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Internode Length in *Pisum*: Biochemical Expression of the *le* and *na* Mutations in the Slender Phenotype

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Abstract. The *le* and *na* mutations in pea block GA biosynthesis and normally cause a marked reduction in internode length. However, neither of these genes influences the growth of plants carrying the *cry^s la* gene combination. Plants of this genotype have long, thin internodes, pale green foliage, and abnormal flower and fruit development, collectively referred to as the "slender" phenotype. [^{13}C,^{3}H]Gibberellin A_{20} is metabolized to GA_1, GA_8, and GA_{29} in slender lines carrying the gene *Le* but only to GA_{29} and GA_{29}-catabolite in *le* lines. Examination of ^{12}C:^{13}C isotopic ratios showed that metabolites were strongly diluted by endogenous [^{12}C]GAs in *Na* lines. However, little if any significant dilution was observed in a line homozygous for the *na* gene. These results confirm that the *le* and *na* mutations are fully expressed at the biochemical level in slender phenotypes of peas and concur with previous reports that internode elongation is entirely independent of GA levels in *cry^s la* (slender) plants.

Mutations that reduce internode extension in plants are relatively common and in several instances have been traced to impaired GA biosynthesis (Hedden and Phinney 1979, Spray et al. 1984, Potts and Reid 1985, Ingram et al. 1983, 1984, Ingram and Reid 1987) or GA sensitivity (Phinney 1957, Radley 1970, Harada and Vergara 1972, Gale and Marshall 1973, Favret et al. 1975, Ho et al. 1981, Stoddart 1984, Reid and Potts 1986). A number of species also contain examples of mutations that increase stem elongation (e.g., De Haan 1927, Lamprecht 1948, Whalen 1964, Favret et al. 1975, Foster 1977). Although these have not been as extensively studied as dwarf mutants, they may provide significant information on the control of stem elongation in higher plants.

The "slender" phenotype of pea (*Pisum sativum* L.) arises from homozygous recessive alleles at the duplicate loci *cry^s* and *la* (De Haan 1927, Lamm

1937). Plants carrying these genes characteristically resemble normal peas treated with saturating doses of GA_3. However, their phenotype is unaffected by treatment with the GA biosynthesis inhibitors AMO-1618 and PP333 (McComb and McComb 1970, Potts et al. 1985) or by the presence of the GA biosynthesis mutations *le* and *na* (Marx 1983, Potts et al. 1985). These features have led to the suggestion that the cry^s *la* gene combination may influence a GA receptor or a normally rate-limiting step between the primary site of reception of the GAs and the phenotypic response (see Potts et al. 1985). The simplest hypothesis remains that slender plants lack an inhibitor (Brian 1957) or repressor present in plants carrying the *Cry* and/or *La* alleles. A third allele, cry^c, is known at the *Cry* locus. This is less severe than cry^s and results in the "crypto" phenotype when combined with *la* (Rasmusson 1927, Lamm 1937, Reid et al. 1983).

The present communication examines the metabolism of GA_{20} in the shoots of slender (cry^s *la*) and crypto (cry^c *la*) plants and confirms that the *le* and *na* mutations are expressed biochemically in these phenotypes.

Materials and Methods

The lines of *Pisum sativum* L. used during this work came from the collection at Hobart (Department of Botany, University of Tasmania). All lines are homozygous for the internode length genes *Lh, Lk, Lm,* and *Ls* (see Reid 1986). Line 8 is a cryptodwarf type (genotype cry^c *la le Na*). Lines 186 (slender), 187 (tall), and 188 (slender) are genetically closely related selections from the F_6 and F_7 of a cross between lines 133 and WL1766. Their genotypes are respectively: cry^s *la Le Na*, *Cry* and/or *La Le Na*, and cry^s *la Le na*. The cry^s *la le Na* plants used were grown from seed of slender plants segregating for a number of noninternode length characters from a cross between lines 2 and 5. Further details of the genetic basis of internode length in peas are discussed by Reid et al. (1983) and Reid (1986). Plants were grown in controlled-environment cabinets under the following conditions: day temperature, 20°C; night temperature, 15°C; day length, 18 h; photon fluence rate, 210 $\mu mol\ m^{-2}\ s^{-1}$ (photosynthetically active radiation). Except for the cryptodwarf line 8, plants were grown in soil-based John Innes No. 3 compost. Line 8 was grown in horticultural-grade vermiculite watered twice a week with Murashige and Skoog (1962) plant salts medium.

Gibberellin Treatments

$[17\text{-}^{13}C,^{3}H_2]GA_{20}$ (88% ^{13}C, 1.27 g Bq $mmol^{-1}$, 97% radiochemically pure) prepared by Ingram et al. (1984) was fed to plants 15–20 days after germination in microdrops of methanol (1 μg GA μl^{-1}) applied to the uppermost pair of fully expanded stipules. In the initial experiment with genotype cry^s *la le Na*, 17 plants were treated with 1 μg $[^{13}C,^{3}H]GA_{20}$ per plant. Forty plants of the cryptodwarf line 8 and 18–23 plants each of lines 186, 187, and 188 were treated with 2 μg $[^{13}C,^{3}H]GA_{20}$ per plant. In each case after 6 days the tissue that had

expanded above the treated node was harvested, frozen in liquid nitrogen, and extracted. The fresh weights of tissue obtained from the *cry^s la le Na* genotype and lines 8, 186, 187, and 188 were, respectively (per plant): 2.17, 2.57, 3.42, 3.91, and 3.86 g.

Gibberellin Extraction and Purification

Plant tissue was homogenized and extracted overnight in methanol:distilled water (4:1 v/v, ~10 ml/g fresh weight). Initial purification of GAs was carried out as described by Ingram et al. (1983). Aliquots (2%) from ethyl acetate and aqueous fractions were routinely examined by liquid scintillation counting to determine the distribution of radioactivity in extracts.

Acidic ethyl acetate fractions were purified by reverse-phase HPLC. In initial experiments with genotype *cry^s la le Na* and the cryptodwarf line 8, polar metabolites were separated from [^{13}C,^{3}H]GA_{20} by semipreparative scale HPLC on a stainless-steel column (250 mm long, 8 mm i.d.) packed with Hypersil ODS (5 μm) (Shandon Southern Products, Runcorn, Cheshire, UK). Extracts, dissolved in 30% methanol, were clarified by filtration and chromatographed with an exponential gradient of methanol in distilled water (containing 0.2% acetic acid), 35–70% in 30 min, followed by isocratic elution with 70% methanol for 10 min, delivered by two pumps controlled by a solvent programmer set on gradient exponent 5 (Laboratory Data Control, Stone, Staffs, UK). The solvent flow rate was 2.5 ml min^{-1}. One-minute fractions were collected, and aliquots (2%) were removed for radiocounting. Fractions containing [^{13}C,^{3}H]GA_{20} metabolites were combined, methylated with ethereal diazomethane, and subjected to analytical reverse-phase HPLC using a column (250 mm long, 4.6 mm i.d.) packed with Spherisorb ODS (5 μm) (Anachem Ltd., Luton, Bedfordshire, UK). The column was eluted with an exponential gradient (exponent 5) of methanol in distilled water, 32–70% in 30 min, followed by isocratic elution with 70% methanol for 10 min, flow rate 1.5 ml min^{-1}. One-minute fractions were collected, and aliquots (4%) were removed for radiocounting.

Samples from lines 186, 187, and 188 were purified using modified HPLC conditions: (1) semipreparative column (250 mm long, 10 mm i.d.) packed with Dynamex C18 (8 μm) (Rainin Instrument Co. Inc., Woburn, MA, USA), eluted with a linear gradient of methanol in distilled water (containing 0.2% acetic acid), 30–100% in 60 min, flow rate 5 ml min^{-1}; (2) analytical column (as above) eluted with an exponential gradient (exponent 5) of methanol in distilled water (containing 0.2% acetic acid), 20–70% over 30 min, followed by isocratic elution with 70% methanol for 10 min, flow rate 1.5 ml min^{-1}. Purified samples from these lines were methylated and repartitioned between ethyl acetate and distilled water to remove sugars and other polar compounds prior to GC-MS.

Throughout the work, radioactivity was determined by liquid scintillation counting following addition of 10 ml of Unisolve E (Koch Light, Haverhill, Suffolk, UK) to the samples. The ^{3}H counting efficiency was ~40% (external standard ratio procedure). Results are adjusted for ^{3}H losses incurred after obtaining the acidic ethyl acetate extracts.

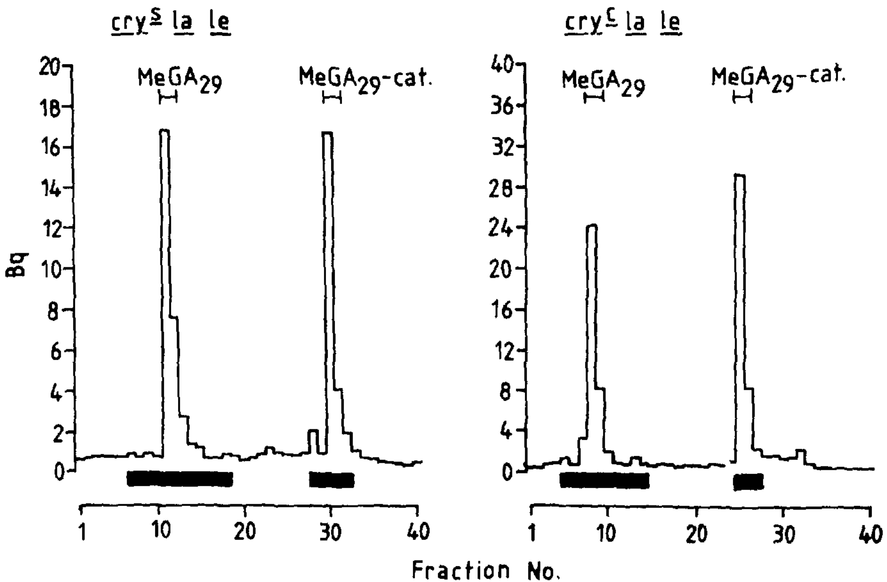

Fig. 1. [^{13}C,^{3}H]Gibberellin A_{20} metabolism in pea genotypes *cry^s la le Na* and *cry^c la le Na* (line 8). HPLC of acidic ethyl acetate extracts from the shoot tissue expanded 6d after treatment with substrate. Radioactivity was determined in aliquots (4%) of each fraction. Barred fractions were combined and analyzed by GC-MS (see Table 1).

Gas Chromatography–Mass Spectrometry

Appropriate fractions from HPLC were combined and reduced to dryness. The trimethylsilyl (TMS) ethers of the methyl esters were prepared immediately prior to gas chromatography–mass spectrometry (GC-MS) by the addition of N-methyl-N-trimethylsilytrifluoroacetamide (MSTFA) or a 3:2:2 mixture of hexamethyldisilazane, trimethylchlorosilane, and pyridine to the extracts dissolved in an equal volume of pyridine. GC-MS was performed as described before (Ingram et al. 1984).

Gibberellin mass spectra were obtained after background subtraction of neighboring scans, and percentages of [^{12}C]- and [^{13}C]GAs were calculated from the relative intensities of ions in the molecular ion (M^+) clusters, using a least-squares procedure to generate a calculated M^+ cluster from the observed spectrum (see Sponsel and MacMillan 1978). Endogenous GA levels were calculated from a knowledge of ^{12}C:^{13}C isotopic ratios and ^{3}H in respective metabolites.

In Table 3 the isotopic composition of GA_{20} was determined by selected ion monitoring under the following conditions: GC oven temperature 200°C; m/e values 418 ([^{12}C]M^+), 419 ([^{13}C]M^+), 420, 421, and 422.

Results

The metabolism of [^{13}C,^{3}H]GA_{20} was initially examined in slender plants of genotype *cry^s la le Na* and the cryptodwarf line 8 (*cry^c la le Na*). In both

Table 1. [^{13}C,^{3}H]Gibberellin A_{20} application to internode length genotypes of pea, *cry^c la le Na* and *cry^s la le Na*. Incorporation of ^{13}C-isotope and endogenous GA levels in the tissue expanded during the 6 days following treatment with substrate.

Metabolite	Percent isotopic composition[a] ^{12}C	^{13}C	Approx. level (ng plant^{-1}) Endogenous	Exogenous[b]	HPLC fractions
cry^c la le Na (line 8)					
GA_{20}	(not analyzed)		—	3	—
GA_{29}	83	17	38	9	(5–14)
GA_{29} catabolite	80	20	27	8	(25–27)
cry^s la le Na					
GA_{20}	85	15	33	7	—
GA_{29}	82	18	50	13	(7–18)
GA_{29} catabolite	82	18	33	8	(28–32)

[a] Substrate ^{13}C composition = 88%.
[b] Estimated from HPLC radioactivity profile (see Fig. 1).

Table 2. Length between nodes 1–4 and 4–7 in the internode length genotypes of pea, *cry^s la Le Na*, *Cry* and/or *La Le Na*, and *cry^s la Le na*.

Line	Genotype	Phenotype	Mean length ± SE (cm) Nodes 1–4	Nodes 4–7	No.
186	*cry^s la Le Na*	Slender	11.8 ± 0.2	25.4 ± 0.3	19
187	*Cry* and/or *La Le Na*	Tall	7.7 ± 0.1	25.7 ± 0.3	23
188	*cry^s la Le na*	Slender	12.7 ± 0.2	26.4 ± 0.2	18

genotypes, [^{13}C,^{3}H]GA_{20} was converted to two major metabolites in the shoots: [^{13}C,^{3}H]GA_{29} and [^{13}C,^{3}H]GA_{29} catabolite (Fig. 1). Both metabolites, and the [^{13}C,^{3}H]GA_{20} in the *cry^s* plants, were strongly diluted by endogenous [^{12}C]GAs (Table 1). No evidence for GA_1 or GA_8 was obtained in either case. The production of significant levels of [^{13}C,^{3}H]GA_{29} catabolite in the shoots is similar to results obtained with the microcryptodwarf line 57 (genotype *cry^c la le lm Na*) but contrasts with feeds of [^{13}C,^{3}H]GA_{20} to lines carrying dominant alleles at the *Cry* and/or *La* loci in which the catabolite is a relatively minor metabolite in the shoots (Ingram et al. 1984).

To examine the influence of the *Le* and *na* genes on GAs in slender plants, the work was extended to the closely related lines 186, 187, and 188. The influence of the *cry^s la* gene combination on internode elongation was evident only in the first four internodes of the slender lines 186 and 188 (Table 2). At the stage at which [^{13}C,$^{3}H_2$]GA_{20} feeds were conducted (node 6 or 7), internode elongation was similar in all lines. The *cry^s la* gene combination was fully epistatic to the *na* gene in line 188 (see also Potts et al. 1985).

[^{13}C,^{3}H]Gibberellin A_{20} was metabolized similarly in all three lines to [^{13}C,^{3}H]-labeled GA_1, GA_8, and GA_{29} (Fig. 2). However, in these feeds no evidence was obtained for the presence of [^{13}C,^{3}H]GA_{29} catabolite. Examination of ^{12}C:^{13}C isotopic ratios in GA_{20} and its metabolites in line 188 showed little if

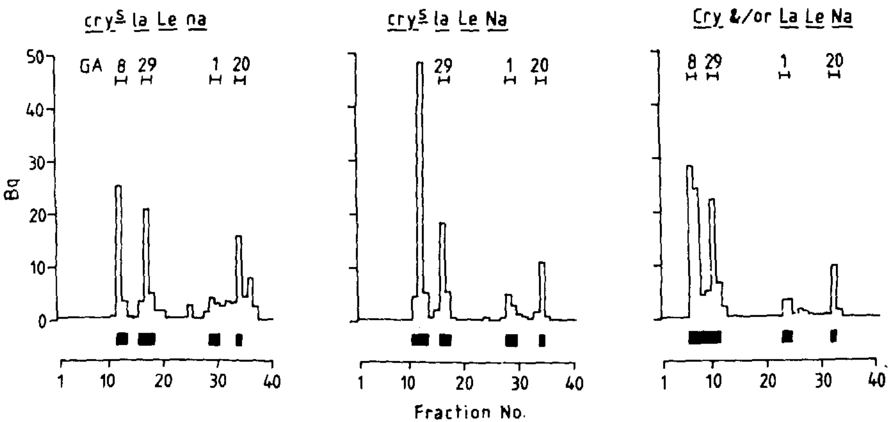

Fig. 2. [^{13}C,^{3}H]Gibberellin A_{20} metabolism in pea genotypes *cry^s la Le na* (line 188), *cry^s la Le Na* (line 186), *Cry* and/or *La Le Na* (line 187). See Fig. 1 for details. Radioactivity was determined in aliquots (2%) of each fraction. Barred fractions were combined and analyzed by GC-MS (see Table 3 and Fig. 3).

any dilution by endogenous [^{12}C]GAs (Fig. 3, Table 3), confirming that the *na* gene almost completely blocks the biosynthesis of GAs in the shoot (Ingram et al. 1984, Reid 1986). In contrast, the [^{13}C,^{3}H]GAs in lines 186 and 187 were markedly diluted by endogenous ^{12}C material.

Discussion

Strong evidence that the *le* and *na* mutations are biochemically expressed normally in *cry^s la* and *cry^c la* genotypes was presented by Potts et al. (1985). Full confirmation has now been obtained following feeds of [^{13}C,^{3}H]GA_{20} to a range of slender lines carrying dominant and recessive alleles at the *Le* and *Na* loci. The absence of the 3β-hydroxylated metabolites GA_1 and GA_8 in the *cry^c la le Na* (line 8) and *cry^s la le Na* genotypes shows that the *le* mutation, which reduces the conversion of GA_{20} to GA_1 (see Ingram et al. 1984, 1986), is fully expressed at the biochemical level in these lines. These compounds were prominent metabolites in *cry^s la Le Na* lines. Furthermore, little if any significant dilution of [^{13}C,^{3}H]GA_{20} or its metabolites by endogenous [^{12}C]GAs was evident in the *na* slender line 188. The *na* gene blocks an early step in GA biosynthesis between *ent*-kaurenoic acid and GA_{12}-aldehyde (Ingram and Reid 1987). However, despite these fundamental differences in GA biochemistry, no major *phenotypic* difference in internode length is evident between slender lines carrying dominant and recessive alleles at the *Le* and *Na* loci (De Haan 1930, Potts et al. 1985) (Table 2). The results therefore concur with previous reports that internode elongation in slender peas is entirely independent of GA levels (McComb and McComb 1970, Potts et al. 1985). The possible action of the *Cry* and *La* loci is discussed by Potts et al. (1985). Unlike the yg_6 mutation in tomato (Perez et al. 1974) and the *gigas* mutation in barley (Favret et al.

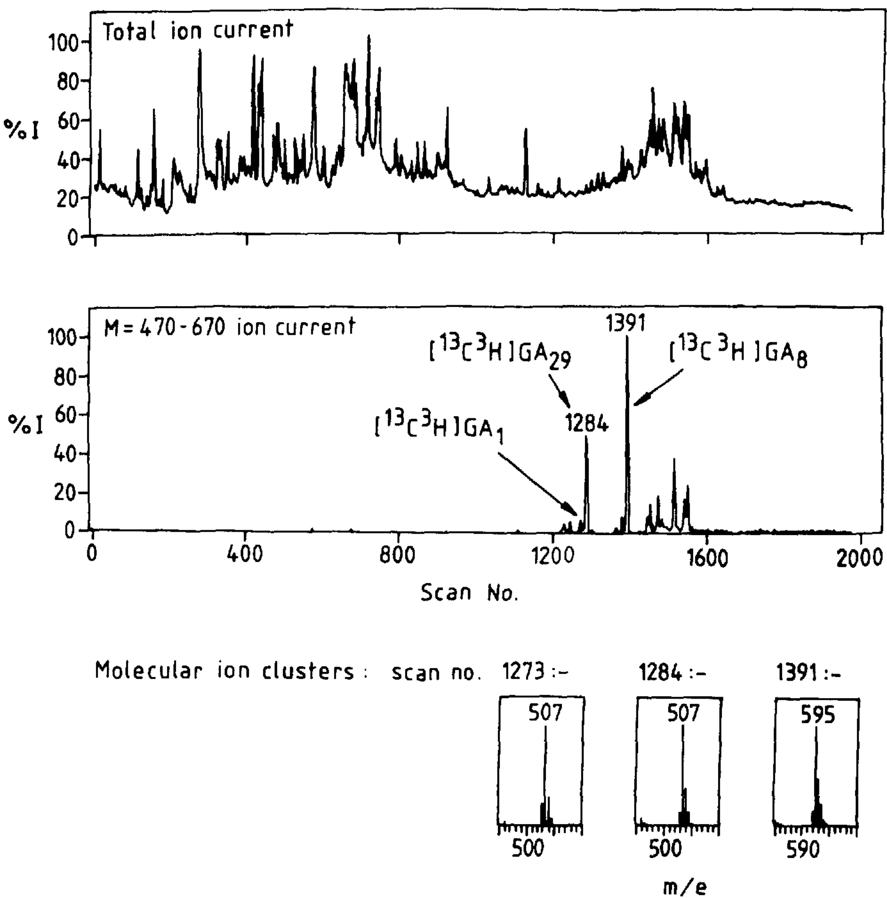

Fig. 3. Combined GC-MS of HPLC fractions cochromatographing with GA_1, GA_8, and GA_{29} from pea genotype *cry^s la le na* (see Fig. 2).

1975), which appear to cause overproduction of GAs, the slender mutant of peas is most simply explained on the basis of an effector/repressor system in which GA_1 acts as the effector and the product of the *Cry* and/or *La* gene acts as the repressor.

In the *cry^c la le Na* and *cry^s la le Na* genotypes, $[^{13}C,^{3}H]GA_{29}$ catabolite formed a significant metabolite of $[^{13}C,^{3}H]GA_{20}$ in the shoot. Similar results were obtained previously with the microcryptodwarf line 57 (genotype *cry^c la le lm Na*) (Ingram et al. 1984). The *lm* gene results in a general reduction in size and vigor of all parts of the shoot ("micro" phenotype), irrespective of the other internode length genes present (Lindquist 1951). These results contrast with *Cry* and/or *La* genotypes, in which the GA_{29} catabolite was a relatively minor metabolite of $[^{13}C,^{3}H]GA_{20}$ in the shoot (Ingram et al. 1984). However, in the present study the catabolite was not observed in *cry^s la Le Na* plants, suggesting that the above difference is not a direct consequence of the *cry^s la*

Table 3. [^{13}C,^{3}H]Gibberellin A_{20} application to internode length genotypes of pea, *cry^s la Le Na*, *Cry* and/or *La Le Na*, and *cry^s la Le na*. Incorporation of ^{13}C isotope and endogenous GA levels in the tissue expanded during the 6 days following treatment with substrate.

Metabolite	Percent isotopic composition[a]		Approx. level (ng plant^{-1})		HPLC fractions
	^{12}C	^{13}C	Endogenous	Exogenous[b]	
cry^s la Le Na (line 186)					
GA_1	54	46	9	10	(28–29)
GA_8	47	53	49	74	(11–13)
GA_{20}	54	46	16	17	(34)
GA_{29}	49	51	24	33	(16–17)
Cry and/or *La Le Na* (line 187)					
GA_1	57	43	6	6	(23–24)
GA_8	50	50	45	59	(6–8)
GA_{20}	67	33	18	11	(32)
GA_{29}	48	52	26	37	(9–11)
cry^s la Le na (line 188)					
GA_1	22	78	1	9	(29–30)
GA_8	12	88	0	36	(12–13)
GA_{20}	17	83	2	30	(34)
GA_{29}	12	88	0	37	(16–18)

[a] Substrate ^{13}C composition = 88%.
[b] Estimated from HPLC radioactivity profile (see Fig. 2).

gene combination. The results may relate to the ability of plants to utilize GA_{20}. However, their basis and significance are unclear at present.

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