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Characterization of anther differentiation in cytoplasmic male sterile maize using a specific isozyme system (esterase)

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Summary. During anther development, characterized in maize plants with N cytoplasm, certain esterase isozymes in non-microspore cells decrease in amount with anther age and new isozymes appear in the developing microspores. In anthers from male sterile plants with cms T or cms C cytoplasm, neither of these changes in esterase patterns occurred. In anthers from plants with cms S cytoplasm, the decrease in the esterases of non-microsporogenous cells was observed but not the appearance of microspore esterases. In lines carrying cms S cytoplasm and nuclear restorer genes, esterase changes during anther development were as in normal fertile anthers. These results are discussed with respect to the phenomenon of cytoplasmic male sterility in the different maize genotypes.

Key words: Maize – Pollen anther – Esterase – Male sterility – Restorer genes

Introduction

The commercial use of hybrid seed has stimulated research into male sterility in many plants, especially maize (*Zea mays*). Male sterility traits display either cytoplasmic or nuclear inheritance. Cytoplasmic male sterility (cms) is particularly interesting because expression of the genotype is influenced by nuclear as well as cytoplasmic genetic components (Duvick 1965; Edwardson 1970).

Cytological investigation of pollen development in different cms lines of maize has shown that pollen abortion occurs at the young microspore stage in cms T (Texas) and cms C (Charrua) lines (Warmke and Lee 1977; Lee et al. 1979; Colhoun and Steer 1981) while in cms S (USDA) lines, the pollen aborts at a later stage (Lee et al. 1980). From the study of plants in which dominant nuclear restorer alleles are present in the heterozygous condition, it can be concluded that cms T and cms C anthers show sporophytic restoration (all the pollen is fertile) while cms S plants have gametophytic restoration (only pollen cells receiving the dominant restorer allele develop normally). These differences may imply that cms S has a different cytoplasmic lesion from cms T and cms C (Duvick 1965).

Comparative studies on the structure of the cytoplasmic genomes in the cms lines using gel electrophoresis of unrestricted and restricted DNAs indicate that all the lines display differences in the structure of their mitochondrial DNAs while the chloroplast DNAs are identical or very similar (Pring et al. 1977; Thompson et al. 1980; Kemble and Bedbrook 1980; Kemble et al. 1980; Lonsdale et al. 1983). These results correlate cytoplasmic male sterility with alterations in mitochondrial DNA structure.

The development of fertile pollen can be defined by a series of cytological changes that imply the existence of a genetically-controlled programme (Fig. 1). Cytological analyses of anthers from the different cms lines have enabled features of aberrant pollen development to be described (Warmke and Lee 1977; Lee et al. 1979, 1980; Colhoun and Steer 1981). However, the biochemical changes underlying pollen development in fertile and sterile anthers need to be established. One means of examining and describing a developmental pathway biochemically is to search for the appearance or disappearance of enzymes or isozymes which characterize particular developmental stages. The system of esterases seemed a potentially useful one in this regard because in maize there are a series of defined esterase isozymes, members of which are differentially regulated during development to create tissue-specific isozyme complements (Schwartz 1962, 1964; MacDonald and Brewbaker 1974).

We therefore carried out a study of anther development in fertile and sterile genotypes of maize using esterase isozymes to help provide a biochemical framework for the normal process of development. Using this framework, aberrations in anther development of the cms genotypes can be described.

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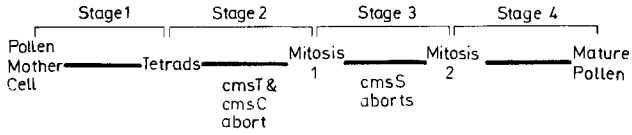

Fig. 1. The major cytological stages of pollen development. *Stages 1–4* were used to define the anthers used in esterase assays

Materials and methods

N, T, S, and C cytoplasms were examined in the nuclear background of the two inbred lines, WF9, B37 and in the hybrid line (CO192) × (WJ).

Enzyme extraction

Anthers were dissected and homogenized in 0.05 M phosphate buffer pH 7.5. The extracts were centrifuged at 12,000 × G for 10 min and the supernatants retained.

Isolectric focusing

Isolectric focusing was performed with Multiphor electrofocusing equipment (LKB) utilizing a self-regulating power supply and Ampholine PAG plates, pH 3.5–9.5 (LKB). A constant power of 1.2 W per cm width of gel was applied with cooling at 4 °C. Small filter paper pieces (Whatman 3 MM, 5 × 10 mm) were soaked with 20 µl of extract and applied to the surface of the gel 2 cm from the cathode. After 30 min, the filter pieces were removed and electrofocusing was resumed for a further 2 h after which time gels were stained for esterase activity.

Enzyme visualization and pH gradient determination

Esterase activity was visualized by incubating the gels at 37 °C in a mixture of 100 mg Fast Blue RR Salt and 50 mg α-naphthyl acetate dissolved in 5 ml acetone made up to 100 ml with 0.05 M phosphate buffer pH 7.5. Excess stain was removed by washing the gels in 7% acetic acid.

pH gradients were determined using a Pye Unicam surface pH electrode.

Results

Changes in esterase isozyme patterns during the development of fertile anthers

In order to describe the normal development of fertile anthers, in terms of the appearance and disappearance of esterase isozymes, anthers at different stages of development were collected and analyzed. The different stages were recognized cytologically by the criteria illustrated in Fig. 1. Three different nuclear genotypes were studied. These were the inbreds B37N and WF9N, and the hybrid CO192 × WJ called 1655N.

Fewer esterase isozymes were detected in B37N anthers than in normal anthers of other genotypes. In anthers containing pollen mother cells at meiosis or premeiosis (stage 1) and at tetrads to mitosis I (stage 2), three isozymes were clearly observed after isofocusing (Fig. 2 A). As anther development proceeded through mitosis I (stage 3) the pattern of esterases changed;

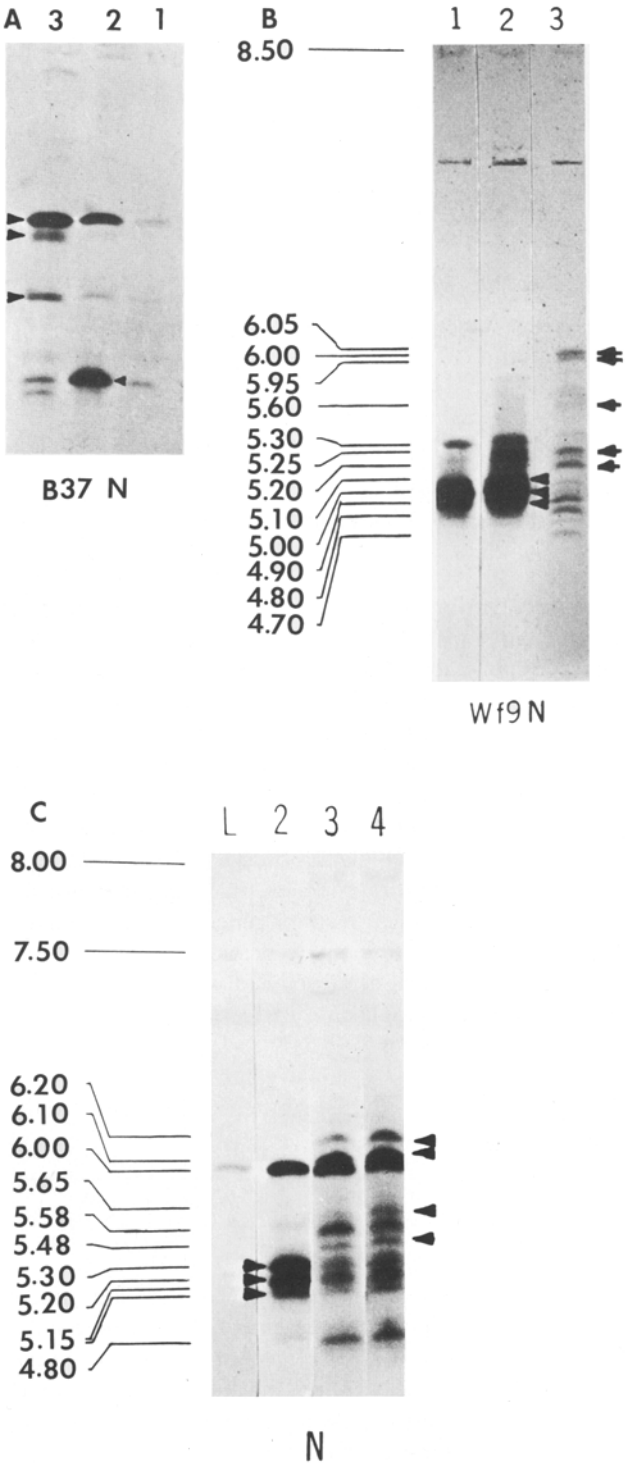

Fig. 2 A–C. Isoelectric focusing gels of N cytoplasm in various nuclear backgrounds. **A** *B37N* inbred nuclear genotype; **B** *Wf9N* inbred nuclear genotype; **C** (C0192) × (WJ) *N* hybrid nuclear genotype. *Arrows* indicate esterase activities which illustrate developmentally timed appearance or disappearance. Numbers at the top of the lanes denote developmental stage of anthers chosen for investigation (Fig. 1). Lane *L* contains mature leaf tissue extract. Where applicable, approximate isoelectric points are listed vertically

some new isozymes appeared and there were changes in the relative concentration of former stage 2 isozymes.

Although the number of esterase isozymes is greater in WF9N than in B37N anthers, a similar developmental pattern was observed (Fig. 2 B). At stage 2, anthers showed a predominance of acidic species (pI 4.9, 5.0, 5.1) and as noted for B37N, developmental progress into stage 3 is marked by the appearance of new esterases (pI 5.2, 5.25, 5.6, 6.0, 6.05).

Of the three nuclear genotypes, the hybrid genotype 1655N has the greatest number of esterase isozymes present at any particular stage of development. However, like the inbred genotypes, anther differentiation shows a distinct transition between stages 2 and 3 (Fig. 2 C). At this point in anther development, the 1655N anthers illustrate loss of some esterase activities (pI 5.15, 5.2, 5.3) and appearance of others (pI 5.48, 5.65, 6.1, 6.2).

The anther contains many cells other than the microspore, and it was therefore important to discover which isozymes were present in microspore cells. The microsporogenous tissue was squeezed out of 1655N anthers at stages 2, 3 and 4 as cleanly as possible and extracted for isoelectrofocusing. The electrophoretic patterns of the esterases from these cells (Fig. 3) suggests that the new isozymes appearing in stage 3 are localised in the microspores. Stage 2 microspores contribute very few esterases to the whole anther pattern suggesting that most stage 2 esterases are from somatic anther cells. The reduction in relative concentration of some stage 2 anther esterase during later development is consistent with the conclusion that they are not formed preferentially in the pollen. The appearance of pollen-specific isozymes by stage 3 is consistent with the conclusion that the pollen nucleus genome has become active by this time.

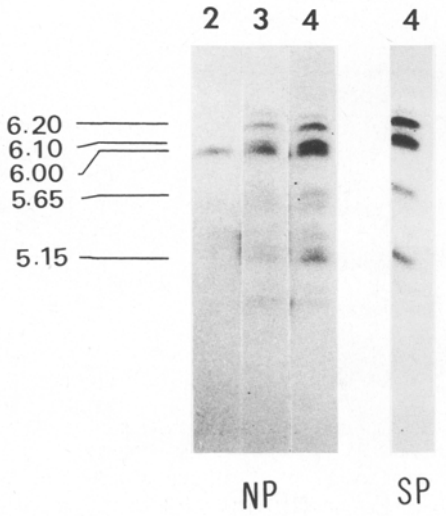

Fig. 3. Isoelectric focusing gel of (C0192)×(WJ) N (*NP*) and (C0192)×(WJ) S (*SP*) microspore extracts. Numbers at the top of the lanes denote developmental stage used for microspore extracts (Fig. 1). Approximate isoelectric points are listed vertically for prominent esterase activities

Esterase isozyme patterns during anther development in plants with male sterile cytoplasms

Having established the patterns of esterase isozymes during the development of fertile anthers, anthers from plants possessing male sterile cytoplasms were studied. Because the inbred WF9 and the CO192×WJ hybrid genotypes showed clearer stage-specific differences in esterase isozymes, the male sterile cytoplasms in these nuclear genotypes were chosen for study. Anthers from WF9 cms T and WF9 cms C had identical esterase patterns which were indistinguishable from WF9N anthers up to stage 2 (Fig. 4). WF9 cms T and cms C anthers did not show, however, the previously described esterase changes in fertile anthers associated with development progression from stage 2 to stage 3. Furthermore, the major stage 2-specific anther isozymes (pI 4.9, 5.0, 5.1) persisted into the later stages of development (near dehiscence) i.e. much longer than in the corresponding male fertile plants. In contrast, some of the isozyme changes found in fertile anthers were detected in anthers from WF9S. The major acidic isozymes decreased in relative concentration but most of the new isozymes characteristic of stage 3 fertile anthers did not appear.

Anthers from the lines in the CO192×WJ background behaved similarly (Fig. 5). None of the changes characteristic of fertile anther development were detected in CO192×WJ cms C or CO192×WJ cms T. The hybrid CO192×WJ cms S is fertile, however, due to the presence of weak restorer genes. In agreement with this, the changes in isozymes patterns were similar to those seen in CO192×WJ N. This illustrates that in the presence of nuclear restorer genes, normal isozyme development is re-established.

Discussion

The results in this paper show that during normal anther development, some esterase isozymes present in the anther before microspore formation decrease in concentration during microspore development and new isozymes appear in the microspores around pollen grain mitosis I. These esterases are specified by nuclear genes and there is variation between genotypes.

Successful completion of the complex pathway of pollen differentiation must rely on tissue interactions between somatic anther tissues and the pollen cells. These interactions are probably most important in early stages when pollen cells are not physically isolated from other anther tissues by the pollen wall. Indeed,

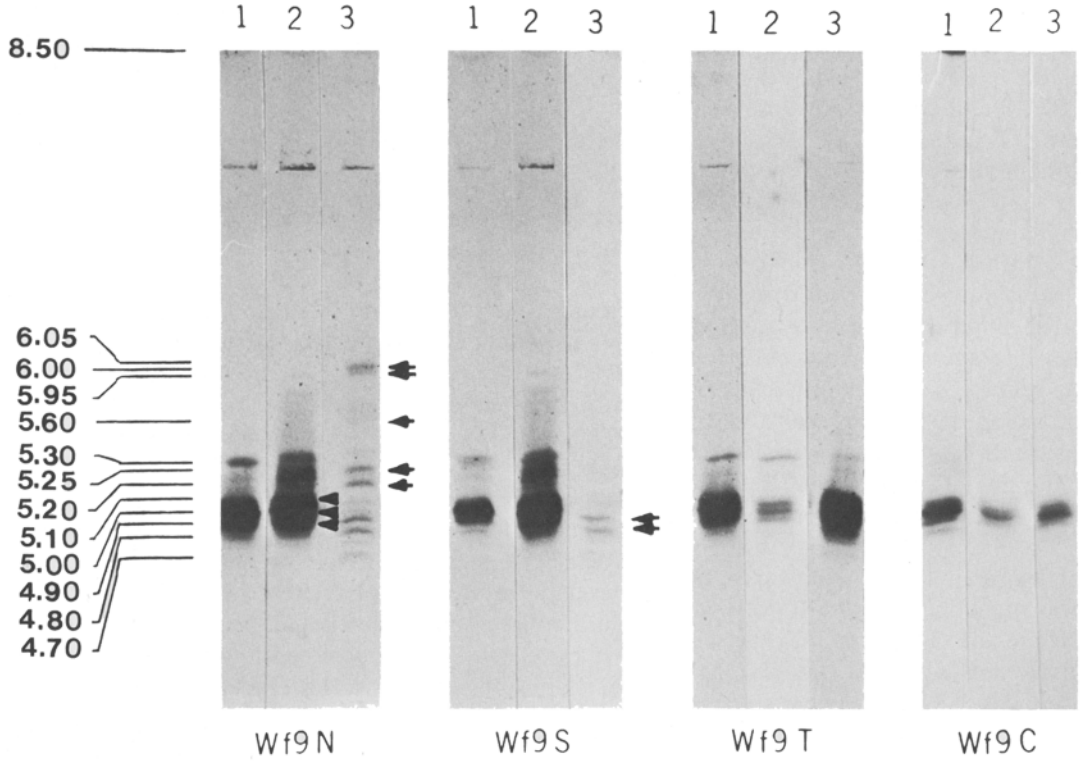

Fig. 4. Isoelectric focusing gel of esterases from *Wf9* anthers with *N, S, T, C* cytoplasms. Numbers above lanes denote anther developmental stages chosen for study (Fig. 1). *Arrows* indicate those esterase species showing developmentally timed appearance or disappearance. Approximate isoelectric points are listed vertically

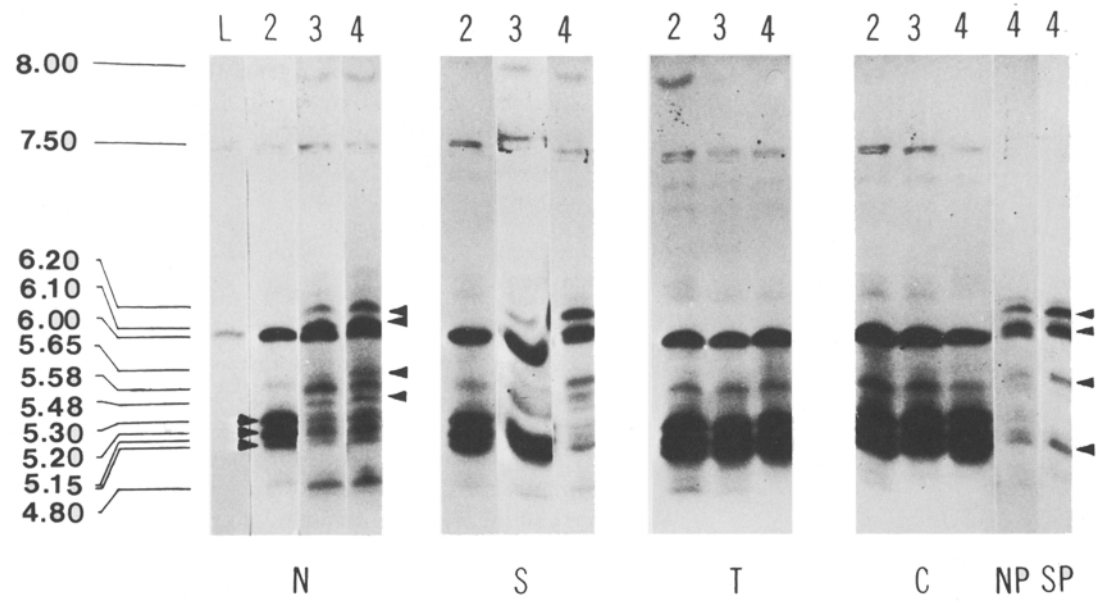

Fig. 5. Isoelectric focusing gel of esterases from (C0192)×(WJ) anthers with *N, S, T, C* cytoplasms. *NP* and *SP* lanes are from pollen extracts. *L* represents mature leaf tissue. Numbers at the top of the lanes denote developmental stage of anthers utilized for the study (Fig. 1). Approximate isoelectric points are listed vertically in composite diagram. *Arrows* indicate those esterases showing developmentally timed appearance or disappearance

the evidence for the early microspore stage as a frequent point in pollen abortion of many male sterile plant species, strongly suggests that this may be a critical stage in pollen differentiation where the penetrance of subtle genetic alterations at the nuclear and cytoplasmic levels is necessarily magnified (Laser and Lersten 1972; Albertson and Phillips 1981).

In accordance with this supposition, we found that the isozyme accumulation, noted for normal development after mitosis I, did not occur in cms T and cms C anthers. Furthermore, cms T and cms C anthers display the persistence of esterase species at stages where they should have diminished. The aberrant persistence of enzymes in non-microspore cells in male sterile anthers has also been observed in barley (Ahokas 1982) and *Petunia* (Izhar and Frankel 1971). The failure of nuclear gene products (esterases) to accumulate in the microspores of cms lines and the abnormal persistence of anther isozymes suggests that the failure of specific nucleocytoplasmic (non-esterase) interactions results in the subsequent failure of all aspects of cell activity and pollen death.

The results obtained with the cms T and cms C anthers do not define the earliest time when nucleocytoplasmic failure occurs but it is clearly before pollen grain mitosis I as also concluded from previous cytological observations by Warmke and Lee (1977); Lee et al. (1979) and Colhoun and Steer (1981). The failure could be before, during or after tetrad formation in cms T and cms C but is not manifested in general cell metabolism until later.

In contrast to cms T and cms C anthers, cms S anthers do not show abnormal persistence of non-microspore esterases although those esterases associated with the microspore failed to develop. Since male sterility in cms S plants is manifested gametophytically, it may be due to aberrant nucleocytoplasmic interaction which occurs after the microspore has become separated from the tapetum by the pollen wall. The explanation is consistent with cytological observations (Lee et al. 1980; Colhoun and Steer 1981) as well as the esterase data presented here which shows that anther development proceeds further than in cms T and cms C plants as judged by normal disappearance of developmentally specific anther esterases. However, in contrast to the normal microspore, cellular activity is conspicuously absent after mitosis I in the genotype WF9S.

S cytoplasm in the hybrid nuclear background CO192×WJ which contains weak restorer genes produces viable pollen. Esterase development was normal in anthers from these plants. This result implies that restorer gene activity overcomes all or most of the pleiotropic effects in the microspores of the male sterile anthers.

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