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Evaluation of Isozyme Systems in *Citrus* to Facilitate Identification of Fusion Products*

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Summary. Ten isozymes were analyzed in nucellar calli of nine *Citrus* species and cultivars and roots of the corresponding apomictic seedlings. The zymograms obtained can be divided into three groups: a) isozyme patterns similar in both calli and roots, b) isozyme patterns similar in calli but variable in roots, and c) isozyme patterns variable in both calli and roots. Analysis of these ten isozyme systems may facilitate identification of fusion products in *Citrus*.

Key words: Enzyme polymorphism – Protoplasts – Callus – Roots – Apomixis

Introduction

Isozymic analysis has proved useful to identify differences in gene expression in various organs of the same plant or to differentiate between closely related cultivars. Gel electrophoretic separation methods have been widely used both in taxonomic and genetic studies of various crops (Shannon 1968; Pierce and Brewbaker 1973).

A number of closely related clones and all hybrids tested have been distinguished by this method in potato (Desborough and Peloquin 1967, 1968), pineapple (Brewbaker 1971), carnation (McCown et al. 1969), beans (Matthews and Williams 1973), *Nicotiana* species (Lo Schiavo et al. 1980), and *Citrus* (Iglesias et al. 1974; Button et al. 1976; Esen and Soost 1976; Spiegel-Roy et al. 1977; Torres et al. 1978 b). We are routinely analyzing banding patterns of peroxidase isozymes from roots in order to distinguish between nucellar and zygotic seedlings in our *Citrus* breeding program (Spiegel-Roy et al. 1977). Phosphoglucoisomerase (PGI) isozyme pattern was also analyzed for the same purpose and proved to be useful (unpublished results). Most studies were performed on enzymes extracted from one organ: roots (Button et al. 1976), leaves (Torres et al. 1978 b), fruit (Wolfe 1976; Torres et al. 1978 a), or pollen (Loukas and Pontikis 1979). However, various organs of the same plant may exhibit different isozyme patterns (Scandalios 1974). Recently it was shown that differences in isozyme pattern also exist between cell culture and leaves of the same plant (Lo Schiavo et al. 1980). It was also demonstrated that the isozyme patterns of plant organs and callus cultures maintained under different growth conditions may vary (McCown et al. 1970). Therefore, consistency should be sought both in the plant stage analyzed and the environment for growth. The relevance of studies of isozyme patterns in callus cultures with respect to growing cells of intact plants was also considered, with the rather discouraging conclusion that cell suspensions are virtually unrelated to the growing cells of intact plants (Zeleneva and Khavkin 1980). However, there are advantages in using isozyme patterns as genetic markers in callus or cell cultures. As fusion experiments are now successfully performed on plant cells, isozyme patterns of both parents may provide a powerful tool to identify somatic hybrids at the earliest possible stage (Lo Schiavo et al. 1980; Lönnendonker and Schieder 1980).

In this paper we report on the analysis of the electrophoretic mobilities of ten different enzymes from nine species and cultivars of *Citrus*. Different isozyme patterns should give us the possibility for selecting the right enzyme, or enzymes, for a given pair of parents in order to confirm the identity of their somatic hybrids. This procedure, employed together with other selective markers, such as selection media (Vardi, Spiegel-Roy and Galun, unpublished results), should increase the efficiency of breeding new combination of *Citrus* via tissue culture techniques.

Materials and Methods

Plant Material and Extraction Procedure

Callus cultures of various *Citrus* species and cultivars (Vardi et al. 1982) were subcultured monthly. In addition, Troyer (*Citrus sinensis* × *Poncirus trifoliata*) callus was obtained from epicotyl explants, as follows: 'Troyer' seeds were peeled and sterilized in 1% sodium hypochlorite for 10 min and then washed three times in sterile water. Embryos were excised under aseptic conditions and placed in petri dishes containing Murashige and Tucker's (1969) medium (M-T), without the addition of plant growth substances. When seedlings reached 1–2 cm in size, the region between the cotyledons and the first

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Table 1. Methods employed in gel electrophoresis of *Citrus* enzymes

Enzyme	Buffer system			Staining solution
	Separation gel	Spacer gel	Electrode	
Phosphoglucoisomerase (PGI)	Tris-HCl, pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.034 M, pH 8.7	Torres et al. 1978 b
Lactate dehydrogenase (LDH)	Tris-HCl, pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.034 M, pH 8.7	[a]
Alcohol dehydrogenase (ADH)	Tris-HCl, pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.034 M, pH 8.7	[a]
Glutamate dehydrogenase (GDH)	Tris-HCl. pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.034 M, pH 8.7	[a]
Glutamate-oxaloacetate transaminase (GOT)	Tris-HCl, pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.034 M, pH 8.7	Brown et al. 1978
Peroxidase	Tris-HCl, pH 7.5	Tris-phosphate, pH 6.8	Sodium borate 0.3 M, pH 8.3	Siegel and Galston 1967
Leucine aminopeptidase (LAP)	Tris-HCl, pH 8.9		Tris-glycine (5 mM Tris and 40 mM glycine), pH 8.3	Torres et al. 1978 a
Malate dehydrogenase (MDH, NADP) or (MDH, NAD)	Tris,HCl, pH 7.5	Tris-phosphate, pH 5.5	Diethylbarbituric acid-Tris (30 mM diethyl-barbituric acid and 8 mM Tris), pH 7.0	[a] [a]
Esterase	Tris-citrate, pH 8.5	Tris-phosphate pH 6.8	Lithium borate, 30 mM, pH 8.2	Scandalios and Sorenson 1977

[a] See text

leaves was sliced into pieces 3–4 mm long and further longitudinal cuts were performed to stimulate callus formation. The epicotyl pieces were then placed on M-T with the addition of 8.9 μM benzyladenine and 60 μM 3-indolacetyl-L alanine.

Calli of lemon species, namely, 'Villafranca' (*C. limon* L.) and 'Volckameriana' (*C. volckameriana*), were obtained from Ms. S. Saad and the late Dr. J. Kochba of this laboratory. In a small mortar held at 0 °C, 1 g fresh weight of callus cells was ground with 1.5 ml of 0.8% NaCl, 0.2% $NaNO_3$ and 20% sucrose. The extract was then centrifuged for 20 min at 1,200 × *g*. Aliquots from the supernatant (15–25 μl) were layered on top of the gel after the addition of bromphenol blue dye. Roots were first washed with water and dried on blotting paper. Enzymes were extracted by grinding 0.2 g roots in 0.5 ml of the solution for callus cells. The extract was then centrifuged for 15 min at about 500 × *g*. Aliquots of the supernatant were treated as above.

Electrophoresis

Polyacrylamide slab gel (anodic) electrophoresis was used. A gel concentration of 7% with various buffer systems was run at a constant voltage of 150–160 volt (about 15–20 mA) at 4 °C (Maurer 1971). All gel separations were run for 3–5 h, until the tracking dye – bromphenol blue – migrated to a distance of 50–70 mm from the origin of the separation gel. The buffer systems were adopted from Maurer (1971) and are summarized in Table 1.

Staining Solutions

Slab gels were twice washed for 20 min at 4 °C with the buffer of the staining solution prior to staining. Staining took place at room temperature in the dark until clear-colored bands appeared (1–6 h). Staining was arrested by 2% acetic acid. As some of the dyes faded quite fast, zymograms were drawn to scale without delay.

The staining solutions for the dehydrogenases, mostly adopted from the literature (Brown et al. 1978), were as follows:

Tris-HCl 0.1 M pH 8.0, 0.32 mM NAD (or NADP), 5 mM $MgCl_2$, 1 mM NaN_3, 0.17 mM phenazine methosulfate (PMS), 0.2 mg ml^{-1} 3-[4.5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) were used in all staining solutions. In addition, MDH, GDH, LDH and ADH contained 0.1 M malate pH 7.0, 0.1 mg glutamate pH 7.0, 0.18 M lactate pH 7.0 and 8% ethanol, respectively.

Results

Nucellar calli of nine *Citrus* species and cultivars established in our laboratory, and roots of the corresponding apomictic seedlings of each cultivar mentioned, were analyzed for ten isozyme patterns. The list includes several dehydrogenases (malate, glutamate, lactate and alcohol), phosphoglucoisomerase, glutamate-oxaloacetate transaminase, leucine aminopeptidase, peroxidases and esterase. Data are presented in Figs. 1–3. The isozyme patterns identified can be divided into three groups.

a) Isozyme Patterns Similar in Both Calli and Roots

This group consists of the enzymes LDH, ADH, PGI, LAP and MDH (NAD). The isozyme patterns of LDH were almost identical in all tested calli and absolutely identical in the roots. Both in calli and roots there was only one distinct band migrating close to the tracking dye. The isozyme patterns of ADH consisted of three bands which were absolutely identical in all tested calli but no enzyme activity was present in any of the roots. The isozyme patterns of LAP were very similar within both the calli and the roots and the differences between the patterns of the calli and the roots were minor (the

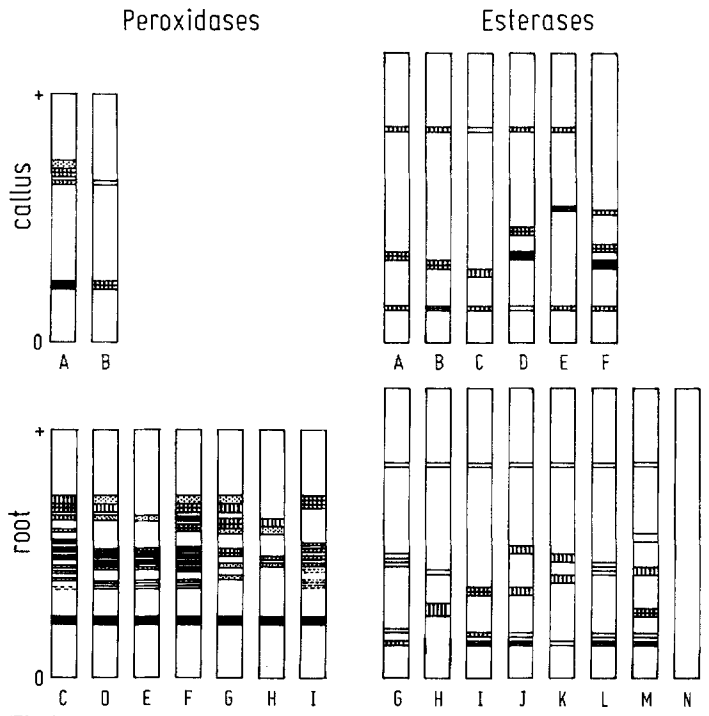

Fig 1.

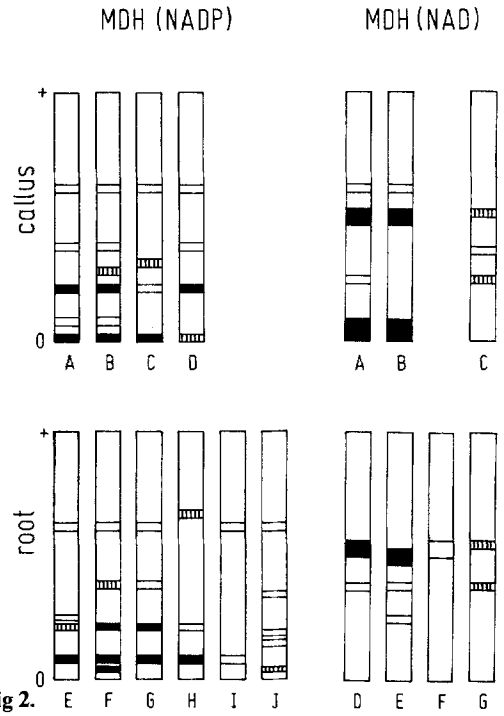

Fig 2.

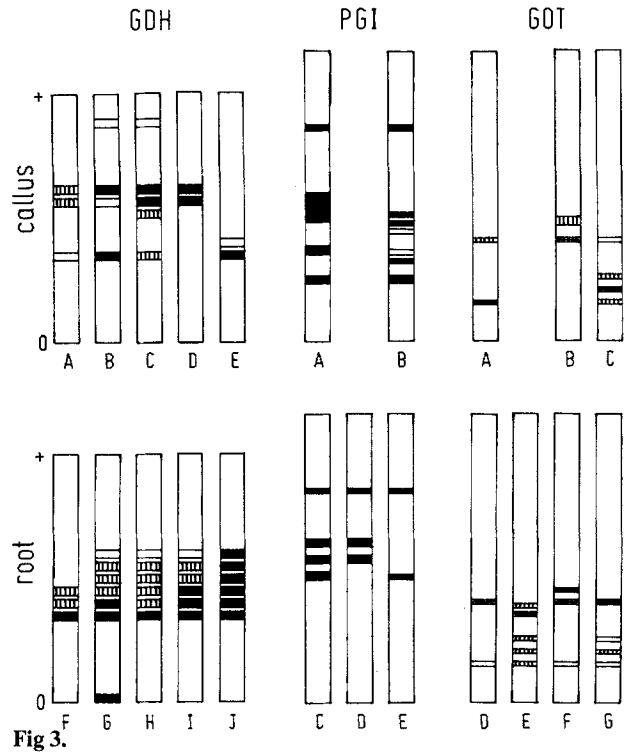

Fig 3.

Fig. 1. Peroxidase and esterase isozyme patterns in *Citrus* callus and roots. The schemes represent the patterns of the following cultivars: 1) Peroxidase: *A* 'Dancy', 'Duncan', 'Murcott', 'Ponkan', 'Nucellar Shamouti', 'Sour orange', 'Villafranca'; *B* 'Volckameriana'; *C–I* Patterns in roots; *C* 'Murcott', 'Nucellar Shamouti'; *D* 'Dancy'; *E* 'Ponkan'; *F* 'Sour orange'; *G* 'Villafranca'; *H* 'Volckameriana'; *I* 'Troyer'. 2) Esterase: *A–F* Patterns in callus; *A* 'Nucellar Shamouti', 'Sour orange', 'Dancy'; *B* 'Villafranca'; *C* 'Duncan'; *D* 'Murcott'; *E* 'Ponkan'; *F* 'Troyer'; *G–N* Patterns in roots; *G* 'Nucellar Shamouti'; *H* 'Sour orange'; *I* 'Dancy'; *J* 'Villafranca'; *K* 'Duncan'; *L* 'Murcott', *M* 'Ponkan'; *N* 'Troyer'. (Horizontal broken line = very weak staining; Dotted box = weak staining; Vertical line box = medium staining; Square box = fairly dark staining; Solid dark box = dark staining)

Fig. 2. NADP- and NAD-specific malate dehydrogenase (MDH) isozyme patterns in *Citrus* callus and roots. The schemes represent the patterns of the following cultivars: 1) MDH (NADP): *A–D* Patterns in callus; *A* 'Nucellar Shamouti'; *B* 'Sour orange', 'Troyer'; *C* 'Murcott'; *D* 'Dancy', 'Ponkan', 'Duncan', 'Villafranca', 'Volckameriana'; *E–J* Patterns in roots; *E* 'Nucellar Shamouti'; *F* 'Sour orange', 'Dancy'; *G* 'Murcott', 'Ponkan', 'Duncan'; *H* 'Villafranca'; *I* 'Volckameriana'; *J* 'Troyer'. 2) MDH (NAD): *A–C* Patterns in callus; *A* 'Nucellar Shamouti', 'Sour orange', 'Murcott', 'Villafranca', 'Duncan'; *B* 'Dancy', 'Ponkan', 'Volckameriana'; *C* 'Troyer'; *D–G* Patterns in roots; *D* 'Nucellar Shamouti', 'Murcott', 'Dancy', 'Ponkan', 'Duncan', 'Villafranca'; *E* 'Sour orange'; *F* 'Volckameriana'; *G* 'Troyer'

Fig. 3. Glutamate dehydrogenase (GDH), Phosphoglucoisomerase (PGI) and glutamate-oxaloacetate – transaminase (GOT) isozyme patterns in *Citrus* callus and roots. The schemes represent the patterns of the following cultivars: 1) GDH: *A–E* patterns in callus; *A* 'Nucellar Shamouti', 'Sour orange'; *B* 'Murcott'; *C* 'Dancy', 'Ponkan'; *D* 'Villafranca', 'Duncan'; *E* 'Troyer'; *F–J* Patterns in roots; *F* 'Nucellar Shamouti'; *G* 'Murcott', 'Dancy', 'Ponkan'; *H* 'Sour orange'; *I* 'Villafranca', 'Duncan'; *J* 'Troyer'. 2) PGI: *A–B* Patterns in callus; *A* 'Nucellar Shamouti', 'Sour orange', 'Murcott', 'Dancy', 'Ponkan', 'Volckameriana'; *B* 'Troyer'. *C–E* Patterns in roots; *C* 'Nucellar Shamouti', 'Sour orange'; *D* 'Dancy', 'Ponkan'; *E* 'Troyer'. 3) GOT: *A–C* Patterns in callus; *A* 'Nucellar Shamouti', 'Sour orange', 'Dancy', 'Villafranca'; *B* 'Murcott', 'Ponkan'; *C* 'Duncan', 'Troyer'; *D–G* Patterns in roots; *D* 'Nucellar Shamouti'; *E* 'Villafranca'; *F* 'Sour orange', 'Murcott', 'Dancy', 'Ponkan'; *G* 'Duncan', 'Troyer'

Table 2. Recognition of fusion products in *Citrus* by isozyme patterns: protoplast fusion combinations in which parental types are clearly differentiated by isozyme patterns[a]

Enzyme	Possible fusion partners	
	Callus extract	Root extract
PGI	'Troyer' with any of the others	'Troyer' with any of the others
GOT	1. 'Duncan' or 'Troyer' with any of the others 2. 'Murcott' or 'Ponkan' with any of the others	1. 'Nucellar Shamouti' with any of the other 2. 'Sour orange', 'Murcott', 'Dancy' or 'Ponkan' with 'Villafranca', 'Duncan' or 'Troyer'
MDH (NADP)	'Murcott' with any of the others	1. 'Villafranca' with any of the others 2. 'Sour orange' or 'Dancy' with 'Nucellar Shamouti'
MDH (NAD)	'Troyer' with any of the others	'Volckameriana' with any of the others
GDH	1. 'Troyer' with any of the others 2. 'Villafranca' or 'Duncan' with any of the others	'Murcott', 'Dancy' or 'Ponkan' with 'Nucellar Shamouti', 'Sour orange, 'Villafranca', 'Duncan' or 'Troyer'
Peroxidase	(none)	Each one with any of the others
Esterase	'Ponkan' with any of the others	'Sour orange' with 'Nucellar Shamouti', 'Dancy', 'Villafranca', 'Duncan', 'Murcott' or 'Ponkan'

[a] 'Nucellular Shamouti orange', 'Sour orange', 'Murcott', 'Dancy' and 'Ponkan mandarin', 'Villafranca lemon', 'Duncan grapefruit', 'Volckameriana' and 'Troyer'

patterns are not shown). The isozyme patterns of PGI were identical for all calli except Troyer, and this was almost so for the roots. The same picture was obtained with isozyme patterns of MDH (NAD), where calli and root isozyme patterns were very similar, with the exception of Troyer. This group of enzymes, with the exception of Troyer, therefore cannot be used as an identification marker for somatic hybrids with PGI and MDH (NAD).

b) Isoenzyme Patterns Similar in Calli but Variable in Roots

This group consists of peroxidases only. The isozyme patterns in calli were almost unvarying, while isozyme patterns of the root seemed highly specific. Thus, peroxidases isozyme pattern in roots from regenerated plants can serve as a very good marker for the identification of any fusion product.

c) Isozyme Patterns Variable in Both Calli and Roots

This group consists of GOT, MDH (NADP), GDH and esterases. The isozyme patterns of these latter enzymes have been grouped according to calli and root extracts, respectively. These enzyme systems can be used as markers for the identification of fusion products between parental protoplast types.

The available enzymes for identification of fusion products resulting from various parental combinations are summarized in Table 2.

Discussion

An analysis of electrophoretic mobilities of ten enzymes in nine different *Citrus* nucellar calli and their corresponding roots from apomictic seedlings has been presented. The analysis was performed in order to find the most suitable system(s) for identifying cell hybrids.

There are three requirements for such a system to be useful. First, isozyme patterns obtained from either callus or plant organ should be constant, namely, independent of age and stage of development. Secondly, the isozyme pattern should consist of a small number of bands, so that additional bands of hybrid cells, if any, can be detected. Third, the parental cells of the interspecific hybrids should have the least similar isozyme pattern possible, so that overlapping of bands is minimal.

All three requirements are met in our system. The age of the calli (subcultured every 4 to 5 weeks) did not affect the isozyme patterns. Isozyme patterns of the roots tested, either from seedlings or cuttings, proved to be identical. Most of the ten enzymes have a small number of bands and the required variability. Therefore, it was possible to find suitable enzymes that will serve for identifying somatic hybrids among the available calli (Table 2).

Cases have been described in which hybrid bands could not be observed in isozyme patterns of both sexual and somatic hybrids. Synthesized amphiploids in *Nicotiana* show hybrid bands of peroxidases and esterases in only 25% of the products (Smith et al. 1970). While in zymograms of somatic hybrids of *Nicotiana* heterokaryons new hybrid bands of esterase did appear (Menczel et al. 1978), no such hybrid bands of amylase isozymes were observed in hybrids of two specis of *Datura* (Lönnendonker and Schieder 1980). Since hybrid bands of some enzymes are not necessarily resolved in sexual or somatic hybrids, it seems rather important to analyze a large number of isozyme

patterns. Only such an analysis will provide a good chance to identify hybrids, as was shown in the case of zygotic seedlings of *Citrus* (Torres et al. 1978 b). Since the *Citrus* calli reported here (Vardi et al. 1982) have a high embryogenic capacity, analysis of isozyme patterns of both calli and roots would increase the probability in the detection of somatic hybrids.

In conclusion, since isozyme systems proved to be a very useful tool for identifying zygotic seedlings obtained by conventional crosses in *Citrus,* we believe that such a system will prove to be as useful for identifying interspecific cell hybrids. This will be particularly important when no other criteria (selection medium, morphological markers or mutant cells) are available to differentiate somatic hybrids from parental types. The small amount of plant material required for analysis and the constancy of expression make the use of isozyme systems rather promising. However, complications due to the possible lack of hybrid bands make it necessary to use simultaneously more than one, and preferably several, isozyme systems. It is hoped that the data presented in this paper will facilitate early confirmation of *Citrus* fusion products. Such experiments are now under way in our laboratory.

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