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Semagenesis and the parasitic angiosperm *Striga asiatica*

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Summary

Over the last several years, intermediates in the reduction of dioxygen have been attributed diverse functional roles ranging from protection against pathogen attack to the regulation of cellular development. Evidence now suggests that parasitic angiosperms, which naturally commit to virulence through the growth of new organs, depend on reduced oxygen intermediates, or reactive oxygen species (ROS), for signal generation. Clearly, the role of ROS in both plant defense and other physiological responses complicates any models that employ these intermediates in host plant recognition. Here we exploit the transparent young *Striga asiatica* seedling to (i) localize the site of H_2O_2 accumulation to the surface cells of the primary root meristem, (ii) demonstrate the accumulation of H_2O_2 within cytoplasmic and apoplastic compartments, and (iii) document precise regulation of H_2O_2 accumulation during development of the host attachment organ, the haustorium. These studies reveal a new active process for signal generation, host detection and commitment that is capable of ensuring the correct spatial and temporal positioning for attachment.

Keywords: semagenesis, *Striga asiatica*, reactive oxygen species, xenognosis, parasitic angiosperms, host–parasite interactions.

Introduction

The fate of obligate parasitic angiosperms depends initially on a successful and intimate physical attachment to another plant. To ensure successful attachment by this group of eukaryotic pathogens, normal developmental programs are often arrested at critical points that include seed germination, differentiation of the host attachment organ (the haustorium), host attachment, and apical meristem initiation. Transition past these arrest points requires specific host-derived signals known as xenognosins (Kuijt, 1969; Lynn and Chang, 1990; Riopel and Timko, 1995; Yoder, 2001; Hirsch et al., 2003; Walker et al., 2003; Palmer et al., 2004). The information content of these xenognosins is proving to be very rich. For example, in *Striga asiatica*, an obligate hemi-parasite of grasses, the hydroquinone sorghum xenognosin for *Striga* germination (SXSg) is necessary and sufficient for inducing *Striga* seed germination (Chang et al., 1986; Lynn and Chang, 1990). Evidence now exists for competing rates of auto-oxidation and diffusion of SXSg at the host surface, creating a steady-state chemical gradient of active hydroquinone around the host seedling (Fate et al., 1990; Fate and Lynn, 1990; Keyes et al., 2000; Keyes et al., 2001; Yoder, 2001; Palmer et al., 2004). The parasite exploits this gradient to enable appropriate spatial and temporal positioning prior to breaking dormancy.

In addition to germination, haustorial development in *Striga* involves recommitment of the terminal root meristem. This transition from vegetative growth (root elongation) to pathogenic growth (haustorium development) could also benefit from positional information for efficient host attachment. Initial experiments established that host cell-wall phenols were oxidatively released to generate the necessary and sufficient haustorial-inducing xenognosins

(Lynn and Chang, 1990; Smith et al., 1990; Smith et al., 1996). More recent studies have now found that H_2O_2 generated by *S. asiatica* is critical for host recognition. For example, enzymatic removal of H_2O_2 in parasite seedling cultures prevented model cell-wall phenols from inducing haustorial development, establishing extracellular H_2O_2 as necessary for haustorial initiation (Kim et al., 1998). These studies suggest that the generation of haustorial xenognosins occurs at the host cell surface where wall phenols are oxidatively released through the combined actions of host peroxidases and *Striga*-generated H_2O_2. In contrast to germination, where the xenognosis process involves passive surveillance for an exuded signal, the accumulation of H_2O_2 appears to be a pro-active search, where the parasite generates its host signal through a process of signal generation, or semagenesis (Keyes *et al.*, 2000; Keyes *et al.*, 2001; Palmer *et al.*, 2004). While the spatial information in germination is defined by the natural auto-oxidation/diffusion rates of SXSg, the commitment to haustorial development may well be a complex function of the chemical reaction dynamics of reactive oxygen species (ROS), e.g. H_2O_2, at the host/parasite interface.

Several constraints are critical to understanding the role of ROS in semagenesis. For example, reactive species such as H_2O_2 function as critical components of the oxidative burst response in plants. Accordingly, these short-lived species generally function at the site of pathogen attachment (Doke, 1983; Apostol et al., 1989; Baker and Orlandi, 1995; Tenhaken et al., 1995; Bolwell and Wojtaszek, 1997). If the parasite's production of the H_2O_2 semagen is localized for proper host/parasite attachment, its accumulation must be proximal to the cells that develop into the haustorium. Moreover, while most plants appear to employ ROS in brief elicitor-induced bursts, semagenesis requires a sustained level of H_2O_2 to be maintained at viable 'pre-haustoria' cells during xenognosin production. As ROS are generally cytotoxic components of the oxidative burst, their accumulation must be managed both within the parasite and at the host surface prior to and during attachment.

We reasoned that these predictions could be evaluated directly by resolving the H_2O_2 localization within the virtually transparent young *S. asiatica* seedlings. In this paper, we utilize laser scanning confocal, fluorescence and electron microscopies that both define the site and reveal the regulation of H_2O_2 accumulation in the young parasite. Moreover, the observed regulation is tightly coupled with the complex plasticity of haustorial development, and the absence of similar ROS accumulation and regulation in Arabidopsis suggests the adaptation may be uniquely associated with pathogenesis by parasitic plants. These findings further underscore the interesting possibility that semagenesis developed from the oxidative burst machinery of one plant to exploit the host defenses of another.

Results

Fluorescent imaging of H_2O_2 accumulation in S. asiatica *seedlings*

Dihydro-dichlorofluorescein diacetate (H_2DCF-DA) has previously been used to visualize cytoplasmic H_2O_2 production in plant cell cultures experiencing mechanical stress (Yahraus *et al.*, 1995), in *Arabidopsis thaliana* guard cells exposed to abscisic acid (Pei *et al.*, 2000), and in parsley cells prior to death by fungal infection (Naton *et al.*, 1996). This reduced, acetylated and non-fluorescent derivative of fluorescein is cell-permeable, and, following hydrolysis by cytoplasmic esterases, remains trapped in the cell as the resulting salt. Subsequent oxidation of H_2DCF by cytoplasmic H_2O_2 yields the highly fluorescent dichlorofluorescein (DCF) (Cathcart *et al.*, 1983). In each previous case, oxidation, rather than hydrolysis, proved to be limiting, providing a marker for cytoplasmic H_2O_2 (Cathcart et al., 1983; Allan and Fluhr, 1997).

As shown in Figure 1(a), a 3 min exposure to H_2DCF-DA resulted in specific fluorescence accumulation within 2–3 min at the root tip. A plot of pixel intensity across the width of the slice, between the white arrowheads in Figure 1(a), suggested that H_2O_2 accumulation was localized to the surface cells (Figure 1b). To determine whether H_2DCF-DA was accessible to all the cells of the seedling, cell-permeable fluorescein diacetate (FDA), which upon esterase cleavage yields the fluorescent probe directly, was loaded into the seedlings under the same conditions. As seen in Figure 1(c), incubation with FDA for 3 min resulted in uniform fluorescence intensity accumulation throughout the root tip, and the level is shown quantitatively in Figure 1(d). Longer incubation times (15 min) and increased dye concentration (50 μM) of H_2DCF-DA did not alter the specific fluorescence localization. Therefore, neither dye accessibility nor significant loss of emission intensity with tissue depth in the seedling appears to account for the differential fluorescence accumulation seen in Figure 1(a).

To determine whether events associated with generalized stress, such as seedling emergence from the seed coat, contributed to oxidant accumulation, the fluorescence was determined on each of 5 days following germination. As shown in Figure 2(a), the arbitrary florescence unit intensity remained constant and localized to the meristem for at least these first 5 days – the time period for which the seedlings remain capable of haustorial development. Although these seedlings responded to 2,6-dimethoxy-*p*-benzoquinone (DMBQ) induction of haustorial development, ROS production in the cells along the root surface could be stress-induced and apoptotic in origin. To specifically evaluate cellular viability in these cells, seedlings were treated with propidium iodide (PI), a fluorescent dye previously used to observe aluminum toxicity in seedlings of *Arabidopsis*

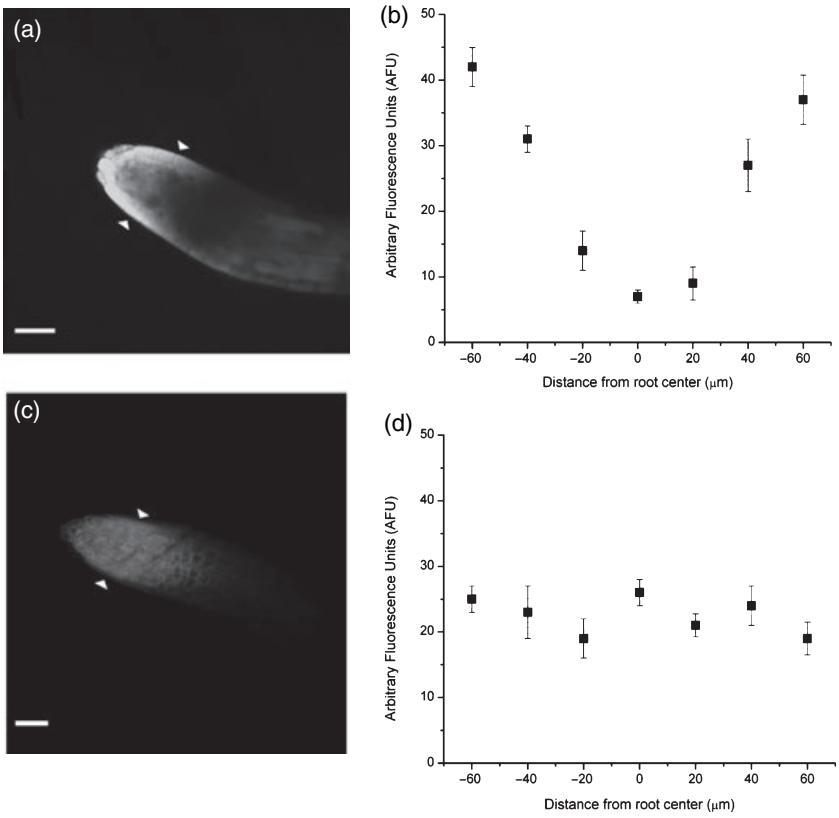

Figure 1. Confocal image of H_2O_2 localization. One-day-old seedlings of *S. asiatica* were incubated in 10 μM H_2DCF-DA or 10 μM DCF for 3 min, washed three times, and scored for fluorescence. The relative fluorescence intensities (b, d) across the seedling root at the white arrowheads highlights the differential localization of oxidation across the root tip. Plots are derived from the fluorescence distribution across five different seedlings (mean ± SD). Bar = 50 μm.

thaliana (Pan *et al.*, 2001; Ortega-Villasante *et al.*, 2005). Healthy cells reject PI, while apoptotic or oxidatively stressed cells allow the dye to accumulate intracellularly; intercalation into DNA results in several hundred-fold increases in fluorescence intensity. As shown in Figure 2(b), PI is excluded by the cells of both 1-day-old Arabidopsis and *Striga* seedlings. However, prior treatment of these seedlings with Al^{3+} resulted in significant root tip-localized PI staining.

Are the sites for cytoplasmic and apoplastic ROS accumulation correlated?

While H_2DCF-DA reports on cytoplasmic H_2O_2, the process of semagenesis depends on the extracellular H_2O_2 made available to host peroxidases. Accordingly, we evaluated apoplastic localization by cerium perhydroxide deposition, exploiting the specific reaction between the cell-impermeable $CeCl_3$ salt and H_2O_2 (Blokhina *et al.*, 2001; Orozco-Cárdenas *et al.*, 2001; Bolwell *et al.*, 2002). *S. asiatica* seedlings treated with $CeCl_3$ were fixed and sectioned, and the insoluble electron-dense cerium precipitates were visualized by transmission electron microscopy (TEM). As shown in the montage of low-magnification electron micrographs in Figure 3(a), extracellular deposits of cerium perhydroxide localized to the cellular interstitial spaces (Figure 3b) along the surface of the meristem. The intensity of the deposits diminished distally along the root axis, always disappearing by at least 8–10 cell lengths from the tip. Multiple deposits within the cellular interstitial spaces, similar to those shown in Figure 3(c), were also apparent. This apoplastic deposition along the surface cells of the meristem correlates with the cells identified as accumulating H_2O_2 by confocal florescence microscopy in Figure 1.

Is ROS accumulation regulated?

The above histochemical analyses confirm and extend previous reports of H_2O_2 accretion in *S. asiatica* (Kim *et al.*, 1998) by localizing the semagen to the cytoplasm and apoplasm of the cells lining the root tip. Moreover, these visualization methods created an opportunity to probe the temporal events of the semagenic process. In sharp contrast with the staining of the vegetative root meristem, seedlings treated with 10 μM DMBQ for 12 h were virtually devoid of cerium perhydroxide deposits (Figure 4a,b). A similar depletion of cytoplasmic H_2O_2 was confirmed by H_2DCF-DA fluorescence (Figure 4c).

The experimental results on cytoplasmic H_2O_2 reported in Figure 4(c) were further explored. Due to the time required for dye loading (3 min), successive washings to remove excess H_2DCF-DA, and positioning onto the microscope, the earliest time point at which DCF fluorescence could be accurately measured was 5 min. Further, the increase in

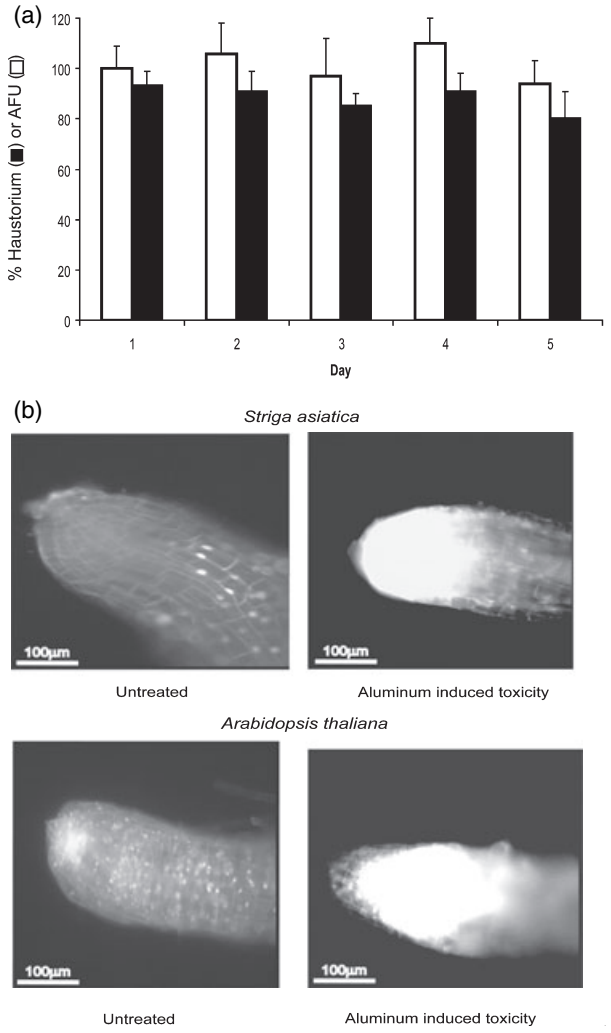

Figure 2. H_2O_2 accumulation and viability.
(a) *Striga* seedlings from 1 to 5 days post-germination were treated with H_2DCF-DA and scored for florescence. The average pixel intensity at the root tip of three seedlings is plotted as arbitrary fluorescence units (AFU) (white bars). The seedlings in each age group were treated with 10 μM 2,6-dimethoxy-*p*-benozquinone (DMBQ) and scored for haustorial development after 24 h (black bars).
(b) One-day-old *Striga* and Arabidopsis seedlings were scored for fluorescence from 10 μM propidium iodide (PI) without (left) or with (right) treatment in 10 mM Al^{3+} for 8 h.

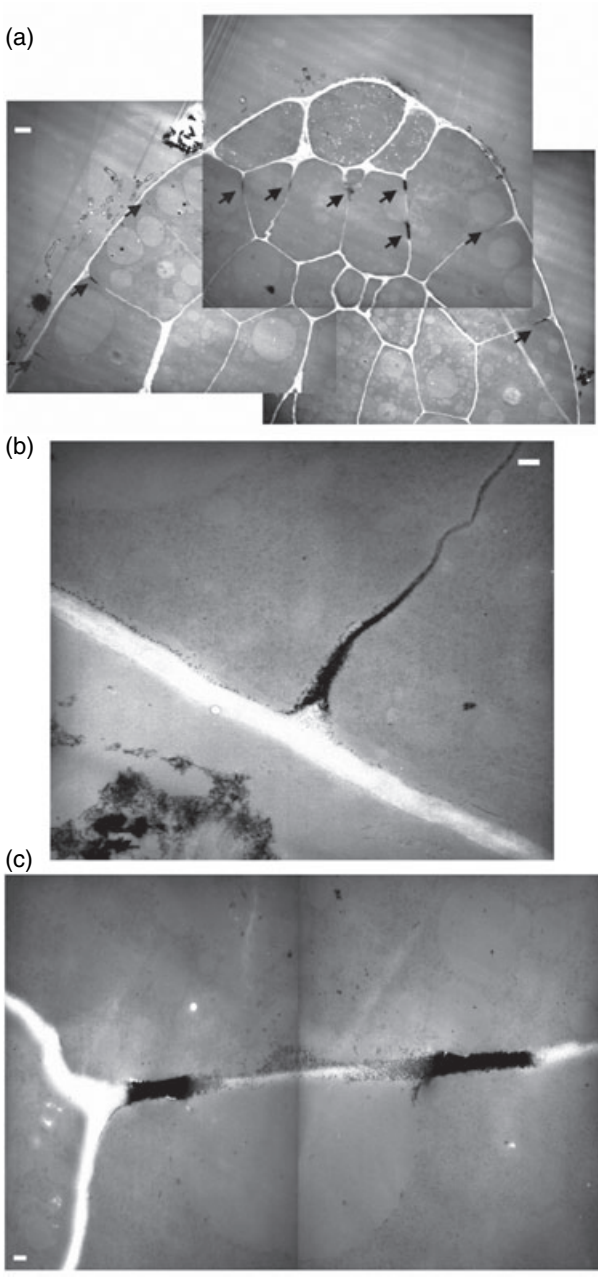

Figure 3. Histochemical localization of H_2O_2.
S. asiatica seedlings (18 h old) were incubated in freshly prepared solutions of $CeCl_3$ for 2 h, fixed, sectioned, and analyzed via TEM.
(a) Electron-dense deposits (arrows) are shown in low-magnification TEMs of a root tip section. Bar = 2 μm.
(b) Enlargement of the cellular junctions of the bottom left corner of (a). Bar = 200 nm.
(c) Enlargement of cells at the root tip of (a). Bar = 200 nm.

fluorescence plateaus after 15 min under these conditions. Increased dye loading times and higher dye concentrations increased the rate of fluorescence accumulation and extended the plateau to longer times, consistent with assigning the plateau to internal dye depletion. Therefore, as shown in Figure 5, the linear range from 5–15 min allowed a comparison of the relative cytoplasmic H_2O_2 concentrations per seedling. This approach established that 10 μM DMBQ depletes approximately 90% of the cytoplasmic H_2O_2 within 2 h, with a steady-state level of the oxidant maintained for at least 16 h when DMBQ is present in the medium (Figure 5b).

Does H_2O_2 regulation correlate with haustorial induction?

As cytoplasmic H_2O_2 levels parallel the apoplastic depletion of H_2O_2 during haustorium development and can be measured more accurately, these florescence assays have been

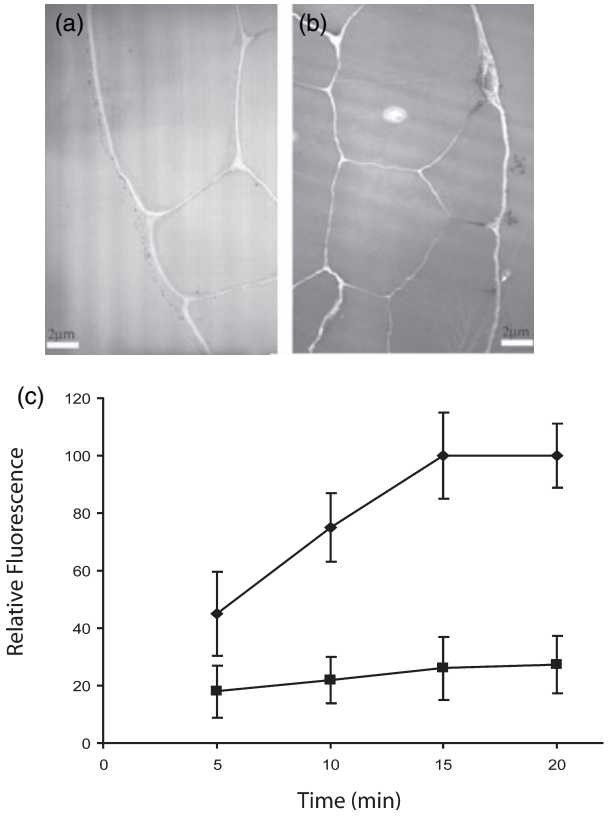

Figure 4. DMBQ perception and H_2O_2 accumulation.
One-day-old seedlings of *S. asiatica* were incubated with (a) or without (b) 10 μM DMBQ for 12 h, transferred to $CeCl_3$ for staining, fixed, sectioned, and analyzed via TEM.
(c) Five seedlings treated with (■) or without (◆) DMBQ were scored for fluorescence at the indicated time periods. Relative fluorescence is the ratio of the pixel intensity of the root tip at the given time to the average intensity in untreated seedlings after 20 min (mean ± SD).

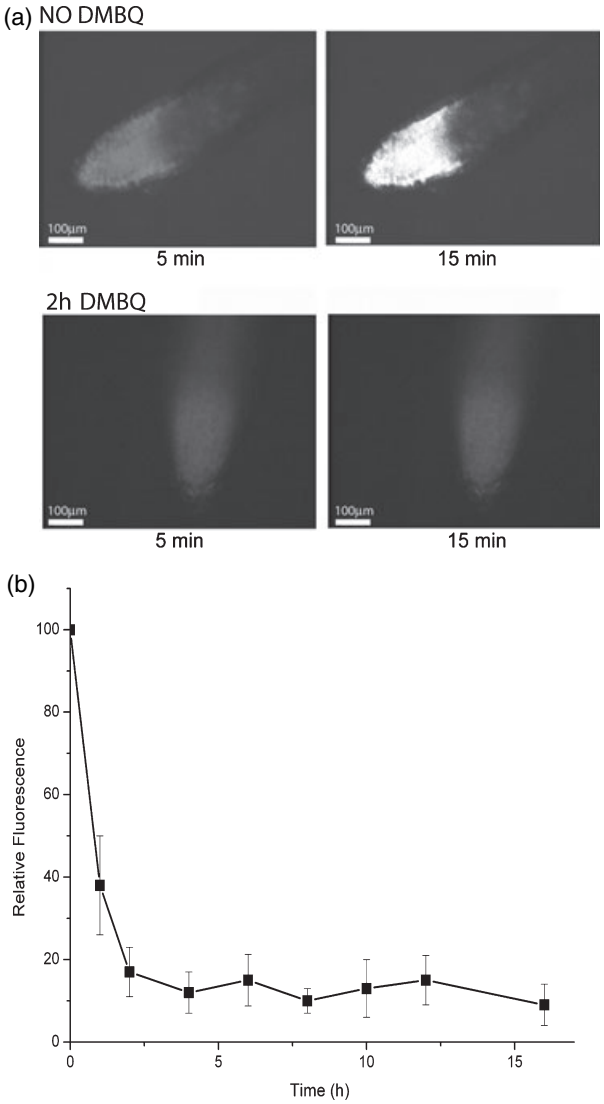

Figure 5. Time dependence of H_2O_2 down-regulation.
One-day-old seedlings of *S. asiatica* were treated with 10 μM DMBQ for the indicated time periods, washed, incubated with 10 μM H_2DCF-DA and scored for fluorescence.
(a) Representative 2 h images with and without DMBQ exposure.
(b) The ratio of pixel intensity of each root tip (15/5 min), the relative fluorescence, was scaled as a percentage and expressed for five replicate seedlings as the mean ± SD.

used to evaluate regulation. The exposure time required for commitment to haustorial development in *S. asiatica* is dependent on both the concentration and structure of the xenognostic quinones (Smith et al., 1990; O'Malley and Lynn, 2000). H_2O_2 depletion proved similarly concentration-dependent. Within the haustoria-inducing range, DCF accumulation was inversely dependent on DMBQ concentration (Figure 6a). Moreover, structurally similar haustoria-inducing *p*-benzoquinone (BQ) downregulated cytoplasmic H_2O_2 at a rate similar to DMBQ, and non-inducers such as tetrafluoro-*p*-benzoquinone and 2-methyl-napthoquinone (Smith et al., 1996) were not effective over a 10 h incubation period (Figure 6b).

Terminal commitment to haustorial development has been shown to have remarkable plasticity, particularly with regard to inducing signal exposure time. For example, previous experiments have established that premature removal of the xenognosin signal prior to terminal commitment to haustorial development leads to altered gene expression, abortion of the developmental transition, and recommitment to vegetative growth (Smith et al., 1990; O'Malley and Lynn, 2000). Figure 7(a) compares the effect of 10 μM DMBQ on both haustorial commitment and the accumulation of H_2O_2 in 2-day-old *S. asiatica* seedlings. At each time point, DMBQ is removed and portions of the seedlings are either scored for fluorescence or for haustorial development after 24 h. The half-maximal exposure times for these two events proved to be different: a 1 h DMBQ exposure was sufficient to reduce DCF accumulation to half maximal levels, whereas a 5 h exposure was required for 50% of the seedlings to commit to haustorial development.

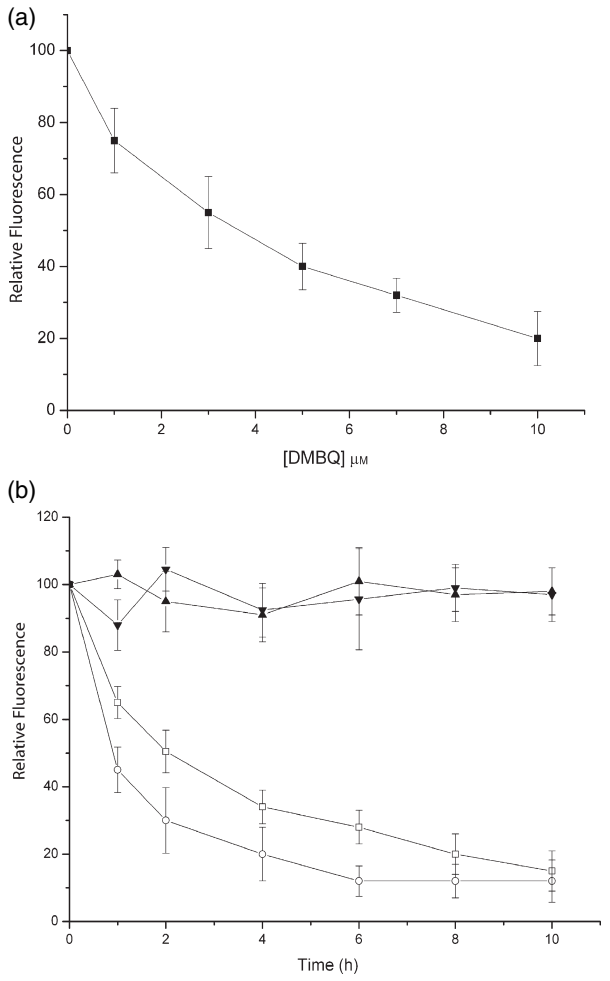

Figure 6. Structural dependence of haustorial inducers on the rate of DCF accumulation.
(a) One-day-old seedlings of *S. asiatica* seedlings were incubated with the indicated DMBQ concentration for 2 h, rinsed, loaded with 10 μM H_2DCF-DA for 3 min, and scored for fluorescence. The results for five seedlings are presented as means ± SD for each concentration of DMBQ.
(b) One-day-old seedlings of *S. asiatica* were incubated with 10 μM concentrations of DMBQ (○), *para*-benzoquinone (□), 2-methyl-*p*-napthoquinone (▼) or tetrafluoro-*p*-benzoquinone (▲) for the indicated times and scored for H_2DCF-DA fluorescence. The results for five seedlings are presented as means ± SD for each time point.

Interestingly, the rate of H_2DCF oxidation completely recovers within 1–2 h in seedlings treated with DMBQ for 1 h, a time point prior to any commitment to haustorium formation (Figure 7b). Re-exposing the seedlings to DMBQ again depleted the H_2O_2 levels, and these rates again recovered after about 1 h following DMBQ removal. These oscillations in oxidant accumulation continued for at least 6 h (Figure 7c) – the critical exposure window necessary for haustorial commitment – establishing the ability of the semagenic process to respond to variable environmental DMBQ exposures.

Upon terminal commitment to haustorial development, removal of the xenognosin does not alter organogenesis, but does permit the resumption of vegetative meristematic growth (Smith et al., 1990; O'Malley and Lynn, 2000). Coincident with the recovery of vegetative growth, the oxidation of H_2DCF is also re-established (Figure 7d). Again, DMBQ re-exposure down-regulates H_2DCF oxidation, and the rate of down-regulation coincides with the extent of commitment to the new growing meristem. The new seedling meristem that develops from the haustorium is able to make a new haustorium (Smith et al., 1990; O'Malley and Lynn, 2000), and also shows equivalent H_2O_2 accrual and DMBQ responsiveness to the initial germinated seedling. Therefore, semagenesis is tightly regulated during xenognosin perception, is sensitive to environmental perturbations in xenognosin availability, and follows a structural and time-dependent program that correlates with the haustorial developmental commitment.

Discussion

Once considered the unfortunate by-products of aerobic life, growing evidence now suggests that ROS play roles in pathogen defense, cell growth and eukaryotic development (Bokoch and Knaus, 2003; Lambeth, 2004). As in other organisms, ROS production in plants can be regulated in response to microbial elicitors, whereby these reactive intermediates function both to toxify the apoplast (Peng and Kuc, 1992) and as a substrate for the oxidative cross-linking of the outer cell-wall barrier (Bradley *et al.*, 1992). In the later case, peroxidases utilize H_2O_2 to catalyze oxidative cross-linking reactions for both primary and secondary wall reinforcement. Similarly, in the nematode *Caenorhabditis elegans*, ROS is apparently required for cross-linking tyrosine residues, a reaction critical to stabilization of the extracellular matrix. Silencing the ROS-producing enzyme Ce-Duox in *C. elegans* results in severe epidermal abnormalities, giving a 'leaky worm' phenotype (Edens *et al.*, 2001).

The small and virtually transparent young seedling of *S. asiatica* offered an opportunity to observe ROS accumulation in the new process of semagenesis. While the underlying mechanisms and many of the reasons for the developmental plasticity of haustorial development remain obscure, the approaches developed here clarify several constraints on ROS accumulation during host recognition. First, H_2O_2 accumulates at the root tip, the tissue whose differentiation ultimately defines the transition to pathogenesis in these organisms. Both H_2DCF-DA and $CeCl_3$ staining localize H_2O_2 accumulation to the surface cells of the primary root meristem. Previous $CeCl_3$ staining in other species identified H_2O_2-mediated deposits in mesophyll cells undergoing the early stages of the hypersensitive response, presumably directed at virulent bacteria lodged in the intercellular space or colonizing the cell surfaces

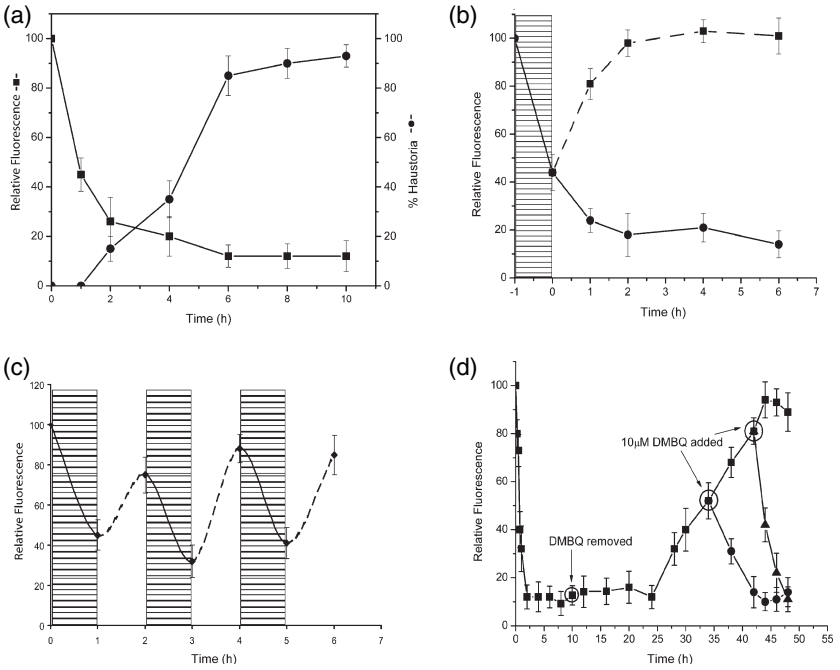

Figure 7. H_2O_2 regulation and haustorial commitment.
(a) Relative fluorescence and haustorial development. One-day-old *S. asiatica* seedlings were incubated with 10 μM DMBQ for the indicated time periods and either scored for fluorescence immediately (■) or for percentage haustoria development after 24 h (●).
(b) Recovery of H_2O_2 depletion. One-day-old *S. asiatica* seedlings were incubated with 10 μM DMBQ for 1 h (hatched area), separated into two fractions and incubated with (●) or without (■) DMBQ for the indicated times, and scored for H_2O_2 production.
(c) Repeat recovery of H_2O_2 depletion. As in (b), 1-day-old *S. asiatica* seedlings were incubated with 10 μM DMBQ for 1 h (solid line), washed and incubated in buffer for a second hour (dashed lines), and this pattern was repeated twice. Equal portions of the seedlings were scored after each hour.
(d) ROS regulation following haustorial commitment. One-day-old *S. asiatica* seedlings were incubated with 10 μM DMBQ for 10 h to ensure commitment to haustorial development, the DMBQ was removed, and at the indicated time points, five seedlings were scored for fluorescence as above. At 34 h (●) and 44 h (▲), fractions of the seedlings were again transferred to 10 μM DMBQ and fluorescence was scored at the indicated time points.

(Bestwick *et al.*, 1997). In constitutive ROS-producing rice mutants, the sites of production are also localized within similar intercellular spaces (Kawasaki *et al.*, 1999). At this point, the cellular localization of H_2O_2 in *Striga* appears to be functionally related to the hypersensitive response.

Second, the apparent insensitivity of the *S. asiatica* terminal root meristem to significant H_2O_2 accumulation is significant. Generally, the production of ROS is tightly regulated to avoid initiation of multiple responses, including programmed cell death (Halliwell and Gutteridge, 1990; Levine et al., 1994). For example, the constitutive production of H_2O_2 in mutant rice plant cells results in spontaneous cell death in both transgenic cultures and regenerated plants (Kawasaki *et al.*, 1999). This insensitivity in *S. asiatica* may arise in part from the small size of the root meristem and the ready diffusion of ROS from the producing cells. Certainly, tight regulation of production and degradation rates is expected to be important in avoiding toxicity.

Finally, and probably of greatest significance for pathogenesis, the xenognosin addition/removal experiments reveal a robust feedback circuit for H_2O_2 accumulation in *Striga*. The haustorial xenognosin down-regulates H_2O_2 accumulation in the parasite, and this suppression is rapidly reversed when DMBQ is removed prior to terminal haustorial commitment. As shown in Figure 8, such phase-shifted oscillating concentrations of semagen (H_2O_2)/xenognosin (DMBQ) at the parasite/host interface may well maintain detectable signal concentrations while minimizing the potential for activating host defensive responses. For example, the haustorial inducing xenognosins generated transiently during semagenesis will diffuse from the host root at a rate similar to that measured for the germination stimulants (Fate et al., 1990; Fate and Lynn, 1990). As shown in Figure 8, if the effective concentration of the signal dropped below the level detectable by the parasite prior to commitment to haustorial development, H_2O_2 production would recommence to generate additional signal.

The number and amplitudes of these oscillations shown in Figure 8 are expected to be modulated by the nature of the host/parasite interface, but some limits as to the expected range can be considered. The toxic concentrations of H_2O_2 and DMBQ for *Striga*, estimated at 1 mM and 50 μM, respectively, in our assays, provide upper limits on the amplitudes, *A*. An effective lower limit on *A* would be the lowest active concentration of DMBQ. Remarkably, this active range spans

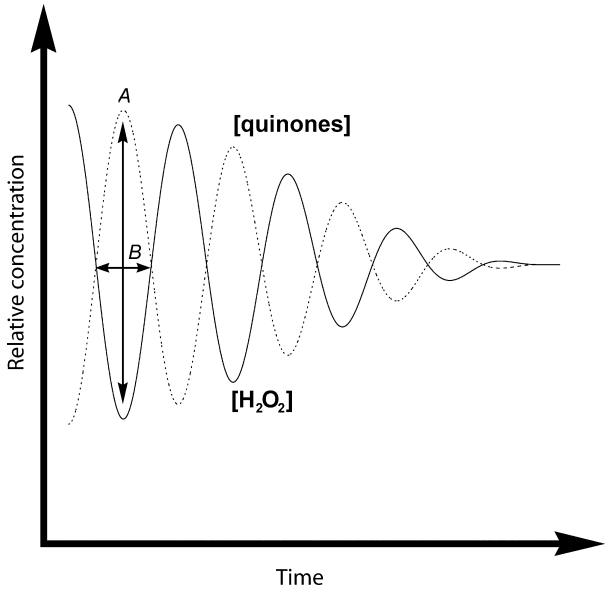

Figure 8. Model for negative feedback of H_2O_2 and xenognostic quinone accumulation at the host/parasite interface.
The reciprocal accumulation of semagenic H_2O_2 and xenognostic quinone is predicted to oscillate with amplitude *A* and a half-period *B*. In this model, the accumulating H_2O_2 (solid line) increases the rate of production of the xenognostic quinones (dashed line). The presence of the xenognosin down-regulates H_2O_2, but, as the xenognosin diffuses away, H_2O_2 accumulation increases. The oscillations are attenuated towards a final steady-state necessary for haustorial commitment.

0.1 to 50 μM (Smith et al., 1990; O'Malley and Lynn, 2000), and the lower concentrations require longer exposure times. These longer times might change the number of 'beats' and/or the oscillation frequency shown in Figure 8. This oscillating frequency, *B*, is expected to be largely defined by both ROS and xenognosin accumulation and diffusion rates. Our studies have now established that the cytoplasmic H_2O_2 concentrations are reversed within 1–2 h, making several damping oscillations possible within the 5 h exposure required for terminal commitment to the DMBQ xenognosin. It is also well established that multiple xenognosin exposures are additive with respect to haustorial commitment (Smith et al., 1990; O'Malley and Lynn, 2000), extending the window to durations much longer than 5 h under oscillating exposures. These data underscore the extraordinary plasticity of both signal concentration and signal exposure time, and the robustness of the semagenesis strategy for accurate temporal and spatial recognition at the host/parasite interface.

In summary, these studies extend our understanding of semagenesis by identifying the localized site of ROS accumulation and correlating ROS regulation with the vegetative/parasitic transition in *Striga asiatica*. Given the general efficacy of the xenognostic benzoquinones in inducing haustorium formation in a number of parasites, the semagenesis strategy could prove to be a general feature in the evolution of parasitic angiosperms. In this model, ROS accumulation by the parasite fulfils a role typically reserved for host-derived ROS during the hypersensitive burst response. H_2O_2, regardless of its origin, is capable of interacting with host-derived phenols and peroxidases to liberate the benzoquinones that *Striga* uses as a cue for the initiation of haustorial development. The functional similarities between host defense and host recognition might well underlie an evolutionary divergence to parasitism, in which the oxidative burst response is hijacked for the purposes of host detection. With obligate parasites such as *Striga asiatica*, the ability to flexibly produce and integrate the xenognostic signal, both as a function of concentration and exposure time, becomes central to a semagenetic strategy, allowing parasitic angiosperms to widely colonize and exploit their brethren.

Experimental procedures

Reagents

All reagents were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com/) except dihydrodichlorofluorescein diacetate (H_2DCF-DA) and fluorescein diacetate (FDA), which were purchased from Molecular Probes (http://www.molecularprobes.com). H_2DCF-DA and FDA were dissolved in dimethylsulfoxide (DMSO) and stored in 100 μl 10 mM aliquots at –20°C. Propidium iodide (PI) was stored as 10 mM stock solutions (in deionized H_2O and 5% DMSO). 2,6-dimethoxybenzoquinone (DMBQ), benzoquinone (BQ), tetrafluorobenzoquinone (TFBQ), methyl-napthoquinone and cerium chloride (C-8016) solutions were prepared as required.

Plant material

Striga asiatica seeds were obtained from Drs R.E. Eplee and R. Norris (US Department of Agriculture, Witchweed Methods Development Laboratory; Oxford, NC, USA), pre-treated and germinated as previously described using 10^{-9} M, strigol (Kim *et al.*, 1998). All work was performed under the auspices of the USDA quarantine licenses awarded to the University of Chicago and Emory University.

Induction experiments

Induction assays required fresh stock solutions of the appropriate inducer (1 or 10 mM) in DMSO. Appropriate aliquots were transferred into wells containing 5 ml 0.1 mM KCl to achieve the final desired concentration. Germinated seedlings (approximately 30 per well) were incubated in these solutions for the desired time and rinsed with three 5 ml washes of 0.1 mM KCl. Seedlings were scored for haustoria after 24 h. All induction assays were performed in triplicate, with error bars representing standard deviation.

Laser confocal scanning microscopy

One-day-old *S. asiatica* seedlings were incubated in a staining solution of either 10 μM H_2DCFH-DA or 10 μM FDA (from aforementioned stock) in 0.1 mM KCl solution for 3 min, washed twice

with 0.1 mM KCl, and then transferred to a custom-made depression cell. The depression slide was constructed by fusing two glass slides together, then drilling a hole approximately 2.5 mm in diameter in the center. This produced a cavity capable of holding two or three seedlings in 30 μl of liquid. The cell was then mounted on a Zeiss LSM 510 laser scanning confocal microscope (http://www.zeiss.com/). The 488 nm line of the Ar laser, set on minimal power and 3% transmittance, was used for excitation, and emission collected in the 500–550 nm window. In order to minimize photobleaching, excitation was limited to approximately 10 sec per sample. Plots of fluorescence distribution across the root tip are derived from five seedlings exposed to either FDA or H_2DCFH-DA.

Transmission electron microscopy

Electron Microscopy Supplies were purchased from Electron Microscopy Sciences (http://www.emsdiasum.com). Cytochemical localization of hydrogen peroxide was carried out according to a procedure based on the production of cerium perhydroxides (Bestwick *et al.*, 1997). *S. asiatica* seedlings 18 h post-germination were incubated in freshly prepared 5 mM $CeCl_3$ in 0.1 mM KCl for 2 h. The seedlings were then fixed in 1.25% v/v glutaraldehyde/1.25% v/v formaldehyde in 50 mM sodium cacodylate buffer (CAB) at pH 7.2 for 1 h. After fixation, the seedlings were washed twice for 10 min in CAB and post-fixed or not, as desired, for 45 min in 1% v/v osmium tetroxide in CAB, followed by two additional washes in CAB (10 min each). The samples were then dehydrated in a graded ethanol series (30, 50, 70, 80, 90% EtOH) with 10 min steps, followed by three changes of 100% dry ethanol and two changes of propylene oxide (PPO), all for 10 min each. The seedlings were then progressively embedded in epoxy plastic (Embed 812; Microscopy Sciences, Inc.), placed in labeled Beem™ capsules, topped off with epoxy plastic and polymerized at 60°C for 48–72 h. Selected blocks were then thin-sectioned (70–90 nm) using a diamond knife, collected onto 200 mesh copper grids or onto Formvar™-covered single-slot copper grids and post-stained or not, as desired. The post-stains used were 3% uranyl acetate and 2% lead citrate. The thin sections were observed with a JEOL JEM-1210 transmission electron microscope at 80 kV. The images were recorded using Kodak™ 4489 EM film (http://www.kodak.com).

Fluorescence microscopy

Fluorescence images were collected on a Leica fluorescence microscope (http://leica-microsystems.com) using an argon lamp source adjusted with the appropriate filter cube. Unless otherwise stated, fluorescence data points are collected in triplicate, with errors bars representing the standard deviation between the images.

PI staining and aluminum toxicity

One-day-old seedlings of *Striga asiatica* and 3-day-old seedlings of *Arabidopsis thaliana* were placed in medium containing 10 μM PI and 0.1 mM KCl for 15 min and transferred to depression slides for scoring, using a blue-light filter cube for excitation. Fluorescence intensities were collected and compared to seedlings previously treated with 10 mM aluminum for 8 h. Aluminum solutions (10 mM) were prepared by slow addition of $AlCl_3$ to H_2O at 4°C to control heat evolution and then allowed to warm to room temperature.

H_2DCFH-DA staining

Sterilized plant seedlings of the indicated age were incubated in 5 ml of staining solution (10 μM H_2DCFH-DA, 0.1 mM KCl and 0.1% DMSO) for 3 min and rinsed three times with 0.1 mM KCl. Seedlings were imaged on a Leica fluorescence microscope in the aforementioned special custom-made depression slide. Excitation with blue light produced fluorescent images collected at set time intervals.

Analysis of fluorescence images

Images were processed using the IDL software package on a Silicon Graphics O_3 desktop (http://www.ittvis.com/idl/). Images were loaded, and the average pixel intensity (PI) at the meristem was determined and corrected for any background fluorescence. To avoid dye translocation, only the initial rate of DCF accumulation was measured (5–15 min). The relative rate of fluorescence accumulation was determined by calculating the ratio of the pixel intensities (RPI) of the entire meristem at 15 and 5 min (RPI = 15/5 min). This method normalizes ROS production to each seedling and the size of each seedling. To determine the effects of each tested compound on DCF fluorescence, the ratios for treated and untreated seedlings were compared and expressed as a percentage of control to create a relative fluorescence (RF) scale ($RPI_{untreated}/RPI_{treated}$) × 100. Each experiment was performed five times, and the results are expressed as means ± SD.

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