Brassinosteroid Deficiency Due to Truncated Steroid 5α-Reductase Causes Dwarfism in the *lk* Mutant of Pea[1]

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The endogenous brassinosteroids in the dwarf mutant *lk* of pea (*Pisum sativum*) were quantified by gas chromatography-selected ion monitoring. The levels of castasterone, 6-deoxocastasterone, and 6-deoxotyphasterol in *lk* shoots were reduced 4-, 70-, and 6-fold, respectively, compared with those of the wild type. The fact that the application of brassinolide restored the growth of the mutant indicated that the dwarf mutant *lk* is brassinosteroid deficient. Gas chromatography-selected ion monitoring analysis of the endogenous sterols in *lk* shoots revealed that the levels of campestanol and sitostanol were reduced 160- and 10-fold, respectively, compared with those of wild-type plants. These data, along with metabolic studies, showed that the *lk* mutant has a defect in the conversion of campest-4-en-3-one to 5α-campestan-3-one, which is a key hydrogenation step in the synthesis of campestanol from campesterol. This defect is the same as that found in the Arabidopsis *det2* mutant and the *Ipomoea nil kbt* mutant. The pea gene homologous to the *DET2* gene, *PsDET2*, was cloned, and it was found that the *lk* mutation would result in a putative truncated PsDET2 protein. Thus it was concluded that the short stature of the *lk* mutant is due to a defect in the steroidal 5α-reductase gene. This defect was also observed in the callus induced from the *lk* mutant. Biosynthetic pathways involved in the conversion of campesterol to campestanol are discussed in detail.

Brassinosteroids (BRs) are steroidal plant growth hormones that are synthesized from plant sterols. The major biosynthetic pathway leading to brassinolide and castasterone, which are deemed biologically active BRs, has been established (Fujioka and Yokota, 2003). BRs are involved in diverse physiological processes, such as stem elongation, tracheary element differentiation, root inhibition, phytohormone synthesis and response, cold and drought stress responses, and regulation of gene expression (Clouse and Sasse, 1998; Li and Chory, 1999; Khripach et al., 2000; Bishop and Yokota, 2001; Bishop and Koncz, 2002; Clouse, 2002). The crucial role played by BRs in plant growth and development has been determined by the identification and characterization of BR mutants in Arabidopsis, pea (*Pisum sativum*), tomato (*Lycopersicon esculentum*), and rice (*Oryza sativa*). To date, the majority of research has been carried out on the Arabidopsis BR biosynthetic mutants, *det2* (Li et al., 1996, 1997; Fujioka et al., 1997; Noguchi et al., 1999b), *dwf4* (Azpiroz et al., 1998; Choe et al., 1998), *cpd* (Szekeres et al., 1996), *dwf5* (Choe et al., 2000), *dwf7* (Choe et al., 1999b), *sax1* (Ephritikhine et al., 1999), *dwf1/dim* (Klahre et al., 1998; Choe et al., 1999a; Takahashi et al., 1995), and *fackel* (Jang et al., 2000), as well as the BR perception and signal transduction mutants, *bri1* (Clouse et al., 1996; Li and Chory, 1997; Noguchi et al., 1999a), *bak1* (Nam and Li, 2002), *brs1* (Li et al., 2001), *bin2* (Choe et al., 2002; Li and Nam, 2002; Perez-Perez et al., 2002), *brz1* (Wang et al., 2002), and *bes1* (Yin et al., 2002). Furthermore, several mutants were also characterized from pea, tomato, and rice. The BR biosynthetic mutants include pea *lkb* (Nomura et al., 1997), tomato *dwarf* (Bishop et al., 1999) and *dumpy*

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(Koka et al., 2000), and rice *brd*s (Hong et al., 2002; Mori et al., 2002) and *d2* (Hong et al., 2003). Mutants that are insensitive to and have defects in perception of BRs include pea *lka* (Nomura et al., 1997, 2003), tomato *cu3* and *abs* (Montoya et al., 2002), and rice *d61* (Yamamuro et al., 2000).

The *lka* and *lkb* mutants of pea show common dwarf phenotypes referred to as erectoides, which are not rescued by GA (Reid and Ross, 1989). The *lka* mutant is defective in the BR receptor kinase PsBRI1 (Nomura et al., 2003). The *lkb* mutant has a defect in the 24-hydrogenase that converts 24-methylenecholesterol to campesterol (Nomura et al., 1999; Schultz et al., 2001). In addition, the *lkc* and *lk* mutants of pea have been classified as erectoides. The *lkc* mutant shows weak dwarfism (Reid et al., 1991) and is slightly insensitive to both BR and GA (T. Yokota, unpublished data), although its genetic lesion has not been clarified. The pea mutant *lk* is the most severe dwarf and is characterized by short internodes, reduced yield, dark green foliage, brittle stems with increased diameter, very short peduncles and petioles, and increased apical dominance (Fig. 1; Reid, 1986). Interestingly, the gene *lk* appears to be largely epistatic to genes involved in the GA response (e.g. *la cry*^{s} slenders; Reid, 1986). In this article, we investigate the effect of BRs on the growth of the *lk* mutant and also analyze the endogenous BRs and sterols, resulting in the conclusion that the *lk* mutant is BR deficient because of impaired sterol biosynthesis. Feeding experiments using synthetic sterol substrates and molecular analyses were conducted to pinpoint the defect in the *lk* mutant.

RESULTS

The Dwarf Phenotype of the *lk* Mutant Was Rescued by Treatment with Brassinolide

Treatment of the fourth internode with as little as 1 ng of brassinolide could restore its growth to that of wild-type plants (Fig. 2). The effect of brassinolide leveled off at a dosage of 10 ng. Such an effective recovery of the dwarfism, together with the earlier finding that changes in GA level or response may not be related to the *lk* mutation (Lawrence et al., 1992), suggests that the *lk* mutant is BR deficient. At a dosage of 33 ng, the fourth internode became thick and twisted, with petioles being epinastic.

The Levels of BRs Are Reduced in *lk* Seedlings

The endogenous levels of castasterone, 6-deoxocastasterone, and 6-deoxotyphasterol in 12-d-old shoots of *lk* seedlings were 4-, 70-, and 6-fold lower than those of wild-type plants, respectively (Table I), indicating that the *lk* mutant has a blockage in BR biosynthesis.

The *lk* Mutant Has a Defect in the Hydrogenation of Campesterol and Sitosterol

In *lk* shoots, campesterol, sitosterol, stigmasterol, and cholesterol, which are bulk sterols considered to be end-pathway sterols (Fig. 3), are present at comparable levels to those of wild-type plants (Table II). However, the level of campestanol, a hydrogenation product of campesterol, was two orders of magnitude lower compared with wild-type plants. Because campestanol is not separable from its 24-epimer (dihydrobrassicasterol), the quantitative data given may express the sum of campestanol and its 24-epimer. Furthermore, the level of sitostanol was also reduced, although to a lesser extent as compared with campestanol (Table II). Thus, the *lk* mutant seemed to have a defect in the hydrogenation of campesterol to campestanol and of sitosterol to sitostanol. It has been demonstrated that, in Arabidopsis, the synthesis of campestanol from campesterol proceeds via three intermediates, campest-4-en-3β-ol, campest-4-en-3-one, and 5α-campestan-3-one (Fig. 4). Therefore, we analyzed these steroids in the shoots of wild-type and *lk* seedlings, revealing that *lk* seedlings contained

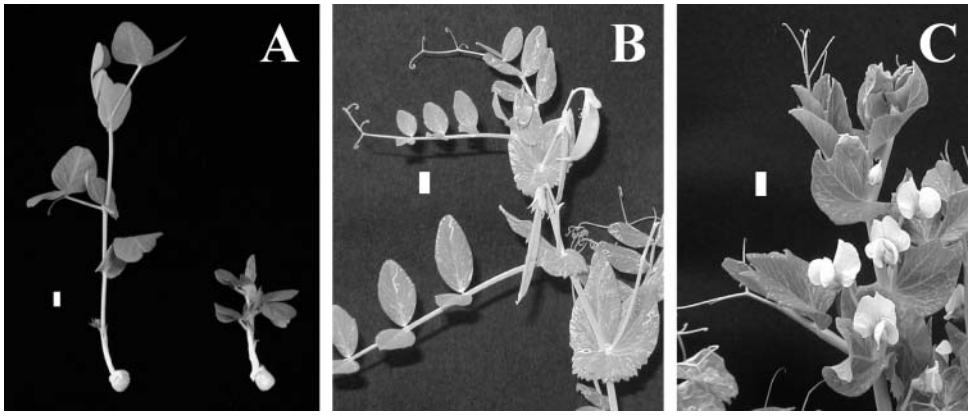

Figure 1. Morphology of the *lk* mutant and wild type. A, 21-d-old seedlings of *LK* (212+; left) and *lk* (212−; right). B, Adult plant of *LK*. C, Adult plant of *lk*. Bar represents 1 cm.

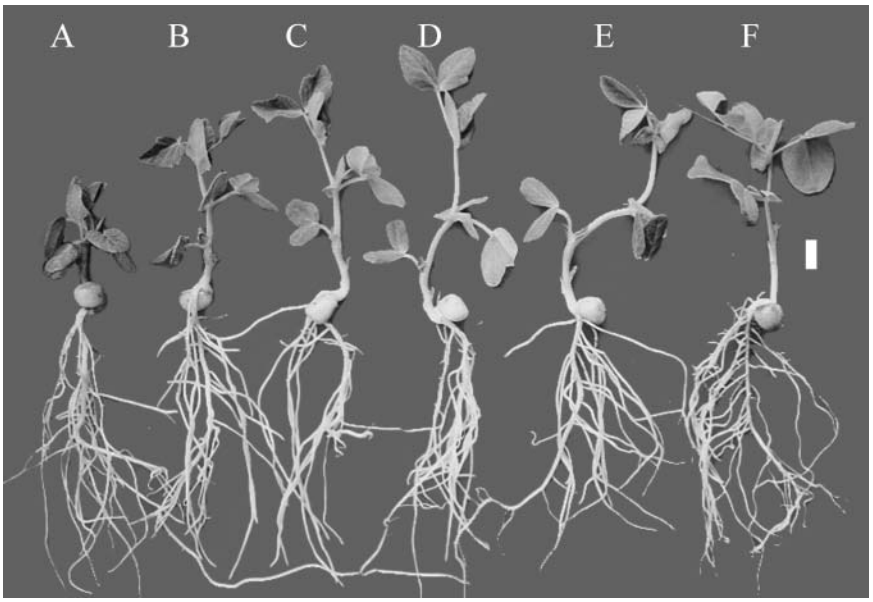

Figure 2. Effect of brassinolide on the growth of *lk* seedlings. Growth profiles were recorded 3 d after the fourth internodes of 8-d-old *lk* seedlings were treated with solvent only (A), 1 ng brassinolide (B), 3.3 ng brassinolide (C), 10 ng brassinolide (D), and 33 ng of brassinolide (E). F, Wild-type seedling treated with solvent only. Bar represents 1 cm.

higher levels of campest-4-en-3β-ol and campest-4-en-3-one, but a lower level of 5α-campestan-3-one compared with wild-type seedlings. These findings suggest that the *lk* mutation results in a loss of 5α-reductase activity.

Calli induced from shoots of *lk* and wild-type seedlings were also examined for the levels of the endogenous sterols (Table II). It was found that the level of campestanol and sitostanol is reduced in the *lk* callus, indicating that the effects of the *lk* mutation were also observable in callus derived from *lk* shoots (Table II).

Steroid 5α-Reductase Activity Is Lost in the *lk* Mutant

To ascertain the reaction lacking in the *lk* mutant, campesterol and the intermediates between campesterol and campestanol were labeled by deuteriums in the side chain (Noguchi et al., 1999b) and fed to apical tissues of *lk* and wild-type plants. In wild-type plants, all of these [$^2H_6$]compounds were converted to [$^2H_6$]campestanol (Table III), indicating the biosynthetic sequence of campesterol → campst-4-en-3β-ol → campest-4-en-3-one → 5α-campestan-3-one → 5α-campestanol is present in garden pea. The fact that [$^2H_6$]4-en-3β-ol and/or [$^2H_6$]3-one were not detected as intermediates in any feeding experiments (Table III) indicates that the lifetimes of 4-en-3β-ol and 3-one are very short.

In *lk* explants, [$^2H_6$]campesterol and [$^2H_6$]4-en-3β-ol were metabolized to [$^2H_6$]4-en-3-one. [$^2H_6$]4-En-3-one was not further metabolized, while [$^2H_6$]3-one was converted to [$^2H_6$]campestanol (Table III). These results clearly show that the *lk* mutation prohibits the conversion of 4-en-3-one to 3-one. Thus the *LK* gene should encode a steroid 5α-reductase, suggesting that it is homologous to the Arabidopsis *DET2* gene (Li et al., 1996).

Table I. *Endogenous levels of BRs in 12-d-old shoots of the pea mutant lk and wild-type plants*

Data (ng kg^{-1} fresh weight) are means of two GC-SIM runs.

BR	Wild Type	*lk*
Brassinolide	nd[a]	nd
Castasterone	446	114
Typhasterol	nd	nd
3-Dehyroteasterone	trace	42
Teasterone	nd	nd
6-Deoxocastasterone	4,302	59
6-Deoxotyphasterol	926	160
6-Deoxo-3-dehydroteasterone	nd	nd
6-Deoxoteasterone	33	180

[a]nd, Not detected, or no reliable data were obtained due to impurities.

The *lk* Mutation Is Due to the Truncation of the Pea DET2 Homolog

The pea steroid 5α-reductase gene homologous to the Arabidopsis *DET2* gene was obtained from wild-type line JI813 and showed 58% identity and 76% similarity with DET2. Primers based on this full-length pea *DET2* sequence were used to clone sequences in the wild-type lines HL107 and 212+, and in the 212− (*lk*) mutant. A mutation in *lk* was found at base 437, where guanine was changed to adenine, which corresponded to an amino acid change from Trp to a stop codon at amino acid position 146 (Fig. 5), resulting in a truncated protein. Loss of function of the LK protein

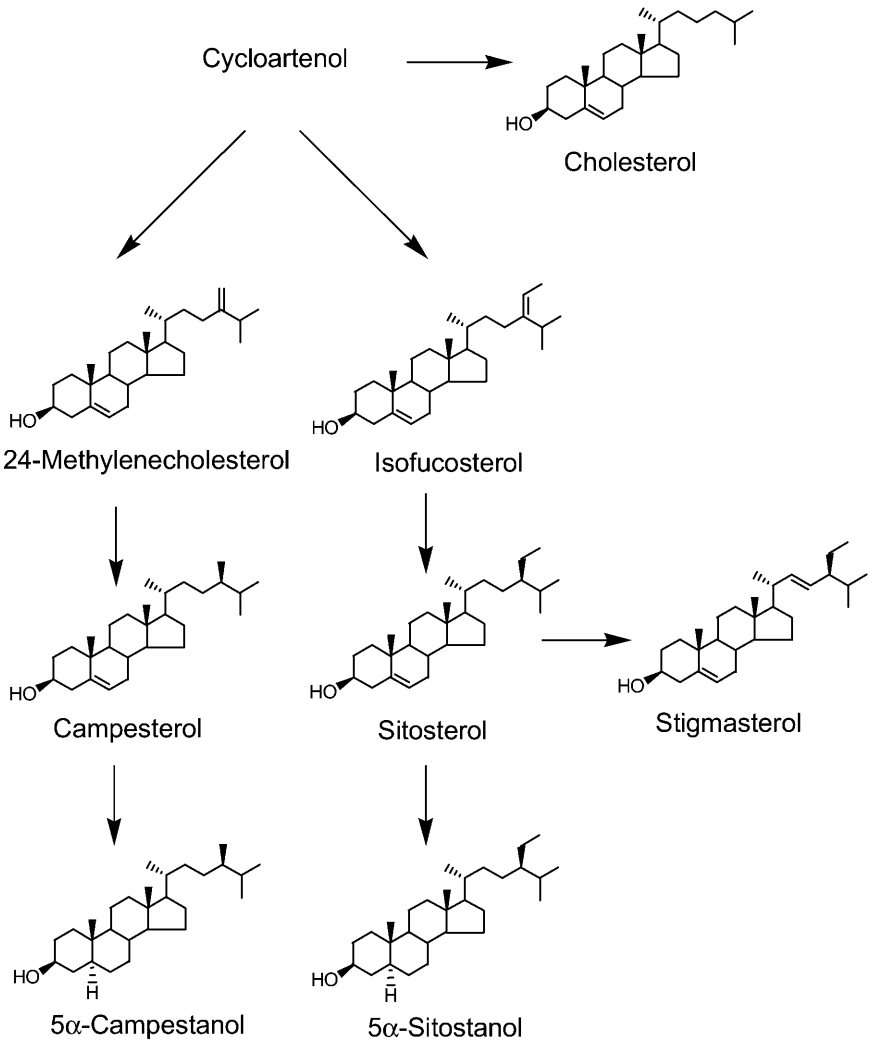

Figure 3. Biosynthetic pathway of plant sterols.

ultimately leads to an early blockage in BR biosynthesis, resulting in a deficiency of the biologically active BRs, thereby explaining the extreme phenotype of the *lk* mutant. The sequence from JI813 differed from the HL107 (and therefore also 212+) sequence at two positions, amino acids 190 and 213. The A-G change in both cases led to a K-E amino acid change that would not be predicted to alter the protein function.

DISCUSSION

This article demonstrates that the pea *LK* gene encodes a steroid 5α-reductase and that a lesion in the *LK* gene causes BR deficiency, resulting in growth suppression. The *det2* mutant of Arabidopsis also has a defect in a steroid 5α-reductase (Li et al., 1996), and recently the *kbt* mutant of *Ipomoea nil* has been shown to have the same defect (Suzuki et al., 2003). The *LK* gene showed 60% identity and 74% similarity with DET2, and 59% identity and 77% similarity with *InDET2*, suggesting that the 5α-reductase gene is well conserved in the plant kingdom. The *lk* mutant is distinct from *det2* and *kbt* in that its leaf blade is not as rugose and curly (Fig. 1). Furthermore, the de-etiolation characteristics of Arabidopsis and tomato mutants are not seen in *lk* (Symons et al., 2002). The *lk* mutation was found to result in a stop codon located close to the midpoint of the gene (Fig. 5). Animal steroid 5α-reductases are membrane bound and require NADPH as the sole cofactor. The human steroid 5α-reductase isozyme-2 has steroid-binding sites at both the N and C termini, as well as NADPH-binding sites in the last half of the protein (Russell and Wilson, 1994; Jin and Penning, 2001). It thus seems that the *lk* mutation depletes the C-terminal steroid-binding site and the NADPH-binding sites. Furthermore, the *lk* mutant protein also may not possess catalytic domains that have been predicted to reside near the C terminus of the rat liver steroid 5α-reductase 1 (Wang et al., 1999). Therefore, 5α-reductase activity is predicted to be seriously reduced in the *lk* mutant.

Table II. *Endogenous levels of sterols in 17-d-old shoots and calli of the pea mutant lk and wild-type plants*
Data are expressed as $\mu g\ g^{-1}$ fresh weight.

Sterol	Shoot		Callus	
	Wild Type	*lk*	Wild Type	*lk*
24-Methylenecholesterol	nd[b]	nd	2.4	nd
Campesterol	30.6	22.8	16.7	20.9
Campest-4-en-3β-ol	nd	0.46	na[c]	na
Campest-4-en-3-one	0.52	1.0	na	na
5α-Campestan-3-one	0.012	0.004	na	na
5α-Campestanol	1.6	0.01	0.7	0.04
Isofucosterol	8.9	5.8	16.7	7.0
Sitosterol	73.2	84.5	41.1	67.4
Stigmasterol	102.0	105.9	17.6	24.5
5α-Sitostanol	3.3	0.3	2.0	0.6
Cholesterol	1.8	2.2	6.0	9.8
Total sterols	221.4	221.5	103.2	130.2
End-pathway sterols[a]	207.6	215.4	81.4	122.6

[a]End-pathway sterols, cholesterol+campesterol+sitosterol+stigmasterol. [b]nd, Not detected. [c]na, Not analyzed.

In both light-grown shoots and dark-grown calli of wild-type plants, the level of campestanol is approximately 5% that of campesterol (Table II), indicating that action of the *LK* gene is not affected by whether the tissue is differentiated or exposed to light (shoots were grown in light and calli in dark). In contrast, the relative amount of stigmasterol to sitosterol is much reduced in callus (Table II), suggesting that the dehydrogenase activity responsible for the conversion of sitosterol to stigmasterol is suppressed in callus. We recently found that the *LK* gene transcript levels are not significantly changed during seed growth and seed germination of pea (T. Nomura and T. Yokota, unpublished data). Expression of the *DET2* gene has been shown to be ubiquitous and constitutive in Arabidopsis seedlings (Li and Chory, 1999; Bancos et al., 2002) and not to be affected by exogenous brassinolide in contrast to P450 genes involved in BR biosynthesis (Mathur et al., 1998; Goda et al., 2002; Hong et al., 2002; Montoya et al., 2002). Altogether, it seems that the 5α-reductase genes are expressed at a steady state throughout the whole plant.

Although *lk* plants have a serious mutation, they still contain campestanol as well as considerable levels of BRs (Tables I and II), suggesting the presence of a partially complementary gene(s). Consistent with this, there also seems to be an alternative steroid 5α-reductase in Arabidopsis (Li and Chory, 1999) and *I. nil* (Suzuki et al., 2003). The *DET2* gene embedded in kidney 293 cells 5α-reduced testosterone, progesterone, and androstenedione (Li et al., 1997). 5α-Reduction of progesterone has also been demonstrated to occur in several plants (Stohs and El-Olemy, 1972; Lin and Heftmann, 1981; Wendroth and Seitz, 1990). So, the *DET2* gene seems to 5α-reduce both C_{27-29} steroids and $C_{19/21}$ steroids. However, steroid 5α-reductases of *Solanum malacoxylon* reduced campest-4-en-3-one and progesterone, but not testosterone and androstenedione (Rosati et al., 2003). Humans and rats have two steroid hormone 5α-reductases, isozymes type 1 and type 2 (Russell and Wilson, 1994; Jin and Penning, 2001). Shefer et al. (1966) demonstrated that, in rat liver, cholest-4-en-3-one was catalyzed by an enzyme different from steroid hormone 5α-reductases. Altogether, we suspect that 5α-reduction of $C_{19/21}$ steroids is catalyzed by an enzyme other than DET2, and this enzyme may partially replace the action of DET2.

This article demonstrates that campest-4-en-3-one, the substrate of LK, is synthesized from campesterol (Fig. 4). Various plants have the same type of enzymatic activity that converts pregnenolone to progesterone (Bennett and Heftmann, 1965; Sauer et al., 1967; Capsi and Hornby, 1968; Stohs and El-Olemy, 1972) and sitosterol to sitost-4-en-3-one (Stohs and El-Olemy, 1971). Furthermore, we found that 4-en-3β-ol is the intermediate of this reaction in pea as observed in Arabidopsis (Noguchi et al., 1999b). However, this intermediate has been investigated only in these two plants. The pathway of 5-en-3β-ol → 5-en-3-one → 4-en-3-one has been proposed for $C_{19/21}$ steroids in bacteria (Talalay and Wang, 1955), mammals (Luu-The et al., 1991), and plants (Seidel et al., 1990; Stuhlemmer and Kreis, 1996; Kreis et al., 1998; compare with Fig. 4). This two-step pathway is catalyzed by 3β-hydroxylsteroid dehydrogenase (3βHSD)/Δ^5-Δ^4 isomerase (Δ^5-3βHSD). Mammalian Δ^5-3βHSDs, members of the aldo-keto-reductase (AKR) superfamily, are membrane bound and require NAD^+ (Rhéaume et al., 1991; Simard et al., 1991). In contrast, Δ^5-3βHSDs of *Digitalis lanata* (Finsterbusch et al., 1999) and *Pseudomonas testosteroni* (Yin et al., 1991) are soluble enzymes belonging to the short-chain dehydrogenase/reductase family. Interestingly, *Pseudomonas* Δ^5-3βHSD was

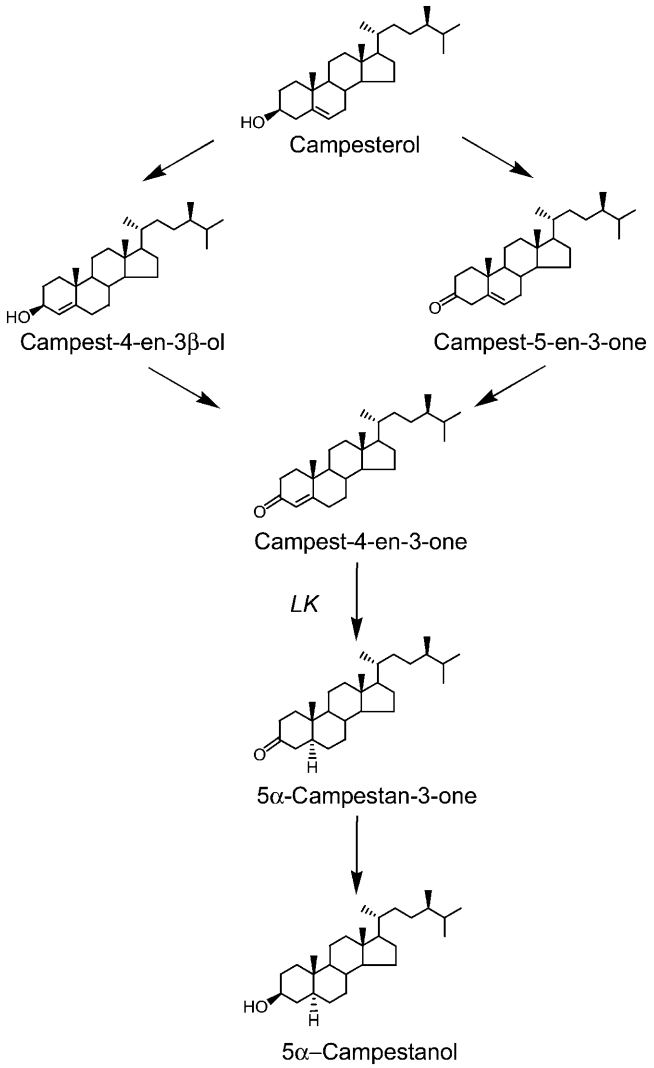

Figure 4. Biosynthetic pathway from campesterol to campestanol.

demonstrated to have no isomerase activity (Talalay and Wang, 1955; Kawahara et al., 1962). We found that the Arabidopsis genome contains three genes homologous to *D. lanata* Δ^5-3βHSD, with 75% similarity, and four genes similar to human Δ^5-3βHSDs, with approximately 50% similarity, indicating the possible presence of 4-en-3-one as the intermediate in plants. It is intriguing to investigate whether Δ^5-3βHSDs catalyze the 5-en-3-one pathway or 4-en-3β-ol pathway or both. Furthermore, it will be interesting to investigate if the substrates of Δ^5-3βHSDs are $C_{19/21}$ steroids or $C_{27–29}$ steroids or both. In rat, hepatic C_{27} steroid Δ^5-3βHSDs are involved in the synthesis of bile acids and cholestanol, but these enzymes were demonstrated to be different from one another and also distinct from $C_{19/21}$ steroid Δ^5-3βHSDs (Björkhem et al., 1972; Björkhem and Karlmar, 1974). The dwarf mutant *sax1* of Arabidopsis was predicted not to convert 22-hydroxycampesterol to 22-hydroxycampest-4-en-3-one resulting in BR deficiency (Ephritikhine et al., 1999), suggesting that *SAX1*, which has not yet been cloned, may play a key role in clarifying the function of plant Δ^5-3βHSDs. On the other hand, *Streptomyces* spp. and Brevibacterium are known to produce flavoproteins that catalyze the conversion of exogenous cholesterol to cholest-4-en-3-one (Horii et al., 1990; Gadda et al., 1997; Yamashita et al., 1998; Venkatramesh et al., 2003). However, a BLAST search indicated that the Arabidopsis genome does not comprise their homologous genes.

We also demonstrated that pea tissue exerts 3βHSD activity converting 5α-campestan-3-one to 5α-campestan-3β-ol (Table III; Fig. 4). Human 3βHSDs have been known to interconvert 5α-androstan-3-ones and 5α-androstan-3β-ols in vitro (Rhéaume et al., 1991). *D. lanata* 3βHSD was demonstrated to catalyze the interconversion of 5α-pregnane-3,20-dione and 5α-pregnane-3β-ol,20-one and of 5β-pregnane-3,20-dione and 5β-pregnane-3β-ol,20-one (Finsterbusch et al., 1999). Recently, mammalian 3α-hydroxysteroid dehydrogenase (3αHSD), a member of the AKR superfamily as is 3βHSD (Jin and Penning, 2001), was found also to have 3βHSD activity that deactivates 5α-dihydrotestosterone into 5α-androstan-3β,17β-diol (Steckelbroeck et al., 2004). Furthermore, *D. purpurea* AKR proteins (DpAR1 and DpAR2) have been shown to reduce C_{21} steroids with 3- and/or 20-carbonyl groups (Gavidia et al., 2002). These findings suggest that 3βHSDs of C_{28} plant steroids are also members of AKR proteins.

Table III. *Metabolism of [$^2H_6$]-labeled sterols in 13-d-old shoots of the pea mutant lk and wild-type plants*

Each substrate (25 μg) was incubated with sliced apical portions of pea. The amounts of metabolites (μg g^{-1} fresh weight) are means of two GC-SIM runs.

Product	Substrate							
	[$^2H_6$]Campesterol		[$^2H_6$]Campest-4-en-3β-ol		[$^2H_6$]Campest-4-en-3-one		[$^2H_6$]5α-Campestan-3-one	
	Wild Type	*lk*	Wild Type	*lk*	Wild Type	*lk*	Wild Type	*lk*
[$^2H_6$]Campesterol	0.94	1.12						
[$^2H_6$]Campest-4-en-3β-ol	nd[a]	nd	3.68	2.82	na[b]	na	na	na
[$^2H_6$]Campest-4-en-3-one	1.27	3.70	3.89	2.47	3.78	13.5	na	na
[$^2H_6$]5α-Campestan-3-one	nd	nd	nd	nd	nd	nd	2.34	2.45
[$^2H_6$]5α-Campestanol	0.82	nd	1.00	nd.	1.98	nd	0.51	1.06

[a]nd, Not detected. [b]na, Not analyzed.

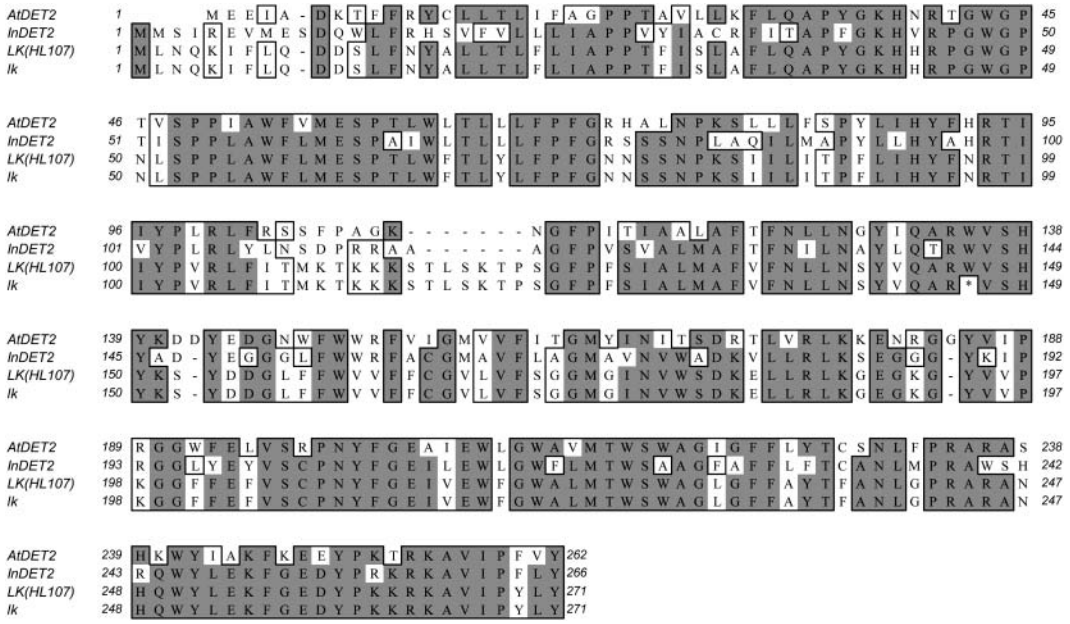

Figure 5. Protein alignment of Arabidopsis *DET2* (GenBank accession no. U53860), *InDET2* (GenBank accession no. AB106360) with pea wild-type HL107 (GenBank accession no. AY573897), and *lk*. Conserved residues are boxed and identical residues are shown by gray shade. The first 12 amino acids of HL107 are based on the pea wild-type JI813 sequence.

MATERIALS AND METHODS

Plant Material and Harvesting

The pure lines of pea (*Pisum sativum*) used in this study were lines 212+ (*LK*) and 212− (*lk*). The *lk* mutation (erectoides) originated as a spontaneous mutation in cv Cefalonia Rogue (John Innes line 885; Reid, 1986). Line 212+ is the wild type for 212−, the *lk* line generated by crossing the original mutant with Hobart line 107.

Unless otherwise stated, pea seedlings were grown either in a growth cabinet or greenhouse under the following conditions. In growth cabinets (Nihon Ikakikai, Tokyo), pea seeds were sown in a tray filled with moist vermiculite and grown under continuous fluorescent light (Toshiba, 40-W daylight-white tube; approximately 240 μmol m^{-2} s^{-1} at top of plant). The temperature was maintained at 25°C for the first 3 d, then lowered to 20°C for further growth. In the greenhouse, pea seedlings were grown at 26°C on Super Mix A (Sakatanotane, Yokohama, Japan) under natural light supplemented with fluorescent light, making a 13-h light/11-h dark regime.

For analysis of genes, seeds of each genotype were germinated and grown under an 18-h photoperiod in a heated greenhouse and shoots were harvested after 14 d of growth, frozen in liquid nitrogen, ground to a fine powder, and stored at −70°C.

Calli of the *lk* Mutant and Wild Type

Wild-type and *lk* seed were surface sterilized with 70% ethanol for 20 s and antiformin (×10) for 15 min, rinsed with sterilized water, and aseptically sown on 0.9% agar medium containing Murashige and Skoog inorganic salts and incubated at 25°C in a growth cabinet under continuous fluorescent light. Calli were induced in the dark at 25°C from the stems of 11-d-old wild-type and *lk* plants on a Murashige and Skoog agar medium containing 0.9% agar, 3% Suc, and 1 mg L^{-1} of 2,4-dichlorophenoxyacetic acid. These calli were subcultured three times under the same conditions before sterol analysis.

Application of Brassinolide

Brassinolide dissolved in 5 μL of ethanol containing 0.15% Tween 20 was applied directly to the fourth internode of 8-d-old seedlings grown in a growth cabinet when the third leaf was fully expanded. Control seedlings were treated just with solvent. Three days after treatment, the lengths of the fourth internodes were measured.

Gas Chromatography-Selected Ion Monitoring

A Jeol JMS-AX 505 instrument equipped with a DB-5 column (0.25 mm × 15 m; 0.25-μm film thickness; J & W Scientific, Folsom, CA) was used. The column oven temperature was set to 170°C for the first 1.5 min, elevated to 280°C at 37°C min^{-1}, and then to 300°C at 1.5°C min^{-1}. The carrier gas was He at the flow rate of 1 mL min^{-1}, the injection port temperature was 260°C, and the samples were introduced by splitless injection.

Extraction and Purification of BRs

Aerial parts of 12-d-old *lk* seedlings grown in a greenhouse (30.7 g fresh weight) were harvested from 66 seedlings whose average height was 5.2 cm, while those of wild-type plants (71.1 g fresh weight) were harvested from 74 seedlings whose average height was 19.6 cm. The materials were extracted with methanol and [$^2H_6$]-labeled internal standards were added to the extract prior to reduction to an aqueous residue. To the extract of the wild-type shoots, the following [$^2H_6$]-labeled internal standards were added: 0.2 μg each of [$^2H_6$]brassinolide, [$^2H_6$]typhasterol, [$^2H_6$]3-dehydroteasterone, [$^2H_6$]teasterone, [$^2H_6$]3-dehydro-6-deoxoteasterone, and [$^2H_6$]6-deoxoteasterone, along with 0.5 μg each of [$^2H_6$]castasterone, [$^2H_6$]6-deoxocastasterone, and [$^2H_6$]6-deoxotyphasterol. Half the amounts of the standards were added to the *lk* shoot extract. The extract was partitioned twice between ethyl acetate and 0.5 M dipotassium hydrogen phosphate (pH 9). The ethyl acetate phase was evaporated to dryness and partitioned three times between *n*-hexane and 80% methanol. The latter phase was evaporated to dryness and purified on silica gel (Wako gel C300, 0.5 g) eluted with chloroform, and then with chloroform containing 0.5%, 7%, 10%, and 20% methanol. The 7% methanol fraction was purified on Sephadex LH-20 (column volume, 500 mL; Pharmacia, Uppsala), using methanol:chloroform (4:1, v/v) with collection of 10-mL fractions. On the basis of the rice lamina inclination bioassay, fractions 31 to 40 (wild type) and 32 to 39 (*lk*), respectively, were combined. These fractions were dissolved in methanol and passed through short columns of diethylaminosilica and octadecylsilica (ODS) successively. Eluates with methanol were subjected to HPLC on an ODS column (8 × 250 mm; Senshu Scientific, Tokyo) eluted with an acetonitrile-water gradient at 40°C at a flow rate of 2.5 mL min^{-1}, fractions

being collected every minute. The mobile phase was programmed as follows: 0 to 20 min, 45% acetonitrile; 20 to 40 min, 45% to 100% acetonitrile; 40 to 60 min, 100% acetonitrile. The following fractions were analyzed for BRs by gas chromatography-selected ion monitoring (GC-SIM): 15 (brassinolide), 21/22 (castasterone), 31 (teasterone), 36 (typhasterol and 3-dehydroteasterone), 39 (6-deoxocastasterone), 43 (6-deoxoteasterone), 45 (3-dehydro-6-deoxoteasterone), and 47 (6-deoxotyphasterol).

GC-SIM Quantitation of BRs

Random aliquots of extracts derived from pooled plant materials were analyzed in duplicate by GC-SIM. BRs were converted to either monomethaneboronates (MB) or bismethaneboronates (BMB), with pyridine-containing methaneboronic acid (2 mg mL^{-1}) at 70°C for 30 min. Typhasterol, teasterone, 6-deoxotyphasterol, and 6-deoxoteasterone were further trimethylsilylated with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide to yield methaneboronate-trimethylsilyl ethers (MB-TMSi). The contents of BRs were calculated from the peak area ratios of $^2H_0$ and $^2H_6$ M^+ ions. 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.

Extraction and Purification of Sterols

For shoots, the aerial parts of *lk* (1.2 g fresh weight; average height, 5.2 cm) and wild-type plants (1.8 g fresh weight; average height, 17.7 cm) were harvested from three 17-d-old seedlings grown in a greenhouse. For calli, 1 g fresh weight of material was used. These materials were extracted with methanol:chloroform (4:1, v/v) with homogenizing. The extract was partitioned twice between ethyl acetate and 0.5 M dipotassium hydrogen phosphate buffer, and the ethyl acetate phases were combined and evaporated to dryness. A portion (100 mg fresh-weight equivalent) of the residual solid was spiked with 1 μg of [$^2H_6$] campestanol and heated with 1 N sodium hydroxide in methanol at 80°C for 1.5 h. After evaporating the solvent, the hydrolysate was partitioned twice between chloroform and water. The chloroform phases were combined and evaporated to dryness, dissolved in chloroform, and passed through a column of silica gel (0.3 g). The eluate with chloroform was evaporated to dryness and analyzed by GC-SIM.

GC-SIM Quantitation of Sterols

Random aliquots of extracts derived from pooled plant materials were analyzed in duplicate by GC-SIM under the conditions used for BRs. Sterols were trimethylsilylated with BSTFA at room temperature. 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.

Feeding Experiments

[$^2H_6$]-labeled precursor sterols were prepared as described elsewhere (Noguchi et al., 1999b). Each sterol (25 μg) was dissolved in 3 mL of acetone and mixed with 10 mL of sterilized 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1% Tween 20. The mixture was placed under a stream of N_2 to give a sterol emulsion. Apical portions including the apex were excised from two 13-d-old seedlings, which were aseptically grown under the same conditions as used for callus production, briefly washed with sterile water, sliced by 1-mm width, and placed in a 30-mL flask that contained 5 mL of an emulsified [$^2H_6$]-labeled sterol. The mixtures were gently stirred on a rotary shaker (90 rpm) for 5 h at 25°C under the same conditions as used for growing the pea seedlings. After incubation, the explants were rinsed with sterilized water and processed under the previously described protocol for extraction and purification of sterols. Quantitation of the metabolites was carried out by GC-mass spectrometry using the absolute calibration curves prepared for the respective metabolites.

Isolation of the *DET2* Homolog from Pea

Total RNA extraction was performed using the QIAquick RNeasy kit (Qiagen, Clifton Hill, Victoria, Australia). To remove contaminating DNA, the total RNA was treated using a DNA-free kit (Ambion, GeneWorks, South Australia). The full sequence of the *DET2* homolog in pea was isolated by screening 200,000 plaques of a Lambda Zap II cDNA library (Stratagene, La Jolla, CA), prepared from RNA isolated from flowering shoot apices of John Innes line 813 (Hobart line 5ly). The probe used to screen the library was based on the *DET2* homolog in HL107 plants, which was partially isolated using degenerate primers (Gregory, 1999).

Sequencing

Reverse transcription (RT)-PCR experiments were performed with 5 μg of total RNA with the GibcoBRL/Life Technologies SuperScript Preamplification system for first-strand cDNA synthesis (Life Technologies, Melbourne, Australia). Standard PCR reactions (Sigma Technical Bulletin; Sigma, New South Wales, Australia) using *Taq* polymerase were subjected to a PCR program consisting of an initial denaturation at 94°C for 1 min and then 35 cycles of 94°C for 5 s, 50°C for 30 s, and 72°C for 1 min, with a final extension step of 15 min at 72°C. PCR reactions were purified using the QIAquick PCR Purification kit (Qiagen). Primers were designed to cover the entire coding region (based on the JI813 sequence). Oligonucleotide sequences are shown 5′ to 3′ (the numbers shown correspond to positions in the cDNA sequence; F refers to the left forward primer while R refers to the right reverse primer): 1F, 5-TGAATCAAAAGATATTCTTACAAGACG-31; 1R, 851-TGCAACACACC-CCAAATTAC-832; 2F, TGACACACAAACATAGTACCTCACA; 2R, 272-AG-AAAGGGGGTGATGAGGAT-253; 3R, 363-TGGGGTTTTCGATAAAGTTGA-343. Sequencing reactions were subjected to a PCR program consisting of 40 cycles of 96°C for 20 s, 50°C for 20 s, and 60°C for 4 min, in a Perkin-Elmer thermal cycler using the quick start CEQ 2000 Dye Terminator Cycle Sequencing (DTCS) kit (Perkin-Elmer, Foster City, CA). Sequencings were performed on the capillary fluorescence Beckman Coulter Sequencer (Beckman Instruments, Fullerton, CA). Sequence data analysis was achieved with Sequencher software and MacVector (Accelrys, San Diego).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY573897.

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