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ETHYL METHANESULFONATE-INDUCED MUTATIONS OF THE Sh_1 PROTEIN IN MAIZE

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SUMMARY

16 sh_1 mutants have been obtained by treating kernels of Sh_1 Wx genotype with the chemical mutagen ethyl methanesulfonate (EMS). Immunochemical analysis of 20-day-old endosperms of newly obtained sh_1 mutants showed that 6 are CRM^+ (form cross reacting material with Sh_1 protein) and 10 are CRM^-. These mutants were further studied by starch gel electrophoresis. The occurrence of a CRM^+ class of sh_1 mutants, in addition to the mutants specifying electrophoretic variants of Sh_1 protein, suggests that the genetic changes induced are true gene mutations at the sh_1 locus. This study indicates that the Sh_1 protein carries out a specific function in the development of the endosperm and is not a non-specific storage-like protein since qualitative changes in the Sh_1 protein are associated with the appearance of the sh_1 phenotype.

INTRODUCTION

The Sh_1 locus on the short arm of chromosome 9 in maize controls the synthesis of Sh_1 protein in the endosperm[17]. This protein, which is readily identifiable even in crude extracts by electrophoretic and immunochemical assays, is the major soluble protein in the endosperm. Nothing is known concerning its function except that it plays a role in the development of the endosperm. The Sh_1 protein is limited to endosperm tissue and has not been detected in other plant parts, not even in the pollen which is a site of active starch synthesis. In kernels homozygous for the recessive sh_1 allele a large cavity forms in the central portion of the endosperm. The outer portion of the endosperm tends to collapse as the kernel dries, giving rise to the shrunken phenotype. SCHWARTZ[17] (and unpublished) analyzed 16 sh_1 mutants, 8 of spontaneous origin and 8 X-ray-induced. The Sh_1 protein was completely lacking in every mutant. It is possible that the 16 mutants were all small deletions which included the Sh_1 locus, and in fact this is a very probable condition for the X-ray-induced mutants. However, an alternative explanation is that the Sh_1 protein does not carry out a specific function,

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Abbreviations: EMS, ethyl methanesulfonate; IKI, iodine–potassium iodide.

characteristic of other proteins such as enzymes, hemoglobins, phytochromes, cytochromes, *etc.*, but is a non-specific storage-like protein whose mere presence, even in altered forms, is sufficient to insure development of plump non-shrunken kernels. According to this alternative, alterations at the Sh_1 locus which alter the structure of the protein would not be detected as mutants, and only gene deletions or nonsense-type mutations would give rise to the shrunken phenotype in the kernel.

To distinguish between these alternatives, a chemical mutagenesis study was undertaken with EMS. EMS is a powerful mutagen which, in addition to causing chromosome breaks, is known to cause base substitution in DNA leading to point mutations[8]. The experimental plan was to treat maize with EMS, screen for sh_1 mutants on the basis of the sh_1 phenotype, and determine if any of the induced mutants contain the Sh_1 protein in the endosperm. If the Sh_1 protein has a specific function one would expect to recover sh_1 mutants which result from loss of the function of the Sh_1 protein, as well as mutants which lack the protein completely.

Most mutation studies in plants have been based on phenotypic expression and at this level it is very difficult to distinguish between point mutations and gene deletions. In fact it is still an open question as to whether or not X-rays produce point mutations in maize[13,20]. At the protein level one could with certainty recognize those point mutations which have altered the activity or electrophoretic mobility of a protein, but it would still be difficult to distinguish between nonsense mutations and deletions of a locus.

METHODS AND MATERIALS

Kernels of the genotype $Sh_1\ Wx/Sh_1\ Wx$ were treated with EMS, planted, grown in the field and used as female parents in crosses with $sh_1\ wx/sh_1\ wx$ tester stocks. The *wx* (waxy) locus governs the composition of the starch in the endosperm; *wx/wx/wx* endosperm stains red with IKI and in the presence of the *Wx* allele stains blue. The F_1 ears were harvested and screened for shrunken kernels. The shrunken kernels were tested for the waxy phenotype by staining a portion of the endosperm with IKI. All shrunken kernels in the F_1 population are classified as mutants at the sh_1 locus since a sh_1 tester stock was used as the male parent and only mutants at this locus were expressed in the F_1. Dominant shrunken mutants at other loci could also be expressed but subsequent crosses with the mutants showed that this had not occurred. The mutant kernels appear in sectors on the F_1 ears since there is a relatively low number of ear primordia cells in the embryo of the mature kernel from which an ear develops. All mutant kernels in a sector carry the same mutation.

Plants grown from the mutant kernels were self-pollinated and crossed with sh_1 *wx* tester stocks. Half of each ear was harvested 16–20 days after pollination for use in electrophoretic and immunochemical analysis, and the other half was allowed to develop to maturity. The immature kernels were stored in plastic bags at −20°. For the electrophoretic analysis the endosperm was separated from the embryo and pericarp. The endosperms were either squashed to moisten the filter paper sections which are inserted in a slit cut in the starch gel, or were macerated, centrifuged at $39000 \times g$ for 15 min, and the supernatant was used for the electrophoretic separations. The supernatants were also used in the Ouchterlony double diffusion tests.

The electrophoretic and immunochemical procedures for the assay of the Sh_1

protein have been described previously[17]. For EMS treatment, the kernels were soaked in an 0.08 *M* aqueous solution of EMS (Eastman Organic Company) for 10 h at room temperature, washed and dried prior to planting according to the procedure of BRIGGS *et al.*[4].

RESULTS

A total of 5700 EMS-treated Sh_1 *Wx*/Sh_1 *Wx* kernels were planted to give female parents for crossing with sh_1 *wx* tester plants. In the F_1 population of 1584 harvested ears, 16 ears were found to contain mutant sh_1 kernels. All of the shrunken kernels were of starchy (*Wx*) phenotype, presumably because large deletions which include both the Sh_1 and *Wx* loci (30% recombination) were not transmissible through the egg. The frequency of shrunken kernels on these ears was quite variable (Table I), due presumably to the different number of cells in the embryonic ear primordia at the time the mutation was produced.

TABLE I

MUTANTS OBTAINED IN CROSSES OF Sh_1Wx ♀♀ (kernels treated with EMS) × sh_1wx ♂♂

Mutant	*Segregation on the F_1 ear*	
	Sh_1 type	*sh_1 type*
sh_1^F	200	4
sh_1^S	97	9
sh_1^A	130	84
sh_1^B	145	120
sh_1^C	89	2
sh_1^D	21	23
sh_1^{null-1}	33	42
sh_1^{null-2}	103	43
sh_1^{null-3}	35	27
sh_1^{null-4}	155	88
sh_1^{null-5}	134	137
sh_1^{null-6}	52	98
sh_1^{null-7}	33	25
sh_1^{null-8}	68	8
sh_1^{null-9}	44	26
$sh_1^{null-10}$	139	95

Electrophoretic and immunochemical analyses of immature endosperms from the mutant F_2 kernels revealed that the 16 mutants, although of identical shrunken phenotype, can be separated into 3 classes (Table II) described below. Since the F_1 kernels were all heterozygous for the standard sh_1 allele of the tester stock, some of the F_2 kernels will be homozygous for this allele, which is immunochemically CRM^- (ref. 17). Thus in the case of CRM^- mutants, no Sh_1 protein would be detected electrophoretically in any of the endosperms. However, for the CRM^+ mutants, the protein would be lacking only in those F_2 kernels which are homozygous for the standard sh_1 tester allele. Because of the nature of the technique used, the immunochemical analysis, unlike the electrophoretic test, could not be done with individual endosperms; this test could be performed only with the supernatant which could be obtained only from the pooled population of (8 to 10 kernels) segregating endosperms of an F_2 ear.

TABLE II

CLASSIFICATION OF EMS-INDUCED sh_1 MUTANTS BASED ON ELECTROPHORETIC AND IMMUNOCHEMICAL ANALYSIS OF 16-DAY-OLD ENDOSPERM

Class	*Characterization of the sh_1 mutants*		*Number of mutants*	*Remarks*
	Electrophoretic behavior on the starch gels	*Immunochemical behavior in Ouchterlony plates*		
I	Protein band with altered migration rate	CRM^+	2	One is faster and another is slower migrating in relation to Sh_1 protein band
II	Sh_1 protein band (unaltered migration rate)	CRM^+	4	Indistinguishable from wild-type protein by these two criteria
III	No protein band	CRM^-	10	Similar to previously analyzed sh_1 mutants

Class I: Two mutants are included in this group, sh_1^F and sh_1^S. Immunochemically these mutants are CRM^+ since they specify a protein product which cross reacts with the Sh_1 protein as determined by the Ouchterlony double diffusion tests[16]. A number of precipitation lines appear in the agar gel between wells which contain an extract from Sh_1 endosperm and rabbit antiserum prepared against a partially purified Sh_1 protein preparation[17]. The Sh_1 precipitation line is most pronounced and is identified as the precipitation line which is lacking when sh_1 extracts are employed in the Ouchterlony test. The major precipitation line which appears when extracts from sh_1^F and sh_1^S endosperm are used coalesces with the Sh_1 protein precipitation line, indicating cross reaction (Fig. 1).

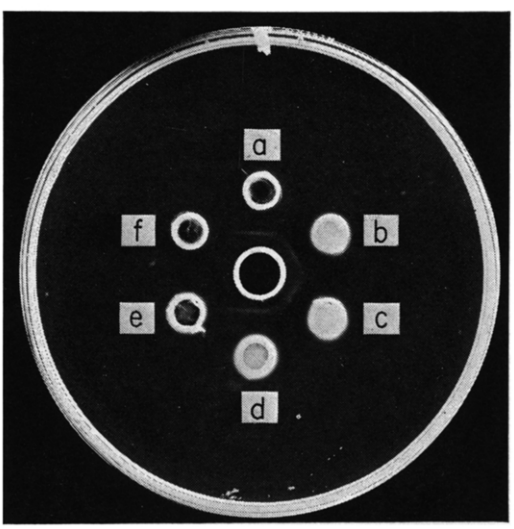

Fig. 1. Ouchterlony precipitation patterns of Sh_1 and the mutant proteins. The antiserum is in the central well and the peripheral wells contain crude extracts of endosperms homozygous for (a) Sh_1, (b) sh_1^F, (c) sh_1^S, (d) sh_1^A, (e) sh_1^{null} and (f) $sh_1^{standard}$ alleles.

In starch gel electrophoresis, these mutants show an intense protein band in the region of Sh_1 protein. The sh_1^F mutant forms a protein which migrates faster (more

anodal), while the sh_1^S mutant forms a protein which migrates slower than the Sh_1 protein (Fig. 2, b and c). Protein bands in these positions are not observed in either Sh_1 or sh_1 endosperm.

Class II: 4 shrunken phenotype mutants, sh_1^A, sh_1^B, sh_1^C, and sh_1^D are included in this group. These mutants specify proteins which cross react (Fig. 1) with and show the same electrophoretic mobility as the Sh_1 protein (Fig. 2d).

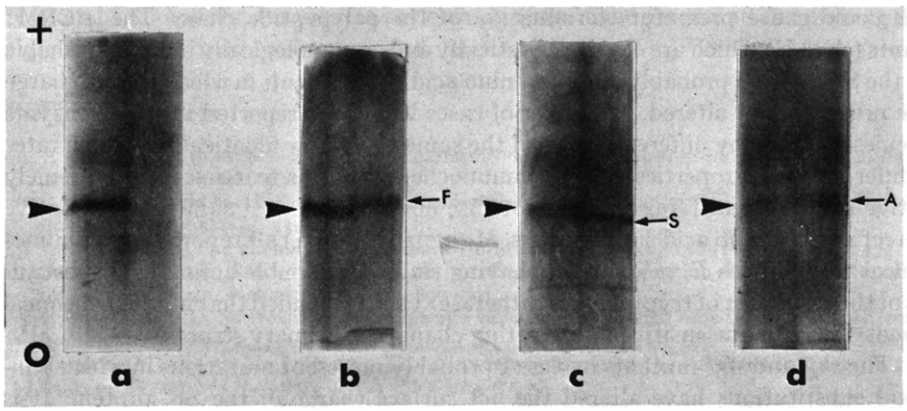

Fig. 2. Starch gel electrophoretic patterns of crude extracts from endosperms of Sh_1 type and its mutant alleles (on the right side of each gel): (a) $sh_1^{standard}$, (b) sh_1^F, (c) sh_1^S and (d) sh_1^A. The arrow on the left side of each gel points to the Sh_1 protein run alongside each extract. O designates the origin.

Class III: The 10 mutants grouped in this class are designated as sh_1^{null}. None of the mutants show the Sh_1 protein or any new protein bands in starch gel electrophoresis. The Ouchterlony double diffusion test shows no precipitation line. These sh_1^{null} mutants seem to be similar to the sh_1 mutants that have been previously analyzed.

All of the EMS-induced sh_1 mutants are recessive to the Sh_1 allele. The Sh_1 phenotype (plump kernel) is produced when Sh_1 is combined in heterozygotes with any of the mutant alleles.

DISCUSSION

The experiments undertaken in this study were designed to test whether or not the Sh_1 protein, the major soluble protein of maize endosperm, is a non-specific storage-like protein, or has a specific function in development of the kernel. This question has been answered by the recovery of 6 CRM^+ mutants, sh_1^F, sh_1^S, sh_1^A, sh_1^B, sh_1^C, and sh_1^D, which specify protein with no or altered function. The sh_1 phenotype is produced even though a normal amount of Sh_1-like protein is synthesized in these mutants. This indicates that Sh_1 protein has a specific function, since slight alteration in the protein, as described, gave the mutant phenotype. Why the mutants of spontaneous origin which were tested previously are all CRM^- is still an open question.

EMS has been shown to be a highly active mutagen in maize[2] and in a number of other organisms including Drosophila[6], Neurospora[21], bacteria[11], and phage[10]. The high mutation frequency accompanied, under certain conditions, by a low incidence of

chromosome aberrations observed in barley[9] and also in *Vicia faba*[12] has stimulated a great deal of work on EMS-induced mutagenesis in higher plants. EMS has been successfully used in maize to induce point mutations or small deletions[1,4,15].

According to FREESE[8] many of the EMS-induced mutations in phage involve transition-type base substitutions. Such base substitutions can cause amino acid replacements in a polypeptide which may or may not alter the net surface charge of a protein. Some base substitutions may result in the formation of amber or ochre codons which could cause premature termination of the polypeptide chain. The 4 CRM^+ mutants (class II) which are electrophoretically and immunologically indistinguishable from the Sh_1 protein probably involve amino acid replacements in which the net charge of the protein is not altered. A number of cases have been reported in which enzyme molecules specified by different alleles of the same gene show identical migration rates but differ in other properties[5,7,18,19]. Immunochemical cross reaction is an extremely sensitive test for identifying similar proteins; however, it is not sufficiently sensitive to detect single amino acid substitutions. MURPHY AND MILLS[14] reported on immunochemical studies with *E. coli* mutants having single and double amino acid substitutions of the α-subunit of tryptophan synthetase. They concluded that immunochemical methods are rather insensitive for detecting changes in primary structure.

The sh_1^F and sh_1^S mutants in class I probably represent mutations in which amino acid substitutions have altered the net surface charge of the Sh_1 protein. It is interesting to note that a correlated change in the Sh_1 protein was obtained while selecting for the sh_1 phenotype after EMS treatment of Sh_1-type kernels. This substantiates SCHWARTZ's[17] conclusion that Sh_1 is the structural gene for the Sh_1 protein.

The majority of the EMS-induced shrunken mutants at the Sh_1 locus are CRM^-. These mutants may have resulted from small chromosome deletions. Alkylating agents such as EMS can cause chromosome breakage and produce chromosome aberrations in addition to point mutations[3]. Depurination, causing a lesion in the DNA strand, has been cited as the basis for EMS-induced chromosome breakage[8]. Recently, ZAMENHOF AND ARIKAWA[22] in a comparative study on alkylation of bacterial DNA *in vivo* and *in vitro*, showed that transforming DNA *in vivo* is more readily inactivated by EMS. This difference has been attributed to an intracellular enzyme which specifically breaks down alkylated DNA. Besides the deletion of genetic material, other possible molecular mechanisms can be considered for the occurrence of the CRM^- mutants: (*i*) A base pair substitution may give rise to an amber or ochre codon which will cause premature chain termination of the nascent polypeptide. A complete lack of the Sh_1 precipitation line with all the sh_1^{null} mutants in the Ouchterlony test would suggest that the chain-terminating codon, if present, is located close to the point of initiation of translation. Thus, not even a fragment of the Sh_1 protein which could cross react, is produced. (*ii*) A protein is formed but its structure is so altered that it cannot be recognized by the techniques employed. (*iii*) It is also possible that amino acid substitutions affect protein stability to the extent that protein is denatured soon after formation.

The electrophoretic and immunochemical investigations with the newly obtained sh_1 alleles indicate that some of these mutants, and particularly those which are CRM^+, are due to the true gene mutations. This interpretation is further substantiated by the positive evidence of interallelic complementation among the CRM^+ mutants

(CHOUREY, in preparation). All the CRM$^-$ mutants are noncomplementing. A drastic alteration of the genetic material, as in the case of deletion, is not likely to produce complementing mutants.

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