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Effect of Gibberellin on Growth, Protein Secretion, and Starch Accumulation in Maize Endosperm Suspension Cells

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Abstract. The physiologic effect of gibberellins (GA) in seed development is poorly understood. We examined the effect of gibberellic acid (GA_3) on growth, protein secretion, and starch accumulation in cultured maize (*Zea mays* L.) endosperm suspension cells. GA_3 (5 and 30 μM) increased the fresh weight, dry weight, and protein content of the cultured cells, but the effect of GA_3 at 50 μM was not significantly different. However, the protein content in the culture medium was increased by these three concentrations of GA_3. The effect of GA_3 on the amount of cellular structural polysaccharides was not significant, but GA_3 had a dramatic effect on the starch content. At 5 μM, GA_3 caused an increase in the starch content, but at 50 μM the starch accumulation was reduced. Chlorocholine chloride (CCC), an inhibitor of GA biosynthesis, significantly increased the starch content and decreased the structural polysaccharide content of the cultured cells. The effects of CCC at 500 μM on the starch and polysaccharide content were partially reversed by 5 μM GA_3 applied exogenously. Based on these results we suggest that GA does not favor starch accumulation in the cell cultures and that the addition of lower concentrations of GA_3 in the medium may provide an improved balance among the endogenous GA in the cultured cells.

Key Words. Gibberellin—Chlorocholine chloride—Maize endosperm—Cell culture—Starch—Protein—*Zea mays*

Plant hormones play pivotal roles in the regulation of plant growth and development (Cao and Chen 1995, Davies 1987) and in the interactions with other organisms (Cao et al. 1993). It is well documented that gibberellins (GA) play essential roles in the breakdown of reserve starch in germinating cereal grains (Jacobsen and Chandler 1987). Accumulation of GA in developing grains of wheat (Radley 1976), barley (Mounla 1978), and *Pharbitis nil* (Barendse et al. 1991) is well correlated with an increase in the fresh weight during early seed development, suggesting a role of GA in cell growth in these fast growing tissues. However, the physiologic significance of the increased GA in seed development is poorly understood (Barendse et al. 1991, Jacobsen and Chandler 1987, Pharis and King 1985). Furthermore, little is known about their role in developing grains in which carbohydrate accumulation is the predominant biochemical event (Pharis and King 1985). Gibberellic acid (GA_3) was reported to cause a decrease in the starch content of isolated chloroplasts (Kazama and Katsumi 1984) and sweet potato suspension-cultured cells (Sasaki and Kainuma 1984). Application of chlorocholine chloride (CCC), an inhibitor of GA biosynthesis (Rademacher 1991), causes an increase in dry matter accumulation in sweet potato tubers (Kuo 1991). The above data suggest that GA do not favor starch accumulation. In contrast, exogenous GA_3 was shown to increase both the dry weight and grain yield of maize inbreds (Rood et al. 1983). Therefore, the relationship between GA and starch accumulation requires further study.

Starch in storage tissues is accumulated in amyloplasts (Shannon and Garwood 1984). The enzymatic reactions leading to the synthesis of starch involve the actions of ADP-glucose pyrophosphorylase (Preiss 1991), starch synthases (Marshall et al. 1996 and references therein), and starch branching enzymes (Cao and Preiss 1996 and references therein). However, the in vivo regulation of starch synthesis and accumulation in storage tissues is more poorly understood (Nelson and Pan 1995, Preiss 1991, Smith et al. 1995), and little is known about the possible roles of hormones in the regulation of starch accumulation during seed development. In vitro cultured

Abbreviations: GA, gibberellins; GA_3, gibberellic acid; CCC, chlorocholine chloride; BSA, bovine serum albumin; MCW, methanol:chloroform:water (13:4:3, v:v).

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maize endosperm suspension cells grown in chemically defined media have been suggested as model systems for in vivo studies of starch biosynthesis (Chu and Shannon 1975, Shannon 1982). However, we recently found that BT1, a putative adenylate translocator (Shannon et al. 1996, Sullivan et al. 1991) in maize amyloplast membranes (Cao et al. 1995, Sullivan and Kaneko 1995) critical for in vivo starch accumulation in developing maize endosperm, is not detected in the maize endosperm suspension cultures (Cao and Shannon 1996) or in the starchy tissues from several other species, including grains of sorghum, wheat, winter barley and oat, potato tubers, pea roots, and cauliflower buds (Cao and Shannon 1997). These data indicate that an alternative pathway of starch biosynthesis may exist in the cultured maize endosperm suspension cells and in the other species. Meanwhile, we found that the starch content in the maize endosperm suspension cultures was increased by supplementation with additional sucrose (Chu and Shannon 1975) or CCC (Cao and Shannon 1996) to the standard media. Since CCC is regarded as an inhibitor of GA biosynthesis, the effect of CCC on starch accumulation suggests that GA may be involved in the regulation of starch accumulation in the maize endosperm suspension cultures. In the present study, we examined the effects of GA_3 on growth, protein secretion, and starch accumulation in maize endosperm suspension cultured cells.

Materials and Methods

Cell Culture

Maize endosperm cell cultures were derived from inbred A636 kernels 10 days postpollination according to the procedure of Shannon (1982) and cultured for about 4 years prior to this study (Cao and Shannon 1996). The standard subculture medium and growth conditions were described previously (Shannon 1982). For this study, filter-sterilized aliquots of GA_3 or CCC were added to the standard subculture medium to final concentrations as indicated in the text.

Determination of Fresh Weight, Dry Weight, and Protein Content

After growth for 8 days, cultures were harvested on Whatman no. 2 filter paper by vacuum filtration and washed twice with distilled water before determining the fresh weight and freeze-dried weight as described previously (Chu and Shannon 1975). For determining the protein content of the cultured cells, freeze-dried cells (100 mg) were homogenized with a Polytron (Brinkmann Instruments) in 15 mL of 50 mM Hepes (pH 7.5). Following filtration through one layer of Miracloth (Calbiochem, CA) and centrifugation at 3,500 ×*g* for 20 min, the protein content of the supernatant was determined by the Bradford assay using the Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA) and BSA as a standard (Cao et al. 1995, Stoscheck 1990). Protein content in the medium was measured as above following removal of the cells by filtration through Whatman no. 2 filter paper.

Extraction and Determination of Starch and Structural Polysaccharides

Starch extraction was modified from previous procedures (Chu and Shannon 1975) and described previously (Cao and Shannon 1996). Briefly, cells (100 mg of freeze-dried cells) were homogenized with a Polytron in 15 mL of cold MCW (methanol:chloroform:water, 13:4:3, v:v). The homogenate was centrifuged at 800 ×*g* for 10 min, the pellet was washed three times by suspension in 10 mL of MCW, and centrifugation was as described above. The final washed pellet was suspended in 15 mL of 10% ethanol and centrifuged again at 3,500 ×*g* for 20 min. The pellet was extracted three additional times by suspension in 10 mL of 10% ethanol and centrifugation at 3,500 ×*g* for 20 min. The final pellet, regarded as total polysaccharides, was suspended in deionized water by heating in a boiling water bath for 15 min. The phenol-H_2SO_4 procedure (Dubois et al. 1956) was used to estimate the content of total polysaccharides. To determine starch content, an aliquot of the total polysaccharides was treated with glucoamylase prior to measuring the quantity of glucose released by Nelson's reducing sugar test (Hodge and Hofreiter 1962). Structural polysaccharide content was calculated as the difference between total polysaccharides and starch.

Table 1. Effect of added GA_3 on fresh weight and dry weight of maize endosperm suspension cultures.

GA_3 (μM)	Fresh weight (g/flask)	Dry weight (mg/flask)
0	3.5 a*	291 a*
5	4.3 c	358 b
30	4.0 b	344 b
50	3.5 a	310 a

*Mean values within columns were analyzed with Duncan new multiple range test (Steel and Torrie 1980). Values in a column with different letters indicate a significant difference among the treatments. Lowercase letters represent significant differences between treatments at the $p = 0.05$ level.

Results and Discussion

The fresh weight and dry weight of maize endosperm suspension cells grown in medium containing 5 and 30 μM GA_3 were significantly higher than those of controls (Table 1). However, cell growth was not significantly affected by the treatment containing 50 μM GA_3. The effect of GA_3 on cellular protein shows a pattern similar to those of the fresh weight and dry weight (Table 2). Cultures containing lower concentrations of GA_3 in the medium had higher amount of protein/flask. Cultures containing GA_3 at 50 μM had an amount of protein similar to that of control (Table 2). However, the protein content in the medium from all GA_3-treated cultures was higher than those from the control (Table 2). The lower concentrations of exogenous GA_3 increased the fresh weight and dry weight and the protein content of the cultured cells, suggesting that added GA_3 favors cell growth. A higher concentration of GA_3 (50 μM) may be superoptimal. The result that the protein content of the medium increased by all three levels of exogenous GA is in agreement with the role of GA in secreting proteins and hydrolytic enzymes during seed germination (Jacobsen and Chandler 1987).

Starch accumulation was dramatically reduced in cells grown in the medium containing 50 μM GA_3. In contrast,

Table 2. Effect of added GA_3 on protein content in the cultured cells and in the medium of maize endosperm suspension cultures.

GA_3 (μM)	Cellular protein (mg/flask)	Medium protein (mg/flask)
0	14.3 a*	8.2 A*
5	18.6 b	9.1 B
30	17.7 b	9.2 B
50	14.4 a	9.3 B

*Mean values within columns were analyzed with Duncan new multiple range test (Steel and Torrie 1980). Values in a column with different letters indicate a significant difference among the treatments. Upper and lowercase letters represent significant differences between treatments at $p = 0.01$ and $p = 0.05$, respectively.

Table 3. Effect of added GA_3 on starch and structural polysaccharide content of maize endosperm suspension cultures.

GA_3 (μM)	Starch (mg/flask)	Structural polysaccharide (mg/flask)
0	29.6 B*	34.7 a*
5	45.4 C	31.4 a
30	29.6 B	31.2 a
50	8.0 A	33.9 a

*Mean values within columns were analyzed with Duncan new multiple range test (Steel and Torrie 1980). Values in a column with different letters indicate a significant difference among the treatments. Upper and lowercase letters represent significant differences between treatments at $p = 0.01$ and $p = 0.05$, respectively.

the starch content was much higher in flasks growing in the medium containing 5 μM GA_3. However, the structural polysaccharide content/flask was not affected by any of the GA_3 concentrations tested (Table 3). The reduction in starch accumulation in cultures containing 50 μM GA_3 is in agreement with earlier reports using other experimental systems such as isolated chloroplasts (Kazama and Katsumi 1984), sweet potato suspension cell cultures (Sasaki and Kainuma 1984), and sweet potato tubers (Kuo 1991). However, the increased starch accumulation in cultures grown in the medium containing 5 μM GA_3 (Table 3) has not been well documented. It was shown that GA_3, a highly active GA species in vegetative maize (Phinney et al. 1991), exists at a very low concentration in developing endosperm (Murofushi et al. 1991). Moreover, Rood et al. (1983) showed that maize inbreds, but not hybrids, sprayed with GA_3 had an increased dry weight and grain yield, and they suggested that inbreds are deficient in GA relative to hybrids. Thus in this study it is possible that the addition of 5 μM GA_3 to the medium may provide an improved balance among the GA in the in vitro cultured maize endosperm cells derived from an inbred, A636, but 50 μM GA_3 may be superoptimal, resulting in the severely reduced starch accumulation.

To test the effect of endogenous GA on growth and starch accumulation in the cultured cells, we examined the effect of CCC, an inhibitor of GA biosynthesis (Rademacher 1991), on fresh weight and on the content of starch and structural polysaccharides in the cultured endosperm cells. CCC significantly increased the starch content of the cultured cells. An increase of starch content by 32.6% and 52.4% was observed when the medium contained 100 μM and 500 μM CCC, respectively (Fig. 1). Meanwhile, the structural polysaccharide content of 100 μM and 500 μM CCC-treated cultures was 144.8 and 57.8% of control (Fig. 1). However, CCC did not have a significant effect on the culture fresh weight (Fig. 1). Exogenously applied GA_3 (5 μM) partially reversed the effects of 500 μM CCC with respect to the content of starch and structural polysaccharides in the cultured cells (Fig. 1). Starch content was reduced from

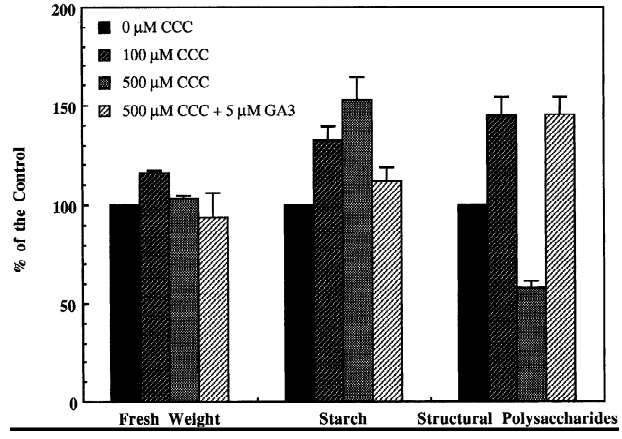

Fig. 1. Effect of CCC and GA_3 on fresh weight and contents of starch and structural polysaccharides in maize endosperm suspension cells. Data represent the percent mean of two replications (flasks) ± percent relative S.D. or coefficient of variation.

152.4% (CCC alone) to 112.0% of the control when cultures contained both GA_3 (5 μM) and CCC (500 μM). Furthermore, structural polysaccharides were increased from 57.8% (CCC alone) to 145.0% of the control when cultures contained both GA_3 (5 μM) and CCC (500 μM).

It is generally accepted that CCC is an inhibitor of the biosynthesis of GA (Rademacher 1991). That starch content was significantly increased by CCC may indicate that CCC directs carbohydrates toward starch accumulation by inhibition of endogenous GA biosynthesis (Fig. 1). The reversible relationship of GA_3 and CCC on carbon partitioning further indicates that GA favors cell growth over starch accumulation in the maize endosperm suspension-cultured cells. Similarly, reversal of the CCC effects by GA_3 has been reported for other systems, including the inhibition of flowering and vegetative growth of the nonrosette long-day plant *Lemna gibba* G3 (Cleland and Briggs 1969). The CCC effect on the promotion of femaleness was reversed by simultaneous application of GA_3 (Chailakhyan and Khryanin 1980). If endogenous GA is inhibitory to starch accumulation as suggested above, what is the explanation for the observation

that large amounts of endogenous GA are synthesized in normally developing maize endosperm cells (Pharis and King 1985)? A reasonable explanation is that much of the GA is conjugated to a storage form during kernel development (Sembdner et al. 1991). Consequently, active GA does not accumulate to levels inhibitory to starch synthesis.

In conclusion, we suggest that GA does not favor starch accumulation in the cell cultures and that the addition of lower concentrations of GA_3 in the medium provides an improved balance among the endogenous GA in the cultured cells. However, the direct relationship between GA and starch accumulation in the maize endosperm suspension cultures requires further study.

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