34. Binding of GroES to a GroEL:polypeptide complex is more rapid in the presence of ATP than ADP (M. K. Hayer-Hartl, unpublished observation).
35. F. U. Hartl, *Nature* **371**, 557 (1994).
36. SPR experiments were performed with the BIAcore apparatus (Pharmacia Biosensor). All protein immobilization was performed at 25°C in buffer A at 0.25 μM GroES and 0.06 μM GroEL. The carboxylated dextran matrix of the 60-nl flow cell (CM5 research grade) was first activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide to allow the subsequent cross-linking of injected protein through primary amine groups (*21*). After cross-linking, the reactive groups were blocked by injection of 1 M ethanolamine (pH 8.5).
37. D. J. O'Shannessy, M. Brigham-Burke, K. K. Soneson, P. Hensley, I. Brooks, *Anal. Biochem.* **212**, 457 (1993).
38. GroEL and GroES proteins were isolated from overproducing strains of *E. coli* as described (*3, 4, 15*), and protein concentrations were determined by quantitative amino acid analysis.
39. GroEL-6His, containing six histidine residues joined to the COOH-terminus of GroEL, was constructed by insertion mutagenesis and the polymerase chain reaction, followed by sequencing from the *groEL* gene obtained in plasmid pOF39 [O. Fayet, J. M. Louran, C. Georgopoulos, *Mol. Gen. Genet.* **202**, 435 (1986)]. His-tagged GroEL was purified from *E. coli* extracts by affinity chromatography on a Ni^{2+}-NTA column (Qiagen). GroEL-6His bound stably to Ni^{2+}-NTA in the absence and presence of nucleotide.
40. Unfolded rhodanese was diluted 100-fold from a solution containing 6 M GdmCl, 30 mM tris-HCl (pH 7.5), and 5 mM DTT into buffer A at 4°C by rapid mixing immediately before initiating injection into the BIAcore flow cell. This technique excludes the possibility of exposing the chaperonin complex to locally high concentrations of denaturant during the dilution step. The solution reached the flow cell after ~30 s. The $t_{1/2}$ for rhodanese aggregation was ~5 min. Allowing aggregation to occur before injection into the flow cell prevented the stimulatory effect of unfolded rhodanese on dissociation of GroEL:GroES.
41. We thank R. Granzow for critical support and discussion. Supported by NIH grant GM 50908.

4 April 1995; accepted 16 June 1995

Growth of Tobacco Protoplasts Stimulated by Synthetic Lipo-Chitooligosaccharides

Horst Röhrig,* Jürgen Schmidt, Richard Walden, Inge Czaja, Edvins Miklaševičs, Ursula Wieneke, Jeff Schell, Michael John

Nodulation (Nod) factors are lipo-chitooligosaccharides (LCOs) secreted by rhizobia to trigger the early steps of nodule organogenesis in leguminous plants. A method to synthesize LCOs in vitro was developed. Synthetic LCOs alleviated the requirement for auxin and cytokinin to sustain growth of cultured tobacco protoplasts. LCOs containing $C_{18:1}$ *trans*–fatty acyl substituents were more effective than those containing *cis*–fatty acids in promoting cell division as well as in activating an auxin-responsive promoter and the expression of a gene implicated in auxin action. These data indicate that LCOs redirect plant growth also in nonlegumes by activating developmental pathways also targeted by phytohormones.

The basic structure of Nod factors produced by rhizobia consists of a β-1,4-linked *N*-acetylglucosamine (GlcNAc) containing tetra- or pentasaccharide, *N*-acylated with different long-chain fatty acids at the nonreducing glucosamine (GlcN) moiety (*1*, *2*). The role of LCOs as signaling molecules in plant development has stimulated interest in their synthesis. Recently, chemical synthesis of the alfalfa-specific Nod Rm-IV factor has been described (*3*). However, this strategy is relatively complex because of the large variety of functional groups requiring numerous coupling reactions, protection, and selective deprotection steps. We have now developed a simplified procedure for synthesis of LCOs. The acetyl group at the nonreducing GlcNAc residue is removed enzymatically from chitooligosaccharides by recombinant NodB (*4*), and a fatty acyl chain is then coupled chemically to the free amino group with fatty acid anhydrides as acylation agents (*5*). With this procedure, we *N*-acylated the tri-*N*-acetyl GlcN tetrasaccharide backbone with a saturated C_{18} fatty acid, as well as with various monounsaturated C_{18} fatty acids. The synthesized LCOs were biologically active, as confirmed by their ability to deform root hairs of vetch (*6*), a specific bioassay for Nod factors (*7*).

Separation of synthetic LCOs on a preparative C_{18} reversed-phase high-performance liquid chromatography (HPLC) column (*8*) yielded two peaks corresponding to α and β anomers of the oligosaccharide backbone. The HPLC profile of an LCO, synthesized by *N*-acylation of the tetrasaccharide with *cis*-11-octadecenoic acid, that co-elutes with *Vicia*-specific NodRIv-IV ($C_{18:1}$) factor (*9*) is shown in Fig. 1A. To verify that the desired lipid had been attached to the tetrasaccharide, we released the corresponding fatty acid from the LCO by alkaline hydrolysis and subjected it to analysis by capillary gas-liquid chromatography (GLC) (*10*) (Fig. 1B). All fatty acids released from the synthetic LCOs by saponification co-chromatographed with authentic lipids. Radioisotopically labeled LCOs were hydrolyzed by chitinase to mono- and disaccharides (GlcNAc and $GlcNAc_2$) and lipid-linked di- or trisaccharides, which migrated on a thin-layer chromatography (TLC) plate faster than nondegraded LCOs (Fig. 1C) (*11*). Together, these results confirmed the presence of a β-1,4 linkage between GlcNAc residues in synthetic LCOs as well as of acyl substituents at the nonreducing terminus of the carbohydrate backbone.

In legumes, LCOs trigger the formation of the root nodule by initiating cell division at

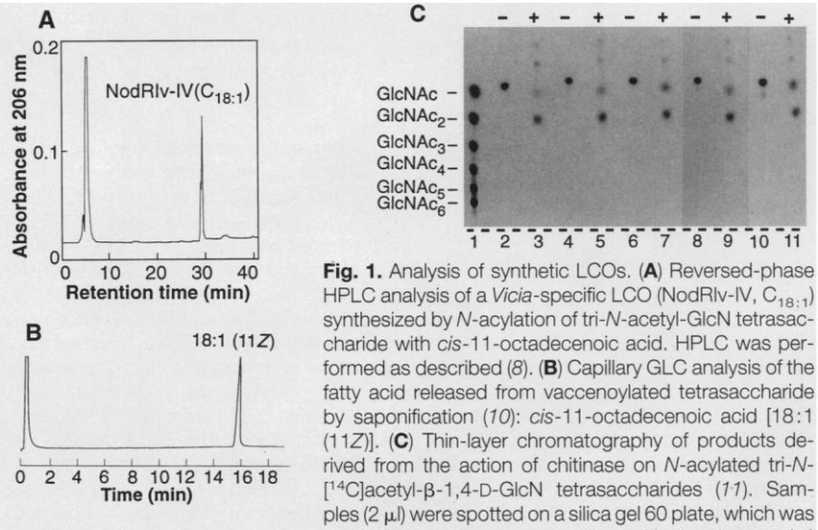

Fig. 1. Analysis of synthetic LCOs. (**A**) Reversed-phase HPLC analysis of a *Vicia*-specific LCO (NodRIv-IV, $C_{18:1}$) synthesized by *N*-acylation of tri-*N*-acetyl-GlcN tetrasaccharide with *cis*-11-octadecenoic acid. HPLC was performed as described (*8*). (**B**) Capillary GLC analysis of the fatty acid released from vaccenoylated tetrasaccharide by saponification (*10*): *cis*-11-octadecenoic acid [18:1 (11*Z*)]. (**C**) Thin-layer chromatography of products derived from the action of chitinase on *N*-acylated tri-*N*-[^{14}C]acetyl-β-1,4-D-GlcN tetrasaccharides (*11*). Samples (2 μl) were spotted on a silica gel 60 plate, which was then developed and subjected to autoradiography (*11*). Incubations were performed in the absence (–) or presence (+) of chitinase. Broken line, the origin of sample application. Lanes: 1, ^{14}C-labeled GlcNAc to hexa-acetylchitohexaose ($GlcNAc_6$); 2 and 3, octadecanoylated tetrasaccharide; 4 and 5, *trans*-9-octadecenoylated tetrasaccharide; 6 and 7, *cis*-9-octadecenoylated tetrasaccharide; 8 and 9, *trans*-11-octadecenoylated tetrasaccharide; 10 and 11, *cis*-11-octadecenoylated tetrasaccharide.

Max-Planck-Institut für Züchtungsforschung, Carl-von-Linné-Weg 10, 50829 Köln, Germany.

*To whom correspondence should be addressed.

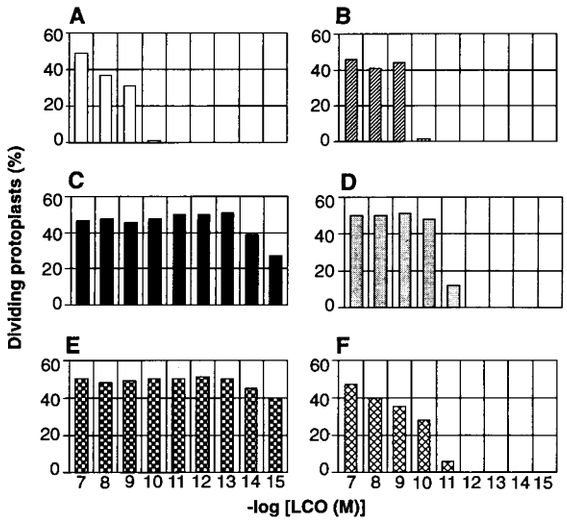

Fig. 2. Comparison of the effects of chitotetraose N-acylated with saturated or unsaturated C_{18} fatty acids on division of tobacco protoplasts in the absence of auxin. The response of protoplasts to different LCOs in a concentration range of 10^{-7} to 10^{-15} M was determined in the absence of auxin and in the presence of 1 μM kinetin (*15*). Data are means of three independent experiments performed in duplicate. Deviation from the mean was ≤5%. The tetrasaccharide backbone carried the following N-acyl substituents: (**A**) no substituent, (**B**) octadecanoyl (18:1), (**C**) *trans*-9-octadecenoyl (18:1, 9*E*), (**D**) *cis*-9-octadecenoyl (18:1, 9*Z*), (**E**) *trans*-11-octadecenoyl (18:1, 11*E*), and (**F**) *cis*-11-octadecenoyl (18:1, 11*Z*).

distinct sites, possibly by affecting regulation of the plant cell cycle (*12*). LCOs may play a general role as plant growth regulators (*13, 14*), and nonleguminous plants may possess at least part of the mechanism targeted by these signaling molecules. We isolated mesophyll protoplasts from wild-type tobacco and determined their response to LCOs (*15*). Under defined culture conditions, tobacco protoplasts require external addition of both auxin and cytokinin for cell growth and division (*16*). The absence of phytohormone could be compensated for by the addition of synthetic LCOs to the culture medium. Whereas auxin, in the presence of cytokinin, promotes cell division in the micromolar range, exogenously supplied synthetic LCOs stimulated auxin-independent growth of protoplasts at nano- to femtomolar concentrations, depending on the fatty acid attached to the oligosaccharide (Fig. 2). Under these conditions, protoplasts were able to divide continuously and form calli in vitro (*17*). LCOs containing monounsaturated C_{18} fatty acids with double bonds in the trans (*E*) configuration (*trans*-9 or *trans*-11) activated maximal rates of cell division (~50%) at a concentration as low as 10^{-15} M, whereas in the cis (*Z*) configuration a concentration of at least 10^{-11} M was needed to observe activity. Lack of a double bond in the acyl moiety, or the absence of an acyl chain on the carbohydrate backbone, resulted in a further decrease in the ability to stimulate protoplast division (Fig. 2). Addition of free fatty acids over the same concentration range to protoplasts did not affect division (*17*).

In the presence of auxin, LCOs could also replace cytokinin to sustain growth of cultured tobacco cells. At 10^{-13} M, LCOs containing $C_{18:1}$ *trans*–fatty acids (9*E* or 11*E*) stimulated maximal levels of cell division in the absence of cytokinin (Fig. 3A). The other compounds tested showed a similar stimulation of cell division only at a concentration of 10^{-10} M. Moreover, LCOs containing *trans*–fatty acyl substituents stimulated division of tobacco protoplasts in the absence of both auxin and cytokinin when added at 10^{-13} M (Fig. 3B).

To investigate whether the ability of synthetic LCOs to substitute for phytohormones might also apply to phytohormone-induced gene expression, we examined the response of a phytohormone-responsive promoter to LCOs. We used the −90–base pair region of the cauliflower mosaic virus 35S RNA promoter linked to the gene encoding β-glucuronidase (GUS) (*15, 18, 19*). This promoter element is responsive to auxin as well as jasmonic and salicylic acid (*20*). Tobacco protoplasts transfected with the reporter gene plasmid (pG35S#13) (*18*) were incubated in the presence of auxin or different LCOs (Fig. 4A). Transient GUS expression was observed in the presence of micromolar concentrations of auxin or picomolar concentrations of LCOs containing $C_{18:1}$ *trans*–fatty acids.

Possible targets of LCOs are genes that mediate auxin-responsive cell division. One such gene, *axi1*, has been isolated by activation of transferable DNA segment (T-DNA) tagging from tobacco (*21*). The *axi1* gene is auxin- and cytokinin-responsive, and its deregulated expression uncouples protoplasts from the normal effects of auxin on cell division (*22*). Northern (RNA) blot analysis revealed the presence of an ~2.1-kb *axi1* transcript in protoplasts cultured in the presence of either auxin or LCOs containing *trans*–fatty acids (Fig. 4B). In contrast, cells treated with chitotetraose or LCOs containing either saturated acyl substituents or monounsaturated *cis*–fatty acids had no or only

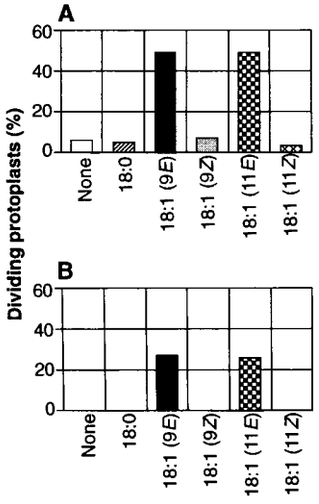

Fig. 3. Response of cultured tobacco protoplasts to LCOs at a concentration of 10^{-13} M. (**A**) Protoplast bioassays were performed in the absence of kinetin but in the presence of 5 μM auxin [α-naphthaleneacetic acid (NAA)]. (**B**) The same experiments were performed as in (A), but without addition of phytohormones to the culture medium. Acyl moieties linked to the oligosaccharide backbone are indicated. Data are means of three independent experiments performed in duplicate.

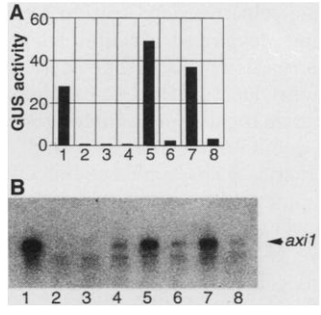

Fig. 4. Effects of chitotetraose and LCOs on the activation of an auxin-responsive element of the cauliflower mosaic virus 35*S* (CaMV35S) promoter and of *axi1* gene expression. (**A**) Transient GUS expression assays. Protoplasts were transfected with plasmid pG35S13, which contains the auxin-responsive region of the CaMV35S RNA promoter linked to the GUS reporter gene (*18*). GUS activity was measured in protoplasts as described (*18*). Data are expressed as nanomoles of 4-methyl-umbelliferone released per microgram of protein per hour and represent means of three independent experiments. Protoplasts grown in the presence of 1 μM kinetin were treated with 5 μM NAA (bar 1), water (control) (bar 2), 10^{-13} M chitotetraose (bar 3), or chitotet- raose *N*-acylated with the following fatty acyl substituents (all 10^{-13} M): $C_{18:0}$ (bar 4), $C_{18:1}$(9*E*) (bar 5), $C_{18:1}$(9*Z*) (bar 6), $C_{18:1}$(11*E*) (bar 7), or $C_{18:1}$(11*Z*) (bar 8). (**B**) Northern blot analysis of *axi1* expression. Total RNA was isolated from 3-day-old protoplasts (6×10^6) grown in the presence of 5 μM NAA, chitotetraose, or *N*-acylated derivatives of chitotetraose (all at a concentration of 10^{-13} M) (*22*). The RNA (30 μg) was fractionated on a 1.5% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled Sph I fragment of *axi1* complementary DNA (0.75 kb). Equal amounts of RNA were shown to be present in each lane by probing the same blot with ^{32}P-labeled ribosomal DNA (not shown). Lane numbers correspond to bar numbers in (A).

a minimal effect on the steady-state concentration of *axi1* transcripts.

Thus, at low concentrations, LCOs containing *trans*–fatty acyl substituents activate the expression of *axi1* in protoplasts of the nonlegume tobacco, conferring on these cells the ability to grow in the absence of auxin. As LCOs mimic auxin in activating the expression of a tobacco gene that apparently mediates auxin action, our data suggest that mitosis of protoplasts triggered by auxin shares steps in signal transduction with LCO-stimulated cell division. At least part of the mechanism necessary for nodule formation in legumes is therefore also present in nonleguminous plants. Thus, LCOs may be considered as plant growth regulators and may be related to as yet unidentified endogenous plant signals.

REFERENCES AND NOTES

1. P. Lerouge *et al.*, *Nature* **344**, 781 (1990); G. Truchet *et al.*, *ibid.* **351**, 670 (1991); J. Dénarié and J. Cullimore, *Cell* **74**, 951 (1993).
2. M. Schultze, E. Kondorosi, P. Ratet, M. Buiré, A. Kondorosi, *Int. Rev. Cytol.* **156**, 1 (1994).
3. K. C. Nicolaou, N. J. Bockovich, D. R. Carcanague, C. W. Hummel, L. F. Even, *J. Am. Chem. Soc.* **114**, 8701 (1992); L.-X. Wang, L. Chuan, R.-W. Wang, Y.-Z. Hui, *Tetrahedron Lett.* **34**, 7763 (1993).
4. M. John, H. Röhrig, J. Schmidt, U. Wieneke, J. Schell, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 625 (1993).
5. Radioisotopically labeled and nonlabeled β-1,4-tri-*N*-acetyl-D-GlcN tetrasaccharides were prepared and purified as described (*8*). Tri-*N*-GlcNAc tetrasaccharide was *N*-acylated with *cis*-9- or *cis*-11-octadecenoic anhydrides (Nu Check Prep, Elysion, MN) as follows. Anhydrides (40 μl) were dissolved in 1.44 ml of 2-propanol. The solution was stirred at 37°C, and 2 mg of tetrasaccharide dissolved in 320 μl of 10% (v/v) acetic acid was slowly added. The mixture was stirred under argon at 37°C for 16 hours and subsequently dried. To remove excess anhydrides, we suspended the residue in 0.5 ml of water and extracted it once with ethyl acetate. The aqueous phase was dried, and the residue was extracted three times with 1-ml portions of 60% (v/v) acetonitrile at 40°C. The combined supernatants were applied to a preparative reversed-phase HPLC column. For the synthesis of *N*-octadecanoyl-tri-*N*-GlcNAc tetrasaccharide, 25 mg of stearic anhydride (Sigma) and 2 mg of tetrasaccharide were dissolved in 3.5 ml of chloroform:2-propanol [1:1 (v/v)] at 37°C. The mixture was stirred under argon at 37°C overnight. After the solvents were evaporated with nitrogen, acylated chitooligosaccharides were extracted with 50% (v/v) acetonitrile at 56°C and subsequently subjected to preparative HPLC. To attach elaidic acid to the free amino group of the tetrasaccharide, we dissolved 2 mg of tri-*N*-GlcNAc tetrasaccharide in 640 μl of 10% (v/v) acetic acid and added the solution to a vial containing 25 mg of *trans*-9-octadecenoic anhydride (Sigma) in 2.9 ml of 2-propanol. The mixture was stirred under argon for 16 hours at 37°C and dried. The residue was extracted with 50% (v/v) acetonitrile at 56°C, and 1-ml portions were applied to a C_{18} column. In the case of *trans*-11-octadecenoic acid, we attached the free acid (Sigma) to the primary amine through the action of 2-chloro-1-methylpyridinium iodide (Sigma) in acetonitrile as described (*3*).
6. U. Wieneke, H. Röhrig, M. John, J. Schmidt, unpublished data.
7. R. Heidstra *et al.*, *Plant Physiol.* **105**, 787 (1994).
8. H. Röhrig *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3122 (1994).
9. The *Vicia*-specific Nod factor from *Rhizobium leguminosarum*, Nod Rlv-IV (Ac, $C_{18:1}$), was deacetylated at C-6 of the nonreducing sugar residue as described [P. Roche, P. Lerouge, C. Ponthus, J. C. Promé, *J. Biol. Chem.* **266**, 10933 (1991)].
10. Fatty acids were released from LCOs by saponification [5% (w/v) KOH, 18 hours, 80°C]. After acidification, fatty acids were extracted with chloroform and analyzed by GLC on a Nukol column (15 m by 0.53 mm, 0.5-μm film thickness; Supelco). After split injection (ratio, 10:1), the column temperature was programmed from 100° to 220°C at a rate of 10°C/min. Helium was the carrier gas, at a flow rate of 30 ml/min.
11. The incubation mixture contained 10 mM sodium phosphate (pH 6.8), *N*-acylated tri-*N*-[^{14}C]acetyl-β-1,4-D-GlcN tetrasaccharide (10 nCi), and 40 μg of recombinant *Serratia marcescens* chitinase (*4*), in a final volume of 20 μl. After incubation at 37°C for 16 hours, the reaction mixture was inactivated by heating. Degradation products were separated by thin-layer chromatography and subjected to autoradiography (*8*).
12. D. P. S. Verma, *Plant Cell* **4**, 373 (1992); G.-H. Miao, Z. Hong, D. P. S. Verma, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 943 (1993); A. Savouré *et al.*, *EMBO J.* **13**, 1093 (1994); W.-C. Yang *et al.*, *Plant Cell* **6**, 1415 (1994).
13. J. Schmidt, R. Wingender, M. John, U. Wieneke, J. Schell, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8578 (1988); J. Schmidt *et al.*, *Plant J.* **4**, 651 (1993).
14. A. D. De Jong *et al.*, *Plant Cell* **5**, 615 (1993).
15. Protoplasts were isolated from tobacco (SR1) as described [R. Walden, I. Czaja, T. Schmülling, J. Schell, *Plant Cell Rep.* **12**, 551 (1993)]. In each cell division assay, protoplasts (5 ml) were cultured at a density of 6×10^4 cells per milliliter. LCOs dissolved in dimethylsulfoxide were diluted in Jensen medium containing 1% dimethylsulfoxide and were added, at various concentrations, in a final volume of 50 μl per assay. Dividing protoplasts were counted microscopically 4 days after addition of LCOs to the medium.
16. T. Nagata and I. Takebe, *Planta* **92**, 301 (1970).
17. R. Walden, I. Czaja, H. Röhrig, unpublished results.
18. R. A. Jefferson, T. A. Kavanaugh, M. Bevan, *EMBO J.* **6**, 3901 (1987).
19. X. Liu and E. Lam, *J. Biol. Chem.* **269**, 668 (1994).
20. S. R. Kim, Y. Kim, G. An, *Plant Physiol.* **103**, 97 (1993); X.-F. Qin, L. Holuigue, D. M. Horvath, N.-H. Chua, *Plant Cell* **6**, 863 (1994); T. Ulmasov, G. Hagen, T. Guilfoyle, *Plant Mol. Biol.* **26**, 1055 (1994); B. Zhang and K. B. Singh, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2507 (1994).
21. H. Hayashi, I. Czaja, H. Lubenow, J. Schell, R. Walden, *Science* **258**, 1350 (1992).
22. R. Walden, H. Hayashi, H. Lubenow, I. Czaja, J. Schell, *EMBO J.* **13**, 4729 (1994); R. Walden, unpublished results.

23. We thank H. Spaink for the gift of Nod factor. Supported by grants from the Alexander von Humboldt Stiftung (E.M.), the Bundesministerium für Forschung und Technologie, and the European Union (J.S.).

17 March 1995; accepted 15 June 1995

Structure of the *Arabidopsis RPM1* Gene Enabling Dual Specificity Disease Resistance

Murray R. Grant,*† Laurence Godiard,*† Esther Straube, Tom Ashfield, Jürgen Lewald, Airlie Sattler, Roger W. Innes, Jeffery L. Dangl‡

Plants can recognize pathogens through the action of disease resistance (*R*) genes, which confer resistance to pathogens expressing unique corresponding avirulence (*avr*) genes. The molecular basis of this gene-for-gene specificity is unknown. The *Arabidopsis thaliana RPM1* gene enables dual specificity to pathogens expressing either of two unrelated *Pseudomonas syringae avr* genes. Despite this function, *RPM1* encodes a protein sharing molecular features with recently described single-specificity *R* genes. Surprisingly, *RPM1* is lacking from naturally occurring, disease-susceptible *Arabidopsis* accessions.

Plants express sophisticated genetic systems to recognize pathogens. Complex *R*-gene loci have been defined in a variety of plant-pathogen interactions. Genetic analyses suggest that each *R*-encoded specificity responds to a single *avr*-dependent pathogen signal (*1*). The recent cloning of four *R* genes (*2–7*) and accumulation of cloned bacterial and fungal *avr* genes (*8*) provide limited insight into the structural basis of specificity, although most models predict that *R* gene products interact with specific pathogen signal molecules produced in an *avr*-dependent manner (*9*).

RPM1 was identified in *A. thaliana* accession Col-0 as conferring resistance to *P. syringae* isolates expressing the *avrRpm1* gene (*10, 11*). Functional homologs of *RPM1* exist in pea, bean, and soybean (*12*). Resistance in *A. thaliana* to the *P. syringae avrB* gene also mapped to the *RPM1* interval (initially termed *RPS3*) (*13*), and genetic analyses of *A. thaliana* mutants have suggested that *RPM1* conferred resistance to *P. syringae* expressing either *avrRpm1* or *avrB* (*14*). Because the sequences of *avrB* and *avrRpm1* are unrelated (*12, 15*), *RPM1* appears to determine a dual specificity.

RPM1 was mapped by restriction fragment length polymorphism (RFLP) analysis

M. R. Grant, L. Godiard, E. Straube, J. Lewald, Max-Delbrück Laboratory, Carl von Linné Weg 10, 50829 Köln, Germany.
T. Ashfield, A. Sattler, R. W. Innes, Department of Biology, Indiana University, Bloomington, IN 47405, USA.
J. L. Dangl, Max-Delbrück Laboratory, Carl von Linné Weg 10, 50829 Köln, Germany, and Department of Biology, University of North Carolina, Chapel Hill, NC 27599–3280, USA.

*These authors contributed equally to this work.
†Present address: Laboratoire de Biologie Moleculaire INRA-CNRS, B.P. 27, 31326 Castanet Tolosan Cedex, France.
‡To whom correspondence should be addressed at Department of Biology, Coker Hall 108, CB 3280, University of North Carolina, Chapel Hill, NC 27599–3280, USA.