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# REGULATION OF α-AMYLASE ISOENZYME EXPRESSION IN *ARAUCARIA ARAUCANA* BY GIBBERELLIC AND ABSCISIC ACIDS

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**Key Word Index**—*Araucaria araucana*; Araucariaceae; α-amylase expression embryos; mega-gametophytes; abscisic acid; β-chloroethyltrimethylammonium; gibberellic acid.

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**Abstract**—Expression of α-amylase (EC 3.2.1.1) isoenzymes present in the embryos and mega-gametophytes of *Araucaria araucana* seedlings was studied under gibberellic acid ($GA_3$) and abscisic acid (ABA) treatments and after 90 hr of imbibition in 20 mM of β-chloroethyltrimethylammonium (CCC), a $GA_3$ biosynthesis inhibitor. When CCC was used, α-amylase activity in the treated embryos decreased to 28% with respect to the controls. The isoenzymes of $R_m$ 0.42 and 0.51 were greatly affected. When the embryos were imbibed simultaneously in the presence of 20 mM CCC and $10^{-5}$ M of $GA_3$, α-amylase activity and the expression of isoenzymes were restored with respect to controls. ABA at $10^{-4}$ and $10^{-5}$ M decreased α-amylase activity of embryos to 67 and 81%, respectively. Expression of isoenzymes of $R_m$ 0.51 was greatly decreased by ABA but the effect on the others was small. Isoenzyme expression of mega-gametophytic α-amylase was not affected by CCC, $GA_3$ or ABA treatment. Copyright © 1997 Elsevier Science Ltd

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## INTRODUCTION

In seeds of the conifer *Araucaria araucana* the principal reserve is starch. This carbohydrate is present in the embryo and mega-gametophyte tissues of the seed [1]. The starch is hydrolysed mainly by the activity of α-amylase enzymes, which are present in both embryo and mega-gametophyte [2, 3]. In the first 90 hr of embryo imbibition, the α-amylase activity and total amount of proteins increase, with a concomitant increase in endogenous gibberellins [2].

Isoelectrofocusing and electrophoretic analyses of native α-amylases extracted from embryos and mega-gametophytes showed the presence of five isoenzymes and other minor bands of activity in quiescent seeds. These isoenzymes were purified by heating the extract to 70° to eliminate other starch-degrading enzymes. After heating, the extract was precipitated with oyster glycogen, resuspended, further purified by affinity column chromatography on CHA-Sepharose 6B column [4] and characterized for amino acid composition, amino terminal sequence, peptide pattern and immunological reactivity with polyclonal antibodies raised against barley α-amylases [5]. The amino acid composition and peptide pattern indicated that the isoenzymes of quiescent seeds are different but related. Antibodies raised against barley α-amylases of high and low isoelectric point [6] immunocross-reacted with *A. araucana* α-amylases, suggesting that the α-amylase isoenzymes of the two species are related [5]. The pattern and expression of these isoenzymes change during germination. After 90 hr of imbibition, there are seven isoforms of α-amylase which correlate with maximum activity of the enzyme [5, 7].

The main objectives of the present work were to investigate whether the increase in the expression of α-amylase isoforms after 90 hr of imbibition is regulated by gibberellin and if abscisic acid is also involved in the regulation of the expression of these isoenzymes. Since *A. araucana* α-amylase is probably evolutionarily related to that of barley, the control of the expression of α-amylase isoforms in this conifer might be similar to that well documented in cereal grains [8–11], where these hormones regulate the expression of two α-amylase gene families [12–14].

## RESULTS

When *A. araucana* seeds were imbibed for 90 hr in the presence of 20 mM CCC, α-amylase activity

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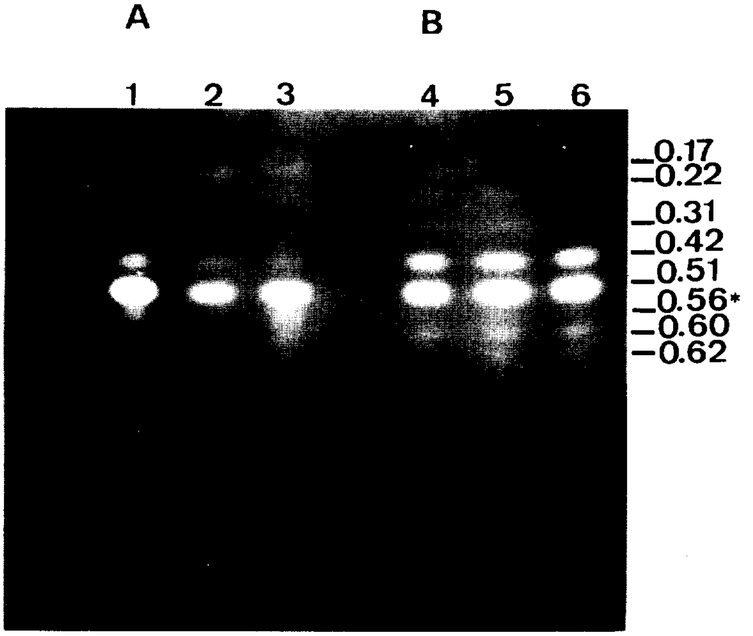

Fig. 1. Electrophoretic pattern of native α-amylase isoenzymes of *A. araucana* seedlings after 90 hr of CCC treatment. Bands were detected by the activity of the enzyme according to the method described in the Experimental. (A) Embryo extracts of imbibed seeds in: (1) water, (2) 20 mM CCC, (3) 20 mM CCC and $10^{-5}$ M $GA_3$. (B) Mega-gametophyte extracts of imbibed seeds in: (4) water, (5) 20 mM CCC, (6) 20 mM CCC and $10^{-5}$ M $GA_3$. The numbers on the right-hand side are the corresponding $R_m$ values for each band. *The $R_m$ of the band present in the embryo but not in the mega-gametophyte.

decreased to 28.2% in the embryo and to 91.3% in the mega-gametophyte, compared with that from embryos and mega-gametophytes of seeds imbibed in water (control samples). When $10^{-5}$ M $GA_3$ was added to 20 mM CCC at the start of seed imbibition, α-amylase activity was restored to 79.5% in the embryo. Activity was not restored in mega-gametophytes (Table 1).

The above results were in agreement with the isoenzyme patterns analysed in zymograms (Fig. 1). In a zymogram, the intensity of the bands indicates the relative activity of each α-amylase isoenzyme. This is the characteristic pattern of α-amylase isoenzymes run in native gel electrophoresis, as demonstrated by Acevedo and Cardemil [5] and Salas and Cardemil [7].

Three of the eight bands present in the extract of the control embryos ($R_m$ 0.22, 0.31 and 0.62) were undetectable when the embryos were treated with CCC (Fig. 1). The activity of the other bands, mainly bands $R_m$ 0.42 and 0.51, decreased considerably under this treatment. In the embryos imbibed with CCC + $GA_3$, the activity and number of α-amylase bands was restored.

Enzyme extracts from mega-gametophytes treated with water, CCC and CCC + $GA_3$ were also analysed

Table 1. α-Amylase activity and total protein concentration of embryos and mega-gametophytes after 90 hr of treatment with CCC and CCC + $GA_3$*

| Treatment | Total activity† | Protein (g protein per g dry wt) | Specific activity† per mg protein | Relative activity (%) |
|---|---|---|---|---|
| *Embryo* | | | | |
| $H_2O$ | 0.28 ± 0.004 | 0.71 ± 0.004 | 0.39 ± 0.003 | 100.0 |
| CCC 20 mM | 0.07 ± 0.002 | 0.66 ± 0.004 | 0.11 ± 0.002 | 28.2 |
| CCC 20 mM + $GA_3$ $10^{-5}$ M | 0.21 ± 0.003 | 0.68 ± 0.004 | 0.31 ± 0.005 | 79.5 |
| *Mega-gametophyte* | | | | |
| $H_2O$ | 0.07 ± 0.004 | 0.30 ± 0.004 | 0.23 ± 0.010 | 100.0 |
| CCC 20 mM | 0.06 ± 0.004 | 0.28 ± 0.004 | 0.21 ± 0.012 | 91.3 |
| CCC 20 mM + $GA_3$ $10^{-5}$ M | 0.06 ± 0.002 | 0.29 ± 0.002 | 0.21 ± 0.010 | 91.3 |

*Values represent the average of three independent experiments and their standard deviations. The control experiment was treated with water.

† Expressed as mg degraded starch $min^{-1}$ $g^{-1}$ dry weight.

Table 2. α-Amylase activity and total protein concentration of embryos and mega-gametophytes after 90 hr of treatment with $10^{-5}$ and $10^{-4}$ M ABA*

| Treatment | Total activity† | Protein (g protein per g dry wt) | Specific activity† per mg protein | Relative activity(%) |
|---|---|---|---|---|
| *Embryo* | | | | |
| $H_2O$ | 1.38 ± 0.008 | 6.46 ± 0.005 | 0.21 ± 0.006 | 100.0 |
| ABA $10^{-5}$ M | 1.09 ± 0.008 | 6.35 ± 0.005 | 0.17 ± 0.003 | 80.9 |
| ABA $10^{-4}$ M | 0.88 ± 0.008 | 6.19 ± 0.005 | 0.14 ± 0.001 | 66.6 |
| *Mega-gametophyte* | | | | |
| $H_2O$ | 0.54 ± 0.006 | 6.27 ± 0.006 | 0.09 ± 0.004 | 100.0 |
| ABA $10^{-5}$ M | 0.54 ± 0.005 | 6.25 ± 0.002 | 0.09 ± 0.006 | 100.0 |
| ABA $10^{-4}$ M | 0.53 ± 0.005 | 6.20 ± 0.000 | 0.09 ± 0.003 | 100.0 |

*Values represent the average of three independent experiments and their standard deviations. The control experiment was treated with water.

†Expressed as mg degraded starch $min^{-1}$ $g^{-1}$ dry weight.

in a zymogram. In the electrophoretic pattern, seven bands of activity were detected in the control imbibed with water. Six bands were visible in the mega-gametophytes treated with CCC; activity of the band with $R_m$ 0.17 disappeared. In mega-gametophytes treated with CCC + $GA_3$, this band was still absent, the pattern being almost identical to that of mega-gametophytes treated with CCC.

When seeds were soaked in $10^{-5}$ and $10^{-4}$ M ABA, α-amylase activity of the embryos decreased to 81% and to 67%, respectively, compared with control samples. However, α-amylase activity of mega-gametophytes was not affected by the ABA treatment (Table 2).

A zymogram of the extracts from embryos imbibed in water, in $10^{-5}$ and in $10^{-4}$ M ABA, is shown in Fig. 2. The intensity of all α-amylase bands appeared to be affected by ABA treatment, being more affected when the ABA concentration increased from $10^{-5}$ to $10^{-4}$ M. The band with $R_m$ 0.51 was the one most affected by the hormone.

## DISCUSSION

Regulation of α-amylase expression by $GA_3$ has been investigated extensively in barley aleurone cells and has been considered as a model system to explain the mode of action of $GA_3$ [8–11, 15]. Hormone effects have also been studied in other cereals, such as rice and wheat [16–19]. However, knowledge of $GA_3$ action in other germination systems is limited [20, 21]. In conifers, the only data available are for *A. araucana*, *Pinus ponderosa* and *P. sylvestris*. In *A. araucana*, a compound with $GA_3$ activity extracted from seed embryos has been reported [2]; there was a positive correlation between the amounts of α-amylase activity and endogenous gibberellins during the first 40 hr of seed imbibition. However, this correlation needs to have stronger experimental support, since $GA_3$ added to the germination media of *A. araucana* seeds in the absence of an inhibitor of gibberellin biosynthesis does not increase α-amylase activity, as occurs in cereals (unpublished results), probably because the system has enough endogenous gibberellin for maximum expression of the enzyme.

That the expression of α-amylases in *A. araucana* could be under gibberellin control was corroborated in embryos by inhibition of enzyme expression produced by CCC, a specific inhibitor of $GA_3$ biosynthesis, and the restitution of α-amylase activity when $GA_3$ was added to CCC. The activity is restored close to 80%. In mega-gametophytes, however, the addition of $GA_3$ and of CCC was ineffective. However, epidermal tissue of mega-gametophytes has a cuticle on the external cell wall and, therefore, it is possible that the applied $GA_3$ and CCC was not absorbed by this tissue [22]. In *Pinus sylvestris* had been found that exogenous $GA_3$ cannot replace the effect of embryo extracts on the expression of α-amylase in mega-gametophytes [23].

In barley, regulation of α-amylase expression by $GA_3$ and ABA does not affect to the same magnitude all of the α-amylase isoenzymes [6]. This is also true for *A. araucana* isoenzymes, where the isoenzymes with $R_m$ 0.42 and 0.51 were more affected in their expression in the absence of gibberellins. The latter was also affected by the ABA treatment.

The antagonistic effects of $GA_3$ and ABA found for cereals are also observed in *A. araucana* embryos, although in a less drastic way. Inhibition of α-amylase expression by ABA mainly affects the isoenzyme with $R_m$ 0.51, while the other isoenzymes are only slightly affected by this hormone. Therefore, the negative ABA effect is not equivalent to the removal of $GA_3$, since when $GA_3$ is not present, three isoforms are not expressed, the isoenzyme of $R_m$ 0.51 being one of them.

As in the case of $GA_3$, ABA appears not to affect the expression of the mega-gametophyte isoenzymes. Thus, we cannot discard the possibility that regulation of α-amylase expression could be different in the mega-gametophyte and in the embryos due to the morphological and physiological differences between these two tissues.

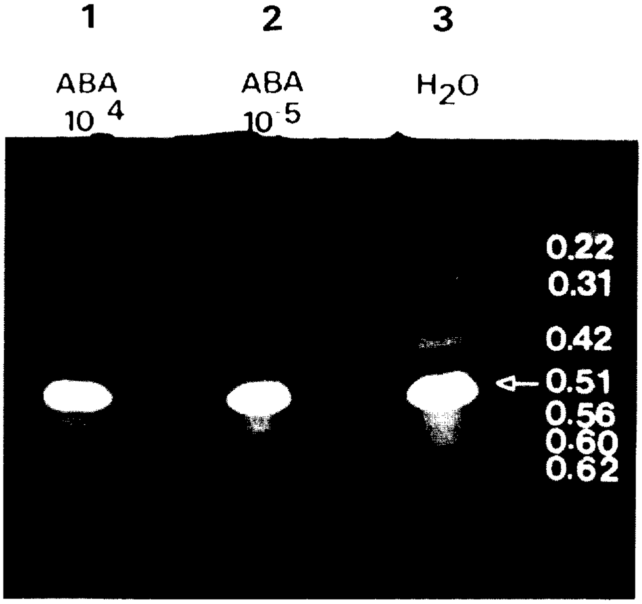

Fig. 2. Electrophoretic pattern of native α-amylase isoenzymes of *A. araucana* seedlings after 90 hr of ABA treatment. Bands were detected by the activity of the enzyme according to the method described in the Experimental. Embryo extracts of imbibed seeds in: (1) $10^{-4}$ M ABA; (2) $10^{-5}$ M ABA, (3) water.

In cereals there is evidence that the antagonistic effects between gibberellins and ABA occur at the transcription stage, with ABA also affecting the translation of α-amylase biosynthesis [24]. From our results it is not clear whether ABA and $GA_3$ control α-amylase expression at the level of transcription or translation in *A. araucana*.

## EXPERIMENTAL

*Material.* Seeds of *A. araucana* (Mol.) Koch were collected in 1989–1990 from March to April in the Chilean forest of Malalcahuello, latitude 37°5′S. After collection, seeds were stored at 5° until used.

*Hormone treatments.* Three groups of 50 seeds each were scarified, sterilized with 10% commercial NaOCl for 15 min and germinated under three different conditions. All groups were incubated for 90 hr in vermiculite moistened with $H_2O$ (group 1); with 20 mM CCC (group 2); with 20 mM CCC and $10^{-5}$ M $GA_3$ (group 3). Each group was maintained at 28° with a 12 hr photoperiod.

For ABA, two groups of 50 seeds were imbibed during 90 hr in the presence of $10^{-4}$ and $10^{-5}$ M ABA at 28° with a 12 hr photoperiod. In control expts, seeds were moistened in $H_2O$ at the same temp.

Under these conditions, seeds germinated 48 hr after the start of imbibition. Therefore, 90 hr after imbibition the radicle had emerged and the embryo axis was easily separated from the mega-gametophyte. All hormone expts were run three times.

*α-Amylase extraction.* After incubation, embryos and mega-gametophytes were sterilized for 15 min in NaOCl and rinsed several times with dist. $H_2O$. Tissues were homogenized using a ratio of 2 ml (g fr wt)$^{-1}$ of extraction buffer. This extraction buffer contained 0.05 M NaOAc, pH 4.8, 0.02 M $CaCl_2$, 0.06 M NaCl and 1 mM phenylmethylsulphonyl fluoride or *N*-tosyl-L-phenylalanine chloromethyl ketone or *N*-*p*-tosyl-L-lysine chloromethyl ketone as protease inhibitors. Six different homogenates were centrifuged at 8000 *g* for 15 min. The supernatants were heated for 15 min at 70°; at this temp., β-amylases are degraded. After heating, they were cooled down in ice and centrifuged for 5 min at 5000 *g*. The supernatants were ppted at 0° with 20% and 40% of $(NH_4)_2SO_4$ to eliminate all other starch-degrading enzymes. Total α-amylase activity was ppted with 60% $(NH_4)_2SO_4$. The corresponding ppts from extracts of each of the treatments, were resuspended in extraction buffer and dialysed at 7° with several changes of buffer during 40 hr. The dialysed extracts were centrifuged at 2000 *g* for 10 min to remove undissolved particles. This partially purified α-amylase extract gives the same isoenzyme pattern as the α-amylase purified by affinity column chromatography with CHA-Sepharose 6B [5].

α-Amylase activity was assayed using the starch–$I_2$–KI method [5, 25]. One enzyme unit is defined as 1 mg of degraded starch min$^{-1}$ g$^{-1}$ dry wt at 37°. Protein concn was determined according to the assay in ref. [26] using BSA as standard; 100 μl portions of enzyme were used.

*Native PAGE analysis.* α-Amylase proteins were analysed using 7% polyacrylamide in the resolving gel [20]. Extracts to be analysed by electrophoresis were partially purified as described for enzyme activity measurements. Each well of gel was charged with 50 μg proteins from the seedlings and 530 μg proteins from the mega-gametophyte. Electrophoresis was run at 90 V for 5 hr at 10°. Isoenzyme bands were detected by the activity of α-amylase. For this, the gel was incubated in 50 mM Tris-maleate pH 5.0, containing 1% starch as substrate and 20 mM $CaCl_2$, for 30 min at room temp. to detect α-amylase activity of the protein band [5, 20]. After incubation, the gel was stained with 10% KI–$I_2$ and fixed in a soln containing 5% HOAc and 30% MeOH.

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