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Brassinosteroid/Sterol Synthesis and Plant Growth as Affected by *lka* and *lkb* Mutations of Pea[1]

Takahito Nomura, Yukiko Kitasaka, Suguru Takatsuto, James B. Reid, Motohiro Fukami, and Takao Yokota*

Department of the Science of Plant and Animal Production, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan (T.N.); Department of Bioproductive Science, Utsunomiya University, Utsunomiya 320-8505, Japan (Y.K., M.F.); Department of Chemistry, Joetsu University of Education, Joetsu-shi, Niigata 943-8512, Japan (S.T.); Department of Plant Science, University of Tasmania, G.P.O. Box 252C, Hobart, Tasmania 7001, Australia (J.B.R.); and Department of Biosciences, Teikyo University, Utsunomiya 320-8551, Japan (Y.T.)

The dwarf pea (*Pisum sativum*) mutants *lka* and *lkb* are brassinosteroid (BR) insensitive and deficient, respectively. The dwarf phenotype of the *lkb* mutant was rescued to wild type by exogenous application of brassinolide and its biosynthetic precursors. Gas chromatography-mass spectrometry analysis of the endogenous sterols in this mutant revealed that it accumulates 24-methylenecholesterol and isofucosterol but is deficient in their hydrogenated products, campesterol and sitosterol. Feeding experiments using ^{2}H-labeled 24-methylenecholesterol indicated that the *lkb* mutant is unable to isomerize and/or reduce the $\Delta^{24(28)}$ double bond. Dwarfism of the *lkb* mutant is, therefore, due to BR deficiency caused by blocked synthesis of campesterol from 24-methylenecholesterol. The *lkb* mutation also disrupted sterol composition of the membranes, which, in contrast to those of the wild type, contained isofucosterol as the major sterol and lacked stigmasterol. The *lka* mutant was not BR deficient, because it accumulated castasterone. Like some gibberellin-insensitive dwarf mutants, overproduction of castasterone in the *lka* mutant may be ascribed to the lack of a feedback control mechanism due to impaired perception/signal transduction of BRs. The possibility that castasterone is a biologically active BR is discussed.

Steroid hormones play important roles in growth and development of various organisms. These include the sex hormones glucocorticoids and mineral corticoids in animals, the molting hormones ecdysteroids in insects and crustaceans, and an antheridiogen, antheridiol, in the microorganism *Achlya bisexualis*. Grove et al. (1979) reported the isolation of brassinolide as a plant growth-regulating steroid, and since then more than 40 analogs have been identified from various plant sources (Sakurai and Fujioka, 1993; Adam et al., 1996; Fujioka and Sakurai, 1997b). These steroids are known collectively as BRs (Mandava, 1988). Structurally, BRs are C_{27}, C_{28}, and C_{29} steroids with different substituents on the A- and B-rings and side chains (Fujioka and Sakurai, 1997b; Yokota, 1997). Brassinolide, the most biologically active BR, is a C_{28} steroid and, along with biosynthetically related compounds, it is distributed widely in the plant kingdom. Recently, brassinolide biosynthetic pathways have been elucidated by feeding ^{2}H-labeled intermediates to suspension cultures of *Catharanthus roseus* (for reviews, see Fujioka and Sakurai, 1997a; Yokota, 1997; Fig. 1). It has long been established that BRs elicit a variety of effects on the growth of higher plants (Mandava, 1988; Sasse, 1997). However, it was the recent discovery of dwarf mutants of Arabidopsis and garden pea (*Pisum sativum*) with BR biosynthesis and sensitivity lesions that provided compelling evidence that BRs are a prerequisite for cell elongation and hence have a defined hormonal role in the regulation of higher plant growth and development (for reviews, see Clouse, 1996, 1997; Yokota, 1997; Clouse and Sasse, 1998).

The roles of three genes in BR biosynthesis have been determined using dwarf mutants of Arabidopsis, *det2* (*detiolated 2*) (Li et al., 1996, 1997), *cpd* (*constitutive photomorphogenesis* and *dwarfism*) (Szekeres et al., 1996), and *dwf4* (*dwarf4*) (Azpiroz et al., 1998; Choe et al., 1998). It has been shown that the *dwf6* mutant (Choe et al., 1998) is an allele of *det2* and that *cpd*, *cbb3* (*cabbage3*) (Kauschmann et al., 1996), and *dwf3* (Choe et al., 1998) are all allelic. *DET2* encodes a steroid 5α-reductase that hydrogenates the (24*R*)-24-methylcholest-4-en-3-one intermediate involved in the conversion of campesterol to campestanol (Fujioka et al., 1997). *CPD* encodes a Cyt P450 enzyme, designated CYP90A1, that catalyzes C-23 hydroxylation (Szekeres et al., 1996), whereas *DWF4* encodes a Cyt P450 enzyme, CYP90B1, that catalyzes C-22 hydroxylation (Choe et al., 1998). In addition, there is evidence that the *dim* (*diminuto*) mutant of Arabidopsis (Takahashi et al., 1995; Klahre et al., 1998), which is an allele of *cbb1* (Kauschmann et al., 1996) and *dwf1* (Choe et al., 1998), and the *dwarf* mutant of tomato (Bishop et al., 1996, 1999), are involved in BR biosynthesis. Severe dwarfism is also observed in the Arabidopsis mutant *bri1* (*brassinosteroid-insensitive1*), the root growth of

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* Corresponding author; e-mail yokota@nasu.bio.teikyo-u.ac.jp; fax 81–28–627–7187.

Abbreviations: BMB, bismethaneboronate; BR, brassinosteroid; MB, monomethaneboronate; SIM, selected ion monitoring; TMSi, trimethylsilyl ether; WT, wild type.

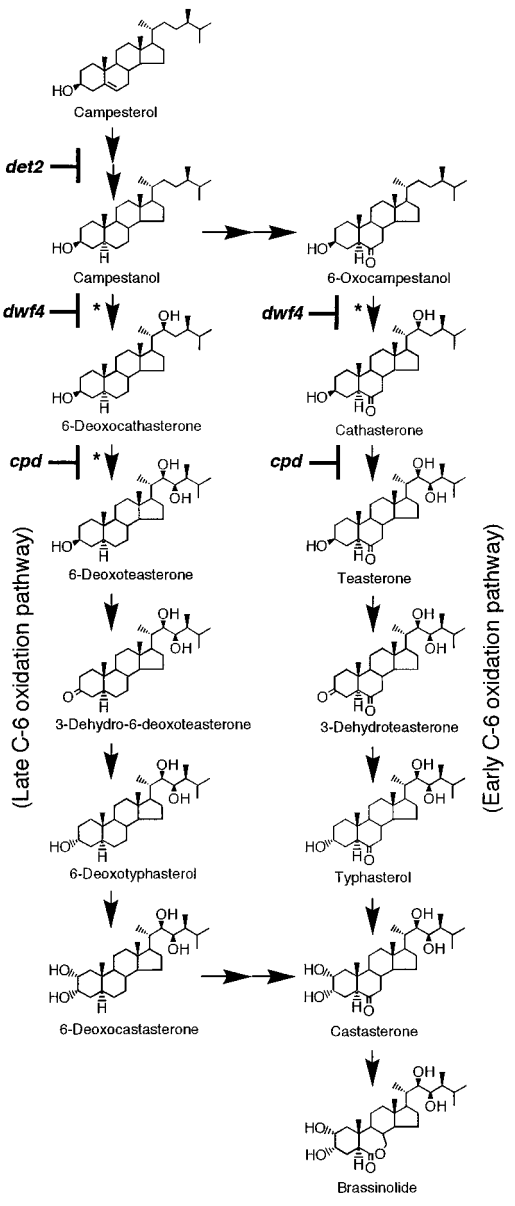

Figure 1. Proposed biosynthesis pathways for brassinolide from campesterol. Conversions marked with asterisks are hypothetical. Lesions in the BR biosynthesis of Arabidopsis mutants *det2*, *dwf4,* and *cpd* are shown.

which is not inhibited by exogenous BRs (Clouse et al., 1993, 1996). The gene *BRI1* was found to encode a putative Leu-rich repeat receptor kinase probably involved in BR signal transduction (Li and Chory, 1997).

In the case of pea, the stunted phenotypes of the *lka*, *lkb*, *lkc,* and *lk* mutants are not fully reversed by treatment with traditional plant hormones such as GA and auxin (Reid and Ross, 1989; Reid et al., 1991; Lawrence et al., 1992; Reid and Davies, 1992; McKay et al., 1994; Yang et al., 1996). It was reported that the dwarfism of the *lkb* mutant is caused by BR deficiency, whereas the *lka* mutant, which has a similar dwarf phenotype, was predicted to be a BR-response mutant (Nomura et al., 1997). Preliminary studies indicate that the *lk* mutant is also BR deficient (Y. Kitasaka, T. Nomura, S. Takatsuto, J.B. Reid, and T. Yokota, unpublished data). In the present work we further analyzed the biochemical lesions in the *lka* and *lkb* mutants by GC-MS analysis of endogenous BRs and their probable precursor sterols, by feeding experiments with isotopically labeled intermediates, and by investigating the effects of different BRs on the growth of the two pea mutants.

MATERIALS AND METHODS

Plant Materials

The pure lines of garden pea (*Pisum sativum*) used in this study were cv Torsdag (WT, tall) and the two single-gene mutant lines derived from this cultivar mutagenized with ethyl methanesulfonate, NGB5865 (*lka*) and NGB5862 (*lkb*), which are held in the University of Tasmania collection at Hobart (Reid and Ross, 1989). Shoots (49 d old; Table I) used for analysis of the endogenous BRs and sterols were sown on October 27, 1997, and grown in a greenhouse under a natural photoperiod. Other plant seedlings used in this study were grown in a growth cabinet, as described by Nomura et al. (1997).

Authentic BRs and Sterols

Brassinolide, castasterone, typhasterol, and teasterone were kindly supplied by Dr. M. Aburatani (Fuji Chemical Industries, Takaoka, Japan). We synthesized the remaining BRs. Campesterol was obtained from Tama Chemicals (Tokyo, Japan). Campestanol was obtained by catalytic hydrogenation (10% palladium on charcoal) of campesterol. 24-Methylenecholesterol was supplied by Dr. T. Akihisa (Nihon University, Japan). Isofucosterol was extracted from bean seeds (Kim et al., 1988). Sitosterol, sitostanol, stigmasterol, and cholesterol were obtained from Fluka, Sigma, Tokyo Kasei (Tokyo, Japan), and Kanto Chemical (Tokyo, Japan), respectively.

^{2}H Standards

26,27-$^2H_6$ labeling of brassinolide, castasterone, typhasterol, and teasterone were reported by Takatsuto and Ikekawa (1986). [$^2H_6$]3-Dehydroteasterone was synthesized as reported by Yokota et al. (1994), [$^2H_6$]6-deoxocastasterone (melting point, 238°C), [$^2H_6$]6-deoxotyphasterol (melting point, 224°C), and [$^2H_6$]6-deoxoteasterone (melting point, 227°C) were synthesized from [$^2H_6$]castasterone, [$^2H_6$]typhasterol, and [$^2H_6$]teasterone, respectively, according to the method of Mori et al. (1984). [$^2H_6$]3-Dehydro-6-deoxoteasterone (melting point, 167°C) was synthesized from [$^2H_6$]6-deoxoteasterone as described by Yokota et al. (1994). [26,27-$^2H_6$]Campestanol was supplied by Dr. S. Fujioka (RIKEN, Saitama, Japan). [25,26,27-$^2H_7$]-24-Methylenecholesterol, [26,27-$^2H_6$]24-methyldesmosterol, and [26,27-$^2H_6$]campesterol were synthesized by the procedures of Takatsuto et al. (1998).

GC-SIM

GC-SIM, in the electron impact mode (70 eV), was carried out on a JMS AX 505 instrument (JEOL) fitted with

Table I. *Growth data for 49-d-old pea shoots*

These plant materials were used for analyses of endogenous BRs (Table II) and sterols (Table III).

Genotype	No. of Plants	Plant Height[a]	No. of Expanded Leaves per Shoot[a]	Total Wt	Averaged Wt per Shoot
		cm			*g*
WT	190	28.9 ± 0.9	7.80 ± 0.1	435	2.29
lka	192	20.0 ± 0.3	7.04 ± 0.1	395	2.06
lkb	218	19.8 ± 0.3	7.48 ± 0.1	481	2.21

[a] Data are means ± SE of 25 plants randomly selected.

either a DB-5 or DB-1 column (0.25 mm × 15 m; 0.25-mm film thickness; J & W Scientific, Folsom, CA). The carrier gas was He at a flow rate of 1 mL min^{-1}, the injection port temperature was 260°C, and the samples were introduced by splitless injection. The column oven temperature was programmed to 170°C for 1.5 min, increased to 280°C at 37°C min^{-1}, and then increased to 300°C at 1.5°C min^{-1}.

Effects of Brassinolide, Its Precursors, and Sterols on Growth

Brassinolide precursors in 5 μL of ethanol containing 0.15% Tween 20 were applied to the fourth internode when the third leaf was almost fully expanded about 8 d after planting in a growth cabinet. Sterols were mixed with a fractionate lanolin (Mitchell and Livingston, 1968), and the lanolin paste was applied to the fourth internode. Control seedlings were treated with the solvent only. After 3 d the lengths of the fourth and fifth internodes were measured.

Effects of Brassinolide on Sterol Content

The procedure of brassinolide application was the same as described above, except that the surface of the expanding third leaf was treated with 100 ng of brassinolide dissolved in 10 μL of the solvent. After 2 d the third leaves were removed, and the apical portions (each with 20 shoots) were harvested by cutting at the third node and used for sterol analysis.

Metabolism of ^{2}H-Sterols

Seeds of the *lkb* mutant were surface-sterilized with sodium hypochlorite solution (0.5% active chlorine) and were placed in 100-mL conical flasks (one seed per flask) containing 15 mL of Murashige and Skoog medium (JRH Biosciences, Lenexa, KS). The seeds were grown for 6 d at 25°C under a 16-h light and 8-h dark regime on a reciprocal shaker (60 rpm); then ethanol solutions (40 μL) of [$^2H_7$]24-methylenecholesterol (40 μg) or [$^2H_6$]24-methyldesmosterol (40 μg) were added to the conical flasks and incubated for an additional 3 d under the same conditions. Roots and shoots were separated and subjected to sterol analysis.

Preparation of Microsomal and Soluble Fractions

All operations were done at 4°C. Ten fresh segments (approximately 5 g) excised at the sixth node from 23-d-old seedlings were homogenized by a Polytron (Kinematica, Littau/Luzern) with 20 mL of 0.1 M Tris-HCl buffer, pH 7.8, containing 0.5 M Suc and 1 mM EDTA, and the homogenate was filtered through four layers of gauze. The filtrate was centrifuged at 6,000*g* for 15 min, and the supernatant was then centrifuged at 100,000*g* for 90 min according to the methods described by Gachotte et al. (1995). The pellet and supernatant obtained by 100,000*g* centrifugation, which represent microsomal membrane and soluble cellular fractions, respectively, were subjected to sterol analysis.

Extraction and Purification of BRs from 49-d-Old Seedlings

The methanol extracts of 49-d-old shoots excised at soil levels (Table I) were spiked with $^2H_6$-labeled internal standards, 0.3 μg of [$^2H_6$]brassinolide, 0.5 μg each of [$^2H_6$]castasterone and [$^2H_6$]typhasterol, and 1 μg each of [$^2H_6$]3-dehydroteasterone, [$^2H_6$]6-deoxocastasterone, [$^2H_6$]6-deoxotyphasterol, [$^2H_6$]3-dehydro-6-deoxoteasterone, and [$^2H_6$]6-deoxoteasterone before reduction to an aqueous residue. The aqueous residue was partitioned against chloroform. The chloroform phase was washed with 0.5 M K_2HPO_4 buffer, pH 9.0, evaporated to dryness, and partitioned between hexane and 80% methanol. The hexane and 80% methanol phases were used for analysis of sterols and BRs, respectively.

The 80% methanol fraction was evaporated to dryness and the residual solid was purified on a column of silica gel (7 g; Wakogel C-300, Wako Pure Chemicals, Osaka, Japan) eluted with chloroform containing 0%, 0.5%, 1%, 2.5%, 5%, 7%, 10%, 20%, and 50% methanol (first, chloroform was washed with water to remove ethanol used for stabilizer, dried, and distilled). Biological activity in the fractions was assayed by the rice lamina inclination test (Yokota et al., 1996). Eluates obtained with 1% to 7% methanol in chloroform were combined, dissolved in 60% methanol, and loaded onto a column of charcoal (chromatography grade, 5 g; Wako Pure Chemicals), then eluted with methanol:water (6:4 and 8:2, v/v), methanol, and methanol:chloroform (9:1, 5:5, 3:7, and 1:9, v/v). The methanol:chloroform (5:5 and 3:7, v/v) fractions were combined and chromatographed on a column of Sephadex LH-20 (bed volume, 500 mL; Pharmacia) using methanol:chloroform (4:1, v/v) as a mobile phase. Successive 10-mL fractions were collected. Fractions 33 to 39 were combined and purified on a column of diethylaminopropyl silica Bondesil (0.2 g; Varian, Palo Alto, CA) using methanol as the mobile phase. The eluate was subjected to reversed-phase HPLC on a Pak

ODS-3251-D column (8 × 250 mm; Senshu Science, Tokyo, Japan) eluted with the following acetonitrile-water gradient: 0 to 20 min, 45% acetonitrile; 20 to 40 min, 45% to 100% acetonitrile; and 40 to 50 min, 100% acetonitrile, at a flow rate of 2.5 mL min^{-1}. Fractions were collected every 1 min. The column oven temperature was maintained at 40°C. The following fractions were collected for analysis by GC-SIM: fractions 14 and 15 (brassinolide), 20 to 22 (castasterone), 30 to 33 (teasterone), 35 to 37 (typhasterol and 3-dehydroteasterone), 38 to 40 (6-deoxocastasterone), and 43 to 47 (6-deoxoteasterone, 3-dehydro-6-deoxoteasterone, and 6-deoxotyphasterol).

Quantitative Analysis of BRs

Random aliquots of extracts derived from pooled plant materials were analyzed by GC-SIM in duplicates. BRs were converted to either MBs or BMBs with pyridine that contains methaneboronic acid (2 mg mL^{-1}) at 70°C for 30 min. Typhasterol, teasterone, 6-deoxotyphasterol, and 6-deoxoteasterone were further trimethylsilylated to yield MB-TMSi derivatives. The $^2H_0/^2H_6$ ions monitored were m/z 528/534 (M^+), 374/374 and 155/161 for brassinolide BMB, m/z 512/518 (M^+), 358/358 and 155/161 for castasterone BMB, m/z 544/550 (M^+), 529/535 and 515/521 for typhasterol MB-TMSi and teasterone MB-TMSi, m/z 470/476 (M^+), 316/316 and 155/161 for 3-dehydroteasterone MB, m/z 498/504 (M^+), 273/273 and 155/161 for 6-deoxocastasterone BMB, m/z 530/536 (M^+), 440/446 and 215/215 for 6-deoxotyphasterol MB-TMSi and 6-deoxoteasterone MB-TMSi, and m/z 456/462 (M^+), 231/231, and 155/161 for 3-dehydro-6-deoxoteasterone MB. The contents of BRs were calculated from the peak area ratios of $^2H_0$ and $^2H_6$ M^+ ions.

Analysis of Sterols

Random aliquots of extracts derived from pooled plant materials were analyzed by GC-SIM in duplicates. Plant tissues were extracted with methanol:chloroform (4:1, v/v), whereas the microsomal membrane pellet was extracted with methanol:dichloromethane (1:2, v/v). In the case of 49-d-old seedlings, the hexane-soluble phase was obtained as described above for sterol analysis. The extracts were partitioned between ethyl acetate and 0.5 M K_2HPO_4 buffer, pH 9.0, and the organic phases were used for sterol analysis. Sterol extracts equivalent to 100 mg fresh weight of tissue were spiked with 1 μg of [$^2H_6$]campestanol as an internal standard and saponified with 1 N sodium hydroxide in methanol at 80°C for 1.5 h. The hydrolysate was partitioned between chloroform and water. The chloroform phase was evaporated to dryness, redissolved in chloroform, and then passed through a short silica gel (Wakogel C-300) column. The eluate was trimethylsilylated at room temperature and subjected to GC-SIM. The levels of sterols were determined using calibration curves constructed from the ratios of the M^+ peak area of [$^2H_6$]campestanol TMSi (m/z 480) to those of cholesterol TMSi (m/z 458), 24-methylenecholesterol TMSi (m/z 470), campesterol/24-epicampesterol TMSi (m/z 472), campestanol/24-epicampestanol TMSi (m/z 474), stigmasterol TMSi (m/z 484), sitosterol TMSi (m/z 486), sitostanol TMSi (m/z 488), and isofucosterol TMSi (m/z 484). Campesterol and 24-epicampesterol (22-dihydrobrassicasterol), as well as campestanol/24-epicampestanol, were analyzed as a mixture because they were not resolved by GC.

RESULTS

Levels of BRs in *lkb* Plants Are Reduced, Whereas Those in *lka* Plants Are Not

Nomura et al. (1997) demonstrated that 36-d-old *lkb* plants contain significantly lower levels of brassinolide, castasterone, and 6-deoxocastasterone than WT plants. To locate the defective biosynthetic step, the levels of the endogenous BRs situated in the early sections of the biosynthetic pathway were analyzed in extracts from shoots of 49-d-old *lkb* plants by GC-SIM. BRs (Table II) were rigorously identified because the relative intensities of the M^+ ion and two daughter ions for each molecule (see "Materials and Methods") were consistent with those of the corresponding ^2H internal standard (data not shown). The contents of BRs, which were calculated from the peak area ratios of $^2H_0$ and $^2H_6$ M^+ ions, are shown in Table II. In the case of 6-oxo-BRs, the levels of castasterone and typhasterol were 23% and 15%, respectively, of the WT levels. The castasterone level was significantly greater than that for the 36-d-old plants, according to previously published results (Nomura et al., 1997). In keeping with this, the size of 49-d-old plants relative to the WT (Table I) was greater than that previously described for 36-d-old plants. It seems that environmental and/or age differences modified the levels of BRs, thereby affecting plant growth. There was no substantial difference in the levels of the earlier precursors, 3-dehydroteasterone and teasterone. Brassinolide was not present at the detectable levels in any genotype. As for 6-deoxo-BRs, the levels of 6-deoxocastasterone and 6-deoxotyphasterol were 7% and 29%, respectively, of the WT levels, whereas no large reduction was observed in the levels of the earlier precursors, 3-dehydro-6-deoxoteasterone and 6-deoxoteasterone (Table II).

In keeping with the data of Nomura et al. (1997), the BR content of *lka* plants was not altered greatly compared with WT seedlings, except for an accumulation of castasterone (Table II).

Brassinolide and Its Biosynthetic Precursor BRs Rescue the Dwarf Phenotype of *lkb* Plants

Previously, Nomura et al. (1997) applied 6-oxo-BRs, including brassinolide, castasterone, typhasterol, 3-dehydroteasterone, and teasterone to the third leaf of *lkb* plants, and this resulted in increased elongation of the fourth and fifth internodes. 6-Deoxocastasterone, however, was inactive. In the current study, the fourth internodes of 8-d-old seedlings were treated with BRs to minimize transportation effects, and under these conditions all of the 6-oxo-BRs and 6-deoxo-BRs exhibited activity when applied in doses of 1 μg and less (Fig. 2). Overall, it is apparent that the biological activities of the BRs tend to

Table II. *Endogenous levels of BRs in 49-d-old shoots of WT, lka, and lkb of pea*

The levels of BRs were determined by GC-SIM using ^{2}H internal standards. Data are means ± SE of duplicate determinations.

BR	BR Level		
	WT	*lka*	*lkb*
	ng kg^{-1} fresh wt		
Brassinolide	ND[a]	ND	ND
Castasterone	491 ± 0.3	3005 ± 2.5	112 ± 0.2
Typhasterol	26 ± 0.1	39 ± 1.5	4 ± 0.0
3-Dehydroteasterone	20 ± 1.4	21 ± 1.9	15 ± 0.4
Teasterone	5 ± 0.1	5 ± 0.5	4 ± 0.2
6-Deoxocastasterone	2937 ± 22	4053 ± 73	208 ± 7.8
6-Deoxotyphasterol	835 ± 2.1	983 ± 17	245 ± 13
3-Dehydro-6-deoxoteasterone	182 ± 1.9	339 ± 4.2	101 ± 4.1
6-Deoxoteasterone	180 ± 2.8	197 ± 1.1	176 ± 2.9

[a] ND, Not detected.

increase with their proximity to brassinolide in the biosynthetic pathway, as reported by Yokota and Mori (1992) and Fujioka et al. (1995). Campesterol and campestanol failed to promote the internode elongation when applied at high doses in a lanolin paste (Fig. 2). This may reflect low-efficiency conversion to brassinolide and/or poor uptake by the plant tissues.

Sterol Content of the Shoots and Seeds Is Drastically Altered in *lkb*, but Not in *lka*

The levels of sterols in the shoots of 49-d-old plants used for BR analysis and mature seeds of WT, *lka*, and *lkb* were analyzed quantitatively by GC-MS, and the data obtained are shown in Table III. All of the sterols listed were rigorously identified by full-scan MS.

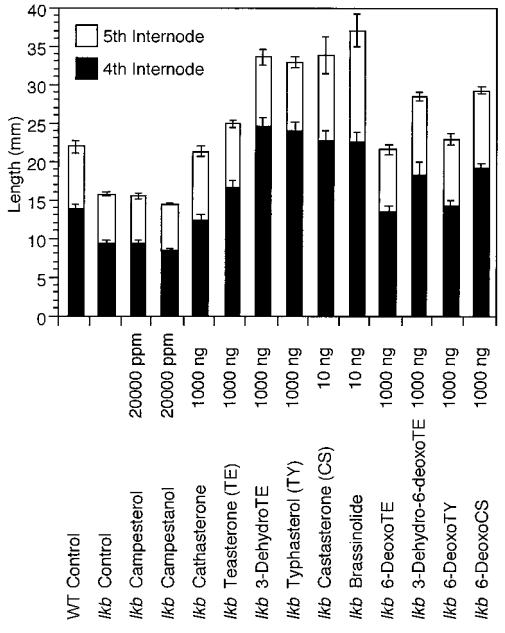

Figure 2. Effects of brassinolide and its biosynthetic precursors on the internode length of *lkb* plants of pea. The lengths of the fourth and fifth internodes were measured 3 d after the fourth internodes were treated. Results are means ± SE (n = 5). Campesterol and campestanol were applied in lanolin paste.

In shoots of WT plants the end-pathway sterols, sitosterol, stigmasterol, campesterol/24-epicampesterol, and cholesterol, account for 87% of the total sterol content, as is the case in most plant species (Nes, 1977). Sitosterol, stigmasterol, and campesterol/24-epicampesterol were the major sterols present (Table III). The levels of 24-methylenecholesterol and isofucosterol were much lower than those of their hydrogenated products, campesterol and sitosterol, respectively. Campestanol/24-epicampestanol and sitostanol were also detected in low levels. The WT and *lka* seedlings had very similar sterol contents. In contrast to the WT and *lka* mutant, the *lkb* plants contained isofucosterol and 24-methylenecholesterol as the major sterols, whereas the levels of the end-pathway sterols, campestanol/24-epicampestanol and sitostanol, were extremely low. It is interesting that the level of cholesterol in the *lkb* shoots was not altered.

The total sterol content of mature seeds of the WT, *lka*, and *lkb* were 4- to 5-fold higher than the levels in the corresponding shoots, although the spectrum of sterols present in the seeds was very similar to that found in shoots (Table III). Thus, in seeds of the WT and *lka* mutant end-pathway sterols occupied about 97% of the total sterol pool, whereas in *lkb* seeds isofucosterol and 24-methylenecholesterol accounted for 98% of the total sterol content.

Conversion of [25,26,27-$^2H_7$]24-Methylenecholesterol to [26,27-$^2H_6$]Campesterol Is Blocked in the *lkb* Mutant

Since campesterol has been reported to be synthesized from 24-methylenecholesterol via 24-methyldesmosterol (Fig. 3; Goodwin, 1985; Benveniste, 1986), [25,26,27-$^2H_7$]24-methylenecholesterol was added to the media in which 6-d-old WT and *lkb* seedlings had been aseptically grown. After 3 d data were obtained by analyzing roots but not by analyzing shoots: It appears that sterols absorbed by the roots that were submerged in the medium did not move to the shoots. The roots of the WT plants were found to contain similar levels (0.5 μg g^{-1} fresh weight) of [25,26,27-$^2H_7$]24-methylenecholesterol and [26,27-$^2H_6$]campesterol,

Table III. *Endogenous levels of sterols in 49-d-old shoots and mature seeds of WT, lka, and lkb of pea*

The levels of sterols were determined by GC-SIM using [$^2H_6$]campestanol as an internal standard. Data are means ± SE of duplicate determinations.

Sterol	Sterol Level					
	WT		*lka*		*lkb*	
	Shoot	Seed	Shoot	Seed	Shoot	Seed
			μg g^{-1} fresh wt			
24-Methylenecholesterol	0.7 ± 0.0	ND[a]	0.5 ± 0.0	ND	17.5 ± 0.2	14.5 ± 0.4
Campesterol/24-epicampesterol	21.1 ± 0.1	85.8 ± 1.0	25.0 ± 0.4	88.2 ± 2.0	2.3 ± 0.0	6.0 ± 0.0
Campestanol/24-epicampestanol	1.3 ± 0.0	5.3 ± 0.1	1.4 ± 0.0	5.7 ± 0.0	0.1 ± 0.0	0.5 ± 0.0
Isofucosterol	20.3 ± 0.2	6.8 ± 0.1	16.5 ± 0.3	6.1 ± 0.1	252.5 ± 2.0	1514.5 ± 20
Sitosterol	122.4 ± 0.8	872.0 ± 17	143.5 ± 0.8	943.2 ± 39	11.7 ± 0.1	21.7 ± 0.2
Stigmasterol	46.9 ± 0.2	42.0 ± 1.1	57.5 ± 0.4	60.4 ± 2.1	0.6 ± 0.0	4.4 ± 0.0
Sitostanol	5.8 ± 0.0	15.9 ± 0.3	6.3 ± 0.0	15.7 ± 0.1	ND	ND
Cholesterol	1.4 ± 0.0	1.4 ± 0.0	1.2 ± 0.0	2.2 ± 0.0	2.0 ± 0.0	1.9 ± 0.0
Total sterols	219.9	1029.2	251.9	1121.5	286.7	1563.5
End-pathway sterols[b]	191.8	1001.2	227.2	1094.0	16.6	34.0

[a] ND, Not detected. [b] Campesterol plus 24-epicampesterol plus sitosterol plus stigmasterol plus cholesterol.

but no [26,27-$^2H_6$]24-methyldesmosterol was present (Fig. 4). In contrast, the roots of the *lkb* plants contained [25,26,27-$^2H_7$]24-methylenecholesterol (0.5 μg g^{-1} fresh weight), but [26,27-$^2H_6$]campesterol and [26,27-$^2H_6$]24-methyldesmosterol were not detected. Unexpectedly, after feeding [26,27-$^2H_6$]24-methyldesmosterol to the WT and *lkb* tissues, neither this compound nor its expected metabolite, [26,27-$^2H_6$]campesterol, was present in root extracts. One of the possible reasons for this result is that [26,27--$^2H_6$]24-methyldesmosterol might be rapidly metabolized to other products before reaching the proper reaction site.

Brassinolide-Treated *lkb* Plants Had Lower Levels of Endogenous Sterols

The effect of exogenous brassinolide on the levels of endogenous sterols was investigated because it was suspected that the abnormal sterol content of the *lkb* plants might be normalized by exogenous brassinolide. WT seedlings (8 d old) were treated with 100 ng of brassinolide. After 2 d elongation was enhanced, and this was accompanied by an increase in the fresh weight of the shoot. The brassinolide treatment also increased the total sterol con-

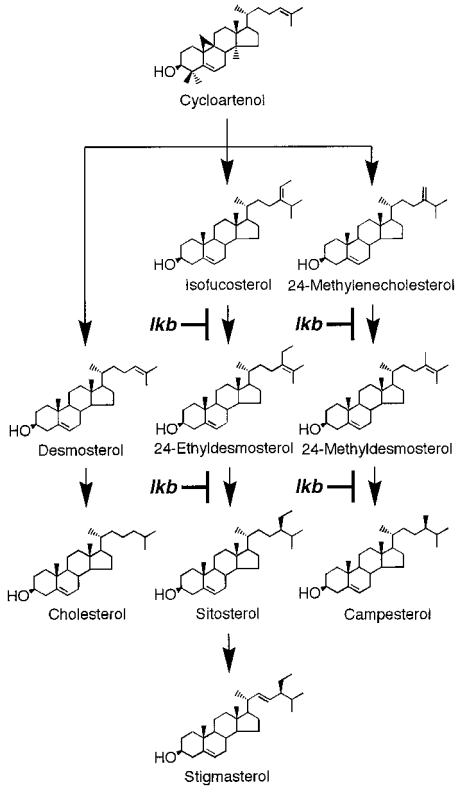

Figure 3. The sterol biosynthesis pathways. Lesions in the BR biosynthesis of the *lkb* mutant of pea are shown.

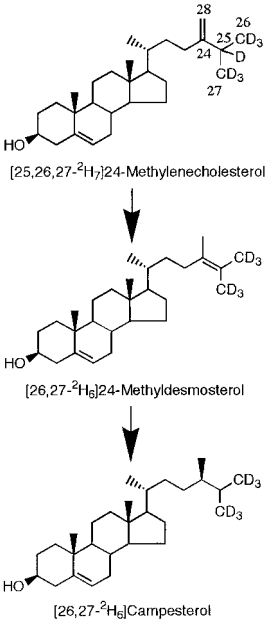

Figure 4. Proposed synthetic pathway from [25,26,27-$^2H_7$]24-methylenecholesterol to [26,27-$^2H_6$] campesterol.

tent per shoot in proportion to the growth increase, although when expressed on a fresh-weight basis this effect was not evident (Table IV). A similar result was obtained in *lka* plants (Table IV).

Treatment of 8-d-old *lkb* seedlings with 100 ng of brassinolide induced an elevated rate of internode elongation that was accompanied by an increase in the fresh weight of the shoot. The endogenous sterol levels of *lkb*, however, tended to decrease (Table IV), with the sterol content on a fresh-weight basis decreasing to 55% and the total sterol content per shoot segment decreasing to 78%.

The *lkb* Mutant Has an Altered Sterol Composition in the Membrane

A microsomal membrane fraction and a soluble fraction were obtained from 23-d-old WT, *lka,* and *lkb* shoots by centrifugation at 100,000*g*. The microsomal fractions sedimented at 100,000*g* are composed of a mixture of vesicles originating from various membrane types (Hartmann and Benveniste, 1987). Sterol compositions in these fractions and whole shoots are shown in Table V. The microsomal membranes of WT contained sitosterol and stigmasterol as the major sterols, whereas those of the *lkb* mutant contained isofucosterol and sitosterol as the principal components. In the WT membranes the level of sitosterol was increased 1.4-fold compared with that of the whole shoots. In the *lkb* membrane the levels of sitosterol and campesterol/24-epicampesterol were elevated 25- and 4-fold, respectively, compared with those of the whole shoots. The levels of cholesterol in the microsomal fraction, soluble fraction, and whole shoots of the *lkb* mutant were nearly comparable to those of the WT. Surprisingly, the sterol compositions of the WT and *lkb* soluble fractions were indistinguishable. Sterol compositions in all samples obtained from *lka* seedlings closely resembled those of the WT.

DISCUSSION

BR Biosynthesis Is Retarded in the *lkb* Mutant

In the biosynthesis of brassinolide, campesterol is first converted to campestanol, which is metabolized to castasterone via either the early or late C-6 oxidation pathway, after which castasterone is converted to brassinolide (Fig. 1). The analysis of endogenous BRs in the present study indicated the presence of components of both the early and late C-6 oxidation pathways in pea (Choi et al., 1997; Table II). Low levels of castasterone, typhasterol, 6-deoxocastasterone, and 6-deoxotyphasterol in shoots of 49-d-old *lkb* plants indicate that both pathways are affected. However, the *lkb* plants had levels of 3-dehydroteasterone and teasterone in the early C-6 oxidation pathway, as well as of 3-dehydro-6-deoxoteasterone and 6-deoxoteasterone in the late C-6 oxidation pathway, comparable to those of the WT. It is possible to speculate that the reduction of the 3-oxo group to 3β-hydroxyl function may be blocked in the *lkb* mutant. However, this possibility is unlikely because both 3-dehydroteasterone and 3-dehydro-6-deoxoteasterone are biologically active (Fig. 2), probably via the conversion to typhasterol and 6-deoxotyphasterol, respectively (Fig. 1). All of the BRs examined could counteract the dwarfism of the *lkb* mutant (Fig. 2), suggesting a blockage at a very early step in BR biosynthesis, possibly even as far back as the sterol biosynthesis pathway.

BR Deficiency in the *lkb* Mutant Is Due to Impaired Synthesis of Campesterol from 24-Methylenecholesterol

Sterol compositions of 10-, 23-, and 49-d-old *lkb* shoots, which are summarized in Tables IV, V, and III, respectively, as well as those of mature seeds (Table III), were almost comparable. Such sterol profiles indicate lesions in the re-

Table IV. *Effects of brassinolide on the growth and sterol levels of WT, lka, and lkb shoots of pea*

Third leaves of 8-d-old seedlings ($n = 20$) were treated with brassinolide. After 2 d growth data and sterol levels were determined. The levels of sterols were determined by GC-SIM using [$^2H_6$]campestanol as an internal standard. Sterol levels are expressed as means ± SE of duplicate determinations.

Growth and Level	WT		*lka*		*lkb*	
	Control	Brassinolide (100 ng)	Control	Brassinolide (100 ng)	Control	Brassinolide (100 ng)
Profile						
Fourth internode length (mm ± SE)	11.2 ± 0.4	17.2 ± 0.6	7.7 ± 0.2	11.5 ± 0.4	8.7 ± 0.2	21.4 ± 0.7
Averaged weight per apical segment (g)	0.18	0.22	0.19	0.22	0.19	0.27
Sterol (μg g^{-1} fresh wt)						
24-Methylenecholesterol	1.2 ± 0.0	1.4 ± 0.0	1.5 ± 0.0	1.9 ± 0.1	43.7 ± 0.4	32.1 ± 0.3
Campesterol/24-epicampesterol	38.3 ± 0.2	39.0 ± 0.1	49.9 ± 0.1	46.0 ± 0.3	2.0 ± 0.1	1.3 ± 0.0
Campestanol/24-epicampestanol	0.9 ± 0.0	0.8 ± 0.0	1.3 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Isofucosterol	32.2 ± 0.3	31.1 ± 0.1	21.4 ± 0.0	26.8 ± 0.3	311.8 ± 8.0	161.1 ± 2.5
Sitosterol	100.5 ± 0.4	100.2 ± 2.2	131.7 ± 1.4	121.1 ± 0.3	9.5 ± 0.0	6.5 ± 0.1
Stigmasterol	132.0 ± 0.0	132.5 ± 1.3	144.9 ± 0.3	137.8 ± 1.1	0.8 ± 0.0	0.5 ± 0.0
Sitostanol	3.3 ± 0.0	3.2 ± 0.0	4.7 ± 0.2	4.4 ± 0.0	ND[a]	ND
Cholesterol	7.2 ± 0.1	8.4 ± 0.1	16.9 ± 0.2	13.9 ± 0.1	7.7 ± 0.0	3.4 ± 0.0
Total sterols (μg g^{-1} fresh wt)	315.6	316.6	372.3	353.3	375.6	205.0
Total sterols (μg per segment)	56.9	69.6	70.8	77.7	71.4	55.5

[a] ND, Not detected.

Table V. *Subcellular distribution of sterols in 23-d-old WT, lka, and lkb seedlings of pea*

Sterols were quantified by GC-SIM using [$^2H_6$]campestanol as an internal standard. Data determined in duplicates were averaged and used to calculate sterol compositions.

Sterol	Sterol Composition								
	Microsomal fraction			Soluble fraction			Whole shoot		
	WT	*lka*	*lkb*	WT	*lka*	*lkb*	WT	*lka*	*lkb*
	% wt of total sterols								
24-Methylenecholesterol	ND[a]	ND	3	ND	ND	ND	ND	ND	9
Campesterol/24-epicampesterol	9	10	4	11	11	11	11	12	1
Campestanol/24-epicampestanol	1	1	0.5	1	5	2	0.5	0.5	0.1
Isofucosterol	8	7	64	ND	ND	2	14	10	88
Sitosterol	52	52	25	73	74	73	38	38	1
Stigmasterol	24	25	0.5	1	1	2	34	37	0.4
Sitostanol	3	3	1	1	1	1	2	2	ND
Cholesterol	3	2	2	13	8	9	0.5	0.5	0.5

[a] ND, Not detected.

ductive conversion of 24-methylenecholesterol to campesterol and of isofucosterol to sitosterol in shoots and seeds of the *lkb* mutant. The inability to metabolize [25,26,27-$^2H_7$]24-methylenecholesterol to [26,27-$^2H_6$]campesterol was confirmed using 6-d-old *lkb* seedlings. This conversion occurred in WT tissues with a loss of the ^2H at C-25, indicating that the $\Delta^{24(28)}$ double bond in 24-methylenecholesterol is first isomerized to a $\Delta^{24(25)}$ double bond, yielding 24-methyldesmosterol, which, in turn, is hydrogenated to campesterol (Fig. 4), as was postulated by Goodwin (1985) and Benveniste (1986). Thus, it is possible that the LKB protein has a dual role in that it catalyzes both isomerization and reduction of the $\Delta^{24(28)}$ double bond in the hydrogenation of 24-methylenecholesterol and isofucosterol. However, we do not exclude the possibility that the *LKB* gene product may act as a regulator influencing gene expression or enzyme activity of one or more sterol isomerase(s)/reductase(s) or that the *LKB* gene may encode either an isomerase or a reductase that act together in a tightly coupled fashion. Nonetheless, we conclude that the dwarfism of the *lkb* mutant is due to BR deficiency caused by blocked conversion of 24-methylenecholesterol to campesterol. Recently, Klahre et al. (1998) found that the dwarf *dim* mutant of Arabidopsis has the same defect as the *lkb* mutant.

[26,27-$^2H_6$]24-Methyldesmosterol could not be detected after feeding [25,26,27-$^2H_7$]24-methylenecholesterol to WT and *lkb* tissues. Furthermore, 24-methyldesmosterol, as well as 24-ethyldesmosterol, which is an intermediate between isofucosterol and sitosterol, could not be detected as endogenous components in either intact shoots or seeds of WT and *lkb*. This suggests that 24-methyldesmosterol, as well as 24-ethyldesmosterol, is synthesized and further metabolized without being released from the surface of the enzyme(s).

24-Methylenecholesterol has been regarded as a putative substrate for BRs having a 24-methylene group, e.g. in seeds of *Lablab purpurea* (L.) Sweet (formerly *Dolichos lablab* L.; Yokota et al., 1984; Yokota, 1997). However, the stunted phenotype of the *lkb* and *dim* mutants indicates that such a pathway is not operative in either pea or Arabidopsis.

Hydrogenation of the $\Delta^{24(25)}$ double bond has been suggested to occur in the reductive synthesis of cholesterol from desmosterol (Fig. 3) in plants (Grunwald, 1975; Goodwin, 1985) and animals (Rilling and Chayet, 1985). The *LKB* gene may not be involved in this conversion because the *lkb* mutant and WT contained similar levels of cholesterol in their shoots, seeds, and microsomal membranes (Tables III and V). Some plant species, such as tomato (Yokota et al., 1997) and *Ornithopus sativus* (Spengler et al., 1995), contain, in addition to C_{28} BRs derived from campesterol, C_{27} BRs having no alkyl substituent at C-24, which are probably synthesized from cholesterol (Yokota, 1997). Thus, such plants may not show a clear dwarf phenotype, even when the gene orthologous to *LKB* is impaired, because they can probably synthesize C_{27} BRs, which may compensate for the loss of campesterol-derived BRs.

Abnormal Sterol Compositions in the *lkb* Mutant May Damage Functions of Membrane

In an attempt to examine the effect of brassinolide on the sterol composition, we found that sterol synthesis was retarded in 10-d-old *lkb* seedlings treated with brassinolide, despite the dwarf phenotype being counteracted with internode elongation being promoted strongly (Table IV). The suppression of sterol synthesis suggests that membranes of the *lkb* mutant may have an unusual sterol composition, which may, in turn, affect adversely the activity of membrane-bound enzymes (Nes, 1977; Benveniste, 1986; Hartmann, 1998), including those involved in sterol biosynthesis. The membranes of 23-d-old *lkb* shoots were most unusual in that they contain isofucosterol as the major sterol (64% in weight), in contrast to the WT and *lka* membranes in which sitosterol is the major component (52% in weight). Surprisingly, the sitosterol content in the *lkb* membranes increased to 25%, although sitosterol in the whole shoot accounts for only 1% of the total sterols (Table V). A similar increase was also observed in the campesterol/24-epicampesterol content. It is well known that sterols affect membrane fluidity (Hartmann, 1998). Sitosterol and campesterol/24-epicampesterol have been found to be the

most efficient sterols for restricting the mobility of fatty acyl chains in soybean phosphatidylcholine bilayers and appear to be very active in reducing the water permeability of the soybean membranes (Schuler et al., 1991). Thus, the partial recovery in the sitosterol and campesterol/24-epicampesterol level in the *lkb* membranes could be due to homeostatic control restoring sterol composition and hence membrane functions. The finding that the soluble fraction of the *lkb* mutant has the same composition as WT also may be due to homeostatic control, although the role of sterols in the soluble fraction seems to be undefined (Gachotte et al., 1995).

A further defect of the *lkb* membranes is the loss of stigmasterol (Table V). In celery cells growth inhibition caused by a sterol biosynthetis inhibitor was restored by either stigmasterol or mixtures of cholesterol and a low concentration of stigmasterol (Goad, 1990). Recently, it was found that stigmasterol and cholesterol stimulate proton pumping in H^+-ATPase of maize, whereas sitosterol and 24-methylcholesterol act as inhibitors (Grandmougin-Ferjani et al., 1997). These findings indicate that stigmasterol has important functions in the metabolism and regulation of plants.

Thus, whereas circumstantial evidence suggests that the changes in sterol composition of the *lkb* membrane may slow biochemical reactions of certain membrane-bound enzymes (Table IV), the phenotypic similarity of the *lka* and *lkb* mutants suggests that the phenotype of both plants is controlled only by their similarly perceived BR levels rather than endogenous sterol levels.

The Level of Castasterone May Be Controlled by a Feedback Mechanism

Although the *lka* mutant has a phenotype similar to the *lkb* mutant, it is not BR deficient (Table II) and its dwarfism is not counteracted by exogenous brassinolide (Nomura et al., 1997). Furthermore, the sterol compositions of the *lka* mutant are almost identical with those of the WT (Tables III–V). Such evidence suggests that the *lka* mutant has a defect in the perception or signal transduction pathway of BRs.

In the dwarf but GA-insensitive mutants *Rht3* (wheat), *D8* (maize), and *gai* (Arabidopsis), the levels of GA_1 are elevated substantially. It was suggested that GA action results in the production of a transcriptional repressor that limits the expression of GA biosynthetic enzymes. Mutants with an impaired response to GA would lack this repressor and have an elevated rate of GA production (Hedden and Kamiya, 1997). In 49-d-old shoots of the *lka* mutant a large accumulation of castasterone was observed. However, no quantitative information was obtained for brassinolide because its levels in both the *lka* mutant and WT plants were below detectable limits. However, it has been demonstrated that the level of brassinolide in the 36-d-old seedlings of the *lka* mutant is not elevated (Nomura et al., 1997). These findings indicate that, at least in pea shoots, synthesis of castasterone rather than brassinolide may be controlled by a feedback mechanism similar to that proposed for GA_1. Recently, it was demonstrated that transcription of the Arabidopsis *CPD* gene is controlled in this manner by BR (Mathur et al., 1998). Brassinolide is the most biologically active BR, and hence, the conversion of castasterone to brassinolide is deemed an activation step. However, such conversion has not been observed in bioassay systems in which castasterone is biologically active (Yokota, 1997). All of the evidence currently available indicates that castasterone, in addition to brassinolide, may be a biologically active BR.

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