extract–mannitol medium (*10*) for 4 to 5 days and then harvested in 10 ml of PBS. The cells were washed once in the same buffer and then in 0.1*M* sodium acetate (*p*H 4.0) before finally being suspended in 10 ml of the acetate buffer. The final cell suspension was adjusted to approximately 12.0 units at an absorbance of 660 nm. The cell suspension (80 μl) was mixed with 80 μl of the seed extract and incubated at 30°C for 15 to 60 minutes.

7. Alfalfa agglutinin was purified by the following method. The crude seed extract was heated at 80°C for 30 minutes. After centrifugation, the supernatant was treated with 60 percent ammonium sulfate; the precipitate was dissolved in 0.1*M* sodium acetate buffer (*p*H 4.0) and loaded onto a Sephadex G-50 column. Active fractions were dialyzed against distilled water. Further purification was achieved by preparative flat-bed isoelectric focusing in Sephadex G-75 (*p*H 3.5 to 10.0). Fractions containing purified agglutinin were pooled and concentrated by ultrafiltration (Amicon, UM2). The ampholytes were removed by gel filtration on a Sephadex G-75 or G-100 column. Biochemical characterization and evidence that the agglutinin is a protein will be presented elsewhere.
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11. Exponentially growing cells of *R. meliloti* 102F51 in yeast extract–mannitol medium were harvested by centrifugation and washed once with 50 m*M* PBS. The cells were suspended in 50 m*M* PBS containing 160 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per milliliter for 30 minutes. They were then pelleted and washed three times with 50 m*M* PBS before being suspended in yeast extract–mannitol medium and incubated overnight at 30°C to allow phenotypic expression. The cells were cultured in minimal mannitol medium for over 30 generations before clones were isolated for testing on alfalfa plants. The mutants grew as well as the wild type on a variety of different media.
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13. The nonnodulating mutants were tested for susceptibility to 20 phage isolates that lyse *R. meliloti* 102F51. Plaque formation on lawns of the mutants in 0.7 percent yeast extract–mannitol agar was noted after incubation at 30°C overnight.
14. Ouchterlony plates contained 0.7 percent agarose (Sigma, type II), 0.05*M* sodium barbital (*p*H 8.4), 0.2 percent NaCl, and 0.05 percent sodium azide. The wells were filled with 20 μl of extract or antiserum, and precipitin bands were allowed to develop at 30°C for 4 to 24 hours.
15. Cotyledons were removed from seedlings, and the cut ends were sealed by dipping them in melted paraffin. The seedling roots were then washed successively in 0.1*M* PBS, 5 mg of bovine serum albumin per milliliter, and 5 mg of bovine gamma globulin per milliliter, followed by two more washings in PBS. Control seedlings were incubated at room temperature with rabbit serum obtained before immunization. Experimental seedlings of matched size were incubated with rabbit antiserum prepared against purified agglutinin. The seedlings were again washed as described above. They were then incubated at 30°C for 1 hour with alkaline phosphatase–conjugated goat antiserum against rabbit IgG (Miles) before being washed as before and placed in 1 ml of 10 percent ethanolamine (*p*H 9.9), containing 1 mg of 2-nitrophenylphosphate per milliliter, at room tempeature for 10 minutes. The reaction was stopped by the addition of 0.17 ml of 3*M* NaOH, and the activity of the alkaline phosphatase was determined at an absorbance of 405 nm.
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Mutator Activity in Maize: Timing of Its Activation in Ontogeny

Abstract. *Mutator activity in maize seems not to be active throughout the development of the plant, but to arise late in ontogeny, and to be developmentally rather than chronologically triggered.*

The mutator system (*Mu*) (*1*) increases the frequency of mutations in outcross progeny 30 to 50 times (*1*, *2*). This *Mu* activity is not expressed throughout the development of the plant but seems to be preferentially expressed late in the development of the germ-line tissue of the sporophyte (*2*). Whether the timing of *Mu* activity is related to total elapsed time from germination (chronologically determined) or is related to a particular developmental stage of the germ line (developmentally determined) that occurs late in ontogeny was investigated in this study.

Several different small sectors (or clusters) of seeds bearing allelic mutants were found in ear maps of plants showing *Mu* activity. The largest sector extended over a minimum of five seeds (*2*). More sectors have now been found in other ear maps, the largest sector containing a minimum of 11 seeds and the smallest, a minimum of 3 seeds. The occurrence of clusters of mutants establishes that some *Mu*-directed mutational events occur in mitotic divisions late in the development of the ear. Solitary mutants on some of the ears may be the result of very late mitotic events giving rise to a sector in which only one of the cells produced a seed with a *Mu*-induced mutant. Alternatively, some or all of these single mutants may be meiotically

Table 1. Frequency of mutants in five serial outcrosses of plants sampled on 5 to 7 consecutive days of pollen shedding, and the distribution of putative clusters in these outcrosses. Heterogeneity χ^2 for totals of each plant outcrossed is 34.0471; d.f. = 4; $P < .01$.

Day	Total plants self-pollinated	Total mutants per outcross	Percent mutants per outcross	Heterogeneity χ^2	Degrees of freedom	*P*	Number of putative clusters	Average putative cluster per plant
				Cross 5062-3				
1	77	8	10.4				1	
2	63	16	25.4				1	
3	52	11	21.2				3	
4	47	10	21.3				1	
5	60	12	20.0				4	
6	71	16	22.5				3	
7	57	7	12.3				2	
Total	427	80	18.7	8.0540	6	.20 to .30		2.5
				Cross 5061-9				
1	49	5	10.2				0	
2	51	7	13.7				1	
3	40	5	12.5				1	
4	44	6	13.6				1	
5	55	7	12.7				2	
6	52	6	11.5				1	
Total	291	36	12.4	0.4038	5	>.99		1.0
				Cross 5062-4				
1	27	2	7.4				0	
2	43	5	11.6				2	
3	32	2	6.2				0	
4	41	2	4.9				0	
5	35	2	5.7				0	
6	30	5	16.7				1	
Total	208	18	8.7	4.3268	5	.50 to .70		0.5
				Cross 5061-8				
1	51	4	7.8				1	
2	53	3	5.7				1	
3	46	5	10.9				1	
4	64	7	10.9				3	
5	52	3	5.8				1	
Total	266	22	8.3	1.9267	4	.70 to .80		1.4
				Cross 5061-6				
1	12	3	25.0				0	
2	10	1	10.0				0	
3	30	8	26.7				2	
4	30	9	30.0				2	
5	19	6	31.6				1	
Total	101	27	26.7	1.8393	4	.70 to .80		1.0

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induced. Mutants also occur in sectors in the tassel (*2*).

The maturity pattern of the tassel allows a test of these alternatives. Pollen matures first in the central axis a little below the tip, and then maturation spreads upward and downward. The pollen of the branches matures a day or so later and also matures first a short distance from the tip, with maturity later spreading in both directions. Pollen in the upper branches matures first and in the basal branches last. Each spikelet has an upper and lower flower. The upper flower matures first, and a day or two later, the lower flower matures. A tassel may shed pollen for up to 7 days, depending on the size of the tassel and conditions such as temperature and moisture during growth (*3*). When samples of pollen are taken throughout the period of shedding, the later samples are drawn from later-maturing germ-line tissue. If total elapsed time is a factor in triggering the activity of *Mu* (chronological triggering), different samples might show different frequencies of mutations. If *Mu* is activated at a certain developmental stage, then pollen samples from different days should exhibit the same mutation frequency.

Tassels of *Mu* plants were bagged on the day that the central axis started shedding. The next day, the pollen in these bags was used to self-pollinate the *Mu* parent and also was outcrossed to standard lines; the tassels were then rebagged. On the following day, the bags were used in outcrosses, and the tassels were again bagged. This procedure was repeated until the tassels had finished shedding. Fifty or more seeds of the outcrosses were sown, and the resulting plants were self-pollinated. Seeds from the resulting ears were seedling-tested, and the number of ears segregating for seedling mutants (the mutant frequency) was noted. Only those outcrosses from *Mu* plants that did not segregate for seedling mutants when self-pollinated were tested.

For the five serial outcrosses (Table 1), the seeds that gave rise to the five plants used were selected from outcrosses that had previously given positive results in tests of *Mu* activity. Tests for heterogeneity were used to determine the homogeneity of each set of outcrosses (*4*). Although there is some variation in the frequencies of mutations in pollen samples taken on different days, it is no greater than that expected from sampling error. The mutant frequency used in these tests is based on the total number of mutants found; no correction was made for the occurrence of clusters. Thus, each member of a cluster, if clusters were present, was counted. The χ^2 heterogeneity test of the total mutant frequencies of the five series of outcrosses indicates that they differ. Thus, the basic mutation frequency can differ from plant to plant.

The frequencies of mutants for each cross for each day were combined, and heterogeneity χ^2 values were calculated for the five crosses for each day and for totals for the various days (Table 2). Again, heterogeneity tests indicate no significant differences in the mutation frequencies among days. Within days, there are significant differences for days 2 and 4 at the 5 percent level and, day 5, at the 1 percent level. For the other days, there are no significant differences. These findings are in general agreement with the significant difference observed for the totals of the five populations in Table 1. The results indicate that there is no significant variation in the *Mu*-induced mutation frequency over the 5- to 7-day interval of pollen shedding, as would be expected if *Mu* is triggered at a particular developmental stage.

It is possible, however, that the triggering is chronological and occurs before the differentiation of all the pollen cells. The frequency of clusters of mutants would then be expected to be greater in later-shedding pollen than in earlier because more intervening cell divisions could occur between the triggering of *Mu* activity and the differentiation of pollen. Putative clusters are recognized as occurring when two or more plants of an outcross progeny segregate for mutants of the same phenotype. Allele tests with the phenotypically similar mutants are required to establish a true cluster. Since these tests were not made, all clusters mentioned are putative. The finding, in an earlier study (*2*), that 72.46 percent of putative clusters are, in reality, not clusters at all suggests that the actual number of clusters is probably considerably less than the number of putative clusters indicated in Table 1. There is no evidence of a higher frequency of clusters in later pollen samples of any of the series of crosses (Table 1).

Table 2. Mutant frequencies combined by days for the five serial outcrosses given in Table 1. Heterogeneity χ^2 for totals of each day is 3.2717; d.f. = 4; P = .50 to .70.

Cross	Total	Total mutants per outcross	Percent mutants per outcross	Heterogeneity χ^2 for each day's crosses	Degrees of freedom	P
			Day 1			
5062-3	77	8	10.4			
5061-9	49	5	10.2			
5062-4	27	2	7.4			
5061-8	51	4	7.8			
5061-6	12	3	25.0			
Total	216	22	10.2	3.4162	4	.30 to .50
			Day 2			
5062-3	63	16	25.4			
5061-9	51	7	13.7			
5062-4	43	5	11.6			
5061-8	53	3	5.7			
5061-6	10	1	10.0			
Total	220	32	14.6	9.8227	4	.02 to .05
			Day 3			
5062-3	52	11	21.2			
5061-9	40	5	12.5			
5062-4	32	2	6.2			
5061-8	46	5	10.9			
5061-6	30	8	26.7			
Total	200	31	15.5	7.2436	4	.10 to .20
			Day 4			
5062-3	47	10	21.3			
5061-9	44	6	13.6			
5062-4	41	2	4.9			
5061-8	64	7	10.9			
5061-6	30	9	30.0			
Total	226	34	15.0	10.9067	4	.02 to .05
			Day 5			
5062-3	60	12	20.0			
5061-9	55	7	12.7			
5062-4	35	2	5.7			
5061-8	52	3	5.8			
5061-6	19	6	31.6			
Total	221	30	13.6	11.9384	4	.01 to .02

If triggering of *Mu* is chronological and occurs before pollen differentiation, the total mutation frequency should also be higher in later pollen samples, inasmuch as *Mu* would have had a longer time to act between triggering and pollen differentiation. Such higher rates are not observed consistently in later shedding pollen. However, a chronological trigger, combined with different maturity rates, might account for the significant difference in total mutant frequency observed among the five crosses (Table 1). In a slow-maturing plant, the chronological trigger would result in an earlier triggering of *Mu* activity, relative to the development of the tassel, than in a faster maturing plant. A slow-maturing plant thus could have more mutants than a faster maturing plant. As indicated in Tables 1 and 2 the five plants were drawn from two different families, designated 5061 and 5062. Family 5061 was planted a week earlier than 5062. As is usually the case with delayed planting, the later plants matured a little faster than the earlier ones. Family 5062 began shedding pollen 4 to 5 days after 5061. Since the 5061 plants matured a little more slowly, they would be expected to have a higher mutation frequency if a chronological trigger were involved. The mutation frequency for the total of the 5061 crosses was 12.9 percent and for the total of the 5062 crosses was 15.4 percent. The faster maturing plants had the higher frequency, although a chi-square test indicated that the two populations are not significantly different. The slower maturing plants also would be expected to have more clusters, and thus an association between higher mutation rate and the occurrence of clusters would be expected. No consistent relation between these two phenomena is seen (Table 1). Thus, the rate of maturation is not a likely explanation for the different mutation frequencies. The reason for the different rates probably will not be known until more is learned about the nature of the *Mu* system.

In sum, these results indicate that a developmentally timed trigger for *Mu* activity is more likely than a chronologically timed trigger.

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$$\chi^2 = \frac{\sum_i (p_i n_{i1} - p N_{\cdot 1})}{\bar{p}\bar{q}}$$

where p_i is the frequency of mutations in each day's outcross, n_{i1} is the total number of mutant plants from each day's outcross, $\bar{p}$ is the frequency of mutations in the total population tested for heterogeneity, $N_{\cdot 1}$ is the total number of plants with mutants in the whole population tested, and $\bar{q} = 1 - \bar{p}$.
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Adenosine Triphosphate–Deficient Erythrocytes of the Egg-Laying Mammal, Echidna (*Tachyglossus aculeatus*)

Abstract. *The erythrocytes of the short-beaked echidna* (Tachyglossus aculeatus), *an egg-laying mammal, were examined for the presence of phosphorylated compounds. The erythrocytes contained only 0.03 ± 0.01 micromoles of adenosine 5′-triphosphate per milliliter of cells. This amount is two orders of magnitude less than that in human cells. Although the echidna erythrocytes had an abundance of 2,3-diphosphoglycerate and other glycolytic intermediates, no other energy-rich pyridine and purine compounds were detected.*

Metabolic machinery in mature mammalian erythrocytes is limited in that the regeneration of adenosine 5′-triphosphate (ATP) depends solely on glycolytic pathways. Although bloodborne glucose is believed to be the key metabolic substrate for mammalian erythrocytes, there are exceptions; for example, in the pig, erythrocytes appear to depend for survival mainly on inosine derived from the liver and other organs (*1–3*). Functions in which ATP plays an essential role include maintaining cell shape (*4, 5*), regulating cell volume by means of active cation transport (*6*), decreasing oxygen affinity of hemoglobin (*7, 8*), and phosphorylating metabolic substrates (*9*). Among vertebrates, the ATP concentration in erythrocytes is highest in the snake (15 μmole per milliliter of cells) (*10*) and lowest in the horse (0.2 μmole/ml) (*11*). About 15 years ago, a curious phenomenon indicating the almost complete absence of ATP in erythrocytes of the short-beaked echidna, an egg-laying mammal, was noted (*12*), but no data were reported. Attempts to corroborate this observation proved to be difficult, owing to the scarcity of echidnas in the United States.

We have now determined the distribution of phosphorylated compounds in erythrocytes of the echidna. Ten echidnas weighing 1.5 to 3.0 kg were studied; some of the animals were collected near Hobart, Tasmania, with permission of the office of the National Parks and Wildlife Services, and others were brought to the University of Tasmania by local residents. The animals were lightly anesthetized with halothane. A blood sample, which varied from 5 to 10 ml, depending on body weight, was taken by cardiac puncture, with EDTA used as an anticoagulant. All animals survived this procedure and remained healthy. A portion of the whole blood sample was taken for measurement of hematological indices, and the rest was used for studies of membrane transport and metabolism (*13*). For the determination of intermediary metabolites, blood samples from four animals were used. The erythrocytes were washed in isotonic NaCl several times, with the usual care taken to aspirate the buffy coat. At least 0.2 ml of freshly packed cells were extracted with 0.56*M* perchloric acid and neutralized with K_2CO_3. The neutralized extract was immediately frozen, packed in dry ice, and transported by air carrier to the University of Alabama. The extract remained frozen until it was assayed for metabolites.

Glycolytic intermediates and cofactors in the extract were determined by Bessman's procedure for anion-exchange column chromatography (*14, 15*). A 3 by 150 mm column partially filled with Dowex AG1-X4 resin and a 3 by 500 mm column packed with equal portions of DA-X4-20 (Durrum) and Aminex A-25 (Bio-Rad) resins were used in tandem. A tenfold dilution of the extract was placed on the columns and eluted with 160 ml of a linear gradient of ammonium chloride (0.1*M* to 0.6*M*) containing 0.5*M* potassium tetraborate. The eluant was monitored at 254 nm with a spectrophotometer (ISCO). Eluant fractions (75 μl) were collected in 48 cups, ashed, and assayed colorimetrically for inorganic phosphate. The temperature of the ashing chamber was gradually increased from 22° to 400°C for complete ashing of organic phosphates. The resulting inorganic phosphate was assayed colorimetrically by addition of 0.5 ml of a solution composed of 20 percent ascorbic acid and 2.5 percent ammonium molybdate in 2.5 percent sulfuric acid to each cup. A fully developed color mixture was monitored

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