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Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*-legume interaction

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SUMMARY

Nod factors secreted by *Rhizobium leguminosarum* bv. *viciae* induce root hair deformation, involving a reinitiation of tip growth, and the formation of nodule primordia in *Vicia sativa* (vetch). Ethylene is a potent inhibitor of cortical cell division, an effect that can be counteracted by applying silver ions (Ag^+) or aminoethoxy-vinylglycine (AVG). In contrast to the inhibitory effect on cortical cell division, ethylene promotes the formation of root hairs (which involves tip growth) in the root epidermis of *Arabidopsis*. We investigate the possible paradox concerning the action of ethylene, putatively promoting Nod factor induced tip growth whilst, at the same time, inhibiting cortical cell division.

We show, by using the ethylene inhibitors AVG and Ag^+, that ethylene has no role in the reinitiation of root hair tip growth induced by Nod factors (root hair deformation) in vetch. However, root hair formation is controlled, at least in part, by ethylene. Furthermore, we show that ACC oxidase, which catalizes the last step in ethylene biosynthesis, is expressed in the cell layers opposite the phloem in that part of the root where nodule primordia are induced upon inoculation with *Rhizobium*. Therefore, we test whether endogenously produced ethylene provides positional information controlling the site where nodule primordia are formed by determining the position of nodules formed on pea roots grown in the presence of AVG or Ag^+.

Key words: *Vicia sativa*, pea, Nod factors, root hair deformation, nodulation, ethylene

INTRODUCTION

The interaction between *Rhizobium* and its legume host plant culminates in the formation of a nitrogen fixing root nodule. The rhizobial signals that initiate development of this organ are specific lipo-chitin oligosaccharides called Nod factors (Lerouge et al., 1990; Carlson et al., 1995). These molecules alter the growth of two cell types in the root as they induce root hair deformation by inducing tip growth in existing root hairs (see below) and, furthermore, activate cortical cells to resume mitosis resulting in nodule primordia that are predominantly formed opposite protoxylem poles (Bond, 1948; Libbenga and Harkes, 1973a; Lerouge et al., 1990; Spaink et al., 1991; Truchet et al., 1991). The phytohormone ethylene has opposite effects on tip growth and cell division in roots of dicotelydonous plants. On the one hand, ethylene promotes tip growth, i.e. root hair formation (Tanimoto et al., 1995), indicating that it may act as a second messenger in Nod factor-induced root hair deformation. On the other hand, ethylene blocks cortical cell division (Grobbelaar et al., 1971; Goodlass and Smith, 1979; Lee and LaRue, 1992). Here we study how *Rhizobium* overcomes this paradox and we examine the role of ethylene in both processes.

Nod factor-induced root hair deformation can be followed when seedlings are grown in so-called Fåhraeus slides. Such studies showed that there is a correlation between the ability of root hairs to deform and their stage of development (Heidstra et al., 1994; N. de Ruijter, personal communication). In the development of root hairs three successive stages can be distinguished, represented by hairs located in adjacent zones on the root (Fig. 1). Zone I contains growing root hairs which do not deform upon Nod factor treatment. They have, just like other tip growing cells (reviewed by Sievers and Schnepf, 1981), a polarly organised cytoplasm. The apical region of these growing root hairs lacks big organelles, including vacuoles, hence it is called a clear zone (Fig. 1). Zone I root hairs show a reverse fountain type of cytoplasmic streaming that does not include the cytoplasm in the tip, and the direction of streaming is reversed below the clear zone. Other characteristics specific for the tip-growing root hairs of zone I are the

occurrence of a spectrin-like antigen and a cytoplasmic Ca^{2+} concentration gradient in the tip (N. de Ruijter, personal communication). In zone II, the clear zone is no longer visible (Fig. 1), cytoplasmic streaming reaches into the tip but is still of the reverse fountain type, and the Ca^{2+} gradient and spectrin-like antigen are absent in the tip. The hairs of zone III are full grown and characterized by the presence of a large vacuole, filling most of the hair, surrounded by a thin layer of cortical cytoplasm (Fig. 1). They show a rotational type of cytoplasmic streaming. Nod factors only induce deformation of root hairs in zone II, where about 80% of the root hairs are able to deform. Upon Nod factor addition, a swelling of the tip is visible within 1 hour, which is followed 1 hour later by the formation of a new cylindrical outgrowth initiated at the swelling (Heidstra et al., 1994). This new outgrowth has the characteristics of a growing root hair since a clear zone is found at the tip (Fig. 1), cytoplasmic streaming is of the reverse fountain type, a cytosolic Ca^{2+} concentration gradient is built up, and the spectrin-like antigen accumulates. Hence, it has been concluded that root hair deformation involves the reinitiation of tip growth in zone II hairs (N. de Ruijter, personal communication). Here we show that, as in *Arabidopsis*, ethylene regulates the initiation of root hair formation in vetch, and we investigate whether ethylene has also a positive regulatory role in Nod factor induced tip growth.

Applied ethylene is a potent inhibitor of nodule formation, blocking the formation of nodule primordia, whereas the number of infections does not decrease (Lee and LaRue, 1992). This inhibitory effect of ethylene can be counteracted by applying silver ions (Ag^+), which restores the ability to form nodules (Grobbelaar et al., 1971; Goodlass and Smith, 1979; Lee and LaRue, 1992). The silver ions are thought to interfere with the functioning of the ethylene receptor, thereby interfering with ethylene action (Burg and Burg, 1967; Beyer, 1976). Endogeneously produced ethylene also has a negative effect on nodulation. Upon treatment with aminoethoxyvinylglycine (AVG), an inhibitor of 1-aminocyclopropane-1-carboxylate (ACC) synthase, nodule formation on alfalfa and vetch was increased twofold (Peters and Crist-Estes, 1989; Zaat et al., 1989). The effect of ethylene as a negative regulator of nodule primordium formation is further demonstrated by a pea mutant (*sym5*) that has lost the ability to form nodule primordia. If *sym5* plants are treated with AVG, the roots regain the ability to form nodules. A similar effect is observed with Ag^+ ions (Fearn and LaRue, 1991; Guinel and LaRue, 1991). Thus, whereas Nod factors may employ ethylene to act as positive regulator in root hair deformation, at the same time ethylene is a negative regulator of primordium formation. Here, we report on the role of ethylene during the induction of both processes.

The developing nodule primordia are predominantly found in areas of the root cortex opposite protoxylem poles (Bond, 1948; Libbenga and Harkes, 1973a). This implies that the plant provides positional information, e.g., by an interplay of positive- and negative-acting factors, controlling the site where the primordia will be formed. Recently, uridine was identified as a positive-acting factor of the stele from pea that, in combination with phytohormones, is capable of inducing cell divisions in the inner cortex of pea root explants (Smit et al., 1995). In this study, we determined where ethylene is most likely produced by locating the site where ACC oxidase mRNA accumulates, and we tested whether endogeneously produced ethylene provides positional information controlling the site where nodule primordia are formed.

MATERIALS AND METHODS

Plant material and root hair deformation assay

Vicia sativa spp. *nigra* seeds were sterilised and germinated as described by Van Brussel et al. (1982). Germinated seeds were transferred to modified Fåhraeus slides (Bhuvaneswari and Solheim, 1985) in small trays containing Fåhraeus medium (Fåhraeus, 1957) and treated as described by Heidstra et al. (1994). Nod factors were purified according to the mrthod of Spaink et al. (1991). The mixture of Nod factors as secreted by *Rhizobium leguminosarum* bv *viciae*, designated NodRlv factors, was used at a concentration of 10^{-10} M in all experiments described. At least 2 Fåhraeus slides, containing 6 plants each, were used for each treatment and deformation was scored in a blind test by 2 persons, 3 hours after addition of NodRlv factors. The average percentage of deformed root hairs in a particular zone was determined. All experiments listed below were performed at least 5 times.

DIC micrographs of root hairs made using a Nikon microscope were imaged with a Panasonic wv-E550 3-CCD camera and digitized in TIFF format using a 756×536 (24 bits) Prysm framegrabber (Synoptics Ltd., Cambridge, UK). The files were contrast enhanced in Adobe Photoshop.

NodRlv factors, AVG, ACC, ethephon or Ag^+ were applied to the roots of vetch plants by replacing the medium with medium containing one or a combination of the above compounds. If the roots were incubated in the presence of Ag^+ ions, the medium was replaced every hour with fresh medium containing Ag^+ ions because silver salts were readily formed due to the presence of chloride and phosphate in the medium, thereby lowering the free Ag^+ concentration. No adverse effect was observed on growth or deformation when the medium of control or Nod factor treated plants was replaced every hour. Stock solutions of AVG, ACC, ethephon and $(Ag)_2SO_4$ (Sigma) were made up in water, filter sterilized, and stored at –20˚C.

In experiments with AVG or Ag^+ ions, root hair growth was determined using an ocular micrometer. Root growth and formation of root hairs was checked during 8 hours at 1-hour intervals by marking the position of the root tip and the emerging root hairs on the coverslip of the Fahraeus slide. After 8 hours, the amount of root hairs on the newly formed part of the root was determined by counting the root hairs found on 50 μm cross sections of the root that were made using a fibratome (BioRad).

Nodulation assay

Pea seeds (*Pisum sativum* cv. Rondo) were grown in gravel as described by Bisseling et al. (1978) and *Rhizobium leguminosarum* bv *viciae* strain 248 was used for inoculation 3 days after planting. When pea plants were grown in the presence of Ag^+ or AVG, 300 ml water or Fåhraeus medium (alternating) containing 50 μM $(Ag)_2SO_4$ or 50 μM AVG was applied 3, 6, 10 and 14 days after planting. After 17 days, the main roots were collected, hand sectioned, and the number and position of the nodules was determined using light microscopy. The position of the nodules was scored as opposite xylem, opposite phloem or opposite the region in between xylem and phloem.

In situ hybridization

Segments of 6-day old pea roots (100 μm) were fixed in PBS containing 4% paraformaldehyde, 0.25% glutaraldehyde, 0.08 M EGTA, 10% DMSO, and 0.1% Tween 20, for 3 hours at room temperature. In situ hybridization was performed essentially as described by Tautz and Pfeifle (1989), with modifications. The heptane washes were

eliminated. After fixation, tissue was kept in ethanol at –20˚C for 2 days. Before the proteinase K treatment, tissue was incubated for 30 minutes in 1:1 ethanol/xylene solution. This treatment was followed by a postfixation step in PBS containing 0.1% Tween 20 and 5% formaldehyde. After the proteinase K treatment the same postfixation step was applied. Prehybridization and hybridization took place at 42˚C. For the posthybridization washes an RNase A treatment for 15-30 minutes was included (40 μg/ml RNase A in 500 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA). Before use, the anti-digoxigenin antibodies coupled to alkaline phosphatase (Boehringer Mannheim) were preabsorbed in an acetone extract of fixed roots (overnight at 4˚C). The final concentration of the antibodies used was 1:2000. Incubation with the antibody took place at 4˚C overnight. The chromogenic reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate; Boehringer Mannheim) and nitroblue tetrazolium (NTB; Boehringer Mannheim) was carried out for 30 minutes to several hours. The cDNA clone PE8 encoding pea ACC oxidase (Peck et al., 1993) was used to make DIG-labeled antisense and sense probes.

RESULTS

Ethylene is a positive regulator of root hair formation in vetch

If vetch roots contained in Farhaeus slides are treated with either AVG or Ag^+, both at concentrations of 50, 10, 1 or 0.1 μM, the rate of root growth (±0.2 mm/hr) is not affected. However, addition of 50, 10 or 1 μM Ag^+ had an immediate effect on the organisation of the cytoplasm in zone I root hairs resulting in the disappearance of the clear zone within 10 minutes (Fig. 1); root hair growth was completely stopped within 1 hour. After adding 50 μM AVG, the clear zone in zone I hairs disappeared within 3 hours (Fig. 1), and root hair growth was stopped within the next hour. After 8 hours, the part of the root formed during the 1 μM Ag^+ treatment contained no root hairs (Table 1). Also the newly formed part of the root in the experiment where root hair growth was blocked with 50 μM AVG contained no root hairs (Table 1). The observation that the area containing the youngest root hairs remained cone shaped confirmed that root hair growth was stopped upon treatment with Ag^+ or AVG (data not shown). The minimal concentrations of 1 μM Ag^+ and 50 μM AVG that completely blocked root hair growth within the 8 hours time period of the experiment were used in further experiments.

To check whether the effect of AVG on root hair formation is caused by blocking ethylene biosynthesis, we preincubated vetch roots in medium with 1 mM ACC for 3 hours before exchanging the medium with one containing 50 μM AVG and 1 mM ACC. If the effect caused by AVG was due to a block of ACC synthase, the addition of ACC should preserve root hair development as it enables the production of ethylene by ACC oxidase. Indeed, on the part of the root formed in the presence of the mixture of AVG and ACC, root hairs developed as on untreated roots (Table 1). Furthermore, the clear zone remained present in zone I hairs. These results show that root hair formation in vetch, i.e. induction of tip growth as well as continuation of tip growth, is controlled, at least in part, by ethylene.

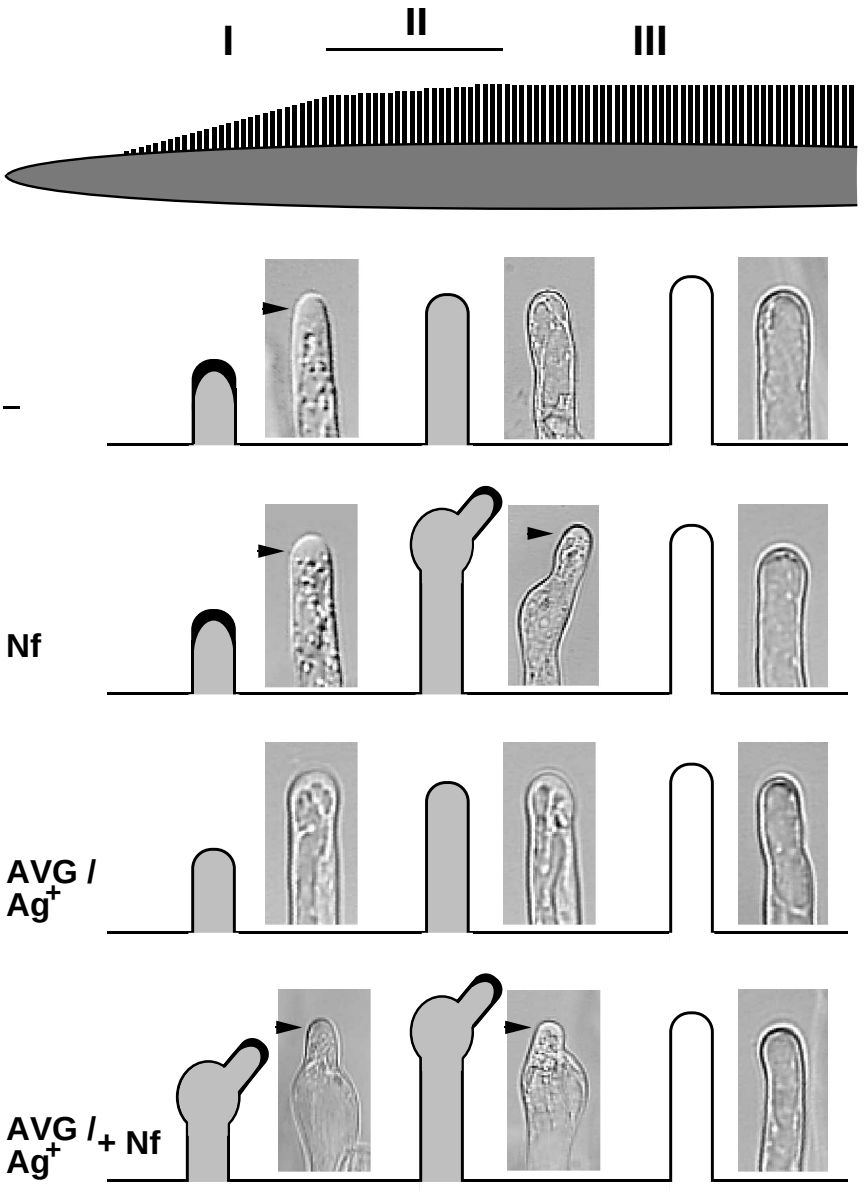

Fig. 1. Root hair deformation is correlated with developmental stage. In the development of root hairs three successive stages I, II and III are distinguished. Zone I root hairs are growing and have a clear zone at the tip (black) and cytoplasmic streaming is of the reverse fountain type (grey). In zone II the clear zone is no longer visible but cytoplasmic streaming is still of the reverse fountain type. After applying Nod factors, only root hairs present in zone II deform. Zone III contains mature root hairs lacking a clear zone and with rotation type cytoplasmic streaming (white). Addition of Ag^+ or AVG results in a disappearing of the clear zone. Subsequent addition of Nod factors leads to deformation of zone II and the altered zone I root hairs. Examples of root hairs are shown next to its schematic representative. All micrographs were made using Nomarski optics.

Table 1. Ethylene triggers vetch root hair formation

Treatment	Root hairs/50 μm
Control	75±7
50 μM AVG	0
1 mM ACC + 50 μM AVG*	79±9
1 μM Ag†	0

For each treatment, 3 roots were sectioned, and the root hairs present on 4 serial sections of 50 μm of the newly formed part of the root 8 hours after treatment were counted and the average amount of root hairs per 50 μm was determined.

*The roots were preincubated for 3 hours in medium containing 1 mM ACC.

†The medium was replaced every hour with fresh medium containing 1 μM Ag^+.

Nod factor-induced root hair tip growth is ethylene independent

Since Nod factors induce tip growth leading to root hair deformation in zone II hairs, we investigated whether ethylene is part of the Nod factor-activated signal transduction pathway. Upon treatment of vetch roots for 0, 3 or 6 hours with either 1, 10, 100 μM or 1 mM ACC or similar concentrations of the ethylene-releasing compound ethephon, neither compound induced root hair deformation at any of the applied concentrations. When 0, 3 or 6 hours later the medium was replaced with medium containing 10^{-10} M Nod factors in addition to the different concentrations of ACC or ethephon, root hair deformation was induced in about 80% of the root hairs in zone II, showing that these root hairs had remained fully competent to deform (Table 2). None of the treatments had any observable effect on the root hairs of zone I and III. These results show that ethylene alone is not sufficient to induce root hair deformation neither does it impede Nod factor-induced root hair deformation.

Subsequently, we tested whether induction of root hair deformation requires ethylene. Since root hair growth could be blocked with 50 μM AVG, this provided an internal control that ethylene-induced tip growth is blocked before adding Nod factors. The roots were pre-incubated for 0, 1, 3 or 6 hours with 50 μM AVG before the medium was exchanged with medium containing 10^{-10} M Nod factors in addition to 50 μM AVG. Even after pretreatment of the roots with AVG for 3 or 6 hours, which brings about a complete inhibition of root hair formation and growth of zone I hairs (see above) Nod factors induced root hair deformation in about 80 and 50% of the zone II root hairs, respectively (Table 2). While zone I root hairs remained usually unaltered, occasionally some zone I root hairs (<5%) also deformed. These results show that ethylene is not required for Nod factor-induced root hair tip growth.

Ag^+ treatment renders zone I root hairs susceptible to Nod factors

The involvement of ethylene in root hair deformation was further tested by using Ag^+ as an inhibitor of ethylene action. The roots were pre-incubated for 0, 15 minutes or 1 hour with 1 μM Ag^+ before the medium was exchanged with medium containing 10^{-10} M Nod factors in addition to 1 μM Ag^+. Addition of 1 μM Ag^+ resulted in the disappearance of the clear zone within 10 minutes and the zone I root hairs stopped growing within 1 hour as found earlier (see above). However, even when the growth of zone I root hairs was blocked at the time Nod factors were added, root hair deformation was induced in about 80% of the root hairs in zone II, just as in the experiments using AVG. Surprisingly, about 50% of the zone I hairs were also deformed (Fig. 1, Table 2), whereas in control plants not treated with Ag^+, zone I root hairs did not deform upon Nod factor addition. As mentioned, zone I hairs are growing hairs unable to deform upon Nod factor treatment, characterized by a clear zone at the tip. When zone I hairs develop into zone II hairs that are able to deform this switch involves the disappearance of this clear zone. Hence, these results indicate that the ability to deform is correlated with the cyto-architecture of the root hair. In addition, these results confirm that ethylene has no required positive role in Nod factor-induced root hair deformation.

Ethylene controls the position where nodule primordia can be formed

Endogeneous ethylene reduces the formation of nodule primordia, as inferred from studies with ethylene inhibitors (Peters and Crist-Estes, 1989; Zaat et al., 1989). As a first step in studying the effect of ethylene on cortical cell division in roots we localized the sites of ACC oxidase mRNA accumulation to determine where endogeneous ethylene may be synthesized.

The region of the root were root hairs just start to emerge is known to be the zone where primordia can be induced by *Rhizobium* and Nod factors. In situ hybridisation on 100-μm-thick cross sections of this zone using DIG-labeled ACC oxidase antisense RNA, showed that ACC oxidase mRNA accumulates in the cell layers opposite the phloem poles (Fig. 2). Interestingly, nodule primordia are usually formed opposite

Table 2. Effect of ethylene and ethylene inhibitors on root hair deformation

Compound	Concentration (μM)	Incubation time (hours)	NodRlv factor* (M)	Deformation† (%) zone II	Deformation† (%) zone I
ACC	1, 10, 100, 1000	0, 1, 3, 6	–	0	0
			10^{-10}	>70	0
ethephon	1, 10, 100, 1000	0, 1, 3, 6	–	0	0
			10^{-10}	>70	0
AVG	50	0, 1	10^{-10}	>70	0
		3, 6	10^{-10}	>70	4±2
Ag^+	1	0, 0.25, 1	10^{-10}	>70	45±5

All experiments were performed at least 5 times. In all cases when deformation was induced in zone II the percentage of deformed hairs was above 70%.

*Mixture of Nod factors secreted by *Rhizobium leguminosarum* bv *viciae*.

†Deformation was scored after the indicated incubation time, or 3 hours after application of NodRlv factor.

Table 3. Effect of Ag^+ and AVG on the position of the nodule on pea roots

Treatment*	Nodules	
	Total	Opposite phloem
–	169	1
100 μM Ag^+	247	23
50 μM AVG	182	15

Eight main roots of 17-day old plants were sectioned in each treatment, and the total number and position of the nodules was determined.

*All plants were inoculated with *Rhizobium leguminosarum* bv *viciae* strain 248 at day 3 and alternately 300 ml water or Fåhraeus medium containing 50 μM $(Ag)_2SO_4$ or 50 μM AVG was applied 3, 6, 10 and 14 days after planting.

protoxylem poles. Since it appears likely that the sites where ACC oxidase mRNA accumulates are also the sites of ethylene production, the ethylene produced in the cell layers opposite the phloem poles may suppress, in this area, cell division leading to nodule primordia.

To test this hypothesis, inoculated pea roots were grown in the presence of Ag^+ or AVG for 17 days. After sectioning the main root, the position of the formed nodules was determined. In the roots of plants grown in the absence of the ethylene inhibitors less than 1% of the nodules is found opposite the phloem poles (Table 3). However, when plants were grown in the presence of the inhibitors, about 10% of the nodules were located opposite a phloem pole (Table 3, Fig. 2). The amount of nodules formed opposite the region between the phloem and xylem poles was similar in all experiments (data not shown). These experiments show that the local production of ethylene by the plant plays a role in positioning nodule primordium formation.

DISCUSSION

By using the ethylene inhibitors AVG and Ag^+, we showed that ethylene has no required positive role in the reinitiation of root hair tip growth induced by Nod factors, i.e. root hair deformation, whereas tip growth in epidermal cells leading to root hair formation is promoted by ethylene. In addition, we show that ethylene is a negatively acting factor controlling the position where nodule primordia are formed.

In the model for the development of root hairs in *Arabidopsis* presented by Tanimoto et al. (1995), it was proposed that ethylene, after being synthesized in the central region of the root, diffuses radially through the apoplast between the walls of the cortical cells towards the epidermis. With in situ hybridization experiments, we showed that ACC oxidase is expressed in the cell layers opposite the phloem in that part of the root where root hairs are being formed. This is most likely the site where ethylene is formed since ACC oxidase catalyzes the last step in ethylene biosynthesis, converting ACC into ethylene. Our data support the proposed model since ethylene produced in the inner layers of the root can subsequently diffuse towards the epidermis where it may induce root hair development. Although ethylene is specifically synthesized opposite the phloem, diffusion will ensure that it also reaches epidermal cells opposite the xylem poles.

Our results in vetch, showing that ethylene is the signal triggering root hair development, confirm the conclusions reached in studies using *Arabidopsis* (Tanimoto et al., 1995). In addition to blocking the induction of root hair formation in vetch, root hair growth was also blocked by AVG and Ag^+, showing that induction as well as maintenance of tip growth in the epidermis in vetch is dependent on ethylene.

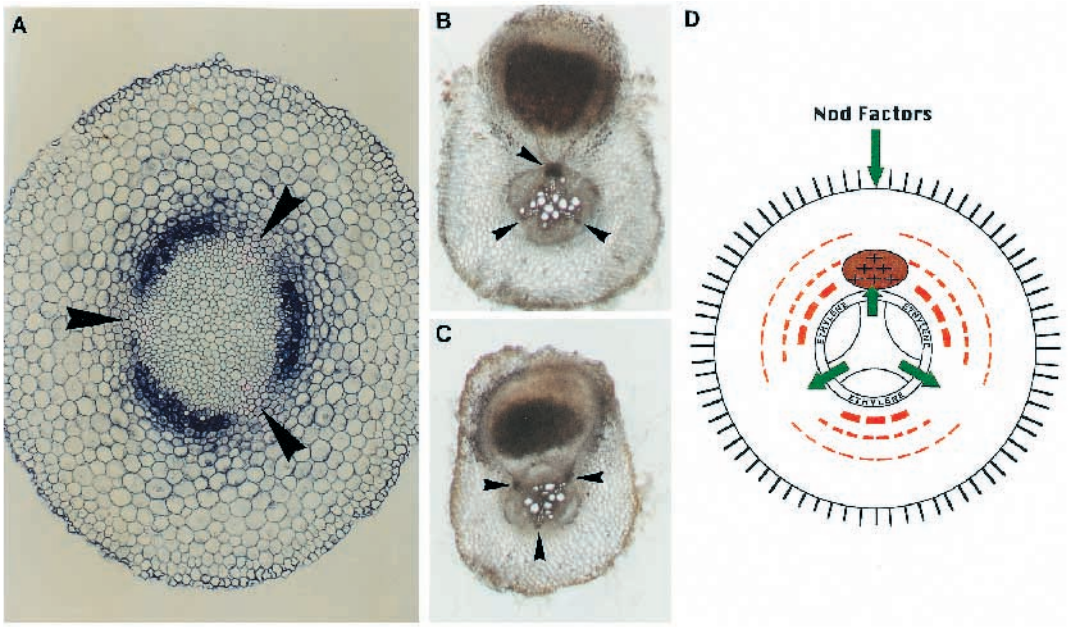

Fig. 2. The role of ethylene in positioning nodule formation. (A) Cross section of a pea root at the region where nodule primordia can be formed showing in situ hybridisation of a DIG-labeled ACC oxidase antisense RNA. ACC oxidase mRNA is seen to accumulate in the cell layers opposite the phloem poles. The position of the protoxylem is indicated with arrowheads. Hybridisation using a sense probe showed no signal. (B,C) Examples of nodules formed opposite the xylem or phloem poles. More than 99% of the nodules on pea roots in the absence of ethylene blockers (Ag or AVG) are formed opposite the xylem poles (arrowheads) whereas in the presence of these inhibitors, approx. 10% of the nodules are located opposite a phloem pole. Characteristic of nodules formed opposite the xylem poles (B) is the bifurcating vascular system coming from this xylem pole whereas with nodules formed opposite the phloem poles (C) the vascular system originates from the two xylem poles adjacent to the nodule. (D) Schematic representation of a gradient model involving positive- and negative-acting factors determining the position where nodule primordia can be formed. The developing nodule primordia are predominantly found opposite the protoxylem poles, implying that positional information is provided by the plant. In the root, a gradient system involving positive (e.g. uridine) and negative acting factors (e.g. ethylene) controls the position where cell division occurs upon stimulation by rhizobial Nod factors.

Growing vetch plants in Farhaeus slides makes it possible to follow root hair deformation continuously and the zones I, II and III are easily recognized. Therefore, it is possible to predict where root hair deformation will be induced and quantify the number of root hairs that respond. This provides an ideal assay for studies on Nod factor action using blockers such as Ag^+ and AVG. In the case of AVG, the root hairs of zone I and zone II on the same root were monitored when ethylene-induced growth of zone I root hairs was stopped after 3 hours. These zone I hairs represented the ideal internal control to show that even when root hair growth was stopped, subsequent application of Nod factors resulted in deformation of zone II root hairs. Although the region of the root where root hairs are emerging is the site where primordia and infection thread formation are induced by rhizobia, there is no reason to assume that the mechanism of root hair deformation induced by rhizobia is different from the root hair deformation induced in zone II by Nod factors in our assay. Therefore, we conclude that ethylene is not involved in root hair deformation and that there is no paradox concerning its action during the early *Rhizobium*-host plant interaction.

Whereas addition of Ag^+ had an immediate effect on the organisation of the cytoplasm in zone I root hairs, where the clear zone disappeared within 10 minutes, the same result was observed only after 3 hours following treatment with AVG. Surprisingly, the altered zone I root hairs deformed upon Nod factor treatment, indicating that the ability to deform is correlated with the cyto-architecture of the root hair. Why the Ag^+-treated zone I hairs deform more efficiently than those treated with AVG is not clear. The slow response of zone I root hairs to AVG compared to Ag^+ may be the reason why only occasionally some of these root hairs deformed. The effect of Ag^+ is fast since probably it blocks the ethylene receptors present in the epidermal cells, whereas AVG has to reach the site where ACC synthase is located, which, in *Arabidopsis* roots was reported to be in the root tip and in the stele (Rodrigues-Pousada et al., 1993). In addition, ACC and ethylene formed before ACC synthesis is blocked can still act until it has diffused out of the system. Alternatively, Ag^+ may, besides blocking ethylene action, affect the growth of root hairs in a way not related to ethylene inhibition. In any case, the fact that upon Ag^+ treatment zone I root hairs are able to deform confirms that ethylene is not involved in root hair deformation.

The developing nodule primordia are predominantly formed opposite the protoxylem poles in the root, implying some sort of positional information provided by the plant. This led Libbenga et al. (1973b) to postulate the presence of a transverse gradient system of endogeneous cell division factors which control the induction of primordium formation in the root cortex. Uridine represents a positive-acting factor since it occurs in the stele of pea roots and, when added to root cortex explants grown in the presence of auxins and cytokinin, induced cell division throughout the cortex (Libbenga et al., 1973b; Smit et al., 1995). These observations indicate that all pea cortical cells have the ability to divide in the presence of the proper cell division signals. However, in a bioassay with complete root explants containing the stele, cell divisions were first observed opposite protoxylem ridges, corresponding to the location where in vivo nodule formation is initiated. This suggests that also inhibiting factor(s) are released from the phloem areas of stele. Interestingly, ACC oxidase is expressed in the cell layers opposite the phloem, in the part of the root where nodule primordia can be formed. This expression pattern together with the inhibitory effect of ethylene on cell division suggested that ethylene can locally suppress the formation of nodule primordia. Indeed, by inhibiting ethylene synthesis or action a significant number (~10%) of the nodules is formed opposite the phloem. That the amount of the nodules opposite xylem and phloem is not equal indicates that ethylene action is not completely blocked and/or that ethylene is not the only factor determining the positioning of nodule primordia. On the one hand, positive-acting factors like uridine, if specifically released from the protoxylem poles, induce cell division in a local manner, whereas on the other hand, negative-acting factors like ethylene, produced opposite the phloem poles, inhibit cell division locally. Both positive- and negative-acting factors could make up gradient systems that determine the position where cell division occurs (Fig. 2).

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