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Soybean Seed Coat Peroxidase

A Comparison of High-Activity and Low-Activity Genotypes

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Peroxidase activity in the seed coats of soybean (*Glycine max* [L.] Merr.) is controlled by the *Ep* locus. We compared peroxidase activity in cell-free extracts from seed coat, root, and leaf tissues of three *EpEp* cultivars (Harosoy 63, Harovinton, and Coles) to three *epep* cultivars (Steele, Marathon, and Raiden). Extracts from the seed coats of *EpEp* cultivars were 100-fold higher in specific activity than those from *epep* cultivars, but there was no difference in specific activity in crude root or leaf extracts. Isoelectric focusing of root tissue extracts and staining for peroxidase activity showed that *EpEp* cultivars had a root peroxidase of identical isoelectric point to the seed coat peroxidase, whereas roots of the *epep* types were lacking that peroxidase, indicating that the *Ep* locus may also affect expression in the root. In seed coat extracts, peroxidase was the most abundant soluble protein in *EpEp* cultivars, whereas this enzyme was present only in trace amounts in *epep* genotypes, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Histochemical localization of peroxidase activity in seed coats of *EpEp* cultivars shows that the enzyme occurs predominately in the cytoplasm of hourglass cells of the subepidermis. No obvious difference in the gross or microscopic structure of the seed coat was observed to be associated with the *Ep* locus. These results suggest that soybean seed coat peroxidase may be involved in processes other than seed coat biosynthesis.

Peroxidases are hemoproteins that catalyze the oxidation of a substrate and the reduction of H_2O_2. Plant peroxidases are typically glycoproteins of 30 to 60 kD (van Huystee, 1987). These enzymes may participate in many processes of plant growth and defense (Gaspar et al., 1982; Greppin et al., 1986). Plants often contain several different peroxidase isozymes that are most readily classified according to their pI values. Both cationic and anionic peroxidases have been described, and the predominant isozyme usually depends on the tissue examined.

Peroxidase activity in the seed coat of soybean (*Glycine max*) has been classified into two broad categories, low activity and high activity (Buttery and Buzzell, 1968). Inheritance of high peroxidase activity in soybean seed coats is attributed to a dominant gene, designated *Ep* (Buzzell and Buttery, 1969). Low activity results from the homozygous recessive condition *epep*. The *Ep* locus, controlling seed coat peroxidase, has been used frequently as an enzyme marker in genetic studies and as a diagnostic tool in the identification of plant cultivars (Griffin et al., 1989; Sahota and Desormeaux, 1990). A previous investigation, measuring total peroxidase activity in soybean tissues at different stages of development, led to the conclusion that seed coat peroxidase activity is unrelated to activity in the root or leaf (Dunleavy and Urs, 1978). However, individual peroxidase isozymes were not measured in this work, and thus the association of the *Ep* locus with a distinct isozyme in the root or leaf may have been overlooked.

The soybean seed coat, or testa, consists of an outer epidermis of palisade cells, a subepidermis of hourglass cells, and an inner layer of compressed parenchyma (Vaughn, 1970; Carlson and Lersten, 1987). In the hilum region hourglass cells are not present, but there is a thick parenchymatous layer containing vascular tissue as well as remains of placental tissue attached to the outer epidermis. There is great variation in the color and luster of the seed coat and hilum; however, the *Ep* locus is not known to be linked to any of these traits (Palmer and Kilen, 1987; Griffin et al., 1989). Indeed, the role of seed coat peroxidase, the consequence of high versus low activity, and the gene product of the *Ep* locus remain unknown.

In the present study, we show that low peroxidase activity in the seed coats of homozygous recessive cultivars *epep* is the result of greatly reduced amounts of peroxidase enzyme present. Comparison of root extracts for peroxidase isozymes shows that the *Ep* locus may also affect peroxidase expression in root tissue. Staining of thin sections of seed coats for peroxidase activity showed that this enzyme is localized mainly in the hourglass cells of plants carrying the *Ep* allele, whereas in *epep* cultivars activity is barely discernible.

MATERIALS AND METHODS

Plant Materials

Low seed coat peroxidase soybean (*Glycine max* [L.] Merr.) cultivars Steele, Marathon, and Raiden, and high seed coat peroxidase cultivars Harosoy 63, Harovinton, and Coles are all yellow seeded, yellow hila cultivars, and were from the collection at the Harrow Research Station. To isolate the seed coat, mature seeds were soaked in water until the seed coat became soft and wrinkled. Seed coats were then dissected

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Abbreviation: pI, isoelectric point.

from the embryo and used for enzyme extraction or for sectioning and histochemical localization of enzyme activity. For analysis of root and leaf tissue, plants were grown in standard potting mix (Promix BX) with a 15-h photoperiod (150–250 $\mu E\ m^{-2}\ s^{-1}$) and a 24°C day/19°C night temperature cycle and were watered daily. Plants were harvested 4 weeks after sowing, and soil was washed from the root tissues with cold running water before extraction.

Enzyme Extraction and Peroxidase Assay

Freshly dissected seed coats, leaves, or root tissues were frozen in liquid N_2, ground with a mortar and pestle, and extracted in buffer containing 10 mM $NaHPO_4$, pH 6.0, 5 mM DTT, and 1% (w/v) PVP-40. After centrifugation at 15,000*g* for 5 min, peroxidase activity was determined on an aliquot of the supernatant fraction by measuring the rate of oxidation of guaiacol to tetraguaiacol. The assay contained a final concentration of 0.05% H_2O_2 and 12 mM guaiacol in 10 mM $NaHPO_4$, pH 6.0. A_{490} was recorded after 1 min. Protein concentration was estimated by the Bradford method (1976) using the Bio-Rad reagent with BSA (Sigma) as standard.

Purification of Seed Coat Peroxidase

Peroxidase was purified from the seed coats of cultivar Harosoy 63 using methods based on published procedures (Sessa and Anderson, 1981; Sesto and van Huystee, 1989; Gillikin and Graham, 1991) and optimized for speed and simplicity. Dry seeds (18 g) were soaked in water until the seed coat was easily removed. This tissue was frozen in liquid N_2, ground to a fine powder, and extracted in 25 mL of 25 mM Tris, pH 7.0, 5 mM DTT, and 1% (w/v) PVP-40. The extract was centrifuged at 12,000*g* for 15 min at 4°C. The supernatant was filtered through two layers of Miracloth, and protein was precipitated from the filtrate by adding four volumes of acetone, prechilled to −20°C. After 1 h at −20°C, protein was collected by centrifugation at 12,000*g* for 15 min at 4°C and was redissolved by addition of 2.0 mL of 25 mM Tris, pH 7.0. A 200-μL aliquot of this concentrated protein solution was separated by gel filtration on a Superose 12 column (Pharmacia), installed in a Pharmacia fast protein liquid chromatography apparatus. The column was pre-equilibrated and eluted with 25 mM Tris, pH 7.0. Fractions were assayed as described above, and 0.5 mL of this Superose 12-purified material was then subject to anion-exchange chromatography on a Mono-Q 5/5 column (Pharmacia). A linear gradient of increasing KCl concentration, in 25 mM Tris, pH 7.0, resulted in the elution of peroxidase at 300 mM KCl.

Electrophoresis

Discontinuous SDS-PAGE followed the method of Laemmli (1970). The 10% polyacrylamide gels were run on a Bio-Rad Mini-Protean apparatus and were silver stained (Blum et al., 1987). Samples from Mono-Q chromatography, containing KCl, were dialyzed against 25 mM Tris, pH 7.0, prior to electrophoresis. IEF was performed on the Phast system (Pharmacia) using pH 3 to 9 PhastGels. Samples of 1 μL were run for 1200 Vh. The gels were then washed with PBS (10 mM $NaHPO_4$, 150 mM NaCl) for 2 min before staining with chloronaphthol solution (Lagrimini and Rothstein, 1987). Chloronaphthol staining solution was prepared by adding 0.5 mL of 4-chloro-1-naphthol (30 mg mL^{-1} in absolute ethanol) and 0.3 mL of 30% H_2O_2 to 50 mL of PBS. This solution was made immediately prior to use and clarified by vacuum filtration through Whatman No. 1 filter paper. After staining for 5 min, gels were washed with water, photographed, and dried.

Histochemistry

Tissue from freshly dissected seed coats was sandwiched between two layers of Styrofoam of 4 to 5 mm thickness, and sections were cut by hand with a razor. Sections were immediately placed in chloronaphthol staining solution, prepared as above, for 10 min, then transferred to water for 2 min before mounting in water for examination by bright-field microscopy.

RESULTS

Peroxidase Activity in the Seed Coat, Root, and Leaf

Seed coat peroxidase activity, controlled by the *Ep* locus, is thought to be unrelated to peroxidase activity in the root or leaf tissues (Dunleavy and Urs, 1978). This possibility was examined by comparing six soybean varieties (three *EpEp* genotypes, Harosoy 63, Harovinton, and Coles; and three *epep* genotypes, Steele, Marathon, and Raiden) for peroxidase activity in cell-free extracts from the seed coat, root, and leaf (Table I). Although specific activity of seed coat extracts were 100-fold higher in *EpEp* cultivars compared with *epep* cultivars, no such difference in peroxidase activity was apparent in root or leaf extracts among the six cultivars.

To determine whether there may be differences in isozyme expression among the cultivars, extracts were separated by IEF and the resulting gels were stained for peroxidase activity with chloronaphthol (Fig. 1). In the seed coat extracts of *EpEp* cultivars, an intense band of pI 3.9 was visible, in addition to activity that is diffuse and not well focused. This smearing of peroxidase activity was especially a problem in seed coat extracts and was not prevented by the addition of nonionic detergents such as Tween-80, Triton X-100, or 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate. Peroxi-

Table I. *Peroxidase activity in six soybean cultivars*

Peroxidase activity was determined from soluble protein extracts by reaction with guaiacol and H_2O_2 as described in "Materials and Methods." One unit of activity is equal to $\Delta A_{490}\ min^{-1}\ (\mu g\ protein)^{-1}$. Means and SE values of three determinations are given.

Cultivar	Specific Activity		
	Seed coat	Root	Leaf
Steele	0.002 ± 0.001	0.73 ± 0.22	0.025 ± 0.007
Marathon	0.031 ± 0.015	0.98 ± 0.60	0.037 ± 0.009
Raiden	0.011 ± 0.008	0.76 ± 0.22	0.040 ± 0.012
Harosoy 63	4.1 ± 2.9	0.79 ± 0.33	0.021 ± 0.007
Harovinton	14.7 ± 5.2	0.99 ± 0.49	0.023 ± 0.008
Coles	6.5 ± 3.6	0.89 ± 0.21	0.024 ± 0.015

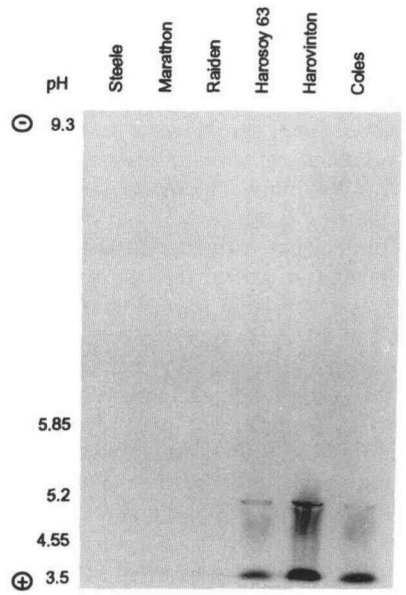

Figure 1. IEF of soybean seed coat extracts and staining for peroxidase activity with chloronaphthol (each sample equal to the supernatant of 0.5 mg fresh weight). The migration of IEF protein standards (Pharmacia) and their corresponding pI values are indicated on the left.

dase activity after IEF of samples from *epep* cultivars was barely discernible.

Analysis of root extracts revealed that at least 10 peroxidase isozymes are present in this tissue, and that the major species is an isozyme with a pI of 5.2 (Fig. 2). The profile of peroxidase isozymes present in root extracts was similar among the six cultivars tested, with one significant exception.

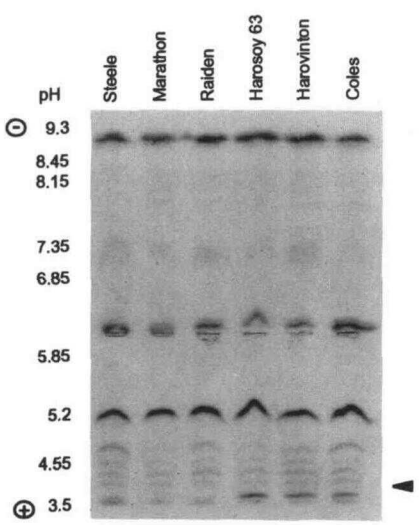

Figure 2. IEF of soybean root extracts and staining for peroxidase activity with chloronaphthol (each sample equal to the supernatant of 0.5 mg fresh weight). The arrow indicates the position of the pI 3.9 peroxidase. The migration of IEF protein standards (Pharmacia) is indicated on the left.

Extracts derived from the *epep* types were lacking a peroxidase of pI 3.9 that was present in the *EpEp* plants, demonstrating that the *Ep* locus may affect peroxidase expression in tissues other than the seed coat.

Leaf extracts had to be concentrated by acetone precipitation before peroxidases could be visualized by cholorona-phthol on IEF gels. Two isozymes, of pI 4.5 and 4.3 and of equal intensity, accounted for most of the peroxidase activity in all cultivars except for Raiden, which had lesser amounts of these anionic peroxidases but contained a predominant cationic peroxidase of pI 9.0 (not shown). Thus, in the leaf there was no difference in the expression of peroxidase isozymes that is associated with the *Ep* locus.

SDS-PAGE of Seed Coat Proteins and Purification of Peroxidase

To determine whether the difference in peroxidase activity of *EpEp* versus *epep* plants is related to changes in protein expression, seed coat extracts of the six soybean varieties were analyzed by SDS-PAGE (Fig. 3). A prominent band of 43 kD was present in extracts of the high seed coat peroxidase cultivars but was reduced in extracts of low seed coat peroxidase cultivars. This difference was also apparent in extracts from seed coats of developing seeds. Since this 43-kD protein band differs by 6 kD from the 37-kD band reported for soybean seed coat peroxidase in previous studies (Sessa and Anderson, 1981; Gillikin and Graham, 1991), we decided to purify the seed coat peroxidase to verify that the 43-kD band present in *EpEp* cultivars examined in this study is the seed coat peroxidase. An SDS-PAGE analysis of samples from each step of the purification is shown in Figure 4. A single

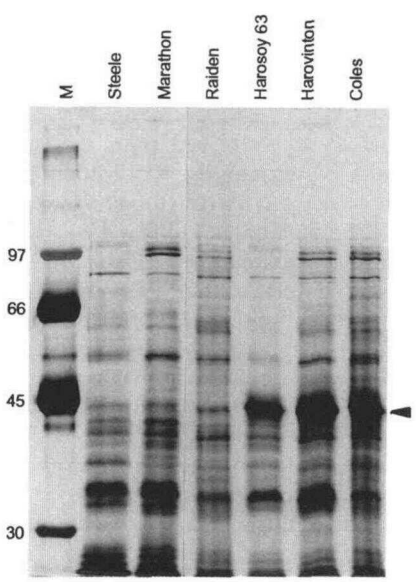

Figure 3. Analysis by SDS-PAGE of soybean seed coat extracts (each sample at approximately 2 μg of protein). The arrow indicates the position of the 43-kD seed coat peroxidase. The migration of protein standards (Bio-Rad) and their corresponding masses in kD are also indicated (lane M).

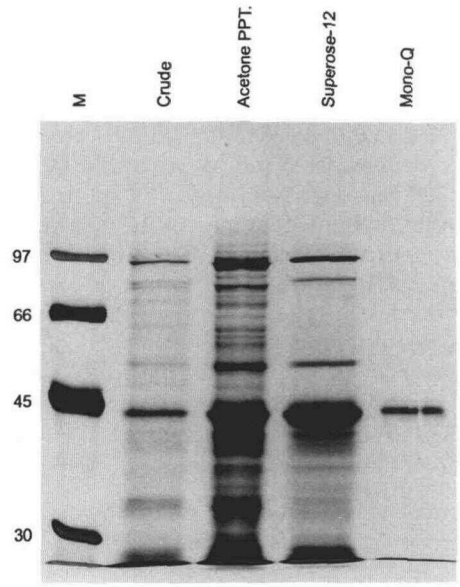

Figure 4. Analysis by SDS-PAGE of soybean seed coat peroxidase at different stages of purity (approximately 1 μg of total protein for Crude, acetone precipitate [Acetone PPT], and Superose-12 samples, approximately 0.2 μg of protein for Mono-Q sample). The migration of protein standards (Bio-Rad) and their corresponding masses in kD are also indicated (lane M).

band of 43 kD was present after the final step of purification, anion-exchange chromatography on a Mono-Q column. However, the native molecular mass of the peroxidase was 37 kD (determined by chromatography on a Superose 12 column and comparison with protein standards), and thus corresponded to the mass determined by SDS-PAGE in previous studies (Sessa and Anderson, 1981, Gillikin and Graham, 1991).

Localization of Peroxidase Activity in the Seed Coat

Thin sections of soybean seed coats were stained with chloronaphthol to determine the localization of peroxidase activity and how this may differ among the six cultivars. In the high seed coat peroxidase cultivar Harovinton, activity was concentrated in the hourglass cells of the subepidermis and in the placental cells of the hilum (Fig. 5). Staining of the hourglass cells is intense and confined to the cytoplasm. The cytoplasm of the hourglass cells appeared fluid and viscous, and slowly exuded from both ends of the cells when the tissue was placed in water. Peroxidase activity in the placental cells was restricted to discrete globular bodies within the cytoplasm, and extracellularly around the point of detachment from the funicle. Staining also occurred in compressed parenchymatous tissue in the region of the aleurone layer and around the bar of tracheid-like cells of the hilum. Seed coats of many other *EpEp* cultivars were examined, including Harosoy 63 and Coles, and the localization of peroxidase activity was identical to that observed in Harovinton.

In contrast to the intense staining of the hourglass cells of Harovinton, staining was faint or not detectable in Steele, Marathon, Raiden, and all other *epep* cultivars examined (Fig. 5). Although the contents of the hourglass cells did not stain for peroxidase in these *epep* cultivars, the cytoplasm was still observed to slowly exude from both ends of the cells upon imbibition. Expression of peroxidase activity in placental cells of the *epep* cultivars was also different from that of *EpEp* plants. The darkly staining globular bodies observed in these cells of *EpEp* plants did not show up in the staining of *epep* cultivars. Staining of parenchymatous tissue in the region of the aleurone layer and around the bar of tracheid-like cells of the hilum was observed in *epep* cultivars, although it was less intense than in *EpEp* types. Many cultivars of both genotypes were compared, and the pattern of staining was consistent within the genotypes. However, the *epep* cultivar Raiden stained consistently for peroxidase activity in the palisade epidermis and the placenta cells of the hilum (Fig. 5). This staining was light and diffuse and was not like the intense staining observed in hourglass and placenta cells of *EpEp* plants.

Aside from the differences observed among the cultivars when staining for peroxidase activity, there were no obvious gross or microscopic features of the seed coat structure, rigidity, or permeability that correlated with the genotype of the *Ep* locus. When seed coat sections were stained for lignin by the test of Mäule ($KMnO_4$/HCl/NH_3; positive for syringyl lignin) or Wiesner (phloroglucinol-HCl; positive for guaiacyl lignins), the bar of tracheid-like cells in the hilum region and the parenchymous tissue around these cells stained positively in both tests, whereas the hourglass cells did not stain, regardless of *Ep*/*ep* genotype (not shown).

DISCUSSION

High peroxidase activity in soybean seed coats is controlled by a single dominant gene, *Ep* (Buzzell and Buttery, 1969). Cell-free extracts from the seed coats of high peroxidase varieties are about 100-fold higher in specific activity than low peroxidase varieties (Buttery and Buzzell, 1968). Neither the *Ep* gene nor its corresponding recessive allele *ep* have been isolated, and hence there are many possible explanations for this variation in seed coat peroxidase activity. The enzyme may be expressed but defective due to mutation, lack of cofactor, or improper processing. Alternatively, low activity may be due to lack of expression of the seed coat peroxidase gene, resulting from mutation of a regulatory protein or transcriptional factor, or from disruption of the coding sequence of the structural gene. By analyzing soluble, cell-free extracts of seed coats from six different soybean cultivars by SDS-PAGE, we have shown that the amount of peroxidase protein present in extracts of *epep* cultivars is greatly reduced compared with that present in *EpEp* genotypes. These observations, together with results from previous investigations (Gillikin and Graham, 1991), also indicate that peroxidase is an abundant protein in soluble extracts of seed coats from *EpEp* cultivars. Thus, it seems that the low peroxidase activity in *epep* cultivars is a result of a lack of expression of the enzyme.

The profile of peroxidase isozymes in root tissue also differed among the six soybean cultivars examined. An additional peroxidase isozyme, of identical pI to the seed coat

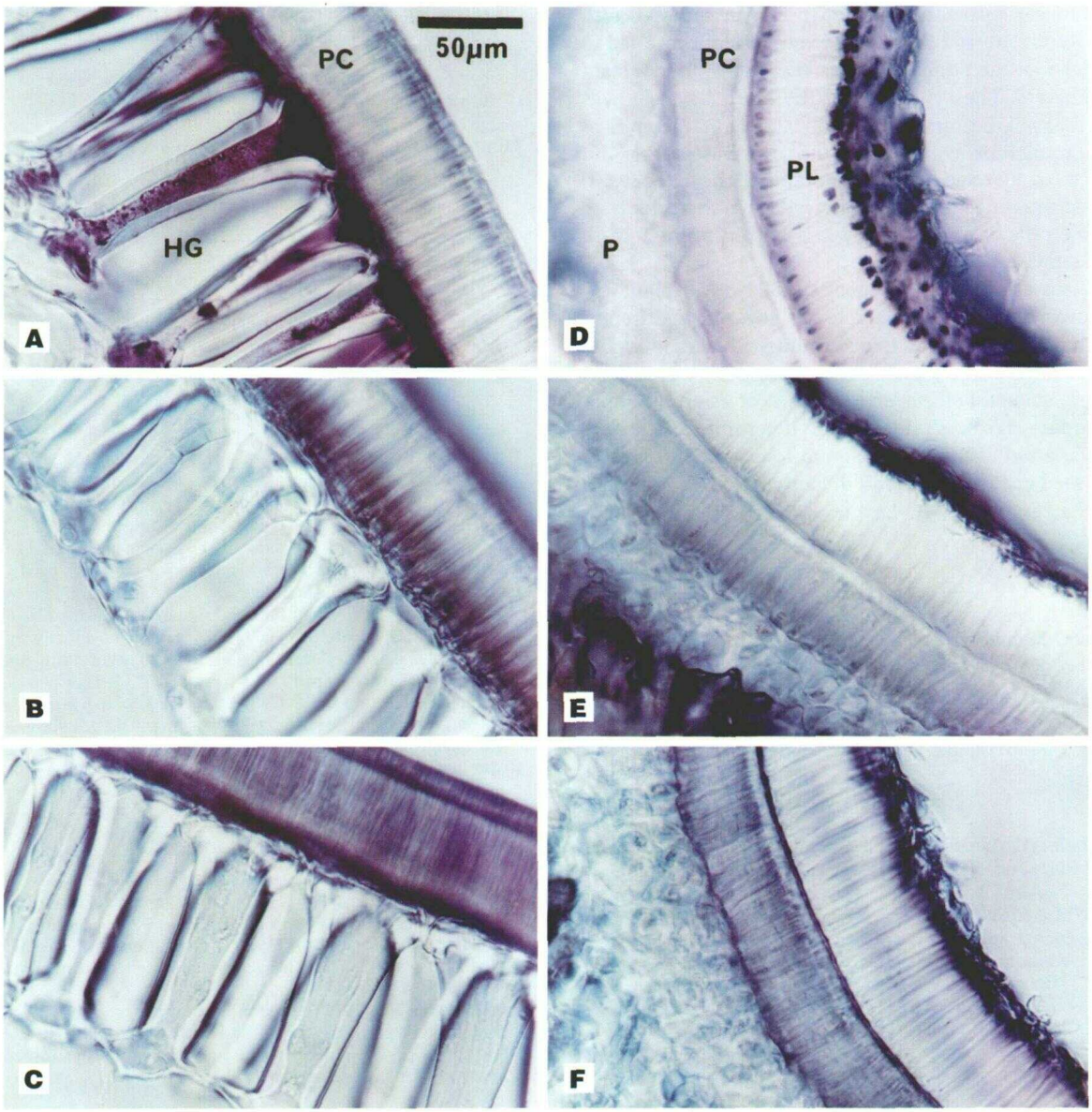

Figure 5. Histochemical localization of peroxidase activity in soybean seed coats. Hand sections of seed coat tissue were stained with chloronaphthol and examined by bright-field microscopy as described in "Materials and Methods." PC, Palisade cells; HG, hourglass cells; P, parenchyma; PL, placenta cells. Epidermis and subepidermis of cultivars Harovinton (A), Steele (B), and Raiden (C). Hilum region of cultivars Harovinton (D), Steele (E), and Raiden (F).

peroxidase, was present only in plants with the *Ep* gene. Apparently, the seed coat peroxidase may be present in other tissues, expression that is nonetheless still controlled by the *Ep* locus.

High peroxidase activity in seed tissues has been observed in plant species other than soybean, including *Sida spinosa* (Egley et al., 1983), *Brassica juncea* (La Beller et al., 1986), and *Araucaria araucana* (Riquelme and Cardemil, 1993). Although the function of peroxidase in seed tissues is not known for certain, it is generally assumed to be important in the process of lignification and the resulting hardness and permeability characteristics of the seed tissues (Egley et al., 1983; La Beller et al., 1986; Riquelme and Cardemil, 1993). Similarly, it has been suggested that soybean seed coat peroxidase is involved in extensin polymerization, lignification, or suberization (Gillikin and Graham, 1991). The hard seed coat, the thick-walled cells that constitute this tissue, and the deposition of extensin in these tissues (Cassab and Varner, 1987) seem to make this an attractive hypothesis. However, if the seed coat peroxidase controlled by the *Ep* locus is important in the biosynthesis of the seed coat, one might expect that the great difference in expression of this enzyme in *EpEp* genotypes compared with *epep* plants would be manifested in some obvious change in seed coat structure, rigidity, permeability, or lignification. No such differences were noted.

Histochemical localization of peroxidase in seed coats of *EpEp* genotypes showed that most activity is concentrated in the cytoplasm of the hourglass cells. These cells did not stain in *epep* cultivars. The cytoplasm of the hourglass cells was observed to swell, become fluid, and exude from the ends of cells upon imbibition with water. Although this process did not seem to be affected by the genotype of the *Ep* locus, it may be that the concentration of peroxidase in the cytoplasm of hourglass cells of *EpEp* genotypes is related to the release of this material during imbibition.

Peroxidases are among the most studied of all plant enzymes; however, the catalytic roles of the many different isozymes have been difficult to determine with certainty. The soybean *Ep* locus will be useful in exploring the function of peroxidases, since this locus affects the expression of a specific peroxidase. Work is underway to determine the genetic cause of variation at the *Ep* locus, and to examine in greater detail the seed coat structure and the antimicrobial properties of seed coat extracts of *EpEp* plants compared with *epep* genotypes.

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