Internode Length in *Pisum:* Gene *lkc*

James B. Reid, John J. Ross, and Omar Hasan

Department of Plant Science, University of Tasmania, Hobart, Tasmania 7001, Australia

Abstract. A new single gene-recessive internode length mutant in *Pisum, lkc,* is characterized. The internodes of *lkc* plants are 30–40% shorter than those of comparable *Lkc* plants, and this is attributable to reductions in both cell length and the number of cells per internode. Dwarfism in the mutant is not due to modified gibberellin (GA) levels, as determined by gas chromatography–selected ion monitoring (GC-SIM) for GA_1 and GA_{20}, and bioassay (rice cv. Tan-ginbozu). Furthermore, *lkc* plants are not as responsive as the wild-type to applied GA_1. The diminished stature of *lkc* plants appears to result from a direct or indirect interference with the transduction of the GA_1 signal.

Genes reducing stem elongation are common in higher plants, and the function of these genes has been examined in several species (see e.g., Phinney 1989, Reid 1989). Two broad categories of mutations have been established. First, there are those that reduce the level of gibberellin A_1 (GA_1) by blocking steps in the GA biosynthetic pathway (Fujioka et al. 1988, Ingram et al. 1986, Phinney 1984, Reid 1989). Second, there are mutants that result in a reduced response to applied GA_1. These fall into the broad-sensitivity category as defined by Firn (1986). The mode of action of the sensitivity mutants has not been determined in detail, although potentially they may block any step between (and including) reception of the GA signal and elongation, either directly or indirectly (Reid 1989). Recent results suggest that this second class of mutants may provide an insight into these largely unknown steps. For example, gene *lv* may increase sensitivity to GA_1 by partially blocking the transduction chain from physiologically light-stable phytochrome (Nagatani et al. 1990), whereas genes *lka* and *lkb* may reduce the sensitivity to GA_1 by mod-

ifying the chemorheological properties of the cell wall (Behringer et al., 1990).

The present paper examines the genetic cause of the reduced elongation observed in a mutant derived from the tall cv. Torsdag. This mutation is the second mutation identified in line K202 (Reid 1986). Results are presented which suggest that this mutation acts by influencing the response to GA_1 rather than by altering GA biosynthesis.

Materials and Methods

Plant Material

The pure lines used during this work are held in the collection at Hobart. Line 206 was selected from a cross between the mutant line K202 and cv. Torsdag. It possesses the less severe of the two dwarf mutations in K202 (Reid 1986). K202 was produced from cv. Torsdag by Dr. K. K. Sidorova (Novosibirsk, USSR). Other internode length lines used during this work were K29 (*lw*), K511 (*lk*), NGB5865 (*lka*), NGB5862 (*lkb*), NEU3 (*le lv cry^c*), and L197 (*la cry^s*). These lines are homozygous recessive for the genes indicated, and homozygous for the dominant wild-type genes at the other internode length loci. Further details about the genotypes and phenotypes of these lines may be found in Reid (1986), Reid and Ross (1988, 1989) and Jolly et al. (1987).

Growing Conditions

All plants were grown in a heated glasshouse as previously described (Reid and Potts 1986). Nodes were counted starting from the cotyledons as 0. The natural photoperiod was extended to 18 h with mixed fluorescent (Thorn, 40 W cool white tubes) and incandescent (Mazda, 100 W pearl globes) light (~30 μmol m^{-2} s^{-1} photosynthetically active radiation at pot top). Cell length and number of cells per internode were determined from epidermal strips as described by Reid et al. (1983). Ten cells were measured from each of six plants per treatment.

Growth Regulator Treatments

The GA-synthesis inhibitor, paclobutrazol (20 μg or 2.7 mM),

was applied to the dry nicked seeds in 5 μl ethanol before planting. A range of GA_1 dosages (0, 0.1, 1, and 10 μg) was applied to the uppermost fully expanded leaf (usually leaf 4, sometimes leaf 5) in 10 μl of ethanol 18 days after planting (four to five leaves expanded).

Harvest and Extraction Procedure

The Torsdag plants grown for these harvests were clearly different from the accompanying line 206 plants in terms of both internode length and plant height. For the gas chromatography–selected ion monitoring (GC-SIM) quantitation of GA levels in line 206 and cv. Torsdag, 38 plants of each genotype were harvested 27 days after sowing (eight to nine leaves expanded). Apical and mature portions were harvested. The former comprised all tissue above the uppermost expanded leaf, whereas the latter consisted of the uppermost expanded leaf and the internode directly below it. Further details are given in Table 3. Plants for analysis by bioassay were 26 days old at harvest. In this case apical portions were excised above node 6 or 7, for plants with eight or nine expanded leaves, respectively. For both genotypes, 97.5 g (fresh wt) of tissue was harvested, from 95 line 206 and 78 Torsdag plants. Harvested material was immediately immersed in cold (−20°C) methanol, followed by addition of $[17,17\text{-}^2H_2]GA_1$ and $[17,17\text{-}^2H_2]GA_{20}$ internal standards provided by Professor L. Mander (Canberra, Australia), to the samples for GC-SIM. The tissue was then homogenized. GAs were extracted in 80% methanol at 4°C for 24 h, followed by filtering. For quantitation by GC-SIM, extracts were then purified by forcing through a Sep-Pak C_{18} cartridge (Waters Associates, Melbourne, Australia) in 60% methanol (Ross and Reid 1989), followed by elution with approximately 15 ml of 60% methanol. After removal of the methanol in vacuo 1 volume of 0.5 M sodium phosphate buffer was added prior to partitioning against ethyl acetate at pH 2.9. The ethyl acetate fraction was then dried (with 5% added toluene) for fractionation by high-performance liquid chromatography (HPLC). The extracts for bioassay were purified using a similar procedure. However, the Sep-Pak step (in 80% methanol) was performed after the ethyl acetate fraction was dried, and initial purification was performed by partitioning against toluene at pH 8.

HPLC

Fractionation was performed by reverse-phase C_{18} HPLC using the system previously described by Potts et al. (1985), and a Waters 10-μm Radial-Pak μ Bondapak C_{18} column (100 × 8 mm ID). The program for fractionation of the extract for bioassay ran from 40–70% methanol in 0.01% (vol/vol) acetic acid over 55 min with the solvent programmer set on curve 9. The flow rate was 2 ml min^{-1} and 1-min fractions were collected and dried. GA-like activity was assayed by applying one sixth of each fraction (in 2 μl methanol) to each of six rice seedlings (cv. Tan-ginbozu; Murakami 1968, Potts et al. 1982). The extract for quantitation by GC-SIM was fractionated using a program which ran from 21–70% methanol in 0.4% acetic acid over 40 min (curve 9), followed by isocratic 70% elution. The flow rate was 2 ml min^{-1}. This program separates GA_{29} and 2-epi GA_{29} from GA_1, thus overcoming problems caused by the close elution of these isomers on gas chromatography (Gaskin et al. 1985, Ross et al. 1990). Broad (9 min) fractions encompassing the retention times

Table 1. Effects of the *Lkc/lkc* gene difference on various shoot and root characteristics of 11-day-old plants.

	Genotype	
	Lkc (cv. Torsdag)	*lkc* (line 206)
Shoot fresh weight (g)	1.01 ± 0.04	0.72 ± 0.03
Shoot dry weight (mg)	91.0 ± 3.7	65.8 ± 2.2
Number of expanded leaves	4.73 ± 0.08	4.93 ± 0.09
Length of internode 3 to 4 (cm)	3.54 ± 0.08	1.95 ± 0.03
Root fresh weight (g)	1.17 ± 0.08	0.95 ± 0.05
Root dry weight (mg)	56.8 ± 2.7	44.1 ± 2.1
Length of tap root (cm)	25.3 ± 0.08	20.7 ± 1.2
Number of lateral roots	71.4 ± 3.7	55.1 ± 3.5

Data are given as means ± SE of eight replicate plants.

of GA_1 (~30 min) and GA_{20} (~40 min) were collected and dried. These fractions were then methylated as before (Ross and Reid 1989) and trimethylsilated in an excess of BSTFA (~10 μl, with up to 2 μl of pyridine to aid dissolution) at 60°C for 10 min.

GC-SIM

GC-SIM was performed using a Hewlett-Packard 5890 GC coupled to a Hewlett-Packard 5970 mass-selective detector. A 25 m × 0.32 mm ID × 0.17-μm film HP1-fused silica column was coupled to the mass-selective detector via an open-split interface. Helium was used as the carrier gas, at an initial flow rate of 2 ml min^{-1} at 60°C. Samples were injected in the splitless mode. The temperature of the column oven was increased from 60–215°C at 30°/min and then at 3°/min. For quantitation of endogenous GA_1 and GA_{20} levels, the peak area ratios (at the correct retention times) for ion pairs 508/506 and 420/418 were measured, respectively, and appropriate corrections were made. Ion pairs 450/448 (GA_1) and 377/375 (GA_{20}) were also monitored to aid identification.

Results

Phenotype of Mutant Line 206

Plants of line 206 are consistently smaller than the parental cv. Torsdag for a wide range of both root and shoot characters (Table 1). Internode length is the most obvious visible difference (Fig. 1) and this appears to be attributable in both the epidermis and cortex to a change in both the number of cells per internode and the length of individual cells (Table 2).

Inheritance of the Line 206 Phenotype

Crosses between line 206 and the internode length mutants K29 (*lw*), K511 (*lh*), NGB 5865 (*lka*), and

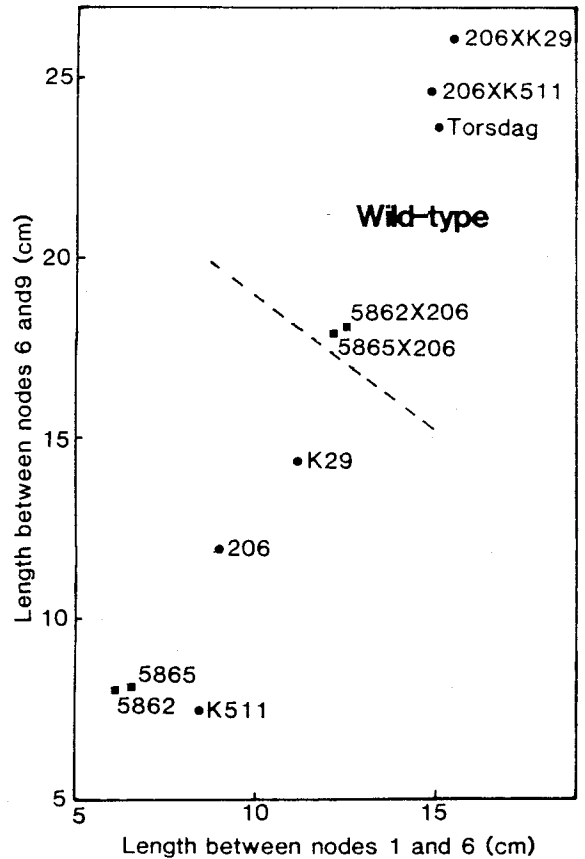Fig. 1. Line 206 (*lkc*) and its parental cv. Torsdag (*Lkc*).

Fig. 2. The length between nodes 1 and 6 plotted against the length between nodes 6 and 9 (cm) for a range of internode-length mutants and the F_1 of crosses between them. Points with the same symbol represent plants grown simultaneously. Six replicate plants were usually grown. The broken line indicates the limits of the wild-type class.

Table 2. Effects of the *Lkc/lkc* gene difference on cell length and the number of cells per internode, for the epidermal and outer cortical cells of internode 8 to 9.

	Genotype	
	Lkc (cv. Torsdag)	*lkc* (line 206)
Cell length (μm)		
Epidermal	329 ± 20	268 ± 14
Outer cortical	72 ± 4	60 ± 4
Number of cells per internode		
Epidermal	264 ± 15	181 ± 12
Outer cortical	1222 ± 115	812 ± 49

Data are given as means ± SE for six replicate plants.

NGB 5862 (*lkb*), all yielded tall (wild-type) F_1 progeny, indicating that the mutation in line 206 is not allelic with the recessive alleles at the *lw, lh, lka,* or *lkb* loci (Fig. 2). In addition, crosses to the elongated mutants NEU3 (*lv*) and L197 (*la cry^s*) yielded tall F_1 plants. Previously reported crosses between K202 (the source of the mutation in line 206) and several other lines indicated that the mutation in line 206 was non-allelic with the genes *Le, Lm, Ls, Na,* or *Lk* (Reid 1986).

The cross between line 206 and the parental cv. Torsdag yielded a tall F_1 along with a relatively clear segregation in F_2 into eight short plants similar to line 206 and 50 tall plants similar to cv. Torsdag (Fig. 3). One plant was intermediate. In the F_3 the eight short plants all bred true (48 progeny). F_3 progeny were grown from eight tall F_2 plants. Three segregated for both short and tall plants, as did the F_3 progeny from the intermediate plant (total: 18 tall, 6 short plants), whereas five bred true (total: 30 tall plants). Although there was a slight deficiency of the short class in the F_2 (χ_1^2 for 3:1 = 3.9), the F_3 data and the allelism tests indicate that the mutant
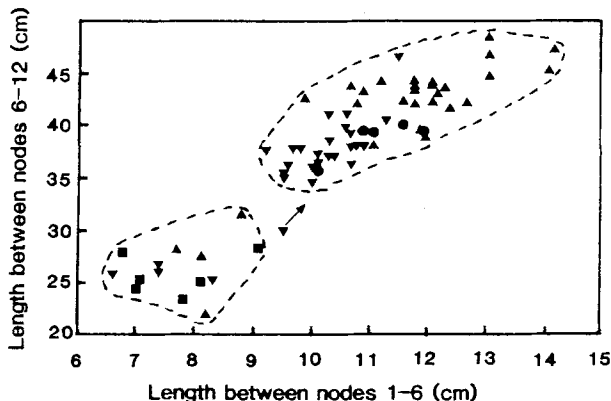
Fig. 3. The length between nodes 1 and 6 plotted against the length between nodes 6 and 12 (cm) for line 206 (■) cv. Torsdag (●) and two families from the F_2 of a cross between line 206 and cv. Torsdag (▲, ▼). Broken lines indicate the limits of the classes. The one intermediate plant was shown in F_3 to be of genotype *Lkc lkc*.

phenotype of line 206 is conferred by a new single recessive gene. It is proposed that the recessive gene carried by line 206 be named *lkc* and that line 206 should be the type line for this mutation.

Response of lkc *Plants to* GA₁

When plants of line 206 (*lkc*), previously dwarfed by treatment with paclobutrazol, were treated with GA_1 they showed pronounced elongation indicating that GA_1 levels are important for growth in this genotype (Fig. 4). However, at all three treatment levels, 0.1, 1, and 10 μg of GA_1, they showed significantly smaller responses (P < 0.001) than comparable plants of cv. Torsdag (Fig. 4). This difference in response was relatively larger at the lower dose rates (0.1 and 1 μg of GA_1), but even at the 10 μg dose the maximum elongation achieved was reduced in the mutant (Fig. 4). The paclobutrazol treatment itself was very effective in dwarfing both genotypes resulting in at least 90% reductions in internode lengths from internode 3 to 4 up to internode 7 to 8. These results suggest that, although the level of GA_1 plays an important role in the elongation of *lkc* plants, the response to GA_1 is reduced in mutant *lkc* compared with *Lkc* plants.

GA Levels in lkc *Plants*

Extracts from the young apical portions of shoots of cv. Torsdag (*Lkc*) and line 206 (*lkc*) contained a qualitatively and quantitatively similar spectrum of GA-like substances (Fig. 5) when bioassayed using rice seedlings (cv. Tan-ginbozu; Murakami 1968,

Potts et al. 1982). Based on previous identifications by GC-mass spectrometry (GC-MS) or GC-SIM of peaks from similar extracts from peas (Davies et al. 1982, Ingram et al. 1983, Potts 1986, Potts et al. 1985), the biological activity in fractions 6 to 8, 16 to 18, 26 to 30, and 32 to 37 may correspond to GA_1, GA_{20}, GA_{44}, and GA_{19}, respectively.

GA_1 and GA_{20} levels were consequently determined by GC-SIM using deuterated internal standards. The overall levels of GA_{20} and GA_1 in extracts from young shoots of line 206 were slightly higher on a per gram fresh weight basis compared to cv. Torsdag (Table 3). The reverse was the case on a per plant basis. When the young shoot was divided into an apical and more mature portion, line 206 possessed marginally less GA_1 in the apical portion than cv. Torsdag, but the reverse was true in the mature portion on a per gram fresh weight basis.

Overall these results suggest that differences in GA levels do not control the difference in elongation between cv. Torsdag (*Lkc*) and line 206 (*lkc*). The minor difference in GA_1 levels between the apical portions of cv. Torsdag and line 206 is unlikely to be biologically meaningful, particularly in view of the difficulty in harvesting material of directly comparable ontogenetic stages between lines that differ substantially in internode length. The analysis of the apical and mature portions confirms earlier work (Potts et al. 1982, 1985), which showed that reduced levels of GA_1-like activity occurred in fully expanded leaf and stem sections compared to young expanding apical tissue.

Discussion

The short phenotype of line 206 is conferred by a single, previously undescribed mutant allele, designated *lkc*. The level of GA_1 appears normal in *lkc* plants, suggesting that their short stature is due to an impaired ability to fully respond to endogenous GA_1 (Table 3). This is supported by the reduced response of *lkc* plants to applied GA_1 compared to that of *Lkc* plants (Fig. 4). Thus, the mutant *lkc* belongs to the GA-sensitivity category of short mutants (Reid 1989) and is the fifth such mutant identified in this species (the others are *lk, lka, lkb* and *lw;* Jolly et al. 1987, Reid and Potts 1986, Reid and Ross 1989). The increasing number of such mutants is consistent with the expected complexity of events between the reception of the GA_1 signal and elongation.

The term sensitivity is used here in the broad sense of Firn (1986) to include aspects relating to "receptivity," "affinity," and "response capacity." Furthermore, the classification of a mutant as
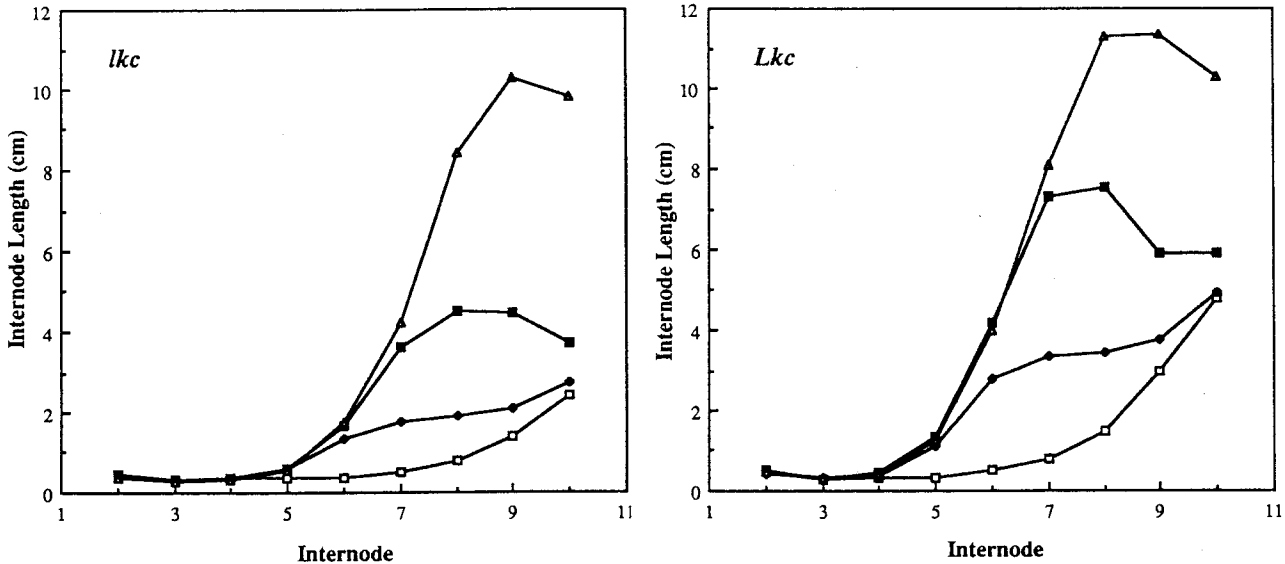
Fig. 4. Internode length plotted against internode number for line 206 (*lkc*) and cv. Torsdag (*Lkc*), treated with 0 (□), 0.1 (♦), 1 (■), or 10 (▲) μg GA_1 in 10 μl ethanol 18 days after sowing. Line 206 and cv. Torsdag plants were dwarfed by treatment with paclobutrazol (20 μg) at the start of germination. Internode 5 is between nodes 5 and 6. SEs for internodes 7 and 8 average 0.16 and 0.22, respectively. N ≥ 10.

a GA-sensitivity mutant does not necessarily imply that the allele involved directly blocks a step in the transduction of the GA_1 signal, in a manner analogous to blocks imposed by the GA synthesis mutants. Rather it is envisaged that in GA-sensitivity mutants the conditions which permit the normal response to GA_1 do not occur. Another substance necessary for "normal" elongation may be deficient, thus reducing the plant's potential to elongate in response to the available GA_1. A GA-sensitivity mutant, then, may be a synthesis mutant with regard to another, hypothetical, substance. However, in the absence of concrete evidence concerning the nature of such a substance, it is convenient to classify short mutants in terms of their response (or lack of it) to GA_1, the only growth factor firmly established as necessary for normal elongation growth in intact peas and probably many other species (see Reid 1990). A relatively indirect effect of GA-sensitivity mutants on the response to GA_1 does not diminish their usefulness for elucidating the partial processes involved in this response and in stem elongation in general.

The comparison of GA_1 levels in *lkc* and *Lkc* plants using the rice seedling bioassay (Fig. 5) produced very similar results to that obtained using internal standards and GC-SIM (Table 3). This may validate previous comparisons between GA-sensitivity mutants and comparable wild-types based solely on bioassay data (e.g., Reid and Potts 1986, Ross and Reid 1989).
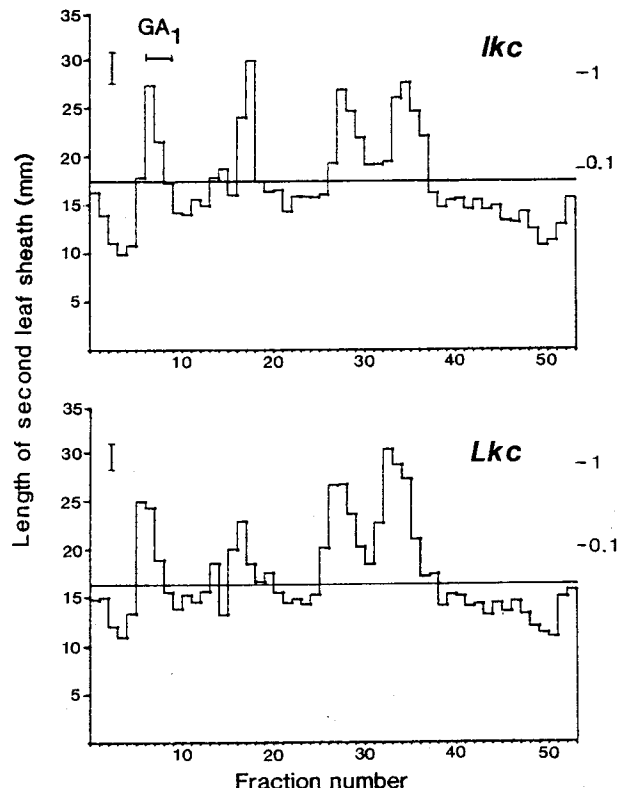

Fig. 5. Gibberellin-like activity in the rice seedling bioassay (cv. Tan-ginbozu) of fractions of extracts from the apical portion of shoots of line 206 (*lkc*) and cv. Torsdag (*Lkc*). The retention time of GA_1, $LSD_{0.01}$ intervals, and the response to 0.1 and 1 ng GA_3 are indicated, as is the growth of control plants (——).

Table 3. Levels of GA_1 and GA_{20} in the "apical" and "mature" portions of 27-day-old *Lkc* (cv. Torsdag) and *lkc* (line 206) plants, determined by GC-SIM using deuterated internal standards.

	Genotype and plant portion					
	Lkc			*lkc*		
	Apical	Mature	Total	Apical	Mature	Total
Fresh weight of tissue (g)	11.4	29.3	40.7	8.2	19.3	27.5
Level of endogenous GA_1						
ng plant^{-1}	2.4	3.7	6.1	1.6	3.4	5.0
ng g^{-1} fresh wt	8.1	4.8	5.7	7.4	6.8	6.9
Level of endogenous GA_{20}						
ng plant^{-1}	2.8	5.1	7.9	2.3	4.6	6.9
ng g^{-1} fresh wt	9.4	6.6	7.4	10.5	9.0	9.5

References

Behringer FJ, Cosgrove DJ, Reid JB, Davies PJ (1990) The physical basis for altered stem elongation rates in intermode length mutants of *Pisum*. Plant Physiol (in press)

Davies PJ, Emshwiller E, Gianfagna TJ, Proebsting WM, Noma M, Pharis RP (1982) The endogenous gibberellins of vegetative and reproductive tissue of G2 peas. Planta 154:266–272

Firn RD (1986) Growth substance sensitivity: The need for clearer ideas, precise terms and purposeful experiments. Physiol Plant 67:267–272

Fujioka S, Yamane H, Spray CR, Gaskin P, MacMillan J, Phinney BO, Takahashi N (1988) Qualitative and quantitative analyses of gibberellins in vegetative shoots of normal, *dwarf-1*, *dwarf-2*, *dwarf-3* and *dwarf-5* seedlings of *Zea mays* L. Plant Physiol 88:1367–1372

Gaskin P, Gilmour SJ, MacMillan J, Sponsel VM (1985) Gibberellins in immature seeds and dark-grown shoots of *Pisum sativum*. Planta 163:283–289

Ingram TJ, Reid JB, MacMillan J (1986) The quantitative relationship between gibberellin A_1 and internode growth in *Pisum sativum* L. Planta 168:414–420

Ingram TJ, Reid JB, Potts WC, Murfet IC (1983) Internode length in *Pisum*. IV. The effect of the *Le* gene on gibberellin metabolism. Physiol Plant 59:607–616

Jolly JC, Reid JB, Ross JJ (1987) Internode length in *Pisum*. Action of gene *lw*. Physiol Plant 69:489–498

Murakami Y (1968) A new rice seedling test for gibberellins, "Microdrop Method," and its use for testing extracts of rice and morning glory. Bot Mag Tokyo 81:33–43

Nagatani A, Reid JB, Ross JJ, Dunnewijk A, Furuya M (1990) Internode length in *Pisum*. The response to light quality, and phytochrome type I and II levels in *lv* plants. J Plant Physiol 135:667–674

Phinney BO (1984) Gibberellin A_1, dwarfism and the control of shoot elongation in higher plants. In: Crozier A, Hillman JR (eds) The biosynthesis and metabolism of plant hormones. Society of Experimental Biology Seminar Series 23. Cambridge University Press, Cambridge, pp 17–41

Phinney BO (1989) The genetics and physiology of dwarfism in *Zea mays* (maize). In: Pharis RP, Rood SB (eds) Plant growth substances, 1988. Springer-Verlag, Heidelberg

Potts WC (1986) Gibberellins in light-grown shoots of *Pisum sativum* L. and the influence of reproductive development. Plant Cell Physiol 27:997–1003

Potts WC, Reid JB, Murfet IC (1982) Internode length in *Pisum*. I. The effect of the *Le/le* gene difference on endogenous gibberellin-like substances. Physiol Plant 55:323–328

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

Reid JB (1986) Internode length in *Pisum*. Three further loci, *lh*, *ls* and *lk*. Ann Bot 57:577–592

Reid JB (1989) Gibberellin synthesis and sensitivity mutants in *Pisum*. In: Pharis RP, Rood SB (eds) Plant growth substances, 1988. Springer-Verlag, Heidelberg

Reid JB (1990) Phytohormone mutants in plant research. J Plant Growth Regul 9:97–111

Reid JB, Murfet IC, Potts WC (1983) Internode length in *Pisum*. II. Additional information on the relationship and action of loci *Le*, *La*, *Cry*, *Na* and *Lm*. J Exp Bot 34:349–364

Reid JB, Potts WC (1986) Internode length in *Pisum*. Two further mutants, *lh* and *ls*, with reduced gibberellin synthesis, and a gibberellin insensitive mutant, *lk*. Physiol Plant 66:417–426

Reid JB, Ross JJ (1988) Internode length in *Pisum*. A new gene, *lv*, conferring an enhanced response to gibberellin A_1. Physiol Plant 72:595–604

Reid JB, Ross JJ (1989) Internode length in *Pisum*. Two further gibberellin-insensitivity genes, *lka* and *lkb*. Physiol Plant 75:81–88

Ross JJ, Reid JB (1989) Internode length in *Pisum*. Biochemical expression of the *le* gene in darkness. Physiol Plant 76:164–172

Ross JJ, Reid JB, Davies NW, Murfet IC (1990) Internode length in *Lathyrus odoratus* L. The involvement of gibberellins. Physiol Plant 79:448–452