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Variation of Glucosephosphate Isomerase and Phosphoglucomutase Isozymes in *Vitis* and Their use in Grape Breeding

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Inheritance and variation of glucosephosphate isomerase (GPI) and phosphoglu - comutase (PGM) isozymes in *Vitis* and their usefulness in triploid grape breeding were investigated using horizontal starch gel electrophoresis. No isozyme variations were observed within cultivars. On electrophoretic analysis of 99 diploid cultivars, 20 diploid plants from 8 wild species, and their progenies, thirteen alleles were found for *Gpi-2* and eleven for ***Pgm-2.*** The data indicate high degree of genetic divergence between species in *Vitis*. Subsequently, genotypes for *Gpi-2* and ***Pgm -2*** were determined in 6 diploid cultivars and 4 tetraploid cultivars used for crossings. Of 98 seedlings from 15 controlled crosses between the diploid and tetraploid cultivars, 92 exhibiting trisomic gene expression could be determined to be triploid hybrids, while 6 exhibiting disomic gene expression were determined to be diploid plants produced by contaminating pollen. These results indicate that the two highly variable enzyme systems are very useful for breeding and phylogenetic study in grape.

INTRODUCTION

The grape is not a convenient plant for genetic analysis because of its long life cycle (about 3 years), large number of chromosomes ($2n=2x=38$), partial sterility of embryo sacs and low seed germination (Einset and Pratt, 1975). Hence, the inheritance of specific characteristics has been worked out for a few characteristics such as fruit color (Baritt and Einset, 1969), disease resistance (Mortensen, 1968) and stenospermocarpy (Stout, 1939), but few of the genes are of any real value to breeder as genetic markers in grape.

Isozyme electrophoresis provides us a new technique to demonstrate marker genes in grape in which a large number of isozyme polymorphisms have been described (Wolfe, 1976; Schwennesen et ***al.,*** 1982; Stavrakakis and Loukas, 1983; Subden et al., 1987; Goldy et al., 1988; Chaparro et al., 1989; Durham et *al.,* 1989; Goldy et *al.,* 1989; Walters et *al.,* 1989; Gray et *al.,* 1990). A large number of polymorphic isozyme loci have been identified in grape (Loukas et *al.,* 1983; Weeden et *al.,* 1988). Genetic analysis of these polymorphic isozyme systems confirmed that grape cultivars are highly heterozygous, and supported the hypothesis that the ancestors of modern grape cultivars were diploidized polyploid with an outcrossing system (Olmo, 1976).

Glucosephosphate isomerase (GPI; EC 5.3.1.9.) and phosphoglucomutase (PGM; EC 2.7.5.1.), the most widely studied enzymes in plants, are known to be polymorphic in many genera and their isozyme loci have been identified (Gottlieb, 1982; Weeden and Wendel, 1989). In grape, polymorphism of GPI was firstly shown by Stavrakakis and Loukas (1983) and two isozymes, a plastidspecific isozyme GPI - 1 and a cytosolic isozyme GPI - 2, have been identified by Weeden et *al.* (1988). Polymorphism of PGM

was firstly shown also by Stavrakakis and Loukas (1983) and a plastid -specific isozyme PGM-1 and a cytosolic isozyme PGM-2 have been identified by Weeden et *al.* (1988). On the basis of banding phenotypes in *145* grape cultivars and results of progeny tests, Parfitt and Arulsekar (1989) described two isozyme loci, *Gpi-2* and *Pgm-2,* with at least *4* (a, b, c, d) and *3* (a, b, c) alleles respectively.

Further data on the genetic basis of polymorphism for the two enzymes in grape and successful screening of seedling progenies for triploid hybrids between diploid and tetraploid cultivars are reported in this paper.

MATERIALS AND METHODS

Plant Material

Sixty-two *Vitis vinifera* cultivars, 33 interspecific hybrid cultivars, *4* cultivars from 3 American species and 20 individuals from 8 with species of *Vitis* were sampled (Table 1). Shoot apices from all the cultivars and wild species were obtained from plants grown in 3 vineyards; Fukuoka Agricultural Research Center, Fukuoka; University Farm of Kyushu University, Fukuoka; Akitsu Branch, Fruit Tree Research Station, Hiroshima.

Seedlings from 5 progenies from 5 controlled crosses were planted in 1986, 1989 and 1990, and were examined in the spring of 1989 and 1990. Of 9 cultivars used in the 5 crosses, 'Alicante - Bouschet', 'Chasselas Rose', 'Flame Tokay', 'Queen' and 'Thompson Seedless' are *V. vinifera* cultivars, 'Russky Concord has *V. labrusca* and *V. amurensis* in its parentage, 'Schuyler' has *V. vinifera, V. labrusca* and *V. amurensis* in its parentage, and 'Muscat Bailey A' has *V. vinifera, V. labrusca* and *V. lincecumii* in its pedigree.

To produce selfed seeds for genetic analysis in the endosperm, 'Mills', which has *V. vinifera* and *V. labrusca* in its parentage, was bagged in 1990 and the endosperm was examined in the winter of 1990.

In order to produce triploid hybrids, 6 diploid cultivars, 'Delaware', 'Muscat Bailey A', 'Muscat of Alexandria', 'Neo Muscat', 'Rizamat' and 'Sekirei', and 4 tetraploid cultivars, 'Cannon Hall Muscat', 'Kyoho', 'Red Pearl' and 'Yufu' were used. 'Muscat of Alexandria', 'Rizamat' and 'Connon Hall Muscat' are *V. vinifera* cultivars. 'Delaware' has *V. vinifera, V. labrusca* and *V. aestivalis* var. *bourquiniana* in its parentage. 'Kyoho' has *V. vinifera* and *V. labrusca* in its parentage. 'Neo Muscat' and 'Sekirei' have *V. vinifera* and uncertain *Vitis* species in their parentage. 'Red Pearl' and 'Yufu' are sports of 'Delaware' and 'Muscat Bailey A' respectively. Fifteen crosses between the diploid and tetraploid cultivars were made from 1985 to 1989. A few days before anthesis, flowers were emasculated carefully, sprayed with water to prevent self -pollination and bagged to avoid outcrossing. Hand-pollination was made at a full bloom stage and bagged again. The seedlings from the 15 progenies were obtained through embryo culture in 1988 and 1989 and by sowing in the other years, and were examined in the summer of 1990. The embryo culture was made at a ripening stage of berry growth on the basal MS medium (Murashige and Skoog, 1962) supplemented with either 100mg/l casamino acid or 250mg/ 1 malt extract.

Table 1. *Vitis* cultivars analyzed.

Species and interspecific hybrid	Cultivar
European species	
V. vinifera	Acma, Aleksandrouli, Alicante-Bouschet, Anab-e-shahi, Avasirhva, Baijixin Baladi, Black Hamburg, Chasselas Rose, Chenin Blanc, Citronelle, Dattier de Beyrouth, Dayuylu, Emperor, Flame Tokay, French Colombard, Heijixin, Hongniuai, Huangkalasi, huotiahong, Hussiene, Iracema, Italia, Itchkimar, July Muscat, Kali Sahebi, Kandahar, Katta Kurgan, Kizliarskij cernyj, Königin der Weingarten, Krimskij, Lunai, Madeleine Angevine, Malbec, Mission, Monukka, Muscat Hamburg, Muscat of Alexandria, Muscat Uzbecistansij, Nehelescol, Nimrang, Niuai, Pannoniakincse, Parkent, Puhljakovskij, Pusa Seedlees, Queen, Red Ohanez, Rich Baba, Rizamat, Rose d'Italia, Ruby Cabernet, Ruby Seedless, Ryugan, Soiaki, Tagobi, Taifei, Taifi Rozovij, Tavriz, Thompson Seedless, Volga Don, Zhana
American species	
V. champini	Salt Creek
V. labrusca	Concord, Fredonia
V. rupestris	St. George
Interspecific hybrid between European and American species	Bronx Seedless, Buffalo, Campbell Early, Canadice, Couderc 1202, Delaware, Diana, Empire State, Fuefuki, Glenora, Golden Muscat, Hanover, Headlight, Hybrid Franc, Iona, Jessica, Mills, Muscat Bailey A, New Niagara, New York Muscat, Niagara, Ontario, Patricia, Pierce, Scarlet, Schuyler, Seneca, Settsu, Sheridan, Steuben, Super Hamburg, Wayne, Yates

Sample preparation and enzyme extraction

In addition to shoot apices, fully expanded young and mature leaves, young and mature twigs, young roots, pollen and endosperms were used for enzyme extraction. The sporophytic tissues were collected in plastic bags to maintain a humid environment and examined immediately or stored in the dark for 1 to 7 days at 5°C until use. Pollen was collected on a plate glass at anthesis by tapping at the bloom cluster, and examined immediately or stored in the dark for 1 to 4 weeks at 5°C until use. Approximately 0.2g of the sporophytic tissues, 0.005g of the pollen and endosperms were respectively homogenized by hand in a pre - chilled mortar and with a pestle with 1.0ml for sporophytic tissue and 0.5ml for pollen and endosperms of chilled extraction

buffer prepared as described by Wendel and Parks (1982) except that 5% PVP K-30 (Nacalai Tesque, Inc. Kyoto) was added instead of 5% PVP-40. Crude enzyme extracts obtained from this procedure were absorbed onto filter paper wicks (Whatman # 3, 11 X 3mm) and used for electrophoresis. All extractions were performed at room temperature (10-20°C).

Electrophoresis and enzyme detection

Horizontal starch gel electrophoresis was conducted using a tris/ citrate buffer system as described by Wendel and Parks (1982), except that 32.40g Starch-hydrolyzed (Nacalai Tesque, Inc. Kyoto, Japan) was used instead of 32.40g electrostarch. The wicks absorbing sample extracts were vertically inserted into pre - cooled gel. Electrophoresis was carried out at 2-4°C for 8 hours at 10 watts. After electrophoresis, the anodal section of the gel was sliced horizontally into lmm thick slices and stained for two enzyme systems, GPI and PGM. Staining of GPI and PGM followed Wendel and Parks (1982).

Allozyme nomenclature

According to the International Union of Biochemistry (Webb, 1984), the locus coding for the products with the most anodal mobility was designated as 1, the next 2, and so forth. Similarly, at each locus, the allele coding for the most anodal or the fastest migrating allozyme was given the alphabetical code a, next b, and so on.

Chromosome observation

To examine the ploidy level of 'Red Pearl', 'Yufu' and seedlings obtained from the 15 crosses between diploid and tetraploid cultivars, root tips obtained from their cuttings were pre - treated with 0.002M 8 - hydroxyquinoline for 24 hours at 10°C, dehydrolyzed in a solution of 1N HCl and 45% acetic acid (1:1) for 1 minute at 60°C, stained with lactic propionic orcein (Dyer, 1963) for 24 hours at room temperature, squashed under a glass cover, and observed with a microscope.

RESULTS

Two enzyme systems from protein extracts from various tissues of grape cultivars and wild *Vitis* species could be resolved by starch gel electrophoresis. They were polymorphic and exhibited allelic variation at one or two enzyme loci.

First, results will deal with GPI extracted from diploid cultivars and wild species (2n=2x=38). Two anodal zones of enzyme activity were detected in all tissues studied. The fast migrating zone GPI-1 was invariant for a single and relatively wide band migrating 9cm form the origin on the gel in all species and cultivars surveyed. When the sporophytic tissues were examined, the slow migrating zone GPI - 2 was variant and exhibited either a single - or three - banded pattern (Fig. 1). The intermediate band of the three bands was stained denser than the other two bands, and was not appeared on the gel when a crashed pollen extract of the same cultivar was used. When triploid endosperms were examined, the slow migrating zone GPI - 2 exhibited either a one-or three - banded pattern. The three-banded pattern was

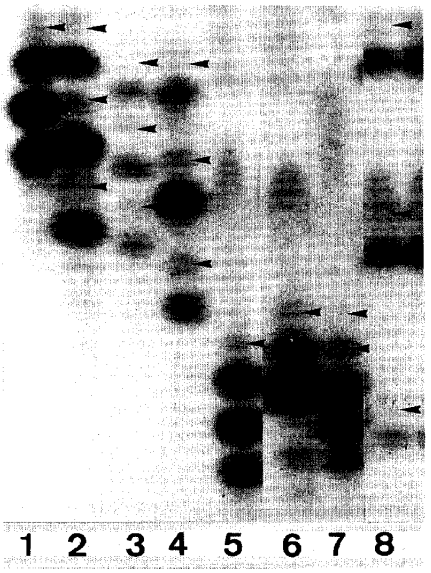

Fig. 1. GPI zymograms of diploid cultivars. 1. 'Jessica'; 2. 'Alicante Bouschet'; 3. 'Mills'; 4. 'Russky Concord'; 5. 'Empire State'; 6. 'St. George'; 7. 'Salt Creek'; 8. 'Delaware'. The arrows indicate ghost bands. Anode is toward the top of the figure.

separated into two types which could be distinguished by their relative intensities of the fast and slow bands. These are the banding patterns typical for a dimeric enzyme as has been reported in grape (Weeden et al., 1988; Parfitt and Arulsekar, 1989) and all other plants examined (e.g., Torres et ***al., 1978;*** Weeden and Gottlieb, 1979; Gottlieb, 1982; Wendel and Parks, 1983; Goldring et ***al.,*** 1985; Parfitt ***et al., 1985;*** Chevreau and Leurens, 1987; Pedersen et ***al.,*** 1987; Suiter, 1988).

In the zymograms of all samples analyzed, each of the bands often appeared with more than one faint bands migrating anodally to the bands with intervals of about 5mm (Fig. 1). Intensity of the faint bands decreased as they migrated from the bands. The extent of intensity of the faint bands was different in different tissues examined; i.e., the occurrence of the faint bands was conspicuous in extracts from mature twigs and fully expanded leaves, but no or a little trace of the faint bands was detected on the gel when pollen, endosperms. very young leaves and shoot apices were used as enzyme sources. These results indicate that the faint bands are break-down products of the isozymes and are negligible for genetic analysis and that the usage of extracts from young tissues provides us consistent zymograms to analyze the isozyme variation in grape.

Ninety-nine diploid cultivars and 20 plants from 8 wild *Vitis* species were analyzed electrophoretically and divided into 31 banding phenotypes for Gpi-2. The mobilities of 13 homodimeric allozymes of GPI found in this study were listed in Fig. 2 with their corresponding hypothetic alleles at *Gpi-2*. Segregation analysis confirmed that ***Gpi-2*** is an isozyme governed by a single locus *Gpi-2* (Table 2). The segregation patterns in F_1 progenies and endosperm did not significantly deviated from the expected ratio at 5%

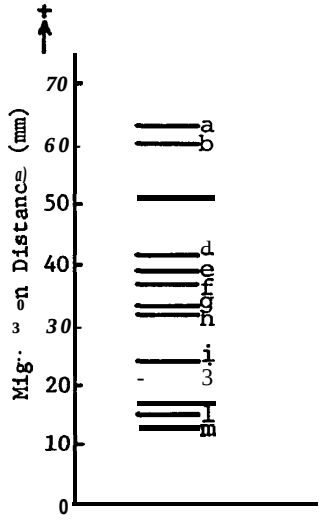

Fig. 2. Position of homodimetric allozymes of Gpi-2. Anode is toward the top of the figure.

Table 2. Goodness-of-fit tests for single locus segregation patterns at ***Gpi-2.***

Cross	Prospected parental Gpi -2 genotypes	Progeny and endosperm genotypes	Expected ratio	X^2	*P*
Alicante-Bouschet X Flame Tokay	ad × bb	24ab : 34bd[a]	1:1	1.72	0.200-0.100
Alicante-Bouschet X Schuyler	ad × bi	10ab : llai : llbd : 17di[a]	1:1:1:1	2.51	0.500-0.300
Chasselas Rose X Schuyler	aa × bi	24ab : 34ai[a]	1:1	1.72	0.200-0.100
Queen **x** Thompson Seedless	aa × ad	25aa : 28ad[a]	1:1	0.17	0.700-0.500
Muscat Bailey A × Muscat Bailey A	aj Xaj	25aa : 44aj : 20jj	1:2:1	0.57	0.800-0.700
Muscat Bailey A X Russky Concord	aj X bg	20ab : 24ag : 16bj : 23gj[a]	1:1:1:1	1.87	0.700-0.500
Mills **x** Mills	be × be	12bbb : 15bbe : 13bee : 10eee[b]	1:1:1:1	1.04	0.800-0.700

a, Progeny; b, Endosperm.

level in all the cross combinations. This indicated that in the diploid sporophytic tissues and triploid endosperms the single-banded and three -banded patterns reflect homozygosity and heterozygosity at *Gpi-2* respectively and that at least 13 alleles exist at *Gpi-2* in *Vitis*. Of the 13 alleles detected, 4 alleles were found in ***V.*** *vinifera*, 6 alleles in 4 American *Vitis* species and 5 alleles in 5 East Asian *Vitis* species, and 9 alleles in American hybrid cultivars (Table 3). On comparing species on different continents, 3 alleles, a, b and h, were exclusively found in ***V.*** *vinifera*, 5 alleles, e, i, k, 1 and m, in American species, and 2 alleles, f and g, in East Asian species.

Second, results will deal with PGM extracted from the diploid cultivars and wild species. Two anodal zones of enzyme activity were detected in all samples analyzed. No differences of banding patterns were detected between the sporophytic tissues of the same cultivar. The fast migrating zone PGM- 1 was variant for 3 to 5 indistinctive bands. The slow migrating zone PGM-2 exhibited either a single -or two-banded pattern (Fig. 3). No differences of banding patterns were seen between sporophytic

Table 3. Alleles of Gpi-2 and ***Pgm-2*** found in species and interspecific hybrids in *Vitis*.

Species and interspecific hybrid	No. of cultivars and individuals analysed	Allele	
		Gpi-2	***Pmg-2***
European species			
V. vinifera	62	abdh	bdehi
North American species			
V. *labrusca*	3	ceij	gh
V. *champini*	1	ji	hk
V. *repestris*	2	j	h
V. vulpina	1	jm	hj
East Asian species			
V. amurensis	1	g	c
V. ***coignetiae***	2	j	ch
V. kiusiana	1	g	c
V. *shiragai*	3	dfj	ace
V. thunbergii	10	cdfgj	ceh
Intercontinental hybrids			
American hybrids[a]	33	abcdeijkm	befghij

a. Interspecific hybrid cultivars with ***V. vinifera*** and North American species in their pedigree.

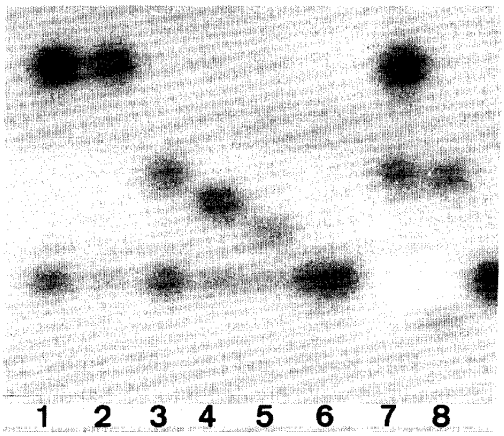

Fig. 3. PGM zymogram of diploid cultivars. 1. 'Wayne'; 2. 'Diana'; 3. 'Jessica'; 4. 'Dr. Collier'; 5. 'Portland'; 6. 'Schuyler'; 7. 'Malbec'; 8. 'Scarlet'. Anode is toward the top of the figure.

tissues and pollen of the same cultivars. When triploid endosperms were examined, PGM-2 exhibited either a single- or two-banded pattern. The two-banded pattern was separated into two types in which staining intensity was very different between the two bands. These were the banding patterns typical for a monomeric enzyme as has been reported in grape (Weeden et al., 1988) and all other plants analyzed (e.g., Wendel and

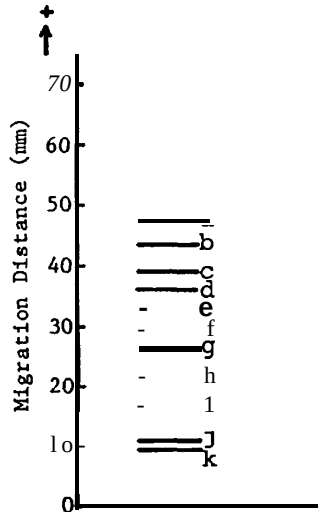

Fig. 4. Position of monomeric allozymes of Pgm-2. Anode is toward the top of the figure.

Table 4. Goodness-of-fit tests for single locus segregation patterns at *Pgm* -2.

Cross	Prospected parental *Pgm* -2 genotypes	Progeny and endosperm genotypes	Expected ratio	X^2	*P*
Alicante-Bouschet x Flame Tokay	bd × bi	14bb :16bd :18bi :10di[a]	1:1:1:1	2.41	0.500-0.300
Alicante-Bouschet x Schuyler	bdxhh	22bh :27dh[a]	1:1	0.51	0.500 -0.300
Chasselas Rose × Schuyler	be x hh	29Bbh :29eh[a]	1:1	0.00	> 0.990
Queen x Thompson Seedless	*ii x ii*	53ii[a]			
Muscat Bailey A x Muscat Bailey A	fi × fi	29ff : 42fi :18ii[a]	1:2:1	3.00	0.300 -0.200
Muscat Bailey AX Russky Concord	fi x ch	17cf : 24ci : 22fh: 20hi[a]	1:1:1:1	1.29	0.800-0.700
Mills x Mills	bixbi	19bbb : llbbi : 12bii : 8iii[b]	1:1:1:1	5.20	0.200-0.100

a, Progeny; b, Endosperm.

parks, 1982; Arus and Orton, 1984; Kahler and Lay, 1985; Suiter, 1988).

The diploid cultivars analyzed were divided into 21 banding phenotypes for PGM-2. The mobilities of 11 monomeric allozymes of PGM found in this study were listed in Fig. 4 with their corresponding hypothetic alleles at *Pgm-2* The segregation test in F_1 progenies and endosperms (Table 4) confirmed that the allozymes of PGM-2 were governed by a second locus *Pgm -2* as suggested by Weeden et ***al.*** (1988). Of the 11 alleles detected, 5 alleles were found in *V.* ***vinifera,*** 4 alleles in North American species, 4 alleles in East Asian species, and 7 alleles in American hybrids (Table 3). On comparing species on different continents, 3 alleles, b, d and i, were uniquely found in *V. vinifera,* 4 alleles, f, g, j and k, in North American species, and 2 alleles, a and c, in East Asian species.

The analysis of linkage relationships confirmed that the 2 enzyme loci *Gpi-2* and

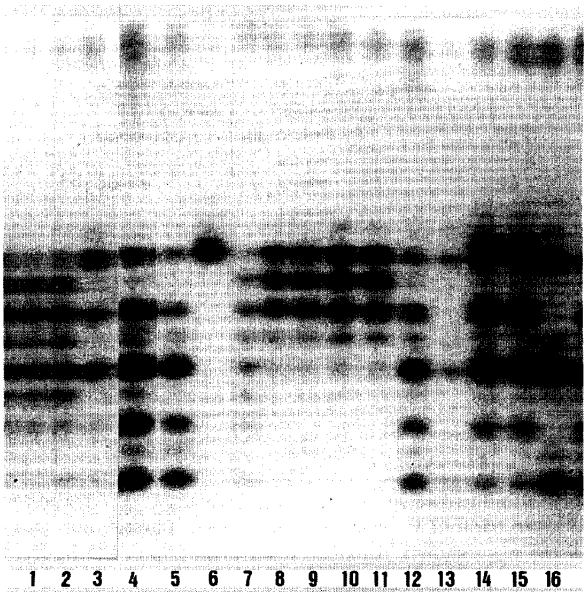

Fig. 5. GPI zymogram of tetraploid cultivars. 1. 'Kyoho'; 2. 'Takasumi'; 3. 'Hakuho'; 4. 'Olympia'; 5. 'Red Queen'; 6. 'Cannon Hall Muscat'; 7. 'Kuroshio'; 8. 'Pioné'; 9. 'Kaiakane'; 10. 'Izunishiki'; 11. 'Beniyamabico'; 12. 'Jasmine'; 13. 'Benifuji'; 14. 'Beniizu'; 15. 'Ryuho'; 16. 'Benizuiho'. Anodo is toward the top of figure.

Pgm-2 segregated independent of each other as indicated by Weeden et al. (1988) and Parfitt and Arulsekar (1989). With the combination of ***Gpi-2*** and ***Pgm*** *-2* genotypes, diploid cultivars and wild species were separated into 82 groups of which 59 groups consisted of only one cultivar or wild species. The genotype of each cultivar and wild species examined will be published elsewhere.

Third, a similar analysis was performed to determine the *Gpi* -2 genotypes responsible for one-to eight-banded patterns which were encountered in 4 tetraploid cultivars (Fig. 5) and 98 seedlings obtained from crosses between the 4 tetraploid cultivars and 6 diploid cultivars. In the tetraploid cultivars, one-, six-and ten- banded patterns must reflect nulliplex, trigenic and tetragenic genotypes respectively, while three banded patterns must reflect simplex or duplex genotypes. In the triploid progenies, one -, three- and six -banded patterns must reflect nulliplex, simplex and trigenic genotypes respectively. The results of GPI isozyme analysis of two triploid hybrids between diploid 'Muscat of Alexandria' with bb genotype for *Gpi* -2 and tetraploid 'Kyoho' exhibiting an eight -banded phenotype on the gel demonstrated the possibility (Fig. 6). The zymogram of one of the two triploid seedlings exhibited a three-banded phenotype reflecting simplex, while that of the another seedling exhibited a five-banded phenotype. The mobilities of allozyme bands and gene dosage confirm that the five-banded phenotype reflects a trigenic genotype; i.e., overlap of an homodimer band dd and heterodimer band bi on the gel resulted in the five-banded phenotype. Hence, the simplex and trigenic genotypes in the two triploid seedlings

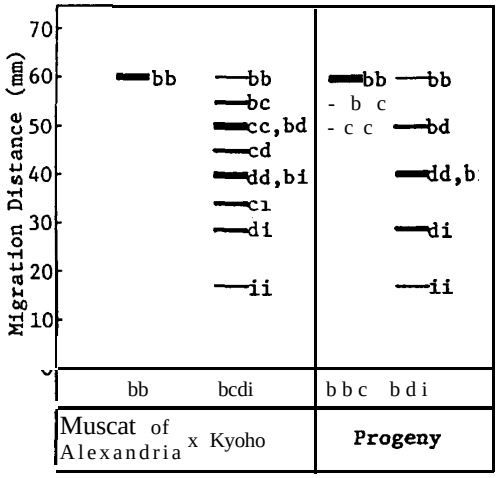

Fig. 6. Schematic illustration of allozymes in 'Muscat of Alexandria', 'Kyoho' and their triploid F_1 progeny for Gpi - 2. Phenotypes and genotypes (below) were shown. Anode is toward the top of the figure.

were determined to be bbc and bdi, respectively. Consequently, genotype of 'Kyoho' exhibiting the eight-banded phenotype was confirmed bcdi. In this tetragenic genotype association of subunits coded by *Gpi-2* yielded ten bands but overlap of the heterodimer band bd and homodimer band cc and that of the heterodimer band bi and homodimer band dd on the gel resulted in the eight-banded pattern.

Not only zymograms of GPI extracts from the sporophytic tissues but those from the pollen also made it possible to confirm duplex genotypes in tetraploid cultivars by the consistent occurrence of the three bands and the consistent relative intensity of the respective allozyme band on the gel. A leaf extract from 'Yufu', a sport of 'Muscat Bailey A' with aj genotype for Gpi-2, and that from 'Red Pearl', a sport of 'Delaware' with ak genotype for *Gpi-2*, exhibited the same three-banded pattern as those from 'Muscat Bailey A' and 'Delaware' respectively, whereas zymograms of pollen extracts from the two tetraploid cultivars also exhibited the same three-banded pattern as those of their leaf extracts. Hence, 'Yufu' and 'Red Pearl' are tetraploid with aajj and aakk

Table 5. *Gpi-2/Pgm-2* genotypes of the seedlings obttained from crosses between tetraploid and diploid cultivars.

Gross	Parental *Gpi-2/Pgm-2* genotype	Number of seedlings examined	Genotypes of seedling produced by successful cross	(N^a)	Genotypes of seedling produced by contaminating pollen	(N^a)
Muscat Bailey A Kyoho	aj/fi	bcdi/bbhh 15	abc/bbi	(1)	aj/ii	(1)
			abd/bfh	(1)		
			abi/bhi	(1)		
			aci/bhi	(1)		
			adi/bfi	(1)		
			bcj/bfh	(1)		
			bcj/bhi	(1)		

				bcj / fhh	(1)		
				bdj/bfh	(1)		
				cij/bhi	(1)		
				dij/bbi	(1)		
				dij/bhi	(2)		
				dij/hhi	(1)		
Muscat Bailey A Red Pearl	aj/fi	aakk/eehh	22	aaa/efh	(1)	aa/fi	(1)
				aaa/ehi	(1)	aj/fi	(1)
				aaa/hhi	(1)	aj/ii	(1)
				aaj/efh	(1)	jj/fi	(1)
				aaj/ehi	(1)		
				aak/eei	(1)		
				aak/efh	(3)		
				aak/ehi	(2)		
				aak/fhh	(1)		
				aak/hhi	(1)		
				ajk/efh	(2)		
				ajk/ehi	(2)		
				jkk/efh	(1)		
Red Pearl Muscat Bailey A	aakk/eehh	aj/fi	27	aak/eef	(1)		(0)
				aak/eei	(2)		
				aak/efh	(2)		
				aak/ehi	(3)		
				aak/fhh	(1)		
				aak/hhi	(1)		
				ajk/eef	(1)		
				ajk/efh	(3)		
				ajk/ehi	(6)		
				ajk/fhh	(1)		
				akk/efh	(2)		
				jkk/eei	(1)		
				jkk/efh	(1)		
				jkk/ehi	(1)		
				jkk/fhh	(1)		
Delaware Yufu	ak/eh	aajj/ffii	7	aaj/eii	(1)		(0)
				aak/efi	(1)		
				ajj/eii	(1)		
				ajk/efi	(2)		
				ajk/ffh	(1)		
				jjk/fhi	(1)		
Yufu Delaware	aajj/ffii	ak/eh	4	aaj/efi	(1)	-	(0)
				aaj/fhi	(1)		
				aaj/hii	(1)		
				ajk/fhi	(1)		
Muscat Bailey A Cannon Hall Muscat	aj/fi	bbbb/bbbi	2	bbj/bbf	(1)		(0)
				bbj/bfi	(1)		
Delaware Cannon Hall Muscat	ak/eh	bbbb/bbbi	6	abb/bbe	(1)		
				bbk/bbh		(1) -	(0)

				bbk/bhi	(4)		
Delaware	Kyoho	ak/eh bcdi/bbhh	1	abc / bbe	(1)	-	(0)
Muscat of Alexandria	Kyoho	bb/bi bcdi/bbhh	1	bbc/bhi bdi/bhh	(1) (1)	-	(0)
Muscat of Alexandria	Red Pearl	bb/bi aakk/eehh	1	bkk/hhi	(1)	-	(0)
Rizamat	Yufu	dd/ii aajj/ffii	2	adj/iii	(1)	dd/ii	(1)
Sekirei	Red Pearl	bb/bi aakk/eehh	4	aab/bee aab/beh abk/beh	(1) (1) (2)	-	(0)
Red Pearl	Rizamat	aakk/eehh bb/hi	3	abk/eei abk/ehi abk/hhi	(1) (1) (1)		
Rea Pearl Neo Muscat		aakk/eehh ab/bb	1	aak/beh	(1)	-	(0)
Yufu	Sekirei	aajj/ffii bb/bi	1	aab/bii	(1)	-	(0)

[a]Number of seedlings.

genotype for *Gpi* -2 respectively. The results of our cytological and morphological studies indicating that both cultivars are periclinal chimera (2x - 4x) denied these conclusions, since activities of GPI in diploid LI (epidermis) is negligible and since their embryo sacs and pollens originate from tetraploid L. Thus, the genotypes of tetraploid cultivars used as a pistillate or pollen parent were determined as listed in Table 5.

In a large number of the seedlings obtained from crosses between the tetraploid and diploid cultivars, overlap of two or three dimeric allozymes with following subunit

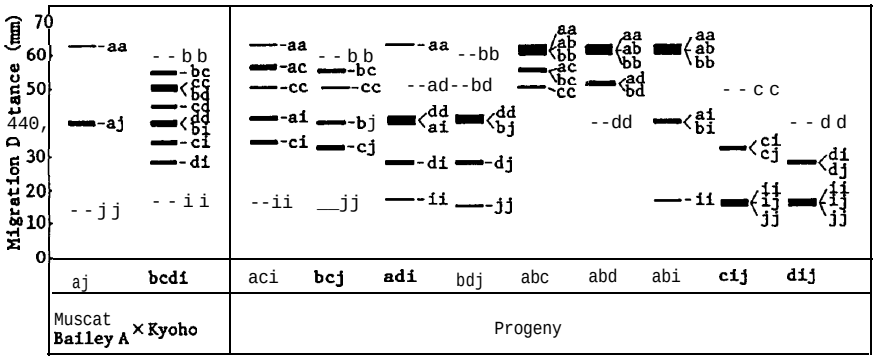

Fig. 7. Schematic illustration of allozymes in 'Muscat Bailey A', 'Kyoho' and their triploid F_1 progeny for Gpi - 2. Phenotypes and genotypes (below) were shown. Anode is toward the top of the figure.

association was seen on the gel: aa, ab and bb; ac and bc; ad, bd and cc; dd and ai; dd and aj; dd and bi; dd and bj; ak and bk; ci and cj; di and dj; ii, ij and jj. In the triploid seedlings from 'Muscat Bailey A', 'Kyoho', overlap of dimeric allozyme bands was frequently observed on the gel and resulted in either a three -, five -or six -banded phenotype in spite of their trigenic genotypes (Fig. 7). These results indicate that ploidy level in a given cultivar or seedling is not directly referred from the banding phenotype even if the *Gpi-2* genotype consists of more than three different alleles. However, it is also indicated that in these cases the Gpi-2 genotype in a given cultivar or seedling is relatively easily determined if the mobilities of allozyme bands and gene dosage are considered carefully.

Of the 98 seedlings analyzed, 89 exhibiting trisomic gene expression were determined to be triploid hybrids, 3 exhibiting disomic gene expression were determined to be diploids, and 5 were determined to be off-types which were produced by contaminating pollen.

Forth, a similar analysis was carried out to determine the *Pgm -2* genotypes responsible for one-, two- and three -banded pattens which were encountered in the 4 tetraploid cultivars and 98 seedlings obtained from crosses between the tetraploid and diploid cultivars. Because of the monomeric enzyme expression of PGM-2, single-, three- and four-banded patterns observed in tetraploid cultivars must reflect nulliplex, trigenic and tetragenic genotypes respectively, while a two-banded pattern with the same intensity must reflect duplex genotypes and that with very different intensity must reflect simplex genotypes, since duplex produces active allozymes in the ratio 1:1 whereas simplex produces them in the ratio 3:1. The genotypes determined for the 4 tetraploid cultivars used for crossing were listed in Table 5.

The *Pgm-2* genotypes of the triploid seedlings obtained from crosses between the tetraploid and diploid cultivars were easily determined, since single - and three-banded patterns reflect nulliplex and trigenic genotypes respectively, and since two two-banded patterns distinguished by relative intensity between the two bands are simplex producing active allozymes in the ratio 2:1. Of the 98 seedlings analyzed, 91 exhibiting trisomic gene expression were determined to be triploid hybrids, 3 exhibiting disomic gene expression were determined to be diploid plants, and 5 without alleles from the pollen parents were determined to be off- types which were produced by contaminating pollen.

On the basis of GPI and PGM analysis, all the genotypes of 98 seedlings obtained from the crosses between the tetraploid and diploid cultivars could be determined for Gpi-2 and *Pgm-2* (Table 5). Subsequently, the genotype data confirmed the hybridity and ploidy in the seedlings; i.e., of the 98 seedlings, 92 could be determined to be triploid hybrids and 6 could be determined to be diploid off-types which presumably produced by self -pollination in the diploid pistillate parents. Our cytological data agreed with these conclusions, since chromosome number in the root tip cells is 57 in the 92 triploid hybrids and 38 in the 6 diploid seedlings.

DISCUSSION

Stability of GPI and PGM isozymes

In recent years, considerable overlap of gametophytic and sporophytic gene expression has been demonstrated by electrophoretic analysis of isozymes in several species (Tanksley et ***al.,*** 1981; Gorla et ***al.,*** 1986; Rajora and Zsuffa, 1986; Weeden, 1986; Pedersen ***et al.,*** 1987). Pedersen *et* ***al.*** (1987) indicated from the results of electrophoresis in barley that 30 out of 50 isozymes including those of GPI and PGM are expressed in both pollen and sporophyte. The extensive overlap of gene expression between various sporophytica tissues in plant also has been demonstrated by electrophoretic analysis of isozymes (Vodkin and Scandalios, 1979; Pedersen and Simonsen, 1987). This situation is quite similar in grape species and cultivars since GPI and PGM isozymes were expressed in the various sporophytic tissues, pollen and endosperms analyzed.

In zymograms of GPI, appearance of ghost bands representing degradation products of the primary allozymes is in accordance with the report that ghost bands tend to be faint, develop more slowly, and typically covary in tandem with and in close proximity of their associated primary bands (Kephart, 1990). Kephart (1990) stated that these secondary bands may result from technical aspects, old tissues, or overheating of gels during runs. In GPI of grape, the fact that development of the ghost bands increased with tissue age indicates that not only technical aspects but physiological conditions of tissues in situ also may result in the development of them, although this mechanism is not known at present.

Allozyme Variation

Among the food crops analyzed electrophoretically, *Vitis* displays high level of allozyme variation both within germ plasm and among cultivars (Weeden, 1989). The presence of high level of allozyme variation in *Vitis* may be very useful for the identification of grape cultivars. Stavrakakis and Loukas (1983) studied pollen isozyme banding patterns in 37 grape cultivars (*V. vinifera*) and found that 11 out of 13 enzyme systems showed different banding patterns and that all cultivars could be identified electrophoretically if more than 2 enzyme systems were combined. Benin et ***al.*** (1988) analyzed leaf isozymes of 40 *V. vinifera* cultivars using 3 enzyme systems and found that combination of 31 variable bands out of 39 bands detected allowed to characterize each cultivar. Although these isozyme analysis were successful for identification of ***V. vinifera*** cultivars, the number of cultivars analyzed was too small to conclude that they were very useful for identification of many grape cultivars. In addition, peroxidase and esterase analyzed in these studies are highly variable enzyme systems but are considered to be highly tissue -specific, or under environmental or developmental control as those reported in other many plants (Gottlieb, 1981; Pedersen et al., 1987). Parfitt and Arulsekar (1989) analyzed leaf isozymes of 145 grape cultivars ***(V. vinifera*** and *Vitis spp.*) and found that they could be separated into 52 groups using stable enzyme systems (GPI and PGM) . They concluded that unique isozyme identities can not be assigned to many of the cultivars using only GPI and PGM, although the cultivars having different GPI or PGM patterns can be shown to be genetically different cultivars. Our results of GPI and PGM isozyme analysis support their conclusion,

although we found higher allozyme polymorphism than that reported by them. This indicates that further identification of cultivars due to GPI and PGM isozyme analysis may be possible.

Examination of the *Gpi-2* and *Pgm -2* data collected in this investigation on the genus *Vitis* reveals that East Asian, European, and North American species exhibit the great intercontinental divergence. This indicates that, in spite of the cross compatibility between these species, a great deal of genetic divergence has occurred during allopatric speciation as has been suggested in genera with extant species in mesic temperate refugia in eastern North America, East Asia, and southerastern Europe or western Asia (Parks and Wendel, 1990; Hoey and Parks, 1991). The higher level of allelic diversity at the two loci in American hybrids than in *V. **vinifera*** reflects introgression of genes from American species to *V. vinifera* since the 1850c to breed for cold tolerance, disease resistance, and *Phylloxera* and nematoda resistance (Snyder, 1937; Einset and Pratt, 1975).

Ploidy and hybridity confirmation

The stability of isozyme number and very high levels of allozyme polymorphism in GPI and PGM of grape allow the determination of the ploidy levels inferred by the number and relative intensity of allozyme bands expressed in these highly conserved enzyme systems in which the variations have been defined genetically. Triploid individuals and endosperms are easily identified by their zymograms of GPI and PGM when their genotypes for *Gpi -2* and *Pgm - 2* are simplex or trigenic. Tetraploid individuals also are easily identified when their genotypes for *Gpi -2* and *Pgm -2* are simplex, trigenic or tetragenic. When the genotypes of tetraploid individuals for *Gpi-2* are duplex, comparison of banding patterns between the sporophytic tissue and the pollen make it possible to identify them to be tetraploid because of the consistent occurrence of heterodimer band. Problem in identification of ploidy level arises when the individuals or endosperms possess nulliplex genotypes for both *Gpi-2* and *Pgm-2* or when the individuals possess duplex genotypes for *Pgm-2* and nulliplex ones for *Gpi-2.* High heterozygosity at *Gpi -2* and *Pgm -2* in diploid cultivars analyzed, however, indicates that these combinations of genotypes for *Gpi-2* and *Pgm -2* are of infrequent occurrence. Therefore, ploidy levels of most grapes, which are usually either diploid, triploid or tetraploid, may be ascertained by banding phenotypes of the two isozymes, GPI-2 and PGM-2.

Of the 92 seedlings from the 15 crosses, 6 had unexpected disomic genotypes which could be explained by accidental self-pollination in the diploid pistillate parents. Of the 6 seedlings, 5 were derived from 2 crosses in which 'Muscat Bailey A' was used as a pistillate parent, and one was derived from 'Rizamat' 'Yufu'. This suggests either that self -pollination had occurred within the buds of 'Muscat Bailey A' and 'Rizamat' before or when emasculation was carried out, or that outcross due to wind- pollination had occurred in the emasculated buds when hand-pollination was carried out. In addition to these reproductive behaviors, the considerable high ratio of 6.5% contamination may be due to the facts that establishement of plants through embryo culture was easy in normally developed diploid embryos but was considerably difficult in abortive triploid embryos from crosses between diploid and tetraploid cultivars. In triploid grape breeding, therefore, GPI and PGM isozymes exhibiting high levels of allozyme

polymorphism are very useful for confirmation of hybridity and exclusion of unexpected plants.

Conclusion

The present analysis has established two marker genes Gpi-2 and Pgm-2 for the confirmation of hybridity and ploidy in grape. The very high polymorphism observed in *Gpi-2* and *Pgm-2* provides a sound base not only for breeding grape but also for analyzing phylogenetic relationships among *Vitis* species and cultivars.

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