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Tissue Level Compartmentation of (*R*)-Amygdalin and Amygdalin Hydrolase Prevents Large-Scale Cyanogenesis in Undamaged *Prunus* Seeds[1]

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Plum (*Prunus domestica*) seeds, which contain the cyanogenic diglucoside (*R*)-amygdalin and lesser amounts of the corresponding monoglucoside (*R*)-prunasin, release the respiratory toxin HCN upon tissue disruption. Amygdalin hydrolase (AH) and prunasin hydrolase (PH), two specific β-glucosidases responsible for hydrolysis of these glucosides, were purified to near homogeneity by concanavalin A-Sepharose 4B and carboxymethyl-cellulose chromatography. Both proteins appear as polypeptides with molecular masses of 60 kD upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but they exhibit different isoelectric points (PH, 5.6–6.0; AH, 7.8–8.2). AH and PH were localized within mature plum seeds by tissue printing, histochemistry, and silver-enhanced immunogold labeling. As was previously shown in black cherry (*Prunus serotina*) seeds (E. Swain, C.P. Li, J.E. Poulton [1992] Plant Physiol 100: 291–300), AH and PH are restricted to protein bodies of specific procambial cells and are absent from the cotyledonary parenchyma, bundle sheath, and endosperm cells. In contrast, the cyanogenic glycosides in both plum and black cherry seeds, which were detected by tissue printing, occur solely in the cotyledonary parenchyma and are absent from the procambium and endosperm. It is concluded that tissue level compartmentation prevents large-scale cyanoglycoside hydrolysis in intact *Prunus* seeds.

In common with the catabolism of several other classes of plant glycosides (e.g. glucosinolates [Bones et al., 1991; Poulton and Møller, 1993] and *o*-coumaric acid glucosides [Oba et al., 1981]), the large-scale degradation of cyanogenic glycosides to HCN occurs only upon tissue disruption (Conn, 1981). It is generally assumed that premature cyanogenesis is avoided in undamaged plants by compartmentalization of the glycoside and its catabolic enzymes at the tissue or subcellular levels. Recent studies involving a wide range of cyanophoric plant species and organs have not yet revealed a common compartmentation strategy (Poulton, 1988). In leaves of 6-d-old light-grown sorghum seedlings, the components of the "cyanide bomb" (i.e. the cyanoglycoside and its catabolic enzymes) are compartmentalized within different tissues. The cyanogenic glycoside (*S*)-dhurrin occurs in vacuoles of epidermal cells, whereas its β-glucosidase and α-hydroxynitrile lyase are located almost exclusively within the underlying mesophyll tissue (Saunders and Conn, 1978; Kojima et al., 1979). For other species, localization data have been interpreted largely in terms of compartmentation at the subcellular level, although in most cases both glycosides and catabolic enzymes have not been unequivocally localized within the same cells (Kojima et al., 1983; Kakes, 1985; Frehner and Conn, 1987; Mkpong et al., 1990).

In recent years, our laboratory has been investigating the catabolism of (*R*)-amygdalin [the β-gentiobioside of (*R*)-mandelonitrile] in rosaceous stone fruits using mature black cherry (*Prunus serotina*) seeds as an experimental system (Poulton, 1993). Upon tissue disruption, this cyanogenic diglucoside is rapidly degraded to mandelonitrile and Glc by the sequential action of AH and PH. Subsequently, mandelonitrile dissociates either enzymically in the presence of MDL or nonenzymically to release HCN and benzaldehyde. Immunocytochemical localization studies, using monospecific polyclonal antisera raised against each deglycosylated enzyme, revealed that these catabolic enzymes have intriguing tissue and cellular distributions (Swain et al., 1992b). The β-glycosidases AH and PH are restricted to protein bodies of specific procambial cells. In contrast, MDL shows highest levels within protein bodies of the cotyledonary parenchyma cells with lesser amounts in procambial cell protein bodies. All three enzymes are absent from the endosperm tissue and from the bundle sheath layer surrounding the procambium. To reveal how untimely cyanogenesis is prevented in intact black cherry seeds now requires the localization of amygdalin, a task made difficult by the low mol wt and high aqueous solubility of this glycoside and by the diminutive size of the seed.

In the present study, the aforementioned polyclonal antisera were used to localize AH and PH in mature plum (*Prunus domestica*) seeds. Furthermore, amygdalin was localized at the tissue level in both black cherry and plum seeds by tissue printing. Taken together, the data from these and earlier studies (Swain et al., 1992b) strongly suggest that premature detonation of the *Prunus* cyanide bomb is prevented in undamaged seeds by tissue level compartmentation.

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Abbreviations: AH, amygdalin hydrolase; GAR, goat anti-rabbit; MDL, (*R*)-mandelonitrile lyase; 4-MUG, 4-methylumbelliferyl-β-D-glucoside; PH, prunasin hydrolase; pI, isoelectric point.

MATERIALS AND METHODS

Plant Materials

Mature black cherry (*Prunus serotina* Ehrh.) and plum (*Prunus domestica*, var Stanley) fruits were obtained locally. Pits (endocarp enclosing seed) were freed from exocarp and fleshy mesocarp tissues, surface sterilized with 0.52% (w/v) sodium hypochlorite for 20 min, and rinsed thoroughly with distilled water. Finally, they were blotted dry on paper towels and stored in plastic containers at 4°C until used.

Chemicals and Biochemicals

Nitrocellulose paper was obtained from Bio-Rad (Richmond, CA). Aldrich Chemical Co. (Milwaukee, WI) provided mercurous nitrate. Almond β-glucosidase (G-0395), which contains high AH, PH, and MDL activities, was purchased from Sigma. All other biochemicals and immunochemicals were obtained as previously described (Li et al., 1992; Swain et al., 1992b).

Antisera

The immunolocalization studies described here utilized two affinity-purified polyclonal antisera with demonstrated monospecificity toward black cherry AH and PH, respectively (Li et al., 1992).

Partial Purification of Plum AH and PH

All operations were undertaken at 4°C. Plum seeds (5 g) were homogenized with a pestle and mortar with 1 g of polyvinylpolypyrrolidone, 2 g of quartz sand, and 30 mL of buffer A (0.1 M His-HCl buffer, pH 6.0). The homogenate was filtered through four layers of cheesecloth and centrifuged for 25 min at 17,600*g*. The clear supernatant was dialyzed overnight against 4 L of buffer B (10 mM His-HCl, pH 6.0, containing 0.17 M NaCl) and then subjected to Con A-Sepharose 4B chromatography as previously described (Li et al., 1992). Fractions displaying AH and PH activities were pooled and dialyzed overnight against 4 L of buffer C (20 mM sodium acetate-HCl buffer, pH 5.0). The resulting preparation was centrifuged for 5 min at 17,600*g* before application to a CM-cellulose (1 × 10 cm) column that had previously been equilibrated with buffer C. After the column was washed with 100 mL of buffer C to remove unbound proteins, bound proteins were eluted by a linear gradient (100 mL) of 0 to 0.3 M NaCl in buffer C. AH and PH fractions were pooled separately, and each pool was concentrated to 5 mL by ultrafiltration.

Gel Electrophoresis

IEF was performed on PhastGel IEF 3/9 gels at 15°C using the Pharmacia Phast System. Total proteins were visualized using a silver-staining kit (Pharmacia) according to manufacturer's instructions. AH and PH were located on native IEF gels by activity staining using the fluorogenic substrate 4-MUG. Gels were examined under UV light after 5 min of incubation in 0.2 M sodium acetate buffer, pH 4.75, containing 5 mM 4-MUG.

Immunochemical Analysis

The specificities of the *P. serotina* anti-AH and anti-PH antisera toward the corresponding plum hydrolases were analyzed by immunoblotting (Swain et al., 1992b). Crude plum seed homogenates as well as highly purified AH and PH were subjected to one-dimensional SDS-PAGE on 10% gels (Laemmli, 1970). Where appropriate, gels were stained with brilliant blue G-colloidal to visualize proteins. For immunochemical analysis, proteins were electroblotted onto nitrocellulose membranes using a Bio-Rad Trans-Blot apparatus and challenged with either purified anti-AH or purified anti-PH antisera (previously diluted 1:100 with blocking buffer (5% [w/v] nonfat dry milk in 10 mM Tris-HCl [pH 7.4], containing 0.9% [w/v] NaCl). In control blots, these antisera were replaced by preimmune serum. Immune complexes were visualized using horseradish peroxidase-conjugated GAR IgG (diluted 1:500 in blocking buffer) and 4-chloro-1-naphthol (Swain et al., 1992a).

Localization of AH by Tissue Print Immunoblots

Tissue printing was performed according to the method of Spruce et al. (1987). Thin seed sections were cut using a razor blade and transferred to a nitrocellulose membrane backed by three layers of Whatman No. 1 filter paper on a table top. A small piece of nonabsorbent paper was placed over the section. Slight finger pressure was applied for 30 s, and the nonabsorbent paper and tissue sections were removed from the membrane. Tissue blots were then immunoanalyzed as described previously (Swain et al., 1992a).

Light Microscope-Level Immunolocalization of *P. domestica* AH and PH

Plum AH and PH were localized at the tissue level by silver-enhanced immunogold labeling using the monospecific *P. serotina* anti-AH or anti-PH antisera as the primary antibody (Swain et al., 1992b). In brief, seed slices were fixed and dehydrated before embedment in LR White resin. Sections (0.5–1.0 μm thick) were cut with a DuPont diamond knife on a Sorvall Porter-Blum MT 2-B ultramicrotome, collected on Superfrost (+) slides (Fisher Scientific), and dried overnight at 42°C. They were subsequently exposed to primary antisera (diluted 1:10) and secondary antiserum (10-nm colloidal gold-conjugated GAR IgG, diluted 1:20) before treatment with a silver enhancement kit (Ted Pella, Inc.) (Swain et al., 1992b). The distribution of colloidal silver was recorded using an Olympus BH-2 light microscope equipped with an Olympus C-35 AD camera.

Localization of Cyanoglucosides by Tissue Printing

The tissue distribution of cyanoglucosides in *Prunus* seeds was determined by tissue printing. Seeds were halved (transverse section) using a double-edged razor blade. Their cut surfaces were applied with slight finger pressure to a nitrocellulose paper previously moistened with 20 mg mL^{-1} of aqueous almond β-glucosidase. After 3 to 10 s, the seeds were removed, and one drop of mercurous nitrate (saturated aqueous solution) was applied to the blot. Areas of the blot

corresponding to cells originally containing amygdalin and/or prunasin appeared black. The distribution of colloidal mercury was recorded using an Olympus BH-2 light microscope (dark-field illumination) equipped with an Olympus C-35 AD camera. This method was modified to locate cells containing both AH and PH by applying halved seeds to papers presoaked in 50 mM amygdalin (rather than β-glucosidase).

Analysis of Cyanogen Content

Qualitative analysis of prunasin and amygdalin content of plum seeds was accomplished according to the method of Poulton and Shin (1983).

Protein Determination and Enzyme Assays

Protein was determined by the Bradford (1976) procedure using a Bio-Rad protein assay kit with BSA serving as the standard. AH and PH activities were assayed as previously described (Kuroki and Poulton, 1986, 1987).

RESULTS AND DISCUSSION

Whereas the flesh of rosaceous stone fruits (e.g. plums, peaches, cherries) is considered acyanogenic and has long been enjoyed by animals including humans, their seeds have been responsible for numerous cases of acute cyanide poisoning (Poulton, 1983). Containing both the cyanogenic diglucoside (*R*)-amygdalin (as major cyanogen) and its catabolic enzymes, these seeds constitute miniature cyanide bombs capable of rapid HCN release upon tissue disruption. Fortunately, such large-scale, suicidal cyanogenesis is apparently avoided in undamaged tissue by compartmentation. For several years, our laboratory has been working to elucidate whether the key components of the *Prunus* cyanide bomb are compartmentalized at the tissue or subcellular levels. We have made extensive use of mature seeds of black cherry (*P. serotina*), a species native to the American Midwest. Employing monospecific, polyclonal antibodies raised against deglycosylated AH, PH, and MDL, each of these catabolic enzymes was immunolocalized at the tissue and subcellular levels (Swain et al., 1992b).

The two β-glycosidases are restricted to the protein bodies of the procambium, which ramifies throughout the cotyledonary storage parenchyma. It is interesting that, whereas AH occurs within most procambial cells, PH is confined to the peripheral layers of this tissue. By contrast, highest levels of MDL are observed in protein bodies of cotyledonary parenchyma cells, with only lesser amounts in procambial cells. The bundle sheath layer surrounding the procambium and the endosperm apparently lack all three catabolic enzymes. As previously noted (Swain et al., 1992b), the absence of the hydrolytic enzymes in bundle sheath, cotyledonary parenchyma, and endosperm cells renders these tissues particularly attractive locations for housing amygdalin. Unfortunately, the already difficult task of localizing amygdalin is compounded by the small size of the black cherry seed. To facilitate our efforts to elucidate the compartmentation in *Prunus* seeds, parallel experiments were undertaken using plums (*Prunus domestica*), an economically important stone fruit crop having much larger seeds. Qualitative analysis showed that (*R*)-amygdalin predominates within these seeds, but the monoglucoside (*R*)-prunasin is also present at far lower levels.

Purification of *P. domestica* AH and PH

For use in immunoblotting analyses (see below), AH and PH were purified to near homogeneity from mature plum seeds by affinity and ion-exchange chromatography (Fig. 1). In common with most other cyanogenic β-glycosidases (Poulton, 1988), these hydrolases are probably glycoproteins as evidenced by their binding to Con A-Sepharose 4B and elution by methyl-α-D-glucoside. The substantially purified enzymes were then resolved by CM-cellulose chromatography. Both β-glycosidases bound to this ion exchanger, with PH eluting at lower (0–50 mM) and AH eluting at higher (100–150 mM) NaCl concentrations. This behavior is consistent with the pI values of 5.6 to 6.0 and 7.8 to 8.2 for PH and AH, respectively, obtained by PhastGel IEF (Fig. 2). Upon SDS-PAGE, PH and AH appeared as major polypeptides of 60 kD (Fig. 1). By comparison, the corresponding *P. serotina* hydrolases had the following pI values and polypeptide molecular masses: PH, pI 4.0 to 4.5, 68 kD; AH, pI 5.8 to 6.4, 60 kD (Poulton, 1993).

Proof of Monospecificity of *P. serotina* Anti-AH and Anti-PH Antisera

To utilize the available *P. serotina* anti-AH and anti-PH antisera to immunolocalize the respective plum hydrolases requires that their monospecificity be demonstrated. This evaluation was made by western immunoblotting after one-dimensional SDS-PAGE of crude plum seed extracts and of highly purified plum AH and PH preparations. As shown in Figure 1, each antiserum is monospecific for its corresponding plum β-glycosidase. In control experiments in which these

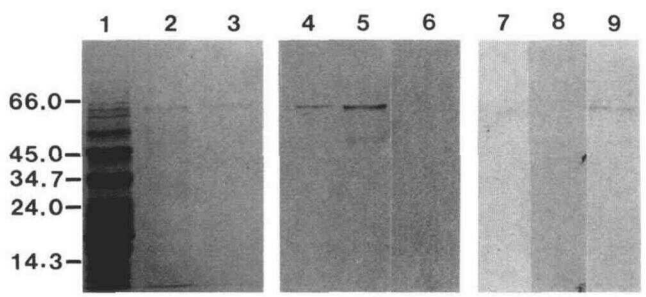

Figure 1. Western immunoblot analysis of the specificity of *P. serotina* anti-AH and anti-PH antisera toward plum AH and PH. Total plum seed proteins (5 μg; lanes 1, 4, and 7) and purified plum AH (0.5 μg; lanes 2, 5, and 8) and PH (0.5 μg; lanes 3, 6, and 9) were subjected to SDS-PAGE (10% gels). Proteins in lanes 1 to 3 were stained with brilliant blue G-colloidal stain. Proteins in other lanes were electroblotted onto nitrocellulose membranes and challenged with *P. serotina* anti-AH (lanes 4–6) or anti-PH antiserum (lanes 7–9). Molecular masses of calibration proteins (Sigma) are indicated in kD.

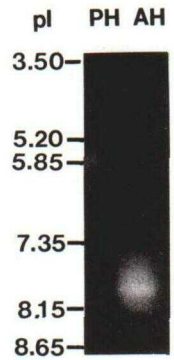

Figure 2. Determination of the pIs of plum AH and PH. The purified enzymes (1 μg each) were subjected to IEF on a PhastGel IEF 3/9 gel and detected by activity staining using the fluorogenic substrate 4-MUG. The pIs of the broad-range pI markers (Pharmacia) are also shown.

active antisera were replaced by preimmune serum, no immunostaining was observed (data not shown). Thus, the two black cherry antisera were deemed suitable for immunolocalization studies with plum seeds.

Localization of Plum AH and PH by Immunochemical and Histochemical Approaches

The localization of AH in transversely sectioned plum cotyledons was first attempted by tissue print immunoblotting (Spruce et al., 1987). Blots challenged with anti-AH antiserum as primary antibody showed two linear series of immunostained spots (Fig. 3A) corresponding to the arrangement of procambial strands within the cotyledons. Control blots exposed to preimmune serum exhibited no immunostaining (Fig. 3B). The procambial location of AH was subsequently confirmed by silver-enhanced immunogold procedures. When LR White-embedded cotyledon sections were exposed to anti-AH antiserum as the primary antibody, immunolabeling was restricted to protein bodies of specific procambial cells (Fig. 4A). The bundle sheath, cotyledonary parenchyma, and endosperm cells remained unlabeled. Likewise, with anti-PH antiserum as the primary antibody, cotyledon sections again exhibited labeling only in procambial cell protein bodies (Fig. 4B). By contrast, no immunolabeling was observed in sections receiving preimmune serum as the primary antibody (Fig. 4C). Close analysis of the serial sections shown in Figure 4, A and B, reveals that, as in black cherry (Swain et al., 1992b), AH and PH exhibit cell-specific distribution within the procambium. Neither enzyme occurs in the smallest procambial cells, which because of their lack of prominent protein bodies are regarded as being more highly differentiated. AH is located in most of the remaining procambial cells, whereas PH appears confined to the larger, peripheral cells. In conclusion, plum AH and PH exhibit tissue- and cell-specific distributions that are identical with those previously described for the corresponding black cherry hydrolases (Swain et al., 1992b).

Additional evidence supporting the procambial location of plum AH and PH was obtained using indoxyl β-D-glucoside as an activity stain. Preliminary experiments had shown that both highly purified plum hydrolases cleave this substrate to the insoluble blue product indican (data not shown). When transversely sectioned plum seeds were appressed to nitrocellulose membranes presoaked with indoxyl β-D-glucoside, the resulting blots showed linear rows of indican spots corresponding to the location of procambial strands across each cotyledon (Fig. 3C). Identical data were obtained with black cherry seeds (data not shown).

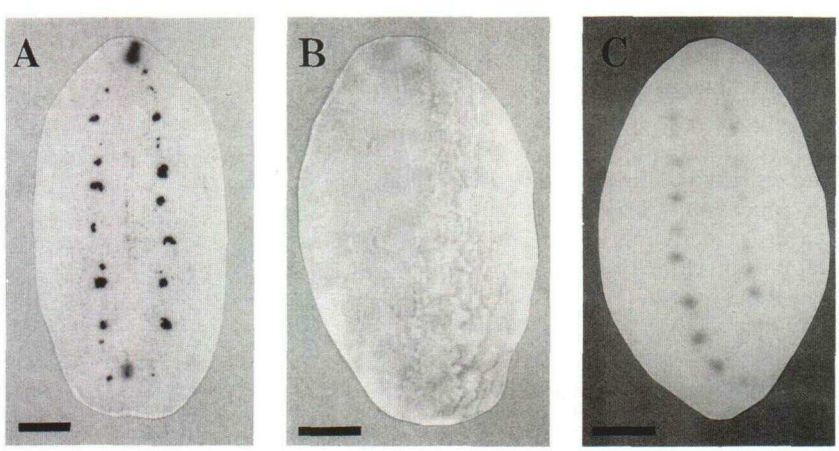

Figure 3. Localization of AH and PH within plum cotyledons by tissue print immunoblots and histochemistry. Tissue prints of transversely sectioned plum seeds were exposed to either anti-AH antiserum (A) or preimmune serum (B) as the primary antibody. Blots were subsequently incubated with horseradish peroxidase-conjugated GAR IgG, and the locations of antigen-antibody complexes were visualized by addition of 4-chloro-1-naphthol and H_2O_2 (Swain et al., 1992a). Histochemical localization of AH and PH is shown in C. Transversely sectioned plum seeds were appressed to nitrocellulose papers presoaked with indoxyl β-D-glucoside. The existence of AH and PH in procambial strands is revealed by the product indican, which appears as two rows of darkly staining spots. Scale bar = 1 mm.

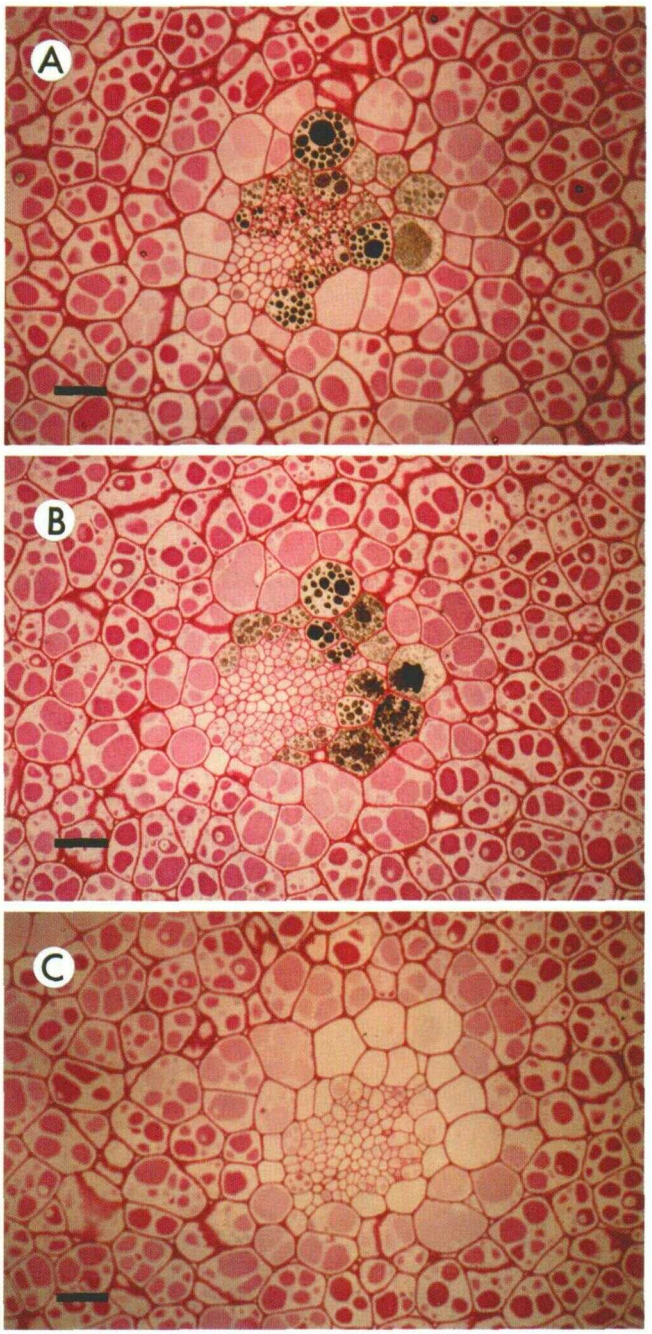

Figure 4. Tissue localization of plum AH and PH by silver-enhanced immunogold labeling. Serial transverse sections of cotyledons embedded in LR White resin were challenged with either anti-AH antiserum (A), anti-PH antiserum (B), or preimmune serum (C) as the primary antibody. After exposure to secondary antibody (colloidal gold-conjugated GAR IgG), poststaining was undertaken with basic fuchsin. Immunogold-labeled antigens appear as black areas after silver enhancement. Scale bar = 35 μm.

Tissue Print Localization of Cyanogenic Glycosides in Plum and Black Cherry Seeds

After we ascertained the distribution of AH and PH in plum and black cherry cotyledons, our attention then focused on localizing amygdalin and prunasin. In common with other unpigmented, water-soluble natural products of low mol wt, the cyanogenic glycosides are notoriously difficult to localize at the tissue and subcellular levels (Brunswick, 1921; Saunders et al., 1977). Our early experiments approached the problem of cyanoglycoside localization through protoplast isolation and subfractionation, a method that was successfully used to locate dhurrin in sorghum leaf blades (Saunders and Conn, 1978; Kojima et al., 1979). Although we optimized techniques to obtain protoplasts in moderate yields from black cherry seeds by cellulase/pectinase digestion (data not shown), our protoplast populations are undoubtedly derived from several tissues (e.g. storage parenchyma, bundle sheath, and procambium). Therefore, we believed that this approach would at best prove ambiguous in localizing these glycosides.

Encouraged by the successful localization of ascorbic acid in plants by tissue printing (Pont-Lezica, 1992), we instead modified this technique to provide a novel approach to cyanoglycoside localization that is dependent on the ability of cyanoglycosides to leak from the cut tissue surface and become bound to nitrocellulose. Preliminary experiments showed that when a drop of mercurous nitrate is applied to a nitrocellulose paper previously spotted with aqueous NaCN, a localized gray-black precipitate of metallic mercury appears at those sites because of the chemical reaction (Svehla, 1987):

$$2CN^- + Hg_2^{2+} \rightarrow Hg \downarrow + Hg(CN)_2$$

When applying this reaction to localize cyanoglycosides in *Prunus* seed slices, one should bear in mind that, depending on how the cyanide bomb components are compartmentalized, the glycoside-containing cells may or may not contain AH and PH. Therefore, for meaningful tissue prints, it is imperative that all cells, whether or not they contain endogenous catabolic enzymes, be first exposed to excess AH, PH, and MDL. This would ensure that all amygdalin- and prunasin-containing cells would liberate cyanide for subsequent detection by mercurous nitrate. For this reason, halved seeds were appressed for several seconds to nitrocellulose membranes that had been presoaked with 20 mg mL^{-1} of almond emulsin. The seeds were then removed and mercurous nitrate was added without delay to react with any liberated cyanide. Those cells originally containing amygdalin and/or prunasin gave rise to easily visible, gray-black zones on such tissue prints.

In tissue prints obtained in this manner, zones corresponding to the cotyledonary parenchyma were intensely labeled, indicating the presence of amygdalin and/or prunasin (Fig. 5A). Zones corresponding to the procambial strands were easily visible as white areas on the rather uniform black background generated by the cotyledonary parenchyma cells. Thus, we conclude that these strands, although rich in AH and PH, lack the cyanoglycosides. Zones corresponding to the endosperm were also white. Taken together with the immunocytochemical data described above, this indicates

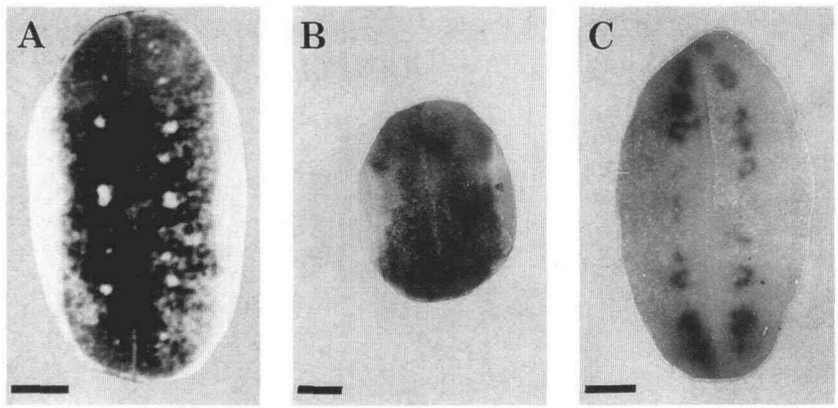

Figure 5. Localization of cyanoglucosides (A and B) and their respective β-glycosidases (C) in mature *Prunus* seeds. Transversely sectioned plum (A) and black cherry (B) seeds were appressed to nitrocellulose papers previously moistened with aqueous almond β-glucosidase. After 3 to 10 s, the seeds were removed, and a drop of mercurous nitrate was applied to the blot to detect liberated cyanide. The procambial location of AH and PH in plum seeds was confirmed by applying transversely sectioned seeds to nitrocellulose papers presoaked with 50 mM amygdalin (C). Soon thereafter, the seeds were removed, and liberated cyanide was detected by applying mercurous nitrate. Scale bar = 1 mm.

that the endosperm lacks both the cyanoglycosides and the hydrolases. Because of technical difficulties encountered with the smallness of the black cherry seeds, tissue prints were more difficult to obtain with this species, and they suffered from poorer resolution. Nevertheless, blots were highly suggestive that amygdalin is similarly distributed within black cherry seed tissues (Fig. 5B).

Although not tested here, it is possible that this tissue-printing technique might prove useful in localizing other cyanoglycosides in organs of different cyanophoric species if the nitrocellulose papers are presoaked in the appropriate catabolic enzymes.

Tissue Print Localization of AH and PH in *Prunus* Seeds

The presence of AH and PH within the procambial strands of plum and black cherry seeds was further confirmed by modifying the above tissue-printing technique in the following manner. Halved seeds were appressed for a brief period to nitrocellulose blots presoaked with high amygdalin concentrations (50 mM) before application of mercurous nitrate. This modification was intended to minimize any effect of endogenous amygdalin levels by providing excess glycoside to all cells. As exemplified by the blot obtained with plum seeds (Fig. 5C), such blots exhibited rows of spots that again correlated with the position of the procambial strands in each cotyledon. Unlike the indoxyl β-D-glucoside blots, however, these spots were ring or horseshoe shaped, rather than solid, in appearance; this observation is fully consistent with the peripheral location of PH within the procambium.

CONCLUSIONS

In the present study, a novel tissue-printing technique was devised allowing the successful localization of the cyanoglucosides in *Prunus* seeds to the cotyledonary parenchyma tissue. Several approaches have now confirmed that AH and PH are restricted in both black cherry and plum seeds to the protein bodies of relatively few specific cells, in this case, certain procambial cells. Having reviewed all available information, it becomes clear that tissue level, rather than subcellular level, compartmentalization prevents premature detonation of the *Prunus* cyanide bomb in undamaged seeds. Although tissue level compartmentation was also observed in light-grown sorghum leaves (Kojima et al., 1979), it remains to be seen whether this strategy is common to all cyanogenic species. Current evidence with other cyanophoric systems (Kojima et al., 1983; Kakes, 1985; Frehner and Conn, 1987; Mkpong et al., 1990) has been interpreted largely in terms of subcellular compartmentation similar to that shown by coumarinyl glucoside and its respective β-glucosidase in sweet clover (Oba et al., 1981). However, further studies will be necessary in these cases to unequivocally exclude tissue level compartmentalization.

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