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AXIAL CONTROL OF AMYLASE SYNTHESIS AND SECRETION IN COTYLEDONS OF AGROSTEMMA GITHAGO

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SUMMARY

In *Agrostemma githago* (Caryophyllaceae) starch reserves are located outside the embryo in the perisperm. One of the main degradative enzymes, α-amylase, is synthesized in and secreted by the cotyledons.

The synthesis and secretion of amylase were shown to be under axial control. Benzylaminopurine (BAP)[1] or gibberellic acid (GA_3) replaced axis requirement. BAP probably acted by a general enhancement of metabolism, whereas GA_3 had, like the axis, a specific effect on the cotyledons. The degradation of storage proteins in the cotyledonary tissue was controlled in a similar way.

Two arguments raised in the literature against hormonal regulation of reserve breakdown in dicots are 1) that the axis withdraws otherwise inhibitory hydrolysis products from the cotyledons and 2) that reduced activity of hydrolytic enzymes in detached cotyledons is an artefact due to lack of oxygen. These arguments were shown to be most likely invalid.

1. INTRODUCTION

Seeds store large amounts of reserves which are hydrolysed after germination to sustain growth of the embryo (BEWLEY & BLACK 1978). In monocots, most of the storage materials are located outside the embryo (MARTIN 1946). The starch reserves in the endosperm are hydrolysed by α-amylase which is secreted by the aleurone layer, and β-amylase which is already present in the endosperm (for recent review see HALMER 1985). α-Amylase is also secreted by the embryo (RANKI & SOPANEN 1984). In many dicots, starch reserves are present outside the embryo (MARTIN 1946). Almost nothing is known about the mode of starch breakdown in these species. Recently, we have shown for one such species, *Agrostemma githago*, that the degradation of the starchy perisperm shows striking parallels with the situation in monocots. Thus, α-amylase did not originate in the storage organ itself but was secreted by the embryo, whereas β-amylase already existed in the perisperm (DE KLERK et al. 1986).

The role of hormones in the regulation of reserve breakdown in seeds is disputed. The reputed gibberellin control of α-amylase in the aleurone layer of monocots (see HALMER 1985) has been challenged (TREWAVAS 1982, AKAZAWA & HARA-NISHIMURA 1985). Axial control of hydrolytic enzymes in cotyledons of

[1] Abbreviations: BAP = 6-benzylaminopurine; c.p. = cotyledon pair; GA_3 = gibberellic acid; KCC-solution = 10 mM KCl, 1 mM $CaCl_2$, 50 $\mu g \cdot ml^{-1}$ chloramphenicol solution.

dicots, which has been ascribed to a hormonal signal (ILAN & GEPSTEIN 1981), may in fact consist of withdrawal from the cotyledons of hydrolysis products which otherwise would inhibit hydrolytic enzymes (DAVIES & SLACK 1981). In addition, it has been maintained that in many cases the observed axial control in dicots is actually an artefact (See Discussion and FORD et al. 1976).

I have initiated a study on axial control of amylase synthesis and secretion in cotyledons of *Agrostemma githago* embryos for two reasons. First, in dry seeds starch is only present in the perisperm and not in the embryo (BORRISS & ARNDT 1956). However, synthesis and secretion of amylase are not reduced by removal of the starchy perisperm (DE KLERK et al. 1986). Axial control of synthesis and secretion of amylase in isolated *Agrostemma* embryos can therefore be studied avoiding a possible negative feedback by starch hydrolysis products. Second, most studies on axial regulation of reserve breakdown in dicots have been carried out in crop species. It is well known that in crop seeds the extent of dormancy is very low. It has been reported that in *Avena sativa* gibberellin requirement of the aleurone layer depends on the extent of dormancy of the seeds (UPADHYAYA et al. 1982). The same may hold for axis requirement in dicots. In *Agrostemma githago* dormancy is well described (BORRISS 1940, DE KLERK 1983).

In the present article it is reported that synthesis and secretion of amylase in *Agrostemma* cotyledons are under axial control, possibly through a hormonal stimulus produced by the axis.

2. MATERIAL AND METHODS

2.1. Plant material

Seeds of *Agrostemma githago* L. (provenance Gatersleben, GDR) were collected from plants cultivated in a glasshouse in Canberra in 1984. After harvest, the air-dried seeds were stored for six months under dry conditions at room temperature to break dormancy, and after that at –20 °C (HÜBEL 1966).

2.2. Incubation procedures

For the determinations on detached cotyledons, the axis and the part of the seed coat covering it were removed from the dry seeds. For the determinations on attached cotyledons, only the part of the seed coat covering the axis was removed (for a diagram of *Agrostemma* seeds, see MARTIN 1946). The seeds were then incubated in the dark at 25 °C in 9 cm Petri dishes on one layer of Whatman 3mmChr paper moistened with 3.5 ml 10 mM KCl, 1 mM $CaCl_2$, 50 $\mu g \cdot ml^{-1}$ chloramphenicol (KCC) solution with or without addition of 10 μM BAP or 10 μM GA_3. After 1.5 h, the cotyledon pairs and the embryos were isolated from the seeds and transferred to other Petri dishes with appropriate fresh solution. Each 24 h, the cotyledon pairs and embryos were transferred to new Petri dishes with fresh solution. In each Petri dish 20 embryos or cotyledon pairs were incubated.

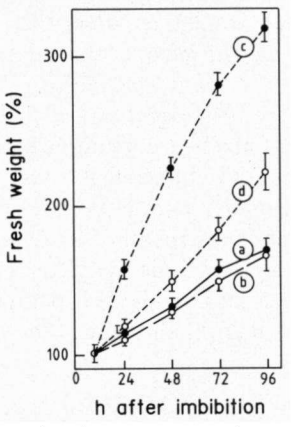

Fig. 1. Fresh weight of cotyledon pairs of *Agrostemma githago* at various times after the start of imbibition. Seedlings were grown in KCl-$CaCl_2$-chloramphenicol (KCC) solution (a). Cotyledon pairs without axis were incubated in KCC-solution (b), KCC-solution with 10 μM BAP (c) or KCC-solution with 10 μM GA_3 (d).

2.3. Amylase secretion

Amylase secretion was determined as described previously (DE KLERK et al. 1986) with the following modification. After rinsing, the 5 cotyledon pairs were incubated in a 25 ml scintillation vial with 400 μl KCC-solution which contained BAP (10 μM) or GA_3 (10 μM) when appropriate, for 20 h (the incubation starting at 4 h after imbibition) or 24 h (the other incubations). Amylase was assayed as described previously (DE KLERK et al. 1986). Each value is the mean of three determinations ± SE.

2.4. Biochemical assays

At the indicated times, 5 cotyledon pairs were homogenized at 0 °C in 1 ml 0.1 M Tris-HCl, pH 8.0 + 0.02% (w/v) Triton X-100. In 100 μl of the homogenate, the total 80% ethanol soluble carbohydrate and free amino acid content were determined according to DE BRUYN et al. (1968) and YEMM & COCKING (1955) respectively. For the carbohydrate determination sucrose was used as reference and for the amino acid determination leucine. Another 100 μl of the homogenate was diluted 1:1 with a solution containing 4% SDS, 20% glycerol and 0.01% Bromphenol blue. SDS-electrophoresis was performed under non-reducing conditions on a 13% gel according to DE KLERK (1984). Finally, in the homogenate amylase activity was determined as described previously (DE KLERK et al. 1986). Each value is the mean of three determinations ± SE.

2.5. Determination of the rate of protein synthesis

At the indicated times, 5 cotyledon pairs were incubated for 1 h in 150 μl KCC-solution with addition of 77 kBq [4,5-^{3}H]leucine (4.15 TBq·mmol^{-1}, Radiochemical Centre, Amersham) and 20 mM unlabeled leucine to swamp the endogenous leucine pool (DE KLERK & SMULDERS 1984). Incorporation into 5% TCA-

insoluble material was determined as described previously (DE KLERK & SMULDERS 1984). Each value is the mean of three determinations ± SE.

2.6. Determination of fresh weight

For the determinations on detached cotyledon pairs, the pairs were weighed individually 6 h after the start of imbibition at 0.1 mg accuracy. On the indicated times, the fresh weight of each cotyledon pair was determined as a percentage of the 6 h-value. Each value is the mean of 20 determinations ± SE. For the determinations on attached cotyledons, cotyledon pairs were separated from the axes at the indicated times and weighed individually. The data are expressed as a percentage of the 6-h value. Each value is the mean of 40 determinations ± SE.

3. RESULTS

3.1. Growth

Removal of the axis from dry *Agrostemma githago* embryos had only a small effect on the growth of the cotyledons (*fig. 1*). Addition of 10 μM BAP or 10 μM GA_3 to detached cotyledons increased the growth rate by 300% or 100%, respectively.

3.2. Amylolytic activity

Cotyledons of *Agrostemma* secrete α-amylase during a short period (24 h–96 h) after the start of imbibition. Secretion occurs both in the presence and the absence of the starchy perisperm (DE KLERK et al. 1986). In the present experiments, amylase secretion occurred mostly between 24 and 48 h (*fig. 2a*). In detached cotyledons, secretion of amylase was barely detectable (*fig. 2b*). Addition of 10 μM BAP or 10 μM GA_3 restored secretion (*fig. 2c, d*).

Amylolytic activity in the cotyledonary tissue increased after imbibition (*fig. 3* and DE KLERK et al. 1986). In detached cotyledons, amylolytic activity also increased, but after a lag (*fig. 3*). After addition of GA_3 or BAP, the course of amylase activity was similar to the course in attached cotyledons (*fig. 3*).

3.3. Content of hydrolysis products

It has been suggested that in detached cotyledons the synthesis and/or activity of hydrolytic enzymes is inhibited by an increased level of soluble end-products of hydrolysis (DAVIES & SLACK 1981). Thus, amylase would be inhibited by low molecular weight carbohydrates. Although in *Agrostemma* embryos starch does not occur until a few days after germination (BORRISS & ARNDT 1956), it might be that low molecular weight carbohydrates, either present in the embryo as storage material or originating from fat degradation (BEWLEY & BLACK 1978) accumulate in detached cotyledons. *Fig. 4* shows that the course of soluble carbohydrates was similar in detached water-incubated and GA_3-incubated cotyledons. This renders the possibility of amylase regulation by low molecular weight carbohydrates unlikely.

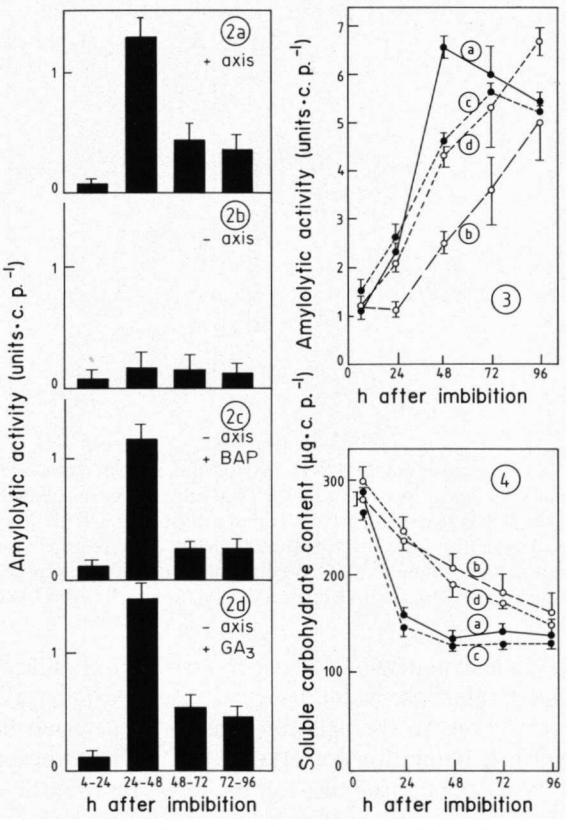

Fig. 2. Secretion of amylase by cotyledon pairs of *Agrostemma githago* during a 20 h or 24 h incubation in liquid medium. Seedlings were grown in KCC-solution (a). Cotyledon pairs without axis were incubated in KCC-solution (b). KCC-solution with 10 µM BAP (c) or KCC-solution with 10 µM GA_3 (d).

Fig. 3. Temporal changes in amylase activity in cotyledon pairs of *Agrostemma githago*. Seedlings were grown in KCC-solution (a). Cotyledon pairs without axis were incubated in KCC-solution (b), KCC-solution with 10 µM BAP (c) or KCC-solution with 10 µM GA_3 (d).

Fig. 4. Temporal changes in 80% ethanol soluble carbohydrate levels in cotyledon pairs of *Agrostemma githago*. Seedlings were grown in KCC-solution (a). Cotyledon pairs without axis were incubated in KCC-solution (b), KCC-solution with 10 µM BAP (c) or KCC-solution with 10 µM GA_3 (d).

It might be that the synthesis of all hydrolytic enzymes is regulated en bloc. and that the accumulation of one hydrolysis product inhibits all hydrolytic enzymes. As storage proteins are, together with fats, the major organic storage materials in cotyledons, I examined free amino acid content. Accumulation of free amino acids has been observed in detached cotyledons of mung bean (KERN & CHRISPEELS 1978) and cucumber (DAVIES & CHAPMAN 1979). *Fig. 5* shows that in *Agrostemma* free amino acid content was lowest in detached cotyledons. When calculated per mg fresh weight, the amino acid content in detached cotyledons was also lower than the content in attached ones.

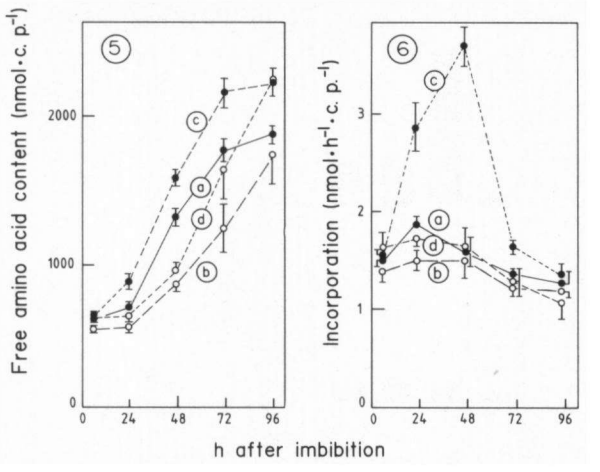

Fig. 5. Temporal changes in free amino acid levels in cotyledon pairs of *Agrostemma githago*. Seedlings were grown in KCC-solution (a). Cotyledon pairs without axis were incubated in KCC-solution (b), KCC-solution with 10 μM BAP (c) or KCC-solution with 10 μM GA_3 (d).
Fig. 6. Temporal changes in the rate of protein synthesis in cotyledon pairs of *Agrostemma githago*. Seedlings were grown in KCC-solution (a). Cotyledon pairs without axis were incubated in KCC-solution (b), KCC-solution with 10 μM BAP (c) or KCC-solution with 10 μM GA_3 (d).

Finally, it is possible that detached cotyledons release into the medium hydrolysis products which otherwise would have been transported to the axis. Transport from the cotyledons to the axis can already be detected between 6 and 24 h after the start of imbibition (DE KLERK 1982). These released hydrolysis products might affect the cotyledons, e.g. by imposing osmotic stress. Amino acid and carbohydrate contents in the medium were, however, always low, viz. not exceeding 4% of the amount present in the cotyledonary tissue (data not shown). Possibly, carbohydrates and amino acids were released and then taken up again. *Agrostemma* cotyledons have an active uptake system for amino acids (DE KLERK & LINSKENS 1979).

3.4. Rate of protein synthesis

The effect of hormones and axis removal may be specific or general. Removal of the axis, for example, might inhibit general metabolism and thereby reduce the synthesis of hydrolytic enzymes. As both axis removal and GA_3-addition had no large effect on the rate of protein synthesis (*fig. 6*), the influences of axis and GA_3 were apparently specific. BAP, however, greatly increased protein synthesis, thus indicating a general effect.

3.5. Degradation of storage proteins

In order to determine whether the degradation of embryonic reserve material is under similar control as perisperm degradation, I examined the degradation of storage proteins. The major storage proteins in *Agrostemma* embryos are 11S legumin-like globulins and 2S globulins and albumins (DE KLERK & ENGE-

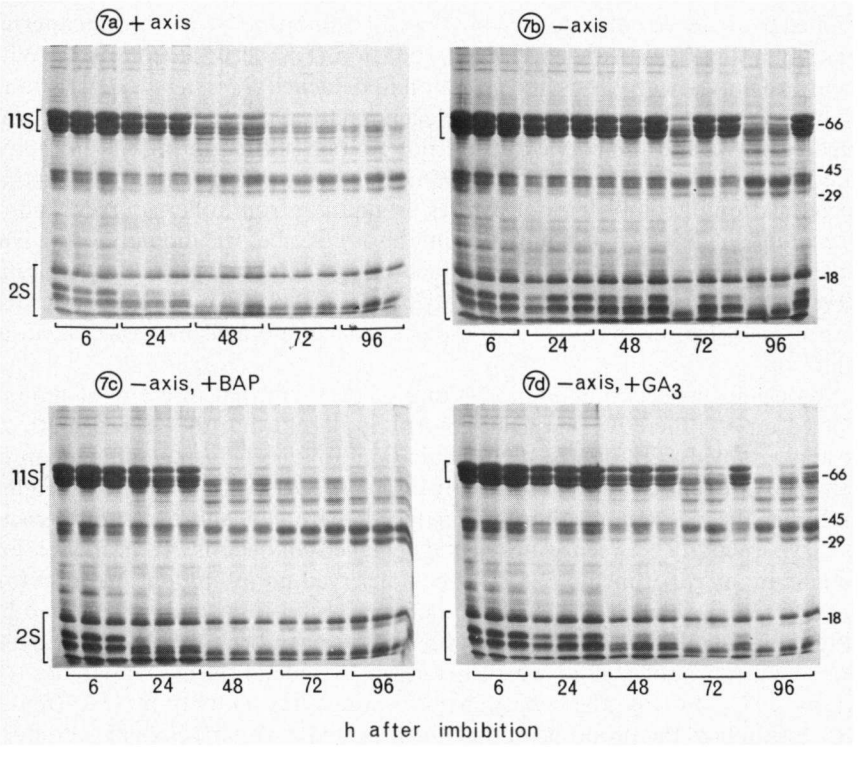

Fig. 7. SDS-electrophoresis under non-reducing conditions of proteins extracted from cotyledons pairs of *Agrostemma githago*. Seedlings were grown in KCC-solution (a). Cotyledon pairs without axis were incubated in KCC-solution (b), KCC-solution with 10 μM BAP (c) or KCC solution with 10 μM GA_3 (d). For each situation three samples of 5 cotyledon pairs were analysed. The 11S and 2S storage proteins and the position of molecular weight markers (in kD) are indicated.

LEN 1985). *Fig. 7* shows that BAP accelerated storage protein degradation, and that axis removal slowed it down. It should be noted that storage protein degradation did occur in detached water-incubated cotyledons. GA_3 only partially compensated for axis removal.

4. DISCUSSION

In *Agrostemma githago* cotyledons, the synthesis and secretion of amylase are undoubtedly under axial control (*figs. 2* and *3*). Replacement of the axis requirement by BAP and GA_3 suggests that the axis produces a hormonal stimulus. Two major arguments have been raised against hormonal control of reserve breakdown in dicots.

First, it has been suggested that the axis withdraws hydrolysis products which otherwise would inhibit hydrolytic enzyme formation and/or action. Hormones

applied to detached cotyledons may offset this inhibition by creating an internal sink, inter alia by causing cotyledon expansion (DAVIES & SLACK 1981). With regard to amylase synthesis and secretion in detached *Agrostemma* cotyledons, no hydrolysis products of starch could accumulate as the starchy perisperm had been removed and the cotyledons do not contain starch during the first days after imbibition (BORRISS & ARNDT 1956). Furthermore, *figs. 4* and *5* show that accumulation of soluble carbohydrates originating from other sources, and of free amino acids which also might be inhibitory (see before) did not occur. This strongly indicates that hydrolysis products did not inhibit amylase synthesis and secretion in detached *Agrostemma* cotyledons. However, it might still be that a specific hydrolysis product, e.g. a particular amino acid or sugar, acted as inhibitor.

Second, it has been maintained (FORD et al. 1976) that in many of the published reports there was no axial control at all and that the observed effects of axis removal were due to high incubation volumes causing submersion of detached cotyledons and consequently a lack of O_2. FORD et al. (1976) found that when the incubation volume was reduced, there were only minor differences in enzyme activity between attached and detached cotyledons. At the incubation volume in my experiments (3.5 ml), they observed no inhibition. Moreover, if the differences between attached and detached cotyledons had been caused by differential aeration, similar differences should have been found for the rates of protein synthesis (*fig. 6*) which is known to depend on O_2 availability (DE KLERK 1981). Finally, the enhancement of amylolytic activity by GA_3 (*fig. 3*) also contradicts the proposition that the incubation volume had an inhibitory effect.

To date, it has not been considered whether axial and hormonal effects on cotyledons are specific or general. As the rate of protein synthesis is approximately the same in detached and attached cotyledons, the axis likely induces specific changes in the pattern of newly synthesized proteins. It is important to note that the synthesis of hydrolytic enzymes also occurs in detached cotyledons but at a much slower rate (*figs. 3* and *7*). Therefore, similar courses of hydrolytic enzyme activity in attached cotyledons and detached hormone-treated ones might be caused only by an acceleration of metabolism by the added hormone and not by a specific effect as the axis has. *Fig. 6* indicates that the effect of BAP is at least partially due to a general enhancement of metabolism. GA_3 though seems to have a specific effect. In this connection, it should be noted that detached cotyledons develop a high level of amylolytic activity by 96 h (*fig. 3*) without, however, secreting significant amounts (*figs. 2b*). This indicates that amylase synthesis and secretion are under different control.

In conclusion, the data presented in this paper strongly indicate that reserve mobilization in *Agrostemma* seeds is under axial control through a mechanism different from the withdrawal of hydrolysis products. Possibly, the control is hormonal. Similar results have recently been obtained for the degradation of β,1-4 mannans and storage proteins in the endosperms of lettuce and castor bean respectively (BEWLEY & HALMER 1981, GIFFORD et al. 1984).

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