LATERAL BRANCHING OXIDOREDUCTASE acts in the final stages of strigolactone biosynthesis in *Arabidopsis*

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Strigolactones are a group of plant compounds of diverse but related chemical structures. They have similar bioactivity across a broad range of plant species, act to optimize plant growth and development, and promote soil microbe interactions. Carlactone, a common precursor to strigolactones, is produced by conserved enzymes found in a number of diverse species. Versions of the MORE AXILLARY GROWTH1 (MAX1) cytochrome P450 from rice and *Arabidopsis thaliana* make specific subsets of strigolactones from carlactone. However, the diversity of natural strigolactones suggests that additional enzymes are involved and remain to be discovered. Here, we use an innovative method that has revealed a missing enzyme involved in strigolactone metabolism. By using a transcriptomics approach involving a range of treatments that modify strigolactone biosynthesis gene expression coupled with reverse genetics, we identified *LATERAL BRANCHING OXIDOREDUCTASE* (*LBO*), a gene encoding an oxidoreductase-like enzyme of the 2-oxoglutarate and Fe(II)-dependent dioxygenase superfamily. *Arabidopsis lbo* mutants exhibited increased shoot branching, but the *lbo* mutation did not enhance the *max* mutant phenotype. Grafting indicated that LBO is required for a graft-transmissible signal that, in turn, requires a product of MAX1. Mutant *lbo* backgrounds showed reduced responses to carlactone, the substrate of MAX1, and methyl carlactonoate (MeCLA), a product downstream of MAX1. Furthermore, *lbo* mutants contained increased amounts of these compounds, and the LBO protein specifically converts MeCLA to an unidentified strigolactone-like compound. Thus, *LBO* function may be important in the later steps of strigolactone biosynthesis to inhibit shoot branching in *Arabidopsis* and other seed plants.

plant | branching | strigolactone | biosynthesis | *Arabidopsis*

Strigolactone synthesis from β-carotene involves 9-*cis*/all-*trans*-β-carotene isomerase, carotenoid cleavage dioxygenase 7 (CCD7), and CCD8 (reviewed in ref. 1). These three proteins can sequentially convert β-carotene into carlactone (CL), an intermediate molecule so far detected in root extracts from both rice and *Arabidopsis* (2, 3). In *Arabidopsis*, the isomerase is encoded by *DWARF27* (*D27*), whereas CCD7 and CCD8 are encoded by *MORE AXILLARY GROWTH 3* (*MAX3*) and *MAX4*, respectively (Fig. 1).

In rice, conversion of CL to the four-ringed canonical strigolactone structure involves the addition of two oxygen atoms to C19 and closure of the B and C rings to produce 4-deoxyorobanchol (4DO; previously known as *ent*-2′-*epi*-5-deoxystrigol) (Fig. 1). This reaction is catalyzed by a member of the MAX1 family of cytochrome P450 proteins (4, 5). A second MAX1 enzyme oxidizes 4DO to make orobanchol (4). In *Arabidopsis*, the situation is less clear, because it has only a single *MAX1* gene, and the encoded protein oxidizes C19 of CL to produce carlactonoic acid (CLA), but it does not apparently catalyze formation of B and C rings (3, 4, 6) (Fig. 1). These studies reported endogenous CLA and methyl carlactonoate (MeCLA), the production of the latter requiring an unknown methyl transferase (6) (Fig. 1). Based on interaction with receptor D14, it was proposed that MeCLA is bioactive, whereas CL and CLA are not bioactive (6). Grafting experiments in *Arabidopsis* suggest that both the upstream and downstream products of *MAX1* are graft-transmissible: that is, the *MAX1* substrate and products can move from the rootstock to the shoot (5). This finding implies that the graft-transmissible mobile products in *Arabidopsis* could be CL- and CLA-related products. Previous reports of other strigolactones in *Arabidopsis* (7, 8) have not been repeated (3, 6).

Strigolactones are thought to be perceived and cleaved by an α/β-fold hydrolase (AtD14 in *Arabidopsis*), possibly within a receptor

Significance

Strigolactone hormones regulate many plant growth and developmental processes and are particularly important in regulating growth in response to nonoptimal conditions. Plants produce a range of bioactive strigolactone-like compounds, suggesting that the biosynthesis pathway is complex. Despite this complexity, only one type of enzyme, the MORE AXILLARY GROWTH1 (MAX1) cytochrome P450, has been attributed to the diversity of strigolactones. Using transcriptomics and reverse genetics, we discovered a previously uncharacterized gene that encodes a 2-oxoglutarate and Fe(II)-dependent dioxygenase involved in strigolactone production downstream of MAX1. Studies with the corresponding mutant have shown that previously identified strigolactone-type compounds in *Arabidopsis* are not the major strigolactone-type shoot branching hormone in this model species.

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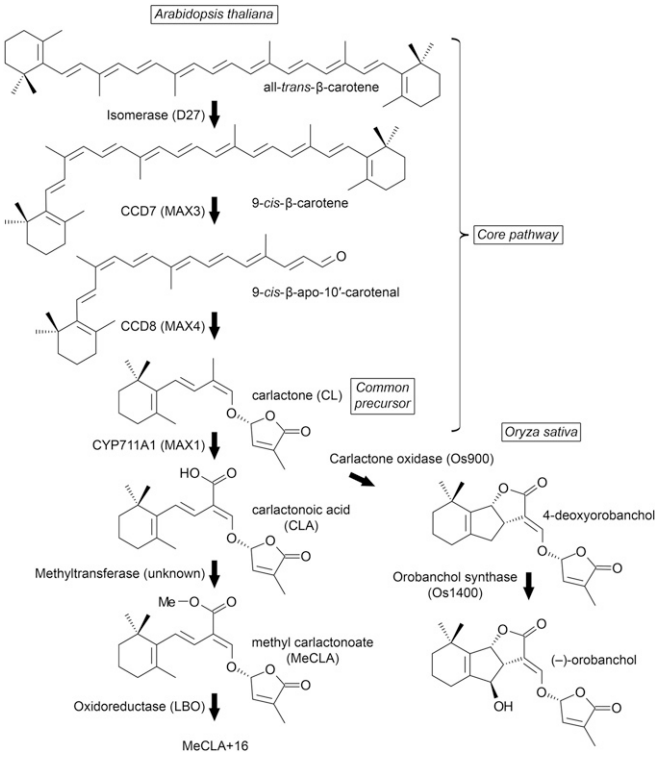

Fig. 1. The strigolactone biosynthesis pathway in *Arabidopsis thaliana*. An isomerase and two CCD enzymes convert β-carotene into CL, the common precursor of diverse strigolactones. In rice, CL is oxidized by two cytochrome P450 enzymes to orobanchol, which functions in the rhizosphere as a signal for mycorrhizal fungi. In *Arabidopsis*, CL is oxidized by the MAX1 cytochrome P450 to CLA, which is converted to MeCLA. Evidence presented in this study suggests that LBO facilitates additional processing to an unknown strigolactone-like product (MeCLA + 16 Da), which is required for the control of shoot branching.

complex that may include an F-box protein (MAX2 in *Arabidopsis*) (reviewed in ref. 1). The active complex targets proteins for degradation, such as SMAX1-like proteins (SMXL6–SMXL8), and promotes expression of transcription factor genes, such as *BRANCHED1*, directly in buds (9–13). The active complex is unusual in that binding with strigolactone triggers destabilization and degradation of the D14 receptor and cleavage of the strigolactone compound (14–16). Negative feedback on some strigolactone biosynthesis genes has been observed in species, including pea and *Arabidopsis* (reviewed in ref. 17).

Forward genetic screens identifying increased branching mutants in several species have led to identification of all of the strigolactone biosynthesis and signaling pathway components to date (reviewed in ref. 18). It is likely that additional enzymes are yet to be discovered because of the diversity of strigolactones observed in plants. Strigolactones of variable structure have recently been isolated from rice (orobanchyl acetate, 7-oxoorobanchyl acetate, and three methoxy-5-deoxystrigol isomers), oats (avenaol), and sunflower (heliolactone) (19–21). If additional enzymes are required for bioactive strigolactones, the genes involved would prove difficult to discover using forward genetics if the corresponding mutants had weak phenotypes and/or enzyme redundancy.

A common feature of *MAX3* and *MAX4* genes in *Arabidopsis* is that their expression is increased in strigolactone biosynthesis and perception mutants, and their transcript levels alter with changes in auxin levels and/or auxin treatments (17). Therefore, we postulated that additional novel strigolactone biosynthesis genes, if present, may be coexpressed with these strigolactone biosynthesis genes under specific conditions. Thus, we used an *Arabidopsis* microarray to identify candidate genes followed by reverse genetics to discover new branching mutants. Here, we describe a previously uncharacterized branching gene in *Arabidopsis*, *LATERAL BRANCHING OXIDOREDUCTASE* (*LBO*), which encodes an oxidoreductase-like enzyme of the 2-oxoglutarate and Fe(II)-dependent dioxygenase (2OGD) superfamily and is represented widely across seed plant species. Our analysis suggests that *LBO* plays an essential role in the production of strigolactone-like compounds downstream of *MAX* genes and CL. This discovery will be vital for identifying additional bioactive strigolactones and increasing our understanding of the components and regulation of the strigolactone biosynthesis pathway and hence, the levels and functions of strigolactones in plants.

Results

Transcriptomics and Reverse Genetics Reveal a Novel Branching Gene. To identify gene(s) involved in strigolactone biosynthesis in *Arabidopsis*, we analyzed changes in gene expression in eight conditions using an *Arabidopsis* microarray. Compared with untreated WT plants, two conditions decreased the expression of *MAX3*: removal of the shoot tip (decapitation) and treatment with an auxin transport inhibitor (*N*-1-naphthylphthalamic acid). Five additional conditions enhanced *MAX3* gene expression: mutants of *max1*, *max2*, *max3*, and *max4* as well as the WT decapitated and treated with auxin (indole-3-acetic acid) (Fig. 2*A*).

Hypocotyl tissue just before bolting was selected for expression analysis, because cell expansion and growth have been completed in this tissue. Furthermore, the hypocotyl is physiologically relevant: it has been shown as a source of the branching inhibition signal, and changes in expression of strigolactone genes have been observed in this tissue (5, 17). Using the Cluster Analysis of Sequences bioinformatics program (22), we were able to identify genes that showed appreciable coexpression with *MAX3*. This analysis led to the identification of the *D27* gene, which we subsequently reported (23), and *LONELY GUY1* (At2g28305), which is involved in the control of shoot branching through a role in cytokinin metabolism (Fig. 2*A*) (24). *D27* had not been previously identified from forward genetic screens in *Arabidopsis*. The analysis also identified an uncharacterized gene At3g21420, which we named *LBO* based on gene phylogeny (Fig. 2) and mutant phenotype (Fig. 3).

The predicted LBO amino acid sequence is similar to 2OGDs, which comprise a family of around 130 members in *Arabidopsis* (25). Related 2OGDs are involved in the biosynthesis of a range of plant hormones and secondary metabolites (26). Like other 2OGDs, LBO has a predicted oxoglutarate/iron-dependent dioxygenase catalytic domain near the C terminus that contains a double-stranded β-helix core fold (Fig. 2*B*). Based on the crystal structure of similar proteins, the LBO catalytic domain contains hallmark amino acid residues His-235, His-293, and Asp-237 for iron binding and Arg-303 for substrate interaction (27–29).

Phylogenetic analyses suggest that *LBO* exists as a highly conserved and mostly single-copy gene in seed plant species (25) (Fig. 2*C*). LBO falls within the DOXC54 clade of 2OGDs, and sequences from the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* do not group in this clade (25), suggesting that the DOXC54 clade evolved after the divergence of lycophyte and seed plant lineages. Clades that cluster near DOXC54 display more diversity, and their functions are generally unknown (25) (Fig. 2*C*). The closest sequence with reported enzymatic function is ETHYLENE-FORMING ENZYME from tomato, which converts 1-aminocyclopropane-1-carboxylic acid to ethylene (Fig. 2*C*) (30).

Analysis of *lbo* Mutants. We identified one transfer-DNA (T-DNA) insertion mutant in At3g21420; FLAG_119G09 from the Versailles *Arabidopsis* Stock Center (31). This line is in the Wassilewskija ecotype (Ws-4) and carries a T-DNA in the second exon of At3g21420 (*lbo-1*) (Fig. 2*B*). Two additional mutant alleles

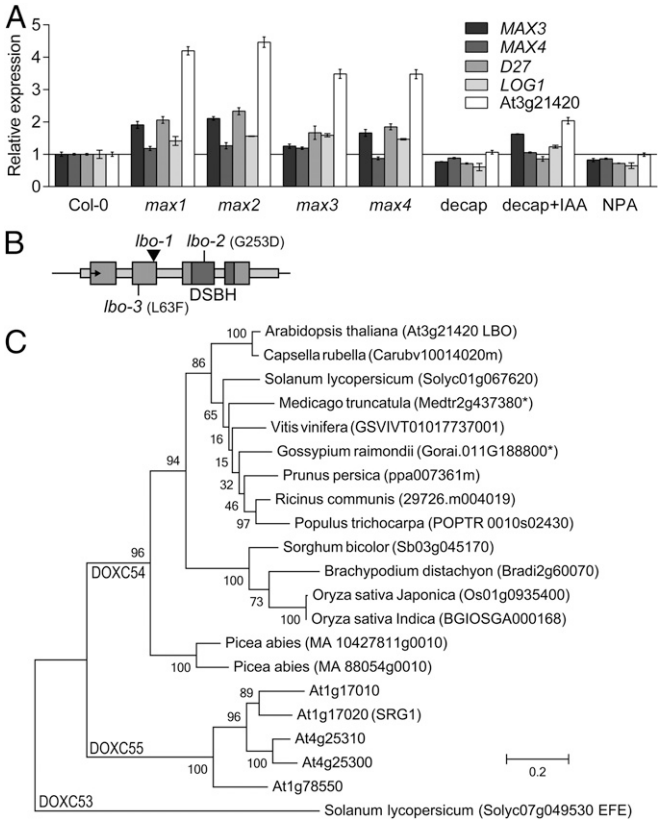

Fig. 2. Discovery of the *LBO* gene by reverse genetics. (*A*) Expression from microarray data revealed genes that followed the distinctive pattern (41) of the *MAX3* gene in hypocotyls dissected from soil-grown WT, *max* mutants, decapitated WT (decap) with or without apical auxin [indole-3-acetic acid (IAA)] added, or WT with *N*-1-naphthylphthalamic acid (NPA) applied locally beneath the rosette. This group included *D27*, *LONELY GUY1* (*LOG1*), and an uncharacterized gene At3g21420 (*LBO*). Microarray expression values are relative to untreated Col-0. Mean ± SEM (n = 2–3; 120–150 hypocotyls per replicate). (*B*) Diagram representing the DNA sequence of the *LBO* locus. The arrow points to the start codon, exons are large boxes, and introns and untranslated regions are narrow boxes. The promoter region spans 3,627 bp to the next gene, and 1,711 bp were used for GUS expression. The positions of the FLAG_119G09 line T-DNA insert (*lbo-1*; arrowhead), other point mutation alleles (*lbo-2* and *lbo-3*), and the double-stranded β-helix conserved catalytic domain (DSBH) are shown. (*C*) A phylogenetic tree of 2OGD amino acid sequences from 2OGD clade DOXC54 (25) from prominent species reveals the similarity of At3g21420 (LBO). For reference, the *Arabidopsis* sequences from the next nearest clade (DOXC55) and the nearest similar sequence with reported functional characterization, ETHYLENE FORMING ENZYME (EFE) from tomato (*Solanum lycopersicum*) in clade DOXC53, are included. Bootstrap values are shown for each branch. Units are amino acid substitutions per site. *Existence of a splice variant.

[*lbo-2* and *lbo-3*; Columbia-0 (Col-0) *erecta* background] were isolated from the University of California, Davis TILLING Core (32). These alleles contain a nonconservative change in the protein sequence; in *lbo-2*, amino acid G253 was changed to D (nucleotide G1200 to A), and in *lbo-3*, amino acid L63 was changed to F (nucleotide C187 to T) (Fig. 2*B*). Homozygous mutant plants exhibited increased rosette branches compared with their respective WTs (Fig. 3 *A* and *B* and Fig. S1*A*). The T-DNA in *lbo-1* would be expected to severely shorten the predicted protein sequence of LBO because of a truncation before the putative double-stranded β-helix catalytic oxygenase domain. The *lbo-3* mutant displays a weaker branching phenotype compared with *lbo-2* (Fig. S1*A*). This finding indicates that the *lbo-3* allele, despite altering a highly conserved amino acid, may not fully disrupt LBO function (Fig. 2*B*).

The increased branching phenotype of *lbo-1* and *max4-9* was mainly observed in the middle nodes of the rosette (Fig. 3*C*). These branches occurred less frequently in *lbo* compared with *max4* (Fig. 3*C*). This subtle but significant phenotype of *lbo* mutants would probably not have been observed in a forward genetics screen. Independent *lbo-1* lines constitutively expressing *LBO* using the cauliflower mosaic virus 35S promoter showed WT levels of branching (Fig. S1*B*). These findings show that *LBO* is required for branching inhibition. Similar to strigolactone biosynthesis genes (5, 23, 33, 34), overexpression of *LBO* was not able to decrease branching below WT levels (Fig. S1*B*).

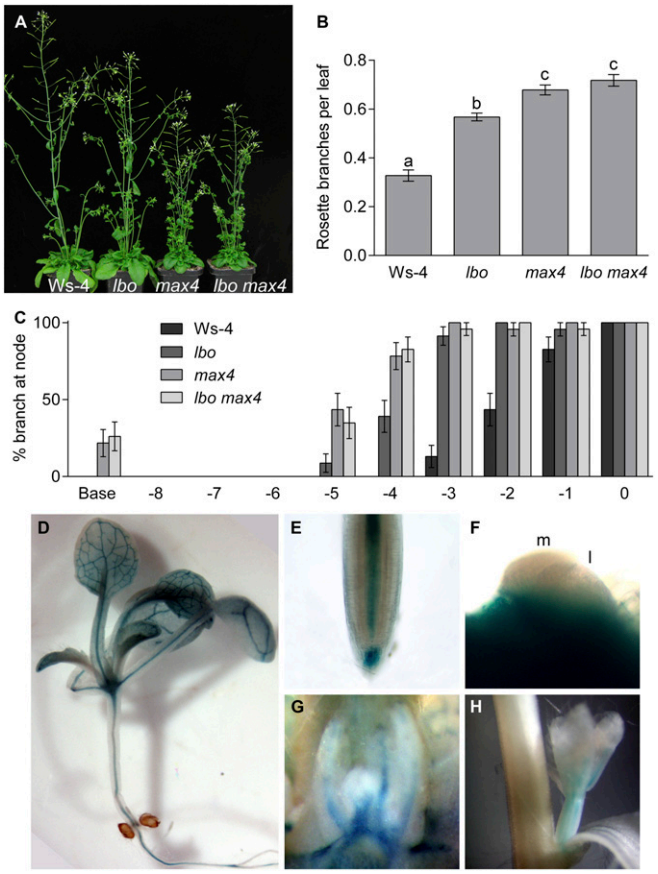

Fig. 3. Mutant phenotype and reporter gene expression of *LBO*. (*A*) The *lbo-1* mutant shoot branching phenotype was intermediate between those of *max4-9* and the Ws-4 WT. The *lbo-1 max4-9* double mutant appeared most similar to *max4-9*. (*B*) These relationships were reflected in the average number of branches (>5 mm) per rosette leaf. Values with different letters are significantly different from each other ($P < 0.05$; t test). Mean ± SEM (n = 23–24). (*C*) Careful examination of the occurrence of a branch in each leaf axil (node) was carried out, with the highest rosette node denoted as zero, counting backward down the rosette. This analysis revealed extra branching in the middle rosette nodes in *max4-9* and *lbo-1 max4-9* plants and mainly the upper middle nodes in *lbo-1* compared with in the WT (Ws-4). Occasionally, branches were seen at the extreme base of the rosette, but it was not possible to determine the precise location. (*D*) To further characterize *LBO*, we generated a pLBO::GUS construct using 1,711 bp of the promoter and examined expression in whole-mount samples (Col-0). Representative lines showed GUS expression in the vasculature throughout the plant. (*E*) Expression extended into the stele of the root tip and was also seen in the root cap and weakly in the epidermis. (*F*) The meristem (m) and tiny leaves (l) of repressed buds from lower nodes did not show expression (although high expression occurred in leaf vasculature just below these tissues). (*G*) Larger repressed buds of higher nodes showed expression in the vasculature that reaches up into the four or five immature leaves/flowers. (*H*) In the inflorescence bolt, most GUS expression was observed specifically in the vasculature of cauline branches and not in the main stem.

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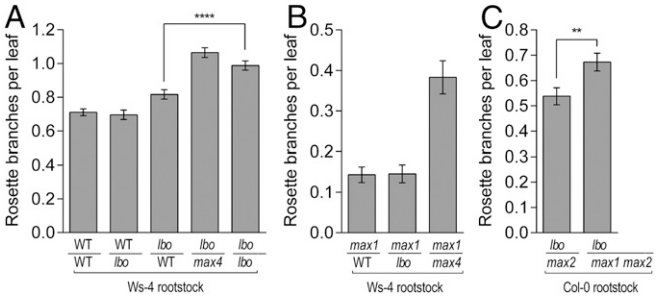

Fig. 4. Grafting with *lbo-1*. (*A*) *lbo* rootstock did not promote branching in Ws-4 shoots. Ws-4 rootstock repressed *lbo* shoot branching nearly to Ws-4 self-graft levels, whereas *max4-9* rootstock did not repress *lbo* shoots. These grafting results mean that the normal enzymatic action of LBO in Ws-4 roots can produce an upwardly mobile product that can repress branching (although not completely) and that this product requires MAX4 enzyme action (i.e., MAX4 is upstream of LBO). Mean ± SEM (n = 19–36). ****P < 0.0001 (t test). (*B*) *lbo* rootstock represses branching in *max1-1* (background Col-0) mutant shoots to the same extent as Ws-4 rootstock compared with *max4-9*. This inhibition by *lbo* rootstocks means that the enzyme action of MAX1 in *lbo* mutant rootstock produces a mobile product that can fully restore *max1* branching and that this product requires MAX4 action. Mean ± SEM (n = 15–20). (*C*) The *max2-1* rootstock reduced branching in the *lbo-1* mutant shoot compared with *max1-1 max2-1* double-mutant rootstock. This finding suggests that LBO (in *max2*) can produce a mobile branching inhibitor and that this product requires MAX1 action upstream of LBO. The rationale for using *max2-1* (in Col-0) rootstock here was that impaired signaling is expected to increase production of strigolactones and mobile intermediates (17) and that *max1* and *max2* were not available in the Ws-4 ecotype. Mean ± SEM (n = 20–23). **P < 0.01 (t test).

To determine the localization of LBO within the plant, *LBO* reporter gene lines expressing β-glucuronidase (GUS) were generated. Lines expressing pLBO::GUS suggest that *LBO* is expressed in the vasculature throughout the plant and in the buds and root tips (Fig. 3 *D*–*H*). Other genes involved in strigolactone production and response are also expressed in vascular tissue (5, 34, 35).

LBO May Act as a Strigolactone Biosynthesis Enzyme Downstream of MAX1. To elucidate the role of LBO in the strigolactone pathway, we made *lbo max4* double mutants. The phenotype of *lbo max4* double-mutant plants seemed similar to that of *max4* single mutants (Fig. 3 *A*–*C*), implying that *LBO* may act in the same pathway as *MAX4*.

Previously, grafting has shown that strigolactone biosynthesis occurs in both the roots and shoots and that the strigolactone signal moves upward in plants (36). Therefore, to further understand the role of LBO, grafting experiments with WT and other strigolactone mutants were carried out. Grafting *lbo* onto the WT (Ws-4) rootstocks significantly reduced branching in *lbo* mutant shoots (Fig. 4*A*). Branching of WT (Ws-4) shoots was not affected by grafting onto *lbo* rootstocks, which is similar to grafting results found with other strigolactone mutants (36–38). Thus, grafting results together with LBO expression patterns suggest that LBO acts in the root and shoot to control a long-distance signal that inhibits branching.

Previous studies showed that mutant *max1* rootstocks can fully restore branching to *max3* or *max4* mutant shoots but that *max3* and *max4* rootstocks cannot inhibit branching in *max1* shoots (5). This grafting shows that the MAX1 enzyme acts downstream of MAX3 and MAX4. In addition, it confirms that MAX1 acts on a mobile substrate produced by MAX3 and MAX4. Using the same logic, our reciprocal grafting suggests that LBO acts downstream of MAX4 and MAX1. Mutant *lbo* rootstocks fully rescued branching in *max1* shoots (Fig. 4*B*). However, neither *max1* nor *max4* rootstocks could reduce branching in *lbo* shoots, despite these rootstocks having the LBO enzyme (Fig. 4*C*). The LBO enzyme may, therefore, be required to convert a mobile product of MAX1 to a product that is either a branching inhibitor or further converted into one.

If LBO acts downstream of MAX1 in strigolactone biosynthesis, then branching in *lbo* mutants should be inhibited by canonical strigolactones but possibly not by CL, which is produced after the action of D27, MAX3, and MAX4 but before MAX1 activity (Fig. 1); *max1* mutants respond to synthetic strigolactone (*rac*-GR24) and not CL (6, 39). As expected, when *rac*-GR24 or CL was supplied hydroponically, branching in *max4* shoots was reduced (Fig. 5*A* and Fig. S2). The *lbo* mutants also responded to *rac*-GR24 in a concentration-dependent manner (Fig. 5*B*). As predicted, similarly to *max1*, there was no significant response to CL in *lbo* single mutants (Fig. S2) or *lbo max4* double-mutant plants (Fig. 5*A*). Gene expression experiments also indicate that *max4* and *lbo* do not differ in strigolactone sensitivity. Gene expression responses to 5-deoxystrigol or 4DO were not different among WT, *lbo*, and *max4* lines (Fig. S3).

Arabidopsis MAX1 has been shown to convert CL into CLA, and then, CLA is thought to be converted by another enzyme into MeCLA (6) (Fig. 1). To clarify further that LBO acts downstream of MAX1, we examined the level of CL and MeCLA in the *lbo* mutant. If LBO acts downstream of MAX1, we might expect one or both of these compounds to be increased in *lbo* mutant plants. Indeed, *lbo* mutant backgrounds had increased levels of CL and MeCLA (Fig. 5*C* and Figs. S4 and S5).

Because *lbo* mutants have increased levels of MeCLA, we predict that the LBO enzyme acts downstream of MeCLA and that it should, therefore, have a reduced response to this compound. We treated *max4* and *lbo max4* double mutants with MeCLA to determine if MeCLA could reduce branching. The results show that MeCLA slightly but significantly reduced branching in *max4* mutants but that it had no effect on the *lbo max4* double mutant (Fig. 5*D*). The differences in the response of *max4* to CL and MeCLA are most likely caused by CL being

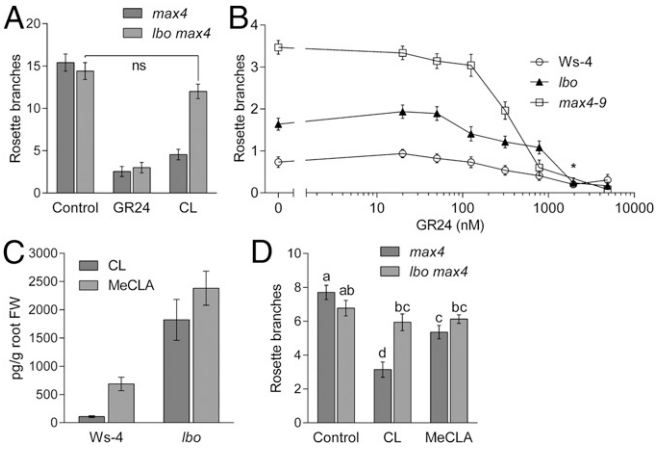

Fig. 5. Strigolactone responses and quantification in *lbo-1*. (*A*) Branching in *max4-9* was reduced with 1 μM *rac*-GR24 or 1 μM CL supplied hydroponically. In contrast, combining the *lbo-1* mutation with *max4-9* as a double mutant specifically inhibits just the response to CL (P > 0.05; t test). Mean ± SEM (n = 10–12). Ns, Not significant. (*B*) *lbo-1* branching responded similarly to Ws-4 across a dose range of *rac*-GR24 added to agar growth medium, suggesting that *lbo* is not defective in strigolactone response. *No *max4-9* data at this concentration. Mean ± SEM (n = 10–34). (*C*) CL and MeCLA were increased in *lbo-1* mutant root tissue extracts compared with the WT (Ws-4). No canonical strigolactones were detected in any *Arabidopsis* sample. Mean ± SEM (n = 20). FW, fresh weight. (*D*) Ten micromolar CL or MeCLA reduced branching in *max4-9* but not in the *lbo-1 max4-9* double mutant. Values with different letters are significantly different from each other (P < 0.05; t test). Mean ± SEM (n = 10–16).

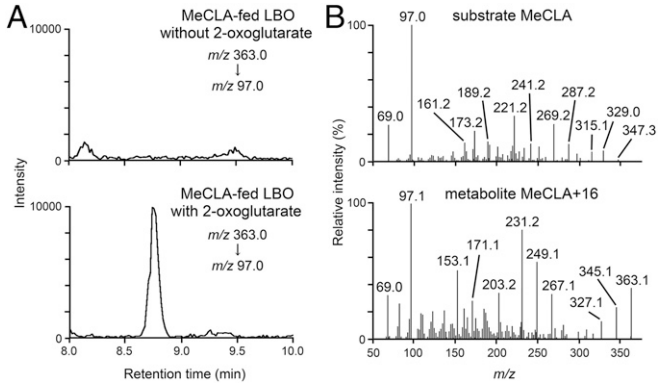

Fig. 6. Conversions of MeCLA to MeCLA + 16 Da (MeCLA+16) by recombinant LBO protein synthesized in *E. coli*. MeCLA was incubated with LBO protein for 15 min, and the extracts were analyzed using electrospray ionization (ESI)-positive mode liquid chromatography–MS/MS. (*A*) Selected reaction monitoring chromatograms for MeCLA-fed LBO (*Upper*) without or (*Lower*) with 2-oxoglutarate. (*B*) Full-scan spectra of product ions from (*Upper*) substrate MeCLA and (*Lower*) metabolite MeCLA+16. The retention times of MeCLA and MeCLA+16 are 15.9 and 8.7 min, respectively.

more stable than MeCLA, thus contributing to the greater inhibition observed with CL than with MeCLA. Nevertheless, these results coupled with the increased endogenous levels of MeCLA in the *lbo* mutants suggest that MeCLA is not very effective at inhibiting branching in plants. This finding is in contrast with a previous report suggesting that MeCLA is the bioactive strigolactone for shoot branching in *Arabidopsis* (6). Although MeCLA interacts with D14, indicating potential importance in strigolactone signaling, this study reveals that MeCLA requires the presence of LBO to be an effective branching inhibitor.

The enzymatic action of LBO was investigated by incubating the soluble protein of *Escherichia coli* cells expressing the LBO recombinant protein (Fig. S6). Protein extracts were incubated with CL, CLA, or MeCLA, and their levels were monitored by liquid chromatography–MS/MS; comparison was made with protein extracts from cells containing the empty vector (Fig. S7) and with or without 2-oxoglutarate (Fig. 6*A*). Levels of MeCLA were reduced within 15 min in the presence of LBO (Fig. 6*A*), whereas levels of CL or CLA were unaffected by LBO (Fig. S7). An ion $[M+H]^+$ of *m/z* 363 was consistently observed from reactions with MeCLA, which correlates to MeCLA + 16 Da (Fig. 6*B*), and as expected for a 2OGD enzyme, the production of MeCLA + 16 Da required 2-oxoglutarate (Fig. 6*A*). The observed mass of MeCLA + 16 Da suggests that LBO has added an oxygen to MeCLA. However, identification of this compound has not yet been possible because of its high instability.

Discussion

Our microarray study was designed to discover potential strigolactone-related genes by coexpression with *MAX3*. We identified *LBO* as a gene with an expression pattern similar to *MAX3* (Fig. 2*A*). The sequence identity of LBO, as an oxidoreductase-like enzyme, made it a good candidate for involvement in hormone biosynthesis. Mutational analysis and reciprocal grafting with strigolactone mutants suggest that LBO likely acts toward the end of the strigolactone biosynthesis pathway, after *MAX1* which is required for CLA production. This suggestion is supported by observation of elevated levels of CLA and MeCLA in the *lbo* mutant (Fig. 5*C*) but not in *max1* (6). Moreover, the *lbo* mutation causes a reduced response to CL and MeCLA but not to canonical strigolactones (Fig. 5*D*). The LBO protein consumes MeCLA but not CL or CLA and converts MeCLA to an unidentified strigolactone-like compound (Fig. 6). Together, these findings suggest a function of LBO in the strigolactone pathway downstream of MAX1 and MeCLA to produce a strigolactone-type signal(s) involved in shoot branching.

The *lbo* mutant may lack production of only a subset of strigolactones, because it can still produce MeCLA and at increased levels (Fig. 5*C*). This hypothesis would explain why the *lbo* mutant shows a relatively weak branching phenotype compared with *max* mutants and validates our approach for finding the additional strigolactone biosynthesis genes by screening gene expression. The weaker phenotype of *lbo*, despite increased MeCLA, implies that endogenous MeCLA is not very effective at repressing branching. In addition, the weak response of *lbo* mutant shoots to WT rootstocks (Fig. 4) suggests that the product(s) of LBO enzyme action may be unstable and/or poorly transported. Thus, the endogenous, LBO-dependent signals that inhibit branching may alter or enhance signaling specificity and localize activity, making it more effective at inhibiting branching compared with MeCLA. Moreover, the existence of CLA and LBO in rice (6, 25) implies that LBO-dependent signal(s) might be important for branching in a broad variety of plant species.

The 2OGD enzymes are extremely versatile and catalyze a wide range of reactions, including oxidations and hydroxylations (26). The products of LBO are not yet known, but detection of a MeCLA + 16 Da compound (Fig. 6) may mean that LBO oxidizes MeCLA to produce an active but unstable MeCLA-related compound and/or that the MeCLA + 16 Da product is further converted to a strigolactone-like compound by LBO and/or other enzymes. Recently, there have been several strigolactones with noncanonical structure found, such as avenaol (20) and heliolactone (21), and the LBO product might be related to those compounds. Moreover, CLA was detected in rice roots (6), and therefore, an MAX1 homolog(s) and LBO may also act to produce such strigolactone-like compounds in rice. Thus, the LBO-dependent signal may represent one of the strigolactones so far identified in rice (19), or perhaps, a completely novel compound. Our studies have not yet revealed compounds from plant tissue that are consistent with the MeCLA + 16 Da LBO product. However, strigolactones are difficult to detect, because they exist in very low concentrations. The effects of high instability and localized strigolactone production combined with strong feedback might render the product of LBO extremely difficult to detect in plants. The most important next step will be to try to prepare enough of this compound in vitro to identify it and then, chemically synthesize it.

The discovery of LBO as a 2OGD most likely involved in strigolactone production is an important step forward for fully elucidating the strigolactone biosynthesis pathway. Additional studies into the precise function of LBO are expected to resolve outstanding questions and provide knowledge and insight into how strigolactones function to regulate a wide range of traits in plants. This study highlights the possibility that multiple endogenous strigolactones within a single species may induce strigolactone signal transduction and response. Future studies should test whether different strigolactones regulate different processes within a single species.

Experimental Procedures

Plants were grown in standard conditions as described (40). ATH1 gene expression chip (Affymetrix) was used for transcriptomics, and after normalizing and filtering, Cluster Analysis of Sequences (41) was used to detect genes with similar expression patterns. Phylogenetic analysis was made using MEGA (42). *Arabidopsis* lines were from laboratory stocks, except *lbo* mutants, which were identified using SIGnAL (43) and provided by the French National Institute for Agricultural Research (INRA) in the Ws-4 ecotype (31) or identified and provided by Davis TILLING in the Col-0 *erecta* background (32). The *max4-9* allele is from the Ws-4 ecotype (44). Branching was defined as total rosette branches >5 mm. Constitutive and reporter transgenes were made using the pMDC162 and pMDC32 binary vectors (45). *Arabidopsis* was transformed by floral dip method (46) and whole-mount GUS staining was as described (47). The abutting method was used for

Arabidopsis grafting (48). Hormones were applied by direct treatment (Fig. 5*D*) (49), through hydroponics (Fig. 5*A* and Fig. S2) (39), in Phytatrays (Sigma-Aldrich) (Fig. 5*B*) (40), or by pipetting solutions directly onto seedlings lying flat on agar (Fig. S3). Strigolactones were produced with the following methods: *rac*-GR24 (50), 5-deoxystrigol (51), 4DO (51), CL (39), and MeCLA (6). Decapitation, auxin, and *N*-1-naphthylphthalamic acid treatments were as described (17). Differences in the level of branching between experiments can be caused by the ecotype that was used or the growth medium and conditions, which are described in the figures and *SI Experimental Procedures*. Quantitative RT-PCR was essentially performed as described (23) using primers listed in Table S1. Analysis of CL and MeCLA from *Arabidopsis* root tissues was performed as previously reported (6). LBO protein was expressed in *E. coli* using the pOPIN-E vector (Oxford Protein Production Facility U.K.) with a polyhistidine tag at the N terminus, and protein was purified by affinity column chromatography for histidine-tagged proteins (His GraviTrap; GE Healthcare). LBO protein reactions were based on the reported method (52) and analyzed by liquid chromatography–MS/MS as described (6). Additional details are provided in *SI Experimental Procedures*.

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