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Short communication

Uridine, a cell division factor in pea roots

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Abstract

Nodulation (root nodule formation) in legume roots is initiated by the induction of cell divisions and formation of root nodule primordia in the plant root cortex, usually in front of the protoxylem ridges of the central root cylinder. We isolated a factor from the central cylinder (stele) of pea roots which enhances hormone-induced cell proliferation in root cortex explants at positions similar to those of nodule primordia. The factor was identified as uridine. Uridine may act as a morphogen in plant roots at picomolar concentrations.

Legume plants such as pea (*Pisum sativum*) form root nodules in symbiotic association with *Rhizobium* bacteria. Formation of nodule primordia is induced by low-molecular-weight mitogenic signals, lipochitin oligosaccharides (LCOs), which are released by the *Rhizobium* bacteria [2, 7, 10, 11]. However, formation of similar primordia can also be induced by, for instance, auxin-transport inhibitors such as 2,3,5-triiodobenzoic acid (TIBA), which suggests that the plant hormone balance contributes to induction of nodule primordia [1]. Significantly, the architecture of the central cylinder (the stele) of the plant root determines the location of primordium initiation. The majority of developing nodule primordia is found in areas of the root cortex opposite one of the types of immature conductive tissue in the central cylinder of the root, the so-called protoxylem ridges [3, 4].

More than two decades ago, Libbenga *et al.* [5] postulated the hypothesis that an endogenous cell division factor from the stele is involved in the cell division response of the pea root cortex. This work proceeded from pilot work by Torrey and co-workers. Normally, cells in the middle and outer cortex of pea root explants, cultured on a synthetic nutrient medium supplemented with auxin and cytokinin, do not respond to auxin and cytokinin by dividing. Libbenga *et al.* [5] showed that addition of a crude alcoholic extract from the stele of pea roots resulted in cell divisions throughout the cortex of pea root explants. Much less activity was found for a root cortex extract.

The present paper reports on the purification

and characterization of a factor from an alcoholic extract from pea root steles, which enhances the cell division response of pea root cortical cells to auxin and cytokinin, as described by Libbenga et al. [5].

We first tested the results of Libbenga *et al.* [5] by determining the activity of pea root stele extract using a similar bioassay with complete root explants of about 1.5 mm long. The explants were transferred onto S2 agar medium of Torrey [9] which contains auxin and cytokinin. Ethanol extracts were obtained from the vascular tissue of pea roots according to Libbenga *et al.* [5]. After 6 to 8 days, cell proliferation was monitored per explant and distinguished into 4 classes, ranging from class 1 (no cell divisions visible) to class 4 (cell proliferation throughout the entire cortical tissue) (Fig. 1). Addition of root stele extract resulted in a shift of cell proliferation towards class 4, with a significance level of $P < 0.001$ as tested with the non-parametric 'comparison of two samples' test. Cell divisions were first observed opposite protoxylem ridges in the root explants, similar to the location where *in vivo* nodule formation is initiated in pea plants. In case of massive cell proliferation (Fig. 1, class 4), cell divisions were observed throughout the whole cortex, indicating that most, if not all, pea cortex cells are able to dedifferentiate into dividing cells in the presence of proper cell division signals. These data confirm the results of Libbenga *et al.* [5], to which paper we refer for detailed microscopic observations. From extracts prepared from different parts of a pea plant, only extracts from root tips and, especially, extracts from root stele tissue appeared to be active in induction of cell proliferation (data not shown).

An active root stele factor was purified by means of reversed-phase HPLC (Fig. 2). Compounds were purified from the extracts using a reversed-phase HPLC column (5 μM, 7 mm × 300 mm, Shandon Hypersil ODS) with a water to methanol gradient as the mobile phase (flow rate 1 ml/min). The compounds were fractionated into compounds of increasing hydrophobicity by elution with a 0 to 100% methanol gradient. The ultraviolet absorbance profile (254 nm) of the eluate is shown in Fig. 2A. This wavelength was chosen since preliminary purification results, based on molecular sieve chromatography, indicated that the most active fraction of root stele extract showed a maximal absorption at about 260 nm. Compounds were collected per peak or group of peaks, concentrated by speed-vac and re-dissolved in 70% ethanol. Subsequently, they were 10^2 to 10^6 times diluted in water and separately tested for activity in the explant assay, with

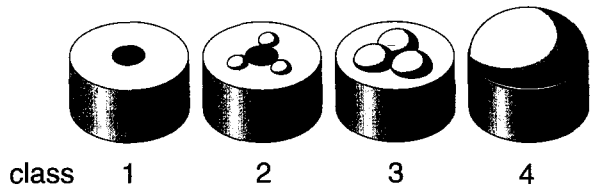

Fig. 1. Schematic representation of the four classes of cell proliferation in pea root explants. Class 1, no visible cell proliferation; Class 2, few cell divisions opposite the protoxylem ridges; Class 3, many cell divisions opposite the protoxylem ridges; Class 4, cell proliferation throughout the root cortex of the explant.

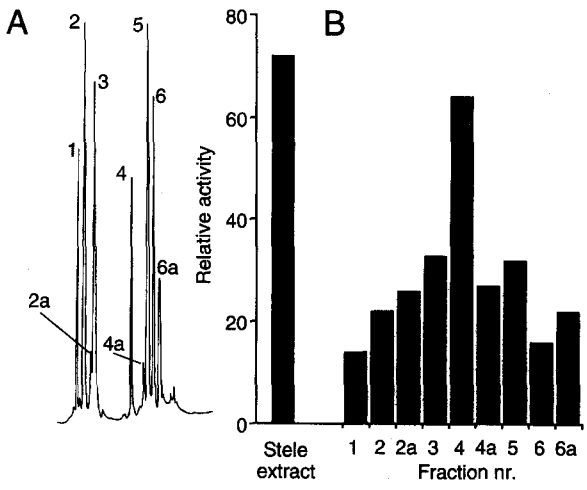

Fig. 2. A. Elution profile of a partially purified extract from the stele of pea roots analysed by reversed-phase HPLC chromatography. B. Relative activity of different fractions in the explant assay. The mean activity of 7 independent isolations and assays are presented together with the activity of stele extract. Relative activity represents the average shift in classes (× 100) in comparison to the control, and is calculated as follows: *relative activity* = (1 × % class 1) + (2 × class 2) + (3 × class 3) + (4 × % class 4) − $act_{control}$.

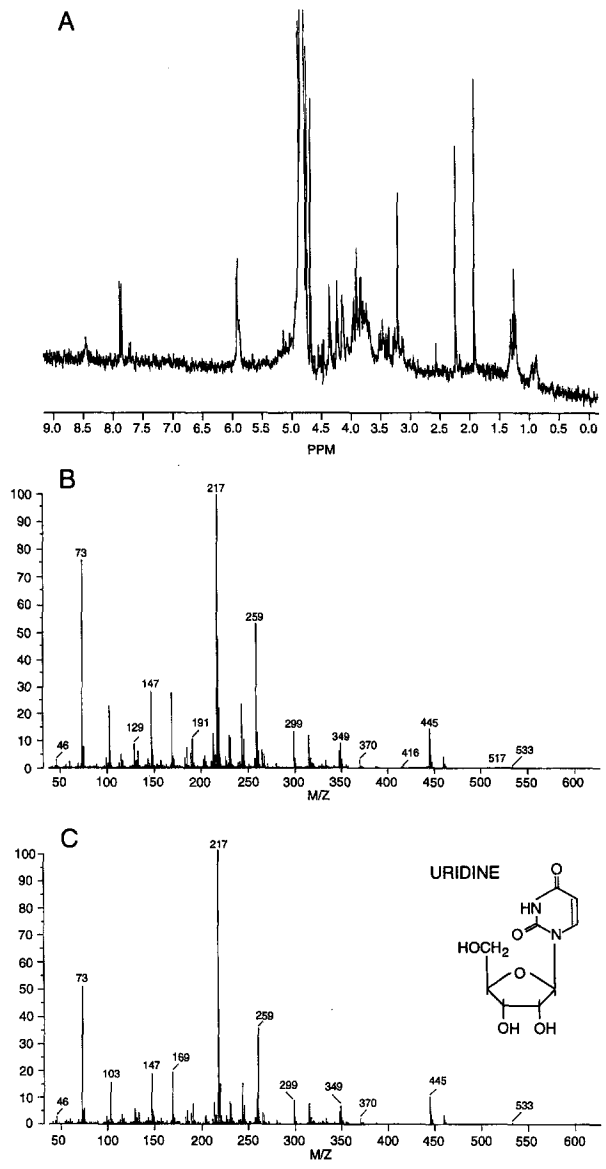

Fig. 3. A. ^{1}H NMR spectrum of compound 4. B. Gas-liquid chromatography-mass spectrometry (GLC-MS) of the trimethylsilyl (TMS) ethers of HPLC fraction 4. (C) The reference compound uridine. Inset: chemical structure of uridine. NMR spectra were recorded on a Bruker WM-300 equipped with an Aspect 2000 data system, operating at 300 Mhz proton resonance frequency. D_2O (Merck, Darmstadt) was used as solvent. The residual solvent signal was used as internal standard for the chemical shifts (4.80 ppm). Samples for GC-MS analysis were dissolved in 50 μl TMS reagent, which was composed of 5 parts bis(trimethylsilyl)trifluoroacetamide (Aldrich), 1 part trimethylchlorosilane (Merck) and 10 parts pyridine (Janssen Chimica). Analyses were carried out with a JEOL JMS-AX505W mass spectrometer fitted with a Hewlett Packard 5890 gas chromatograph (Bijvoet Center, Mass Spectrometry, Utrecht University) using an on-column injector and helium as the carrier gas at a flow rate of 2 ml/min. The components were separated on a CPSil5 column (0.32 mm × 25 m, Chrompack) with the following temperature program: 90 °C for 2 min, 40 °C/min to 200 °C for 4 min, 5 °C/min to 300 °C and finally holding the temperature at 300 °C. Mass spectra, obtained under electron ionization conditions (70 eV), were recorded by linear scanning from *m/z* 35–800 at an accelerating voltage of 3 kV.

the activity of the unfractionated extract as a positive control. As shown in Fig. 2B, the major activity was found in the fraction corresponding to peak 4.

Spectral analysis revealed that the maximal absorption of fraction 4 was at 262 nm. Figure 3A shows the ^{1}H NMR spectrum of fraction 4. The spectrum revealed two doublets at 7.85 and 5.87 ppm, respectively, with coupling constants of 8.2 Hz, characteristic for the presence of a uracil moiety in the molecule. The more upfield signal was overlapping with another doublet with a coupling constant of 4.2 Hz. Additional signals were present around 4 ppm, indicating the presence of a sugar moiety. Uridine (uracil riboside) was found to show ^{1}H NMR signals identical to fraction 4 (data not shown). Additional signals from this fraction are presumably due to minor impurities.

Trimethylsilyl (TMS) derivatives were used to analyze the isolated pea root factor into more detail. GC-MS analysis of the trimethylsilylated fraction 4 revealed one major component at retention time (RT) 17.59 min. The mass spectrum at RT 17.59 min is given in Fig. 3B. Ions resulting from simple losses from the molecular ion ($M^{+\cdot}$) of trimethylsilylated compounds are usually observed at [M-15] and [M-90]. The characteristic formation of [M-15] is useful to allow the assignment of the molecular ion at *m/z* 460. Several sugar-derived ions from a pentose-containing nucleoside are presented in Fig. 3A. The presence of a series of fragment ions at *m/z* 349, *m/z* 259, *m/z* 245, *m/z* 243, *m/z* 217, and *m/z* 169 is characteristic of a pentose-$(TMS)_3$. The ion at *m/z* 349 can be assigned to ribosyl-$(TMS)_3$. Subtraction of the latter *m/z* value from *m/z* 460

of the molecular ion yields 111 for a uracil (MW 112) base of the nucleoside. These results point to a uridine nucleoside with 3 TMS groups (MW 460). The reference mass spectrum of uridine-$(TMS)_3$ is given in Fig. 3C. Figures 3B and 3C show strong similarity, and these results are in good agreement with the mass spectrum of uridine-$(TMS)_3$ as described [6]. It was concluded that the component from fraction 4 is uridine.

In order to test whether uridine is the only nucleoside present in pea root stele extract, we analysed the extract for the presence of other nucleosides. As judged from HPLC retention time values, none of the other nucleosides could be detected, with the possible exception of adenosine which has a retention time similar to that of peak 6 (Fig. 2A). However, GC-MS characterization of fraction 6 revealed that spectra of this fraction did not correspond to that of adenosine. Uridine is apparently the only nucleoside present in detectable amounts in an alcoholic extract from the vascular tissue of pea roots.

In addition, we tested the presence of uridine in stele extracts of pea roots, inoculated with the pea symbiont *R. leguminosarum* biovar *viciae*. HPLC analyses of these stele extracts appeared to be indistinguishable from those of extracts obtained from uninoculated control plants (data not shown). This indicates that synthesis and transport of uridine are not significantly affected by inoculation. Similar results were obtained for plants treated with either mitogenic LCOs from *R. leguminosarum* biovar *viciae* (5×10^{-7} M) or TIBA (5×10^{-4} M), both added to the medium.

Commercially available uridine was tested for activity in the explant assay. As shown in Fig. 4, uridine was highly active in the explant assay even at concentrations as low as 4×10^{-12} M. As judged from peak integration of HPLC chromatograms, stele extract from 2.5 mg of pea stele tissue, as prepared according to Libbenga *et al.* [5], contains about 4×10^{-9} M uridine and shows a comparable activity in the explant bioassay. Libbenga mentioned in his Ph.D. thesis that stele extract can be significantly diluted without losing its activity [3]. Interestingly, uridine appeared to

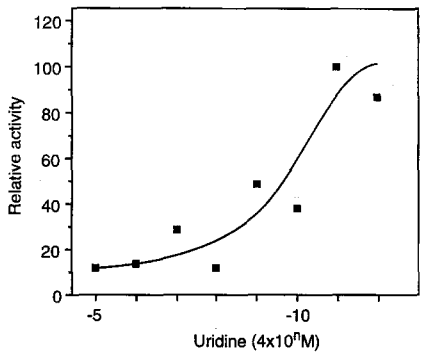

Fig. 4. Dose-response curve of uridine (■) on cell division activity of pea explants. Uridine was added to the explants similar to the addition of root stele extracts. Relative activity is described in Figure 2.

be inactive at concentrations higher than found to be present in stele extract.

Taken together, our results identify uridine as a cell division factor for pea root cortical cells. Most probably, uridine represents the factor $\times 2$, as described by Libbenga [3]. In view of the (lower) activity of other root stele fractions (Fig. 2B), other components of stele extract may contribute to the action of uridine. Preliminary results of Spaink *et al.* [8] indicate that uridine is also involved in induction of nodule primordia by rhizobial LCOs, since ballistic microtargeting experiments with vetch roots (*Vicia sativa* ssp. *nigra*) demonstrated that induction of root cortical cell divisions by oligochitin derivatives was dependent on co-introduction of uridine in the roots.

Referring to Libbenga *et al.* [5], we hypothesize that (1) auxin and cytokinin, at an appropriate ratio, (2) rhizobia (through LCOs), or (3) auxin transport inhibitors such as TIBA trigger physiological responses leading to induction of cortical cell divisions in pea roots in the presence of appropriate amounts of uridine. By forming a gradient from the protoxylem ridges into the root cortex in pea plants, uridine may create a morphogenetic pre-pattern in which formation of nodule primordia is induced. Of course, we do not exclude that other components of stele extract are additionally involved in nodule primordium initiation. At present, the interaction of uridine and plant hormones is being studied by use of various other bioassays.

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