mutant plants appeared normal, suggesting that thiamine from heterozygous maternal tissue was able to diffuse into the homozygous mutant embryos. A defective kernel mutant of corn produced by pollen mutagenesis was observed to grow normally only in the presence of proline (Gavazzi et al., 1975). The first auxotrophic cell cultures of higher plants were reported in haploid Nicotiana tabacum cells lacking nitrate reductase activity (Müller and Grafe, 1978). Cultured haploid cells have proven to be a valuable source of plant auxotrophs. For example, mutants of Hyoscyamus muticus isolated from haploid leaf protoplasts and characterized in subsequent studies have been identified to be auxotrophic for histidine, leucine,

nicotinamide and tryptophan (Gebhardt et al., 1981, 1983). In addition, mutants deficient in pantothenate (Savage et al., 1979) and isoleucine and valine (Horsch et al., 1983) have been recovered in haploid cell suspension cultures of Datura innoxia. Recently, leaf mesophyll protoplasts isolated from N. tabacum nitrate reductase deficient, streptomycin resistant mutants of N. tabacum have been fused with cell suspension protoplast of the wild-type species, N. rustica (Pental et al., 1984).

Thiamine conditional-lethal mutants were the first auxotrophic mutants reported in Arabidopsis (Langridge, 1955; Rédei, 1960, 1962). The single most extensive study of auxotrophs in Arabidopsis described 65 thiamine-requiring mutants defective in four different loci controlling thiamine biosynthesis (Li and Rédei, 1969). Heterozygous plants from these mutant lines produced phenotypically normal homozygous mutant embryos that germinated but were unable to grow and develop normally without added thiamine. In addition, some of these mutants proved to be leaky and/or temperature-sensitive. Arabidopsis seedling mutants deficient in nitrate reductase activity have also been isolated by selection for chlorate resistance, and characterized in detailed studies (Ostindiër-Braaksma and Feenstra, 1973). Koornneef and van der Veen (1980) analyzed mutants of Arabidopsis isolated following EMS seed mutagenesis that are only able to grow normally in the presence of giberellin.

Mutant 122G-E was studied in detail because the differential response produced by mutant embryos cultured on

basal and enriched 2,4-D media suggested that this mutant might be an auxotroph. However, the response produced by mutant embryos cultured on the modified media proved to be confusing because extensive amounts of callus were produced on all of the modified enriched media instead of significant callus growth on two of the media and only slight growth on the other one. In addition, only slight amounts of callus were observed on all of the modified basal media when what was expected was significant growth on one medium and only slight growth on the other two. It is possible that mutant 122G-E is an auxotroph that requires a combination of various organic components instead of just one single component. Alternately, the response produced by mutant embryos on enriched callus media could be a result of a pH change, an osmotic potential difference, or some other undetermined factor.

Regardless of whether mutant 122G-E is an auxotroph or not, interesting conclusions about expression of the mutant gene in this line can be drawn. Homozygous mutant plants rescued *in vitro* on enriched NAA media and transplanted to pots with vermiculite consistently become pale at the onset of flowering. This response suggests that the same mutant gene that causes embryo lethality is also expressed during flower formation. If this were true, homozygous mutant plants supplied with enriched liquid NAA medium at the time of bolting would be expected to flower normally and produce siliques with normal seed set. This response was probably

not observed *in vivo* because either (1) enough of the enriched medium was not originally supplied to the homozygous mutant plants during watering; or (2) the large population of microorganisms observed growing on the soil surface was depleting the essential component(s) of the enriched medium that allows normal development. When this same experiment was performed aseptically in deep dish containers to eliminate the microbial contamination, homozygous mutant plants on basal media consistently turned pale and produced abnormal buds. However, homozygous mutant plants grown on enriched NAA media and wild-type plants grown on both enriched NAA and basal media grew normally, bolted, and flowered but did not set seed. These results suggest that the mutant gene is being expressed during embryogenesis and flower formation because only those homozygous mutant plants subcultured to an enriched NAA medium were able to flower normally. One possible explanation for the lack of seed set in these plants is that the excessive moisture inside these deep dish containers was preventing normal dehiscence of the mutant and wild-type anthers. Older flowers produced from homozygous mutant plants on enriched NAA media were analyzed, but pollen was not observed on the anthers. Whether the absence of pollen was a result of the older age of the flower or the high moisture content of the container has not been determined.

Mutant 122G-E is an interesting mutant because (1) it appears to be defective in a pleiotropic gene that is expressed during embryogenesis as well as flower formation;

and (2) it has some characteristics that resemble an auxotrophic mutant. Whether the enhanced growth of mutant embryos on the enriched media is a result of a combination of specific organic components, a change in pH, an osmotic potential difference or some other unrecognized factor has not been determined. In future experiments, it may be possible to identify whether mutant 122G-E is indeed an auxotrophic mutant by culturing arrested embryos on different types of modified basal and enriched callus media that have altered pH levels or contain more specific combinations of organic supplements.

CHAPTER V

GAMETOPHYTIC GENE EXPRESSION IN MUTANT 126E-B

Introduction

Following self-pollination, heterozygous siliques of Arabidopsis thaliana (L.) Heynh. segregating for a single embryo-lethal mutation normally contain approximately 25% aborted seeds that are distributed randomly along the length of the silique. Occassionally, a non-random distribution is observed causing a greater number of aborted seeds to be located in the top half of the silique (Table VI). Meinke (1982) observed this same response in an earlier group of embryo-lethal mutants and hypothesized that the non-random distribution of aborted seeds was caused by a detrimental effect of the mutant allele on either pollen formation, pollen germination, or pollen-tube growth. Those pollen grains carrying the mutant allele were therefore less likely to grow to the base of the silique. Eight of 32 embryo-lethal mutants of A. thaliana recently isolated following EMS seed mutagenesis were also observed to have a non-random distribution of aborted seeds in heterozygous siliques (Meinke, 1985). Mutant 126E-B was one of these eight mutants that had significantly more aborted seeds located in the top half of the silique (Table VI). I chose to test the

hypothesis presented by Meinke (1982) by performing reciprocal crosses with heterozygous 126E-B plants and homozygous wild-type plants. Mutant 126E-B was chosen for these crosses because the non-random distribution of aborted seeds was extremely pronounced in the heterozygous siliques. Results of these experiments will be presented in this chapter.

Materials and Methods

Crosses Between Mutant 126E-B and Wild-Type Plants

Homozygous wild-type and heterozygous mutant plants of Arabidopsis thaliana (L.) Heynh. strain "Columbia" were maintained as described in Chapter II. After the plants were approximately 6 weeks old, wild-type and heterozygous plants were identified by screening siliques for the presence of aborted seeds. Four different types of crosses were performed with these plants (Table XXIII). To perform the first cross, mature flowers were removed from 126E-B heterozygous plants and placed on a slide under a Wild stereomicroscope. With fine-tipped forceps, intact stamens with dehiscent anthers were removed from the flowers and placed on the slide in an upright position. This slide was then removed from the stereomicroscope. Wild-type plants that contained buds in which the stigma surface was just slightly protruding through the sepals (Figure 16) were selected for the female parent. The inflorescence with the appropriate bud was carefully laid on the stage of the

TABLE XXIII

CROSSES BETWEEN WILD-TYPE AND 126E-B MUTANT PLANTS

	Male Parent		Female Parent	Anthers
1.	126E-B (+/m)	X	Wild-type (+/+)	Intact
2.	Wild-type (+/+)	X	126E-B (+/m)	Intact
3.	126E-B (+/m)	X	Wild-type (+/+)	Removed
4.	Wild-type (+/+)	X	126E-B (+/m)	Removed

Figure 16. Inflorescence of Arabidopsis thaliana (L.) Heynh. The white arrow indicates the stage of development of the buds used in the controlled crosses.


V

stereomicroscope and held in place with a bent paper clip. The sepals were slightly pulled back to further expose the stigma surface. One at a time, isolated stamens from the 126E-B flowers were picked-up with fine-tipped forceps and used to dust the stigma surface of the wild-type bud. After the stigma surface appeared to be saturated with pollen, a small portion of one of the petals from this bud was torn and the buds immediately above and below the cross-pollinated bud were removed to mark the bud. In addition, the position of the marked bud was determined by counting the siliques on the stem starting with the oldest and recording this number. Finally, the stem containing the crossed bud was labelled with a small piece of paper and tape.

The second cross was performed identically to the first cross except that wild-type stamens were used to saturate the stigma surface of 126E-B heterozygous flowers. The third and fourth types of crosses were identical to the first and second except that all of the stamens were carefully removed from the female parent of each cross to insure that no self-pollination occurred. The buds were checked after 24 and 48 hours to confirm the location of the bud used for pollination. The siliques produced by these crosses were carefully harvested when they turned yellow-green, approximately two weeks after pollination. Harvested siliques were then stored in labelled vials until needed.

Planting F-1 Seeds

To determine the genotype of the F-1 individuals, it was necessary to plant the F-1 seeds and screen the resulting F-1 plants. The siliques produced from each cross were first removed from the vials, placed in a petri plate on very moist Whatman filter paper, and allowed to soak for 15 minutes to loosen the valves of the siliques. The petri plate was then placed under a Wild stereomicroscope, and each silique was carefully opened along both sides of the central septum to insure that the original position of each seed was maintained. A map of each silique was then drawn on a piece of graph paper by designating one box per seed. The position of spontaneously aborted seeds was also noted on the silique. Each seed from the silique was then carefully transferred in consecutive order with fine-tipped forceps to a different petri plate containing moistened, Whatman filter paper. The most efficient method of seed transfer occurred if the tips of the forceps were first dipped in water. The seeds were then carefully transferred in consecutive order to the moist soil surface of 3" pots. The pots were labelled with stakes, placed in tubs, and misted throughly with distilled water. The tubs were then partially covered with Saran wrap and a wet piece of cheesecloth to provide sufficient moisture for germination. The tubs were placed under fluorescent lights on 16 hour/8 hour light/dark cycles at 24°C $\pm$ 2°C. The resulting F-1 plants were maintained as described in Chapter II.

Screening Siliques Produced by F-1 Plants

After 6 to 8 weeks, 5 to 8 mature siliques were harvested from each of the mature, F-1 plants. These siliques were then screened to determine the genotype of the F-1 seed that produced each F-1 plant. If the siliques on a given F-1 plant contained all phenotypically normal seeds, then the seed that produced that plant was recorded on the original map as being homozygous wild-type (+/+), but if both phenotypically normal and mutant seeds were present, then the seed was recorded on the original map as being heterozygous (+/m). Using this method, the genotypes of the seeds in the siliques produced by cross-pollination could be determined. Each valve of the mapped siliques was then divided in half perpendicular to the central septum and the segregation ratio and distribution of heterozygous seeds was determined as described in Chapter II.

Wild-Type Controlled Crosses

Additional crosses with wild-type plants were performed to better understand the factors that control the direction of normal pollen-tube growth. Under a Wild stereomicroscope, dehiscent stamens were removed from wild-type flowers and rubbed across the surface of the slide to disperse the pollen grains. An inflorescence containing a young, wild-type bud in which the stigma surface was slightly protruding through the sepals was positioned on a slide under a second stereomicroscope and again held in place with a paper clip.

The anthers were carefully removed from the bud, and three to six pollen grains were transferred with forceps to the stigma surface of the wild-type bud. The bud was then marked as described previously and allowed to grow for approximately 2 weeks. The resulting wild-type siliques from these crosses were harvested and the position of each seed was mapped on a piece of graph paper by designating 2 squares for each mm of the silique, and placing a symbol in the box corresponding to the position of each seed. Each silique was then divided into four equal parts, and the number of seeds in each section was determined. In addition, naturally occurring short siliques from wild-type plants were screened and mapped to determine where the first seeds develop in flowers that only receive a limited number of pollen grains during self-pollination.

Controlled Crosses With Mutant 111H-2B and Wild-Type Plants

No seed set was observed in the majority of the wild-type crosses described above; therefore an alternate method involving the putative male-sterile plants observed segregating in the 111H-2B mutant line was attempted. Although seed set is rarely observed in these mutant plants, siliques with good seed set can be produced if wild-type pollen is added to the stigma surface of the mutant flowers. Three to six wild-type pollen grains were carefully transferred to the stigma surface of 111H-2B male-sterile flowers at various stages of development. The buds were

marked and allowed to grow for 2 weeks. The resulting siliques were then harvested and mapped as before.

Results

Controlled Crosses With Mutant 126E-B and Wild-Type Plants

The results obtained from the first and third types of crosses (i.e. when mutant 126E-B is the male parent) are summarized in Table XXIV. If the non-random distribution of aborted seeds observed in 126E-B heterozygous siliques was caused by an effect of the mutant allele on the male side prior to fertilization, there should be a non-random distibution of heterozygous and homozygous wild-type seeds along the length of the siliques. In addition, < 50% heterozygous seeds should be observed throughout the siliques. These results were exactly what were obtained for the first and third types of crosses.

The results obtained for the second and fourth group of crosses (i.e. when mutant 126E-B is the female parent) are presented in Table XXV. In these two groups of siliques, there should be a random distribution of heterozygous and homozygous wild-type seeds along the length of the silique and approximately equal numbers of heterozygous and homozygous seeds throughout the siliques. These results were observed in the second group of crosses. In the fourth group of crosses a random distribution of heterozygous seeds was observed as expected, but significantly less than 50% heterozygous seeds was observed thoughout the siliques. The

TABLE XXIV

RESULTS FROM CONTROLLED CROSSES BETWEEN 126E-B HETEROZYGOUS PLANTS (♂) AND WILD-TYPE PLANTS (♀)*

Cross	Number of Siliques Produced	Number of Plants Screened	% of +/m Seeds in Top Half	Chi-Square	% of +/m Seeds in Siliques	% of +/+ Seeds in Siliques	% of Seeds not Screened**
#1	18	827	85.5	82.5***	20.1	68.9	11.0
#3	9	321	76.6	17.0***	19.9	64.8	15.3

* A non-random distribution of heterozygous and homozygous wild-type seeds with < 50% of the heterozygous seeds distributed throughout the siliques is expected.

** Seeds did not develop into plants that could be screened.

*** Significantly different from 50% at P = 0.001.

TABLE XXV

RESULTS FROM CONTROLLED CROSSES BETWEEN WILD-TYPE PLANTS (♂) AND 126E-B HETEROZYGOUS PLANTS (♀)*

Cross	Number of Siliques Produced	Number of Plants Screened	% of +/m Seeds in Top Half	Chi-Square	% of +/m Seeds in Siliques	% of +/+ Seeds in Siliques	% of Seeds not Screened**
#2	9	357	52.0	0.16	42.0	41.7	16.3
#4	5	205	53.6	0.16	27.3	54.6	18.1

* A random distribution of heterozygous (+/m) and homozygous (+/+) wild-type seeds with approximately equal numbers of heterozygous and homozygous seeds distributed throughout the siliques is expected.

** Seeds did not develop into plants that could be screened.

results from this last group of crosses suggested that the defective gene of mutant 126E-B might be reducing the number of mutant ovules produced by the female parent. This same result was not observed in the other three groups of crosses in which more siliques were mapped.

Controlled Crosses with Mutant 111H-2B and Wild-Type Plants

Twelve attempts to place three to six pollen grains on the stigma surface of emasculated wild-type flowers were successful in only three cases. Evidently, the excessive amount of manipulation required to remove the stamens and carefully add pollen grains to the stigma surface of wild-type buds prevented seed set. The alternate method utilizing male-sterile mutant plants, however, was successful. Results from these controlled crosses indicated that those pollen grains that were added to the stigma surface of flowers that were very young (i.e. sepals completely closed or just beginning to open) more often fertilized ovules that lie in the middle or base of the silique, while pollen grains that were added to the stigma surface of flowers at a later stage of development (i.e. completely open or beginning to deteriorate) more often fertilized ovules in the middle portion to the top half of the silique (Table XXVI). These results suggested that the first pollen grains that landed on the stigma surface of the flowers did not preferentially fertilize ovules at the base of the silique. Instead, the position reached by the pollen tubes appeared to be related to the age of the flower.

TABLE XXVI

PERCENT OF SEEDS FOUND IN DIFFERENT PARTS OF 111H-2B SILIQUES AT DIFFERENT STAGES OF DEVELOPMENT WHEN POLLINATED WITH 3 TO 6 WILD-TYPE POLLEN GRAINS

Description of bud	Number of Siliques Screened**	Total Number of Seeds	% Seeds in Each Quadrant*				% of Seeds in Top 1/2	Chi-Square
			1st	2nd	3th	4th		
Young; sepals completely closed; stigma surface may be protruding through sepals	16	38	29	29	32	11	42.1	0.66
Just beginning to open; stigma hairs elongated	11	28	25	43	21	11	32.1	2.90
Flower completely open	27	68	9	26	47	18	64.7	5.30***
Older; stigma hairs beginning to deteriorate	12	38	5	32	42	21	63.2	2.14

* The 1st quadrant is located at the base of the silique and the 4th is at the top of the silique. The 2nd and 3rd quadrants lie between these two portions.

** Does not include siliques in which no seed set was observed.

*** Significantly different from 50% at P = 0.05.

Short Wild-Type Siliques

The percent of wild-type seeds found in a specific position of naturally occurring short siliques is presented in Table XXVII. Significantly more aborted seeds were found in the center portion of the siliques while approximately equal numbers of aborted seeds were found in the top and bottom halves of the siliques.

Discussion

The non-random distribution of aborted seeds observed when mutant 126E-B is the male parent clearly indicates that the non-random distribution of aborted seeds is caused by a disruption of gametogenesis on the male side of mutant 126E-B and not the female side. Whether the mutant allele is affecting pollen formation, pollen development, pollen germination or pollen-tube growth has not been determined. Many pollen grains from mutant 126E-B were measured with a stage micrometer under a dissecting microscope and graphed according to size (Patton and Meinke, unpublished results). A bimodal distribution of pollen grain sizes was not observed indicating that the mutant allele does not significantly alter the size of the mutant pollen grains. These results do not eliminate the possibility that the mutant allele is reducing the concentation of a product that is essential for pollen formation but does not significantly affect the phenotype of the mutant pollen grains. Another possibile

TABLE XXVII

DISTRIBUTION OF SEEDS IN DIFFERENT PARTS OF SHORT WILD-TYPE SILIQUES*

Number of Siliques	Number of Seeds	% of Seeds in Each Quadrant**				% of Seeds in Top Half	Chi-Square
		1st	2nd	3rd	4th		
89	334	18.8	32.3	31.4	17.4	48.8	0.14

* Only those siliques containing 1 to 6 seeds were used.

** The 1st quadrant is located at the base of the silique and the 4th is located at the top of the silique. The 2nd and 3rd quadrants lie between these two portions.

explanation is that the 126E-B mutant allele is affecting pollen germination such that those pollen grains carrying the mutant allele are less likely to germinate as readily as wild-type pollen grains. Alternately, the 126E-B mutant allele may be affecting the rate of pollen-tube growth. Those pollen grains carrying the mutant allele would be less likely to fertilize ovules at the base of the silique. However, unsuccessful attempts to induce a high frequency of *in vitro* pollen germination and pollen-tube growth of wild-type pollen grains suggest that it my be very difficult to further test these explanations with studies involving *in vitro* pollen germination and pollen-tube growth of mutant 126E-B (Dwyer and Meinke, unpublished results).

Although a random distribution of heterozygous seeds is observed in the fourth group of crosses (i.e. when mutant 126E-B is the female parent), the segregation ratio of heterozygous seeds is extremely low. These results suggest that something may be occurring on the female side to reduce the number of mutant ovules within heterozygous siliques. The majority of the data from the other three types of crosses, however, do not support this conclusion. It could be that if more siliques from the fourth cross were screened, the segregation ratio would more closely approximate 50%.

Although it appears that the same mutant allele blocking embryogenesis is disrupting pollen-tube growth in mutant 126E-B, the data obtained from these crosses cannot disprove the possibility that two separate genes are being affected. However, many genes that are expressed during both the

gametophytic and sporophytic stage of development have been identified. For example, Tanksley et al. (1981) compared tomato isozyme profiles of the male gametophyte (e.g. pollen) with those of the sporophyte and found that many of the structural genes coding for these enzymes were being expressed in both generations of the life cycle. In addition, Ottaviano et al. (1981) studied the rate of pollen-tube growth in inbred lines of corn and found that those pollen tubes with an increased rate of growth generally produced a higher quality sporophytic generation. Recently, Willing et al. (1984) compared the mRNAs of mature pollen grains and vegetative shoots of Tradescantia paludosa by hybridization of cDNA to poly(A) RNA and determined that many of the genes expressed during the sporophytic stage of development are also expressed in the gametophytic stage.

In order to more fully understand the non-random distribution of aborted seeds in heterozygous siliques, it was necessary to determine what directed the growth of wild-type pollen tubes. Naturally occurring short siliques were screened to determine where seeds would develop if only a limited number of pollen grains were present on the stigma surface. The results from 89 siliques screened indicated that approximately equal numbers of aborted seeds were found in each half of the silique. These results suggest that the first pollen grains that land on the stigma surface do not always grow to either the tip or the base of the silique. It was not clear from this study whether (1) ovules at the

base of the silique become receptive before ovules at the tip of the silique; or (2) mutant pollen tubes preferentially grew toward the basal portion of the silique in young flowers and toward the top portion in older flowers, because the stage of development when the pollen grains landed on each stigma could not be determined. It was possible, however, to determine the stage of development of the 111H-2B male-sterile flowers that were experimentally cross-pollinated with 3 to 6 wild-type pollen grains. The results from these controlled crosses suggest that the first pollen grains present on young buds tend to fertilize ovules toward the middle or base of the silique, while the first pollen grains added to older flowers tend to fertilize ovules in the center or the top half of the silique. Seeds nevertheless developed in all parts of the silique regardless of the stage of development of the flower. Although general patterns of pollen-tube growth in Arabidopsis have been identified in this study, the specific factor(s) controlling the direction of pollen-tube growth have not been clearly determined. In future experiment, it may be possible to further characterize wild-type pollen-tube growth by staining pollen grains to analyze the timing of germination and improving the conditions for the growth of pollen-tubes in vitro.

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Master of Science

Thesis: EMBRYO-LETHAL MUTANTS OF ARABIDOPSIS THALIANA: RESPONSE OF MUTANT EMBRYOS IN CULTURE AND ANALYSIS OF THE DISTRIBUTION OF ABORTED SEEDS IN HETEROZYGOUS SILIQUES

Major Field: Botany

Biographical:

Personal Data: Born in Tulsa, Oklahoma, August 19, 1961, the daughter of Dr. Donald E. and Mrs. Jalayne I. Dirksen. Married to Mark L. Baus on August 4, 1984.

Education: Graduated from Northwest High School, Wichita, Kansas in May, 1979; received Bachelor of Arts degree in Spanish and Biology from McPherson College in May 1983; completed requirements for the Master of Science degree at Oklahoma Stage University in July, 1985.

Professional Experience: Tutor, Department of Biology and Department of Foreign Languages, McPherson College, September, 1981, to December, 1982; Teaching Assistant, Department of Biology, Oklahoma State University, August, 1983 to May, 1984, and August, 1984, to May 1985; Graduate Research Assistant, Department of Botany, May, 1984 to July, 1984.