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Isolation and Characterization of 51 *embryo-specific* Mutations of Maize

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A plant embryo consists of an embryonic axis, which eventually grows into the adult body, and one or two nutritive structures, the cotyledons. In the grasses embryo morphogenesis can be divided into three periods: during the first the embryo is regionalized into an embryo proper and suspensor, during the second the embryonic axis is established, and during the third vegetative structures are elaborated. Maize, with its well-characterized embryogenesis, powerful genetics, and transposon tagging stocks, offers an attractive system for mutational analysis of these events. We have isolated 51 *embryo-specific* (*emb*) mutations from active Robertson's *Mutator* maize stocks. These are single-gene recessive lethals that represent at least 45 independent mutation events. Each of the 25 mutations was located to a chromosome arm using a B-A translocation set that uncovers approximately 40% of the genome; the same test failed to locate 20 others. The embryo phenotype of 27 mutations was characterized by examining mature mutant embryos in fresh dissection: the various *emb* mutations differ in phenotype and each is consistent in its expression. All 27 mutations result in retarded embryos that are morphologically abnormal. Nine mutants are blocked during the first period; 10 mutants are blocked during the second period; and eight mutants are blocked during the third period. Based on both the genetic and developmental data, it is likely that there are many loci that can mutate to give the *emb* phenotype and that these genes are crucial to the morphogenesis of the embryo.

INTRODUCTION

A plant embryo consists of an embryonic axis, which eventually grows into the adult plant body, and one or two massive nutritive structures, the cotyledons. In many plants, including most dicots, the mature embryo is structurally simple, with the embryonic axis being defined by the shoot and root meristems at either end, and little or no organ differentiation within the embryonic axis. In the grasses, including maize, embryogenesis is elaborate and results in the formation of a large embryo consisting of a massive cotyledonary structure, the scutellum, and a well-differentiated miniature plant, the embryonic axis. All of the novel morphogenetic events in the embryo occur during the first quarter of embryogenesis; during this period the structures unique to the embryo, namely the suspensor, scutellum, and coleoptile, are formed. The embryonic axis is also established at this time, and morphogenetic programs of the vegetative development of the plant are initiated. The latter part of embryogenesis is devoted to the reiterative initiation and elaboration of structures that first appear during the early period and deposition of storage products.

Maize, with its well-characterized embryogenesis and powerful genetics, offers an attractive system for mutational analysis of embryo development (Coe et al., 1988; Sheridan, 1988). Chemical mutagenesis has already yielded thousands of mutations, approaching saturation in the case of kernel mutants (Neuffer, 1978; Neuffer et al., 1986; Neuffer and Chang, 1989), and there are several transposable element systems that have been used for transposon tagging of genes and facilitating their molecular cloning (Walbot and Messing, 1989).

The maize *defective kernel* (*dek*) mutants, in which both embryo and endosperm are defective, have already yielded important insights into the genetic programs governing embryogenesis. Studies of ethylmethane sulfonate-induced *dek* mutants (summarized in Sheridan and Clark, 1987) have revealed that the program governing shoot morphogenesis is genetically separable from that governing root morphogenesis (*dek1*, Sheridan and Neuffer, 1981; *dek23*, Clark and Sheridan, 1986; *cp*-1418*, Sheridan and Thorstenson, 1986), that programs governing morphogenesis (*dek31*, Sheridan and Thorstenson, 1986) can be uncoupled from the genetic regulation of cellular division and growth, and that there are genes that contribute to the maintenance of organizational integrity of the embryo (*rgh*-1210* and *f1*-1253B*, Clark and Sheridan, 1988).

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Several *dek* genes (e.g., *rgh*-1210*, *fl*-1253B*, *dek31*, and *bno*-747B*) appear to be expressed and coordinately regulated in the embryo and aleurone, and several are expressed in the gametophyte as well as the sporophyte generation (Clark and Sheridan, 1988; W.F. Sheridan, unpublished data). The *dek* mutations are pleiotropic in that both embryo and endosperm are altered in morphology. Here we report on a little-studied class of mutations whose morphogenic effects are specific to the embryo. We have designated them *embryo-specific* (*emb*) mutations.

In the *emb* mutants of maize, morphogenesis of the embryo is profoundly disturbed, whereas that of the endosperm is apparently normal. The *emb* kernel phenotype consists of a kernel that appears otherwise normal but has a depression on the adaxial face that marks the site of the defective embryo. Because of their embryo specificity and because of their effects on morphogenesis, mutations of this type are likely to identify many genes that play an essential role in embryo morphogenesis. Furthermore, some of these mutations are likely to identify regulatory genes that make crucial decisions in directing the course of morphogenesis. Despite their potential significance, maize *emb* genes have not been investigated, save for two reports published more than 50 years ago defining the phenotype (designated as *germless* [*gm*] by Demerec, 1923) and describing transmission patterns of a few mutations of this type (Wentz, 1930). This lack of study is understandable because these mutations are difficult to screen and handle because of their embryo lethality and their normal endosperm phenotype. Unfortunately, all of the *gm* mutations studied by Demerec and Wentz have been lost. We have screened on a large scale for embryo-specific mutations that are likely to be transposon tagged with one of the *Mutator* transposable elements. Inasmuch as the term *germless* is confusing because it implies the lack of a germ (embryo), while in fact this is rarely the case, and because this term has been used to designate certain *dek* mutants with defective endosperms and tiny embryos (Sass and Sprague, 1950), we prefer the term *embryo-specific* (*emb*) for these mutations.

Robertson's *Mutator* transposable element system (Robertson, 1978) is an attractive tagging system for embryo-lethal mutations because of its high mutation rate. Mutator stocks whose mutagenic activity has been confirmed by Robertson's standard sandbench test for seedling mutations can be expected to produce new mutations at a particular locus at a rate of about 1 in 10^4 to 10^5 gametes per generation (Robertson, 1978). Although the *Mutator* transposable element family is molecularly complex (Talbert et al., 1989), most *Mutator*-induced mutations isolated to date have been caused by elements that cross-hybridize with internal sequences of the 1.4-kb *Mu1* element (Walbot, 1991; V. Chandler and K. Hardeman, manuscript in preparation).

We report herein on our isolation of 51 *emb* mutations from active *Mutator* stocks. We present evidence that these loci represent many genes that are crucial to the morphogenesis of the embryo.

RESULTS

Recovery of *emb* Mutations

A total of 1000 self-pollinated ears of D.S. Robertson's active *Mutator* plants were screened for ears segregating for kernels with a fully developed endosperm and a defective embryo. From these ears 51 mutations were recovered, representing at least 45 independent mutation events. These mutations are listed in Table 1. Pedigree analysis has identified three cases in which mutations from a previous generation were carried in several plants in a family, and may have been recovered twice in two cases and five times in a third case. All 51 mutations were crossed onto standard stocks and subsequently self-pollinated. All proved to be heritable single-gene recessive mutations. Thus, the mutation frequency to the phenotype conferred by *emb* in active Robertson's *Mutator* stocks was approximately 5×10^{-2} (51 per 1000 gametes tested per generation).

Segregation Ratios

Segregation ratios on self-pollinated ears were determined for 49 of the *emb* mutations (Table 1). The majority, 36 mutations, segregated at a near 3:1 ratio (19% to 29% mutant kernels). Twelve mutations were significantly reduced in transmission. We have propagated the *emb* mutations by outcrossing and subsequent selfing for two to six generations, and their segregation ratios appear to be stable.

Chromosome Arm Location

A total of 45 *emb* mutations were crossed by the standard B-A tester stocks for chromosome arms *1S*, *1L*, *2L*, *3L*, *4L*, *5S*, *9S*, and *10L*. These B-A stocks uncover about 40% of the genome (see Methods). The 25 *emb* mutations that were located were distributed on six of the eight arms tested and are shown in Table 2; the unlocated mutations presumably are located on other chromosome arms in proximal chromosome regions not uncovered by the B-A stocks. Taken together, these data suggest that the *emb* loci are distributed throughout the genome. However, significant clusters of mutations were found on *1S*, *3L*, and *4L*. These may represent either clusters of functionally related genes or one or a few loci per arm that serve as hot spots for *Mu* element insertion. Allelism tests are underway to distinguish between these alternatives.

Table 1. Embryo-Specific Maize Mutations Used in This Study

Mutation	Segregation			Germination	
	Number of Kernels Examined	% Mutant	Chromosome Arm Location	Number of Kernels Planted	Number of Seedlings
emb-8501*	100	24	*4L*	109	5
emb-8502*[a]	130	24	*1S*	100	1
emb-8503*[a]	120	20	*1S*	100	3
emb-8504*	214	22	*5S*	16	0
emb-8505*	214	22	NT[b]	NT	
emb-8506*	NT		NL[c]	NT	
emb-8507*	94	19	NL	100	7
emb-8508*	224	23	NL	70	5
emb-8509*	100	29	*4L*	80	5
emb-8510*	117	23	NL	75	2
emb-8511*	124	23	NT	NT	
emb-8512*	75	21	*3L*	100	3
emb-8513*	103	12[d]	*4L*	35	2
emb-8514*	150	23	*3L*	100	6
emb-8515*	104	20	*3L*	35	0
emb-8516*	212	28	NT	NT	
emb-8517*	51	23	*1S*	45	2
emb-8518*	67	18	*2L*	45	2
emb-8519*[e]	93	19	*1S*	50	2
emb-8520*[e]	183	17[f]	*1S*	103	8
emb-8521*	100	13[f]	*3L*	100	2
emb-8522*	140	21	*9S*	105	5
emb-8523*	180	14[d]	NL	40	0
emb-8524*	187	18[g]	NL	NT	
emb-8525*	150	11[d]	NL	44	1
emb-8526*	160	13[d]	NT	NT	
emb-8527*	133	20	NL	75	1
emb-8528*	99	19	NL	15	0
emb-8529*	78	14[g]	NL	95	3
emb-8530*	157	10[d]	NT	NT	
emb-8531*	118	28	*1S*	100	1
emb-8532*	206	23	*3L*	25	0
emb-8533*	105	14[f]	NL	20	0
emb-8534*	396	22	*4L*	100	2
emb-8535*	141	21	NL	50	0
emb-8536*[h]	166	22	NL	NT	
emb-8537*[h]	167	19	*4L*	20	1
emb-8538*[h]	NT		*4L*	30	2
emb-8539*[h]	139	28	NL	50	2
emb-8540*[h]	197	18[g]	*4L*	50	0
emb-8541*	48	27	NT	NT	
emb-8542*	184	19	NL	60	15
emb-8543*	123	20	NL	65	4
emb-8544*	186	21	NL	25	5
emb-8545*	64	23	*1S*	75	8
emb-8546*	163	26	NL	100	4
emb-8547*	111	23	*4L*	100	0
emb-8548*	190	26	NT	100	0
emb-8549*	86	23	*1S*	100	1
emb-8550*	194	15[f]	NL	100	3
emb-8551*	120	22	*3L*	10	5

[a] Each may be a single mutation event, based on pedigree analysis.
[b] NT, not tested.
[c] NL, tested but not located.
[d] Significantly different from 25% at P = 0.001.
[e] Each may be a single mutation event, based on pedigree analysis.
[f] Significantly different from 25% at P = 0.01.
[g] Significantly different from 25% at P = 0.05.
[h] Each may be a single mutation event, based on pedigree analysis.

Table 2. Location of 45 *emb* Mutations among the Eight Chromosome Arms Tested

1S	*1L*	*2L*	*3L*	*4L*	*5S*	*9S*	*10L*	Not Located
8502[a]		8518	8512	8501	8504	8522		
8503[a]			8514	8509				
8517			8515	8513				
8519[b]			8521	8534				20 mutations
8520[b]			8532	8537[c]				
8531			8551	8538[c]				
8545				8540[c]				
8549				8547				
8	0	1	6	8	1	1	0	
Total: 25 mutations								

[a,b,c] May be same mutation event, based on pedigree analysis.

Lethality

Mature mutant kernels of 42 of the *emb* mutants were tested for germination in the sandbench (Table 1). The level of germination was low (about 3%) for all but seven mutants, *emb*-8507*, *emb*-8514*, *emb*-8520*, *emb*-8542*, *emb*-8544*, *emb*-8545*, and *emb*-8551*. The germination events seen in most of the tests probably represent misscored normals. However, the seven mutants with higher frequencies appeared to be able to germinate at a relatively low frequency. Developmental analysis of three of these mutants (see below) indicated that they proceeded far enough in development to possess an embryonic axis that could be capable of germinating.

Development of the *emb* Mutants

Embryos of 27 of the *emb* mutants were examined in fresh dissection at kernel maturity to assess their maximal developmental capacity. For each mutant, 10 embryos from the same ear were examined to determine the stage of developmental block, the degree of morphological abnormality, and the variability in mutant expression.

All 27 of the mutants were retarded in development and were disturbed in morphogenesis. Many underwent disorganized growth and tissue proliferation. Individual mutants were blocked stage specifically or over a narrow range of embryo stages and were consistent in phenotype. Figure 1 shows that the mutants ranged over a wide range of stages, from very early in embryogenesis to near physiological maturity. However, a large majority were blocked early in embryogenesis. The stages of embryo development are illustrated in Figure 1 and are based on the classification scheme of Abbe and Stein (1954). (For a detailed description of maize embryo development, see Randolph [1936] and Kiesselbach [1949].) The *emb* mutants may be divided into three groups, reflecting three major periods of embryo morphogenesis.

Mutants Blocked during the Period of Setting Apart of the Embryo Proper and Suspensor—Proembryo and Transition Stages

During the first part of embryogenesis the zygote divides to form first an ovoid and then a club-shaped proembryo. These early divisions do not occur in a precisely ordered pattern; nevertheless, the proembryo is progressively regionalized into an upper, small-celled embryo proper and a lower, large-celled suspensor region. The embryo proper will eventually form the mature embryo. The suspensor, which may have an anchoring and transfer function, will persist until structural elaboration and storage product deposition are under way (see below). The suspensor of the proembryo continues to elongate, and the embryo proper continues cell division, yielding the "ice cream cone"-shaped transition stage embryo. Nine of the 27 *emb* mutants studied were blocked during this period of embryo development (Figure 1). Most suffered failure in development of the embryo proper and concomitant enlargement of the suspensor.

Mutants Blocked during the Period of Establishment of the Embryonic Axis—Late Transition Stage through Stage 1

During this part of early embryo development the scutellum expands, the coleoptile differentiates, and the organs of the adult plant make their first appearance in the embryonic axis. The embryonic axis is established during the late transition stage when the shoot apical meristem appears as a dome of meristematic cells visible on the face of the embryo proper (Figure 1) and the root apical meristem forms within the embryo. During the ensuing coleoptilar stage the remainder of the embryo proper broadens and expands to form the scutellum, and the coleoptile appears as a doughnut-shaped bulge surrounding the shoot apical meristem on the face of the scutellum. At this time, the

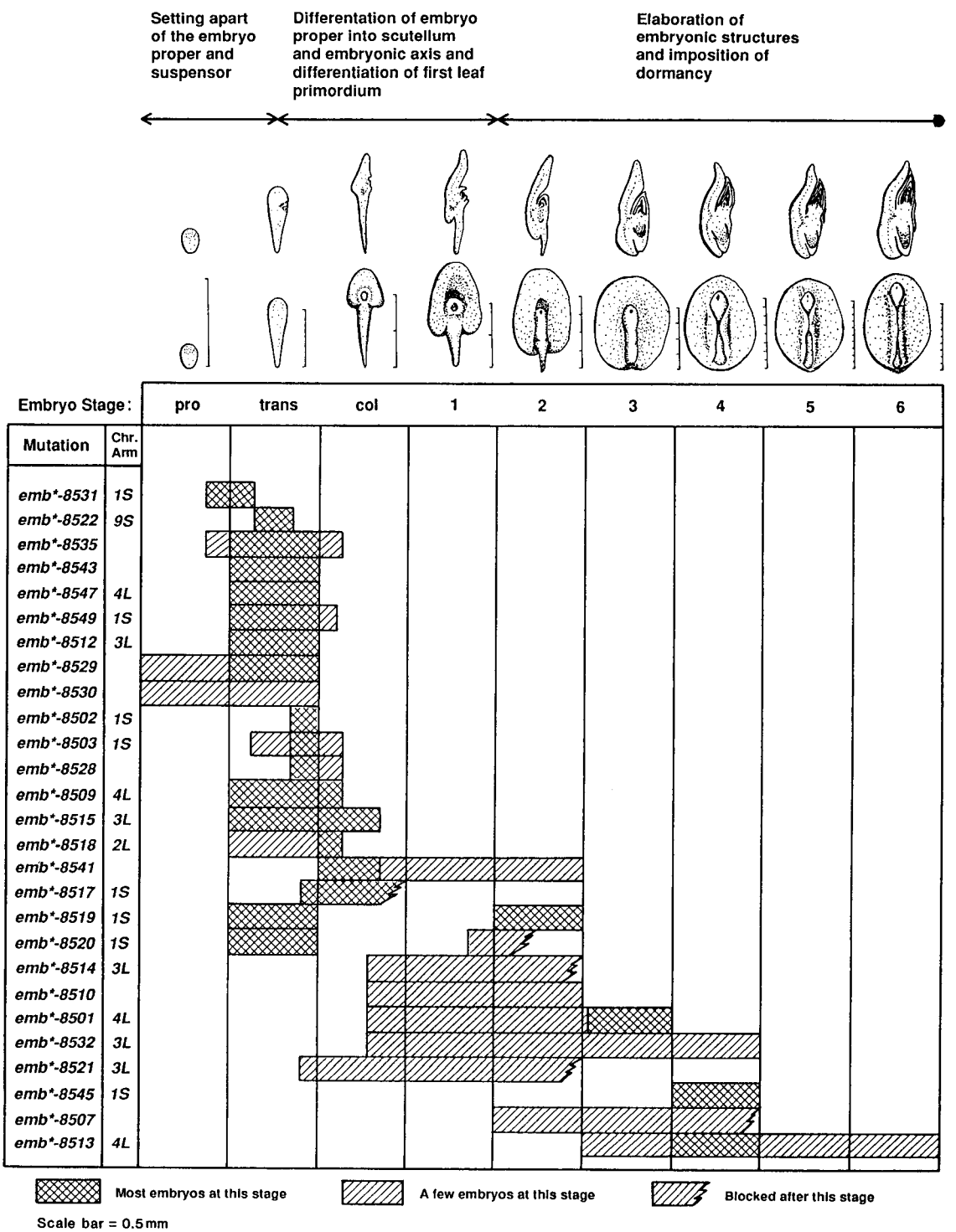

Figure 1. Summary of the Development of the *emb* Mutations.

Normal embryo development is shown at the top of the figure. Embryo stages are based on Abbe and Stein (1954) and Clark and Sheridan (1986). Each division of the scale bar equals 0.5 mm. The stage of developmental block for 27 *emb* mutations is shown at the bottom.

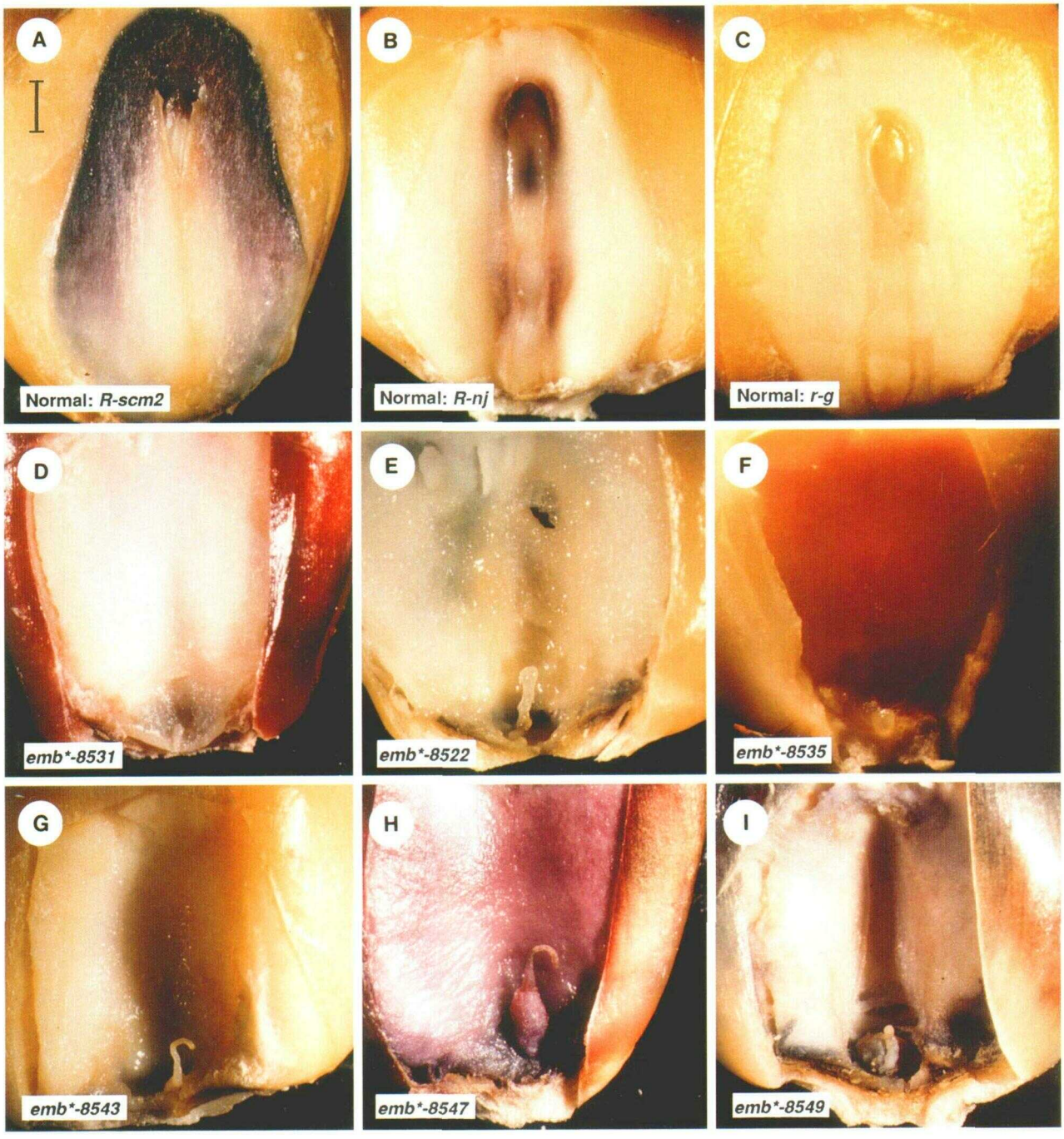

Figure 2. Normal and *emb* Mutant Embryos in Fresh Dissection at Kernel Maturity.

(A) Normal embryo carrying the *R-scm2* allele, which colors the scutellum.
(B) Normal embryo carrying the *R-nj* allele, which colors portions of the embryonic axis.
(C) Normal embryo carrying the *r-g* allele, which produces a colorless embryo.
(D) *emb***-8531*; embryos of this mutation are blocked at a late proembryo to early transition stage.
(E) *emb***-8522*, blocked at an abnormal transition stage.
(F) *emb***-8535*, blocked at a late proembryo to early coleoptilar stage.
(G) *emb***-8543*, blocked at an abnormal transition stage, with stunted embryo proper.

vascular system begins to differentiate within the embryo. At the close of this period (stage 1; Figure 1), the first leaf primordium differentiates at the base of the shoot apical meristem. Thus, by the end of this phase of embryogenesis all of the tissue types and structures of the vegetative adult plant have made their first appearance. Continued development, of both the embryonic axis and the adult plant, will consist of repetitions of the genetic programs first switched on during this crucial developmental period. Ten of the 27 *emb* mutants studied were blocked in this period (Figure 1). Although embryos of all 10 mutants developed some sort of scutellar structure, this structure was misshapen. In some mutants no embryonic axis was evident, although the overall shape of the embryo and the degree of differentiation of the scutellum and suspensor placed the embryo in this developmental class.

Mutants Blocked during the Period of Elaboration of Embryonic Structures—Stage 2 through Stage 6

This period (stages 2 to 6; Figure 1) marks the completion of embryogenesis and the imposition of dormancy. During this period the scutellum continues rapid growth and storage product deposition, forming the massive shield-shaped cotyledonary structure that comprises the bulk of the mature embryo. The embryonic axis differentiates up to six leaf primordia, continues elaboration of the vascular system, and gives rise to the first true root. The coleoptile grows to ensheath the shoot apex and leaf primordia. At the close of this developmental phase, the rate of growth and leaf production by the embryonic axis slows and finally halts as dormancy is imposed upon the embryo (Abbe and Stein, 1954). Eight of the 27 *emb* mutants studied were blocked during this period (Figure 1). These eight mutants varied greatly in phenotype. One mutant was scutellum specific, one underwent premature greening of the coleoptile, and a third suffered permanent dormancy. Many of the mutants in this class developed irregular protrusions; two of these produced sectors of colored tissue in the scutellum. Both of these features suggest that the embryos may be chimeras of mutant and normal tissue.

Profiles of 27 *emb* Mutants

Mature normal embryos marked by the color alleles *R-scm2* (conditions color in the scutellum), *R-nj* (conditions color in the embryonic axis), and *r* (no color in the embryo) are shown in Figures 2A, 2B, and 2C. The *R* markers are useful for determining the identity of abnormal organs and also for assessing whether physiological maturity of a tissue has been reached despite being blocked morphologically at an early stage. Sectors of colored tissue in a colorless background may be evidence that an embryo consists of a chimera of mutant and normal tissue.

A representative embryo of each of the 27 mutants is shown in Figures 2, 3, and 4. All embryos in these figures are shown at the same magnification.

Mutants Blocked during the Period of Setting Apart of the Embryo Proper and Suspensor—Proembryo and Transition Stages

emb**-8531 (1S). The mutant is uniformly blocked at a late proembryo to early transition stage. Four of 10 embryos had a swollen suspensor; the embryo proper of three of these was necrotic. Most of the embryos were of a small size typical of the early transition stage. One embryo had elongated greatly but remained blocked in differentiation. Apparently, a normal *emb*-8531* allele is not required for the establishment of the embryo proper but is required very early in embryogenesis for its differentiation.

emb**-8522 (9S). The mutant is uniformly blocked at the midtransition stage but is variable in size. In four of 14 embryos the embryo proper had undergone extensive development into a large irregular mass and was necrotic to varying degrees. In all embryos the suspensor retained normal morphology, and in kernels carrying the *R-scm2* allele it developed the deep blue color characteristic of color-expressing tissues at kernel maturity. As with the previous mutant, the normal *emb*-8522* allele is apparently required for differentiation of the embryo proper, but not for its general growth. In the *emb*-8522* mutant, however, the suspensor remained normal, but continued growth of the embryo proper was accompanied by necrosis and breakdown of tissue organization.

emb**-8535. The mutant is blocked variably from a late proembryo to early coleoptilar stage, with four of eight embryos at the transition stage. The embryos were irregular in shape; however, none were necrotic. Apparently, the *emb*-8535* gene product is not required for the com-

Figure 2. (continued).

(H) *emb*-8547*, blocked at an abnormal transition stage with swollen suspensor.
(I) *emb*-8549*, blocked at an abnormal late transition to early coleoptilar stage.
All embryos are shown at the same magnification. Bar = 1 mm.

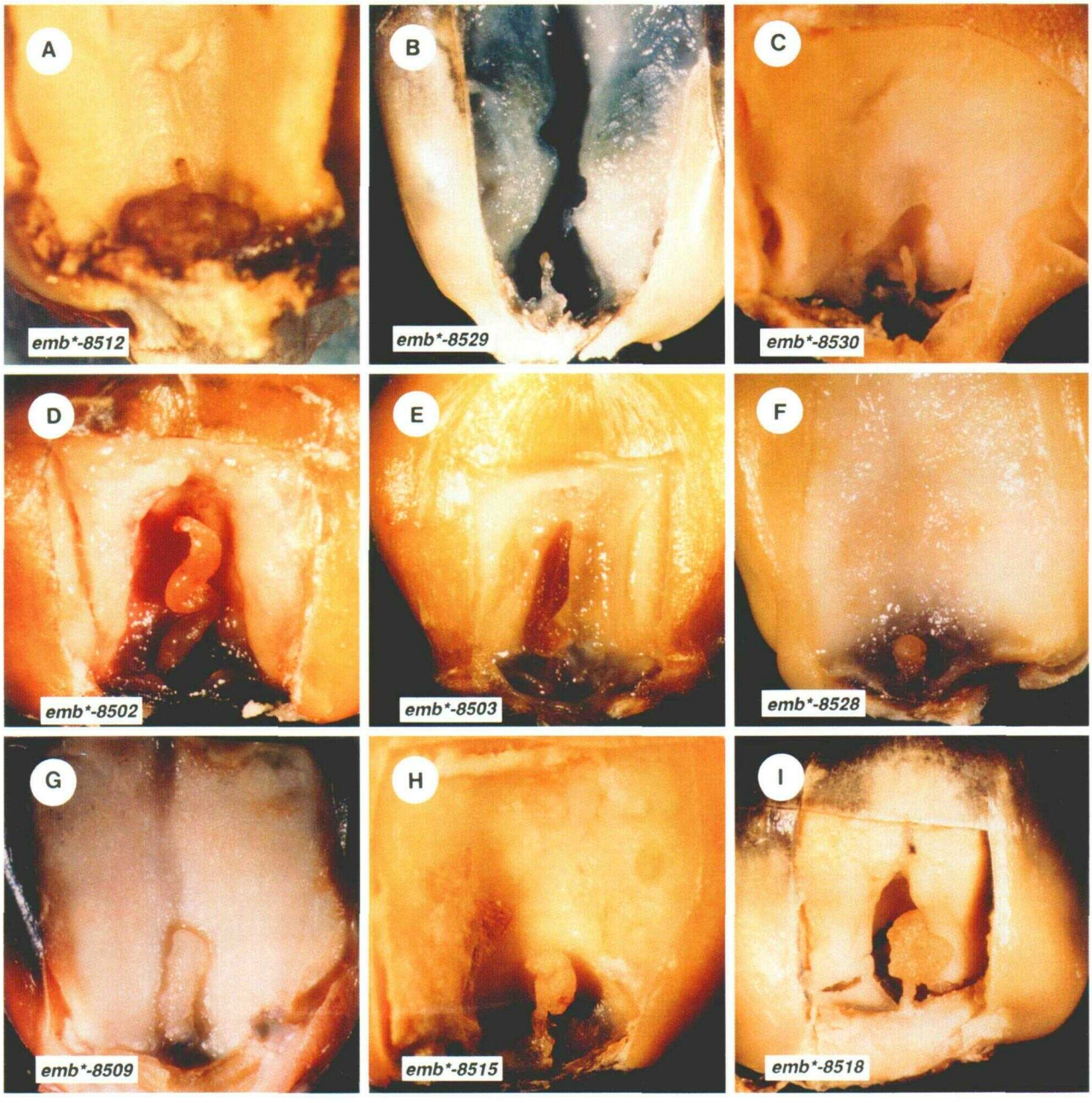

Figure 3. Mutant *emb* Embryos in Fresh Dissection at Kernel Maturity.

(A) *emb*-8512*; embryos of this mutation are blocked at the transition stage.
(B) *emb*-8529*, blocked from the proembryo to the transition stage.
(C) *emb*-8530*, blocked at a proembryo to transition stage.
(D) *emb*-8502*, blocked at an abnormal late transition stage with twisted scutellum.
(E) *emb*-8503*, blocked at an abnormal midtransition to early coleoptilar stage.
(F) *emb*-8528*, blocked at an abnormal transition to early coleoptilar stage.
(G) *emb*-8509*, blocked at an abnormal transition to early coleoptilar stage, with tissue proliferation throughout the embryo.
(H) *emb*-8515*, blocked at an abnormal transition to coleoptilar stage.
(I) *emb*-8518*, blocked at an abnormal transition to coleoptilar stage, with tissue proliferation localized in the embryo proper.
All embryos are shown at the same magnification as Figure 2.

pletion of a specific step or the morphogenesis of a particular structure, but lack of its proper function early in development resulted in developmental retardation and the loss of organization throughout the embryo.

emb*-8543. The mutant is uniformly blocked at the transition stage. In three of nine embryos the suspensor continued to elongate after the arrest of the embryo proper. These suspensors did not reach physiological maturity, as evidenced by their failure to develop blue color in the presence of the *R-scm2* allele. There was no evidence of necrosis or tissue proliferation in the embryo proper. Therefore, this locus may be involved specifically in suspensor development; the retarded and abnormal suspensor may then be unable to support continued growth of the embryo proper.

emb*-8547 (4L). The mutant is uniformly blocked at the transition stage. Embryos were abnormal in having a bulbous suspensor and a stunted embryo proper that had elongated but not expanded. This structure was necrotic in seven of 10 embryos. In *R-scm2* kernels the suspensors were pink or blue, indicating that these tissues had reached some sort of functional maturity. The mutant defect in *emb*-8547*, therefore, results in a complete failure of the embryo proper to expand and differentiate but does not interfere with growth, expansion, or maturation of tissues within the suspensor. It is not clear whether the abnormal morphology of the suspensor is the cause or the effect of the abnormal growth and developmental arrest of the embryo proper.

emb*-8549 (1S). The mutant is blocked variably from the transition to early coleoptilar stage. Seven of 10 embryos were at the transition stage, with a small necrotic embryo proper. The remaining embryos had advanced to an abnormal coleoptilar stage, with a swollen suspensor and a knobby, necrotic scutellum. Apparently this gene is required for normal completion of the transition stage, and its failure in the *emb*-8549* mutant resulted in the loss of organization and eventual death of the embryo proper, which precluded the formation of any of its derived organs.

emb*-8512 (3L). The mutant is uniformly blocked at the transition stage. Embryos had a stunted, necrotic embryo proper. The suspensor was buried in a mass of proliferated tissue. The pad of proliferated tissue at the base of the embryo is a novel structure and may represent localized disorganized growth either of the suspensor or of the chalazal cells of the endosperm. In this mutant the embryo proper died early, but suspensor development remained normal.

emb*-8529. The mutant is blocked variably from the proembryo to the transition stage. Six of eight embryos were at a morphologically normal transition stage but with a degenerating embryo proper. The two remaining embryos had differentiated abnormally: one as a greatly elongated but well-formed suspensor-like structure devoid of an embryo proper, and the other as a large amorphous mass. Apparently this mutation acts early in development but is somewhat leaky. The embryos that escaped the block at the transition stage were unable to assert normal controls over growth and differentiation.

emb*-8530. The mutant is blocked variably from the proembryo to the transition stage. Eight of 10 embryos examined were at the early proembryo stage and appeared to be blocked soon after fertilization. The remaining embryos were at the transition stage, and one of these had an irregular-shaped embryo proper. The *emb*-8530* gene appears to be required for continued development of the early proembryo, and those embryos that escaped blockage at this stage could not proceed past the transition stage.

Mutants Blocked during the Period of Differentiation of the Embryo Proper into an Embryonic Axis and Scutellum, and Initiation of the First Leaf Primordium—Late Transition Stage through Stage 1

emb*-8502 (1S). The mutant is uniformly blocked at a late transition stage. Embryos were elongated and spirally twisted, with a narrow, pointed scutellum. Seven of 10 embryos had a depression or fold at the expected site of the coleoptilar ring. All embryos examined were necrotic. The suspensor was normal in morphology and was blue in kernels that contained the *R-scm2* allele. This combination of phenotypic characters is strikingly like that of *dek23* mutant embryos, suggesting that these two loci, located on different chromosome arms, may be functionally related.

emb*-8503 (1S). The mutant is variably blocked from the midtransition to the early coleoptilar stage. *emb*-8503* was allelic to the preceding mutant but was more variable in expression. Two of the seven embryos examined were at a midtransition stage, three were at a late transition stage and resembled the allelic mutant *emb*-8502*, and two were at an abnormal early coleoptilar stage with a rounded scutellum and a depression at the site of the shoot apex. All of the embryos were necrotic. This allele appears to be phenotypically similar to *emb*-8502*, but was leaky in expression.

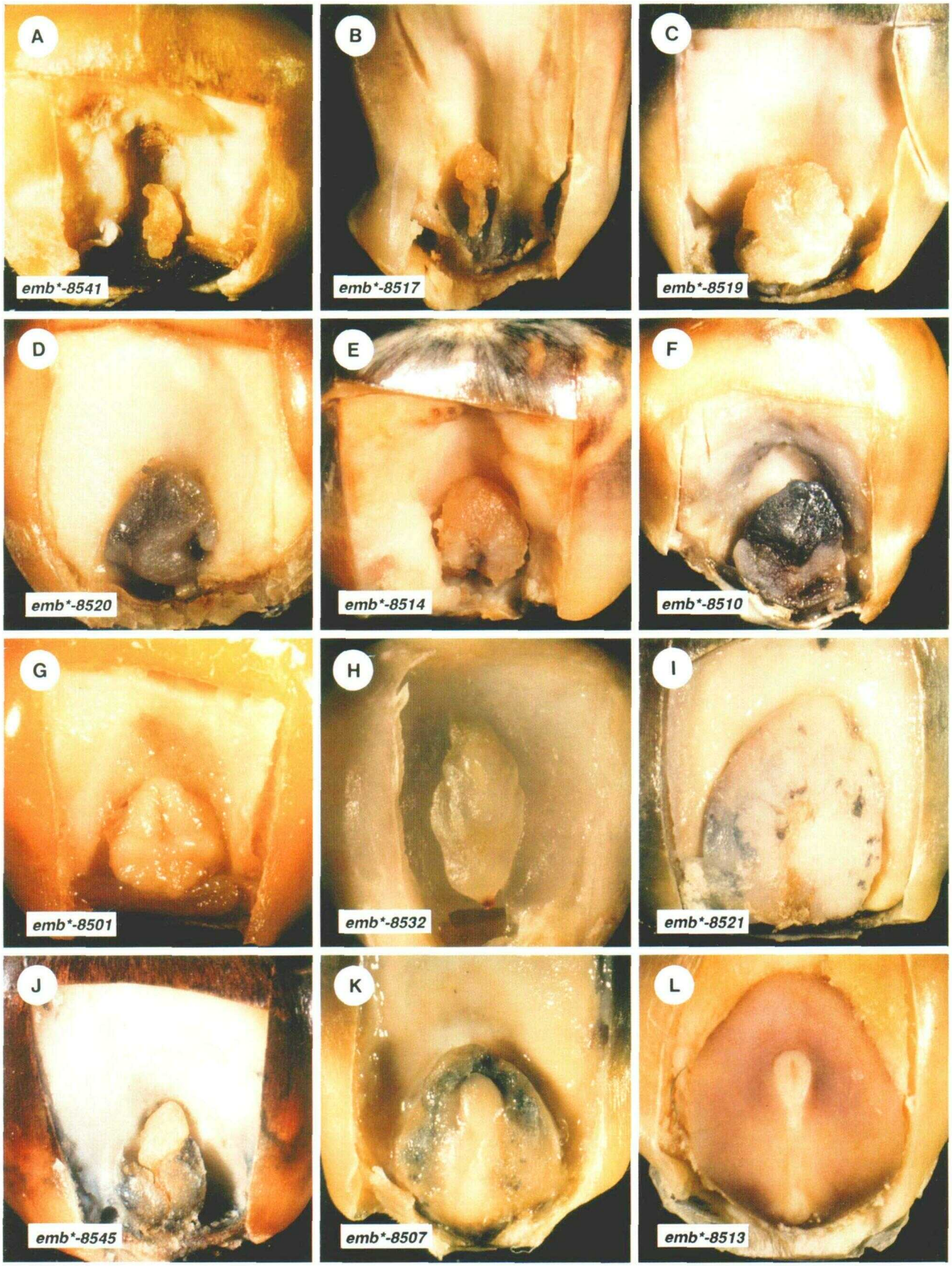

Figure 4. Mutant *emb* Embryos in Fresh Dissection at Kernel Maturity.

emb**-8528. The mutant is blocked variably from the late transition to early coleoptilar stage. Seven of 10 embryos were blocked at the transition stage. The embryo proper of these was enlarged and necrotic. The remaining three embryos had advanced beyond the transition stage by enlargement of the embryo proper to form an irregular-shaped scutellum. There was no evidence of a coleoptilar ring. The area normally occupied by the shoot apical meristem was depressed or folded. The phenotype of *emb*-8528* embryos was similar to that of *emb*-8502* and *emb*-8503*; however, embryos of *emb*-8528* did not undergo the extreme elongation characteristic of these two. Although the mutation in *emb*-8528* apparently affects the differentiation of the shoot apical meristem, it did not interfere with the ability of the embryo proper to undergo expansion in all directions to form a scutellum.

emb**-8509 (4L). The mutant is blocked variably from the transition to early coleoptilar stage. Five of 10 embryos were at the transition stage and were normal in morphology. The remaining five embryos had advanced to the coleoptilar stage, as evidenced by broadening of the embryo proper to form an irregular scutellum. Four of these had a coleoptile and shoot apical meristem that was partially overgrown by proliferating scutellar tissue. The *emb*-8509* gene does not appear to be involved with differentiation of any of the fundamental structures of the embryo; rather, the mutant condition resulted in either early death or later breakdown in the organization of developmental patterns.

emb**-8515 (3L). The mutant is blocked variably from a morphologically normal but necrotic transition stage to an abnormal midcoleoptilar stage, with the latter characterized by an extreme degree of disorganized growth and tissue proliferation in the scutellum, coleoptile, and shoot apex area. The 20 embryos examined formed an evenly graded series with regard to the stage of their developmental arrest. Therefore, it is likely that the product of the *emb*-8515* gene is involved with processes that maintain organized patterns of growth rather than the differentiation of specific structures.

emb**-8518 (2L). The mutant is blocked variably from the transition to an early coleoptilar stage. Eight of 10 embryos had reached an abnormal early coleoptilar stage, with an irregular scutellum lacking a coleoptilar ring and shoot apex. Embryos were uniform in size and shape for each stage. This mutant underwent tissue proliferation in the scutellum but not the suspensor, suggesting that the *emb*-8518* gene may act specifically to determine the patterns of growth in the embryo proper.

emb**-8541. The mutant is blocked variably from the coleoptilar stage to stage 2, with seven of nine embryos at an early to midcoleoptilar stage. The scutellum of all embryos had expanded at the base, but the upper portion was attenuated or missing altogether. In two of the most advanced embryos the embryonic axis protruded out from the back of the reduced scutellum. Proliferative growth was evident in the scutellum. Apparently the *emb*-8541* gene is not needed for morphogenesis and growth of the shoot apex and embryonic axis, but is involved with maintaining the organized growth and development of the scutellum.

emb**-8517 (1S). The mutant is variably blocked from a late transition stage to a late coleoptilar stage or greater. The 18 embryos examined formed a graded series without any sharp stage specificity. All embryos showed thickening and irregular protrusions in all regions of the embryo. It was not possible to discern whether a coleoptile or embryonic axis had differentiated because these regions were obscured by proliferation of scutellar tissue. Normal function of the *emb*-8517* gene is clearly required for organized growth of embryonic structures; its role in morphogenesis is not clear.

Figure 4. (continued).

(A) *emb*-8541*; embryos of this mutation are blocked from the coleoptilar stage to stage 2, with an abnormal scutellum.
(B) *emb*-8517*, blocked from an abnormal transition stage to the coleoptilar stage.
(C) *emb*-8519*, blocked at the transition stage or at an abnormal stage 1 or beyond.
(D) *emb*-8520*, blocked at the transition stage with necrosis, or blocked at an abnormal stage 1 or beyond.
(E) *emb*-8514*, variably blocked from the coleoptilar stage to stage 2, with reduced development of the embryonic axis but excessive growth of the scutellum.
(F) *emb*-8510*, blocked at an abnormal coleoptilar stage through stage 2.
(G) *emb*-8501*, blocked from an abnormal coleoptilar stage to stage 3, with multiple embryonic axes.
(H) *emb*-8532*, blocked from an abnormal coleoptilar stage to stage 4, with premature greening.
(I) *emb*-8521*, blocked from an abnormal transition stage to stage 2 or greater.
(J) *emb*-8545*, blocked at an abnormal stage 4, with reduced scutellum.
(K) *emb*-8507*, blocked from an abnormal stage 2 to stage 4 or greater.
(L) *emb*-8513*, blocked from stage 3 to stage 6, with a miniature embryo.
All embryos are shown at the same magnification as Figure 2.

emb*-8519 (1S). Four of eight embryos were blocked uniformly at the transition stage. The other four embryos had developed a scutellar structure characteristic of a stage 2 embryo but irregular in shape. These embryos lacked a well-defined shoot apex, and there was a deep cleft in the scutellum at the site normally occupied by the embryonic axis. The *emb*-8519* gene may be required at two stages of embryogenesis: at first during early growth of the embryo proper, and again during differentiation of the embryonic axis. The embryos that escaped the early block suffered a breakdown in organization of the growing scutellum and were unable to differentiate a shoot apical meristem.

emb*-8520 (1S). Seven of 10 embryos examined were blocked uniformly at the transition stage and were degenerating. The remainder had extensive development of the embryonic axis, including a shoot apical meristem. However, the scutellum failed to develop beyond a rudimentary structure, and in several embryos it had developed irregular lobes of proliferated tissue. The three most advanced embryos appeared to consist entirely of enlarged, highly vacuolated necrotic cells. The *emb*-8520* gene is apparently required at two stages of embryogenesis. The embryos that were caught by the early block simply died; those that escaped it underwent extreme hypertrophy of cells throughout the embryo, suggesting that this gene is involved with some aspect of general cellular metabolism as well as the maintenance of growth and organizational integrity throughout the scutellum.

Mutants Blocked during the Period of Elaboration of Embryonic Structures and the Imposition of Dormancy—Stage 2 through Stage 6

emb*-8514 (3L). The mutant is variably blocked from the late coleoptilar stage to stage 2 or greater, with a broad scutellum having an abnormal, pebbly surface. The embryonic axis in all eight embryos examined was either missing or rudimentary and had been partially overgrown by the scutellum. This mutation is apparently associated with a partial or complete failure of the newly differentiated embryonic axis to develop further, while the scutellum continues to grow to an abnormal extent.

emb*-8510. The mutant is variably blocked from the late coleoptilar stage through stage 2. In all embryos the scutellum had enlarged into an irregular-shaped mass. Five of 10 embryos had an embryonic axis typical of their stage. In the remaining embryos the central region of the embryo was necrotic and deeply depressed, suggesting either that the embryonic axis had degenerated or had never developed. In kernels carrying the *R-scm2* allele, the entire embryo proper and the suspensor were strongly colored. The *emb*-8510* gene is required for the growth and development of the embryonic axis. It is also expressed in the scutellum; in this tissue, defective gene action led to irregular growth and tissue proliferation.

emb*-8501 (4L). The mutant is blocked variably from a late coleoptilar stage to an abnormal stage 3. Seven of 10 embryos had the overall morphology characteristic of stage 3 but had undergone extensive growth of the scutellum in the region surrounding the embryonic axis. Two of these embryos had multiple horizontal protrusions that may be either ectopic embryonic axes or secondary embryos. A third embryo, blocked earlier at the late coleoptilar stage, consisted of twin embryos joined at the base of the suspensor. At least one embryo had developed in reverse orientation within the kernel. *emb*-8501*, therefore, seems to be involved in a fundamental way with the setting of the developmental "clock" or the interpretation of positional information.

emb*-8532 (3L). The mutant is blocked variably from an abnormal coleoptilar stage to an abnormal stage 4, but consistent in having a reduced scutellum and an upward-projecting green embryonic axis. Neither anthocyanin nor carotenoid synthesis was affected by the mutation. Mutant embryos that reach an advanced stage laid down abundant amounts of scutellar storage products (the scutellums were thick, heavy, and opaque) and developed normal *R-scm2* coloration in a miniature, slow-growing scutellum; the embryonic axis enlarged enormously, developed chlorophyll, and began to germinate within the kernel. Embryos blocked earlier continued to grow into giants with the morphology of an early stage; they, too, developed chlorophyll within the kernel. Thus, in the *emb*-8532* mutant the programs of growth, differentiation, and germination appear to have been uncoupled.

emb*-8521 (3L). The mutant is blocked variably from an abnormal transition stage to an abnormal stage 2 or greater, with necrosis in the region of the embryonic axis. All embryos had undergone abnormal tissue proliferation, as evidenced by thickened suspensors in the transition stage embryos and large, irregular-shaped scutellums in the more advanced embryos. Two of the advanced embryos had colored sectors in the scutellum, indicating that this mutation may be unstable and that the embryos may include sectors of normal tissue able to express *R-scm2* color. The *emb*-8521* gene was similar to the *dek* gene *fl*-1253B* in being involved in the maintenance of organizational integrity in the embryo (Clark and Sheridan, 1988).

***emb**-8545 (1S).** The mutant is uniformly blocked at an abnormal stage 4. Embryos had a well-developed, morphologically normal embryonic axis, but a scutellum that was reduced in size because its upper portion failed to develop. Despite its small extent, the scutellum became filled with starch and had the full *R-scm2* coloration characteristic of normal embryos late in development. This indicates that the mutation is specific to scutellum morphogenesis, and apparently does not interfere with the organ-specific metabolic functions of the scutellar tissue once it has differentiated.

***emb**-8507.** The mutant is blocked variably from an abnormal stage 2 to an abnormal stage 4 or greater. In all of the embryos the embryonic axis had developed normally, but the scutellum consisted of large irregularly shaped lobes. Embryos blocked early in development consisted of twisted masses of scutellar and embryonic axis tissue, whereas the most advanced embryos showed only a small amount of scutellar proliferation. Embryos carrying the *R-scm2* marker had developed extensive colored sectors in the scutellum, suggesting that this mutation is unstable and that embryos may consist of chimeras of mutant and normal tissue.

***emb**-8513 (4L).** The mutant is blocked variably from stage 3 to stage 6; most embryos were at stage 4. Embryos of this mutant were normal in appearance but were retarded in stage and reduced in size for their stage. Embryos marked with *R-scm2* developed full purple coloration of the scutellum. Despite their apparent normal morphology, only two of 35 mature mutant embryos tested germinated in sandbench tests. This mutant, therefore, suffers both from a retardation in rate of development and also from permanent dormancy. In this respect it was like the nongerminating *dek* mutants. It differed from them, however, in that the *emb*-8513* mutation did not affect the endosperm, whereas in the nongerminating *dek*s the endosperm is severely defective.

DISCUSSION

Many Loci Can Mutate To Produce the *emb* Phenotype

Three lines of evidence support this conclusion. First, the *emb* mutations were recovered at a relatively high frequency. Second, *emb* loci appear to be dispersed throughout the genome. Third, the wide range in stage of blockage and the diversity of mutant phenotypes observed is consistent with a large group of loci rather than a limited number of loci mutating repeatedly. Limited allelism tests of mutants on chromosome arm *1S* indicates that there may be at least four different loci on that arm (data not shown).

The *emb* Loci Are Essential to Embryo Development

Two lines of evidence support this conclusion. The first is the developmental arrest of mutant embryos before maturity. The second is their failure to germinate at kernel maturity in sandbench tests. The few instances of germination observed among mutants blocked during the early morphogenesis period are likely to represent misscored normals because these embryos never developed enough to be capable of germination. In contrast, germination in the three mutants blocked at the most advanced stage indicate that they are leaky mutations because these embryos possess embryonic axes and leaf primordia capable of germination.

The *emb* Loci Are Involved in Morphogenesis in a Fundamental Way

All of the *emb* mutations produce morphologically abnormal embryos. The mutations differ in phenotype and are consistent in mutant expression, indicating that they are involved in a wide range of developmental events.

These genes are not likely to represent housekeeping genes involved in general cellular growth and metabolism because in mutant kernels the endosperm is able to grow normally. For none of the 27 *emb* mutations studied by dissection of mutant embryos in mature kernels did it appear that the normal allele was required for the early cleavage of the zygote because none of the mutations were zygotic lethals. Because many *emb* loci have been sampled in this group and no zygotic lethal mutants have been found among the *dek* mutants characterized to date, it is likely that such genes are very rare. Likewise, none of these 27 *emb* mutants were blocked in establishing the fundamental polarity of the embryo. These developmental events are likely to be established in the egg under the control of the maternal genome; therefore, the relevant genes will be defined by female gametophyte mutants.

Nine of the 27 mutants studied by dissection represent genes that are crucial for the full development of the proembryo into an embryo proper and suspensor. Many of these underwent degeneration of the embryo proper and concomitant enlargement of the suspensor. In this regard they are similar to the *50B* mutant of Arabidopsis (Marsden and Meinke, 1985). Enlargement of the suspensor is not an obligate consequence of death of the embryo proper because mutants in which death of the embryo proper is not accompanied by death of the suspensor have been found among *dek* mutants in maize (Clark and

Sheridan, 1986) and among embryo-lethal mutants in Arabidopsis (Marsden and Meinke, 1985). For further consideration of how embryo-lethal mutations in maize compare with this type of mutation in Arabidopsis, see Meinke (1991).

Some of the *emb* mutants appear to represent genes specifically involved in the morphogenesis of specific organs or structures (e.g., the scutellum in *emb*-8545*, the embryo proper in *emb*-8522*, the suspensor in *emb*-8543*), whereas others involve a breakdown of organization of patterns of growth and differentiation (e.g., generalized tissue proliferation in *emb*-8535*, polyembryony in *emb*-8501*). Eleven embryo-specific mutants that are disturbed in organ differentiation have been described in rice (Nagato et al., 1989). These mutations result in structural abnormalities in the embryonic axis and produce embryos that either abort late in embryogenesis or germinate but die as seedlings.

The *emb* Genes May Be Required in More than One Tissue and at More than One Time during Kernel Development

The triploid endosperm of maize is a complex structure, divided morphologically and functionally into three distinct regions: the massive starchy storage region, comprising the bulk of the tissue; the epidermal aleurone layer, which is normally a single cell layer covering the endosperm and enclosing the embryo; and the transfer region at the base of the kernel (Kiesselbach, 1949; Schel et al., 1984). The *emb* genes are not required for the full development of the massive endosperm; indeed, this selectivity is the basis for their identification. Some of the *emb* mutations are associated with a thickened aleurone, indicating that the gene may be involved in aleurone development. These same mutants also undergo tissue proliferation in the embryo (data not shown). This pattern of gene expression has been observed in *dek* mutants with phenotypes that include tissue proliferation (Clark and Sheridan, 1986). It appears likely, therefore, that the aleurone layer differs from the rest of the endosperm in sharing some genetic programs and regulatory pathways with the embryo. It is not clear from our data whether any of the *emb* mutations are expressed in the basal transfer cells of the endosperm, although this may be the case, as evidenced by necrosis observed in this region of the endosperm in *emb*-8515* (data not shown). One interesting aspect of endosperm development emerged from our studies: in normal kernels the embryo was situated in a depression in the endosperm. It has been suggested that this depression results from digestion of the endosperm by the developing embryo (Smart and O'Brien, 1983). However, *emb* mutant kernels, even those with minuscule embryos, had an extensive cleft in the face of the endosperm. This indicates that the genetic component of endosperm morphogenesis controls its shape independently from the embryo.

Expression of the *emb* genes in the gametophyte generation is suggested by altered transmission ratios. Of 49 mutations tested, 12 were significantly reduced in transmission. In early studies of mutants of this class, Demerec (1923) described several lines containing *gm* mutants that segregated at reduced ratios. Wentz (1930) obtained only two lines segregating consistently at a 3:1 ratio from 52 *gm* lines tested. Progenies of most lines were inconsistent in segregation ratio through five generations of outcrossing. In contrast, we have observed that the transmission patterns of the *emb* mutants in our collection have remained stable through two to six generations of propagation. We recognize that it is possible that any case of reduced transmission may be the result of a closely linked gametophyte factor; however, we believe that in the majority of cases here the transmission patterns reflect the expression of the *emb* genes themselves.

The *emb* Mutants Are Attractive Candidates for Molecular Analysis

The *emb* mutants described here arose in active *Mutator* stocks, so many should be *Mu* tagged. Robertson's *Mutator* stocks contain a complex transposable element family consisting of a number of elements that have only their inverted terminal repeat sequences in common. The *Mu1* element has been responsible for most of the *Mutator* insertion mutations characterized to date; screening can reasonably begin with this element.

We conclude, therefore, that by screening *Mutator* populations we have been able simultaneously to identify and transposon tag many new loci crucial to embryogenesis. These *emb* mutations should facilite the genetic dissection and molecular analysis of this complex but important aspect of plant development.

METHODS

Source and Propagation of Mutants

The *emb* mutations were obtained by screening 1000 self-pollinated ears kindly made available by D.S. Robertson, Iowa State University, Ames. These ears were produced on F_1 plants. The parents of the F_1 were genetic stocks of D.S. Robertson's, at least one of which was known to have *Mutator* activity.

A sample of approximately 50 kernels was removed from each ear, and those kernels with a normal-appearing, fully developed endosperm were turned germinal-side-upward and examined for the presence of a reduced or absent embryo. The ears segregating for such kernels were selected as potential sources of embryo-specific mutations. Kernel samples were then taken to test for heritability and for propagation of the mutations.

We have propagated the mutations by planting normal kernels, self-pollinating the resulting plants, and simultaneously crossing these same plants to early maturing, vigorous genetic stocks in

our summer and winter nurseries. Inasmuch as all of the mutations under investigation are embryo lethals as homozygotes, it has been necessary to propagate them in the heterozygous condition. The *emb* mutations have been crossed onto two genetic stocks homozygous dominant for all of the aleurone color factors, one of which carries the *R-nj* allele and the other of which carries the *R-scm2* allele. The *R-nj* allele conditions anthocyanin color in the coleoptile of the mature embryo (Coe et al., 1988). The *R-scm2* allele arose from *R-mb* (Weyers, 1961) and conditions anthocyanin coloration in the scutellum of the embryo (Birchler, 1983, 1991; Sheridan, 1988). The original accessions of these mutations were obtained in colorless aleurone stocks homozygous for *c1* and *r1*. Conversion of the mutations into our *R-nj* and *R-scm2* stocks not only conferred earliness and vigor but also facilitated the eventual allelism tests.

Location of Mutations to Chromosome Arm

The *emb* mutations were tested for chromosome arm location by the use of B-A translocations (Beckett, 1978, 1991). The procedure is the same as that used by Neuffer and Sheridan (1980) to locate *dek* mutations except that in our study we could not utilize the presence of a defective endosperm to reveal the arm locations.

Forty-five of the *emb* mutations were tested for chromosome arm locations. Because of the scope of the effort required to test such a large number of mutations of this type (embryo-lethal and lacking a mutant endosperm phenotype), eight of the 20 chromosome arms were selected for testing. These arms and their respective tester stocks were: *1S*, *+/dek1*; *1L*, *bz2/bz2*; 2L, *+/dek23*; *3L*, *a1/a1*; *4L*, *c2/c2*; *5S*, *a2/a2*; *9S*, *c1/c1*; and *10L*, *r1/r1*. The *+/dek23* tester was a colorless aleurone stock, and all of the other tester stocks were homozygous dominant for all of the other anthocyanin factors except for the tester locus listed above. The eight B-A translocation stocks were *1S*, TB-*1Sb*; *1L*, TB-*1La*; *2L*, TB-*1Sb-2L4464*; *3L*, TB-*3La*; *4L*, TB-*4Lf*; *5S*, TB-*5Sc*; *9S*, TB-*9Sb*; and *10L*, TB-*10Lb*. The break points for the A chromosome arms of these eight stocks are listed in Coe et al. (1988). They range from 0.05 for *1S* to 0.4 for *9S*. This set of eight translocations includes both arms of chromosome 1 and the long arms of chromosomes 2, 3, 4, and 10, as well as the short arms of 5 and 9; an examination of the cytological map and arm lengths indicates that these arms comprise nearly half the total physical length of the 10 chromosomes (Neuffer et al., 1968; Sheridan, 1982). After considering that the arms tested comprise about half the total chromosome arm length, i.e., 714 of a total of 1454 arbitrary units (Sheridan, 1982), and that, on average, about the distal 80% of the chromosome arms are uncovered by B-A translocations, we estimated that approximately 40% of the total genome is uncovered by the eight B-A translocations used in this study.

The arm-locating tests were conducted by planting 100 normal kernels from self-pollinated or F_1 ears for each of the 45 mutations. The self-pollinated ears were selected because they were segregating for the mutation, and the F_1 ears were selected because either the male or the female parent plant was known to be carrying the mutation. Usually two to four ears were utilized as sources of kernels for each of the different mutations. In the case of kernels from self-pollinated ears, two-thirds of the normal kernels are expected to be heterozygous, whereas one-half of the kernels from F_1 ears should be heterozygous for the mutation of interest, assuming nonreduced transmission of the mutant allele. Each of the eight B-A translocation stocks and their respective tester stocks were planted at a series of planting dates before and after the single planting date of the *emb* stocks to assure the availability of pollen from the B-A stocks at the time that the ears of the mutation cultures were ready for pollination. Pollinations were performed by standard procedures (Neuffer, 1982). The goal was to cross at least five plants in each of the 45 mutation cultures by pollen from each of the eight B-A stocks. When a TB parent was first used as a source of pollen, it was crossed onto at least one tester plant, except that at least three tester plants were crossed onto whenever the *dek1* or *dek23* testers were being used. This was done because the aleurone testers can be grown as homozygotes, but the two *dek* testers can only be grown as heterozygotes, and the genetic identity of any individual plant is uncertain at the time of pollination. In this way the presence of the B-A translocation was confirmed for the pollen sources.

At harvest time the tester ears were scored for confirmation of the presence of the B-A translocation in each of the TB stocks. To score the crossed ears of the 45 mutation families, about half of the kernels were removed from each of the approximately 2000 ears resulting from crosses by the B-A stocks. This usually comprised about a 200- to 250-kernel sample. Each of these samples was poured out onto a well-lit surface, the kernels were turned to have their embryo side upward, and they were visually examined for kernels having a reduced embryo. When mutant kernels were identified, the total number of kernels and the number of mutant kernels in the sample were recorded. Ordinarily, on an arm-locating ear about one-eighth of the kernels would be expected to have a hypoploid mutant embryo (Beckett, 1978). A finding of at least 10 kernels with mutant embryos per 100 kernels examined was required to conclude that an arm-locating cross had been accomplished.

Developmental Analysis

Segregating self-pollinated ears were harvested at maturity but before drying, generally 40 to 60 days after pollination. A sample of 10 mutant kernels was taken from the middle of the ear. It was necessary to avoid sampling from the tip of the ear because genetically normal kernels with an *emb* phenotype resulting from a physiological accident can frequently occur in this area (Mangelsdorf, 1926). The kernels were dissected, and their embryos were photographed with Ektachrome 50T film using a Wild 400 photomacroscope (Wild Heerbrugg Ltd., Heerbrugg, Switzerland). The classification scheme of Abbe and Stein (1954), based on the stage of shoot apex development, was used in this study (see Figure 1).

Germination Tests

For each mutant, 25 to 100 mutant kernels from dried ears were planted in the sandbench and grown with bottom heat. Germination was scored after 21 days. A total of 50 normal kernels from nonsegregating ears of *emb* families were planted as controls. These had 100% germination, indicating that growing conditions were adequate to test the mutants.

Segregation Analysis

For each of the segregating self-pollinated ears examined in the developmental analysis, usually a sample of 50 to 200 kernels from the middle of the ear was removed and scored for frequency of mutant kernels. χ^2 values were calculated using the G test of goodness of fit (Sokal and Rolf, 1981).

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