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EFFECT OF GIBBERELLIC ACID ON STARCH DEGRADING ENZYMES IN LEAVES OF *DIGITARIA DECUMBENS**

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Abstract—Tropical 'Pangola' digitgrass was treated with gibberellic acid (GA) and subjected to 30° or 10° nights. The starch degrading enzymes of the leaves were separated by polyacrylamide gel electrophoresis. Densitometer tracings of gels negatively stained by starch–iodine showed the presence of seven bands regardless of treatment of plants. GA treatment increased starch degrading enzyme activity in plants subjected to 10° to the level of activity found in 30° untreated (control) plants and, additionally, enhanced enzyme activity in plants at 30°. GA treatment of 10° plants decreased sucrose and starch levels when compared to levels found in untreated 10° plants. The action of GA in reversing the effects of 10° nights on 'Pangola' leaves was found to be the result of a quantitative increase in activity of existing enzyme forms rather than production of isozymes.

INTRODUCTION

EXPOSURE of the tropical pasture grass 'Pangola' digitgrass (*Digitaria decumbens* Stent.) to 10° nights causes reductions in growth and photosynthetic capacity,[1–5] immobilization of assimilate starch in leaf mesophyll chloroplasts,[6,7] and decreased activity of starch degrading enzymes.[8–10] This diminished enzyme activity is believed to be responsible for the accumulation of starch granules, the presence of which may contribute to reduced yields by interfering with photosynthesis.[1,9,10] Application of 10 μM gibberellic acid (GA) to leaves of 'Pangola' prior to 10° nights reversed the deleterious effects of 10° nights by increasing the activity of starch degrading enzymes, thereby mobilizing accumulated starch.[11] Previously, using gel electrophoresis, we have separated the starch degrading enzymes of some temperate and tropical grasses and have compared their properties.[12] We have attempted

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during this study to determine whether the GA-induced increase in starch degrading enzyme activity is the result of activation of isozymes not active in the leaf before GA treatment (qualitative change), an increase in activities of existing enzymes (quantitative change), or both.

RESULTS AND DISCUSSION

Following electrophoresis and incubation of gels, subsequent negative staining with buffered iodine solution produced bands depleted of starch to varying degrees by the starch degrading enzymes. For reference purposes, plant treatments will hereafter be designated with regard to night temperature received and presence or absence of GA application as follows: 30° (control), 10°, 30° + GA, and 10° + GA.

No additional enzyme bands were detected as a result of treatment with GA. All gels exhibited seven bands with migration patterns which were very similar regardless of plant treatment. These bands separated into a fast-migrating pair (bands 1 and 2) and a slow-migrating group (bands 3–7). We found previously only three bands characteristic of 'Pangola' enzyme extracts, one fast-migrating protein and two slow-migrating proteins.[12] Of the four additional bands observed in this study, three were in the slow-moving group, and one exhibited rapid movement. These bands appeared after a longer gel migration path was provided to allow overlapping enzyme forms to separate more completely. Bands 3–7 separated and developed much more gradually than bands 1 and 2. Therefore, it was necessary to increase migration and incubation times to allow development of bands 3–7. As determined previously, under these conditions incubation time was not long enough to result in substrate limitation for enzymes of the most active bands.[12]

Areas of portions of densitometer tracings corresponding to each gel band were used as measurements of each enzyme's activity, referred to as 'relative activity'. This term is intended to be a measure of both specific activity and enzyme concentration for purposes of comparing enzymes of plants subjected to different treatments. In unpublished work, we have found relative activity measurements from densitometer scans to be proportional to activity present in the extracts. In this study, enzyme activities of the same extract were comparable when expressed as per cent of control whether determined colorimetrically or measured from densitometer tracings. Enzyme activities thus expressed were also found to be comparable to those determined colorimetrically by Karbassi.[11]

The 10° treatment reduced total relative activity of enzymes to 60–65% of control. However, total relative activity of the 10° + GA treatment increased to 92–100% of control. On the average, the 30° + GA treatment increased total relative activity above that of control by 16%. GA treatment of 10° plants reduced starch and sucrose levels by about 50 and 25%, respectively. GA treatment of 30° plants reduced starch and sucrose levels by about 60 and 75%, respectively. An example of the relationships among the values of total relative activity ascertained from tracings, colorimetrically determined activity, and starch and sucrose levels for the four treatments is given in Table 1.

While all bands generally reflected the trends shown by total relative activity values of the treatments indicated, certain bands were more or less responsive to treatments than others. The activity in band 2 was more reduced by 10° (52% of control) than any of the major bands and most enhanced by GA (15% above control). The activity in band 7 was least influenced by any treatment. Over the three treatments, the activity of bands of the 3–7 group represented 13% of the total relative activity. Of this less active group, the enzyme of band 3 was most responsive to GA treatment, and that of band 5, while being the most reduced by 10°,

was the least enhanced by GA. An example of the relative activities of each band is shown in Table 2.

TABLE 1. EFFECT OF SUBOPTIMAL TEMPERATURE AND GIBBERELLIC ACID ON ENZYME ACTIVITY AND CARBOHYDRATE CONTENT IN 'PANGOLA' DIGITGRASS LEAVES

	30°	10°	30° + GA	10° + GA
Total relative activity (cm^2)*	24·5	15·1	25·4	24·4
Total activity†				
(ΔA/30 min/0·6 g fr. wt)	0·797	0·499	0·841	0·723
mg Starch/g fr. wt	0·7	15·7	0·3	7·9
mg Sucrose/g fr. wt	0·8	5·5	0·2	4·3

* Determined from densitometer tracings of 3 replicate gels per treatment.
† Determined colorimetrically from 2 replicate enzyme extracts per treatment.

In brief, it has been shown that in 'Pangola' the effect of GA on the starch degrading enzymes of the leaf is quantitative rather than qualitative, since no new enzymes were formed in plants treated with GA. The fact that GA did not cause production of additional isozymes may be unique to chloroplasts, as it has been demonstrated that GA-enhanced starch degrading enzyme activity in seeds is accompanied by production of multiple isozymic forms.[13–15] In the present study, GA enhanced total relative activity of the starch degrading enzymes in plants subjected to 30° nights and increased total relative activity of plants subjected to 10° nights to as much as 98% of control plants. Certain bands showed greater or less response to treatments. Application of GA to 10° plants reduced starch levels to 50% of the untreated 10° level. This reduction is not reflected, however, in increased sucrose levels, as the sucrose content of 10° plants dropped 25% when treated with GA.

TABLE 2. EFFECT OF SUBOPTIMAL TEMPERATURE AND GIBBERELLIC ACID ON AVERAGE RELATIVE ACTIVITY OF INDIVIDUAL ENZYME BANDS*

	Relative activity (cm^2)					Relative activity (cm^2)			
Band	30°	10°	30° + GA	10° + GA	Band	30°	10°	30° + GA	10° + GA
1	16·2	10·1	14·2	15·2	5	0·4	0·1	0·2	0·3
2	7·0	3·6	8·0	6·9	6	1·2	0·5	0·7	0·7
3	0·6	0·4	0·7	0·7	7	1·0	1·0	1·0	1·1
4	0·5	0·3	0·5	0·6					

* Values for bands 1 and 2 are averages of 6 replicates; values for bands 3–7 are averages of 3 replicates.

This decrease in sucrose may have been caused by an increase in invertase activity. GA-induced increases in invertase have been observed in other plant species.[16–19] The obser-

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vation that neither starch nor sucrose were reduced to control levels suggests that some process not influenced by GA may be involved in some phase of immobilization of starch in the chloroplasts and associated growth reduction.

EXPERIMENTAL

Plant material. 'Pangola' plants were grown in plastic pots containing steam-treated potting soil in a greenhouse between 27 and 33°. Plants were watered daily and received nutrients weekly. For each experiment the tillers of 12 plants (3–4 weeks regrowth after topping) were staked upright and the plants placed in growth chambers. 4 treatments were applied to 3 replicates: 30 or 10° 12-hr night temp. regimes with or without application of 10 μM GA_3. Either 10 μM GA_3 or dist. H_2O was applied 2× with a hand sprayer, 12 and 8 hr before imposition of the night temp. regimes. All plants received 12-hr days at 30° with 30 klx illumination. Temp. treatments were continued 3 consecutive days and nights. The fourth morning, equal numbers of first and second collared leaves of each treatment were cut into 0·3 cm lengths, pooled and mixed. All samples were taken at random from each treatment pool.

Preparation of extracts. Samples (0·6 g) were immediately ground for 10 min in an ice-cooled glass homogenizer containing 5·0 ml cold 1·0 mM $CaCl_2$. The homogenate was washed with an additional 5·0 ml 1·0 mM $CaCl_2$ and placed at 0° on a shaker for 1 hr to permit lysis of cells and leakage of enzymes into solution. This procedure was found to extract into solution 95% of the starch degrading enzymes present in the leaf. The homogenate was then centrifuged in the cold at 8×10^4 *g* for 90 min. The centrifugate was decanted and sucrose added to 20%.

Electrophoresis. Small pore polyacrylamide gels (without spacer or sample gels) were made in glass gel tubes 100 × 5 mm (i.d.) by a modification of the method of Davis.[20] Amylose solution (1 g amylose/100 ml deionized dis. H_2O, with boiling and filtration) was substituted for the H_2O phase. Davis' Tris-glycine buffer, pH 8·3, was used in both reservoirs of the vertical disc type electrophoresis apparatus. The apparatus was prechilled and used in a cold box (2–4°). Samples (50 μl) of each extract were applied to 3 replicate gels. The current was 1 mA/tube for 1 hr and 2 mA/tube thereafter. The period of electrophoresis varied (*ca.* 4 hr) and was terminated 35 min after the natural extract pigment streamed from the bases of the gels.

Band development and staining. Gels were removed from the glass tubes and rinsed with dis. H_2O, then incubated in 200 mM acetate buffer, pH 4·8, at 30° for 2·5 hr. After incubation, gels were rinsed again and placed in cold 10% TCA overnight. The following morning they were stained 1·5 hr in an iodine–KI acetate buffer solution (4×10^{-3}% I_2 and 1·5% KI in 200 mM acetate buffer, pH 4·8), then held in deionized dis. H_2O.

Densitometer tracings and relative activity measurements. Optimum wavelength for densitometer scanning was found previously to be 575 nm.[12] Measurement of the area of each peak was made with a planimeter.

Colorimetric determination of enzyme activity. Starch degrading enzyme activity of extracts was determined also by colorimetric measurement of iodine-staining starch remaining after 30° incubation with aliquots of extracts used on gels.[11]

Starch and sucrose determinations. Leaf samples (0·1 g fr. wt) were dropped into 30 ml boiling 80% EtOH, boiled for 30 sec., and removed from heat. After 1 hr at room temp., the alcohol was decanted, and the boiling procedure was repeated 3× at 1 hr intervals. The combined alcohol extracts of each replicate were heated until free of EtOH, then brought to 10 ml with dis. H_2O. These solutions were centrifuged 1 hr at 27×10^3 *g* and the centrifugate used for assay of sucrose. Sucrose content was calculated from the difference in reducing sugar contents measured before and after action of invertase. Reducing sugar content was colorimetrically determined by the method of Somogyi and Nelson.[21,22] Starch content was determined using the alcohol-extracted leaf pieces. These were oven-dried at 70° for 72 hr, then ground in a glass homogenizer in 5·0 ml 100 mM acetate buffer, pH 4·8. The homogenate was washed with 5·0 ml more buffer into a 25-ml Erlenmeyer flask and incubated 90 min with 20 mg amyloglucosidase (from *Rhizopus*, Sigma Chemical Co., 1200–3000 units/g) at 55° with agitation. The contents were centrifuged at 27×10^3 g for 30 min, and glucose content of the centrifugate was determined by the glucose oxidase method. Starch was calculated from glucose content.

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