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Genetics of the Peroxidase Isoenzymes in Petunia

Part 1: Organ Specificity and General Genetic Aspects of the Peroxidase Isoenzymes

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Summary. The peroxidase system in Petunia can be separated into a considerable number of bands by starch gel electrophoresis. The electrophoretic pattern obtained is not only dependent on the genotype of the plant but also on the organ investigated, the state of development of that organ, and the age of the plant. Several structural and regulatory genes seem to be involved in the production of the peroxidase isoenzymes. In Petunia at least three structural genes can be distinguished by their independent segregation. It is suggested that a few more structural genes code for the peroxidase isoenzymes in Petunia. In addition, post-transcriptional modification may contribute to the number of peroxidase isoenzymes.

Key words: Petunia – Peroxidase – Isoenzymes – Post-transcriptional modification

Introduction

Isoenzymes of peroxidase have been reported in various Solanaceae:
Petunia (Hess 1967; Natarella and Sink 1975; Power et al. 1976), Potato (Borchert 1974), Tomato (Fobes 1980; Tanksley and Rick 1980), Datura (Conklin and Smith 1971), Tobacco (Sheen 1969, 1973; Smith et al. 1970; Bredemeijer and Blaas 1980; Mäder et al. 1977, 1980; Mäder 1980). In all these plants the peroxidases show variation in electrophoretic mobility, while the number and relative activities of the isoenzymes are specific for the tissue investigated. In Tomato, a thorough genetic analysis of the peroxidase system revealed that the peroxidase isoenzymes are coded for by at least six or seven structural genes (Tanksley and Rick 1980). The function of the peroxidase isoenzymes in higher plants is not yet well understood, although a few suggestions have been made in the literature. Among these, a role of the peroxidase system in the biosynthesis of lignin, production of hydrogen peroxide, biosynthesis of ethylene, metabolism of flavonoids, degradation of auxin, and resistance against microflora and toxic substances, are examples mentioned by several authors (Endo 1968; Sheen and Rebagay 1970; Seevers et al. 1971; Poovaiah 1974; Mäder et al. 1977, 1980; Halliwell 1978; Fielding and Hall 1978; Patzlaff and Barz 1978; Zmhral and Machackova 1978).

As an introduction to a comprehensive genetic analysis of peroxidase in Petunia, we give in this report a survey of the peroxidase isoenzymes, describing the variation and organ specificity as detected by starch gel electrophoresis. In addition we deal with general aspects of the genetics and function of peroxidase isoenzymes, and the molecular interpretation of the complex zymographic pattern of the peroxidase system.

Materials and Methods

Plant Material

The inbred line S2 has been derived from seeds of *P. axillaris* collected in the wild in Uruguay in 1958 (Bianchi, pers. comm.), and is now in its eighth generation of inbreeding. S2 was used to describe the organ specificity of the peroxidase isoenzymes in this paper. Other inbred lines from the Petunia collection of the Institute of Genetics were: S1, *P. axillaris*, received in 1954 from the Royal Botanic Gardens, Kew; S6, *P. inflata* and S9, *P. violacea*, sent by Dr. K.C. Sink in 1974; R51, V23 and M1, *P. hybrida*, derived from the cultivars 'Royal Ruby', 'Blauzwerg' and 'Rose of Heaven', respectively; and Vu6, *P. hybrida* of recombinant descent. Plants were grown from seeds of *P. cf. inflata*, collected in the wild in Prov. Corrientes, Argentina, and kindly sent by Dr. T.M. Pedersen.

Sample Preparation

Plant material was sampled the day before the electrophoretic experiment and stored at −20°C. Storage of the material, even for several days, had no influence on the peroxidase patterns. Plant tissue was homogenized in the cold with pestle and mortar. The following additions were used, the amount depending on the weight of the sample: acid washed quartz sand, insoluble polyvinylpyrrolidone and a few drops of a solution containing 1% NaCl and 0.02 M 1.4.dithioerythritol. After homogenization the homogenate was covered with tissue paper to prevent insoluble material

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to attach itself to the paper wick (Whatman 3 MM) laid on top of the tissue paper. The paper wicks were inserted in a slit in the starch gel within 30 minutes after homogenization.

Electrophoresis

Two gel systems were employed in the separation of the peroxidase isoenzymes. System I was modified after Siciliano and Shaw (1976) and system II was modified after Brewer (1970). System I consisted of a gel containing 12% starch, 0.05 M Tris, 0.0016 M Na_2EDTA and 0.05 M boric acid (pH 8.5) and electrode buffer containing 0.5 M Tris, 0.016 M Na_2EDTA and 0.65 M boric acid (pH 8.0). Electrophoresis was performed during 5-6 hours under a constant voltage of 400 V at 4°C. Gel system II consisted of a gel containing 14% starch, 0.015 M Tris and 0.004 M citric acid (pH 7.8) and electrode buffer containing 0.3 M boric acid adjusted to pH 8.1 with NaOH. For unambiguous identification of the peroxidase isoenzymes we used the mobility of the peroxidase bands relative to the borate front in system II. For reproducible results in relative mobilities it was necessary to develop a standard electrophoretic procedure: the Shandon Universal electrophoresis apparatus was used with gel size 0.5 × 18.5 × 10 cm and the paper wicks were inserted in the middle of the gel; electrophoresis was performed at 4°C under a constant current of 30 mA during 5-6 hours and was stopped when the borate front reached the anodic end of the gel.

Staining for Peroxidase Activity

After electrophoresis the gels were cut horizontally and stained for peroxidase activity in a solution containing 50 mg 3-amino-9-ethylcarbazole, 5 ml N,N,-dimethyl-formamide, 100 µl 2 M $CaCl_2$, 100 µl 30% hydrogen peroxide and 95 ml 0.05 M sodium acetate buffer, pH 5.0. After incubation in the cold for less than 30 minutes the staining was stopped by fixing the gel in 50% methanol.

Nomenclature of the Peroxidase Genes and Enzymes

As mentioned earlier the peroxidase isoenzymes will be given a number corresponding to their mobility relative to the migrational distance of the borate front using gel system II; e.g., PRX82. When after genetic analysis structural genes can be distinguished, the genes will be symbolized with three letters and a capital, and the alleles will be numbered in order of discovery, e.g., the structural gene prxA, the allele prxA1. The peroxidase isoenzymes can subsequently be designated through reversal of lower case and capital symbols of the corresponding allele: the allele prxA1 codes for the enzyme PRXa1 (≡ PRX82). When more than one enzyme band is coded for by one allele, the enzymes will be numbered arbitrarily as follows: PRXa1.1, PRXa1.2 etc.

Results

Electrophoretic Analysis of the Peroxidase Isoenzymes

Separation of the peroxidase isoenzymes in Petunia was carried out by starch gel electrophoresis using the two gel systems as described in the Materials and Methods section.

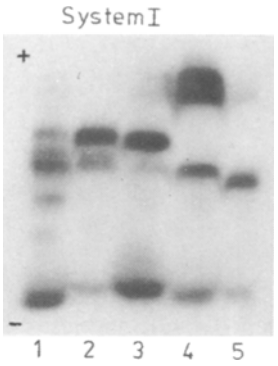

Fig. 1. Starch gel showing peroxidase isoenzymes in different organs of *P. axillaris* S2 analyzed with gel system I. (*1*) root; (*2*) flower limb; (*3*) flower tube; (*4*) old leave; (*5*) young leave

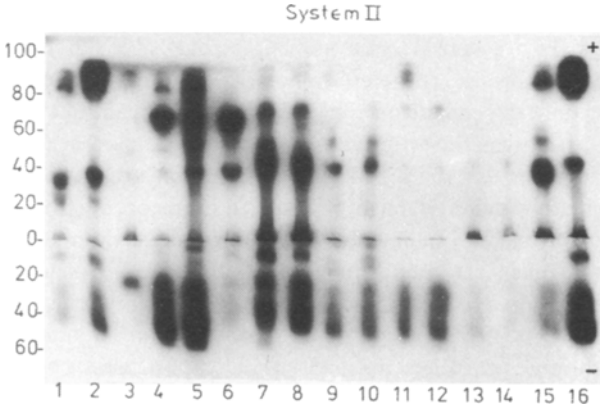

Fig. 2. Starch gel showing peroxidase isoenzymes in different organs of *P. axillaris* S2 analyzed with gel system II. *1* young leave; *2* old leave; *3* flower bud; *4* flower tube; *5* flower tube in wilted state; *6* flower limb; *7* young root; *8* old root; *9* young pistil; *10* old pistil; *11* young filaments; *12* old filaments; *13* young anthers; *14* mature anthers; *15* young stem; *16* old stem

Electrophoretic results for extracts of different organs of *P. axillaris* obtained with gel system I and II are shown in Figs. 1 and 2, respectively. The differential resolution of the peroxidase isoenzymes is apparent in the following observations: first, the cathodal isoenzymes are separated more clearly in system II; second, a slowly migrating anodal isoenzyme, which is specific for root tissue, could only be detected in system I; and third, the anodal PRX22 was only detected in system II (also compare Fig. 4a, b).

Gel system I was used to analyze the fast moving anodal group of isoenzymes around position 86 because it allowed better visualization. Since system II gave better results as to the number and distinctness of bands, it was used for the other electrophoretic separations presently reported on, and to designate the enzymes corresponding to their relative mobility.

Organ Specificity of the Peroxidase Isoenzymes

In the present report the peroxidase isoenzymes are described as found in different organs of full grown plants of *P. axillaris* S2, but the situation is representative for the organ specificity of the peroxidase isoenzymes in all species of Petunia investigated. Figures 1 and 2 show the electrophoretic patterns obtained by electrophoresis of crude extracts of different parts of the plant. Two featuring anodal bands, PRX65 and PRX75 (Fig. 2), are specific for flower and root tissue, respectively, and absent from leaves and stems.

PRX65, the flower specific enzyme, is only present in a few of our various inbred lines of *P. hybrida* and these were all derived from the cultivar 'Pendula Cyanea'. At present, in material of the *P. violacea/inflata* complex, inbred lines as well as wild material, the flower specific band has only been found in two inbred lines of *P. violacea*, among these S9. Rather complex is the situation for the anodal triplet around position 86, which can only be detected in green tissue (see also Figs. 3, 4, 5). In young plants before flowering, only the slowest band is apparent. Upon aging, the two faster moving bands appear, but first in older tissue (cf. Fig. 4a). Only in plants that have flowered for months, with a high degree of lignification, can they also be detected in young tissue. The genetic background may play a role, but vitality of the plants seems to be a more important factor, as illustrated by the much earlier appearance of additional bands in trisomics and inbred lines than in F1 hybrids between inbred lines.

In general, as can be seen from the pattern in Figure 2, the organ specificity is twofold. First, differences in the number of bands between organs can be detected, and second, changes in relative intensity of bands. These two types of variation can also occur as a consequence of aging of organ and/or plant. Both types of organ specificity, temporally and spatially determined, have been reported by others (Scandalios 1974).

Possibly these features of the peroxidase system reflect its functioning in a multitude of biochemical processes in higher plants, and it suggests that several structural and regulatory genes might be involved in the production of all peroxidase isoenzymes.

Genetic Interpretation of the More Prominent Bands

Our search for structural genes that code for the peroxidase isoenzymes in Petunia started with studying those coding for the major bands that can be detected in mature leave extracts. Figure 3 shows a composite drawing of the more prominent isoenzymes present in leaves of selected inbred lines and hybrids. So far we have detected three variants of the anodal triplet (Fig. 5), three variants of the

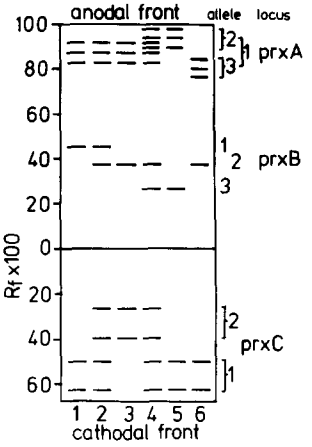

Fig. 3. Composite drawing showing the more prominent peroxidase isoenzymes present in mature leaves. (*1*) *P. axillaris* S1; (*2*) F1 S1 × S2; (*3*) *P. axillaris* S2; (*4*) F1 S2 × S6; (*5*) *P. inflata* S6; (*6*) *P. violacea* S9. The R_f value indicates the mobility relative to the migrational distance of the borate front

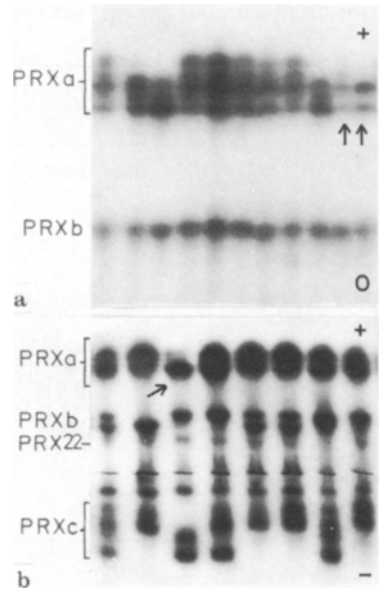

Fig. 4a and b. Starch gels showing segregations for prxA, prxB and prxC. a Segregation for prxA in B1 (Vu6 × V23) × Vu6, analyzed with system I in the period of flowering. Genotype: prxA1/A2 × prxA2/A2. b Segregation for prxB and prxC in F2 S1 × R51, analyzed with system II. Genotype: prxB1/B1, prxC1/C1 × prxB2/B2, prxC2/c2.

The juvenile types not yet having the PRXa mozymes are indicated by arrows

anodal group of isoenzymes with moderate mobility, the allozymes PRX28, 36 and 44, and two variants of the cathodal doublet (Fig. 4).

From F1 analysis it can be concluded that all peroxidase variants listed in Fig. 3 are monomers, and that the genes which code for the enzymes are co-dominant. All segregations are shown to be monohybrid, including the triplet and doublet segregations. This suggests that, if

Table 1. Linkage analysis of prxB and prxC showing independent segregation

Cross: S1 x R51 Genotype: prxB1/B1, prxC1/C1 x prxB2/B2, prxC2/C2	
PRX phenotypes	Number of progeny
b1/b1+b1/b2, c1/c1+c1/c2	111
b1/b1+b1/b2, c2/c2	48
b2/b2, c1/c1+c1/c2	33
b2/b2, c2/c2	13
Segregation chi square tests	
prxB $X^2_{3:1} = 0.72$	$P = 0.39$
prxC $X^2_{3:1} = 2.47$	$P = 0.12$
Test on independent segregation	
$X^2_{9:3:3:1} = 3.31$	$P = 0.35$

Table 2. Linkage analysis for prxA and prxC showing independent segregation

Cross: (M1 x V23) x V23 Genotype: prxA1/A2, prxC1/C2 x prxA2/A2, prxC2/C2	
PRX Phenotypes	Number of progeny
a2/a2, c2/c2	29
a2/a2, c1/c2	22
a1/a2, c2/c2	19
a1/a2, c1/c2	24
Segregation chi square tests	
prxA $X^2_{1:1} = 0.68$	$P = 0.41$
prxC $X^2_{1:1} = 0.04$	$P = 0.95$
Test on independent segregation	
$X^2_{1:1.1:1} = 2.25$	$P = 0.52$

more than one structural gene codes for the triplet or doublet of bands, such genes have to be closely linked (Table 1, 2; Fig. 4). As described later on, we presume that only two genes, named prxA and prxC, code for the triplets and doublets, respectively.

The gene that codes for the triplets, prxA, and the structural gene that codes for the other prominent anodal group of enzymes in leaves, prxB, have been located on different chromosomes (van den Berg and Wijsman, in prep.).

The gene coding for the cathodal doublets, prxC, shows independent segregation in regard to prxA and prxB (Table 1, 2; Fig. 4). Therefore, we can conclude that at least three structural genes are involved in the production of the major peroxidase isoenzymes in Petunia.

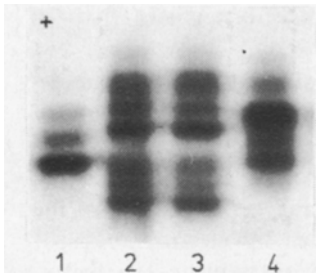

Fig. 5. System I starch gel showing the three PRXa triplets. (*1*) *P. axillaris* S1; (*2*) and (*3*) F1 *P. inflata* × *P. violacea*; (*4*) *P. hybrida*, line R51

One Allele – More Enzymes

As shown in Fig. 3 we detected three allelic triplets of enzyme bands that migrate towards the anode. It is more likely that the triplet shifts (Fig. 5) are caused by one mutation in one structural gene than by three mutations in three tightly linked structural genes. We suppose that post-transcriptional modification takes place; whether pre- or post-translational processing is involved remains to be investigated. Therefore, we conclude that one structural gene codes for the fast migrating anodal group of enzymes: prxA. Observations supporting the hypothesis are the following:

1. Young plants, homozygous for prxA and not yet flowering only show the slowest band of the PRXa triplet. This suggests that the two faster migrating bands of the triplets can be regarded as processed forms of the slower ones. With regard to the two faster moving bands in relation to the slower moving band, we propose to speak of mozymes, alluding to some form of modificational process. They can be designated as allomozymes when compared with allelic modification products of other triplets. It is interesting that in mature flowers, even on an old plant, the primary band is found but not the mozymes (cf. Fig. 2).

2. In B1 and F2 segregations only triplets and hexaplets were observed in a total of 1042 plants, which suggests that, if three genes were involved, they would have to be very highly linked.

Similar arguments can be applied to the cathodal doublet in which one of the two bands is suspected to be a mozyme since in crosses in which the doublets segregate no crossovers have been detected in a total of 534 plants.

Discussion

On the basis of isoenzyme patterns as observed in different species and cultivars of Petunia and in progeny of crosses between plants with different genotypes, it can be con-

cluded that only a limited number of genes is responsible for the considerable number of peroxidase isoenzymes in certain parts of the Petunia plant, even if the plant sampled is homozygous for the peroxidase genes.

The results of other authors (Power et al. 1976) seem to fit the pattern as established in the present paper. Minor differences can be the result of using other gel systems. As a consequence of the different ionic nature of the various gel systems, a band formed by the same enzyme in the one system may occupy a different position in another. The position of a peroxidase band, irrespective of the gel system used, could potentially be shared by more than one enzyme. Allelic replacement of bands may prove that one band represents one protein.

The observation that 'mozymes' are found only in older plants together with the fact that they seem to co-migrate with the more primary gene product, provides evidence for their post-transcriptional modification. In fact, there is no reason to assume the existence of three linked genes, responsible for the three bands of the triplets, as long as no isoenzyme patterns of crossover type have been found.

We investigated the two putative parent species of *P. hybrida*, viz., *P. axillaris* and *P. violacea*. In 1911, Fries described as *P. inflata* what he calls 'a geographic species locally replacing' *P. violacea* in inland areas. When we consider our inbred lines S2, S9 and S6 as representative for the three parental species and compare them with *P. hybrida* cultivars, their different alleles for prxA, prxB and prxC can be found in other combinations. But they seem to be subject to the same process of modification. The nature of the process is a point of future investigation.

Apart from the conspicuous bands specific for flower and root we can say that only three structural genes are responsible for the more prominent bands: prxA, prxB and prxC. In addition to these major bands, a number of minor bands could be detected. The localization of the genes – or gene– that code(s) for the root and flower specific major bands and for the minor bands is currently under investigation.

Rick et al. (1979) suggests that in Tomato post-translational modification is responsible for the production of the co-migrating combinations of bands. The cases in Tomato involve quadruplets rather than triplets. However, in Petunia, from time to time we found traces of a fourth enzyme in the PRXa group, which was the fastest of all (cf. Fig. 5). In one plant of *P. inflata* grown from seed collected in Argentina the four bands were always detected. The situation might then be homologous to the Tomato case.

Mäder and coworkers (1977, 1980) investigated the molecular origin of the fast migrating anodal group of peroxidase isoenzymes in Tobacco, which they call Group I. Group I is possibly homologous to the PRXa isoenzymes of Petunia, in view of the inducible character of the mozymes and the similar mobility. Mäder et al. (1977) found that the Group I isoenzymes have a broad substrate specificity and higher affinities for artificial substrates than the other peroxidase isoenzymes in Tobacco. As a function they put forward resistance against toxic substances and a role in lignin synthesis. The increase of PRXa activity during aging and the absence of mozymes in young tissue are not contradictory to the suggestion.

The organ specificity of the peroxidase isoenzymes found in Petunia seems to point to the action of several structural and regulatory genes. These differential gene activities of the peroxidase system provide the basis for an investigation of the regulation of the activity of the peroxidase isoenzymes in Petunia. The availability of inbred lines with qualitative differences as described and in addition quantitative differences (unpublished results) offers excellent material for such a study. The present contribution can be considered a basis for future publications on the genetics and biochemistry of the peroxidase enzyme system in Petunia.

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