Soluble Cell Wall Compounds from Carrot Roots Induce the *picA* and *pgl* Loci of *Agrobacterium tumefaciens*

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Crude extracts from carrot roots induce the *picA* chromosomal locus of *Agrobacterium tumefaciens*. The inducer is a complex pectic polysaccharide whose activity can be destroyed by incubation with a highly purified cloned pectate lyase (PelE protein of *Erwinia chrysanthemi* EC16). Oligogalacturonates of degree of polymerization (dp) 6–17 induce the *picA* locus, with peak activity at dp 8. However, the inducing compound partially purified from carrot root extracts is about 100-fold more active (per uronic acid content) than is the most active oligogalacturonate in inducing the *picA* locus. Chemical linkage analysis of a QAE Sephadex fraction containing peak inducing activity from the carrot extract indicates that it contains a complex mixture of acidic and neutral sugars. The inducer is not simply a rhamnogalacturonan but requires both arabinose and galacturonic acid for activity. De-esterification by alkali treatment of the carrot inducer also increases activity. Partially purified inducing compound from carrot root extracts can induce the *picA* locus at galacturonate concentrations of 5–10 μM, suggesting that it may act as a signal molecule from the plant. Compounds from carrot root extracts also induce the *pgl* locus, which encodes a predicted protein with homology to known polygalacturonases.

Additional keywords: pectin, plant cell walls, plant signal molecules.

Crude extracts from carrot roots induce the *picA* locus on the *Agrobacterium tumefaciens* chromosome. The locus was initially identified by MudI1681 transposon mutagenesis (Rong *et al.* 1990) and subsequently characterized by DNA sequence analysis (Rong *et al.* 1991). Another gene, called *pgl*, which encodes a predicted protein with considerable (67–72% conserved amino acid) homology to polygalacturonases from several bacterial and plant species, is located directly upstream of the *picA* locus (Rong *et al.* 1991). We previously showed that the inducing activity in crude carrot root extracts is sensitive to digestion by pectinase (Worthington), a purified polygalacturonase, and is, therefore, most likely derived from the pectic portion of plant cell walls (Rong *et al.* 1990). PicA is only one of at least 10 proteins that is induced in *A. tumefaciens* cells incubated in media containing crude extracts from carrot roots (Rong *et al.* 1990).

We report here the partial purification and characterization of the inducer found in extracts from carrot roots. The compound is a complex pectic polysaccharide and induces both the *picA* and the *pgl* loci. Although oligogalacturonates of degree of polymerization (dp) 6–17 are effective in inducing the *picA* locus, the inducing compound from carrot root extracts is approximately 100-fold more potent (per uronic acid content) in inducing *picA* expression. We speculate that it is a signal molecule from plant cells that defines a new regulon of *A. tumefaciens* chromosomal genes.

RESULTS

Acetone precipitation of the inducer in carrot root extracts.

In our original experiments, epidermal shavings from the tips of fresh carrot roots were used to prepare crude carrot root extracts that gave the best induction of β-galactosidase activity (a reporter enzyme to measure the transcriptional activity of the *picA* locus) in *A. tumefaciens* At156 (Rong *et al.* 1990). We observed that shaving extracts prepared from market-purchased carrots gave very different degrees of induction. Because the shavings protocol was time-consuming and nonreproducible, we examined other methods for a more efficient preparation of carrot root extract. We found that acetone precipitation of homogenates of whole carrot roots resulted in a greatly enhanced and more reproducible induction of the *picA* locus. For example, the incubation of *A. tumefaciens* At156 in carrot root extract prepared from one batch of epidermal shavings resulted in 2,300 Miller units of β-galactosidase activity after acetone precipitation, but only 186 units before acetone precipitation. Similarly, incubation of *A. tumefaciens* At156 in AB glucose medium with whole carrot root homogenates resulted in 949 Miller units of β-galactosidase activity after acetone precipitation, but only 46 units before acetone precipitation. It appeared that the precipitation of material from carrot root extracts with acetone removed an activity inhibitory of *picA* induction. The identity of this inhibitor, however, has not been determined. The dif-

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ference in induction of β-galactosidase activity in extracts from epidermal shavings (2,300 units) and extracts from whole carrots (949 units) may reflect the fact that metabolically active tissues such as carrot tips contain a higher concentration of inducer per unit of weight. Regardless, acetone precipitation greatly improved our previous method for preparation of crude carrot root extracts, making it much less time-consuming and more reproducible.

Optimal degree of polymerization of PGA for induction of the *picA* locus.

We reported previously that the incubation of carrot root extracts with pectinase greatly reduced their ability to induce the *picA* locus. Treatment of the inducer with cellulase, however, did not destroy inducing activity (Rong *et al.* 1990). In addition, commercial polygalacturonic acid (PGA) at a concentration of 0.1–1.0% could act as an inducer of the *picA* locus. We also observed that monogalacturonic acid was not an inducer of the *picA* locus under the same assay conditions (data not shown). These results suggested to us that the degree of polymerization of galacturonosyl units determines the extent of induction of the *picA* locus. We therefore used oligogalacturonate mixtures of DEAE Sephadex chromatography–fractionated PGA to induce β-galactosidase activity in *A. tumefaciens* At156. Figure 1 shows that trimers and tetramers were not good inducers. Neither were oligomers of dp greater than 15. The fraction eluted with 350 mM KCl induced the *picA* locus of *A. tumefaciens* At156 to the greatest extent. This fraction contained mostly oligomers of dp 5–12.

We next tested a number of individual oligomers (dp 3–19) of PGA for their ability to induce the *picA* locus. Figure 2 shows that PGA oligomers of dp less than 6 or greater than 17 were not effective inducers of the *picA* locus. However, oligomers of dp 6–17 were effective inducers. Induction assays using individual oligogalacturonides of dp 6–9 (separated by high-pressure liquid chromatography [HPLC] as shown in Fig. 4A) indicated that the octamer was the most effective inducer (Fig. 2). These results are consistent with those described above.

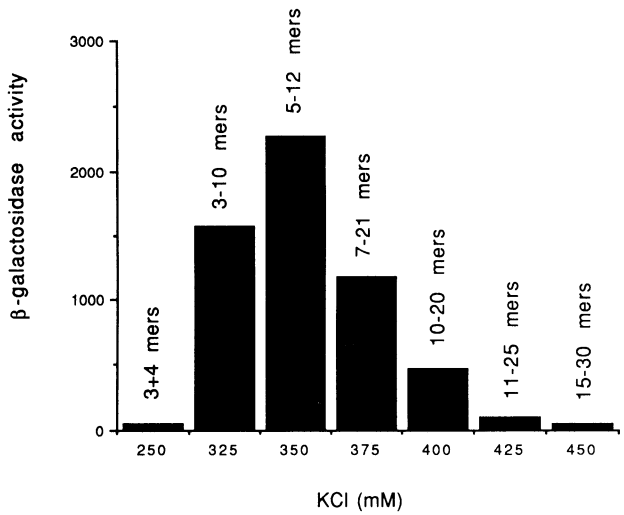

Fig. 1. β-Galactosidase activity of *Agrobacterium tumefaciens* At156 incubated with mixed galacturonic acid oligomers. *A. tumefaciens* At156 was incubated with galacturonic acid oligomers fractionated by DEAE Sephadex chromatography, and the β-galactosidase activity of the *picA*::*lacZ* fusion was determined as described in Materials and Methods. The abscissa indicates the concentration of KCl used to elute the galacturonic acid oligomers from the DEAE Sephadex column.

Purification of the *picA* inducer from carrot root extracts by QAE Sephadex chromatography.

To characterize the inducing compound or compounds in carrot root extracts responsible for the induction of the *picA* locus of *A. tumefaciens* At156, acetone-precipitated carrot root extract was subjected to QAE Sephadex A-25 anion exchange chromatography. A single peak of β-galactosidase–inducing activity appeared in fraction 24 (550 units) of the fractionated carrot root extract (Fig. 3A). Other fractions, except for fractions 25 and 26, did not induce much β-galactosidase activity (20–50 units). Except for a peak of uronic acid–containing material eluted early from the column, the induction profile of β-galactosidase activity correlated well with the uronic acid concentration profile of the fractions (Fig. 3A), which suggested that the inducer in carrot root extract contains galacturonic acid. This hypothesis was supported by a similar experiment with commercial PGA fractionated in a QAE Sephadex column (Fig. 3B). Again, a single peak of β-galactosidase–inducing activity was eluted at fraction 26 (0.3 M NaCl), and the induction profile and uronic acid concentration profile correlated well with each other. Furthermore, the fact that the inducers from carrot root extracts and commercial PGA were eluted at the same NaCl concentration indicates that, at this pH, they are similarly charged. Galacturonic acid oligomers from fraction 26 of the QAE Sephadex column were fractionated by HPLC. Figure 4B shows that the predominant sizes of oligomers from this active inducer fraction ranged from dp 6 to dp 16, confirming the results described above.

Comparison of β-galactosidase induction by carrot root extracts, PGA oligomers, and model pectins.

The inducing activity from carrot roots is not a simple oligouronide. Although PGA oligomers of specific sizes were able to induce β-galactosidase activity, the peak activities were much lower than those induced by carrot extracts.

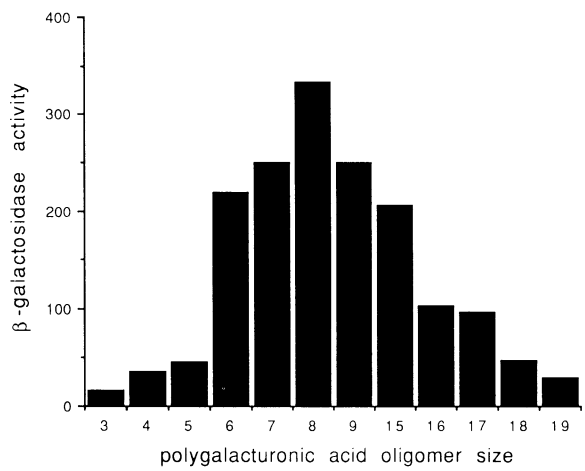

Fig. 2. β-Galactosidase activity of *Agrobacterium tumefaciens* At156 incubated with individual galacturonic acid oligomers. *A. tumefaciens* At156 was incubated with purified galacturonic acid oligomers fractionated by HPLC, and the β-galactosidase activity of the *picA*::*lacZ* fusion was determined as described in Materials and Methods.

Furthermore, the carrot extracts purified by QAE Sephadex chromatography contained only trace amounts of PGA oligomers (Fig. 4C). Unlike the HPLC-fractionated PGA oligomers, none of these fractions contained inducing activity (data not shown). This result suggests that the inducing activity from QAE-fractionated carrot roots is not a simple oligogalacturonide. Comparison of the inducing activity of the QAE Sephadex–purified carrot extracts (fraction 24) and that of the PGA oligomers (fraction 26) showed that the carrot extract was approximately 100-fold more active per uronic acid content (Fig. 5). Plant pectic polysaccharides comprise the most complex polysaccharides known, and their functional significance has only begun to be appreciated (Carpita and Gibeaut 1993). Two distinct rhamnogalacturonans are spliced into PGA, and several kinds of neutral sugar side chains are attached to many of the rhamnosyl units. We tested several model pectins fractionated from cotton (Table 1) and found that, relative to uronic acid content, the carrot extract was approximately eightfold more active than rhamnogalacturonan (RG) II in inducing the *picA* locus. Neither RG I (Table 1) nor alginic acid (data not shown) induced the *picA* locus. Even though the carrot extract was a more active inducer by two orders of magnitude than were the PGA oligomers, uronic acids were still important for activity. Incubation of purified carrot extract with pectate lyase (a cloned PelE protein from *Erwinia chrysanthemi* EC16) abolished the inducing activity completely (Table 1). Chemical reduction of uronic acids in the carrot extract or fractionated PGA solution to their respective neutral sugars also abolished inducing activity (Table 1). Selective removal of arabinosyl units by anhydrous HF at −73° C lowered the inducing activity (Table 1). Treatment of the carrot extract with pectin methylesterase did not affect the inducing activity, and de-esterification by NaOH increased the activity (Table 1). These data indicate that unesterified uronic acids and arabinosyl units are both important for full inducing activity, but the specific chemical nature of the inducing molecule is still not known.

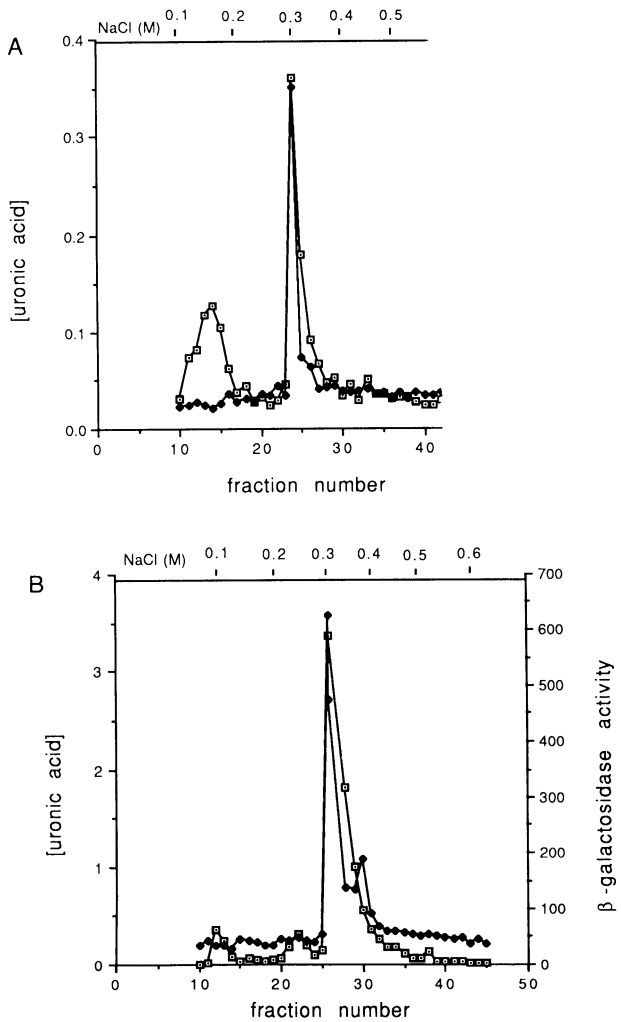

Fig. 3. Fractionation of extracts (acetone-precipitated) from carrot roots (**A**) and autoclaved polygalacturonic acid (**B**) by QAE Sephadex A-25 chromatography. Solutions were loaded onto a QAE Sephadex column, and the compounds were eluted with a linear NaCl gradient (indicated at the top of each graph). *Agrobacterium tumefaciens* At156 was incubated with individual column fractions, and the β-galactosidase activity of the *picA*::*lacZ* fusion was determined as described in Materials and Methods. □, Relative uronic acid concentration; ◆, β-galactosidase activity (Miller units).

Linkage analysis of the polysaccharides in carrot root extracts.

Methylation of the polysaccharides with peak inducing activity purified by QAE Sephadex chromatography (fraction 24) was performed after chemical reduction of carbodiimide-activated uronic acids with $NaBD_4$. Linkage analysis showed that GalA was the more abundant uronic acid (12%) and was virtually all 4-linked (Table 2). Nearly equal amounts of Rhm

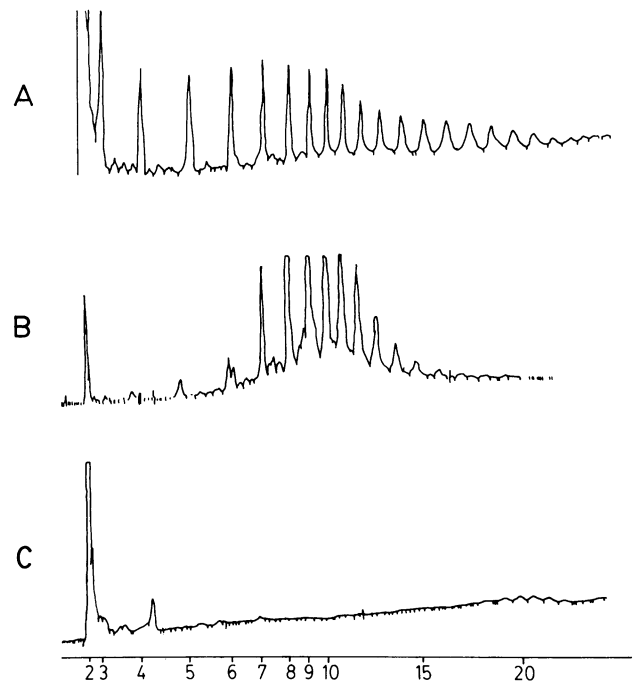

Fig. 4. High-pressure liquid chromatography fractionation of galacturonic acid oligomers from an autoclaved solution of polygalacturonic acid before QAE Sephadex chromatography (**A**), autoclaved polygalacturonic acid fraction 26 from the QAE Sephadex column (**B**), and carrot root extract fraction 24 from the QAE Sephadex column (**C**). The numbers below the profiles indicate the degree of polymerization of the galacturonic acid oligomers.

and GalA were found, indicating that RG I was a predominant uronic acid–rich polymer, its backbone constituting about 25% of the total sugar. The ratio of branched (2,4-Rhm) to unbranched (2-Rhm) rhamnosyl units was only 1:7, unusually low compared to the 1:1 ratio typical of most RG I from other plant pectins (Bacic *et al.* 1988) and the 1:2 ratio of total water-soluble pectins from carrot storage roots (Stevens and Selvendran 1984). Further, t-Rhm also constitutes a substantial portion of the total Rhm units. Given that RG I backbones are constructed of ...→4)-α-D-Gal-(1→2)-α-L-Rhm-(1→... disaccharides (Lau *et al.* 1985), our methylation analyses indicate that the RG I backbones of the carrot extract average about 10 glycosyl units (Table 2). The lack of 4-Rhm units and virtual lack of t-GalA units indicates that the RG I terminates with or is cleaved only at GalA units preceding unsubstituted Rhm units. The carrot extract, even after QAE Sephadex chromatography, is a complex mixture of polymers. Arabinosyl and galactosyl units typical of type I and type II arabinogalactans and arabinans, polymers commonly associated with RG I, make up an additional 41% of the sugar units in purified carrot extract. Linkages indicative of glucomannan (4-Man and 4-Glc), xyloglucan (t-Fuc; t- and 2-Xyl; t- and 2-Gal; and 4- and 4,6-Glc), and glucuronoarabinoxylan (t-GlcA; t-Ara; and 4-, 2,4-, and 3,4-Xyl) make up the remaining 34%.

Induction of the *pgl* locus by carrot root extracts.

To identify other *A. tumefaciens* chromosomal genes that may be inducible by extracts from carrot roots, we mutagenized *A. tumefaciens* A348 with the transposon Tn*phoA'-1*. This transposon is derived from Tn*5* and contains a promoterless *lacZ* gene near one of the IS50 elements (Wilmes-Riesenberg and Wanner 1992). Approximately 1,000 kanamycin-resistant transconjugants were plated on media containing X-Gal and either containing or lacking extracts from carrot roots (acetone-precipitated fraction). Colonies showing a more intense blue color on media containing the carrot extract were quantitatively assayed for β-galactosidase activity using ONPG and QAE Sephadex–purified carrot root extract (fraction 24). One transconjugant (*A. tumefaciens* At707) consistently showed inducible (12- to 13-fold) β-galactosidase activity (an average of 225.1 Miller units induced versus 18.0 Miller units uninduced). With the same preparation of inducer, the *picA* locus of *A. tumefaciens* At156 was induced 16- to 20-fold (an average of 385.5 Miller units induced versus 21.3 Miller units uninduced). Total DNA was isolated from *A. tumefaciens* At707 and digested with *Eco*RI, *Hin*dIII, or *Pst*I. DNA blot analysis, using as a hybridization probe cosmid 7, which contains the *A. tumefaciens* chromosomal region including the *pgl* and *picA* loci, indicated that a specific *Eco*RI–*Pst*I fragment was disrupted (data not shown). This 1,435-bp fragment is almost entirely composed of the *pgl* open reading frame (Rong *et al.* 1991). The orientation of the *lacZ* gene in Tn*phoA'-1* is the same as that of the *pgl* gene (Rong *et al.* 1991).

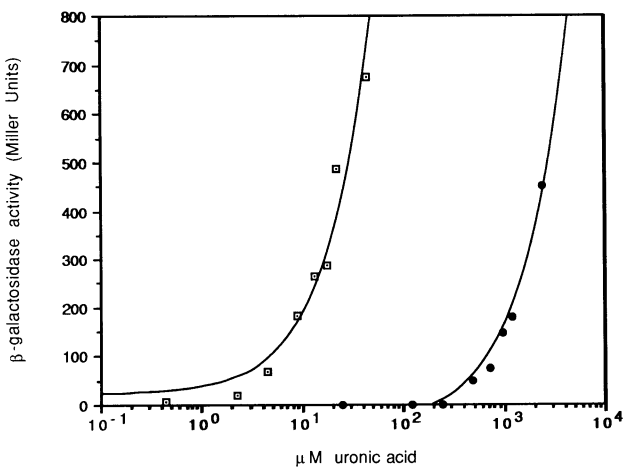

Fig. 5. Dose-response curves of β-galactosidase induction with varying concentrations of carrot root extract or polygalacturonic acid. *Agrobacterium tumefaciens* At156 was incubated with various concentrations of purified carrot root extract (QAE Sephadex fraction 24, □) or polygalacturonic acid (QAE Sephadex fraction 26, ●), and the β-galactosidase activity of the *picA*::*lacZ* fusion was determined as described in Materials and Methods.

DISCUSSION

We have partly characterized an extract of carrot roots that induces the *picA* locus of *A. tumefaciens*. We previously reported that commercial PGA induces the *picA* locus (Rong *et al.* 1990). We show here that galacturonic acid oligomers ranging from dp 6 to dp 17 are effective as inducers, with dp 8 the optimal degree of polymerization of oligomer for this induction. We substantially purified the inducer from carrot root extracts by QAE Sephadex chromatography. The prop-

Table 1. Induction of β-galactosidase activity in *Agrobacterium tumefaciens* At156 by extracts from carrot roots or by various model pectins

Treatment of carrot root extract or model pectin	β-Galactosidase activity (Miller units per nanomole of uronic acid)	(Miller units)
Carrot root extract[a]	152	
Lyophilized	76	
Lyophilized and de-esterified with NaOH	224	
Lyophilized and treated with HF at −73° C	22	
Cotton RG I[b]	0	
Cotton RG II	9.6	
Uninduced		0
Carrot root extract[a]		588
Plus PelE protein:		
3.6 μg		558
36 μg		407
360 μg		0
Uninduced		16
Polygalacturonic acid[c]		425
Reduced fraction		22
Carrot root extract[a]		532
Reduced fraction		25
Uninduced		5.5
Carrot root extract[a]		564
Plus pectin methylesterase:		
30 units		595
120 units		555
260 units		580

[a] QAE Sephadex fraction 24.
[b] RG = rhamnogalacturonan.
[c] QAE Sephadex fraction 26.

erties of this inducer suggested that it is a pectic polysaccharide. This conclusion is based upon the following observations. First, both polygalacturonase (Rong *et al.* 1990) and a highly purified cloned pectate lyase (Table 1) could destroy the inducibility of crude root extracts. Commercial polygalacturonases can contain contaminating glycosylhydrolase activities. We therefore used a highly purified cloned enzyme to establish the importance of oligogalacturonides for inducing activity. The pectate lyase encoded by the *pelE* gene of *Erwinia chrysanthemi* EC16 catalyzes the endolytic cleavage of PGA predominantly to digalacturonic acid moieties (Preston *et al.* 1992). Second, the inducer from carrot root extracts was eluted from a QAE Sephadex column in a manner virtually identical to that of the inducer from commercial PGA, which indicates that they are similarly charged. The fact that the inducer was eluted at 0.3 M NaCl is consistent with our previous conclusion that the inducing molecules in crude carrot root extracts are acidic (Rong *et al.* 1990). Third, the induction profile of the *picA* locus of *A. tumefaciens* At156 correlated very well with the concentration of uronic acid in the carrot root extract fractions after QAE Sephadex chromatography; this was also true for the induction profile of commercial PGA. However, the fact that the inducer from carrot root extracts was approximately 100-fold more potent than was the inducer from commercial PGA indicates that the inducer in carrot root extracts is different from PGA. Methylation of PGA is probably not responsible for this difference. The fractions purified from carrot root extracts by QAE Sephadex chromatography with strong inducing activity represent a broad range of oligomer sizes and are rich in both neutral sugars and uronic acid. Methylation analysis of the peak inducing fraction showed that galacturonic acid was only about 12% of the total sugar in a fraction rich in pectic RG, arabinan, and type I and type II arabinogalactans.

Although still a complex mixture, some features of the inducer are particularly noteworthy. First, the amounts of Rhm and GalA are nearly equal, which indicates that RG is enriched relative to PGA oligomers. From comparison of the ratio t-Rhm to 2- and 2,4-Rhm and an equal amount of 4-GalA, the RG backbones are about 10 residues long. The RG may have been solubilized by selective cleavage of α-D-GalA-(1→2)-α-L-Rhm–linked disaccharide units. Our finding of substantial amounts of t-Rhm but not t-GalA or 4-Rhm indicates that the α-L-Rhm-(1→4)-α-D-GalA disaccharide unit is rarely cleaved, if at all, and that attachment of side groups to Rhm protects the (1→2)-α-L-Rhm linkage. The potential induction by a specific carrot RG needs further clarification, but regardless, the structure contrasts with that of the growth-promoting lepidimoide, a disaccharide of 4,5-unsaturated α-D-GalA-(1→2)-α-L-Rhm, possibly cleaved from RG by the action of a polygalacturonate lyase and rhamnosidase (Hasegawa *et al.* 1992).

The side groups of the Rhm units are diverse (Table 2). Cotton RG II has greater inducing activity than does RG I. Selective cleavage of arabinosyl units from the carrot RG also results in a loss of inducer activity. Additional work will be necessary to determine the precise structure of carrot RG I, RG II, or any of the other minor polymeric constituents that may possess the true activity.

The compound or compounds purified from carrot root extracts by QAE Sephadex chromatography could induce the *picA* locus at a galacturonate equivalent concentration of 5–10 μM (Fig. 5). The most active polygalacturonate oligomer (the octamer) may represent the molecular size of the active component of the complex pectic inducer. The inducer may have a formula weight considerably greater than that of this most active octamer region, and hence the effective concentration must be lower than 1 μM. *A. tumefaciens* A136, the wild-type parental strain of At156, cannot utilize commercial PGA as a sole carbon source for growth (L. Rong and S. Gelvin, unpublished). Because the inducer from carrot root extracts can induce the *picA* locus at such a low concentration, we hypothesize that the inducer is a signal molecule, rather than merely a metabolite. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins from *A. tumefaciens* A136 suggests that the *picA* locus is just one of many *A. tumefaciens* genes that responds to this plant signal (Rong *et al.* 1990). Transposon mutagenesis of the *A. tumefaciens* A348 chromosome identified another locus that was inducible by purified pectic compounds from the extracts of carrot roots. DNA blot analysis identified this gene as *pgl*, which is located directly upstream from *picA*. Although the *pgl* and *picA* genes are transcribed in the same direction, a transcription start site of *picA* has been localized to a region down-

Table 2. Linkage analysis of the carrot inducing factor enriched by QAE Sephadex chromatography

Sugar and linkage[a]	mole %[b]
t-Fuc	1.6
t-Rhm	1.8
2-Rhm	9.1
2,4-Rhm	1.2
t-Ara	6.5
2-Ara	0.4
3-Ara	3.0
5-Ara	1.1
2,5-Ara	tr
3,5-Ara	tr
t-Xyl	1.3
2-Xyl	0.4
4-Xyl	4.1
2,4-Xyl	0.3
3,4-Xyl	tr
4-Man	5.7
6-Man	7.5
2,4-Man	0.5
t-Gal	5.0
2-Gal	1.8
3-Gal	0.7
4-Gal	0.5
6-Gal	4.6
3,4-Gal	3.0
3,6-Gal	3.4
4,6-Gal	1.3
t-GalA	0.3
4-GalA	11.7
t-Glc	1.7
2-Glc	0.3
4-Glc	14.6
4,6-Glc	3.1
t-GlcA	0.3
4-GlcA	3.0

[a] Uronic acids were determined by mass spectrometry of their 6,6-dideuterio alditols after chemical reduction with $NaBD_4$.
[b] tr = Trace amounts.

stream of the *pgl* gene (Rong *et al.* 1991). Thus, we consider it unlikely that *pgl* and *picA* form an operon. Rather, it is more likely that they belong to a regulon of genes that responds to the same plant signal. Mutagenesis of cosmid 7 (containing the *picA* and *pgl* loci and the surrounding DNA) with the *lacZ* fusion transposon Tn*3*-HoHo1 has so far failed to reveal any other pectin-inducible genes in the vicinity of *picA* and *pgl* (S. Gelvin, unpublished). The identification and characterization of other pectin-inducible genes in the chromosome of *A. tumefaciens* may help us understand the significance of this regulon.

It is also interesting to note that PGA derivatives from the pectic portion of plant cell walls can act both as inducers of *A. tumefaciens* genes and as elicitors of plant defense responses. The synthesis of certain proteinase inhibitors and phytoalexins, which are important for plant defense against microbial infection, is induced by PGA released from plant cell walls (Bruce and West 1982; Lee and West 1981a,b; Walker-Simmons *et al.* 1983, 1984). Furthermore, this induction is dependent upon the size of galacturonic acid oligomers. The synthesis of proteinase inhibitors is induced by galacturonic acid oligomers of dp less than 10, whereas the synthesis of some phytoalexins is induced by larger fragments of PGA (Nothnagel *et al.* 1983). Carrot extracts contain carbohydrate inducers that are at least two orders of magnitude more active than galacturonic acid oligomers (Fig. 5). Galacturonic acids and arabinofuranosyl units are apparently both integral to activity. Hence, the carrot extract may contain complex oligomeric signals that are far more active than are simple oligogalacturonides.

The view of the cell wall as a responsive structure, particularly with respect to it as a source of signal molecules, will continue to be bolstered by new discoveries. It is possible that similar pectin-derived signals in the environment are recognized both by plants and by *A. tumefaciens*, but serve different purposes. For *A. tumefaciens*, the presence of such molecules may signal a favorable condition for bacterial infection and result in the activation of genes that allow the bacteria to take advantage of such environments. For plant hosts, on the other hand, such molecules may signal microbial invasion and result in the activation of host defense systems.

MATERIALS AND METHODS

Bacterial strains, growth and induction conditions, and β-galactosidase activity assays.

A. tumefaciens At156 was used to monitor the induction of the *picA* locus. This strain contains a *picA*::*lacZ* transcriptional fusion, created by transposon MudI1681 mutagenesis, that is inducible 50- to 100-fold by crude extracts from carrot roots (Rong *et al.* 1990). *A. tumefaciens* At156 was grown in AB minimal medium (Lichtenstein and Draper 1986) containing glucose (0.5%) and kanamycin (100 μg/ml). For β-galactosidase activity assays, *A. tumefaciens* At156 was grown to mid-log phase, diluted 100-fold into media containing kanamycin (100 μg/ml) and various inducing compounds, and incubated at 30° C for 20 hr. β-Galactosidase activity was measured as previously described (Miller 1972).

Preparation of extracts from carrot roots.

Carrot roots from a local market were soaked in a dilute detergent solution, washed (with a plastic scrub pad) in deionized water, ground in a blender, and shaken at 60 rpm in sterile AB minimal medium plus 0.5% glucose (0.1 g of carrot per milliliter of AB minimal medium) at room temperature for 6–8 hr. The extract was first passed through a Büchner funnel and then filtered through one layer of Whatman 3 MM paper three times. The crude carrot root extract was mixed with acetone at a ratio of 100 ml of crude carrot root extract to 900 ml of acetone and incubated at 4° C overnight. The supernatant solution was decanted, the remaining acetone was evaporated, and the thin layer of precipitate on the bottom of the flask was suspended in 50 ml of 10 mM sodium phosphate buffer (pH 7.0). This extract was dialyzed against either deionized water or 10 mM sodium phosphate buffer for 24 hr at 4° C; it was then centrifuged for 10 min at 8,000 rpm. The pellet was discarded, and the supernatant solution was stored at –20° C.

Preparation of commercial PGA.

A 1.0% (w/v) PGA solution was prepared as follows: 1) PGA (Polysciences, Inc.) was dissolved in AB minimal glucose medium, the pH adjusted to 7.0 with NaOH, and the solution filter-sterilized, or 2) PGA was dissolved in water (pH adjusted to 4.5 with NaOH) and autoclaved at 121° C for 40 min.

Mixed oligomeric and polymeric sizes of uronans from PGA were purified by DEAE Sephadex chromatography and eluted stepwise with KCl solutions of 250, 325, 350, 375, 400, 425, and 450 mM. The fractions were dialyzed and lyophilized. The purity of each fraction was determined by uronic acid analysis, and the degree of polymerization of the galacturonic acid oligomers in each fraction was determined by HPLC. These fractions were added separately to AB glucose medium at a concentration of 0.1% (w/v) for β-galactosidase activity assays using *A. tumefaciens* At156. Galacturonic acid oligomers of dp 3–19 were similarly used for β-galactosidase activity assays. Because of their limited quantity, only a 0.02% solution was made for each of these oligomers.

Separation of uronide oligomers by HPLC.

An oligomeric and polymeric uronate series was generated from PGA (Polysciences, Inc.). Two grams of PGA was suspended in about 70 ml of water and brought into solution by adjustment of the pH to 4.0 with NaOH. The volume was brought to 100 ml (2%, w/v) and autoclaved at 121° C for 45 min. Samples of the autoclaved PGA solution were taken for separation of the active components by QAE Sephadex chromatography.

The oligomeric and polymeric uronate series, ranging from dp 3 to dp 25, were separated according to a method modified from that of Hotchkiss and Hicks (1990). Autoclaved PGA solution (25 μl) was loaded on a CarboPac PA-1 anion exchange column (0.4 × 25 cm) (Dionex, Sunnyvale, CA), equilibrated in 0.45 M Na acetate (pH 5.0) at 0.8 ml/min, and eluted in a linear-step gradient produced by the following program: t (min) = 0, 0.45 M Na acetate; t = 2, 0.45 M Na acetate; t = 30, 0.8 M Na acetate; t = 60, 0.9 M Na acetate, t = 70, 1.0 M Na acetate; t = 80, 1.0 M Na acetate. The sugars were assayed following postcolumn addition of 0.5 M NaOH (0.5 ml/min) by pulsed amperometric detection (Dionex). For large-scale production of specific oligomers, 1-ml fractions

from four separate 0.5-ml injections of the autoclaved PGA solution were collected without postcolumn addition of alkali and dialyzed against water, and each fraction was tested for β-galactosidase activity in *A. tumefaciens* At156. The degree of polymerization of each fraction was verified by comparison of total sugar (DuBois *et al.* 1956) to reducing end. The active fractions were reassayed by HPLC as described above to verify the size of the most active oligomers.

QAE Sephadex chromatography of carrot root extracts and PGA.

QAE Sephadex A-25 anion exchanger (Sigma Chemical Company, St. Louis) was treated with 0.2 M NaCl (in 10 mM sodium phosphate buffer, pH 7.0) for 10 min. An excess of phosphate buffer was used for swelling, either at room temperature for 24 hr or in a boiling water bath for 2 hr, with frequent changes of the supernatant solution. A column (1.6 × 33 cm) was packed with the resin and equilibrated with phosphate buffer until the pH of the eluted solution reached 7.0. About 35–40 ml of carrot root extract (in phosphate buffer after acetone precipitation) was applied to the column. The column was washed with 100 ml of phosphate buffer and eluted with a linear NaCl gradient (0–2.0 M in phosphate buffer), and 4-ml fractions were collected. Similarly, 20 ml of autoclaved 1% PGA (pH adjusted to 7.0 with NaOH) was applied to a QAE Sephadex column and eluted as described above. The conductivity of each fraction was measured to determine the NaCl concentration, with 0–2.0 M NaCl as standards.

Following QAE Sephadex chromatography, 100 μl of each fraction of either carrot root extract or 1% PGA was added to 1.9 ml of AB glucose minimal medium, and the β-galactosidase activity of *A. tumefaciens* At156 was measured (Miller 1972).

Each fraction was also assayed for total sugar concentration by a phenol-H_2SO_4 method (DuBois *et al.* 1956) and for uronic acid concentration according to a method in which sulfamate and *m*-hydroxydiphenyl were used to eliminate interference by neutral sugars (Filisetti-Cozzi and Carpita 1991).

Induction dose response assays of carrot root extract and PGA.

The QAE Sephadex fractions that induced the highest β-galactosidase activity in *A. tumefaciens* At156 (fraction 24 of carrot root extract and fraction 26 of PGA) were dialyzed against water and concentrated by rotary evaporation at –20° C. The uronic acid concentration of each fraction was determined (Filisetti-Cozzi and Carpita 1991). To assay the dose response of the bacteria to the inducers, carrot root extract fraction 24 (with uronic acid concentration in the range of 0–50 μM) and PGA fraction 26 (with uronic acid concentration in the range of 0–3 mM) were added to AB glucose minimal medium, and the β-galactosidase activity of *A. tumefaciens* At156 was determined after 20 hr incubation.

Treatment of purified carrot root extract with pectate lyase.

Purified carrot root extract (fraction 24 from the QAE Sephadex column) was adjusted to 60 mM Tris-HCl (pH 8.5), 3 mM $CaCl_2$, and incubated at 25° C for 30 min with 0, 3.6, 36, or 360 μg of purified PelE protein. PelE protein, a generous gift from Alan Collmer, was purified from the periplasmic fraction of *E. coli* containing a cloned *pelE* gene of *Erwinia chrysanthemi* EC16 (Tamaki *et al.* 1988) by isoelectric focusing on a granulated gel bed (Sheng-Yang He, personal communication). Following incubation of the carrot root extract with pectate lyase, the reaction was terminated by incubation at 68° C for 10 min, followed by the addition of additional 60 mM Tris-HCl, pH 7.0. *A. tumefaciens* At156 (1.5 ml in AB glucose minimal medium containing kanamycin [20 g/ml]) was added to 0.5 ml of treated carrot root extract and incubated at 30° C for 20 hr, and then β-galactosidase activity was determined.

Treatment of carrot root extract with pectin methylesterase or de-esterification with NaOH.

Carrot root extract after acetone precipitation (dialyzed against deionized water) was pH-adjusted to 7.5, and 30, 120, or 360 units of pectin methylesterase (Sigma) was added to different tubes containing 2 ml of carrot root extract. The reactions were incubated at 30° C for 1.5 hr, and then the enzyme was heat-inactivated. A 1-ml sample of the enzyme-treated carrot root extract was added to 1 ml of 2× AB glucose minimal medium for β-galactosidase assays of *A. tumefaciens* At156.

Compounds in acetone-precipitated and dialyzed carrot root extract were de-esterified by the addition of 1 M NaOH to a final pH of 12.0 and incubation at 0° C for 4 hr. NaOH was added during this period to maintain the pH. The solution was acidified to pH 5.2 by the addition of glacial acetic acid and then dialyzed against water.

Treatment of carrot root extract with HF.

Carrot extract was treated with anhydrous liquid HF under conditions in which only furanosyl linkages are cleaved (Qi *et al.* 1993), with the apparatus described by Mort (1983) but with extra precautions to maintain the reaction mixture at –73° C or below until the reaction was quenched.

Reduction and linkage analysis of the inducer from carrot root extract.

The active fractions of carrot root extract eluted from the QAE Sephadex column were pooled and dialyzed against deionized water. The sample was diluted to 10 ml with water, and the pH adjusted to 4.75 with dilute HCl. 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate powder (250 mg) was added, and the pH was kept at 4.75 by dropwise addition of dilute HCl (Taylor and Conrad 1972). The pH stabilized after about 2 hr, the solutions were chilled to ice temperature, and 2.5 ml of ice-cold 4 M imidazole (HCl), pH 7.0, was added (Maness *et al.* 1990). Two batches of $NaBD_4$ (200 mg each) were added to the inducer solutions, and the mixtures were stirred in an ice bath for at least 1 hr. The excess borodeuteride was destroyed by dropwise addition of glacial acetic acid, and the mixtures were dialyzed against deionized water for at least 40 hr. Samples were frozen and lyophilized.

A sample (1 mg) of the lyophilized powder was suspended in 1 ml of dry dimethyl sulfoxide (DMSO) and incubated in a sonic bath for 3 hr. The polymers were partially methylated by the method described by Kvernheim (1987) as modified by Gibeaut and Carpita (1991a). After purging the solution

with continuous dry N_2, 0.5 ml of 2.5 M *n*-butyllithium was slowly added by syringe to the suspension, and the anion was permitted to develop for 2 hr. Methyl iodide (0.5 ml) was then added dropwise, and the clear solution was stirred for an additional 1 hr. The partially methylated polymers were partitioned into chloroform, and the chloroform phase was washed extensively with water. The chloroform was evaporated under a stream of N_2, and the residue was hydrolyzed in 1.0 ml of 2 M trifluoroacetic acid containing 1 mmol *myo*-inositol (internal standard) at 120° C for 90 min in 4-ml glass vials sealed with caps lined with Teflon (PTFE). After the vials cooled to ambient temperature, 1.0 ml of *tert*-butyl alcohol was added, and the mixture was evaporated under a stream of N_2. The partially methylated monomers were then reduced with $NaBD_4$ in DMSO and acetylated with acetic anhydride in a reaction catalyzed by 1-methylimidazole, by the method described by Blakeney *et al.* (1983) as modified by Gibeaut and Carpita (1991a). Phthalate esters and other impurities were removed by partitioning with 40% methanol against CCl_4 (Gibeaut and Carpita 1991b).

The partially methylated alditol acetates were separated and identified by gas-liquid chromatography and electron-impact mass spectrometry as described by Carpita and Shea (1989). The derivatives were separated on a vitreous silica capillary column of SP-2330 (0.25 mm × 30 m) temperature-programmed from 160 to 210° C at 1.5° C/min and then to 240° C at 5° C/min with a 10-min hold at the upper temperature. A 3-µl sample in CH_2Cl_2 was on-column injected in a helium flow of 1.5 ml/min. Derivatives were identified after electron-impact mass spectrometry (Carpita and Shea 1989) with a Hewlett-Packard quadrupole MSD autotuned at 70 eV. Uronosyl units were identified as 6,6-dideuterio derivatives formed during reduction to their respective sugars with $NaBD_4$, resulting in a shift of 2 amu in appropriate primary and secondary fragments (Carpita and Shea 1989).

Isolation of RG I and RG II from cotton.

RG I and RG II were prepared from cotton suspension culture cell walls. The walls were digested with a purified endopolygalacturonase, and the solubilized material was fractionated on a Toyopearl HW50 (S) gel filtration column. The RG I eluted in the void volume, and the RG II as a distinct peak immediately following it (A. J. Mort, J. An, X. Qi, P. Komalavilas, F. Qiu, G. Otiko, P. West, and N. O. Maness, unpublished).

Transposon mutagenesis of *A. tumefaciens* A348 and screening for induction by extracts from carrot roots.

A. tumefaciens A348 (rif^r, kan^s), containing the Ti plasmid pTiA6 and the *A. tumefaciens* C58 chromosomal background (the same chromosomal background as that contained in *A. tumefaciens* At156), was mutagenized with the transposon Tn*phoA'-1* (Wilmes-Riesenberg and Wanner 1992), which contains a promoterless *lacZ* gene and a kanamycin resistance gene. A freshly grown culture of *A. tumefaciens* A348 was incubated with *E. coli* BW13696 containing Tn*phoA'-1* (rif^s, kan^r) on a yeast extract–peptone plate (Lichtenstein and Draper 1986) overnight at 30° C. The bacterial cells were washed from the plate with a 0.9% NaCl solution, and 10-fold serial dilutions were plated onto AB-sucrose plates containing rifampicin (10 µg/ml) and kanamycin (100 µg/ml). Individual colonies were patched onto AB-sucrose plates containing rifampicin, kanamycin, and X-Gal and either containing or lacking carrot root extract (acetone-precipitated fraction). The plates were incubated at 30° C for 2–3 days, and the development of blue color of the colonies was monitored. Colonies that developed a more intense blue color upon growth in the presence of carrot root extract, relative to growth in the absence of carrot root extract, were reassayed for β-galactosidase activity as described by Miller (1972) in AB-glucose medium containing or lacking QAE Sephadex–purified inducer from carrot root extracts.

Total DNA from one highly inducible colony (*A. tumefaciens* At707) was isolated and digested with *Eco*RI, *Pst*I, or *Hin*dIII, and 1 µg of the DNA was fractionated by agarose gel electrophoresis. The DNA in the gel was denatured with NaOH and was transferred to a nitrocellulose membrane. The membrane was hybridized (Gray *et al.* 1992) with ^{32}P-labeled cosmid 7 containing the *picA* and *pgl* region of the *A. tumefaciens* C58 chromosomal DNA (Rong *et al.* 1990).

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