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Identification of a Ca^{2+}-Pectate Binding Site on an Apoplastic Peroxidase

Sabine Carpin,[1] Michèle Crèvecoeur, Mireille de Meyer, Patrice Simon, Hubert Greppin, and Claude Penel[2]

Laboratoire de Biochimie et Physiologie Végétales, Université de Genève, Place de l'Université 3, 1211 Genève 4, Switzerland

An apoplastic isoperoxidase from zucchini (APRX) was shown to bind strongly to polygalacturonic acid in their Ca^{2+}-induced conformation. By homology modeling, we were able to identify a motif of four clustered arginines (positions 117, 262, 268, and 271) that could be responsible for this binding. To verify the role of these arginine residues in the binding process, we prepared three mutants of APRX (M1, R117S; M2, R262Q/R268S; and M3, R262Q/R268S/R271Q). APRX and the three mutants were expressed as recombinant glycoproteins by the baculovirus–insect cell system. This procedure yielded four active enzymes with similar molecular masses that were tested for their ability to bind Ca^{2+}-pectate. Recombinant wild-type APRX exhibited an affinity for the pectic structure comparable to that of the native plant isoperoxidase. The mutations impaired binding depending on the number of arginine residues that were replaced. M1 and M2 showed intermediate affinities, whereas M3 did not bind at all. This was demonstrated using an in vitro binding test and on cell walls of hypocotyl cross-sections. It can be concluded that APRX bears a Ca^{2+}-pectate binding site formed by four clustered arginines. This site could ensure that APRX is properly positioned in cell walls, using unesterified domains of pectins as a scaffold.

INTRODUCTION

Higher plant peroxidases (EC 1.11.1.7) are oxidoreductases that catalyze the reduction of hydrogen peroxide and the concomitant oxidation of various hydrogen donors, such as phenolic compounds. Most of the functions attributed to plant peroxidases occur in cell walls. These functions can be divided into two main categories. The first is the oxidative cross-linking or coupling of many aromatic molecules by using hydrogen peroxide as an electron acceptor. This leads to the formation of lignin (Harkin and Obst, 1973; Ros Barceló et al., 1998) or suberin (Espelie and Kolattukudy, 1985) and also to the establishment of covalent bonds between hydroxycinnamate ester moieties or flavonoids associated with pectins or hemicellulose (Fry, 1986). By catalyzing these reactions, peroxidases are involved in the construction of cell walls and in the control of cell wall plasticity. They also participate in the cross-linking reactions that occur in cell walls upon infection by pathogens (Moerschbacher, 1992).

On the other hand, it has been shown that some peroxidases produce reduced oxygen species such as hydrogen peroxide (Bestwick et al., 1999) or hydroxyl radical (Schweikert et al., 2000) through complex free radical reactions. Indoleacetic acid oxidation by specific peroxidases also is likely to occur (Gazaryan et al., 1996). Many different isoperoxidases are present simultaneously in apoplast. This has been shown in several plant materials by using vacuum infiltration followed by centrifugation (Castillo et al., 1984; Bernal et al., 1993; Carpin et al., 1999). These apoplastic enzymes have often been classified in three different categories—soluble, ionically bound, and covalently bound—depending on the treatment necessary for their release from cell walls (McDougall and Morrison, 1995). This suggests that different peroxidase molecules may exhibit different interactions with the various constituents of the extracellular matrix.

Despite the potential importance of these interactions for the control of peroxidase action, little is known about their nature or the cell wall polymers that could be involved. One of the few known examples of specific interaction between a cell wall protein and a cell wall polymer is the binding of some isoperoxidases to the homogalacturonan domains of pectins. This has been shown in lupin (Ros Barceló et al., 1988), in zucchini (Penel and Greppin, 1994, 1996), and in horseradish (Penel et al., 1996). In zucchini, three cationic isoperoxidases and one anionic isoperoxidase bind to homogalacturonan if calcium ions are present (Penel and Greppin, 1994, 1996). Actually, the binding occurs only to pectic chains cross-linked by Ca^{2+} (Ca^{2+}-pectate). Cationic amino acid residues such as arginine and lysine that expose

[1] Current address: Laboratoire de Biologie des Ligneux et des Grandes Cultures, Université d'Orléans, BP 6759, 45067 Orléans cedex 2, France.

[2] To whom correspondence should be addressed. E-mail claude.penel@bota.unige.ch; fax 41-22-329-77-95.

their positive charges at the surface of peroxidases are involved in this binding. The anionic Ca^{2+}-pectate binding isoperoxidase from zucchini (APRX) was shown to be an apoplastic enzyme that can be recovered from hypocotyl apoplast by vacuum infiltration with a buffer containing EGTA (Carpin et al., 1999). It has been purified and microsequenced, allowing the recovery of its cDNA from a zucchini cDNA library. The open reading frame corresponded to a deduced mature protein of 309 amino acids after cleavage of a signal peptide of 16 amino acids. *APRX* transcripts were found in all organs of zucchini seedlings but were particularly abundant in roots and more generally in epidermal and some vascular tissues (Carpin et al., 1999).

The broad distribution of *APRX* mRNA suggests a probable important function for this peroxidase. In this study, site-directed mutagenesis was used to identify the cationic amino acids responsible for the Ca^{2+}-pectate binding properties of APRX.

RESULTS

Production of Recombinant APRX

Spodoptera frugiperda (Sf9) cells transfected with pVLAPRX synthesized and secreted a peroxidase that could be detected in the culture medium by measuring its activity with guaiacol/hydrogen peroxide, whereas control cells did not. The addition of polygalacturonic acid (PGA) and $CaCl_2$ followed by centrifugation allowed quantitative recovery of the peroxidase activity in the pellet associated with the pelleted Ca^{2+}-pectate gel (Table 1). This procedure corresponded to a 70-fold purification of the peroxidase in one step. PGA was then removed from the peroxidase preparation by chromatography through heparin–Sepharose, as described previously (Penel and Greppin, 1996). Figure 1 shows a comparison of this recombinant peroxidase (rAPRX) and the corresponding enzyme purified from zucchini seedlings (Penel and Greppin, 1996). It appeared that recombinant and plant APRX had approximately the same molecular mass and the same isoelectric point. Thus, the baculovirus–insect cell expression system was very suitable for the production of this plant glycoprotein. In addition, the recombinant peroxidase exhibited an affinity for the Ca^{2+}-pectate structure, as did the plant enzyme (Table 1). Its catalytic characteristics were similar as well (data not shown).

Production of Mutants

Previous work has shown that the affinity of APRX for Ca^{2+}-pectate was the result of the presence of cationic amino acids (Penel and Greppin, 1996). Because higher plant class III peroxidases are structurally well conserved (Schuller et al., 1996; Gajhede et al., 1997; Mirza et al., 2000), it was possible, using the known crystal structure of the cationic peanut peroxidase (1SCH; Schuller et al., 1996), to generate the homology three-dimensional model of APRX shown in Figure 2. A cluster of four arginines (117, 262, 268, and 271), present on the opposite side of the substrate channel entry APRX, appeared to be the best candidate for the interaction with Ca^{2+}-pectate. The arginines or lysines located around the substrate channel entry, which appear as blue areas in Figure 2B, were most likely not responsible for the binding, because the interaction with Ca^{2+}-pectate did not reduce the activity of APRX.

Figure 3 shows the configuration of the putative binding site. R268 and R271 were located on, and R262 was located close to, α-helix J. R117 was placed on a coil between α-helices D and D′. The arginines were substituted with polar amino acids to generate three mutants, M1 (R117S), M2 (R2G2Q and R268S), and M3 (R262Q, R268S, and R271Q) (Figure 3). Wild-type APRX and its three variants were produced by Sf9 cell cultures transfected with pVLAPRX, pVLM1, pVLM2, and pVLM3. After four to five rounds of culture, all four media contained peroxidase activity equiva-

Table 1. Binding of Recombinant Peroxidases Present in Sf9 Cell Culture Medium to Ca^{2+}-Pectate

	Culture Medium[a]			Pellet[b]		
Transfection Vector	ΔA470 nm min^{-1}	Protein (μg)	Specific Activity[c]	ΔA470 nm min^{-1} (% Medium)[d]	Protein (μg)	Specific Activity
pVLAPRX	13.4	1311	10.2	15.6 (116)	21.5	725
pVLM1	16.4	1677	9.8	7.1 (43)	30.2	235
pVLM2	18.8	1882	10.0	3.8 (20)	47.2	80.5
pVLM3	13.4	1684	7.9	0.18 (1)	38.4	4.7

[a] Peroxidase activity (ΔA470 nm min^{-1}) and protein amount in 1 mL of culture medium.
[b] Peroxidase activity (ΔA470 nm min^{-1}) and protein content of pellets obtained by centrifugation of 1 mL of culture medium after the addition of 10 μg of PGA and 2 mM $CaCl_2$.
[c] ΔA470 nm min^{-1} mg protein^{-1}.
[d] Percentage of activity initially present in the medium recovered in the pellet.

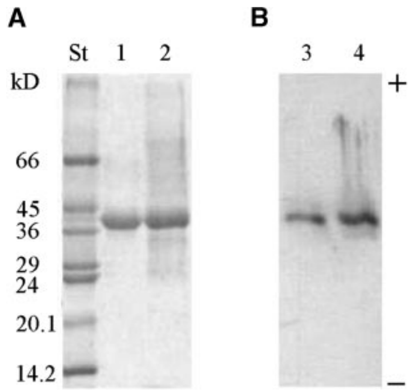

Figure 1. Comparison of APRX Extracted from Plants and rAPRX Produced by Transfected Sf9 Cells.

Both enzymes were purified by coprecipitation with a Ca^{2+}-pectate gel followed by chromatography through heparin–Sepharose (Penel and Greppin, 1996).

(A) SDS-PAGE separation. Lane St, molecular mass markers with units indicated at left in kilodaltons; lane 1, 6 μg of plant APRX; and lane 2, 6 μg of rAPRX.

(B) Isoelectric focusing separation on a Servalyt Precotes gel, pH range 3.0 to 10.0. Lane 3, 40 ng of plant APRX; and lane 4, 40 ng of rAPRX. (+), anode; (−), cathode.

lent to ~5 μg/mL. An attempt to precipitate these peroxidases by adding PGA and $CaCl_2$ and centrifuging showed that the mutants exhibited reduced affinity, depending on the number of arginines that were replaced. As shown in Table 1, the recovery of the activity present initially in the medium was <50% for M1, ~20% for M2, and near 0% for M3. This result indicated that the mutagenesis had the expected effect on the binding capacity of the peroxidase.

The consequence of the loss of affinity exhibited by the mutants was that they could not be purified from the insect cell culture medium by simple coprecipitation with Ca^{2+}-pectate. For this reason, another procedure was used that included chromatography through a column of concanavalin A–Sepharose followed by preparative isoelectric focusing. The active peroxidases obtained in each case were used for molecular mass and isoelectric point determinations. Figure 4 shows that the three mutants and APRX had approximately the same mass, whereas the isoelectric points of the mutants were shifted toward more acidic pH in relation to the number of substituted arginines.

In Vitro Binding to Ca^{2+}-Pectate

Purified rAPRX and APRX mutants were tested for their capacity to bind to Ca^{2+}-pectate. Figure 5 shows typical binding curves obtained when increasing amounts of peroxidases were mixed with 5 μg of PGA in the presence of calcium ions. After centrifugation of the samples, peroxidases associated with the Ca^{2+}-pectate pellets were quantified. This experiment showed that the binding profile of rAPRX was very similar to that of plant APRX described previously (Penel et al., 1999). In the same conditions, M1 exhibited an affinity similar to that of rAPRX for the low peroxidase concentrations but slightly reduced affinity for higher concentrations. M2 bound poorly, and M3 did not bind at all. These results confirmed the role of the four arginines in the binding of APRX to Ca^{2+}-pectate. The level of affinity of rAPRX, M1, and M2 was also tested by increasing the ionic strength with NaCl. It was shown previously that the binding of APRX was

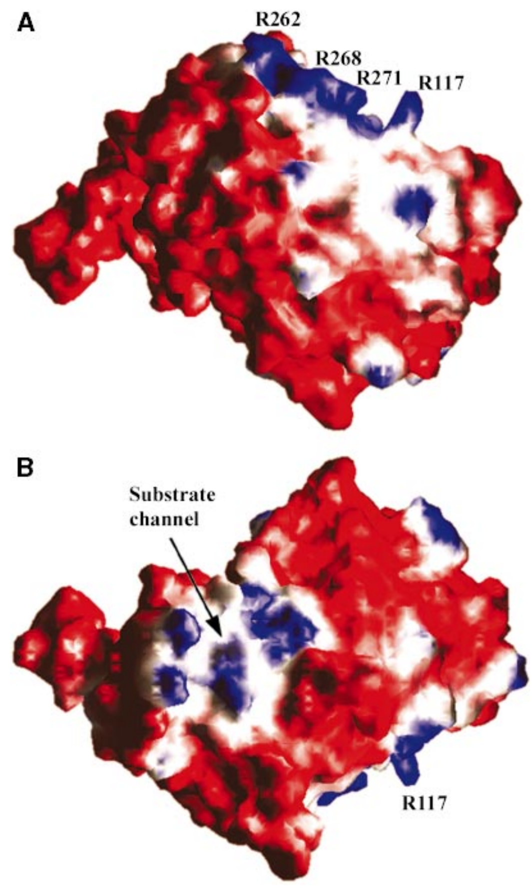

Figure 2. Structure of APRX Showing the Electrostatic Potentials at the Surface of the Protein.

Regions of positive and negative potential are shown in blue and red, respectively. The figure was prepared by homology modeling with the cationic peanut peroxidase as a template (Schuller et al., 1996), using SWISS-MODEL and the Swiss-Pdb Viewer (Guex and Peitsch, 1997), which are available at www.expasy.ch/spdbv/.

(A) Front view. Clustered arginines forming the putative binding domain are marked.

(B) Back view. The entry of the substrate channel is indicated.

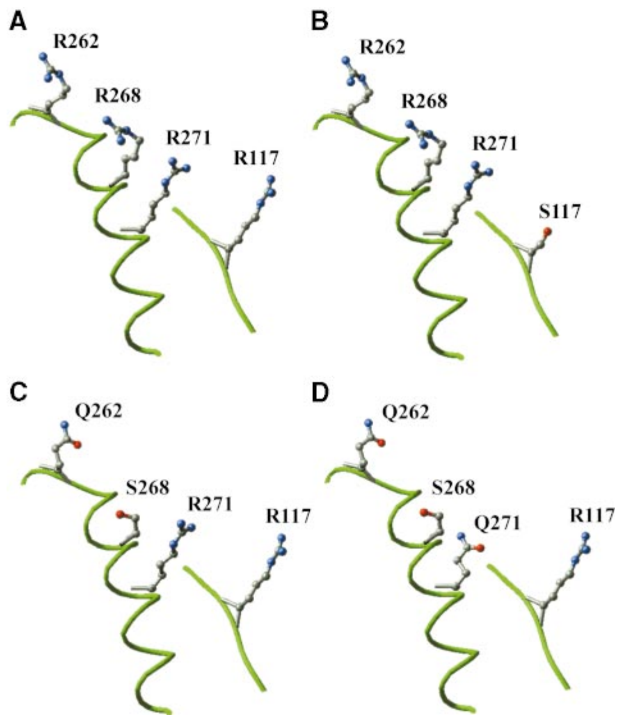

Figure 3. Close-up Views of the Putative Pectin Binding Motif and Its Variants Obtained by Mutagenesis.

The $C\alpha$ trace is shown in green, and arginine, serine, and glutamine residues are shown as stick models and are labeled.
(A) Wild-type APRX.
(B) R117S mutation (M1).
(C) R262Q and R268S mutations (M2).
(D) R262Q, R268S, and R271Q mutations (M3).

sensitive to NaCl concentrations >200 mM, meaning that it resulted from ionic interactions (Penel and Greppin, 1996). The concentration of NaCl necessary to prevent the binding of APRX and the mutants was determined and is shown in Figure 6. The binding of rAPRX and M1 was unaffected by NaCl concentrations up to 100 mM. Higher salt concentrations released M1 more efficiently than did rAPRX from Ca^{2+}-pectate. This difference showed that rAPRX has a stronger affinity for Ca^{2+}-pectate than does M1. M2, which bound only partially in the absence of NaCl, also was sensitive to the addition of salt.

Binding to Cell Walls

It was shown that APRX binds to cell walls of hypocotyl sections after elimination of the endogenous peroxidase activity by treatment with trichloroacetic acid (Penel et al., 1996). In the present work, free-hand sections through zucchini hypocotyls were cleared with Na hypochlorite, a treatment that does not alter the cell wall structure but denatures proteins (Cutler, 1978). Such pretreated sections, which did not exhibit peroxidase activity detectable with 4-chloronaphthol/hydrogen peroxide, were used to assess the capacity of the different recombinant peroxidases to bind to cell walls. They were incubated in a solution containing 10 nM of each of the recombinant peroxidases in the presence of either EGTA or Ca^{2+}. In these conditions, as shown in Figure 7, APRX exhibited a Ca^{2+}-dependent binding to cell walls of several tissues, mainly epidermis but also collenchyma, parenchyma, and some vascular bundles (Figures 7A and 7C). When Ca^{2+} ions were chelated by EGTA, no binding of rAPRX was observed (Figure 7B). Staining of similarly pretreated sections with ruthenium red, a dye of acidic pectins (Sterling, 1970), showed that the tissue distribution of acidic pectins and bound rAPRX was similar (Figure 7G). Another indication that pectins were necessary for the binding of APRX to cell walls was obtained by incubating hypochlorite-treated sections with a pectinase before incubation in the peroxidase-containing solution. This treatment completely abolished the Ca^{2+}-dependent binding of APRX (Figure 7H). The two mutants M1 and M2 behaved like APRX when tested for binding to cell walls in the presence of Ca^{2+} (Figures 7D and 7E), but M3 was unable to bind to cell walls (Figure 7F).

DISCUSSION

Pectin is the main charged component of plant cell walls. It consists partially of PGA chains bearing evenly distributed negative charges. This structure is suitable for interactions with positively charged molecules, such as polyamines or cations (Messiaen et al., 1997). It also should be able to attract some proteins exposing positive charges in a favorable orientation. This is the case with APRX. One of the major characteristics of the interaction of APRX with PGA is that it occurs only in the presence of Ca^{2+}. This probably indicates that the positive charges responsible for APRX binding do not match spatially with the negative charges aligned on a single PGA chain but match with the negative charges located on two or more chains linked together by Ca^{2+}.

It is known that PGA chains are aggregated in the presence of calcium ions, forming supramolecular structures. In these structures, the PGA chains may be under two main helical conformations, 2_1 and 3_1, depending on the concentration of the Ca^{2+}-pectate gel (Grant et al., 1973; Kohn, 1975). Goldberg et al. (1996) showed that these two conformations coexist in plant cell walls, providing a complex structure and offering many negative charges or oxygen atoms for electrostatic interactions or hydrogen bond formation with proteins. Because the binding of APRX occurs in vitro at a PGA concentration as low as 10 mg/L, it can be hypothesized that the 2_1 helical conformation, cor-

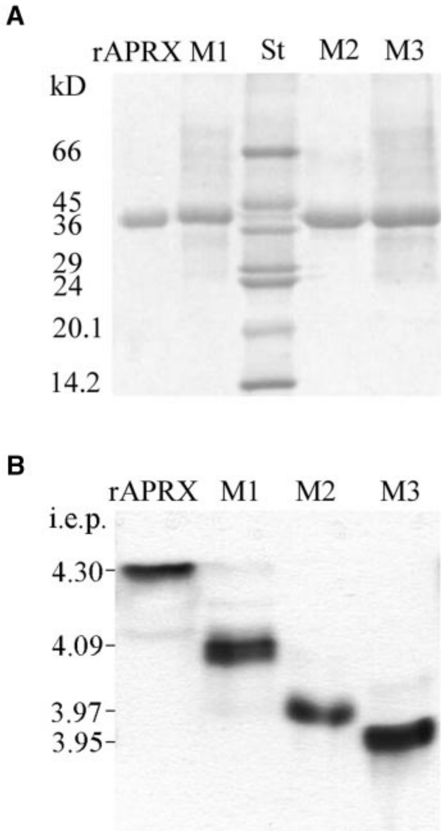

Figure 4. Electrophoretic Separations of the Recombinant Peroxidases.

rAPRX, M1, M2, and M3 were purified by affinity chromatography on concanavalin A–Sepharose followed by preparative isoelectric focusing.

(A) SDS-PAGE separation. Lane St, molecular mass markers with units indicated at left in kilodaltons.

(B) Isoelectric focusing separation on a Servalyt Precotes gel, pH range 3.0 to 6.0. Forty nanograms of each enzyme was deposited on the gel. The pH gradient was determined at the end of the electrophoresis procedure by measuring the pH of pieces of gel (5 × 5 mm) in 300 μL of deionized water. i.e.p., isoelectric point.

responding to the so-called egg box structure of Ca^{2+}-pectate (Morris et al., 1982), is the structure suitable for APRX binding. This does not preclude the possibility of a binding to 3_1 helix. Alternately, Ca^{2+} could modify the conformation of the pectate chains by linking adjacent uronic acid moieties in the same pectate helix. This has been observed in the complex formed at pH 9.5 by pectate lyase C and its substrate, an oligogalacturonate (Scavetta et al., 1999).

The binding of APRX to pectin is based on electrostatic interactions, as indicated by the inhibitory effect of high NaCl concentrations (Figure 6). However, Ca^{2+}-PGA does not behave like a simple cation exchanger, retaining proteins according to the density of their positive charges. For example, the most cationic isoperoxidase of zucchini does not bind Ca^{2+}-pectate (Penel and Greppin, 1996), despite its numerous positive charges. APRX is an anionic protein with an isoelectric point of 4.3 (Penel and Greppin, 1994) that carries a net negative charge at neutral pH and should be unable to bind to a polyanion such as PGA. The interaction between APRX and Ca^{2+}-pectate therefore most likely results from the exact spatial fitting of a few APRX positive charges to a negatively charged motif of the pectic structure. Similar interactions have been described in animals. Many proteins have been shown to interact with anionic polymers of the extracellular matrix, such as heparin, heparin sulfate, or chondroitin, mainly through cationic amino acids (Hileman et al., 1998).

The present study showed that the replacement of three arginines by serine and glutamine in M3 abolishes completely the affinity of APRX for the Ca^{2+}-PGA structure. This observation confirms previous results on the involvement of cationic amino acids in the binding process (Penel and Greppin, 1996). These results showed that the chemical modification of cationic amino acids with phenylglyoxal or sulfo-N-hydroxysuccinimide-acetate suppressed the binding and that polylysine, polyarginine, and polyornithine, but not polyglucosamine, were efficient inhibitors of APRX binding to Ca^{2+}-pectate. Therefore, it can be assumed that the binding mainly results from electrostatic interactions between the positively charged guanidinium group of arginines and the negatively charged carboxylate group of uronic acids. However, the existence of Ca^{2+} bridges between APRX and

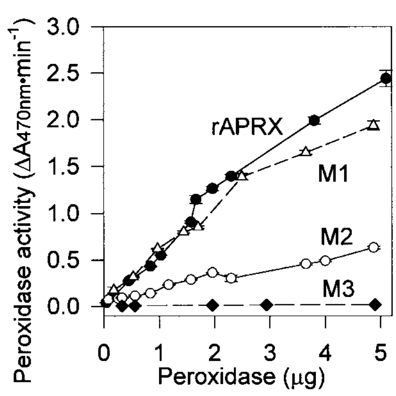

Figure 5. Binding of the Recombinant Peroxidases to Ca^{2+}-Pectate.

Binding tests were performed with increasing concentrations of rAPRX or the three mutants in the presence of 5 μg of PGA and 2 mM $CaCl_2$ in a total volume of 100 μL. After centrifugation, the peroxidase activity associated with Ca^{2+}-pectate pellets was measured. The data are means of three determinations ±SD. rAPRX, closed circles; M1, open triangles; M2, open circles; M3, closed diamonds.

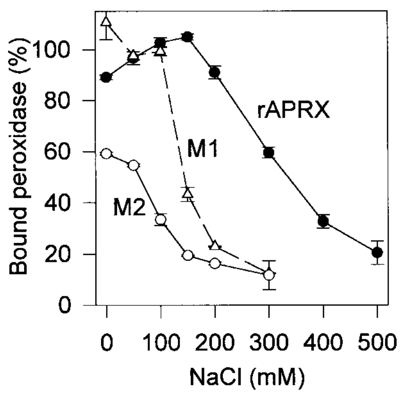

Figure 6. Effect of NaCl on the Binding of the Recombinant Peroxidases to Ca^{2+}-Pectate.

Binding tests were performed with 500 ng of rAPRX, M1, or M2 in the presence of 10 μg of PGA, 2 mM $CaCl_2$, and increasing concentrations of NaCl. The data are expressed as percentages ±SD of the initial peroxidase activity found in the pellets. rAPRX, closed circles; M1, open triangles; M2, open circles.

PGA or the existence of hydrogen bonds between arginines and hydroxyl groups as described for pectin lyase C and its substrate (Scavetta et al., 1999) cannot be ruled out. The contributions of the four arginines in the interaction process were partially assessed by designing two other mutants, one lacking R117 (M1) and another lacking R262 and R268 (M2). M1 exhibited an affinity apparently similar to that of APRX, but its binding was much more susceptible to increasing salt concentration and was incomplete when the concentration of Ca^{2+}-pectate was low. Therefore, it can be concluded that the group of three arginines (262, 268, and 271) located at the level of α-helix J forms a Ca^{2+}-pectate domain. R117 further stabilizes the binding. The mutant exposing only R117 and R271 (M2) had reduced affinity, although it still bound to cell walls, as shown in Figure 7. This observation indicates that two arginines may confer some affinity to the Ca^{2+}-pectate structure if they are in an adequate spatial orientation. The results obtained with M2 and M3 clearly implicate a significant role for R271 in the binding but do not resolve the question as to whether R262 or R268 or both are essential for binding to Ca^{2+}-pectate.

There are many examples of the binding of animal proteins to extracellular matrix polymers, which show that arginine residues mediate a tight binding (Hileman et al., 1998). This can be explained by the presence of a flexible side chain ending in a guanidinium cation highly suitable to form hydrogen bonds with oxygens of hydroxyl and carboxyl groups of polysaccharides (Fromm et al., 1995). It was shown that the affinity for heparin, a repeating linear copolymer of uronic acid and sulfated glucosamine, was not significantly increased for peptides having a cluster greater than four arginines (Fromm et al., 1997). The Ca^{2+}-pectate binding domain of APRX also apparently consists of four clustered arginines, which confer to this plant peroxidase a strong affinity for heparin (Penel and Greppin, 1996). During this study, it was found that the M1 and M2 mutations reduced the affinity for heparin and that M3 canceled it (data not shown).

APRX binds to Ca^{2+}-pectate through an epitope remote from its substrate channel entry (Figure 2). This explains why the binding has absolutely no effect on its catalytic activity. APRX anchoring to homogalacturonan-rich domains of the cell wall is likely to control the spatial distribution of APRX and the orientation of reaction products released by peroxidase. It can be hypothesized that pectins, in addition to being a constituent of the physical frame that surrounds the plant cell, may exert a biological activity through the localization and stabilization of interacting proteins. It has been shown that in addition to some peroxidases, there are other proteins that interact with pectins (Penel and Greppin, 1996). The observation that this binding process occurs in the cell wall of many different tissues (Figure 7) and the facts that the recovery of APRX from apoplast requires the presence of EGTA (Carpin et al., 1999) and that APRX displays in vitro a high affinity for Ca^{2+}-pectate all strongly suggest that the binding occurs in vivo.

The exact function of APRX in cell walls remains to be elucidated. In vitro, APRX was shown to catalyze the oxidation of ferulic acid or coniferyl alcohol in the presence of hydrogen peroxide, but it has no extensin peroxidase activity (M.D. Brownleader, personal communication) and does not produce hydrogen peroxide in the presence of reducing molecules such as cysteine or NADH, as do some other peroxidases (Bolwell et al., 1995; Overney et al., 1998). These catalytic characteristics indicate that APRX could be involved in cross-linking reactions or in lignin or suberin deposition. Pectin binding isoperoxidases have been found in other plants (Penel et al., 1996), and recently, we have identified a group of four Arabidopsis peroxidase sequences that encode an amino acid motif, including three lysine residues aligned as R262, R268, and R271 in APRX. The recombinant peroxidases encoded by these sequences exhibit an affinity for Ca^{2+}-pectate.

The affinity for Ca^{2+}-pectate exhibited by APRX may provide a smart control mechanism for the spatial distribution of the enzyme within the cell wall matrix. Ca^{2+}-pectate is a particular conformation of pectins occurring mainly in middle lamella and cell corners but also in other sites of cell walls (Carpita and Gibeaut, 1993; McCann and Roberts, 1996). It may be generated enzymatically by in muro deesterification of esterified PGA, but it also can be formed or broken down by changes in Ca^{2+} or H^+ apoplastic concentrations. In vitro experiments have shown that the attraction of Ca^{2+}-pectate for APRX was dependent on Ca^{2+} concentration (Penel et al., 2000). The presence of APRX within particular cell wall domains could be modulated by these parameters.

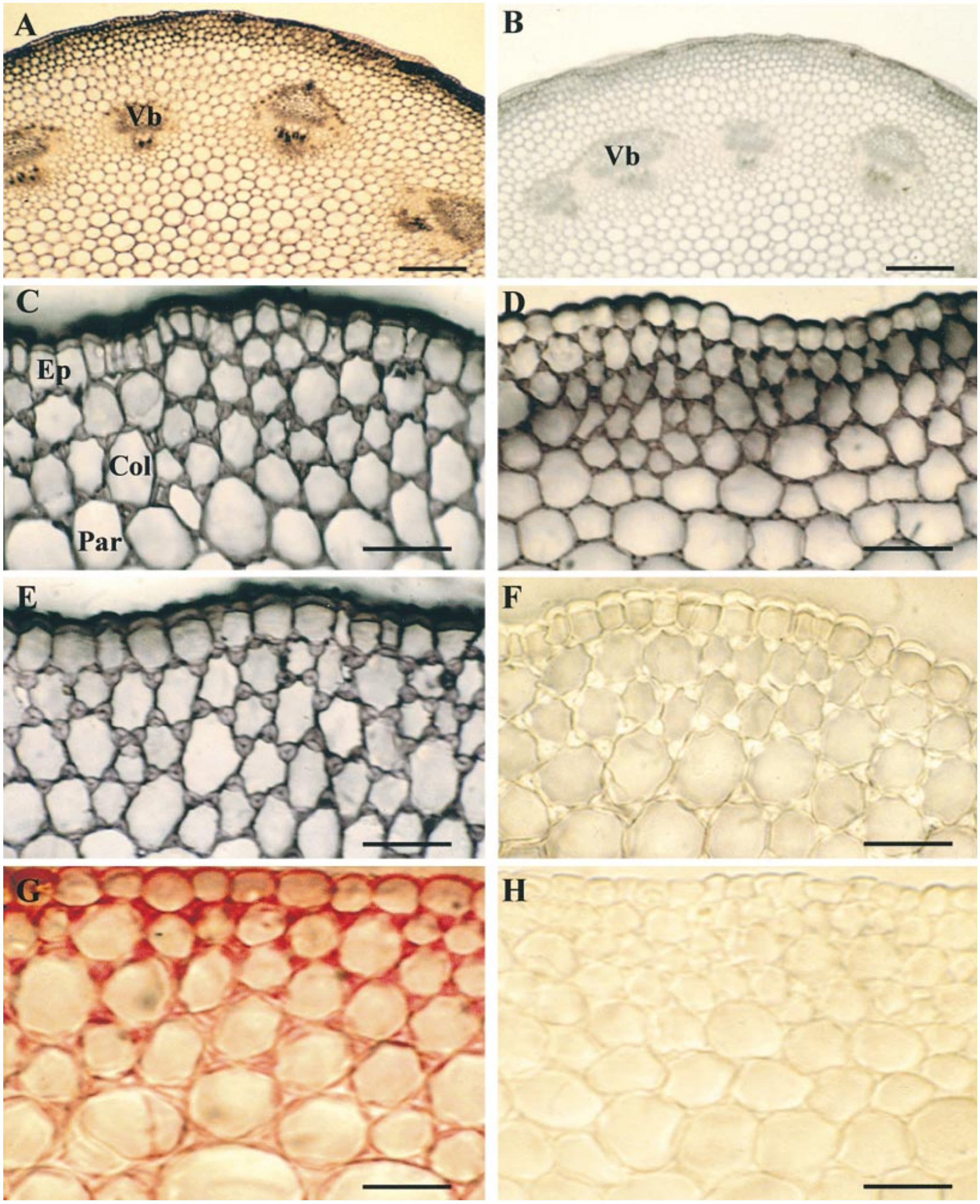

Figure 7. Binding of the Recombinant Peroxidases to Cell Walls of Hypocotyl Cross-Sections.

The sections were pretreated with Na hypochlorite before incubation with the different peroxidases.
(A) and **(C)** Incubation with rAPRX in the presence of Ca^{2+}.
(B) Incubation with rAPRX in the presence of EGTA.
(D) Incubation with M1 in the presence of Ca^{2+}.
(E) Incubation with M2 in the presence of Ca^{2+}.
(F) Incubation with M3 in the presence of Ca^{2+}.
(G) Section stained with ruthenium red after Na hypochlorite treatment.
(H) Incubation with rAPRX in the presence of Ca^{2+} after treatment of the section with Macerozyme R-10.
Col, collenchyma; Ep, epidermis; Par, parenchyma; Vb, vascular bundles. Bars in **(A)** and **(B)** = 250 μm; bars in **(C)** to **(H)** = 50 μm.

METHODS

Production of Recombinant Peroxidase

The anionic isoperoxidase of zucchini (*Cucurbita pepo*) APRX (GenBank accession number Y17192) was expressed in the baculovirus–insect cell expression system. For this purpose, *APRX* sequence, previously cloned in pGEM-T Easy (Promega, Wallisellen, Switzerland) (Carpin et al., 1999), was digested to obtain a SpeI/NotI fragment. This fragment, including the 5′ untranslated leader, was ligated into pVL1392 transfer vector (Pharmingen, Becton Dickinson, Basel, Switzerland) and expressed in *Spodoptera frugiperda* (Sf9) cells, according to the manufacturer's instructions (Gruenwald and Heitz, 1993). Sf9 cells were cultured in 9-cm Petri dishes and were initially cotransfected with 5 μg of pVLAPRX and 0.25 μg of linearized BaculoGold DNA (Pharmingen). After 5 days at 27°C, the culture medium was collected. One milliliter of this spent medium was used to infect a new Petri dish containing a confluent plate of Sf9 cells. Peroxidase activity in the culture medium became detectable after two rounds of culture. The successive culture media after four to five rounds were kept at 4°C and used for peroxidase purification.

Mutations

Site-directed mutagenesis was performed to modify the arginines predicted to be critical for the binding of APRX to Ca^{2+}-pectate. Three mutants were designed. In the first mutant (M1), Arg-117 was replaced with Ser (R117S). In the second mutant (M2), Arg-262 and Arg-268 were replaced with Gln and Ser, respectively (R262Q and R268S). The third mutant (M3) was similar to M2, with the additional substitution of Ser for Arg-271. Mutagenesis was achieved with the GeneEditor in vitro site-directed mutagenesis system from Promega. The mutations were introduced into the *APRX* sequence inserted in pGEM-T vector by using the following primers: 5′-GGTGGGCCC-AGCTGGAG**C**(A)GTTTTATTCGG-3′ for M1, 5′-CCGTCTTGGGTC-CC**A**(G)AGAAGGAACCTTCTTTAG**C**(A)CAATTTCGGGTGTCC-3′ for M2, and 5′-CCGTCTTGGGTCCC**A**(G)AGAAGGAACCTTCTTTA-G**C**(A)CAATTTC**A**(G)GGTGTCCATGATTAAG-3′ for M3 (mutated bases are in boldface; replaced wild-type bases are within parentheses). The three resulting plasmids, p117APRX, p262-268APRX, and p262-268-271APRX, were cloned into *Escherichia coli* strain JM109. The M1, M2, and M3 constructs were sequenced with the dideoxy method, using T7 Sequenase (version 2.0; Amersham Pharmacia, Dübendorf, Switzerland), to confirm the correct introduction of the mutations. The three mutated sequences were subcloned into pVL1392 to obtain three vectors, pVLM1, pVLM2, and pVLM3, which were used to transfect Sf9 cells.

Purification of Peroxidases

The recombinant peroxidases were purified from the insect cell spent media by affinity chromatography through concanavalin A–Sepharose (Amersham Pharmacia). Usually, 100 to 150 mL of medium, supplemented with 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 M NaCl, and 0.1% Tween 20, was passed through a 1-mL column of concanavalin A–Sepharose. After extensive washing with 20 mM Mes buffer, pH 6.0, containing 1 mM $MnCl_2$, $CaCl_2$, and $MgCl_2$ and 1 M NaCl, the column was eluted with 500 mM methyl-α-D-glucopyranoside. The fractions containing peroxidase activity were pooled and desalted by ultrafiltration with Centricon YM-10 (Millipore, Bedford, MA). This preparation was then subjected to preparative isoelectric focusing in a column, as described previously (Penel and Greppin, 1994), except that the pH range was between 3.0 and 6.0. Once the separation was completed, fractions were collected and assayed for peroxidase activity. The active fractions were pooled and kept for additional assays. Plant APRX was purified from etiolated zucchini hypocotyl, as described previously (Penel and Greppin, 1996).

Binding Tests

The capacity of recombinant APRX (rAPRX) and mutants to bind to Ca^{2+}-pectate was assessed using a centrifugation test described previously (Penel and Greppin, 1996). In brief, 100-μL samples containing polygalacturonic acid (PGA) (Na salt; Sigma), 2 mM $CaCl_2$, and a peroxidase in 20 mM Hepes, pH 7.0, were incubated for 60 min at room temperature in Eppendorf tubes. The samples were then centrifuged in a Sorvall microfuge at 9,500*g* for 5 min. The supernatants were removed carefully, and the pellets were resuspended in 200 μL of Hepes, pH 7.0, containing 2 mM EGTA and 0.1% Tween 20. Peroxidase activity was measured before and after centrifugation.

The ability of the various recombinant peroxidases to exhibit Ca^{2+}-dependent binding to cell walls was determined on hypocotyl sections. For this purpose, free-hand sections were made through etiolated zucchini hypocotyls at 1 cm below the hook. The sections were incubated in 2.5% Na hypochlorite for 60 min at 0°C to denature the proteins. After four washes in Hepes, pH 7.0, with either 1 mM EGTA or 1 mM $CaCl_2$, the sections were incubated for 60 min in Hepes, pH 7.0, containing 400 ng/mL pure APRX or mutants (∼10 nM) and either 1 mM EGTA or 1 mM $CaCl_2$. The sections were then washed four times in the same buffer without peroxidase and stained for 2 min for peroxidase activity with 2 mM 4-chloronaphthol and 2 mM hydrogen peroxide in acetate buffer, pH 4.0. All of these operations, except for the hypochlorite treatment, were performed at room temperature. Some hypochlorite-treated sections were incubated with 1% Macerozyme R-10 (Yakult Pharmaceutical, Tokyo, Japan) in Hepes, pH 7.0, for 40 min and then used for their ability to bind rAPRX in the presence of $CaCl_2$. Pectins were stained with 0.02% aqueous ruthenium red (Sterling, 1970). The various experiments were repeated three to 10 times with similar results.

Assays and Electrophoresis

Peroxidase activity was measured by following the oxidation of 8 mM guaiacol at 470 nm in the presence of 2 mM hydrogen peroxide. The results were expressed as increased absorbance at 470 nm min^{-1}. Purified APRX and mutants were quantified by converting their activity assayed in standard conditions to nanograms (Penel and Greppin, 1994). Proteins were assayed with the Coomassie Brilliant Blue R 250 reagent (Spector, 1978), using BSA as a standard. SDS-PAGE followed by Coomassie blue staining was used for protein separation (Hames and Rickwood, 1981). Peroxidases were separated by isoelectric focusing, using Servalyt Precotes (Catalys, Wallisellen, Switzerland). After electrophoresis, the gels were stained for peroxidase with 0.04% *o*-dianisidine and 10 mM hydrogen peroxide in 100 mM acetate buffer, pH 4.5.

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