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# The *SLENDER* Gene of Pea Encodes a Gibberellin 2-Oxidase[1]

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**The amount of active gibberellin (GA) in plant tissues is determined in part by its rate of catabolism through oxidation at C-2. In pea (*Pisum sativum* L.) seeds, GA 2-oxidation is controlled by the *SLN* (*SLENDER*) gene, a mutation of which produces seedlings characterized by a slender or hyper-elongated phenotype. We cloned a GA 2-oxidase cDNA from immature pea seeds by screening an expression library for enzyme activity. The clone contained a full-length open reading frame encoding a protein of 327 amino acids. Lysate of bacterial cultures expressing the protein converted the $C_{19}$-GAs, $GA_1$, $GA_4$, $GA_9$, and $GA_{20}$ to the corresponding 2$\beta$-hydroxy products. $GA_9$ and $GA_{20}$ were also converted to $GA_{51}$ and $GA_{29}$ catabolites, respectively. The gene appeared to be one member of a small family of GA 2-oxidases in pea. Transcript was found predominantly in roots, flowers, young fruits, and testae of seeds. The corresponding transcript from *sln* pea contained a point mutation and did not produce active enzyme when expressed heterologously. RFLP analysis of a seedling population segregating for *SLN* and *sln* alleles showed the homozygous mutant allele co-segregating with the characteristic slender phenotype. We conclude that *SLN* encodes GA 2-oxidase.**

Gibberellins (GAs) are involved in many aspects of plant development, particularly stem elongation. $GA_1$, which is biosynthesized by the early 13-hydroxylation pathway (Fig. 1), is the principal GA regulating stem length in pea (Ingram et al., 1984). The amount of hormone available and the plant's response to it determine the extent of elongation.

$GA_1$ content depends on its relative rate of synthesis and catabolism in plant tissue. The net result hinges in part on two reactions at the end of the pathway: 3-oxidation, which converts $GA_{20}$ to $GA_1$, and 2-oxidation, which inactivates both precursor and hormone. The *le* (length) and *sln* (slender) mutants illustrate the effect of these reactions on stem length. *LE* encodes a 3-oxidase in pea shoots (Lester et al., 1997; Martin et al., 1997), and *SLN* controls 2-oxidation in seeds (Ross et al., 1995). Generally, mutations in *LE* produce dwarf plants and mutations in *SLN* produce hyper-elongated plants. In a double mutant, *le* is epistatic to *sln*. The *sln* mutation should not be confused with *la crys*$^s$, also known as slender, which exhibits an overgrowth of internodes due to constitutive expression of the GA signaling pathway (Potts et al., 1985).

The original slender mutant in pea was produced by $\gamma$-radiation. It was first described by Jaranowski (1976) as being "characterized by a very rapid growth rate, especially at the initial period of development. . . stems are thin, the internodes are long. . . . With the passage of time the plants assimilated to normal ones." Explaining the genetics of slender was complicated by an unusual pattern of inheritance. In crosses, the trait did not appear until the $F_3$ generation, because of an epistatic effect of the maternal testa on seeds. Jaranowski attributed the trait to a combination of two recessive genes, *cel* and *cres*, whereas Reid et al. (1992) attributed it to a single recessive gene, *sln*. Subsequent experiments revealed the effect on GA metabolism. In feeds of radiolabeled GAs to seed, the mutation(s) blocked conversion of $GA_{20}$ to $GA_{29}$ in cotyledons, and conversion of $GA_{20}$ to $GA_{29}$ and $GA_{29}$ to $GA_{29}$-catabolite in testae, suggesting that two genes were involved after all (Ross et al., 1993, 1995). To reconcile this observation and the genetic data, Ross et al. (1995) suggested that *SLN* was a regulatory gene controlling both metabolic steps.

These experiments provided the basis for understanding the slender phenotype. Pea seeds contain micrograms of $GA_{20}$, $GA_{29}$, and $GA_{29}$-catabolite during development, although at maturity only $GA_{29}$-catabolite remains in quantity (Frydman et al., 1974; Sponsel, 1983). The presence of such large amounts of GAs is unusual and their function in seeds is unknown. When the slender mutation disrupts normal catabolism, mature seeds retain large amounts of $GA_{20}$, which, on germination, is metabolized to excess $GA_1$, producing plants with a characteristic slender or hyper-elongated phenotype. The effect dissipates as the supply of $GA_{20}$ declines and normal growth resumes.

We cloned a GA 2-oxidase from pea seed and present evidence to show that it is encoded by *Sln*. Recently, cDNAs encoding similar GA 2-oxidases were cloned from runner bean and Arabidopsis (Thomas et al., 1999).

## MATERIALS AND METHODS

### Plant Material

Seedlings of pea (*Pisum sativum* L.) were grown as described previously (Martin et al., 1996). Lines used in experiments were Progress No. 9 (*le*, *SLN*); $I_3$ (a selection of cv Alaska; *LE*, *SLN*) from the late G.A. Marx (New York Agricultural Experiment Station, Geneva); NGB6074 (*LE*, *sln*) from the Nordic GenBank (Alnarp, Sweden); and line

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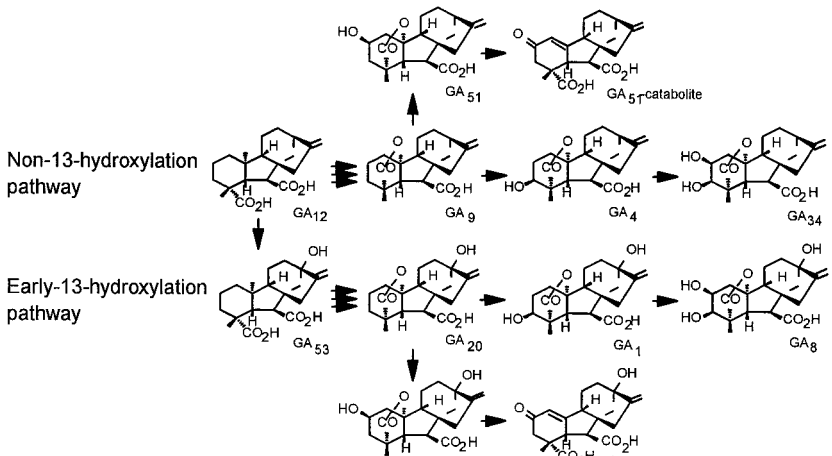


**Figure 1.** GA-biosynthetic pathways from $GA_{12}$ showing the non-13- and early-13-hydroxylation pathways.

178 (*la*, *cry*$^s$, *SLN*) from I.C. Murfet (Department of Plant Sciences, University of Tasmania, Hobart, Australia).

### In Vitro Translation

Poly($A^+$) RNA was translated in vitro using a Rabbit Reticulocyte Lysate System (Promega, Madison, WI) according to the supplier's instructions. Reactions consisted of 4 $\mu$g of poly($A^+$) RNA, 1 $\mu$L of RNasin (40 units/$\mu$L), 1 $\mu$L of a complete amino acid mixture (1 mM), 35 $\mu$L of reticulocyte lysate, and water to 50 $\mu$L total volume. Samples were incubated for 2 h at 30°C and subsequently assayed for enzyme activity as described below, substituting 20 $\mu$L of in vitro translation reaction and 65 $\mu$L of water for 85 $\mu$L of bacterial lysate.

### cDNA Library Construction

An expression library was constructed using the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (GIBCO-BRL, Grand Island, NY). Poly($A^+$) RNA was isolated as described previously (Martin et al., 1997) from fresh, whole seed of line 178 (*la cry*$^s$) 20 d after flowering (DAF). cDNA was prepared according to the kit's instructions, with one exception: Microcon-100 concentration units (Amicon, Beverly, MA) were used to change buffers between reactions to circumvent sample losses associated with phenol extraction and ethanol precipitation. Poly($A^+$) RNA (5 $\mu$g) yielded 2.3 $\mu$g of cDNA, as measured with a TKO 100 fluorometer (Hoefer, San Francisco) using calf thymus DNA as a standard. cDNA was collected in six fractions from the kit's sizing column; only cDNA from the first fraction was used for ligation and cloning.

cDNA was ligated directionally into the *Sal*I-*Not*I site of expression vector pET23a (Novagen, Madison, WI). Plasmid (approximately 20 ng) was introduced to host bacteria by electroporation using 40 $\mu$L of DH12S cells (GIBCO-BRL; competency $>1.0 \times 10^{10}$) and a TransPorator Plus (BTX, San Diego; 16.6 kV/cm). The transformation produced $2.1 \times 10^6$ independent colony-forming units, which were amplified once in 2 L of semi-solid SeaPrep agarose (FMC BioProducts, Rockland, ME). Amplified plasmid was isolated from bacteria using a Plasmid Midi Kit (Qiagen, Valencia, CA); total yield, 50 $\mu$g of DNA. In an analysis of 16 clones from the unamplified library, insert sizes ranged from 1 to 4.5 kb (average 1.9 kb), and one clone had no insert.

### Library Screening

The library screening protocol was adapted from Lange (1997). Electrocompetent BL21(DE3) cells were prepared as described by Miller (1994) using superbroth. Their transformation efficiency was $10^8$ transformants/$\mu$g of plasmid using 10 pg of pUC19 monomer, 20 $\mu$L of cells, 1-mm cuvettes, and a setting of 1.5 kV on the transporator. Voltages higher than 1.5 kV were detrimental. Growth of transformants on 2× yeast-tryptone (2YT) medium was superior to growth on 2× Luria-Bertani medium.

Competent cells (40 $\mu$L) were transformed with 20 ng of amplified library plasmid by electroporation. A typical transformation produced $10^7$ colony-forming units, a number somewhat higher than expected from the efficiency rating. A single transformation reaction could be kept in a 1.5-mL microfuge tube on ice for up to 1 week for use in several experiments; the number of cells surviving after 1 week's storage was about one-third the initial number.

The transformation reaction was titered and a portion was diluted to 100 colony-forming units/mL in 2YT and 100 $\mu$g/mL carbenicillin. Pools of 100 clones (1-mL aliquots) were pipetted into 12- × 75-mm glass culture tubes, grown overnight at 37°C, with shaking, and stored at 4°C until used. Overnight cultures were stable for 2 to 3 weeks. For analysis of expression products, overnight cultures were organized in groups of six tubes; 600 $\mu$L from each group (100 $\mu$L from each tube) was used to inoculate 250-mL flasks containing 50 mL of 2YT and 100 $\mu$g/mL of carbenicillin. Cultures were grown at 30°C and 275 rpm, and expression was induced at $A_{600}$ 0.6 by the addition of isopropyl-$\beta$-D-thiogalactopyranoside to 0.4 mM. Bacterial lysates were prepared from cultures as described previously (Martin et al., 1997).

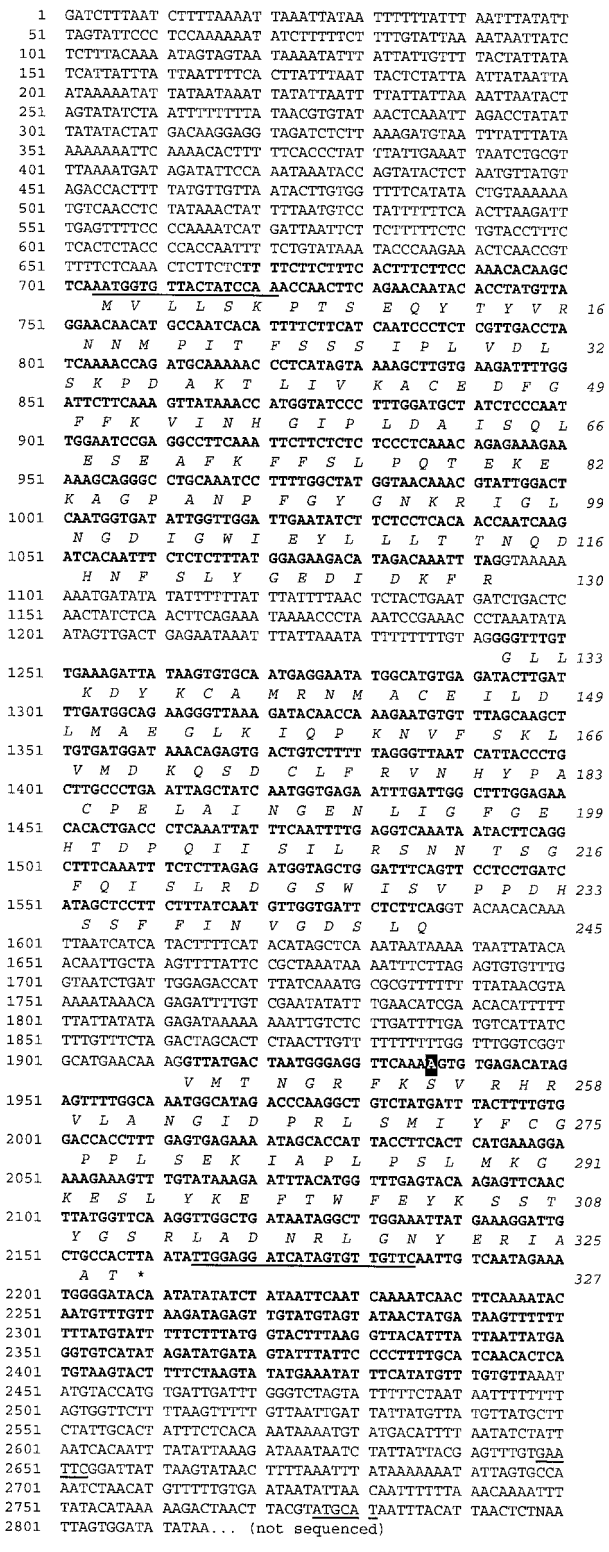

**Figure 2.** GA 2-oxidase genomic clone from cv Alaska pea. cDNA sequence is shown in bold type; the deduced amino acid sequence is shown in italics. The missing nucleotide in *sln* cDNA is shaded. Primers used in PCR and two restriction sites for enzymes used in the Southern analysis are underlined. Sequences are registered under GenBank accession nos. AF101383 (*SLN* genomic clone), AF056935 (*SLN* cDNA), and AF101382 (*sln* cDNA).

Lysates were assayed for enzyme activity to identify pools with GA 2-oxidase clones. Assays consisted of 85 $\mu$L of lysate, 5 $\mu$L of 20× cofactors (MacMillan et al., 1997), and 10 $\mu$L of [2,3-$^3H_2$]$GA_9$ (166 Bq, 1.74 × $10^{15}$ Bq/mol in 100 mM Tris, pH 7.6) in capped 1.5-mL tubes. After incubating overnight at room temperature, 1 mL of charcoal slurry (5%, w/v) was added to each reaction, vortexed, and adsorbed for 10 min. (Allowing the sample time to equilibrate produced more consistent results.) Samples were centrifuged for 5 min, and 0.5 mL of supernatant counted in a liquid scintillation counter. GA 2-oxidase activity was detected as liberated $^3H_2O$ in the supernatant (Smith and MacMillan, 1984).

Positive clones were purified from overnight cultures by repeated subdivision and assay of smaller and smaller clone pools, using the same technique. Intervening vector and 5′ untranslated sequence was removed from pure clones prior to enzyme analyses using a Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the kit's instructions. Oligonucleotides (Ransom Hill Biosciences, Ramona, CA) for the procedure were reverse phase desalted and used without further purification. Changes effected by site-directed mutagenesis were confirmed by sequencing.

## Identification of Products

Recombinant enzyme was characterized by incubations with a range of $^2H$ substrates. Each incubation contained one of the following substrate pairs: 500 ng of [17-$^2H_2$]$GA_1$ and 800 Bq of [1,2-$^3H_2$]$GA_1$ (1.39 × $10^{15}$ Bq $mol^{-1}$); 500 ng of [17-$^2H_2$]$GA_4$ and 300 Bq of [1,2-$^3H_2$]$GA_4$ (1.24 × $10^{15}$ Bq $mol^{-1}$); 500 ng of [15,17-$^2H_4$]$GA_9$ and 666 Bq of [17-$^{14}C$]$GA_9$ (2.10 × $10^{12}$ Bq $mol^{-1}$); 500 ng of [17-$^2H_2$]$GA_{20}$ and 666 Bq of [1,2,3-$^3H_3$]$GA_{20}$ (1.11 × $10^{15}$ Bq $mol^{-1}$). Substrate in methanol was evaporated to dryness and resuspended in 95 $\mu$L of lysate from recombinant clones and 5 $\mu$L of 20× cofactors as described previously (Martin et al., 1997). Samples were incubated overnight at 20°C. Products were separated by HPLC and identified by gas chromatography-mass spectrometry (Gaskin and MacMillan, 1992).

## Genomic Clones

A full-length cDNA clone (our no. 170) was used to screen an EMBL3 genomic library of the pea cv Alaska (CLONTECH, Palo Alto, CA) according to the supplier's instructions. Twenty 100-mm plates of 20,000 plaques each were screened. Plaque lifts were made in duplicate onto nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH). Lifts were hybridized overnight at 42°C in hybridization solution (50% [v/v] formamide, 0.25 M NaCl, 7% [w/v] SDS, and 0.12 M sodium phosphate, pH 6.5), washed 10 min each in 5×, 1×, and 0.2× SSC plus 0.1% (w/v) SDS at 42°C, and autoradiographed. Two positive clones were isolated and subcloned in pBluescript II (Stratagene) for sequencing.

### Isolation of 2-Oxidase Clone from NGB6074 (*LE, sln*)

The GA 2-oxidase transcript from slender mutants was cloned by PCR using primers based on the sequence of the clone from line 178. PCR consisted of 1 μL of reverse-transcribed poly($A^+$) RNA from roots of NGB6074, 0.5 μL of 5′ primer (CTAGAATGGTGTTACTATCCAA) (50 μM), 0.5 μL of 3′ primer (GAACAACACTATGATCCTCCAA) (50 μM), 1 μL of dNTPs (10 mM), 0.2 μL of *Taq* polymerase (5 units/μL), 5 μL of 10× buffer (Promega), water to 50 μL, plus two drops of mineral oil. Sample was denatured 5 min at 94°C, cycled 40 times for 30 s at 94°C, 30 s at 60°C, and 3 min at 72°C, and extended for 10 min at 72°C. Products were cloned initially in pBluescript II and subcloned in pET23a for enzyme analyses. These manipulations added three residues (Met·Ala·Arg) to the beginning of the plant protein when expressed in pET23a.

### Sequence Analysis

DNA was sequenced at the Center for Gene Research and Biotechnology at Oregon State University (Corvallis) on an automated sequencer (model 370, Applied Biosystems, Foster City, CA) using 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 9 software (Genetics Computer Group, Madison, WI).

### Southern- and Northern-Blot Analysis

Genomic DNA was isolated by the method of Doyle and Doyle (1990); poly($A^+$) RNA was isolated by the method described previously (Martin et al., 1997). Blots were prepared with Hybond-N nylon membranes (Amersham, Arlington Heights, IL) (Sambrook et al., 1989). Radiolabeled probe was made from cloned, full-length, 1.2-kb 2-oxidase cDNA using Ready-To-Go DNA Labeling Beads (-dCTP) (Pharmacia, Alameda, CA) and [α-$^{32}$P]dCTP (NEN Life Science Products, Boston). Southern blots were hybridized overnight at 60°C in hybridization solution (probe, 5× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, and 100 μg/mL fish sperm DNA) and washed at both low and high stringencies to detect related sequences (Phillips et al., 1995). Northern and RNA slot blots were hybridized overnight at 68°C in hybridization solution, washed 10 min each in 5× and 1× SSC plus 0.1% (w/v) SDS at 42°C and 10 min in 0.1× SSC plus 0.1% (w/v) SDS at 68°C. Blots were sealed in 4-mil polyethylene bags and exposed to X-OMAT AR film (Kodak, Rochester, NY) with intensifying screens at −80°C. Some results were quantified using a phosphor imager and imaging software (ImageQuaNT, Molecular Dynamics, Sunnyvale, CA).

## RESULTS

### Cloning GA 2-Oxidase from Pea Seeds

Because pea seeds contain microgram quantities of $GA_{29}$ and $GA_{29}$-catabolite, products of 2-oxidation of $GA_{20}$, we surmised that 2-oxidase transcript would be abundant in this tissue. In vitro translation products of poly($A^+$) RNA from seeds of line 178 (20 DAF) and Progress No. 9 (28 DAF) pea exhibited weak 2-oxidase activity (about 3-fold over background), as measured by the release of $^3H$ from [$^3H_2$]$GA_{20}$.

In a modification of the protocol described by Lange (1997), clones from a cDNA expression library prepared from seed were divided into pools and screened for GA 2-oxidase activity, using the $^3H$-release assay with [$^3H_2$]$GA_9$ as substrate. This assay was capable of detecting activity equivalent to one clone in 6,000 in preliminary trials with a GA 3-oxidase clone (Martin et al., 1997). In practice it was less sensitive and detection was limited at most to one positive in an initial pool of 600 to 800 clones. Approximately $4 \times 10^4$ library clones were screened; one to two positives were encountered per $10^4$ clones.

Several positives were purified and sequenced; they appeared to be full-length with 5′ leaders. cDNA from the longest clones was 1,324 bp long and the longest ORF encoded a protein of 327 amino acids, $M_r$ 36,800, and pI

**Table I.** *Identification of products from incubation of GA 2-oxidase with $C_{19}$-GAs*

| Substrate | Product | Mass Spectra of Products |
|---|---|---|
| | | *m/z (% relative abundance)* |
| [$^2H_2$]$GA_{20}$ | $GA_{29}$ | $M^+$ 508(100), 493(8), 479(5), 449(7), 391(8), 377(9), 305(21), 237(6), 209(33), 169(14) |
| | $GA_{29}$-catabolite[a] | $M^+$ 520(100), 477(89), 447(7), 431(32), 311(10) |
| [$^2H_4$]$GA_9$ | $GA_{51}$ | $M^+$ 422(13), 407(11), 390(35), 374(9), 332(45), 300(31), 289(93), 288(95), 272(55), 24?(37), 229(100), 228(92), 202(22), 184(32) |
| | $GA_{51}$-catabolite[a] | $M^+$ 434(100), 419(4), 390(9), 375(13), 357(9), 343(3), 315(35), 285(7), 269(12), 244(10), 225(3) |
| [$^2H_2$]$GA_1$ | $GA_8$ | $M^+$ 596(100), 581(7), 538(6), 506(3), 450(16), 381(7), 379(7), 331(3), 313(5), 283(5), 240(18), 209(20) |
| [$^2H_2$]$GA_4$ | $GA_{34}$ | $M^+$ 508(100), 476(30), 461(2), 388(5), 376(7), 357(10), 315(10), 290(13) |

[a] As the trimethylsilyl *enol*.

**Table II.** *Effect of enzyme concentration on metabolism of [$^{14}$C]$GA_{20}$*

Lysate of bacteria expressing GA 2-oxidase was diluted with inactive control lysate and incubated overnight at 20°C with substrate and cofactors. Products were separated by HPLC. Results are expressed as percentages of total counts in substrate and product fractions.

| Fraction | Relative Enzyme Concentration | | |
|---|---|---|---|
| | 100× | 10× | 1× |
| $GA_{20}$ | 6.4 | 6.9 | 24.5 |
| $GA_{29}$ | 66.6 | 79.5 | 74.0 |
| $GA_{29}$-catabolite | 26.4 | 13.6 | 1.4 |

7.38. The protein sequence was similar to GA 2-oxidases from runner bean (67% similarity) and Arabidopsis (62%, 63%, and 65% similarity) (Thomas et al., 1999). One of the cDNA clones was used to isolate a genomic clone from an EMBL3 library (Fig. 2).

### Function of PsGA2ox1

Uncertainty about the site of translation initiation prompted construction of two new expression clones, with translation beginning at the first and second in-frame Met codons. Both clones possessed 2-oxidase activity when expressed in *Escherichia coli* and, surprisingly, catalyzed not one reaction but two. In heterologous expression assays each clone converted $GA_{20}$ to $GA_{29}$ and $GA_{29}$ catabolite. The shorter clone converted $GA_{29}$ to $GA_{29}$ catabolite only half as well as the longer one (data not shown). We presumed the longer clone (*PsGA2ox1*) encoded the native protein. In additional assays, PsGA2ox1 converted the $C_{19}$-GAs, $GA_1$, $GA_4$, $GA_9$, and $GA_{20}$ to the corresponding 2$\beta$-hydroxy products (Table I). $GA_9$ and $GA_{20}$ were also converted to $GA_{51}$- and $GA_{29}$-catabolite, respectively.

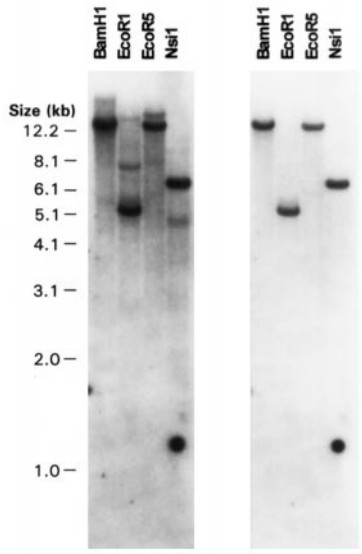


**Figure 3.** Southern analysis of Progress No. 9 pea. The blot was probed with a *PsGA2ox1* cDNA clone and washed sequentially at low (left) and high (right) stringency.

The formation of catabolite depended strongly on enzyme concentration catabolite and was adversely affected by dilution. For example, dilutions of recombinant enzyme up to 100-fold reduced $GA_{29}$-catabolite formation 20-fold, but had a much smaller effect on $GA_{29}$ formation (Table II).

### Southern- and Northern-Blot Analysis

A number of bands appeared on a Southern blot probed with *PsGA2ox1* and washed at low stringency (Fig. 3). Higher stringency washes left only one band in most lanes. *PsGA2ox1* transcript was detected in a variety of organs, including young roots, flowers, fruits, and seeds (Fig. 4A), and was particularly abundant in testae, which accounted for most of the signal detected in seed (Fig. 4B). In Progress No. 9 seeds, the expression of transcript increased as the seed matured, peaking around 30 DAF (Fig. 4B).

### The slender Phenotype Is Caused by a Point Mutation in PsGA2ox1

We cloned and sequenced the corresponding cDNA from seeds of *sln* plants, because of the attenuated 2-oxidase activity associated with this mutation. Compared with *PsGA2ox1*, the sequence contained a single base deletion (Fig. 2) and encoded a truncated product. GA 2-oxidase cloned from *sln* seeds did not metabolize $GA_9$, $GA_{20}$, or $GA_{29}$ in vitro. We could distinguish the wild-type and mutant genes in pea seedlings by a *Nsi*1 RFLP. In a *sln* ×

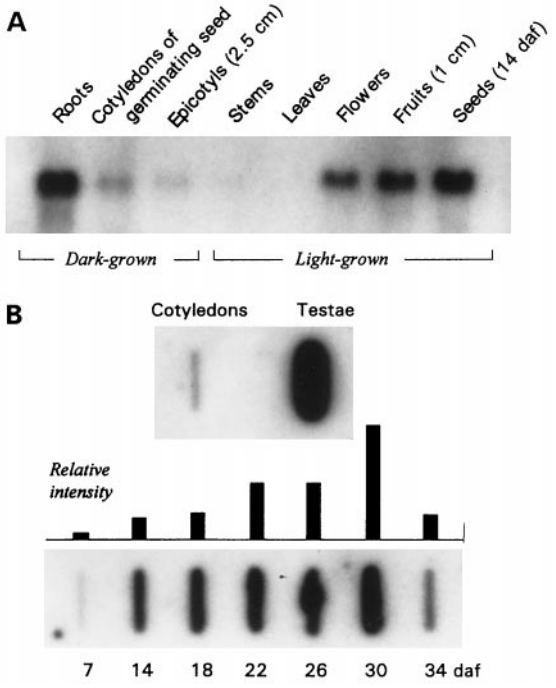


**Figure 4.** RNA analysis of Progress No. 9 pea. A, Northern blot of poly($A^+$) RNA isolated from various organs (3 $\mu$g/lane). B, Slot blot of poly($A^+$) RNA from dissected seed 26 DAF (top) and whole seed (bottom) (1 $\mu$g/slot). Relative intensities were corrected for background and quantified on a phosphor imager. Band intensities for cotyledons and testae differed by 1,000-fold.

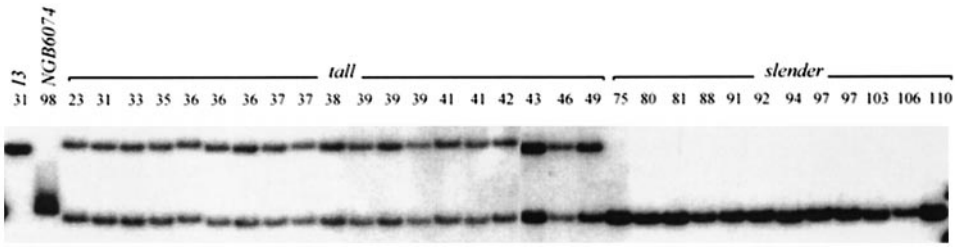


**Figure 5.** RFLP analysis of slender backcross NGB6074 × (NGB6074 × I3). Progeny are arranged by size and segregate into two groups based on the length of stem between the first bract and first true leaf (shown in millimeters above each lane). Wild-type (tall) progeny are heterozygous for both alleles; slender progeny are homozygous for the mutant allele. Figure is a composite of two Southern blots probed with *PsGA2ox1* cDNA.

(*sln* × *Sln*) backcross, the slender phenotype co-segregated with the homozygous mutant allele (Fig. 5).

## DISCUSSION

Several factors made cloning of the 2-oxidase practical. Foremost was the publication of a technique in which pools of clones from an expression library were screened for enzyme activity (Lange, 1997). Our adaptation of the technique was less sensitive than the original and assays were limited to smaller pools of clones. Choice of vector (pET23a versus pMOSE1ox) and changes in culture conditions (e.g. induction at $A_{600}$ 0.6 versus 0.8) may have been the cause. However, the lower sensitivity was offset by the abundance of GA 2-oxidase message in pea seed and the use of a simple $^3$H release assay (Smith and MacMillan, 1984) that facilitated screening.

GA 2-oxidase from pea seed is a multifunctional enzyme catalyzing 2$\beta$-hydroxylation and 2-ketone formation of the $C_{19}$-GA substrates $GA_9$ and $GA_{20}$. GAs $A_1$ and $A_4$ were also 2$\beta$-hydroxylated by the recombinant enzyme but were not oxidized further. The enzyme is homologous to GA 2-oxidases from runner bean and Arabidopsis, which have similar substrate specificities but do not produce $GA_{29}$ catabolite (Thomas et al., 1999). In our experiments, $GA_{29}$ catabolite formation was attenuated when the first 18 amino acids were removed from PsGA2ox1, and translation was initiated at the second in-frame Met codon. Although not shown here, one distinct difference in PsGA2ox1 occurs in a highly conserved region of these GA 2-oxidases, where $Pro_{266}$ is found in place of Ser. Pro is associated with kinks and bends in proteins. The residue is just downstream of $His_{257}$, one of the three iron-binding residues conserved among dioxygenases. The calculated molecular mass of PsGA2ox1, 36.8 kD, is lower than the 45 kD determined by gel filtration for the partially purified 2-oxidase activity from pea seeds (Smith and MacMillan, 1986), although the pI of 7.38 calculated for the gene product is similar.

Southern analysis and the cloning of a second GA 2-oxidase cDNA from pea (J.L. García-Martínez, personal communication) indicate that *PsGA2ox1* is a member of a small family of GA 2-oxidase genes in pea. Three GA 2-oxidase genes have been identified in Arabidopsis (Thomas et al., 1999). Like GA 20-oxidase genes from pea (GenBank accession no. AF138704) and Arabidopsis (GenBank accession no. U20873; Xu et al., 1995), *PsGA2ox1* has two introns. Interestingly, they occur at the same relative positions in all three genes. Furthermore, the single intron in a GA 3-oxidase gene from pea (GenBank accession no. U93210; Lester et al., 1997) is located at the same position as the first intron in the other genes. All of these genes belong to a class of enzymes known as 2-oxoglutarate-dependent dioxygenases.

The *sln* mutation apparently arose from a base deletion in the 2-oxidase gene. There has been some confusion in the past over the number of mutated genes involved. Jaranowski (1976) concluded, "A test cross with the initial form, in $F_2$ segregated in the ratio 15:1, so the [trait was determined] by two recessive genes." However, in crosses with other lines the trait "began to segregate only from the $F_3$ generation" (Jaranowski, 1977). Indeed, the original mutant appeared first in $M_3$, the third generation after mutagenesis, where mutations initially obscured by the maternal genotype in $M_2$ would surface. This unusual inheritance pattern is consistent with data published by others (Reid et al., 1992). Our data support the single gene hypothesis. Evidence comes from the apparent mutation in *PsGA2ox1* cDNA from *sln*, inactivity of the mutant enzyme expressed in *E. coli*, and RFLP analysis of a population segregating for slender and wild-type alleles. Barring tight linkage to a second mutation, we conclude that the trait is determined by mutation of a single gene, *PsGA2ox1*.

*PsGA2ox1* transcript is found predominantly in roots, flowers, young fruits, and testae of seeds. The change in message abundance in developing seeds resembles the change in major GA metabolites. The amount of message peaks at about 30 DAF. Relative to earlier data (Frydman et al., 1974; Sponsel, 1983), this is after the peak in $GA_{29}$ content (24–27 DAF) and before the peak in the $GA_{29}$ catabolite (36 DAF). The high level of expression in testae relative to cotyledons may explain why testae produce mainly $GA_{29}$-catabolite and cotyledons produce mainly $GA_{29}$ (Sponsel, 1983). This and other findings led Ross et al. (1995) to suggest that two distinct enzymes were involved. However, what appears to be the action of two different enzymes may in fact be due to one enzyme present at much higher concentration in testae than in cotyledons. As we have shown (Table II), catabolite formation requires a high enzyme concentration. If transcript abundance is any indication, the concentration of 2-oxidase in these tissues could differ by two to three orders of magnitude, which would

explain the low catabolite content of cotyledons (Sponsel, 1983; Ross et al., 1995). Natural desiccation would increase the concentration of enzyme in maturing seed and favor catabolite formation. $GA_{29}$ may be drawn to the testa by both biochemical and moisture gradients established between testa and cotyledon.

Expression of *PsGA2ox1* is high in roots, as is that of GA 3-oxidase (*PsGA3ox1*) (Martin et al., 1997). Ingram et al. (1985) measured large amounts of $GA_{29}$- and $GA_8$-catabolite in pea roots and suggested that their accumulation in roots was analogous to that in testae. By feeding labeled $GA_{20}$ and $GA_{29}$ to leaves, Ross et al. (1995) observed only small effects of *sln* on 2-oxidation products found in roots. The mutation had no effect on the conversion of $GA_{29}$ to $GA_{29}$-catabolite or of $GA_1$ to $GA_8$ in shoots, although it reduced the conversion of $GA_{20}$ to $GA_{29}$ (Ross et al., 1995). These results and the fact that *sln* plants revert to normal growth over time suggest that other GA 2-oxidases are active in vegetative tissues. The accumulation of $GA_{20}$ in seeds of *sln* plants suggests that PsGA2ox1 is the only GA 2-oxidase active in seed. Furthermore, it is clear from the physiological effects of *sln* that germinating seeds cannot control the effect of large quantities of $GA_{20}$. Because of its dual action at the end of the biosynthetic pathway, *PsGA2ox1* plays a pivotal role in maintaining active GAs and their $C_{19}$ precursors at appropriate levels.

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## LITERATURE CITED

**Doyle JJ, Doyle JL** (1990) Isolation of plant DNA from fresh tissue. BRL Focus **12:** 13–15

**Frydman VM, Gaskin P, MacMillan J** (1974) Qualitative and quantitative analyses of gibberellins throughout seed maturation in *Pisum sativum* cv. Progress No. 9. Planta **118:** 123–132

**Gaskin P, MacMillan J** (1992) GC-MS of the Gibberellins and Related Compounds: Methodology and a Library of Spectra. Cantock's Enterprises, Bristol, UK

**Ingram TJ, Reid JB, MacMillan J** (1985) Internode length in *Pisum sativum* L: the kinetics of growth and [$^3$H]gibberellin $A_{20}$ metabolism in genotype *na Le*. Planta **164:** 429–438

**Ingram TJ, Reid JB, Murfet IC, Gaskin P, Willis CL, MacMillan J** (1984) Internode length in *Pisum*: the Le gene controls the 3$\beta$-hydroxylation of gibberellin $A_{20}$ to gibberellin $A_1$. Planta **160:** 455–463

**Jaranowski JK** (1976) Gamma-ray induced mutations in *Pisum arvense*. Genet Pol **17:** 478–495

**Jaranowski JK** (1977) New genotypes of *Pisum* sp. derived from hybridization of mutants and cultivars. Genet Pol **18:** 337–355

**Lange T** (1997) Cloning gibberellin dioxygenase genes from pumpkin endosperm by heterologous expression in *Escherichia coli*. Proc Natl Acad Sci USA **94:** 6553–6558

**Lester DR, Ross JJ, Davies PJ, Reid JB** (1997) Mendel's stem length gene (Le) encodes a gibberellin 3$\beta$-hydroxylase. Plant Cell **9:** 1435–1443

**MacMillan J, Ward DA, Phillips AL, Sánchez-Beltrán MJ, Gaskin P, Lange T, Hedden P** (1997) Gibberellin biosynthesis from gibberellin $A_{12}$-aldehyde in endosperm and embryos of *Marah macrocarpus*. Plant Physiol **113:** 1369–1377

**Martin DN, Proebsting WM, Hedden P** (1997) Mendel's dwarfing gene: cDNAs from the Le alleles and function of the expressed proteins. Proc Natl Acad Sci USA **94:** 8907–8911

**Martin DN, Proebsting WM, Parks TD, Dougherty WG, Lange T, Lewis MJ, Gaskin P, Hedden P** (1996) Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. Planta **200:** 159–166

**Miller J** (1994) Bacterial transformation by electroporation. Methods Enzymol **235:** 375–385

**Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttly AK, Gaskin P, Graebe JE, Hedden P** (1995) Isolation and expression of three gibberellin 20-oxidase cDNA clones from Arabidopsis. Plant Physiol **108:** 1049–1057

**Potts WC, Reid JB, Murfet JC** (1985) Internode length in *Pisum:* gibberellins and the slender phenotype. Physiol Plant **63:** 357–364

**Reid JB, Ross JJ, Swain SM** (1992) Internode length in *Pisum*: a new slender mutant with elevated levels of $C_{19}$ gibberellins. Planta **188:** 462–467

**Ross JJ, Reid JB, Swain SM** (1993) Control of stem elongation by gibberellin $A_1$: evidence from genetic studies including the slender mutant *sln*. Aust J Plant Physiol **20:** 585–599

**Ross JJ, Reid JB, Swain SM, Hasan O, Poole AT, Hedden P, Willis CL** (1995) Genetic regulation of gibberellin deactivation in *Pisum*. Plant J **7:** 513–523

**Sambrook J, Fritsch EF, Maniatis T** (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

**Smith VA, MacMillan J** (1984) Purification and partial characterization of a gibberellin 2$\beta$-hydroxylase from *Phaseolus vulgaris*. J Plant Growth Regul **2:** 251–264

**Smith VA, MacMillan J** (1986) The partial purification and characterisation of gibberellin 2$\beta$-hydroxylases from seeds of *Pisum sativum*. Planta **167:** 9–18

**Sponsel VM** (1983) The localization, metabolism and biological activity of gibberellins in maturing and germinating seeds of *Pisum sativum* cv. Progress No. 9. Planta **159:** 454–468

**Thomas SG, Phillips AL, Hedden P** (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci USA **96:** 4698–4703

**Xu Y-L, Li L, Wu K, Peeters AJM, Gage DA, Zeevaart JAD** (1995) The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. Proc Natl Acad Sci USA **92:** 6640–6644