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Oligogalacturonides Prevent Rhizogenesis in *rolB*-Transformed Tobacco Explants by Inhibiting Auxin-Induced Expression of the *rolB* Gene

Daniela Bellincampi,[a] Maura Cardarelli,[b] Daniela Zaghi,[a] Giovanna Serino,[b] Giovanni Salvi,[a] Christiane Gatz,[c] Felice Cervone,[a] Maria Maddalena Altamura,[a] Paolo Costantino,[b] and Giulia De Lorenzo [a,1]

[a] Dipartimento di Biologia Vegetale, Università di Roma "La Sapienza," Piazzale Aldo Moro 5, 00185 Rome, Italy

[b] Dipartimento di Genetica e Biologia Molecolare, Università di Roma "La Sapienza," Piazzale Aldo Moro 5, 00185 Rome, Italy

[c] Institut für Genetik, Universität Bielefeld W6, 33501 Bielefeld, Germany

Oligogalacturonides elicit several defense responses and regulate different aspects of growth and development in plants. Many of the development-related effects of oligogalacturonides appear to be amenable to an auxin antagonist activity of these oligosaccharins. To clarify the role of oligogalacturonides in antagonizing auxin, we analyzed their effect on root formation in leaf explants of tobacco harboring the plant oncogene *rolB*. We show here that oligogalacturonides are capable of inhibiting root morphogenesis driven by *rolB* in transgenic leaf explants when this process requires exogenous auxin. Because *rolB* expression is induced by auxin and dramatically alters the response to this hormone in transformed plant cells, the inhibiting effect of oligogalacturonides could be exerted on the induction of *rolB* and/or at some other auxin-requiring step(s) in rhizogenesis. We show that oligogalacturonides antagonize auxin primarily because they strongly inhibit auxin-regulated transcriptional activation of a *rolB*-β-glucuronidase gene fusion in both leaf explants and cultured leaf protoplasts. In contrast, oligogalacturonides do not inhibit rhizogenesis when *rolB* transcriptional activation is made independent of auxin, as shown by the lack of inhibition of root formation in leaf explants containing *rolB* driven by a tetracycline-inducible promoter.

INTRODUCTION

α-1,4-Linked oligogalacturonides derived from the plant cell wall have been shown to elicit several defense responses (Côté and Hahn, 1994) and to induce the transcriptional activation of defense-related genes, such as those encoding phenylalanine ammonia-lyase, chalcone synthase, and the pathogenesis-related proteins PR-1 and PR-2 (Messiaen and Van Cutsem, 1993), as well as the accumulation of polygalacturonase-inhibiting protein transcripts (Bergmann et al., 1994). Oligogalacturonides have also been reported to affect several aspects of plant growth and development (Darvill et al., 1992), such as elongation in pea stem segments (Branca et al., 1988), and flower (Marfà et al., 1991) and root organogenesis in tobacco explants (Bellincampi et al., 1993). However, the effect of oligogalacturonides on the expression of development-related genes has not been investigated.

The majority of defense responses and developmental effects exerted by oligogalacturonides require a degree of polymerization (DP) between 10 and 15; shorter oligogalacturonides generally do not exhibit any biological activity (Bellincampi et al., 1994; Côté and Hahn, 1994). The reason for the observed size dependence of the responses to oligogalacturonides is not known. It has been proposed that oligogalacturonides require 10 or more galactosyluronic acid residues to assume a biologically active conformation in solution. Evidence that oligogalacturonides undergo a conformational transition at a DP of >10 has been obtained by binding studies with a monoclonal antibody that reacts preferentially with oligogalacturonides with a DP ≥10 (Liners et al., 1992).

Many, if not all, of the effects of oligogalacturonides on plant development appear to be associated with the activity of auxin (Branca et al., 1988; Bellincampi et al., 1993, 1995) and suggest an antagonism between oligogalacturonides and auxin. The relationship between the activity of auxin and oligogalacturonides is intriguing, and the interplay between these molecules might be crucial in the regulation of growth and morphogenesis in plants. It is not clear whether the capability of oligogalacturonides to antagonize the effects of auxin is related to their ability to induce defense responses.

[1] To whom correspondence should be addressed.

To shed light on the role played by oligogalacturonides in antagonizing auxin in plant development, we analyzed the effects of oligogalacturonides on the rhizogenesis brought upon by the *Agrobacterium rhizogenes* plant oncogene *rolB*. This gene is capable of inducing root differentiation in all plants tested (Cardarelli et al., 1987; Capone et al., 1989), and its effects in transgenic plants and tissues suggest a role in enhancing auxin responsiveness (Spanò et al., 1988; Barbier-Brygoo et al., 1991; Maurel et al., 1994) of transformed cells via an as yet unknown mechanism. *rolB*, recently associated with tyrosine phosphatase activity (Filippini et al., 1996), acts as a powerful morphogene in auxin-mediated organogenesis (Altamura et al., 1994), and auxin also plays a role in its transcriptional activation. In fact, tobacco leaf protoplasts from plants containing the β-glucuronidase (*GUS*) reporter gene under the control of the *rolB* promoter show a severalfold increase in GUS activity after incubation with auxin (Maurel et al., 1990; Capone et al., 1991).

Here, we show that oligogalacturonides inhibit auxin-requiring root organogenesis in leaf explants from *rolB*-transformed tobacco and inhibit the transcriptional activation of the gene *rolB* by auxin. We also show that at least in this type of organogenesis, their auxin-antagonizing effect is exerted primarily, if not solely, on *rolB* induction. In fact, we show that oligogalacturonides have no effect on *rolB*-driven root organogenesis when *rolB* expression is under the control of a tetracycline-induced heterologous promoter.

RESULTS

Oligogalacturonides Inhibit the Formation of Roots Induced by Auxin in *rolB* Leaf Miniexplants

When cultured for 15 days on basal medium containing indoleacetic acid (IAA), leaf miniexplants—small leaf explants devoid of primary and secondary veins (Maurel, 1991)—from transgenic tobacco plants harboring *rolB* produced roots. The number of roots increased with increasing concentrations of auxin, as shown in Figure 1A. Roots could be observed only occasionally in *rolB* miniexplants cultured in the absence of IAA. In contrast, no roots formed in leaf miniexplants from untransformed SR1 tobacco plants when cultured in the absence or presence of up to 60 μM IAA (data not shown).

We tested the effect of oligogalacturonides (DP of 9 to 18) on the auxin-requiring formation of roots in *rolB* miniexplants at two different concentrations of auxin. As shown in Figure 1B, a substantially complete inhibition of root formation was observed with a concentration of oligogalacturonides as low as 0.3 μg/mL at an IAA concentration of 0.6 μM. A concentration of oligogalacturonides of 10 μg/mL was needed to obtain complete inhibition of root formation in the presence of 3 μM IAA (Figure 1B).

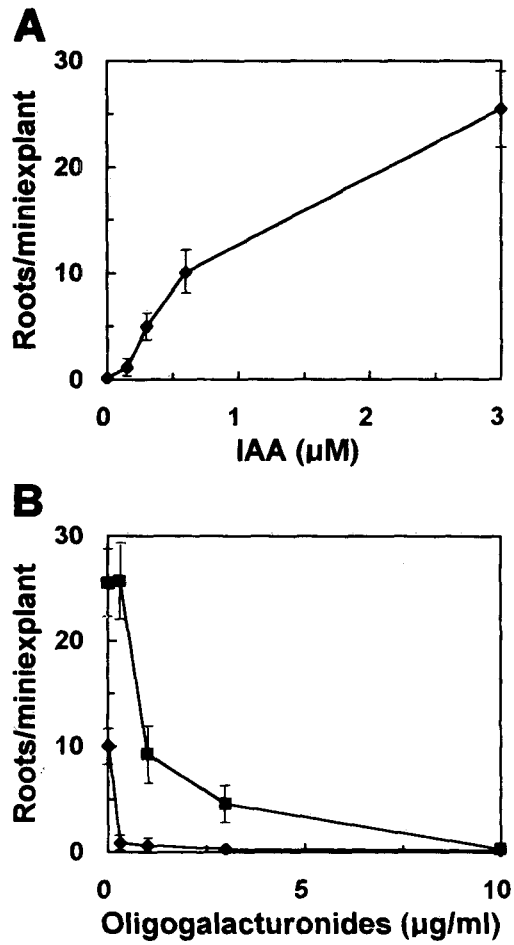

Figure 1. Effect of IAA and Oligogalacturonides (DP of 9 to 18) on the Formation of Roots in *rolB* Transgenic Tobacco Leaf Miniexplants.

(A) Dose–response curve for IAA-induced root formation.
(B) Effect of oligogalacturonides (DP of 9 to 18) on *rolB* miniexplants cultured in the presence of 0.6 μM (diamonds) or 3 μM (squares) IAA. The number of roots per miniexplant was scored after 15 days of culture. Each data point is the mean number of roots per miniexplant (± SE).

Oligogalacturonides Inhibit Auxin-Induced *GUS* Expression Driven by the *rolB* Promoter in Leaf Miniexplants

In a previous work, it was reported that leaves of transgenic tobacco plants harboring the *GUS* reporter gene under the control of the *rolB* promoter (B1185-GUS plants) (Capone et al., 1991) show detectable levels of GUS activity only in the vascular tissue of the main veins (Altamura et al., 1991a; Capone et al., 1991). Accordingly, we found a low level of GUS activity in leaf miniexplants from B1185-GUS plants both immediately after excision (121 ± 14 pmol of 4-methylumbelliferone per mg of protein per min) and after 48 hr of

incubation on auxin-free medium (118 ± 20 pmol of 4-methylumbelliferone per mg of protein per min). Figure 2A shows that after a 48-hr incubation of the B1185–GUS miniexplants with IAA, a strong induction of GUS activity occurred.

To test whether oligogalacturonides could act by inhibiting the auxin-dependent transcriptional activation of *rolB*, we measured the GUS activity of B1185–GUS leaf miniexplants incubated for 48 hr with IAA in the presence of different concentrations of oligogalacturonides (DP of 9 to 18). As shown in Figure 2B, 0.3 μg/mL oligogalacturonides inhibited by 65% the induction of GUS activity induced by auxin; inhibition was complete at a concentration of 3 μg/mL.

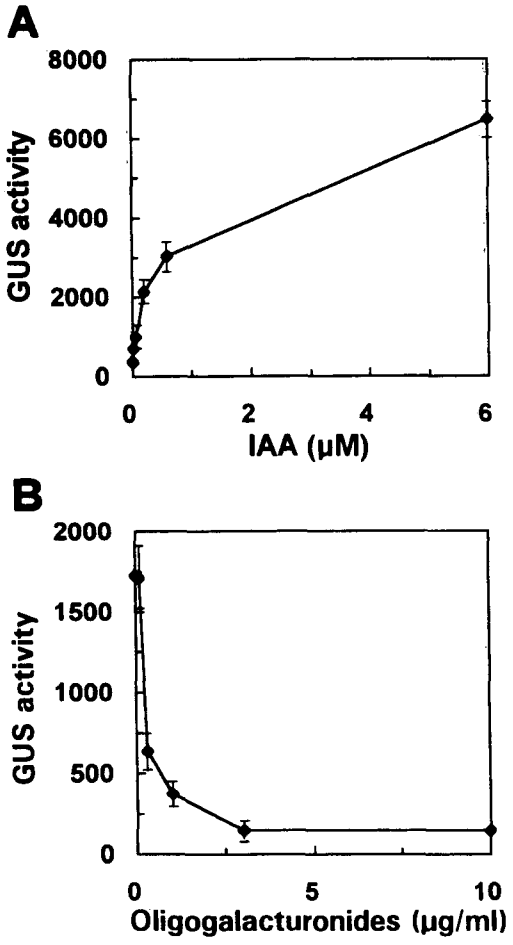

Figure 2. Auxin-Induced GUS Activity in Transgenic B1185–GUS Tobacco Leaf Miniexplants Cultured in the Presence or Absence of Oligogalacturonides.

(A) Dose–response curve for IAA-induced GUS activity. Leaf miniexplants were cultured for 48 hr in MS liquid medium at the IAA concentrations indicated.

(B) Effect of oligogalacturonides (DP of 9 to 18) on GUS activity. B1185–GUS miniexplants were cultured for 48 hr in liquid culture medium in the presence of 0.6 μM IAA and oligogalacturonides at different concentrations.

Each data point is the mean value of GUS activity (±SE) expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute.

Inhibition of Auxin Induction of the *rolB* Promoter Is Due to Size-Specific Oligogalacturonides

In contrast to the inhibitory effect of oligogalacturonides with a DP of 9 to 18, incubation of B1185–GUS miniexplants with a mixture of short oligogalacturonides (DP of 1 to 8), a mixture of oligomannuronides (DP of 9 to 20), or polygalacturonic acid at a concentration of 10 μg/mL resulted in a limited or no inhibitory effect on *GUS* expression, as shown in Figure 3A.

To identify the active oligogalacturonides in the mixture with a DP of 9 to 18, size-homogeneous oligogalacturonides were separated by high-performance anion exchange chromatography and added to B1185–GUS miniexplants at a final concentration of 1 μg/mL. As shown in Figure 3B, almost complete inhibition of *GUS* expression was obtained by adding oligogalacturonides with DP values from 11 to 16, whereas ~50% inhibition was obtained by adding oligogalacturonides with a DP of 17. In contrast, oligogalacturonides with DP values of 9, 10, and 18 exhibited limited or no inhibitory activity, confirming the well-established size requirement for the biological activity of the oligogalacturonides.

Oligogalacturonides Inhibit Auxin-Induced *GUS* Expression Driven by the *rolB* Promoter in Leaf Protoplasts

Because auxin strongly induces GUS activity in protoplasts from leaves of B1185–GUS plants (Maurel et al., 1990; Capone et al., 1991), the effects of oligogalacturonides on auxin-dependent *rolB* promoter activation were assessed further in leaf protoplasts. Freshly prepared protoplasts from B1185–GUS leaves were cultured on an IAA-containing medium in the presence or absence of oligogalacturonides. As a control for auxin-independent gene expression, leaf protoplasts from plants containing the *GUS* gene under the control of the cauliflower mosaic virus 35S promoter (35S protoplasts) were used. Figure 4 shows a time course of GUS activity in B1185–GUS protoplasts cultured with auxin up to 24 hr. The presence of oligogalacturonides inhibited the auxin-induced increase in GUS activity in these protoplasts. Concentrations of oligogalacturonides higher than those active on leaf miniexplants were necessary to inhibit *rolB* expression in protoplasts; this may be due to the presence in the suspension of residual pectinase used for protoplast preparation that would depolymerize active oligogalacturonides. In contrast, GUS activity in 35S–

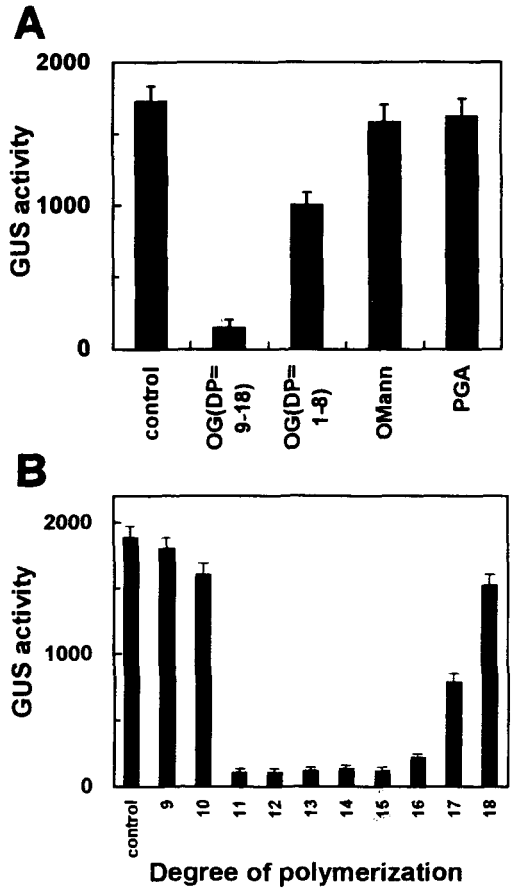

Figure 3. Auxin-Induced GUS Activity in Transgenic B1185-GUS Tobacco Leaf Miniexplants Cultured in the Presence or Absence of Different Uronides.

Shown are the effects of oligogalacturonides (OG; DP of 9 to 18), short oligogalacturonides (DP of 1 to 8), oligomannuronides (OMann; DP of 9 to 20), polygalacturonic acid (PGA), and size-homogeneous oligogalacturonides on GUS activity in B1185-GUS tobacco miniexplants. **(A)** Oligogalacturonides (DP of 9 to 18), short oligogalacturonides, oligomannuronides, and polygalacturonic acid were added separately at a concentration of 10 μg/mL to the medium containing 0.6 μM IAA. **(B)** Size-homogeneous oligogalacturonides were added separately to the liquid culture medium at a concentration of 1 μg/mL in the presence of 0.6 μM IAA.

The controls contained only IAA. GUS activity was determined after 48 hr of incubation. Each data point is the mean value of GUS activity (±SE) expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute.

GUS protoplasts did not increase when exposed to IAA and was not affected by the addition of oligogalacturonides to the medium. Figure 4 also indicates that the auxin-antagonistic effect of oligogalacturonides does not present any lag relative to the effect of auxin itself.

Oligogalacturonides Do Not Inhibit Rhizogenesis if *rolB* Expression Is Made Independent of Auxin

Leaf miniexplants from tobacco plants harboring the gene *rolB* under the control of a tetracycline-inducible derivative of the 35S promoter (pTX*rolB*/F15) (Röder et al., 1994) also were analyzed. In contrast with *rolB* miniexplants, leaf miniexplants from pTX*rolB*/F15-transformed plants did not show any root formation when cultured in the presence of auxin, as shown in Figure 5. Incubation with 0.5 μg/mL tetracycline during the first 30 hr of culture in the presence of auxin had no effect on leaf miniexplants from untransformed tobacco but resulted in root production on pTX*rolB*/F15 miniexplants. A much less conspicuous root formation was observed when pTX*rolB*/F15 miniexplants were cultured in the presence of tetracycline alone. Oligogalacturonides at concentrations as high as 100 μg/mL did not inhibit root formation in tetracycline-treated pTX*rolB*/F15 miniexplants in the absence or presence of auxin (Figure 5).

Oligogalacturonides Do Not Affect the Rate of Disappearance of IAA in the Culture Medium of Tobacco Leaf Miniexplants

To ascertain whether the observed effects of oligogalacturonides on rhizogenesis and *rolB* expression could be explained

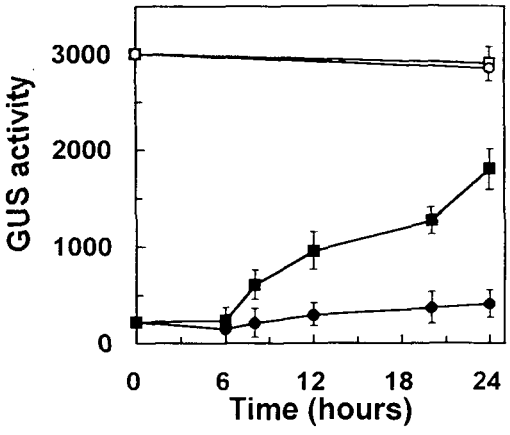

Figure 4. Effect of Oligogalacturonides (DP of 9 to 18) on GUS Activity in Mesophyll Protoplasts.

Protoplasts from B1185-GUS and 35S-GUS plants were isolated in the absence of hormones and cultured in medium containing 0.25 μM IAA in the presence or absence of oligogalacturonides (100 μg/mL). Shown are B1185-GUS protoplasts in medium containing IAA (solid squares), B1185-GUS protoplasts in medium containing IAA plus oligogalacturonides (solid circles), 35S-GUS protoplasts in medium containing IAA (open squares), and 35S-GUS protoplasts in medium containing IAA plus oligogalacturonides (open circles). Each data point is the mean value of GUS activity (±SE) expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute.

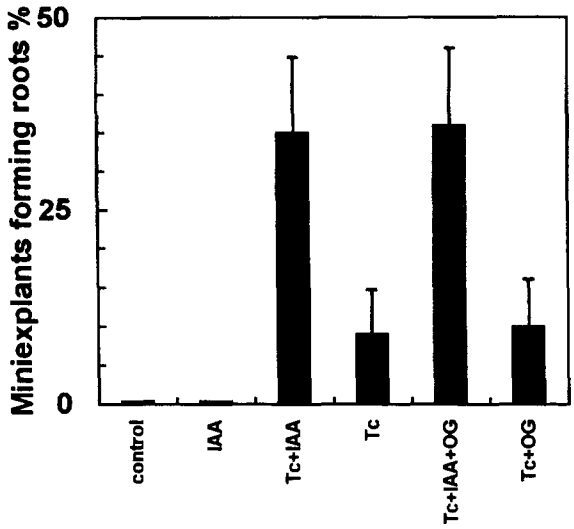

Figure 5. Effect of Oligogalacturonides (DP of 9 to 18) on Root Formation in Tobacco Miniexplants from pTX*rolB*/F15 Transgenic Plants.

Miniexplants were incubated in medium containing 0.5 μg/mL tetracycline (Tc), 0.6 μM IAA, and 100 μg/mL oligogalacturonides (OG). The control is the untransformed miniexplants incubated in medium containing tetracycline plus IAA. Each data point is the mean of the number of miniexplants forming roots (±SE).

by a faster depletion (uptake, modification, or breakdown) of IAA from the culture medium, a time course analysis of the concentration of radiolabeled ^{3}H-IAA in the medium of untransformed tobacco leaf miniexplants in the presence or absence of oligogalacturonides (DP of 9 to 18; 10 μg/mL) was performed. ^{3}H-IAA was separated by HPLC, and its radioactivity was counted. As shown in Figure 6, oligogalacturonides do not significantly affect the disappearance rate of IAA from the medium.

Histological Analysis of Root Formation in *rolB* and Untransformed Leaf Explants

Because oligogalacturonides inhibit the root formation driven by the plant oncogene *rolB* as well as that of untransformed explants (Bellincampi et al., 1993), we decided to compare the two rhizogenic processes histologically. Explants containing main veins were used, because leaf miniexplants from untransformed plants do not form roots in the presence of auxin. The dose–response curve of IAA on root formation in untransformed SR1 and *rolB* explants with main veins is shown in Figure 7A. The histological analysis was performed on untransformed and *rolB* leaf explants cultured on either basal medium or root-inducing medium (containing 0.6 μM IAA) in the presence or absence of oligogalacturonides (DP of 9 to 18; 3 μg/mL) at day 5 and at day 15, when elongated roots were evident macroscopically.

At day 5, the formation of direct meristemoids (originating from the phloem parenchyma cells of the veins; Figures 8A to 8C) is complete in both untransformed and *rolB* explants and is independent of exogenous IAA. The number of direct meristemoids was not significantly different in the two types of explants and ranged from an average of 20 to 35. Indirect meristemoids (originating from callus cells, as shown in Figure 8D) were observed only in the presence of IAA. Their number was much larger in *rolB* explants (16 ± 1.4 compared with 3 ± 0.5 observed in normal explants; $P < 0.05$). A few direct root primordia, shown in Figure 8E, were present in *rolB* explants cultured in the presence of auxin.

By day 15, as reported in Table 1, the effect of exogenously added IAA is evident in the number of indirect meristemoids in both untransformed and *rolB* explants. There were more than twice as many indirect meristemoids ($P < 0.01$) in *rolB* explants than in untransformed explants. The roots of normal explants in the presence of IAA and of *rolB* explants in the absence or presence of IAA did not exhibit significant anatomical differences. The number of indirect roots in *rolB* explants in the presence of IAA was four times higher than that of direct origin, pointing to a prevalent effect of auxin in stimulating indirect roots. Indirect meristemoids and root primordia in *rolB* explants are shown in Figure 8F.

Together, these data indicate that the differences in root organogenesis between untransformed and *rolB* leaf explants are merely quantitative and that the processes go through the same morphogenetic steps.

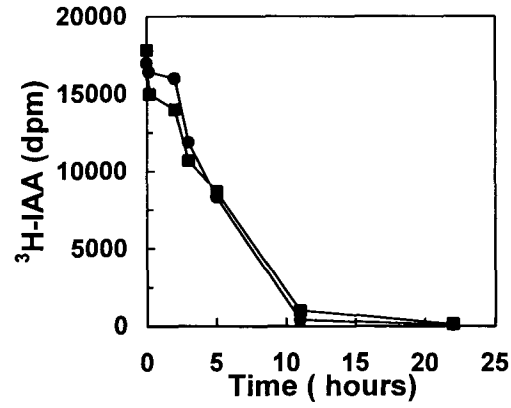

Figure 6. Effect of Oligogalacturonides on the Rate of Disappearance of ^{3}H-IAA in the Culture Medium of Leaf Miniexplants.

Leaf miniexplants were cultured for different periods of time in liquid culture medium containing ^{3}H-IAA in the absence (circles) or presence (squares) of 10 μg/mL oligogalacturonides (DP of 9 to 18). Each data point is the mean value of radioactivity counted in 50-μL aliquots of the HPLC fractions containing IAA.

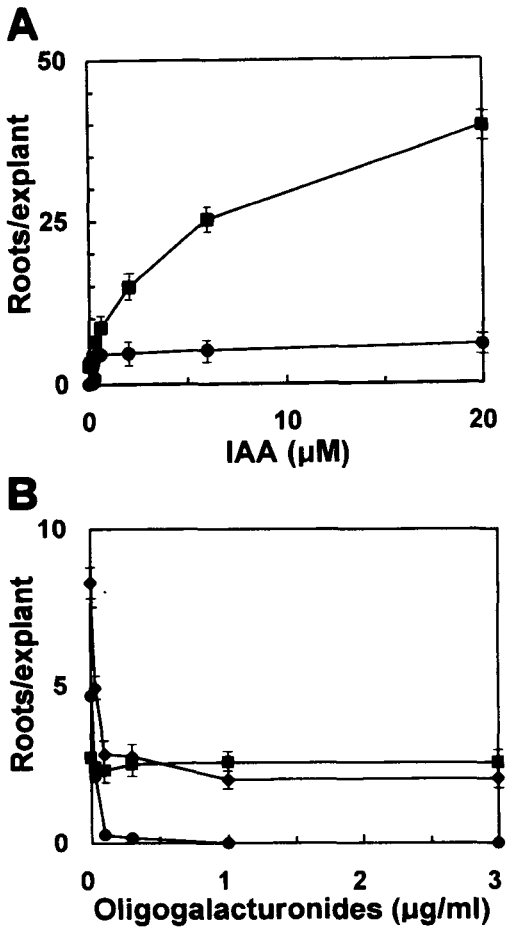

Figure 7. Effect of the Concentration of IAA and Oligogalacturonides (DP of 9 to 18) on the Formation of Roots in Untransformed and *rolB* Leaf Explants with Main Veins.

(A) Dose–response curve for IAA-induced root formation in untransformed (circles) and *rolB* (squares) tobacco leaf explants.

(B) Effect of oligogalacturonides (DP of 9 to 18) on untransformed leaf explants cultured in the presence of 0.6 μM IAA (circles) and *rolB* transgenic tobacco leaf explants cultured in the absence (squares) or presence (diamonds) of 0.6 μM IAA.

The number of roots per explant was scored after 15 days of culture. Each data point is the mean number of roots per explant (±SE).

Effects of Oligogalacturonides on Rhizogenesis of Leaf Explants

We also compared the effects of oligogalacturonides on the root formation in leaf explants with main veins from untransformed SR1, *rolB*, and pTX*rolB*/F15 plants. Figure 7B shows the dose–response curve of oligogalacturonides (DP of 9 to 18) on root formation in untransformed and *rolB* leaf explants cultured in the presence of 0.6 μM IAA. Similar concentrations of oligogalacturonides inhibited the root formation induced by IAA in both explant types. In pTX*rolB*/F15 explants, roots are induced by incubation in the presence of tetracycline alone (data not shown). In tetracycline-treated explants, the presence of oligogalacturonides (at concentrations up to 100 μg/mL) in the culture medium did not affect root formation (data not shown).

Oligogalacturonide-treated untransformed SR1 and *rolB* explants with main veins were analyzed histologically. As shown in Table 1, oligogalacturonides had no effect on the number of meristemoids (in untransformed and *rolB* explants), primordia, and roots (in *rolB* explants) formed in the absence of exogenous auxin. In contrast, oligogalacturonides completely inhibited ($P < 0.01$) the increase in the number of indirect meristemoids (in untransformed and *rolB* explants) and of primordia and roots (in *rolB* explants) induced by auxin. Oligogalacturonides also completely inhibited the formation of primordia and roots induced by auxin in untransformed explants (Table 1).

DISCUSSION

An increasing body of evidence suggests that oligogalacturonides and auxin may act in an antagonistic manner to modulate growth and morphogenesis of plant tissues (Branca et al., 1988; Darvill et al., 1992; Bellincampi et al., 1993). In this study, we show that oligogalacturonides inhibit the auxin-dependent formation of adventitious roots not only in normal leaf explants but also in explants from transgenic plants containing the oncogene *rolB* from *A. rhizogenes*. We show here that oligogalacturonides act by inhibiting the auxin-dependent transcriptional activation of *rolB* expression.

Unlike leaf miniexplants from normal tobacco, *rolB* miniexplants form roots when cultured in the presence of auxin (Maurel, 1991; this work). Root formation is inhibited by micromolar concentrations of oligogalacturonides, and the effect can be reversed by increasing the concentration of auxin.

Because the transcriptional activation of *rolB* is under the control of auxin (Maurel et al., 1990; Capone et al., 1991), we addressed whether oligogalacturonides could inhibit *rolB* induction. Oligogalacturonides with a DP between 11 and 16 completely inhibited IAA-induced expression of the *GUS* reporter gene driven by the *rolB* promoter. This size-specific effect is consistent with previous data regarding auxin-induced growth (Branca et al., 1988) and root formation (Bellincampi et al., 1993).

The auxin-antagonist action of oligogalacturonides probably is not exerted on other auxin-requiring steps downstream of *rolB* expression during rhizogenesis. Several observations support this interpretation. Oligogalacturonides do not inhibit root formation in TX*rolB*/F15 explants, in which *rolB* expres-

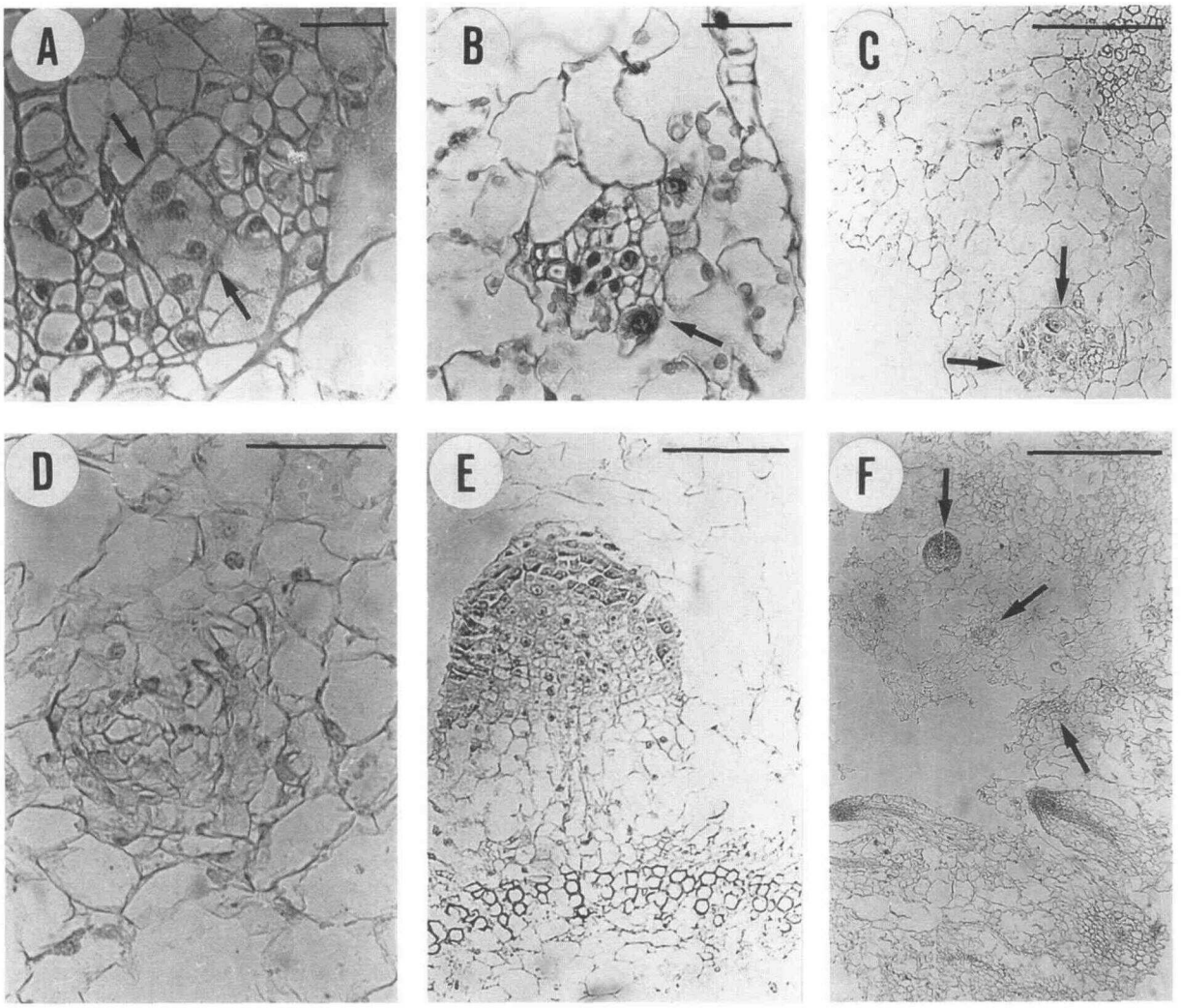

Figure 8. Histological Time Course of Root Formation from Untransformed and *rolB* Tobacco Leaf Explants with Main Veins.

(A) to **(C)** Formation of the first meristematic groups (arrows) after 5 days of culture due to the reactivation of cell division of the phloem parenchyma cells of the veins. **(A)** shows the cells of a *rolB* leaf explant cultured in basal medium. **(B)** shows cells of a normal leaf explant cultured in medium containing 0.6 μM IAA. **(C)** shows cells of meristemoids of direct genesis in normal explants cultured for 5 days in medium containing 0.6 μM IAA.
(D) Meristemoid of indirect genesis in a *rolB* leaf explant cultured for 5 days in basal medium plus 0.6 μM IAA; callus region.
(E) Root primordium of direct genesis in a *rolB* leaf explant cultured for 5 days in medium containing 0.6 μM IAA; midrib region.
(F) Meristemoids (arrows) and root primordia of indirect genesis in a *rolB* leaf explant cultured for 15 days in medium containing 0.6 μM IAA; callus region.
Bars in **(A)** and **(B)** = 30 μm; bar in **(C)** = 200 μm; bar in **(D)** = 40 μm; bar in **(E)** = 300 μm; bar in **(F)** = 800 μm.

sion is induced by tetracycline. They also do not affect rhizogenesis occurring in the absence of exogenous auxin in *rolB* midrib-containing leaf explants. These roots originate from cells of the leaf vasculature, in which auxin is present and *rolB* is strongly expressed in the intact leaf (Altamura et al., 1991a; Capone et al., 1991). Thus, in the explants, once the RolB protein is expressed, oligogalacturonides have no effect on rhizogenesis. Moreover, the timing of the effect of oligogalacturonides and auxin in normal explants also suggests the inability of these oligosaccharins to interfere with auxin-requiring steps later during rhizogenesis: auxin is necessary for a minimum of 4 days for roots to form; oligogalacturonides, however, exert their inhibitory effect only when added during the first 2 days (Bellincampi et al., 1993).

Competence for root formation has been defined as the ability of cells within tissues to form roots as a response to specific

Table 1. Formation of Roots in Untransformed SR1 and *rolB* Leaf Explants Cultured for 15 Days under Different Conditions[a]

Differentiation Stages	MS	MS + IAA	MS + OG	MS + IAA + OG
SR 1				
Meristemoids				
D	36.0 ± 2.0	33.5 ± 3.5	31.0 ± 5.0	31.5 ± 6.5
I	5.0 ± 2.0	36.5 ± 2.5[b]	2.0 ± 0	9.0 ± 0
Primordia				
D	0	3.0 ± 1.0	0	0
I	0	4.0 ± 1.0	0	0
Roots				
D	0	3.0 ± 1.0	0	0
I	0	4.5 ± 1.5	0	0
rolB				
Meristemoids				
D	43.5 ± 4.5	25.5 ± 2.5	25.0 ± 3.5	22.5 ± 2.5
I	9.5 ± 2.5	76.5 ± 2.5[b]	11.5 ± 1.5	7.5 ± 2.5
Primordia				
D	0	3.5 ± 1.5	0	0
I	1.0 ± 0	20.0 ± 2.0[b]	1.0 ± 0	0
Roots				
D	1.0 ± 0	7.0 ± 1.0[c]	1.0 ± 0	1.0 ± 0
I	1.0 ± 0	8.5 ± 2.5[b]	2.0 ± 0	1.0 ± 0

[a] The mean number of root meristemoids and root primordia with direct (D) or indirect (I) origin per explant (± SE) was scored in leaf explants cultured in MS medium alone (MS), in MS plus 0.6 μM IAA, in IAA plus oligogalacturonides (OG) (DP of 9 to 18; 3 μg/mL), and in MS plus both compounds.

[b] $P < 0.01$.

[c] $P < 0.05$.

root-inducing stimuli (Mohnen, 1994). In the leaf explants analyzed here, the gene product of *rolB* plays the role of a competence factor, because this protein confers on leaf tissue devoid of main veins the ability to go through rhizogenesis in response to an auxin-inducive stimulus. Our comparative histological analysis shows that normal and *rolB*-mediated rhizogenic processes are identical qualitatively, because adventitious roots have the same origin, their development proceeds through similar steps, and roots exhibit no obvious differences in structure. These data support the hypothesis that *rolB* mimics a physiological process leading to rhizogenesis in normal explants (and possibly plants). Thus, the conclusions drawn here on the role of oligogalacturonides on *rolB*-driven rhizogenesis may be of more general relevance.

The effect of oligogalacturonides on the auxin-dependent expression of *rolB* may be due to interference with processes along the transduction pathway leading from the perception of the auxin signal to the activation of the *rolB* promoter. It could be hypothesized that the same transduction pathway as that leading to the activation of the oncogene *rolB* is activated by auxin to induce the expression of endogenous *rolB*-like functions necessary for initiation of root formation in normal explants.

The auxin-dependent induction of the expression of the *rolB* promoter can now be used as a tool to elucidate the step(s) at which the oligogalacturonide–auxin antagonism occurs. Studies on the transduction of the oligogalacturonide signal have shown that size-specific oligogalacturonides induce several rapid cell surface responses, including the following: stimulation of a transient K^+ efflux concomitant with membrane depolarization, alkalinization of the incubation medium, and an influx of Ca^{2+} in suspension-cultured tobacco cells (Mathieu et al., 1991); elevation of cytosolic-free Ca^{2+} levels in carrot cells and protoplasts (Messiaen and Van Cutsem, 1994); in vitro phosphorylation of a 34-kD protein associated with potato and tomato plasma membranes (Farmer et al., 1990; Reymond et al., 1995); and induction of an H_2O_2-mediated oxidative burst in soybean cells (Apostol et al., 1989; Legendre et al., 1993a; Chandra and Low, 1995). H_2O_2 production is preceded by several signaling events, including activation of GTP binding proteins (Legendre et al., 1992), stimulation of phospholipase C (Legendre et al., 1993b), and changes in protein phosphorylation (Chandra and Low, 1995; Reymond et al., 1995). These rapid responses have not been correlated with any of the known plant responses induced by oligogalacturonides.

Some of these surface responses to oligogalacturonides are also likely to occur in our tobacco miniexplants. The ability of oligogalacturonides to block the action of auxin has been ascribed to the stimulation of the oxidative modification (degradation) of the hormone (Aldington and Fry, 1993). However, our results show that the rate of disappearance of IAA in the

culture medium is not affected by oligogalacturonides. The possibility that the oligogalacturonide-induced oxidative burst determines modification and consequently depletion of auxin in the medium can therefore be ruled out. Whether some or all of the early responses induced by oligogalacturonides are essential for their auxin-antagonistic activity and whether they represent common or specific initial transduction steps for defense-related and developmental effects remain to be elucidated.

METHODS

Preparation of Oligogalacturonides

Oligogalacturonides with degrees of polymerization (DP) between 1 and 8 and 9 and 18, size homogeneous oligogalacturonides, and heterodisperse oligouronides enriched in oligomannuronides (β-1,4-oligo-D-mannosyluronic acid) (DP of 9 to 20) were prepared as described by Bellincampi et al. (1993). Oligogalacturonides, oligomannuronides, and polygalacturonic acid Na^+ salt were dissolved in distilled water and sterilized by filtration through Millipore (Bedford, MA) membranes (pore size is 0.2 μm).

Plant Material

Nicotiana tabacum cv Petit Havana SR1 (Maliga et al., 1973) plants harboring the *rolB* gene under the control of a 1185-bp segment of its 5′ upstream noncoding region (*rolB*) (Capone et al., 1989) or the β-glucuronidase (*GUS*) gene fused to the same promoter (B1185–GUS) (Cardarelli et al., 1987) were used. *N. tabacum* cv Wisconsin 38 plants harboring *rolB* under the control of a modified, tetracycline-inducible promoter (pTX*rolB*/F15) (Röder et al., 1994) were propagated routinely by cuttings and grown aseptically on Murashige and Skoog (MS; Murashige and Skoog, 1962) basal medium (Sigma, St. Louis, MO) supplemented with 2% sucrose and 1% Oxoid (Unipath LTD, Basingstoke, Hampshire, UK) agar in Magenta boxes at 25°C under a 16-hr illumination period. The irradiance was 100 E μm^{-2} sec^{-1}, measured with a quantum radiometer photometer (model LI-1858; LI.COR, Lincoln, NE) equipped with a pyranometer sensor (model LI-2005B; LI.COR).

Leaf Miniexplants and Explants with Main Veins

Leaves ~5 cm long from plants 4 weeks after propagation were selected. The two apical leaves and the two basal ones were excluded. Miniexplants (squares of ~2 × 2 mm) were excised with a razor blade from leaves, carefully avoiding the primary and secondary veins, as described by Maurel (1991). Ten miniexplants were placed in each well of a multiple-well culture plate containing 5 mL of liquid MS basal medium (Sigma) supplemented with 2% sucrose and indoleacetic acid (IAA) at the concentration specified below. Explants (0.3 × 0.6–cm rectangles), with the midrib medially placed, were excised from leaves. In each experiment, 10 leaf explants were placed abaxial side down in Petri dishes containing 10 mL of MS supplemented with 2% sucrose, 0.8% agar, and IAA at the concentrations specified below. Leaf (mini)explants and explants with main veins were incubated at 25°C under low-intensity light (27 μE m^{-2} sec^{-1}) with an 8-hr illumination period. Oligogalacturonides, dissolved in distilled water at a concentration of 1 mg/mL and filter sterilized, were added to the culture medium. When appropriate, explants were incubated in the presence of 0.5 μg/mL tetracycline in the first 30 hr of culture.

The number of roots per (mini)explant or the number of explants forming roots were scored after 15 days of culture by using a dissecting microscope. Each data point was the mean value (±SE) of three independent experiments (in each experiment, three replicates of 10 leaf (mini)explants were used for each treatment). The significance of the differences between the means was evaluated using Student's *t* test.

GUS Assay in Leaf Explants

GUS activity was estimated from a pool of 30 miniexplants cultured in Petri dishes, each containing 10 explants plunged into 5 mL of liquid culture medium. Extracts were prepared from miniexplants that had been frozen in liquid nitrogen and kept at −80°C until processing. Plant tissue was scratched with liquid nitrogen and homogenized in extraction buffer (100 μL per 100 mg of tissue). The extracts were transferred to microcentrifuge tubes and centrifuged at 14,000 rpm for 10 min in a refrigerated Eppendorf microcentrifuge. Aliquots from the supernatant were incubated at 37°C for 15, 30, and 60 min in extraction buffer containing 1 mM 4-methylumbelliferyl β-D-glucuronide (Sigma). GUS activity was determined fluorometrically by using known concentrations of 4-methylumbelliferone (Sigma) with a fluorometer (Kontron, Basel, Switzerland) and expressed as picomoles of 4-methylumbelliferone per milligram of protein per minute. Protein content was determined with a commercial kit (Protein Assay; Bio-Rad, Hercules, CA), using BSA as a standard. Each data point was the mean value (±SE) of three independent experiments.

Protoplast Isolation and Culture

Mesophyll protoplasts from B1185–GUS transgenic leaves were prepared as described by Capone et al. (1991) and cultured in the presence of IAA and oligogalacturonides at the concentrations specified in the legend to Figure 4. GUS activity was assayed fluorimetrically as described by Capone et al. (1989). Each data point was the mean value (±SE) of three independent experiments.

Determination of IAA in the Culture Medium

For each treatment, 40 leaf miniexplants, corresponding to ~80 mg of tissue fresh weight, were cultured in 0.5 mL of MS liquid medium with 296 KBq/mL ^{3}H-IAA (Amersham, Little Chalfont, Buckinghamshire, UK; 962 GBq/mmol) and 0.6 μM unlabeled IAA in the presence or absence of oligogalacturonides (DP of 9 to 18; 10 μg/mL) under the light and temperature conditions described above. At the different incubation times, 20 μL of culture medium was loaded on a reverse-phase HPLC column (model Nova-Pak C18, 3.9 × 150 mm; Millipore), using methanol:1% acetic acid (30:70 [v/v]) as an eluent at a flow rate of 1 mL/min. Unlabeled IAA was used as an internal standard and eluted at a retention time of 7.0 min. Fifty microliters of each fraction (0.5 mL) was counted for radioactivity in a counter (model LS 6000; Beckman, Fullerton, MA). Each data point was the mean value of three independent experiments. No degradation of IAA in MS medium in

the absence of the miniexplants was detected, in agreement with data reported by Yamakawa et al. (1979).

Histological Analysis

Ten randomly chosen explants per plant type and treatment were fixed in FAA (70% ethanol–glacial acetic acid–formalin 18:1:1 [v/v]) on days 5 and 15, dehydrated, embedded in paraffin (melting point 52 to 54°C; Merck, Darmstadt, Germany), and sectioned at 8-μm intervals with a microtome (model Topsuper S-150; Pabish, Milano, Italy). Sections were stained by using a Topstainer LX-100 (Pabish) with eosin and Carazzi haemalum (Carlo Erba, Milano, Italy) as described by Altamura et al. (1991b).

The counts of meristemoids and root primordia were expressed as means (±SE) per explant; the statistical analysis was performed with Student's *t* test.

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REFERENCES

Aldington, S., and Fry, S.C. (1993). Oligosaccharins. Adv. Bot. Res. **19,** 1–101.

Altamura, M.M., Archilletti, T., Capone, I., and Costantino, P. (1991a). Histological analysis of the expression of *Agrobacterium rhizogenes rolB*–GUS gene fusion in transgenic tobacco. New Phytol. **118,** 69–78.

Altamura, M.M., Capitani, F., Serafini-Fracassini, D., and Falasca, G. (1991b). Root histogenesis from tobacco thin cell layers. Protoplasma **161,** 31–42.

Altamura, M.M., Capitani, F., Gazza, L., Capone, I., and Costantino, P. (1994). The plant oncogene *rolB* stimulates the formation of flower and root meristemoids in tobacco thin cell layers. New Phytol. **126,** 283–293.

Apostol, I., Heinstein, P.F., and Low, P.S. (1989). Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: Role in defense and signal transduction. Plant Physiol. **90,** 109–116.

Barbier-Brygoo, H., Ephritikhine, G., Klämbt, D., Maurel, C., Palme, K., Schell, J., and Guern, J. (1991). Perception of the auxin signal at the plasma membrane of tobacco mesophyll protoplasts. Plant J. **1,** 83–93.

Bellincampi, D., Salvi, G., De Lorenzo, G., Cervone, F., Marfà, V., Eberhard, S., Darvill, A., and Albersheim, P. (1993). Oligogalacturonides inhibit the formation of roots on tobacco explants. Plant J. **4,** 207–213.

Bellincampi, D., De Lorenzo, G., and Cervone, F. (1994). Oligogalacturonides as signal molecules in plant–pathogen interactions and in plant growth and development. Int. Assoc. Plant Tissue Cult. Newslett. **73,** 2–4.

Bellincampi, D., Forrest, R., Nuss, L., Salvi, G., De Lorenzo, G., and Cervone, F. (1995). Extracellular accumulation of an auxin-regulated protein in *Phaseolus vulgaris* L. cells is inhibited by oligogalacturonides. J. Plant Physiol. **47,** 367–370.

Bergmann, C., Ito, Y., Singer, D., Albersheim, P., Darvill, A.G., Benhamou, N., Nuss, L., Salvi, G., Cervone, F., and De Lorenzo, G. (1994). Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors, and fungal infection. Plant J. **5,** 625–634.

Branca, C., De Lorenzo, G., and Cervone, F. (1988). Competitive inhibition of the auxin-induced elongation by α-D-oligogalacturonides in pea stem segments. Physiol. Plant. **72,** 499–504.

Capone, I., Spanò, L., Cardarelli, M., Bellincampi, D., Petit, A., and Costantino, P. (1989). Induction and growth properties of carrot roots with different complements of *Agrobacterium rhizogenes* T-DNA. Plant Mol. Biol. **13,** 43–52.

Capone, I., Cardarelli, M., Mariotti, D., Pomponi, M., De Paolis, A., and Costantino, P. (1991). Different promoter regions control level and tissue specificity of expression of *Agrobacterium rhizogenes rolB* gene in plants. Plant Mol. Biol. **16,** 427–436.

Cardarelli, M., Mariotti, D., Pomponi, M., Spanò, L., Capone, I., and Costantino, P. (1987). *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. Mol. Gen. Genet. **209,** 475–480.

Chandra, S., and Low, P.S. (1995). Role of phosphorylation in elicitation of the oxidative burst in cultured soybean cells. Proc. Natl. Acad. Sci. USA **92,** 4120–4123.

Côté, F., and Hahn, M.G. (1994). Oligosaccharins: Structures and signal transduction. Plant Mol. Biol. **26,** 1379–1411.

Darvill, A., Augur, C., Bergmann, C., Carlson, R.W., Cheong, J.-J., Eberhard, S., Hahn, M.G., Ló, V.-M., Marfà, V., Meyer, B., Mohnen, D., O'Neill, M.A., Spiro, M.D., van Halbeek, H., York, W.S., and Albersheim, P. (1992). Oligosaccharins: Oligosaccharides that regulate growth, development and defence responses in plants. Glycobiology **2,** 181–198.

Farmer, E.E., Moloshok, T.D., and Ryan, C.A. (1990). *In vitro* phosphorylation in response to oligouronide elicitors: Structural and biological relationships. Curr. Top. Plant Biochem. Physiol. **9,** 249–258.

Filippini, F., Rossi, V., Marin, O., Trovato, M., Costantino, P., Downey, P.M., Lo Schiavo, F., and Terzi, M. (1996). A plant oncogene as a phosphatase. Nature **379,** 499–500.

Legendre, L., Heinstein, P.F., and Low, P.S. (1992). Evidence for participation of GTP-binding proteins in elicitation of the rapid oxidative burst in cultured soybean cells. J. Biol. Chem. **267,** 20140–20147.

Legendre, L., Rueter, S., Heinstein, P.F., and Low, P.S. (1993a). Characterization of the oligogalacturonide-induced oxidative burst in cultured soybean (*Glycine max*) cells. Plant Physiol. **102,** 233–240.

Legendre, L., Yueh, Y.G., Crain, R.C., Haddock, N., Heinstein, P.F., and Low, P.S. (1993b). Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J. Biol. Chem. **268,** 24559–24563.

Liners, F., Thibault, J.-F., and Van Cutsem, P. (1992). Influence of the degree of polymerization of oligogalacturonates and of esterifi-

cation pattern of pectin on their recognition by monoclonal antibodies. Plant Physiol. **99,** 1099–1104.

Maliga, P.A., Sz-Breznovits, A., and Marton, L. (1973). Streptomycin-resistant plants from callus culture of haploid tobacco. Nature New Biol. **244,** 29–30.

Marfà, V., Gollin, D.J., Eberhard, S., Mohnen, D., Darvill, A., and Albersheim, P. (1991). Oligogalacturonides are able to induce flowers to form on tobacco explants. Plant J. **1,** 217–225.

Mathieu, Y., Kurkdijan, A., Xia, H., Guern, J., Koller, A., Spiro, M., O'Neill, M., Albersheim, P., and Darvill, A. (1991). Membrane responses induced by oligogalacturonides in suspension-cultured tobacco cells. Plant J. **1,** 333–343.

Maurel, C. (1991). Sensibilité à l'auxine de plantes de tabacs transformées par *Agrobacterium rhizogenes.* PhD Dissertation (Centre d'Orsay: Université de Paris-Sud).

Maurel, C., Brevet, J., Barbier-Brygoo, H., Guern, J., and Tempé, J. (1990). Auxin regulates the promoter of the root-inducing *rolB* gene of *Agrobacterium rhizogenes* in transgenic tobacco. Mol. Gen. Genet. **223,** 58–64.

Maurel, C., Leblanc, N., Barbier-Brygoo, H., Perrot-Rechenmann, C., Bouvier-Durand, M., and Guern, J. (1994). Alterations of auxin perception in *rolB*-transformed tobacco protoplasts: Time course of *rolB* mRNA expression and increase in auxin sensitivity reveal multiple control by auxin. Plant Physiol. **105,** 1209–1215.

Messiaen, J., and Van Cutsem, P. (1993). Defense gene transcription in carrot cells treated with oligogalacturonides. Plant Cell Physiol. **34,** 1117–1123.

Messiaen, J., and Van Cutsem, P. (1994). Pectic signal transduction in carrot cells: Membrane, cytosolic and nuclear responses induced by oligogalacturonides. Plant Cell Physiol. **35,** 677–689.

Mohnen, D. (1994). Novel experimental systems for determining cellular competence and determination. In Biology of Adventitious Root Formation, T.D. Davis and B.E. Haissig, eds (New York: Plenum Press), pp. 87–98.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Reymond, P., Grünberger, S., Paul, K., Müller, M., and Farmer, E.E. (1995). Oligogalacturonide defense signals in plants: Large fragments interact with the plasma membrane *in vitro.* Proc. Natl. Acad. Sci. USA **92,** 4145–4149.

Röder, F.T., Schmülling, T., and Gatz, C. (1994). Efficiency of the tetracycline-dependent gene expression system: Complete suppression and efficient induction of the *rolB* phenotype in transgenic plants. Mol. Gen. Genet. **243,** 32–38.

Spanò, L., Mariotti, D., Cardarelli, M., Branca, C., and Costantino, P. (1988). Morphogenesis and auxin sensitivity of transgenic tobacco with different complements of Ri T-DNA. Plant Physiol. **87,** 479–483.

Yamakawa, T., Kurahashi, O., Ishida, K., Kato, S., Kodama, T., and Minoda, Y. (1979). Stability of indole-3-acetic acid to autoclaving, aeration and light illumination. Agric. Biol. Chem. **43,** 879–880.