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Pollen gene expression analysed by micro-isoelectric focusing of proteins from isolated pollen grains in *Cucurbita pepo* L.*

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Summary. Isoelectric focusing of proteins from single pollen grains of *Cucurbita pepo* L. has been developed for large scale study of pollen grain populations' heterogeneity. Forty to forty-five protein bands from one pollen grain are revealed after silver staining. Applications of this technique to pollen grain populations from different genotypes are described in this paper. Possible applications and limits of this technique are discussed with respect to plant breeding especially for the measure of gene frequencies in pollen grain populations.

Key words: *Cucurbita pepo* L. – Isoelectric focusing – Proteins – Single pollen grain

Introduction

Extensive pollen gene expression in higher plants has been demonstrated (Mulcahy et al. 1981; Willing and Mascarenhas 1984; Zamir et al. 1982).

It seems that quite a significant fraction of the plant genome is expressed during both the male gametophytic and the sporophytic phases of plant life cycles (Willing and Mascarenhas 1984; Zamir et al. 1982). This implies that a selection pressure applied to the pollen, characterized by large populations and haploid genotypes, is likely to modify the frequency of sporophytic genes. Indeed, the incidence of pollen competition on sporophytic gene frequencies was revealed after a chilling stress during pollen function in a tomato species (Zamir et al. 1982). Mulcahy et al. (1979) have demonstrated that the electrophoretic analysis of proteins from single pollen grains of *Cucurbita* species was possible. Mulcahy et al. (1981) produced the first enzyme visualization after isoelectric focusing (IEF) of proteins from single pollen grains. We have adapted and improved this technique for a large scale comparison of isolated pollen grains from *Cucurbita pepo* L. plants (Gay et al. 1986).

This paper describes examples of pollen grain population heterogeneity for major proteins. Possible applications and limits of this technique are also discussed in terms of pollen gene expression and pollen competition.

Material and methods

Squash plants were grown in the field in summer for lines (cv. 'Black Beauty' (BB), cv. 'Ronde de Nice' (RN)) and for F1 hybrids (cv. 'Seneca', cv. 'Black Beauty' × 'Ronde de Nice' (BB × RN)). The staminate flower buds were attached in the evening in order to prevent insects' entrance. Pollen was harvested at 8 a.m. and immediately assayed for quality. Pollen samples were in all cases kept in sealed tubes at 6 °C in order to avoid dehydration or rapid ageing.

Pollen quality was assessed by means of the fluorochromatic reaction (FCR) (Heslop-Harrison and Heslop-Harrison 1970; Heslop-Harrison et al. 1984). Pollen grains were scored as "viable" when they showed an acceptable fluorescence intensity by analogy to pollen grains with high germinative capacity. These characteristics defined the FCR positivity (FCR+). Each observation was done by scoring a population of 400 pollen grains. Only, those samples which exhibited more than 98% FCR+ were retained for experiments.

For fractionation of proteins from the cell contents, pollen grains were placed on a polyacrylamide gel one by one and quickly crushed with fine forceps before isoelectric focusing. Several repetitions were done with pollen grains from separated flowers and different plants, during two successive years. Gel preparation, focusing conditions and silver nitrate staining procedures were performed according to Gay et al. (1986). For the interpretation of gels, a semi-quantitative estimation of staining intensity of the proteins bands was made visually (+: faint band, ++: well marked band, +++: strong band, 0: no detectable staining).

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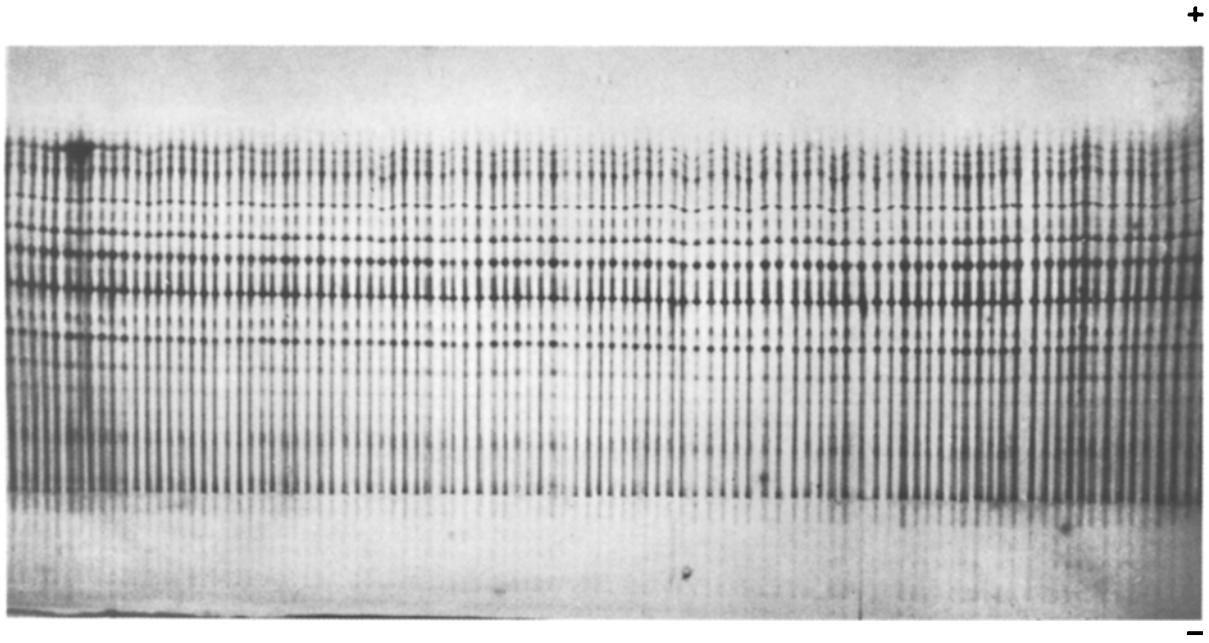

Fig. 1. Protein isoelectrofocusing patterns after silver staining of single pollen grains (cv. 'Seneca'). One hundred pollen grains may be compared on one gel

Table 1. Repetitions of the estimation of Z character frequency in pollen grain populations of the 'Seneca' F1 hybrid

Experiment	No. of pollen grains of each phenotype	
	Z+	Z−
1	50	49
2	3	3
3	15	16
4	25	26
5	3	2

Results

The first results were obtained with pollen grains from the F1 hybrid 'Seneca'. Large size populations were examined (Fig. 1).

These populations were homogeneous for major proteins. Only one case of segregation for one character was detected for major proteins (Fig. 2, arrow). This segregation occurred with a classical 1/1 Mendelian ratio in all experiments (Table 1). This extremely low level of segregation of characters had not been expected so we decided to apply the same technique to individual pollen grains from two distant and well-characterized lines (precocity, fruit shape and color, . . .) and their F1 hybrid.

Several major proteins segregated in the pollen grain population from the F1 hybrid (Fig. 3). The A, B, C and D bands showed a typical disjunction at the haploid stage, in the BB×RN plant with a 1/1 ration. The E, F, G and H characters' inheritance cannot be explained directly by Mendel's law.

Discussion

Technical discussion

Both the high resolution of IEF (Righetti 1983) and the high sensitivity of silver nitrate staining (Gay et al. 1986) permit the staining of 40 to 45 protein bands from one squash pollen grain (200 μm). An attempt was made to extend this technique to smaller pollen grains. Corn pollen (100 μm) showed 16 bands while wheat pollen (50 μm) only showed 4 bands (Gay, unpublished data). The IEF patterns are extremely reproducible between separated squash pollen grains and are not reproducible at all for smaller pollen grains. The apparent limit of this technique may be correlated with the impossibility to crush smaller pollen grains in a precise and reproducible manner with fine forceps. However, this technique could probably become feasible for pollen species such as corn if a laser microbean was used to perforate the pollen wall (Stanford 1983). In addition, the non-denaturing conditions for protein fractionation preserve their biological activities.

Genetic pollen analysis

The very low level of character segregation observed for pollen grains populations from the F1 hybrid 'Seneca' seemed to contradict the existence of an extensive gametophytic transcription and a large overlap of sporophytic and gametophytic genome expression as had been mentioned in earlier papers (Zamir et al. 1982; Willing and Mascarenhas 1984). In fact, this F1 hybrid shows a remarkable aptitude to bear fruit under greenhouse conditions in the cold season. In this

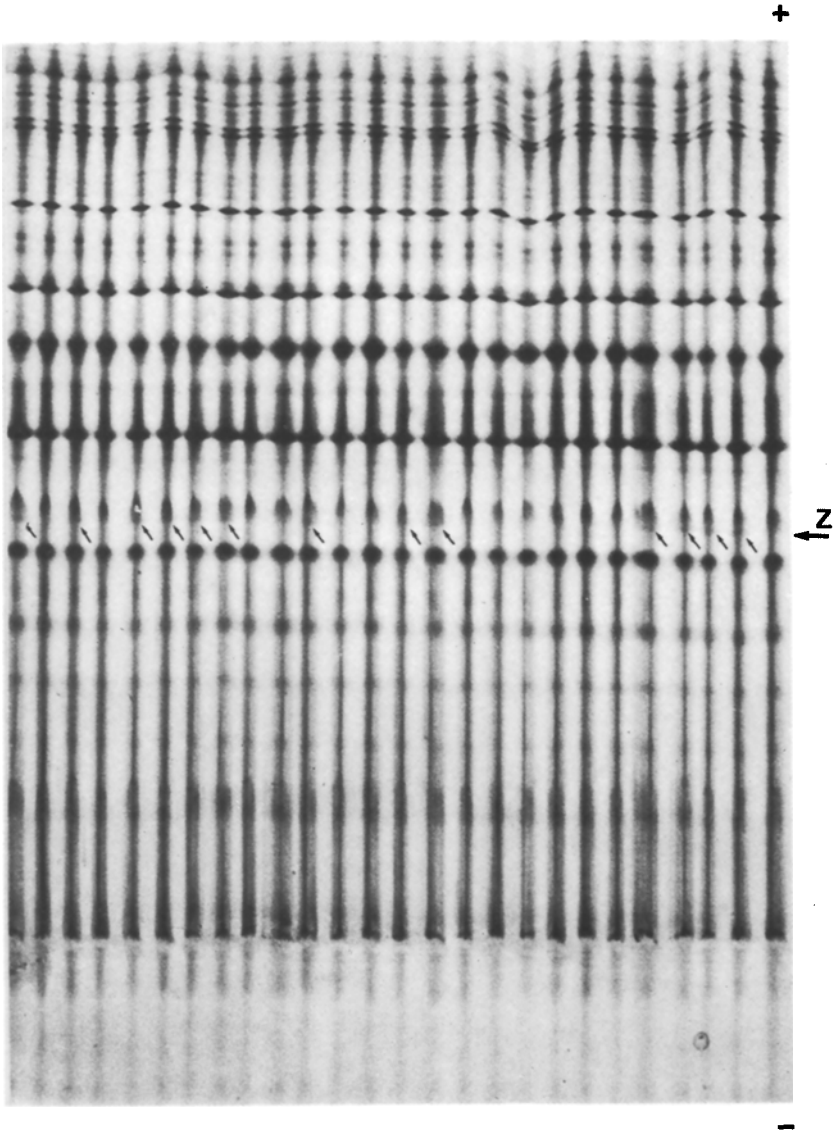

Fig. 2. Enlargement of Fig. 1. Pollen grain populations from 'Seneca' are extremely homogeneous for major proteins. Only one case of segregation was observed (Z character, *arrows*)

context, pollination success could be a critical limiting step. The selection pressure (especially low night temperatures) may have been very important for the pollen grains and may account for the great homogeneity of pollen grain populations from this F1 hybrid at the level of major proteins. Further investigations would be interesting, especially to compare several tolerant and non tolerant genotypes. On the other hand, the typical disjunction observed in pollen populations from BB×RN plant supports previous data reported by Mulcahy et al. (1981) (Table 2).

The abnormal inheritance of characters G and H can be explained by the fact that these lines are not genetically fixed for these characters (Table 2). In fact, the F1 hybrid individual chosen in this experiment did not necessarily belong to the progeny of the cross between the two individuals chosen among plants of the two lines. So, the G and H band patterns expressed the heterogeneity of the characters among the population lines. This suggests that selection for agronomical characters has no influence on the frequency of these genes. For the F character the disjunction of characters at the haploid stage with a ration 1/1 is then masked by the overlapping of a protein related to the H character at this pHi (Table 2).

In contrast to previous observations, no segregation of the E character was observed in pollen grains from the hybrid plant, and a differential expression rate of this gene was observed between BB×RN pollen grain grains (haploid organism). At least two hypothesis may be formulated: (a) the lines are not fixed for this character (b) this character is under sporophytic control (Fig. 4). The (a) hypothesis is not supported by the difference in the expression rate of this gene between BB and BB×RN pollen grains (haploid organisms). The (b) hypothesis implies that all pollen grains from a

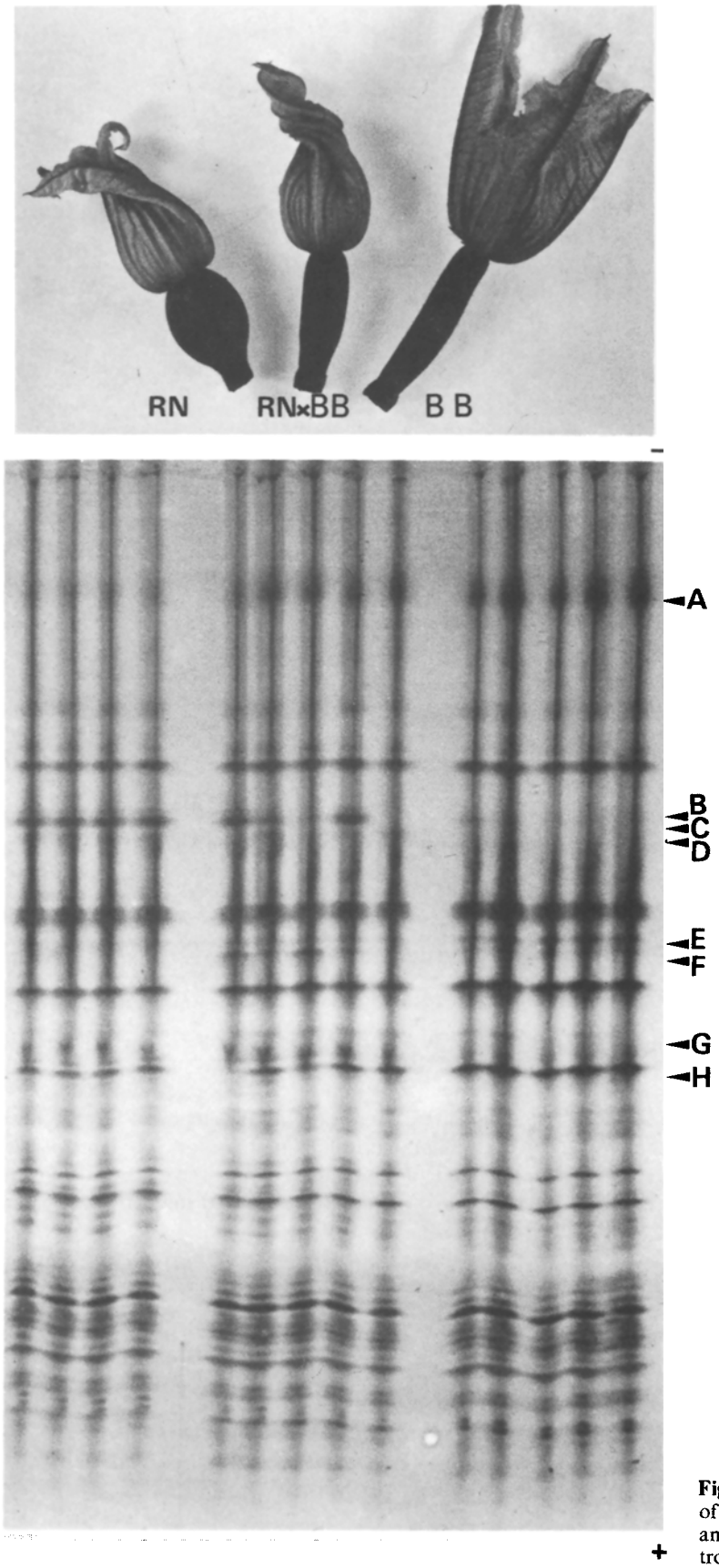

Fig. 3. Pistillate flowers and major protein patterns of isolated pollen grains populations from two lines and their F1 hybrids (silver staining after isoelectrofocusing)

Table 2. Comparison of the isoelectrofocusing patterns of the two lines and their F1 hybrid for populations of isolated pollen grains. 0 = no detectable staining, + = faint band, + + = well marked band, + + + = strong band

Character \ Genotype	BB	BB×RN	RN	Observations
A	+ + +	+ + + or +	+	Classical disjunction
B	0	0 or +	+	of characters
C	0	0 or + +	+ +	at meiosis with
D	0	0 or +	+	the 1/1 ratio
E	+ +	+	0	No segregation (see text)
F	+	+ or 0 (or + + +)	0	Segregation but interaction with H
G	0 or +	+	0 or +	Character non fixed
H	+ + +	+ + + or 0	+ + +	(see text)

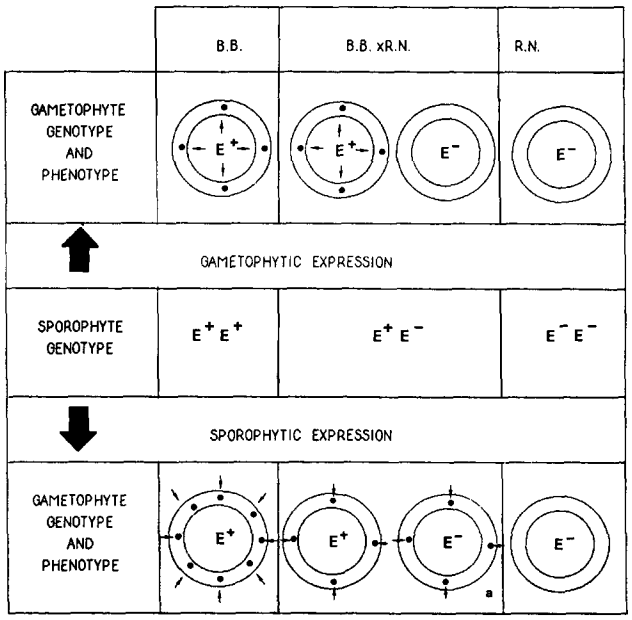

Fig. 4. Interpretation of the results of E character inheritance. The hypothesis of sporophytic expression of the *E* gene and transmission of the product of this gene to the pollen wall supports the experimental results. [a] In this case, 50% of pollen grains do not posses the E^+ allele but possess the product of the expression of E^+

plant possess the same pattern for the E character. At the sporophytic level, BB plants could possess two dominant alleles of the E gene, BB×RN plants one dominant allele only and RN plants no dominant allele at all (Fig. 4). This hypothesis is consistent with our experimental observations: differential expression of this gene at the haploid stage and no segregation in pollen from the hybrid plant. The accumulation of sporophytic proteins in the pollen wall is well-known (Heslop-Harrison et al. 1975; Knox 1984; Tsinger and Perovskaya-Baranova 1967). However, this hypothesis should be confirmed with larger pollen grain and plant populations. This special case of inheritance could be important in plant breeding strategies using pollen selection, since some pollen grains from heterozygous plants may benefit from alleles which they do not own. Pollen selection in this case would then be inefficient because of the maternal effect (Fig. 4a).

Perspectives

Intra species identification. We noticed that some protein bands, such as the A band, showed different kinetics of silver nitrate staining for each cultivar, indicating a differential rate of expression of this character in pollen grains from each cultivar. Differences were also observed in the presence or absence of characteristic bands between cultivars. This technique was used for six squash genotypes. In each case the IEF protein patterns of single pollen grains were sufficient to identify the genotypes and to discriminate hybrids from lines.

Gene linkage. Another possibility of this technique is the determination of linkage between segregating genes. The IEF gel presented in Fig. 3 indicates that A, B and C bands are related to linked genes. A, D and G bands correspond to genes carried by three different chromosomes.

Estimation of pollen selection: application to breeding. The micro IEF on single pollen grains is a highly performing tool for the estimation of gene frequencies in a pollen grain population. Under normal conditions the segregation ratio in a pollen grain population from an F1 hybrid is 1/1. However a selection pressure applied during pollen development or function is likely to induce perturbations of the segregation ratio (Zamir et al. 1982). The perturbation of the segregation ratio

indicates that the marker gene observed is linked to selection sensitive genes. In this connection, pollen grain populations from the F1 hybrid may be analysed to try to obtain correlations between marker gene frequency and agronomic characters (chilling, tolerance, disease resistance, . . .).

The presence of marker genes could then be used as a screening test for the sorting of convenient plants in breeding programs. This technique could provide a new and very rapid test applicable especially in cases where the characterization of interesting agronomic characters with classical breeding techniques seems difficult.

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