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HORMONAL AND LIGHT REGULATION OF THE MULTIPLE AMYLASE ISOZYMES DURING SEED GERMINATION AND IN VEGETATIVE TISSUES OF *ZEA MAYS*

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The molecular forms of amylase present in germinating maize seeds (*Zea mays*), and in vegetative tissues (radicule, coleoptile, leaf and root from adult plants), have been studied. Twelve different isozymes showing different mobilities under non-denaturing polyacrylamide gel electrophoresis could be recognized. A time-dependent pattern of expression for the different amylase forms during the germination process has been observed. The isozyme pattern of amylases present in vegetative tissues is much simpler than for germinating seeds.

The effects of hormones, gibberellic acid (GA_3) and abscisic acid (ABA) on the expression of amylase isozymes during germination were studied. In the presence of GA_3 or ABA, an increase or decrease, respectively, in the total amylolytic activity was observed. Moreover, GA_3 induced a selective increase in the amylolytic activity of particular isozymes while others were not affected. On the other hand, ABA induced a decrease in the activity of all amylase isozymes.

In addition to the hormonal regulation of the amylase isozymes observed in germinating seeds it is shown that expression of particular amylase isozymes can also be light-regulated in vegetative tissues.

Key words: abscisic acid; amylase; gibberellic acid; isozymes; light-inducibility; *Zea mays*.

Introduction

Germination of many seeds, including those of important food grains, requires production of amylases to degrade starch in the seed endosperm for its use by the developing plant embryo. Many reports have been published about α-amylase production in barley and it has been amply demonstrated the presence of hormonal regulation in the system [1—6]. The germinating barley embryo produces the hormone gibberellic acid which, in turn, stimulates aleurone cells covering the endosperm to produce α-amylases. The synthesis of α-amylase in barley has received attention and the study of amylase expression in isolated barley aleurone layers has been used as a model system to study hormonal regulation of gene expression in plants [7]. In barley, α-amylase is the most abundant and the best characterized of the aleurone hydrolases. There are two families of α-amylase isozymes, differing in charge but not in molecular weight (44 kDa): the high p*I* group (p*I* 5.9—6.6) and the low p*I* group (p*I* 4.6—5.2) [8,9]. The synthesis of these two isozyme groups has been shown to vary over time, to be differentially affected by gibberellic acid, and to require different calcium concentrations [7,10,11].

On the other hand, knowledge concerning the nature and physiological role of amylases in vegetative tissues is scarce and often even contradictory. Thus, α-amylase has been found in the chloroplasts of spinach [12,13], however, several studies with pea and barley indicate that α-amylase is either absent or displays very low activity in the chloroplast [14—16]. Regardless of whether α-amylase is present or absent in the chloroplast, it appears that the largest amount of the total α-amylase activity in leaf tissues is extrachloroplastic [12,15—17].

Contrary to the well characterized barley

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amylases, very little information is available for other germinating cereal seeds, on the relative amounts present in different vegetative tissues, the existence of different amylase isozymes, or the factors affecting the regulation of amylase expression. Separation of multiple α-amylase components in some germinating cereal grains by isoelectric focusing and chromatophocusing has been published [18], and recently, the purification and characterization of α-amylase forms from endosperm of germinating maize has been reported [19].

We have characterized in the present study the different isozyme forms expressed during the germination process of maize seeds, and compared them with amylase isozymes present in vegetative tissues. We have also analyzed the effect of hormones and light on the expression of the different isozymes.

Material and Methods

Plant material and germination conditions

Maize (*Zea mays* pure imbred line W64A) was used as the experimental material. Leaves and roots were obtained from plants grown in a greenhouse for about 6 weeks; coleoptiles and radicules were separated from 3-day germinated seedlings. All tissues were frozen in liquid N_2 and stored at $-70\,°C$ until needed. To study changes in amylase isozyme expression during germination, maize seeds were germinated at 25 °C in the dark for three days followed by a daily cycle of 15 h illumination for the required time. At all stages of germination, seeds were harvested, frozen in liquid N_2 and stored at $-70\,°C$. Maize seeds were also germinated in the presence of hormones. Hormones, GA_3 (Merck) and ABA (Fluka) were added at a final concentration of 10 μM to the germination trays. Fresh hormone was added daily.

To determine whether light affects the expression of the different amylase isozymes, seeds were germinated in the dark for three days followed by a period of 8 h of light (control seeds were allowed to continue germination in the dark for the same period of time). Amylase isozymes present in radicules and coleoptiles from seeds germinated in complete darkness or in the presence of light at the late period of germination were analyzed.

Preparation of enzyme extracts and assay of amylase activity

For the preparation of crude extracts used for the amylolytic activity determinations, frozen seeds or vegetative tissues (coleoptile, radicule, leaf and root from adult plants) were powdered in the presence of liquid nitrogen with a prechilled mortar and pestle. Chilled extraction buffer (0.05 M Tris – HCl pH 7.0, 0.05 M NaCl, 4 °C) was added to this powder (1 ml/g fresh weight tissue). The extraction was carried out at 4 °C for 45 min with continuous and mild stirring in a rotatory wheel. Samples were centrifuged at 10 000 rev./min for 15 min at 4 °C. Supernatants recovered were made 5 mM in $CaCl_2$ and were assayed for amylolytic activity or were used for analysis and characterization of amylase isozymes by non denaturing polyacrylamide gel electrophoresis.

Amylase activity was also assayed by the 'in solution' assay as described by Crispeels and Varner [3] using soluble starch (Merck) as substrate and a 10-min period of incubation. The decrease in optical density at 620 nm caused by the action of the enzyme was measured. Total amylolytic activity is expressed as the change in optical density per 10 min. Protein content of extracts was estimated by a modification of the Lowry method [20] using bovine serum albumin as standard.

Characterization of amylase isozymes

Electrophoretic techniques. Aliquots from extracts were studied by using non-denaturing polyacrylamide gel electrophoresis. The separating gel contained 7% acrylamide and 0.2% bisacrylamide. The discontinuous buffer system of Davis [21] was used. Visualization of protein bands with amylolytic activity was carried out as follows. After electrophoresis, the gel was washed in distilled water and incubated in 2% soluble starch, 0.1 M sodium succinate (pH

5.0) at 37 °C for 60 min. The gel was then washed with distilled water to remove the excess starch solution and the gel left in a capped container for 30 min at 37 °C. The gel was then stained overnight in acidified iodine-potasium iodide solution (0.2 M HCl, 6 mM I_2, 50 mM KI). Amylolytic activity appears as a light zone against a deep blue background.

Electrophoresis in SDS-polyacrylamide gel electrophoresis (SDS- PAGE) and the subsequent activation of amylases was also carried out for the characterization of maize amylases. The method, based on the recovery of amylolytic activity following SDS removal, has been sucessfully applied previously for detection of different enzymatic activities (nucleases, proteases) [22,23]. Electrophoresis was performed according to Laemmli [24] by using a 12.5% acrylamide separating gel. To detect amylolytic activity, SDS was exchanged with Triton X-100 for 60 min at room temperature. Afterwards, gels were incubated with soluble starch solution, and stained as described above.

Results

Characterization of amylase isozymes in germinating maize seeds

Non-denaturing gel electrophoresis has been used to analyze developmental changes in amylase isozymes during maize seed germination, between days 1 and 7. Amylase activity was resolved into twelve different isozymes (Fig. 1) named groups A to E in terms of decreasing mobility, whose expression varies during the germination period. Thus, the high p*I* isozymes (slow migrating bands, D and E) are present at the earliest stages, being dominant at day 1

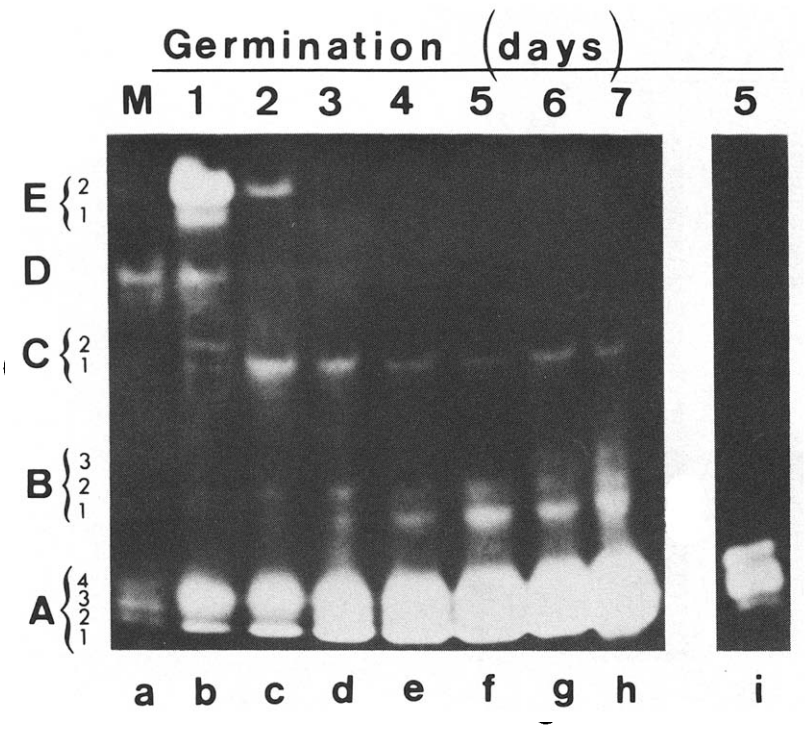

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of amylase isozymes from mature and germinating maize seeds. Aliquots of extracts containing the same amount of protein (50 μg) for each stage of germination (except track i) were analyzed. After electrophoresis, the gel was stained for amylolytic activity using soluble starch as a substrate. Track a, mature seed; tracks b – h, germinating seeds (days 1 – 7); and track *i*, 5-day germinated seeds (5 μg of protein aliquot). Isozymes have been named as groups A – E in terms of decreasing mobility (increasing p*I*).

(see band E-2). From day 2 and later on, amylase activity is mainly found in the low p*I* isozyme group (group A) with a gradual increase of total group activity during germination. However, examination of the time course of amylase activity production within this group (group A) shows that not all forms appear at the same time. Differences in the intensities and time of appearance of the different amylase isozymes present in group A are better seen when low amounts of protein are analyzed (see Fig. 4 below). Amylase activity at isoform A-4 is detected before than amylase activity at isoforms A-2 and A-3 (Fig. 4A day 2 of germination), which in turn are the major isozymes present later on (see Fig. 1, track i). Isoform A-1 is the latest appearing activity.

Although non-denaturing gel electrophoresis indicates an important heterogeneity on the pattern of isozymes in germinating maize seeds, when amylolytic activity was analyzed after separation of proteins in extracts by SDS-PAGE and subsequent reactivation of amylases by SDS exchange with Triton-X100, the pattern is much simpler (Fig. 2). Amylase activity appears as a major band for all stages of germination (an example is shown in Fig. 2, track i) with an apparent molecular weight of approx. 40 kDa. When larger amounts of extract are analyzed (Fig. 2, tracks a—h) some additional bands with higher molecular weights and a minor band with lower molecular weight (day 3 and after) are visible. This later one is constantly present until the latest stage of germination analyzed in the present work. The former bands (high molecular weight amylase forms) could represent aggregates of amylase with other proteins. The low molecular weight

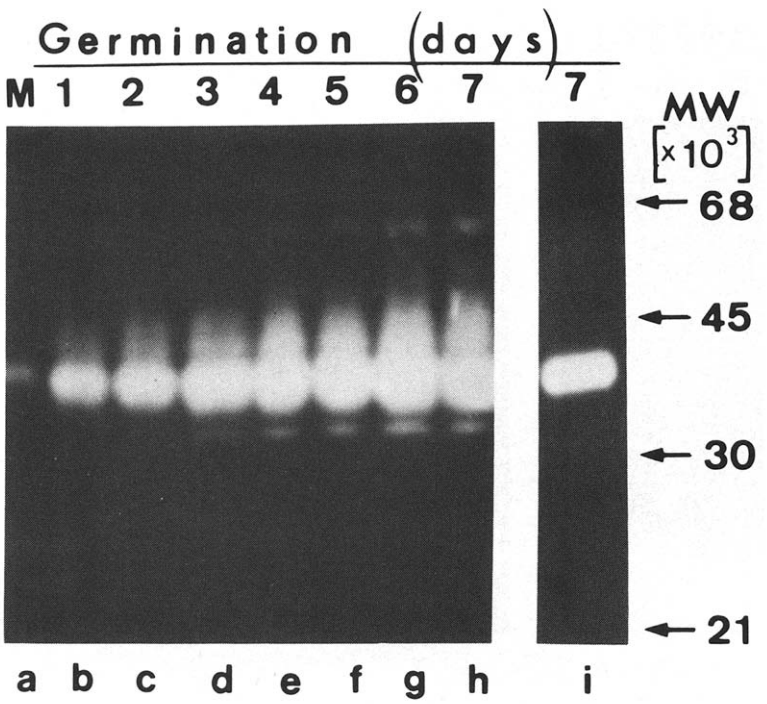

Fig. 2. Analysis by SDS-polyacrylamide gel electrophoresis of maize amylases from mature and germinating seed. After SDS-polyacrylamide gel electrophoresis, SDS was exchanged with Triton X-100 followed by detection of amylolytic activity using soluble starch as substrate. Aliquots of extracts containing the same amount of protein (40 μg) for each stage of germination (except track i) were analyzed. Track a, mature seed; tracks b—h, germinating seeds (days 1—7) ; track i, 7-day germinated seeds (10μg of protein aliquot).

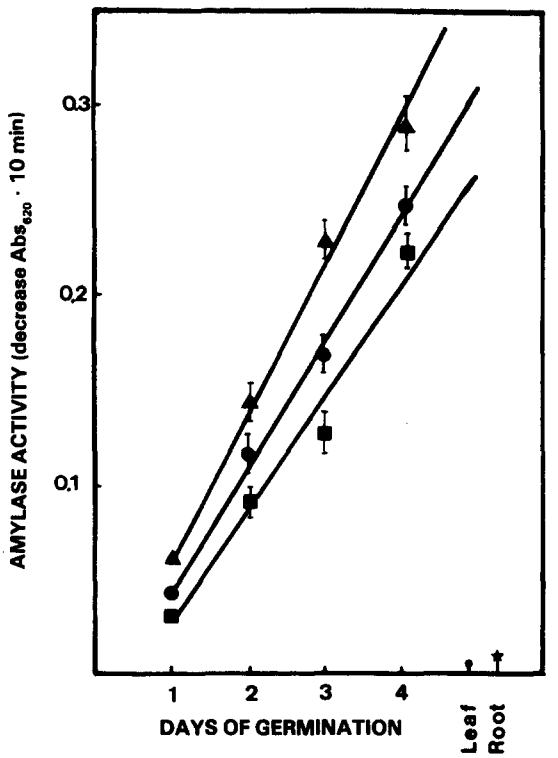

Fig. 3. Total amylolytic activity present in extracts prepared from germinating maize seeds (days 1—4) and vegetative tissues (leaf and root from adult plants). The 'in solution' assay for amylolytic activity was performed by measuring the amount of starch hydrolyzed during a 10-min period of incubation and using the KI/I reagent (3). Seeds were germinated with GA_3 (▲—▲) or ABA (■—■) (10 μM each), or without added hormone (●—●). Results are the average of four different germination experiments and three separate preparations of seed extracts for each germination experiment. Bars indicate maximum and minimum values obtained. Equal amount of protein (7 μg) from each extract, both from germinating and adult tissues, was used for each assay.

amylase form could correspond either to a newly synthesized amylase isozyme or to posttranslational modifications of a pre-existing amylase isozyme form. The nature and/or relationship of the different molecular weight bands with the different isozymes separated by non denaturing gel electrophoresis will need further analysis.

Response of the amylase isozymes profile to hormones

Amylolytic activity present in extracts from seeds germinated in the presence of hormones, GA_3 and ABA, was analyzed by a conventional solution assay (see Material and Methods), and by non denaturing gel electrophoresis. In the first case, we can measure the effect of these hormones in the total amylase enzyme activity, while the second method allow us to observe the effect on particular amylase isozymes, separately.

When the solution assay for amylase activity determination was performed in extracts prepared from germinating seeds (days 1—4), we observed that germination was accompanied by an increase in the total amylolytic activity along the germination process (Fig. 3). Compared with the activity found in seeds germinated in the absence of hormone, the presence of GA_3 caused an increase in the total amylase activity at each day of germination. Presence of ABA, however, decreased that activity. Figure 3 also shows the level of total amylase activity present in vegetative tissues, leaf and root from adult plants. The activity found in extracts prepared from these tissues is much lower than the activity found in germinating seeds.

To further characterize the response of amylase isozymes to hormones, extracts from seeds germinated in the presence or absence of hormones were analyzed by non denaturing gel electrophoresis. If the amount of enzyme loaded in the gel was kept low, the intensities of the amylase bands are an indication of the relative amounts of activity present in each band. In this study, we present only the hormonal effects on the amylase isozyme group with lower p*I* (fast moving bands or group A), because no detectable effects could be found for the high p*I* isozymes. Results are shown in Fig. 4. Germination in the presence of GA_3 causes a significant increase in activity at specific isozymes. For instance, at days 1 and 2 of germination the activity of the amylase isozyme A-4 band is higher when GA_3 is present. The same effect is observed for the amylase isozyme A-2 band at day 3 of germination.

Moreover, GA_3 treatment during seed germination induces an increment in the total amylolytic activity present in germinating seeds

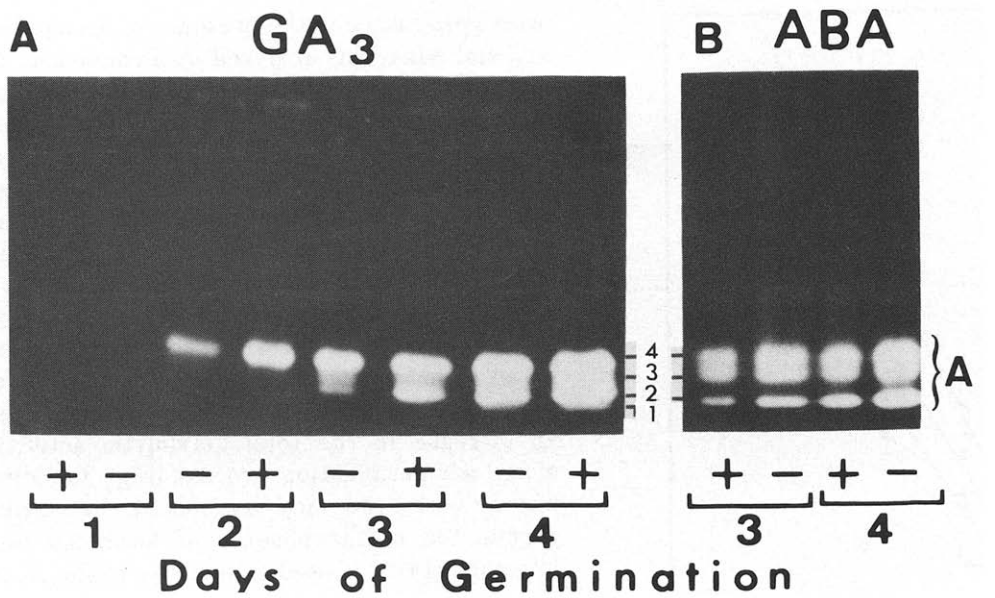

Fig. 4. Effect of hormones, GA_3 (A) and ABA (B), on the expression of the amylase isozymes during germination of maize seeds. Seeds were germinated with the hormone (10 μM each) (+) or without added hormone (−). Non-denaturing polyacrylamide gel electrophoresis, followed by amylolytic activity detection was carried out. Aliquots of each extract containing the same amount of protein (8 μg) were applied to each lane. Numbering refers to the low pI isozyme group (group A).

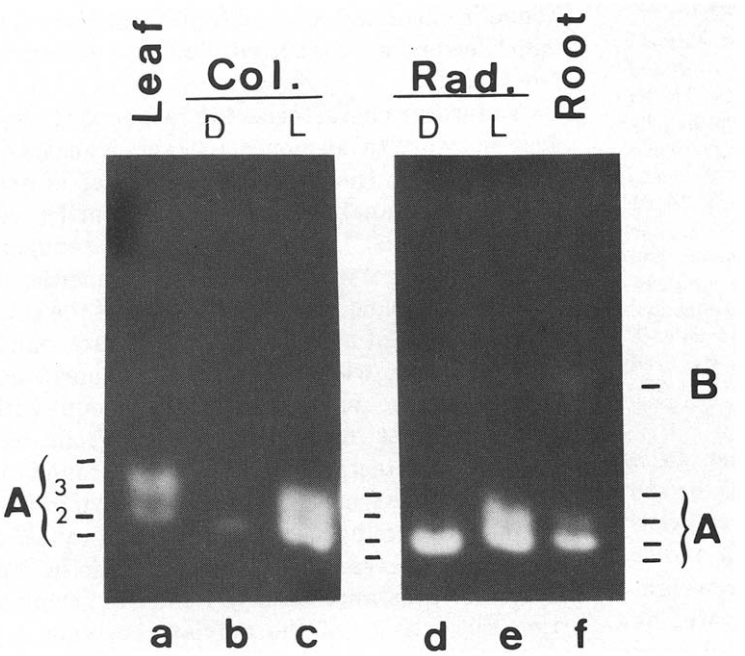

Fig. 5. Amylase isozymes in vegetative tissues. Non-denaturing polyacrylamide gel electrophoresis, followed by amylolytic activity detection was carried out. Track a, adult leaf (120 μg of protein in aliquot); track b, coleoptile from 3-day seedlings germinated in complete darkness (60 μg); track c, coleoptile from 3-day seedlings germinated in the dark and transferred to light conditions (8 hours) (60 μg); track d, radicule from 3-day dark germinated seeds (60 μg); track e, radicule from 3-day dark-germinated seeds, transferred to light conditions (8 hours) (60 μg); and track f, adult root (60 μg); Col, coleoptile; Rad, radicule; D, dark; and L, light.

but stimulating preferentially the amylase activity at particular isozymes rather than increasing uniformly the amylase activity at the different isozymes. Besides, the presence of GA_3 during seed germination does not cause the appearance of new isozyme forms as compared with the pattern of isozymes present in seeds germinated in absence of the hormone.

When extracts of seeds germinated in the presence of ABA were analyzed by non denaturing gel electrophoresis no drastic effects were observed when compared with seeds germinated in the absence of hormone (Fig. 4B). There is a slight decrease in activity for all isozymes present in the low p*I* group (group A) but no particular activity band seems to be preferentially affected.

Results presented in Figs. 3 and 4 suggest that the final effects caused by the presence of hormones, GA_3 and ABA, are an increase or decrease, respectively, in the total amylolytic activity. However, when analyzing the effect of these hormones at the isozyme level it is seen that GA_3 induces a preferential stimulatory effect on the activity of specific amylase isozymes while ABA uniformly reduces the mesurable activity for all amylase isozymes along the germination period (4 days) studied.

Amylase isozymes in vegetative tissues

Analysis of the total amylolytic activity present in adult tissues (leaf and root) demonstrates that the levels of amylase activity in these tissues are low when compared with the activity present in germinating seeds (see Fig. 3).

The isozyme pattern of amylases present in adult tissues is much simpler than for germinat-

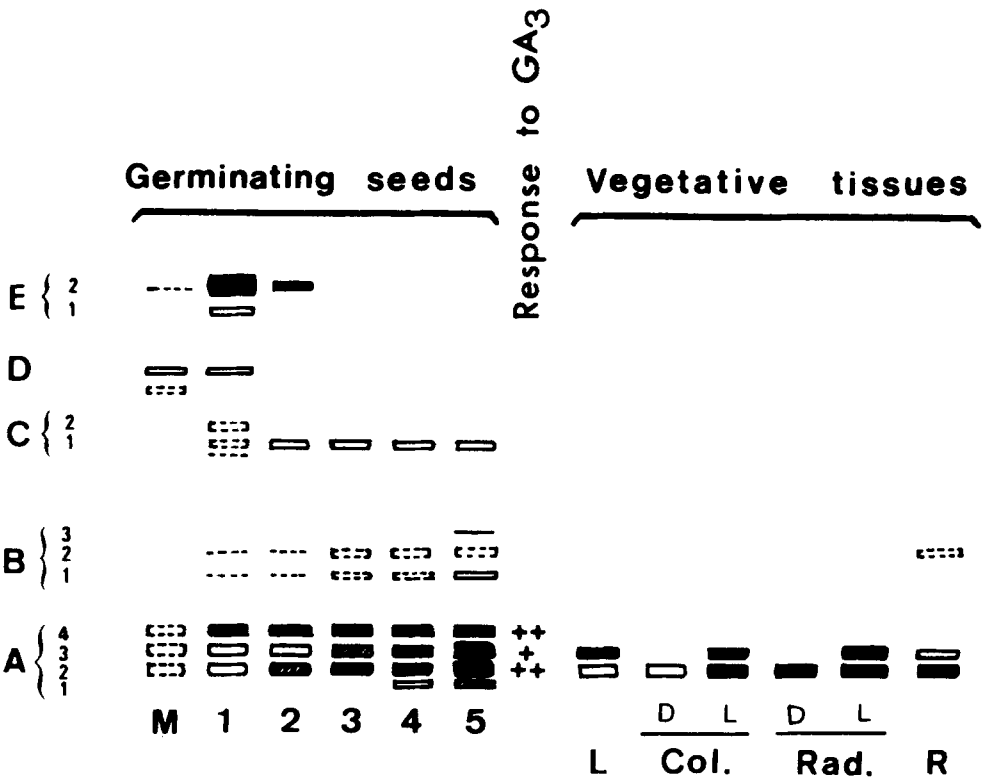

Fig. 6. Summary of the amylase isoforms identified by non-denaturing polyacrylamide gel electrophoresis in germinating seeds and vegetative tissues in maize. The expression behavior of the different isozymes during germination (days 1 to 5) is also indicated. Isozymes are numbered sequentially according to their mobility during electrophoresis. Col. = coleoptile, Rad. = radicule, L. = adult leaf and R. = adult root. Strong bands positions are marked as filled black rectangles. Weaker bands, in order of decreasing band intensity, are marked with open rectangles, dotted rectangles and dotted lines.

ing seeds (Fig. 5). Two amylase isozymes are found in extracts prepared from leaves and roots from adult tissues (Fig. 5, tracks a and f) but with different relative intensities (the fast moving isozyme band is more intense in root while the slow moving isozyme is more intense in leaf). These two amylolytic activities comigrate with isozymes A-2 and A-3 from germinating seeds.

An interesting feature was observed when analyzing amylase isozymes present in extracts from coleoptile and radicule from seedlings which were grown for 3 days in complete darkness and then exposed to light for the last 8 h of germination. One single isozyme form is present in both tissues when seeds are germinated in darkness (Fig. 5, tracks b and d). However, when they are exposed to light, expression of a second amylase isozyme is induced (Fig. 5, tracks c and e). The mobility of this light-induced amylase form is equal to the more intense isozyme form present in leaves from adult plants and its expression is induced both in coleoptile and in radicule. When analyzed by SDS-PAGE followed by amylase activity detection, all vegetative tissues show a single band with the same mobility than the major band (40 kDa band) found in germinating seeds (results not shown). We have not studied the subcellular localization of the amylase activities detected in adult tissues, accordingly we cannot discard the possibility that the light-induced amylase activity occurs in the chloroplasts.

Finally, we have summarized in Fig. 6 the nomenclature for the different isozyme forms found by non denaturing polyacrylamide gel electrophoresis and also the changes in their pattern of expression among tissues, and under the influence of light and gibberellic acid treatment.

Discussion

Our study demonstrates the existence of multiple forms of amylase isozymes with a specific pattern of expression during seed germination and in vegetative tissues of maize. Total amylase activity present in vegetative tissues is very low compared with activity present in germinating seeds, as expected due to the important role of this hydrolase in the mobilization of sugars from the starch granules stored in the endosperm of seeds. Starch degradation is the result of the action of many different enzymes: α- and β-amylases, limit dextrinases, 'debranching' enzymes, phosphorylases, etc. Several methods have been developed for the detection of amylolytic enzymes, however, none of them provides an easy identification of differing amylolytic enzymes. Particularly, the specific determination of α-amylase activity in crude plant extracts is difficult because of the presence of β-amylase activity in the tissues that interferes directly in most assay methods. The most commonly used procedure involves the selective inactivation of β-amylase by heating or by the addition of $HgCl_2$. However, response to these treatments varies from one tissue to another or even in the analysis of amylases from a single tissue (seeds) among different species. In contrast to those strategies, we have not made a distinction between α- and β-amylases (soluble starch has a very short chain length and many fragments contain few if any [1 — 6] branch points and can be hydrolyzed by α-amylase as well as β-amylase).

A complete understanding of the response of maize amylases to hormones, or other possible factors affecting their expression, requires a complete characterization of all forms of maize amylases and the identification of their tissues of origin. We report in this work our contribution to this goal.

Non-denaturing gel electrophoresis has been routinely used to characterize the isozyme forms of amylases. We have used this technique to follow changes in the expression of the different amylase isozymes during germination of maize seeds and to compare the different molecular forms of amylase present in germinating seeds and in vegetative tissues (radicule, coleoptile, and leaf and root from adult plants). Hormonal regulation and light inducibility of amylase isozymes was also studied by using this electrophoretic technique.

Moreover, the existence of multiple amylase isozymes with the same molecular weight has been previously demonstrated in barley. We should mention that the electrophoretic analysis of amylases in polyacrylamide gels containing SDS followed by removal of the SDS from the polyacrylamide slab by incubating it in aqueous Triton X-100 is of great value for distinguishing different amylases on the basis of their relative sizes and to follow developmental changes. However, it cannot be used to assign precise molecular weights as the technique relies upon preservation of enzyme activity and the samples cannot be heated in SDS before electrophoresis (and should not contain β-mercaptoethanol). Our results, however, are in agreement with those reported by Warner et al. [19] on the purification and characterization of α-amylase forms from germinating maize. These authors report the existence of a major amylase form with a molecular weight of 40 kDa and other minor amylase forms with molecular weights of 94, 67, 42 and 31 kDa.

In plants, knowledge about the mechanisms by which hormones control gene expression is very scarce. Results obtained in barley, exemplify the complexity of GA_3 and ABA action in the aleurone system. For instance, it has been shown that GA_3 has multiple effects in barley aleurone, increasing the concentration of some mRNAs and proteins and decreasing the concentration of others [25]. In particular, the stimulation of α-amylase expression by gibberellic acid and the inhibition of this effect by abscisic acid have been well documented (in barley) [7,25,26]. Thus, it has been widely demonstrated that regulation of the barley α-amylase system is exerted at the transcriptional level [25]. In rice, induction of aleurone α-amylase isozymes by GA_3 has been recently reported [27]. In this species, it has been also found that gibberellic acid induces the production of a factor, presumably a protein, in the aleurone tissue that binds to a specific region of the upstream regulatory sequence of a rice α-amylase gene [28]. On top of that, Nolan and Ho [7] have also proposed that in barley, α-amylase expression can be also posttranscriptionally regulated by gibberellic acid and abscisic acid in addition to its demonstrated transcriptional regulation.

Analysis of the effect of hormones, GA_3 and ABA, on the amylase expression has been hampered by the existence of multiple amylase isozymes in germinating barley seeds and different amylase gene families which are not expressed in a coordinated manner and which are differentially regulated by these hormones [26,29].

We have demonstrated in this work that in maize various amylase isozymes are present both in germinating seeds and in vegetative tissues. Accordingly, we suggest that, in maize, amylase studies should be performed at the isozyme level. Maize amylase isozymes have been shown here to display time-dependent expression during germination. Furthermore, their responses to gibberellic acid treatment can vary among them. In addition to differences in the isozyme pattern of amylases present at each stage of germination, and in the time and level of response to gibberellic acid, we have also shown that the expression of amylases is differentially controlled by light in vegetative tissues. Indeed, the effect of light on β-amylase expression has been described before in another species (mustard) [30]. Additional experiments should be carried out to elucidate the possible existence of different populations of amylase mRNAs encoded by several structural genes and differentially affected by hormones. For this purpose, obtention of cDNA probes specific for different amylase mRNAs would be particularly useful. In addition to the analysis of the number of genes coding for amylases, and consequently of the existence of different amylase mRNAs, additional experiments should be carried out in order to analyze the contribution of posttranslational modifications in the appearance of different amylase isozymes. Results obtained from these type of studies will allow us a finer analysis of the expression of maize amylase isozymes and of the effect of hormones (and other factors, such as light) on specific amylase isozymes.

In conclusion, results here presented on the

light-inducibility, tissue-specificity and hormonal regulation of the expression of the different amylase isozymes reveals this system as an attractive model for studying the regulatory elements required for gene regulation during plant development.

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