

# Characterization of Two Brassinosteroid C-6 Oxidase Genes in Pea<sup>1</sup>[W][OA]

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C-6 oxidation genes play a key role in the regulation of biologically active brassinosteroid (BR) levels in the plant. They control BR activation, which involves the C-6 oxidation of 6-deoxocastasterone (6-DeoxoCS) to castasterone (CS) and in some cases the further conversion of CS to brassinolide (BL). C-6 oxidation is controlled by the CYP85A family of cytochrome P450s, and to date, two CYP85As have been isolated in tomato (*Solanum lycopersicum*), two in Arabidopsis (*Arabidopsis thaliana*), one in rice (*Oryza sativa*), and one in grape (*Vitis vinifera*). We have now isolated two CYP85As (CYP85A1 and CYP85A6) from pea (*Pisum sativum*). However, unlike Arabidopsis and tomato, which both contain one BR C-6 oxidase that converts 6-DeoxoCS to CS and one BR C-6 Baeyer-Villiger oxidase that converts 6-DeoxoCS right through to BL, the two BR C-6 oxidases in pea both act principally to convert 6-DeoxoCS to CS. The isolation of these two BR C-6 oxidation genes in pea highlights the species-specific differences associated with C-6 oxidation. In addition, we have isolated a novel BR-deficient mutant, *lke*, which blocks the function of one of these two BR C-6 oxidases (CYP85A6). The *lke* mutant exhibits a phenotype intermediate between wild-type plants and previously characterized pea BR mutants (*lk*, *lka*, and *lkb*) and contains reduced levels of CS and increased levels of 6-DeoxoCS. To date, *lke* is the only mutant identified in pea that blocks the latter steps of BR biosynthesis and it will therefore provide an excellent tool to further examine the regulation of BR biosynthesis and the relative biological activities of CS and BL in pea.

Brassinosteroids (BRs) are now widely recognized as essential regulators of plant development. Brassinolide (BL) has been shown to be the most active member in the BR biosynthetic pathway (Yokota, 1997; Wang et al., 2001). However, because BL is not present in some tissues of some species, the penultimate compound in BR biosynthesis, castasterone (CS), is also thought to be biologically active (Nomura et al., 2005). This notion is supported by the fact that CS is also able to bind to the BR receptor (Wang et al., 2001). The major precursor to CS and the most abundant BR in a number of species, 6-deoxocastasterone (6-DeoxoCS; Nomura et al., 2001), shows no significant biological activity per se. The fact that 6-DeoxoCS levels are at least one order of magnitude higher than

levels of its metabolite, CS (Nomura et al., 2001), also indicates that the conversion of 6-DeoxoCS to CS is an important rate-limiting step. Together, this suggests that the conversion (C-6 oxidation) of 6-DeoxoCS to CS and BL is the crucial activation step in the BR biosynthesis pathway (Fig. 1) and is important for the regulation of endogenous bioactive BR levels.

The first C-6 oxidation gene isolated was the *Dwarf* gene in tomato (*Solanum lycopersicum*), which was found to be a cytochrome P450 monooxygenase (Bishop et al., 1996). Indeed, we now know that the conversion of 6-DeoxoCS to CS and BL in a wide range of species is catalyzed by the CYP85A family of P450s (Bishop et al., 1999; Shimada et al., 2001). To date, two CYP85As (CYP85A1 and CYP85A3) have been isolated in tomato (Bishop et al., 1996; Nomura et al., 2005), two (CYP85A1 and CYP85A2) in Arabidopsis (*Arabidopsis thaliana*; Shimada et al., 2001, 2003; Kim et al., 2005), one (CYP85A1) in rice (*Oryza sativa*; Hong et al., 2002; Mori et al., 2002), and one (CYP85A1) in grape (*Vitis vinifera*; Symons et al., 2006).

Arabidopsis CYP85A1 (*AtBR6ox1*), rice CYP85A1 (*OsBR6ox1*), and tomato CYP85A1 (*LeBR6ox1*) all catalyze the conversion of 6-DeoxoCS to CS in these species. The detection of BL in tomato fruit indicated the presence of another enzyme catalyzing the C-6 oxidation of 6-DeoxoCS (Nomura et al., 2005). This led to the isolation of CYP85A3 (*LeBR6ox3*), which is able to convert 6-DeoxoCS into BL via CS (Nomura et al., 2005). CYP85A3 is expressed only in tomato fruit

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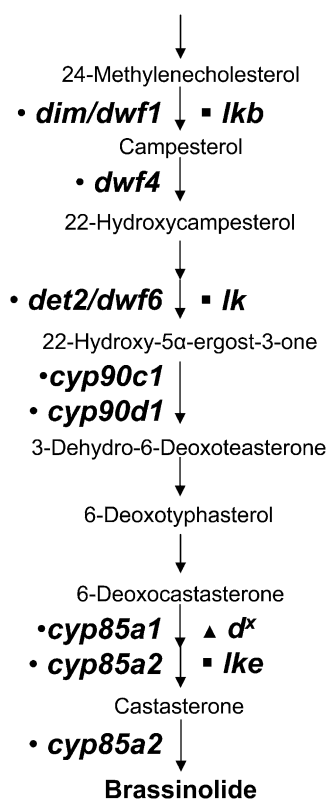
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**Figure 1.** The possible dominant BR biosynthetic pathway in plants (Fujita et al., 2006; Ohnishi et al., 2006; Szekeres and Bishop, 2006), showing Arabidopsis (●), tomato (▲), and pea (■) mutants.

(Montoya et al., 2005), which explains why BL is detected in only this tissue. Arabidopsis CYP85A2 (*AtBR6ox2*) has the same function as tomato CYP85A3, and therefore converts 6-DeoxoCS to BL, but unlike the situation in tomato, CYP85A2 is expressed in all tissues (Castle et al., 2005; Kim et al., 2005; Nomura et al., 2005). Similar genes involved in the formation of BL in rice or grape have not been reported, to our knowledge. In addition to their activity in the BR activation steps, the CYP85A family of enzymes also catalyze multiple reactions in which the C-6 position of 6-deoxytyphasterol, 3-dehydro-6-deoxoteasterone, and 6-deoxoteasterone are oxidized (Shimada et al., 2001).

The isolation of mutants blocking the final steps of BR biosynthesis has been a crucial step in understanding C-6 oxidation in plants. Identification of the *Dwarf* gene in tomato was made possible by the isolation of the extreme dwarf mutant, *d<sup>x</sup>* (Bishop et al., 1999). Vegetative tissues of *d<sup>x</sup>* plants have no detectable BL or CS and increased levels of 6-DeoxoCS (Bishop et al., 1996), as they are unable to convert 6-DeoxoCS to CS (Fig. 1). However, CS and BL are present in *d<sup>x</sup>* fruit, due to the fact that CYP85A3 is still able to convert 6-DeoxoCS into BL, via CS, in this tissue (Nomura et al., 2005). Arabidopsis CYP85A1 and CYP85A2 are expressed in all tissue types (Shimada et al., 2003; Castle et al., 2005). Therefore, neither of the loss-of-

function *cyp85a1* and *cyp85a2* mutants exhibits a similar phenotype to the *d<sup>x</sup>* mutant in tomato. In fact, *cyp85a1* mutants do not display a mutant phenotype at all, because CS levels are maintained due to the functional redundancy of the CYP85A1 and CYP85A2 genes (Kim et al., 2005). However, the *cyp85a2* mutant exhibits a weak phenotype and CS levels are only 80% of wild-type levels (Kim et al., 2005), despite the fact that CYP85A1 is converting 6-DeoxoCS to CS in this mutant (Fig. 1). As expected, the double mutant, *cyp85a1cyp85a2*, displays a severe dwarf phenotype (Kwon et al., 2005; Nomura et al., 2005).

Interestingly, in rice, genetic redundancy occurs at multiple steps earlier in the BR pathway (Hong et al., 2003; Sakamoto et al., 2006), yet only one CYP85A has been reported (Hong et al., 2002; Mori et al., 2002). In Arabidopsis, redundancy does occur at the C-6 oxidation step and it has recently been reported that there are also multiple genes controlling C-23 hydroxylation (Ohnishi et al., 2006). However, there are other steps in the Arabidopsis BR pathway that only appear to be controlled by one gene (Azpiroz et al., 1998; Choe et al., 1998; Fujita et al., 2006). It requires investigation to see if in other dicotyledonous species there are similar differences in relation to genetic redundancy.

The results presented here from pea (*Pisum sativum*) further highlight the differences in the regulation of C-6 oxidation genes between species. We show that, unlike tomato and Arabidopsis (which both contain a BR C-6 oxidase that converts 6-DeoxoCS to CS and a BR C-6 Baeyer-Villiger oxidase that converts 6-DeoxoCS to BL), pea contains two CYP85As, CYP85A1 (*Pisum sativum* BR C-6 oxidase 1 [*PsBR6ox1*]) and CYP85A6 (*Pisum sativum* BR C-6 oxidase 6 [*PsBR6ox6*]), which act primarily as CS synthases by converting 6-DeoxoCS to CS. The isolation of a novel mutant, *lke*, which blocks the function of CYP85A6, has allowed us to examine and discuss the role of C-6 oxidation and genetic redundancy in this species.

## RESULTS

### Phenotypic Characterization of *lke*

The *lke* mutant was produced through ethyl methanesulfonate mutagenesis of cv Torsdag. F<sub>1</sub> crosses of *lke* with *lk*, *lka*, *lkb*, *lkc*, and *lkd* (all BR biosynthetic or response mutants) showed that *lke* was not allelic with any of these genes.

The *lke* mutant exhibits an intermediate phenotype between wild-type plants and previously characterized BR mutants (*lk*, *lka*, and *lkb*; Schultz et al., 2001; Nomura et al., 2003, 2004) in terms of height (Fig. 2A) and internode length (Fig. 2B). The phenotype of *lke* when grown in the light has many similar traits to other pea BR mutants, including smaller, darker leaves, reduced internode length, and thicker stems (compared with wild type; Fig. 2). Like *lka* and *lkb*, *lke* plants also exhibit epinastic leaves and stem banding



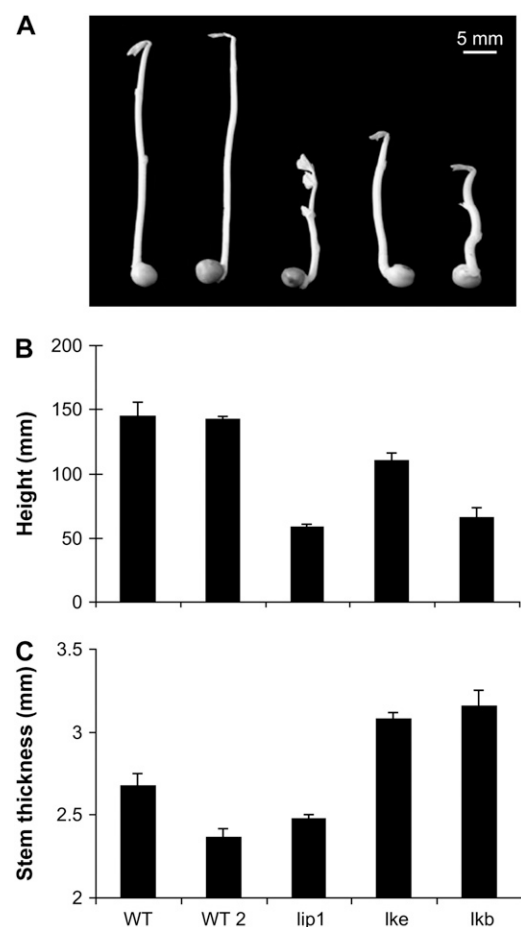
**Figure 2.** Morphology of 18-d-old wild-type, *lke*, and *lkb* plants (A) and internode 4 (between nodes 3 and 4) of wild-type, *lke*, and *lkb* plants (B), showing the intermediate phenotype of *lke*. The average height of an 18-d-old wild-type plant is 30 cm, while the average length of internode 4 is 43 mm.

(Reid and Ross, 1989). The phenotype of *lke* was also investigated after being grown in the dark for 7 d to determine whether *lke* has a similar phenotype to other BR mutants when grown in the dark. Unlike reports for many *Arabidopsis* and tomato BR mutants, BR mutants in pea do not exhibit a de-etiolated phenotype when grown in the dark (Symons et al., 2002). The *lke* plants did not develop expanded leaves and still possessed an apical hook when grown in the dark for 7 d (Fig. 3A). This is similar to other pea BR mutants but in contrast to the fully de-etiolated *lip1* mutant (Frances et al., 1992), which exhibits a de-etiolated phenotype in the dark. This shows that the phenotype of *lke* is similar to other pea BR mutants when grown in the dark. The height of dark-grown *lke* plants was intermediate between the wild type and *lkb* mutant (Fig. 3B), as occurs in light-grown plants. The stem thickness of *lke* was significantly ( $P < 0.001$ ) greater than the wild type (Fig. 3C). Together, these results show that *lke* exhibits a weak dwarf phenotype in both

the light and the dark and its phenotype is consistent with previously characterized pea BR mutants *lk*, *lka*, and *lkb* (Schultz et al., 2001; Nomura et al., 2003, 2004).

#### 6-DeoxoCS Levels Are Increased in the *lke* Mutant

Endogenous CS levels in *lke* were decreased to 70% of wild-type levels (Table I, experiment 1). However, in some situations, CS levels in *lke* are not reduced to the same extent as in *lkb* plants, which is consistent with the intermediate phenotype exhibited by *lke* (Fig. 2). It is of particular interest that endogenous 6-DeoxoCS levels in *lke* are 25 times higher than *lkb* levels and are also 40% higher than wild-type levels (Table I, experiment 1). A second experiment analyzing CS and 6-DeoxoCS levels in wild-type, *lke*, *lkb*, and *lka* plants also confirmed that CS was decreased and 6-DeoxoCS was significantly ( $P < 0.01$ ) increased in *lke* compared with the wild type (Table I, experiment 2). As expected (Nomura et al., 1997), 6-DeoxoCS and CS levels were decreased in BR-deficient *lkb* plants compared with the wild type, and 6-DeoxoCS and CS were increased in



**Figure 3.** Seven-day-old dark-grown wild-type (HL107) and wild-type 2 (*LIP1*) plants compared to a de-etiolated mutant (*lip1*) and the BR-deficient mutants, *lke* and *lkb*, showing differences in morphology (A), height (B), and stem thickness (C). Data are shown as means  $\pm$  SE ( $n = 7$ ).

**Table I.** Endogenous BR levels (as determined by GC-MS-selected ion monitoring) in wild-type plants and BR-deficient and -response mutants

For experiment 1, the tissue harvested included the apical bud and the three internodes below, without any expanded leaf tissue from 26-d-old seedlings. For experiments 2 and 3, the tissue harvested consisted of the whole shoot from 14-d-old and 25-d-old seedlings, respectively. Data are shown as means  $\pm$  SE ( $n = 2$ ). nd, Not detected; NA, not analyzed.

	Level			
	Wild-Type	<i>lke</i>	<i>lkb</i>	<i>lka</i>
	<i>ng/g fresh weight</i>			
Experiment 1				
6-DeoxoCS	3.70 $\pm$ 0.48	5.25 $\pm$ 0.17	0.21 $\pm$ 0.04	NA
CS	0.44 $\pm$ 0.01	0.31 $\pm$ 0.05	0.09 $\pm$ 0.01	NA
BL	nd	nd	nd	NA
Experiment 2				
6-DeoxoCS	2.39 $\pm$ 0.06	3.64 $\pm$ 0.10	0.09 $\pm$ 0.01	4.50 $\pm$ 0.29
CS	0.56 $\pm$ 0.07	0.26 $\pm$ 0.00	0.27 $\pm$ 0.07	1.80 $\pm$ 0.29
BL	nd	nd	nd	nd
Experiment 3				
6-DeoxoCS	3.50 $\pm$ 0.60	4.22 $\pm$ 0.03	NA	NA
CS	0.17 $\pm$ 0.01	0.10 $\pm$ 0.01	NA	NA
BL	nd	nd	NA	NA

BR-response *lka* plants compared with the wild type (Table I, experiment 2). A third experiment further confirms that CS levels are decreased and 6-DeoxoCS levels are significantly ( $P < 0.05$ ) increased in *lke* compared to the wild type (Table I, experiment 3). A two-way ANOVA of the combined data showed that both the decrease in CS levels and the increase in 6-DeoxoCS levels in *lke* are significant ( $P < 0.001$  and  $P < 0.01$ , respectively). A buildup of 6-DeoxoCS in *lke* plants is indicative of a blockage in the biosynthetic pathway after this compound, preventing its conversion to CS.

#### Indole-3-Acetic Acid and GA<sub>1</sub> Levels in *lke* Are Consistent with Other BR Mutants

In addition to BRs, endogenous indole-3-acetic acid (IAA) and GA<sub>1</sub> levels were measured in the oldest unexpanded internode of wild-type, *lke*, and *lkb* plants. The level of IAA in *lke* and *lkb* internodes was significantly ( $P < 0.05$  and  $P < 0.01$ , respectively) decreased compared with the wild type (Fig. 4A). The reduction in IAA in *lkb* is consistent with findings from McKay et al. (1994). Interestingly, the IAA level in *lke* internodes was not decreased to the same extent as in *lkb* (Fig. 4A). This result is once again consistent with the intermediate phenotype of *lke* plants (Fig. 2). There was no significant difference in GA<sub>1</sub> levels in either *lke* or *lkb* internodes compared with the wild type (Fig. 4B). This is consistent with previous findings that show that GA<sub>1</sub> levels are unaltered in *lkb* plants (Jager et al., 2005).

#### 6-DeoxoCS Application Does Not Result in a Growth Response in *lke* Plants

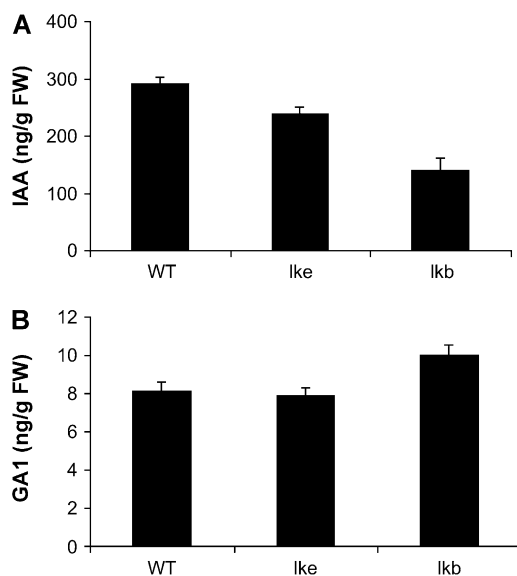
6-DeoxoCS, CS, and BL were applied to *lke* and *lkb* plants to examine growth responses. *lke* plants showed

a significant growth response when treated with BL ( $P < 0.01$ ) and CS ( $P < 0.02$ ). A small but nonsignificant growth response was recorded when the *lke* plants were treated with 6-DeoxoCS (Fig. 5). 6-DeoxoCS may not cause a significant growth response in *lke* plants possibly because its conversion to the bioactive compounds CS and BL is reduced. The application of 6-DeoxoCS, CS, and BL to BR-deficient *lkb* plants resulted in significant ( $P < 0.001$ ) growth responses in relation to control *lkb* plants (Fig. 5).

#### Isolation of CYP85A1 and CYP85A6 from Pea

The phenotypic characterization and feeding studies, along with the endogenous BR levels, all suggest that the *lke* mutation reduces the conversion of 6-DeoxoCS to CS. Based on the results from other species (Bishop et al., 1996; Shimada et al., 2001, 2003; Hong et al., 2002; Mori et al., 2002; Kim et al., 2005; Nomura et al., 2005), the enzyme(s) responsible for C-6 oxidation in pea is expected to be in the CYP85A family. To identify the CYP85A gene(s) from pea, we designed oligonucleotide primers based on the conserved nucleotide sequences among legume expressed sequence tags (from *Glycine max*, *Lotus japonicus*, and *Medicago truncatula*) and the CYP85A genes of Arabidopsis and tomato (Supplemental Table S1). We succeeded in isolating two full-length cDNAs for CYP85A homologs from pea using a PCR-based approach. Sequence analysis revealed that those cDNAs encode amino acid sequences that show 77% identity and 86% similarity to each other and show 80% to 88% similarity with the CYP85A proteins of Arabidopsis and tomato. Therefore, CYP numbers CYP85A1 and CYP85A6 were given to those pea sequences (Dr. David Nelson, personal communication).

A phylogenetic tree for the newly isolated CYP85As in pea and CYP85As from several other species was



**Figure 4.** Endogenous IAA and GA<sub>1</sub> levels (as determined by GC-MS-selected ion monitoring) in 25-d-old wild-type, *lke*, and *lkb* plants. Tissue harvested consisted of the oldest unexpanded internode, which was approximately 30% expanded at the time of harvest. Data are shown as means  $\pm$  SE ( $n = 3$ ).

constructed using those full-length protein sequences (Fig. 6). Pea CYP85A1 was closest to pea CYP85A6 in sequence. This is similar to the pairing of CYP85As in other species. This observation suggests that the CYP85A genes duplicated independently in pea as in Arabidopsis and tomato.

#### Both CYP85A1 and CYP85A6 of Pea Encode BR C-6 Oxidases with CS Synthase Activity

To examine whether pea CYP85A1 and CYP85A6 catalyze the conversion of 6-DeoxoCS to CS or BL, these proteins were expressed functionally in yeast (*Saccharomyces cerevisiae*). The cDNAs of CYP85A1 and CYP85A6 were cloned into the plasmid YeDP60 that allows Gal-inducible expression of P450 in yeast (Pompon et al., 1996). As a positive control, Arabidopsis CYP85A2 cDNA that encodes BL synthase in pYeDP60 was used (Nomura et al., 2005). Yeast strains WAT11 and WAT21 were used as hosts, because Arabidopsis NADPH P450 reductase 1 and 2 are integrated, respectively, and are induced by Gal to donate the electrons required for plant P450 activity in yeast (Pompon et al., 1996). Induced cultures of the transformants were incubated with 6-DeoxoCS or CS, and the metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS).

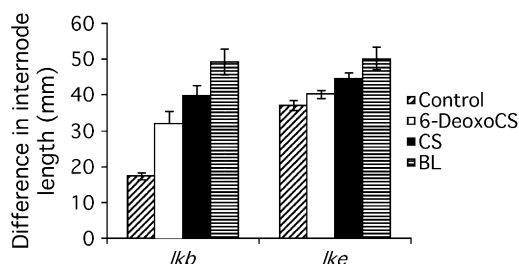
A large fraction of added 6-DeoxoCS was metabolized to produce CS in both WAT11 and WAT21 strains producing pea CYP85A1 protein (Fig. 7; Supplemental Fig. S1A; Supplemental Table S2). This result indicates that pea CYP85A1 has BR C-6 oxidation activity like the previously characterized CYP85A1 members of

tomato, Arabidopsis, and rice (Bishop et al., 1999; Shimada et al., 2001; Hong et al., 2002), and Arabidopsis NADPH P450 reductase 1 and 2 have the same activity for pea P450 function in yeast. In less than one-half of the replicates, pea CYP85A1 also produced a small amount of BL from 6-DeoxoCS (Supplemental Fig. S2A). When CS was incubated with pea CYP85A1 recombinant yeast, a trace level of BL was also detected (data not shown). The BL synthase activity (Baeyer-Villiger reaction) of pea CYP85A1 was very low compared with that of Arabidopsis CYP85A2 (Supplemental Fig. S2) and tomato CYP85A3 (Nomura et al., 2005). Therefore, pea CYP85A1 does not possess a perfect domain for the Baeyer-Villiger reaction to produce BL, which results in pea CYP85A1 producing CS as the predominant C-6 oxidation product from 6-DeoxoCS.

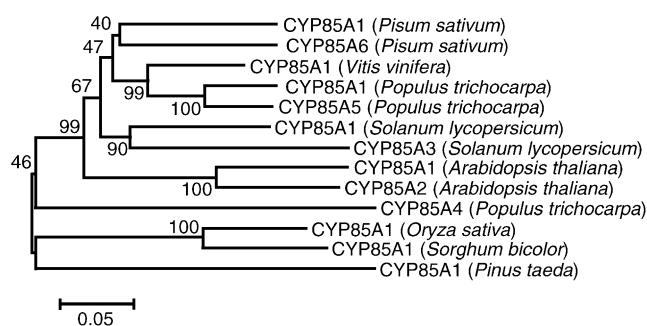
The recombinant yeast expressing pea CYP85A6 also catalyzed the formation of CS from 6-DeoxoCS (Fig. 7; Supplemental Table S2) but did not produce BL from 6-DeoxoCS or CS (data not shown). These data indicate that both pea CYP85A1 and CYP85A6 are C-6 oxidases involved in the production of CS from 6-DeoxoCS. Therefore, the CYP85A1 and CYP85A6 of pea were named as *PsBR6ox1* (*Pisum sativum* BR C-6 oxidase 1) and *PsBR6ox6* (*Pisum sativum* BR C-6 oxidase 6), respectively.

#### The *lke* Mutation Is Due to a Base Pair Change in the Start Codon of *PsBR6ox6*

We examined the two genes controlling C-6 oxidation of 6-DeoxoCS, *PsBR6ox1* and *PsBR6ox6*, to determine if there was a mutation in either of these genes in *lke* plants. Primers were designed to amplify the entire coding sequences of *PsBR6ox1* and *PsBR6ox6* from the wild type and the *lke* mutant (Supplemental Table S3). No differences were found in the coding region for *PsBR6ox1* for these two genotypes (data not shown). However, a difference was found in the *PsBR6ox6* gene. A mutation in *lke* was found at base 3 (Fig. 8A), where guanine was changed to adenine, which corresponded to the loss of the putative start codon. Hence, translation in *lke* is likely to begin at the next ATG



**Figure 5.** Growth of the oldest unexpanded internode of 24-d-old *lkb* and *lke* plants in the 24 h following the application of 200 ng of BL, CS, or 6-DeoxoCS (applied to the oldest unexpanded internode). Data are shown as means  $\pm$  SE ( $n = 7$ ).



**Figure 6.** Phylogenetic relationships among pea CYP85A1 and CYP85A6 and related CYP85A proteins. The phylogenetic tree was constructed using the deduced full-length protein sequences of pea CYP85A1 and CYP85A6 with the CYP85A family members from the Web site of Dr. Nelson (<http://drnelson.utmem.edu/CytochromeP450.html>). Bootstrap values in percentage are shown at each branch point.

downstream at base 66 (Fig. 8A). This potential start codon is out of frame with the translation in the wild type. If translation in *lke* does begin at base 66, then the LKE protein may be severely truncated, as the frame shift results in a stop codon (TGA) occurring after only the second amino acid (Fig. 8A), suggesting that *lke* may be a null mutation.

#### The Mutation in *PsBR6ox6* Cosegregates with the *lke* Semierectoides Phenotype

The mutation in the *PsBR6ox6* sequence results in the introduction of an *AluI* restriction site by altering the putative start codon from ATG to ATA. This polymorphism was used in a cosegregation analysis on the basis of PCR RFLP. Sixty-four F<sub>2</sub> individuals obtained by crossing the *lk* (erectoides) and *lke* (semierectoides) mutants segregated into 29 wild type, 15 semierectoides (*lke*), and 20 erectoides (*lk*, *lk/lke*) in agreement with a 9:3:4 ratio ( $\chi^2 = 3.1$ ,  $0.3 > P > 0.2$ ).

A 349-bp fragment spanning the restriction site for *AluI* was amplified by PCR from each isolate and then digested with *AluI*. The PCR product of the *LKE* plant remained intact after *AluI* digestion, whereas two bands of 239 and 110 bp resulted from the *lke* product, confirming the presence of a *AluI* polymorphism between wild-type and *lke* plants. RFLP analysis of the PCR products from a random sample of 26 wild-type and *lke* F<sub>2</sub> plants demonstrated cosegregation of the *AluI* polymorphism with the wild type/semierectoides *lke* phenotypic difference. Wild-type segregates produced either one band of 349 bp (*LKELKE*) or three bands of 349, 239, and 110 bp (*LKElke*), while the *lke* segregates produced two bands of 239 and 110 bp (Fig. 8B).

#### Expression Analysis of BR Genes in the *lke* Mutant

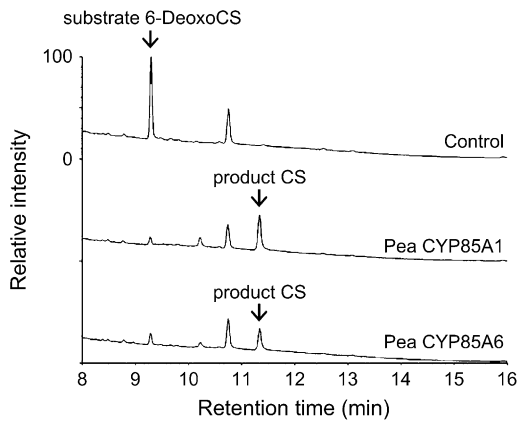
Semiquantitative, real-time reverse transcription (RT)-PCR analysis revealed that the two CS synthase genes, *PsBR6ox1* and *PsBR6ox6*, were both expressed

in a range of shoot tissues (including the apical bud, young expanding internode tissues, and mature leaves; Fig. 9A). To investigate the effect of the *lke* mutation on BR biosynthesis, metabolism, and response pathways, we also conducted PCR expression analysis of the BR genes *LKB*, *LK*, *PsCPD1*, *PsCPD2*, *PsDWF4*, *PsBR6ox1*, and *PsBR6ox6* (BR biosynthesis); *PsBAS1* (BR metabolism); and *LKA* (BR perception) genes in wild-type and *lke* plants. Results show that *PsBR6ox1*, *PsBR6ox6*, *PsCPD1*, and *PsCPD2* transcript levels were all elevated in *lke* plants (Fig. 9B). This pattern is suggestive of feedback regulation of these genes in response to the reduction in endogenous BR levels (Table I) and is consistent with similar results in other species (Nomura et al., 2001; Bancos et al., 2002; Goda et al., 2002; Castle et al., 2005; Tanaka et al., 2005). In contrast, expression levels of genes known to undergo feedback regulation in Arabidopsis (Tanaka et al., 2005), such as *PsDWF4* and *PsBAS1*, were not significantly altered in *lke*. This may be because the relatively small reduction in BR levels in the *lke* mutant may not reach the threshold level required to trigger the feedback regulation of these genes. While we propose that *PsBR6ox1* and *PsBR6ox6* are primarily CS synthases, it is interesting that the elevated expression of *PsBR6ox1* in the *lke* mutant does not fully compensate for the loss of *PsBR6ox6* function, as CS levels in *lke* tissues are still lower than in the wild type (Table I). This could be a consequence of differences in the tissue specificity of the two genes (Fig. 9A) and suggests the two genes are not fully redundant as shown by the phenotype, even though there is clear evidence of feedback regulation of the transcript level for *PsBR6ox1* in *lke* plants.

#### DISCUSSION

C-6 oxidation genes play a key role in the BR activation step(s) and are therefore crucial for the regulation of biologically active BR levels in the plant. The C-6 oxidation of 6-DeoxoCS to CS is also an important rate-limiting step in BR biosynthesis (Nomura et al., 2001, 2005). C-6 oxidation is controlled by the CYP85A family of P450s (Bishop et al., 1999; Shimada et al., 2001), and to date, two CYP85As have been isolated in tomato (Bishop et al., 1996; Nomura et al., 2005), two in Arabidopsis (Shimada et al., 2001, 2003; Kim et al., 2005), one in rice (Hong et al., 2002; Mori et al., 2002), and one in grape (Symons et al., 2006). Here we have established that pea also contains at least two CYP85A genes (*CYP85A1* [*PsBR6ox1*] and *CYP85A6* [*PsBR6ox6*]). However, unlike tomato and Arabidopsis, *PsBR6ox1* and *PsBR6ox6* in pea both function principally as CS synthases by converting 6-DeoxoCS to CS, but do not appear to carry out Baeyer-Villiger oxidation to produce significant amounts of BL when expressed in yeast.

In addition, we have identified and characterized a novel BR biosynthesis mutant in pea, *lke*. Based on



**Figure 7.** Functional assay of pea CYP85A1 and CYP85A6 in yeast. One microgram of 6-DeoxoCS was incubated in 5 mL of transformed yeast cultures. Control yeast has an empty vector. Total ion chromatograms (mass-to-charge ratio 50–600) of bismethaneboronate derivatives on GC-MS are shown. Product CS was identified by the mass spectra listed in Supplemental Table S2. The ratio of relative intensity on the same amount of BRs is 1 (6-DeoxoCS):0.6 (CS):0.5 (BL).

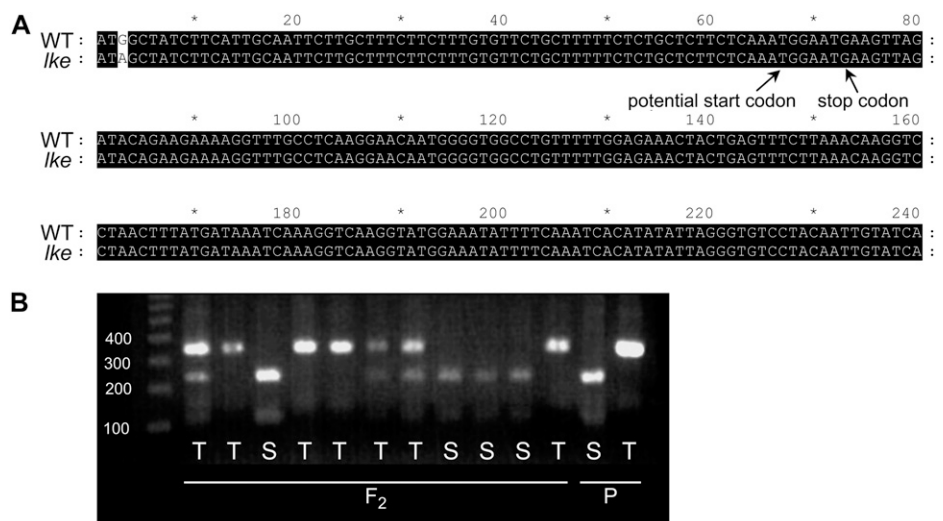
phenotypic evidence and the reduction in CS levels (Table I), it is reasonable to conclude that *lke* is indeed a BR-deficient mutant similar to the previously characterized BR mutants, *lkb* and *lk* (Schultz et al., 2001; Nomura et al., 2004). Interestingly, while *lkb* plants contained lower 6-DeoxoCS levels, *lke* plants had significantly increased 6-DeoxoCS levels compared with the wild type (Table I). A buildup of 6-DeoxoCS in *lke* plants is indicative of a blockage in the BR biosynthetic pathway after this compound. Furthermore, the fact that CS levels were reduced suggested that *lke* may block the conversion of 6-DeoxoCS to CS, but not the further metabolism of CS. The application of 6-DeoxoCS, CS, and BL to *lke* plants is consistent with this suggestion. While all three compounds caused a growth response in BR-deficient *lkb* plants, which blocks at an early step in the pathway (Fig. 5),

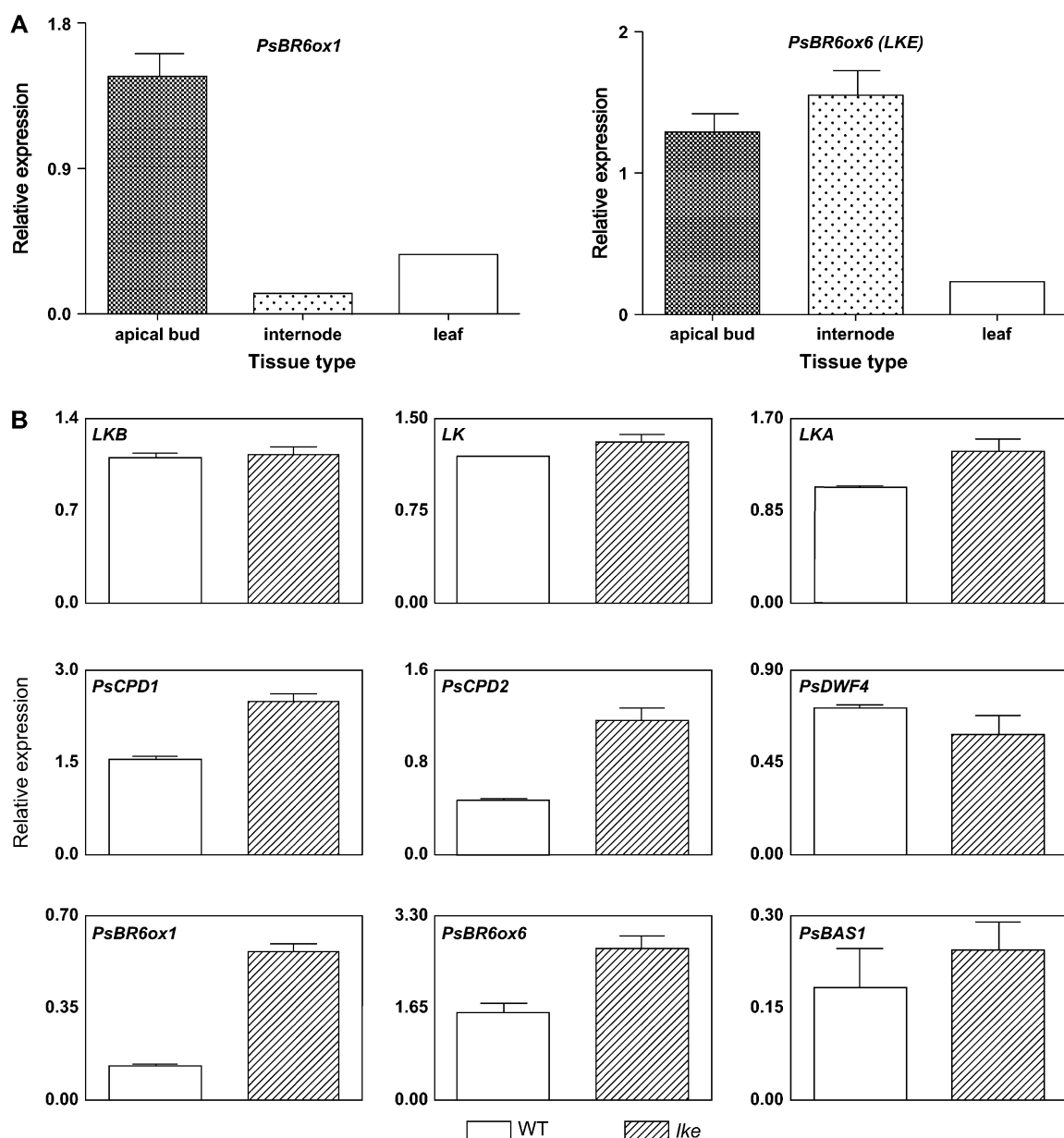
only BL and CS caused a significant ( $P < 0.02$ ) growth response in *lke* plants, but 6-DeoxoCS did not (Fig. 5). Together, these results strongly suggest that the *lke* mutation impairs or blocks the conversion of 6-DeoxoCS to CS (Fig. 1).

To confirm this hypothesis, sequence analysis of the *BR6ox* genes in pea was undertaken. First, the coding sequence of the *lke* gene was isolated and compared to *PsBR6ox1* (*CYP85A1*). However, no differences were found between the mutant and wild-type sequences. Sequence comparison between wild-type and *lke* plants for the second BR C-6 oxidase in pea, *PsBR6ox6*, revealed a base pair change in the start codon from the *lke* mutant (Fig. 8A), which cosegregated with the BR-deficient phenotype (Fig. 8B). Taken together, the physiological, biochemical, and molecular data strongly suggest that *lke* blocks BR biosynthesis after 6-DeoxoCS because of a mutation in the *PsBR6ox6* gene.

The characterization of the two pea *BR6ox* genes and the isolation of a mutation in the *PsBR6ox6* gene have provided insights into BR activation in pea and have also highlighted the species-specific differences that exist in relation to C-6 oxidation. Like Arabidopsis, pea contains at least two BR 6-oxidases that are expressed throughout the various shoot tissue types (Fig. 9A). However, in relation to the BR activation steps, the BR 6-oxidases isolated from pea both appear to be principally CS synthases, whereas a CS synthase and a BL synthase are present in Arabidopsis. Tomato also possesses both a CS and a BL synthase (Nomura et al., 2005). However, unlike Arabidopsis, they show tissue specificity, with the BL synthase (*LeBR6ox3*) only expressed in the developing fruit (Montoya et al., 2005). Hence, CS is considered to be the major biologically active BR during vegetative growth in tomato (Nomura et al., 2005). This may also be the case in pea, because BL has rarely been detected in vegetative tissues from mature pea plants (Nomura et al., 1997, 1999; Symons and Reid, 2004). However, BL has been

**Figure 8.** A, Sequence alignment of the first 240 bp of *PsBR6ox6* in pea wild-type (HL107) and *lke* (a BR-deficient mutant) plants. Black areas represent 100% homology. B, Cosegregation analysis of  $F_2$  progeny of *lke*  $\times$  *lk* by PCR-RFLP. An ethidium bromide-stained agarose gel shows the results of the RFLP analysis of PCR products from *LKE* and *lke* parents (P) and 11 individuals from four  $F_2$  segregating families ( $F_2$ ). Cosegregation of the *AluI* RFLP with the tall (T)/semierectoides (S) phenotypic difference is shown.





**Figure 9.** Semiquantitative, real-time RT-PCR expression analysis of *CYP85A1* and *CYP85A6* genes in shoot tissues of wild-type (L107) plants (A). Tissues are the apical bud, internode (oldest expanded internode), and leaf (mature leaf from two nodes below the apical bud). B, Genes involved in BR biosynthesis, metabolism, and response in wild-type and *lke* mutant plants. Transcript levels of genes involved in BR biosynthesis (*LKB*, *LK*, *PsCPD1*, *PsCPD2*, *PsDWF4*, *PsBR6ox1*, and *PsBR6ox6*), metabolism (*PsBAS1*), and perception (*LKA*) were determined in young expanding internodes of wild-type and *lke* plants that had 10 leaves fully expanded. All results were obtained from three independent replicates, each consisting of tissue from three separate plants. Note that gene expression values are relative, not absolute, and comparisons of expression levels between different genes are not valid.

detected in pea seeds (Nomura et al., 2007) and the shoots of young pea seedlings (Symons et al., 2002), suggesting that either another enzyme capable of C-6 oxidation occurs in pea that is able to carry out the Baeyer-Villiger oxidation, or that in a different cellular environment, one of the two identified C-6 oxidation genes possesses the capacity for significant Baeyer-Villiger oxidation (BL synthase).

The isolation of *PsBR6ox1* and *PsBR6ox6* in pea not only emphasizes species-specific differences in the regulation of C-6 oxidation, but it also highlights the evolutionary history of the BR 6-oxidases, as shown in a phylogenetic tree constructed with *CYP85As* from several species (Fig. 6). *PsBR6ox1* is most closely related to *PsBR6ox6* (Fig. 6). This is also the case for *CYP85A* pairs in other dicot species, including Arabidopsis,



tomato, and poplar (*Populus trichocarpa*). This suggests that the CS synthase and BL synthase activities of BR 6-oxidases evolved independently in Arabidopsis and tomato. Further, in pea, one of the BR 6-oxidation genes, *PsBR6ox1*, has incipient BL synthase activity when expressed in yeast (Supplemental Fig. S2A). It will be interesting to determine the nature of the genetic changes needed for BL synthase activity to occur in addition to CS synthase activity. In contrast to the dicot species, only one BR 6-oxidase occurs in monocots.

In conclusion, the isolation of two BR C-6 oxidases with CS synthase activity in pea (*PsBR6ox1* and *PsBR6ox6*) has highlighted the species-specific differences that exist in relation to C-6 oxidation in the latter stages of BR biosynthesis. In addition, the characterization of *lke* has revealed a novel BR-deficient mutant that blocks BR biosynthesis after 6-DeoxoCS through a mutation in the start codon of the *PsBR6ox6* gene. To date, *lke* is the only mutant identified in pea that blocks the latter steps of BR biosynthesis and it will therefore provide an excellent tool to further examine the regulation of BR biosynthesis and the relative biological activities of CS and BL in pea.

## MATERIALS AND METHODS

### Plant Material and Growing Conditions

The pure lines of pea (*Pisum sativum*) used in this study were HL107 (wild type) and the single-gene BR mutant lines NGB5862 (semierectoides; *lkb*) and AF48 (semierectoides; *lke*). The *lke* mutant line was derived from pea cv Torsdag by mutagenesis with ethyl methanesulfonate and was shown to be nonallelic with the existing BR mutants. Seeds were germinated and grown three per pot in a heated glasshouse with the natural photoperiod extended to 18 h before dawn and after dusk with a 1:1 mixture of fluorescent (Sylvania 40 W cool-white tubes) and incandescent (Thorn 100 W pearl globes) lights. The photoperiod extension provided an irradiance of 25 to 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at the pot top. Glasshouse temperatures generally ranged from 13°C to 21°C during the coolest month and 17°C to 30°C during the warmest month. The average daily maximum temperature was 25°C. All node counts began from the cotyledons as zero.

### Isolation and Sequencing of Pea *CYP85A1* and *CYP85A6*

Primer sequences used for cloning the *CYP85A1* and *CYP85A6* genes from pea are shown in Supplemental Table S1. First primers were designed based on highly conserved nucleotide sequences between legume expressed sequence tags and the *CYP85A* genes of Arabidopsis (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*). PCR amplification was carried out using the Expand High Fidelity PCR system (Roche). Templates were from single-strand cDNA libraries that were made from immature seeds or 7-d-old shoots of pea (cv Torsdag). The resulting products were sequenced using Long-Read Tower sequencer (Amersham Biosciences). Based on those sequences, gene-specific primers were designed to amplify the 5' and 3' end of each gene. Primers listed in Supplemental Table S1 were used sequentially for 5'- and 3'-RACE reactions according to 5'/3' RACE kit (Roche). The resulting products were sequenced and these fragments were assembled to construct an open reading frame. Primers were designed to amplify the full-length cDNA, and the full-length clones were sequenced to check the PCR errors. Sequence analysis was performed using MacVector 7.1 software (Oxford Molecular). Amino acid sequences of the *CYP85A* family members were obtained from the Cytochrome P450 Homepage (<http://dmelson.utmem.edu/CytochromeP450.html>). The phylogenetic tree was constructed by neighbor joining with p-distance and bootstrap replication (1,000 replications) using MEGA version 3.1 software (<http://www.megasoftware.net/>).

### Functional Assay

The protein-coding regions of pea *CYP85A1* and *CYP85A6* were ligated into the *Bam*HI/*Kpn*I and *Bam*HI/*Eco*RI site of plasmid YeDP60, respectively. Those plasmids were transformed into yeast (*Saccharomyces cerevisiae*) WAT11 and WAT 21 strains. Two culture methods were used for this functional assay: Low Density Procedure that produces a high specific P450 content and High Density Procedure that produces a large amount of P450, according to Pompon et al. (1996). BR feeding and extraction were performed using the same method described by Nomura et al. (2005).

### Extraction, Purification, and GC-MS Quantification of BRs

For experiment 1, approximately 30 g of tissue was harvested from 26-d-old seedlings. The tissue harvested included the apical bud and the three internodes below, without any expanded leaf tissue. For experiments 2 and 3, approximately 70 g of tissue was harvested from whole shoots of 14-d-old and 25-d-old seedlings, respectively. Tissue was homogenized and extracted with 80% (v/v) methanol. Purification and quantification of endogenous hormones was carried out as previously described in Symons and Reid (2003).

### Extraction, Purification, and GC-MS Quantification of IAA and GA<sub>1</sub>

Endogenous IAA and GA<sub>1</sub> levels were measured in the oldest unexpanded internode (approximately 30% expanded at the time of harvest) of 25-d-old wild-type, *lke*, and *lkb* plants. Purification and quantification of IAA and GA<sub>1</sub> was carried out as previously described in Jager et al. (2005).

### BR Application

A total of 200 ng of BL, CS, or 6-DeoxoCS was applied (in 2  $\mu\text{L}$  of 100% ethanol) to the oldest unexpanded internode of 24-d-old *lkb*, *lke*, and wild-type plants, which was approximately 30% expanded at the time of application. Control plants were treated with 2  $\mu\text{L}$  of ethanol only. Internode length was measured prior to and 24 h after BR application. Growth was determined by the difference in internode length over the 24 h after BR application.

### Isolation and Sequencing of *lke*

Total RNA extraction was performed using the QIAquick RNeasy kit (Qiagen). Genomic DNA was removed with an on-column DNase digest during the RNA extraction, as per the manufacturer's protocol (Qiagen). Primers were designed to cover the entire coding regions of *PsBR6ox1* and *PsBR6ox6*. To specifically isolate *PsBR6ox2*, primers were based on differences between the two *PsBR6ox* genes. The primers used are listed in Supplemental Table S3. RT-PCR experiments were performed with 5 mg of total RNA with the GibcoBRL/Life Technologies SUPERScript III Preamplification system for first-strand cDNA synthesis. Standard PCR reactions (Qiagen) using Taq polymerase were subjected to a PCR program consisting of an initial denaturation at 94°C for 3 min and then 35 cycles of 94°C for 10 s, 54°C to 58°C for 20 s, and 72°C for 2 min, with a final extension step of 5 min at 72°C. PCR reactions were purified using the QIAquick PCR Purification kit (Qiagen). 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 kit (Beckman Coulter). Sequencing was performed on a capillary fluorescence Beckman Coulter Sequencer (Beckman Coulter Instruments). Sequence data analysis was achieved with Sequencher software and MacVector (Accelrys).

### Cosegregation Analysis on the Basis of PCR-RFLP

Genomic DNA was extracted from 5-mm<sup>2</sup> leaf sections of 26 wild-type and *lke* plants from the F<sub>2</sub> of a cross between lines AF48 (*lke*) and 212 (*lk*). The leaf sample was placed in an Eppendorf tube with a tungsten carbide bead and shaken in a bead mill for 1 min. A total of 140  $\mu\text{L}$  of extraction buffer (2 M NaCl; 0.2 M Tris, pH 8; 0.7 M Na<sub>2</sub>EDTA; 3.8 g L<sup>-1</sup> Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) and 40  $\mu\text{L}$  of 5% (w/v) sarcosyl were added to the sample and shaken for 1 min. The tissue was incubated at 60°C for 1 h and then centrifuged at 14,000 rpm for 15 min. The supernatant was removed to a new tube and equal volumes of ammonium

acetate and isopropanol were added to the supernatant. The samples were left at room temperature for 15 min and centrifuged at 14,000 rpm for 5 min. After removal of the supernatant, the pellet was rinsed in cold 70% (v/v) ethanol and dissolved in 100  $\mu$ L of Tris-EDTA. A 2- $\mu$ L aliquot was used in a 50- $\mu$ L PCR reaction. A 349-bp fragment covering the mutation was amplified using the forward primer BR6ox6F<sub>1</sub> and reverse primer BR6ox6R<sub>cs</sub> (Supplemental Table S3) and then digested with *AluI* (Promega). The digested products were separated on a 2.0% agarose gel and visualized by ethidium bromide staining.

### PsBR6ox1 and PsBR6ox6 Expression Studies

Sequence data of *PsCPD1* (CYP90A9), *PsCPD2* (CYP90A10), *PsDWF4* (CYP90B8), and *PsBAS1* (CYP734A11) used in these expression studies were obtained from Nomura et al. (2007).

### RNA Extraction, Quantification, and Integrity

Shoot tissues were harvested and frozen in liquid nitrogen. Three replicates of three plants were harvested. RNA was extracted from approximately 100 mg of frozen tissue using Qiagen's Plant RNEasy mini kit as per the manufacturer's instructions and included the on-column DNase digestion. RNA concentration was measured using a Bio-Rad UV Smart Spec, and RNA concentration and integrity was confirmed by running 5  $\mu$ g of RNA on a 1% denaturing formaldehyde gel.

### cDNA Synthesis and Semiquantitative, Real-Time RT-PCR Conditions and Analysis

RT was performed on 1  $\mu$ g of RNA using Qiagen's Quantitect RT kit according to the manufacturer's protocol, using the supplied mix of random hexamers and oligo(dT). Semiquantitative, real-time RT-PCR reactions were carried out on 20 ng equivalent RNA in duplicate. Reactions contained 5  $\mu$ L iQ SYBR Green 2 $\times$  Supermix (Bio-Rad) and 500 nM primer in a final volume of 10  $\mu$ L. The reaction profile was an initial denaturing at 95°C for 5 min, followed by 40 cycles of 95°C for 5 s and 60°C for 40 s, followed by a melt from 72°C to 95°C. The reactions were carried out on a Rotorgene 3000 (Corbett Research). Initial experiments confirmed the presence of a single amplicon by gel electrophoresis and melt curve analysis. The identity of the PCR amplicons was confirmed by sequencing with Dye Terminator Cycle Sequencing kit (Beckman-Coulter). The primers used are listed in Supplemental Table S4.

### Data Analysis

The threshold cycle was determined using the Rotorgene 3000 software. Standard curves over 4 orders of magnitude, with cDNA pooled from the experiment as the template, were imported into each run to determine the relative (not absolute) concentrations.

Four housekeeping genes were used for normalization: actin (Foo et al., 2005), ubiquitin (Albrecht et al., 1998), EF-1 (Foucher et al., 2003), and 18sRNA (Ozga et al., 2003). BESTKeeper (Pfaffl et al., 2004) determined that all four housekeeping genes were appropriate genes to use for normalization. The housekeeping gene concentrations were adjusted so that the first sample had a relative concentration of 1 and each housekeeper made an equal contribution to a normalizing index (mean of all four genes) for each sample.

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank data libraries under accession numbers AB218759 (pea CYP85A1) and AB218760 (pea CYP85A6).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Functional assay of pea CYP85A1 and Arabidopsis CYP85A2 in yeast WAT11 and WAT21 strains.

**Supplemental Figure S2.** BL production from 6-DeoxoCS by pea CYP85A1 and Arabidopsis CYP85A2.

**Supplemental Table S1.** Primer sequences used for cloning CYP85A1 and CYP85A6 from pea.

**Supplemental Table S2.** GC-MS identification of CS converted from 6-DeoxoCS in the recombinant yeast producing pea CYP85A1 or CYP85A6.

**Supplemental Table S3.** Primer sequences used for cloning *lke* from pea.

**Supplemental Table S4.** Primer sequences used for semiquantitative, real-time RT-PCR.

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