On becoming a parasite: evaluating the role of wall oxidases in parasitic plant development

Dongjin Kim, Remigiusz Kocz, Laural Boone, W John Keyes and David G Lynn

Background: The temporal and spatial control of the transition from vegetative to parasitic growth is critical to any parasite, but is essential to the sessile parasitic plants. It has been proposed that this transition in *Striga* spp. is controlled simply by an exuded oxidase that converts host cell-surface phenols into benzoquinones which act as developmental signals that mediate the transition. An understanding of this mechanism may identify the critical molecular events that made possible the evolution of parasitism in plants.

Results: PoxA and PoxB are identified as the only apoplastic phenol oxidases in *Striga asiatica* seedlings, and the genes encoding them have been cloned and sequenced. These peroxidase enzymes are capable of oxidizing the 60 known inducing phenols into a small set of benzoquinones, and it is these quinones that induce parasitic development. Analysis of the reaction requirements and comparisons to host enzymes, however, lead us to argue that PoxA and PoxB are not necessary for host recognition.

Conclusions: A new model is proposed where constitutive production of an activated oxygen species (in the case of *Striga*, H_2O_2) mediates host recognition. This strategy would allow a parasite to exploit abundant host enzymes to produce the diffusible recognition signals by converting a standard host defense into a parasitic offense.

Address: Searle Chemistry Laboratory, The University of Chicago, Chicago, IL 60637, USA.

Correspondence: David G Lynn
E-mail: d-lynn@uchicago.edu

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Introduction

One percent of all known flowering plants exploit parasitic associations with other angiosperms [1,2]. This parasitism is broadly distributed, with species across at least 16 families in habitats that range from the poles to the dry and humid tropics and in annuals and perennials in the form of trees, shrubs, vines, or herbs [3]. Such a broad and diverse distribution has suggested that the parasitic strategy was repeatedly discovered throughout angiosperm evolution [4].

Searcy and MacInnis [5,6] first proposed three general developmental phases for the molecular evolution of the parasitic plants. The first phase involved the development of the specialized organ that forms the physiological bridge to the host — the haustorium. The second and third phases involved specialization and adaptation of the parasite to the host. There is significant evidence in support of the idea that the parasite loses both biochemical pathways and morphological structures that become redundant upon attachment to the host [7–11], and for the accrual of more complex adaptations specific to an obligate parasite, such as host specialization [1,12–14] and mechanisms which overcome host defenses [15–18]. In contrast, less is known about the development of the host attachment organ. There are differences in anatomical features of mature haustoria across the parasitic plants, although there appear to be common elements in the early initiation events [1,18].

Lateral haustoria in the rhinanthoid Scrophulariaceae are initiated by early rounds of cell division and radial swelling in the pericycle and inner cortex layers of the root to give rise to the haustorial primordia. In the obligate hemi-parasite *Striga asiatica*, which can survive for only 5 days without host attachment, a transformation of the terminal root meristem occurs: cells in the root primordia stop dividing and radial swelling occurs just distal to the root tip, ultimately giving rise to the pre-attachment organ [18]. Both processes are very rapid (development of the haustorium is complete within 12–16 h), and are tightly coupled to host chemistry [12,18,19]. The same host recognition compound or xenognosin, 2,6-dimethoxy-*p*-benzoquinone (DMBQ), was identified as a natural signal that was necessary and sufficient for induction of both lateral and terminal haustoria in several of the Scrophulariaceae [12,20].

Prior to the discovery of DMBQ, a diverse array of simple phenols had been found to induce haustorial development in the Scrophulariaceae [12,19]. The discovery of DMBQ came from the observation that host cell wall fragments removed from sorghum root surfaces were found to induce haustorial development in *S. asiatica* [19]. During the incubation DMBQ accumulated, and when the concentration reached 10^{-6} M, haustorial development was induced. Syringic acid, used as a model cell wall phenolic precursor, was oxidized to DMBQ by *S. asiatica* seedlings

and induced haustoria only when the quinone concentration reached the micromolar minimal threshold. These results suggested that a parasite enzyme was required to oxidatively liberate the inducing signal, sorghum xenognosin for *Striga* haustoria (SXSh), from the host cell surface [12,19]. Consistent with that model, extensive washing of *S. asiatica* seedlings prior to placing them on host roots prevented haustorial development. This inhibition of haustorial development was reversed, however, when a solution of *Pyricularia oryzae* laccase (benzenediol:O_2 oxidoreductase; EC 1.10.3.2) was added to these washed seedlings while on the host surface, presumably because the enzyme reconstituted the oxidizing activity.

The model of a parasite phenol oxidase mediating host detection was appealingly simple and motivated the investigation reported here. We report the isolation of two apoplastic oxidases from *S. asiatica*, and the cloning and sequencing of their encoding genes. These oxidases account for the wide range of structural variability acceptable for the xenognostic signal, as they catalyze the oxidation of diverse phenols to a common benzoquinone. We argue, however, that these parasite enzymes need play no role in host recognition; rather, the parasite can enlist homologous host enzymes for that task.

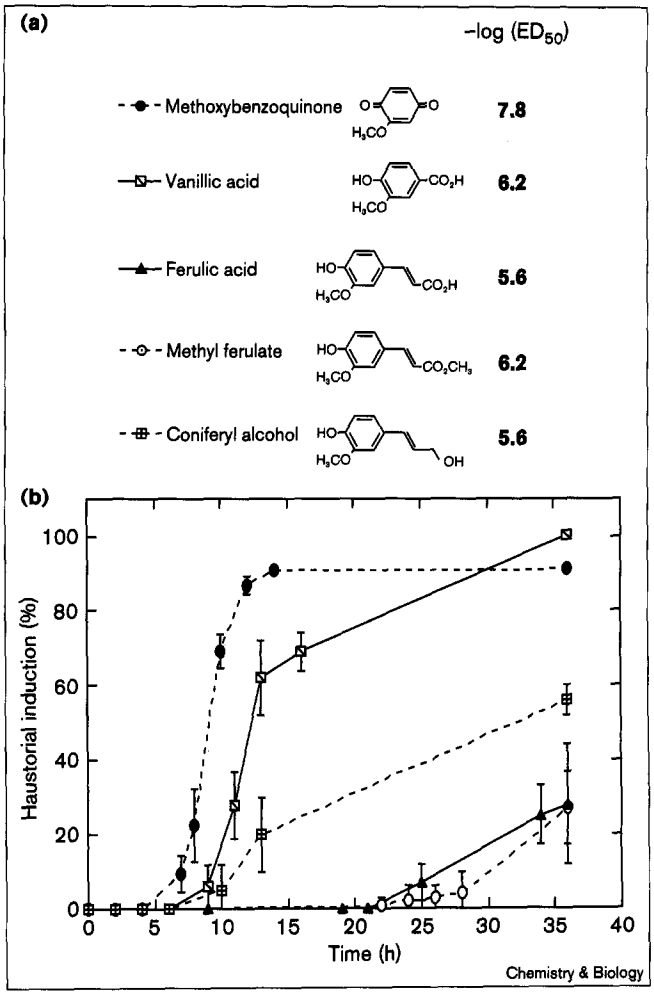
Results

Source of SXSh

Root surface abrasion experiments had previously implicated the plant cell wall as the source of SXSh [12,19]. Cell walls were isolated from sorghum roots and this fraction induced, depending on the isolate, 20–40% of the *S. asiatica* seedlings to develop haustorial primordia. Further fractionation of the walls demonstrated that all the haustorial-inducing activity was associated with the pectin-containing fraction. After saponification of this fraction, all the inducing activity partitioned from H_2O into Et_2O. Both the organic soluble fraction and the initial pectic fraction required a 30–40 h incubation time for half-maximal haustorial induction, consistent with phenyl propanoid esters in the pectic fraction being responsible for the induction.

Plant cell walls are known to contain specific phenyl propanoid-derived phenolics that differ in degree of methoxylation [21]. Oxidation of the aryl–Cα bond of these phenyl propanoids would give methoxybenzoquinone, 2,6-dimethoxybenzoquinone, and the unsubstituted *p*-benzoquinones, all of which have been shown to be active haustorial inducers [22]. More than 60 other synthetic phenolics were further investigated [23,24], and those that contained the appropriate substitution pattern to be oxidized to an active quinone induced haustoria. These active phenolics, however, required a concentration that was at least an order of magnitude higher than the similarly substituted quinones themselves (Figure 1a). For example, the half-maximal concentration for the monomethoxy

Figure 1



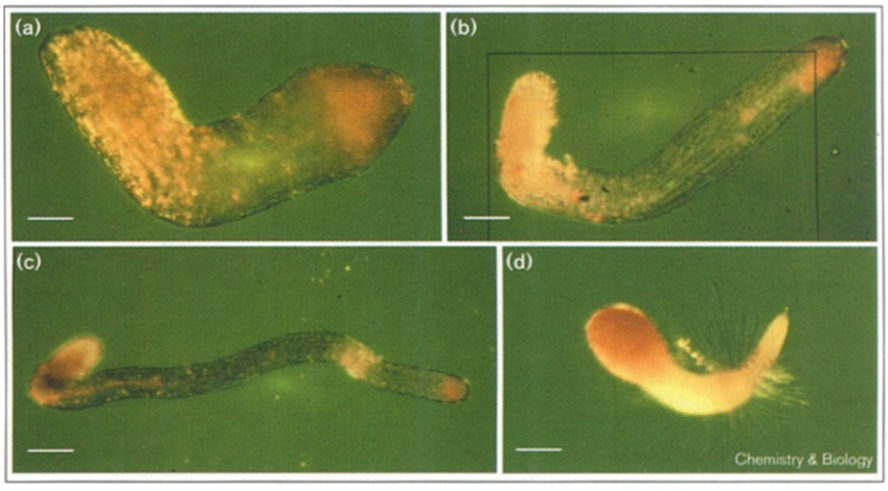
Induction of haustorial development in *S. asiatica* seedlings. **(a)** Dependence on concentration. **(b)** Dependence on time at 10^{-6} M inducer.

phenols varies between $10^{-5.6}$ M and $10^{-6.2}$ M whereas monomethoxybenzoquinone shows half-maximal activity at $10^{-7.8}$ M. The seedling exposure time required for haustorial induction by the active phenols was also consistently longer than that of the analogous quinones. Compounds with an oxygenated carbon *para* to the phenol hydroxyl group, such as vanillic acid, required relatively short exposure times. The phenyl propanoids, ferulic acid and methyl ferulate, required exposure times of 30–40 h, similar to the pectic fractions (Figure 1b). A similar substrate dependence has been reported for the rate of peroxidase-catalyzed oxidations of phenyl propanoids [25,26].

Histochemical staining

The broad structural range of the active phenols suggested that chromophoric substrates could be used to localize the oxidase(s). Both syringaldazine and pyrogallol identified oxidase activity concentrated at the meristematic root tip

Figure 2



Histochemical staining of *S. asiatica* and *Arabidopsis thaliana* seedlings. **(a)** One day-old *Striga* seedling. **(b)** Two day-old *Striga* seedling. **(c)** Three day-old *Striga* seedling. **(d)** Two day-old *Arabidopsis* seedling. Scale bars: (a) 40 μm; (b) 80 μm; (c) 150 μm; (d) 250 μm.

of one, two and three day-old *S. asiatica* seedlings (Figure 2a–c). The reddish coloration from the pyrogallol took several seconds to develop and was most strongly detected in the presence of added H_2O_2. Several other monocot and dicot seedlings were also screened with these substrates and the 2 day-old *Arabidopsis* seedling shown in Figure 2d is typical. The *Arabidopsis* root hair zone had very strong oxidase activity, much stronger than *Striga*, and the color developed immediately upon addition of H_2O_2, but not at all in the absence of the H_2O_2 co-substrate. Peroxidase activity has been shown to be localized in the walls of the root cap and epidermal cells in older wheat seedlings [27]. *S. asiatica*, however, has no root cap and no root hairs; only the walls of the root meristem cells appeared to accumulate the stain. Some stain did develop around the site of emergence from the seed coat, which might be attributed to transient wound-induced peroxidase expression. Removal of the seed coat (Figure 2a–c) revealed the orange-pigmented embryo that did not accumulate stain from either substrate.

Extraction of oxidases bound to the cell wall

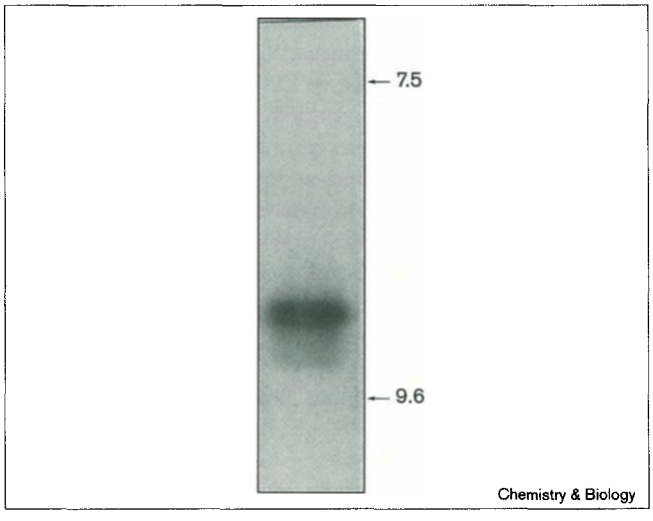
The identified oxidase activity was removed by extensive washing of the *S. asiatica* seedlings as previously reported [12,19]. Because the histochemical staining suggested that the oxidase activity was concentrated within the cell walls, an isolated cell wall fraction was extracted with 1M NaCl, using the methods of Kay and Basile [28] to enrich the activity. When the extract was desalted and analyzed by isoelectrofocusing gel electrophoresis, two diffuse basic peroxidase bands that depended on added H_2O_2 were typically detected, both with pI's of ~9 (Figure 3).

The wall extract, under the conditions used in the whole seedling experiments — with the exception that H_2O_2 was not added — catalyzed the oxidative cleavage of the aryl–Cα bond of phenols. At pH 5.5, the half-life of syringic acid was shorter, 4 h as compared to 27 h, and the conversion of syringic acid to DMBQ was much higher, 17% as compared to to 2%, than found with intact seedlings. The rate and yield increases *in vitro* may be attributed to higher oxidase concentration, an apparently greater stability of the quinones *in vitro*, and/or the greater availability of H_2O_2.

Dependence of haustorial induction on pH

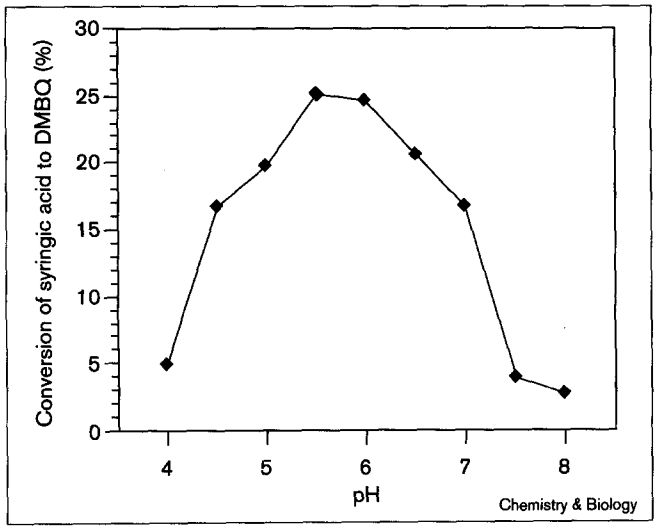
We used several approaches to evaluate the involvement of the identified enzymatic activity in haustorial induction. Oxidase inhibitors, such as the peroxidase inhibitors N_3 and

Figure 3



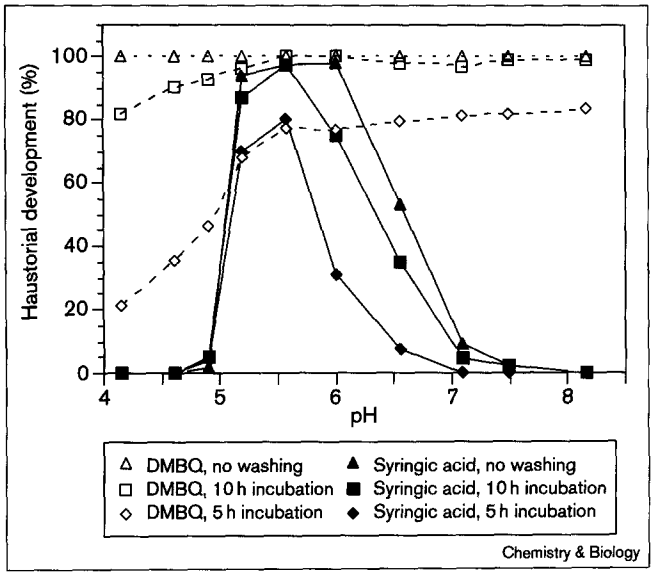
Isoelectrofocusing gel of *S. asiatica* cell wall proteins. The peroxidase activity on the gel was detected with a peroxidase assay solution, and the pI's of standard proteins are as marked.

Figure 4



Dependence of the conversion of syringic acid to DMBQ *in vitro* on pH. The percent conversion was calculated from the ratio [DMBQ]/([DMBQ] + [syringic acid]), from a 4 h time point. The 4 h time point was chosen because the half-life of the syringic acid to DMBQ reaction is 4 h at pH 5.5.

Figure 5



Dependence of haustorial induction with syringic acid and DMBQ on pH. *S. asiatica* seedlings (1.5 days old) were placed in 2 mM potassium phosphate buffer over a pH range of 4.0 to 8.5. The seedlings were incubated at 27°C with either 10^{-4} M syringic acid or 10^{-5} M DMBQ for 5 h or 10 h before being washed with three volumes of the designated buffer and further incubated with fresh buffer. Haustoria were counted and photographed after 2 days of incubation.

CN, are generally metabolic toxins that interfere with haustorial development. The pH dependence of the oxidase activity proved most valuable in our analyses, however. Figure 4 shows the pH dependence of the conversion of syringic acid to DMBQ by the extracted oxidases. DMBQ was stable under the reaction conditions, and the conversion required the presence of H_2O_2 throughout the explored pH range. The pH optima occurred between 5.5 and 6; at pH ≤ 4 and ≥ 7.5 there was no significant oxidation of syringic acid to DMBQ.

Figure 5 shows the pH-dependence of haustorial development. *S. asiatica* haustorial induction is independent of pH when seedlings are exposed to DMBQ for long periods of time (>10 h). The time-dependence of quinone exposure, however, has been shown to be crucial [22,29], and longer exposure times are required at low pH. At pH 4.2, only 22% of the seedlings formed haustoria after a 5 h exposure to DMBQ, but at 10 h induction approached that of the unwashed control. The morphology of the haustoria was not significantly altered across this pH range (Figure 6c,d).

At pH 5.6, haustoria induced by syringic acid were morphologically indistinguishable from those induced by DMBQ. At pH 5.2, however, swelling and haustorial hairs form, but haustorial morphology was abnormal (Figure 6b). Below pH 5, 10^{-4} M syringic acid was toxic to the seedlings (Figure 6a); the root tips showed a pronounced darkening and the seedlings were no longer viable even after syringic acid removal. Between pH 6 and 7, the longer the exposure time to the syringic acid, the higher the percentage of haustorial induction, but beyond pH 7, few haustoria were induced.

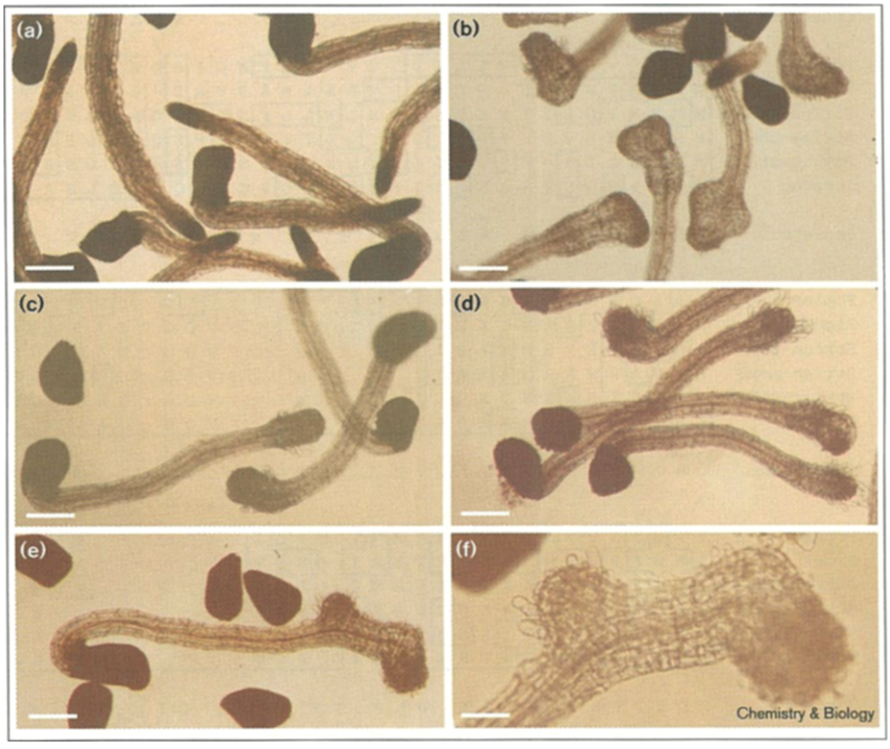
When syringic acid was not removed, many of the seedlings formed double haustoria (Figure 6e,f). Such double haustoria have been described in previous experiments [29] after multiple exposures to DMBQ. The exposure of seedlings to DMBQ for 6–10 h is sufficient to induce full haustorial development. Following removal of DMBQ, the seedlings were found to develop a new root primordium, one that was subsequently responsive to haustorial induction by DMBQ. Seedlings exposed to syringic acid for long periods of time were similar in appearance to these seedlings that saw multiple DMBQ exposures [29], as though the inducing signal had been removed to allow for meristem development and then reapplied.

The role of H_2O_2 in haustorial induction

There are several classes of oxidases. In order to determine what kind of oxidase was involved in *Striga* haustorial induction, we investigated the requirement of H_2O_2 in the induction process. A distinguishing feature of peroxidases, in comparison to other phenol oxidases, is a dependence on H_2O_2 as a co-substrate. An haustorial-induction model that involves conversion of phenols to benzoquinones, catalyzed by wall peroxidases would require the H_2O_2 co-substrate to be supplied to the parasite apoplast. We therefore evaluated the requirement for H_2O_2 by the exogenous

Figure 6

Dependence on pH of *S. asiatica* haustorial development induced by syringic acid or DMBQ. **(a)** With 10^{-4} M syringic acid at pH 4.6 for two days. **(b)** With 10^{-4} M syringic acid at pH 5.2 for two days. **(c)** With 10^{-5} DMBQ at pH 4.2 for two days. **(d)** With 10^{-5} M DMBQ at pH 8.0 for two days. **(e, f)** With 10^{-4} M syringic acid at pH 5.6 for two days. Scale bars: (a–e) 100 μm; (f) 30 μm.



application of catalase (an enzyme that breaks down H_2O_2). Bovine liver catalase (EC 1.11.1.6) proved to be very efficient, rapidly removing H_2O_2 from the *in vitro* assays and blocking peroxidase-catalyzed phenol conversion. In the presence of DMBQ, 10^{-2} to $>10^3$ units of catalase per ml, had no effect on haustorial induction. At $10^{3.2}$ units per ml, however, catalase completely inhibited haustorial induction by 10^{-4} M syringic acid, indicating a requirement for peroxidase in generating the signaling molecule from syringic acid. The magnitude of the inhibition gradually diminished with lower catalase concentrations, such that below 10^{-2} units per ml, normal haustorial induction ensued.

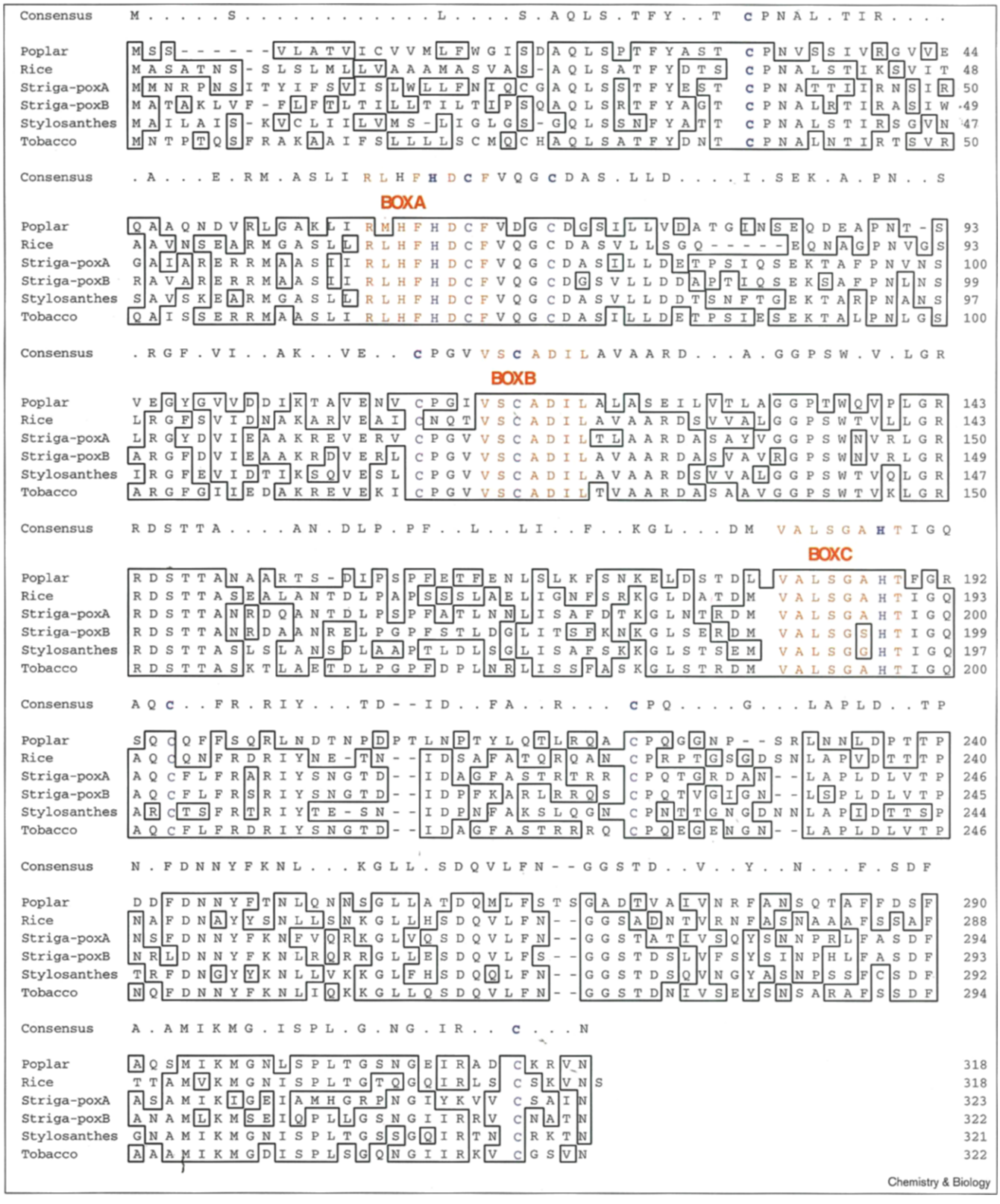
Cloning of *Striga* peroxidase cDNAs

Although the accumulated evidence established that the extracted peroxidases were essential for the oxidation of phenols to quinone inducers, a role for these enzymes in host recognition had not yet been established. Genes encoding peroxidases from several plants have been sequenced [30,31] and two amino-acid stretches from conserved regions, around the catalytic domain (box A; Figure 7) and the heme-binding domain (box C; Figure 7), were used to design degenerate oligonucleotide primers. The first primer (P-I) was based on the peptide sequence RLHFHDCF (using single-letter amino-acid code) of box A, and the second primer (P-II) was based on the sequence VALSGAHT of box C. Polymerase chain reaction (PCR) amplification using these degenerate primers with cDNA prepared from total RNA of 2 day-old *S. asiatica* seedlings yielded a band of the expected size, approximately 420 base pairs. Several independent candidate clones were sequenced from these and other degenerate primers and only two contained the conserved box B sequence. The deduced amino-acid sequences of the two different fragments strongly resembled each other, as well as those of other plant peroxidases. Repeated sequencing runs were used to confirm the base pair assignment. In order to isolate full-length cDNAs, gene-specific primers (PA-1, -2, -3, -4, PB-1, -2, -3, -4) were designed (Figure 8 shows their sequence origins). Following successful amplification, the products of 5′- and 3′- random amplification of cDNA ends (RACE) were gel-purified, subcloned and sequenced. These procedures enabled us to isolate the full-length *S. asiatica* cDNAs designated *poxA* and *poxB*.

Characterization of the *Striga* peroxidase genes

The eight conserved cysteines and two invariant histidines of the plant peroxidases [30,31] are encoded in the *poxA* and *poxB* genes (Figure 8). Histidine 68/69 has been predicted to be involved in acid/base catalysis and belongs to the highly conserved subdomain box A. Histidine 195/196 in the highly conserved subdomain box C is predicted to serve as the fifth ligand to the heme iron. Between these two conserved subdomains, there is another conserved region of seven amino acids, VSCADIL, designated box B. Putative

Figure 7



Multiple alignment of the amino-acid sequences of the plant peroxidases and the derived *Striga* peroxidase A (PoxA) and peroxidase B (PoxB) sequences. The highly conserved regions are marked Box A, Box B and Box C.

sites of *N*-glycosylation (asparagine-X-threonine/serine, where X is any amino acid) were found at amino-acid positions 40 and 213 of PoxA and at positions 180, 212, 236, and 318 of PoxB (Figure 8). The diffuse nature of the bands seen in the isoelectric focusing experiments in Figure 3 might be due to differential glycosylation of the peroxidase proteins.

The full-length cDNAs isolated using RACE had 110 base pairs of 5′- and 150 base pairs of 3′-untranslated sequences, each including the possible polyadenylation signal AATAAA. This sequence is located 28 nucleotides before the poly(A) stretch in *poxA* (Figure 8). The deduced amino-acid sequences each contain an amino-terminal signal sequence with a hydrophobic core and the predicted cleavage site at an alanine [32] (Figure 8). The length of the signal sequences are 27 and 26 amino acids, respectively, for *poxA* and *poxB*. The mature proteins for peroxidase A and peroxidase B each contain 295 amino acids with predicted molecular weights of 35,250 Da and 35,252 Da and calculated pI's of 8.6 and 9.3, respectively. Although there is a very broad range of homology throughout the known plant peroxidases, from 30% to 70%, the two cDNAs share 67% identity with each other in their coding sequence and their predicted amino acid sequences are 67.1% homologous. Several closely related genes are known and are compared in the dendrogram in Figure 9.

Expression of *poxA* and *poxB* during *Striga* development

The relative expression levels of the *Striga* peroxidase genes during development were determined using quantitative PCR. In order to quantify the amounts of *poxA* and *poxB* mRNAs, gene-specific primers (PA-1, PA-3, PB-1, PB-3) were used to amplify different-sized products, a 316 base-pair product from *poxA* (using PA1/PA3) and a 333 base-pair product from *poxB* (using PB-1/PB-3). The recombinant plasmid DNAs were used to prepare standard curves for quantitative PCR with the 5′ end-labeled primer. The amount of the first-strand cDNAs primed by oligo(dT) from total RNA of different stage *S. asiatica* seedlings was adjusted to fall within the linear range of the PCR experiments. Neither *poxA* nor *poxB* mRNA was detectable in the mature plant tissues or in preconditioned seeds prior to addition of the germination stimulant (Figure 10). Following addition of the SXSg germination stimulant, expression of both peroxidases increased within the first 24 h, roughly the time required for emergence of the seedling from the seed coat, and remained constant for several days. This expression, however, was reduced within hours of exposure of the seedling to DMBQ. Clearly, the expression of *poxA* and *poxB* is therefore regulated during early seedling growth and during haustorial induction in *S. asiatica* and appears not to occur in the ariel parts of the plant. This pattern of regulation was consistent with the histochemical staining data.

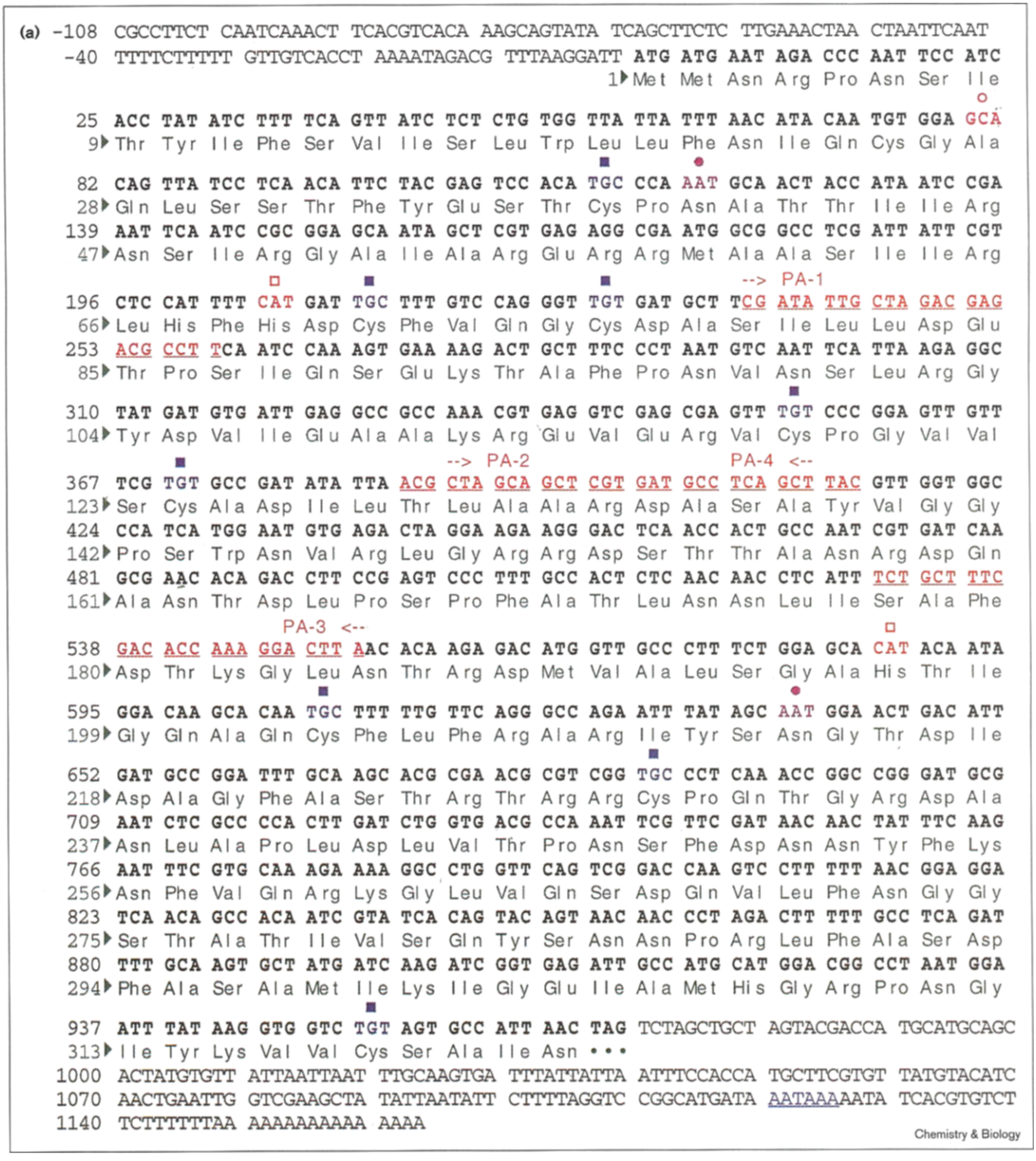
Discussion

Striga spp. represent the major biotic constraint to grain production in sub-Saharan Africa and, as a result, have been studied extensively. Accordingly, *Striga* spp. are the best characterized parasitic plants with respect to the chemistry of host recognition and therefore are the most readily analyzed mechanistically. Parasitic phenol oxidases were implicated in host recognition as a result of two principal findings: washing *S. asiatica* seedlings rendered them unable to develop haustoria in response to their hosts, and, this loss of function was reconstituted by laccase. In this study, we show that the apoplastic oxidases removed by washing are peroxidases with broad substrate specificity, capable of oxidizing known haustorial-inducing phenols to benzoquinones, and that these enzymes are dependent on pH in a pattern that correlates precisely with the pH dependence required for phenol induction of haustoria. Moreover, removal of the peroxidase co-substrate, H_2O_2, abolishes phenol induction of haustoria. Because quinone haustorial induction is independent of both pH and H_2O_2, the cloned peroxidases must function upstream of quinone detection in the conversion of wide array of phenol inducers into a small subset of quinone signals.

A more important question is whether these apoplastic oxidases function to mediate host detection. In *S. asiatica*, the major commitment to the host is at the level of germination, but the transition from vegetative to parasitic growth is no less critical. Haustorial development in *S. asiatica* occurs at the root meristem and successful attachment of the parasite to the host requires that haustorial development is initiated only after host–parasite contact [12,19,29]. Histochemical studies clearly showed that the oxidase activity is localized uniquely to the meristem where such contact must occur. Localization of the oxidases in this fashion could appropriately restrict the host detection chemistry to the host–parasite interface, but issues regarding the control of oxidase expression, the nature of the reactions being catalyzed, and the uniqueness of the oxidases to the parasite must be resolved in order to address the issue of host recognition fully.

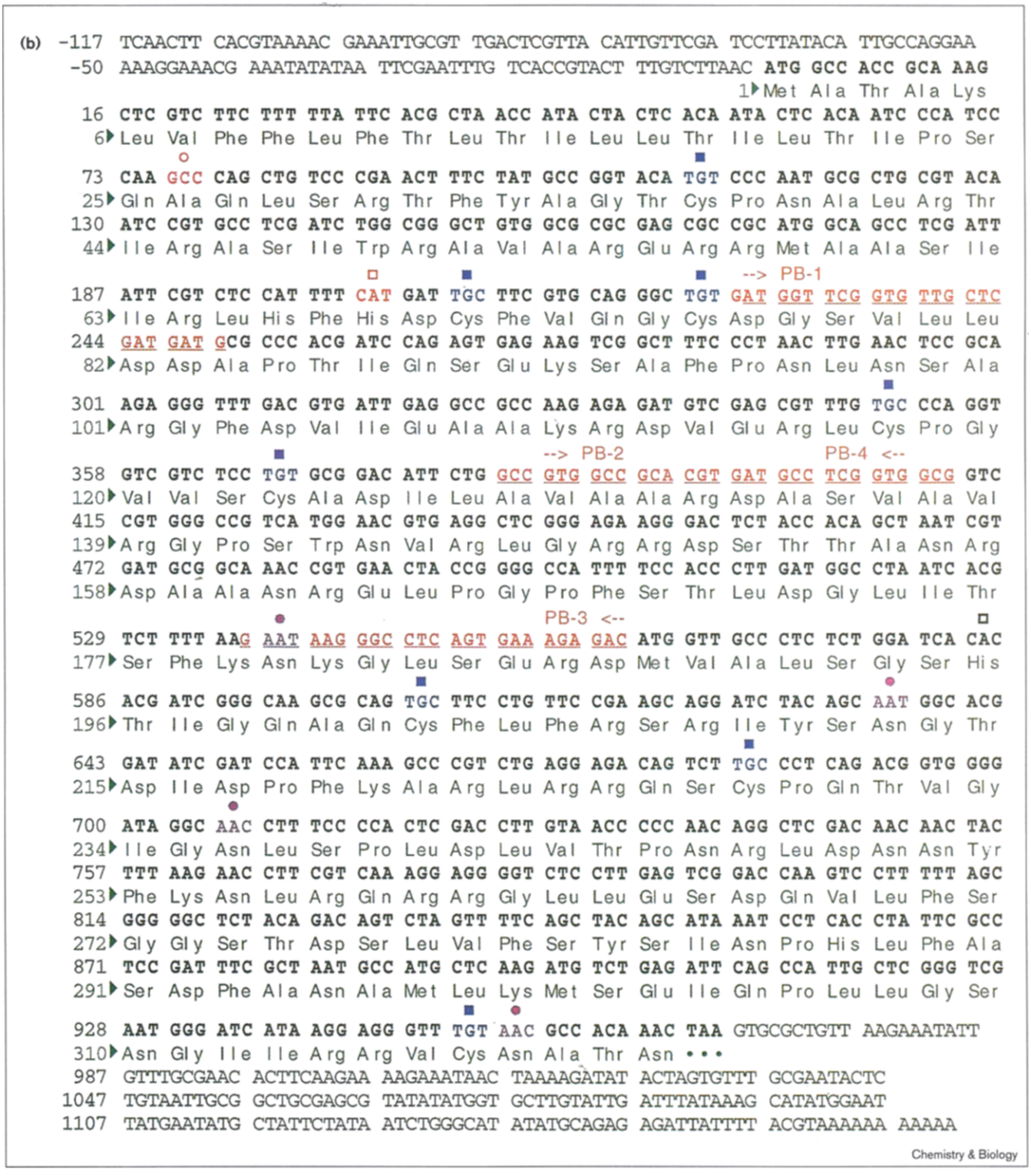
We found that *poxA* and *poxB* were the only oxidase genes expressed in young *Striga* seedlings. Both peroxidase genes contain leader sequences that are consistent with their apoplastic localization, and their predicted pI's are identical to those of the peroxidases found in the *S. asiatica* apoplast. The expression of *poxA* and *poxB* is activated by exposure to the germination signal, SXSg, and is suppressed by DMBQ. The speed and level of *Striga* peroxidase induction and repression represents a dramatic example of peroxidase regulation. There are now several examples of plant peroxidase gene regulation which ensure highly specific spatial and temporal localization of peroxidases. The peroxidases from barley [33], wheat [34] and rice [35] are induced by fungal pathogens; a cationic peroxidase from

Figure 8a



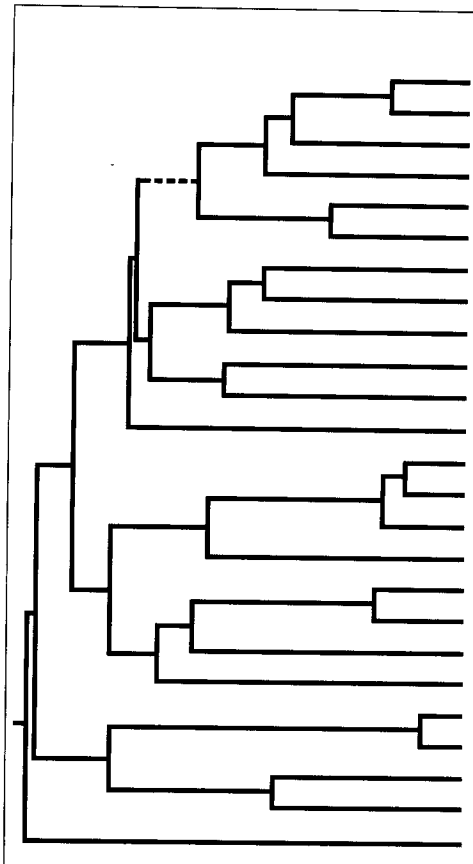
Nucleotide sequences, and deduced amino acid sequences, of *S. asiatica* peroxidase cDNAs. **(a)** PoxA and **(b)** PoxB. The gene-specific primers (PA-1,-2,-3,-4, PB-1,-2,-3, and -4) for RACE were designed from the cDNA sequnces underlined. Solid lines designate the sense strand and the broken arrows the nonsense strand. The plant peroxidase conserved cysteines are marked with a filled blue box and histidines with an open red box. Putative asparagine residues for *N*-glycosylation are marked with the filled purple circle, and the predicted cleavage site at alanine of the amino-terminal signal sequnces are marked with the open red circle. The likely polyadenylation site AATAAA is underlined.

Figure 8b



Medicago truncatula is induced by its symbiotic *Rhizobium* spp. [36]; and an anionic cucumber peroxidase is induced by ethylene [37]. A cationic peroxidase of the tropical forage legume *Stylosanthes humilis*, which is highly homologous to *poxA* and *poxB* (Figure 9), is strongly induced by the fungal phytopathogen *Colletotrichum gloeosporioides*

Figure 9



	Gene	Gene bank accesion no.	Homology to *striga poxA*	Homology to *striga poxB*	pI	Reference
Barley		X58396	47.0	47.9	6.4	[33]
Wheat		X56011	47.8	49.4	5.7	[34]
Rice	*pir3*	X66125	49.1	50.9	5.7	[35]
Wheat	*pox4*	X85230	48.3	50.2	8.5	[66]
Peanut	*pnc1*	M37636	51.6	55.4	7.8	[67]
Stylosanthes	*Shpx6*	L36110	50.5	55.8	8.8	[38]
Striga	*poxA*	AF043234	100	**67.1**	**8.6**	This study
Tobacco		M74103	**71.4**	**64.9**	6.2	[41]
Striga	*poxB*	AF043235	**67.1**	100	**9.3**	This study
Linum		L24120	47.7	50.3	4.7	[68]
Turnip	*tp7*	B23116	53.0	55.7	8.8	[69]
Medicago	*rip1*	U16727	41.2	45.3	9.4	[36]
Arabidopsis	*prxca*	M58380	40.2	40.4	7.2	[70]
Arabidopsis	*prxcb*	X71794	40.9	40.7	7.9	[71]
Horseradish	*prxC1a*	M37156	40.2	40.7	5.7	[72]
Arabidopsis		P24102	41.5	40.4	6.1	
Tobacco		L02124	35.5	35.5	4.1	[73]
Tobacco		J02979	40.9	40.1	4.4	[74]
Poplar	*prxA1*	D11102	41.2	41.2	4.3	[75]
Cucumber		M32742	37.3	39.4	6.1	[37]
Tobacco		D42064	35.0	35.7	8.6	[76]
Tobacco		D42065	35.0	34.8	8.8	[76]
Tomato	*tpx1*	L13653	32.8	34.8	8.7	[77]
Tomato	*tpx2*	L13654	34.7	37.9	7.6	[77]
Barley		M73234	32.5	34.5	7.2	[78]

Chemistry & Biology

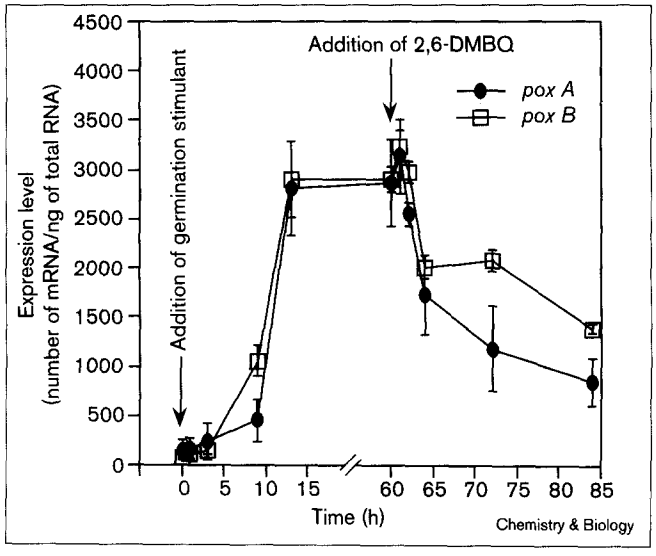
Dendrogram showing the relationships among the deduced amino-acid sequences of the PoxA and PoxB proteins from *S. asiatica* and those of 23 other plant peroxidases. Percentage identity to the *Striga* PoxA and PoxB are shown, and the pI is calculated from the deduced amino-acid sequence of each mature protein.

within 4 h of inoculation [38]. The peroxidase from *Nicotiana sylvestris* that is most similar to *Striga poxA* and *poxB* is highly expressed at the beginning of *Nicotiana sylvestrisis* protoplast regeneration and moderately in roots [39]. It appears therefore that the regulation of peroxidases at the transcriptional level is common and certainly important for their function.

The regulation of *poxA* and *poxB* may explain the formation of the multiple haustoria under certain conditions (shown in Figure 6e,f). DMBQ has been shown to be cleared *in vivo* [12] and, once cleared, new root primordia develop from the committed haustorium [29]. This new meristematic growth would activate expression of *poxA* and *poxB*, and, in the presence of excess phenol, the accumulating enzymes could catalyze DMBQ generation, which would start haustorial development over again. The formation of multiple haustoria, (Figures 6e,f), could be controlled through such a cyclic process; in the presence of excess syringic acid, oscillating peroxidase expression would result in sequential haustorial inductions. The oscillation in expression of *poxA* and *poxB* could provide a mechanism for controling the development of multiple haustoria *in vitro* [40,41].

The *Striga* PoxA and PoxB proteins are plant peroxidases that contain a ferriprotein porphyrin prosthetic group, four conserved disulfide bridges, an invariant aspartic acid–arginine salt bridge, and two calcium ions. Across the three different peroxidase classes, bacterial, mitochondrial, and chloroplast (Class 1), fungal (Class 2), and plant (Class 3), there is less than 20% sequence identity, but the catalytic residues in the heme cavity are highly conserved [31] and the reaction mechanism is shared. The broad redox range of peroxidases has been attributed to the relative position of the porphyrin within the protein [42], but it is likely that the broad product distribution is much more a function of the reaction pathways open to the phenoxy-radical intermediate generated by the one-electron oxidation chemistry of the reaction [25,26,43–45]. Certainly the pectic fractions from host cell walls contain phenolic substituents that are substrates for enzymes like PoxA and

Figure 10



The expression levels of *poxA* and *poxB* as determined using quantitative PCR during seedling development.

PoxB, and the products include quinones. Just as we have shown here for the model phenol, syringic acid, the host pectic wall fractions are oxidized to DMBQ [12].

PoxA and PoxB are, however, not unique to the *Striga* parasite, but are members of a large gene family. A survey of the literature suggests that the plant peroxidase genes are numerous, as many as 40 per plant [46]. This number of peroxidases would allow Fenton chemistry to be targeted to virtually every cellular compartment where a specific role of maintaining or adapting structures and functions of the plant cell to its environment must be met. In both *Arabidopsis*, which has a seed and radicle of similar size to *Striga*, and grass hosts, including maize and sorghum, the root hair zone has intense peroxidase activity. In the panicoid grasses, both epidermal cells in the later regions of root elongation and root cap cells have apoplastic peroxidases in significantly higher abundance [27] than we found in *S. asiatica*. These host enzymes, which are homologous to PoxA and PoxB, are capable of the same generic Fenton chemistry, are abundant in the host walls where parasite attachment occurs, and are therefore quite capable of converting wall-localized phenols into quinones.

If PoxA and PoxB are not unique to the parasite and not involved in host recognition, how did the washing experiments, which removed these enzymes, remove host detection activity? Addition of horseradish peroxidase did not reconstitute the oxidase activity [12], but we have now found that the addition of H_2O_2 to maize and sorghum seedlings does release significant haustorial-inducing activity (R.K., W.J.K and D.G.L., unpublished observations). Differentiation between host and parasite therefore lies in the availability of the oxidant, not the oxidase. This conclusion is supported by the observation that chromophoric substrate coloration (confirmation of oxidase activity) develops slowly without added H_2O_2 in *S. asiatica*, but not at all in the other plants tested.

The required oxidant, H_2O_2, is common in plants and has been assigned a variety of biological roles that range from a general antimicrobial agent to a specific signal molecule in systemic acquired resistance [47–49]. There is even evidence that it accumulates in the plant cell wall during pathogen invasion [50]. Several enzymes have been implicated in H_2O_2 production, including apoplastic peroxidases [51,52], lipoxygenases and oxalate oxidase [53], each of which requires a reducing substrate to be supplied to the apoplast [54–57]. Alternatively, redox activity across the plasma membrane [58] could produce an active oxygen species directly. Oxidase complexes homologous to the human neutrophil oxidative burst machinery have been detected in plants [59–62]. Although the specific function of these oxidase complexes is unclear [63], the possibility of such a system is particularly interesting as xenognostic quinones were recently shown to be detected through a benzoquinone-dependent oxidoreductase whose terminal acceptor, based on the discovered redox range, is likely to be O_2 [22]. An autocatalytic cycle connecting H_2O_2 generation with quinone detection could greatly enhance the sensitivity of host detection and suggests that a single transmembrane oxidase would be sufficient for both activities.

Given the model that the oxidant is the limiting factor, there must be another difference between host and parasite, that is, the phenolic substrates must be available in the host wall, and absent from the parasite. In the grass hosts of *Striga*, the phenolic content in the primary walls of nonvascular tissues varies greatly, from 1/50 to 1/2,400 phenols per sugar residue, depending on the tissue type [27]. The finding that H_2O_2 added to sorghum and maize roots released significant haustorial-inducing activity suggests that host phenols are accessible to the host peroxidases; these oxidation products must be further characterized to better understand the result, however. Addition of H_2O_2 to *Striga* roots does not induce haustoria, suggesting that either the phenolic substrates are not present in the parasite wall or they are not available to PoxA/PoxB.

In *Agalinis purpurea*, a related hemi-parasitic Scrophulariaceae in which meristem-localized oxidase activity has also been detected [19], haustoria are induced by phenols in agar plate cultures [64]. In two week-old cultures, however, spontaneous haustoria begin to develop and self-attachment is common in these as well as in other Scrophulariaceae [20]. In *S. asiatica*, the roots never mature more than 5 days past germination because of limited food reserves in the seed [12]. Initial extraction

experiments have shown that these young seedlings have reduced phenolic content, and the isolated walls are devoid of haustorial-induction activity. The parasitic plants may therefore capitalize on the low phenolic epithelial wall content of the juvenile roots in order to exploit the critical difference between the hosts and the parasites, namely the constitutive production of H_2O_2.

Significance

The loss of autonomy and the acquisition of traits that enforce the dependence of one organism on another is a central feature of the evolution of genome structure. In the parasitic plants, elements of genome autonomy are lost and it appears possible to understand the molecular events that have driven this evolution by characterizing the events that initiate attachment organ development. From a practical point of view, parasitic plants represent a serious constraint on agricultural productivity and insight into the mechanism of initiating parasite development in one organism might open general strategies for control.

Striga asiatica **respond to a diverse array of known molecular structures in order to induce the parasitic mode. The two peroxidases that we have identified in *Striga* root cell walls can account for the diversity of the inducing signals. Identification of these peroxidases does not, however, provide support for the hypothesis that a parasitically exuded oxidase mediates host detection. We propose instead a new model which holds that the critical evolved difference between host and parasite is the constitutive production of an activated oxygen species, via reduction of O_2. Furthermore, both host and parasite play an active role in host detection. Detection of host root surfaces exploits both host-wall phenolic content, which may be a function of organ maturity, and host enzymes. The parasite is therefore asking two questions — Do the walls contain the phenol substrate? And are there enzymes available to convert the substrates to the quinone signal? — which increase the precision of its commitment to the parasitic mode. Finally, the model is consistent with the idea that H_2O_2 production is coupled with detection of the haustorial induction signal in an autocatalytic cycle to enhance host detection sensitivity.**

Materials and methods

Plant materials

Striga seeds were sterilized as previously described [22] and pretreated in H_2O at 27°C in the dark for 10 days. Pretreated seeds (100 mg) were used for germination and were collected after incubation (0 h, 1 h, 3 h, 9 h, and 13 h) with the SXSg germination stimulant [13,14]. Two-day-old germinated seedlings were exposed to 2×10^{-6} M of DMBQ and were collected after a series of incubations (0 h, 1 h, 2 h, 4 h, 12 h, or 24 h).

Sorghum Sudan Grass Hybid seeds (20 g) were grown aseptically as described previously [13] and after 12 days the roots (4 g) were removed, frozen in liquid nitrogen and lyophilized. The resulting brown powder was resuspended using sonication and washed 4 × @ 1,800 g with 30 ml Tris-maleate buffer, pH 7.8, containing 1% Triton X100, washed with EtOH, and lyophilized to a gray powder (0.5 g). Extraction in boiling H_2O overnight solubilized the pectic fraction. Saponification in 0.2 M KOH under N_2, neutralization with dilute HCl, and Et_2O extraction gave the organic soluble fraction [28,65].

Histochemical staining

Striga seedlings were taken from the germination media and transferred to the peroxidase assay solution containing 10 mM sodium phosphate, 0.03% H_2O_2, and 0.2% pyrogallol. Oxidation of pyrogallol results in reddish coloration of the tissue that is visible within 2–5 min. Following color development, seedlings were rinsed with ddH_2O. Oxidation of syringaldazine results in a red–purple color. When the seedlings were stained with syringaldazine they were transferred to microscope slides and a solution of the dye in 0.1% in EtOH (1–2 drops), followed by 0.3% H_2O_2 (1–2 drops) was added. The red–purple color tended to fade after 15 min.

RNA isolation and first strand cDNA synthesis

Total RNAs were isolated from 2 day-old *Striga* seedlings (100 mg) by phenol/chloroform extraction (Promega Total RNA isolation kit). The yield of total RNA obtained was determined spectrophotometrically at 260 nm, where one A260 unit = 40 µg of single stranded RNA/ml. First strand cDNAs were synthesized from 2 µg of total RNA with 0.5 µg of oligo(dT)12–18 using SuperScript II RNase H–Reverse Transcriptase (Life Technologies). The RNA was digested with *E. coli* RNase H and the resulting single stranded cDNA was used for PCR amplification.

PCR amplification of the peroxidase genes

PCR reactions were carried out in 100 µl of buffer containing the first strand cDNA from 2 µg of total RNAs, 75 pmol each of the degenerate primers (P–I, P–II), 5 units of AmpliTaq DNA polymerase (Perkin-Elmer), 20 mM $Tris^-HCl$ (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 ug BSA, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.5 mM dTTP, and 10 mM dithiothreitol (DTT). Following an initial 5 min denaturation at 95°C, 35 cycles of PCR amplification were performed with 1 min denaturation at 94°C, 1 min annealing at 45°C, and 2 min extension at 72°C that was increased by 6s in each subsequent cycle.

Subcloning and DNA sequencing

The amplified PCR products were analyzed by gel electrophoresis in 4% Nusieve agarose. PCR products were purified from the agarose gel and subcloned using the pCR-ScriptTM SK(+) Cloning kit (Stratagene) according to the manufacturer's instructions. Putative recombinant plasmids carrying inserts of the appropriate size were used for DNA sequencing. All DNA sequencing was performed on double-stranded plasmid templates by the dideoxy chain termination method with Sequenase version 2.0 kit (United States Biochemical Co.).

Cloning of full-length peroxidase cDNAs

5′-RACE and 3′-RACE experiments utilized the materials from Life Technologies. The gene-specific primers were designed from the cDNA sequences of cloned genes. PCR reactions were conducted with AmpTaq polymerase (Perkin Elmer) according to the manufacturer's conditions. For 3′-RACE, first strand cDNAs were synthesized from total RNA using the chimeric adaptor primer (AP), which contained both oligo(dT)17 and the amplification primer (UAP), and PCR-amplified using the gene specific primers, PA-1 or PB-1, and UAP. Second round PCR was carried out with a nested gene specific primer, PA-2 or PB-2, and UAP. For 5′-RACE, first strand cDNAs were synthesized from total RNA using gene specific primers PA-3 or PB-3. After RNAse treatment, an anchor sequence, oligo dC, was added to the 3′ end of the cDNA using terminal transferase and dCTP. The cDNA products were PCR-amplified using nested gene specific primers PA-4 or PB-4 an a chimeric anchor primer ANP. Following amplification, the products of 5′- and 3′- RACE were gel-purified, subcloned, and sequenced as described above.

Quantitative PCR

In order to quantify the amplified products, primers (10 pmol) were labeled with 50 µCi of [γ-^{32}P]-ATP (6,000 Ci/mmol, Amersham) by T4 polynucleotide kinase (Life Technologies,Inc). The optimized buffer condition for each primer set was determined using the PCR Optimization kit (Stratagene). With the optimized conditions, standard curves were constructed for each clone–primer combination using labeled primers (100 µCi/pmol of primer per 25 µl reaction volume). Control reactions contained a mixture of two peroxidase clones ranging from 0.1 pg to 10 pg of plasmid. After the initial denaturation of the template DNA at 94°C for 5 min, 25 cycles of PCR amplification were performed with 0.5 min of denaturation at 94°C, 1 min of annealing at 45°C and 1 min of extension at 72°C. After PCR, the amplified products were resolved on a 4% Nusieve-agarose gel and the gel was dried at 55°C in vacuo for 2 h. The amplified products on the dried gel were visualized by the use of a Phosphor Imager system. Standard curves were determined by plotting the counts of the amplified band vs. the initial template DNA. First strand cDNAs primed by oligo (dT) were used to measure the expression level of *poxA* and *poxB* in *Striga* seedlings.

Oligonucleotide synthesis

Two degenerate oligonucleotides, P-I 5′-CAGGTCGACCGI(T/C)TICA (C/T)TT(C/T)CA(C/T)GA(C/T)TG(C/T)TT-3′ and P-II 5′-GCCGGATCC-TT(G/A)TGI GCICCI(G/C)(A/T)IA(G/A)IGCIAC-3′ were synthesized as primers for PCR amplification of peroxidase genes. The gene specific primers, PA-1 5′-CGATATTGCTAGACGAGACGCCTT-3′, PA-2 5′-CT-AGCAGCTCGTGATGCCTCAGCTTAC-3′, PA-3 5′-TAAGTCCTTTG-GTGTCGAAAGCAGA-3′, and PA-4 5′-AGCTGAGGCATCACGAGCT-GCTAGCGT-3′ were designed from the sequences of the cloned cDNA of peroxidase-A. PB-1 5′-ATGGTTCGGTGTTGCTCGATGATG-3′, PB-2 5′-GTGGCCGCACGTGATGCCTCGGTGGCG-3′, PB-3 5′-GTCTCT-TTCACTGAGGCTCTTATTC-3′, and PB-4 5′-CACCGAGGCATCAC-GTGCGGCCACGGC-3′ were designed from the sequences of peroxidase-B cDNAs. The adaptor primer, AP 5′-GGCCACGCGTCGACT-AGTAC(T)$_{17}$-3′, the amplification primer, UAP 5′-CUACUACUACU-AGGCCACGCGTCGACTAGTAC-3′ and the anchor primer, ANP 5′-CUACUACUACUAGGCCACGCGTCGACTAGTACGGGIIGGGIIG-GGIIG-3′ for 5′- and 3′-RACE were purchased from Life Technologies.

Fractionation of oxidases bound to the cell wall from Striga *seedlings*

Frozen *Striga* seedlings (100 mg wet weight) were ground in a pre-chilled mortar and pestle in 1 ml of Extraction Buffer (25 mM Tris·HCl pH 7.0, 2 mM EDTA, 2 mM β-mercaptoethanol). The slurry was centrifuged at 15,000 g for 10 min and the pellet was washed twice in 500 µl of Extraction Buffer containing 1% Triton. The insoluble cell wall fraction was recovered by retention on an 0.22 µm filter. Proteins were extracted from the cell wall fraction by incubation in 500 µl of Extraction Buffer containing 1 M NaCl for 1.5h on ice with gentle shaking. The extracted protein was recovered by passage through a 0.22 µm filter. The filtrate was de-salted against Extraction Buffer and concentrated by amicon ultrafiltration. Protein concentration was determined by Coomassie protein assay (PIERCE) and the individual proteins were resolved by isoelectrofocusing (Novex precast gel; pI 3.0–10.0). After electrofocusing, the gel was incubated in 10 mM sodium phosphate buffer (pH 6.0) for 10 min and the peroxidase activity was determined by 10 min immersion in peroxidase assay solution (PIERCE). The reaction was stopped by rinsing with water.

Peroxidase assays

Peroxidase activity in cell wall extracts was quantified by conversion of syringic acid to DMBQ. Cell wall extracts were incubated at 30°C for various times in 100 µl of buffer containing 20 µM syringic acid, 25 µM H_2O_2 and 50 mM sodium phosphate (pH 6.0 to 8.0) or sodium acetate (pH 4.0 to 5.5) and a portion of the reaction mixture was applied directly to a high performance liquid chromatography (HPLC) column (Zorbax ODS 0.46 i.d. × 25 cm, AcOH/MeOH/H_2O, 3.0/24.7/75.0, 1.0 ml/min, detection at 284 nm). The conversion was determined by comparing integrated peak areas with standard curves constructed from syringic acid and DMBQ standards. Background oxidation without the peroxidase or without H_2O_2 was insignificant.

Catalase assays

Striga seeds (15–30) were placed in each of the 24 wells of a microtiter plate and germinated with a 10^{-9} M solution of strigol over a 12 h period. Following a wash with sterile deionized water, the seedlings were allowed to grow for another 12 h before the addition of bovine liver catalase (EC 1.11.1.6), from 1.7×10^{3} to 1.7×10^{-2} units per ml, and syringic acid (10^{-4} M). The percentage of haustoria was determined after an additional 36 h of incubation. Each assay was performed in triplicate and expressed as ± SD. One unit of catalase is defined as the amount of solid that will decompose 1.0 µmol of H_2O_2 min^{-1} at pH 7.0 and 25°C, with an initial concentration of H_2O_2 ≥ 10.0 mM.

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