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Isozymes as molecular markers for gametophytic selection-preferential transmission in apples (*Malus domestica* Borkh.)

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In order to investigate the use of isozymes of glutamic oxalate transferase (GOT; EC 2.6.1.1), peroxidase (PRX; EC 1.11.1.7) and phosphoglycomutase (PGM; EC 5.4.2.2) as markers for gametophytic selection, cross-fertilization of two apple cultivars ('Cox' and 'Fallstaff') with 'Early Victoria' was performed at two temperatures (5 and 20°C). *GOT-2* locus could be used as a marker for gametophytic selection, while under certain circumstances, *PRX-2* locus could be used as a marker for preferential transmission. Deviations from the expected genotypes due to the rate of pollen elongation at relatively low or high temperatures could be detected by the GOT-2 isozymes profile but not by the PRX-2 profile. The observed segregation of two apple progenies (E 508: 'Fiesta' x SA572/2 and E 513: 'Fiesta' x 'Priscilla') in the zone of phosphoglycomutase (PGM-I) was deviated from the expected 1:1 ratio at 20°C. Since our results concerning PGM are in agreement with those of other authors studying the above zone, it could be assumed that, *Pgm-1* could be used as a marker for preferential transmission.

Key words: apple (*Malus domestica* Borkh.), oxalate transferase, peroxidase, phosphoglycomutase, pollen, temperature.

INTRODUCTION

There are many reports dealing with the physiology of the angiosperm gametophyte from pollen germination and tube growth to ovule fertilization (Ottaviano *et al.* 1988; Ottaviano & Mulcahy, 1989). As a result of these studies, it has been established that both pollen and ovule act as transmission vectors for the genome and as independent organisms expressing their own behavior (Hormaza & Herrero, 1992). Genetic irregularities have been described by analyzing the segregation of molecular markers (including isozymes) in various loci and various crops (Chevreau *et al.* 1985; Weeden & Lamb 1985; Torress *et al.* 1986; Wendel *et al.* 1987; Paredes & Gepts, 1995; Boskovic *et al.* 1997). The observed irregularities between selected (intra- and inter- specific) crosses had led to the theory of gametophytic selection. The gametophytic selection has been considered as one of the evolutionary mechanisms of species (Ottaviano *et al.* 1988). According to this theory, haploid male gametophytes, which were influenced by several biotic and abiotic factors, could be positively correlated with changes in the next sporophytic generation (Mascarenhas, 1990; Hormaza & Herrero, 1992). However, loci controlling male and female gametophytic selection have been localized in specific chromosomes (Devaux *et al.* 1995; Faris *et al.* 1998).

A segregation of the distortion, as a result of gametophytic selection, has been recorded in avocado (Torress *et al.* 1986), *Citrus* (Torress *et al.* 1985) and maize (Wendel *et al.* 1987). Almost half of the isoenzyme markers examined showed abnormal segregation ratio in *Lycopersicon*, *Capsicum* and lens, indicating that some loci are closely linked to genes or chromosomal segments which were exposed to strong selection pressures during gametophytic and/or post-zygotic development (Zamir & Tadmor, 1986).

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At 5°C or 20°C the apple cultivars 'Cox' and 'Falstaff' showed different stylar receptivity and rate of pollen elongation (Chevreau *et al.* 1985) and many irregularities in the transmission of various alleles have been observed. Since segregation irregularities for consecutive years were observed in apples, three isoenzymic profiles (GOT-2, PRX-2, PGM-1) were examined in the present study in specific apple progenies in order to evaluate the potential of using them as molecular markers for gametophytic selection.

MATERIALS AND METHODS

Plant material

Flowers (1-2) from all clusters at the balloon stage were selected from four healthy potted apple trees of 'Cox' (*Got-2bb*, *Prx-2bc*) and 'Falstaff' (*Got-2bb*, *Prx-2cc*) cultivars and were pollinated with pollen from 'Early Victoria' (*Got-2ab*, *Prx-2cn*). Immediately after pollination, one tree from each cultivar was placed at 20°C in a greenhouse. The rest were placed at 5°C for 72 h, and subsequently in a greenhouse at 20°C.

Sampling and enzyme extraction

Embryos of seeds from mature fruits (after removing the seed-coat and the endosperm) were electrophoretically analysed for glutamate-oxalate transferase (GOT) and peroxidase (PRX) isozymes. For the electrophoretic analysis of the phosphoglycomutase isozymes (PGM), vigorously grown leaves from the following apple progenies were used: E 508: 'Fiesta' (*Pgm-1*, *dd*) x SA572/2 (*Pgm-1*, *cd*) (Maypole x SA 18/83), E 511: 'Fiesta' x SA 18/83 (*Pgm-1*, *cd*) (O-521 x 3573 (*M. zumi* open pollinated), E 513: 'Fiesta' x 'Priscilla' (*Pgm-1*, *cd*).

The plant tissues were homogenized with a pestle in a cold mortar containing an extraction solution of 50 mg dithiothreitol, 100 mg ascorbic acid, 4.5 g insoluble polyvinyl polypyrollidone and 8 g sucrose in 0.05 M sodium phosphate buffer. pH was 7.2 and total volume was adjusted to 100 ml. Just before use, 75 μl mercaptoethanol were added.

Electrophoretic procedure

Sample preparation and electrophoretic procedure were conducted according to Boscovic *et al.* (1997). Electrophoresis was performed on 9% polyacrylamide gels using the Tris-glycine buffer system (pH 8.3). Electrophoresis was run for at least 1/2 hour pre-electrophoresis at 100 V, loaded then with samples and run for an hour at 100 V and about five hours at 350 V. Quantification of protein concentration of the samples was done according to Lowry *et al.* (1951) and equivalent protein quantities were loaded onto each gel lane.

Activity, staining and incubation of gels

After electrophoresis, gels were stained for PRX activity according to Vinterhalter & James (1983) and for PGM activity according to Quick *et al.* (1974). For GOT activity, gels were stained according to the technique of Siciliano & Shaw (1976), slightly modified. A solution of 75 mg L-aspartic acid and 50 mg a-ketoglutaric acid was freshly prepared and stirred for at least 20 min in a flask (wrapped in aluminum foil) containing 50 ml of 0.2 M Tris-HCl (pH 7.5). Briefly, 200 mg of fast blue BB and 5 mg of pyridoxal-5-phosphate were added and stirred for 30 sec. Gels were incubated at 30 oC for about 30 min. They were then washed and fixed in a solution containing 7% acetic acid.

RESULTS AND DISCUSSION

The electrophoretic separation of GOT revealed the existence of four zones of activity assigned as GOT-I, GOT-II, GOT-III and GOT-IV (Fig. 1). A single band was observed in homozygote plants while in heterozygote plants three bands with an intermediate 'hybrid' band were observed. The results of the *Got-2* screening for both progenies are presented in Table 1.

The *Got-2* patterns of the progeny 'Falstaff' x 'Early Victoria' (Table 1) showed that the *ab* genotypes were predominant when fertilization occurred at 5°C. However, when the fertilization occurred at 20°C, the *bb* genotype was predominant. The genotype *bb* was also predominant in 'Cox' x 'Early Victoria' cross at 20°C, whereas at 5°C the ratio between the two genotypes *ab* and *bb* was 1:1.

As concerns PRX, embryos were expressed only for PRX-II and PRX-III (data not shown). However, the lack of secondary bands in the banding patterns of PRX (obtained from the seeds) facilitated the distinction of the various genotypes. The distinction of *cn* from *cc* genotypes was based on the intensity and thickness of the bands in the appropriate positions. All combinations gave distorted isoenzymic ratios at both temperatures, except for the segregation pattern of *Prx-2* of the cross 'Falstaff' x 'Ear-

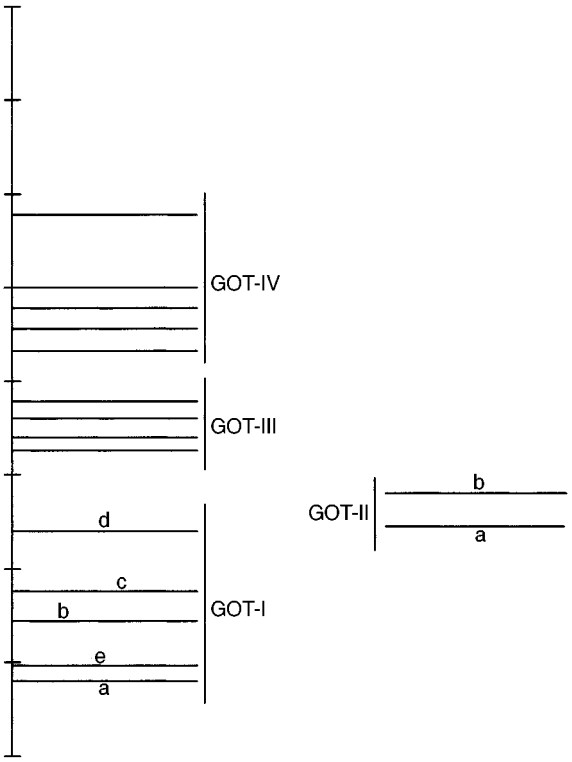

FIG. 1. Diagram showing the bands of the four GOT active zones detailing I and II. Zero value is at the top of the graph. The unit of the scale is 0.05.

ly Victoria' (Table 1). However, the cross 'Cox' x 'Early Victoria' expressed the *Prx-2* genotype *bn*, which was predominant at both temperatures.

All expected genotypes were represented according to the genotypes of the parents (Manganaris & Alston, 1987). The seed screening technique employed in this work proved to be a valuable method for the isoenzyme profile determination, as well as the accomplishment of genetic studies in the zones of PRX-II and PRX-III and in all of the electrophoretic zones of GOT. Therefore, for genetic studies concerning the isozymes of PRX and GOT, it is not necessary to grow seedlings from the appropriate crosses (method less costly). Furthermore, the polymorphism of GOT observed in this work was consistent with that described by other authors (Manganaris & Alston, 1987).

When fertilization of the ovule occurred at 20°C, *Got-2 bb* seeds represented a much greater number than *ab* ones. When fertilization between 'Cox' and 'Early Victoria' occurred at 5°C, the ratio of the two observed genotypes was 1*ab*:1*bb*, while the observed ratio for 'Falstaff' x 'Early Victoria' was 2*ab*:1*bb*. It could be assumed that at 5°C, *a* pollen had a higher probability to fertilize *b* ovules compared to *b* pollen. It also appeared that at 20°C, *a* pollen had lost this capacity and *b* pollen had greater chances to fertilize more *b* ovules. Factors related to pollen mobility, pollen tube formation and pollen-pistil interaction could be influenced by temperature. Fertilization of 'Cox' and 'Falstaff' in different temperatures, with pollen originated from other cultivars with *Got-2ab* genotype, could further clarify to what extent the phenomenon of pollen capacity to fertilize an ovule is influenced by temperature. It could thus be assumed that the transmission of *Got-2* was influenced by temperature.

A decisive factor responsible for the deviation affecting the *Got-2* locus might be temperature. This locus could be linked to a *Pl* gene, which is resistant to powdery mildew (Brown, 1975). Manganaris & Alston (1987) have found a linkage between *Got-2* and *Lap-2*, and Batlle *et al*. (1995) confirmed the linkage between *Lap-2* and the gene *Plw* for powdery mildew

TABLE 1. Segregation of two apple progenies for Got-2 and Prx-2 after pollen germination and fertilization in two different temperatures

			Got-2		
Progeny		Expected ratio	Observed ratio	χ^2	P
Cox (bb) x Early Victoria (ab)	5°C	1bb:1ab	21ab: 26bb	0.574	0.5-0.25
Cox (bb) x Early Victoria (ab)	20°C	1bb:1ab	9ab:23bb	6.1	0.025-0.01
Falstaff (bb) x Early Victoria (ab)	5°C	1bb:1ab	31ab:15bb	5.56	0.025-0.01
Falstaff (bb) x Early Victoria (ab)	20°C	1bb:1ab	15ab:29bb	4.63	0.05-0.025
			Prx-2		
Progeny		Expected ratio	Observed ratio	χ^2	P
Cox (bc) x Early Victoria (cn)	5°C	1bc:1cn:1cc:1bn	3bc:10cn:11cc:23bn	17.25	<0.005
Cox (bc) x Early Victoria (cn)	20°C	1bc:1cn:1cc:1bn	1bc:5cn:5cc:21bn	29.5	<0.005
Falstaff (cc) x Early Victoria (cn)	5°C	1cn:1cc	24cn:22cc	0.087	0.995-0.99
Falstaff (cc) x Early Victoria (cn)	20°C	1cn:1cc	21cn:23cc	0.099	0.995-0.99

resistance derived from 'White Angel'. All these suggest a potential for manipulating temperature at pollination to increase the proportion of resistant plants. It is also possible to obtain different proportions of resistant seedlings (i.e. in a crossing program where 'White Angel' is used as the resistant parent to powdery mildew), from year to year, according to the ambient temperature during pollination.

As concerns *Prx-2* locus, in the cross 'Cox' x 'Early Victoria, the Prx-2*bn* genotype was predominant at both temperatures, compared to the other three genotypes. *Prx-2n* pollen showed higher frequency in fertilizing ovules of 'Cox' and furthermore *Prx*-2*b* ovules appeared to be more preferable than *Prx*-2*c* ovules. Thus, it seemed that *Prx-2n* pollen was more active than *Prx-2c* pollen of 'Early Victoria'. It was also observed that the *bc* genotype was the least frequent. Both pollen alleles showed the same mobility on the pistil of 'Falstaff'. Factors related to the recognition system on the pistil or in the ovary of the recipient cultivar may be involved in this case. Thus, gametophytic selection depends on an interaction between pollen tube and style and/or the ovary of the recipient cultivar. A crossing program with pollen donors of appropriate genotypes might clarify this point.

Two *Pgm-1* alleles (*c* and *d*) were detected corresponding to the two relative slow migrating bands. In families E508 and E513, the observed ratio deviated significantly from the expected one (1*cd*:1*dd*), while in family E511, the observed ratio was not identical to the expected one (Table 2).

The electrophoretic separation, revealed four zones of activity for PGM (Fig. 2), the same as described by Manganaris & Alston (1987). The *Pgm-1dd* genotype was dominant in progenies E508 και E513 (Table 2). Weeden and Lamb (1985) found skewed *Pgm-1* ratios in apples. The potential of using *Pgm-1* as a marker for preferential transmission in apples should be further examined. The deficiency of *Pgm-1* genotypes that have been observed could be explained by the theory of preferential transmission. It is well established by Manganaris & Alston (1987) that *Pgm-1* is linked to *Vf* (resistance to apple scab). It would be reasonable to identify whether *Pgm-1* is linked to *Prx-2* and/or *Got-2* and to perform crosses in high and low temperatures to test the proportion of seedling resistant to scab.

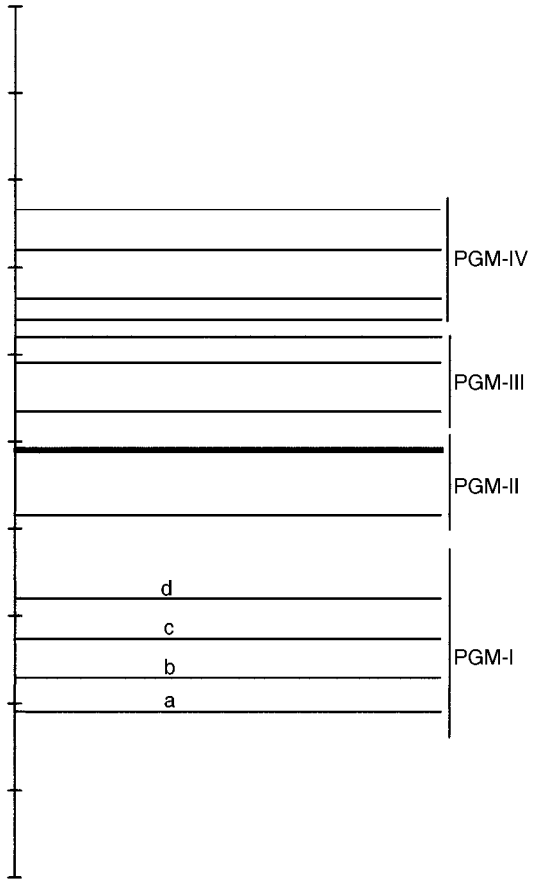

FIG. 2. Diagram showing the bands of the four PGM active zones detailing zone I. Zero value is at the top of the graph. The unit of the scale is 0.05.

According to the results of this work, it could be assumed that genetic differences among pollen may lead to gametophytic competition and selection that

TABLE 2. Segregation of Pgm-1 in three apple progenies

Progeny	Parental genotype	Expected ratio	Observed ratio	x^2	P
E508	cd x dd	1cd:1dd	35cd:70dd	11.6	0.01-0.005
E511	cd x dd	1cd:1dd	46cd:51dd	0.17	0.75-0.5
E513	cd x dd	1cd:1dd	15cd:33dd	7	0.02-0.01

E 508: 'Fiesta' (Pgm-1, dd) x SA572/2 (Pgm-1, cd) (Maypole x SA 18/83)
E 511: 'Fiesta' x SA 18/83 (Pgm-1, cd) (O-521 x 3573 (M. zumi open pollinated)
E 513: 'Fiesta' x 'Priscilla' (Pgm-1, cd)

result in non-random fertilization. Consequently, the genes *Got-2*, *Prx-2*, and *Pgm-1* could be circumstantially used as markers for gametophytic selection-preferential transmission in apples, as in few cases they showed distorted ratios while in others, not.

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