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Genetics of the Peroxidase Isoenzymes in Petunia

Part 3: Location and Developmental Expression of the Structural Gene prxA

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Summary. As detected by starch gel electrophoresis, the fast moving anodal group of peroxidase isoenzymes, the PRXa complex, of a Petunia homozygous for the encoding gene can be made up of one to four bands, depending on the tissue sampled, the age of the tissue and of the plant, and the genetic background. Additional evidence is presented showing that the PRXa complex is encoded by one structural gene, prxA, rather than by tandem duplicated genes. On the basis of electrophoretic variation in *Petunia hybrida* and related species, five prxA alleles were found. A prxA internal site mutation was found recognized by the absence of recombination between the mutation that affected the temporal programme of the gene and the mutation that altered the mobility of the enzyme. By a three-point test, the gene prxA was located on chromosome III and found to be linked to the genes *Mfl* and *Htl* in the order prxA-*Mfl*-*Htl*. The construction of a trisomic III triply heterozygous for prxA confirmed the location of prxA.

Key words: Petunia – Peroxidase – Gene localisation – Developmental expression

Introduction

Electrophoretic analysis of the peroxidase isoenzymes in higher plants showed that the peroxidase system consists of a considerable number of isoenzymes (for reviews see Shannon 1968; Scandalios 1974).

For several higher plant species it was found that the peroxidases are encoded by several structural genes (Benito et al. 1980; Tanksley and Rick 1980; Pai et al. 1973; van den Berg and Wijsman 1981; Quiros and Morgan 1981). However, the considerable number of peroxidase isoenzymes in plant tissue may also be due to the production of more than one enzyme by one allele, as was shown for peroxidase loci in Tomato as well as Petunia (Rick et al. 1979; van den Berg and Wijsman 1981).

As to the fast-moving anodal group of peroxidases in higher plants, inheritance in a monogenic fashion and shifting of the group as a unit, as if the group were encoded by one gene, has been reported for a number of species. In the case of *Lycopersicon pimpinellifolium,* a shift of four bands, encoded by the gene Prx-2, has been mentioned by Rick et al. (1979). For *Curcubita pepo,* the existence of the Px_1^1 allele controlling a cluster of four bands was reported, whereas in Curcubita species the cluster was found to shift up or down relative to that of *Curcubita pepo* (Denna and Alexander 1975). Hirano and Naganuma (1979) described for Mulberry species two isoenzyme groups, consisting of three bands each, controlled by the codominant alleles Px_1^1 and Px_1^2. Finally, for the PRXa complex in Petunia, monogenic inheritance and shifting of a cluster of three bands was shown (van den Berg and Wijsman 1981).

Here, further evidence is presented suggesting that all PRXa bands are encoded by the structural gene prxA, based on analysis of differential allelic expression and on the discovery of new prxA alleles in Petunia species related to *P. hybrida.*

Materials and Methods

Plant Material

The following inbred lines were used: *P. axillaris:* S1, S2, S8; *P. inflata:* S6, S9, S10 (the origin of the *P. axillaris* and *P. inflata* inbred lines has been given elsewhere, van den Berg and Wijsman 1982); *P. hybrida:* V23, M1, W4 (derived from the cultivars 'Blauzwerg', 'Rose of Heaven', 'Pendula Cyanea', respectively), and V35, Vu6, R3 (lines of recombinant descent). For breeding triply heterozygous trisomics, and analysis of trisomic segregation for prxA, the trisomic III PY3003A-49, isolated by the late Dr. F. J. Smith (Institute of Genetics, Amsterdam), was used. Plants, grown from seeds of wild species of Petunia related to *P. hybrida* were studied to find new alleles of prxA: *P. inflata:* D580, from Mun. S. Lourenco do Sul, Brazil; D582, from Dep. Mburucuya, Prov.

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Corrientes, Argentina; *P. integrifolia:* D579, from Mun. Dom Pedrito, Brazil and D659, D660, from Arroio dos Ratos, Rio Grando do Sul, Brazil. The seeds were kindly collected and sent by Dr. T. M. Pedersen (D579, D582) and Sr. K. Hagelund (D659, D660).

Electrophoretic Analysis and Zymogram Scanning

Sample preparation, electrophoretic separation of the peroxidase isoenzymes using gel system I, and staining for peroxidase activity were essentially as described previously (van den Berg and Wijsman, 1981). The procedure was modified in that the electrode buffer was re-used several times. Though this influenced the mobility of the anodal moving peroxidases in a different extent relative to the tracking dye bromphenol blue, it improved the separation of the fast-moving anodal bands. Zymograms were scanned as described in van den Berg and Wijsman (1982).

Nomenclature of the Peroxidase Genes and Enzymes

As described previously the gene encoding the PRXa complex is termed prxA, and the alleles and enzymes are numbered, e.g., the allele prxA1 encodes the enzyme PRXa1.1 and the mozymes PRXa1.2, PRXa1.3, and PRXa1.4 (van den Berg and Wijsman 1981). An internal site mutation is defined as a cis-acting mutation of the prxA locus that bears the structural gene of which the expression in enzyme activity is affected, whereas an external site mutation is located at another locus (that can be separated by crossing-over). To detect internal site mutations the line R51 was taken as a reference, having the standard allele prxA1.

Determination of Flower Colour Gene Segregation

Determination of genotypes for *Mf1* and *Mf2*, methylation genes controlling substitution at the 5′ position of anthocyanins, and *Ht1*, a hydroxylation gene controlling substitution at the 3′ position of anthocyanins, was carried out as described by Wiering (1974).

Characterization of Trisomics III

Trisomics III can be recognized by their characteristic flower morphology: a longer tube and larger corolla compared to *P. hybrida* diploids (Maizonnier 1976). In addition, routinely chromosome counts were made as described by de Jong and de Bock (1978).

Results

Electrophoretic Variation of the PRXa Complex

The fast-moving anodal group of peroxidases in Petunia, the PRXa complex, can only be detected in green tissue and consists of one to four bands, even if the plant is homozygous for the encoding gene. The number of bands present depends also on the age of the tissue and the plant.

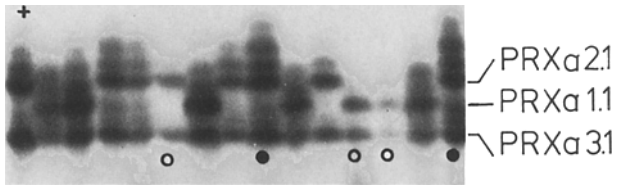

Fig. 1. Starch gel showing segregation for prxA involving the alleles prxA1, prxA2 and prxA3 in the progeny of (W4×S6)×S6 (genotype: prxA1/A2×prxA3/A3). Mature leaves of young flowering plants were analysed. Only symbols for the first bands of the PRXa complex are given. Lanes with open circles indicate leaves not yet having PRXa mozymes; filled circles indicate the presence of the third mozyme PRXa2.3

Amongst the collection of inbred lines of *P. hybrida* and related species three electrophoretic variants of the PRXa complex were found (Fig. 1). Nearly all of our *P. hybrida* inbred lines, representing most of the classical cultivars, are homozygous for prxA1; the only inbred line containing a different mobility allele is line V23, which is homozygous for prxA2. In *P. inflata* the alleles prxA2 and prxA3 can be found. By inbreeding the lines S6, S9 and S10 were obtained, which were originally heterozygous, containing both alleles. The lines S9 and S10 are now homozygous for prxA3 and S6 for prxA2. All three *P. axillaris* inbred lines, S1, S2 and S8 are homozygous for prxA1.

Developmental Expression of prxA in Mozyme Activity

In young leaves of young plants only the first band of the PRXa complex (the one with the lowest mobility) can be detected. Normally, two additional bands, equally spaced towards the anode, called the mozymes, come up during further development. The timing of mozyme synthesis seems to depend on the vitality of the plants since trisomy and inbreeding increases the appearance of the mozymes (van den Berg and Wijsman 1981). The following observations indicate that differences in the development of mozymes do not show simple Mendelian segregation.

First, in line S1 the second mozyme, PRXa1.3, cannot clearly be detected (cf. Fig. 5, van den Berg and Wijsman 1981), even in old leaves of plants that have flowered for several months. But in progenies of which S1 was one of the parents, the second mozyme was always clearly detected.

Second, until now two plants have been found that did not show any mozyme activity, even after they had flowered for one year. But among the progeny obtained by selfing mozyme activity was found.

Third, in some crosses a third mozyme (normally detectable by prolonged staining) can be detected in mature leaves (Fig. 1).

Differential Expression of prxA Alleles in Inbred Lines

Analysis of leaves of different age from young flowering hybrids between line V23 and other *P. hybrida* inbred lines (R51, M1, Vu6, R3) with genotype prxA1/A2 showed a higher expression level of the prxA2 allele in young tissue; in mature tissue the expression of both alleles is similar, whereas in old tissue the prxA1 allele shows a higher expression level (Figs. 2a, 4). In B1 and F2 progenies no recombination among 560 plants was observed between the prxA2 temporal programming mutation and the mobility mutation: in young tissue a higher activity of the PRXa2.1 enzymes was always found. There may be differences in the temporal programming of the prxA1 alleles from different *P. hybrida* inbred lines, but as yet they remain undetectable.

The prxA2 allele of line S6 shows a similar behaviour of differential expression as the prxA2 allele of V23 compared with the expression of the prxA1 allele. No recombination has been observed among 156 plants between the temporal programming mutation and the mobility mutation. Thus, we assume that the prxA alleles of V23 and S6 have the same internal site mutation.

The prxA3 alleles of the lines S6 (originally heterozygous prxA2/A3), S9 and S10 show a similar expression level throughout development of the plant with regard to that of the prxA2 allele of S6 and V23, although minor, but yet genetically undefined, differences in the temporal expression of the prxA2 and prxA3 alleles can be noted (Figs. 1, 2b, 4).

Concomitant Differential Expression of First Bands and Mozymes

At an early stage of development where differential allelic expression can be easily detected, it can be shown that the expression of the prxA2 mozymes is also influenced by the internal site mutation involved in the expression of the primary band encoded by prxA2. This can be observed in Figure 2a and 2c: a higher activity of the PRXa2.1 allozyme goes hand in hand with a higher activity of the PRXa2 mozymes. Also for the differential expression of the allomozymes no recombination between the internal site mutation and the structural gene mutation has been found.

The concomitant differential expression of first bands and allomozymes can also be observed in prxA1/A3 and prxA2/A3 heterozygotes (Fig. 2b, c). In addition to the monogenic inheritance of the PRXa complex and complex shifting by mutation of the structural gene, these results indicate that the PRXa complex is encoded by one gene.

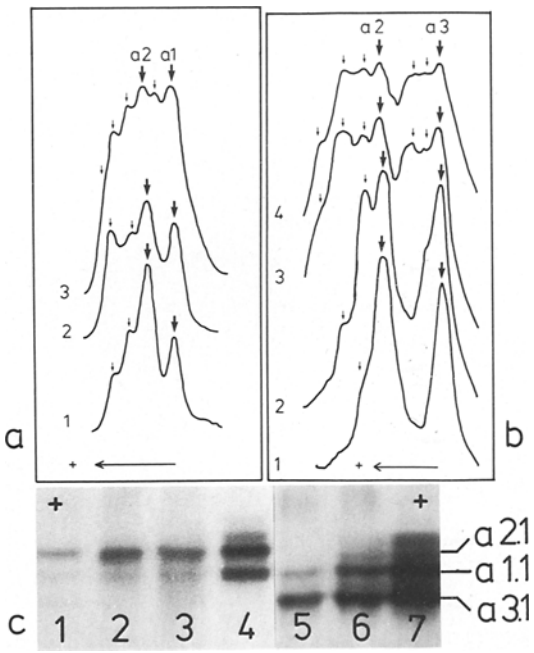

Fig. 2a–c. Zymogram scannings (**a, b**) and zymogram (**c**) showing the differential expression of the prxA1, prxA2 and prxA3 alleles in first bands and mozymes in a young (1, 5), an intermediate (2, 6) and mature leave (3, 4, 7; 4 older than 3) of young flowering plants. The small arrows indicate the presence of mozymes. **a** Differential expression of the alleles prxA1 (from trisomic III PY3003A-49) and prxA2 (from line V23). **b** Differential expression of the alleles prxA2 and prxA3 (from line S9). **c** Differential expression of the alleles prxA1, prxA2 and prxA3. 1–4, young to mature leave of a young flowering plant with genotype prxA1/A2; 5–7, young to mature leaves of a young flowering plant with genotype prxA1/A3. The leaves were taken from F1 (V23×R51) and from the F1 (R51×S6), respectively. Only symbols for the primary bands of the PRXa complex are given

Location of prxA by Linkage Analysis

From the analysis of a backcross (Vu6×V23)×Vu6, linkage of prxA with one of the two polymeric genes *Mf1* and *Mf2* can be deduced (Table 1). The genotype of line V23 as to the *Mf* genes was unknown, but in view of the segregation for *Mf* it can be concluded that V23 donated only one dominant *Mf* allele to the F1 (Vu6×V23) used, since Vu6 is homozygous and recessive for both *Mf* genes.

The gene *Mf2* has been located on chromosome V (Maizonnier and Moessner 1979), closely linked to the gene *Po* (Cornu et al. 1980). From the analysis of B1 and F2 progenies segregating for both *Po* and prxA there was no reason to assume linkage of the genes. Therefore, it can be assumed that the dominant gene involved in the B1 (Vu6×V23)×Vu6 was *Mf1*. The gene *Mf1* has been assigned to chromosome III on the basis of its linkage to the gene *Ht1*, at 10.1±2.4 map units (Wiering and de Vlaming 1977).

Table 1. Backcross segregation analysis showing linkage of *Mf1* and prxA.

Cross	B1 (Vu6 × V23) × Vu6		
Genotype	$\frac{\text{prxA1, } mf1}{\text{prxA2, } Mf1} \times \frac{\text{prxA1, } mf1}{\text{prxA1, } mf1}$		
Genotypes and number of progeny			
	prxA1/A1	prxA1/A2	
Mf1mf1	7	38	
mf1mf1	28	19	
Segregation chi square tests (df = 1)			
prxA	35 : 57	$\chi^2_{1:1} = 5.3$	$P = 0.02$
Mf1	45:47	$\chi^2_{1:1} = 0.04$	$P = 0.84$
Linkage chi square test (df = 1)			
$\chi^2_{2\times2} = 18.9$	$P < 10^{-3}$		

Table 2. Segregation for prxA, *Mf1* and *Ht1* showing linkage of the three genes

Cross	{(Vu6 × V23) × Vu6} × V35		
Genotype	$\frac{\text{prxA1, } mf1, Ht1}{\text{prxA2, } Mf1, ht1} \times \frac{\text{prxA1, } mf1, ht1}{\text{prxA1, } mf1, ht1}$		
Genotypes and number of progeny			
	prxA1/A1	prxA1/A2	
Ht1ht1Mf1mf1	0	6	
Ht1ht1mf1mf1	64	27	
ht1ht1Mf1mf1	27	58	
ht1ht1mf1mf1	6	1	
Segregation chi square tests (df = 1)			
prxA	97 : 92	$\chi^2_{1:1} = 0.13$	$P = 0.28$
Ht1	97 : 92	$\chi^2_{1:1} = 0.13$	$P = 0.28$
Mf1	91 : 98	$\chi^2_{1:1} = 0.26$	$P = 0.61$
Linkage chi square tests (df = 3)			
prxA-*Mf1*	27 : 70 : 64 : 28	$\chi^2_{1:1:1:1} = 33.4$	$P < 10^{-3}$
Mf1-*Ht1*	6 : 85 : 91 : 7	$\chi^2_{1:1:1:1} = 141.0$	$P < 10^{-3}$
prxA-*Ht1*	64 : 33 : 33 : 59	$\chi^2_{1:1:1:1} = 17.5$	$P < 10^{-3}$
Linkage			
prxA-*Mf1*	29.1 ± 3.3 cM		
Mf1-*Ht1*	6.9 ± 1.8 cM		
prxA-*Ht1*	34.9 ± 3.4 cM		

To carry out a three point test involving the genes prxA, *Mf1* and *Ht1*, a plant from the B1 (Vu6 × V23) × Vu6, with established genotype prxA1/A2, *Mf1mf1, mf2mf2, Ht1ht1,* was crossed to line V35, known to be homozygous and recessive for all three flower colour genes. The three point analysis of the progeny showed that the genes prxA, *Mf1* and *Ht1* are in that order

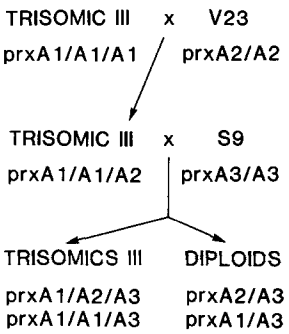

Fig. 3. Crossing programme for breeding the triple-banded trisomic III with genotype prxA1/A2/A3

located on chromosome III (Table 2). On the basis of both crosses mentioned in Table 1 and 2 combined, a recombination percentage of 28.8 ± 2.7 cM between prxA and *Mf1* was calculated.

Location of prxA by Use of Trisomics

To confirm the location of prxA on chromosome III, a triply heterozygous trisomic III was bred following the crossing programme as shown in Figure 3. The available trisomic III PY3003A-49 is homozygous for prxA1.

From the progeny of trisomic III PY3003A-49 × V23, a trisomic III with double-banded PRXa phenotype (as observed in young tissue) was isolated. An allelic dosage effect cannot properly be shown due to the differential expression of the alleles prxA1 and prxA2.

The trisomic III with assumed genotype prxA1/A1/A2 was crossed to S9. Among the progeny only one trisomic III was found, fortunately with genotype prxA1/A2/A3 (Fig. 4).

To analyse trisomic segregation for prxA and *Ht1* a trisomic III with genotype prxA1/A1/A2, *Ht1ht1ht1* was crossed to V23. The observed segregation for the genes prxA and *Ht1* among the diploid progeny seems to confirm the location of both genes on chromosome III (Table 3; see discussion).

Table 3. Trisomic segregation for prxA and *Ht1*

Cross	Trisomic III × V23			
Genotype	$\frac{\text{prxA1, } Ht1}{\text{prxA1, } ht1} \times \frac{\text{prxA2, } ht1}{\text{prxA2, } ht1}$			
Genotypes and numbers of diploid progeny				
prxA1/A2 : prxA2/A2 = 119 : 34		(exp: 2 : 1)		
Ht1ht1 : *ht1ht1* = 53 : 100		(exp: 1 : 2)		
Segregation chi square tests				
prxA	$\chi^2_{1:1} = 47.2$	$P < 10^{-3}$	$\chi^2_{2:1} = 8.5$	$P = 0.004$
Ht1	$\chi^2_{1:1} = 14.4$	$P < 10^{-3}$	$\chi^2_{1:2} = 0.12$	$P = 0.73$

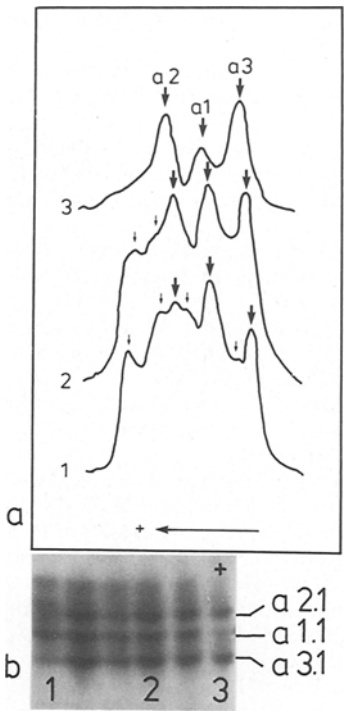

Fig. 4a and b. Densitometric scannings (**a**) and zymogram (**b**) showing the PRXa phenotype of the triply heterozygous trisomic III. A three month old cutting was analysed: 1, young leave; 2, intermediate leave; 3, old leave. Only symbols for the first bands of the PRXa complex are indicated; the small arrows indicate the mozymes. The densitometric tracings are taken from the corresponding number on the zymogram, which shows the analysis of young to old (from right to left) leaves of the three month old cutting

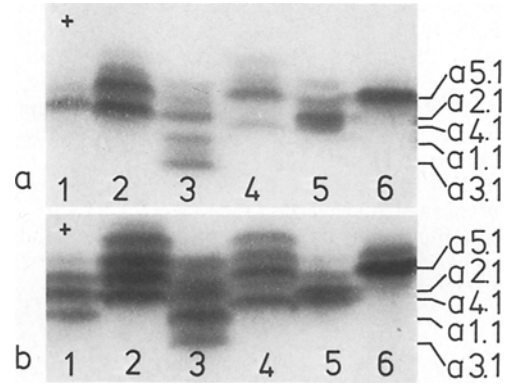

Fig. 5a and b. Starch gel showing the PRXa phenotypes of a triply heterozygous trisomic III and plants derived from seeds from South-America. **a** Young leaves of flowering plants; **b** mature leaves of flowering plants. (1) *P. axillaris* S2×*P. inflata* D580-2; prxA1/A2. (2) *P. integrifolia* D660-1; prxA2/A5 (3) trisomic III; prxA1/A2/A3. (4) *P. integrifolia* D659-3× *P. inflata* D582-1; prxA4/A5. (5) *P. inflata* D582-1×*P. integrifolia* D579-1; prxA2/A4. (6) *P. integrifolia* D659-1; prxA5/A5. Only symbols for the primary bands of the PRXa complex are given

The Alleles prxA4 and prxA5

Some populations of Petunia species from South America became available for further study. On the basis of electrophoretic variation two new alleles were found. Populations of *P. intergrifolia* from Brazil contained the alleles prxA2 and prxA5. A *P. inflata* population from Argentina contained three alleles, namely prxA2, prxA4 and prxA5 (Fig. 5).

Three features of the expression of the alleles prxA4 and prxA5 can be seen from Figure 5. First, just as for prxA1, prxA2 and prxA3, the alleles prxA4 and prxA5 form mozymes, equally spaced towards the anode, during the development of leaves. Second, the combinations A2/A5, A1/A5, A4/A5 and A2/A4 show differences in the activity of allozymes, especially in young tissue. Third, concomitant differential expression of first bands and mozymes can be observed.

Discussion

The structural gene prxA could be assigned to chromosome III by linkage tests, the isolation of a trisomic III with a triple-banded phenotype, and trisomic segregation. An analysis of telotrisomics and progeny of crosses with telotrisomics are now underway to determine a more precise location of the genes *Htl, Mfl* and prxA on chromosome III.

The segregation for prxA in the progeny of trisomic III×V23 fits the 2:1 segregation better than the 1:1 segregation (Table 3). However, there is still a significant deviation from the 2:1 segregation. In the case of prxA segregations a large deviation from the expected B1 and F2 segregation was often found. For instance, in the F2 (V23×R51) a segregation of 71:100:1 was found for prxA1/A1, prxA1/A2 and prxA2/A2, respectively. The deviation from the expected 1:2:1 segregation may be caused by certation, but linkage of prxA to deleterious mutations may also be involved. Although the homozygous tester was always used as the pollen parent, most B1 crosses gave distortions of the expected 1:1 segregation. The deviation of the prxA segregation in the diploid progeny of trisomic III×V23 (Table 3) from the normal backcross segregation might be explained by these observations. However, we never found such a large deviation among several B1 crosses with V23.

As to the high number of peroxidase isoenzymes in higher plants, two general origins can be envisaged. First, several structural genes can be involved in encoding the peroxidases, and second, one gene may encode more than one enzyme. With respect to the fast-moving anodal complex in Petunia, gene duplication must be considered. Several arguments stand against

the possibility. First, among progenies consisting of more than two thousand plants, no crossovers have been found between the assumed duplicated genes. Second, now that five allelic mobility variants have been found, we can say that mutation of the gene prxA, causing an altered mobility of the enzymes, involves shifting of the complex as a whole, which indicates that mutation of one gene is involved. Third, the expression of all enzymes encoded by one allelic gene are influenced by the same internal site mutation.

There may be analogous situations among a number of higher plant species with respect to the complexity and genetics of the fast-moving anodal group of peroxidases. Monogenic inheritance of the group as a unit as well as group shifting has been reported by several authors (Rick et al. 1979; Denna and Alexander 1975; Hirano and Naganuma 1979; van den Berg and Wijsman 1981).

Among the Solanaceae species investigated so far a number of similarities can especially be shown. The monogenic inheritance and group shifting has been reported for Tomato (Rick et al. 1979) and Petunia (this paper, van den Berg and Wijsman 1981). For both species the results presented argue against gene duplication. For Datura and Tobacco group shifting may be apparent from the figures given by Conklin and Smith (1975) and Sheen (1970), respectively. Finally, also for Potato a fast-moving three-banded group of peroxidases has been reported (Borchert and Decedue 1978).

The location and developmental expression of the gene prxA, and especially the differential expression of five mobility alleles may provide the material for a molecular study of the regulation of the expression of the gene prxA.

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