Short Communication

Initiation of Postmeiotic β-Galactosidase Synthesis during Microsporogenesis in Oilseed Rape

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ABSTRACT

The synthesis of β-galactosidase during *Brassica campestris* pollen development results from the transcription of the haploid genome. A quantitative cytochemical method has been developed in which 5-bromo-4-chloro-3-indoxyl-β-D-galactoside is used as substrate giving a blue-green final reaction product. We have recently detected oilseed rape plants which are heterozygous for the β-galactosidase locus, in which 50% of the pollen grains produced are *Gal* (having enzyme activity), while the other 50% are *gal* (enzyme deficient). The *gal* pollen grains served as a built-in control during microspectrophotometric determinations of enzyme activity. The present study has identified the developmental phase at which synthesis of the enzyme commenced. Activity is absent in microsporocytes, tetrads, and at microspore release. Enzyme activity is first detected in the young microspores and, by early vacuolate period, there is an increase in the rate of enzyme activity. A second period of increased enzyme synthesis occurred prior to generative cell division, although the rate is reduced in mature pollen.

Pollen grains, the male gametophytes of flowering plants, are products of meiotic division. Being haploid, genetic segregation and recombination during meiosis may reveal genotypic differences among the pollen grains of an individual plant. Since pollen grains are very much reduced in size and function, differences among pollen grains, as reflected by differential enzyme activities or accumulation of chemical constituents, are difficult to detect.

To date, the only examples of biochemical segregation detected at the gametophytic level are waxy *versus* nonwaxy starch (1, 3) and the presence or absence of alcohol dehydrogenase (ADH) in maize pollen (4), and segregation for β-galactosidase activity in oilseed rape pollen (9). These markers all appear to be products of postmeiotic transcription of the haploid genome. However, the exact stage of pollen development at which synthesis of gametophytic proteins is initiated is not known. In the present report, we have used a recently developed quantitative cytochemical method (10) in combination with cytological stains to study the pattern of synthesis of β-galactosidase.

Plants of oilseed rape, *Brassica campestris*, which are heterozygous for the *Gal* locus, produce pollen grains where 50% carry the mutant *gal* allele and are therefore deficient for β-galactosidase activity (9). Anthers from such plants are particularly useful when studying the initiation of postmeiotic genetic activity because the enzyme-deficient pollen grain serve as an internal control for the visual observation and quantitation of the en-zyme-specific reaction product. The presence of enzyme-deficient pollen enables ready identification of the first developmental stage where *Gal* grains show enzyme activity.

MATERIALS AND METHODS

Brassica campestris, oilseed rape, plants of *Gal/gal* genotype (9, 10) were raised to flowering under constant growth conditions with a 25°/18° C day/night temperature regime and a 14-h photoperiod.

Buds at various stages of development were removed from an inflorescence of one of these plants and the six anthers were dissected from each bud. Bud and anther lengths were recorded. For the assessment of pollen quality, two of the anthers from each bud were kept in Petri dishes lined with moist filter paper, prior to being used for the FCR[1] test (5). This same method was employed for identification of microspore and pollen developmental periods (12). The FCR method enables precise definition of the vacuolate period of microspore development. To identify the occurrence of pollen grain and generative cell mitosis, a modification of the method of Coleman and Goff (2) was employed. Two anthers were fixed in 3:1 ethanol:acetic acid for 2 h, washed in 70% ethanol, rinsed in water, and stained in DAPI, a nucleic acid fluorochrome (0.5 μg/ml) and the grains viewed by incident light fluorescence microscopy.

The remaining two anthers from each bud were employed for enzyme cytochemistry. The anthers were placed in polythene Eppendorf tubes and teased open to release the pollen grains. The tubes were then transferred to a water bath set at 35°C where 200 μl of incubation medium was added to each tube. The incubation medium was prepared by dissolving the substrate, 5-bromo-4-chloro-3-indoxyl-β-D-galactosidase in *N,N*-dimethyl-formamide (50 μl solvent/mg substrate). Fifty mM acetate buffer (pH 4.8) was added to give a final substrate concentration of 3 mM. Potassium ferrocyanide and potassium ferricyanide were then added to give a final concentration of 2 mM each. This medium also contained 5% sucrose (w/v). After 15 min incubation, the pollen was pelleted by low speed centrifugation, the reaction medium decanted off, and pollen grains washed three times with phosphate-buffered saline. The washed pellet was then resuspended in 50% glycerol and aliquots of this suspension were applied to microscope slides and sealed with cover slips.
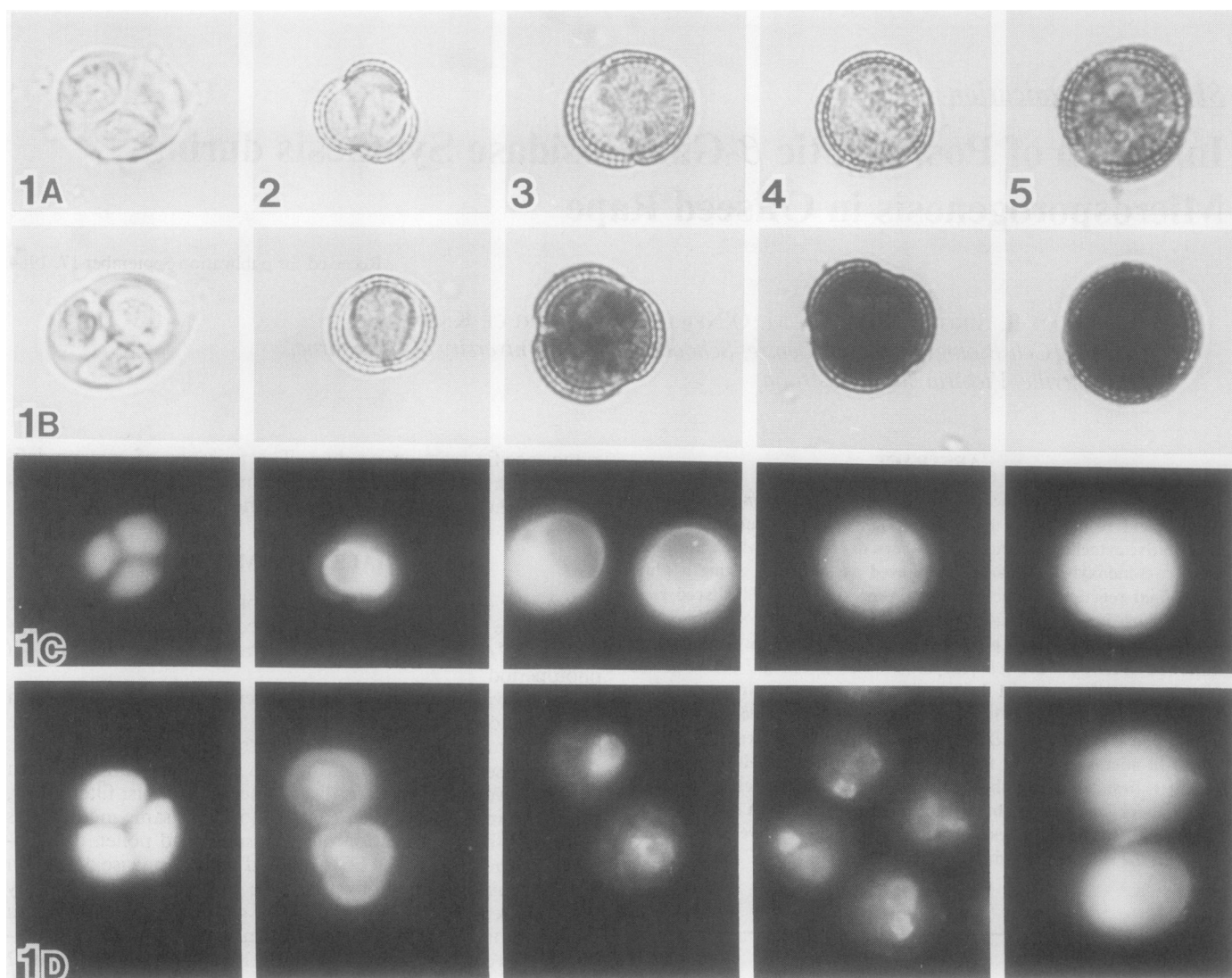
The absorbance of the reaction product deposited in each pollen grain was measured using a Zeiss Scanning Microspectrophotometer (Zeiss SMP-05) equipped with a grating monochromator using the method described by Singh and Knox (10).

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[1] Abbreviations: FCR, fluorochromatic reaction; DAPI, 4,6-diamidino-2-phenylindole.

Figs. 1–5. Assessment of stages of microspore and pollen development, and cytochemistry of β-galactosidase in *B. campestris*. A and B, microspores and pollen showing reaction product for β-galactosidase. A, *gal* genotype; B, *Gal* genotype. C, FCR test in microspores and pollen from an anther of same flower as A. D, DAPI fluorescence showing cytological state of nuclei of microspores and pollen, from anther of same flower as A. 1, period II; 2, period IV.2; 3, period IV.2; 4, period IV.3; 5, period V.1.

Measurements were made of both normal *Gal* and mutant *gal* grains. The validity of the cytochemical method for obtaining relative quantitative data was checked by using β-galactosidase (*E. coli*) coupled with CNBr-activated Sepharose 4B (Pharmacia) beads (see 10).

The absorbances of at least 20 pollen grains were recorded for each developmental stage, 10 each for *Gal* and *gal* grains at stages where the distinction could be made. The mean absorbance of *gal* pollen for each stage was subtracted from each of the 10 *Gal* readings. The means and standard errors of these adjusted *Gal* absorbances were calculated and plotted against bud lengths.

RESULTS AND DISCUSSION

Present procedures for defining the periods of microspore and pollen development are based on the FCR test (see 6, 12). By means of this method, the state of vacuolation is determined where the vacuolate period corresponds to the major growth period of the developing grain (see 7, 8). A problem which the FCR test is that the nuclear divisions within the grain, which provide useful developmental guide lines, are difficult to define.

We have employed the DNA fluorochrome DAPI to detect the occurrence of pollen and generative cell division. By taking into account the relationship between pollen development and flower bud length, we have been able to define the following developmental periods (in parentheses) for *B. campestris*: (I) microsporocytes; (II) tetrad of microspores held within a callose special wall (Fig. 1, A–D); (III) spore release or prevacuolate period; (IV) vacuolate period comprising: (IV.1) early (Fig. 2, A–D); (IV.2) mid (Fig. 3, A–D); (IV.3) late, bicellular grains (Fig. 4, A–D). (V) maturation period, comprising: (V.1) early (Fig. 5, A–D); (V.2) mid (Fig. 6, A–D); (V.3) generative cell mitosis, bi- and tricellular pollen (Fig. 7, A–D); (V.4) tricellular pollen (Fig. 8, A–D); (V.5) late, 1 d before anthesis (Fig. 9, A–D); (V.6) mature, day of anthesis (Fig. 10, A–D).

Using the quantitative cytochemical tests for β-galactosidase, the reaction product is first evident at the early vacuolate period of development (*i.e.* period IV.1; Fig. 2, A–D). In an initial experiment, using only the FCR test to monitor microspore development, enzyme activity appeared to be biphasic. There was an initial small peak, approx 16% of maximum activity, at the vacuolate period, with the maximum activity at the early
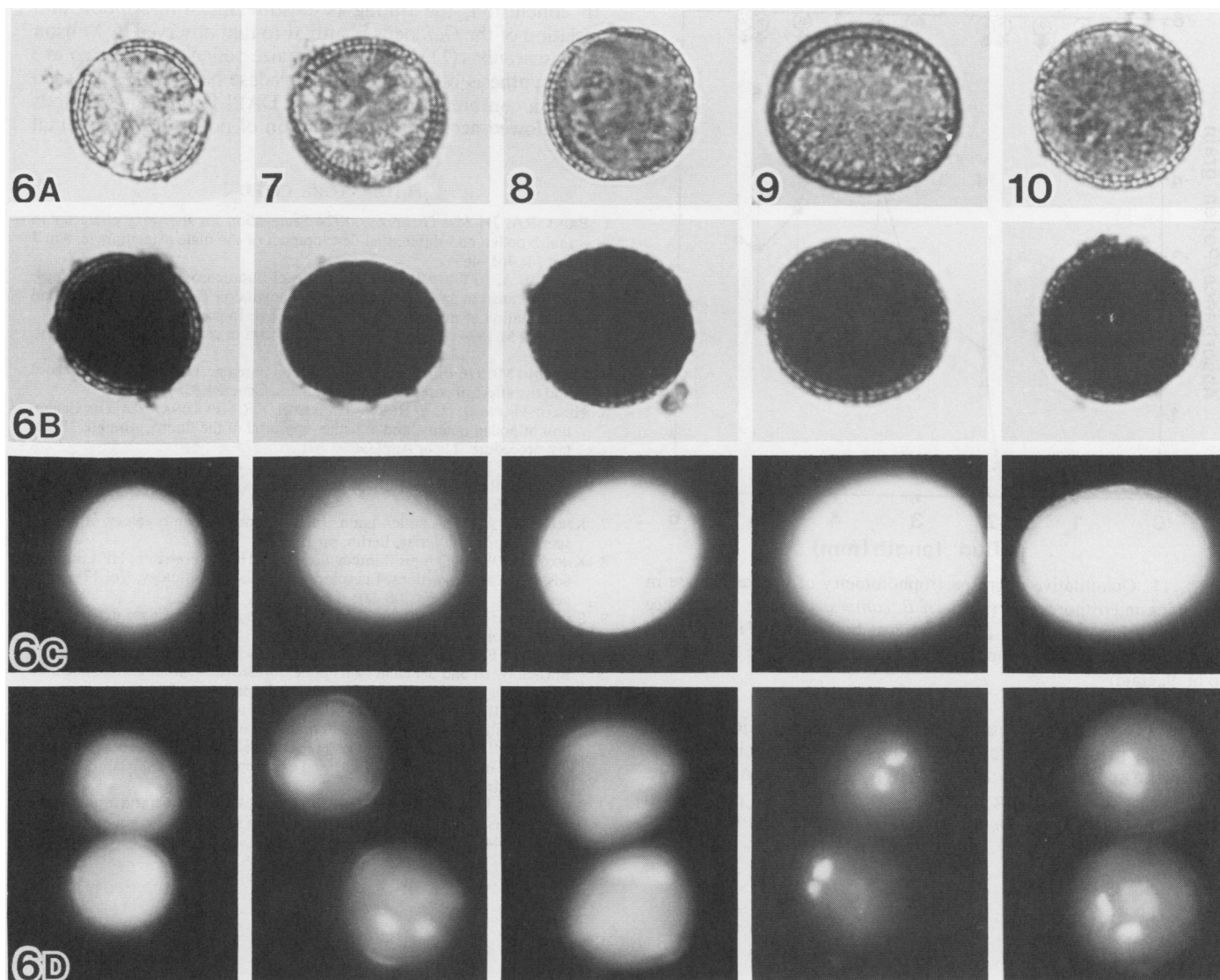
FIGS. 6-10. Assessment of stages of microspore and pollen development, and cytochemistry of β-galactosidase in *B. campestris*. A–D, same as in legend to Figures 1 to 5. 6, period V.2; 7, period V.3; 8, period V.4; 9, period V.5; 10, period V.6.

maturation period. A feature of these results is that no activity was detectable in the premeiotic microsporocytes or in microspores within the tetrad.

In a subsequent experiment, the combined FCR and DAPI method was employed to determine development periods. Quantitative estimation showed that there was no activity detectable in early microspores, but a small amount of reaction product was present at the early vacuolate period, in 2 mm buds (Fig. 11). A sharp increase in the activity (up to approximately 36% of maximum) accompanied the first mitotic division of the microspore nuclei (Figs. 3D and 4D). This increased activity continued until the second mitotic division of the generative cell into the two sperm cells (Fig. 7D) where the enzyme activity was found to peak (Fig. 11). After this period, enzyme activity dropped by about a third and leveled off towards the final maturation stages.

These results suggest that the genes for β-galactosidase are transcribed a short time after meiosis. In an accompanying report, Stinson and Mascarenhas (11) used corn plants heterozygous for alcohol dehydrogenase (*Adhl*) to study the onset of enzyme synthesis in developing pollen. A similar pattern of synthesis to that of β-galactosidase in oilseed rape pollen has

been found using quite different methodology. A difference between the Adhl system in maize pollen and the β-galactosidase system in oilseed rape pollen concerns the cellular location of the enzymes. *Adhl* is considered to be a cytoplasmic enzyme (4) whereas β-galactosidase is present both in cytoplasmic and extracellular sites, as shown by its rapid diffusion from moistened pollen (Singh *et al.*, unpublished data). In addition, preliminary cytochemical studies showed the reaction product to be present both in the peripheral cytoplasm and the intine layer of the pollen wall (10).

The pattern of activity for β-galactosidase is similar to that observed previously for acid phosphatase (12, 13) in *B. oleracea* and *Helianthus annuus* pollen. In *B. oleracea*, the first increase in activity occurred during the vacuolate period, and was interpreted as reflecting the activity of the enzyme destined to be incorporated in the intine, which is laid down at this time. A subsequent increase was considered to reflect cytoplasmic synthesis associated with the maturation of the pollen. The same interpretation may be applied to the β-galactosidase data for *B. campestris* which is an enzyme of gametophytic transcription. The leveling off of enzyme activity in buds of 2 to 3 mm (Fig. 11) may reflect the intine incorporation. The peak observed in
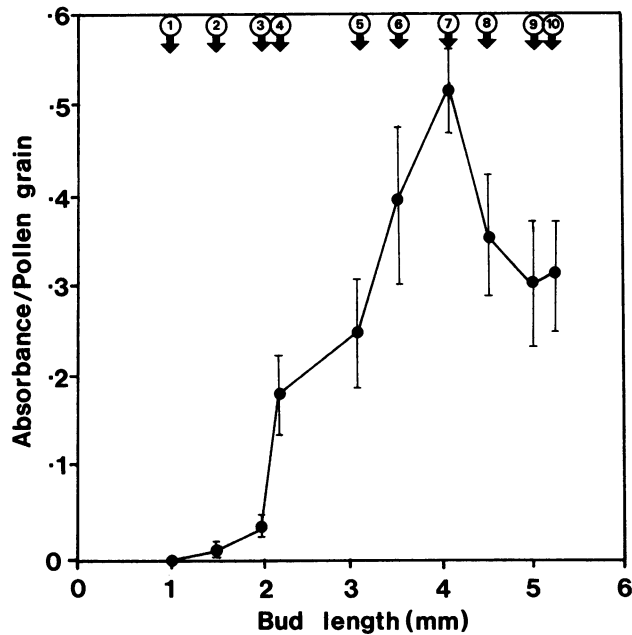
FIG. 11. Quantitative microspectrophotometry of β-galactosidase in developing microspores and pollen of *B. campestris*. Mean absorbance per pollen grain ($\pm$ 1 SE) is plotted against bud length. The stages of pollen development corresponding with Figures 1 to 10 are indicated at top of diagram.

buds of 3 to 4 mm may reflect the major cytoplasmic synthesis that occurs prior to generative cell division. A possible explanation for the apparent reduction in activity in nearly mature grains (Fig. 11), is that the enzyme may be condensed, perhaps in vesicles, in preparation for the period of dormancy commencing at anthesis.

In conclusion, the timing of postmeiotic transcription and translation of the *Gal* locus is similar to that observed by Stinson and Mascarenhas (11) for ADH in maize pollen. The time course for the synthesis of pollen β-galactosidase has been established utilizing a combination of FCR and DAPI fluorescent methods which allowed accurate determination of pollen developmental stages.

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