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Starch Synthesis in Shriveled and Plump Triticale Seeds[1]

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ABSTRACT

Seven lines of triticale (X *Triticosecale* Wittmack) with either shriveled or plump seed characteristics were planted in the field, and seed developmental changes in weight, starch content, the activity of starch biosynthetic and degradative enzymes, and ATP content were studied in three consecutive years in Oregon. Experimental results varied among genotypes and with growing environment, but overall indicated that: (a) amylase activity was higher in shriveled lines, but was not directly synchronized with the occurrence of shriveling at later stages of seed formation; (b) ADP-glucose starch synthase contributed to starch accumulation in triticale, but it appeared not to be associated with shrivelledness as no stage-related changes were observed; and (c) ADP-glucose pyrophosphorylase activity was 2- to 3-fold higher in plump lines than that of shriveled lines, indicating that this enzyme may play an important role in the degree of plumpness or stach accumulation of triticale seeds.

The man-made intergeneric hybrid, triticale (X *Triticosecale* Wittmack), is generally high in protein content and balanced in essential amino acids, has better tolerance than wheat and other small grains, to sandy, dry, saline, high aluminum, and nutrient-poor soils, and resists low and high temperatures and many diseases (1, 12, 14). Shriveled seed, however, continues to be a problem to plant breeders around the world, reportedly being related to α-amylase activity occurring at the latter stages of developing grain (7, 20), low translocation and substrate supply to the grain (24), mitotic aberrations occurring in 9× (hexaploid triticale) or 12× (octaploid) endosperm cells at early stages of the seed development (24), and necrosis in endosperm tissue and failure of filling endosperm cavity with cells at later stages (7, 16). Enzymes participating in starch synthesis may play a positive role in starch accumulation (19) and seed plumpness. To date, little information is available regarding starch synthesis in triticale and this study was undertaken to fill the void.

MATERIALS AND METHODS

Materials. Seven triticale lines with their average plant height, seed test weight, and seed number/100 ml shown in Table I were used for this study. They were selected for their marked difference in phenotypic seed characteristics, *i.e.* S[2] and P. Lines F and G originated from a cross of male sterile Red Bobs wheat with a primary octaploid triticale synthesized from Daws wheat (6X) and Snoopy rye (2X). Following several generations of selfing and some outcrossing, many phenotypes were produced. Sister lines designated S and P were selected for this study in 1981 because they have similar genetic backgrounds, but with phenotypic extremes in seed conformation. All plantings followed standard cultural practices. Lines A, B, C, and D were planted at Hyslop Farm, Corvallis, OR, in October 1979. Spikes were tagged daily at 20% anthesis in May and June 1980, and spike samples were taken every week after anthesis for 9 weeks. Lines A, B, C, D, and E were planted in December 1980 at the East Farm, Corvallis. Irrigation and additional fertilizer were applied in the spring of 1981. Irrigation was stopped 3 weeks prior to harvest. Spikes were tagged daily in early June and weekly samples of spikes taken to maturity. Line A was not sampled due to excessive infection of root rot in that year. Lines A, C, D, E, F, and G were planted in October 1981 at the Hyslop Farm, and spikes were tagged in late May and early June. Spike samples of lines F and G were taken every 3 to 7 d to maturity, instead of once a week, due to the hot and dry weather conditions that hastened maturation. Other lines were spot checked only.

Generally, 6 to 10 spikes/sample were collected and stored in 1% sucrose and 0.1% glycine to provide a continuous simulated supply of photosynthate while being transported to the laboratory (4). Six to 10 seeds from the middle section of each spike were dissected on ice. Four replications of 10 seeds were used to determine fresh and dry weight.

Enzyme Extraction and Assays. Seed coats and embryos were removed from two replications of 10 seeds each and the peeled endosperms were placed in 10 ml of ice-cold GB containing Tris-HCl, 50 mM, K_2HPO_4, 10 mM, DTT, 2 mM, pH 8. All of the subsequent procedures were conducted at 0 to 5°C. The endosperms were ground in GB in a mortar with pestle and the slurry was centrifuged at 30,000*g* for 10 min. The supernatant was collected in a tube and the pellet was washed with 10 ml of GB and centrifuged. The wash was combined with the first supernatant. The pellet was used for the assay of insoluble starch synthase.

Ten ml of the combined supernatant was dialyzed in GB for 4 h with four changes of 250 ml each and then used for the assay of the following four enzymes. 1) Glucose-1-phosphate adenylyltransferase (EC 2.7.7.27, ADPglucose pyrophosphorylase, ADP-G-PPase) was assayed by the procedures of Ching (4). An aliquot of 0.1 to 0.4 ml (for older to younger seeds, respectively) of dialyzed extract was incubated with 0.5 μmol ADP-G and 1 μmol of PPi in a total volume 0.5-ml reaction mixture containing 50 mM Hepes and 5 mM Mg acetate (pH 8.0) at 30°C for 0 and 10 min. After incubation, the reaction mixture was boiled for 1 min and 5 ml of luciferase reaction buffer containing 25 mM each of Hepes and Mg acetate, pH 8.0, was added and ATP content in the reaction mix was determined by the luciferin-luciferase method (4). The results of this assay procedure are highly correlated with that of the commonly used [^{14}C]glucose-1-P assay method (10). Based on 16 extracts of maturing endosperms of both shriveled and plump seed types assayed in the

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[2] Abbreviations: S, shriveled; P, plump; WAA, weeks after anthesis; DAA, days after anthesis; ADP-G-PPase, ADP-glucose pyrophosphorylase; UDP-G-PPase, UDP-glucose pyrophosphorylase, GB, grinding buffer; 3-PGA, 3-phosphoglycerate.

Table I. *Genetic Pedigree, Average Plant Height, Seed Test Weight, and Seed Number per 100 ml of the Material*

	Plant Height	Seed Test Wt.	Seed Number
	cm	*g 100 ml^{-1}*	*100 ml^{-1}*
A. Kiss/193 × 803–358 (S)	78.3	63.4	1359
B. URSS 3310/BVA (S)	101.7	60.7	1498
C. 274 358 (P)	135.5	71.2	1243
D. 6TA 876 (P)	142.8	78.7	1480
E. 6TB 163 (S)	68.3	54.9	1491
F. $\frac{\text{Red Bobs}}{\text{Daws/Snoopy}}$ (S)	72.6	62.7	1716
G. $\frac{\text{Red Bobs}}{\text{Daws/Snoopy}}$ (P)	74.9	71.9	1331

presence of 2 mM 3-PGA, the regression equation was: $Y = -0.21 + 0.034\,X$ where Y = radiotracer assay results and X = luciferase assay results. The correlation coefficient was $r^2 = 0.868$** (highly significantly correlated at 1% level). The addition of 3-PGA in reaction mixture did not elevate the results of the ADP-G-PPase assay by the luciferase method (4) on extracts of early or middle stages of seed development, but a stimulation of 150 to 350% was observed in extracts of 7 to 9 WAA materials in both P and S typed seeds. 2) Glucose-1-phosphate uridylyltransferase (EC 2.7.7.9, UDPglucose pyrophosphorylase, UDP-G-PPase) was assayed by the enzyme cycling method (11). 3) ADP-G sucrose synthase and 4) UDP-G sucrose synthase activity (EC 2.4.1.13) were assayed in reverse direction using ADP or UDP as substrate following the method of Shannon and Dougherty (23).

Starch synthase (EC 2.4.1.21, ADPglucose:1,4-α-D-glucan 4-α-D-glucosyl transferase) was assayed by the procedures of Ching (5). The insoluble pellet was suspended in a reaction buffer containing Tricine-Mg acetate-K acetate-DTT (25:5:5:2 mM, pH 7.5). An aliquot of 0.2 ml was taken while the suspension was vigorously mixing and incubated with 0.5 μmol ADP-G, 1 μmol P-enolpyruvate, and 50 μg pyruvate kinase at 30°C for 10 min. The reaction mixture was then boiled for 1 min, 5 ml luciferase reaction buffer was added, and the ATP content was assayed by the luciferin-luciferase system (5). Based on 16 extracts of both plump and shriveled types, the regression equation of the radiotracer and luciferase assay results was: $Y = -0.033 + 0.368\,X$ where Y = ADP-[U^{14}C]glucose incorporated into methanol-insoluble glucan (17), X = luciferase assay results, and a correlation coefficient, $r^2 = 0.821$** (highly significantly correlated at 1% level). The soluble starch synthase was fractionated from the supernatant with $(NH_4)_2SO_4$ (40% saturation) to exclude the interference of amylase (19), and then assayed as above.

All buffers were freshly prepared to ensure a constant concentration and to avoid the oxidation of DTT.

For the assay of amylolytic enzyme activity, two replications of 10 endosperms/sample were extracted in 10 ml of Ca acetate buffer (10 mM, pH 6.0) using mortar and pestle. The slurry was centrifuged at 30,000*g* for 10 min, and the supernatant was used to assay the total amylolytic activity using the Bernfeld method (2). An aliquot of the supernatant was heated to 70°C for 20 min, centrifuged at 30,000*g* for 10 min, and the supernatant was used as α-amylase extract (3) and assayed (2).

Starch Content Determination. An aliquot of the suspension of the washed pellet was first extracted by 85% ethanol to remove lipids and soluble sugars. Then the starch pellet was suspended in 10 ml water and boiled for 10 min with stirring. Three replications of 1 ml each were taken from the gelatinized starch solution. Fifty mg of amyloglucosidase (α-1,4- and α-1,6-glucan hydrolase from Rhizopus Mold, Sigma Chemical Co.) were added to each aliquot. After adding 2 ml sodium acetate buffer (50 mM, pH 4.5), the samples were incubated at 50°C for 20 min. The reaction mixture was boiled, diluted to 10 ml, and centrifuged. The supernatant was collected. The precipitant was washed twice, and the wash was combined with the supernatant. The glucose in the combined supernatant was determined by the dinitrosalicylic acid method (2).

Seed ATP Content. ATP extraction was conducted using liquid N_2-preserved seeds and analyzed using the luciferin-luciferase system (6).

RESULTS AND DISCUSSION

Based on current knowledge, the synthetic enzymes directly related to starch accumulation in cereal seeds are starch synthase, ADP-G-PPase, UDP-G-PPase, ADP-G sucrose synthase, UDP-G sucrose synthase, and phosphorylase (9, 19). Phosphorylase activity was extremely low in a preliminary study and was not subsequently studied. Others were assayed in cell-free extracts of P and S type seeds.

The experimental results of 1980 are summarized in Figure 1. The seed weight increased rapidly in all four lines during the first four weeks after anthesis; then a distinct slow-down in weight gain was observed in S lines compared to that of P lines (Fig. 1A). The increase of starch (Fig. 1A) paralleled seed weight with an average of 21, 36, and 56% of seed dry weight, respectively, for 2, 3, and 4 WAA. The P lines continued to gain starch to 65, 70, 73, 76, and 77% of seed dry weight for 5, 6, 7, 8, and 9 WAA, respectively. The increase of starch content was slower in the S than the P lines being 58, 60, 62, 66, and 68% of seed dry weight, respectively. Seed weight increase was sigmoid in shape in all lines and the only difference between the S and P appears to be the starch content which was slower in accumulating and less in total quantity in S lines.

The α-amylase activity was very low in the endosperm throughout the developmental period and little difference was observed among P and S lines. The α-amylase activity comprised up to 1% of the total amylolytic activity; thus, the total activity was plotted in Figure 1B. The S lines had consistently higher activity than that of P line 274–358 throughout the whole seed developmental period. Another P line 6TA-876 had as high amylase activity as the S lines during the first 6 weeks, then declined rapidly thereafter. Even though these *in vitro* differences between P and S lines were observed, the availability of substrate (starch) apparently is very limited in the *in vivo* situation. Otherwise, the high hydrolytic rate of the amylase of 10 mg endosperm^{-1} min^{-1} (6 WAA) would digest all the starch synthesized during a 24-h period (Fig. 1C) within 1 min, resulting in no weight gain during seed formation. All lines, however, gained weight in spite of the amylase activity. Starch is synthesized in the amyloplast and this compartment may segregate the starch from amylases in the cytosol (9, 14, 19). It is also possible that immature starch granules are resistant to the amylases of developing endosperm as they seem to be resistant to those derived from the germinated grain (9). Using scanning electron microscope, only three of the eight shriveled hexapoid triticales showed pitted starch granules that might be caused by the hydrolytic action of amylases (16). In the same study, the lines with high frequency of shriveledness all contained small starch granules, but no evident relationship between α-amylase activity and starch content or surgar content in grains (15). Therefore, the activity of amylase in endosperm probably has no direct bearings on the starch accumulation or reduction in developing cereals.

The activity of granular or amyloplast bound starch synthase was detected in the endosperm at 2 WAA in all lines; it rapidly increased to a peak at 6 WAA, and then declined gradually (Fig. 1C). The activity under the optimum assay conditions was not apparently related to S or P characters even though differences in starch synthase activity have been observed by others in barley

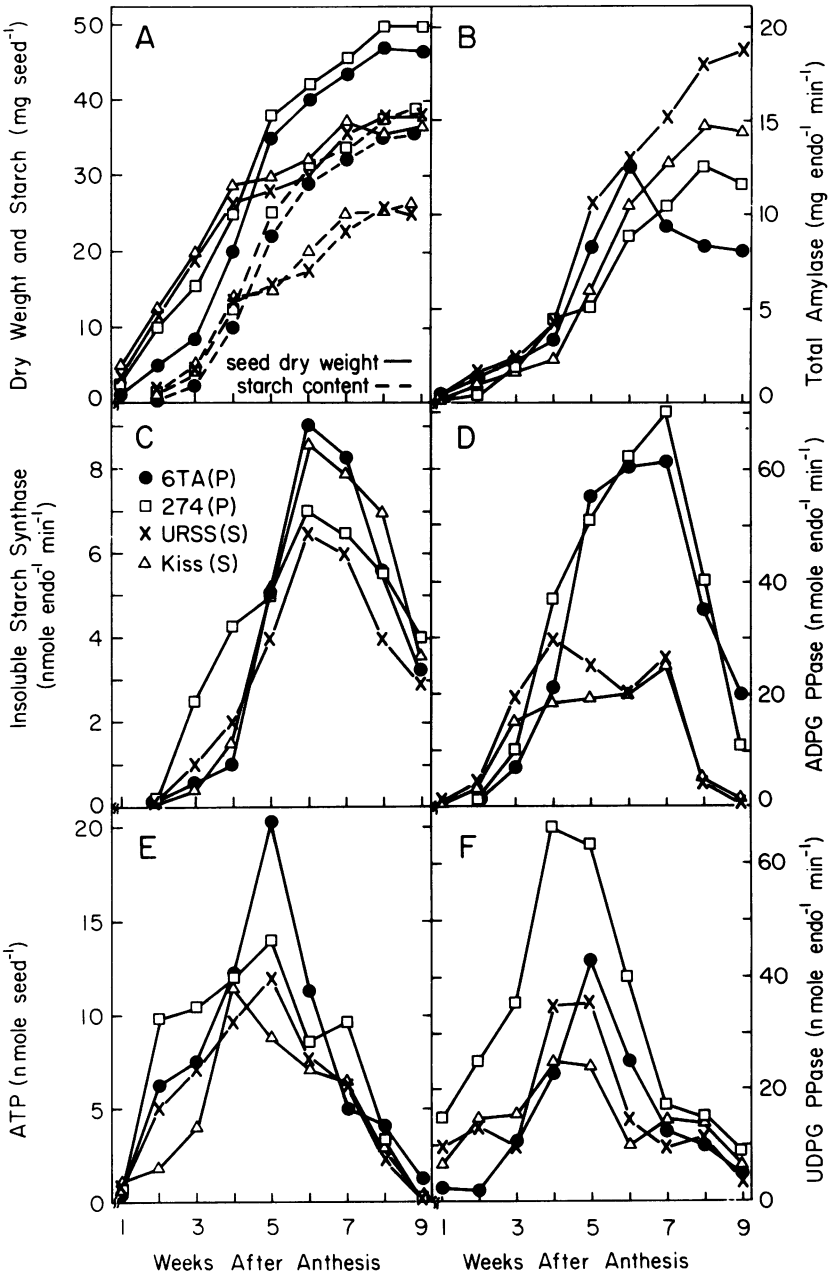

FIG. 1. Changes in seed dry weight and starch content (A), total amylolytic activity in endosperm (B), endosperm granular starch synthase activity (C), endosperm ADP-G-PPase activity (D), ATP content in seed (E), and endosperm UDP-G-PPase activity (F) in two plump lines, D (6TA-876) and C (274-358), and two shriveled lines, A (Kiss 193 × 803-358) and B (URSS-3310/BVA) of triticale during seed formation in 1980. Data are the average of two to four replications.

cultivars with varied seed weight and degree of shriveledness (22). The *in vitro* rate of starch synthase (Fig. 1C) amounted to 20 times that of *in vivo* starch accumulation at 6 WAA (Fig. 1A) indicating a constrained condition *in situ*. The limitation of substrate supply may be one of the constraints as found in corn kernels (8).

The substrates for starch synthesis in cereals are mainly ADP-glucose and UDP-glucose which can be provided by ADP-G- and UDP-G-PPase or the reverse reaction of sucrose synthase (8, 17, 21). The changes in the activity of the first two enzymes during seed formation are summarized in Figures 1, D and F. All lines increased ADP-G-PPase activity during the first 3 weeks of development (Fig. 1D). By 4 WAA, the two P lines showed about 3-fold ADP-G-PPase activity compared to the S lines. The activity of P lines peaked at 7 WAA and remained higher than S lines throughout the development. The difference observed in triticale lines, though significant, was not as great as those found in corn mutants when compared to their control material (8). This lowered ADP-G-PPase activity may result in diminutive sink force which cause a low translocation rate of photosynthate and low substrate supply for starch synthesis in shriveled triticale lines (24), as observed in corn mutants (13). The activity of UDP-G-PPase (Fig. 1F) exhibited a different pattern with an early peak at 4 to 5 WAA for all lines and only line 274 had a significantly higher activity than the other three lines. The peak earlier than that of starch synthase and ADP-G-PPase may indicate that UDP-G-PPase activity is mainly related to pectic substance synthesis during endosperm cellularization at the early stages of

seed formation and not to the major event of starch accumulation which started at 5 WAA and peaked at 7 WAA. The regulation of ADP-G-PPase in starch biosynthesis is well known in maturing corn kernels (8, 10, 19) and the experimental results presented here indicate that ADP-G-PPase plays a similar role in triticale; thus, the S lines are all low in ADP-G-PPase activity. Further characterization of the purified enzyme may elucidate whether the activity differences are because of a structural genetic variation or a functional alteration in these two seed types.

All the differences in enzymic activities were not due to differences in endosperm weight as the pattern of enzymic specific activities was comparable to the total activity per endosperm.

Since ATP is one of the substrates of ADP-G-PPase and the energy supply for cellular activity (8), the change in ATP content in seeds was traced and plotted in Figure 1E. It would be more reasonable to analyze the ATP content in endosperm following removal of the seed coat, but the ATP content declined too rapidly during transporting and dissecting to be of value. The liquid N_2-perchloric extraction recovered about 96% of the ATP in fresh material; thus, the whole seed was used. A parabolic curve in reference to WAA and total biosynthetic capacity of grains was observed for all lines. A significant difference between P and S seeds was not evident, probably indicating that the average cellular status of different seed tissues is not sensitive enough to reveal any relationship of starch synthesis in endosperm.

The general trends of seed developmental changes in 1981

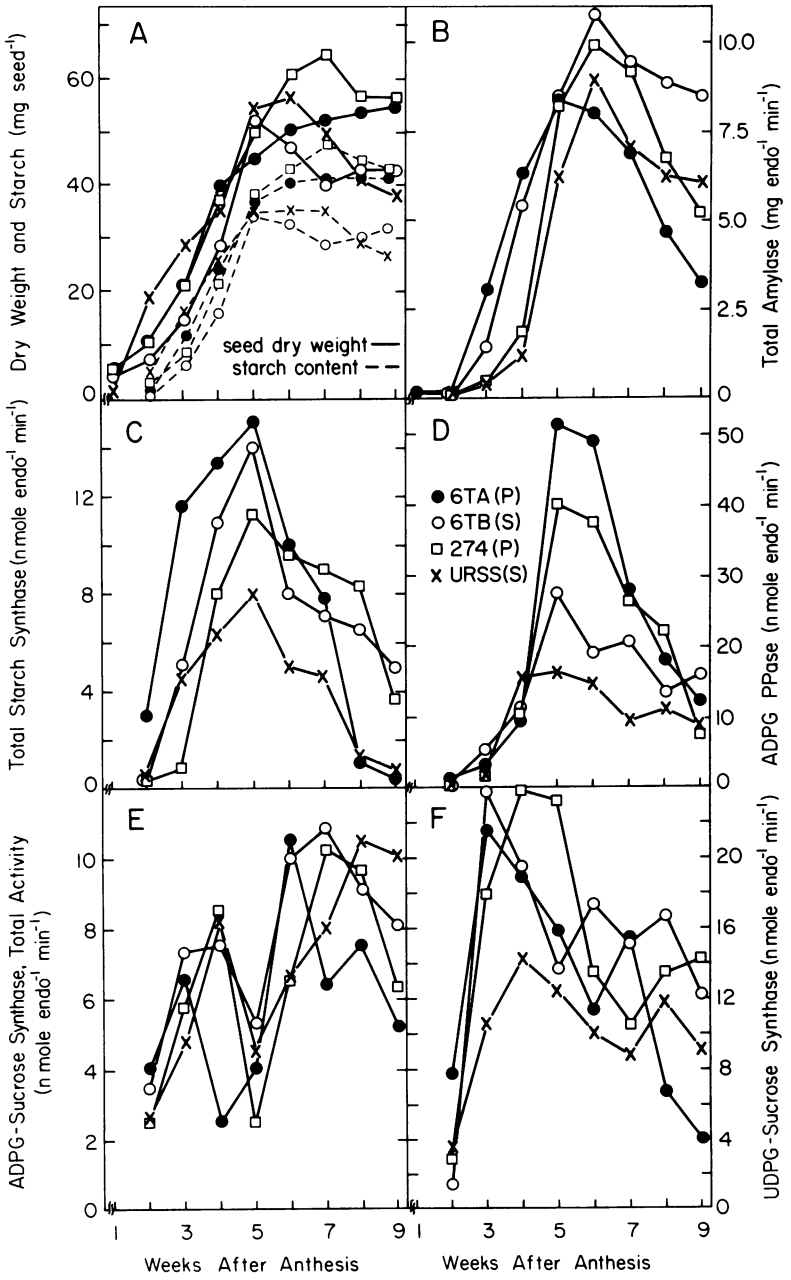

FIG. 2. Changes in seed dry weight and starch content (A), total amylolytic activity in endosperm (B), total starch synthase activity in endosperm (C), ADP-G-PPase activity in endosperm (D), ADP-G sucrose synthase activity in endosperm (E), and UDP-G sucrose synthase activity in endosperm (F) in two plump lines, D (6TA-876) and C (274-358), and two shriveled lines, E (6TB-163) and B (URSS-3310/BVA) of triticale during seed formation in 1981. Data are the average of two to four replications.

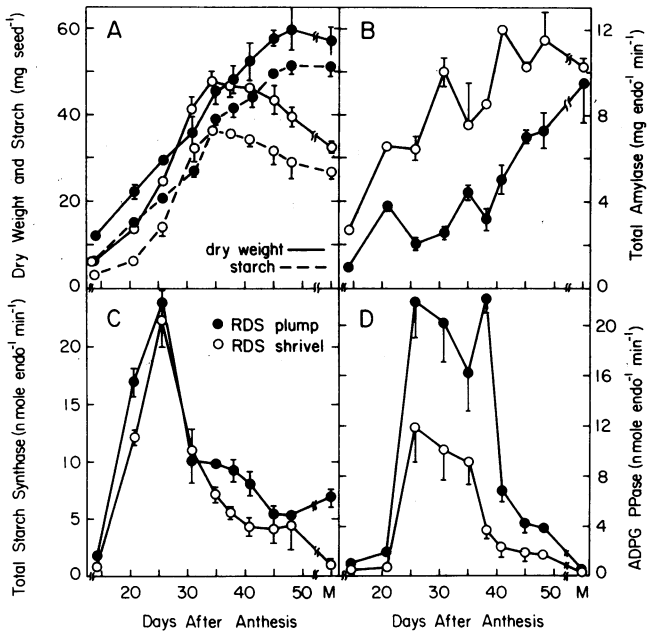

FIG. 3. Changes in seed dry weight and starch content (A), total amylolytic activity in endosperm (B), total starch synthase activity in endosperm (C), and ADP-G-PPase activity in endosperm (D) in one each of plump (G) and shriveled (F) phenotype of an octaploid triticale (ms Red Bobs//Daws/Snoopy) during seed formation in 1982. Data are the mean ± SD of two to four replications.

(Fig. 2, A–D) were comparable to those of 1980 except a marked reduction of seed weight occurred after 5 WAA in S lines and the peaks of amylase, starch synthase, and ADP-G-PPase occurred earlier. These differences might be brought about by the termination of irrigation at 6 WAA. Soluble starch synthase was assayed in 1981. The activity was about 1% of the granular bound starch synthase at the early stages and 8% at the mid-stages for all lines. Then at later stages, the soluble starch synthase increased to 14% of the bound starch synthase in S lines and to 10% in P lines while the total soluble and bound enzyme activity was decreasing with maturation. These results might indicate a differential solubilization of the enzyme in maturing amyloplasts in S lines or more soluble starch synthase was synthesized in S lines.

Two additional enzymes, ADP-G and UDP-G sucrose synthase, were assayed and the results are summarized in Figure 2, E and F, respectively. ADP-G sucrose synthase activity showed two peaks during the seed development with one peak at 4 WAA and the other after 6 WAA. All lines then maintained high activity throughout the maturation period, indicating that the particular enzyme is equally capable in all lines of supplying the ADP-G for the starch accumulation if ADP is equally available. The lack of correlation between the activity and the quantity of starch accumulated, however, probably negates the enzyme's possible participation in the regulation of starch biosynthesis or the cause of seed shriveledness. The peak of UDP-G sucrose synthase activity reached early at 3 to 4 WAA during the seed formation and the lack of difference between P and S lines lead one to conclude that the enzyme probably is not related to starch accumulation in these triticale lines. The same conclusion was derived in another study of other triticale lines (21). Again, as in 1980, the activity of ADP-G-PPase was significantly higher in P lines (Fig. 2D) and the higher activity was somewhat correlated with starch accumulation (Fig. 2A). In general, the developmental pattern was not as clear-cut between the P and S lines as observed in 1980. Irrigation and extra fertilizer might have altered enzymic activity differently in these lines.

In 1982, two new secondary octaploid triticale lines (F and G) were studied while the other lines were spot checked at mid- and late stages. The persistent drought conditions in the spring of 1982 resulted in shorter plants and hastened maturity from the normal 70 DAA to 50 d in Corvallis.

The general trend of seed developmental pattern observed in 1981 was also observed in 1982 using the Red Bobs lines (Fig. 3) and others (not shown). The P line continued to increase in seed weight throughout developmental period while the S line gained weight only up to 35 DAA. Thereafter dry weight and starch content declined in the S line (Fig. 3A), indicating lesions had been developed in endosperm tissue which is characteristic of shriveling in triticales (24). Amylase activity was consistently and significantly higher in the S line than the P line, but the developmental trend of the enzyme (Fig. 3B) had no bearing on the starch quantity (Fig. 3A). Again, these results indicated that amylase activity is not directly related to shriveledness. The total starch synthase activity was similar in the two lines and reached a peak at 21 DAA (Fig. 3C) instead of the usual 35 DAA in other lines during two earlier years. The activity was much higher than the two previous years, probably indicating an adaptive adjustment made by plants under drought conditions. After 30 DAA, the starch synthase activity in the P line was higher than that of the S line and this probably contributed to the continuous accumulation of starch in the P line. The soluble starch synthase activity amounted to 0.5, 1.2, 6.5, and 18% of the total starch synthase activity, respectively, for 21, 31, 38, and 48 DAA in the S lines, while the P line had 0.3, 0.7, 2.4, and 8.3%, respectively. This would indicate, as in 1981, a differential solubilization or greater synthesis of the enzyme at later stages in the S line. The ADP-G-PPase activity was double in rate in the P line throughout the developmental period and the high plateau occurred at the rapid starch accumulation stage of 25 to 40 DAA (Fig. 3D). The developmental pattern of UDP-G-PPase activity was similar to that of ADP-G-PPase but peaked earlier and the S line was slightly higher than the P line (not shown). The activity of ADP-G sucrose synthase or UDP-G sucrose synthase was not different between S and P lines (not shown), indicating again that they may not be related to shriveledness.

Based on 3 years' experimental results collected on seven related or unrelated lines of S and P materials and other observations in the literature, the following conclusions can be made. (a) Even though most S lines have higher amylase activity than P lines at the later stages of seed development, the activity is probably not related to shriveledness because of the cellular compartmentation which encloses starch within amyloplast and out of the reach of amylases in the cytosol. When abnormal mitosis, to the extent of several per cent, occurs in the endosperm of S lines at the early stage of seed formation, localized cell necrosis follows at the middle stage (7, 24). The cell necrosis is probably a result of the activity of lysosomal hydrolases that may break down the cellular compartments and allow amylases to degrade starch grains within the amyloplast. Consequently, amylase activity appears to be secondary as far as shriveledness is concerned. (b) ADP-G starch synthase activity contributes to the accumultion of starch in triticale grain as in other cereals (19), but is not associated with shriveledness because the enzyme activity was not evidently different between the S and P lines when exogenous substrate was amply provided and the reduction of activity at the later stages of maturation was not specific to S lines. (c) UDP-G-PPase and UDP-G sucrose synthase were possibly responsible for supplying substrate for the biosynthesis of pectic substances for cellularization at the early stages of seed formation in both P and S lines. (d) ADP-G sucrose synthase possibly participated in providing substrate for cellularization at the early stages and in starch accumulation at the later stages of

seed formation. No evident difference in the enzyme activity was observed between P and S lines. (e) ADP-G-PPase appears to regulate starch synthesis in triticale and to be a definite contributing factor to the degree of plumpness in grains. The uniform and consistent activity difference between S and P lines observed in all 3 years warrants a detailed investigation of the structural and functional characteristics of this enzyme so that their chromosomal inference might be clarified. Furthermore, the genomic origin and influences on ADP-G-PPase in triticale has not been explored. In corn, mutation at *shrunken-2* (*sh-2*, chromosome 3) and at *brittle-2* (*bt-2*, chromosome 4) abolishes 92 to 95% of endosperm ADP-G-PPase activity (10). In triticale, the difference in the enzyme activity may be attributed to the cooperation or incompatibility of rye and wheat genomes (structural genes), or regulated by different effectors such as 3-phosphoglycerate, Pi, and others (19). ADP-G-PPase, however, may not be entirely responsible for grain shriveledness; different causes, such as low translocation capacity (24), mitotic aberration in endosperm development (24), and endosperm necrosis (7, 16) may all be involved.

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