Ribonuclease Activity in the Developing Seeds of Normal and Opaque-2 Maize

Abstract. *During development, the ribonuclease activity of maize endosperm homozygous for the* opaque-2 *gene increased earlier and, after a longer period of exponential increase, was much higher finally than in normal endosperm. The maximum difference was about sixfold and occurred 22 to 25 days after pollination. Heterozygotes on day 23 did not show a comparable increase in endosperm activity. No difference in the activity ratio (*pH *6.0 : pH 5.2) was found between normal and* opaque-2 *endosperm, indicating that the increase in activity in* opaque-2 *was due solely to an increase in ribonuclease A. The ribonuclease activity of* opaque-2 *embryos increased more rapidly than that of normal embryos, but the final activity was the same in each case.*

The *opaque-2* (o_2) mutant in maize was discovered by Singleton and Jones (*1*). It is a recessive gene which manifests itself phenotypically only in the homozygous state in the triploid nucleus of maize endosperm, where it gives an opaque character to the usually translucent mature seed. Mertz, Bates, and Nelson (*2*) reported that *opaque-2* also produced a very marked modification in the amino acid composition of the mature endosperm, resulting in a striking improvement in the nutritional quality of maize seed as a source of protein. The modified amino acid composition occurs only in kernels that are $o_2/o_2/o_2$ (*3*).

The mutant gene causes a large reduction in the amount of the complex of alcohol-soluble proteins found in maize endosperm known as zein, both relative to other proteins (*2*, *4*–*7*) and on an absolute basis (*4*, *5*, *7*). The relative amounts of the proteins of the zein complex are changed also (*4*, *6*), but there is some conflict concerning reported changes in the amino acid composition of zein (*2*, *6*). Other groups of proteins are also changed in amount and in amino acid composition in mature (*4*) and developing (*5*, *7*) seed. These changes, together with the reduced zein synthesis, seem primarily responsible for the improved nutritional quality of the seed. A second mutant gene, *floury-2*, isolated by Mumm (*1*), modifies the endospermal amino acid composition in a direction similar to that of *opaque-2* (*8*).

We now report changes in the ribonuclease content of *opaque-2* and normal seed during development and discuss their relation to the mutant gene and to the modifications in endospermal protein and amino acid composition that the gene causes. An inbred line of maize W64A, homozygous nonopaque (normal), and the corresponding mutant W64A, homozygous *opaque-2*, were grown on adjoining plots at the Purdue University Agronomy Farm, Lafayette, Indiana, and self-pollinated during the summer of 1965. The W64Ao_2 was originally isolated as a spontaneous mutant from W64A+ and presumably differs from the parent line only at the o_2 locus. Hence, any phenotypic differences between parent and mutant arise solely as a consequence of the allelic state at the o_2 locus. Ears were harvested at various times after pollination, and the kernels were cut from the ears in the field and immediately placed in dry ice. The frozen kernels were subsequently stored at −20°C.

The W64A material (23 days after pollination) used in the dosage-series experiment was grown in the greenhouse during the early months of 1966 and stored frozen. The homozygous *opaque-2* and the two heterozygous *opaque-2* samples were harvested the same day; the homozygous nonopaque sample was the same age, but was pollinated and harvested 13 days later.

Except for the use of whole kernels (10 and 15 days after pollination), all enzyme extracts were prepared either from endosperm or from embryo tissue dissected from the frozen grain. Either 50 endosperms or 50 kernels (10- and 15-day) were homogenized in 0.05*M* tris(hydroxymethyl)aminomethane (tris) buffer, *p*H 7.5, containing 0.5*M* KCl in a VirTis 45 homogenizer at top speed for 2 minutes at 0°C. The homogenate was made to an appropriate volume and centrifuged at 500*g* for 15 minutes in the cold, and the supernatants, suitably diluted with tris-KCl buffer, were used for assay. It was necessary to soak the more mature (greater than 40 days) endosperm tissue for several hours in tris-KCl buffer at 0°C to obtain adequate disintegration of the tissue. The overall procedure is essentially the same as that of Ingle and Hageman (*9*).

For embryo extracts, 100 embryos were washed three times with tris-KCl buffer and ground in the same buffer with a chilled pestle and mortar. The resulting paste was further triturated in an all-glass, Duall tissue grinder (Kontes Glass Company), made to volume, and centrifuged as for endosperm tissue.

No appreciable difference in ribonuclease activity was noted between whole homogenates and the corresponding 500*g* supernatants. We used the ribonuclease assay of Tuve and Anfinsen (*10*) as modified by Wilson (*11*), except that we replaced uranyl nitrate with uranyl acetate in the perchloric acid solution used to stop the reaction. In addition, a volume of enzyme equal to that in the sample tube was added to the control tube immediately after the addition of perchloric acid. Thus, each sample had a corresponding control; both sample and control were run in duplicate. Normal and *opaque-2* samples of the same age were always assayed simultaneously and, in the case of endosperm, the assays were performed at *p*H 5.2, 5.8, and 6.0 in the

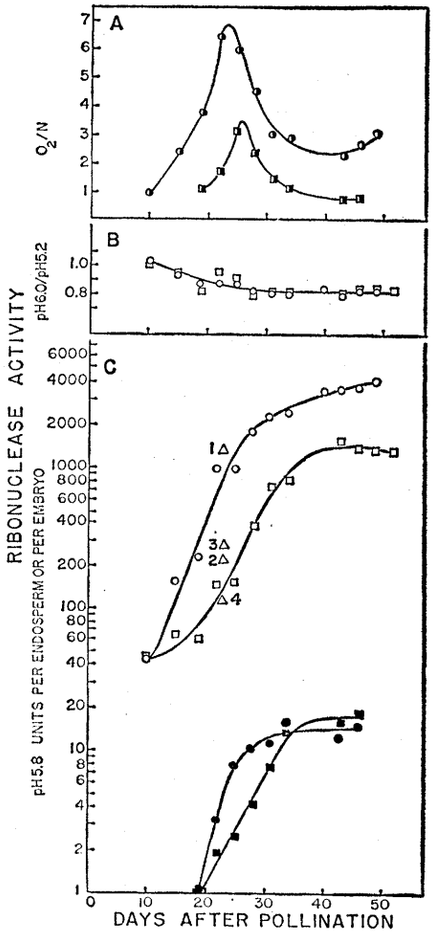

Fig. 1. Ribonuclease activity of normal (+/+/+) and *opaque-2* ($o_2/o_2/o_2$) maize tissue during development. (A) Endosperm, ◑——◑; embryo, ◧——◧. (B) *Opaque-2* endosperm, ○——○; normal endosperm, □——□. (C) *Opaque-2* endosperm, ○——○; normal endosperm, □——□; *opaque-2* embryo, ●——●; normal embryo, ■——■; dosage series (△), 1($o_2/o_2/o_2$), 2(o_2/o_2/+), 3(+/+/o_2), 4(+/+/+).

same experiment. The activity at *p*H 5.8 was used as a measure of total ribonuclease (*9*). The substrate ribonucleic acid (Nutritional Biochemicals) was dialyzed for 48 hours against four changes of deionized water before use. The unit of ribonuclease activity is the amount of enzyme which, under conditions of the assay, produces a change in absorbance of 0.1 when measured at 260 $m\mu$ in a 1-cm cuvette (*11*).

The pattern of ribonuclease activity (*p*H 5.8) in the developing endosperm and embryo of normal and *opaque-2* maize is shown in Fig. 1C, together with the ratio of the activities of *opaque-2* to normal for each tissue (Fig. 1A).

Normal endosperm was characterized by a sigmoid curve showing an initial phase of accelerating synthesis, a short exponential phase, and finally a period of decreasing synthetic rate, with some evidence of a loss in total activity as the seed dried out. In contrast, *opaque-2* endosperm immediately entered a prolonged phase of exponential increase at a rate somewhat higher than that of normal endosperm. The final phase also differed from normal in that the ribonuclease activity in *opaque-2* reached a considerably higher level and was still increasing as late as 49 days after pollination; at 25 days, the activity was that achieved by normal endosperm at its maximum, more than 15 days later. The largest difference between normal and *opaque-2* was in excess of sixfold and occurred between 22 and 25 days after pollination, as shown by the ratio of activities of *opaque-2* to normal (Fig. 1A).

Figure 1B shows the ratio of the ribonuclease activity measured at *p*H 6.0 to the activity at *p*H 5.2 for both normal and *opaque-2* endosperm for the entire experimental period. No notable difference in this ratio is evident between the mutant and normal endosperm, and the fall between 10 and 28 days to a contant value of 0.82 is characteristic of each.

The ribonuclease activity of both normal and *opaque-2* embryos increased exponentially from the start of the experimental period at day 19 and reached approximately the same final value (Fig. 1C). However, the rate of accumulation in *opaque-2* was two to three times greater than in normal embryos. The difference in activity (ratio of *opaque-2* to normal) was never as great as the difference shown by endosperm, and the maximum difference occurred somewhat later (between 25 and 28 days).

Table 1. Ribonuclease activity in endosperm (day 23) of the *opaque-2* dosage series.

Genotype	Ribonuclease activity (unit/endosperm)	Ratio of activities to normal (+/+/+)
$o_2/o_2/o_2$	1270	11.3
$o_2/o_2/+$	218	1.9
$+/+/o_2$	283	2.5
+/+/+	112	1.0

Because *opaque-2* is a recessive gene, it was important to establish whether or not the level of ribonuclease in endosperm reflected this recessive character. The ribonuclease content of 23-day-old endosperm isolated from all four members (+/+/+, $+/+/o_2$, $o_2/o_2/+$, $o_2/o_2/o_2$) of the *opaque-2* dosage series is shown in Table 1. Although not part of the same experiment, the values have also been marked in Fig. 1C for purposes of comparison. The results clearly suggest that the high ribonuclease level is only found when the *opaque-2* gene is in the homozygous condition. We are investigating the question of whether the marked difference between the homozygous normal value and the values of the two heterozygous states is genetically determined or is the result of physiological variation.

Our results raise a number of fundamental questions to which only tentative answers may be offered at the present time. The first of these concerns the nature of the increased ribonuclease activity in *opaque-2* endosperm. At least two ribonucleases, designated A and B, have been described for maize (*11*). Only one of these, ribonuclease A, was reported as being present in the endosperm; this was based on evidence from chromatographic analysis (*12*) and the ratio of the ribonuclease activity at *p*H 6.0 to that at *p*H 5.2 (*9*). The ratios for purified ribonuclease A and B were given as 0.60 and 1.7, respectively, although Ingle and Hageman (*9*) pointed out that the actual value of the ratio is subject to variation depending upon the salt concentration and the source of the RNA used as the substrate. The ratios that we observed in both genotypes were somewhat higher than 0.60; this suggests either that the endosperms contained some ribonuclease B, or that the ratio of 0.60 for ribonuclease A did not hold for our experimental conditions. Of most significance, however, is the fact that the two genotypes did not show different ratios. This leads to the conclusion that the increased ribonuclease activity of *opaque-2* endosperm is not the result of an excessive production of ribonuclease B.

It seems unlikely that *opaque-2* lacks an inhibitor which is present in normal endosperm, in view of the fact that mixed extracts of normal and *opaque-2* material (22-day) shows the activity expected from the sum of the individual activities.

A further question concerns the relation between the alleles at the *opaque-2* locus and the observed changes in the kinetics and extent of synthesis of ribonuclease in maize endosperm. The simplest explanation is that the *opaque-2* locus is a regulator of ribonuclease synthesis. Hence, the normal allele produces a repressor substance which is absent or defective in the mutant. Additional evidence is necessary to establish the existence of such a direct relation, however. The increase in ribonuclease may simply reflect a more general increase in non-zein protein synthesis which seems to account for the higher lysine content of *opaque-2* endosperm (*5*). We found that the phosphatase activity (*p*H 5.5) of immature *opaque-2* is also increased over normal by a factor of about two.

Because there is evidence that the endospermal ribonuclease of maize is active in vivo (*13*), an increase of ribonuclease activity occurring at an unusually early stage of endosperm maturation, as in *opaque-2*, could probably modify the protein composition of the endosperm extensively. Thus, it has been suggested that the synthesis of storage proteins in seeds is dependent upon stable messenger RNA's (*14*). Such synthesis would be reduced if it coincided with a period of elevated ribonuclease activity. We have demonstrated earlier that zein synthesis in both genotypes occurs between 10 and 30 days after pollination (*5*, *7*), the period when the excess production of ribonuclease is most apparent in *opaque-2* (Fig. 1). Therefore, the altered ribonuclease activity in *opaque-2* may account, at least in part, for the observed reduction in zein synthesis and the modification of the amino acid composition of the endosperm.

Although the difference between the embryos in the rate of development of ribonuclease activity suggests that the *opaque-2* gene is active there also, no difference in amino acid composition between normal and *opaque-2* embryos has been found (*15*). This in itself may not be significant, however, as the embryo is not primarily a protein-

storage tissue and it contains little alcohol-soluble protein (*16*). An alternative explanation—that there is some passage of ribonuclease from the endosperm into the embryo—gains some support from the observation that the ratio of the activities of *opaque-2* to normal in the embryo reaches a peak several days after that of the endosperm.

ARTHUR DALBY
IOLO AB I. DAVIES*
Departments of Biochemistry
and *Botany and Plant Pathology,*
Purdue University,
Lafayette, Indiana 47907

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* Present address: Department of Biological Chemistry, University of Manchester, Manchester 13, England.

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Ribonuclease Activity in Normal and Opaque-2 Mutant Endosperm of Maize

Abstract. *Three strains of maize heterozygous for the* opaque-2 *mutant gene were self-pollinated to produce ears bearing both normal and opaque kernels. The ribonuclease activity of the* opaque-2 *endosperm was two to more than four times as high as the activity in the normal endosperm.*

The *opaque-2* mutant gene of maize drastically alters the amino acid composition of the endosperm proteins by greatly increasing the lysine content (*1*). The change comes through a replacement of zein by other proteins containing higher amounts of lysine. This finding is of great practical importance because the *opaque-2* seed possesses a higher food value than normal maize does (*2*). There is also a fundamental interest in the operation of a mutant gene that may act either by repressing the synthesis of a single storage protein or by preventing the normal development of the protein granules that are thought to be the site of zein (*3*).

Increasing ribonuclease activities are often associated with increasing concentrations of RNA in rapidly growing plant tissues (*4*). The soluble ribonuclease of maize, which liberates 2′,3′-cyclic nucleotides (*5*), is present in high concentrations in mature endosperm and reportedly increases during the development of the endosperm (*6*). The maximum activities of ribonuclease in endosperm range from about 500 units/g (fresh weight) up to about 1200 units/g for a number of hybrids, while activities of up to 1700 units/g were found in Illinois Low Protein, a strain with low amounts of zein (*7*).

The ribonuclease activities of normal and opaque endosperm were compared in three strains which were segregating for the *opaque-2* gene. The 07N strain was backcrossed three times, the others one time, after it was crossed with a source of the *opaque-2* gene. Then the self-pollinated plants were examined for ears containing both normal and opaque kernels soon after the time that a visible distinction could be made. Fifteen endosperms of each phenotype were separated from hulls and embryos. The endosperms were homogenized in ten times their weight of 0.05*M* sodium citrate buffer, *p*H 5.0, with 0.5*M* KCl, and the homogenates were then centrifuged to remove debris. Ribonuclease was assayed as described previously (*5*), with the units being defined in terms of the increase in the absorbancy at 260 mμ of acid-soluble material as RNA is digested. Dry weights were determined on a sample of ten kernels. The developing endosperms of the opaque kernels contained from 1.7 to 4.4 times as much enzyme per gram of fresh weight as did the normal endosperms from the same ear (Table 1). The lowest ribonuclease activity in the mutants was higher than any yet found in nonopaque kernels. The opaque endosperms had a higher water content at this stage of development than the normal endosperms did, so the differences are even greater when activities are based on units per dry weight. The dry weights of the opaque endosperms are less than those of the normals in all cases.

High activities of ribonuclease were also characteristic of the endosperm from plants homozygous for the *opaque-2* gene in backgrounds of five other strains as compared to the normal endosperm of the same strains. Large differences in activity were noticeable about a month after pollination. The differences between ribonuclease acitvities of normal and opaque endosperm are maintained even after the seed is put through normal drying procedures after harvest, but the activity may be considerably reduced.

The results of a few assays on lots of seed known to be heterozygous for the *opaque* gene and on individual normal-appearing seeds from one of the segregating ears assayed above suggest that there is no dosage effect of the *opaque-2* mutation on activities of ribonuclease; only the homozygous opaque kernels have the high concentration.

Preliminary experiments with disc polyacrylamide-gel electrophoresis (*8*) indicate that the ribonuclease in opaque endosperms is the same as that in normal endosperms.

The role of ribonucleases in normal development is unknown, and we may only speculate on the significance of the high activity in the *opaque-2* mutant. The enzyme might prevent zein synthesis by destroying a specific messenger RNA. However, its concentration in the normal endosperm is high enough to destroy all the RNA within 10 minutes, unless the enzyme is normally inhibited or separated from the RNA.

Table 1. Ribonuclease content of normal and opaque kernels from self-pollinated plants segregating for the *opaque-2* mutant gene. Each pair of assays was made on seed from a single ear.

Strain	Days after pollination	Ribonuclease (units/g*)		Dry weight (%)	
		Normal	Opaque	Normal	Opaque
07N	43	1390	2340	64	53.5
07N	43	1560	3540	66	58
Oh43	47	900	3600	73	69
Oh43	52	730	3250	68	59
Oh43	52	1380	3720	71.5	64
Oh45b	54	510	2110	73	66

* Fresh weight.