At the maize/*Agrobacterium* interface: natural factors limiting host transformation

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Background: *Agrobacterium tumefaciens* has been successfully harnessed as the only natural vector for the incorporation of foreign genes into higher plants, but its use in the grain crops is often limited. Low transformation efficiency has been partly attributed to a failure in the initial events in the transformation process, specifically in the capacity of the VirA/VirG two-component system to induce expression of the virulence genes.

Results: Here we show that the root exudate of *Zea mays* seedlings specifically inhibits virulence gene expression, determine that 2-hydroxy-4,7-dimethoxybenzoxazin-3-one (MDIBOA), which constitutes $>98\%$ of the organic exudate of the roots of these seedlings, is the most potent and specific inhibitor of signal perception in *A. tumefaciens*-mediated gene transfer yet discovered, and develop a model that is able to predict the MDIBOA concentration at any distance from the root surface. Finally, variants of *A. tumefaciens* resistant to MDIBOA-mediated inhibition of *vir* gene expression have been selected and partially characterized.

Conclusions: These results suggest a strategy in which a plant may resist pathogen invasion by specifically blocking virulence gene activation and yet ensure that the 'resistance factor' does not accumulate to levels sufficient to impose toxicity and selection pressure on the pathogen. The data further establish that naturally occurring inhibitors directed against signal perception by the VirA/VirG two-component regulatory system can play an important role in host defense. Finally, selected variants resistant to specific MDIBOA inhibition may now be used to extend the transformation efficiency of maize and possibly other cereals.

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Keywords: *Agrobacterium tumefaciens*; Maize; Transformation

Received: **15 February 2000**
Revisions requested: **29 March 2000**
Revisions received: **19 May 2000**
Accepted: **26 May 2000**

Published: **1 August 2000**

Chemistry & Biology 2000, **7**:611–621

1074-5521/00/$ – see front matter
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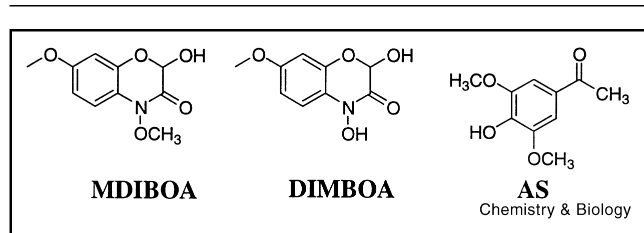
PII: S1074-5521(00)00007-7

Introduction

Agrobacterium tumefaciens is the causative agent of crown gall disease in plants and remains the only known natural vector for inter-kingdom gene transfer [1–3]. The biotechnological importance of the unique DNA transfer process and the efforts directed at its optimization have resulted in *Agrobacterium* becoming a model pathogenic system [4–6]. The genetic elements required for the production of the transferred DNA intermediate, and the membrane-bound DNA transfer elements are encoded within the virulence (*vir*) regulon of the resident tumor-inducing (Ti) plasmid. The expression of the *vir* regulon is controlled by *virA* and *virG*, two genes homologous to 'two-component' regulatory systems utilized by seemingly all bacteria to mediate responses to environmental stimuli [7,8]. VirA serves as the membrane-localized histidine autokinase transmitter and VirG as the response regulator. When the appropriate xenognosins (host recognition factors) accumulate at the infection site they are perceived, either directly or indirectly, by VirA which then phosphorylates VirG. Phosphorylated VirG binds to specific regions of the *vir* promoters and induces the expression of all the *vir* genes, including *virA* and *virG*.

The Ti-encoded DNA transfer elements have been productively harnessed as a natural vector for the incorporation of foreign genes into higher plants. Maize and, in fact, most cereal crops, are more resistant to transformation, seriously limiting the biotechnological use of this vector [9,10]. Repeated approaches to overcome this resistance have identified signal-induced expression of the gene transfer elements as limiting in maize transformation [3,11–13], and the seedling root as the most recalcitrant tissue [14]. Over-expression of multiple *vir* genes in *trans* and/or co-cultivation in the presence of high levels of the xenognosin, acetosyringone (AS, Scheme 1), has resulted in an increase in maize transformation efficiency, particularly when embryogenic cultures are utilized [15,16].

The inability of maize root tissues to be transformed by *Agrobacterium* suggests that at least one of several critical steps in the transformation process is blocked. In this



Scheme 1. Structures of MDIBOA, DIMBOA and AS.

study, we show that the maize seedlings specifically inhibit *vir* expression within a well-defined zone proximal to the root surface. 2-Hydroxy-4,7-dimethoxybenzoxazin-3-one (MDIBOA) is shown to be a critical, but unstable, constituent of maize seedling exudate, and to be the most potent and specific inhibitor known for the VirA-mediated induction of *vir* gene expression. In addition, mutant strains of *Agrobacterium* possessing significant resistance to inhibition of *vir* gene expression by MDIBOA have been isolated. MDIBOA is the first naturally occurring inhibitor of a histidine kinase shown to block xenognosin perception rather than kinase activity and, as such, serves as a model for alternative strategies in the specific disruption of two-component systems involved in the regulation of pathogenesis [17].

Results

Co-cultivation

To determine whether maize roots retarded *vir* expression in a physiologically significant manner, *A. tumefaciens* strain 348 carrying a *virB::lacZ* reporter construct in the kanamycin resistant pSW209 plasmid was diluted into soft agar and layered across an agar plate containing 10 μM AS and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), a substrate for β-galactosidase that, when hydrolyzed, yields the blue indolyl aglycone. Sterile 5-day-old maize seedlings were positioned on the plates under sterile conditions and incubated for 3 days at room temperature. During this time many lateral roots were formed, and those that grew into the top agar, came in contact with the bacterial suspension. While colonies growing throughout the plate expressed β-galactosidase, those located close to the root surface displayed a significant reduction in the accumulating blue coloration (Figure 1, arrows). These experiments established that *A. tumefaciens* was not restricted from freely colonizing the maize root surface, but that β-galactosidase expression was reduced in those proximal colonies. At lon-

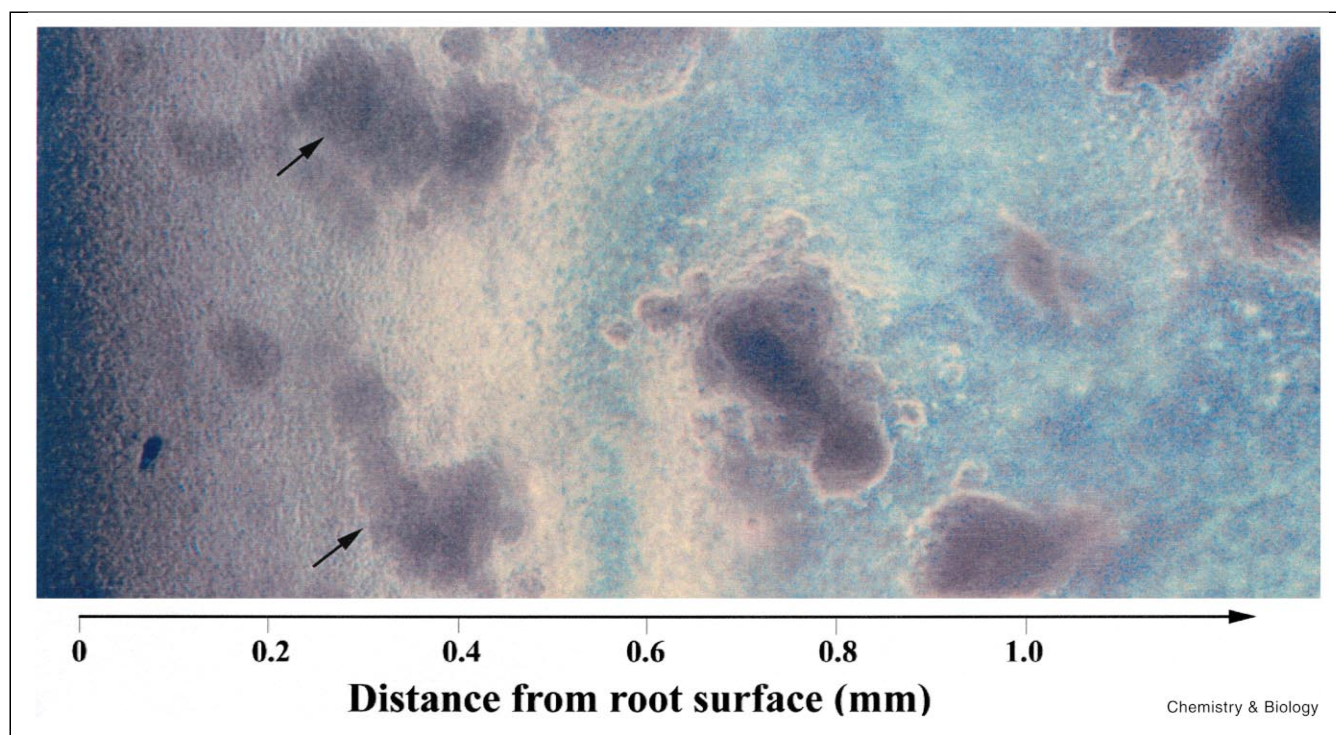


Figure 1. Co-cultivation of maize seedling with A348/pSW209. Photograph of 8-day-old maize seedling after growing on the agar plate containing 10 μM AS, 80 μg/ml X-gal, and 50 μg/ml kanamycin for the final 3 days. This magnification (122.5×) shows the surface of the maize root along the left border and some of the individual colonies indicated by the arrows. After several additional days the blue color shown towards the right side of the plate spreads to the entire area. The blue background did not appear in control plates containing no bacteria.

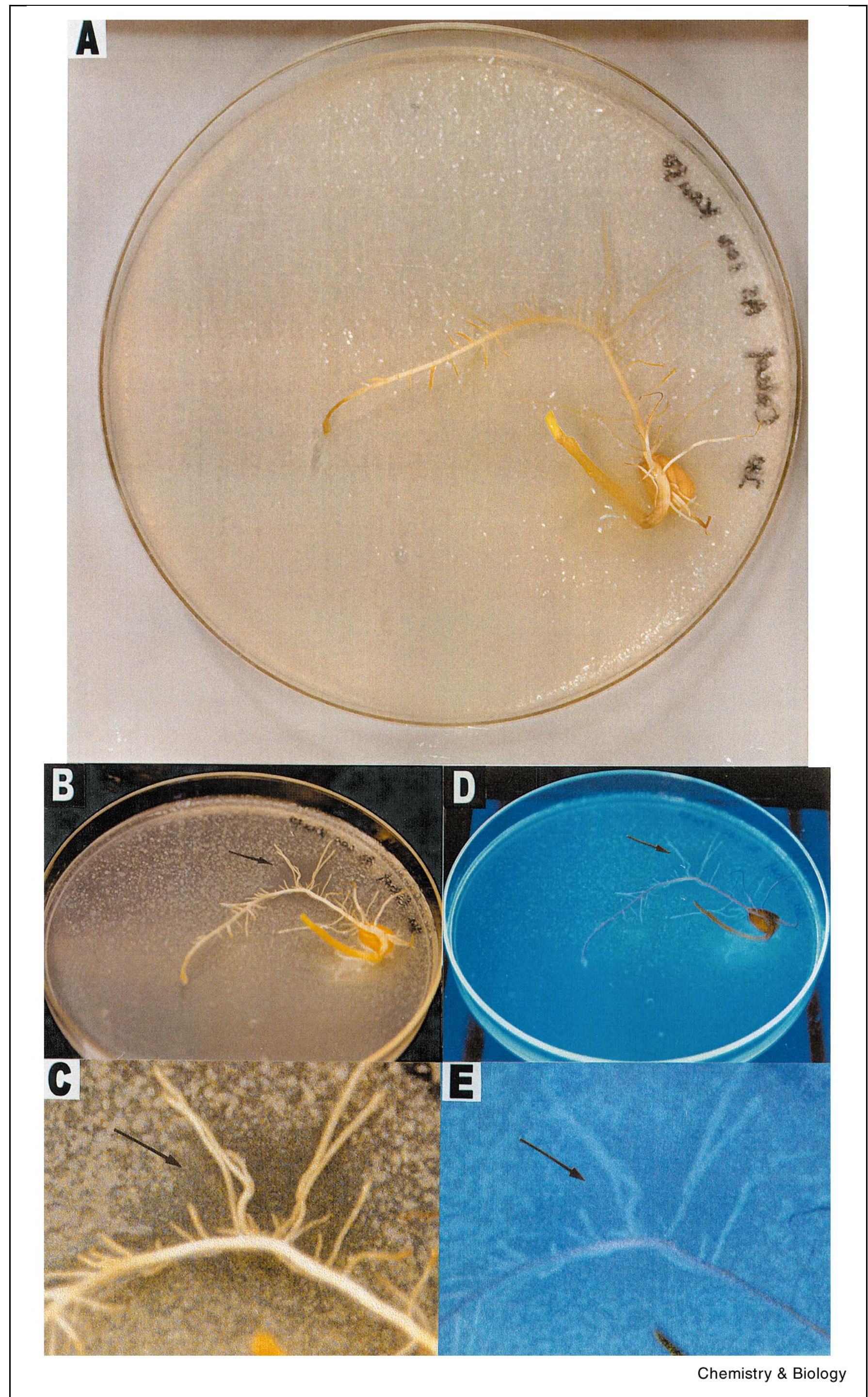


Figure 2. Co-cultivation of maize seedling with A348/pAC2. **(A)** Photograph of 10-day-old maize seedling after growing on the agar plate containing 100 μM AS, 0.2 μg/ml Calcofluor (1 μg/ml Calcofluor in top agar), and 50 μg/ml kanamycin for the final 5 days. **(B,C)** Expansion of the roots embedded in the agar with the arrow indicating the zone of depleted bacterial colonies, plated at 10^5 CFU/ml in the top agar. **(D,E)** UV illumination of the same expanded region (arrow) highlighting both the maize roots that were embedded in the agar and the bacterial colonies.

ger culture times, the blue hydrolysis product did accumulate around the more distant blue colonies in the plate due to diffusion of the hydrolysis product (Figure 1, background). Nevertheless, an inhibition zone could be visualized around the roots growing within the top agar.

In an alternate approach, *A. tumefaciens* strain A348/pAC2, carrying the P*virB*/*npt*II fusion [18], was distributed on induction plates containing AS (100 μM) and kanamycin (50 μg/ml). This strain survives on these plates in the presence of 100 μM AS, but die if <30 μM AS is present, whereas vector controls died at all AS concentrations.

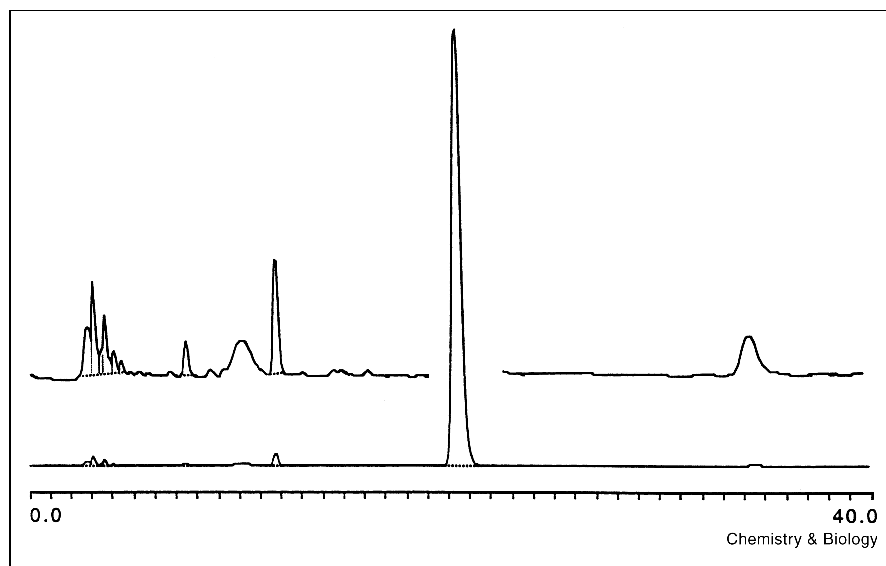


Figure 3. Maize root exudate. Exudate was collected by dipping 7-day-old maize seedlings in CH_2Cl_2 containing 0.5% HOAc and analyzed by HPLC (Microsorb SiO_2, cyclohexane/ethyl acetate (67:33 v/v)). Insert contains expansions of the minor components present in the exudate. Detection at 254 nm.

Thus, kanamycin resistant growth of this strain requires the activity of the VirA/VirG system. After 5 days of co-incubation, bacterial growth in regions proximal to the newly growing lateral roots was reduced (Figure 2), and a clearly defined zone was detected around those roots growing into the agar. The inhibited growth was not the result of general toxicity to A348 in that the experiments using A348/pSW209 established that this strain freely colonized the maize root surface (Figure 1). The inability of A348/pAC2 to grow must be due either to the depletion of AS from the media and/or to a factor released from the roots that specifically blocks AS induction.

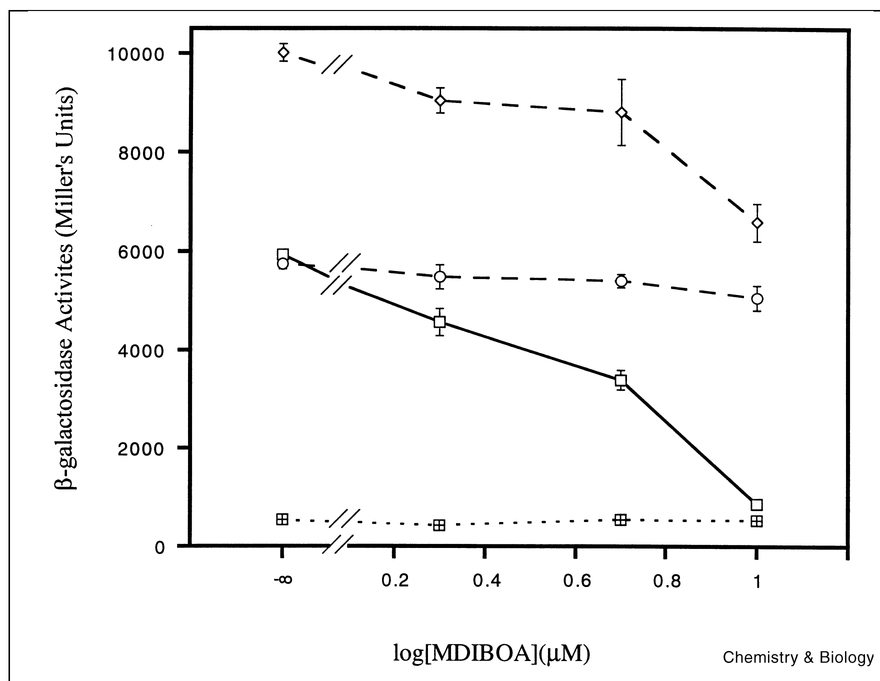
Exudate analysis

Freshly collected exudate from sterile-grown 9-day-old maize seedlings both stimulated cell growth and strongly inhibited AS-induced *vir* gene expression. However, the inhibitory activity was extremely labile, even to standing at room temperature (data not shown). Following a previous protocol [19–21], the organic exudate was collected in CH_2Cl_2 containing 0.5% HOAc and by RP-HPLC found to consist essentially of a single compound, $>98\%$ of the detected components (Figure 3). ^1H NMR (acetone-d_6) of this material was also clean, showing two methyl singlets, δ 3.78 (3H) and 3.91 (3H), a 1,2,4-substituted aromatic nucleus, δ 7.16, 1H, d, $J=8.8$ Hz, δ 6.68, 1H, d, $J=2.5$ Hz, δ 6.70, 1H, dd, $J=2.5$, 8.8 Hz, and a broad singlet at δ 5.4 (1H). The IR ($CHCl_3$) spectrum contained a carbonyl stretch at ν 1686 cm^{-1} and a broad OH stretch at ν 2958 cm^{-1}. MS (EI, 70 eV) gave a possible molecular ion at m/z 225 which was confirmed both by the M+H ion, m/z 226, obtained by CI (isobutane) analysis and by acetylation (Ac_2O, pyridine, 0°C) giving a monoacetate, CI (isobutane) m/z 268 (M+H). Exact mass measurements on the molecular ion established a molecular formula of $C_{10}H_{11}NO_5$. Taken together, these data were most consistent with an aromatic ring fused to a six-membered lactam. Placement of the additional methoxy group on the N atom suggested MDIBOA as the structure. Final proof was obtained via diazomethane methylation of 2,7-dihydroxy-4-dimethoxybenzoxazin-3-one (DIMBOA), isolated from whole root extracts of the same maize seedlings [22]. Several benzoxazinones are known to be present in grasses, including the 2-*O*-β-glycoside of MDIBOA first identified in maize roots [23]. MDIBOA proved to be hydrolytically very labile, giving a $t_{1/2} < 4$ h at pH 5.5, and <10 min at pH 7, with the major product being 6-methoxy-2*H*-benzoxalin-2(3*H*)-one (MBOA) based on full spectroscopic analyses [22]. This hydrolytic instability prevented the free aglycone from being isolated and conclusively identified either in the aqueous exudate or with the previously employed extraction conditions [24–26].

Inhibition of *vir* gene expression

Both the organic exudate and the synthesized MDIBOA displayed the same dose-dependent inhibition of *virB::lacZ* gene expression. The potency was evaluated directly in strain A348-3 (Δ*virA*) carrying a wild type version of *virA* (pAB123-22) and a *virB::lacZ* reporter (pSW209Ω). MDIBOA strongly inhibited *vir* gene expression induced by 100 μM AS (Figure 4). Inhibitor specificity is evaluated in two ways. First, the inhibition is shown to be significantly rescued by high AS concentrations, 1 mM in Figure 4. Second, tests using strain A348-3 (pMutAG665D, pSW209Ω), capable of constitutive vir gene expression in the absence of AS [27], demonstrate that *vir* gene expression is unaffected by concentrations of MDIBOA that completely inhibit such expression in the wild type strain. Therefore, the MDIBOA-mediated inhibition of *vir* gene

Figure 4. Inhibition of *vir* expression by MDIBOA. β-Galactosidase induced by 1 mM AS (◇), 100 μM AS (□) and without AS (⊞) in A348-3/pAB123-22, pSW209Ω and without AS in A348-3/pMutA G665D, pSW209Ω (○).



expression occurs upstream of VirA activation and phosphorylation of VirG, most probably at the site of phenol perception.

Diffusion–reaction model

The demonstrated hydrolytic lability of MDIBOA suggests that the compound must be produced continuously along the root axis of the maize seedling to create the zone shown in Figure 2. Moreover, the zone was found to be stable over several days, a result attributable to the attainment of a steady state influx, the rate of diffusion from the root surface, and efflux, the rate of hydrolytic decay. To the extent that MDIBOA accounts for all the observed inhibition, the result of these two competing rates at steady state would generate a limiting boundary for both constructs at the MDIBOA IC_{50} in agar.

To further evaluate this hypothesis, the diffusion from the seedling root in the 1 cm thick agar plate was modeled as radial diffusion from a cylinder. As the rates of diffusion and decomposition are equal at steady state, the following expression can be written [21,28].

$$D\frac{\mathrm{d}^2[\mathrm{MDIBOA}]}{\mathrm{d}r^2}+\frac{D}{r}\frac{\mathrm{d}[\mathrm{MDIBOA}]}{\mathrm{d}r}-k[\mathrm{MDIBOA}]=0$$

D is the diffusion coefficient and k is the pseudo-first order rate constant for the decomposition of MDIBOA. Rearrangement and setting $\alpha^2 = k/D$ gives a Bessel differential equation [29],

$$\frac{\mathrm{d}^2[\mathrm{MDIBOA}]}{\mathrm{d}r^2}+\frac{1}{r}\frac{\mathrm{d}[\mathrm{MDIBOA}]}{\mathrm{d}r}-\alpha^2[\mathrm{MDIBOA}]=0$$

one that can be solved as a linear combination of modified Bessel functions.

$$[\mathrm{MDIBOA}] = A * I_0(\alpha r) + B * K_0(\alpha r)$$

Here, $I_0(\alpha r)$ and $K_0(\alpha r)$ are modified Bessel functions of the first and second kind, respectively [30]. Under the boundary condition where as $r \to \infty$, $[\mathrm{MDIBOA}] \to 0$, A must be 0, simplifying the expression to:

$$[\mathrm{MDIBOA}] = B * K_0(\alpha r).$$

The measurements of these reaction constants and the determination of B would therefore allow the concentration of MDIBOA to be defined around the root surface.

Molecular diffusion coefficients in water are inversely proportional to the molecular mean radius, and in agar, where the value is little changed for neutral molecules, can be assigned as 1.0×10^{-6} cm^2/s for MDIBOA [21,31]. The hydrolytic decomposition of MDIBOA was monitored directly with ^1H NMR, establishing a pseudo-first order decomposition constant at pH 5.5 of 5.5×10^{-5} s^{-1}; and the ratio of k/D giving a value of $\alpha = 7.4$. The edge of the inhibition zone was assigned the estimated IC_{50} value of MDIBOA in agar, e.g., 1 μM for A348/pAC2, existing r cm from the seedling root surface. The range of r across multiple roots in each plate fell between 0.3 and 0.5 cm, varying

with the age of the root and its position in the agar. Using these limits, $K_0(\alpha r)$ [30] lies between 0.087 and 0.015, and B is determined to be between 12 and 70 μM.

With this limiting range for B, we sought to estimate the MDIBOA concentration at the surface of the root, that region critical for bacterial attachment and ultimate DNA transfer. At $r = 0.05$ cm, the surface region for a lateral root of 1 mm diameter, the MDIBOA concentration would be between 15 and 80 μM, a concentration range below the 150 μM bacteriostatic concentration estimated for MDIBOA in agar. Therefore, consistent with the observation made in Figures 1 and 2, the released MDIBOA is sufficient to restrict *vir* expression at great distances from the root, but not sufficient to inhibit bacterial growth even in those regions at the root surface.

Isolation of resistant mutants

This striking difference between the MDIBOA concentration necessary to inhibit virulence and that required to restrict bacterial growth around the seedling may be significant for pathogenesis. Specific inhibition of *vir* expression, without altering vegetative growth, would not establish as severe a selection pressure for bacterial mutations that could avoid the MDIBOA defense. We therefore sought to determine whether variants of *Agrobacterium* resistant to inhibition of *vir* gene expression by MDIBOA could be selected. To reproduce the conditions that existed around the maize root, the more stable DIMBOA was incorporated into media on which A348/pAC2 variants were selected. Under these conditions, the viability in kanamycin is directly dependent on *vir* gene expression. Preliminary studies showed that colonies grew on induction plates with 40 μg/ml kanamycin at AS concentrations greater than 30 μM, but did not survive with 100 μM DIMBOA present. In this study, DIMBOA resistant colonies were selected on AB induction medium (ABIM) containing 100 μM DIMBOA, 50 μM AS, and 50 μg/ml kanamycin. The selected colonies were secondarily screened on kanamycin plates without AS, and those colonies that grew were classified as AS independent (constitutive) strains and not further characterized. Four stable colonies, JZ101-104, showed AS dependent *virB::lacZ* expression, as demonstrated by JZ103 and JZ104 in Figure 5A, that was little affected by 10 μM MDIBOA and reduced by $\leq 30\%$ at 100 μM DIMBOA (data not shown). Each of these variants proved to be ~ 10 times more sensitive to the [AS] (Figure 5B), suggesting that at least part of the resistance was mediated by an increase in the sensitivity of the two-component system to the xenognosin. The genetic basis of the MDIBOA resistance is under current investigation.

Discussion

Previously in sorghum [19–21], and now in maize, and possibly more generally among higher plants [32], a single physically unstable metabolite has been shown to constitute $> 90\%$ of their organic exudate. Strategically, the commitment of precious carbon resources by the young seedling to the secretion of large concentrations of an antibiotic seems both wasteful and dangerous. In the case of MDIBOA however, the compound's instability defines a transient localized zone, one that avoids both the accumulation of molecules toxic to the producer, and reduces potential selection pressure that could result in the occurrence of resistance in potential pathogens. This result is significant, at least in the case of *A. tumefaciens*, where variants more resistant to MDIBOA inhibition can be selected.

MDIBOA, a new member of the benzoxazinones, (i) is a major component of maize root exudate, (ii) functions as a specific and very potent inhibitor of *vir* gene expression in *A. tumefaciens*, (iii) exists at a physiologically significant concentration at the organ surface, and (iv) has sufficient hydrolytic lability such that elevated concentrations do not accumulate in the agar during seedling growth. At neutral pH, MDIBOA decomposes cleanly and rapidly to the inactive MBOA. While the observed IC_{50} in solution is ~ 1 μM, the actual value is considerably lower due to its decomposition over the 8 h assay time period. Therefore, MDIBOA becomes the most potent and specific inhibitor yet discovered for any environmental sensing two-component signal transduction system [22,33–39].

Benzoxazinones related to MDIBOA are produced in several of the Gramineae, including maize [23–26], and DIMBOA, the best known and most abundant of these structures, has been shown to inhibit both *A. tumefaciens* growth and *vir* gene induction [22]. DIMBOA exists primarily as the inactive glucoside, and only vacuole rupture exposes the glucosidase capable of releasing the toxic aglycone. In contrast, the methylated hydroxamic acid MDIBOA is uncharged at neutral pH and is released in large quantities along the root surface, that part of the seedling in direct contact with soil-borne bacteria. Certainly for *Agrobacterium*, the continual production of MDIBOA significantly reduces the effectiveness of any xenognostic signals required for induction of pathogenesis [3,11–13], but only locally as the hydrolytic lability ensures the observed 'halo of protection' around the root axis.

Independent estimates of the rates of diffusion and hydrolysis of MDIBOA made it possible to develop a linear combination of modified Bessel functions for predicting inhibitor concentration at specific distances from the seedling in agar media. The accuracy of these predictions is dependent on multiple factors. First, the measured distances to the outer boundary of the halo in the culture plate are dependent on attaining an equilibrium between influx and hydrolytic decomposition. Depending on the age of the root and its position in the reporting layer of the

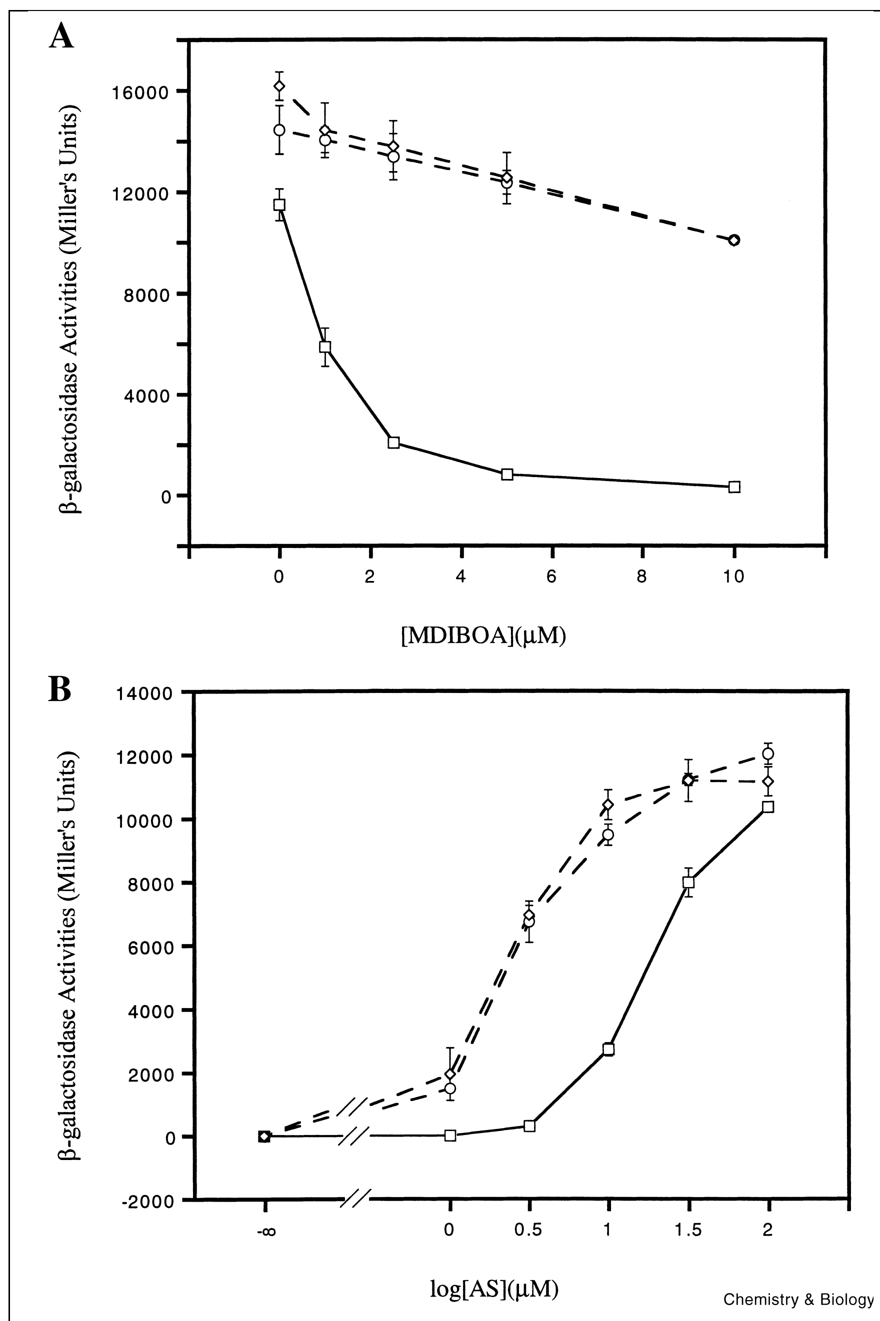


Figure 5. Mutant analysis. **(A)** Effect of various [MDIBOA] on *virB::lacZ* gene expression induced by 10 μM AS with 100 mM glucose in wild type A348 (□), JZ103 (○), and JZ104 (◇) carrying pSW209 (*virB::lacZ*). **(B)** β-Galactosidase induction by AS in wild type A348 (□), JZ103 (○), and JZ104 (◇) carrying pSW209 (*virB::lacZ*). Mean ± standard deviation ($n = 3$).

agar, the outer boundary limit, *r*, can vary. While the actual data for the pSW209 reporter was not as clearly defined as that for pAC2, this reporter was also critical for evaluating the inhibitory zone. The use of both pAC2 and pSW209 reporter constructs were therefore essential for defining the range of values for *r*. Second, the MDIBOA hydrolysis rates and diffusion are sensitive to the physical environment, e.g., pH, temperature, and certainly the H_2O concentration, the importance of which was highlighted with the organic exudate methods. Our specific rate measurements control for these physical variables in agar plates, and it is unlikely that any other components of the maize exudate will alter either MDIBOA diffusion or hydrolysis. However, the bacteria may respond differently to the complex mixtures that comprise an aqueous seedling exudate [40]. For example, the concentrated fresh aqueous exudate was found to provide a superior growth media for *A. tumefaciens* relative to the minimal induction media. Around the surface of the roots, however, no detectable effect on growth rate was observed. Third, the inducing phenol AS

was added to the large agar reservoir in these assays as AS depletion from the media would affect the measured MDIBOA IC_{50}. AS was selected as the inducer because it is both physically and biologically stable, in fact it was isolated as a stable metabolite from plant cell cultures [41]. Consequently, over the period of the assay, the AS concentration is not expected to change, certainly not at the zone boundary several millimeters from the root surface. Even if the AS reservoir concentration in the plate were depleted by the seedling, the largest effect would occur at the root surface where the inhibitor IC_{50} would be reduced making MDIBOA a more effective resistance factor.

Therefore, the steady state MDIBOA concentration at the surface of 1-week-old maize seedlings can be placed in the range of 15–80 μM. The MDIBOA concentration necessary to inhibit maize seedling growth appears to be comparable with DIMBOA, where concentrations well above 500 μM are necessary [42]. The upper end of this concentration range is also below the bacteriostatic threshold for *A. tumefaciens*, consistent with the data provided in Figure 1 that this microbe is able to readily colonize the root surface. The lower end of the determined concentration range is sufficient to restrict *vir* expression by any wall-attached Agrobacteria, and therefore retard DNA transfer. Significantly, there is greater than two orders of magnitude between the minimal concentrations for *vir* inhibition at high AS concentrations and the bacteriostatic threshold. In most maize cell transformations, the inducer concentration appears to be limiting [3], making this difference between specific inhibition and toxicity even greater. Therefore, in *A. tumefaciens*, MDIBOA is able to function as a specific virulence resistance factor, not merely as an antibiotic, and its release suggests a novel and highly specific defense strategy.

In the general context of bacterial plant pathogenesis, maize is not a host for *Agrobacterium*. The presence of complex soil surfaces, the depletion of H_2O in arid environments, and the presence of other colonizing organisms could alter both the MDIBOA production rates and as well as the compound's rate of hydrolysis. Nevertheless, it is possible to transform maize with *A. tumefaciens*, and understanding the natural limitations on the host range could be biotechnologically useful and pathologically interesting. The results presented here argue that virulence can be regulated through inhibition of the ubiquitous two-component signal transduction systems. The fact that constitutively active VirA molecules are not sensitive to inhibition by MDIBOA demonstrates that the mode of action of MDIBOA is upstream of VirA transmitter activity, most likely at the xenognostic phenol receptor. Recent biochemical and genetic data suggest that this receptor is not encoded within the infectious Ti plasmid that contains the *vir* genes, but within the *Agrobacterium* chromosome [18,43]. Therefore, phenol recognition may be a more general feature of related soil microbes, particularly plant pathogens and symbionts, e.g., *Rhizobium* spp., as their first encounter with the plant is at the plant cell wall, a structure uniquely laden with phenols. The fact that the *vir* regulon is induced by more than 80 simple phenols [35], and MDIBOA specifically inhibits this broad substrate recognition system, may provide a general mechanism for inhibiting phenol recognition by plant pathogens. Further studies attempting to explore the broader significance of such an inhibitory strategy are in progress.

Significance

There are three aspects of the studies that are particularly noteworthy. First and foremost, this analysis of *A. tumefaciens* suggests a novel strategy in which the release of MDIBOA disrupts pathogenic activation in a manner that avoids toxicity to either host or pathogen. The importance of this claim is demonstrated by the fact that conditions are here reported under which variants resistant to MDIBOA-mediated inhibition of *vir* gene expression can be selected. The extent to which the initial signaling events limit transformation efficiencies of maize, and/or other grasses, can now be explored with these MDIBOA resistant variants. Certainly definition of the mechanism of inhibition by MDIBOA and protocols designed to overcome inhibition could greatly expand the biotechnological utility of this bacterium. Likewise, the identification of MDIBOA opens the possibility for further engineering these biosynthetic pathways into other organisms to expand their arsenal of self-defense.

Secondly, taken together, these results indicate that signal recognition components upstream of the histidine kinase autophosphorylation event can serve as targets for the development of inhibitors that block critical two-component system activation in pathogenic organisms. Given the ubiquitous nature of these signaling pathways among eubacterial pathogens of plants and animals, the further development of new inhibitors directed at these targets can now build on a biologically proven strategy to control infectious diseases.

Finally, the methods described here for collecting the organic exudate provide access to extremely labile molecules released from plant organs. In the two seedlings investigated with these methods, maize and sorghum, our understanding of the chemical biology of the organisms that colonize plant surfaces has changed radically. To the extent that these two systems can be generalized, the continued use of these simple methods could profoundly alter our understanding of the chemical ecology at the surface of eukaryotic organs.

Materials and methods

^{1}H NMR and ^{13}C NMR spectra were either obtained on a Bruker Avance/DRX500 or a Bruker Avance/DRX400 spectrometer. Low-reso-

Table 1
Strains and plasmids.

Strains/plasmids	Relevant genotype	Source/reference
Strains		
A136	Strain C58 cured of pTiC58	[45]
A348	A136 containing pTiA6	[46]
358mx	A348 derivative harboring insertion of the transposon Tn3-HoHo1 in the *virE2* gene of pTiA6 creating a *virE::lacZ* fusion	[47]
A348-3	VirA deletion derivative of A348, Km^r	[33]
JZ101-104	MDIBOA resistant mutants derived from A348	This study
Plasmids		
pMutA-G665D	4.5 kb *Kpn*I fragment containing a mutant *virA* (G665D) from pTiA6 in pMutG-very low copy number in *A. tumefaciens*; Cb^r	[27]
pAB123-22	4.5 kb *Kpn*I fragment containing wild type *virA* from pTiA6 in pMutG-very low copy number in *A. tumefaciens*; Cb^r	This study
pSW209	IncP, *virB::lacZ* fusion derived from pSW243cd, Km^r	S.C. Winans, Cornell University
pSW209Ω	2 kb *Bam*H1 fragment from pHP45 cloned into *Bam*HI site of pSW209; IncP Sp^r, Km^r, *virB::lacZ*	[48]
pSM102	IncP, *occ::lacZ*, Cb^r	[49]
pAC2	IncP, P*virB/npt*II, Sp^r	[18]

lution mass spectra (MS) were obtained using a UG 70-250 mass spectrometer. High performance liquid chromatography (HPLC) employed a Rainin Model HPXL (pressure module max; 8700 psi), dynamax Absorbance detector (model UV-D) and a Macintegrator software installed on a Macintosh computer. Ultraviolet (UV) spectra were recorded with a Perkin-Elmer Lambda 6 UV–VIS spectrophotometer in quartz cuvettes with an optical pathway of 1 cm. Sterilization was afforded by treatment in a Market Forge Sterilmatic at 15 lb/in^2 and 121°C for 25 min.

Seeds, reagents and methods
Flats seed corn was surface sterilized with 30% commercial bleach (20 min), washed with double distilled H_2O, and planted in an autoclavable box (12″×24″×6″) containing sterile moist vermiculite for 7–11 days. The roots of the seedlings were dipped for 3 s in ABIM media and this aqueous exudate was either immediately frozen or used directly in virulence assays (see below). Alternatively, the organic exudate was obtained by dipping the roots in either EtOAc or CH_2Cl_2 containing 0.5% HOAc for 3 s, and the solvent removed in vacuo. The organic exudate was analyzed directly by HPLC (Microsorb SiO_2, cyclohexane/ethyl acetate (67:33 v/v)) on a Rainin MPXL dual-pump with detection at 254 nm.

Syntheses
AS was purchased from Aldrich and used directly. HPLC grade solvents were purchased from Fisher Chemical Co. or J.T. Baker Chemical Co. Diethyl ether was distilled from sodium/benzophenone and dichloromethane (CH_2Cl_2) was distilled from calcium hydride. H_2O was double distilled from all glass stills.

Flash column chromatography employed EM Science silica gel, 230–400 mesh, 40–63 Å in diameter. Thin layer chromatography was performed with Baker-flex silica gel IB2-F plastic backed plates with a 0.25 mm silica gel layer. Plates were visualized by 254 nm illumination, and/or treatment with Aldrich phosphomolybdic acid reagent with two-fold dilution with absolute ethanol.

Following a previous procedure [22], 10-day-old corn seedlings (200 g) were frozen in N_2 (l), homogenized in a Waring blender in 400 ml of water, and agitated at room temperature for 2 h to allow for enzymatic hydrolysis of DIMBOA glucoside. The suspension was clarified twice at 4000 rpm for 15 min in a Sorval RC-5B with a GSA rotor, or simply filtered through celite twice. The brown aqueous layer was extracted 3× with an equal volumes of EtOAc, and the organic layer was dried over $MgSO_4$, concentrated and recrystallized from EtOAc. The product gave a positive $FeCl_3$ test and ^{1}H NMR (400 MHz, C_3D_6O) δ 7.25 (d, 1H, $J=8.8$ Hz, C5-H), 6.7 (dd, 1H, $J=2.6$, 8.8 Hz, C6-H), 6.6 (d, 1H, $J=2.6$ Hz, C8-H), 5.7 (s, 1H, C2-H), 3.8 (s, 3H, OCH_3).

DIMBOA (0.05 mmol) was dissolved in 5 ml of anhydrous diethyl ether and treated with an excess of alcohol free diazomethane ethereal solution (0.33 mmol) at 0°C. Alcohol-free diazomethane prepared in situ in diethyl ether limited nonselective methylation of the phenol ring-chain tautomer. The reaction was monitored by the disappearance of both the yellow CH_2N_2 and the positive $FeCl_3$ test. The crude mixture was concentrated in vacuo and rapidly chromatographed (SiO_2, hexane/ethyl acetate, 1:1) to afford MDIBOA (40%). ^{1}H NMR (500 MHz, acetone-d_6) δ 7.16 (d, 1H, $J=8.8$ Hz, C5-H), 6.70 (dd, 1H, $J=2.6$ Hz, C6-H), 6.68 (d, 1H, $J=2.6$ Hz, C8-H), 5.69 (s, 1H, C2-H), 3.91 (s, 3H, OMe) and 3.78 (s, 3H, OMe). ^{13}C NMR (500 MHz, $CDCl_3$) 157.5, 156.9, 141.9, 119.7, 114.0, 108.5, 104.0, 92.3, 62.8, 55.7. MS (CI) *m/z* (relative intensity) 226 (10, M+H), 208 (10), 194 (20), 180 (10), 166 (100); HRMS (EI) calculated for $C_{10}H_{11}NO_5$ 225.0637, observed 225.0664.

Bacterium strains and media
The strains and plasmids used are listed in Table 1. All strains were maintained on LB plates or AB minimal medium [44] supplemented with antibiotics as appropriate. ABIM, pH 5.5 and containing 1% glucose or 1% glycerol ± 0.1% arabinose, was used for *vir* gene expression studies.

Vir induction assay
A. tumefaciens strains were grown overnight at 27°C to an OD of 0.3–0.6 in LB medium supplemented with appropriate antibiotics. Cells were

Table 2
Co-cultivation conditions.

	ABIM plate	ABIM top agar
A348/pSW209	AS 10 μM	Bacterial suspension (ca. 10^5 CFU/ml)
	X-gal 80 μg/ml Kanamycin 50 μg/ml	Kanamycin 50 μg/ml
A348/pAC2	AS 100 μM	Bacterial suspension (ca. 10^5 CFU/ml)
	Calcofluor 0.2 μg/ml	Calcofluor 1 μg/ml
	Kanamycin 50 μg/ml	Kanamycin 50 μg/ml

then pelleted by centrifugation and resuspended to an OD_{600} of 0.1 per ml in sterilized induction medium (ABIM, pH 5.5) supplemented with either 1% glucose or 1% glycerol. The various inducer and inhibitor compounds tested were dissolved in a minimal amount of DMSO in water as mM stocks, and diluted with ABIM to the desired concentration (final DMSO concentration was no greater than 0.1%). The bacteria were incubated at 28°C for 8 or 16 h and subsequently assayed for β-galactosidase activity by the method of Miller [50]. The concentrations necessary for 50% inhibition (IC_{50} values) were determined from the dose response analyses. Each assay value reported is the mean of three replicates; error bar indicates one standard deviation from the mean. Data are representative of three similar independent experiments.

Co-cultivation assays

A. tumefaciens stains were grown to mid-log phase in LB media and pelleted by centrifugation and resuspended in sterilized induction medium (ABIM, pH 5.5). Strains were then serially diluted in induction broth; 4 ml volumes of these mixtures (ca. 10^5 CFU/ml) were added to 100 ml of ABIM top agar (induction medium containing 0.7% agar) and overlaid on ABIM plates (150×15 mm) containing additions as shown in Table 2 such that the top agar/plate agar = 1/5 (v/v) [51]. Two different AS concentrations were used due to the different threshold sensitivities of the two reporter constructs. The 5-day-old maize seedling was placed on the top agar, and the plates were incubated right-side-up at room temperature for 3–5 days. Photographs were taken on the third day for A348/pSW209 and on the fifth day for A348/pAC2. Calcofluor was added to both agar layers and was used to enhance bacterial detection [52,53], whereas the β-galactosidase substrate, X-gal, was only included in the agar plate. The IC_{50} of MDIBOA inhibition of *PvirB/npt*II in the presence of 100 μM AS on agar plates could not be measured directly due to the instability of this compound, but was estimated to be 1μM based on the fact that it was 10 times more potent than DIMBOA under all conditions tested.

Mutant selection

DIMBOA resistant mutants were selected as follows. Individual colonies of A348/pAC2 were grown overnight in LB broth plus spectinomycin, and the progeny from each colony kept separate throughout the procedure. The bacteria were subsequently washed in AB medium, resuspended to an OD_{600} of approximately 0.1 and induced overnight in AB media containing 100 μM AS. The next day the cells had grown to an approximate OD_{600} of 1.0 and 10^5 CFU/ml bacteria were plated onto plates containing ABIM medium plus AS at 50 μM, DIMBOA at 100 μM and kanamycin at 50 μg/ml. Colonies that grew on this selection medium were subsequently assayed for growth on plates containing kanamycin but no phenol; those that could not survive in this screen were characterized further.

Acknowledgements

We thank S.C. Winans for providing pSW209, R. Banovich and R. Skurauskis for graphic arts, and NIH GM47369 for support.

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