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Rhizobium Lipooligosaccharides Rescue a Carrot Somatic Embryo Mutant

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At a nonpermissive temperature, somatic embryos of the temperature-sensitive (ts) carrot cell mutant ts11 only proceed beyond the globular embryo stage in the presence of medium conditioned by wild-type embryos. The causative component in the conditioned medium has previously been identified as a 32-kD acidic endochitinase. In search of a function for this enzyme in plant embryogenesis, several compounds that contain oligomers of *N*-acetylglucosamine were tested for their ability to promote ts11 embryo formation. Of these compounds, only the *Rhizobium* lipooligosaccharides or nodulation (Nod) factors were found to be effective in rescuing the formation of ts11 embryos. These results suggest that *N*-acetylglucosamine-containing lipooligosaccharides from bacterial origin can mimic the effect of the carrot endochitinase. This endochitinase may therefore be involved in the generation of plant analogs of the *Rhizobium* Nod factors.

INTRODUCTION

An intriguing observation made in several recent studies is that somatic embryogenesis appears to be highly dependent on proteins that either promote (Cordewener et al., 1991; De Jong et al., 1992; Kreuger and Van Holst, 1993) or inhibit (Gavish et al., 1992) somatic embryogenesis. These proteins most likely represent cell wall proteins, some of which may have a function in the control of cell expansion (Van Engelen and De Vries, 1992, 1993). However, the cell wall substrates for these secreted proteins remain to be identified.

The carrot cell line ts11, obtained after chemical mutagenesis (Giuliano et al., 1984), was originally isolated on the basis of its temperature-sensitive arrest in the transition of globular to heart stage somatic embryos. This arrest could be overcome by the addition of a mixture of proteins secreted by embryogenic wild-type cell lines (Lo Schiavo et al., 1990). The extracellular protein 3 (EP3), which was responsible for the observed ts11 embryo rescue, was identified as an acidic endochitinase of 32 kD (De Jong et al., 1992). The effect of the 32-kD endochitinase on the development of ts11 embryos appeared to be to promote the formation of proembryogenic masses and globular embryos from ts11 suspension cells at the nonpermissive temperature as well as to relieve the temperature arrest of ts11 embryos at globular stage. These results suggested that the 32-kD endochitinase affects ts11 embryo development from proembryogenic mass formation until the transition from the globular to heart stage embryo. In contrast with the aberrantly formed protoderm of ts11 globular embryos, ts11 globular embryos formed in the presence of 32-kD endochitinase exhibited a properly formed protoderm. This suggested that the 32-kD endochitinase has a positive effect on protoderm formation of ts11 globular embryos (De Jong et al., 1992).

In view of the presumed absence of the only known natural substrate for endochitinases, polymers of β-1,4-linked *N*-acetylglucosamine (chitin), in the cell walls of higher plants, it is unlikely that the endochitinase affects an abundant structural plant cell wall polymer. As put forward previously (De Jong et al., 1992), the assumption must be made that the substrate specificity of plant endochitinases is restricted to at least three β-1,4-linked *N*-acetylglucosamines. However, this has only been shown so far for a wheat germ endochitinase (Molano et al., 1979).

Recent reports have described the structure of signal molecules produced by *Rhizobium* bacteria as *N*-acetylglucosamine–containing lipooligosaccharides or nodulation (Nod) factors. These molecules have been identified on the basis of their ability to induce nodule organogenesis in roots of compatible legume host plants (Lerouge et al., 1990; Spaink et al., 1991; Truchet et al., 1991). They consist of an oligosaccharide backbone of four or five β-1,4-linked *N*-acetyl-D-glucosamines with a C16 or C18 fatty acid group attached to the nonreducing end. Modifications of the sugar moiety such as addition of an O-acetyl group or a sulfate group and the degree of unsaturation of the fatty acid moiety determine the host range

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specificity of the rhizobia (reviewed by Fisher and Long, 1992; Spaink, 1992).

An alternative hypothesis for the role of the carrot 32-kD endochitinase would be that the enzyme releases a signal molecule analogous to the *Rhizobium* Nod factors from a larger and so far unidentified *N*-acetylglucosamine–containing precursor present in a low amount in the plant cell wall. To test this hypothesis, several compounds that contain oligomers of *N*-acetylglucosamine were tested for their ability to promote proembryogenic mass and globular embryo formation in ts11. Of these, the *R. leguminosarum* bv *viciae* lipooligosaccharide NodRlv-V(Ac, C18:4) was found to be as effective as the 32-kD endochitinase.

RESULTS

Rescue of ts11 Embryo Formation by the *Rhizobium* Lipooligosaccharide NodRlv-V(Ac, C18:4)

In ts11 at the permissive temperature of 24°C, 1.6 somatic embryos per 10,000 cells are formed. This is ~1% of the wild-type level of 135 somatic embryos per 10,000 cells when incubated under the same conditions (De Vries et al., 1988). At 32°C, only very few embryos are formed in the absence of the 32-kD endochitinase. In the presence of an optimal amount of endochitinase, the number of somatic embryos formed at 32°C is slightly higher than in unsupplemented cultures at the permissive temperature, as shown in Table 1. These results confirm and extend the previously described dependence of ts11 embryogenesis on the presence of the 32-kD endochitinase. No effect was found of solubilized high molecular mass chitin or *N*-acetylglucosamine pentamers (Table 1) or of *N*-acetylglucosamine tetramers (results not shown) on ts11 embryo formation. In contrast, the *Rhizobium* lipooligosaccharide NodRlv-V(Ac, C18:4) at concentrations between 10^{-7} and 10^{-9} M was able to promote the formation of proembryogenic masses and globular embryos in the ts11 line, as shown in Table 1 and Figures 1C and 1D. At the optimal concentration (10^{-8} M) of NodRlv-V(Ac, C18:4), which is similar to the concentration used to induce nodule organogenesis in legume roots (Spaink et al., 1991), the *Rhizobium* molecules were as effective as the 32-kD endochitinase (Table 1 and Figures 1B to 1D). NodRlv-V(Ac, C18:1), differing only in the presence of one double bond instead of four in the fatty acid chain, is less active in the rescue of ts11 embryo formation (Table 1). This suggests that the fatty acid moiety of the lipooligosaccharides is important for its effect on ts11.

At reduced concentrations, the synthetic plant growth regulators 2,4-D and 6-benzylaminopurine (6-BAP) in combination promote the formation of proembryogenic masses and globular embryos in wild-type carrot cultures (Nomura and Komamine, 1985). Employed at the same concentrations, 6-BAP and/or 2,4-D did not promote ts11 proembryogenic mass and embryo formation at 32°C (Table 1). These results suggest that the effects of the 32-kD endochitinase and NodRlv-V(Ac, C18:4) cannot be mimicked by conventional plant growth regulators. Except for the cultures supplemented with 6-BAP and/or 2,4-D, in which cell proliferation was observed, all cultures were found to be similar to untreated controls with respect to the total number of cells present after 3 weeks (results not shown).

Table 1. Rescue of ts11 Embryos by Endochitinase and by *N*-Acetylglucosamine–Containing Compounds

Compound	Concentration (M)	Mean No. of ts11 Embryos per 10,000 Cells (SE)[a]	n[b]	P Values Compared to Control at 32°C[c]
Control (no addition) at 24°C	–	1.6 (0.5)	13	0.001
Control (no addition) at 32°C	–	0.3 (0.1)	34	–
32-kD endochitinase	5×10^{-8}	2.4 (0.5)	32	0.000
Glycol chitin	0.0001% w/v	0.0 (0.0)	4	0.256
N-Acetylglucosamine pentamer	10^{-9}	0.0 (0.0)	4	0.410
	10^{-8}	0.0 (0.0)	4	0.410
	10^{-7}	0.3 (0.3)	4	0.867
NodRlv-V(Ac, C18:4)	10^{-9}	1.0 (0.5)	16	0.043
	10^{-8}	2.4 (0.8)	24	0.000
	10^{-7}	1.6 (0.6)	20	0.001
NodRlv-V(Ac, C18:1)	10^{-9}	0.0 (0.0)	4	0.339
	10^{-8}	1.5 (1.5)	4	0.086
	10^{-7}	0.8 (0.8)	4	0.455
2,4-D	2×10^{-7}	0.3 (0.3)	3	0.946
2,4-D + 6-BAP	$2 \times 10^{-7}/6 \times 10^{-8}$	0.0 (0.0)	3	0.339

[a] The effect of addition of an optimal amount of the 32-kD endochitinase and of various *N*-acetylglucosamine–containing compounds is expressed as the number of proembryogenic masses and globular embryos obtained per 10,000 suspension cells. The standard error of the mean (SE) is included within parentheses.

[b] The number of individual assays (n) was obtained in 13 independent experiments.

[c] P values less than 0.05 are regarded as significantly different from the untreated control.

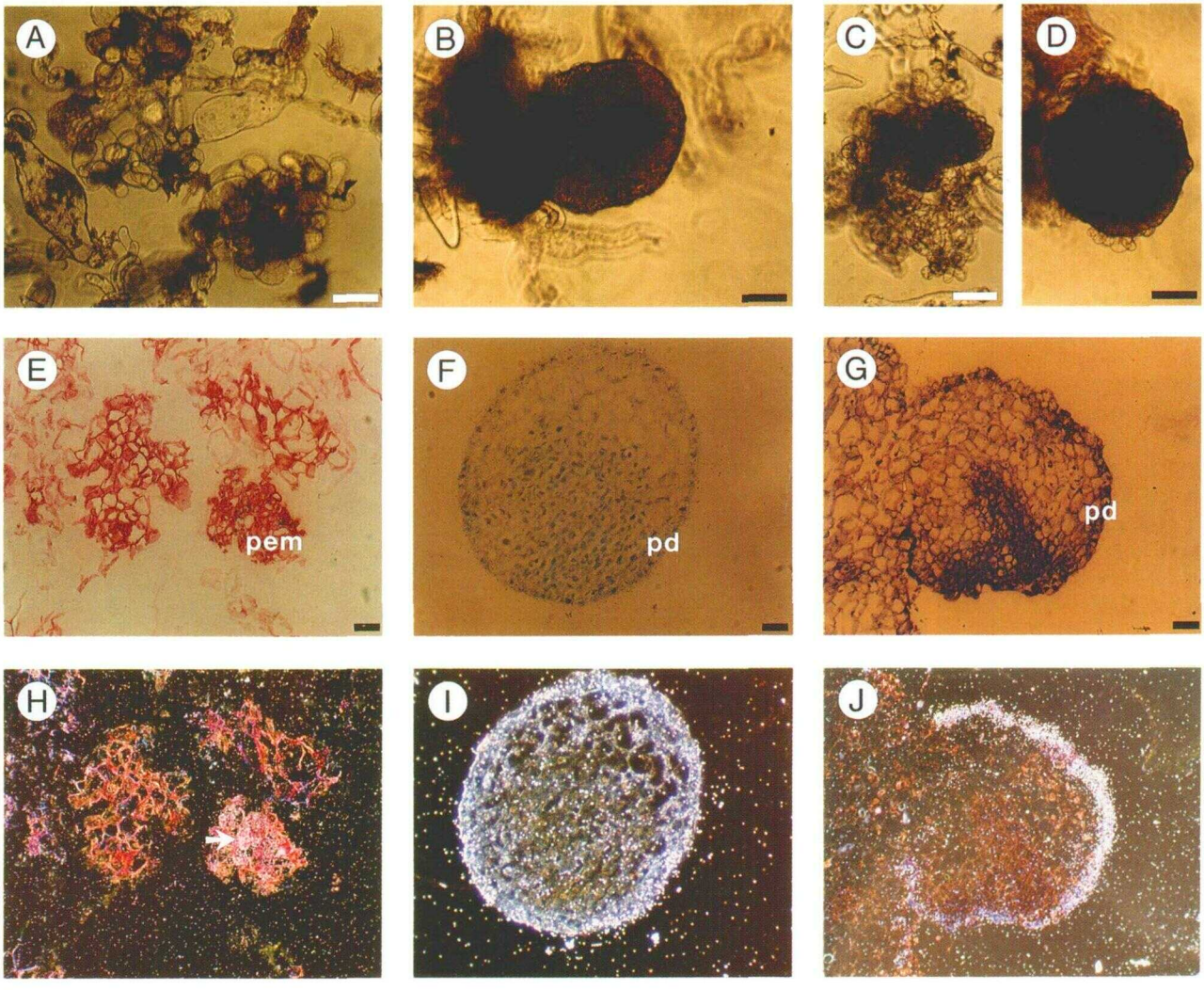

Figure 1. Rescue of ts11 Embryos.

Cells and embryos were photographed by bright-field microscopy. Sections were photographed by bright- and dark-field microscopy. Silver grains are visible as bright white dots in dark-field images.

(A) Control ts11 embryo culture at 32°C. Bar = 100 μm.

(B) Globular embryo in a ts11 embryo culture with 5 × 10^{-8} M of 32-kD endochitinase. Bar = 100 μm.

(C) Proembryogenic mass in a ts11 embryo culture with 10^{-8} M of NodRlv-V(Ac, C18:4). Bar = 100 μm.

(D) Globular embryo in a ts11 embryo culture with 10^{-8} M of NodRlv-V(Ac, C18:4). Bar = 100 μm.

(E) Bright-field photograph of sectioned ts11 control cells at 32°C. pem, proembryogenic mass. Bar = 50 μm.

(F) Bright-field photograph of a sectioned ts11 globular embryo formed in the presence of the 32-kD endochitinase. pd, protoderm. Bar = 100 μm.

(G) Bright-field photograph of a sectioned ts11 globular embryo formed in the presence of NodRlv-V(Ac, C18:4). pd, protoderm. Bar = 50 μm.

(H) In situ localization of EP2 mRNA in the section shown in **(E).** The arrow points to cells that express the EP2 gene.

(I) In situ localization of EP2 mRNA in the section shown in **(F).**

(J) In situ localization of EP2 mRNA in the section shown in **(G).**

Rescued ts11 Embryos Exhibit a Wild-Type EP2 Expression Pattern

To confirm that intact ts11 somatic embryos are formed after exposure to NodRlv-V(Ac, C18:4), expression of the carrot EP2 gene, previously identified as a marker for protodermal differentiation in carrot proembryogenic masses and embryos (Sterk et al., 1991), was examined by in situ mRNA localization. The results show that, in contrast to arrested ts11 embryos that exhibit either a uniform (Figure 1H) or a diffuse subepidermal pattern of expression (Sterk et al., 1991), ts11 embryos formed in the presence of the 32-kD endochitinase as well as

in the presence of NodRlv-V(Ac, C18:4) express the EP2 gene in its normal protoderm-specific fashion (Figures 1I and 1J, respectively).

DISCUSSION

The carrot 32-kD endochitinase EP3 has been identified based on its ability to promote proembryogenic mass and globular embryo formation and its ability to rescue arrested globular embryos of the temperature-sensitive mutant ts11 (De Jong et al., 1992). In this work, several compounds were tested that contain oligomers of *N*-acetylglucosamine for their ability to promote ts11 proembryogenic mass and globular embryo formation. Of these compounds, only the metabolic products of the *R. leguminosarum nod* genes, which were identified as *N*-acetylglucosamine–containing lipooligosaccharides (Lerouge et al., 1990; Spaink et al., 1991; Truchet et al., 1991), were found to be active. The products of the *Rhizobium nod* genes, therefore, appear to have a biological effect on in vitro embryogenesis in a nonleguminous plant.

At first glance, root nodule development in legumes and somatic embryogenesis in carrot are two disparate systems. However, a parallel may exist in that both require reinitiation of meristematic cell division in previously nondividing plant cells. In the *Rhizobium*–legume interaction, purified *Rhizobium* Nod factors elicit root hair deformation and initiation of cortical cell division in compatible interactions (Spaink et al., 1991). One explanation for the effect of the Nod factors on ts11 is that they may initiate meristematic cell divisions. In somatic embryogenesis, meristematic cell divisions, characteristic of proembryogenic mass formation, are generally initiated by exogenous auxins. In view of the multitude of physiological effects of auxins (Palme and Schell, 1991), other signal molecules are undoubtedly also involved. In line with this is the fact that no significant effect on ts11 embryo formation at the nonpermissive temperature was observed by commonly used plant growth regulators. Morphological observations, which were confirmed by the expression pattern of the protodermal marker EP2, reveal a properly formed protoderm of ts11 globular embryos formed in the presence of the 32-kD endochitinase or NodRlv-V(Ac, C18:4), which confirms previous morphological observations (De Jong et al., 1992). Because, similar to zygotic embryogenesis (Jürgens and Mayer, 1993), the protodermal cell layer of a somatic embryo is established well before the globular stage, it is plausible that the positive effect observed on protoderm formation of the 32-kD endochitinase and NodRlv-V(Ac, C18:4) is a consequence of their promotive effect on the initiation of meristematic cell divisions giving rise to proembryogenic masses.

Although both NodRlv-V(Ac, C18:4) and NodRlv-V(Ac, C18:1) elicit root hair deformation in *Vicia sativa* subsp *nigra*, only NodRlv-V(Ac, C18:4) is able to induce nodule meristems (Spaink et al., 1991). These results correlate with our finding that NodRlv-V(Ac, C18:1) is less active in the rescue of ts11 embryo formation than NodRlv-V(Ac, C18:4) and may indicate that the secondary structure of the fatty acid moiety is of importance in both nodule formation and in somatic embryogenesis. However, it is not known whether the fatty acid chain has a similar function in legume roots and carrot suspension cells.

In *Rhizobium*, the *nodABC* or common *nod* genes are essential for the synthesis of the core lipooligosaccharide structure (reviewed by Fisher and Long, 1992; Spaink, 1992). Introduction of the *Rhizobium nodA* and *nodB* genes in tobacco resulted in plants with altered flower and leaf morphology (Schmidt et al., 1991); this is postulated to be due to the generation of mitosis-stimulating compounds (Schmidt et al., 1988, 1991). The *Rhizobium* NodB protein has been identified as a chitooligosaccharide deacetylase (John et al., 1993). This deacetylase causes characteristic morphological alterations in transgenic tobacco plants; therefore, in tobacco, substrates are apparently present that can be modified by NodB to form specific plant growth signals (Schmidt et al., 1991). There are only a few indications to suggest that plant cells indeed contain oligomers of *N*-acetylglucosamine, other than those present in the carbohydrate moiety of glycoproteins. The results of Benhamou and Asselin (1989) indicate that chitin derivatives may occur in secondary plant cell walls of various plant species. Lipophilic molecules that can be degraded by chitinase appear to be present in uninfected Lathyrus plants (Spaink et al., 1993), but their biological relevance remains to be determined. The Xenopus gene *DG42*, transiently expressed during early embryogenesis (Rosa et al., 1988), was found to have sequence homology with the *Rhizobium nodC* gene and with the yeast chitin synthase II (*CHS2*) and catalytic subunit of chitin synthase III (*CSD2*) genes (Sandal and Marcker, 1990; Bulawa, 1992), suggesting that *N*-acetylglucosamine–containing signal molecules may be fairly common.

It is tempting to speculate that at least one member of the large family of endochitinases has a function in the generation of plant-produced signal molecules from larger precursors. In the case of ts11, this would imply that the *Rhizobium* Nod factor mimics the activity of these putative endochitinase-produced signal molecules. Although the correlations that may exist between the carrot 32-kD endochitinase and the *Rhizobium* Nod factors remain to be elucidated, it will now be of interest to identify biologically active, plant-produced molecules that have a similar effect as the *Rhizobium* Nod factors, with the aid of responsive in vitro systems such as that described here.

METHODS

ts11 Embryo Rescue Assays

Purification of the 32-kD endochitinase and embryo rescue assays at 32°C with the temperature-sensitive mutant ts11 were performed with newly initiated ts11 embryo cultures, as described by De Jong et al. (1992). *N*-Acetylglucosamine tetramers were purchased from

Sigma and *N*-acetylglucosamine pentamers from Seikagaku Kogyo, Tokyo, Japan, and were used without further purification. Lipooligosaccharides were purified to homogeneity from *Rhizobium* culture supernatants, as described by Spaink et al. (1991), and dissolved to 5×10^{-5} M in 50% (v/v) acetonitrile in water. Concentrations were determined, as described by Spaink et al. (1991). Dilution to 2×10^{-6} M was done in basal B5 plant culture medium. After autoclaving for 15 min, appropriate amounts were added to 2 mL of a resuspended 70- to 170-μm fraction of a 6-day-old ts11 suspension culture at 2500 clusters per mL. Control experiments, performed with appropriate serial dilutions of solvents (acetonitrile for the lipooligosaccharides and Tris-KCl for the 32-kD endochitinase), did not show any adverse effects on ts11 embryogenesis (results not shown).

A generalized linear model (Aitkin et al., 1991) was used to relate the number of somatic embryos both to treatment and experiment number. Because the number of somatic embryos obtained did not follow a normal distribution, the Poisson distribution was used as error distribution and the logarithm as the link function to relate the scale of measurement to the linear scale. The overall effect of treatment and experiment number was assessed by means of F tests. Significant differences between treatments ($P < 0.001$) and experiments ($P = 0.006$) could be demonstrated. Comparison of individual treatments to the unsupplemented control at 32°C resulted in the P values listed in Table 1.

In Situ Hybridization

In situ hybridization with ^{35}S-labeled antisense extracellular protein 2 mRNA was performed on fixed, paraffin embedded, and sectioned 3-week-old ts11 embryo cultures, as described previously (Cox and Goldberg, 1988; Sterk et al., 1991). Hybridization was visualized as bright white dots by dark-field illumination.

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REFERENCES

Aitkin, M., Anderson, D., and Francis, B. (1991). Statistical Modelling in GLIM (Oxford, England: Oxford University Press).

Benhamou, N., and Asselin, A. (1989). Attempted localization of a substrate for chitinases in plant cells reveals abundant *N*-acetyl-D-glucosamine residues in secondary walls. Biol. Cell **67,** 341–350.

Bulawa, C.E. (1992). *CSD2, CSD3* and *CSD4*, genes required for chitin synthesis in *Saccharomyces cerevisae*: The *CSD2* gene product is related to chitin synthases and to developmentally regulated proteins in *Rhizobium* species and *Xenopus laevis.* Mol. Cell. Biol. **12,** 1764–1776.

Cordewener, J., Booij, H., Van der Zandt, H., Van Engelen, F., Van Kammen, A., and De Vries, S. (1991). Tunicamycin-inhibited carrot somatic embryogenesis can be restored by secreted cationic peroxidase isoenzymes. Planta **184,** 478–486.

Cox, K.H., and Goldberg, R.B. (1988). Analysis of plant gene expression. In Plant Molecular Biology: A Practical Approach, C.H. Shaw, ed (Oxford, England: IRL Press), pp. 1–34.

De Jong, A.J., Cordewener, J., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., Van Kammen, A., and De Vries, S.C. (1992). A carrot somatic embryo mutant is rescued by chitinase. Plant Cell **4,** 425–433.

De Vries, S.C., Booij, H., Meyerink, P., Huisman, G., Wilde, H.D., Thomas, T.L., and Van Kammen, A. (1988). Acquisition of embryogenic potential in carrot cell-suspension cultures. Planta **176,** 196–204.

Fisher R.F., and Long, S.R. (1992). *Rhizobium*-plant signal exchange. Nature **357,** 655–660.

Gavish, H., Vard, A., and Fluhr, R. (1992). Suppression of somatic embryogenesis in *Citrus* cell cultures by extracellular proteins. Planta **186,** 511–517.

Giuliano, G., Lo Schiavo, F., and Terzi, M. (1984). Isolation and developmental characterization of temperature sensitive carrot cell variants. Theor. Appl. Genet. **67,** 179–183.

John, M., Röhrig, H., Schmidt, J., Wieneke, U., and Schell, J. (1993). *Rhizobium* NodB protein involved in nodulation signal synthesis is a chitooligosaccharide deacetylase. Proc. Natl. Acad. Sci. USA **90,** 625–629.

Jürgens, G., and Mayer, U. (1993). *Arabidopsis.* In A Colour Atlas of Developing Embryos, J. Bard, ed (London: Wolfe Publishing), in press.

Kreuger, M., and Van Holst, G.J. (1993). Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. Planta **189,** 243–248.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.C., and Dénarié, J. (1990). Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature **344,** 781–784.

Lo Schiavo, F., Giuliano, G., De Vries, S.C., Genga, A., Bollini, R., Pitto, L., Cozzani, G., Nuti-Ronchi, V., and Terzi, M. (1990). A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. Mol. Gen. Genet. **223,** 385–393.

Molano, J., Polacheck, I., Duran, A., and Cabib, A. (1979). An endochitinase from wheat germ. Activity on nascent and preformed chitin. J. Biol. Chem. **254,** 4901–4907.

Nomura, K., and Komamine, A. (1985). Identification and isolation of single cells that produce somatic embryos at a high frequency in a carrot suspension culture. Plant Physiol. **79,** 988–991.

Palme, K., and Schell, J. (1991). Auxin receptors take shape. An end to the long search for phytohormone receptors is firmly in sight. A better understanding of how plants respond to environmental changes should follow. Curr. Biol. **2,** 228–230.

Rosa, F., Sargent, T.D., Rebbert, M.L., Michaels, G.S., Jamrich, M., Grunz, H., Jonas, E., Winkles, J.A., and Dawid, I.B. (1988). Accumulation and decay of *DG42* gene products follow a gradient pattern during *Xenopus* embryogenesis. Dev. Biol. **129,** 114–123.

Sandal, N.N., and Marcker, K.A. (1990). Some nodulin and Nod proteins show similarity to specific animal proteins. In Nitrogen Fixation: Achievements and Objectives, P.M. Gresshoff, L.E. Roth, G. Stacey, and W.E. Newton, eds (New York: Chapman and Hall), pp. 687–692.

Schmidt, J., Wingender, R., John, M., Wieneke, U., and Schell, J. (1988). *Rhizobium meliloti nod*A and *nod*B genes are involved in generating compounds that stimulate mitosis of plant cells. Proc. Natl. Acad. Sci. USA **85,** 8578–8582.

Schmidt, J., John, M., Wieneke, U., Stacey, G., Röhrig, H., and Schell, J. (1991). Studies on the function of *Rhizobium meliloti* nodulation genes. In Advances in Molecular Genetics of Plant–Microbe Interactions, H. Hennecke, and D.P.S. Verma, eds (Dordrecht: Kluwer Academic Publishers), pp. 150–155.

Spaink, H.P. (1992). Rhizobial lipo-oligosaccharides: Answers and questions. Plant Mol. Biol. **20,** 977–986.

Spaink, H.P., Sheeley, D.M., Van Brussel, A.A.N., Glushka, J., York, W.S., Tak, T., Geiger, O., Kennedy, E.P., Reinhold, V.N., and Lugtenberg, B.J.J. (1991). A novel highly unsaturated fatty acid moiety of lipo-oligosaccharide signals determines host specificity of *Rhizobium*. Nature **354,** 125–130.

Spaink, H.P., Aarts, A., Bloemberg, G.V., Folch, J., Geiger, O., Schlaman, H.R.M., Thomas-Oates, J.E., Van De Snade, K., Van Spronsen, P., Van Brussel, A.A.N., Wijfjes, A.H.M., and Lugtenberg, B.J.J. (1993). Rhizobial lipo-oligosaccharides: Their biosynthesis and their role in the plant. In Advances in Molecular Genetics of Plant–Microbe Interactions, Vol. 2, E. Nester, ed (Dordrecht: Kluwer Academic Publishers), pp. 151–162.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot EP2 lipid transfer protein gene. Plant Cell **3,** 907–921.

Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., De Billy, F., Promé, J-C., and Dénarié, J. (1991). Sulphated lipo-oligosaccharide signals of *Rhizobium meliloti* elicit root nodule organogenesis in alfalfa. Nature **351,** 670–673.

Van Engelen, F.A., and De Vries, S.C. (1992). Extracellular proteins in plant embryogenesis. Trends Genet. **8,** 66–70.

Van Engelen, F.A., and De Vries, S.C. (1993). Secreted proteins in plant cell cultures. In Markers of Plant Morphogenesis, NATO-ASI Series, K.A. Roubelais-Angelakis, ed (New York: Plenum Press), in press.