Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6986–6991, July 1996
Biochemistry

A mechanism for inducing plant development: The genesis of a specific inhibitor

(induction/redox reaction/*Striga*/plant parasite/haustorial development)

CHRISTOPHER E. SMITH, THOMAS RUTTLEDGE*, ZHAOXIAN ZENG, RONAN C. O'MALLEY, AND DAVID G. LYNN†

Searle Chemistry Laboratory, The University of Chicago, 5735 Ellis Avenue, Chicago, IL 60637

Communicated by Josef Fried, The University of Chicago, Chicago, IL, March 25, 1996 (received for review February 1, 1996)

ABSTRACT **Parasitic strategies are widely distributed in the plant kingdom and frequently involve coupling parasite organogenesis with cues from the host. In *Striga asiatica*, for example, the cues that initiate the development of the host attachment organ, the haustorium, originate in the host and trigger the transition from vegetative to parasitic mode in the root meristem. This system therefore offers a unique opportunity to study the signals and mechanisms that control plant cell morphogenesis. Here we establish that the biological activity of structural analogs of the natural inducer displays a marked dependence on redox potential and suggest the existence of a semiquinone intermediate. Building on chemistry that exploits the energetics of such an intermediate, cyclopropyl-*p*-benzoquinone (CPBQ) is shown to be a specific inhibitor of haustorial development. These data are consistent with a model where haustorial development is initiated by the completion of a redox circuit.**

Plant apical meristems consist of persistent embryonic tissue maintaining root or shoot elongation. All of the information necessary to define plant ontogeny is established within the meristem, a space of just a few millimeters. These embryonic cells also remain acutely responsive to external stimuli; a dramatic example is found in the parasitic plants (1, 2). In *Striga asiatica* (Schrophulariceae), the host attachment organ, haustorium, originates from the cells of the root apical meristem. Generated at the host root surface by *Striga* enzymes, 2,6-dimethoxy-*p*-benzoquinone (DMBQ) is necessary and sufficient to induce the transition from vegetative growth to haustorial development (3, 4). Induction consists of rapid cell cycle arrest, redirection of cellular expansion from longitudinal to radial dimensions, and, ultimately, the formation of haustorial hairs on the periphery of the swelling root tip. Electron micrographs showing the extent of the growth before haustorial attachment are shown in Fig. 1.

Parasitic plants occur in at least 16 families (5); members include the mistletoe, which is responsible for extensive timber destruction in North America, and *Striga spp.*, which are the major limiting agent to grain production in sub-Saharan Africa (2, 6). The common origin and morphology of the haustorium among these individuals as well as the wide distribution in the plant kingdom argue for a common, readily evolved mechanism controlling commitment to this structure. The simple nature of the inducing signal and the speed of the transition from vegetative growth to parasitic mode focused our attention on the mechanism of this developmental induction. Here we present data consistent with the completion of a redox circuit initiating haustorial induction. Exploring predictions made by this model has enabled the development of the first specific irreversible inhibitor of haustorial induction.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Reagents, Seeds, and Methods. Unless otherwise stated, all reagents were obtained from Aldrich. *Sorghum bicolor* seeds were grown and the germination stimulant prepared as previously reported (4). *S. asiatica* seeds, obtained from R. E. Eplee (U. S. Department of Agriculture Witchweed Methods Development Laboratory, Whiteville, NC), were pretreated and germinated as previously described (4). Redox potentials were measured by previously published methods (7) or obtained directly from the authors (P. L. Dutton, R. C. Prince, and K. Warncke, unpublished results). Electron micrographs are provided courtesy of Vance Baird (Clemson University, Clemson, SC).

Syntheses. In most cases, the quinones were prepared by oxidation of the appropriately substituted phenol with Fremy's salt (Aldrich) and the structure was confirmed by complete chemical and spectroscopic assignments. The chloromethylene- and hydroxymethyl-substituted benzoquinones were synthesized by described methods (8). Cyclopropyl-*p*-benzoquinone (CPBQ) was prepared as yellow crystals (9): ^{1}H NMR ($CDCl_3$) δ 6.80 (d, J = 10 Hz, 1 H, H-3) 6.65 (dd, J = 2.4 Hz, 10 Hz, 1 H, H-4), 6.11 (d, J = 2.4 Hz, 1 H, H-6), 2.15–2.11 (m, 1 H, H-1′), 1.08–1.04 (m, 2 H, H-2′ and H-3′), 0.80–0.75 (m, 2 H, H-2′ and H-3′); UV/visible (methanol), λmax (nm) (ε, (M^{-1} cm^{-1})), 250 (10,000), 345 (1, 490); LRMS (EI^+, 70 eV), *m/z* 148 (53%, M^+), 120 (80%, M - CO), 105 (21%), 91 (100%, $C_7H_7^+$); HRMS calculated for $C_9H_8O_2$ 148.0524, found 148.0507; Anal calculated for $C_9H_8O_2$: C, 72.96; H, 5.44. Found: C, 72.23; H, 5.56.

Synthesis of Oxirane-*p*-benzoquinone. 2,5-Dimethoxybenzaldehyde (554 mg, 3.34 mmol) and trimethylsulfonium methyl sulfate [$(CH_3)_3S^+CH_3SO_4^-$, 755 mg, 4.01 mmol] were combined in 1.68 ml 50% NaOH and 3.36 ml $CH2Cl_2$. After 3 hr, the reaction mixture was washed two times each with 20 ml of water and 20 ml of saturated NaCl; the organic layer was dried over $MgSO_4$ and the solvent was removed *in vacuo*. The residue was then dissolved in wet CH_3CN with excess Ag(I-I)dipicolinate (1.5 g, 10.1 mmol) and, after 30 min, the reaction was diluted with ether and the layers were separated and filtered. The aqueous layer was extracted three times with 20 ml of ether and the solvent was evaporated *in vacuo*. Chromatography through SiO_2 (10% ethyl acetate in hexane) afforded 2-oxirane-*p*-benzoquinone. ^{1}H NMR ($CDCl_3$) δ 2.65, 2.66 (dd, 1H, cis oxirane, J = 2.4 Hz, 5.9 Hz), 3.19, 3.20 (dd, 1H, trans oxirane, J = 4.3 Hz, 6 Hz), 3.96 (m, 1H, benzylic), 6.62 (d, 1H, H-3, J = 1.6 Hz), 6.75, 6.77 (dd, 1H, H-5, J = 2.37, 10 Hz), 6.82 (d, 1H, H-6, J = 10 Hz).

Induction Assays. As previously described (4), germinated 2-day old *Striga* seedlings (30–50) were placed in each of 24

Abbreviations: CPBQ, cyclopropyl-*p*-benzoquinone; DMBQ, 2,6-dimethoxy-*p*-benzoquinone.

*Present address: Department of Chemistry, Earlham College, Richmond, IN 47374.
†To whom reprint requests should be addressed. e-mail: d-lynn@uchicago.edu

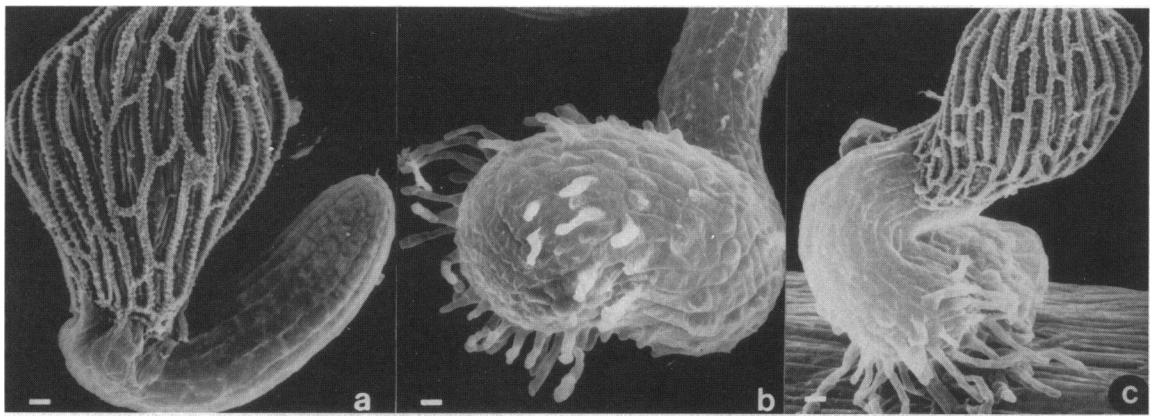


FIG. 1. Electron micrographs of the early developmental stages of *S. asiatica*. (*a*) Radicle, 12 hr postgermination; radicle growth is sustained in the absence of a haustorial stimulus from the host root. (*b*) Primary haustorium at 24 hr postgermination induced by host stimulant. (*c*) Three-day-old haustorium attached to a corn root. (*a* and *b*, bar = 20 μm; *c*, bar = 15 μm.)

culture wells of a microtiter plate and cultured with the inducers (10^{-4} to 10^{-9} M) for 24 hr before being counted with a dissecting scope. Inhibitors were added with inducers or at various points before and after the induction as specified. Each assay was done in triplicate and expressed as ± SD.

RESULTS

Structural Analyses. Significant evidence that the xenognostic quinone is oxidatively released from the host cell wall by *S. asiatica* enzymes has been presented (3). From cell wall compositional analyses (10), benzoquinone **16**, methoxybenzoquinone **13**, and 2,6-dimethoxybenzoquinone **12** are possible products, and these show ED_{50} for induction of 6.3×10^{-11} M, 1.6×10^{-8} M, and 6.3×10^{-7} M, respectively. The addition of substituents to the quinone reduced biological activity, which is consistent with this general trend. Of the other dimethoxy regioisomers **11** and **15** and the dihydroxy compound **18**, only regioisomer **15** showed high activity. Alkyl substituents were less tolerated; dihydroxy naphthaquinone **10** showed weak activity but all other dialkyl structures, e.g., **3**, **20**, **21**, **22** and **23**, and trisubstituted benzoquinones investigated were inactive. A similar preference for methoxy substituents in receptor agonists has been reported for *vir* gene induction in Agrobacterium (11), but a reason for this preference is not yet available.

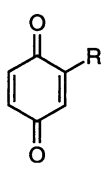
Correlation with Redox Potential. Whereas quinone analogs with a remarkably wide range of first half-wave reductive potentials were evaluated, the active analogs fall in a narrowly defined window between −280 and +20 mV (Fig. 2). At both ends of this range, compounds **10**, **11**, **17**, and **18**, only partial induction was possible. Variations in the structure of the monoalkyl substituent generally showed little effect on the redox potential or the biological activity. Methyl-, ethyl-, propyl-, isopropyl-, butyl-, and isobutylbenzoquinone showed half-maximal inducing activity that ranged between 1 and 5×10^{-9} M and their redox potentials fall within the window. The biphenyl compound **17** has a redox potential that was shifted to the edge of the window and only showed partial activity. Therefore, redox potential appears to be a critical indicator of inducing activity.

Structural analogs distributed at the extremes of the redox window were further examined. Three close structural analogs not reduced within this window, hydroquinone **29**, 2,6-dimethoxyhydroquinone **30**, and cyclohexanedione **31**, are not inducers, nor do they block induction by DMBQ (data not shown). The inactivity of the hydroquinones suggests that the two-electron reduced product is not responsible for the induction but rather that it is the process of reduction that is important.

The strongly electropositive tetrafluorobenzoquinone **8** is predicted to be easily reduced but not re-oxidized within the limits of the window. This quinone is not an inducer, but reversibly inhibits induction by 10^{-5} M DMBQ half-maximally at 10^{-7} M (data not shown). It is possible that tetrafluorobenzoquinone **8** is sterically accommodated in the site, reduced to the semiquinone, and, therefore, binds competitively with inducer. Induction would then require exposure to a compound capable of both one-electron reduction and re-oxidation. The one-electron carrying quinones of both the oxidative phosphorylation and photosynthetic electron transport chains show similar characteristics (12).

Exploiting the Semiquinone Intermediate. The extended exposure time necessary for induction (4) suggests that a semiquinone intermediate must either be repeatedly generated or have an extended lifetime. Two independent strategies were developed to harness the potential energy of this intermediate. The first strategy was built on previous observations that several antitumor antibiotics unmask specific rearrangements after reduction (13). For example, the aziridine ring of mitomycin C preferentially opens through the semiquinone and the products of this rearrangement are readily detected (14, 15). Simple benzoquinones with benzylic leaving groups also give first order decomposition rates from the semiquinone (8) and several such leaving groups, alcohol **25** and acetate **26** specifically, are compatible with the structural requirements of the inducers. Both of these compounds are good inducers, having ED_{50}s comparable with the other monoalkyl quinones, and are not likely to be structurally altered during the induction process. The previous studies (8) found specific pH and substituent requirements for the rearrangement and these may not be accommodated within this receptor site.

Attempts to increase the rearrangement potential led to the preparation of the chloride **27** and the oxirane **28**, but neither compound was stable under the assay conditions. The chloride **27** was clearly toxic to the seedlings; however, the oxirane **28**, most analogous to the mitomycin aziridine, inhibited induction half-maximally when added at 10^{-6} M. The oxirane's instability, as illustrated by its complete hydrolysis under the assay conditions within 24 hr, severely complicated the *in vivo* analyses. Clearly in the mitomycins, the unmasking is greatly attenuated by insulation of the aziridine from the quinone by the intervening animal.



R = CH_3, **14**
R = CH_2OH, **25**
R = CH_2OCOCH_3, **26**
R = CH_2Cl, **27**

, **28**

, CPBQ

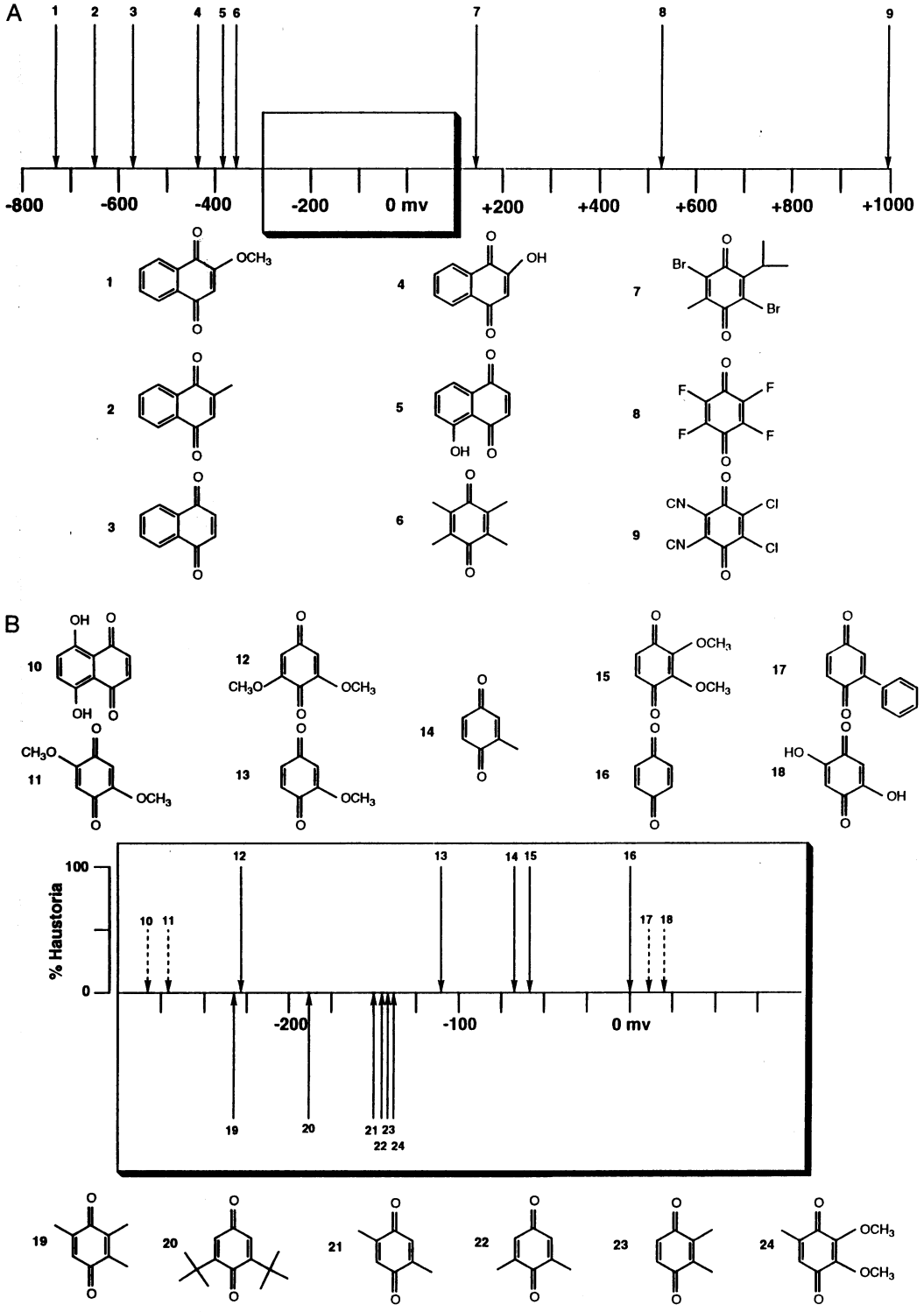


FIG. 2. (*A*) Full redox window tested by structural analogs. (*B*) Expansion of the redox range containing the active haustorial inducers (**10-18**) plotted above the redox scale. The half-wave reductive potential of the quinones was measured with a saturated Calomel reference electrode and the relative potentials are compared with that of benzoquinone, which was set at zero (7). The abscissa represents the haustorial induction percentage referenced to DMBQ at the maximal inducing concentration; the solid arrows indicate 100% of control that was >90% with DMBQ and dashed arrows partial haustoria induction that was between 40 and 50%.

The second strategy builds upon the known reactions of the ketyl anions of aryl cyclopropyl ketones. These electrochemically generated intermediates delocalize radical character into the cyclopropane and exist in the ring-opened form long enough to undergo isomerization and radical recombination reactions (16). The semiquinone of CPBQ was found to undergo similar reactions. Whereas CPBQ was stable to the assay conditions, it has now been shown to undergo reversible ring-opening through the semiquinone (9) and to exist as the ring-opened intermediate at least long enough for bond isomerization. *In vivo*, CPBQ proved to be a potent and specific inhibitor of the induction process. Fig. 3*A* shows the concentration dependence for DMBQ induction and Fig. 3*B* shows the concentration dependence for the inhibition of 10^{-5} M

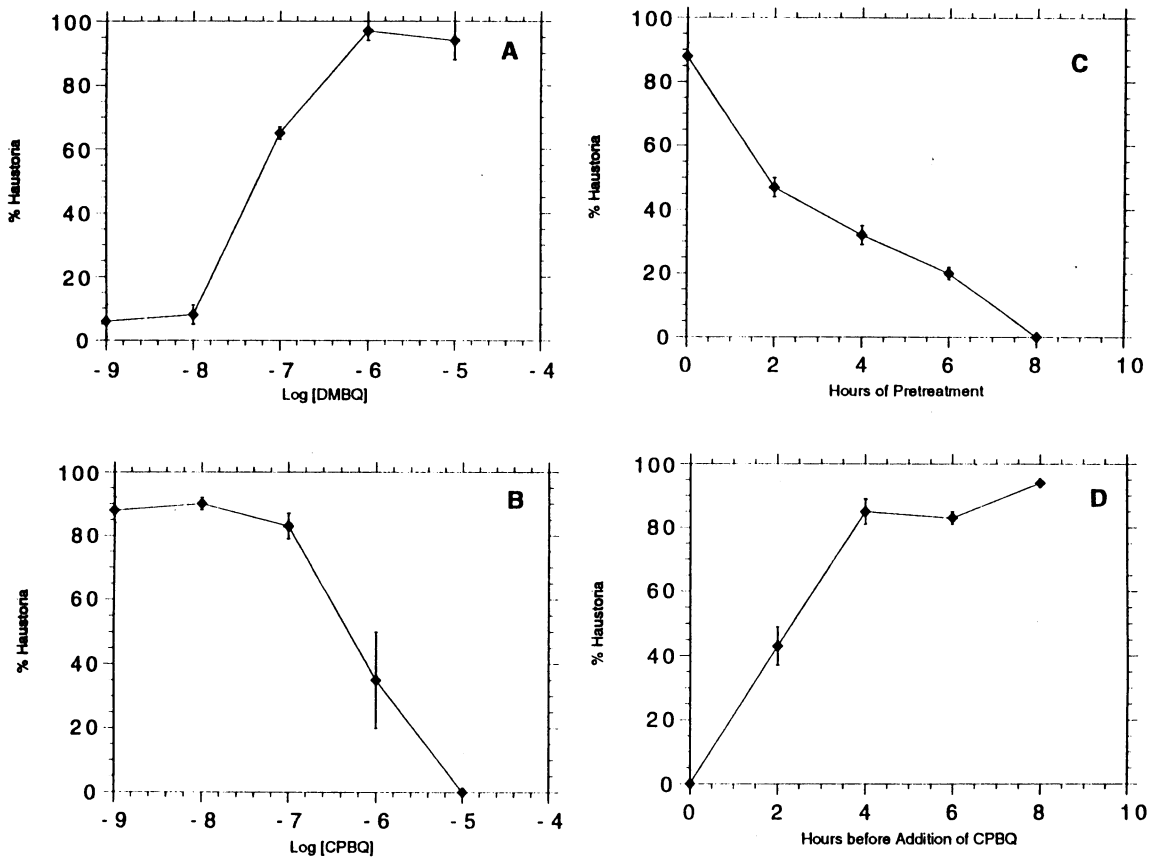


FIG. 3. Induction and inhibition of haustorial development. Germinated 2-day-old *S. asiatica* seedlings were cultured for 24 hr at 24°C with DMBQ from 10^{-4} to 10^{-9} M (*A*) and 10^{-5} M DMBQ and CPBQ from 10^{-4} to 10^{-9} M (*B*). (*C* and *D*) The effects of inhibitor treatment before (*C*) and after (*D*) haustorial induction. (*C*) The seedlings were incubated with CPBQ (10^{-5}M) for the indicated time before the inhibitor was removed by washing (3×) and further incubation with 10^{-5}M DMBQ. (*D*) A solution containing 10^{-5}M DMBQ was applied to the seedlings at time zero and a stock solution of CPBQ was added directly to the well to give a final concentration of 10^{-5}M at the indicated times. In all experiments, the % haustoria were counted with a dissecting scope and the results expressed as ± SD of triplicates.

DMBQ induction by CPBQ. The reduced product, cyclopropyl-*p*-hydroquinone, did not inhibit DMBQ induction consistent with the lack of inducing activity of the hydroquinones.

The inhibition by CPBQ is not immediate as shown in Fig. 3*C*. Haustorial induction requires an exposure to DMBQ of ≥ 6 hr (4) and CPBQ must be present throughout this induction period to completely block development. The resulting inhibition is irreversible; the washing experiments that effectively removed DMBQ (4) did not reverse the effect of the inhibitor. Only after 2 days of further growth, a time frame consistent with the appearance of new responsive cells in the meristem, could haustorial growth be initiated. A further test for the effects of CPBQ on the initiation event is shown in Fig. 3*D*. If DMBQ is added for ≥ 4 hr before the addition of CPBQ, normal growth and differentiation of the haustorium occur. Therefore, CPBQ is specific for the induction process (i.e., does not affect the metabolic processes contributing to growth of the haustoria) and it blocks induction irreversibly.

DISCUSSION

In recent years, the morphogenetic signals that regulate the developmental fate of various embryonic tissues have been discovered (17–20), yet the mechanisms that control these changes have remained obscure. In *Striga*, a structurally simple quinone signal arises from the host to induce one of the fastest organogenic events known, a terminal differentiation of the embryonic meristem into the host attachment organ (4). Here we show that a range of structures are capable of haustorial induction and that the biological activity correlates with signal redox potential. Structure activity analyses suggest a model that requires both reduction and reoxidation of the quinone signal for induction.

The existence of a long-lived semiquinone lies at the core of this model and the attempts to legitimize its intermediacy led to the discovery of CPBQ as a specific inhibitor of induction. Based on the chemical reactivity of this compound (9), three pathways for inhibition can be proposed (Fig. 4). First, quinone protonation activates the cyclopropane for nucleophilic attack, as in pathway A, and CPBQ derivatives accept two nucleophile equivalents (9). This pathway is neither dependent on redox events nor does it occur under reducing conditions (e.g., BF_3·MeOH/$NaBH_3CN$); however, it provides a possible mechanism for inactivation.

Second, the cyclopropane should become more basic after quinone reduction and a reaction through pathway B would be most analogous to that seen for mitomycin (14, 15). However, both the alcohol **25** and the acetate **26** contain substituents more basic than the cyclopropane and both are potent inducers, not part of the inhibition pathway. At this point, it is unlikely that pathway B explains the inhibition.

Third, the cyclopropane of CPBQ does open following reduction as in pathway C (9). Based on the chemistry of related radical anions (16), either radical recombination in the redox binding site (21, 22) or nucleophilic addition, as in pathway A and B, provides the most consistent model for this inactivation. Each of these pathways predicts a distinctly different product distribution that can be used to evaluate these mechanisms *in vitro*. CPBQ will aid both in purification of the receptor protein and in defining the reactions that are specific to the receptor site.

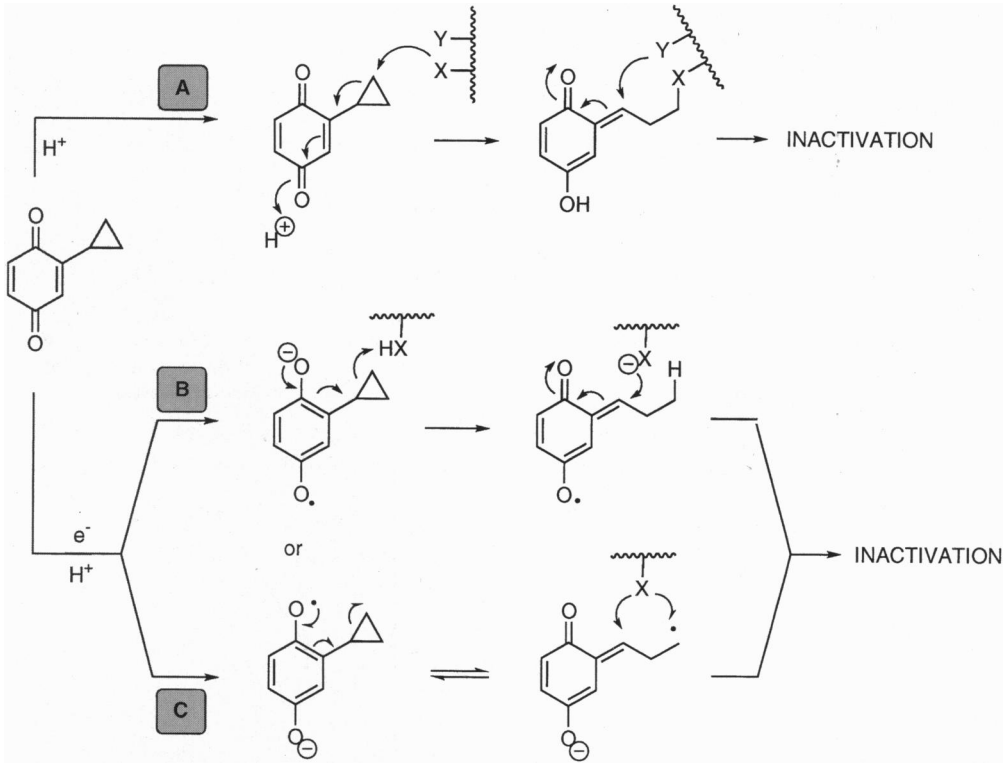


FIG. 4. Three proposed pathways for the irreversible inactivation of haustorial development. (*A*) Receptor surface residues, X and Y, add to the cyclopropane following quinone protonation. (*B*) The semiquinone methide is generated through cyclopropane rearrangememt. (*C*) The radical character of the semiquinone induces ring-opening and either radical recombination or nucleophilic addition leads to inactivation.

An association between redox events and cellular development has been recognized for many years (23), yet neither the initiating events nor the mechanisms have been defined. Now transcriptional factors from viral, bacterial, and mammalian sources are known to be under redox control (24–29) and in plants, chloroplast genome expression (30–32) is regulated by redox reactions. The influence of membrane potential on voltage-gated Ca^{2+} channels is also well known in many plant cells (33), particularly with regard to cell growth. For example, the cytosolic [Ca^{2+}] is central to the control of guard cell swelling (34, 35) and pollen tube growth (36) and such swelling events are central to haustorial formation. Access to this class of irreversible inhibitors should make it possible to localize the primary site of quinone induction of organogenesis or at least act as a first step to differentiate between specific transcription factors or a more global control of membrane potential.

This work is dedicated to Koji Nakanishi of Columbia University who is in his 71st year. We thank the Department of Energy (Grant DE-FG02–91ER20024.A002) for financial support of this work.

1. Kuijt, J. (1969) *The Biology of Parasitic Flowering Plants* (Univ. California Press, Berkeley).
2. Press, M. C. & Graves, J. D., eds. (1995) *Parasitic Plants* (Chapman & Hall, London).
3. Lynn, D. G. & Chang, M., (1990) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **41,** 497–526.
4. Smith, C. E., Dudley, M. W. & Lynn, D. G. (1990) *Plant Physiol.* **93,** 208–215.
5. Molau, U. (1995) in *Parasitic Plants*, eds. Press, M. C. & Graves, J. D. (Chapman & Hall, London), pp. 141–176.
6. Nour, J., Press, M., Stewart, G. & Tuohy, J. (1986) *New Sci.* **9,** 44–48.
7. Prince, R. C., Lloyd-Williams, P., Bruce, J. M. & Dutton, P. L. (1986) *Methods Enzymol.* **125,** 109–119.
8. O'Shea, K. E. & Fox, M. A. (1991) *J. Am. Chem. Soc.* **113,** 611–615.
9. Zeng, Z., Cartwright, C. H. & Lynn, D. G. (1996) *J. Am. Chem. Soc.* **118,** 1233–1234.
10. Siquiera, J. O., Nair, M. G., Hammerschmidt, R. & Safir, G. R. (1991) *Crit. Rev. Plant Sci.* **10,** 63–121.
11. Duban, M. E., Lee, K. & Lynn, D. G. (1993) *Mol. Microbiol.* **7,** 637–645.
12. Gunner, M. R., Braun, B. S., Bruce, J. M. & Dutton, P. L. (1985) in *Antennas and Reaction Centers of Photosynthetic Bacteria,* Chemical Physics Series No. 42, ed. Michel-Beyerle, M. E. (Springer, Berlin), pp. 298–304.
13. Moore, H. W. & Czerniak, R. (1981) *Med. Res. Rev.* **1,** 249–280.
14. Danishefsky, S. J. & Egbertson, M. (1986) *J. Am. Chem. Soc.* **108,** 4648–4650.
15. Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G. L. & Nakanishi, K. (1987) *Science* **235,** 1204–1208.
16. Tanko, J. M. & Drumright, R. E. (1990) *J. Am. Chem. Soc.* **112,** 5362–5363.
17. Thaller, C. & Eichele, G. (1987) *Nature (London)* **327,** 625–628.
18. Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendriks, H. F. J., de Vries, N. J., Heideveld, M. & Nieuwkoop, P. D. (1989) *Nature (London)* **340,** 140–144.
19. Morris, H. R., Taylor, G. W., Maasento, M. S., Jermyn, K. A. & Kay, R. R. (1987) *Nature (London)* **328,** 811–814.
20. Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J. C. & Dénaríc, J. (1990) *Nature (London)* **344,** 781–784.
21. Silverman, R. B. & Zieske, P. A. (1985) *Biochemistry* **24,** 2128–2138.
22. Vazquez, M. L. & Silverman, R. B. (1985) *Biochemistry* **24,** 6538–6543.
23. Crane, F. L., Morre, D. J. & Low, H., eds. (1988) *Plasma Membrane Oxidoreductases in Control of Animal and Plant Growth* (Plenum, New York), Vol. 157.
24. Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, H., Hamuro, J., Brown, N., Arai, K.-I., Yokota, T., Wakasugi, H. & Yodoi, I. (1989) *EMBO J.* **8,** 757–764.
25. Abate, C., Patel, L., Rauscher, F. J., III & Curran, T. (1990) *Science* **249,** 1157–1161.
26. Storz, G., Tartaglia, L. A. & Ames, B. N. (1990) *Science* **248,** 189–194.

27. Staal, F. J. T., Roederer, M., Herzenberg, L. A. & Herzenberg, L. A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9943–9947.
28. McBride, A. A., Klausner, R. D. & Howley, P. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7531–7535.
29. Allen, J. F. (1993) *FEBS Lett.* **332,** 203–207.
30. Buchanan, B. B. (1991) *Arch. Biochem. Biophys.* **288,** 1–9.
31. Danon, A. & Mayfield, S. P. (1994) *Science* **266,** 1717–1719.
32. Allen, J. F. (1994) *Photosynth. Res.* **36,** 95–102.
33. Bush, D. S. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 95–122.
34. McAinsh, M. R., Webb, A. A. R., Taylor, J. E. & Hetherington, A. M. (1995) *Plant Cell.* **7,** 1207–1219.
35. Kinoshita, T., Nishimura, M. & Shimazaki, K. (1995) *Plant Cell.* **7,** 1333–1342.
36. Malhó, R., Read, N. D., Trewavas, A. J. & Pais, M. S. (1995) *Plant Cell.* **7,** 1173–1184.