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PERSISTENCE OF DIFFERENCES BETWEEN PEROXIDASE ISOENZYMES OF FLAX GENOTROPHS IN TISSUE CULTURE

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Abstract—Anionic peroxidase isoenzymes from seedling root, hypocotyl and cotyledon regions of the large (L) and small (S) flax genotrophs were separated on acrylamide gels. Tissue cultures were initiated from each of these regions of the seedlings, and maintained for a 200-day period with six transfers. The differences in electrophoretic mobility of the peroxidase isoenzymes between L and S noted in seedlings, and also in main stems of adult plants, were still present in the tissue cultures.

INTRODUCTION

Environmentally induced heritable changes have been produced in flax and tobacco which can be completely inbred [1, 2]. Contrasting L and S flax genotrophs resulted from one generation of treatment of a completely inbred flax genotype with large concentrations of inorganic nutrients, in particular NPK (yielding L) and NK (yielding S). In subsequent generations grown in uniform environmental conditions, progeny from NPK and NK differed markedly in plant weight, and the progeny differences remained essentially stable over many generations. The *Rm*s of anionic peroxidase isoenzymes from main stem tissue differ in the stable progeny, i.e. the L and S flax genotrophs [3].

The four major anionic peroxidase isoenzymes in flax main stem tissue have been numbered 1 to 4 in order of increasing *Rm*. The four isoenzymes from L showed *Rm*s which were all slightly faster than the four from S. Isoenzymes 1 from L and from S have been referred to as 'corresponding' isoenzymes on the basis that, prior to induction in the original stock genotype, only a single *Rm* would be demonstrable for this slowest moving isoenzyme. The same argument is applicable to isoenzymes 2, 3 and 4 from L and from S. The *Rm* difference between, for example, isoenzyme 2 from L and 2 from S would indicate molecular differences ascribable to the original treatments with NPK and NK. The distinction between an isoenzyme 1 from L or from S is made by labelling in tables, etc.; in general, 'corresponding' isoenzymes is used for brevity.

Peroxidase activity differed between L and S main stem tissue; the amount of isoenzyme synthesized per unit fr. wt, or, alternatively, the amount of breakdown, differed such that the ultimate amount per unit fr. wt was higher in S than in L [4].

The aim of this study was to check whether L–S differences in isoenzyme *Rm* remained when root, hypocotyl and cotyledon tissues from L and S genotrophs were actively growing in tissue cultures. The *Rm*s were also examined in the root, hypocotyl and cotyledon tissues of 7-day-old seedlings of L and S, that is, seedlings at the same age as those from which tissue was removed to start cultures.

Table 1. Seven-day-old seedling material; mean values for fr. wt, gross peroxidase activity (P.A.) and adjusted *Rm*

Region	Genotroph	Wt (g)	P.A. (ΔA/min/ml)	Isoenzyme *Rm* 1	2	2	4
Root	L	0.13	1.17	0.071	0.107	0.153	0.400
	S	0.15	1.29	0.070	0.131	0.145	0.383
Hypocotyl	L	0.10	0.74	0.075		0.167	0.398
	S	0.11	0.53	0.071		0.155	0.386
Cotyledon	L	0.14	0.41	0.066		0.157	
	S	0.14	0.49	0.065		0.150	

RESULTS

Seven-day-old seedling material

Table 1 gives the mean fr. wts, gross peroxidase activities and major isoenzyme *Rm*s for the three seedling regions of both genotrophs. Gross activity was obtained prior to electrophoretic separation, and represented the activity of all isoenzymes present. Gross activity was higher in roots than in hypocotyls and cotyledons; an analysis of variance for the hypocotyl alone showed a significant genotroph (L − S) difference, with L being higher than S.

The isoenzyme patterns of the three regions were distinctive; the patterns, comparative staining intensities (from areas and shapes of recorded absorbance peaks), and the *Rm*s, strongly suggested that three of the isoenzymes corresponded to isoenzymes 1, 2 and 4 from mature (30 to 40-day-old) main stem tissue. Cotyledon samples contained isoenzymes 1 and 2, while roots and hypocotyls displayed all three. However, in roots the relative activity of isoenzyme 4 compared to isoenzymes 1 and 2 was considerably lower than that in hypocotyls. The appearance of some isoenzyme 4, albeit in small amounts, in the roots of 7-day-old seedlings could have been due to the point of cutting between the root and hypocotyl regions of these seedlings, i.e. the accidental incorporation of some hypocotyl tissue in the root tissue sample. In addition, roots showed an isoenzyme with *Rm* between 1 and 2; this isoenzyme is designated 2′. For isoenzymes 1, 2 and 4, the L *Rm*s were always faster than those for S; this was not the case for isoenzyme 2′. In an analysis of variance of the isoenzyme 1 and 2 data from all three regions, the overall genotroph difference was significant, as were the overall differences amongst regions and between isoenzymes. There were no significant interactions between genotroph, region and isoenzyme in this analysis. L *Rm* was significantly faster than S *Rm* in all three regions, and the mean *Rm* of the hypocotyl was significantly faster than that of roots or cotyledons.

Tissue cultures

All cultures grew well throughout the six transfers; as well as abundant growth, root hair production also occurred, and was much more pronounced in all S cultures compared to L cultures. This root hair difference was, therefore, consistent over the two replicates of the set of three regions, the two genotrophs, and the three culture ages after initiation. Media and environmental conditions were identical for all 18 combinations in the experiment.

Two of the isoenzymes detected in the tissue cultures had *Rm*s similar to those of isoenzymes 1 and 2 from mature main stem tissue, and were presumably closely related, if not identical. The mean adjusted *Rm*s for these two isoenzymes are shown in Table 2. There were some other isenzymes detected with no consistency and in small amounts amongst the 18 cultures, but attention here is focused simply on isoenzymes 1 and 2. Each value shown in Table 2 was the mean of two estimations. The variances for the 18 means for isoenzyme 1 data, as well as those for isoenzyme 2, were not homogeneous, making an analysis of variance for the main effects and their interactions insensitive.

To test the data for significant effects, a model was fitted to the data by a weighted least squares procedure [5]. The weights were the reciprocals of the variances of the *Rm*

Table 2. Tissue culture material; mean adjusted *Rm* values for isoenzymes (Iso.) 1 and 2

		Culture ages = days after initiation of first culture						Means over all ages	
		68 (2nd transfer)		154 (5th transfer)		208 (6th transfer)			
Region	Genotroph	Iso. 1	Iso. 2	Iso. 1	Iso. 2	Iso. 1	Iso. 2	Iso. 1	Iso. 2
Root	L	0.083	0.151	0.081	0.156	0.078	0.153	0.080	0.153
	S	0.072	0.142	0.072	0.143	0.076	0.142	0.073	0.142
Hypocotyl	L	0.081	0.149	0.078	0.147	0.079	0.156	0.079	0.150
	S	0.073	0.149	0.075	0.141	0.073	0.146	0.073	0.145
Cotyledon	L	0.080	0.143	0.076	0.139	0.078	0.148	0.078	0.144
	S	0.074	0.151	0.076	0.149	0.070	0.148	0.073	0.150
							L =	0.079	0.149
							S =	0.073	0.146

		Culture age			Region		
Isoenzyme	Genotroph	68	154	208	Root	Hypocotyl	Cotyledon
1	L	0.081	0.078	0.078	0.080	0.079	0.078
	S	0.073	0.074	0.073	0.073	0.073	0.073
2	L	0.148	0.148	0.152	0.153	0.151	0.144
	S	0.147	0.144	0.146	0.142	0.148	0.147

Table 3. Tissue culture material; analyses of adjusted *Rm* values for isoenzymes 1 and 2

Comparison†	Parameter estimate‡	Standard error	*t*
Isoenzyme 1			
(L − S)	0.00381	±0.00021	18.44**
(R − C)	0.00117	±0.00042	2.79*
($A_1 + A_3 + 2A_2$)	0.00063	±0.00014	4.50**
Interaction: genotroph with age			
= ($LA_1 - SA_1$) − ($LA_3 - SA_3$)	0.00093	±0.00023	4.04**
	[$\chi^2 13 = 16.25$, P = 0.3–0.2]		
Isoenzyme 2			
(L − S)	0.00202	±0.00047	4.30**
Interaction: genotroph with region			
= (LR − SR) − (LC − SC)	0.00403	0.00064	6.30**
Interaction: genotroph with age			
= ($LA_1 - SA_1$) − ($LA_3 - SA_3$)	−0.00240	±0.00062	3.87**
	[$\chi^2 14 = 11.49$, P = 0.7–0.5]		

* Significant at probability 0.05, ** significant at probability 0.01.
† L,S = genotrophs; R, C = roots, cotyledons; A, etc. = culture ages; LA_1 = genotroph L at age 1 (68 days after initiation), etc.
‡ Overall mean, accounting for 1 degree of freedom in model, is omitted for brevity.

means shown in Table 2. Isoenzyme 1 data were examined separately from isoenzyme 2 data. The main effects, and the interactions which were significant, are shown in Table 3.

Isoenzyme 1. *Rm*s for L were significantly faster than those for S. However, the summary in Table 2 of genotroph and age values indicates that the *Rm* difference between L and S decreased from age 1 to age 3, resulting in the significant G/T interaction. There was a faster overall *Rm* in roots compared to cotyledons, but no indication that this difference was affected by age.

Isoenzyme 2. *Rm*s for L were again faster than those for the S genotroph. With this isoenzyme, the genotroph difference increased from age 1 to age 3, as can be seen from the summary shown in Table 2. As well as a genotroph interaction with age, there was a genotroph interaction with region. This meant that, for L, *Rm*s were faster in the cotyledons than in the roots and hypocotyls, whereas for S the converse was true. *Rm* for S in the cotyledons was faster than *Rm* for L.

DISCUSSION

The principal interest here was the reappearance in tissue culture of the *Rm* shifts between the L and S peroxidase isoenzymes seen in the 7-day-old seedlings and in mature plants. For isoenzymes 1 and 2, the mean *Rm*s across the various regions and culture ages suggested that culturing did not result in complete and immediate de-differentiation of the genotroph isoenzyme differences. For both isoenzymes 1 and 2, however, the size of the *Rm* shift may have been changing with time in the cultures. Also, in cultures from cotyledons the S isoenzyme 2 *Rm* was faster than that for L isoenzyme 2 for at least 154 days after culture initiation.

The changes seen for L and S with culture age may be yet another facet of the more or less continuous *Rm* modifications which have been indicated in comparisons of isoenzyme *Rm* from stem base to apex [6], and in comparisons of mean main stem *Rm* over the 16 to 36-day period in the growth of flax plants [7]. In both these latter cases, the degree of *Rm* shift was much smaller than that seen in the overall comparison between L and S. The smoothness of the *Rm* shift also suggested that modification of the molecule in a continuous fashion might be occurring, rather than the switching on and off of members of an array of isoenzyme genes. Part of the changes in *Rm* seen here with culture age must be ascribed to sampling error, and to selective effects of the culture environment. Whether the *Rm* changes over culture age are due to such continuous modification, most likely in the enzyme's carbohydrate moiety, would be clarified by increasing the number of time points sampled over the same time range, an approach equally applicable to comparisons between tissues and between times in the normally growing plant.

To the extent that these tissue culture data confirm the continuing integrity of the L and S genotrophs, they are in line with earlier experiments on reciprocal grafts with L and S. It has been shown [8] that a scion of L or S grafted onto a stock of the other genotroph retained its integrity, and its progeny were unaffected by such a passage.

EXPERIMENTAL

Seedling material. Seedlings of L and S were grown to 7 days after sowing, in vermiculite, with 50 seedlings of each genotype in each of 2 replicates. Each seedling was cut into cotyledon, hypocotyl and root sections, and the seedling sections within genotrophs and within replicates were pooled for the preparation of 12 seedling extracts, i.e. (2 × 2 × 3) = replicates × genotrophs × sections.

Tissue culture material. Callus cultures were initiated and maintained using standard procedures [9]. 12 callus cultures were initiated from 7-day-old seedlings by excising material from the 3 seedling sections of 2 seedlings (=replicate) of each genotroph. Cultures were initiated on 1% agar medium [10] supplemented with 2 mg indole acetic acid, 22 μg kinetin, 100 mg

myo-inositol and 3% sucrose, and transferred at regular intervals onto fresh medium of the same composition, but with 0.6% agar. Cultures were maintained in the dark, and subcultured every second transfer. One of the initial explants failed to grow (L, hypocotyl, replicate 2) reducing the initial number of callus types from 12 to 11. Thus, only 33 of 36 callus extracts were prepared by removing callus samples of the 11 callus types at the 2nd, 5th, and 6th transfers at 68, 154, and 208 days, respectively after initiation.

Extract preparation. All extracts were prepared by homogenizing plant material in 0.1 M Pi buffer, pH 8 in the presence of anion exchange resin (Amberlite 1X8, 200–400 mesh) to absorb phenolic peroxidase inhibitors. The homogenizing ratio was 1:1:2 = g plant material:g dry wt resin:ml buffer. Homogenized extracts were strained through nylon mesh, centrifuged at 17000 *g* and stored at −20°.

Peroxidase activity and isoenzymes. Both peroxidase activity and the examination of peroxidase isoenzymes by anionic electrophoresis were conducted as detailed elsewhere [11]. Each electrophoresis run contained the samples of 1 replicate and 3 tracks of a standard [12]. *Rm* values of the isoenzymes of each sample were obtained as means from 3 electrophoresis tracks. Values for the callus type which failed were fitted by appropriate missing plot techniques.

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