Mendel’s dwarfing gene: cDNAs from the Le alleles and function of the expressed proteins

(2-oxoglutarate-dependent dioxygenase/gibberellin 3β-hydroxylase/Pisum)

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ABSTRACT The major gibberellin (GA) controlling stem elongation in pea (Pisum sativum L.) is GA1, which is formed from GA20 by 3β-hydroxylation. This step, which limits GA1 biosynthesis in pea, is controlled by the Le locus, one of the original Mendelian loci. Mutations in this locus result in dwarfism. We have isolated cDNAs encoding a GA 3β-hydroxylase from lines of pea carrying the Le, le, le-3, and leb alleles. The cDNA sequences from le and le-3 each contain a base substitution resulting in single amino acid changes relative to the sequence from Le. The cDNA sequence from leb, a mutant derived from an le line, contains both the le “mutation” and a single-base deletion, which causes a shift in reading frame and presumably a null mutation. cDNAs from each line were cloned in Escherichia coli. The expression product for the clone from Le converted GA20 to GA1, and GA10 to GA20, with Km values of 1.5 μM and 13 μM, respectively. The amino acid substitution in the clone from le increased Km for GA10 100-fold and reduced conversion of GA20 to almost nil. Expression products from le and le-3 possessed similar levels of 3β-hydroxylase activity, and the expression product from leb was inactive. Our results suggest that the 3β-hydroxylase cDNA is encoded by Le. Le transcript is expressed in roots, shoots, and cotyledons of germinating pea seedlings, in internodes and leaves of established seedlings, and in developing seeds.

In his experiments on plant hybridization, Gregor Mendel (1) examined seven pairs of traits in pea. One of these traits was “difference in the length of the stem,” and Mendel demonstrated the dominance of tall over dwarf. White (2) noted that this pair of alleles represented “the presence and absence of a factor for tallness” and introduced the gene symbol Le (length). The factor for tallness in peas was first associated with gibberellins (GAs) by Brian and Hemming (3), who stimulated stem elongation in dwarf peas by applying GA1 to the seedlings. Brian (4) proposed that tall peas normally produce a product for the clone from Le, whereas GA1, with Km values of 1.5 μM and 13 μM, respectively. The amino acid substitution in the clone from le increased Km for GA10 100-fold and reduced conversion of GA20 to almost nil. Expression products from le and le-3 possessed similar levels of 3β-hydroxylase activity, and the expression product from leb was inactive. Our results suggest that the 3β-hydroxylase cDNA is encoded by Le. Le transcript is expressed in roots, shoots, and cotyledons of germinating pea seedlings, in internodes and leaves of established seedlings, and in developing seeds.

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in. min, respectively, in ca. 10 volumes of water. Gel was excised from the region of the anticipated product, chopped into small pieces, and eluted in water overnight at 4°C. The second reaction of nested PCR included 0.2 μl eluate as template and 2 μM forward and reverse primers. Cycle parameters: denature 5 min at 94°C; 40×, denature 30 s at 94°C, anneal 30 s at 50°C, extend 1 min at 72°C; extend 10 min at 72°C; soak 4°C.

**PCR of full-length cDNA.** In addition to components listed above, PCR included 0.5 μl reverse transcription reaction as template and 0.5 μl each 3′ end and 3′ end primers. Cycle parameters: denature 5 min at 94°C; 5×, denature 30 s at 94°C, anneal 3 min at 52°C, extend 1 min at 72°C; 35×, denature 30 s at 94°C, anneal 30 s at 62°C, extend 1 min at 72°C; extend 10 min at 72°C; soak 4°C.

**Cloning of PCR Products.** Standard cloning procedures were used (26). Primary PCR products were purified on 2% NuSieve GTG agarose gels in 1× TBE before cloning. To minimize interference by EDTA in subsequent reactions, gels were stained and destained 30 min and 10 min, respectively, in ca. 10 volumes of water. Gel bands (~250 μg) were excised over longwave UV light, digested overnight with 0.5 units Gelase (Epitect Technologies, Madison, WI), and precipitated according to the manufacturer’s instructions. Pellets were dissolved in 10 mM Tris, pH 8, cloned into Bluescript T-vector (Stratagene), and transformed in E. coli DH5α (BRL).

Full-length products were added without purification directly to ligation mix: 1 μl PCR products, 0.5 μl T-vector (100 ng/μl), 0.5 μl T4 DNA ligase (400,000 units/ml; New England Biolabs), and 2 μl 10× buffer (with 10 mM ATP, included with enzyme) in a volume of 20 μl, overnight at 16°C. Clones were sequenced and checked against sequences of partial clones for spurious mutations. The ca. 1.2-kb XbaI fragment of bona fide clones was subcloned into the NdeI site of expression vector pET23a. This manipulation added 3 codons (met-sla-arg) at the beginning of the coding sequence. The beginning of selected clones was sequenced to verify insertion into proper reading frame. Purified plasmid from DH5α clones was used to transform E. coli expression hosts HMS174(DE3) or BL21(DE3) (Novagen).

**Sequence Analysis.** DNA was sequenced at the Center for Gene Research and Biotechnology at Oregon State University (Corvallis) on Applied Biosystems Model 370/373A machines using dye-primer/dye-terminator chemistry. Plasmid for sequencing was prepared with QIAprep Spin Miniprep and Plasmid Mini Kits (Qiagen). Sequences were analyzed with WISCONSIN SEQUENCE ANALYSIS PACKAGE 8 software from Genetics Computer Group, Madison. Figures of aligned sequences were produced by BOXSHADE shareware (K. Hofmann, Swiss Institute for Experimental Cancer Research, Epalings, and M. D. Baron, Institute for Animal Health, Pirbright, U.K.).

**Expression in E. coli and Enzyme Assays.** Clones for expression analysis were grown on plates at 37°C for 24 h the day before culturing. Flasks (250 ml) containing 50 ml prewarmed (30°C) medium (2YT + 50 mg/l ampicillin) were inoculated with one to two large (2- to 3-mm) colonies. Cultures were grown at 30°C, 300 rpm (2 cm eccentric). Expression was induced at OD₆₀₀ 0.6–0.65 by addition of 0.2 ml isopropyl β-D-thiogalactoside (0.1 M), and cultures were harvested 2 to 3 h later. After cooling on ice, cells were collected by centrifugation for 5 min at 5,000 × g, 4°C, and washed twice in cold, freshly prepared buffer (100 mM Tris, pH 7.6/20 mM DTT). Cell pellets were resuspended in 0.95 ml buffer, mixed with 95 ml buffer, mixed with 0.95 ml buffer, mixed with 0.95 ml buffer, and incubated 45 min on ice. After freezing in liquid nitrogen and thawing in water at room temperature, lysates were centrifuged 30 min at 50,000 × g, 4°C, and supernatants were stored in aliquots at −75°C.

**General assays for enzyme activity contained [17,14C]GA₉ (170 Bq, 2.1 Tbg/mol) or [17,14C]GA₂₀ (170 Bq, 1.8 Tbg/mol), prepared as described (22) and added in methanol (5 μl), supernatant (10 μl), cofactors (4 mM 2-oxoglutarate, 4 mM

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**Table 1. Primers used in the PCR experiments**

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Peptide sequences are shown in bold above nucleotide sequences. Arrows indicate forward/reverse primers. Significant differences within a primer group are boxed. Redundancies are in standard IUPAC code: D = A,G,T; N = AGCT; R = AG; S = GC; W = AT; Y = CT.
ascorbate, 4 mM DTT, 0.5 mM FeSO4, 2 mg/ml BSA, 1 mg/ml catalase), and 0.1 M Tris-HCl, pH 7.5, in a total volume of 0.1 ml. Samples were incubated overnight at 20°C. Products were separated by HPLC and identified by GC-MS (12, 15).

For kinetic studies with the expression product from Le cDNA, different concentrations of [14C]GA0 (0.5–8 μM) or [14C]GA20 (0.5–7.1 μM) were incubated for 15 min at 30°C with cofactors and, respectively, 2 μl cell extract in 0.2 ml or 20 μl cell extract in 0.1 ml. In corresponding incubations with the le product, [14C]GA0 (4.7 μM–2.3 mM) was incubated with 25 μl cell extract in 0.1 ml total volume. To achieve the higher concentrations necessary for the latter study, substrate was diluted with unlabeled GA0. Reaction rates were based on the separation of products by HPLC with online radiomonitoring (27). Michaelis–Menten curves and Km values were obtained by nonlinear regression using Enzfitter (Biosoft, Milltown, NJ).

### Results

A GA 3β-hydroxylase cDNA from Le Pea. We used cDNA from stems of GA-deficient line B686–67–(3) (naLe) as template for our PCR experiments. In this mutant, GA biosynthesis is blocked at a step prior to that in le (28). Choice of template was based on the knowledge that 3β-hydroxylase activity is relatively high in internodes (29) and the possibility that 3β-hydroxylase transcript in pea, like arabidopsis (15), would be more abundant in a GA-deficient mutant.

Nested PCR using primers 1c and 3ab, followed by 2d and 3cd (Table 1) generated a single product of 1.2 kb cloned 3β-hydroxylase cDNA from stems of GA-deficient line B686–67–(3) (naLe) (46). DNA was electrophoresed in LE agarose (FMC) and transferred to Hybond-N (NJ).

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Southern and Northern Blot Analysis. DNA was electrophoresed in LE agarose (FMC) and transferred to Hybond-N (NJ).

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### Southern and Northern Blot Analysis

DNA was electrophoresed in LE agarose (FMC) and transferred to Hybond-N (NJ). Poly(A)+ RNA was either electrophoresed in formaldehyde gels and transferred to nylon membranes (Amersham) (26). Poly(A)+ RNA was either electrophoresed in formaldehyde gels and transferred to nylon membranes (Amersham) (26) or applied directly to nylon membranes using a Bio-Dot SF blot apparatus (Bio-Rad). Probe was prepared from 1.2 kb cloned 3β-hydroxylase (Le) template by random priming with Ready-To-Go beads (Pharmacia). Unincorporated nucleotides were removed by spin column through Sephadex G-50 (Pharmacia). Blots were hybridized overnight at 42°C in hybridization solution (50% formamide/0.25 M NaCl/7% SDS/0.12 M sodium phosphate, pH 6.5), washed once each in 5×, 1×, and 0.2× SSC at 42°C, washed twice in 0.1× SSC at 42°C (60°C for RNA blots), and autoradiographed.

### Homologous Clones from le, le4, and le-3 Lines

Homologous 3β-hydroxylase cDNA clones were isolated from le, le4, and le-3 lines by PCR, and their sequences were compared with that from Le (Fig. 2). In sequences from le and le4 lines, substitution of A for G at base position 685, relative to the start codon, converts alanine-229 to threonine. Additionally in the le4 line, deletion of base G276 produces a shift in reading frame. In the le-3 line, substitution of T for C at base position 826 converts histidine-276 to tyrosine.

When expressed in E. coli, enzyme activity of clones from le, le4, and le-3 lines was lower than that from the Le line. Based on the conversion of [14C]labeled GA0 and GA20 by crude cell lysates in overnight incubations, their relative activities were Le > le-3 > le > le4 for both substrates (Table 2). Kinetic parameters for the 3β-hydroxylases from the Le and le lines were determined (Fig. 5). The expression product from Le gave Km values of 1.5 μM (Vmax = 0.29 nmol/min per mg protein) and 13 μM (Vmax = 0.10 nmol/min per mg protein) for GA0 and GA20, respectively. A much higher Km value for GA0 (118 μM; Vmax = 1.06 nmol/min per mg protein) was obtained for the le expression product. Enzyme activity with GA20 was too low in this case to measure reaction rates. The relative abundance of the expressed protein in E. coli, as estimated by Northern blots, was found to be too low in this case to measure reaction rates. The relative abundance of the expressed protein in E. coli, as estimated by Northern blots, was found to be too low in this case to measure reaction rates.
Fig. 3. Analysis of line I3 (Le). (A) Southern blot of genomic DNA (30 μg per lane). PsI cut poorly, and signal was not detected in this lane. (B) Northern blot of poly(A)+ RNA isolated from different organs (3 μg per lane; overnight exposure). Signal was not detected in flowers and fruits even after 1-week exposure. Arrow indicates direction of electrophoresis.

determined by SDS/polyacrylamide electrophoresis, was very low and was similar for both the Le and le clones.

DISCUSSION

Because the arabidopsis GA 3β-hydroxylase sequence (15) was the only one of its kind available, we relied on regions of the amino acid sequence that are conserved within groups of plant 2-oxoglutarate-dependent dioxygenases, as well as on regions conserved across the dioxygenases, when designing primers for PCR (24). We selected primers to limit redundancy and to take advantage of nested PCR (Table 1). Systematic screening of primer combinations yielded a product that led to the isolation of a GA 3β-hydroxylase cDNA clone from Le pea.

We investigated the possibility that the enzyme was encoded by Le, one of the original Mendelian loci. Four alleles at this locus are known: Le, le (1, 2), led (31), and le-3 (23). They affect internode length differentially, Le > le-3 > le > led. The stature of Le mutants can be restored by application of GA1, but not by GA20 (31, 32). This and other observations discussed in the Introduction led to the proposal that Le encoded a 3β-hydroxylase (7, 14).

Sequences of clones isolated from pea lines homozygous for mutant alleles differed slightly from that of our original 3β-hydroxylase clone. However, we could not be sure whether these differences were significant or simply coincidental, be-

![Table 2. Conversion of [14C]GA9 and [14C]GA20 to products [14C]GA4 and [14C]GA1, respectively, by lysate of E. coli expressing GA 3β-hydroxylase clones](image)

Values are percent of total counts in substrate and product fractions after overnight incubations at 20°C. Cultures were grown simultaneously and adjusted to approximately equal cell numbers prior to lysis.

*Products were identified by comparison of their mass spectra with published spectra (30): [17-14C]GA4 mass/charge (% relative abundance), 420(23), 392(12), 388(14), 360(10), 330(22), 298(20), 291(48), 286(100), 263(26), 235(42), 227(84), 225(84), 203(37), 129(59); [17-14C]GA1, 508(100), 493(10), 450(18), 435(5), 418(5), 378(15), 359(5), 315(10), 237(9), 209(32).

cause the clones came from lines with different genetic backgrounds. The sequence of the clone isolated from led suggested that these differences were significant. Because the led mutant had originated from an le line, it was likely to be a more severe, double mutant. Consistent with the origin of the allele, the 3β-hydroxylase clone isolated from led contained two changes. One was the same as that found in the clone from le; the other was a single-base deletion that shifted reading frame.

An additional indication of a link between Le and the 3β-hydroxylase clones came from in vitro enzyme assays. Activity of the recombinant enzymes under our conditions reflected the effects of the Le alleles on stem elongation and GA4 content (12, 13, 23, 33). Their relative enzyme activities were Le > le-3 > le > led for both substrates, GA9 and GA20.

![Fig. 4. Variation in expression of the GA 3β-hydroxylase gene among pea lines (1 μg poly(A)+ RNA per slot). Average length of internode (i-node) 5–6 is given for reference.](image)

![Fig. 5. Michaelis–Menten plots for GA 3β-hydroxylase by cell lysates from E. coli expressing cDNA clones from Le (A) or le (B) lines. [14C]GA9 or [14C]GA20 was incubated for 15 min at 30°C with lysate and cofactors. The amount of lysate used in A was 2 μl (19 μg protein) and 20 μl (192 μg protein) with GA9 and GA20, respectively; in B, 25 μl (240 μg protein) was used with GA9. Lysate of the clone from le had no activity on GA20.](image)
Supporting this result was the high $K_m$ for GA$_20$ with the recombinant enzyme from $le$, nearly 100-fold higher than the corresponding value for the enzyme from $Le$. $K_m$ values are independent of variations in enzyme concentration inherent in heterologous gene expression systems.

Based on this evidence, we propose that $Le$ encodes the GA $\beta$-hydroxylase. $Le$ transcript is found in highest amounts in germinating seedlings, in which GA$_20$, stored in the mature seed or produced de novo, would be converted to GA$_1$ to promote rapid stem elongation. Indeed, in seeds of the slender mutant $sln$, which contain abnormally large amounts of GA$_20$, conversion to GA$_1$ on germination results in hyperelongation (34). Much less transcript is found in shoots of established plants and in developing seeds. Although GA$_1$ is also found in young seeds, conversion of GA$_20$ to GA$_1$ in this tissue has not been demonstrated (35). In pea as in arabidopsis (15), transcript is more abundant in GA-deficient mutants, suggesting that expression of the GA $\beta$-hydroxylase gene, like that of the GA 20-oxidase (17, 18, 21), is subject to feedback regulation.

The effect of $le$ and $le$-3 mutations may be due to their proximity to the active site of the enzyme. The $le$ mutation is near his-231 and asp-233, residues that are conserved in $Le$-3-hydroxylases, in plants and in developing seeds. Although GA$_1$ is also found in young seeds, conversion of GA$_20$ to GA$_1$ in this tissue has not been demonstrated (35). In pea as in arabidopsis (15), transcript is more abundant in GA-deficient mutants, suggesting that expression of the GA $\beta$-hydroxylase gene, like that of the GA 20-oxidase (17, 18, 21), is subject to feedback regulation.

The discovery that $le^{rd}$ is a null mutation was unexpected. The small amount of GA$_1$ found in these plants (37) suggests that other $\beta$-hydroxylases may exist. The $le^{rd}$ mutation should be a very useful tool for studying the role of GA$_1$ in plant growth and development.

In summary, the cloned $\beta$-hydroxylase fits the description of $Le$. Our assertion is based on several pieces of evidence. Mutations in the $\beta$-hydroxylase are found in cDNAs isolated from lines carrying recessive $Le$ alleles. Furthermore, the cDNA from $le^{rd}$, purportedly a double-mutant, has two mutations. The effect of the mutations on the $\beta$-hydroxylase activity of recombinant enzymes reflects the relative effects of the $Le$ alleles on plant growth and GA$_1$ content. On the basis of this evidence, we conclude that $Le$ encodes the $\beta$-hydroxylase.

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