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Short Communication

Onset of Alcohol Dehydrogenase Synthesis during Microsporogenesis in Maize[1]

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ABSTRACT

During male gametophyte development the synthesis of several proteins occurs from transcripts of the haploid genome. Alcohol dehydrogenase (ADH1), a developmentally regulated protein, was chosen for study to determine the stage at which its synthesis was initiated and the pattern of its synthesis during microsporogenesis. The ability of ADH to reduce *p*-nitro blue tetrazolium chloride *in situ* was used as an indicator of enzyme activity. Maize strains heterozygous for *adh1* were utilized to provide an internal control, 50% of the grains being *adh1*$^+$ and 50% being *adh1*$^-$. No ADH activity was detectable when tetrads were first formed after meiosis. Activity was initially detected soon after the tetrads began to break apart but before the microspores in the tetrads had completely separated. The transcription of the *adh1* gene from the haploid genome must thus occur very soon after meiosis is completed. ADH activity increases at a constant rate thereafter until microspore mitosis when an increase in the rate takes place which lasts until generative cell division. Thereafter, there is a marked decrease in the rate of accumulation of ADH activity.

The synthesis of several proteins during the development of the male gametophyte of angiosperms is dependent on the transcription of the haploid genome. ADH[2] in maize is one such protein that has been conclusively shown to be transcribed and translated after meiosis (4, 8). Similar evidence has been obtained for other enzymes in corn (1, 3, 6, 7), in *Clarkia dudleyana* (14), in tomato (13), and in *Cucurbita* (5). The precise stage after meiosis during which the synthesis of these proteins is initiated is not known, however. Our study was undertaken to determine the stage in pollen development when the synthesis of ADH, a cytoplasmic protein that is known to be under the control of the haploid genome, was initiated, and also to determine the pattern of accumulation of the enzyme in the developing microspore. These results have been briefly reported (12).

MATERIALS AND METHODS

Two strains of *Zea mays* L., CH701 × A36-3-1-3 and T232 × IL14H, heterozygous for *adh1* (adhF/null) were used (seed kindly provided by Dr. Major M. Goodman). Plants were grown in the greenhouse and in the field. Anthers were collected from tassels dissected out from the plant before their emergence or for later stages, after tassel emergence. The length of the anthers from each floret was used to identify the approximate stage of pollen development. Each floret contains three anthers at the same developmental stage (2). One anther was squashed and stained with Trypan Blue (10) to rapidly determine its stage, a second was fixed in acetic acid:ethanol (1:3) for 4 h, then placed in 70% ethanol for a more permanent staining by the Feulgen reaction. The third anther was processed immediately for ADH activity.

The ADH assay used was basically that described by Freeling (4). Anthers of the same developmental stage slit open to release the microspores, were placed in a Petri dish with 30 ml of 0.1 M Na phosphate buffer (pH 7.3). The samples were frozen at −20°C for 3 to 4 h on a metal tray. They were then thawed at room temperature while shaking at 30 rpm. The buffer was replaced with 20 ml of staining solution containing 86 mM Na phosphate (pH 7.3), 0.3 M *p*-nitroblue-tetrazolium chloride (Sigma Chemical Co.), 1.0 mM NAD+ (ICN Nutritional Biochemicals), and 9.5% ethanol. The microspores were stained in the dark for 3 to 4 h at 30 rpm. The reaction was stopped by replacing the staining solution with 100% methanol. The preparations were photographed with a Zeiss CS-matic 35 mm camera attachment on a Zeiss Universal Microscope using a planachromat 6.3/0.16 or an achromat 40/0.85 (oil immersion) objective and Kodak Ektachrome ASA 160 (3200 K) slide film. Bright field illumination was used with a 12 v, 100 w Halogen lamp operated at 12 v to give a color temperature of 3200 K. The film was processed commercially. To determine the density of ADH staining, each pollen grain on the color slides was scanned across its diameter in three different directions in an LKB 2202 Ultroscan Laser Densitometer and Integrator and the values averaged. To determine whether the stain density in the color slides was proportional to enzyme concentration, different amounts of ADH (yeast, crystallized and lyophilized, containing >90% protein; activity, 200 units/mg protein; Sigma) were placed in wells of a horizontal 11% starch gel and the enzyme caused to enter the gel by electrophoresis. The conditions of electrophoresis and assay for ADH activity were as described by Schwartz and Endo (9). The stained gel bands were photographed with Ektachrome ASA160 (3200 K) film. After processing the film, the stained bands were scanned in the densitometer.

RESULTS AND DISCUSSION

Developing microspores and pollen were collected at different stages starting with cells during meiosis and including stages up to mature pollen grains. Strains heterozygous for *adh1* were utilized because they provided an internal control, 50% of the grains being *adh* positive and 50% *adh* negative (Fig. 1, E and F). These results also confirm the specificity of the assay for ADH activity. The assay for ADH activity is based on the ability of the enzyme to reduce *p*-nitroblue tetrazolium chloride *in situ*. The results with both strains were identical, hence only the results

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[2] Abbreviation: ADH, alcohol dehydrogenase.

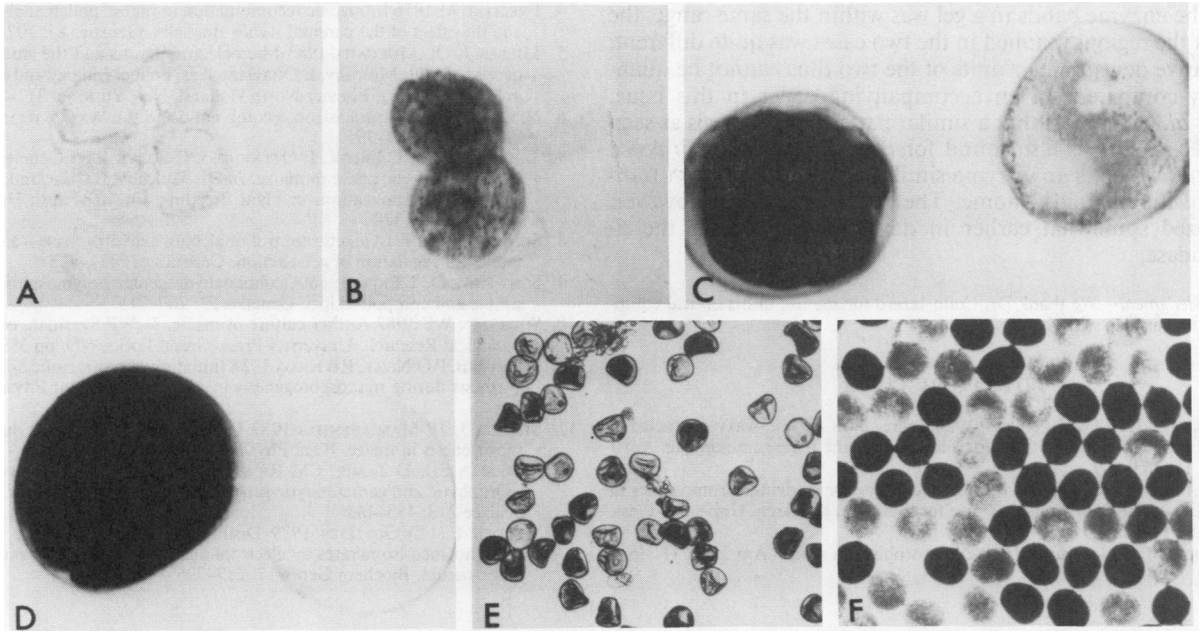

FIG. 1. Microspores at different stages of development stained for ADH activity. A, tetrads; B, tetrads in the process of separation; C, microspore mitosis; D, mature pollen grains. A to D are at the same magnification. Low magnification view (E) of young microspores in interphase prior to microspore mitosis and (F) mature pollen grains, showing 50% adh^+ and 50% adh^- grains.

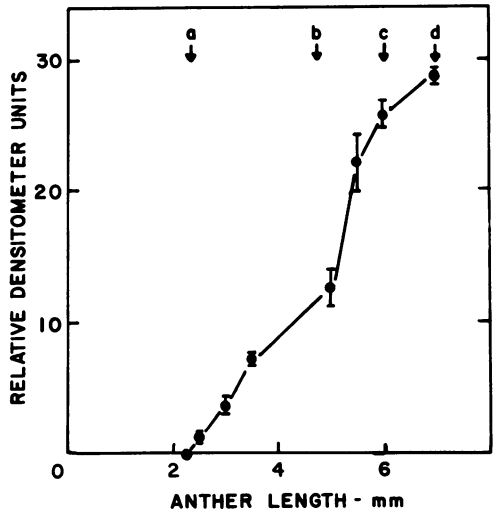

FIG. 2. Changes in ADH activity during microsporogenesis; average values of three to five grains ±SE; a, tetrads; b, microspore mitosis; c, generative cell division; d, mature pollen.

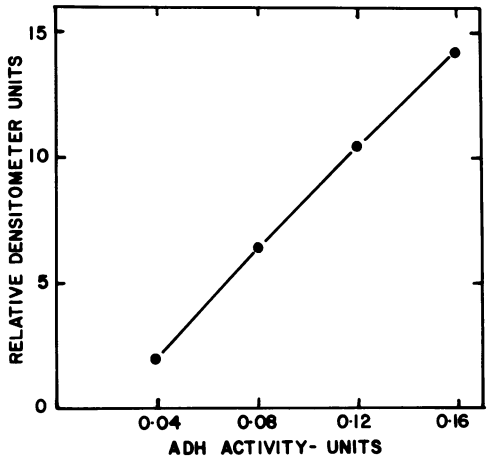

FIG. 3. Relationship between enzyme concentration and density of stain. Purified ADH was electrophoretically introduced into a starch gel, stained, photographed, and the stained bands then scanned in the densitometer.

with T232X IL14H are presented. No staining for ADH activity was seen in microsporocytes (results not presented) or when tetrads were first formed after meiosis (Fig. 1A) or when the microspores were still within the callose wall. Low levels of staining were seen soon after the tetrads began to break apart but before the microspores in the tetrads had completely separated (Fig. 1B). This would indicate that the transcription of the *adh* gene from the haploid genome must occur very soon after meiosis is completed. The ADH activity increased at progressively older stages reaching a maximum in mature pollen grains (Fig. 1, C–F).

Densitometer scans (Fig. 2) of the stained grains showed a constant rate of increase in intensity of stain (*i.e.* ADH activity) from soon after the beginning of tetrad dissolution until the microspores became vacuolate prior to microspore mitosis. Soon after microspore mitosis, the rate of ADH accumulation increased dramatically until generative cell division when it rapidly leveled off. The intensity of the stained reaction as measured by the densitometer appears to be an accurate reflection of enzyme content of the grains. Different amounts of the crystalline enzyme were introduced into a starch gel by electrophoresis and the enzyme activity assayed (9). There is a linear relationship between enzyme concentration and density (results not presented). The stained bands on color slides obtained by photographing the gel also show a linear relationship between enzyme concentration and density (Fig. 3). The linear relationship between enzyme concentration and optical density of the stained reaction product is thus maintained in the photographic reproduction on color film. One is justified, therefore, in assuming that the densitometric scans of stained microspores are a measure of the relative amount of enzyme present in the grains at the different stages of development and reflect the rate of synthesis and accumulation of the ADH enzyme. Although the intensity of the stain in pollen

and in the enzyme bands in a gel was within the same range, the width of the regions scanned in the two cases was quite different. The relative densitometer units of the two thus cannot be quantitatively compared. In an accompanying paper in this issue, Singh *et al.* (11) report that a similar pattern of synthesis as seen for ADH in corn is also found for β-galactosidase in *Brassica campestris* which is an enzyme similarly synthesized from transcripts of the haploid genome. The ADH enzyme is, however, synthesized somewhat earlier in development than is the β-galactosidase.

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LITERATURE CITED

1. BRINK RA, JH MACGILLIVRAY 1924 Segregation for the waxy character in maize pollen and differential development of the male gametophyte. Am J Bot 11: 465–469
2. BURNHAM CR 1982 Details of smear technique for studying chromosomes in maize. *In* WF Sheridan, ed, Maize for Biological Research. University Press, Grand Forks, ND, pp 107–118
3. DEMEREC M 1924 A case of pollen dimorphism in maize. Am J Bot 11: 461–464
4. FREELING M 1976 Intragenic recombination in maize: pollen analysis methods and the effect of the parental *Adh1*+ isoalleles. Genetics 83: 707–717
5. MILLER J, DL MULCAHY 1983 Microelectrophoresis and the study of genetic overlap. *In* DL Mulcahy, E Ottaviano, eds, Pollen: Biology and Applications in Plant Breeding. Elsevier-North Holland, New York, pp 317–321
6. NELSON OE 1958 Intracistron recombination in the Wx/wx region of maize. Science 130: 794–795
7. SARI-GORLA M, C FROVA, E OTTAVIANO, C SOAVE 1983 Gene expression at the gametophytic phase in maize. *In* DL Mulcahy, E Ottaviano, eds, Pollen: Biology and Applications in Plant Breeding. Elsevier-North Holland, New York, pp 323–328
8. SCHWARTZ D 1971 Genetic control of alcohol dehydrogenase—a competition model for regulation of gene action. Genetics 67: 411–425
9. SCHWARTZ D, T ENDO 1966 Alcohol dehydrogenase polymorphism in maize-simple and compound loci. Genetics 53: 709–715
10. SHERIDAN WF 1982 Anther culture of maize. *In* WF Sheridan, ed, Maize for Biological Research. University Press, Grand Forks, ND, pp 389–396
11. SINGH MB, P O'NEILL, RB KNOX 1984 Initiation of postmeiotic β-galactosidase synthesis during microsporogenesis in oilseed rape. Plant Physiol 77: 225–228
12. STINSON J, JP MASCARENHAS 1983 Onset of ADH1 synthesis during microsporogenesis in maize. Plant Physiol 72: S-31
13. TANKSLEY SD, D ZAMIR, CM RICK 1981 Evidence for extensive overlap of sporophytic and gametophytic gene expression in *Lycopersicon esculentum*. Science 213: 453–455
14. WEEDEN F, LD GOTTLIEB 1979 Distinguishing allozymes and isozymes of phosphogluco-isomerases by electrophoretic comparisons of pollen and somatic tissues. Biochem Genet 17: 287–296