Plant Physiol. (1981) 67, 1026–1031
0032-0889/81/67/1026/06/$00.50/0

Characterization of a Gibberellin-Insensitive Dwarf Wheat, D6899[1]

EVIDENCE FOR A REGULATORY STEP COMMON TO MANY DIVERSE RESPONSES TO GIBBERELLINS

Received for publication March 4, 1980 and in revised form November 24, 1980

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ABSTRACT

The effects of gibberellin in two wheat varieties, Nainari 60 and D6899, have been studied. D6899 is a dwarf wheat having a single locus mutation, the Rht 3 gene ("Tom Thumb" gene), which is located on chromosome 4A. When compared with the standard height wheat variety, Nainari 60, D6899 does not have a slowdown in cellular metabolism such as respiration rate, protein content, rate of protein synthesis, and uptake of amino acids. The content of ATP is even higher in D6899. However, all of the gibberellin-mediated physiological processes that we have studied, including leaf elongation, synthesis and release of hydrolytic enzymes, and secretion of phosphate ions and reducing sugars in aleurone layers, are retarded in D6899. D6899 and Nainari 60 have essentially the same uptake and metabolism of gibberellin and their levels of endogenous inhibitors such as abscisic acid do not differ drastically. The dosage-response curve of the gibberellin-mediated α-amylase production indicates that a rate-limiting step, which is common to many of the diverse gibberellin responses, is partially blocked in D6899 wheat.

Various responses of plant tissues to GA have been well documented (12). The most intensively studied responses are the induction of hydrolytic enzymes in the aleurone layers of cereal grains and the elongation of stem internodes, leaves, hypocotyls, and epicotyls (12). Despite the fact that much is known about these GA responses a basic problem remains unsolved. Is there any single regulatory step which is common to all the diverse physiological responses to GA? To address this problem, information obtained from biochemical analysis of different GA responses has been compared. It is generally accepted that both hydrolytic enzyme induction and the maintenance of long-term elongation growth depend on protein and RNA synthesis (12). However, the complexities of proteins and RNA synthesized in plant tissues do not permit an easy search for regulatory molecules which may be common to different GA-mediated physiological processes.

Another approach to this problem is the use of genetic mutants which do not respond to GA to determine whether there are genetic loci that are capable or regulating all the diverse GA effects. Dwarf varieties of cereal plants, which do not respond to GA, appear to be suitable for this approach. Numerous dwarf varieties of cereal plants exist, but most of these respond to exogenous GA and are likely to be GA synthesis-deficient mutants. In the case of d-5 dwarf corn, Phinney (10) has demonstrated that the production of GA is deficient. Fick and Qualset (5), as well as Gale and Marshall (6), tested the effect of GA on several dwarf wheat varieties. Two of them, D6899 and Minister dwarf, did not respond to GA in coleoptile and leaf elongation tests, nor in the production of α-amylase during germination. Genetic analysis indicated that D6899 and Minister dwarf have a single locus mutation in Rht 3 gene ("Tom Thumb" gene) located on chromosome 4A (5, 17). Their work seemed to indicate that Rht 3 gene codes for information which is required for both types of GA responses, elongation and enzyme induction.

The lack of GA response in a tissue could be attributed to many causes, such as the presence of inhibitors and a general slowdown in cellular metabolism. To determine whether a specific regulatory step is affected by the mutation in Rht 3 gene, we asked the following questions. First, does D6899 wheat have gross aberrations in basic cellular metabolism which cause its failure to respond to GA? Second, are all of the many diverse GA effects in the aleurone layers of cereal grains retarded in D6899 wheat? Third, does D6899 wheat have the same rates of uptake and metabolism of GA as the standard height wheat? Fourth, does D6899 wheat have a higher endogenous level of inhibitors which prevents most of the GA effect (*e.g.* AbA) than wild-type wheat?

MATERIALS AND METHODS

Chemicals and Seeds. GA_3, AbA, azoalbumin, phosphatase substrate, and the reconstituted firefly luciferase were purchased from Sigma. [^{14}C]Gibberellic acid (3.44 mCi/mmol) and [^{35}S]methionine (specific radioactivity > 1,000 Ci/mmol) were supplied by Amersham. All of the other chemicals were reagent grade.

Plant Materials. Seeds of D6899 wheat and Nainari 60, a standard height wheat, were obtained from Dr. C. O. Qualset's lab, Department of Agronomy and Range Science, University of California, Davis. Wheat seeds were planted in vermiculite moistened with half-strength Hoagland solution. Aleurone layers were prepared from water-imbibed endosperm half-seeds and treated as described by Ho and Varner (8).

Respiration Rate and ATP Level. Respiration rate was determined by measuring O_2 uptake of five aleurone layers in 10 ml of air-saturated buffer (20 mM Na-succinate, pH 5.0, and 20 mM $CaCl_2$) at 25 C with a Clark O_2 electrode and monitor (Yellow Springs Instrument Co.). ATP was extracted by boiling five to 10 intact aleurone layers in 50 mM K-glycinate (pH 7.5) for 1 min. Virtually all of the cellular ATP (90–98%) went into the solution after boiling. The K-glycinate solution was decanted and stored

[1] This research was supported by National Science Foundation Grant PCM 78-16143 to T. H. D. H.

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on ice. ATP levels were assayed with the reconstituted firefly luciferase by using an Amico Chem-Glo photometer as described by Gronwald *et al.* (7).

Enzyme Assays. α-Amylase was assayed according to Varner and Mense (20) and Ho and Varner (9), except that the phosphate buffer was replaced by 20 mM Na-succinate buffer (pH 5.0). The substrate for protease assay, azoalbumin (1 mg/ml), was dissolved in 20 mM Na-succinate (pH 5.0), containing 20 mM $CaCl_2$ and 10 mM β-mercaptoethanol. One ml of azoalbumin was mixed with 1 ml of enzyme preparation and incubated at 30 C for 1 h. One half ml of 50% trichloroacetic acid was then added to the assay solution. After storing on ice for 10 min, the assay solution was filtered through Whatman No. 1 filter paper. Three-tenths ml of 5 M NaOH was added to 1.5 ml filtrate and the A_{440} nm was determined. The unit of protease was defined as ΔA_{440} nm/h. Acid phosphatase was assyaed according to Ashford and Jacobsen (1). The unit of acid phosphatase was defined as ΔA at 440 nm/10 min.

Gel Electrophoretic Analysis of Proteins. Isolated aleurone layers were treated with or without 1 μM GA_3 for 24 h and then labeled with 25 μCi [^{35}S]methionine/ml for 2 h. Proteins were extracted and analyzed as described by Ho *et al.* (9) except that slab gels were used. After electrophoresis the gel was dried and the profile of radioactivity on the gel was determined by autoradiography with Kodak SB-5 film.

Phosphate Ion Release. Five aleurone layers were incubated in 2 ml of 20 mM $CaCl_2$ with or without 1 μM GA_3 at 25 C for 24 h. Phosphate levels present in the incubation medium were then assayed by a modified Fiske-Subbarow method described by Clark and Switzer (3).

Release of Reducing Sugars. Isolated aleurone layers were rinsed thoroughly with cold distilled H_2O and incubation medium to wash off the attached starch granules. The layers were then incubated with or without GA_3 at 25 C for 24 h. The media were assayed for reducing sugar with the Nelson test as described by Clark and Switzer (4).

Uptake and Metabolism of [^{14}C]GA_3. Isolated aleurone layers were incubated without GA_3 for 12 h before 0.05 μCi/ml of [^{14}C]GA_3 was added. After 1 h of further incubation, the layers were rinsed several times with 1 mM cold nonradioactive GA_3 solution. The radioactivity in the layers was measured by scintillation counting according to Musgrave *et al.* (18).

The procedure of Kende (14) was adopted to study the metabolism of [^{14}C]GA_3. Twenty-five aleurone layers were incubated in 4 ml of Na-succinate buffer containing 0.5 μCi [^{14}C]GA_3 for 12 to 30 h. After being thoroughly rinsed with 1 mM nonradioactive GA_3 the layers were ground with 10 ml methanol in a mortar. The homogenate was stirred at 4 C for an additional 3.5 h, and then centrifuged at 12,000*g* for 10 min. The pellet was washed with 5 ml methanol. The combined methanol supernatant was dried in vacuum at 40 C. The residue was redissolved in 15 ml 0.25 M phosphate buffer (pH 8.5). The buffer was extracted three times with 15 ml ethyl acetate, the extracts were combined and designated basic ethyl acetate fraction. The aqueous phase was adjusted to pH 2.5 with 2 N HCl, and then extracted seven times with 15 ml ethyl acetate. The resulting organic phase was designated acidic ethyl acetate fraction. The radioactivity in each fraction was counted and dpm was calculated by the internal standard method. ACS scintillation fluid (Amersham) and a Beckman LS-230 scintillation counter were used in this procedure.

The acidic ethyl acetate fraction was further analyzed by TLC on Silica G plates (Brinkmann). The solvent system used was chloroform:ethyl acetate:acetic acid (5:5:1). After development, silica gel was scraped off the plate in 1-cm sections. The radioactivity in each fraction was determined by scintillation counting. Authentic GA_3 was analyzed in a separate channel on the same TLC plate. The location of GA_3 on the plate was visualized by spraying the plate with ethanol:H_2SO_4 mixture (95:5) followed by heating the plate at 110 C for 10 min.

Extraction and Bioassay of AbA and Other Inhibitors. Two-week-old wheat seedlings were homogenized with 80% methanol (20 ml/g fresh weight) in a mortar. The homogenate was stirred at 4 C for 24 h. After centrifugation at 10,000*g* for 10 min, the methanol supernatant was dried in a vacuum at 40 C. The residue was redissolved in 5 ml distilled H_2O. For the bioassay of AbA, 2% agar plates containing 0.2% potato-soluble starch, 10^{-8} M GA_3, and various concentrations of AbA (10^{-5}–10^{-10} M) or wheat extracts were prepared according to Ho *et al.* (10). Surface-sterilized barley endosperm half-seeds were placed on top of the agar plates, which were incubated at 28 C for 24 h. The plates were then flooded with I_2 solution (0.72 g KI + 72 mg I_2/100 ml) in 0.2 N HCl. Transparent halos were formed in the agar plates due to the synthesis and secretion of α-amylase from the half-seeds. The concentration of AbA in wheat extracts was estimated by comparing the size of the halos in plates containing wheat extracts with those in plates containing standard AbA.

RESULTS

Many Diverse GA_3 Responses Are Partially Blocked in D6899. The leaves of D6899 were about half as long as those of Nainari 60 and they elongated only slightly when GA_3 was applied. The difference between these two wheat varieties in terms of GA-mediated leaf elongation was even more apparent when they were treated with CCC[3] to suppress partially the synthesis of GA. As shown in Figure 1 and Table I, the leaves of Nainari 60 elongated by at least 50% of the original length in response to exogenous GA_3, whereas the leaves of D6899 did not elongate as much as those of Nainari 60, and remained short after GA_3 treatment. The GA-mediated elongation still took place in Nainari 60 seedlings whose endosperm had been dissected out (Table I). This indicates that the GA_3-mediated elongation is independent of ability of the seeds to mobilize endosperm nutrients.

We have also studied other GA_3 effects in the aleurone layers. In the absence of GA_3 the amount of α-amylase produced by isolated D6899 aleurone layers was about the same as that produced by Nainari 60 aleurone layers (Table II). In the presence of 1 μM GA_3, D6899 aleurone layers increased their α-amylase production by only 3-fold, whereas there was more than 100-fold enhancement in Nainari 60. The release of α-amylase to the medium was also enhanced by GA_3 in Nainari 60, but not in D6899.

Analyzing newly synthesized proteins in aleurone layers with SDS gel electrophoresis we have observed that D6899 aleurone layers failed to increase drastically the rate of synthesis of α-amylase protein after GA_3 treatment (Fig. 2). This observation indicated that the low α-amylase activity in GA_3 treated D6899 aleurone layers was due to the low rate of α-amylase synthesis, rather than the inactivation of α-amylase. Time course studies (Fig. 3) revealed that the production of α-amylase in D6899 not only had a slower rate but also had longer lag periods than Nainari 60 (about 8 h for Nainari 60 and 24 h for D6899).

Table III summarizes the other GA_3 effects that we have studied in these two types of wheat aleurone layers. Production of protease is the result of *de novo* synthesis of enzyme molecules in barley aleurone layers (10), and the same probably applies to wheat aleurone layers. Using azoalbumin as substrate, we showed that GA_3 enhanced the production of protease in Nainari 60 but not in D6899. The release of protease, phosphate ion (13), and sucrose (2) is likely to be membrane related phenomena. GA_3 significantly affected all of these processes only in Nainari 60 wheat aleurone layers. The release of phosphatase has been shown to involve the

[3] Abbreviation: CCC, chlorocholine chloride.

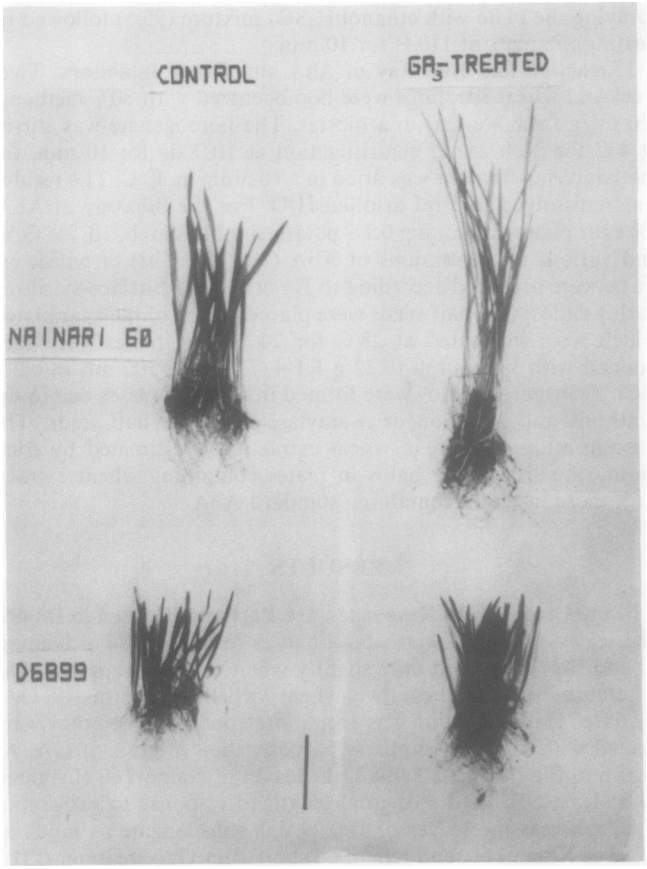

FIG. 1. Effect of exogenous GA_3 on wheat leaf elongation. The plants were grown in vermiculite watered with half-strength Hoagland solution containing 1 mM CCC, a known GA biosynthesis inhibitor. For the GA treated plants 1 μM GA_3 was also included in the Hoagland solution. Plants were grown at room temperature (22 C) and the length of the oldest leaf of each plant was measured on the 10th day after germination. The vertical bar is 5 cm.

Table I. *Effect of GA_3 on Leaf Elongation Growth in Wheat*

The experimental procedures are the same as described under Figure 1.

	Length of Oldest Leaf Blade (Mean ± SE)		
	Nainari 60	Nainari 60 (seedlings from seeds whose endosperm had been cut off)	D6899
		cm	
Experiment 1			
Control	11.4 ± 0.6	ND[a]	6.0 ± 0.3
GA_3-treated	17.4 ± 1.2	ND	7.4 ± 0.2
Experiment 2			
Control	10.4 ± 0.4	6.8 ± 0.5	5.5 ± 0.4
GA_3-treated	19.7 ± 0.7	12.0 ± 0.8	6.0 ± 0.6

[a] Not determined

transfer of preexisting enzyme molecules from cell wall to the incubation medium (1). Again, the GA_3-mediated process did not function effectively in D6899 wheat.

Comparison of D6899 and Nainari 60 Wheat at Different Concentrations of GA_3. Most of the above experiments were performed with the optimal GA_3 concentration, 1 μM. When lower concentrations of GA_3 were used, the amounts of α-amylase decreased in both varieties (Fig. 4A). Nainari 60 appeared to be more sensitive to the changes of GA_3 concentration. As a consequence, the difference between D6899 and Nainari 60 was gradually minimized as concentration of hormone was decreased (Fig. 4B). At zero or very low GA_3 concentrations (10^{-10} M) there was actually no significant difference between them (Fig. 4B).

Table II. *Induction of α-Amylase in Aleurone Layers of Nainari 60 and D6899 Wheat*

	α-Amylase Activity		
	Medium	Layer	Total
		units/layer	
Nainari 60			
Control	0.04	0.04	0.08
+GA (10^{-6} M)	6.68	2.47	9.15
D6899			
Control	0.02 (0.03)[a]	0.03 (0.05)	0.05 (0.08)
+GA (10^{-6} M)	0.06 (0.09)	0.09 (0.13)	0.15 (0.21)

[a] Numbers in parenthesis are the values corrected for fresh weight differences of the two wheat varieties.

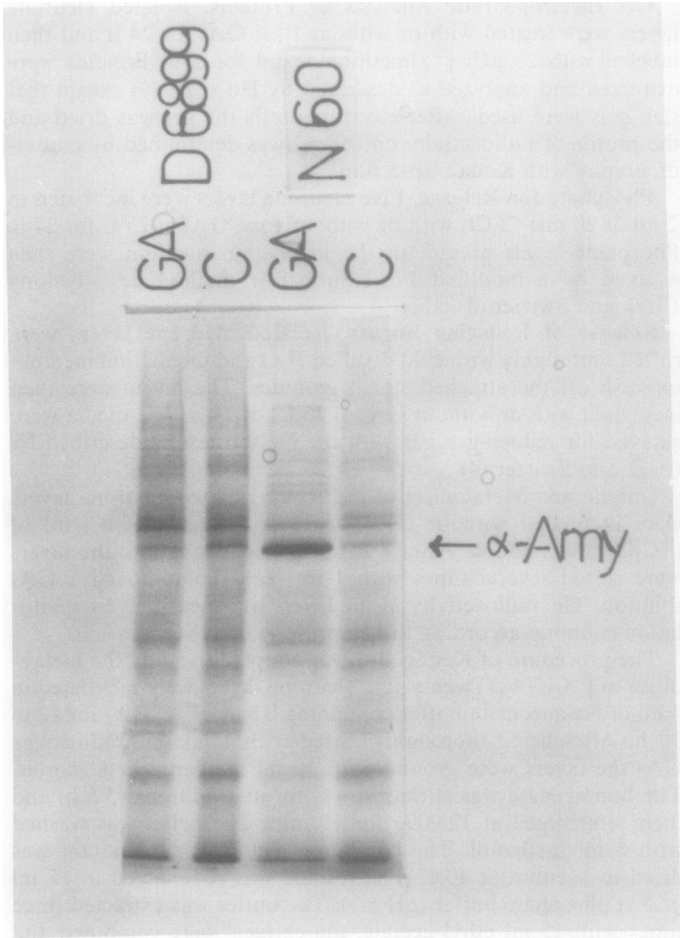

FIG. 2. SDS gel analysis of newly synthesized proteins in wheat aleurone layers. The portions were prepared and analyzed as described. The autoradiogram of the gel is shown in this figure; GA, GA_3 treated; C, control, without GA_3 treatment. The arrow indicates the position of α-amylase.

D6899 Wheat Does Not Have a General Slowdown in Cellular Metabolism. D6899 wheat has significantly shorter leaves and smaller seeds than Nainari 60. Therefore, the data of basic cellular metabolism were presented on equal fresh weight basis. The

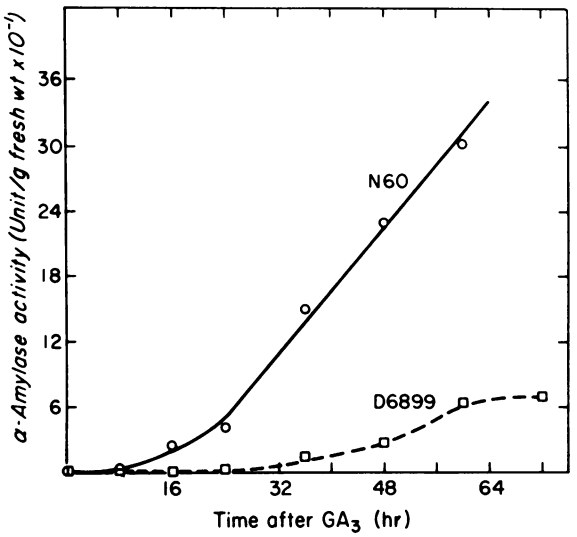

FIG. 3. Time course of GA_3-mediated α-amylase production in wheat aleurone layers. N60, Nainari 60.

Table III. *Other GA_3 Effects That Are Partially Blocked in D6899 Wheat Aleurone Layers*

	Nainari 60			D6899		
	−GA	+GA	+GA/−GA	−GA	+GA	+GA/−GA
	unit/g fresh wt					
Protease						
Medium	1.7	6.3	3.71	1.9	1.9	1.00
Layer	2.3	4.3	1.87	2.5	2.3	0.92
Total	4.0	10.6	2.65	4.4	4.2	0.96
Phosphatase						
Medium	27.1	78.5	2.90	31.5	41.7	1.32
Layer	94.5	114.2	1.21	99.3	101.3	1.02
Total	121.6	192.7	1.58	130.8	143.0	1.09
	μmol Pi released/g fresh wt					
Release of phosphate ion	8.4	30	3.57	15	16	1.07
	mg glucose equivalent/g fresh wt					
Release of reducing sugar	0.35	2.54	7.26	0.26	0.28	1.04

aleurone layer of D6899 was about two-thirds of that of Nainari 60 in fresh weight as well as in dry weight (Table IV). The water content of both aleurone layers was about 70 to 75%, a bit lower than other tissues probably due to the presence of attached seed coat. The respiration rate was nearly the same for both aleurone layers, yet D6899 had a higher ATP content. The uptake of GA_3 and amino acid (methionine) did not differ in these two wheat varieties. The same rate of GA_3 uptake for both varieties is particularly important, bacause it rules out the possibility that D6899 is a "hormone-uptake" mutant. Both types of aleurone layer had a soluble protein content of 2 to 3% of the fresh weight, and the rate of protein synthesis was also similar. In general, the presence of "Tom Thumb" gene in D6899 wheat does not cause gross aberrations in cellular metabolism.

Metabolism of [^{14}C]GA_3. In this study wheat aleurone layers were incubated with [^{14}C]GA_3 followed by methanol extraction. A significant portion of the radioactivity remained to be methanol unextractable. The proportion of methanol unextractable radioactivity increased with the time of incubation with [^{14}C]GA_3, and after 24 h of incubation it amounted to almost half of the radioactivity. The identity of the methanol unextractable compounds

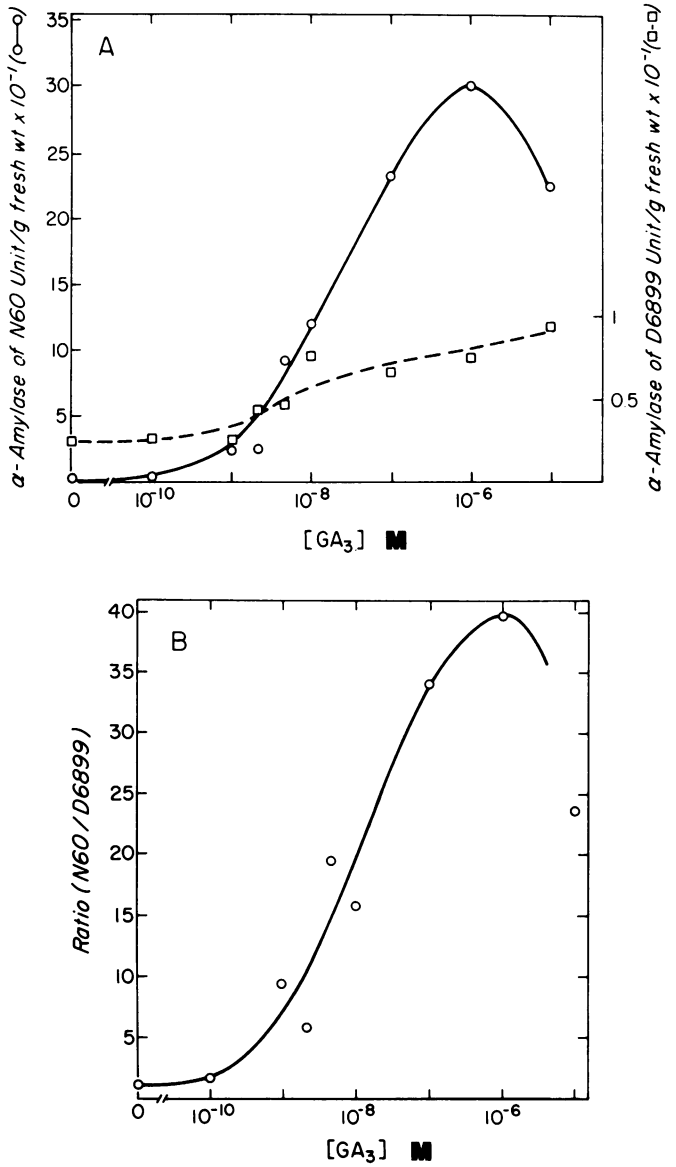

FIG. 4. Dosage-response curve of GA_3-mediated α-amylase production in wheat aleurone layers. A, absolute values of α-amylase; O– – –O, Nainari 60; □– – –□ D6899; B, ratio of Nainari 60 to D6899. Note that the two curves in A are on different scales.

Table IV. *Basic Metabolism of Aleurone Layers of Nainari 60 and D6899 Wheat*

	Nainari 60	D6899
Fresh weight, mg/layer	15.4	10.0
Dry weight, mg/layer	4.6	2.8
Respiration, μmol O_2/g fresh wt·h	17.5	16.4
ATP content, nmol/g fresh wt	19.0	27.5
Uptake of [^{14}C]GA, cpm/g fresh wt × 10^{-3}	1.66	1.98
Uptake of [^{35}S]methionine, cpm/g fresh wt × 10^{-9}	1.34	1.33
Protein synthesis, incorporation of [^{35}S]methionine, cpm/g fresh wt × 10^{-7}	2.67	2.55
Protein content,[a] mg/g fresh wt	28.9	25.9

[a] Protein content was measured according to the Lowry method.

is not yet known. The methanol extract was dried and redissolved in phosphate buffer, which was partitioned with ethyl acetate at different pH values. The distribution of radioactivity among all

Table V. *Metabolism of [^{14}C]GA$_3$ in Aleurone Layers of Nainari 60 and D6899 Wheat*

Fractions	Nainari 60	D6899
	% of total radioactivity	
Methanol extractable		
Basic ethyl acetate	2.3	2.0
Acidic ethyl acetate	11.0	14.6
Aqueous	41.3	35.4
Methanol unextractable	45.4	48.0

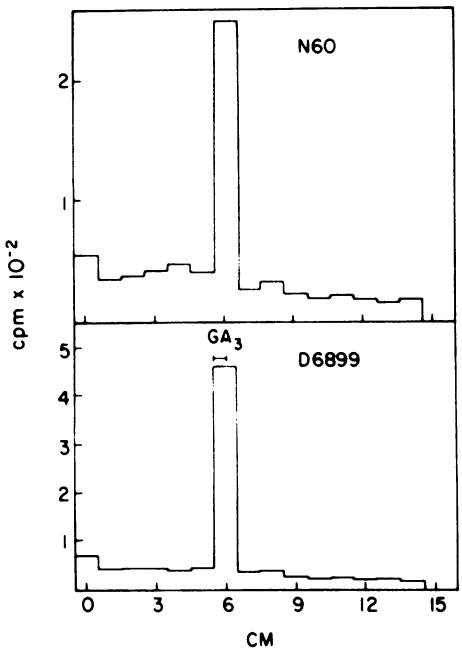

FIG. 5. TLC analysis of [^{14}C]GA$_3$. Only the acidic ethyl acetate fraction was analyzed. The short horizontal bar labeled GA$_3$ is the position of authentic GA$_3$ on the plate. The total radioactivity applied to the channel for D6899 was twice that for Nainari 60.

the fractions was similar for both wheat varieties (Table V). The acidic ethyl acetate fraction, further analyzed in this fraction, was in the form of GA$_3$ (Fig. 5). The aqueous fraction was not further analyzed. Kende (15) analyzing GA$_1$ metabolism in pea has found that the aqueous fraction consisted of compounds which are biologically inactive. Although detailed GA$_3$ metabolism has to rely on further analysis, our results indicate that there may be no apparent major difference in GA$_3$ metabolism between D6899 and Nainari 60 wheats.

Level of Endogenous Inhibitors of GA$_3$ Action. The effects of GA$_3$ in many plant tissues could be inhibited by AbA (16), and potentially by other inhibitors that have not yet been identified. To determine whether the lack of GA response in D6899 is due to the presence of high levels of endogenous inhibitors in this variety, we used the inhibition of α-amylase induction in barley aleurone layers as a bioassay for the presence of inhibitors. Our results indicated that the level of endogenous inhibitors in both varieties of wheat was within the range of 20 to 50 ng of AbA equivalents/g fresh tissue. Although the level of inhibitors might still differ by 2.5-fold in these two varieties, this is not likely to be the major cause of the lack of GA response in D6899, because the difference in hormone level would have to be more than 10-fold in order for significant physiological changes to be observed.

DISCUSSION

In this work we have characterized a GA insensitve dwarf wheat, D6899, which carries a mutation in the Rht 3 gene ("Tom Thumb" gene). All the diverse GA responses we have studied, including elongation of leaves, synthesis, and release of hydrolytic enzymes in aleurone layers, are retarded in this dwarf wheat. D6899 does not have a general slowdown in cellular metabolism in terms of respiration rate, content of ATP and protein, or uptake and incorporation of amino acids.

Uptake and metabolism of GA$_3$ in D6899 do not differ significantly from the standard height wheat, Nainari 60. The level of endogenous inhibitors in these two varieties of wheat also does not differ drastically. Therefore, the partial blockage of GA effects in D6899 is probably the consequence of a specific lesion rather than the failure of some general cellular metabolism or gross aberrations in hormone metabolism. The specific lesion in D6899 appears to be a regulatory step which is common to most, if not all, of the diverse GA effects in wheat.

What could this common regulatory step be? Based on the following considerations, we suggest that this step is probably related to the receptor molecules of GA. First, a large variety of cellular processes, such as enzyme induction, secretion, and cell wall weakening, are involved in the diverse GA effects we have studied in this work. It is unlikely that these cellular processes *per se* share a common regulatory step, except that they are all mediated by GA. The ability to recognize hormone molecules may be the only common step shared by these diverse GA responses. Second, despite several published papers concerning GA receptors, rigorous identification of a GA receptor in plant tissues is still lacking (15). Therefore, we have chosen an indirect way to study the possibility that D6899 may have a lesser amount of, or less active (*e.g.* lower affinity) GA receptors than Nainari 60. One may expect that when the concentration of GA$_3$ is high (saturating), the rate of binding between GA and its receptor would be determined by the concentration (or affinity) of the receptor, and when the concentration of GA is very low the rate of hormone-receptor complex formation would be determined mainly by the concentration of hormone. In the latter case the difference in receptor concentration (or affinity) between two wheat varieties would have little impact on the synthesis of α-amylase. Experimentally, this was exactly what we observed when we measured α-amylase production at different concentrations of GA$_3$ (Fig. 4). Nainari 60 produced about 40 fold more α-amylase than D6899 at the optimal GA$_3$ concentration (1 μM). The difference between the two varieties was much less at lower GA$_3$ concentrations. The definite proof of this suggestion requires direct evidence on binding to GA receptors. Nevertheless, our observations strongly point to a rate-limiting step, which is common to many GA mediated processes and is partially blocked by a mutation in the Rht 3 gene of wheat.

It is worth noting that the dwarfism in D6899 is caused by the reduced response to GA while many other dwarfism cases in corn, rice, wheat, pea, etc. are caused by lack of GA production. It is not understood why D6899 can survive without normal responses to GA. Probably, there are reduced responses to GA in D6899 which still allow it to complete its life cycle and to yield viable progeny. Another possibility would be that responses to GA are not essential for the survival of wheat. The consequence of having reduced GA responses is merely a reduction in plant size, such as the length of leaves and the size of seeds.

Acknowledgments—We are grateful to Dr. C. O. Qualset for sending us the wheat seeds used in this work.

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