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Genetics of the Peroxidase Isoenzymes in Petunia

Part 2: Location and Developmental Expression of the Structural Gene prxB

B. M. van den Berg and H. J. W. Wijsman
Institute of Genetics, University of Amsterdam, Amsterdam (the Netherlands)

Summary. Three electrophoretic variants of the peroxidase b isoenzymes in Petunia have been found. The encoding gene prxB is shown to be located on chromosome I by its linkage with the gene *Hf1*. Analysis of prxB heterozygotes showed a gradual increase of the electrophoretic mobility of all three PRXb allozymes during development and differential expression in enzyme activity of three prxB alleles. The location of prxB on chromosome I was confirmed by an allelic dosage effect in trisomics I, trisomic segregation and the construction of trisomics I with triple-banded PRXb phenotype. From telotrisomic analysis it was concluded that prxB and *Hf1* are located on the same arm of chromosome I. The unexpected linkage of prxB and *Hf1* with the gene *F1* in one of the crosses was suggested to be caused by a translocation in line S1, involving the gene *F1*.

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

Introduction

In a first paper dealing with the genetics of the peroxidase isoenzymes in Petunia (the garden Petunia, *Petunia hybrida,* as well as other related species) the existence of three genes coding for a number of the peroxidases was reported (van den Berg and Wijsman 1981). Here we report the location of prxB, the gene coding for the PRXb enzymes.

Using the zymogram technique, structural genes can be located in several ways. In addition to the mapping of genes by linkage analysis, the study of aneuploids carrying an aberrant number of alleles has been used for locating genes of several higher plants (e.g. Wheat: Hart and Langston 1977; Wolf et al. 1977; Maize: Nielsen and Scandalios 1974; McMillin et al. 1979; Birchler 1979; Roupakias et al. 1980; Barley: Nielsen and Frydenberg 1971; Tomato: Fobes 1980). Whether an additional gene copy gives rise to more gene products may be dependent on the regulatory mechanism involved (Birchler 1979). In locating isozymic genes the problem is circumvented by demonstrating an allelic dosage effect using electrophoretic variation. Trisomics with the hypothetical genotype E1 E1 E2 might show a higher e1/e2 ratio of enzyme activity when compared to the E1 E2 diploid. Comparison of double-banded phenotypes of diploids and trisomics can then demonstrate the allelic dosage effect for gene E. The E1 E2 E3 trisomic containing three variants of enzyme e offers qualitative evidence for the location of the gene E on the chromosome in triplicate (Nielsen and Frydenberg 1971; Nielsen and Scandalios 1974; Fobes 1980; Lewis et al. 1980).

The morphological and cytogenetic characterization of all seven primary trisomics of Petunia (Maizonnier 1976) makes it possible to locate isozymic genes by the allelic dosage effect.

However, differences in temporal programming of the expression of the different alleles may interfere with scoring an allelic dosage effect, since it is impossible to sample organs of diploids and trisomics in the same state of development for comparing double-banded phenotypes. Therefore, the developmental expression of each of the prxB alleles had to be studied. Subsequently, the chromosomal location of the gene prxB could be confirmed by an allelic dosage effect study.

Materials and Methods

Plant Material

The following inbred lines were used: *P. axillaris:* S1 (Royal Botanic Gardens, Kew, 1954), S2 (collected in the wild, Uruguay, 1958), S8 (Michigan State University, 1974); *P. inflata:* S6 (Botanic Gardens, Stockholm, 1974), S9 (I.N.R.A.,

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Dijon, France, 1976), S10 (Michigan State University, 1977); *P. parodii:* S7 (Michigan State University, 1974); *P. hybrida:* R51 (derived from the cultivar 'Royal Ruby').

In addition, plants of *P. inflata* and *P. integrifolia,* grown from seeds collected in Uruguay and Brazil, were investigated. The name *P. integrifolia* is used since *P. violacea* is a junior synonym (cf. Skan 1918). For the allelic dosage effect study the trisomic I PX3018B-85, isolated by the late Dr. F. J. Smith (Institute of Genetics, Amsterdam), and the telotrisomic I C7521-1, a gift from Drs. Maizonnier and Cornu (I.N.R.A., Dijon, France) were used.

Electrophoretic Analysis

Sample preparation, electrophoretic separation of the peroxidase isoenzymes using starch gel system I, and staining for peroxidase activity were carried out as described previously (van den Berg and Wijsman 1981). The nomenclature of the peroxidase isoenzymes and the encoding genes as given in the same report is followed.

Zymogram Scanning

To determine relative activities of the PRXb allozymes in plants heterozygous for prxB, staining was stopped as soon as possible (within 10 minutes) to obtain initial rate enzyme kinetics. After staining the gel was washed, and fixed in 50% methanol for at least 24 hours. Gels were scanned at 470 nm using the Zeiss Chromatogramm Spektralphotometer KM3 coupled to the PMQ3 system. Dilution experiments demonstrated a linear relationship between staining density and enzyme concentration within the concentration range normally used.

Determination of Genetic Segregation of two Flower Colour Genes

Genotypes for the gene *Hf1,* involved in the hydroxylation at the 3′ and 5′ positions of anthocyanins in the flower of Petunia, and for the gene *F1,* which in the dominant form stimulates flavonol formation, were determined as described by Wiering (1974). The marker *hf1-1,* an allele of *Hf1,* is only expressed in the flower tube, not in the limb.

Characterization of Trisomics I

Trisomics I can be recognized from their characteristic plant morphology – narrow and pointed leaves, retarded elongation of the internodes – but in addition chromosome counts were made from root tips following the lacto-propionic orcein method as described by de Jong and de Bock (1978). The telotrisomic I C7521-1 cannot be characterized on the basis of the chromosome morphology since chromosome I contains two arms of equal length. However, the telotrisomic I used has more or less the same characteristic plant morphology as the primary trisomic I.

Results

Electrophoretic Variation of the Peroxidase b Isoenzymes

The peroxidase b enzymes are present in all parts of the Petunia plant; they can routinely be detected by electrophoresis of crude extracts of mature leaves from full-grown flowering plants.

In inbred lines from the Petunia collection of the Institute of Genetics, Amsterdam, until now three electrophoretic variants of the PRXb enzymes have been found. All our inbred lines of *P. hybrida,* representing most of the classical cultivars of Petunia, are homozygous for prxB2. But in inbred lines of the related species of *P. hybrida (P. inflata, P. axillaris, P. integrifolia* and *P. parodii)* two other alleles have been found. The prxB1 allele is present in lines S1, *P. axillaris,* and S7, *P. parodii* (both homozygous). The prxB3 allele was found in lines S6, S9, S10 (*P. inflata;* originally all three lines were heterozygous containing the alleles prxB2 and prxB3). *P. inflata* and *P. integrifolia* from several sources in South America contain both the prxB2 and prxB3 alleles (unpubl. observ.). At present the occurrence of the B1 allele is restricted to the whiteflowering *P. axillaris* and *P. parodii* and the B3 allele to the violet-flowering *P. inflata* and *P. integrifolia.* The prxB2 allele can be found in all four related species of *P. hybrida* (*P. axillaris* S2 and S8 are both homozygous for prxB2).

Developmental Modification

The analysis of leaves of different age from a full-grown flowering plant suggests that in young leaves the PRXb enzyme has a lower electrophoretic mobility than in old leaves (cf. Figs. 1, 2, van den Berg and Wijsman 1981). Analysis of young, flowering plants, heterozygous for prxB shows a concomitant difference in mobility of the allozymes (Fig. 1). Moreover, the increase in mobility is gradual: the PRXb1b2 doublet in Fig. 1 shifts towards the anode during the development of leaves. This shift, or aging effect, is not only

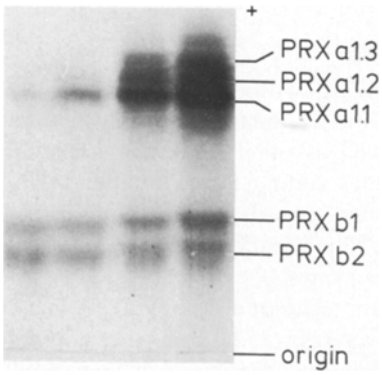

Fig. 1. Zymogram showing the gradual increase in electrophoretic mobility of the prxb1b2 doublet concomitantly with age. From left to right analysis of leaves of increasing age of a flowering plant of F1 (S1×R51), with genotype prxA1/A1, prxB1/B2

dependent on the age of the leave but also on the age of the plant. In older plants the final mobility of the PRXb enzymes is reached at a younger age of the leaves. As shown below (cf. Figs. 5, 6), in trisomics the final mobility is reached at a younger age of leaves when compared to diploids.

Location of prxB by Linkage Analysis

To locate the structural gene prxB by linkage analysis the inbred tester line R51 (prxB2/B2) was crossed with lines S1 (prxB1/B1) and S6 (prxB3/B3).

In the F2 (S1×R51) progeny, linkage of prxB with *hf1-1* and *F1* was observed (Table 1; for a typical zymogram see Fig. 4b, van den Berg and Wijsman 1981). Due to the small number of crossovers the order of the genes cannot be established.

The linkage of the three genes is surprising since *hf1-1* and *F1* have been located on different chromosomes. According to Wiering (1974), *hf1-1* is an allele of the gene *Hf1*, which has been located on chromosome I by Maizonnier and Cornu (1971). *F1* is reported to be highly linked with *Lu* on chromosome II (Maizonnier and Moessner 1979). Crosses of S1 with other *P. hybrida* inbred lines resulted in the same linkage of *hf1-1* with *F1*, whereas crosses of S2 (another *P. axillaris* inbred line) with *P. hybrida*, and crosses of different lines of *P. hybrida*, show independent inheritance of the genes *Hf1* and *F1* (Wiering and de Vlaming, pers. comm.). This suggests that S1 contains a translocation involving either *hf1-1* or *F1*. It can be concluded that prxB is located on chromosome I or II.

A plant from the progeny F2 (S1×R51), with an aberrant flower morphology, was crossed with R51. All flower limbs of the progeny showed variegation and a frayed circumference (Fig. 2). In view of the findings of Maizonnier and Cornu (1971) – the observation of variegation in relation to a translocation – the translocation hypothesis seems to be corroborated.

In the F2 (S6×R51) progeny, prxB is shown to be linked with *Hf1*, even if for both genes the deviation from the expected F2 segregation is large (Table 2). It can now be concluded that the gene prxB is situated on chromosome I.

Fig. 2. Photograph of two flowers from the progeny F3 (S1×R51)R51, showing the variegation and retarded growth of sectors as typical for each plant of the progeny

Table 1. Segregation for prxB, *hf1-1* and *F1* showing linkage of the three genes

Cross: F2 (S1 × R51)

Genotype: $\frac{\text{prxB1, } hf1\text{-}1, F1}{\text{prxB2, } hf1, f1} \times \frac{\text{prxB1, } hf1\text{-}1, F1}{\text{prxB2, } hf1, f1}$

Genotypes and number of progeny	prxB1/B1	prxB1/B2	prxB2/B2
hf1-1 – F1 –	19	81	4
hf1-1 – f1f1	0	0	0
hf1hf1 F1 –	1	1	4
hf1hf1 f1f1	1	4	27

Segregation chi square tests

prxB: $\chi^2_{1:2:1} = 10.90$ (df=2) P=0.004
hf1-1: $\chi^2_{3:1} = 0.23$ (df=1) P=0.63
F1: $\chi^2_{3:1} = 0.46$ (df=1) P=0.50

Linkage chi square tests

prxB – *hf1-1*: $\chi^2_{3\times2} = 90.7$ (df=2) $P < 10^{-3}$
prxB – *F1*: $\chi^2_{3\times2} = 79.4$ (df=2) $P < 10^{-3}$
hf1-1 – F1: $\chi^2_{2\times2} = 113.1$ (df=1) $P < 10^{-3}$

hf1-1 – and *F1* – stand for the heterozygous and the homozygous dominant forms

Table 2. Segregation for prxB and *Hf1* showing linkage of the genes

Cross: F2 (S6 × R51)

Genotype: $\frac{\text{prxB, } Hf1}{\text{prxB2, } hf1} \times \frac{\text{prxB3, } Hf1}{\text{prxB2, } hf1}$

Genotypes and number of progeny	prxB3/B3	prxB3/B2	prxB2/B2
Hf1 –	3	14	2
hf1hf1	0	6	25

Segregation chi square tests

prxB: $\chi^2_{1:2:1} = 25.0$ (df=2) $P < 10^{-3}$
Hf1: $\chi^2_{3:1} = 32.2$ (df=1) $P < 10^{-3}$

Linkage chi square test

prxB – *Hf1*: $\chi^2_{3\times2} = 24.3$ (df=2) $P < 10^{-3}$

Hf1 – stands for the heterozygous an the homozygous dominant form

Developmental Expression of the prxB Alleles

To take into consideration differential expression of prxB alleles in studying an allelic dosage effect in trisomics, several F1 hybrids between inbred lines differing in prxB genotype were investigated.

For all three prxB alleles a difference in the degree of increase in activity of allozymes during development of leaves was observed. Most pronounced is the difference of the developmental programme of prxB1 from S1 with regard to prxB2 alleles from several *P. hybrida* inbred lines and the prxB3 allele from line S6 (Fig. 3, cf. Figs. 5, 6). In leaves of young plants, not yet flowering, prxB1 has a lower initial expression level, but relative to the prxB2 expression the level of prxB1 increases during development of leaves. In plants that have flowered for months the expression level of the B1 allele even surpasses that of the B2 allele (Fig. 3).

The B2 allele from several *P. hybrida* inbred lines shows a slightly retarded increase in expression level

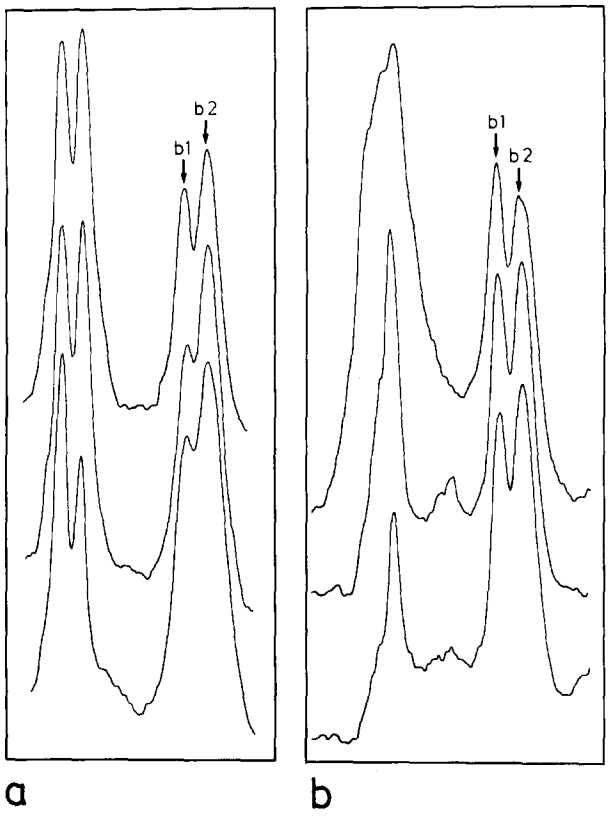

Fig. 3a and b. Zymogram scannings showing differential expression in enzyme activity of the prxB1 and prxB2 alleles in two different genetic backgrounds. The origin of the gel is at the right of each scanning. Both scanning sets represent analysis of a young, intermediate, and old leave (from bottom to top, respectively). **a** Analysis of a plant not yet flowering with genotype prxA1/A2, prxB1/B2. **b** Analysis of a plant flowering for three months with genotype prxA1/A1, prxB1/B2

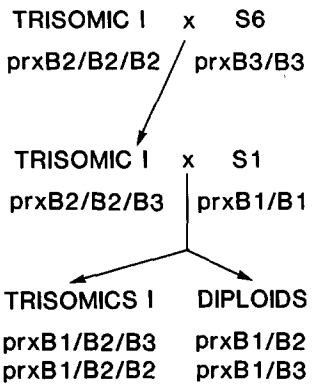

Fig. 4. Crossing programme for breeding triple-banded trisomics I with genotype prxB1/B2/B3

with regard to that of the B3 allele from S6. In mature leaves of flowering plants only the difference in enzyme activity of about a factor 2 is observed (cf. Figs. 5, 6), which persists during further developmental of organ and plant.

With regard to the allelic dosage effect these results suggest that for scoring the dosage effect in double heterozygous trisomics b2-b3 makes the better combination.

Location of prxB by Use of Trisomics

To confirm the location of prxB on chromosome I a crossing programme was set up for the construction of triple-banded trisomics I (Fig. 4).

The available trisomic I, the primary trisomic I PX3018B-85 is homozygous for prxB2. Though independently isolated in a different genetic background

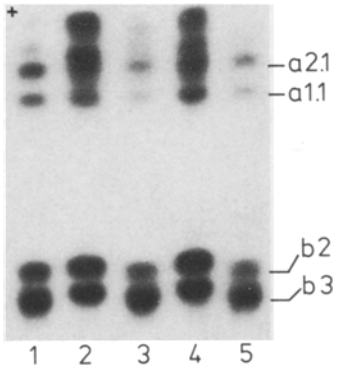

Fig. 5. Starch gel showing the anodal peroxidase isoenzymes of some plants of comparable age of the progeny of trisomic I PX3018B-8B-85 × S6 (genotype prxB2/B2/B2 × prxB3/B3). Leaves of intermediate age of young plants, not yet flowering were analysed. Diploids: 1, 3, 5; trisomics: 2, 4. Note the alternate shift of the PRXb2b3 doublet and the presence of the PRXa mozymes only in leaves of trisomics

the trisomic I has the same characteristic plant morphology as those described by Maizonnier (1976).

The progeny of the cross trisomic I PX3018B-85 × S6 showed two kinds of PRXb double-banded phenotypes (Fig. 5). All 13 diploids tested showed a PRXb2 band that was about half as active as the PRXb3 band. The 8 trisomics tested showed equally active allozymes. The relative doubling of the PRXb2 activity represents the double dosage of the prxB2 allele in the trisomics I (Fig. 5).

Among the progeny trisomic I D7634B-5 × S1 (prxB2/B2/B3 × prxB1/B1) the three expected PRXb phenotypes were found, one of these the triple-banded phenotype (Fig. 6). Among the diploid progeny the segregations for prxB and *Hf1* deviated, through in opposite directions, from the expected trisomic segregation. However, the segregation among the trisomic progeny fitted the expected 2 : 1 segregation (Table 3).

The telotrisomic C7521-1, homozygous for prxB2, was crossed with S6. Among the progeny seven telotrisomic with the characteristic trisomic I plant morphology were found; they did not show a double dosage for prxB, as deduced from their PRXb2b3 phenotype. This indicates that prxB is not located on the extra arm of chromosome I.

According to Maizonnier (pers. comm.) the extra arm of the telotrisomic I does not carry an *Hf1* allele. This means that prxB and *Hf1* are located on the same arm of chromosome I.

The Translocation in Line S1

As pointed out above the inbred line S1 is suggested to carry a translocation involving either *hf1-1* and prxB1 or *F1*. To exclude one of the possibilities, either location of prxB1 and *hf1-1* on chromosome II, or location of *F1* on chromosome I, one of the triple-banded trisomic I was crossed with line R51 (genotype prxB1/B2/B3 × prxB2/B2). If the allele prxB1 is situated on chromosome II, then among the diploid progeny triple-banded PRXb phenotypes would be predicted. However, only PRXb1b2, PRXb2 and PRXb2b3 phenotypes have been found among 113 diploids, in a ratio of 24:36:53, respectively. The absence of a triple-banded phenotype among the diploid progeny suggest that prxB1 is located on chromosome I, and, consequently, that *F1* is translocated to chromosome I.

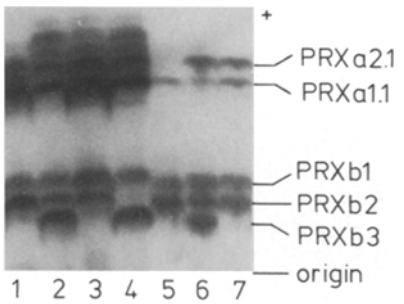

Fig. 6. Starch gel showing anodal peroxidase isoenzymes of flowering plants from the progeny trisomic I (prxB2/B2/B3) × S1 (prxB1/B1), showing the expected PRXb phenotypes among trisomics and diploid progeny. Diploids: 1, 4, 7; trisomics I: 2, 3, 5, 6; in the case of 1, 2, 3, 4 old leaves and in the case of 5, 6, 7 young leaves of young shoots were analyzed. Note the difference in mobility of the PRXb enzymes of 1–4, compared to 5–7, together with the differences in PRXa phenotypes

Table 3. Trisomic segregation for prxB and *Hf1*

Cross: Trisomic I D7634B-5 × S1

Genotype: $\frac{\text{prxB2, } \textit{Hf1}}{\text{prxB2, } \textit{hf1}} \times \frac{\text{prxB1, } \textit{hf1-1}}{\text{prxB1, } \textit{hf1-1}}$ / prxB3, *Hf1*

Expected and found segregations

		Expected	Found
Diploids:	prxB1/B2	2	67
	prxB1/B3	1	61
	Hf1 hf1-1	2	107
	hf1 hf1-1	1	21
Trisomics:	prxB1/B2/B3	2	33
	prxB1/B2/B2	1	18

Segregation chi square tests (df = 1)

Diploids:	prxB	$\chi^2_{1:1} = 0.28$	$P = 0.60$
		$\chi^2_{2:1} = 11.80$	$P < 10^{-3}$
	Hf1	$\chi^2_{1:1} = 57.80$	$P < 10^{-3}$
		$\chi^2_{2:1} = 16.50$	$P < 10^{-3}$
Trisomics:	prxB	$\chi^2_{1:1} = 4.40$	$P = 0.04$
		$\chi^2_{2:1} = 0.09$	$P = 0.76$

Discussion

From standard linkage tests and trisomic analysis, and, especially, the isolation of triple-banded trisomics I, it can be concluded that the structural gene prxB is located on chrosome I.

Although both F2 progenies analyzed show linkage of prxB with *Hf1*, the genetic distance cannot be estimated due to distortion of the expected F2 segregation, and possible interference of the translocation in the case of the F2 (S1 × R51). Further precision of the location of prxB on chromosome I must wait for the introduction of the prxB3 allele in the *P. hybrida*

genome, or other markers on chromosome I to become available.

Though only in mature tissue, an allelic dosage effect could be assessed in trisomics I. In telotrisomics I lacking a third dosage of *Hf1* an allelic dosage effect for prxB was absent, indicating that the genes prxB and *Hf1* are located on the same arm of chromosome I.

The trisomic segregation for prxB and *Hf1* among the diploid progeny deviated from the expected 2:1 segregation, but for these linked genes they deviated in opposite directions, indicating specific loss of the prxB2-*hf1* combination. Possibly on the relevant chromosome a deleterious mutation limits transfer to the progeny. The fact that among the trisomic progeny the expected trisomic segregation was found may be easiest explained by genetic complementation at the n+1 gamete level.

As to the translocation in line S1, the absence of triple-banded PRXb phenotypes among the progeny of triple-banded trisomic I×R51 suggests that in line S1 the gene *F1* is located on chromosome I. As argued by Sybenga (1972), no quantitative expectations can be made as to the inheritance of the translocation chromosome and, therefore, of prxB, but the low number of PRXb1b2 phenotypes among diploids may be explained by reduced transmission of the translocation linked to prxB1.

The gradual increase in electrophoretic mobility of the PRXb enzymes represents an interesting phenomenon, although an artifact cannot be excluded. As shown by the zymograms the PRXb bands from younger leaves are more diffuse than in older leaves (cf. Fig. 6). Perhaps this can be ascribed to the cells of the younger leaves being in a different state of development. Even so, their mean mobility shifts upward. It may be that a multi-step process is involved in the increase in mobility. If the shift relates to the attachment of sugar moities, the system may provide a model for studying glycoprotein biosynthesis.

As to differential expression of the prxB alleles we wish to stress the following considerations. Initially, different patterns of increase of activity of the PRXb allozymes were investigated in F1 hybrids between inbred lines. Two differences could be distinguished between the expression of the prxB1 allele from line S1 and the prxB3 allele from line S6 with regard to the prxB2 allele from several *P. hybrida* inbred lines.

First, the rate of prxB1 expression changes with regard to that of prxB2 throughout the development of organ and plant, whereas the change in the prxB3 expression rate is only apparent in an early stage of development. Thus, three different temporal programmes can be viewed in the expression of the gene prxB.

Second, when a constant b2/b3 activity ratio has been reached at a certain stage in the development, a difference in expression of the alleles prxB2 and prxB3 persists during further development of the plant, the b3 allozyme being twice as active.

Since the expression of the temporal programmes may be dependent on the genetic background it is not easy to investigate by B1 or F2 crosses whether the different temporal programmes are specified by different internal site mutations – defined as mutations near or within the structural gene of which the expression is effected –, or by external site mutations – defined as mutations in other genes acting in trans –, or by a combination of both internal and external site mutations. The results concerning the developmental patterns as presented here mean that only an investigation of crosses at an early stage of development may ascertain whether internal site, external site, or both types of mutations are involved.

For systemic difference between the prxB2 and prxB3 expression the situation is genetically more defined. In the crosses mentioned in this paper no crossovers between the mutation causing the systemic difference and the mutation in the structural gene have been found, indicating that the systemic mutation represents an internal site mutation.

Further search will be dedicated to the sites and the molecular mechanisms causing the differential expression of the prxB alleles.

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Drs. B. M. van den Berg
Dr. H. J. W. Wijsman
Institute of Genetics
Kruislaan 318
1098 SM Amsterdam (the Netherlands)