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Genetic relationship between *o6* and *pro-1* mutants in maize

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Summary. This paper reports that the *opaque-6 (o6)* mutation of maize, which causes seedling lethality and interferes in the endosperm with the synthesis of zeins and b-32 protein, is a proline requiring mutant functionally allelic to *proline-1 (pro-1)*. Furthermore, immunological studies on the b-32 content of ten independently originated *o6* and *pro-1* alleles demonstrated that four alleles contain an apparently normal b-32 protein while the others are either devoid of it or contain trace amounts of cross-reacting proteins of lower molecular weight.

Key words: Proline requiring mutants – b-32 protein – Maize endosperm

Introduction

Zein, the storage protein of maize endosperm, is a mixture of polypeptides encoded by at least five major families of structural genes (see Soave and Salamini 1984, for a review). Zein synthesis commences in the endosperm around 15 days after pollination (DAP) and continues linearly until 40 DAP. Several loci exert a positive control on the rate of zein deposition; the mutant alleles at these loci reduce zein level to a different extent and confer an opaque or floury phenotype to the endosperm. These mutants improve the nutritional value of maize flours by reducing the zein level so that the contribution of the other endosperm proteins (relatively richer in essential amino acids) to the total amino acid content is higher than in the wild-type (reviewed in Nelson and Burr 1973). The molecular mechanism regulating the rate of zein deposition is still unknown. In principle, however, the mutations can influence either the transcription of zein genes, or the translation of the corresponding mRNAs, or the availability of some amino acids required in large amount for zein synthesis.

Previous studies proved that two of these mutants, *opaque-2 (o2)* and *opaque-6 (o6)*, in addition to decreasing the amount of zein, lack an endosperm specific protein (b-32 protein, MW = 32,000) which is a non zein monomeric protein located in the soluble cytoplasm, temporally and quantitatively coordinated in its expression with zeins (Soave et al. 1981; Di Fonzo et al. 1986). The biological function of b-32 is presently unknown. Genetic study had shown that *O6* might be the structural gene for b-32 (Soave et al. 1981). The finding, however, that *o6/o6* seeds are seedling lethals (Ma and Nelson 1975), while *o2/o2* are completely vital, suggests that the lack of b-32 is not in itself the cause of the lethality.

In the present work we demonstrate that *o6/o6* seedlings can be repaired by L-proline and that the *o6* mutation is allelic to *pro-1*, a proline-requiring mutant already described by Gavazzi et al. (1975). In addition, we discovered that, out of 10 independently originated *o6* and *pro-1* alleles, some are devoid of b-32 while others contain normal levels of the protein.

Materials and methods

Stocks

The *o6* and *o2* alleles used in this work were from the collection of the Istituto Sperimentale per la Cerealicoltura, Sezione di Bergamo; the *pro-1* alleles were from the Department of Genetics, University of Milano. The *o6* allele previously studied (Soave et al. 1981) and here indicated as *o6-1* was received from the Maize Genetic Cooperative Stock Center, Urbana, Illinois and converted by repeated backcrosses to the inbred A69Y. *o6-11* and *o6-15* are two independently originated *o6* alleles; the first arose as a spontaneous mutation in an Italian variety and was converted to A69Y and the second

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was detected in the M2 population of A69Y seeds treated with ethylmethanolsulphonate (EMS). The origin of the *pro-1* and *o2* alleles studied in this work has been previously reported (Racchi et al. 1981; Soave et al. 1981; Motto et al. 1986). The location of *pro-1* on chromosome 8 was ascertained by means of crosses with the complete set of TB-A translocations (Gavazzi et al. 1978).

Cultures

Mature wildtype and mutant seeds from segregating ears obtained by selfing +/*o6-1*, +/*o6-11* and +/*o6-15* plants were sterilized with 4% calcium hypochlorite and germinated in Petri dishes for 48 h at 30 °C. Embryos were then dissected and cultivated under the conditions described by Gavazzi et al. (1975) in the presence or absence of L-proline. After eight days of culture the seedling length and dry weight were measured.

Allelism test

Plants grown from non mutant seeds obtained upon selfing +/*o6-1* and +/*pro-1* individuals were crossed inter sè. Complementation was ascertained by endosperm and leaf morphology, seedling lethality and recovery on L-proline enriched medium.

Protein extraction and immunological assays

Saline soluble proteins were extracted from 100 mg powdered mature seeds (deprived of embryos) with 1 ml of PBS (10 mM sodium phosphate buffer pH 7.2, 15 mM NaCl, 1 mM phenylmethylsulfonylfluoride) at 4 °C for 2 h under continuous shaking. Extracts were centrifuged at 30,000 × g for 15 min and the supernatants frozen. Protein concentration was determined according to Peterson (1977).

b-32 antibody preparation, immunoelectrophoresis and purification of b-32 related material on b-32 antibody bound Sepharose 4 B columns was done as previously described (Soave et al. 1981). SDS-polyacrylamide gel electrophoresis was performed on 15% polyacrylamide gel slabs according to Laemmli (1970) and the gels were stained with Coomassie R-250.

Results

o6 is a proline requiring mutant allelic to pro1

o6-1, *o6-11* and *o6-15* are recessive alleles characterized, at the seed level, by an opaque phenotype associated with a collapsed endosperm morphology. Zein level always appears to be reduced but in varying degrees in the three alleles (around 10% of the corresponding wildtype for *o6-1*, 50% for *o6-11* and 30% for *o6-15*). No preferential inhibition of some zein polypeptides is observed. After germination, the seedlings exhibit striation of leaves, reduced growth rate and lethality at the second or third leaf stage.

Since this phenotype resembles that of *pro-1*, a proline requiring mutant, we tested the effect of increasing amounts of L-proline in the culture medium on the growth of homozygous *o6-1* embryos (Fig. 1). In the presence of increasing amounts of proline a restoration of the phenotype is observed. A complete recovery was obtained in the presence of 320 mg/l of L-proline. Table 1 indicates the shoot length and dry weight of three *o6* alleles cultivated in the presence of 320 mg/l proline. In the absence of proline the shoot length and weight of *o6-1* are severely reduced as in *o6-11* and *o6-15* even if at a lower extent. All three alleles showed the abnormal striated phenotype. In the presence of proline, the three mutants recover both in terms of growth and greening of leaves.

The demonstration of the proline requirement of the *o6* mutants prompted us to test the allelism between *o6* and *pro1*. Plants grown from non-mutant seeds obtained upon selfing +/*o6-1* and +/*pro1-1* plants were crossed inter se. Out of 40 crosses, 24 gave ears with a 3 to 1 segregation for normal and opaque-

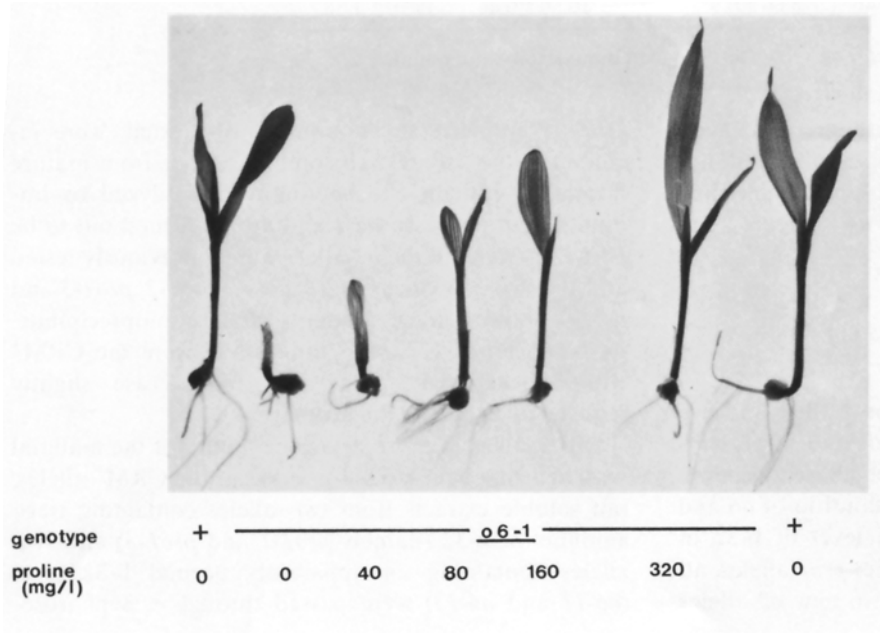

Fig. 1. Normal and mutant seeds, obtained upon selfing +/*o6-1* plants, were sterilized with hypochlorite and germinated in Petri dishes for 48 h. Excised embryos were cultivated for eight days in D-medium (Gavazzi et al. 1975) supplemented with various amounts of proline

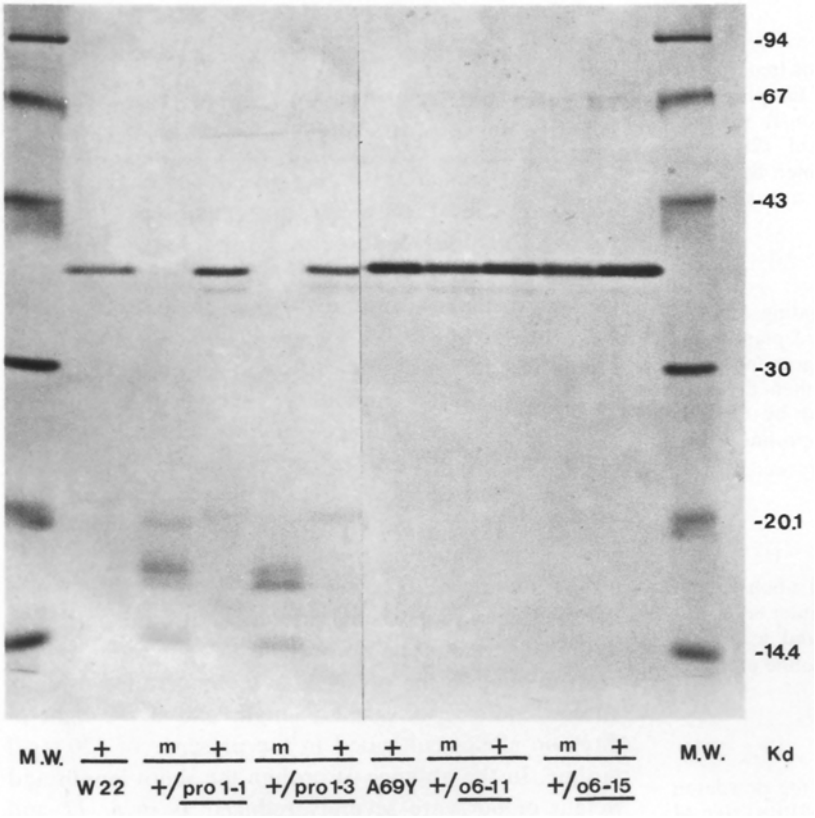

Fig. 2. Fifty mature endosperms from normal and mutant seeds, obtained upon selfing *+/pro1-1* and *+/pro1-3* (W22 background) and *+/o6-11*, *+/o6-15* (A69Y background) plants, were powdered and extracted with PBS. The extract was passed on a Sepharose-bound b-32 antibody column and retained protein material was analyzed by SDS gel electrophoresis. Seven μg samples were loaded on each lane. The gel was stained with Coomassie Brillant Blue R-250. m = mutant seeds; + = normal seeds. W22 and A69Y refer to extracts from the standard inbred lines (not segregating mutant seeds). On the *left and right borders*, MW marker

Table 1. Effects of L-proline on the growth of *o6* mutant maize embryos

		Shoot length (cm)		Shoot dry wt (mg)	
	L-proline	0	320 mg/l	0	320 mg/l
o6-1	normal	10.27±0.47	11.00±0.37	26.67±1.17	21.88±0.11
	mutant	1.12±0.09	5.26±0.45	2.61±0.34	8.90±0.30
o6-11	normal	10.15±0.33	9.93±0.41	20.50±0.35	19.40±1.00
	mutant	5.85±0.28	10.22±0.44	16.20±0.60	17.85±0.42
o6-15	normal	12.73±0.50	13.37±0.50	21.00±0.96	23.50±0.93
	mutant	3.58±0.46	8.66±0.97	10.50±0.46	15.70±1.38

[a] Not less than 30 embryos per experiment were grown as described in materials and methods

collapsed seeds yielding seedlings with striated leaves, reduced growth and lethality. In addition, seedlings turn green by growing embryos on L-proline enriched medium. This result is expected if *o6* and *pro1* are functionally allelic.

b-32 protein in pro1 (o6) and in o2 alleles of independent origin

Soave et al. (1981) reported that the *o6-1* allele and five *o2* alleles of independent origin are devoid of proteins cross reacting with b-32 antibodies in the endosperm. Having demonstrated the allelic relationship of *o6* and *pro1* mutations, we investigated the level of b-32 in *o6-1*, *o6-11*, *o6-15* alleles and in 7 other *pro1* alleles, all of independent origin. In addition, two new *o2* alleles [*(o2Ref* and *o2m(r)*], not previously tested, were included in the survey. Salt soluble extracts from mature normal or mutant endosperms were analyzed by immunoelectrophoresis: *o6-1* and *pro1-5* turned out to be CRM^- as were all the *o2* alleles (the 5 previously tested and the two new ones) while *pro1-1*, *pro1-2*, *pro1-3* and *pro1-7* showed trace amounts of immunoprecipitate. *pro1-342*, *pro1-1121*, *o6-11* and *o6-15* were the CRM^+ with a peak area similar or, in some case, slightly reduced in respect to the normal.

To analyze in more detail the nature of the material cross reacting with b-32 antibodies in the CRM^+ alleles, salt soluble extracts from two alleles containing trace amounts of b-32 (namely *pro1-1* and *pro1-3*) and two alleles containing an apparently normal b-32 level (*o6-11* and *o6-15*) were passed through a Sepharose-

bound b-32 antibody column. Cross-reacting material was retained by the column in amounts of 15–25 μg per seed for normal, *o6-11* and *o6-15* seeds and 3–5 μg for *pro1-1* and *pro1-3* seeds. When the retained proteins were analyzed by SDS gel electrophoresis, in *pro1-1* and *pro1-3* a band comigrating with b-32 was absent and only some lower MW peptides appeared. In *o6-11* and *o6-15* retained protein, however, an apparently normal b-32 was present (Fig. 2). Western blotting analyses of extracts from different *o6* and *pro-1* alleles essentially confirmed the previous results (not shown).

Discussion

The major findings reported in this paper are: 1) the demonstration that *o6* is a proline requiring mutant functionally allelic to *pro-1*; 2) the stringent control of *O2* on b-32 production; and 3) the separation of the *o6* and *pro1* alleles tested on the basis of their b-32 content: some being devoid of the protein while others contain it (at least judging by the presence of a immunoreacting protein comigrating with b-32).

The lesion at the basis of the *o6* and *pro1* and the action of proline in resuming the growth of the mutant seedlings is still unclear. Obvious defects in proline metabolism in the mutants have been ruled out since they appear able to synthesize proline from either glutamic acid and ornithine and to incorporate proline in proteins (Bertani et al. 1980; Dierks-Ventling and Tonelli 1982). Proline respiration is also apparently unaltered in *pro-1* and *o6* seedlings (Dierks-Ventling and Tonelli 1982 and our unpublished observations for *o6*). Addition of L-proline to the culture medium results, however, in the resumption of growth of the mutant as proved by embryo, shoot, root and callus cultures (Gavazzi et al. 1975; Racchi et al. 1978; Tonelli et al. 1982). When, however, developing mutant kernels, dissected from immature ears as described by Gengenbach (1977), were cultivated in presence of proline no repair of endosperm phenotype was achieved (Tonelli et al. 1984). This result could be due to the poor transport of proline through the cob and pedicel tissues to the developing seed as indicated by Shimamoto and Nelson (1981). Further studies are then needed to establish the role of proline in the mutant.

The relationships between b-32 protein and the *O2* and *O6-Pro-1* genes indicate that a functional *O2* allele is an absolute requirement for b-32 production. As a matter of fact, none of the 7 independently originated *o2* alleles produced b-32 even in trace amounts.

The picture for *O6-Pro1* is more complex since four out of ten *o6* and *pro1* alleles studied contain b-32 and the others are either completely CRM^- or contain trace amounts of cross-reacting proteins of lower molecular weight. This finding could be consistent with the hypothesis of *O6-Pro1* being the structural gene for b-32, admitting that the normal b-32 level in some *o6* and *pro1* alleles is a non functional protein while, in the others, b-32 is either absent or produced in an unstable form. The major objection to this hypothesis remains, however, the lethality of all the *o6* and *pro1* alleles up to now isolated. A lack of b-32 (or of its function) in fact can not account for seedling lethality because 1) *o2* seeds lack b-32 in the endosperms but are completely viable and 2) b-32 protein is not found in wildtype seedlings even in trace amounts.

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