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19. The microtubule nucleation assay was done essentially as described [T. Stearns and M. Kirschner, *Cell* **76**, 623 (1994)]. Briefly, rhodamine-labeled bovine tubulin (from T. Stearns, Stanford University) and *Xenopus* sperm were added to extracts to yield final concentrations of 120 μg/ml and 200 nuclei per microliter, respectively. Extracts were incubated at room temperature for 10 min. Samples were diluted in 9 vol of glutaraldehyde (0.25%), centrifuged through a 25% glycerol cushion onto coverslips, and stained with DAPI. The DAPI-stained sperm and rhodamine-labeled tubulin were examined by fluorescence microscopy.
20. Addition of purified *Xenopus* or rat MAPK to interphase extracts can be sufficient to produce mitotic-like microtubules under some circumstances [Y. Gotoh *et al.*, *Nature* **349**, 251 (1991)]. One difference between their experiment and ours is the way the interphase extracts were prepared. We used cycloheximide-soaked eggs, and prepared the interphase extracts in cycloheximide-containing buffers, which prevents cyclin synthesis (*17*). Apparently they prepared interphase extracts in the absence of cycloheximide, and as a result their extracts may have contained substantial amounts of cyclins. The mitotic-like microtubules they observed therefore may have resulted from a combination of MAPK and Cdc2–cyclin activities.
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23. Supported by a grant from the National Institutes of Health (GM46383). We thank T. Stearns for providing rhodamine-labeled tubulin and for advice; N. Ahn for providing MEK plasmids and for sharing unpublished data; G. Gorbsky and M. Weber for sharing unpublished data; M. Murakami and G. Vande Woude for providing Mos plasmids; and K. Cimprich and members of the Ferrell laboratory for comments on the manuscript. T.M.G. is a Special Fellow of the Leukemia Society of America. J.E.F. is a Howard Hughes Junior Faculty Scholar.

6 July 1998; accepted 14 October 1998

Catalytic Plasticity of Fatty Acid Modification Enzymes Underlying Chemical Diversity of Plant Lipids

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Higher plants exhibit extensive diversity in the composition of seed storage fatty acids. This is largely due to the presence of various combinations of double or triple bonds and hydroxyl or epoxy groups, which are synthesized by a family of structurally similar enzymes. As few as four amino acid substitutions can convert an oleate 12-desaturase to a hydroxylase and as few as six result in conversion of a hydroxylase to a desaturase. These results illustrate how catalytic plasticity of these diiron enzymes has contributed to the evolution of the chemical diversity found in higher plants.

All higher plants contain one or more oleate desaturases that catalyze the O_2-dependent insertion of a double bond between carbons 12 and 13 of lipid-linked oleic acid ($18:1^{\Delta 9}$) to produce linoleic acid ($18:2^{\Delta 9,12}$) (*1*). In contrast, only 14 species in 10 plant families have been found to accumulate the structurally related hydroxy fatty acid, ricinoleic acid (D-12-hydroxyoctadec-*cis*-9-enoic acid) (*2*), which is synthesized by an oleate hydroxylase that exhibits a high degree of sequence similarity to oleate desaturases (*3*). The oleate desaturases and hydroxylases are integral membrane proteins, which are members of a large family of functionally diverse enzymes that includes alkane hydroxylase, xylene monooxygenase, carotene ketolase, and sterol methyloxidase (*1*). These nonheme iron–containing enzymes use a diiron cluster for catalysis (*4*) and contain three equivalent histidine clusters that have been implicated in iron binding and shown to be essential for catalysis (*1*). This class of proteins exhibits no significant sequence identity to the soluble diiron-containing enzymes which represent a similar diversity of enzymatic activities that include plant acyl-ACP desaturases, methane monooxygenase, propene monooxygenase, and the R2 component of ribonucleotide reductase (*1*, *5*). The catalytic activities of these enzymes has been mimicked by a synthetic diiron-containing complex with a coordination sphere composed entirely of nitrogen ligands (*6*).

The oleate hydroxylase from the crucifer *Lesquerella fendleri* has about 81% sequence identity to the oleate desaturase from the crucifer *Arabidopsis thaliana* and about 71% sequence identity to the oleate hydroxylase from *Ricinus communis* (*7*). The observation that these crucifer desaturase and hydroxylase enzymes are more similar than the two hydroxylases, and the presence of ricinoleic acid in a small number of distantly related plant species, suggests that the capacity to synthesize ricinoleate has arisen independently several times during the evolution of higher plants, by the genetic conversion of desaturases to hydroxylases.

Comparison of the amino acid sequences of the hydroxylases from *L. fendleri* and *R. communis* with the sequences for oleate desaturases from *Arabidopsis*, *Zea mays*, *Glycine max* (two sequences), *R. communis*, and *Brassica napus* revealed that only seven residues were strictly conserved in all of the six desaturases but divergent in both of the hydroxylases. The role of these seven residues was assessed by using site-directed mutagenesis to replace the residues found in the *Lesquerella* hydroxylase, LFAH12, with those from the equivalent positions in the desaturases (*8*, *9*). In a reciprocal experiment, we replaced the seven residues in the *Arabidopsis* FAD2 oleate desaturase with the corresponding *Lesquerella* hydroxylase residues (*10*). The activity of the modified and unmodified genes was then determined by expressing them in yeast and transgenic plants before analyzing the composition of the total fatty acids. Technical difficulties limited the utility of direct measurements of enzyme activity in cell extracts (*11*).

The mutant hydroxylase and desaturase genes containing all seven substitutions (designated m_7LFAH12 and m_7FAD2, respectively) were expressed in yeast cells under transcriptional control of the GAL1 promoter. Transgenic cells were harvested after induction and their total fatty acid composition determined by gas chromatography. Wild-type yeast cells do not accumulate detectable concentrations of diunsaturated or hydroxylated fatty acids (*12*). Expression of FAD2 caused the accumulation of about 4% diunsaturated fatty acids (16:2 and

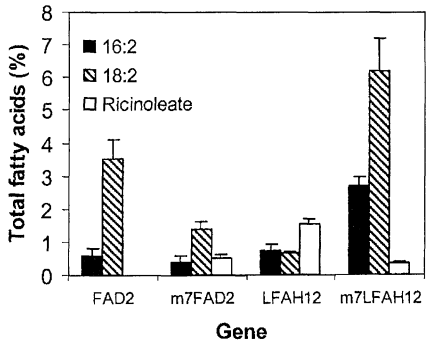

Fig. 1. Fatty acid composition of yeast cells expressing desaturase and hydroxylase genes. Cultures were induced in growth medium containing galactose, ~2 × 10^8 cells were harvested, and fatty acids were extracted and modified for analysis by gas chromatography, as described (*7*). Values are the averages (±SE) obtained from five cultures of independent transformants.

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18:2) but no detectable hydroxy fatty acids (Fig. 1). Expression of LFAH12 caused the accumulation of about 1.4% diunsaturated fatty acids and 1.5% ricinoleic acid, confirming the mixed function of this enzyme (*7*). Cells expressing m_7FAD2 accumulated ricinoleic acid to ~0.5% of total fatty acids and had ~50% reduction in the accumulation of diunsaturated fatty acids (Fig. 1). Thus, replacement of the seven residues (*10*) converted a strict desaturase to a bifunctional desaturase-hydroxylase comparable in activity to the unmodified *Lesquerella* hydroxylase.

The amount of desaturase activity of the LFAH12 enzyme is relatively low compared with its hydroxylase activity (*7*). However, yeast cells expressing LFAH12 accumulated linoleic and ricinoleic acids to similar concentrations, possibly because linoleic acid is more stable than ricinoleic acid in yeast cells. In cells expressing m_7FAH12, the ratio of 18:2 diunsaturated fatty acid to ricinoleic acid was, on average, 43 times that in cells expressing LFAH12. There was also a 16-fold increase in the ratio of 16:2 diunsaturated fatty acid to ricinoleic acid. Notwithstanding the quantitative limitations of the assay system, noted above, these results indicate a major increase in desaturase activity and a decrease in hydroxylase activity upon introduction of the seven desaturase-equivalent residues into LFAH12.

The activity of the mutant enzymes in planta was examined by using the corresponding genes to produce stable transgenic plants in an *Arabidopsis fad2* mutant, which is deficient in oleate desaturase activity (*13*). Expression of LFAH12 under transcriptional control of the constitutive cauliflower mosaic virus (CaMV) 35*S* promoter resulted in accumulation of high concentrations of hydroxy fatty acids in seeds (*7*), but no detectable suppression of the *fad2* mutant phenotype in leaves (Fig. 2). In contrast, expression of m_7LFAH12 under the same circumstances resulted in complete suppression of the *fad2* phenotype in 8 out of 10 transgenic plants analyzed (Fig. 2). There was an average 21-fold increase in the ratio of linoleate to oleate in leaf fatty acids and a small increase in the amount of linolenic acid. These results, which are consistent with the results of the yeast assays, confirm that expression of m_7LFAH12 in plants deficient in oleate desaturation has identical phenotypic consequences to expressing a wild-type desaturase such as FAD2 (*13*).

To evaluate the effect of the seven mutations on the activity of the gene encoding FAD2, we expressed FAD2 and m_7FAD2 in the *Arabidopsis fad2* mutant under the control of the strong seed-specific promoter from the *B. rapa* napin gene. As expected from previous studies (*7*), none of the 15 transgenic lines expressing the FAD2 gene accumulated detectable hydroxy fatty acids, although the ratio of linoleate to oleate accumulation was increased an average of 10-fold as compared with untransformed controls. In the transgenic lines expressing m_7FAD2, the amount of hydroxylated fatty acids, which included ricinoleic, densipolic, and lesquerolic acids, composed up to 9.4% of total seed fatty acids (Fig. 3). The ratio of seed linoleate to oleate contents was increased an average of 6.4-fold (*14*), which indicated that m_7FAD2 exhibited significant desaturase activity, albeit less than the wild-type FAD2 gene. The high concentrations of hydroxy fatty acid accumulation observed in transgenic plants expressing m_7FAD2 indicated that the modified desaturase had comparable levels of hydroxylase activity, in the in planta assay, to the native *Lesquerella* hydroxylase enzyme.

To determine whether any single amino acid residue of the seven had a major effect on the ratio of hydroxylase to desaturase activities, we introduced each of the seven FAD2-equivalent residues (*8*) individually into the LFAH12 enzyme. None of the enzymes containing single amino acid substitutions had activities that differed significantly from the wild-type hydroxylase enzyme when expressed in yeast (*14*). We also tested seven modified LFAH12 genes containing all combinations of six desaturase-equivalent residues (Fig. 4). Each of the seven constructs produced a ratio of diunsaturated to hydroxylated fatty acids that was similar to the ratio produced by the m_7FAH12 enzyme. Thus, as few as six residues principally determine the ratio of desaturation or hydroxylation activity. All lines showed somewhat reduced levels of desaturase activity, with the largest reductions of ~40% seen in F218Y and G105A. Therefore, we made a construct in which both these changes were combined (xF218Y/G105A). This construct exhibited similar activity to the individual F218Y and G105A mutants (*14*), suggesting that their effects are redundant and that the observed changes in activity result from interactions of more than two of the seven residues. Considered together, these results indicate that no single amino acid position plays an essential role in catalytic outcome. Rather, changes in activity result from a combined effect of several amino acid positions that have partially overlapping effects.

Because four of the seven amino acids are adjacent to histidine residues that have been identified as essential to catalysis (*1*), we hypothesized that these four residues may be of greatest importance to the outcome of the reaction. A modified FAD2 enzyme, designated m_4FAD2, was constructed in which these four amino acids

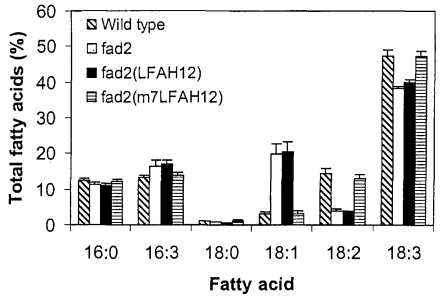

Fig. 2. Genetic complementation of the *Arabidopsis fad2* mutation with the m_7LFAH12 gene. Measurements were made of the fatty acid composition of leaf lipids from wild-type, the *fad2* mutant, and transgenic *fad2* plants expressing LFAH12 or m_7LFAH12, under the control of the CaMV 35*S* promoter. Values are means ± SE (n = 3).

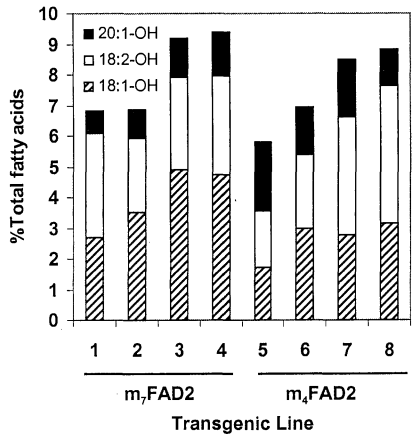

Fig. 3. Fatty acid content of seed lipids from independent transgenic *Arabidopsis* lines expressing m_7FAD2 or m_4FAD2 under control of the *B. napus* napin promoter. Abbreviations: ricinoleic acid (18:1-OH), densipolic acid (18:2-OH), and lesquerolic acid (20:1-OH).

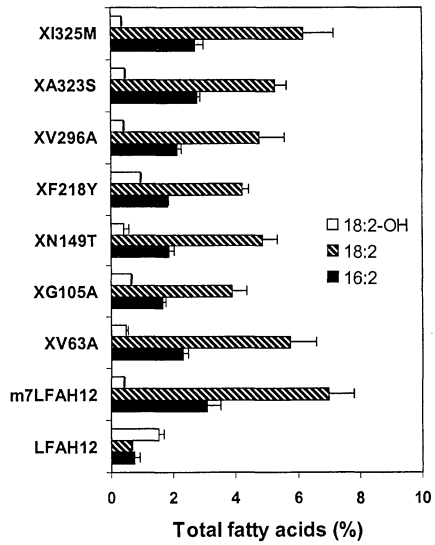

Fig. 4. Contribution of individual amino acid substitutions to the activity of the modified *Lesquerella* hydroxylase. Seven derivatives of the m_7LFAH12 gene containing all combinations of six out of seven substitutions were introduced into yeast cells, and the fatty acid composition of five independent cultures was measured. The "X" designation refers to the unmodified amino acid (that is, enzyme XI325M contains all of the seven substitutions except I325M).

were replaced by their equivalents from the *Lesquerella* hydroxylase (T148N, A296V, S322A, M324I). Expression of m_4FAD2 in seeds of wild-type *Arabidopsis* resulted in the accumulation of average concentrations of hydroxy fatty acids that were similar to those obtained with m_7FAD2 (Fig. 3). Thus, only four changes are required to convert a strict desaturase to an enzyme that retains some desaturase activity but is also an efficient hydroxylase.

Biochemical and structural similarities between the desaturase and hydroxylase, in addition to recent kinetic isotope experiments, suggest that there is a common initial oxidation event at C-12 for both enzymes (*15*). Thus, it seems likely that the different functional outcomes represent a partitioning between two reaction pathways that diverge after initial C-12 hydrogen abstraction such that one pathway favors a second hydrogen abstraction whereas the other favors oxygen transfer. We envision that because no specific single amino acid change is required, and in view of the substantial effect of the four residues that abut the active site histidines, the differences between desaturase and hydroxylase outcome is influenced by changes in active site geometry. Examples of such changes might include the relative positioning of the substrate with respect to the iron center, the coordination geometry of the iron ions, or the active site hydrogen bonding network. Whatever the case, this mode of evolving new catalytic activity differs from the more general case in which the evolution of new activities involves the incorporation of new catalytic groups into the active site (*16*).

Acetylenic and epoxy fatty acids are produced by desaturation and epoxidation of double bonds by enzymes that are structurally similar to the enzymes described here (*17*). Thus, variations of the same catalytic center can catalyze the formation of at least four different functional groups in fatty acids. Because various combinations of these four functional groups define most of the chemical complexity found among the hundreds of different fatty acids that occur in higher plants (*2*), it is now apparent that most of the chemical complexity of plant fatty acids can be accounted for by divergence of a small number of desaturases. Extrapolating from the results described here, it also seems very likely that a small number of amino acid substitutions will account for the functional divergence of desaturases, hydroxylases, expoxgenases, and acetylenic bond–forming enzymes.

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8. The seven mutations were V63A, G105A, N149T, F218Y, V296A, A323S, and I325M numbered relative to the LFAH12 sequence, where the first residue at the indicated position was mutated to the second residue. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
9. Mutagenic oligonucleotides were used to introduce nucleotide substitutions into cloned genes by overlap-extension polymerase chain reaction (PCR) [W. Ito, H. Ishiguro, Y. Kurosawa, *Gene* **102**, 67 (1991)]. In a first step, we amplified overlapping fragments in separate PCR reactions using primer pairs designed to introduce mutations. The products were gel purified, then assembled in a PCR reaction primed with terminal primers only. Modified LFAH12 genes containing one or seven substitutions were constructed with pLFAH12-1 as template (*7*) and primers mH1 through mH5 or mH67. Modified LFAH12 genes containing only six mutations were constructed with m_7LFAH12 as a template and one of the primers H1 to H7 to revert one of the mutations. The 5′ end of terminal primers was modified to allow the introduction of convenient restriction sites for the cloning of PCR products. The m_7FAD2 was constructed with oligonucleotides D1 to D5 and D67. The PCR conditions were as follows: 10 ng of plasmid DNA, 200 μM deoxynucleotide triphosphates, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 200 mM tris-HCl (pH 8.8), 20 mM $MgSO_4$, 1% (v/v) Triton X-100, bovine serum albumin (1000 μg/ml), 3 mM $MgCl_2$, 5% (v/v) dimethyl sulfoxide, 125 pmol of each primer, 1.25 U of Pfu polymerase (Stratagene), to a final volume of 50 μl. Amplifications conditions were as follows: 4 min denaturation step at 94°C, followed by 30 cycles of 92°C for 1 min, 50°C for 1 min, and 72°C for 2 min, concluded with a final extension step at 72°C for 5 min. PCR products were purified from agarose or polyacrylamide gels. For the second PCR step, 10 ng of purified overlapping fragments were used as templates in PCR reactions as above except that only 15 cycles were used. PCR fragments encoding modified LFAH12 enzymes were cloned into pLFAH12-1 cut with Pst I and one of Sma I or Eco RV or Sac I. All inserts were sequenced. Yeast expression vectors containing wild-type or modified LFAH12 genes were constructed by excising inserts from the above constructs with the enzymes Hind III and Sac I and cloning them into the Hind III–Sac I sites of pYESII (Invitrogen). Constructs for plant transformation were made by cloning the Stu I–Sac I fragment from modified LFAH12 genes into the Sma I–Sac I sites of pBI121. The FAD2 cDNA clone 146M12T7 was amplified with Pfu DNA polymerase by using primers D5′ and D3′ to introduce restriction sites for Kpn I and Sma I immediately upstream of the initiation codon, and Sac I and Eco RI restriction sites following the terminator codon. The fragment was cloned into the Eco RV site in the vector pZErO (Invitrogen). For expression of FAD2 in yeast, the insert was excised by restriction with Kpn I and Eco RI and cloned into the corresponding sites in pYESII, resulting in plasmid pYESII-F2. Binary Ti-vector pDN was constructed for seed-specific expression of FAD2 genes. In a first step, the napin promoter was amplified from rapeseed DNA with primers ggcgtcgacaagcttctgcggatcaagcagcctttca and ggttttgagtagtgatgtcttgtatgttctagatggtaccgtac. A Hind III–Bgl II fragment was cloned into the Hind III–Bgl II sites of pBI121 (Clontech), replacing the 35*S* promoter. FAD2 coding sequences were excised from pYESII-F2 with Sma I and Sac I and cloned into pDN with corresponding restriction sites. The construction of the m_7FAD2 cDNA encoding a modified FAD2 enzyme containing seven amino acid substitutions was achieved with overlap extension PCR. After the second round of assembly-amplification with the primers D5′ and D3′, the PCR products were treated exactly as the amplified wild-type FAD2 sequence described above.

Plant expression constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 by electroporation and used to transform *Arabidopsis fad2* mutant plants by vacuum infiltration [D. Bouchez, C. Camilleri, M. Caboche, *C. R. Acad. Sci. Ser. Iii* **316**, 1188 (1993)]. The oligonucleotides used were mH1f atcactttagcttcttgcttct, mH1r agaagcaagaagctaaagtgat, mH2f ctgggcattgccatgaatgtggtcacc, mH2r ggtgaccacattcatgggcaatgacccag, mH3f caccattccaacactggatcctagaa, mH3r ttctagggatcccagt gttggaatggtg, mH4f catgcacctatctataaggaccgtg, mH4r cacggtccttatagataggtgcatg, mH5f agaggagcttggctacggtagac, mH5r gtctaccgtagccaaagctcctct, mH67f catctctttcaactatgccgcatt, mH67r aatgcggcatagttgaeaaagagatg, mH6f catctctttcaactataccgcatt, mH6r aatgcggtatagttgaaaagagatg, mH7f catctctttgcaactatgccgcatt, mH7r aatgcggcatagttgcaaagagatg, H1f atcactttagtttcttgcttct, H1r agaagcaagaaactaaagtgat, H2f ctgggcattggccatgaatgtggtcacc, H2r ggtgaccacattcatggccaatgacccag, H3f caccattccaacaatggatccctagaa, H3r ttctagggatccattgttggaatggtg, H4f catgcacctatctttaaggaccgtg, H4r cacggtccttaaagataggtgcatg, H5f agaggagctttggttacggtagac, H5r gtctaccgtaaccaaagctcctct, H6f catctctttgcaactataccgcatt, H6r aatgcggtatagttgcaaagagatg, H7f catctctttgcaactatacgcgcatt, H7r aatgcggtatagttgcaaagagatg, mD1f gacatcattatagtctcatgcttctact, mD1r agtagaagcatgagactataatgatgtc, mD2f ctgggtcataggccacgaatgcggtc, mD2r gaccgcattcgtggcctatgacccag, mD3f caccattccaacaatggatccctcgaa, mD3r ttcgagggatccattgttggaatggtg, mD4f ccccaacgctcccatcttcaatgaccgaga, mD4r tctcggtcattgaagatgggagcgttgggg, mD5f cagggagctttggttaccgtagacagag, mD5r ctctgtctacggtaaccaaagctcccctg, mD67f cacctgttcgcgacaataccgcattataacgc, mD67r gcgttataatgcggtattgtcgcgaacaggtg, H5′ tatcga**aggcct**gatgggtgct, H3′ ctcgcagtatc**gagctc**ataacttattgtt, D5′ **gatcggtacccggg**atgggtgcaggtggaagaatgccgg, and D3′ **gatcgaattcgagctc**tcataacttattgttgtaccagtacacacc. The bold and underline letters indicate altered nucleotides and codons, respectively.
10. The seven mutations were A63V, A104G, T148N, Y217F, A295V, S322A, and M324I, designated on the basis of the numbering of the *Arabidopsis* FAD2 sequence.
11. The enzymes are integral membrane proteins that act on fatty acids esterified to lipids and require cytochrome b_5 reductase and cytochrome b_5 for activity. The difficulty of quantitatively incorporating labeled lipids into isolated membranes and ensuring that cytochrome b_5 and b_5 reductase are not limiting, restricts the utility of direct measurements of enzyme activity. Our best estimates of oleate desaturase or oleate hydroxylase activities in crude microsomal preparations from *Arabidopsis* roots indicated specific activities of 1.2 and 0.3 pmol/mg of protein per minute, respectively.
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18. We thank S. Stymne for communicating results before publication, and B. Behrouzian for helpful discussion. This work was supported in part by grants from the U.S. Department of Energy Office of Basic Energy Sciences to C.R.S. (DE-FG02-97ER20133), and to J.S. and E.J.W. The Monsanto Company awarded P.B. a fellowship.

31 July 1998; accepted 9 October 1998