Defense-Related Gene Induction in *Brassica campestris* in Response to Defined Mutants of *Xanthomonas campestris* with Altered Pathogenicity

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We have studied the induction of β-1,3-glucanase (BGL) in turnip following inoculation with pathovars of *Xanthomonas campestris* and derived mutants. BGL transcript accumulated more rapidly in leaves in the incompatible interactions with *X. c.* pv. *armoraciae* and *X. c.* pv. *raphani* than in the compatible interaction with *X. c.* pv. *campestris*. No accumulation was seen in response to wounding or inoculation with water, salicylic acid, or *Escherichia coli*. Deletion of the *hrp* cluster from the *X. campestris* pathovars caused a reduction in the level of transcript accumulation; these effects were much more pronounced in the incompatible than in the compatible interaction, in which bacterial growth was also affected. In the compatible interaction, bacterial growth and BGL transcript accumulation were not altered by mutation of bacterial genes involved in the regulation of the synthesis of extracellular enzymes or their export from the cell, or by mutation of the structural genes for extracellular endoglucanase and serine protease. Mutation of genes involved in the synthesis of extracellular polysaccharide or lipopolysaccharide reduced bacterial survival *in planta*, so that the numbers were between two and three orders of magnitude lower than the number of wild-type bacteria. However, total BGL transcript accumulation after inoculation with these mutants was about 80% of that seen after inoculation with the wild-type bacteria, suggesting that one aspect of the role of extracellular polysaccharide and lipopolysaccharide in pathogenesis is to mask the presence of bacteria in the plant. Our results are discussed in the context of work on other plant-microbe interactions.

Additional keywords: bacterial pathogenicity gene mutants, β-1,3-glucanase induction.

Plants in incompatible interactions with pathogens respond with the rapid induction of a coordinated defense response, which can comprise the synthesis of antimicrobial compounds and hydrolytic enzymes to directly attack the pathogen, the synthesis of inhibitors of microbial enzymes, and cell wall modifications (most recently reviewed by Bowles [1990], Dixon and Lamb [1990], Lamb *et al.* [1989], and Cutt and Klessig [1992]). Many plant genes involved in these defense responses have been described. However, defense-related genes are also induced in the late phases of compatible interactions, so that in the diseased state the host plant cannot be regarded as passive partner. Some workers have suggested that bacterial pathogens in compatible interactions or symbionts actively suppress the early plant defense response (Jakobek *et al.* 1993; Kamoun *et al.* 1992; Niehaus *et al.* 1993; Palva *et al.* 1993), as has been suggested for a number of fungal pathogens and mycorrhizal fungi (Ziegler and Pontzen 1982; Yamada *et al.* 1989; Lambais and Mehdy 1993). In a few cases bacterial genes and gene products responsible for these effects have been described (Kamoun *et al.* 1992; Niehaus *et al.* 1993). These bacterial genes have been viewed as belonging to the set of basic pathogenicity or compatibility genes of bacteria. Although considerable progress has been made in identifying bacterial genes involved in plant pathogenesis (reviewed by Daniels *et al.* [1988]), the expression of host defenses in response to bacteria carrying mutations in these genes has been relatively little studied. More often the interactions are characterized only in terms of host symptoms and bacterial numbers. More detailed analysis of the plant response to defined bacterial mutants could increase our understanding of the complex of interactions between the two organisms.

Our model system for studying bacterial pathogenesis is the interaction between turnip (*Brassica campestris*) and *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson, the causal agent of black rot disease of crucifers (Williams *et al.* 1980). Using this system, we have described a number of bacterial genes required for pathogenicity (reviewed by Daniels *et al.* [1993]); interactions with mutants having lesions in these genes result in decreased symptoms or bacterial numbers *in planta*. Specific changes in plant gene expression accompany the resistance response of turnip in incompatible interactions with pathovars of *X. campestris* (Collinge *et al.* 1987). At a biochemical level we have described the properties of a β-1,3-glucanase (BGL) which is more rapidly induced in incompatible than in compatible interactions but is not induced by *Escherichia coli*, heat-killed bacteria, or wounding (Conrads-Strauch *et al.* 1990). This induced BGL is indistinguishable from the basal enzyme present in uninoculated leaves. In this paper we further characterize this defense-related induction of BGL in turnip. We characterize a

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cDNA clone which we have used to study changes in BGL gene expression and localization of the gene product by immunogold electron microscopy. Having established the parameters of induction in compatible and incompatible interactions with different pathovars, we then describe the effects of mutation in a number of bacterial genes on the kinetics of BGL mRNA accumulation in response to challenge by the mutants. These experiments suggest no simple correlation between bacterial growth, symptom production, and defense-related gene expression.

RESULTS

cDNA clone for a defense-related BGL from *Brassica*.

Antisera to BGL were used to screen a lambda-Zap cDNA library made from RNA extracted from turnip leaves undergoing an incompatible interaction with *X. c.* pv. *vitians*. Several positive clones were identified, and inserts were sequenced. All the clones shared the same sequence (Fig. 1); the predicted polypeptide has good sequence homology to BGL from other sources and is a basic protein of pI 8.36 and 38.2 kD. Experimental values for purified BGL protein are 36.5 kD and (for the native enzyme) pI 8.5. Determination of the amino acid sequence of purified BGL (for comparison with the sequence predicted by the cDNA) was hampered, as the protein was N-terminally blocked. However, peptides for amino acid sequencing were generated from the protein by treatment with *N*-chlorosuccinimide, which cleaves after tryptophan residues. Three of the peptides thus generated had the N-terminal sequence V?DNVQ?YNDVRF?YISVG, which is as predicted by the nucleotide sequence for amino acid residues 99–117. No C-terminal extension was observed, as is

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   1  CTCGTGCCGCATTCGGCACGAGGGTTAGGATGTTAGCATCATCACCAATGTTGCTGTTTC   60
                                  M  L  A  S  S  P  M  L  L  F  L
  61  TTCTCAGCCTTCTAATGGCTTACAACTTCGACACCACAGCTGGACAAATCGGAGTATGCT  120
      L  S  L  L  M  A  Y  N  F  D  T  T  A  G  Q  I  G  V  C  F
 121  TCGGGCAGATGGGAAATAACATACCAAATCCATCGGAAGTTGTGGCTATGTTCAAGCAGT  180
      G  Q  M  G  N  N  I  P  N  P  S  E  V  V  A  M  F  K  Q  Y
 181  ATAGCATCCCGCGAATGCGGATGTACGGTCCCAACCCCGACGCTCTCAACGCTCTCCGTG  240
      S  I  P  R  M  R  M  Y  G  P  N  P  D  A  L  N  A  L  R  G
 241  GCTCCAACATCGAGTTCATCCTCGACGTTCCCAATGGCGACTTAAAACGTCTCGCAGACA  300
      S  N  I  E  F  I  L  D  V  P  N  G  D  L  K  R  L  A  D  S
 301  GCCAAGCGGAGGCCAACACATGGGTCCGAGACAACGTCCAGAAGTATAACGATGTCAGAT  360
      Q  A  E  A  N  T  W  V  R  D  N  V  Q  K  Y  N  D  V  R  F
 361  TCAAGTACATCTCGGTCGGAAACGAGGTGAAAACCAGGGGAACCGGGGGCGGCGGCTCTCA  420
      K  Y  I  S  V  G  N  E  V  K  P  G  E  P  G  A  A  A  L  I
 421  TCCAGGCGATGCAGAACATAGATAGAGCGCTTTCCGCAGCAGGCCTCAGCAATATAAAGG  480
      Q  A  M  Q  N  I  D  R  A  L  S  A  A  G  L  S  N  I  K  V
 481  TCTCCACGACTACATTCATGGGACCTTCGCGGAACACGTATCCTCCGTCGCGCGGAAGAT  540
      S  T  T  T  F  M  G  P  S  R  N  T  Y  P  P  S  R  G  R  F
 541  TCAAGGATGAGTATAGAAACTTTCTACAACCGGTGATAGGTTTCTTGGTGAACAAGCGAT  600
      K  D  E  Y  R  N  F  L  Q  P  V  I  G  F  L  V  N  K  R  S
 601  CCCCTCTGCTCGTGAATATCTACACTTACTTCGGCTACATGAACCGCGACGTCTCTCTAC  660
      P  L  L  V  N  I  Y  T  Y  F  G  Y  M  N  R  D  V  S  L  Q
 661  AATTCGCTCTGTTGCAACCGAATAGTAATAATGAATTCACTGACCCCAACAACCAGCTCC  720
      F  A  L  L  Q  P  N  S  N  N  E  F  T  D  P  N  N  Q  L  R
 721  GTTACCTAAACTTCTTCGACGCCAATCTCGACTCAGTTTACGCGGCACTGGAGAAATCGG  780
      Y  L  N  F  F  D  A  N  L  D  S  V  Y  A  A  L  E  K  S  G
 781  GCGGGGGATCGTTGGATGTCGTGGTGTCGGAGAGCGGTTGGCCCACGCAGGGAGGACCCG  840
      G  G  S  L  D  V  V  V  S  E  S  G  W  P  T  Q  G  G  P  G
 841  GGGCAAGTGTGCCGAATGCGGAGGCTTATGTTAACAATTTGAGACTACATGTTAATAAGA  900
      A  S  V  P  N  A  E  A  Y  V  N  N  L  R  L  H  V  N  K  N
 901  ATGGATCTCCGAAAAGGCAGGAAGCTATAGAGACTTACATATTCGCCATGTTCGATGAGG  960
      G  S  P  K  R  Q  E  A  I  E  T  Y  I  F  A  M  F  D  E  A
 961  CACCGAGGCAGACGTCGCCTAATGATGAGTATGAGAAGTATTGGGGGATGTTTTCTCCTA 1020
      P  R  Q  T  S  P  N  D  E  Y  E  K  Y  W  G  M  F  S  P  T
1021  CTACTAGACAGCTTAAATATGGTGTTAAGTTTAACTAATCTCCTTGGAGAGACTTCTTGA 1080
      T  R  Q  L  K  Y  G  V  K  F  N
1081  AGAAGCAAGGGCGATGCGGTAGATGGTATTTGTCTATTTGAGGTTTTTTTTTATGTGTCT 1140
1141  TAATTAAAAATGTGTGAACTTGTTGTAATAATAAGGACTAATGAGCCTTAACCGTTGCAC 1200
1201  TTCATCTGTTTCAATAATGTAATGAGAGTTGTTCGATTATAGTAAACAAAAACTTTTAAA 1260
1261  TAAAAAAAAAAAAAAAAAACTCGA  1284
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Fig. 1. Nucleotide sequence of the insert of a cDNA clone for β-1,3-glucanase from *Brassica campestris*. The deduced amino acid sequence is written beneath the nucleotide sequence.

also the case for the basic glucanases from *Arabidopsis,* in contrast to the basic glucanases of tobacco (reviewed by Meins *et al.* [1992]). The predicted protein has a potential glycosylation site NGS toward the C-terminus, although no glycosylation of the purified protein could be detected.

The cDNA clone was used to probe restriction digests of turnip genomic DNA (Fig. 2). The multiple bands seen, particularly in the digest with *Hin*dIII (which does not cut within the insert of the cDNA clone), suggest that several copies of the gene or closely related genes are present. Preliminary experiments using this cDNA as a probe in Northern blots of poly(A)$^+$ RNA showed a dramatic increase in BGL transcript level in leaves inoculated with *X. c.* pv. *vitians* over the barely detectable level in control leaves. This probe was used in subsequent analysis of BGL transcript levels in response to the range of wild-type and mutant *X. campestris* pathovars listed in Table 1.

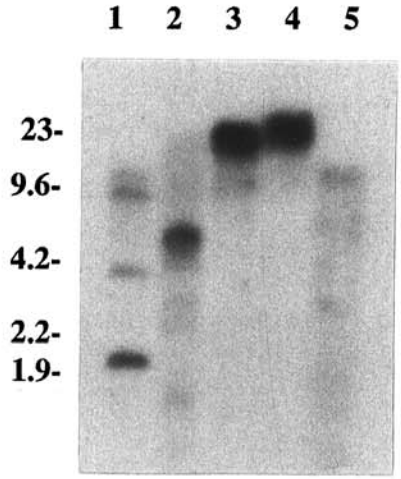

Fig. 2. Southern blot of restriction enzyme digests of turnip genomic DNA probed with the β-1,3-glucanase cDNA clone. The DNA was cut with *Hin*dIII (lane 1), *Eco*RI (lane 2), *Pst*I (lane 3), *Sal*I (lane 4), and *Pvu*II (lane 5). Molecular weight markers are given in kilobases.

Induction of BGL gene expression in turnip in compatible and incompatible interactions with pathovars of *X. campestris* and localization of the response.

Transcript levels of BGL were estimated by Northern analysis of total RNA extracted at different times after inoculation with bacteria and were quantified by use of the Bio-Imaging Analyzer, as described in Materials and Methods. Some representative blots are shown in Figure 3, and the full analysis of all the Northern blot data is given in Figure 4.

No detectable level of transcript was present in total RNA of uninoculated plants. In the compatible interaction with *X. c.* pv. *campestris* strain 8004, accumulation of BGL transcript was first detectable at low levels at 12 hr after inoculation, reached a maximum at 24 hr, then dropped at 72 hr (Figs. 3 and 4A). In contrast, *X. c.* pv. *raphani* strain 1946 and *X. c.* pv. *armoraciae* strain 1930, which are both incompatible with turnip cultivar Just Right, caused a much earlier accumulation of BGL transcript, which could be detected as early as 4 hr after inoculation in both cases. Maximum accu-

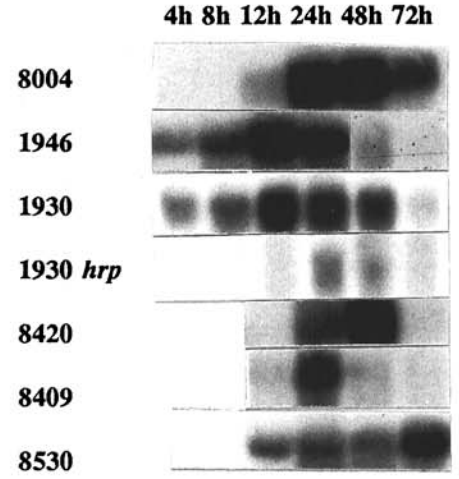

Fig. 3. Northern blots of total RNA extracted from turnip leaves after inoculation with bacteria probed with the insert of the β-1,3-glucanase cDNA clone. The strains are described in Table 1.

Table 1. Bacterial strains used in this study

Bacterium	Relevant characteristics	Source or reference
Xanthomonas campestris pv. *campestris*		
8004	Wild type	Daniels *et al.* 1984
8420	*hrp* deletion mutant of 8004	Liddle 1992
516-10	8004::Tn*5* extracellular protease$^-$	Tang 1989
8409	8004::Tn*5* extracellular endoglucanase$^-$	Gough *et al.* 1988
8403	8004::Tn*5* intracellular endoglucanase$^-$	Gough *et al.* 1988
8522	8004::Tn*5* rpfC$^-$ (regulatory gene)	Tang *et al.* 1991
8315	8004::Tn*5* enzyme export$^-$	Dow *et al.* 1987
8397	8004::Tn*5* extracellular polysaccharide$^-$	Barrère *et al.* 1986
8530	8004::Tn*5* path$^-$; altered lipopolysaccharide	Osbourn *et al.* 1990; Dow *et al.*, unpublished
X. campestris pv. *armoraciae*		
1930	Wild type	NCPPB[a]
1930 *hrp*	*hrp* deletion mutant of 1930	Liddle 1992
X. campestris pv. *raphani*		
1946	Wild type	NCPPB
X. campestris pv. *vitians*		
9000	Wild type	NCPPB
Escherichia coli		
ED 8767	...	Murray *et al.* 1977

[a] National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.

mulation of transcript was seen at 8–12 hr in the case of *X. c.* pv. *armoraciae* and 12–24 hr for *X. c.* pv. *raphani* (Figs. 3 and 4A). *X. c.* pv. *vitians* strain 9000, which is also incompatible with turnip, similarly caused this earlier increase (data not shown). In all of these incompatible interactions, the bacteria caused a hypersensitive response (HR) characterized by tissue collapse (Table 2) and showed no growth or attenuated growth compared to the compatible interaction with *X. c.* pv. *campestris* strain 8004 (Fig. 5A). The number of recoverable bacterial cells of *X. c.* pv. *armoraciae* actually dropped after 24 hr after inoculation, a decrease associated with the strong HR in the host. Surprisingly, strain 9001, a spontaneous rifampicin-resistant mutant of *X. c.* pv. *vitians* strain 9000, was not able to grow in leaves, produced no HR, and did not induce BGL transcript accumulation. The reasons for this are unknown, although similar behavior of this particular strain in *Arabidopsis* has been reported (Lummerzheim *et al.* 1993). Inoculation of leaves with water, wounding them by crushing or cutting or by rubbing them with Carborundum powder, and inoculation with *E. coli,* heat-killed or antibiotic-killed *X. campestris,* or 0.15 mM salicylic acid all did not induce BGL transcription. The changes in the level of BGL transcript in response to the various treatments are completely consistent with previously reported changes in levels of extractable enzyme activity (Conrads-Strauch *et al.* 1990), indicating that regulation was at the level of transcription. The induction of BGL was relatively localized and appeared not to be a systemic response. This was shown by experiments in which leaves were inoculated with *X. c.* pv. *campestris* strain 8004 or *X. c.* pv. *raphani* strain 1946 only in an area 5 cm to either side of the midrib. The inoculated and uninoculated areas of the leaf were processed separately. Only weak induction of BGL was seen in the uninoculated parts of the leaf up to 72 hr after inoculation with either bacterial strain.

The cellular location of BGL was determined by immunoelectron microscopy of tissue from uninoculated leaves and from the area immediately surrounding the collapsed tissue in leaves inoculated with *X. c.* pv. *vitians*. The antisera used recognized a single protein band, with the same molecular weight as purified BGL, in Western blots of total homogenates of control leaves and inoculated leaves. In both control leaves and inoculated leaves, the BGL was located almost exclusively in the vacuole (Fig. 6). Only in areas of cellular disintegration in the inoculated plant tissue were other locations of BGL observed.

BGL transcript accumulation in response to *hrp* mutants of *X. campestris*.

Deletion of the *hrp* gene cluster from *X. c.* pv. *campestris* strain 8004 reduced the level of BGL transcript but did not

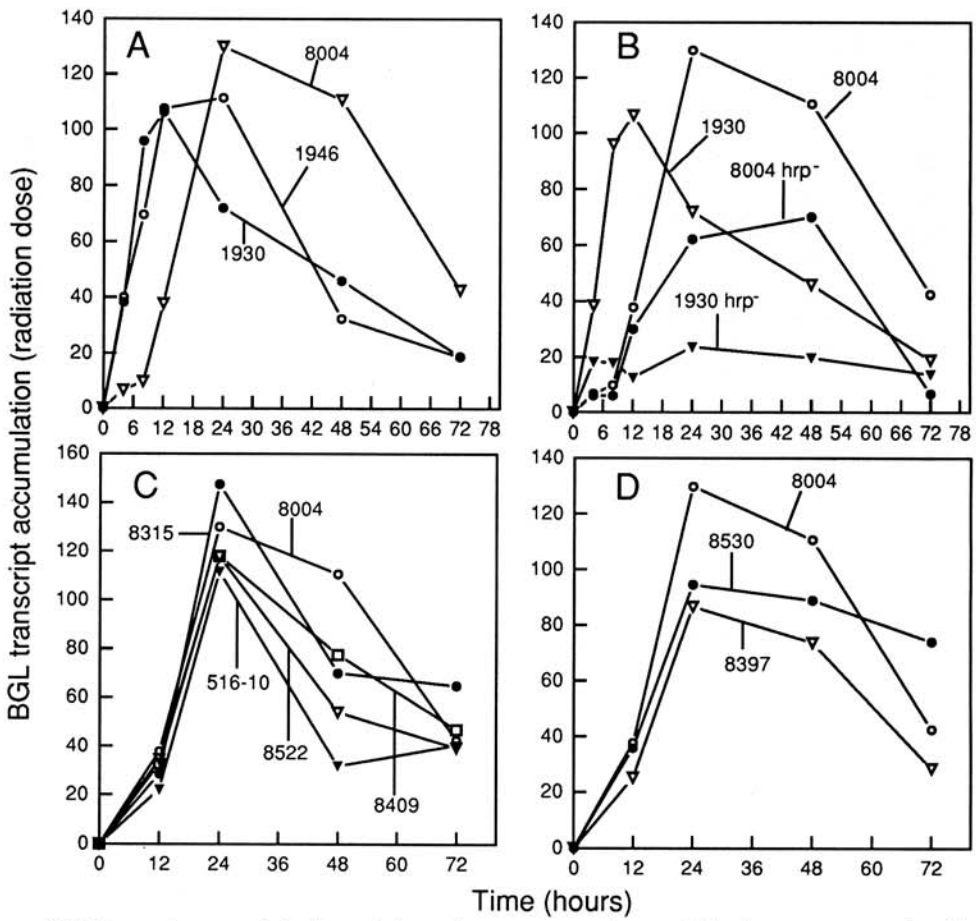

Fig. 4. β-1,3-Glucanase (BGL) transcript accumulation in turnip leaves in response to pathovars of *Xanthomonas campestris* and mutant strains, as measured by the Bio-Imaging Analyzer. The strains are described in Table 1. The profile for *X. c.* pv. *campestris* strain 8004 is included in each panel for comparison.

change the time course of accumulation. At 24 hr after inoculation, the level of BGL mRNA was approximately 50% of that induced by the wild type (Figs. 3 and 4B). This was associated with reduced bacterial growth and symptoms (Fig. 5B and Table 2). The effects of deletion of the *hrp* cluster from *X. c.* pv. *armoraciae* strain 1930 were much more pronounced. At 12 hr after inoculation, the level of BGL transcript was 10-fold lower than that induced by the wild type (Figs. 3 and 4B). This mutant did not cause an HR and did not grow *in planta* (Fig. 5B and Table 2).

BGL transcript accumulation in response to other mutants of *X. c.* pv. *campestris*.

Mutation of different pathogenicity genes of *X. c.* pv. *campestris* strain 8004 caused specific alterations in symptoms, the growth of the bacteria *in planta,* and the pattern of BGL induction in this compatible interaction.

Mutation of a number of genes affecting the production of extracellular enzymes had similar effects on symptoms, bacterial growth, and BGL induction. Strains with Tn*5* mutations in a serine protease gene (*prt* mutant 516-10), in an endoglucanase gene (*eng* mutant 8409), in a cluster of genes required for enzyme export (*xps* mutant 8315), and in a gene regulating enzyme synthesis (*rpf*C mutant 8522) all showed reduced growth *in planta* compared to the wild-type strain 8004; the mutant strains produced approximately 10-fold fewer bacteria after 72 hr (Fig. 5C). A reduction of symptoms was also observed (Table 2). For the first 24 hr after inoculation, BGL transcript accumulated to the same level as that observed after inoculation with the wild type, but it appeared to be more transient, decreasing to levels lower than that observed with the wild type at 48 hr (Fig. 4C). These differences were not simply due to the presence of Tn*5* in the mutant strains, because strain 8403 (a mutant with a Tn*5* insertion in a gene for an intracellular endoglucanase) showed growth identical to that of the wild-type strain and induced BGL transcript to the same level (not shown).

In contrast to the above-described mutants, which were capable of considerable growth in the plant, the *eps* mutant strain 8397 was found to have very limited growth, producing numbers over two orders of magnitude lower than the wild type after 48 hr (Fig. 5D). This mutant produced almost no symptoms on turnip leaves (Table 2). However, BGL transcript accumulated with the same time course and to 70% of the level seen with the wild type. This level of accumulation was higher than that observed with the *hrp* mutant, whose bacterial numbers were 10-fold higher. Similar behavior is seen with mutant 8530, which carries a lesion in a pathogenicity gene of unknown function (Osbourn *et al.* 1990), and which we have recently shown to be defective in lipopolysaccharide (LPS) but not extracellular polysaccharide (EPS) synthesis (J. M. Dow, unpublished data). Again the level of transcript accumulation was nearly 75% of that seen with the wild type, although the bacterial numbers were up to three orders of magnitude lower and symptoms were markedly reduced (Figs. 4D and 5D and Table 2). The BGL transcript persists in response to this LPS-defective mutant, considerable transcript levels still being present 72 hr after inoculation.

Table 2. Symptom development in turnip after infiltration with *Xanthomonas campestris* and mutants of *X. campestris*[a]

Strain	Time after inoculation					
	4 hr	8 hr	12 hr	24 hr	48 hr	72 hr
8004	–	–	–	–	+++	++++
8420	–	–	–	–	+	+
516-10	–	–	–	–	+	++
8409	–	–	–	–	+	++
8403	–	–	–	–	+++	++++
8522	–	–	–	–	++	+++
8315	–	–	–	–	+	++
8397	–	–	–	–	+	+
8530	–	–	–	–	+	+
1930	–	–	**	***	****	****
1930 *hrp*	–	–	–	*	*	**
1946	–	–	**	****	****	****
9000	–	–	*	***	***	****
E. coli	–	–	–	–	–	–
H_2O	–	–	–	–	–	–

[a] Symptom development was scored on a scale from – to ++++ in the compatible interaction (+: leaf shiny; ++: chlorosis, start of vein blackening; +++: chlorosis, vein blackening; ++++: total tissue collapse, vein blackening, chlorosis spreading away from the site of inoculation) and on a scale from – to **** in the incompatible interaction (*: leaf shiny; **: limited vein blackening and tissue collapse; ***: vein blackening, tissue collapse; ****: complete collapse, vein blackening, and drying out of tissue.). The beginning concentration of bacterial suspension was 10^7 cfu ml^{-1}. The strains are described in Table 1.

DISCUSSION

The plant hydrolases BGL and chitinase have attracted considerable interest as defense-related gene products in a wide variety of plants (Cutt and Klessig 1992; Meins *et al.* 1992). Although this paper is concerned with the induction of BGL by bacteria, this enzyme probably does not have antibacterial activity. We consider it to be one component of the defense-related responses in turnip and have used it in this work as an indicator of the triggering of those responses.

Most plants elaborate several acidic and basic isoforms of the hydrolase enzymes. Although we have characterized the cDNA for a basic glucanase in *Brassica,* Southern analysis suggests that there are likely to be several other genes present. In *Arabidopsis thaliana,* another crucifer, there are at least five genes for BGL (Dong *et al.* 1991; Delp *et al.* 1993; Uknes *et al.* 1992). Two of these genes (*bgl1* and *bgl3*) encode basic enzymes, whereas *bgl2* encodes an acidic isoform. Several lines of evidence suggest that the cDNA we have isolated encodes the protein we have previously purified. Although several cDNAs were derived by screening the expression library from plants in an incompatible interaction, these were of identical sequence (but different lengths). In these plants only one glucanase protein can be detected, which is indistinguishable from that present (at higher levels) in control plants (Conrads-Strauch *et al.* 1990). We have shown that the cDNA encodes a polypeptide which is basic, that the N-terminal amino acid sequence of peptides derived from the mature protein is predicted by the cDNA sequence, and that the changes in enzyme levels are predicted by the accumulation of transcript detected with the cDNA as probe.

In general the acidic isoforms of chitinase and glucanase are found extracellularly, whereas the basic proteins are intracellular, usually located in the vacuole. The vacuolar location of the basic BGL in *Brassica* as determined by immunoelec-

tron microscopy is consistent with this generality. However our analysis of the cDNA clone for this glucanase shows that it does not possess the C-terminal extension which in tobacco is considered to contain the targeting information for the vacuole (Melchers *et al.* 1993). The basic glucanases from *Arabidopsis* (bgl1 and bgl3), likewise do not have a C-terminal extension and, in common with the turnip enzyme, have a conserved potential glycosylation site (Dong *et al.* 1991). This may indicate a mechanism of targeting in crucifers that is different from that in other plant families. In plants challenged with *X. c.* pv. *vitians,* the location of the BGL in the intact tissue immediately surrounding the collapsed region remained vacuolar. In the collapsed and disorganized tissue, gold labeling was seen across the whole grid, and no localization was evident. Mauch and Staehelin (1989) have suggested that BGL acts as a last line of defense when plant cells are lysed, and these observations are consistent with this view.

Differential induction of BGL in compatible and incompatible interactions with pathovars of *X. campestris*.

The differential induction of BGL in compatible and incompatible interactions with pathovars of *X. campestris* is similar to the differential induction of other defense-related genes in bean in compatible and incompatible interactions with races of *Pseudomonas syringae* pv. *phaseolicola* (Voisey and Slusarenko 1989; Meier *et al.* 1993) and in *Arabidopsis* in compatible and incompatible interactions with strains of *P. s.* pv. *maculicola* (Dong *et al.* 1991; Ausubel *et al.* 1993). However it contrasts in detail with the BGL induction in the *Arabidopsis–P. s.* pv. *maculicola* interaction. Here only a gradual induction of *bgl2* transcript occurs in incompatible interactions, although in compatible interactions a strong but gradual accumulation of *bgl1, bgl2,* and *bgl3* transcript is found (Dong *et al.* 1991). Jakobek and Lindgren (1993) have reported differential accumulation of chitinase in bean in incompatible and compatible interactions with bac-

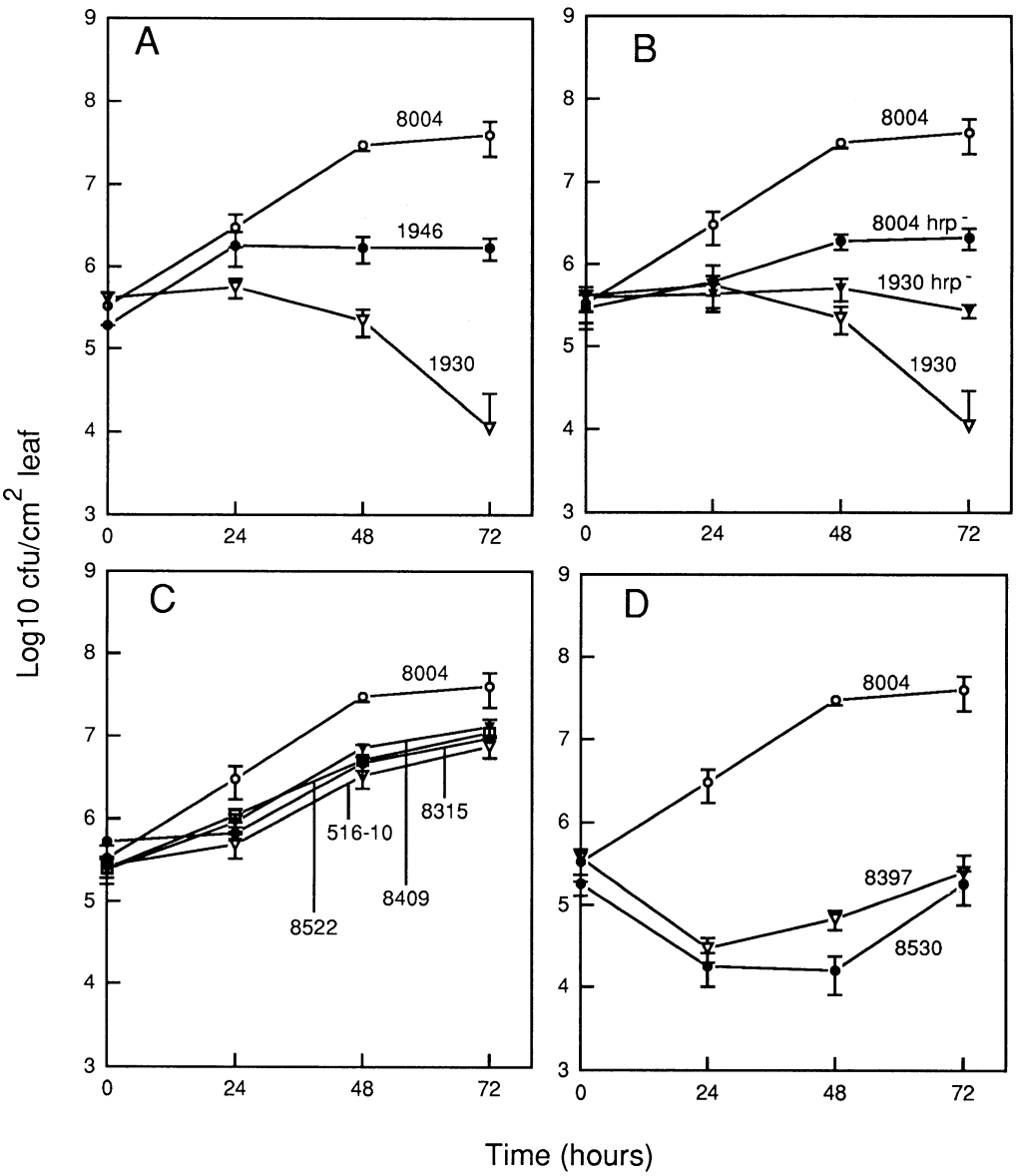

Fig. 5. Growth of *Xanthomonas campestris* strains in turnip leaves. The strains are described in Table 1. The mean and standard deviation of at least four separate measurements at each time point are given.

teria. However, in this case, other defense-related genes (phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase) were not induced at later stages of the compatible interaction. All these bean defense-related genes were induced by *E. coli* and saprophytic bacteria, which has prompted the suggestion that, in the compatible interaction, the bacteria suppress the bean defense responses which are triggered by a general mechanism. We have no evidence in *Brassica* for such a general induction mechanism involving BGL. *E. coli* and heat-killed *X. campestris* did not induce BGL activity or increase BGL transcript levels. These treatments do induce chitinase activity, but this resides in a different isoform than that induced in an incompatible interaction (Conrads-Strauch *et al.* 1990).

The induction of BGL in turnip is relatively localized and occurs in response to pathogens, but not *E. coli,* salicylic acid, or wounding. Ethylene is a weak inducer (unpublished data). In a number of plants differential induction of BGL isoforms occurs in response to these agents. Some isoforms in *Arabidopsis* are induced under the same conditions as for the turnip enzyme (Delp *et al.* 1993).

Induction of BGL in response to *hrp* mutants of *X. campestris*.

Deletion of the *hrp* cluster from *X. c.* pv. *armoraciae* had a more dramatic effect on BGL transcript accumulation than deletion from *X. c.* pv. *campestris*. In the compatible interaction, it is clear that defense gene induction does not depend entirely on *hrp* gene expression. However, we cannot distinguish between a direct and an indirect role for the *hrp* gene products in BGL transcript accumulation. The reductions in BGL transcript accumulation could be simply due to the lower numbers of bacteria in the leaf. Here the role of the *hrp* gene products in BGL induction would be indirect, allowing nutrition of the bacteria within the plant, so that higher bacterial numbers are attained. Other bacterial products would elicit the still considerable defense gene induction. Consonant with a role in nutrition are our own observations that *hrp* gene expression in *X. c.* pv. *campestris* is triggered by starvation (S. A. Liddle and M. J. Daniels, unpublished data). Alternatively, the plant may respond directly to the *hrp* gene products to enhance the level of transcript accumulation. In their absence the response may be quantitatively reduced.

No differences in bacterial numbers are seen in *X. c.* pv. *armoraciae* and its *hrp* mutant at 8, 12, and 24 hr after inoculation. This may suggest that, in this incompatible interaction, BGL transcription is triggered directly by the *hrp* gene products or by molecules secreted from the bacteria by an *hrp*-encoded process (Gough *et al.* 1992; Fenselau *et al.* 1992). BGL transcript accumulation does not appear to be a consequence of the HR collapse, since accumulation precedes the appearance of macroscopically visible symptoms, which are first detectable at 12 hr and reach completion at 24 hr after inoculation.

These results contrast with those described in other plant-bacterial interactions. Lummerzheim *et al.* (1993) have es-

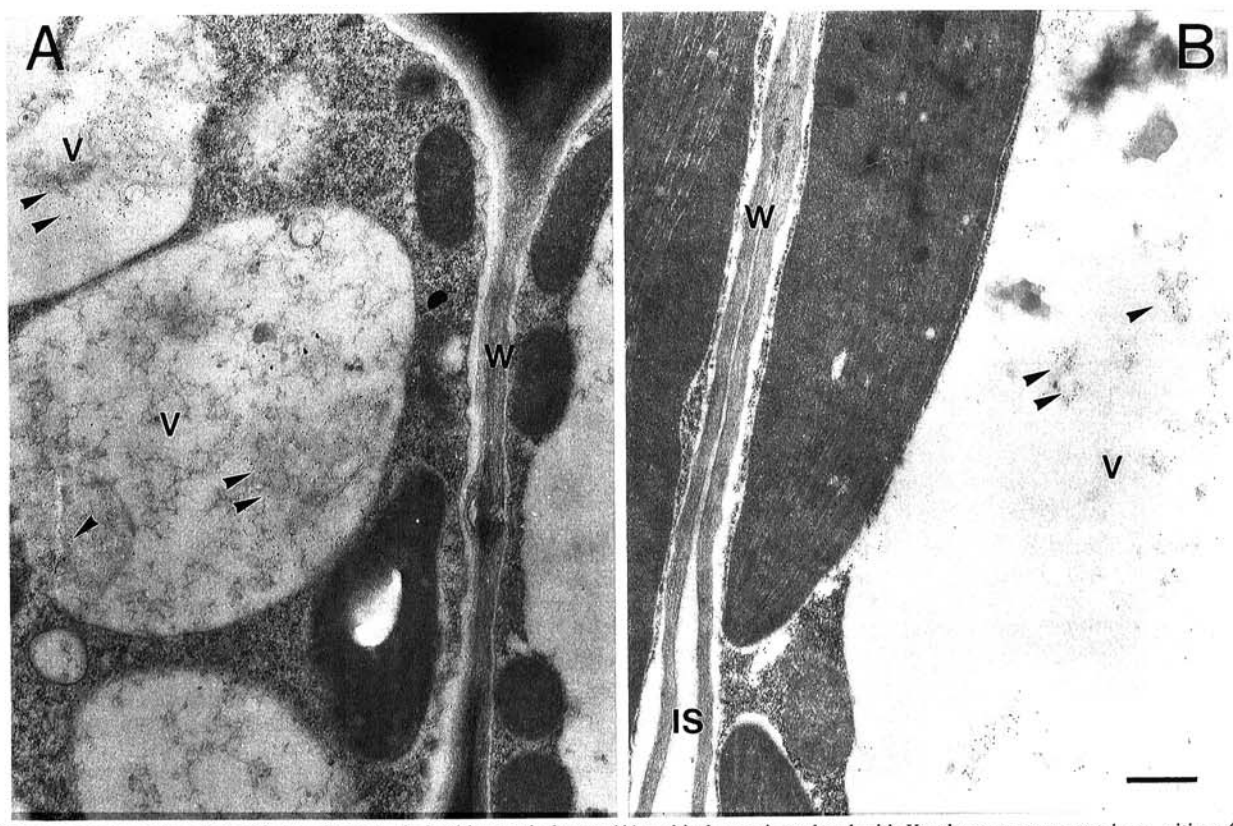

Fig. 6. Immunogold localization of β-1,3-glucanase in healthy turnip leaves (**A**) and in leaves inoculated with *Xanthomonas campestris* pv. *vitians* (**B**). In both cases the majority of the gold particles (arrows) are associated with the plant vacuole (V) and not with the plant cell wall (W) or the intercellular spaces (IS). Both micrographs are at the same magnification (×10 000; bar = 500 nm).

tablished that *X. c.* pv. *campestris* strain 8004 is compatible with *A. thaliana,* whereas *X. c.* pv. *campestris* strain 147 is incompatible. In the incompatible interaction there is a late induction of the bgl2 transcript, 48 hr after inoculation, whereas the compatible strain gives a very weak induction of bgl2, again at 48 hr. However, the *hrp* deletion mutant 8420, which grew as well as the wild type, did not induce bgl2 or any other defense-related transcripts tested (phenylalanine ammonia lyase, chitinase, and peroxidase). The effects of deletion of the *hrp* gene cluster from strain 147 were not reported. Jakobek and Lindgren (1993) demonstrated that an *hrp* mutant of *P. s.* pv. *tabaci,* which was incompatible with bean, was still able to induce defense-related transcripts (for phenylalanine ammonia lyase, chalcone synthase, and chitinase) in bean to the same level as that induced by the wild type.

Induction of BGL in response to mutants of *X. c.* pv. *campestris* defective in extracellular enzyme production.

Extracellular enzymes have been implicated as pathogenicity determinants in a number of phytopathogenic bacteria (reviewed by Daniels *et al.* [1988]). Paradoxically, some of these (in particular, pectic enzymes), have been shown to induce a number of plant defense responses, including the production of phytoalexins (Davis *et al.* 1984; Hahlbrock and Scheel 1987), phenylalanine ammonia lyase (Yang *et al.* 1992), and BGL (Davis and Ausubel 1989). Most recently Palva *et al.* (1993) have shown that BGL can be induced in tobacco in response to *Erwinia carotovora* subsp. *carotovora* infection and pectic enzymes of *E. c.* subsp. *carotovora.* Enzyme export–defective mutants of *E. c.* subsp. *carotovora* were unable to grow *in planta* and caused no induction of BGL transcript. The weak and transient induction of BGL in response to the wild-type bacteria has led to the suggestion that, in this system, the wild-type bacteria suppress the plant response (Palva *et al.* 1993). One implication of the work on the *E. c.* subsp. *carotovora*–tobacco system is that extracellular enzymes are the sole determinants for triggering the plant defense response.

In our system, BGL transcript accumulation in response to strains defective in the production of specific extracellular enzymes (endoglucanase or protease) or pleiotropically defective in all tested extracellular enzymes, including pectic enzymes (export-defective or regulatory mutants), did not differ considerably from the wild-type response in the first 24 hr after inoculation. Transcript levels after 48 hr, however, lower than those seen with the wild type. These results are consistent with a role for the *Xanthomonas* enzymes in the later phases of this compatible interaction with *Brassica.* We do not know the time course of extracellular enzyme production in *X. c.* pv. *campestris*–infected *Brassica* leaves. However, in a compatible interaction between *Brassica* and *X. c.* pv. *armoraciae,* bacterial protease production can only be detected at 48 hr after inoculation with bacteria at 10^8 cfu/ml (an inoculum level 10-fold higher than that used here), although it rises dramatically after this time (Dow *et al.* 1993). The differences between our results and those of Palva *et al.* (1993) may be a consequence of the considerable difference in growth of the export-defective mutants of *Erwinia* and *Xanthomonas* in their respective hosts.

Induction of BGL transcript in response to *eps* and *lps* mutants of *X. c.* pv. *campestris*.

For both the *eps* mutant strain 8397 and the LPS-defective mutant strain 8530, the bacterial numbers were up to three orders of magnitude lower than those seen with the wild type, although BGL transcript levels were between 70 and 80% of that seen with the wild type. These results demonstrate that EPS and LPS of *X. c.* pv. *campestris* have a role in bacterial survival and growth *in planta* and suggest that one aspect of this role is to mask the bacterial presence to prevent induction of defense-related genes. Similar conclusions were drawn for the role of EPS-I in the symbiotic relationship between *Rhizobium meliloti* and alfalfa (Niehaus *et al.* 1993) and for LPS in the *R. leguminosarum*–pea interaction (Brewin *et al.* 1993). *Xanthomonas* genes involved primarily in EPS biosynthesis have not previously been reported to affect virulence. Recently, however, Kingsley *et al.* (1993) have characterized a locus in *X. c.* pv. *citrumelo* where mutations affect both the biosynthesis of EPS and LPS and virulence. Since at least some of the mutant bacteria appear to be killed in the plant, it is also possible that products released from these dead bacteria trigger the responses seen. This seems less likely, since bacteria killed by heat treatment or by antibiotics do not induce BGL. Although isolated EPS from *Xanthomonas* is not thought to induce plant defense responses (Fett and Osman 1985), isolated LPS preparations have been shown to be active as weak elicitors of phytoalexin accumulation (Barton-Willis *et al.* 1984) and as inducers of increased resistance to bacteria in a number of plants (Mazzucchi *et al.* 1979). Further work will address the question of whether isolated LPS from wild-type and mutant *X. c.* pv. *campestris* can induce BGL transcription in *Brassica.*

Our results together with those of others cited in this paper suggest that defense-related gene expression can be triggered by a number of bacterial gene products. Although some of our results differ from those of other workers using different plant-bacterial interactions, we believe that strict parallels between different pathosystems should not necessarily be expected. Variation in the sensitivity of different plants to the array of bacterial products presented to them could readily account for the differences reported. In addition, the biology of the infection process is different for the different bacteria. It must always be borne in mind, however, that effects of mutation of some pathogenicity genes on defense gene induction may be indirect, affecting processes critical to the growth and survival of the pathogen rather than altering or eliminating a component that directly influences plant gene expression.

MATERIALS AND METHODS

Growth of bacteria and inoculation of plants.

Bacterial strains used in this study are listed in Table 1. *Xanthomonas* cultures were grown overnight in NYGB medium and *E. coli* in L broth (Daniels *et al.* 1984). The bacteria were harvested by centrifugation and resuspended in sterile distilled water to a final concentration of 10^7 cfu ml^{-1}. These suspensions were introduced in a patchwise fashion into mature leaves of 4- to 5-wk-old turnip plants of the cultivar Just Right, and the plants were maintained at 25° C with 15 hr of lighting as described previously (Conrads-Strauch *et al.*

1990). The numbers of viable bacteria in leaves were determined by punching leaf disks from the middle of the inoculated area (with a cork borer, 0.4 cm in diameter), homogenizing the disks in distilled water, and plating serial dilutions on NYGA plates containing the appropriate antibiotic. The growth data for each strain is an average value determined for four different leaves at each time point. In order to directly compare bacterial growth, symptoms, and defense gene induction in the host, the same bacterial inoculation levels were used for each type of experiment.

BGL purification, peptide sequencing, and antisera.

BGL was purified from turnip leaves inoculated with *X. c.* pv. *vitians* as described by Conrads-Strauch *et al.* (1990). Peptides were derived from the mature protein by treatment with *N*-chlorosuccinimide (Lischwe and Ochs 1982), separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis on 11% gels, and blotted onto Immobilon-P polyvinylidene difluoride membrane. Sequencing was performed by the Protein Sequencing Facility of the Department of Biochemistry, University of Cambridge. Antisera to the purified protein were raised in rats. In Western blots of total extracts of healthy and *X. c.* pv. *vitians*–inoculated turnip leaves the antisera recognized a single band of the same molecular weight as the purified protein. Glycosylation of the protein was examined with the DIG glycan detection kit (Boehringer, Mannheim, Germany).

Immunoelectron microscopy.

Segments of leaf material (approximately 2 mm square) were cut with a razor blade from healthy tissue and tissue immediately surrounding the collapsed area in *X. c.* pv. *vitians*–inoculated leaves. These samples were fixed as described by Scofield *et al.* (1992). Dehydration and resin infiltration were carried out at –20° C as described by Wells (1985). Procedures for polymerization of the resin, sectioning, immunogold labeling, and staining with uranyl acetate and lead citrate are all described in Scofield *et al.* (1992). The anti-glucanase antiserum was used at a 1:200 or 1:500 dilution. The grids were examined using a JEM-1200EX transmission electron microscope (JEOL U.K., Welwyn Garden City, Herts., U.K.)

cDNA clone for BGL.

Antisera were used to screen a cDNA library from *X. c.* pv. *vitians*–inoculated leaves which was constructed in lambda-Zap (Stratagene, La Jolla, CA) according to the protocol recommended by the manufacturer. Several positive clones were identified and sequenced by the dideoxy method with the use of a Sequenase kit (United States Biochemical, Corp., Cleveland, OH). All the clones had the same sequence but were of different lengths. Both strands of the longest clone were sequenced to give the sequence shown in Figure 1. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession number X77990.

Southern blotting.

Turnip DNA was extracted with urea extraction buffer and purified by CsCl–ethidium bromide gradient centrifugation as described by Shure *et al.* (1983). Purified DNA was digested with a range of restriction enzymes, and the fragments were separated on agarose gels, transferred to Genescreen (New England Nuclear, Boston, MA), and probed with the full-length BGL probe, which was labeled with ^{32}P by means of a random priming kit (Amersham, Little Chalfont, U.K.). Highly stringent conditions were used, with final washes at 65° C with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 1% (w/v) SDS.

RNA isolation and characterization.

Total RNA was extracted from leaf material as described by Collinge *et al.* (1987) at various time points after inoculation. For Northern blots, RNA samples (10 μg of total RNA) were separated on formaldehyde-agarose gels and transferred to Hybond-N nylon membranes (Amersham) according to the manufacturer's protocol. The integrity of the RNA was assessed by visualization of ribosomal RNA with ethidium bromide staining.

The filters were prehybridized in 6× SSC , 10× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone plus 0.02% bovine serum albumin), 1% (w/v) SDS, and 50 μg of salmon sperm DNA per milliliter at 65° C for 2 hr. ^{32}P-labeled BGL probes were synthesized with a random priming kit (Amersham), and hybridization with the denatured probe was carried out overnight in 6× SSC and 1% (w/v) SDS at 65° C. After hybridization, the filters were washed with 6× SSC and 0.1% (w/v) SDS for 5 min at room temperature, then with 1× SSC and 1% (w/v) SDS for 20 min at 65° C, and finally with 0.1× SSC and 1% (w/v) SDS for 1 hr at 65° C.

Autoradiography was performed with Fuji X-ray film and an intensifier screen at –70° C. Alternatively, the hybridized filters were exposed on a Fuji Imaging Plate and analyzed on a FUJIX BAS 1000 Bio-Imaging Analyzer. The amount of [^{32}P]-BGL probe hybridized to the filter was quantified after defining the region on the image (the BGL mRNA band on the Northern) to be analyzed. To directly compare the levels of transcript, Northern blots of all the RNA preparations were hybridized at the same time and exposed to the Fuji Imaging Plate for the same period. This procedure was repeated twice with essentially the same result. One of these experiments is presented in detail. Furthermore the whole experiment (with a different set of inoculations) was repeated with essentially the same outcome.

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