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Water Stress Enhances Expression of an α-Amylase Gene in Barley Leaves

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ABSTRACT

The amylases of the second leaves of barley seedlings (*Hordeum vulgare* L. cv Betzes) were resolved into eight isozymes by isoelectric focusing, seven of which were β-amylase and the other, α-amylase. The α-amylase had the same isoelectric point as one of the gibberellin-induced α-amylase isozymes in the aleurone layer. This and other enzyme characteristics indicated that the leaf isozyme corresponded to the type A aleurone α-amylase (low pI group). Crossing experiments indicated that leaf and type A aleurone isozymes resulted from expression of the same genes.

In unwatered seedlings, leaf α-amylase increased as leaf water potential decreased and ABA increased. Water stress had no effect on β-amylase. α-Amylase occurred uniformly along the length of the leaf but β-amylase was concentrated in the basal half of the leaf. Cell fractionation studies indicated that none of the leaf α-amylase occurred inside chloroplasts.

Leaf radiolabeling experiments followed by extraction of α-amylase by affinity chromatography and immunoprecipitation showed that increase of α-amylase activity involved synthesis of the enzyme. However, water stress caused no major change in total protein synthesis. Hybridization of a radiolabeled α-amylase-related cDNA clone to size fractionated RNA showed that water-stressed leaves contained much more α-amylase mRNA than unstressed plants. The results of these and other studies indicate that regulation of gene expression may be a component in water-stress induced metabolic changes.

Plant cells and tissues show numerous metabolic responses to water stress, some of which may have adaptive significance (9). These responses are usually described at one or another of three levels: (a) perturbation of whole metabolic pathways leading to accumulation or depletion of metabolites, (b) alterations in activities of conveniently assayable enzymes, and (c) changes in the pattern of synthesis of proteins of unknown function. Examples of (a) include the accumulation of carbohydrates, organic and amino acids, quaternary ammonium compounds, and ABA. Since the enzymology of these responses is, in most cases, poorly understood, little can be deduced about the type of metabolic regulation involved. Among instances of (b) are stress-related increases in extractable activity of a number of hydrolytic enzymes (32). Although summing (a) and (b) indicates that stress-induced changes in intermediary metabolism may involve regulation of enzyme activity, differences in extractable enzyme activity could result from a number of causes. They may be artifacts of tissue disruption involving, for example, differential solubilization or differential enzyme inactivation during extraction. Alternatively, the differences may reflect genuine enzyme regulation which, in turn, may entail changes in enzyme activity, or changes in the absolute level of enzyme through regulation of synthesis or degradation. For most reports of stress-induced enzyme changes, there is little evidence to indicate which of the above possibilities apply (9), although it is clear that water stress can provoke specific changes in the overall pattern of protein synthesis (12).

In this paper, we first characterize the amylases of barley leaves. We then describe a promotive effect of water stress on the activity of α-amylase. We demonstrate that the activity increase is due to an enhanced rate of synthesis of a single α-amylase isozyme and that this is accompanied by an increase in the level of the corresponding mRNA.

MATERIALS AND METHODS

Plant Material. Unless otherwise stated, *Hordeum vulgare* cv Betzes was used. In addition to cv Betzes, the comparison of genotypes (Fig. 3) employed cv Himalaya and three accessions of *H. spontaneum* shown previously to have different aleurone α-amylase isozyme spectra (2).

Plants were grown in potting mix (6 parts river loam, 2 parts Australian peat moss, 2 parts coarse mixture containing river sand, vermiculite, and perlite) in 10-cm pots (usually 7 seedlings/pot) in a growth cabinet with a day/night regime of 21°C (12 h)/16°C (12 h). The daytime humidity was controlled at about 55% RH. The pots were watered each day, alternating nutrient solution (Hoagland No. 2 solution) with demineralized water.

Identification of α- and β-Amylases. Second leaves of Betzes barley were homogenized in 0.05 M sodium succinate buffer (pH 5.0) (200 μl/leaf). The homogenate was centrifuged and the supernatant subjected to cycloheptaamylose-Sepharose affinity chromatography as described previously (4). The run-off and bound amylase fractions were used for IEF[2] (see below) and amylose hydrolysis studies. The latter were performed as described previously (16).

Leaves were homogenized in 0.05 M sodium succinate buffer (pH 5.5) containing 10 mM $CaCl_2$ (200 μl/leaf) and the homogenates were heated at 70°C for different times, cooled in ice, and centrifuged. The supernatants were used in IEF studies.

Isoelectric Focusing of Amylases. Isozymes were separated by IEF using an LKB Multiphor apparatus and LKB-prepared gels of pH range 4 to 6.5. The gels were prefocused and run according to the maker's instructions. After prefocusing, two LKB sample

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[2] Abbreviations: IEF, isoelectric focusing; CHA, cycloheptaamylose; pCMB, *p*-chloromercuribenzoate; HSP, heat shock protein; ANP, anaerobic protein; WSP, water stress protein.

application papers soaked in each solution (approximately 40 μl solution total) were applied to the gel near the cathode. At the end of run, the gel was routinely incubated in a 2% aqueous solution of soluble starch (Stärke löslich, Merck) for 30 min, washed several times in water to remove the excess starch solution, the last wash was decanted, and the gel left at room temperature in a closed Petri dish for about 1 h. The gel was then washed once in water and stained overnight in the acidified iodine-potassium iodide solution used for α-amylase assay (5). Amylase isozymes appear as light zones against a deep blue background. This technique detects β-amylase isozymes as well as α-amylase: because the soluble starch has a very short chain length and many fragments contain few if any α-(1 → 6) branch points, it can be hydrolyzed extensively by β-amylase. The blue color of the gel is stable in acid solution and gels can be stored for long periods sealed in plastic bags in the presence of 0.01 N HCl.

α-Amylase Assay. α-Amylase was assayed as described by Chrispeels and Varner (5) but because leaves contained very little enzyme, reactions were allowed to proceed for 30 to 60 min at 35°C in order to obtain sufficient color change. Because of the low amounts of enzyme, activity is expressed as milliunits (units $\times 10^{-3}$).

Incubation of Aleurone Layers. Aleurone layers were prepared and incubated essentially as described by Chrispeels and Varner (5). De-embryonated seeds were allowed to imbibe water on moist sterile sand for 3 d at 20°C and single aleurone layers were incubated for 40 h at 25°C in 200 μl of solution containing 10 mM $CaCl_2$ and 1 μM GA_3. The incubation medium was assayed for α-amylase and diluted to about 0.2 units/ml which was an appropriate activity for IEF studies.

Water Stress Experiments. The activity of α-amylase was followed in well-watered control plants and in plants subjected to a cycle of water withholding and rewatering. Seed was sown in 13 pots and grown as described above until the second leaves were fully expanded (13–14 d). Pots were assigned to one block of five which were no longer watered, and one block of three which was continuously watered daily. One d after the last watering, five second leaf blades were harvested from one control pot, and divided as follows: the basal 10 cm segment was designated 'base,' the next 2 cm segment was used for relative water content determination, the next 1.2 cm segment was used for leaf water potential measurement using a Wescor HR-33T Dew Point Microvoltmeter (20), and the rest of the leaf (average total leaf blade length = 25.1 cm) was designated 'tip.' The five base segments were bulked and the five tip segments were also bulked for α-amylase assay. Each lot was homogenized in a total of 1.5 ml 0.05 M sodium succinate buffer (pH 5.0) containing 10 mM $CaCl_2$. The homogenates were heated at 70°C for 15 min as described above, centrifuged, and α-amylase was assayed (in triplicate) in the supernatants. On d 2, the process was repeated on five second leaves from one block of unwatered pots, one leaf from each pot, and on d 3 it was repeated on five second leaves from the other block of pots. The experiment continued using leaves from alternating blocks of pots so that leaf area in each pot (and hence rate of water loss by transpiration) changed in an orderly way during the experiment. On d 6, after stressed and control (from a second control pot) leaf samples had been collected, all pots were watered and the leaf sampling continued up to d 13. A third control pot was sampled on d 11.

A linear relationship was obtained between the leaf water potential and relative water content data from the first experiment. In the second experiment only relative water content was measured; this relationship plot was used to convert relative water content values to leaf water potential.

Analyses of variance were performed on base α-amylase, tip α-amylase, and leaf water potential, and least significant differences were derived from them.

Assay of ABA. ABA was assayed as described by King (17) in the second leaves of wilted (6 d without water as described above) and turgid plants.

Radioactive Labeling and Analysis of Leaf Proteins. *[^{35}S] Methionine Feeding of Leaves.* Wilted (6 d without water) and turgid plants were prepared as described above and two attached second leaves in each of two pots were radiolabeled essentially as described by Hanson and Scott (10). Leaf blades were trimmed to 10 cm above the ligule and 25 μCi of L-[^{35}S]methionine (Amersham, approx. 1400 Ci/mmol) in 5 μl were absorbed into the cut surface of each leaf. The labeled plants were left for 24 h in the normal growth chamber conditions. The excised leaf tips were examined for α-amylase content by IEF to ensure that the enzyme content was elevated in wilted tissue as usual.

Extraction of Proteins and SDS-PAGE. After 24 h, the labeled 10 cm leaf blades were harvested and the four control segments (two from each of two pots) were combined as were the four wilted leaf segments. Leaves were homogenized in 1 ml of 50 mM Tris·HCl buffer (pH 7.4) containing 150 mM NaCl and 5 mM EDTA (NET buffer) supplemented with 0.05% Nonidet P40, 5 mg/ml BSA, 2 mM methionine, and 3 units of α-amylase purified from cv Himalaya aleurone as described by Chandler *et al.* (4). The homogenates were clarified by centrifugation at 4,000*g* for 10 min and the supernatants were then centrifuged again at 100,000*g* for 45 min to remove ribosomes. The supernatants (each about 1.15 ml) were divided in four portions as follows: (a) 5 μl was counted to determine total radioactivity retained in the leaves. (b) The protein in 100 μl of each sample was precipitated by adding 4 volumes of acetone, and allowing them to stand at −20°C overnight. The precipitates were recovered by centrifugation, washed three times with 80% acetone containing 2 mM methionine, and then dissolved in SDS-PAGE sample buffer ready for analysis by SDS-PAGE. (c) 500 μl were used for CHA-Sepharose affinity chromatography as described above. The bound fraction of protein was precipitated by adding 4 volumes of acetone. The CHA was removed from the precipitate by washing it twice with 10% TCA and then removing the TCA with two washes of 80% acetone. The pellets were dissolved in SDS-PAGE sample buffer. (d) 500 μl were used for immunoprecipitation of α-amylase using the *Staphylococcus aureus* protein A-antibody adsorption technique of Kessler (1975 J Immunol 115: 1617–1624). *S. aureus* cells were stored frozen in PBS-azide as a 10% suspension. To prepare them for use, cells were thawed, pelleted by centrifugation, and incubated in NET buffer containing 0.5% Nonidet P40 for 15 min at 23°C. The cells were pelletted and washed in NET buffer containing 0.05% Nonidet P40. Finally, they were brought to a 10% suspension in NET buffer containing 0.05% Nonidet P40 and 2 mM methionine. Before immunoprecipitation of α-amylase, the leaf extracts were subjected to a nonspecific precipitation as described previously (11). To 500 μl of leaf extract was added 3 μg of ovalbumin followed by 25 μl of sheep antiovalbumin serum. After 15 min at room temperature, 200 μl of *S. aureus* cells were added and after another 15 min, the mixture was centrifuged to remove the cells. The supernatant was used for α-amylase precipitation. Fifteen μl of sheep anti-α-amylase serum was added and after 15 min at room temperature, 200 μl of *S. aureus* cells were added. After a further 15 min, the cells were recovered by centrifugation and washed three times in 2 ml of NET buffer containing 0.05% Nonidet P40 and 2 mM methionine. Pelleted cells were resuspended in SDS-PAGE sample buffer, heated at 100°C for 2 min, and then centrifuged again. The supernatant was used for SDS-PAGE.

Proteins dissolved in SDS-PAGE sample buffer were separated by SDS-PAGE using gradient gels as described by Spencer *et al.* (27) and radioactivity was detected by fluorography.

Measurement of Rate of Total Protein Synthesis. Leaves were cut and labeled with L-[^{35}S]methionine essentially as described above. However, in this case a much larger dose of low specific activity methionine was supplied to each leaf, in order to swamp the endogenous methionine pool and to provide a methionine pool of known specific activity that would remain unchanged over a 6.5 h labeling period. From published values of a free amino acid content of unstressed and stressed barley leaves (26) we calculated that 10 cm of leaf contained about 3 nmol methionine. Accordingly, leaves were fed 1 μmol methionine (about 300 times the leaf content) in 5 μl containing about 1 μCi [^{35}S] methionine. Labeled amino acids are rapidly absorbed from the free space of barley leaves (>75% absorption in 1 h [13]) when supplied as described above.

Six control and six wilted leaves were radiolabeled. After 3.5 h and 6.5 h, three leaves from each treatment were harvested. Leaves were ground individually in NET buffer containing 0.05% Nonidet P40, 2 mM L-methionine, and 5 mg/ml BSA. The homogenates were centrifuged at 10,000*g* for 10 min, and the supernatant recentrifuged at 100,000*g* for 1 h to remove ribosomes. Soluble protein was precipitated by adding four volumes of acetone and the precipitate was washed and counted.

The second experiment used an abbreviated procedure for leaf processing following radiolabeling (13). The leaf lamina were boiled for 10 min in 10 ml 80% ethanol containing 5 mM methionine. The solution was sampled for total soluble counts and the tissue was reextracted as above until no more radioactivity was solubilized. The tissue was dried and ^{35}S incorporation into the ethanol-insoluble fraction (protein) was estimated directly by scintillation counting of the dried leaf material.

The following assumption is involved in interpreting ^{35}S incorporation data as a measure of the rate of protein synthesis: that throughout the experiment the pool of methionine used for protein synthesis had the same specific activity in the unstressed and stressed leaf tissues.

Analysis of variance was performed on ^{35}S incorporation for each experiment.

RNA Hybridizations. Total RNA was isolated as described previously (3) from second leaves of well-watered plants and plants which had not been watered for 5 and 6 d (Fig. 5). Samples of RNA (20 μg/lane) were electrophoresed in formaldehyde gels and blotted onto nitrocellulose filter paper (31). The papers were hybridized and washed as described previously (3) using nick translated insert of clone E of Rogers and Milliman (24). The insert in this plasmid is derived from an α-amylase mRNA which probably codes for an isozyme of the group A α-amylase (4, 15).

Localization of Leaf α-Amylase. Plants were grown for 11 d, the water was withdrawn for 6 d, and then reapplied for 4 d. At this time, 32 second leaves were harvested and protoplasts were prepared from them essentially as described by Edwards *et al.* (7). After the purified protoplasts had been pelleted, they were resuspended in 0.60 ml of 0.4 M sorbitol, 50 mM Hepes-KOH (pH 7.0), and 1 mM EDTA (no $NaHCO_3$). Half of this suspension was taken for total protoplast amylase and RuBP carboxylase analysis and the rest of the protoplasts were lysed. The chloroplasts were pelleted and the supernatant kept. The chloroplasts were resuspended in 0.3 ml of the above buffer and lysed either by freezing and thawing twice or by making the suspension 0.1% in Triton X-100. The intact protoplast suspension and the lysate supernatant were also made 0.1% in Triton. The amylases in each fraction were compared by IEF.

Intactness of chloroplasts was estimated by assaying RuBP carboxylase in the lysate supernatant and in the chloroplasts as described by Perchorowicz *et al.* (22).

Addition of Triton did not interfere with the IEF pattern of amylase isozymes or the RuBP carboxylase assay.

RESULTS

Characterization of Leaf Amylases. Total amylase from fully expanded second leaves of Betzes barley was resolved into eight isozymes (the two broad bands are doublets) by IEF (Fig. 1). A number of criteria were used to identify isozymes as either α- or β-amylases.

(a) It has been shown previously that α-amylose is bound to CHA-Sepharose while β-amylase is not (15). CHA-Sepharose chromatography of total leaf amylase from control or wilted (see later) leaves resulted in only one amylose isozyme, the most anodic, being bound and all others passing through the column (Fig. 1).

(b) If the CHA-Sepharose bound and unbound amylase fractions were incubated with amylase and the hydrolysis products separated using paper chromatography, different patterns of products were obtained (Fig. 2). The CHA-Sepharose-bound amylase gave products which are typical of α-amylase (16). The unbound amylase reaction mixture contained fructose, glucose, and maltose but because fructose and glucose were already present in the mixture at the start of the reaction (see 0 reaction time, not bound to CHA, Fig. 2) in amounts approximately equal to those after 20 h of reaction, it appears that the unbound amylase liberated primarily maltose which is characteristic of β-amylase.

(c) In crude preparations, α-amylase is usually heat stable whereas β-amylase is not. Holding crude extracts of control or wilted leaves at 70°C inactivated all isozymes, except the most

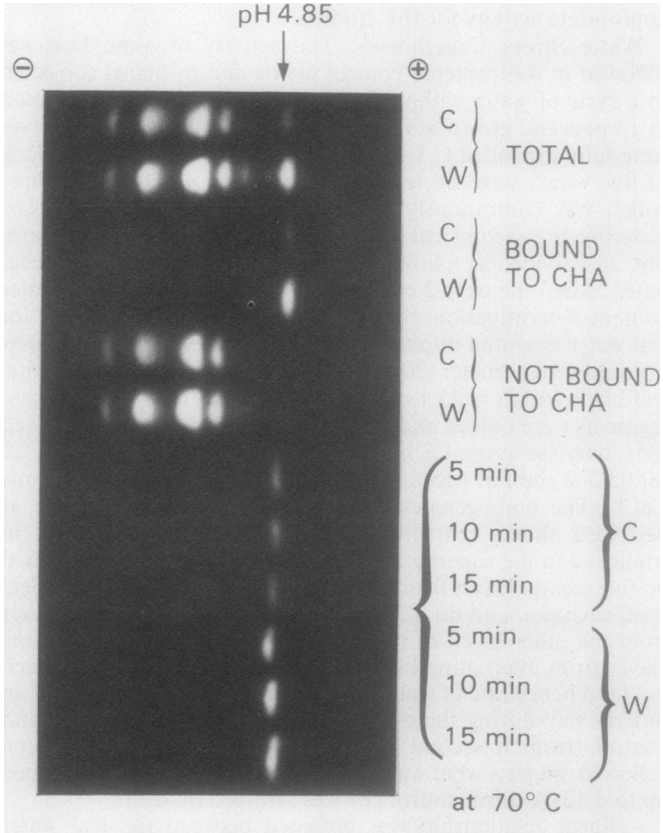

FIG. 1. Isozymes of barley leaf amylase separated on an IEF gel. The top part of the gel compares the total isozymes extracted from control (C) and wilted (W) leaves, the center part shows the fractionation of these isozymes using CHA-Sepharose affinity chromatography, and the bottom part shows the isozyme remaining in homogenates of control and wilted leaves after heating at 70°C for different periods of time.

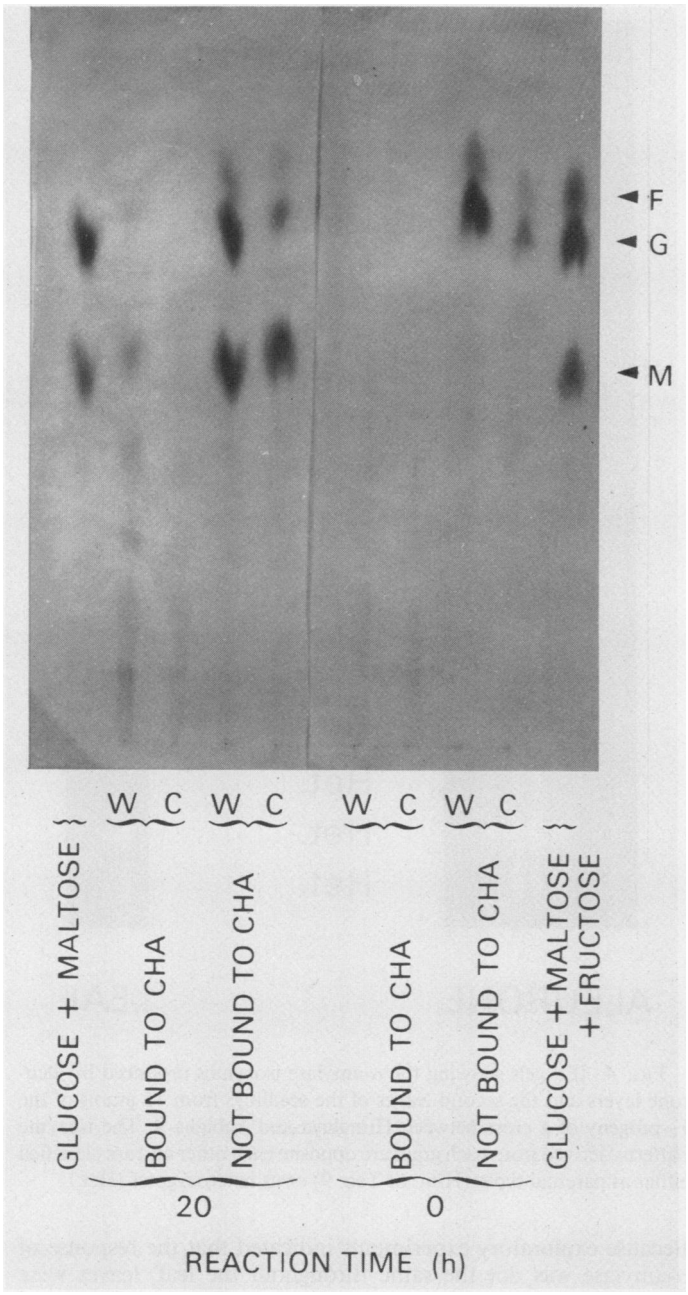

FIG. 2. Paper chromatograms of the products of hydrolysis of amylose by the CHA-Sepharose fractionated amylases shown in Figure 1. The panel on the right shows the components of the enzyme-substrate mixtures at the beginning of the reaction (0 h) and the panel on the left shows the components after 20 h of reaction. F, Fructose; G, glucose; M, maltose.

anodic one (Fig. 1). Evidence of the heat stability of this isozyme comes from the time-of-heating study. Judging from band strength on IEF gels (Fig. 1), it appears that whereas all other isozymes were inactivated after 5 min at 70°C, little or none of the activity of anodic isozymes was lost between 5 and 15 min at 70°C. *In vitro* assay of amylase remaining in heated solutions (data not given) verified this result. Clearly, the most anodic amylase isozyme is much more heat stable than the other isozymes.

(d) If a focused gel containing all amylase isozymes was immersed in 0.4 mM pCMB for 15 min before the starch incubation procedure used to detect amylase activity, none of the isozymes appeared on a stained gel indicating that all isozymes were sulfhydryl requiring (data not shown). β-Amylase is very sensitive to sulfhydryl reagents and whereas α-amylase is not usually thought of as being sensitive to these reagents, there is at least one type, the group A isozymes of barley aleurone, which is pCMB sensitive (16). The characteristics of group A α-amylase isozymes (pI around pH 4.7) and group B isozymes (pI around pH 6.3) are summarized in Jacobsen (14).

(e) In an experiment similar to that described in (d), a gel was soaked in 5 mM EDTA for 15 min before the starch incubation. None of the leaf isozymes was inactivated (data not shown). On the same gel, the activity of the group A α-amylase of Betzes aleurone was also stable but the type B enzyme was not. The two α-amylase types from Himalaya aleurone show a similar differential sensitivity to EDTA (16).

On the basis of these results (a–e), we identify the most anodic isozyme as an α-amylase, probably of the aleurone group A type, and all the others as β-amylase.

Comparison of Leaf and Aleurone α-Amylases of Different Barley Genotypes. Because the leaf α-amylase isozyme has characteristics similar to the aleurone group A α-amylase, it was of interest to examine the relationship of the aleurone and leaf α-amylases further. Accordingly, the isozymes of heated samples

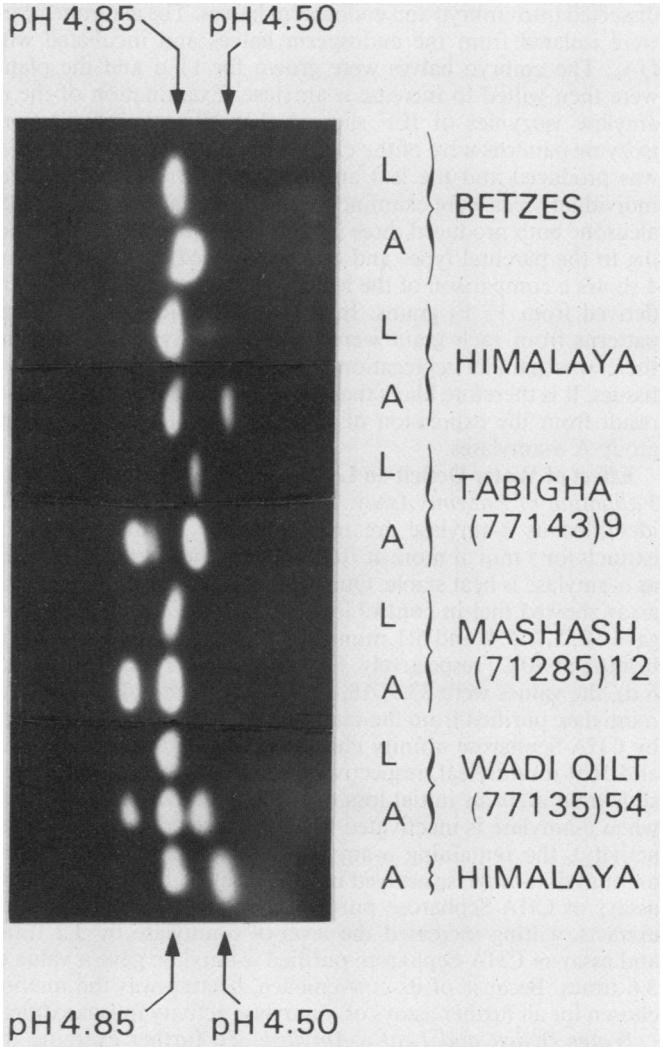

FIG. 3. IEF gel comparing the α-amylase isozymes obtained from leaves (L) and aleurone (A) of different genotypes of barley.

(10 min at 70°C) of leaf extracts and of heated incubation media from GA_3-treated aleurone layers were compared using IEF. A number of barley genotypes were compared; they were selected because they had different group A α-amylase isozymes (2).

Figure 3 shows the leaf and aleurone isozymes in the group A zone of an IEF gel. The arrows point to the major isozymes of Himalaya barley with isoelectric points of 4.85 and 4.50 (15). The group B aleurone isozymes which have isoelectric points near pH 6 are not shown because we have not been able to detect any leaf α-amylase isozymes other than those in the group A zone as shown in Figure 3 (see also Fig. 6). Leaves of all genotypes contained only one α-amylase (heat stable) isozyme whereas aleurone layers produced 1, 2, or 3 isozymes, but in all cases, the leaf isozyme matched one of the aleurone isozymes. This constituted further evidence that leaf α-amylases were related to aleurone enzymes. However, some doubt remained because of the differences between the leaf and aleurone isozyme patterns.

Further evidence of the relationship was sought from crossing studies. Figure 3 shows that the line Tabigha 9 and Himalaya barleys have different leaf and aleurone IEF patterns. In exploratory studies, it was found that all isozymes could be clearly distinguished by IEF in mixtures of leaf extracts and of aleurone incubation media (results not shown) indicating that leaves and aleurone of the progeny of a Tabigha 9 × Himalaya cross could be easily typed. Accordingly, some F_1 seed from this cross was dissected into embryo and endosperm halves. The aleurone layers were isolated from the endosperm halves and incubated with GA_3. The embryo halves were grown for 11 d and the plants were then wilted to increase α-amylase. Examination of the α-amylase isozymes of IEF showed that all leaf and aleurone isozyme patterns were of the expected heterozygote type. F_2 seed was produced and the leaf and aleurone isozyme patterns for individual seeds were examined as described above. Leaves and aleurone both produced three isozyme patterns, two corresponding to the parental types and one the heterozygote type. Figure 4 shows a comparison of the leaf and aleurone isozyme patterns derived from 15 F_2 grains. In all cases, the leaf and aleurone patterns from each grain were of the same type showing that there was parallel segregation of α-amylase genes in the two tissues. It is therefore likely that the leaf and aleurone α-amylases result from the expression of the same set of genes specifying group A α-amylases.

Effect of Water Deficit on Leaf α- and β-Amylase. *α-Amylase. Validation of Enzyme Assay.* Figure 1 shows that all isozymes identified as β-amylase are inactivated by heating crude leaf extracts for 5 min or more at 70°C and that the isozyme identified as α-amylase is heat stable. Quantification of amylase by *in vitro* assay showed that in control leaf extracts, the assays of amylase gave 18, 5.7, 5.4, and 5.1 munits/leaf after 0, 5, 10, and 15 min heating at 70°C, respectively. For wilted leaves (not watered for 6 d), the values were 33.9, 18, 18, and 17 munits/leaf. Assay of α-amylase purified from the same control and wilted leaf extracts by CHA-Sepharose affinity chromatography gave values of 4.5 and 16.4 munits/leaf, respectively. Taken together, these results show that after an initial loss of amylase activity from extracts when β-amylase is inactivated (the assay used detects β-amylase activity), the remaining α-amylase is heat stable. Further, the amount of α-amylase assayed in heated samples agrees well with assays of CHA-Sepharose purified α-amylase. Assaying heated extracts, wilting increased the level of α-amylase by 3.2 times and assay of CHA-Sepharose purified α-amylase gave a value of 3.6 times. Because of its convenience, heating was the method chosen for all further assays of α-amylase activity in leaf extracts.

Water Deficit and Leaf α-Amylase. To further examine the effect of water deficit on α-amylase level, we determined water potential and α-amylase level in the second leaves of seedlings from which water was withheld for 6 d, followed by rewatering.

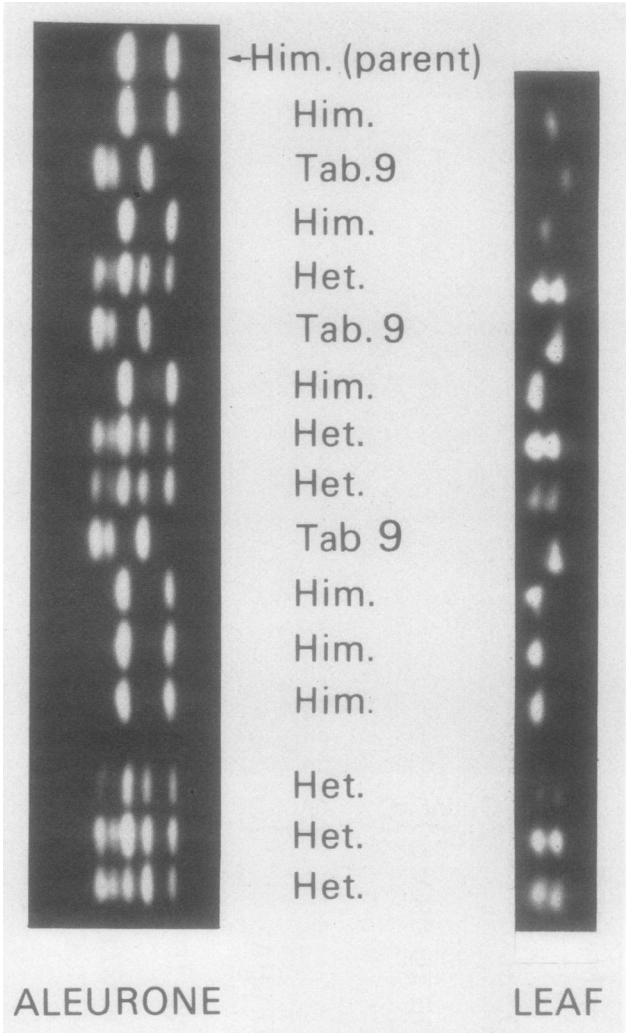

FIG. 4. IEF gels showing the α-amylase isozymes produced by aleurone layers and the second leaves of the seedlings from 15 grains of the F_2 progeny of a cross between Himalaya and Tabigha 9. The isozyme patterns derived from each grain are opposite each other and are classified either as parental types (Him. or Tab. 9) or as heterozygotes (Het.)

Because exploratory experiments indicated that the response of α-amylase was not the same throughout the leaf, leaves were divided for assay into base and tip sections. Figure 5 shows that after 4 d of water-withholding, the α-amylase in leaf tips had begun to increase and ψ_{leaf} had begun to fall. As leaf water potential continued to fall, α-amylase level continued to increase. On the 5th d, seedlings showed signs of wilting and on the 6th d, wilting was severe. When watering was resumed on the 6th d, the plants rapidly regained full turgor, but the level of α-amylase remained high and had not returned to pre-stress levels 7 d after rewatering. For leaf bases, the results were similar except that α-amylase was always at a lower level than in tips and apparently continued to increase for 1 day after watering was resumed.

Water Deficit and Leaf β-Amylase. Figure 2 shows that both control and wilted leaf extracts contain fructose and glucose (see track NOT BOUND TO CHA, W and C) although they contain different amounts. After 20 h reaction with amylose, the amount of maltose was greatly increased using both control and wilted leaf extracts but the amounts of fructose and glucose were apparently unchanged. Although the detection of reducing sugars on the chromatogram is only semiquantitative, these results were considered adequate to indicate that any increase in reducing

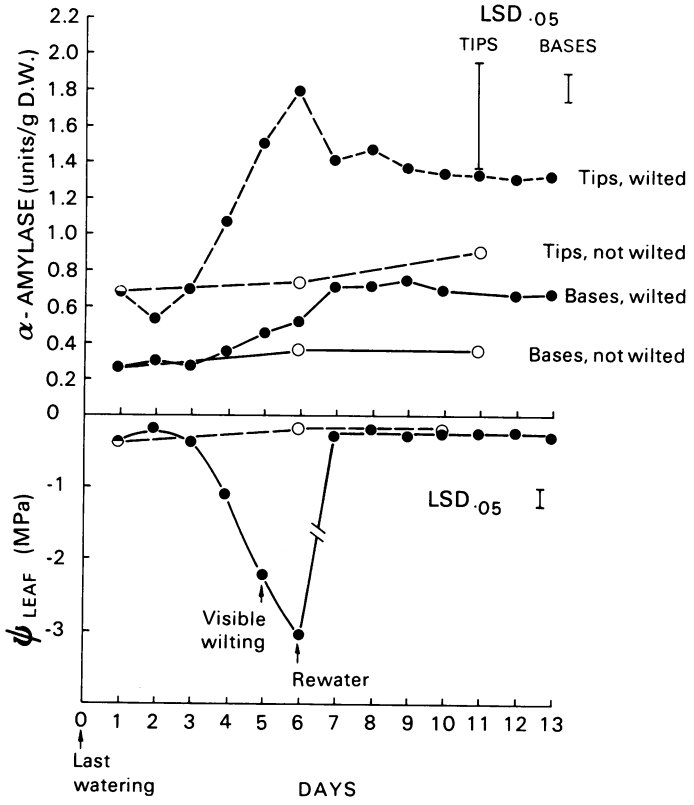

FIG. 5. Relationship between the α-amylase content of barley leaves (top) and their water potential (ψ leaf) (bottom). Plants were grown for 11 d and then divided into two groups. One group was continuously watered (not wilted, ○). Water was withheld from the second group for 6 d and then reapplied (wilted, ●). Leaves were divided into tip and basal sections for enzyme assay.

sugars in enzyme reaction mixtures containing amylose and enzyme not bound to CHA-Sepharose columns (CHA-Sepharose binds only α-amylase) was due primarily to maltose and that a reducing sugar assay was a reasonable estimate of β-amylase activity. Using such an assay and measuring reducing sugars as described by Nelson (19), the amounts of β-amylase in control and wilted tissues were found to be the same (data not shown).

Distribution of Amylases along the Second Leaf. The development of a procedure to separate α- and β-amylase and for obtaining a semiquantitative assay of them on IEF gels, enabled us to determine the distribution of the two enzymes along the blade of leaf 2. Seedlings were grown for the usual 11 d and water was withheld from some but not others. After 6 d, when the unwatered plants were wilted, leaf blades were harvested and cut into quarters lengthwise. Amylases were extracted and separated by IEF. The gel was loaded on the basis of equal dry weight of leaf per sample. Figure 6 shows that α-amylase is fairly uniformly distributed along the blade in both control and wilted leaves although the wilted leaves contain much more α-amylase than turgid leaves. However, β-amylase is not distributed evenly. In both turgid and wilted leaves, β-amylase is concentrated in the lower part of the leaves, particularly in the second segment from the blade base. The enzyme occurs in relatively low concentration in segment 3 and is barely detectable in the apical quarter of the leaves. Therefore, it appears that the ratio of α- to β-amylase varies considerably along the leaf lamina both in turgid and wilted leaves.

Figure 6 also shows the group A and B α-amylases of aleurone layers. As in Figure 3, the leaf α-amylase matches the group A α-amylase of aleurone layers but whereas group B aleurone α-amylase is clearly evident on the gel, no such isozymes are detectable either in turgid or wilted leaves.

ABA Content of Leaves. Wilting is usually associated with elevated ABA content of leaves and, at least in aleurone, ABA inhibits the production of α-amylase. Therefore, it was of interest to determine if the increase in leaf α-amylase occurred in spite of elevated ABA content. Accordingly, ABA was assayed in control second leaves and in leaves from plants which had not been watered for 6 d and were visibly wilted. The control leaves contained 8.4 ng ABA/leaf while wilted leaves contained 30.1 ng/leaf.

The Mechanism of the Increase in α-Amylase Level. During wilting, levels of proteases, phenols, and so forth may change so that the observed increase in α-amylase might have been due to changes in factors which modify enzyme activity during the extraction procedure, rather than to changes in enzyme level. To test for such artifacts, turgid and wilted leaves were extracted separately and together and the α-amylase levels were assayed. The amount of enzyme in the mixture was equal to the sum of the individual turgid and wilted leaf samples indicating that the increase in enzyme activity was likely to be due to changes in enzyme level.

Support for this result was obtained from radiolabeling experiments designed to determine if α-amylase arose by new synthesis. On the morning of the 6th d after the last watering, control and wilted leaves were fed with [^{35}S]methionine for 6 h. Water soluble proteins were extracted, and α-amylase was purified both by CHA-Sepharose affinity chromatography and by immunoprecipitation using *Staphylococcus aureus* cells. Comparison of total radioactive proteins from control and wilted leaves by SDS-PAGE (Fig. 7A) showed that wilting caused a number of changes in protein synthesis, apparently involving both reductions and increases in the synthesis of various polypeptides. In particular, there were decreases in the syntheses of the two major polypeptides with $M_r \sim 53{,}000$ and 13,000, tentatively identified as the large and small subunits of ribulose bisphosphate carboxylase, and of a polypeptide with $M_r \sim 21{,}000$. There was increased synthesis of a polypeptide with $M_r \sim 85{,}000$. In the control protein preparation, there were no labeled proteins detectable which bound to CHA-Sepharose but in the wilted leaf protein preparation, there was one labeled polypeptide which co-migrated with the upper band of the doublet given by CHA-Sepharose-purified α-amylase from the aleurone of Betzes barley. The two Betzes aleurone α-amylase components were separated by carboxymethylcellulose chromatography (Fig. 7B). The upper (or lower mobility) component corresponding to the leaf α-amylase was identified as group A α-amylase and the lower (or higher mobility) component as group B α-amylase by IEF (data not presented). The group A components have isoelectric points around pH 4.7 and the group B components around pH 6.3 (15).

The immunoprecipitation experiments gave results which supported the CHA-Sepharose studies. The material immunoprecipitated from control leaf protein extracts contained two major polypeptides, one of them corresponding to the polypeptide identified as the large ribulose bisphosphate carboxylase subunit ($M_r \sim 53{,}000$) and the other having $M_r \sim 40{,}000$, and a very small amount of a polypeptide corresponding to the slower mobility band of the Betzes aleurone α-amylase. The protein immunoprecipiated from wilted leaf extract contained three major polypeptides, two of them the same as in control protein and the third corresponding to α-amylase. There was much more of this third component in wilted then in control leaves. The two major components common to both control and wilted leaf protein immunoprecipitates were precipitated using anti-ovalbumin as well as anti-α-amylase serum and therefore represent cases of nonspecific precipitation.

Taken together, these results indicate that the increase in α-

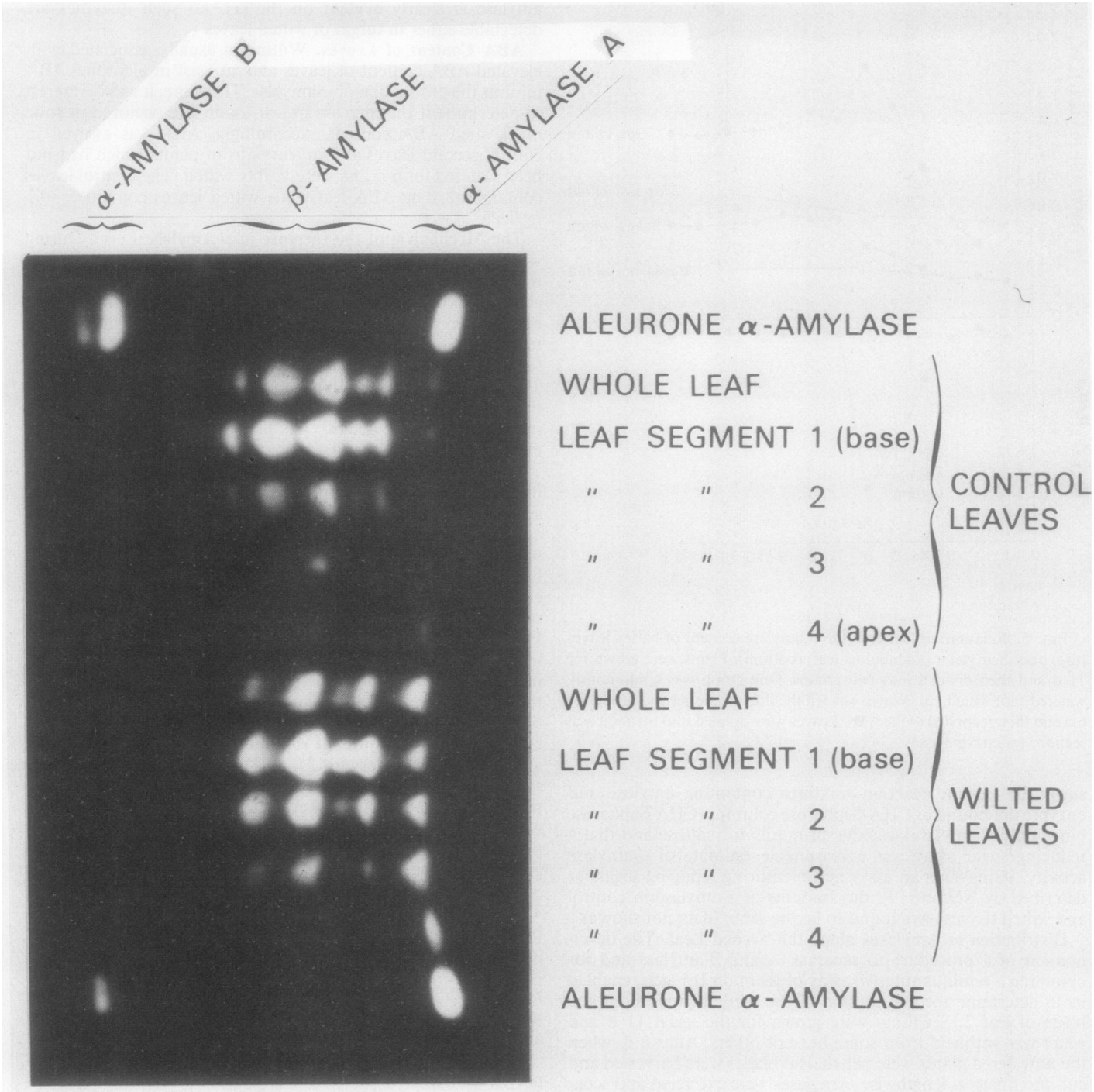

FIG. 6. IEF gel showing the distribution of amylase isozymes along the blades of turgid (control) and wilted leaves. The leaves were divided lengthwise into quarters. The leaf isozymes are compared with those from aleurone.

amylase activity provoked by water deficits is associated with *de novo* synthesis of α-amylase protein, and provide additional evidence that the leaf α-amylase is of the A type. In stressed leaves, the α-amylase constitutes only a very small proportion (less than 0.05%) of the total radioactivity incorporated into protein; this accounts for the fact that no differences can be seen in the α-amylase regions of the control and wilted leaf total protein tracks.

The relative increase in [^{35}S]methionine incorporation into α-amylase protein, caused by water stress could arise in various ways: (a) in wilted leaves, the absolute rate of α-amylase synthesis could remain constant while the synthesis of most other proteins decreases. (b) The absolute rate of α-amylase synthesis in wilted leaves could increase. (c) The rate of degradation of newly-synthesized amylase could decrease. To test the first possibility, estimates of the rates of total protein synthesis in control and wilted leaves were made by supplying leaves with a large dose of low specific activity [^{35}S]methionine, and measuring the incorporation of ^{35}S into protein. Table I shows the results of two experiments. In the first, the estimated protein synthesis rates in turgid and stressed leaves were the same and in the second, the rates in stressed leaves were significantly higher than in turgid

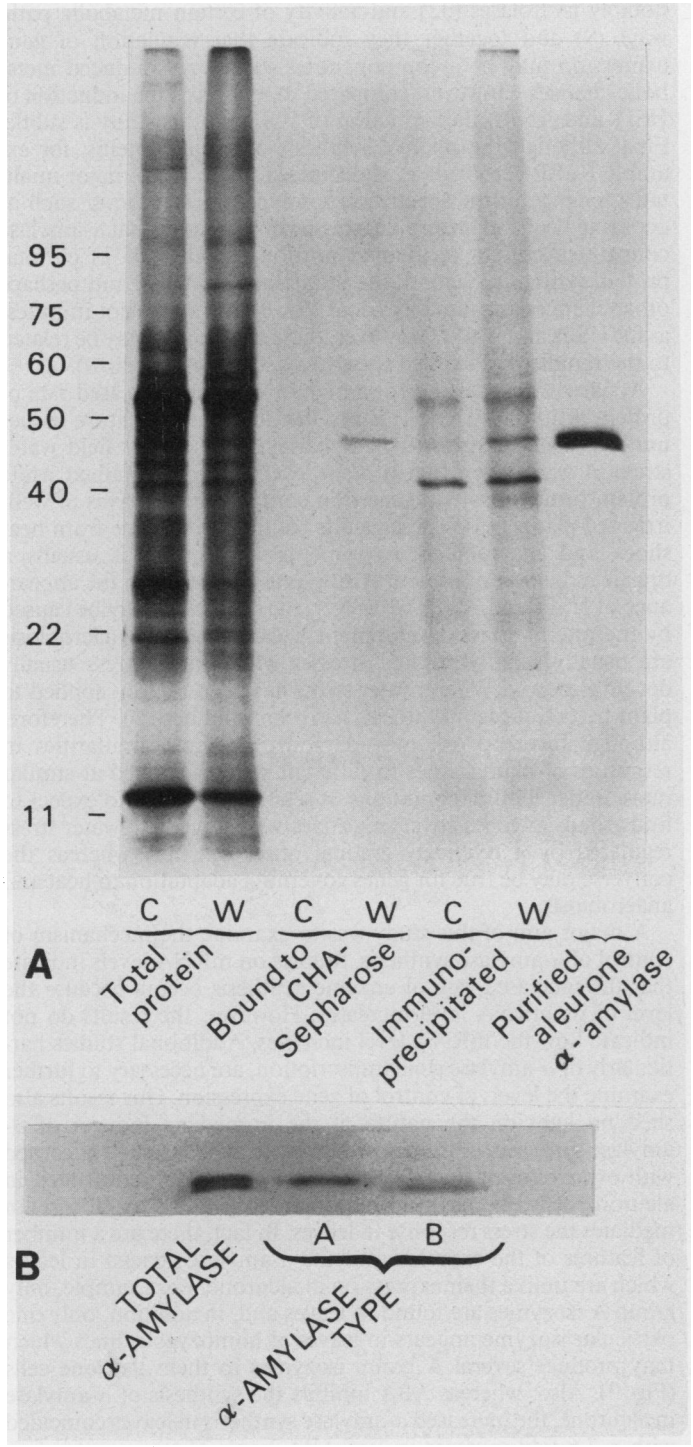

FIG. 7. A, Fluorograph of SDS-PAGE separations of [^{35}S]methionine labeled polypeptides from control (C) and wilted (W) leaves. The total polypeptides are compared to those extracted by CHA-Sepharose chromatography and immunoprecipitation. The position of α-amylase on the gel was determined by running purified aleurone α-amylase which is resolved into two major components. The total protein tracks were loaded with protein from equal amounts of tissue and the protein contained approximately equal amounts of radioactivity. The numbers ($\times 10^{-3}$) indicate the positions of mol wt markers. B, SDS-PAGE of total purified aleurone α-amylase (as shown in A) and of the type A and B components separated by carboxymethylcellulose chromatography.

Table I. *Incorporation of [^{35}S]Methionine into Total Protein in Turgid and Water-Stressed Second Leaves of Barley*

	Labeling time			
	3.5 h		6.5 h	
	Turgid	Wilted	Turgid	Wilted
	nmol methionine/leaf			
Experiment 1	7.9	8.3[a]	12.9	14.1[a]
Experiment 2	9.7	11.9[b]	13.8	19.2[b]

[a] Wilted not significantly different from turgid at 5% level. [b] Wilted significantly greater than turgid at 1% level.

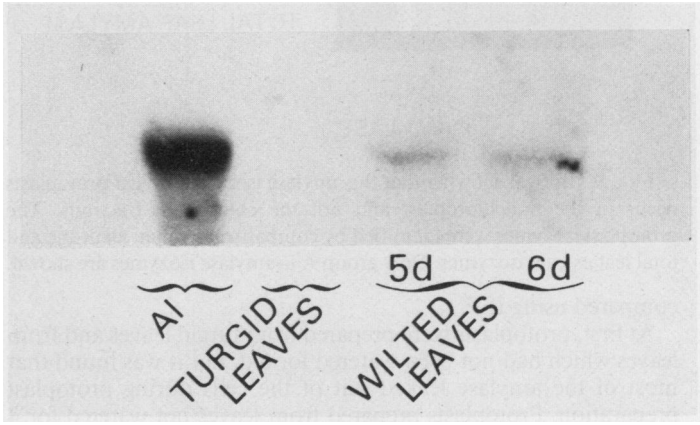

FIG. 8. Hybridization of ^{32}P-labeled-insert of 'clone E' (Rogers and Milliman, 1983) to size-fractionated RNA. The first track (left) contains 40 ng of aleurone RNA from GA_3-treated (24 h) aleurone layers plus 20 μg of pea cotyledon RNA which contains no α-amylase-related sequences. The other tracks contain 20 μg of RNA from turgid leaves and from leaves not watered for 5 and 6 d.

leaves. There is thus no evidence that wilting depresses total protein synthesis, leading to the conclusion that the relatively greater incorporation of label into α-amylase in wilted leaves shown in Figure 7A represents accumulation of newly synthesized enzyme. This is likely to result from an increased rate of enzyme synthesis; an alternative, but less likely, possibility is decreased degradation.

Assay of α-Amylase mRNA. Because it was possible that there was an increase in the rate of α-amylase synthesis, it was of interest to determine if there was an increase in the amount of α-amylase mRNA. α-Amylase mRNA assay was facilitated by the availability of the α-amylase cDNA clone E (24) which was made from barley aleurone RNA and which probably corresponds to the group A of α-amylases (4).

Figure 8 shows that no α-amylase mRNA was detected in RNA from turgid leaves but that it was detected in RNA from wilted leaves. However, as for α-amylase protein, the amount of α-amylase mRNA in wilted leaf RNA is very small compared to GA_3-treated barley aleurone RNA. From the results shown in Figure 8, it can be seen that the amount of α-amylase mRNA in aleurone RNA diluted 500-fold with pea cotyledon RNA, is still much more than that in wilted leaf RNA. We estimate that RNA from wilted leaves contains about 1/5000 of the α-amylase mRNA in RNA from aleurone treated with GA_3 for 24 h.

Localization of Amylases in Leaf Cells. Because leaf α-amylase is commonly thought to be involved in starch hydrolysis in chloroplasts and because starch breakdown is a feature of water stressed plants (28 and reference therein), it was of interest to determine if the stress-induced enzyme occurred in chloroplasts. This was done by preparing protoplasts from second leaves of barley, lysing them, and separating chloroplasts from cytoplasm by centrifugation. Chloroplast and nonchloroplast amylases were

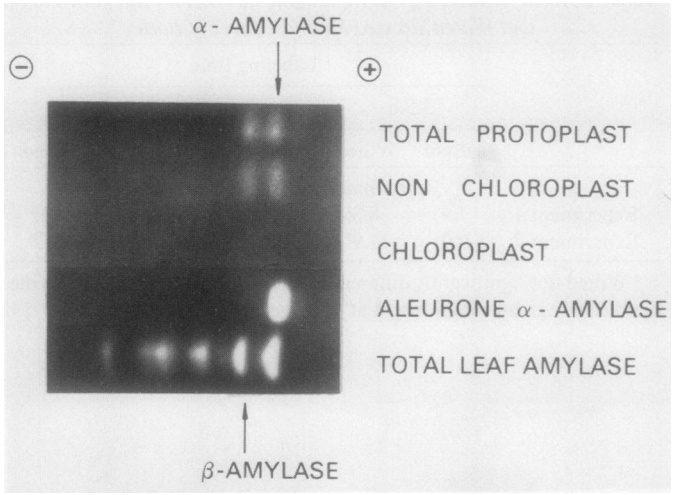

FIG. 9. IEF gel showing that the amylase isozymes in leaf protoplasts occur in the nonchloroplast and not the chloroplast fractions. The protoplast isozymes were identified by comparison with an aleurone and total leaf extract isozymes. Only group A α-amylase isozymes are shown.

compared using IEF.

At first, protoplasts were prepared from turgid leaves and from leaves which had not been watered for 6 d, but it was found that most of the amylase leaked out of the cells during protoplast preparation. Protoplasts prepared from leaves not watered for 4 d were also leaky and it became evident that we were unable to use water-stressed leaves. As an alternative approach, we repeated the experiment using leaves which had been loaded with α-amylase by allowing them to wilt as usual and then rewatering. When harvested 4 d after rewatering, the leaves contained elevated levels of α-amylase (see Fig. 5, d 10), they were turgid, and amylase-containing protoplasts could be prepared. Figure 9 shows that α-amylase and the most anodic (and dominant) β-amylase were detected in total protoplast extracts and in the nonchloroplast fraction but no amylase activity could be detected in the chloroplast fraction. Of the RuBP carboxylase detectable in the two fractions, 87% were in the chloroplasts indicating that they were relatively intact. This experiment was done three times and in each case, there was very little chloroplast breakage as determined by RuBP carboxylase assay and yet most or all of the amylase activity occurred in the nonchloroplast fraction. However, in all of the carboxylase assay experiments, the amount of enzyme assayed in total protoplast lysates fell short of that assayed in the component chloroplast and nonchloroplast fractions by about 40%. We have no explanation for this but it probably does not change the conclusion that most of the carboxylase occurred in the isolated chloroplasts.

DISCUSSION

This study shows that water stress in barley leaves causes changes in the pattern of protein synthesis involving relative increases and decreases in polypeptide synthesis and in particular it shows that regulation of α-amylase activity probably results from regulation of its synthesis. This, in turn, probably results from accumulation of α-amylase mRNA levels which may be due to regulation of transcription. These results are similar in principle to those from studies on water stressed *Avena* coleoptiles and maize mesocotyls in which it was shown that there were also a number of relative increases and decreases in polypeptide synthesis (6, 12). Taken together, these results constitute evidence that, just as there are HSPs (1) and ANPs (25) which are polypeptides whose synthesis is induced by stress so there are WSPs, one of which is α-amylase. These results complement those which have measured stress-induced increases in activity of enzymes, notably hydrolases (32) and activity of certain metabolic pathways (9) and together they indicate that regulation of gene expression may be a component in water stress-induced metabolic changes. However, compared to reports of the induction of HSPs and ANPs, the induction of WSPs in this study is subtle. First, although the relative synthesis of some proteins, for example RuBP carboxylase, is decreased, there is no major qualitative change in the spectrum of polypeptide synthesis, such as occurs in heat and anaerobic stress, with the result that α-amylase constitutes only a very small portion (about 0.05%) of total protein synthesis. Second, the inductions of WSPs, and perhaps other changes, presumably occur slowly over days (not minutes) as for HSPs and ANPs. However, these differences may be related to the rapidity with which the stress develops (see below).

Apparent maintenance of a high or even an increased rate of protein synthesis (Table I) is not uncommon for mature tissues under long-term stress (9). For barley leaves under field water stress, it was shown that protein levels were maintained while protein turnover rate increased in comparison to leaves of well-irrigated plants (13). These results contrast with those from heat shock and anaerobiosis experiments where there is usually a drastic reduction of protein synthesis associated with the appearance of HSPs and ANPs. However, the difference may be caused by the rate of stress development. Heat stress and anaerobiosis are usually rapidly applied stresses while water stress usually develops slowly. Where water stress has been rapidly applied to plant tissues, protein synthesis has been inhibited (9). Therefore, although further work may demonstrate more similarities in responses of plant tissues to different stresses applied at similar rates, under natural conditions, it seems reasonable to expect to find groups of genes governing adaptive responses to water stress regulated by a relatively gradual onset of stress whereas the converse may be true for genes governing adaptation to heat and anaerobiosis.

A major aim of this study was to examine the mechanism of control of α-amylase synthesis. Studies on mRNA levels indicate that the increased rate of enzyme synthesis occurs because the level of its mRNA level increases. However, the results do not indicate how the mRNA level increases. Additional studies particularly of α-amylase gene transcription, are necessary to further examine the levels of control of gene expression. Our results also shed no light on the nature of the immediate inducer of α-amylase synthesis. Although in leaves we appear to be concerned with expression of the same gene(s) that are GA_3-controlled in aleurone cells, we have no reason to conclude that gibberellin mediates the stress response in leaves. In fact, there are a number of features of the expression of the α-amylase gene(s) in leaves which are unlike their expression in aleurone. For example, only group A isozymes are found in leaves and, in addition, only one particular isozyme appears in leaves of homozygous lines which may produce several A group isozymes in their aleurone cells (Fig. 3). Also, whereas ABA inhibits the synthesis of α-amylase in aleurone, the increased α-amylase synthesis in leaves coincided with a 3.6-fold increase in leaf ABA level. These aspects of α-amylase indicate that, in leaves, the control mechanism may be different from that in aleurone.

In studies of leaf α-amylases, it has commonly been assumed that the enzymes have the characteristics of the classical plant α-amylase from cereal endosperm, including a requirement of Ca^{2+} ions and sensitivity to EDTA (for reviews see 8, 23, 30), and identification of amylolytic activity as either α- or β-amylase has been made on this basis. However, it is now known that there are two kinds of cereal endosperm α-amylase, type B with the classical EDTA sensitivity but insensitivity to sulfhydryl reagents, and type A, which is insensitive to EDTA but sensitive to sulfhydryl reagents (14 and references therein) and that the

assumptions made above are therefore not justified. In fact, α-amylases identified in spinach leaves (21) and now in barley leaves (this study) are of the A type. It is also of interest to note that the α-amylases of the photosynthetic alga, *Chlamydomonas reinhardtii* are also of the A type (18). However, α-amylaes isolated from *Phaseolus* cotyledons (33) and from *Pisum* cotyledons (29) are of the B type. These data indicate that, in general, there may be two types of plant α-amylases based on inhibitor characteristics and that occurrence of the types may be tissue specific. These studies also highlight the difficulty in identifying amylolytic activity on the basis of inhibitor characteristics since β-amylase has the same characteristics as type A α-amylase.

Finally, the localization of the α-amylase activity outside of the chloroplasts, provides little insight into its physiological significance. Based on the assumption that the enzyme plays some role in water stressed leaves, possibilities are (a) that there is regulated movement of α-amylase into and out of chloroplasts or (b) that the enyzme only functions if starch or dextrins leak from chloroplasts under mild stress or (c) if under severe stress, there is complete breakdown or cellular permeability barriers and mixing of cell contents occurs. The last possibility is attractive because α-amylase seems to be one of a group of hydrolases which increase during stress (32) perhaps in preparation for mobilizing polymers from severely stressed cells and transporting the products to parts of the plant which are more likely to survive.

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