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The effect of the etched (et) mutation on the amylolytic enzyme activities in germinating kernels and seedlings of *Zea mays*

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Summary. The etched (*et*) mutation in maize causes distinct depressions and structural gaps in the endosperm and also gives rise to virescent seedlings. α- and β-Amylase activities were observed to be higher in et^+ et^+ kernels and seedlings as compared to that of the *et et* mutant. The total amylase and β-amylase trends during germination also differed between normal and mutant kernels and seedlings (it increases in the wildtype and decreases in *et et*). On the contrary, the overall α-amylase trend was found to be similar in both genotypes (slight decrease during germination). The native gel electrophoresis of crude enzyme extracts did not reveal any qualitative differences in α and β amylases during germination. The germinating *et et* kernels initially showed lower levels of starch compared with the wild type kernels, whereas no such difference was found at later stages of germination. It is concluded that *et* gene associated endosperm lesions lead to an impairment of starch degradation in germinating kernels resulting in virescent seedlings.

Key words: Etched – Virescence – Starch – Amylolytic enzymes

Introduction

In maize, of the many known endosperm mutants, the etched (*et*) mutant (Stadler 1940) is unique for its distinct kernel and seedling phenotypes. Kernels homozygous recessive for *et* allele (3L; 153) exhibit the so-called etched phenotype, i.e., cracks or depressions in the endosperm, whereas the seedlings express virescence during early stages of growth. (Neuffer et al. 1968; Coe and Neuffer 1977; Coe et al. 1983). The characteristic virescence phenotype of *et et* seedlings has been recorded both under field and laboratory conditions. Under certain defined conditions of light and temperature, *et et* seedlings begin their growth with leaves devoid of chlorophyll and gradually turn normal green in about 10 days after germination (Ramesh 1983). At this stage, both normal (et^+ et^+) and mutant (*et et*) seedlings are phenotypically indistinguishable. Standard in vitro procedures as well as photoacoustic spectral analysis revealed significant quantitative differences in chlorophyll and carotenoids during the greening process under light (Ramesh et al. 1984; Sangeetha 1985). It was observed that *et et* seedlings accumulate reduced levels of chlorophyll and carotenoids up to the 8th day. Transmission electron microscopy of chloroplast preparations revealed that chloroplastogenesis is delayed in mutant seedlings. Further, SDS-PAGE analysis of solubilized thylakoid membrane extracts of virescent leaves showed reduced levels of chlorophyll-protein complexes (Sangeetha 1985; Sangeetha et al. 1986).

The association of *et* mutation with both kernel as well as the seedling phenotypes is interesting. The inseparability of these phenotypes by genetic crossing-over led to the conclusion that these two traits are controlled by the *et* gene itself. Although an absolute correlation between endosperm defect and seedling virescence in *et* mutant has been unequivocally established, the underlying biochemical and physiological basis is not yet understood.

The present study attempts to look into the nature of the relationship between kernel and seedling phenotypes of the *et et* mutant. We report here on starch-degrading enzyme activities in *et et* kernels during germination as well as greening of seedlings.

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Materials and methods

The isogenic maize lines of homozygous normal (et^+ et^+) and mutant (*et et*) genotypes are in the background of line R168. Mature kernels were soaked in water for 8–10 h, surface sterilized with sodium hypochlorite and germinated in vermiculite. The seedlings were maintained at 25±2 °C under continuous illumination with cool fluorescent lights (4–5 watts/m^2).

Determination of starch content in germinating kernels and seedlings

Germinating kernels of both genotypes were ground in 75% ethanol. The suspension was heated for 30 min in a boiling water bath and centrifuged at 10,000 × g for 15 min. The starch precipitate was suspended in 0.2NKOH and boiled for 30 min and neutralized to pH 5.5 with glacial acetic acid. The suspension was incubated with amyloglucosidase (E.C. 3.2.1.3, Sigma) at 37 °C for 18 h. After centrifugation of the suspension, the supernatant was used for glucose estimation (Nelson 1944). Starch content in leaf tissue was estimated according to the procedure of Mac Rae (1971).

Estimation of total amylase, α-amylase and β-amylase in germinating kernels and leaves

The amylolytic enzyme activities were estimated in normal and mutant kernels as well as leaves. The day of soaking the seeds for germination is considered day 1 and the age of seedlings is referred to as days after germination (DAG). Kernel or leaf material was homogenized in 2 ml of chilled extraction buffer (20 mmol sodium citrate), pH 6.1. The homogenate was diluted to 7 ml with extraction buffer and then spun at 30,000 × g for 30 min. All steps were performed at 4 °C. Total amylase activity was assayed in a reaction mixture (4 ml) containing 240 μmol of sodium-citrate buffer pH 6.1, 8 mg soluble starch, 6 μmol sodium flouride and 500 μl of enzyme extract. Aliquots of 500 μl were withdrawn at 0, 30 and 60 min and the reaction was stopped by adding 500 μl of 3,5 dinitrosalicylic acid reagent (Bernfield 1955) and boiling for 5 min in a water bath. After cooling, the mixture was diluted with distilled water to a final volume of 6 ml. Reducing sugars were determined colorimetrically at 540 nm using maltose as a standard. α-Amylase was assayed under the above mentioned reaction conditions, except β-limit dextrin was the substrate instead of soluble starch.

For estimation of β-amylase, the enzyme extract was preincubated with 5 m*M* EDTA for 48 h and then assayed as described. Micromoles of maltose liberated/second was taken as the unit of enzyme activity. Enzyme activity was expressed as nkat/mg seed and nkat/gm leaf. Protein estimations were according to the procedure of Lowry et al. (1951).

Native gel electrophoresis of amylases in crude enzyme extracts of kernels and leaves was performed at 4 °C according to Davis (1964). Amylases were visualized on gels by the negative staining method of Work and Work (1972).

Results

Total α- and β-amylase activities in germinating kernels

The total amylase and specific activity profiles of mutant and normal kernels (3, 4 and 5 DAG) are shown in Fig. 1 a. The total amylase levels and specific activity of

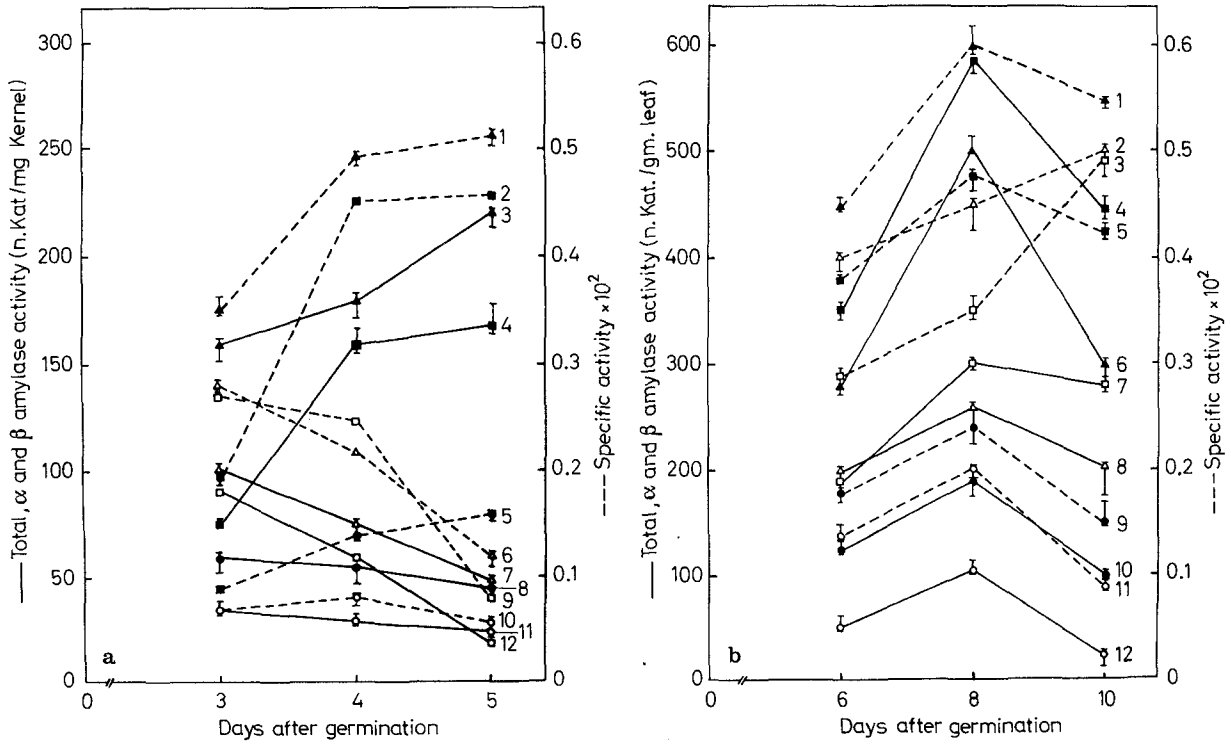

Fig. 1 a–b. Total (T), α and β amylase profiles. SA-specific activity. Total amylase: ▲=et^+ et^+ △=*et et*; α amylase: ●=et^+ et^+ ○=*et et*; β amylase: ■=et^+ et^+ □=*et et*. (**a**) Germinating kernels: *1*=T, SA; *2*=β, SA; *3*=T; *4*=β; *5*=α, SA; *6*=T, SA; *7*=T; *8*=α; *9*=β, SA; *10*=α, SA; *11*=α; *12*=β. (**b**) Developing seedlings: *1*=T, SA; *2*=T, SA; *3*=β, SA; *4*=β; *5*=β, SA; *6*=T; *7*=β; *8*=T; *9*=α, SA; *10*=α; *11*=α, SA; *12*=α. Each data point represents an average of 5–6 experiments

amylase in et^+ et^+ kernels increased between the 3rd and 5th day after soaking, while in the *et et* kernels they decreased during the same period. The total amylase content was always significantly higher in the et^+ et^+ kernels as compared with the *et et* kernels.

α-Amylase activity profiles are shown in Fig. 1 a. The α-amylase level of et^+ et^+ kernels was observed to be significantly higher than that of *et et* kernels at all three stages studied. However, both in the mutant and normal kernels α-amylase related data follow the same trends, i.e., they decreased slightly during germination.

The β-amylase activity profiles are also shown in Fig. 1 a. The β-amylase levels increased from the 3rd to the 5th day after germination in et^+ et^+ kernels, whereas its levels actually decreased in the *et et* kernels. Further, β-amylase levels were observed to be significantly higher in et^+ et^+ kernels of 4 and 5 DAG compared with that of *et et*.

Total α- and β-amylase activities in leaves

Total amylase profiles of seedlings (6, 8 and 10 DAG) are shown in Fig. 1 b. It was observed that amylase levels were significantly higher in normal (et^+ et^+) leaves than that of virescent (*et et*) leaves. Total amylase levels in leaves of both genotypes peaked at 8 DAG. Similarly, the specific activity of total amylase peaked on the 8th day in et^+ et^+ seedlings and then decreased. On the contrary, specific activity of total amylase in *et et* seedlings increased from the 6th to the 10th DAG.

The α-amylase profiles of leaves harvested from seedlings of 6, 8 and 10 DAG are shown in Fig. 1 b. The α-amylase level was significantly higher in normal leaves as compared with the virescent leaves at 6, 8 and 10 days after germination. The α-amylolytic profiles and specific activity profiles peaked on the 8th day in both et^+ et^+ and *et et* leaves.

The β-amylase profiles in seedlings (6, 8 and 10 DAG) are shown in Fig. 1 b. β-Amylase activity was significantly higher in normal leaves as compared with virescent leaves. β-Amylase activiy peaked on the 8th day in et^+ et^+ leaves, whereas it increased up to the 10th day in *et et* leaves.

Electrophoretic analysis

Electrophoretic analysis of amylases in et^+ et^+ and *et et* kernels during germination and in the seedlings during greening did not reveal any qualitative differences in the isozyme pattern (data not shown). It was concluded that the *et* mutation affects only the level but not the isozymic pattern of amylases.

Correlation between amylase levels and starch content

The mature *et et* kernels accumulated significantly lower levels of starch (42.84% fresh weight) compared with that of et^+ et^+ kernels (68.31% fresh weight). Similarly, germinating kernels of the 3 DAG stage also showed the same trend (et^+ et^+ −56.39%; *et et* −40.22%). On the contrary, there was no difference between *et et* (4 DAG −39.86%; 5 DAG −41.86%) and et^+ et^+ kernels (4 DAG −43.11%; 5 DAG −42.30%) at 4 and 5 DAG stages. Mutant kernels consistently showed the same amount of starch during germination (1−5 DAG), whereas normal kernels indicated a decrease. Leaves of both *et et* and et^+ et^+ seedlings at 6, 8 and 10 DAG exhibited similar levels of starch. Further, the starch profiles of growing seedlings were the same for both genotypes.

Discussion

The present data on starch hydrolyzing enzymes during germination of kernels suggest that the total amylase levels are significantly reduced by *et* mutation. This could be due to an impaired movement of amylases or of their reaction products caused by structural gaps in the endosperm, as revealed by Scanning Electron Microscopic analysis (data not shown). Similarly, β-amylase levels also show such a reduction during germination. In *et et* kernels, α-amylase also shows significantly reduced activity. Taken together, these data suggest that during germination of *et et* kernels, the starch-hydrolyzing enzymes are somehow affected by the structural discontinuity in the endosperm, which might in turn lead to an early virescence of seedlings. It is unlikely that the *et* gene has any direct effect on the expression of genes encoding amylases.

The amylolytic enzyme activity profiles in the growing seedlings under light did not show any significant deviations from that of the germinating kernels, indicating that the *et* effect continues up to 10 DAG. These observations lead to the suggestion that the virescence of *et et* seedlings has its origin in the endosperm lesions caused by *et* allele during kernel development. This conclusion is supported by the following observations: (1) the mutant kernels consistently show the same amount of starch during germination (1−5 DAG), whereas normal kernels show a decrease; (2) leaves of both et^+ et^+ and *et et* seedlings were found to have similar levels of starch at all three stages studied; (3) the leaves of young virescent seedlings (6−8 DAG), when grown under light, accumulate greatly reduced amounts of chlorophyll pigments as shown by in vivo and in vitro procedures (Ramesh 1983; Ramesh et al. 1984); and (4) chloroplastogenesis is delayed in virescent seedlings as shown by transmission electron microscopy and this transient delay is reversed by 10 DAG (Sangeetha et al. 1986).

In summary, we show here that there is a positive correlation between the impairment of starch degrada-

tion during germination of *et et* kernels and virescence of seedlings. However, there could be several other factors leading to virescence, besides the *et* mediated lesions in the endosperm. It has been reported that the virescent mutant of maize *v16* has a deficiency of chloroplastic 16s and 23s rRNA (Hopkins and Elfman 1984). Interestingly, the kernels of *v16* are phenotypically normal as in the case of other virescent mutants known in maize. The availability of a number of non-allelic virescent mutants simplifies the task of elucidating the causes leading to virescence. A comparative analysis of several such non-allelic virescent mutants that do not show endosperm lesions is in progress.

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