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Genetics of superoxide dismutase in apple

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Abstract Four zones of superoxide dismutase (SOD), activity were detected in apple. Genetic studies confirmed the presence of three genes *Sod-1*, *Sod-3* and *Sod-4* with three, two and two alleles respectively, including one null allele. One of these genes, *Sod-1*, was found to be loosely linked to *Prx-2* and *Prx-3*. The *Sod-1a* allele predominated in all the three groups studied, cultivars, rootstocks and *Malus* species. The distinct achromatic bands produced by SOD after electrophoresis facilitate its important role with peroxidase (PRX), glutamate oxalacetate transaminase (GOT) and phosphogluconate dehydrogenase (PGD) in the discrimination of apple cultivars.

Key words *Malus pumila* Mill · Superoxide dismutase · Genes *Sod-1*, *Sod-3*, *Sod-4* · Linkage · Cultivar identification

Introduction

Superoxide dismutase (EC1.15.1.1; SOD) belongs to a class of metalloproteins that catalyse the dismutation of the superoxide radical to hydrogen peroxide and oxygen.

Isoenzymic variation for SOD has been reported in a wide range of crops including citrus cultivars (Almansa et al. 1989), rice (Pan and Yau 1991) and the grass genus *Elymus* (Jaaska 1992). Genetic studies have been made in soybean (Kiang and Gorman 1985), maize (Baum and Scandalios 1982) and grape (Loukas et al. 1983). Some of the isoenzymic bands observed for SOD in soybean coincided with the position of certain peroxidase (PRX) bands (Kiang and Gorman 1985).

In apple, Chevreau and Laurens (1987) observed complex SOD zymograms in extracts from leaves. The two phenotypes in the fastest zone were explained on the basis of disomic determination through two homoeologous genes with common alleles.

The purpose of the present study was to investigate SOD polymorphism in a wider range of apple cultivars and *Malus* species and derivatives and to clarify the genetics of variation occurring in the zones of SOD activity.

Materials and methods

Plant material

Seedling progenies from controlled crosses were studied. Dormant flower buds, bark, leaves, cotyledons and pollen provided extracts for analysis. In addition, scion cultivars, rootstocks and *Malus* species were examined from the gene banks of Horticulture Research International, East Malling and the National Fruit Collection, Brogdale.

Sample preparation

Tissues were frozen, using liquid N_2, then ground into a fine powder, and immediately added to the extraction solution (0.05 *M* Na phosphate buffer pH 7.2, 10% w/v PVP insoluble, 0.4 *M* sucrose and 14 m*M* 2-mercaptoethanol). The extracts were ground until the tissue was reduced to a fine suspension then centrifuged at 35,000 *g* for 30 min at 4°C. The resulting supernatant was used for electrophoretic separation. When pollen was sampled the extraction solution included the buffer 0.1 *M* TRIS - 0.025 *M* KCl adjusted to pH 8.0 with HCl, 0.4 *M* sucrose and 0.05% Triton X-100.

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Electrophoretic procedure

Acrylamide gels, $180 \times 140 \times 2$ mm were prepared according to Davis (1964) with slight modifications. The sample gel was omitted and TRIS-glycine pH 8.8 (0.19 *M*) was incorporated into the running gel in place of TRIS-HCl. Electrophoresis was performed at a constant 100 V until the tracking dye entered the stacking gel, when the voltage was raised to 150 V. When the tracking dye entered the running gel the voltage was further increased to 350 V. The duration of electrophoresis was 4 h (1 h after the dye reached the bottom of the gel).

Zones of SOD activity were marked by achromatic bands after electrophoresis (Baum and Scandalios 1979). Gels were incubated in the dark for 20 min in a 0.05 *M* TRIS-HCl pH 8.2 solution containing 0.003% riboflavin, 2×10^{-4} *M* EDTA and 0.01% nitro-blue tetrazolium and were then illuminated for 15–20 mn. When clear achromatic bands were apparent, the staining solution was drained off and the gels were rinsed with tap water and fixed in an alcohol gel wash.

Gels were also stained for PRX according to the method described by Manganaris and Alston (1992a).

Statistical analysis

The program LINKAGE-1 (Suiter et al. 1983) was used for analyses of single-gene segregations and linkage.

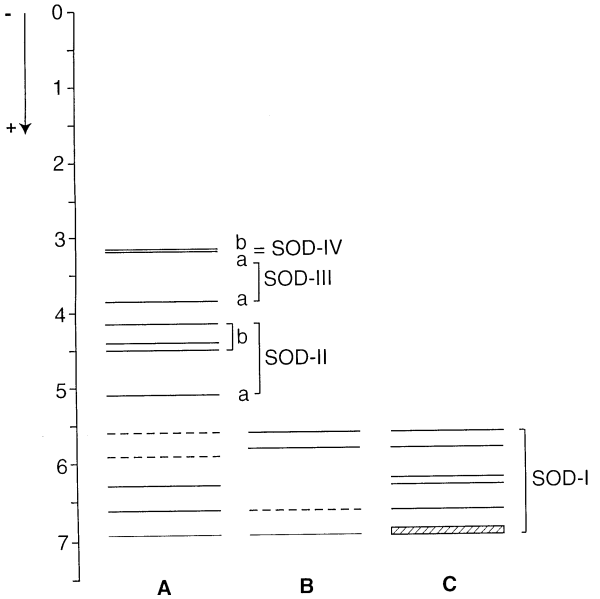

Fig. 1A–C Schematic representation showing the relative migration distance of SOD electrophoretic bands. For SOD-I, **A** and **B** denote the homozygous *aa bb* genotypes, while **C** denotes the heterozygous genotype *ab*

Results

Description of zymograms

Four zones of SOD activity were detected in the extracts of leaves, bark, cotyledons and flower buds and identified from the anode as SOD-I, SOD-II, SOD-III, and SOD-IV (Fig. 1). Two additional fast zones were detected from pollen extracts; one occasionally showed polymorphism, while the other showed no polymorphisms.

In the SOD-I zone three phenotypes were detected, A, B, and C, that were characterised by the presence of five, four and six bands, respectively.

Although the SOD-II zone showed polymorphism, discrimination was difficult with many bands appearing close together. The trailing band of this zone, SOD-IIb, was always present. In a few cases, after prolonged electrophoresis this band separated into three bands which showed polymorphism amongst cultivars.

One band position was observed in the SOD-III zone to consist of a major band accompanied by secondary bands. In some samples the band observed in the SOD-IV zone was slower or thicker than in others. After prolonged electrophoresis two bands, a and b, appeared in this position; these were more clearly resolved when cotyledons or leaves were sampled.

Genetic control

The segregation of bands in the SOD-I zone was recorded in 19 progenies (Table 1). These results are in agreement with a hypothesis that activity in this zone is determined by a single gene, *Sod-1*, with two alleles, *a* and *b*. When homozygous they determine phenotypes A and B, respectively. Plants heterozygous for *a* and *b* show the C phenotype. All three expected phenotypes were observed; phenotypes A and C correspond respectively, to phenotypes [1. 3. 5] and [1. 2. 3. 4. 5] described by Chevreau and Laurens (1987). In 3 progenies (F36, 49, F101 and F140), while all the expected phenotypes were observed, significant deviations from the expectation did occur. No significant departure from homogeneity was observed amongst either the two-class (*aa* : *ab*) segregations ($\chi^2 = 8.49$, 9 *df*) or the three-class (*aa* : *ab* : *bb*) segregations ($\chi^2 = 12.63$, 14 *df*). However, the combined segregations for each of these classes deviated significantly from expectation (198 *aa* : 153 *ab* $\chi^2 = 5.77$, 1 *df*, 64 *aa* : 144 *ab* : 50 *bb* $\chi^2 = 5.00$, 2 *df*). There was an excess of the *a* allele in these progenies. In contrast the only progeny which gave a *bb* : *ab* segregation (F140 'Glengyle Red' *Sod-1bb* × 3762 *Sod-1ab*) showed a significant excess of the *b* allele.

An intermediate or 'hybrid' band which was present in heterozygous (*ab*, phenotype C) plants suggests that SOD-I functions as a dimeric enzyme (Fig. 1). This hybrid band was not observed in zymograms prepared from pollen extracts.

The variable and unclear separation of the three bands sometimes associated with the b position in the SOD-II zone made it difficult to score these bands for genetic analyses.

Table 1 Segregations for *Sod-1*

Family	Parental genotypes	Progeny genotypes	Expected ratio	χ^2	P
F5, 6[a] ('Idared' × T31-12)	*aa* × *ab*	20*aa*:22*ab*	1:1	0.09	0.75
F18 ('Cox' × 'Fiesta')	*ab* × *aa*	5*aa*:8*ab*	1:1	0.69	0.40
F23 ('Vista Bella' × 'Katja')	*ab* × *aa*	4*aa*:4*ab*	1:1	0.00	1.00
F27, 29[a] ('Delprim' × 'Katja')	*ab* × *aa*	11*aa*:13*ab*	1:1	0.17	0.68
F36, 49[a] (Northern Spy' × 'Winter Majetin')	*aa* × *ab*	56*aa*:37*ab*	1:1	3.88	0.05*
F71 (A140-7 × A172-2)	*aa* × *ab*	12*aa*:7*ab*	1:1	1.31	0.25
F72 (604 × A853-1)	*ab* × *aa*	9*aa*:9*ab*	1:1	0.00	1.00
F135 ('Idared' × A679-12)	*aa* × *ab*	48*aa*:37*ab*	1:1	1.42	0.23
F101 (A723-6 × 'Jester')	*aa* × *ab*	22*aa*:8*ab*	1:1	6.23	0.01*
F69 (3759 × 'Baskatong')	*aa* × *ab*	11*aa*:8*ab*	1:1	0.47	0.49
F3 ('Cox' × A463-70)	*ab* × *ab*	2*aa*:16*ab*:4*bb*	1:2:1	4.90	0.09
F9, 10[a] ('Golden Delicious' × A463-70)	*ab* × *ab*	15*aa*:19*ab*:5*bb*	1:2:1	5.14	0.08
F12, 17[a] ('Cox' × T30-24)	*ab* × *ab*	4*aa*:17*ab*:7*bb*	1:2:1	1.93	0.38
F13 (T32-3 × 'Cox')	*ab* × *ab*	3*aa*:7*ab*:4*bb*	1:2:1	0.14	0.93
F41 ('Cox' × 'Baskatong')	*ab* × *ab*	12*aa*:35*ab*:9*bb*	1:2:1	3.82	0.15
F70 (A172-2 × A814-137)	*ab* × *ab*	7*aa*:14*ab*:7*bb*	1:2:1	0.00	1.00
F37 ('Granny Smith' × 'Kent')	*ab* × *ab*	14*aa*:23*ab*:8*bb*	1:2:1	1.62	0.44
F7, 8[a] ('Golden Delicious' × 'Cloden')	*ab* × *ab*	7*aa*:13*ab*:6*bb*	1:2:1	0.08	0.96
F140 ('Glengyle Red' × 3762)	*bb* × *ab*	9*ab*:20*bb*	1:1	4.17	0.04*

[a] Pooled data from reciprocal crosses
* $P < 0.05$

Table 2 Segregation for *Sod-3*

Family	Parental genotypes	Progeny genotypes	Expected ratio	χ^2	P
F11, 21[a] ('Idared' × 'Fiesta')	*an* × *nn*	18*an*:24*nn*	1:1	0.86	0.35
F12 (T30-24 × 'Cox')	*an* × *nn*	8*an*:6*nn*	1:1	0.28	0.59
F48 ('Jonathan' × 'Idared')	*nn* × *an*	17*an*:9*nn*	1:1	2.46	0.11
F140 ('Glengyle Red' × 3762	*an* × *nn*	19an:13nn	1:1	1.12	0.29
F633 ('Spencer Seedless' × 'George Carpenter')	*an* × *nn*	39*an*:29*nn*	1:1	1.47	0.23
F6 (T31-12 × 'Idared')	*an* × *an*	8 (*aa* + *an*)[b]:6*nn*	3:1	2.38	0.12
F101 (A723-6 × 'Jester')	*an* × *an*	17 (*aa* + *an*)[b]:4*nn*	3:1	0.40	0.53

[a] Pooled data from reciprocal crosses
[b] Genotypes within brackets not distinguished

Results shown in Table 2 accord with the hypothesis that activity in the a band of SOD-III is coded by an allele *a* of another gene, *Sod-3*, and a recessive null allele *n* which in the homozygous state does not show band a. In some progenies, band a was always present, which suggested that at least one of the parents was homozygous for the *a* allele. Plants without the SOD-IIIa band usually showed less activity in the SOD-IV zone position. This was apparent in progeny F11 ('Idared' × 'Fiesta') and its reciprocal F21 ('Fiesta' × 'Idared'). When band SOD-IIIa was observed in these progenies, it was accompanied by the SOD-IVb band, and when SOD-IIIa was absent the SOD-IVb band was also absent. However in another progeny F93 ('Jonathan' × A849-7), where neither parent carries *Sod-3a*, SOD-IVb was clearly represented in some seedlings. The segregation observed, 11 with band SOD-IVb and 8 without band SOD-IVb, suggests that a gene-determining activity in the SOD-IV zone is involved. In a few cases, where cotyledons were used, the two close migrating bands (a, b) were clearly resolved in the SOD-IV zone, and their segregation was recorded in 2 progenies as follows:

'Fiesta × 3762 ab × aa 57aa:48ab $\chi^2 = 0.77$ $P = 0.37$
'Fiesta × SA572-2 ab × aa 34aa:41ab $\chi^2 = 0.65$
$P = 0.42$

This was particularly clear in the Fiesta × 3762 progeny where both parents are homozygous for the *Sod-3n* allele, and the two *Sod-4* bands could be observed clearly in the absence of bands in the SOD-III zone.

These results suggest that a gene, *Sod-4*, codes for activity in the SOD-IV zone.

When gels were stained consecutively for SOD and PRX, both achromatic SOD and red PRX bands appeared in a blue background. Although PRX-IIIa and SOD-IIIa bands migrated to the same position, other

Table 3 *Sod-1* genotypes and presence (+) and absence (−) of SOD-IIIa band in apple cultivars

	Sod-1	SOD-IIIa		*Sod-1*	SOD-IIIa
'Akane'	*aa*	+	'Jester'	*ab*	+
'Andre Briollay'	*ab*	+	'Jonagold'	*ab-*	+
'Ashmead's Kernel'[a]	*ab-*	+	'Jonathan'	*aa*	−
'Beauty of Bath'	*ab*	−	'Jupiter'[a]	*ab-*	+
'Blenheim Orange'[a]	*aaa*	+	'Katja'	*aa*	−
'Bountiful'	*ab*	+	'Kent'	*ab*	−
'Bramley's Seedling'[a]	*ab-*	+	'Lane's Prince Albert'	*ab*	+
'Cloden'[a]	*ab-*	+	'Laxton's Superb'	*aa*	?
'Court Pendu Plat'	*ab*	−	'Leonie de Sonnaville'	*aa*	−
'Cox'	*ab*	−	'Lodi'	*bb*	−
'Crawley Beauty'	*bb*	+	'Louiton'	*ab*	+
'Delcorf'	*ab*	+	'McIntosh Starkspur'	*ab*	+
'Delprim'	*ab*	+	'McIntosh Wijcik'	*ab*	+
'Discovery'	*aa*	+	'Marie Joseph d'Othee'	*ab*	+
'Elstar'	*ab*	−	'Newton Wonder'	*aa*	+
'Early Victoria'	*aa*	−	'Northern Spy'	*aa*	+
'Edward VII'	*aa*	+	'Reinette Clochard'	*ab*	+
'Fiesta'	*aa*	−	'Reinette de France'[a]	*ab-*	+
'Falstaff'	*ab*	−	'Rhode Island Greening'[a]	*aaa*	+
'Gala'	*aa*	+	'Ribston Pippin'[a]	*ab-*	+
'George Carpenter'	*aa*	−	'Rome Beauty'	*bb*	+
'Gloster 69	*ab*	+	'Glengyle Red'	*bb*	+
'Golden Delicious'	*ab*	+	'Spartan'	*aa*	+
'Golden Noble'	*ab*	−	'Spencer Seedless'	*bb*	+
'Granny Smith'	*ab*	+	'Starkrimson'	*aa*	+
'Greensleeves'	*ab*	+	'Suntan'[a]	*ab-*	−
'Holstein'[a]	*ab-*	−	'Vista Bella'	*ab*	+
'Howgate Wonder'	*ab*	+	'Wagener'	*aa*	+
'Idared'	*aa*	+	'White Transparent'	*ab*	−
'Ingrid Marie'	*ab*	−	'Winter Majetin'	*ab*	+
'James Grieve'	*aa*	−	'Worcester'	*aa*	+

[a] Triploid

bands did not coincide, making it difficult to distinguish bands of the two zones.

Peroxidase genes *Prx-2* and *Prx-3* are closely linked ($r = 0.002$) (Manganaris and Alston 1992a). Tests for linkage between these two genes and *Sod-1* established a rather looser linkage between *Sod-1* and *Prx-2* ($r = 0.25 \pm 0.035$) and *Prx-3* ($r = 0.23 \pm 0.039$). No linkage was detected between *Sod-3* or *Sod-4* and *Prx-2* or *Prx-3*.

Distribution of SOD alleles in apple

The genotypes of 62 cultivars, 39 rootstocks and 31 *Malus* species and derivatives are presented in Tables 3, 4 and 5 respectively. All three possible phenotypes were observed in the SOD-I zone (Fig. 2) corresponding to genotypes, *Sod-1aa*, *Sod-1bb* and *Sod-1ab* with one exception. *Malus yunnanensis* showed a band which was faster than that corresponding to the *Sod-1a* allele. This fast band may be determined by a rare third allele, *c*. In all three groups of plants, the *Sod-1a* allele predominated in about 60% of the accessions (Table 6). The *Sod-3a* allele was absent from all the *M. baccata* varieties and derivatives except *M* × *robusta* Erecta.

Table 4 *Sod-1* genotypes and presence (+) and absence (−) of SOD-IIIa band in apple rootstocks

	Sod-1	SOD-IIIa		*Sod-1*	SOD-IIIa
M1	*ab*	+	M21	*aa*	+
M2	*ab*	−	M23	*bb*	+
M3	*ab*	+	M24	*ab*	+
M4	*aa*	+	M25	*aa*	+
M5	*bb*	+	M26	*ab*	−
M6	*ab*	+	M27	*ab*	−
M7	*ab*	+	MM101	*ab*	+
M8	*aa*	+	MM102	*ab*	+
M9	*aa*	+	MM104	*ab*	+
M10	*bb*	−	MM105	*ab*	+
M11	*ab*	+	MM106	*aa*	+
M12	*ab*	+	MM109	*aa*	+
M13	*bb*	−	MM110	*aa*	?
M14	*ab*	+	MM111	*aa*	+
M15	*ab*	+	MM112	*aa*	+
M16	*ab*	+	MM113	*aa*	+
M17	*bb*	+	MM115	*ab*	+
M18	*bb*	?	Merton 793	*aa*	+
M19	*aa*	+	Merton 789	*aa*	+
M20	*aa*	+			

Table 5 *Sod-1* genotypes and presence (+) and absence (−) of SOD-IIIa band in *Malus* species

	Sod-1	SOD-IIIa		*Sod-1*	SOD-IIIa
M. baccata flexilis	*bb*	−	*M. fusca*	*aa*	+
M. baccata 'Gracilis'	*ab*	−	*M. glaucescens*	*bb*	−
M. baccata jackii	*bb*	−	*M. hupehensis*	*aa*	+
M. × *arnoldiana*	*ab*	−	*M. hupehensis robusta* o.p. (3759)	*aa*	−
M. × *hartwigii*	*ab*	−	*M. hupehensis theifera*	*aa*	+
M. × *robusta* '*Erecta*'	*ab*	+	*M. prunifolia Locinea*	*ab*	−
M. × *robusta* o.p. (3760)	*ab*	−	*M.* × *purpurea* 'Aldenhamensis'	*aa*	+
M. × *robusta* o.p. (3762)	*ab*	−	*M.* × *atrosanguinea*	*ab*	−
M. brevipes (A 1535)	*ab*	−	*M.* × *moerlandsii*	*ab*	+
M. brevipes (A 1710)	*bb*	−	*M.* × *soulardii*	*ab*	+
M. coronaria 'Charlottae'	*aa*	+	*M. trilobata*	*aa*	−
M. × *platycarpa*	*aa*	+	*M. yunnanensis*	*ac*	+
M. × *floribunda*	*ab*	−	*M.* 'Baskatong'	ab	−
M. × *scheideckeri* 'Exzellenz Theil'	*ab*	−	*M.* 'Red Jade'	*bb*	?
M. × scheideckeri 'Hillieri'	*ab*	−	*M.* 'White Angel'	*aa*	−
M. × *florentina*	*aa*	−			

Table 6 Distribution of *Sod-1* genotypes and SOD-IIIa band amongst apple cultivars, rootstocks and *Malus* species

	Sod-1						SOD-IIIa	
	Numbers per genotype				Percentage of alleles		Presence	Absence
	aa	*ab*	*bb*	*ac*	*a*	*b*	(+)	(−)
Cultivars	20	37	5	–	62.1	37.9	42	19
Rootstocks	16	18	6	–	62.5	37.5	33	5
Malus species	10	15	5	1	58.0	40.3	10	20

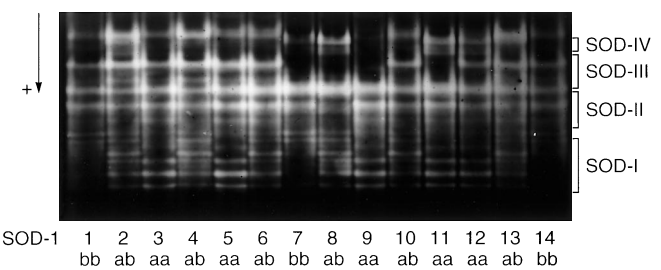

Fig. 2 Superoxide dismutase zymogram from flower bud extracts of fourteen cultivars (*left to right*) *1* 'Glengyle Red', *2* 'Reinette Clochard', *3* 'Northern Spy', *4* 'Vista Bella', *5* 'Akane' *6* 'Delprim', *7* 'Lodi', *8* 'White Transparent', *9* 'Jonathan', *10* Delcorf', *11* 'Leonie de Sonnaville', *12* 'Rhode Island Greening', *13* 'Winter Majetin', *14* 'Rome Beauty'. Note the absence of SOD-IIIa band in 'Lodi' (*7*), 'White Transparent' (*8*), 'Jonathan' (*9*) and 'Leonie de Sonnaville' (*11*)

Discussion

It was established that a single gene *Sod-1* with two alleles *a* and *b* controls activity in the SOD-I zone. Most crosses in the testcross mode (homozygote × heterozygote) and the F_2 mode (heterozygote × heterozygote) gave segregations close to expectation. However 1 *bb* × *ab*(F140) and 2 *aa* × *ab* (F30, 49 and F101) testcrosses had significantly distorted segregations, showing an excess of plants with the genotypes of the homozygous parents *Sod-1bb*(F140) *Sod-1aa*(F30, 49 and F101). It is possible that preferential fusion of gametes with common *Sod-1* alleles was responsible. Gametophytic selection of this nature has been observed in the segregation of other isoenzyme genes in apple (Manganaris and Alston 1987).

The polymorphism observed for SOD-I and SOD-III amongst cultivars, rootstocks and species was sufficient to establish the value of this enzyme as a support to the other enzyme polymorphisms predominant in cultivar discrimination, PRX (Manganaris and Alston 1993) and glutamate oxalacetate tranzaminase (GOT) (Manganaris and Alston 1989), each with a wide range of polymorphism. However, PRX and GOT cannot be used satisfactorily to discriminate between 'Greensleeves' and 'Falstaff', each derived from 'Golden Delicious' × 'James Grieve'. This can be achieved through SOD since 'Greensleeves' carries *Sod-3a*, which is not carried by 'Falstaff'. In a comprehensive study of isoenzyme analysis for the reliable identification of apple cultivars and rootstocks Manganaris (1989) established that efficient identification could be achieved through the application of only three highly polymorphic enzymes PRX, GOT and phosphogluconate

dehydrogenase (PGD), supported by the clearly recognised SOD-I and SOD-III bands. Rootstocks MM102 and MM106, which showed identical PRX genotypes, were distinguished through the *Sod-1* genotype. Further confirmation of the distinctiveness of SOD and PRX banding patterns derived from linkage studies where *Sod-1* appeared to be linked only loosely with *Prx-2* and *Prx-3*. In addition, the independent segregation of *Sod-3* and *Sod-4* from *Prx-2* and *Prx-3* suggests that these isoenzymes are not associated in apple.

Eleven triploid cultivars were included in this study (Table 3). When homozygous for a *Sod-1* allele the single band produced did not differ in intensity from that produced by a single allele. Similarly, when two allozymic bands were present no differences in intensity were observed between them, as might be expected when one allele is carried in duplicate and can be observed with some other enzymic genes, *Pgd-1* (Manganaris 1989), *Lap-1* (Manganaris and Alston 1992b), *Est-1* (Manganaris and Alston 1992c) and *Enp-1* (Manganaris 1989). Thus, the *Sod-1* locus is not suitable for the discrimination of polyploids.

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