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### **Citation**

Schlaman, W. R. M., Gisel, A. A., Quaedvlieg, N. E. M., Bloemberg, G. V., Lugtenberg, E. J. J., Kijne, J. W., ... Sautter, C. (1997). Chitin oligosaccharides can induce cortical cell division in roots of *Vicia sativa* when delivered by ballistic microtargeting. *Development*, 124(23), 4887-4895. Retrieved from <https://hdl.handle.net/1887/62707>

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## Chitin oligosaccharides can induce cortical cell division in roots of *Vicia sativa* when delivered by ballistic microtargeting

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### SUMMARY

Rhizobia, bacterial symbionts of leguminous plants, produce lipo-chitin oligosaccharide (LCO) signal molecules that can induce nodule organogenesis in the cortex of legume roots in a host-specific way. The multi-unsaturated fatty acyl and the *O*-acetyl moieties of the LCOs of *Rhizobium leguminosarum* biovar *viciae* were shown to be essential for obtaining root nodule induction in *Vicia sativa* plants. We have used ballistic microtargeting as a novel approach to deliver derivatives of the nodulation signal molecules inside the roots of *V. sativa*. This method offers the unique ability to introduce soluble compounds into the tissue at a small area. The mitogenic effect of microtargeting of chitin oligosaccharides, including an analysis of the influence of the chain length and modifications, was tested in a qualitative assay. The role of a cell division factor from the root stele, uridine, has also been examined in these

experiments. The results show that *O*-acetylated chitin oligosaccharides can induce root cortical cell divisions when delivered by microtargeting. For this effect it is essential that uridine is co-targeted. The foci of cortical cell division were often similar to root nodule primordia. Anatomical examination also revealed chimeric structures that share characteristics with lateral root and nodule primordia. Our data favour a model in which the oligosaccharide moiety of the rhizobial LCO induces cortical cell division and the fatty acyl moiety plays a role in transport of the LCO into the plant tissue.

Key words: root nodulation, microtargeting, microprojectile bombardment, ballistics, *Rhizobium*, lipo-chitin oligosaccharide, uridine, cell division, plants, Nod factor

### INTRODUCTION

Bacteria of the genus *Rhizobium* are symbionts of legume roots in which they induce de novo formation of a new organ, the root nodule. On the basis of their anatomical structure, root nodules are classified as determinate or indeterminate nodules. The latter are characterized by the presence of a persistent apical meristem (which is lacking in a determinate nodule) contributing to continuous growth of the nodule (Corby, 1981). Nodule formation starts with cell divisions in the inner or outer cortical cell layers for indeterminate and determinate nodules, respectively (for a recent review see Spaink, 1996). The type of root nodule that is formed depends on the plant species. Indeterminate nodules are mainly formed by legumes of temperate zones, such as *Vicia* and *Medicago*, whereas determinate nodules are found mainly in (sub)tropical legumes, such as *Phaseolus*. The initial step of root nodule formation is elicited by bacterial lipo-chitin oligosaccharides (LCOs). In low concentrations, purified LCOs can induce the formation of root nodule primordia, as has been demonstrated for a large set of leguminous plants (Ardourel et al., 1994; Bauer et al., 1996; Bloemberg et al., 1995; Cardenas et al., 1995; López-Lara et

al., 1995a,b; Minami et al., 1996; Relic et al., 1993; Sanjuan et al., 1992; Schultze et al., 1992; Spaink et al., 1991; Truchet et al., 1991). *Rhizobium leguminosarum* biovar (bv.) *viciae*, the symbiont of *Vicia sativa*, produces LCO molecules that are composed of a chitin oligosaccharide backbone consisting of five or four  $\beta$ -1,4-linked *N*-acetyl-glucosamine residues to which a C18 fatty-acyl chain, containing one (C18:1) or four (C18:4) double bonds, and an *O*-acetyl group are attached. The highly unsaturated C18:4 fatty acyl chain, together with the *O*-acetyl substituent at the C6 position of the non-reducing terminal sugar, were shown to be essential for mitogenic activity (Spaink et al., 1991). Chitin oligosaccharides have been shown to be unable to elicit nodule primordia when externally added to *V. sativa* roots.

A role of (lipo)chitin oligosaccharides in processes other than nodulation is suggested by the following data. (1) The rhizobial NodC, which is involved in the production of the glucosamine backbone of the LCO, has significant similarity with the animal DG42 protein, which could play a role in early embryogenesis (Bakkers et al., 1997; Bulawa and Wasco, 1991; Semino and Robbins, 1995; Semino et al., 1996). (2) A carrot mutant arrested at a defined stage during somatic

embryogenesis can be rescued either by the addition of rhizobial LCOs or by an endochitinase purified from wild-type embryos (de Jong et al., 1993). (3) Tobacco plants transgenic for *Rhizobium nodA* and/or *nodB*, which are involved in LCO production, are severely affected in development (Schmidt et al., 1993). (4) External addition of LCOs or chitin tetraose stimulates cell division in tobacco protoplasts (Röhrig et al., 1995). (5) The expression of the early nodulin gene *ENOD40* is transiently induced in soybean roots by external treatment of the plants with chitin pentaose (Minami et al., 1996). These data support a hypothesis that (lipo)chitin oligosaccharides have mitogenic activity in various organisms. We were interested to know whether an intact LCO molecule is a prerequisite to obtaining cortical cell divisions when it is delivered inside the plant tissue or whether a substructure is sufficient in such a situation. To approach this question, the well-described *V. sativa*-LCO system was chosen as a model and we used the method of ballistic microtargeting (Sautter et al., 1991) for the delivery of signal molecules and analogs into the legume root tissue. Ballistic microtargeting as described by Sautter et al. (1991) has been shown to be an excellent tool for delivery of DNA into particular cells of interest because a very small area covering a few cells only is precisely targeted. Penetration of the projectiles can be controlled by varying particle size and pressure (Bilang et al., 1993; Iglesias et al., 1994). Another big advantage of this method is that it offers the unique possibility of introducing compounds in solution into the tissue, since coating of the particles is not required in contrast to conventional ('biolistic') micro-bombardment.

We tested the mitogenic effect of microtargeting of chitin oligosaccharides, including an analysis of the influence of modifications and chain length of the chitin oligosaccharide, in a qualitative assay. In addition, the effect of uridine was tested. Uridine, identified as a component of an extract of the root stele of pea plants, is able to enhance hormone-induced cell proliferation of pea root cortex explants at picomolar concentrations (Smit et al., 1995). Our results show that micro-targeted *O*-acetylated chitin oligosaccharides are able to induce cortical cell divisions provided that uridine is present. Hence, the fatty acyl chain of the rhizobial LCO can be omitted for its mitogenic activity, suggesting that this LCO moiety is required for transport of the LCO into or through the root tissue of the host plant.

## MATERIALS AND METHODS

### Plant material

Seeds of *V. sativa* L. subsp. *nigra* (L.) were surface sterilized, vernalized and germinated on Jensen medium as described earlier (van Brussel et al., 1982). Seedlings 10-14 days post-sterilization were used.

### Signal molecules

LCOs of *R. leguminosarum* bv. *viciae* strain LPR5045.pIJ1089 were isolated and purified according to Spaink et al. (1991). Purified compounds were dissolved in 100% dimethyl sulfoxide (DMSO) or 5 mM cyclodextrin (Sigma Chemical Co., St Louis, MO, USA) in water. Chitin fragments composed of 4, 5 or 6 glucosamine residues were obtained from Seikagaku (Tokyo, Japan) and dissolved in water. Chitin fragments were *O*-acetylated at the C6 position of the non-reducing terminal sugar using the NodL enzymatic assay, purified

using amino-HPLC as described previously (Bloemberg et al., 1994), and dissolved in water. Uridine used in the high- and low-pressure experiments was obtained from Sigma Chemical Co. and Fluka Chemie (Bornem, Belgium), respectively, and dissolved in water.

### Microtargeting and microscopy

Two sets of experiments were performed at different laboratories and under slightly different conditions. For the experiments performed in Zürich, Switzerland, the so-called high-pressure experiments, the seedlings were mounted on 2% agarose supplemented with 10 mM CaCl<sub>2</sub> and exposed for about 2 milliseconds to an aerosol jet by the microtargeting microprojectile accelerator (Sautter et al., 1991). The jet targeted maximally 20 nl of a suspension containing 10<sup>4</sup> gold particles (mean diameter of 2.3 µm) prepared according to Sautter et al. (1991), and in the liquid phase chitin oligosaccharides and/or uridine, as indicated. Each root, 5-10 mm in length, was microtargeted with one shot at the area of the just-emerging root hairs, using a pressure of 130 bar nitrogen and a restriction of 140 µm in diameter. After the microprojectile treatment, the plants were grown aseptically on steel grids in liquid Jensen medium supplemented with 0.1 mg/ml aminoethoxyvinylglycine (AVG) (Sigma Chemical Co.) to inhibit ethylene synthesis (Zaat et al., 1989) at 21°C, for 2 days under dim light conditions (<0.2 µE m<sup>-2</sup> second<sup>-1</sup>, 3770 True-Lite 20 W TH12 fluorescence tubes (Duro-Test Int. Inc.)), followed by 5 days each with a 12-hour light period (2 µE m<sup>-2</sup> second<sup>-1</sup>, 3770 True Lite 20 W TH12 fluorescence tubes). Subsequently, the roots were cleared in 3% sodium hypochlorite (Truchet et al., 1989) and examined under the transmission light microscope using Nomarski interference contrast. To enhance contrast, methylene blue was used in some experiments.

For the experiments performed in Leiden, The Netherlands, the so-called low-pressure experiments, several parameters were different from the ones described above. Plants were mounted on Jensen medium containing 2% agarose and supplemented with 2% maltose as osmoticum 2-3 hours prior to microprojectile treatment. The microtargeting accelerator was a modified version (Gisel et al., 1996). Microtargeting was performed at 70 bar pressure with a load of 60 nl suspension per shot containing approximately 3×10<sup>4</sup> gold particles of 1.8-2.0 µm average diameter, and dissolved compounds in the liquid phase as indicated. The use of lower pressure, smaller projectiles and a larger suspension volume per shot in these low-pressure experiments compared to the high-pressure experiments, may have resulted in less penetration of the projectiles both in terms of depth and number (Bilang et al., 1993). LCOs were dissolved in 5 mM cyclodextrin and mixed with gold particles in 5 mM cyclodextrin. Roots 13-15 mm in length were microtargeted once at approximately 1, 2 or 4 mm from the root tip, corresponding to root-hair zones that are just emerging, growing longer and almost full length, respectively. After the microprojectile treatment plants were left on the same osmoticum for another 3 hours. Subsequently, plants were grown aseptically with the roots through perforated lids in dark glass bottles containing Jensen medium at 20°C, 70% relative humidity, under 15,000-20,000 lux light (Philips TLF 60W/33 fluorescence tubes) measured at the surface of the table, for 7 days each with a 16-hour light period. Growth of *V. sativa* plants with the roots in dark glass bottles is equivalent to growth in the light in the presence of AVG (van Workum et al., 1995). For microscopic analysis, root material was collected and chemically fixed overnight using 2.5% glutaraldehyde and 2.0% formaldehyde in 0.1 M cacodylate buffer (pH 7.2), or 3.7% formaldehyde, 50% ethanol, 3% propionic acid and 3% glacial acetic acid. Staining for nucleic acids was performed with Feulgen reagent (Libbenga, 1970), and subsequently roots were cleared in methyl benzoate at least 24 hours prior to microscopic examination. Root segments of interest were embedded in Technovit resin following the manufacturers' instructions (Heraeus and Kulzer, 1992), sectioned and observed in a transmission light microscope (Zeiss ICM 405) using Nomarski interference contrast optics. Micrographs were taken

on Kodak EPY 64 T film. All microscopic examinations were conducted blindly.

## RESULTS

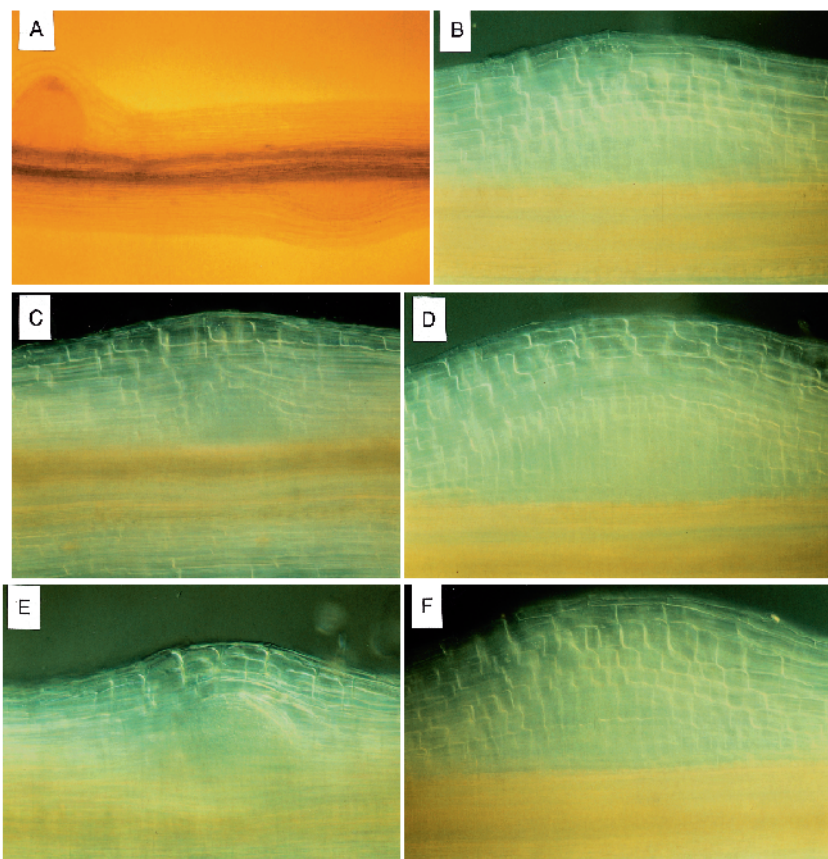
To determine whether chitin oligosaccharides have mitogenic activity in plants, chitin tetraose, pentaose and hexaose with or without an *O*-acetyl group ( $5 \times 10^{-7}$  M or  $1 \times 10^{-5}$  M in the gold particle suspension) were introduced into the root of *V. sativa* by microprojectile bombardment. *O*-acetyl chitin oligosaccharides were enzymatically synthesised using *R. leguminosarum* bv. *viciae* NodL, which acetylates chitin oligosaccharides at the non-reducing C6 position (Bloemberg et al., 1994) (results not shown). Uridine ( $1 \times 10^{-7}$  M or  $5 \times 10^{-7}$  M in the gold particle suspension) was included in part of the experiments. Two series of completely independent experiments were performed, one at the laboratory in Zürich and the other in Leiden, using comparable methods but with slightly different parameters adapted to local laboratory facilities. The use of high or low pressure during bombardment was the major difference between the two sets of experiments. For reasons of clarity, the results will be presented separately in the following sections.

### High-pressure experiments

Light microscopy was used to examine treated plants for foci of cell divisions with a morphology comparable to root nodule primordia, which can be distinguished from lateral root primordia (reviewed in Spaink, 1996). Nodule primordia in *V.*

*sativa* result from cell divisions in the inner cortex, which start concomitantly in several cells, giving rise to a broad and elevated zone at the surface of the root. Lateral root primordia start with cell divisions in the pericycle with a clearly recognizable outgrowth of the central cylinder, resulting in a much sharper elevated zone (Fig. 1A,C,E). Nodule and lateral root primordia are found opposite xylem poles, but for nodule primordia this is not always the case, as is illustrated in Fig. 3A. In the positive control, the condition and responsiveness of the plants were tested by the addition of low concentrations of *R. leguminosarum* bv. *viciae* LCOs to the growth medium of *V. sativa* seedlings. In 93–100% of the plants, nodule primordia developed as described previously (Spaink et al., 1991). The average number of nodule primordia per root obtained was 1.7 and 0.4 using  $2 \times 10^{-6}$  and  $10^{-7}$  M LCOs, respectively (Table 1A).

Microtargeting of *O*-acetylated chitin pentaose together with uridine yielded induction of cell divisions at a relative high frequency (Table 1A). Pilot experiments using a concentration range from  $10^{-7}$  to  $10^{-3}$  M indicated that  $10^{-7}$ – $10^{-5}$  M was the optimal concentration of *O*-acetylated chitin pentaose in combination with  $10^{-7}$  M uridine (data not shown). There was no difference between the results obtained with either  $10^{-7}$  M or  $10^{-5}$  M *O*-acetylated chitin pentaose. Foci of cortical cell division observed from the outside were found in up to 25% of the treated roots, and such structures averaged 0.7 per root. The cell division foci were observed in different developmental stages that were similar to stages of normal root nodule primordia induced by externally added LCOs (Fig. 1). These cell divisions were present in the region of developing lateral



**Fig. 1.** Light microscopic examination of treated *V. sativa* roots by the method used in the high-pressure experiment and comparison of developmental stages of nodule primordia-like cortical cell division foci and lateral roots. (A) An event of cortical cell division after microtargeting of *O*-acetylated chitin pentaose in combination with uridine, which occurred at close proximity to the site of a developing lateral root. (B,D,F) Successive developmental stages of foci of cortical cell division induced by microtargeted *O*-acetylated chitin pentaose in combination with uridine. (C,E) Successive developmental stages of a lateral root.

roots, as is the case with nodule primordia. In a few cases they were found in very close proximity to developing lateral roots (Fig. 1A). Histological analysis of several roots revealed that the formation of small foci containing only a few dividing cortical cells was induced as well. Because not all treated roots were analysed for these small foci, their number is not included in Table 1A. Surprisingly, the cortical cell divisions were never found at exactly the same position where microtargeting was performed but just acropetal of that position (1–10 mm). If externally added, the combination of *O*-acetylated chitin pentaose and uridine did not lead to cortical cell divisions, nor did microtargeting of *O*-acetylated chitin pentaose alone (Table 1A). Control experiments, using only water or uridine, excluded non-specific effects of ballistic microtargeting as none of these treatments elicited cortical cell divisions (Table

1A). These controls showed also that possible wounding by the ballistic microtargeting did not alter normal root development. Quantitatively, the number of cortical cell division centres per plant induced by microtargeting the combination of approximately  $10^{-13}$  moles *O*-acetylated chitin pentaose and  $10^{-15}$  moles uridine corresponds to the number of nodule primordia elicited by external addition of  $10^{-7}$  to  $2 \times 10^{-6}$  M LCO.

We also tested whether the size of the oligosaccharide backbone or chemical modifications are important factors for elicitation of cortical cell divisions. The results, presented in Table 1A, show that *O*-acetylated chitin tetraose and hexaose can also elicit nodule primordia-like cell divisions if co-targeted with uridine. However, the efficiency is 4.5-fold less compared to *O*-acetylated chitin pentaose, considering the number of cortical cell division foci per root. Microtargeting

**Table 1. Development of cortical cell division foci after microtargeting of chitin oligosaccharide derivatives, uridine or combinations of these, to roots of *V. sativa***

**(A) High-pressure experiments**

Compound (M) <sup>a</sup>	Uridine (10 <sup>-7</sup> M)	Delivery method <sup>b</sup>	Number of positive plants per tested total <sup>c</sup>	Mean number of cortical cell division foci per plant
<i>O</i> -Acetylated-chitin pentaose (10 <sup>-7</sup> or 10 <sup>-5</sup> )	–	mt	0/30	0
	–	ext	0/60	0
	+	mt	8/30	0.7
	+	ext	0/45	0
<i>O</i> -Acetylated-chitin tetraose (10 <sup>-5</sup> )	+	mt	5/30	0.16
<i>O</i> -Acetylated-chitin hexaose (10 <sup>-5</sup> )	+	mt	5/30	0.16
Chitin pentaose (10 <sup>-7</sup> or 10 <sup>-5</sup> )	+	mt	0/45	0
Chitin tetraose (10 <sup>-5</sup> )	+	mt	0/15	0
Chitin hexaose (10 <sup>-5</sup> )	+	mt	0/15	0
H <sub>2</sub> O <sup>d</sup>	–	mt	0/60	0
	+	mt	0/60	0
LCO <sup>e</sup> (2×10 <sup>-6</sup> ) (10 <sup>-7</sup> )	–	ext	14/15	1.7
	–	ext	15/15	0.4

**(B) Low-pressure experiments**

Compound (M) <sup>a</sup>	Uridine (5×10 <sup>-7</sup> M) <sup>a</sup>	Delivery method <sup>b</sup>	Number of positive plants per tested total <sup>c</sup>	Mean number of cortical cell division foci per plant		
				Class I	Class II	Class III
<i>O</i> -Acetylated-chitin pentaose (5×10 <sup>-7</sup> )	–	mt	3/58	0.05	0	0
	–	ext	0/15	0	0	0
	+	mt	23/123	0.11	0.07	0.03
	+	ext	0/19	0	0	0
<i>O</i> -Acetylated-chitin tetraose (5×10 <sup>-7</sup> )	+	mt	0/12	0	0	0
<i>O</i> -Acetylated-chitin hexaose (5×10 <sup>-7</sup> )	+	mt	2/22	0.05	0.05	0
Chitin pentaose (5×10 <sup>-7</sup> )	+	mt	0/12	0	0	0
Chitin tetraose (5×10 <sup>-7</sup> )	+	mt	1/12	0.08	0	0
Chitin hexaose (5×10 <sup>-7</sup> )	+	mt	2/18	0	0.05	0.05
H <sub>2</sub> O <sup>d</sup>	–	mt	0/16	0	0	0
	–	ext	0/12	0	0	0
	+	mt	0/39	0	0	0
	+	ext	0/11	0	0	0
LCO <sup>e</sup> (10 <sup>-7</sup> ) (10 <sup>-7</sup> )	–	mt	6/8	0.13	0.50	0.50
	–	ext	10/10	0	0.33	3.65

<sup>a</sup>The concentration of the compound is given in the suspension of which 20 nl (high-pressure experiments) or 60 nl (low-pressure experiments) was loaded for microtargeting or which was present in the growth medium when the compound was externally added.

<sup>b</sup>mt, microtargeting with projectiles; ext, externally added.

<sup>c</sup>Number of roots with cortical cell division per total number of treated roots.

<sup>d</sup>Water, either supplemented with uridine or not, was used as negative control.

<sup>e</sup>LCO, NodR1v-V(C18:4,Ac) (Spaink et al., 1991) dissolved in DMSO in the high-pressure experiments and in cyclodextrin in the low-pressure experiments.

of chitin oligosaccharides consisting of 4, 5 or 6 residues lacking an *O*-acetyl group did not induce cortical cell division foci observable from the outside (Table 1A).

### Low-pressure experiments

Using a modified microtargeting device that was established in the laboratory of Leiden, comparable experiments to the ones described above were performed. The major difference between the two sets of experiments was the use of a twofold lower pressure during bombardment, since high-pressure experiments are technically difficult to perform. Microscopic analysis revealed that with the low-pressure technique the microprojectiles were mainly targeted to the epidermis and the first two cortical cell layers (data not shown). Pilot experiments indicated that the penetration depth of the particles did not alter the qualitative data obtained with *O*-acetylated chitin oligosaccharides and uridine in the high-pressure experiments described above (data not shown). Treated *V. sativa* roots were examined using light microscopy after fixation, histochemical staining of the nuclei and clearing. Cortical cell divisions are indicated by accumulation of nuclei in the cortex, whereas young lateral root primordia can be distinguished by a much more intensely stained and strictly bordered region of accumulating nuclei in the stele (Fig. 2B, lower part). Because of the staining method used, a resolution much higher than with the results presented in Fig. 1 could be achieved, resulting in the discovery that both LCO and chitin oligosaccharide molecules can induce various different patterns of cortical cell division. The observed structures were ordered into three classes. Class I represents small foci of cortical cell division; class II represents larger foci, morphologically inbetween nodule and lateral root primordia (designated as hybrid structures), and class III represents broad regions of dividing cells in the inner cortex, which closely resemble classical nodule primordia of the indeterminate type. It is worth noting that the class II structures sometimes seem to result from cell divisions in all cortical cell layers. Representative examples of the different classes are illustrated in Fig. 2.

In the positive control experiment *V. sativa* plants were exposed to LCOs in growth medium. Cortical cell divisions were found in the roots of all treated plants (Table 1B). Predominantly found were nodule primordia, indicated as class III (Table 1B), and consistent with earlier observations (Spaink et al., 1991). Class II structures were observed as well, though at much lower frequency (Table 1B).

Microtargeting was performed at various positions in the root with respect to the distance from the root tip. Since these variations did not result in obvious differences, each data set is not separately presented. From Table 1B it can be concluded that *O*-acetyl chitin pentaose can induce cortical cell division when delivered by microtargeting. When uridine was co-targeted the frequency of cell division foci and positively responding plants increased considerably. In most positive roots one centre of cortical cell division was present with a maximum of up to three belonging either to the same or to different classes. The structures belonging to class I were found most frequently; those classified as class II or III were found in lower numbers (Table 1B). No cortical cell divisions were observed when *O*-acetyl chitin pentaose and uridine, separately or in combinations, were added externally to the plants (Table 1B). Statistical analysis using a non-parametric Wilcoxon test

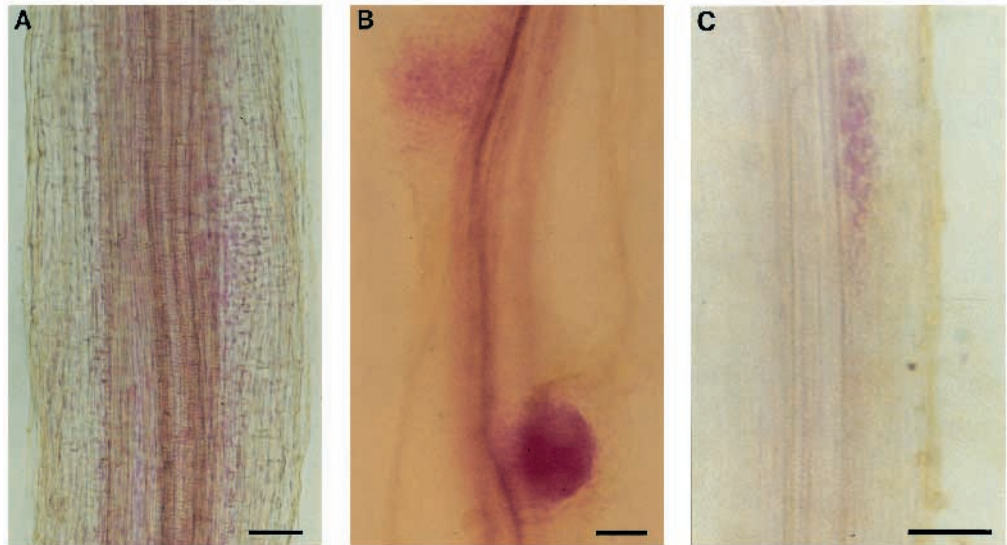
showed that the observed effect of *O*-acetylated chitin oligosaccharides and uridine was significantly different from all the negative controls ( $P=0.01$ ). Microtargeting of purified LCOs gave rise to cortical cell division in six out of eight plants. A lower number of cortical cell division centres and smaller foci belonging to class I were detected when LCOs were microtargeted rather than added externally (Table 1B). Cortical cell divisions induced either by LCO or by *O*-acetyl chitin pentaose were observed 1-10 mm acropetal from the position of targeting.

The importance of the *O*-acetyl decoration as well as the length of the sugar backbone for the induction of cortical cell division in *V. sativa* roots was examined by microtargeting of chitin oligosaccharides of different chain length carrying or lacking an *O*-acetyl group. The negative results with microtargeted chitin pentaose (Table 1B) suggest that the presence of an *O*-acetyl group at the chitin pentamer is a prerequisite to obtaining cortical cell division. However, one event of cortical cell division was found with chitin tetraose. Furthermore, this modification seems not to be required when a chitin hexasaccharide is introduced into the root tissue, since microtargeting of chitin hexaose gives rise to the same number of cortical cell divisions as *O*-acetyl chitin hexaose, provided that uridine is co-targeted. The lower efficiency of *O*-acetylated chitin hexaose and tetraose compared to *O*-acetylated chitin pentaose in induction of cortical cell divisions confirms the results obtained in the high-pressure experiments.

### Anatomical examination

Cortical cell division foci belonging to class I were small and sometimes hard to distinguish from very young lateral root primordia. To confirm cortical cell divisions in such structures they were studied by anatomical analysis after embedding and sectioning of the root tissue. Nomarski interference contrast microscopy of the sections enabled us to observe the Casparian strip and thereby to identify the endodermis without any additional staining. It was found that the class I foci were often positioned opposite xylem poles, but in contrast to the situation with lateral roots, not always. Moreover, the results confirm that the class I foci showed cell division in the endodermis and in adjacent cells of the inner cortex (Fig. 3B-D). In addition, however, cell divisions in the pericycle were evident when these structures further developed (Fig. 3D). This may either be a similarity with normal nodule primordia, which at later stages become invaded by vascular bundles, or alternatively, many of the observed class I foci are chimerae, inbetween an indeterminate nodule and a lateral root primordium. To distinguish between these possibilities two control experiments were performed. Firstly, nodule primordia induced by externally added LCOs were microscopically examined. These primordia were found most frequently opposite xylem poles or, in several cases, opposite phloem poles as shown in Fig. 3A. They clearly showed cortical cell divisions and, in addition, mitogenic activity in the pericycle (Fig. 3A). Secondly, to obtain a detailed anatomical picture of lateral root primordia under our experimental conditions, *V. sativa* roots were examined microscopically after microtargeting with a control solution that did not contain chitin oligosaccharides or LCOs. The results show that in lateral root primordia cortical cell divisions were never observed and that all cell divisions occurred exclusively inside the endodermis (Fig. 3E). Sections of further developed lateral

**Fig. 2.** Light microscopic examination of *V. sativa* roots (after fixation, staining and clearance) treated by low-pressure microtargeting of *O*-acetylated chitin pentaose in combination with uridine. Representative examples of cortical cell divisions belonging to different classes are shown. (A) Class I, small foci of cortical cell division. (B) Top part: class II, hybrid structure. Bottom part: lateral root primordium, shown for comparison. (C) Class III, broad region of dividing cells in the inner cortex. Bars, 200  $\mu$ m.



roots clearly showed that the growing lateral root breaks through the endodermis, which does not participate in its growth (Fig. 3F). These observations led us to conclude that mitogenic activity in the inner cortex, including the endodermis, accompanied by divisions in the pericycle caused by the microtargeting of *O*-acetylated chitin oligosaccharides, is in all respects different from the formation of lateral root primordia. Most likely, the induced cortical cell division foci are comparable to normal nodule primordia.

## DISCUSSION

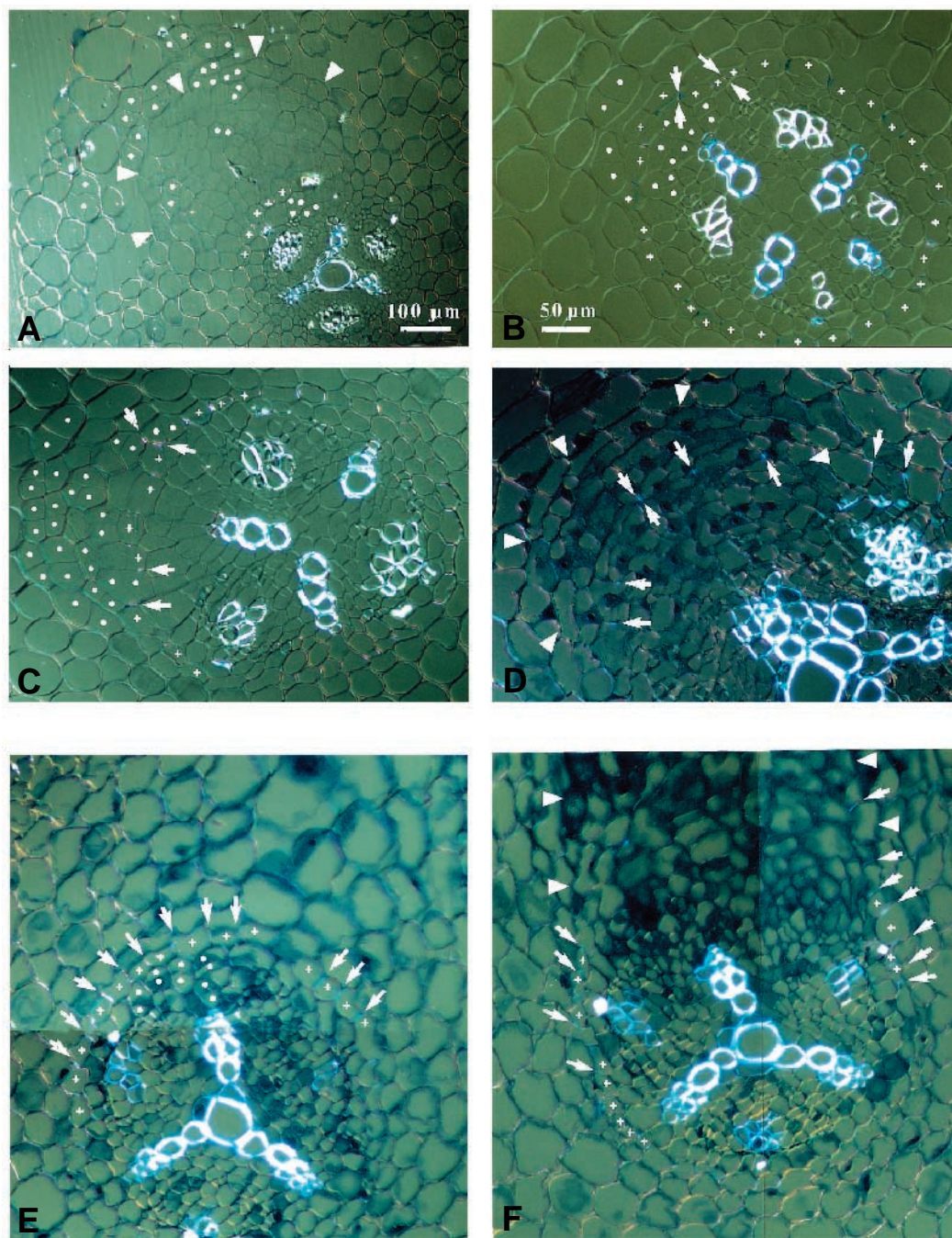
Chitin oligosaccharides have been shown to be elicitors of various plant responses suggested to be related to mechanisms for defence against pathogens (Aldington and Fry, 1993; Ren and West, 1992; Shibuya et al., 1993; Staehelin et al., 1994a). However, here we show for the first time that *O*-acetylated chitin oligosaccharides are able to trigger cell division in the root cortex of *V. sativa* when delivered by ballistic microtargeting. These results were repeatedly obtained in two different laboratories using slightly different conditions. The novel approach of microtargeting devised by Sautter et al. (1991) enabled us to introduce compounds in solution into plant tissue at a very localized position. A modified version of microtargeting was used in the so-called low-pressure as compared to the high-pressure experiments, which led to a change of several technical parameters. A combination of the parameters used in the high-pressure experiments might have led to a higher frequency of clearly detectable foci of cortical cell division that look like large nodule primordia, as compared to the low-pressure experiments which predominantly led to the formation of small foci of cortical cell division (indicated as class I in Table 1).

The presence of an *O*-acetyl substituent at the C6 position of the non-reducing terminal sugar of microtargeted chitin pentaose is a prerequisite for the induction of cortical cell divisions in *V. sativa* roots (Table 1), consistent with the results of earlier studies on the specific requirements of LCOs for mitogenic activity (Spaink et al., 1991). In contrast, chitin

hexaose and tetraose showed mitogenic activity even when lacking the *O*-acetyl moiety, but their efficiency was much lower than that of *O*-acetylated chitin pentaose (Table 1). The differences in mitogenic activity of the various chitin oligosaccharides may reflect different degradation rates by plant chitinases, as has been suggested previously (Staehelin et al., 1994b).

The cell divisions induced by chitin oligosaccharides were observed in the inner cortical cell layers even when the projectiles were mainly targeted to the epidermis and the first two cell layers of the cortex. Furthermore, we have shown that these cortical cell division events were always found just acropetal to the position that was microtargeted. These observations suggest that chitin oligosaccharides act indirectly on their target cells *via* a secondary signal transduction pathway, which is activated as soon as the oligosaccharide enters the root tissue. The results of Heidstra et al. (1994), who report an accumulation of LCOs in the epidermis, may also suggest that LCOs act indirectly on the cells of the inner cortex. Inner cortical cell division may result from a local alteration in the auxin/cytokinin hormone balance, for instance by influencing hormone transport across the endodermis, giving rise to local cell divisions. Support for this hypothesis comes from different investigations. Hirsch et al. (1989) showed that auxin transport inhibitors induce pseudo-nodules in alfalfa. Externally added cytokinin appeared to induce foci of inner cortical cell divisions comparable to LCO-induced nodule primordia (Bauer et al., 1996). LCOs mimic auxin action in that they stimulate cell division and enhance the expression of the auxin-responsive promoter *axi1* (Röhrig et al., 1995). Furthermore, most recently it has been shown, using spotinoculation and microtargeting bombardment techniques, that LCOs and *O*-acetyl chitin oligosaccharides can induce auxin transport inhibition locally in the pericycle exactly opposite the site of application in transgenic white clover plants. This phenomenon is accompanied by a local increase of auxin concentration immediately acropetal of that position in the pericycle and in the inner cortical cells (Mathesius et al., 1998). Interestingly, the location where the cortical cell divisions were observed corresponds with this region of

**Fig. 3.** Root cross sections of *V. sativa* showing cell divisions in nodule and lateral root primordia and in cortical cell division foci belonging to class I. (A) Nodule primordium induced by externally added LCO of *R. leguminosarum* bv. *viciae*: cell divisions in the cortex and inside the endodermis. This is an example of a nodule primordium which is not present opposite a xylem pole, as is mostly the case. (B-D) Class I cell division foci induced by microtargeted *O*-acetyl chitin pentaose in combination with uridine in three successive stages of development. Besides the apparent cell divisions in the cortex, cell divisions in the endodermis are present. This is very clear in D, where the distance between two subsequent Casparian strips is about two cell lengths. (E) Very young lateral root primordium. Cell divisions occur exclusively inside the endodermis. (F) Older lateral root. The lateral root breaks through the endodermis. Sections D-F were made after the nuclei were stained with Feulgen reagents. White spots, some cells generated by recent cell divisions; +, identified endodermis cells; arrows, examples of Casparian strip in radial cell walls of the endodermis; arrowheads, approximate outer border of the nodule primordium or lateral root.



enhanced auxin levels. The results suggest that the cause of the local activation of the cell cycle in nodule primordia formation is a higher auxin/cytokinin ratio induced by LCOs or chitin oligosaccharides.

The results (Table 1) show that in our test system uridine is required for induction of cortical cell division. This supports a role of uridine in nodulation, as has been indicated previously by the results of Smit et al. (1995). Uridine enhances hormone-induced proliferation of root cortical cells, and thus may amplify the response to chitin oligosaccharides. Uridine is expected to be a normal component of the plant root, although its intracellular concentrations has never been determined. Therefore, the requirement of uridine in our system seems

paradoxical. The most likely explanation is that at the site of microtargeting the concentration of uridine is rate-limiting. We performed our microtargeting in young parts of the root in which no lateral root development was observed. Such young roots might have very low concentrations of free uridine in this particular part of the root. Since it is impossible to calibrate the volume that is actually transported by the microprojectiles into the plant cells, it is difficult to test the required absolute doses of uridine concentrations while maintaining a constant *O*-acetyl chitin pentaose concentration by microtargeting. We showed that extremely low doses of added uridine, which can be roughly estimated to be less than  $10^{-16}$  moles per shot, are effective in our system.

The question remains as to whether the effects of chitin oligosaccharides and uridine that we observed are identical to the early stages in the process of *Rhizobium*-induced nodule formation in leguminous plants. The cortical cell divisions were always found in the same region, where nodule primordia are normally present, namely in the regions of developing lateral roots. Like nodule primordia they were mostly, but not always, present opposite xylem poles. However, the numbers anatomically analysed were too low for us to perform a reliable statistical analysis of this positioning. On the anatomical level, cortical cell division foci belonging to class I induced by microtargeting of *O*-acetylated chitin pentaose are identical to early nodule primordia induced by externally added LCO. Cell division in the inner cortex was accompanied by cell division in the pericycle and in the stele (Fig. 3A-D), suggesting that class I cortical cell division foci are ontogenetically related to class III structures, which resemble indeterminate nodule primordia. In contrast, lateral root primordia never showed cortical cell division in our test system (Fig. 3E), which is in agreement with published studies on lateral root formation (Laskowski et al., 1995). Nodule-related cell division has also been shown to be accompanied by cell divisions within the endodermis in several other leguminous plants, such as clover (C. Díaz, personal communication). Hence, our results clearly show that the foci of cortical cell division induced by chitin oligosaccharides are different from lateral roots and, at an anatomical level, indistinguishable from nodule primordia. Due to technical restrictions, *in situ* hybridization, e.g. using the early nodulin *ENOD40* and *ENOD12* probes, could not be used as a tool for further characterization of foci of cortical cell division induced by microtargeted chitin oligosaccharides. Furthermore, *ENOD12* and *ENOD40* have been shown to be expressed in lateral root primordia and might therefore be not sufficient as decisive markers for nodule primordium formation (Asad et al., 1994; Bauer et al., 1996, 1997; Papadopoulou et al., 1996). Interestingly, *ENOD40* is transiently induced when soybean roots are externally treated with chitin pentaose or non-mitogenic LCOs (Minami et al., 1996).

To obtain cell divisions caused by *O*-acetylated chitin oligosaccharides it is necessary that the molecules are delivered internally in the plant tissue. This is in contrast to LCOs, which are also able to trigger the cell cycle machinery after external addition. Considering the fact that at least one apparent result of both treatments, cortical cell division, is identical, it is tempting to speculate that both chitin derivatives activate a common receptor, which is involved in cell cycle regulation. All locations inside the cell wall, i.e. intra- and extracellularly, are potential receptor sites, since bombardment results in entry at all these sites. A location inside the cell wall is supported by the data of Röhrig et al. (1995), who showed that tobacco protoplast cell division was stimulated by chitin oligosaccharides, albeit at a  $10^6$ -fold higher concentration than LCOs containing *trans*-unsaturated fatty acyl groups. Savouré et al. (1997) showed that chitin oligosaccharides induce the expression of isoflavonoid biosynthetic genes in *Medicago* cell cultures, an effect that was not observed with treatment of the intact roots of the entire plant. These results could be explained by the assumption that a receptor for chitin oligosaccharides is easier to reach in suspension-cultured cells than in target cells in the root. In summary, our results may indicate that the acyl group of LCOs is involved in the transport of an active part of

the molecule inside the plant tissue, and we are encouraged to search for a proposed receptor for chitin oligosaccharides. A transport role of the LCO lipid is supported by the data recently described by Philip-Hollingsworth et al. (1997). Our novel approach, of using ballistic microtargeting for delivery of signal molecules, could be of great importance for further elucidation of the mechanism of nodule meristem formation.

H.R.M.S. and N.E.M.Q. were supported by a Pionier grant from the Netherlands Organisation for Scientific research (NWO) awarded to H.P.S. G.B. was supported by the Netherlands Foundation of Chemical research (SON) with financial aid of the NWO. We are grateful to Teun Tak who supplied us with *V. sativa* seedlings and we wish to thank Dr Ton van Brussel for critical discussion and help in part of the experiments. We gratefully acknowledge the skilful technical assistance of Elke Fenner, Marga Hartevelde and Daphne Meijer (NWO-Pionier).

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