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Genetics of the peroxidase isoenzymes in *Petunia*

9. Immunological investigation into differential expression of *prxA* alleles

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Summary. Antibodies were raised against the peroxidases encoded by the allele *prxA1* to determine the specific activities of the peroxidases encoded by the alleles *prxA1, prxA2, prxA3,* and *prxA5*. The results from double diffusion experiments indicated that all peroxidases encoded by the four alleles are antigenically identical. By rocket immuno electrophoresis it was shown that the peroxidases encoded by the alleles *prxA1, prxA2, prxA3,* and *prxA5* have different specific activities. The results presented are discussed in relation to differential expression of the alleles involved.

Key words: *Petunia* – *prxA* alleles – Specific peroxidase activities

Introduction

In previous papers we have described differential temporal expression of five *prxA* alleles that can be distinguished by differences in electrophoretic mobility of the encoded peroxidases (van den Berg and Wijsman 1982; van den Berg et al. 1983). The temporal character of the differences indicates that different quantities of the allozymes are present during development of the plants. The differences in allozyme activity might be caused by differential transcription of the alleles involved, but post-transcriptional events (e.g., processing of RNA, synthesis of the protein) may also be involved (Scandalios 1979).

The present investigation was carried out to determine whether, in addition to differences in quantity of the allozymes, possible differences in specific activity also contribute to the temporal differences in allozyme activity which were described previously (van den Berg et al. 1983).

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Materials and methods

Plant material

The inbred lines used are listed in Table 1. The origin of the *P. axillaris s.l* and *P. integrifolia s.l.* lines has been given earlier (van den Berg and Wijsman 1982).

Purification procedures

PRXa1 was purified as described by van den Berg and van Huystee (1984) from stem tissue of old flowering plants from the progeny of the cross (W115 × Vu6) × Vu6.

Partial purification of the PRXa variants was carried out to obtain PRXa fractions that are devoid of peroxidases other than PRXa. Leaf tissue was ground in a mortar with a pestle and a little sand. The homogenate was centrifuged at 7,000 g for 10 min, and the protein in the crude extract was precipitated in 80% acetone. The protein was resolubilized in 0.01 M Tris-HCl, pH 8.0, and layered onto a DEAE-cellulose column (0.5 × 3 cm) equilibrated with the same Tris buffer. After washing the column with 10 column volumes of Tris buffer, the partially purified PRXa could be eluted with 0.1 M NaCl in Tris buffer. Absence of other peroxidases in this fraction was shown by starch gel electrophoresis using gel system II (van den Berg and Wijsman 1981).

Separation of the triplet peroxidases was carried out by starch gel electrophoresis (gel system I, van den Berg and Wijsman 1981) of the pure PRXa1 triplet. After staining the gel, the three bands were cut from the gel and squeezed through a glasswool stoppered syringe. The gel suspension was centrifuged at 7,000 g for 5 min, and the supernatant was used for the assay.

The peroxidase assay and the determination of the concentration of pure peroxidase was carried out as described by van den Berg and van Huystee (1984).

Antiserum production

A new Zealand White rabbit was immunized by injecting it three times (in three weeks time) with about 130 μg pure PRXa1. Inoculations were made subcutaneously at four different sites on the animal. Blood was tapped from the marginal ear vein 28 days after the first inoculation.

Immunological experiments

Double diffusion analysis in agarose gels was performed as described by Ouchterlony and Nilsson (1978). PRXa and antibodies (applied to 6 mm wells) were incubated for 2 or 3 days at room temperature in a medium of 1% agarose in 75 mM Tris, 25 mM diethylbarbituric acid buffer, pH 8.6, containing 0.5 mM Ca-lactate and 3.5 mM NaN_3. The gels were washed for 3 days, stained for peroxidase (van den Berg and Wijsman 1981), and dried.

Rocket immuno electrophoresis was carried out essentially as described by Laurell (1966). To 1% agarose in standard Tris-barbituric acid buffer heated to 55 °C, 0.1% antiserum was added. The gel was poured onto a 12×12 cm glass plate. Samples were added to 6 mm wells. Electrophoresis was carried out at 5 °C for 16 h at 150 V. After electrophoresis the gel was stained for peroxidase, washed, and dried.

Results

Purification of PRXa1

PRXa1 was purified from the progeny of the cross (W115×Vu6)×Vu6. The pure PRXa1 obtained, containing all three triplet bands, had a specific activity of 51 μkat/mg. This is in good agreement with the value of 47 μkat/mg found for pure PRXa1 isolated from the progeny of (W115×R4)×W15 (van den Berg and van Huystee 1984).

Antiserum specificity

Double diffusion of the three speparate triplet bands with the PRXa1 antibodies showed complete fusion of the different precipitation lines, without spur formation (Fig. 1a). This indicates that the three PRXa1 triplet peroxidases in the precipitates exhibite the same antigenic specificity towards the PRXa1 antibodies. This was confirmed by the fact that double diffusion of the three PRXa1 peroxidases with the antibodies gave rise to one precipitation line, and that rocket immuno electrophoresis of the pure PRXa1 triplet resulted in the formation of only one rocket (Figs. 1b and 2). Using the same technique it was shown that the three peroxidases of the PRXa1 triplet have the same specific activity (data not shown).

The results from double diffusion experiments with several triplet variants (Fig. 1b) suggest that variants tested share the same antigenic determinants. It was therefore possible to quantitate, and, consequently, to determine the specific activities of the triplet variants by rocket immuno electrophoresis.

Specific activity of PRXa variants

To determine the specific activities of the PRXa variants, partially purified fractions were subjected to rocket immuno electrophoresis using pure PRXa1 (51 μkat/mg) as reference material. The specific activities could be determined by dividing the peroxidase activities present in the applied fractions by the quantity of protein found by measuring the height of the rockets. The specific peroxidase activities calculated are presented in Table 1. In addition to line R51, carrying the standard *prxA1* allele, several other lines with genotype *prxA1/A1* as well as lines homozygous for *prxA2*, *prx-A3*, and *prxA5* were analysed. The results presented show small, but significant differences in specific activities between the various allozymes tested.

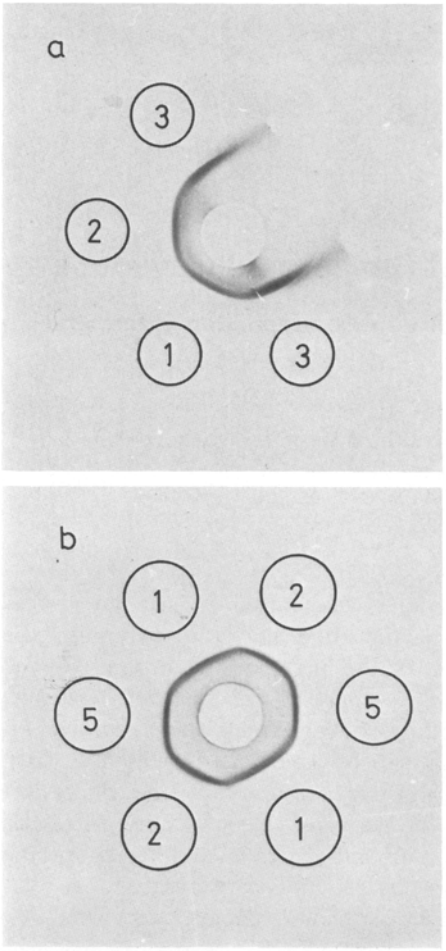
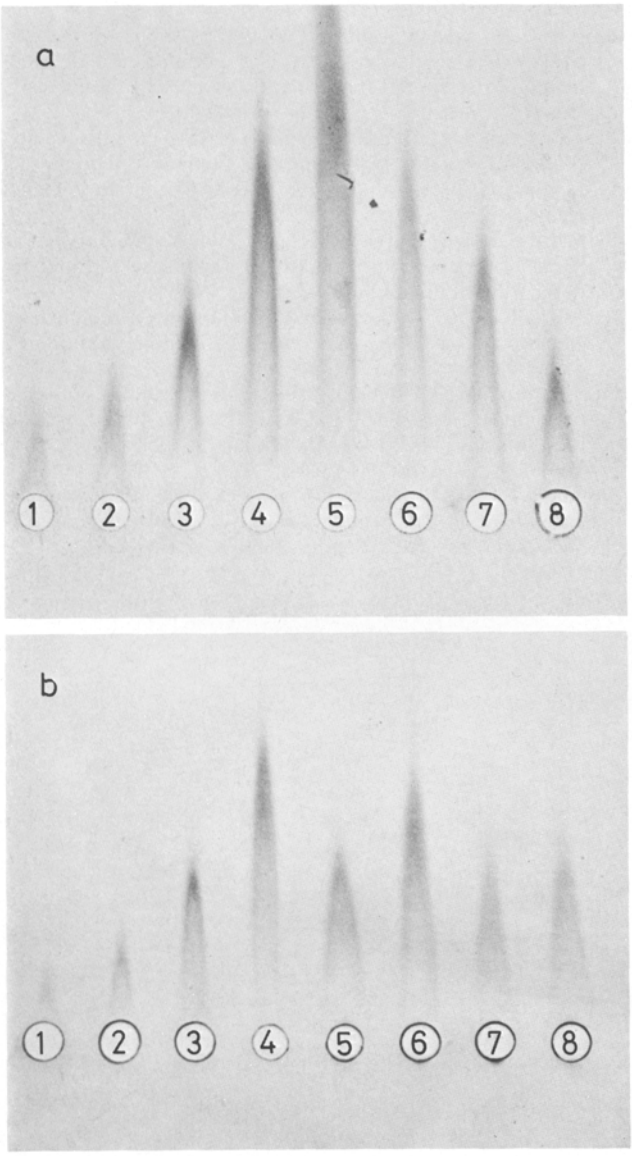


Fig. 1 a, b. Ouchterlony double diffusion gels stained for peroxidase activity showing serological similarity of all PRXa enzymes tested. In the central hole 50 μl of a 1:10 dilution of antiserum was applied. **a)** Double diffusion of the three triplet bands separately. The number indicates PRXa1.1 *(1)*, PRXa1.2 *(2)*, and PRXa1.3 *(3)* from the progeny of the cross (W115×Vu6)×Vu6 *(1)*: 18.3 nkat, *(2)*: 18.9 nkat, *(3)*: 9.0 nkat peroxidase. **b)** Double diffusion of PRXa variants. *(1)* 11.7 nkat PRXa1 from R51, (2) 15.3 nkat PRXa2 from V23, *(5)* 47.2 nkat PRXa5 from S12.

Table 1. Specific activities of PRXa variants as determined by rocket immuno electrophoresis

Line	Cultivar/species	Genotype	SA[a] ± SD[b]	n[c]
R51	'Royal Ruby'	*prxA1/A1*	62 ± 6	(8)
R4	'Admiral', 'Rose of Heaven'	*prxA1/A1*	51 ± 1	(3)
W115	'Rose of Heaven', *P. axillaris s. s.*	*prxA1/A1*	46 ± 6	(4)
W15	'Fire Chief'	*prxA1/A1*	56 ± 6	(3)
Vu6	'Rose of Heaven', 'Fire Chief'	*prxA1/A1*	61 ± 3	(3)
V23	'Blauzwerg'	*prxA2/A2*	51 ± 6	(4)
S9	*P. integrifolia* ssp. *inflata*	*prxA3/A3*	40 ± 1	(5)
S12	*P. integrifolia* ssp. *integrifolia*	*prxA5/A5*	42 ± 2	(4)

[a] SA – specific peroxidase activity in μkat/mg
[b] SD – standard deviation
[c] n – number of determinations



Discussion

The present paper deals with the specific activities of *PRXa* mobility variants in relation to differential temporal expression of *prxA* alleles. The immunological experiments to determine specific peroxidase activities were carried out with crude extracts of old leaf tissue. Young tissue may contain peroxidases that have a higher electrophoretic mobility than the PRXa enzymes. These peroxidases were supposed to be precursors of the PRXa enzymes (van den Berg et al. 1983). The precursors could not be separated from the PRXa enzymes by DEAE-cellulose chromatography, as was the case for all other peroxidases present in leaf tissue. Preliminary experiments indicated that the precursors can react with the PRXa1 antibodies, but their specificity is less compared to PRXa1. This observation supports the hypothesis that these peroxidases are indeed precursors. This also means that young tissue cannot be used in determining specific peroxidase activity by rocket immuno electrophoresis. Old leaf tissue can be used since it is virtually devoid of precursors (possibly due to the much higher PRXa activity, the precursors can barely be detected).

The specific activities of peroxidase variants could only be determined properly provided the variants were antigenically identical. That this was the case was shown

Fig. 2 a, b. Rocket immuno electrophoresis of pure PRXa1 from the progeny of (W115 × Vu6) × Vu6, and partially purified fractions from several inbred lines. **a)** × 1–5: pure PRXa1, 0.29–0.58, 1.15, 2.31, and 4.62 nkat, respectively; 6: 1.89 nkat PRXa5 from line S12; 7: 1.53 nkat PRXa2 from line V23; 8: 1.17 nkat PRXa1 from line R51. **b)** *1–4*: same as in **a)** *5*: 1.44 nkat PRXa1 from line Vu6; *6*: 1.71 nkat PRXa1 from line W115; *7*: 1.35 nkat PRXa1 from line W15; *8*:– 1.17 nkat PRXa1 from line R4

by the double diffusion experiments. All three PRXa triplet peroxidases appeared to be antigenically identical. Since the precipitation lines from the different PRXa triplet variants were completely fused with the PRXal antibodies, we can also conclude that, in the case of PRXa variants other than PRXal, the three triplet bands are antigenically identical.

The fact that all three triplet bands share the same antigenic determinants, and the fact that they have the same specific activity – only proved in the case of the PRXal peroxidases but probably also true for the other triplets – supports the hypothesis that the PRXa triplets are encoded by the same gene (van den Berg and Wijsman 1981, 1982).

From the differences in temporal expression of the *prxA* alleles described earlier (van den Berg et al. 1983), one could speculate that during the initial stage of development different quantities of the allozymes are present; at the final stage of development, the differences in allozyme activities would then be caused by differences in specific activities (see Fig. 9 in van den Berg et al. 1983). In that case, PRXal should have a specific activity two to three times as high as the other allozymes, for at the final stage of development the PRXal activity is two to three times higher. However, the slightly higher value found for the specific activities of PRXal (Table 1) can not account for this difference. At the final stage of development there are no clear differences in the activities between PRXa2, PRXa3, and PRXa5, which contrasts with the difference between the specific activity of PRXa2 (51 μkat/mg) on the on hand, and that of PRXa3 (40 μkat/mg) and PRXa5 (42 μkat/mg) on the other hand. These data show that, in addition to differences in quantities between the allozymes, differences in specific activities also determine the balance of allozyme activity. It follows that both mutation of the structural as well as the regulatory part of the gene are involved.

The results from immuno electrophoresis of a crude extract, followed by double diffusion of the separated peroxidases and PRXal antibodies indicated that all peroxidases other than PRXa do not react with the PRXal antibodies. This means that further research can be carried out with the different mutants that were described earlier; first at the RNA level, and, subsequently, at the DNA level. In that way we may establish on what level of gene expression the temporal programming mutations interfere.

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