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Effect of RNase on Zein Synthesis in Endosperms of *brittle-2;opaque-2* Maize Double Mutant[1]

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ABSTRACT

Posttranscriptional degradation of zein mRNAs in *Zea mays*, presumably caused by high RNase activity, may be involved in reducing zein synthesis in the endosperms of zein-deficient mutants. Zein proteins prepared from mature endosperms of zein-deficient double mutants such as *brittle-2;opaque-2* showed an almost complete blockage of the synthesis of two major zein components, Z1 and Z2. However, these two components were present prior to 12 days postpollination. The changes in quantity and quality of zein synthesis in these double mutants correlated well with the rising RNase levels in the developing endosperm. In addition to its effect on zein synthesis, high RNase activity also influenced the stability of rRNAs in the *brittle-2;opaque-2* double mutant at the later stages of endosperm development. These results suggest that RNase played a role in regulating zein synthesis in developing zein-deficient mutant endosperms.

Zein proteins, major storage proteins of maize, may account for more than 60% of total endosperm protein (11). When characterized by SDS-polyacrylamide gel electrophoresis, the proteins are found to be composed of six components with 22,000 (Z1) and 19,000 (Z2) mol wt proteins as the two major components (2, 15, 19). These proteins are synthesized from membrane-bound polyribosomes (2, 14) and are coded for by a complex multigene family (3, 9, 20, 28, 29).

A mutation at the *o2* locus reduces total zein synthesis (17) and strongly suppresses the synthesis of Z1 component (15, 19). Accordingly, membrane-bound polyribosomes (12) and zein mRNA (21) isolated from *o2* mutants reduced synthesis of this component in a cell-free system because there was less mRNA specific for the Z1 component (21).

Maize starch-deficient mutants, such as *brittle*-2 (*bt2*), and *shrunken*-2 (*sh2*), which have low starch content and high sucrose accumulation in the endosperm (24), also decrease zein synthesis (27). These mutants contained as little zein as the *o2* genotype (6, 18). When each of these starch-deficient mutants was combined with *o2*, the double mutant combinations synergistically reduced zein synthesis; the double mutants accumulated very little zein (18, 25). The fact that all these double mutants act synergistically to prevent zein synthesis make it doubtful that a specific transcriptional control is involved, especially since *sh2* and *bt2* have been established as structural genes for ADP-glucose pyrophosphorylase (10, 24). However, all these double mutants contain high sucrose concentrations and high RNase activity in their endosperms. The specific effect of high sucrose concentration in maize endosperm on protein synthesis is unknown. Tsai et al. (25) suggested that a high sucrose concentration in the endosperm may affect the interaction of polyribosomes with the ER, hence the zein mRNAs are more sensitive to high RNase activity. Other mRNAs specific for enzymes are presumably actively engaged in translation and are translated at early stage of development before the rapid increase of RNase activity (26), thus are less affected.

To test the hypothesis that high levels of RNase activity affect zein synthesis, changes in RNase activity in developing endosperms of *bt2;02* double mutant were correlated with its zein quality and quantity. In addition, the stability of rRNA in the developing endosperms of normal and double mutant was determined.

MATERIALS AND METHODS

Collection of Maize Materials and Dry Weight Determination. Maize inbred Oh43 and its isogenic mutants *bt2;o2* were grown at the Purdue Agronomy Farm in 1979, 1980, and 1981. All plants were self-pollinated. Ear samples were harvested at 8 through 50 d after pollination. Ears were frozen in liquid nitrogen immediately after harvesting in the field. Intact kernels were removed from cobs, bulked (at least 10 ears for each stage) and stored at −80°C.

Zein Protein Extraction and Determination. Endosperm samples were lyophilized to constant weights, ground to powder, and then defatted for 48 h with hexanes in a Soxhlet apparatus. The defatted samples were sequentially extracted into four protein fractions (albumin, globulin, zein, and glutelin) according to the method of Tsai (26). Protein in various fractions was determined by the Lowry method (16).

Measurement of RNase Activity. Frozen endosperms were weighed, homogenized in three parts (w/v) of 0.05 M Tris-HCl buffer, pH 7.5, with a Polytron homogenizer and centrifuged at 500*g* for 15 min (4). RNase was assayed as described previously (25). One unit of activity was defined as the amount of enzyme preparation which caused a change in absorbance of 0.1 o.d. units/30 min.

Gel Electrophoresis. Extracted zein was lyophilized, dissolved in sample buffer (0.025 M Tris-glycine, pH 8.3, 1% SDS, 1% 2-mercaptoethanol, 0.004% bromophenol blue, and 10% glycerol), and analyzed on a slab gel by an SDS-polyacrylamide gel electrophoresis system similar to that of Laemmli (13). Samples containing approximately 40 μg of endosperm zein protein were placed in one of the sample slots of slab gel. Gels were 1 mm thick and consisted of an 8.5 cm running gel of 12.5% acrylamide (acrylamide/bisacrylamide = 75/1) in a 0.375 M Tris-HCl (pH 8.9), 0.058 mM Temed, and 0.075% SDS. The running gel was overloaded with a 1.5 cm stacking gel of 5% acrylamide. Freshly prepared ammonium persulfate solution was added to a final concentration of 0.035% immediately before each gel layer was poured. Electrophoresis was done at room temperature by using a 15-mamp constant current through the stacking gel and a 25-mamp constant current through the running gel until the tracking dye reached the bottom of the gel. Gels were stained overnight

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in a solution of 0.1% Coomassie blue, 45% methanol, and 9% acetic acid and destained in 15% methanol-7% acetic acid.

***In Vitro* Translation.** Isolation of polyribosomes and *in vitro* protein synthesis were carried out according to the procedures described previously (14).

Total RNA Extraction and Characterization. Ten g of endosperm tissue were added to 15 ml of cold 0.1 M glycine, 0.3 M NaCl, 5 mM K_2HPO_4, pH 9.4, (GPS buffer) (8) and ground with a Polytron homogenizer before being centrifuged at 17,000*g* for 15 min. The supernatant was mixed with one volume of phenol saturated with GPS buffer and centrifuged at 17,000*g* for 15 min. The aqueous upper phase was repeatedly extracted with buffer-saturated phenol until the interphase became clear after centrifugation. RNA was precipitated twice with ethanol. Total RNA isolated was denatured by methylmercuric hydroxide and characterized with agarose gels (1).

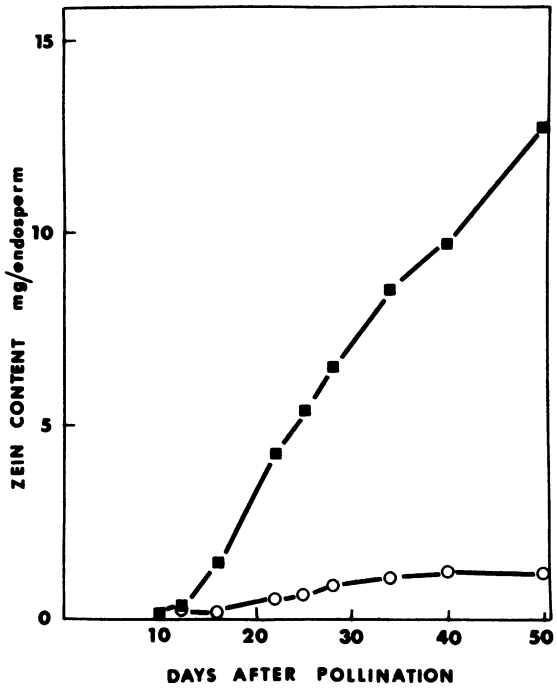

FIG. 1. Zein content of normal (■) and *bt2;o2* (○) endosperms during development.

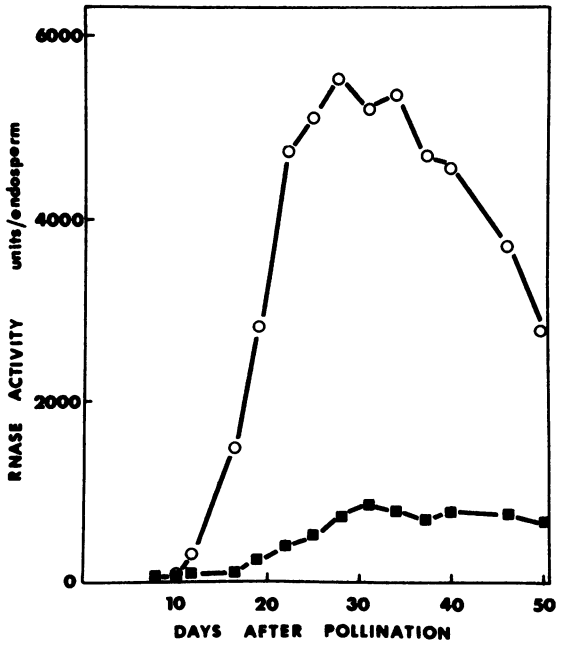

FIG. 2. RNase activities of normal (■) and *bt2;o2* (○) endosperms during development.

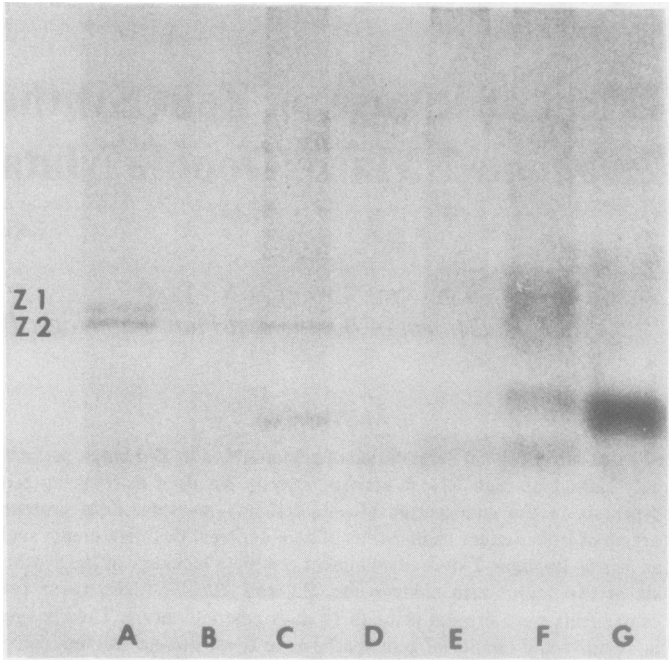

FIG. 3. Gel electrophoretic pattern of zein prepared from developing endosperms of 10-d-old normal (A), and *bt2;o2* mutants at 10 d (B), 11 d (C), 12 d (D), 16 d (E), 22 d (F), and 50 d (G) after pollination.

Hybridization of RNAs with Cloned Complementary DNAs. Zein mRNAs in the developing kernels were located by Northern blot hybridization with ^{32}P-labeled clones of pZ 19.1 (22). The specific activity was 8×10^7 cpm/μg, and about 10 ng/ml was used for hybridization. Total RNAs or total polyribosomal RNAs from five stages of endosperm development were extracted as explained previously, then subjected to electrophoresis on 1.4% agarose gels containing 5 mM methylmercuric hydroxide, as described by Bailey and Davidson (1). The RNA was then transferred to nitrocellulose. The nitrocellulose was prewashed overnight, prehybridized 8 h and then hybridized at 42°C for 18 h in 50% formamide, 5 × SSC (1 × SSC = 0.03 M NaCl, 0.003 M sodium citrate), 20 mM sodium phosphate (pH 6.8), 0.02% BSA, 0.02% Ficoll, 0.02% PVP, 0.1% SDS, 100 μg/ml sheared calf thymus DNA and 5% dextran sulfate (23). After hybridization, the filter was washed two times at room temperature in 1 × SSC and 0.1% SDS followed by two times at 65°C in the same buffer. The filter was blotted dry and autoradiographed for 7 d (21).

RESULTS

Zein Protein Contents in Developing Endosperms. Zein synthesis in normal endosperm started rapidly at 12 d after pollination, while the double mutant *bt2;o2* contained small amounts of zein protein throughout development (Fig. 1). At 50 d after pollination, *bt2;o2* had only 10% of normal amount of zein.

RNase Activity in Developing Endosperms. RNase activities in endosperms of normal and *bt2;o2* double mutant were similar prior to 12 d postpollination. For the normal genotype, in contrast to the double mutant, RNase activity increased only slightly throughout endosperm development (Fig. 2), and RNase activity in the double mutant was about 6 to 10 times higher than those of the normal genotype.

Table I. *In Vitro Translation of Developing Normal and bt2;o2 Membrane-Bound Polyribosomes*

Genotype	Time after Pollination	Total TCA Insoluble		Ethanol Soluble	
	d	*cpm/A_{260}·30 min*	*cpm/kernel·30 min*	*cpm/A_{260}·30 min*	*cpm/kernel·30 min*
Normal	10	17960	1370	2720	207
	22	38635	7265	19630	3690
bt2;o2	10	16970	1410	2070	175
	22	13530	1735	2810	360

Table II. *Total RNA Contents and Ratio in the Developing Normal and bt2;o2 Endosperms*

Time after Pollination	Normal	*bt2;o2*	*bt2;o2*/Normal
d	*A_{260}/kernel*		
10	0.63	0.76	1.2
12	1.04	0.85	0.82
16	4.40	3.30	0.75
22	5.17	3.51	0.67
28	4.48	2.95	0.66
34	4.72	2.43	0.51
40	3.79	1.53	0.40

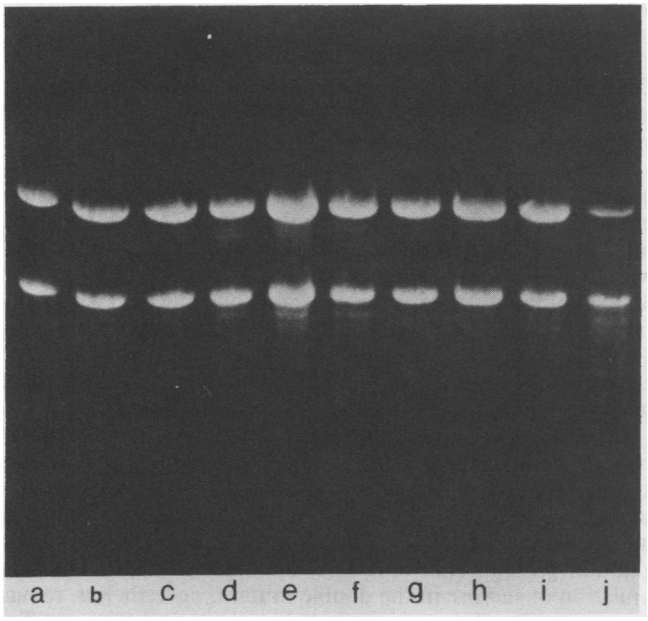

FIG. 4. Gel electrophoretic pattern of total RNAs extracted from normal endosperms at 10 d (a), 12 d (c), 16 d (e), 22 d (g), and 34 d (i) after pollination; and *bt2;o2* mutant endosperms at 10 d (b), 12 d (d), 16 d (f), 22 d (h), and 34 d (j) after pollination.

SDS-polyacrylamide Gel Electrophoretic Patterns of Zein Prepared from Developing Endosperms. Zein proteins extracted from developing normal endosperms showed two major bands, Z1 and Z2 (Fig. 3). The *bt2;o2* double mutant endosperms, on the other hand, had zein patterns different from the normal genotypes with the exception of early stage development (10 and 11 d postpollination), which showed a pattern characteristic of *o2* (Fig. 3, B and C). The synthesis of these two major components in the double mutant was suppressed 12 d after pollination and thereafter, and the double mutant accumulated alcohol-soluble proteins with smaller mol wt (Fig. 3, D, E, F and G).

***In Vitro* Translation of Polyribosomes.** *In vitro* translation of polyribosomes in the wheat germ system showed that normal and *bt2;o2* endosperms at 10 d after pollination had similar amounts (cpm/A_{260} or cpm/kernel) of translatable messages for total protein and ethanol-soluble proteins. However, at 22 d after pollination, the *bt2;o2* endosperms contained only about 20% of translatable messages for total protein and 15% for ethanol-soluble protein of the normal genotype, respectively (Table I).

Total RNA in Developing Endosperms. Total RNA content of the developing endosperms of *bt2;o2* and normal increased from 10 d postpollination and reached maxima at about 22 d postpollination. However, the RNA ratio of *bt2;o2* to normal decreased progressively as the endosperm developed (Table II).

When total RNA extracted from these two genotypes was separated on methylmercuric hydroxide gel, both genotypes revealed numerous bands, and appeared to be identical in pattern prior to 22 d postpollination (Fig. 4). The two major bands were 28S and 18S rRNAs, and the ratio of 28S to 18S was about 2 to 1. However, at 34 d postpollination, rRNAs in the double mutant (Fig. 4j) were degraded as indicated by changes in intensities and ratio of the rRNAs. Several smaller mol wt RNA components appeared concomitantly. The degradation of rRNAs observed in the later stages of mutant endosperm was not an artifact due to preparation. At 40 d postpollination, the double mutant did not contain appreciable amounts of rRNA. The RNA gel profile from the co-extracted sample that contained 22-d-old normal and 40-d-old mutant kernels did not reveal rRNA degradation (data not shown).

Hybridization of RNAs with Cloned Zein Complementary DNAs. Northern blot hybridization of nick translated pZ 19.1 plasmid DNA probe with total RNAs prepared from normal or *bt2;o2* endosperms showed that zein mRNAs of normal genotype were first detected 10 d after pollination. Sharply increased levels of hybridization were shown for the normal genotype at 12, 16, 22, 34, and 40 d after pollination (Fig. 5). Because of the homologous nature of zein mRNAs, the probe hybridized with different sizes of zein mRNAs. The hybridizable smear may be caused by the over-exposure of autoradiograph which could also account for the reaction with rRNA. The double mutant, on the other hand, contained small amounts of zein mRNA, like normal, at 10 d postpollination but no zein mRNA was detectable thereafter. Both 18S and 28S rRNAs showed some ability of hybridization with the probe. A high mol wt nucleic acid that hybridized with the probe was observed in both normal and *bt2;o2* mutant at all stages of endosperm development (Fig. 5).

DISCUSSION

The mutation at the *o2* locus has been shown to produce pleiotropic effects in increasing RNase activity (4, 30). This high RNase activity in the *o2* was further enhanced by the presence of high levels of sucrose; therefore, the double mutant combination between *o2* and each of the starch-deficient mutants had RNase activity about 2 times higher than *o2* alone or 10 times greater than the normal (Fig. 2). Although it was previously suggested that an elevated RNase activity might not be the key to the total reduction of zein synthesis in the *o2* mutant (5), the possibility that a high RNase activity may cause the early termination of zein synthesis in this mutant cannot be ruled out. Unlike albumin, the bulk of zein is synthesized at the later stages of endosperm development which coincides with an abrupt

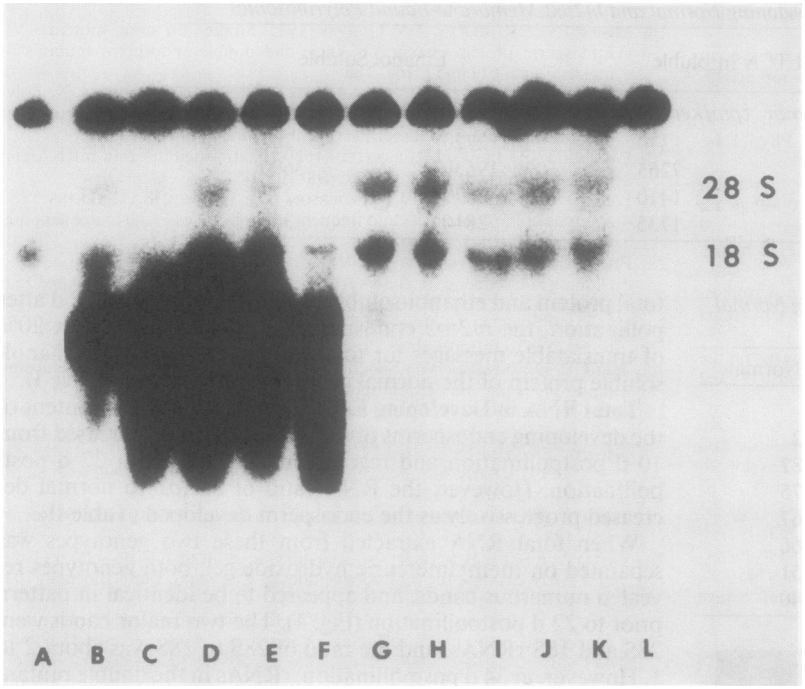

FIG. 5. Autoradiogram of Northern blot hybridization of plasmid pZ 19.1 DNA and total RNAs of normal and *bt2;o2* mutant at 10 d (A and G), 12 d (B and H), 16 d (C and I), 22 d (D and J), and 34 d (E and K), and 40 d (F and L) after pollination.

increase in RNase activity of the mutants (26).

In all the starch-forming mutants tested, *i.e. bt2*, *bt2;o2*, *bt*, *bt;o2*, *sh2*, *su*, *su;o2*, *du*, *du;o2*, and *wx;o2*, the RNase activity at 22 d postpollination correlated negatively ($r = -0.79$) with amounts of zein accumulation (Lee and Tsai, unpublished data). These observations further support the notion that a high RNase activity may affect zein synthesis in the endosperm of *bt2;o2*.

The *bt2;o2* mutant had an amount of translatable zein mRNAs similar to that of the normal genotype at 10 d after pollination, but the mutant had only 15% of normal translatable zein mRNAs at 22 d after pollination (Table I). This agrees with a previous observation that the amount of ethanol-soluble proteins synthesized *in vitro* by the 22-d-old membrane-bound polyribosomes of the *bt2;02* double mutant was about 10% of the normal control (25). Prior to 12 d postpollination, the *bt2;o2* double mutant contained a normal level of RNase and this double mutant produces a "normal" quantity and quality of zein (Figs. 1 and 3). As the mutant endosperm developed, RNase activity increased rapidly, and zein synthesis in the endosperm was substantially reduced, especially the synthesis of Z1 and Z2 components. This observation was consistent with the notion that high RNase activity affected zein synthesis in the endosperm.

In the normal genotype, zein mRNAs were first detected at 10 d after pollination. The increase of hybridization levels in the normal genotype coincided with the synthesis of zein proteins. However, in the *bt2;o2* mutant, zein mRNAs were only detected at 10 d postpollination, and later on, when RNase activity in the double mutant was much higher than in its normal genotype, zein mRNAs in the double mutant were hardly detected. However, a high mol wt nucleic acid was hybridized with zein cDNA as shown in Figure 5. The nature of this high mol wt nucleic acid remains unknown. Although zein genes do not contain an intervening sequence (22, 29), they exist in multiple copies in the genome (21, 28); therefore, this high mol wt nucleic acid may represent heterogenous nuclear RNA (hnRNA). If so, the observation that the double mutant *bt2;o2* contained this hnRNA in all samples tested further support the notion that the deficiency of Z1 and Z2 in the double mutant was not due to transcriptional control. However, we cannot rule out the possibility that the high mol wt band seen in all tracks may be due to DNA contamination. The RNA extraction procedure employed may not remove DNA from the samples although there is no ethidium bromide staining in Figure 4 at this position.

Attempts to identify the presence of fragmented zein mRNAs, probable degradation products of zein mRNAs, at the early stages of the double mutant endosperm development were unsuccessful. Although no detectable hybridization of small mol wt RNAs with zein cDNAs was observed, degradation of rRNAs in the double mutant were observed (Fig. 4), and coincided with reduction in total RNA content of the developing endosperms. This degradation of rRNA in the double mutant also coincided with an early cessation of zein and glutelin synthesis, which are normally synthesized at later stages of development.

Because the *bt2* gene conditions a low ADP-glucose pyrophosphorylase activity (7, 10, 24), the double mutant *bt2;o2* produces a small amount of starch but accumulates a large quantity of sucrose in the endosperm (24). This change in carbohydrate metabolism causes little effect on the synthesis of albumin and globulin proteins (Lee and Tsai, unpublished data). The accumulation of sucrose in the double mutant, nevertheless, reduces the movement of the amino acids into the kernel (Lee and Tsai, unpublished data). Since zein is the only protein fraction in the endosperm dynamically responding to the amino acid supplies (27), the reduced movement of amino acids may in part explain a reduction of zein content in the double mutant. However, this study suggests that a high RNase activity in the double mutant may play a primary role in preventing zein synthesis by degrading nontranslocatable zein mRNAs and rRNA.

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