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Acid Phosphatases and Seed Shriveling in Triticale[1]

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ABSTRACT

Seed shriveling in the man-made intergeneric hybrid, triticale (× *Triticosecale* Wittmack) appears to be related to increased activity of endosperm acid phosphatases including para-nitrophenyl phosphatase, ATPase, ADPase, phosphatidic phosphatase, and glucose-1-phosphatase that occur specifically at later stages of seed development. These hydrolases may reduce endogenous substrates for starch synthesis, deplete energy supply for maintenance and biosynthesis of tissue growth, and deassemble membrane structures resulting in a partially filled endosperm and localized necrosis. Electrophoretic isozyme patterns of endosperm acid phosphatase exhibited distinctive differences between lines producing plump and shriveled seeds indicating a divergent role of the isozymes in these two different seed conformations.

Plumpness and high starch content in triticale seeds is related to the activity of ADP-G PPase[2] (EC 2.7.7.27) in the endosperm (5). The morphological structure of shriveled kernels was not different from that of plump ones at early stages of the seed formation, though patches of mitotic aberrations were observed in dividing endosperm cells of the shrivel type (12, 16). At the latter stages, cavities or localized necrosis were formed in the endosperm tissue of shriveled kernels, and aleurone cells were disrupted in most of the area surrounding the cavities (12, 16). A premature cessation of grain-filling was found to be a common phenomenon of shrunken seed types in cereals (8, 12, 16). What causes the necrosis and the premature cessation of normal tissue development is of importance in discerning the mechanism of cereal seed shriveling, particularly in early generation hybrids of inter- and intra-specific or intergeneric crosses.

It is known that leaf senescence and degradation are related to increased hydrolase activity, and the tissue senescence is genetically programmed and environmentally modulated (15). Hydrolases may play an important role in the grain shriveling, particularly in the man-made intergeneric hybrids of wheat and rye, triticale (× *Triticosecale* Wittmack). This study characterizes the developmental pattern of several acid hydrolyases in plump and shriveled endosperm of triticale lines.

MATERIALS AND METHODS

Materials. Four winter triticale lines, 6TA 876 and Red Bobs//Daws/Snoopy-P with plump seed characteristics and 6TB 163 and Red Bobs//Daws/Snoopy-S with shriveled seed conformation (5) were used. Seeds were planted using standard cultural practices at the Hyslop Agronomy Farm, Corvallis, OR in October 1981 and 1982. Spikes were harvested weekly after anthesis in the year following planting. Samples collected in 1982 were stored at −80°C in heavy polyethylene bags prior to use. In 1983, samples were collected in 1% sucrose and 0.1% glycine and were used immediately for the determination of seed weight and starch content, and for enzyme extraction and assay (5).

Enzyme Extraction and Assay. All procedures were conducted at 0 to 5°C, except those specially noted. Two seeds from each of 10 spikes were collected each week, and their seed coat was peeled off and the embryo was removed by forceps to obtain endosperms. Two replications of 10 endosperms were each ground in 10 ml grinding buffer containing 50 mM Tris-HCl and 2 mM DTT(pH 7.0) in a mortar with pestle; the slurry was centrifuged at 30,000*g* for 10 min. The supernatant was used for the assays of ADP-G PPase (3, 5) and acid hydrolases (detailed below), and the pellet was assayed for ADP-G starch synthase (EC 2.4.2.21) (4, 5).

Assay of Hydrolases. In a preliminary test, enzyme extracts were assayed at pH 5.5, 7, and 8, with pNPP (artificial substrate), ATP, ADP, AMP (endogenous substrate and potential energy supply), 3-PGA (ADP-G PPase effector), glucose-1-P (substrate for ADP-G PPase), phosphatidylcholine (membrane component), and BSA (soluble protein) as substrates. The rational in selecting these particular pH ranges for testing was that: (a) most acid hydrolases are active at pH 5 to 5.5 (1, 7, 9, 11); (b) the sap of most endosperm tissue of triticale had an average pH of 6.6 to 7.2; (c) the optimal pH for enzymes related to starch synthesis is 8.0 (3–5, 13); and, (d) it is impossible and impractical to test such a large number of substrates at all the pH ranges for eight samples at weekly intervals. Among the three pH conditions, the highest activity was obtained at pH 5.5 for all substrates tested and, therefore, acid hydrolases were assayed in all samples. The activity of AMPase was extremely low and was not studied subsequently.

pNPPase. Two replications of 50 to 100 μl enzyme extract were incubated with rapid shaking at 30°C in 2 ml of 50 mM Na-acetate (pH 5.5) with 0.4 mg pNPP for 5 min and another two replications were incubated for 10 min. After incubation, 1 ml of 0.5 N NaOH was added to each tube to stop the reaction and develop the color. The reaction mixtures were centrifuged at 3,000*g* for 1 min to precipitate protein, and the supernatant was read against a blank at 400 nm. The quantity of *p*-nitrophenol produced was then determined from a standard curve (7).

ATPase, ADPase, 3-PGAase, and G-1-Pase. Six aliquots of 0.25 ml enzyme extract and 1 ml 50 mM Na-acetate containing 5 mM substrate at pH 5.0 were prepared. Two replications each were incubated for 0, 5, and 10 min at 30°C with rapid shaking. The reaction was stopped by adding 1 ml 20% TCA; after 10 min, the reaction mixture was centrifuged at 30,000*g* for 5 min and the supernatant was assayed for Pi produced by the enzyme using the Fiske-Subbarow method (7). The rate of linear increase against the blank was used for calculations; and the blank was

[1] Oregon Agricultural Experiment Station Technical Paper No. 7110.

[2] Abbreviations: ADP-G PPase, ADP-glucose pyrophosphorylase; WAA, weeks after anthesis; G-1-Pase, glucose-1-phosphatase; 3-PGAase, 3-phosphoglycerate phosphatase; pNPP, para-nitrophenyl phosphate.

assayed for each sample using boiled enzyme extract.

Phospholipase D and Phosphatidic Acid Phosphatase. It was reported that crude extract of mung bean cotyledons contained both phospholipase D and phosphitidic acid phosphatase and degraded phosphatidylcholine to free choline, Pi, and 1,2-diacylglycerol (9). The crude extract of triticale endosperm also may contain both enzymes. Several preliminary tests verified this prediction. A simplified assay was then developed to handle a large number of samples. One g of phosphotidylcholine was dissolved in 10 ml of chloroform-methanol (1:2 by vol) with constant stirring for 24 h at room temperature. One ml of the solubilized substrate was suspended in 20 ml Na-acetate (50 mM, pH 5.0) and this suspension was used as substrate. Six aliquots of 0.5 ml of enzyme extract and 1 ml substrate suspension were prepared for each sample. Two tubes each were incubated 0, 30, and 60 min at 30°C, and the hydrolyzed Pi was determined as for other phosphatases (7). The 1% methanol contained in the reaction mixture did not affect the enzyme activity, as a linear increase in Pi was observed with respect to the time of incubation or the enzyme quantity.

Acid Protease. BSA (1 mg/1 ml of 0.1 M sodium acetate, pH 5.0) was used as substrate for 0.1 ml enzyme extract. Two replications each of the reaction mixture were incubated at 35°C for 0, 30, and 60 min, and the reaction was stopped by adding TCA to a final concentration of 10%. The supernatant was assayed for the amino acid formed (17).

Seed Weight and Starch Content. Fresh weight was determined by weighing four replications of 10 seeds for each sample. Dry weight was obtained by drying at 100°C for 24 h and reweighing. The dried seeds were ground to pass a 0.75-mm screen. An aliquot of the seed meal was extracted with 85% ethanol to remove soluble sugars and amino acids, and the residue was hydrolyzed in 0.2 N H_2SO_4. The glucose in the hydrolysate was determined by the nitrosalicylic acid method (2). A conversion factor of 0.9 was used for the calculation of the starch content from hydrolyzed glucose.

Electrophoresis and Isozyme Detection of Acid Phosphatase. Fresh enzyme extracts containing 100 μg protein each were electrophoretically separated in 7.5% polyacrymide gel tubes using the Davis method (6). Acid phosphatase was stained by the method of Scandalios (14) using α-naphthyl acid phosphate as substrate and fast Garnet GBC as dye. All samples were incubated at 30°C for 2 h with rapid shaking. Then the gels were destained in 50% ethanol for 1 h and fixed in 7% acetic acid.

The stained gels were scanned in a Cary 219 spectrophotometer with a gel scanner accessory at 560 nm with 0.05 mm aperture, scan rate of 4 $s \cdot mm^{-1}$ of gel, zero suppress of 1.3 *A*, and a chart range of 2 *A*.

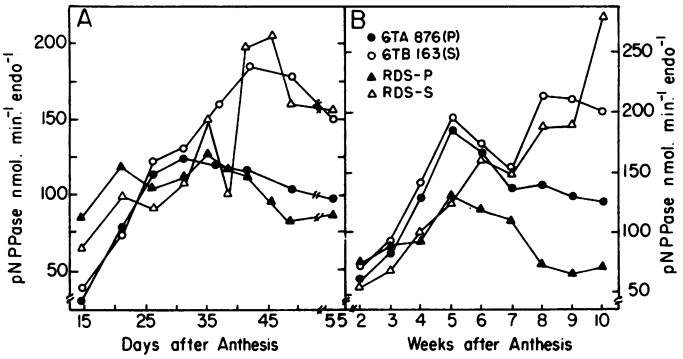

FIG. 1. Changes in acid phosphatase activity in endosperm of field-grown triticale of two plump (6TA 876, RDS-P) and two shriveled lines (6TB 163, RDS-S) during seed formation in 1982 (A) and 1983 (B). Data are the mean of four replications.

RESULTS AND DISCUSSION

In both 1982 and 1983, acid phosphatase (pNPPase) activity was 2-fold greater during the later stages of seed filling in lines

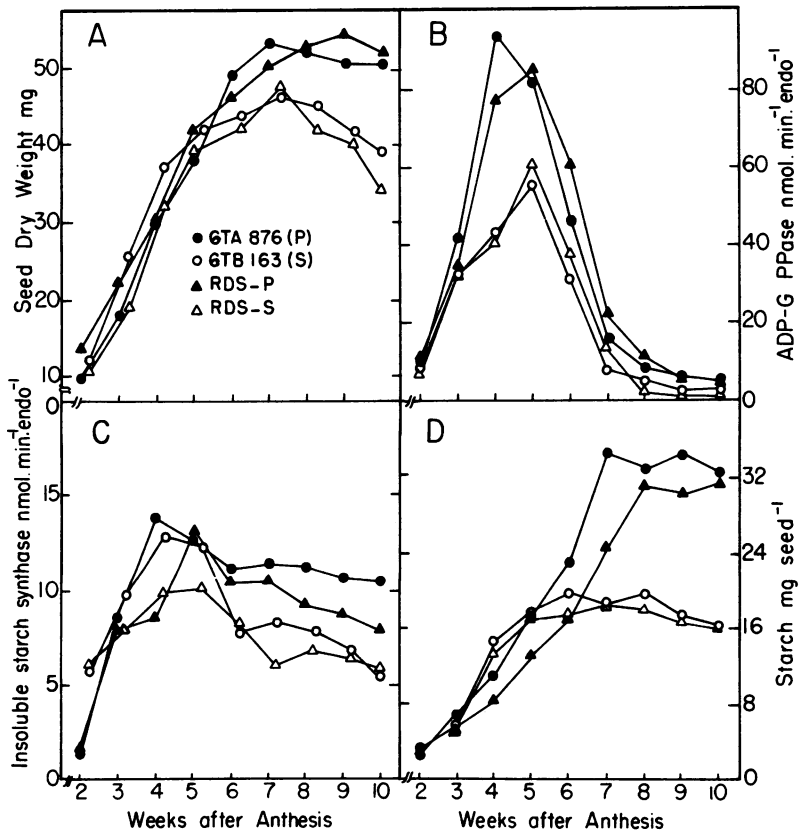

FIG. 2. Changes in seed dry weight (A), activity of ADP-glucose pyrophosphorylase (B) and insoluble starch synthase (C) of endosperm, and starch content in seed (D) of field-grown triticale of two plump (6TA 876, RDS-P) and two shriveled lines (6TB 163, RDS-S) during seed formation in 1983. Data are the mean of four replications.

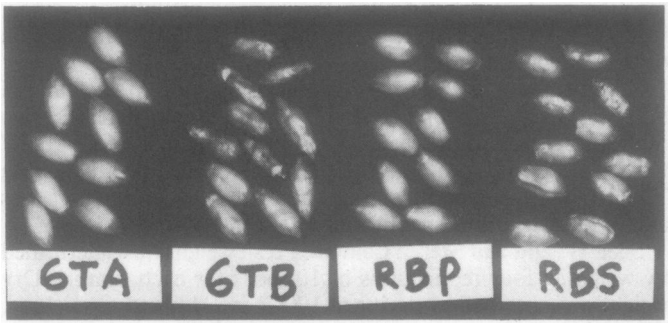

FIG. 3. Morphological characteristics of two lines of plump seed (6TA 876, RDS-P) and two lines of shriveled seed (6TB 163, RDS-S) harvested at 6 weeks after anthesis.

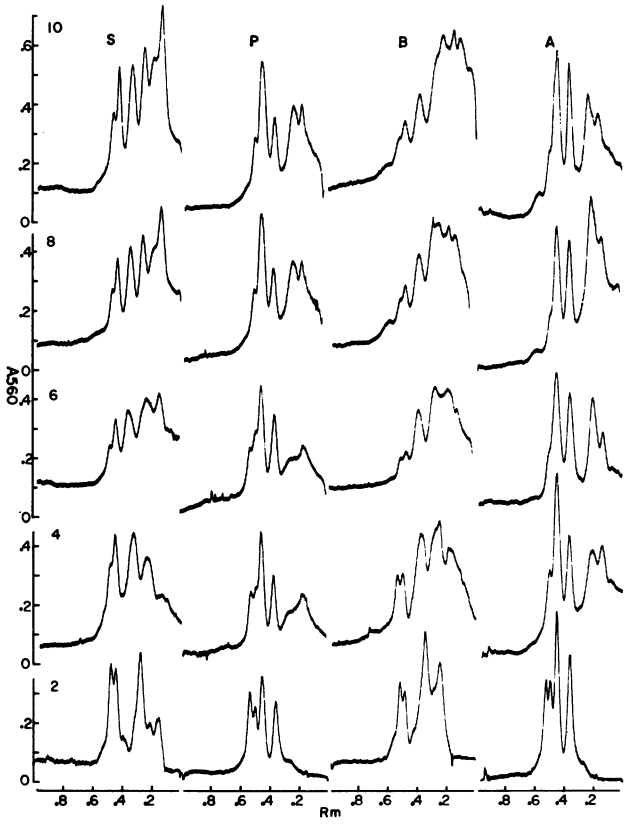

FIG. 4. Changes in isozyme patterns of endosperm acid phosphatase in two plump lines (6TA 876, RDS-P) and two shriveled lines (6TB 163, RDS-S) during seed formation in 1983. The numbers on the left side of profiles represent the number of weeks after anthesis samples in the row were taken. The letters at the top of columns represent the line: A, 6TA 876; B, 6TB 163; P, Red Bobs//Daws/Snoopy-P; S, Red Bobs//Daws/Snoopy-S. Gels were charged with 100 μg of soluble protein each which represents 0.79, 0.20, 0.08, 0.11, and 0.09 endosperm for 6TA 876 (A) at 2, 4, 6, 8, and 10 WAA, respectively; for 6TB 163 (B) 0.22, 0.09, 0.08, 0.07, and 0.05 endosperm; for RDS-P (P) 0.62, 0.11, 0.09, 0.13, and 0.07 endosperm; and for RDS-S (S) 0.24, 0.11, 0.10, 0.08, and 0.04 endosperm.

that produced shriveled seeds when compared with lines producing plump seeds (Fig. 1). This increase correlated with the reduction of seed dry weight (Fig. 2A) and the leveling off of starch accumulation (Fig. 2D) in lines producing shriveled seeds (5). These data indicate that increased acid phosphatase activity may enhance seed shriveling. It was reported shriveled triticale seeds often contain internal cavities and large channels by the soft dough stage (middle stage of seed formation) (12, 16), and prior to maturity, irregular dark, necrotic patches are observed in the endosperm tissue. We have observed these same seed morphological characteristics in our lines that produced shriveled seeds (Fig. 3). These cavities and necrotic spots could be the result of lysosomal enzyme digestion that reduced seed weight and caused shriveling. Acid phosphatase is the major hydrolase or digestive enzyme found in cytoplasmic vesicles (lysosomes) that instigate cellular autophagy to form vacuoles and heterophagic digestion for turnover of cellular organelles and for cellular differentiation (11). In the normal course of grain development of lines with plump seeds, acid phosphatase may be needed for turnover of cellular organelles and for differentiation to a storage tissue. Thus, the enzyme activity (Fig. 1) increased with increasing cell numbers and cell content (dry wt, Fig. 2A) in early stages, and decreased gradually with maturation. In the abnormal situation of shriveling grain, special degradative phosphatases produced at later stages of maturation resulted in necrosis and, consequently, in shriveled seed (Fig. 3).

Developmental age-related changes in the electrophoretic isozyme patterns of acid phosphatase were observed (Fig. 4). At 2 WAA (lowest row), the endosperm of each line exhibited a distinctive number of isozymes as shown by their relative mobilities (Rm) and activities (A_{560}). New isozymes of lower mobility (less anodic and/or larger molecules) were synthesized at 4 WAA in all four lines (second row from the bottom). Continued increase in activity of all isozymes in each line was observed at 6 WAA, and by 8 WAA the total phosphatase activity (area under the curve) in lines producing shriveled seeds exceeded that of lines producing plump seeds. At 10 WAA, the difference in total activity was more clearly shown, though variation existed among various isozymes. The substrate used for isozyme detection in gels, α-naphthyl acid phosphate, was different from those used for enzyme activity assays (Figs. 1 and 5). A general trend, however, that increased acid phosphatase activity in shriveled seeds occurred specifically at the later stages of development is evident in the isozyme profile (Fig. 4) as well as in the activity of various acid phosphatases (Figs. 1 and 5).

Among the slow moving isozymes synthesized during and after 4 WAA in all four lines (Fig. 4), those of <0.30 Rm with the highest activity at 10 WAA were primarily from lines producing shriveled seeds (Red Bobs//Daws/Snoopy-S and 6TB 163). Whether these isozymes are responsible for the tissue degradation in shriveled seeds is not certain, but the similarity in isozyme pattern between the two lines producing shriveled seeds suggests a common involvement. In a parallel manner, the developmental pattern of acid phosphatase isozymes in the two lines producing plump seeds (6TA 873 and Red Bobs//Daws/Snoopy-P) is very similar. This may indicate that the two sets of isozymes, one for plump seeds and the other for shriveled seeds, play divergent, functional roles. One is used for the normal differentiation in plump seeds and the other for degradation of tissues and cells, as well as some differentiation.

Since the enzyme preparation for the acid phosphatase assay was a crude extract and the substrate (pNPP) was exogenous, some endogenous substrates related to starch biosynthesis and organellar integrity were tested for enzyme specificity and temporal changes in reference to shriveling. The results (Fig. 5) indicated that the activity of ATPase, ADPase, phosphatidyl phosphatase, and G-1-Pase were all significantly higher at the later stages of shriveled seed formation than at comparable stages of development of plump kernels.

ATP and G-1-P are the substrates of ADP-G PPase for the synthesis of ADP-G which in turn provides the substrate for starch synthesis. The more active ATPase and G-1-Pase in the shriveled seeds at later stages of seed formation may reduce the *in situ* concentration of respective substrates drastically; thus, starch accumulation could be hampered in shriveling seeds as

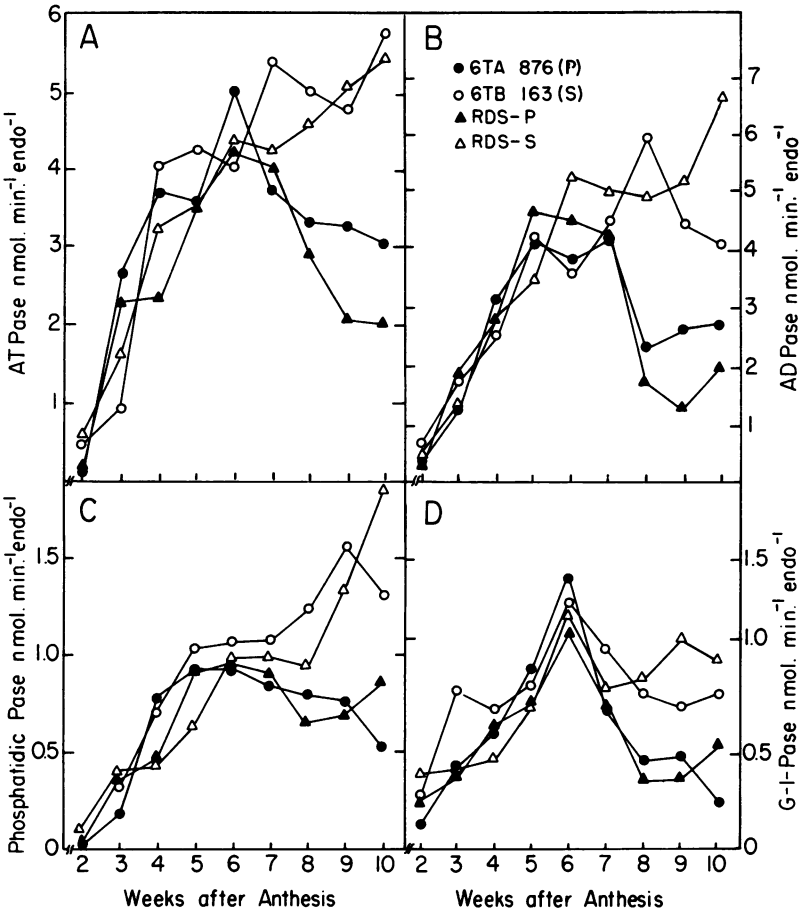

FIG. 5. Changes in activity of acid phosphatases using ATP (A), ADP (B), phosphotidylcholine (C), and glucose-1-phosphate (D) as substrate in triticale endosperm of two plump lines (6TA 876, RDS-P) and two shriveled lines (6TB 163, RDS-S) during seed formation in 1983. Data are the mean of four replications.

observed (Fig. 2D). In 1983 the mild, moist spring and early summer altered the activity pattern of insoluble starch synthase (Fig. 2C) and ADP-G PPase (Fig. 2B) during seed formation from what was observed in the previous 3 years (5). The peak activity shifted from 5 to 6 WAA to 4 to 5 WAA, and the starch synthase activity was maintained at a high level in the later stages of seed formation in 1983. Nevertheless, the lines with plump seeds, 6TA 876 and RDS-P, still exhibited higher activity than that of lines producing shriveled seeds as noted previously (5).

ADP is the substrate for ATP synthesis via oxidative phosphorylation or substrate phosphorylation in the endosperm. When ADP is quickly hydrolyzed by ADPase in shriveled seeds (Fig. 5B) at the later stage of seed formation, ATP needs for starch synthesis and basic cellular maintenance were limited as evidenced by the reduced dry weight and starch accumulation in Figure 2, A and D.

Phosphatidylcholine is one of the cellular membrane components. The activity of degradative enzymes of this compound was higher in lines producing shriveled seeds at the later stages of seed formation (Fig. 5C). As a result of this membrane degradation, tissue necrosis ensued. Even though histological development was not studied in these materials, extensive tissue degradation has been shown in other shriveling lines of triticale (12).

The activity pattern of 3-PGA phosphatase and acid protease was bell shaped with a peak at 4 to 5 WAA and appeared to be similar in all four lines (data not shown). Perhaps 3-PGA, an effector of ADP-G PPase in corn and spinach leaves (13), was not involved in seed shriveling. BSA was used as the substrate for acid protease assay and it may not be suitable for assaying endosperm necrosis on account of its animal origin. When endogenous substrates were used for assaying the extract of several developmental stages, comparable results as that of using BSA were obtained. The membrane-bound acid protease (1% Triton X-100 extracted) (7) also did not differ significantly among lines producing plump and shriveled seeds. Since the cell-free extract contained many enzymes, it was difficult to analyze any further without purification of the specific enzyme.

The developmental patterns of the activity of various hydrolases of 1982 materials (data not shown) were similar to those of 1983, indicating that tissue necrosis and grain shriveling were probably related to the activity of acid hydrolases. Whether these active acid hydrolases are newly synthesized at the later stage of development in shriveled seeds in response to the increase of ABA (7) or GA (1, 10), or in accordance to another specific genomic program (1, 15) is not clear. More research will be needed to identify the mechanism of shriveling in cereal seeds.

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