Hydroxyl carlactone derivatives are predominant strigolactones in *Arabidopsis*

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26 Author contributions

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30 M.U., and C.B. analyzed data; and Kaori Yoneyama, K.A, P.B., and Koichi Yoneyama wrote
31 the manuscript.

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ABSTRACT

Strigolactones (SLs) regulate important aspects of plant growth and stress responses. Many diverse types of SL occur in plants, but a complete picture of biosynthesis remains unclear. In *Arabidopsis thaliana*, we have demonstrated that MAX1, a cytochrome P450 monooxygenase, converts carlactone (CL) into carlactonoic acid (CLA), and that LBO, a 2-oxoglutarate-dependent dioxygenase, converts methyl carlactonoate (MeCLA) into a metabolite called [MeCLA+16] Da. In the present study, feeding experiments with deuterated MeCLAs revealed that [MeCLA+16] Da is hydroxymethyl carlactonoate (1'-HO-MeCLA). Importantly, this LBO metabolite was detected in plants. Interestingly, other related compounds, methyl 4-hydroxycarlactonoate (4-HO-MeCLA) and methyl 16-hydroxycarlactonoate (16-HO-MeCLA) were also found to accumulate in *lbo* mutants. 3-HO-, 4-HO- and 16-HO-CL were detected in plants, but their expected corresponding metabolites, HO-CLAs, were absent in *max1* mutants. These results suggest that HO-CL derivatives are predominant SLs in *Arabidopsis*, produced through MAX1 and LBO.

Key words: *Arabidopsis thaliana*, hydroxyl carlactone derivative, lateral branching oxidoreductase.

INTRODUCTION

Strigolactones (SLs) were originally identified as germination stimulants for root parasitic plants (Cook et al., 1966) and then as hyphal branching factors for symbiotic arbuscular mycorrhizal (AM) fungi (Akiyama et al., 2005). SLs were thought to function only as rhizosphere signals until the discovery of their role as a plant hormonal signal that inhibits lateral shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008).

Shoot branching involves the formation of axillary buds in the axil of leaves. The level of dormancy in buds is an essential determinant of plant architecture. Defects in the SL pathway correspond with loss of bud dormancy and excessive shoot branching as displayed by SL mutants that include *ramosus* (*rms*) of pea (*Pisum sativum*), *decreased apical dominance* (*dad*) of petunia (*Petunia hybrida*), *dwarf* (*d*) of rice (*Oryza sativa*) and *more axillary growth* (*max*) of *Arabidopsis* (*Arabidopsis thaliana*).

Natural SLs are carotenoid-derived compounds consisting of a butenolide D ring linked by an enol ether bridge to a less conserved moiety. These SLs can be classified into two structurally distinct groups: canonical and non-canonical SLs. Canonical SLs contain the ABCD ring formation, and non-canonical SLs lack the A, B, or C ring but have the enol ether-D ring moiety (Al-Babili and Bouwmeester, 2015). During biosynthesis, the initial compound that contains the D ring is carlactone (CL), an endogenous precursor for SLs, which is produced by the sequential reactions of 9-*cis*/all-*trans*-β-carotene isomerase and two carotenoid cleavage dioxygenases (CCD7, CCD8) (Alder et al., 2012). In *Arabidopsis*, the isomerase is encoded by *DWARF27* (*D27*), and CCD7 and CCD8 by *MAX3* and *MAX4*, respectively (Fig. 1). We have demonstrated that recombinant MAX1, (a cytochrome P450 monooxygenase) expressed in yeast, converts CL to carlactonoic acid (CLA) by oxidations at C-19 (Abe et al. 2015). This function was also observed in MAX1 homologs of other plant

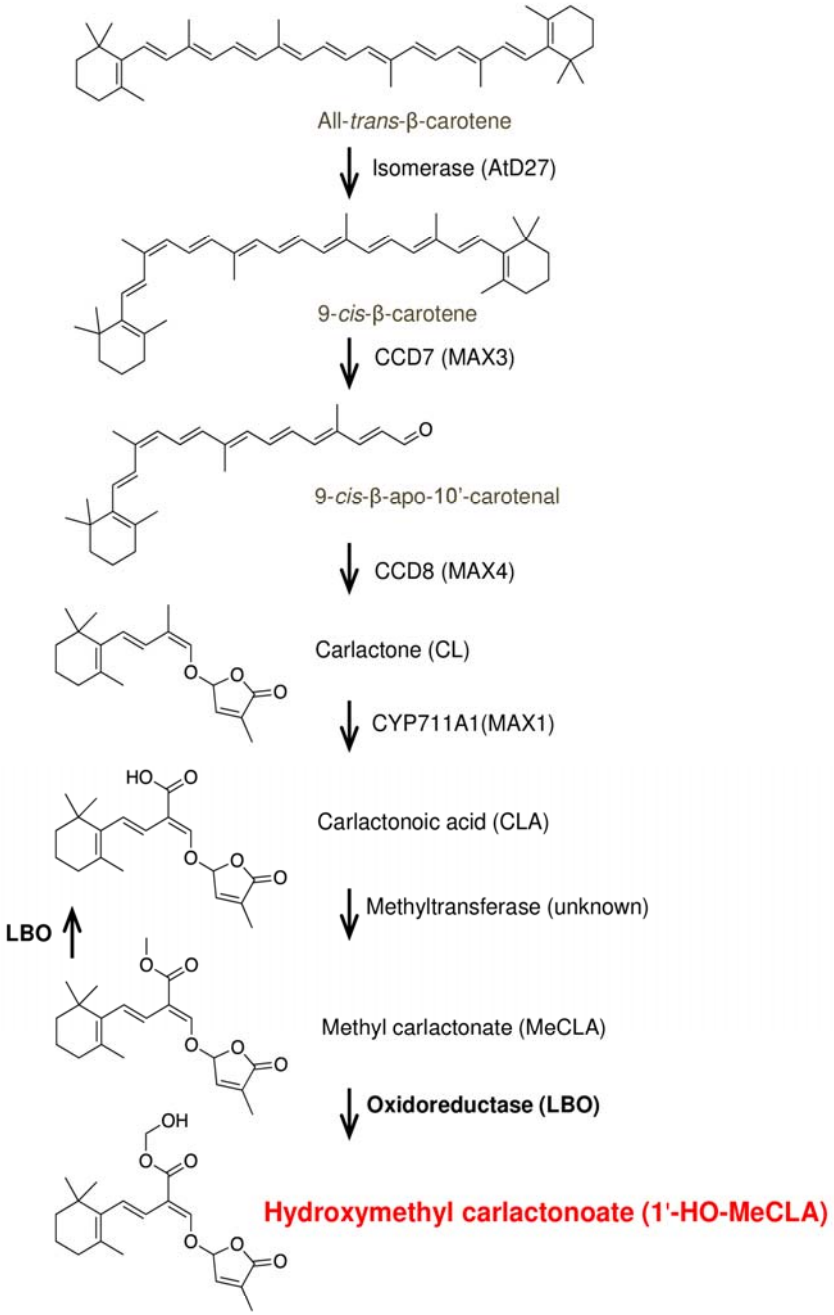

Figure 1. Proposed strigolactone (SL) biosynthesis pathway in *Arabidopsis*. An isomerase (AtD27) and two CCD enzymes (MAX3 and MAX4) convert β-carotene into carlactone (CL), an endogenous common precursor for diverse SLs. CL is then oxidized by cytochrome P450 (MAX1) to carlactonoic acid (CLA), which is converted into MeCLA by unknown methyltransferase. The present study showed that 2-oxoglutarate-dependent dioxygenase LBO converts MeCLA into 1'-HO-MeCLA, which is essential for regulating shoot branching.

77 species including rice, maize, tomato, a model tree poplar, and a lycophyte spike moss,

78 suggesting this conversion of CL to CLA is highly conserved in the plant kingdom

(Yoneyama et al., 2018). It was also shown that CL, CLA, and methyl carlactonoate (MeCLA) are present in *Arabidopsis* root tissues (Seto at al., 2014; Abe et al., 2014). Furthermore, differential scanning fluorimetry and hydrolysis activity tests showed that, among CL, CLA, and MeCLA, only MeCLA could interact with the SL receptor, AtD14, suggesting MeCLA may be biologically active in the inhibition of shoot branching in *Arabidopsis* (Abe et al., 2015). *Arabidopsis max1* mutants display a highly increased lateral shoot branching phenotype, and yet accumulate CL (Seto et al., 2014), indicating that CL is not active in repressing shoot branching.

As a novel SL biosynthetic gene, *LATERAL BRANCHING OXIDOREDUCTASE* (*LBO*), encoding a 2-oxoglutarate and Fe (II)-dependent dioxygenase was identified by using a transcriptomic approach, and was shown to function downstream of MAX1 (Brewer et al., 2016). *Arabidopsis lbo* mutant shoot branching is increased compared to WT (Ws-4), but its phenotype is intermediate between WT and *max4* mutants. LC-MS/MS analysis of SLs revealed that CL and MeCLA accumulate in root tissues of *lbo* mutants (Brewer et al., 2016). Because the active shoot branching inhibitor MeCLA accumulates in *lbo* mutants, the intermediate branching phenotype of *lbo* mutants may be explained by the presence of MeCLA. Thus, it was suggested that LBO is necessary for complete suppression of shoot branching in plants by converting the partly bioactive MeCLA to a compound with greater bioactivity for branching. We then showed that the LBO enzyme expressed in *E. coli* only consumed MeCLA when fed with CL, CLA, or MeCLA, and converted MeCLA into a product of [MeCLA+16] Da. However, complete characterization of this LBO metabolite had not yet been conducted.

In the present study, we have determined the structure of the [MeCLA+16] Da compound produced by LBO from MeCLA by feeding experiments using deuterated MeCLAs. In addition, we could identify this LBO metabolite as an endogenous compound from not only

104 roots, but also basal parts of *Arabidopsis* shoot tissues. Since two additional *lbo* mutant
105 alleles, *lbo-2* and *lbo-3,* exist, and homozygous mutant plants exhibited increased shoot
106 branching (Brewer et al., 2016), recombinant proteins of LBO-2 and LBO-3 were produced
107 and the correlation between their enzymatic activities in the conversion of MeCLA to
108 [MeCLA+16] Da and their shoot branching phenotypes was investigated to further examine
109 the importance of the LBO metabolite for shoot branching. Then, biochemical functions of
110 LBO homologs in other plant species including tomato, maize, and sorghum were examined
111 to clarify if the conversion of MeCLA to [MeCLA+16] Da is conserved among these plant
112 species. Furthermore, endogenous SLs in *Arabidopsis max1* and *lbo* mutants were carefully
113 analyzed in search of other potential substrates for MAX1 and LBO to better understand the
114 SL biosynthetic pathway in *Arabidopsis*.
115
116

RESULTS

LBO catalyzes the conversion of methyl carlactonoate (MeCLA) into hydroxymethyl carlactonoate (1'-HO-MeCLA)

To characterize the structure of [MeCLA+16] Da, LBO enzyme reactions were performed repeatedly. Both the substrate MeCLA and the metabolite [MeCLA+16] Da were highly unstable and the yield of the metabolite was extremely low. We tried to optimize enzyme assay conditions but the maximum yield of the LBO metabolite did not exceed 0.1%. Although more than 500 µg of synthetic MeCLA has been used for LBO enzyme assay, the amount of the metabolite after purification by DEA, silica, and HPLC was not enough for NMR spectroscopy measurement.

The observed mass of [MeCLA+16] Da (Brewer et al. 2016) suggests that LBO has simply added an oxygen to MeCLA. Therefore, feeding experiments with using deuterated MeCLAs were conducted to identify the site of oxidation of MeCLA (Nomura et al., 2013). When MeCLA was fed to LBO, the metabolite was detected by the transition of *m/z* 363 to 97 (Fig. 2). When 18-d_3-MeCLA was fed, the metabolite was detected by the transition of *m/z* 366 to 97 (Fig. 2), clearly indicating that 18-d_3 remained unaffected and thus oxidation did not occur at C-18. By contrast, when 1'-d_3-MeCLA, in which ester methyl group had been labeled with deuterium was fed, major metabolite was detected by the transition of *m/z* 365 to 97 (Fig. 2), apparently showing that ester methyl group was oxidized. Consequently, it was demonstrated that LBO converts MeCLA into hydroxymethyl carlactonoate (1'-HO-MeCLA) (Fig. 1).

On the other hand, when MeCLA was incubated with LBO, most MeCLA was converted to CLA; the ratio of CLA to 1'-HO-MeCLA was 100: 1 based on the peak areas in the LC-MS/MS chromatograms of LBO reaction products (Fig. 3), indicating that the LBO

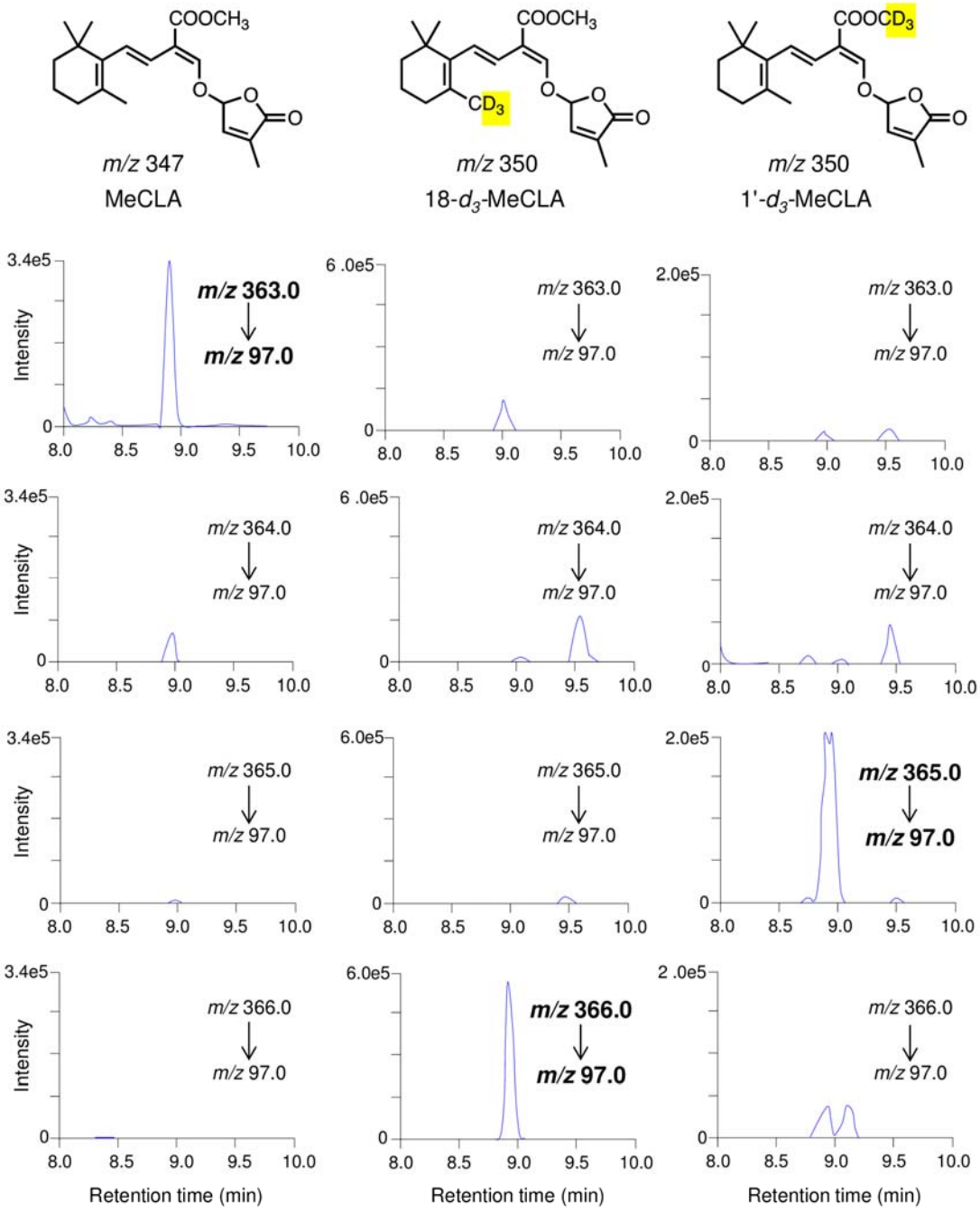

Figure 2. LBO converted [18-d_3]-MeCLA to [MeCLA+16+3] and [1'-d_3]-MeCLA to [MeCLA+16+2]. To characterize the structure of [MeCLA+16], [18-d_3]-MeCLA (*Middle*) and [1'-d_3] MeCLA (*Light*) were fed as substrates to recombinant LBO proteins and incubated for 15 min. Products were identified by LC-MS/MS (MRM).

142 protein assay mainly produces CLA.

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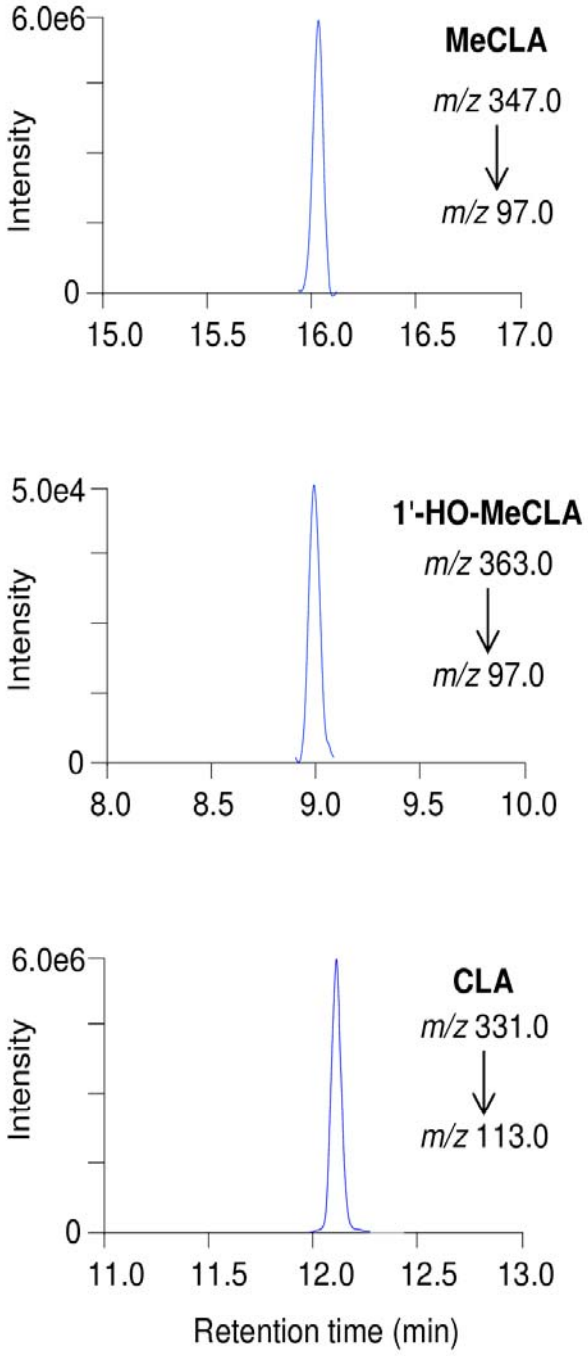

Figure 3. Most MeCLAs were converted to CLA. MeCLA was incubated with recombinant LBO proteins for 15 min. The extracts were analyzed by LC-MS/MS (MRM).

144 **1'-HO-MeCLA is present in *atd14* mutants but not in *lbo* mutants**

It is important to clarify if 1'-HO-MeCLA is an endogenous compound in plant tissues because there is a possibility that 1'-HO-MeCLA would only be produced in the heterologous expression system. Identification of 1'-HO-MeCLA was conducted using *atd14* mutant plants, because they lack a functional SL receptor and accumulate SLs due to negative feedback on the biosynthesis pathway. As a negative control, *lbo* mutant plants were also used. 1'-HO-MeCLA was detected from the basal part of shoots, and also root tissues of *atd14* mutants (Fig. 4). By contrast, CL and MeCLA, but not 1'-HO-MeCLA, were detected from both tissues of *lbo* mutants (Fig. 4, Brewer et al. 2016). These results clearly indicate that LBO may act to convert MeCLA into 1'-HO-MeCLA in plants.

Production of 1'-HO-MeCLA correlates with shoot branching

We previously described additional alleles of mutation in the *LBO* gene (Brewer et al. 2016). *lbo-2* plants have a point mutation in the predicted catalytic domain and display significant extra branching. *lbo-3* plants have a point mutation elsewhere in the gene and have a branching phenotype that is much weaker than *lbo-2* (Brewer et al., 2016). LBO-2 and LBO-3 proteins were produced in *E. coli* heterologous expression system and enzymatic activities to produce 1'-HO-MeCLA were examined. The very low conversion of MeCLA to 1'-HO-MeCLA by LBO-2 enzyme activity (Fig. 5) relates well to the mutant shoot branching phenotype. However, LBO-3 appears to have normal function in our assay (Fig. 5).

Conversion of MeCLA into 1'-HO-MeCLA is conserved among different plant species

Tomato, maize, and sorghum have one LBO homolog each and their recombinant LBO

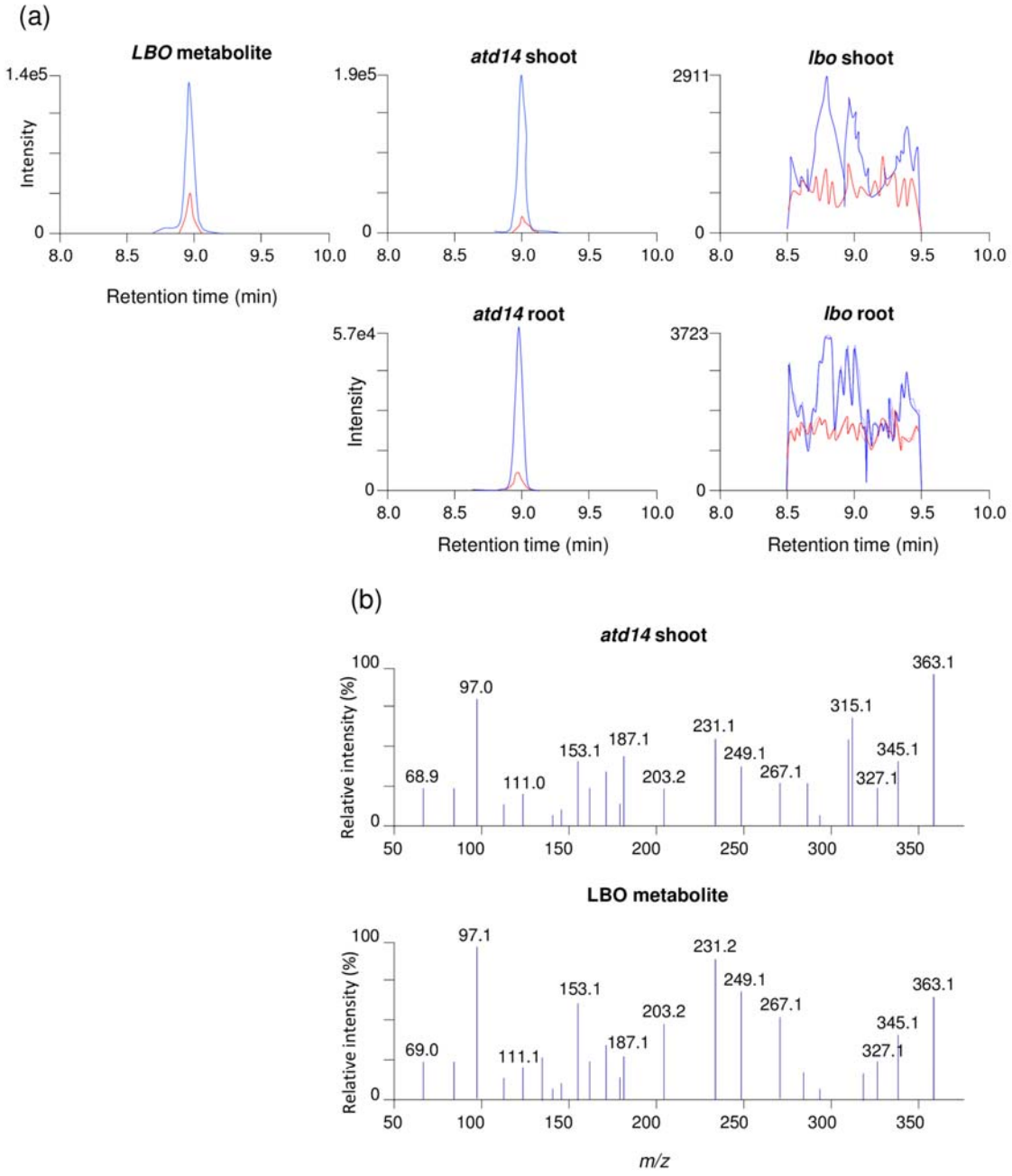

Figure 4. 1'-HO-MeCLA was found from *atd14* shoot. Identification of endogenous 1'-HO-MeCLA in basal parts of shoot and root tissues was conducted. (a) MRM of chromatograms (363.0/97.0; *m/z* in positive mode) of *atd14* mutants (*Middle*) and *lbo* mutants (*Light*). (b) Product ion spectra derived from endogenous 1'-HO-MeCLA in basal parts of shoot of *atd14* mutants.

170 proteins were expressed in *E. coli*. Not only *Arabidopsis* LBO but also the other LBO

171 proteins examined converted MeCLA into 1'-HO-MeCLA (Fig. 6), where the major reaction

172 product was CLA (Supplemental Fig. S1).

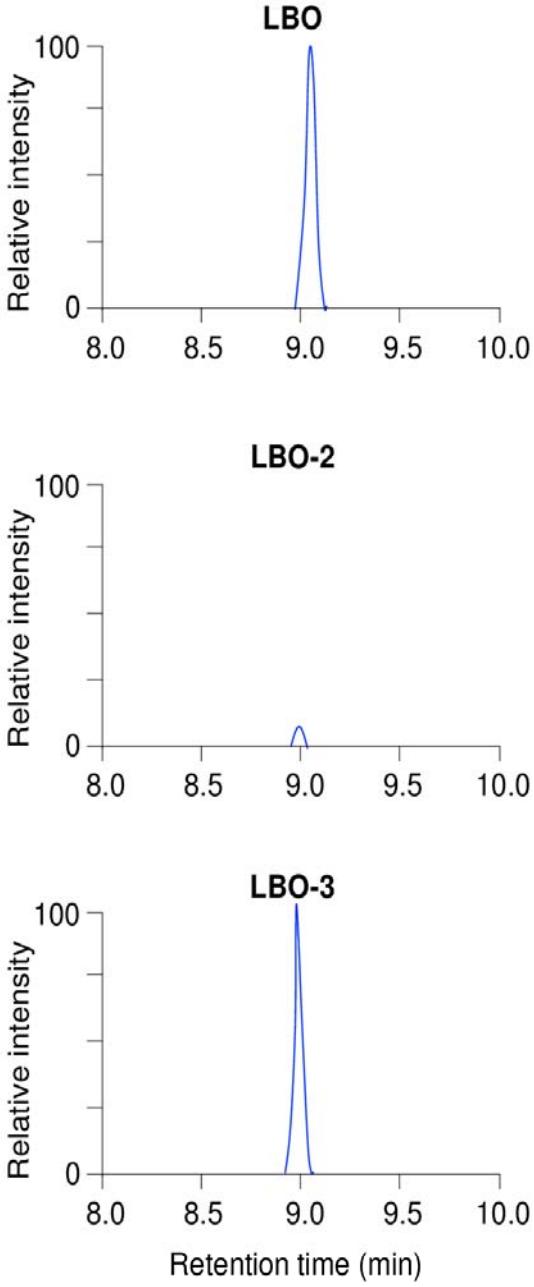

Figure 5. Production of 1'-HO-MeCLA is very low in LBO-2. MeCLA was incubated with each recombinant protein for 15 min and extracts were analyzed by LC-MS/MS. MRM chromatograms of 1'-HO-MeCLA (363.0/97.0; *m/z* in positive mode) are shown.

173 It is intriguing to test if LBO has an ability to produce canonical SLs or not. Tomato plants

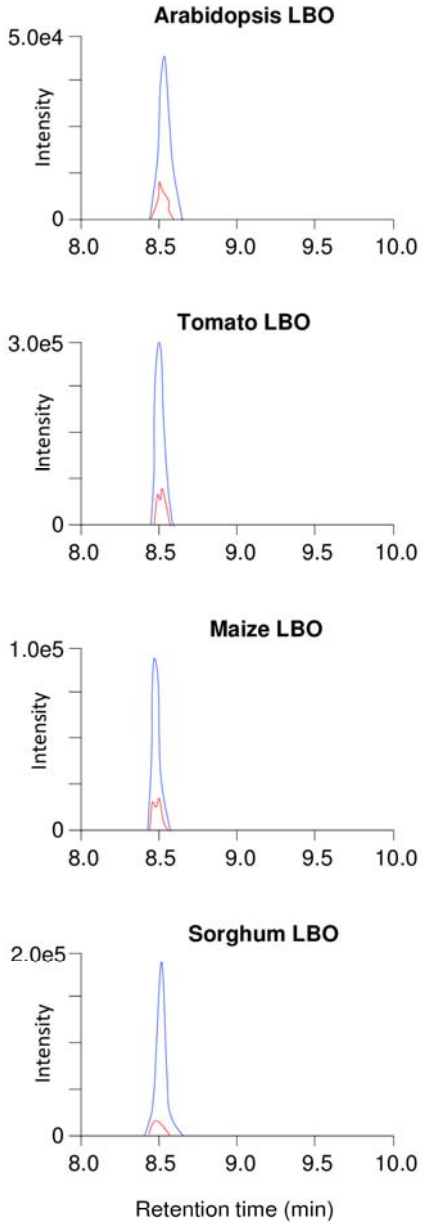

Figure 6. Conversion of MeCLA into 1'-HO-MeCLA is conserved among different plant species. MeCLA was incubated with each recombinant protein for 15 min and extracts were analyzed by LC-MS/MS. MRM chromatograms of 1'-HO-MeCLA (363.0/97.0; *m/z* in positive mode) are shown.

174 produce canonical SLs such as solanacol and orobanchol. Tomato MAX1 expressed in yeast
175 cannot produce canonical SLs from CL (Yoneyama et al., 2018). Accordingly, there is a
176 possibility that tomato LBO produces canonical SLs including solanacol and orobanchol.
177 However, tomato LBO produced neither solanacol nor orobanchol from MeCLA (Data not

178 shown). In addition, tomato LBO did not convert 4DO into solanacol or orobanchol, either
179 (data not shown). Similar results were obtained with sorghum or maize LBOs. Sorghum LBO
180 produced neither 5-deoxystrigol (5DS) nor sorgomol, two major canonical SLs of sorghum
181 (cv Hybrid), from MeCLA. Although it was proposed that sorgomol is produced from 5DS
182 (Motonami et al., 2013), LBO did not produce sorgomol from 5DS (data not shown). Maize
183 plants produce zealactone (Charnikhova et al., 2017; Xie et al., 2017) and zeapyranolactone
184 (Charnikhova et al., 2018), non-canonical SLs with unique structures. Maize LBO did not
185 produce these SLs from MeCLA (Data not shown).
186
187 **Endogenous non-canonical SLs in *Arabidopsis***
188
189 CYP711A2, one of rice MAX1 homologs, produces 4-deoxyorobanchol (4DO) via
190 18-HO-CLA from CL (Yoneyama et al., 2018). This suggests that not only 1'-HO-MeCLA
191 but also other HO-CL derivatives including HO-CLs, HO-CLAs, and HO-MeCLAs are
192 endogenous compounds in *Arabidopsis,* and some of them may be substrates for MAX1 and
193 LBO. Therefore, endogenous SLs in *atd14*, *max1* and *lbo* mutants were investigated in detail.
194 Synthetic standards of 2-, 3-, 4-, 16- and 18-HO-CL (Fig. 7) were prepared and used for
195 LC-MS/MS analyses. HO-CLAs (Fig. 7) were obtained by conversion of the corresponding
196 HO-CLs by MAX1 expressed in yeast. HO-MeCLAs (Fig. 7) were obtained by methylation
197 of the corresponding HO-CLAs with diazomethane.
198 Basal parts of *Arabidopsis* shoot were harvested when the shoot branching phenotype was
199 clearly observed (Supplemental Fig. S2). From *atd14* mutants, 3-, 4-, and 16-HO-CLs, 3-, 4-,
200 and 16-HO-CLAs, and 4- and 16-HO-MeCLAs, in addition to CL, CLA, and MeCLA, were
201 detected (Fig. 8).
202 3-, 4-, and 16-HO-CLs and CL were detected from basal parts of *max1* mutants (Fig. 8).

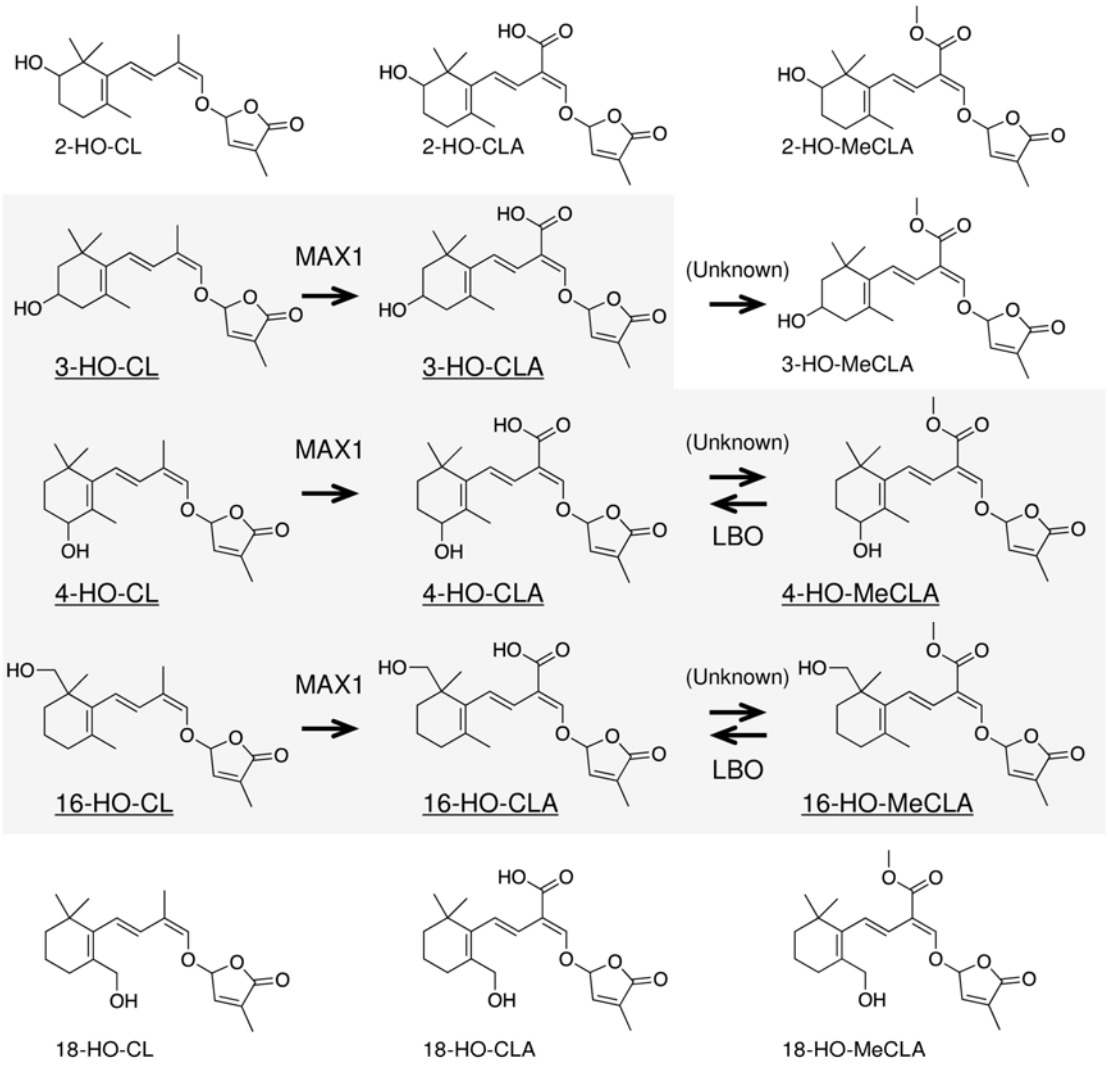

Figure 7. Structures of HO-CLs, HO-CLAs and HO-MeCLAs and a proposed strigolactone biosynthesis pathway in *Arabidopsis*. The present study shows that 3-, 4-, and 16-HO-CL derivatives are predominant and produced through MAX1 and LBO in *Arabidopsis*.

Although 3-, 4-, and 16-HO-CLAs were detected, even from Col-0 plants (Supplemental Fig. S3), these HO-CLAs were not detected in *max1* mutants (Fig. 8). By comparing peak areas of MRM chromatograms between *atd14* and *max1* mutants (Fig. 8), 3-, 4-, and 16-HO-CLs appeared to accumulate in *max1* mutants.

4- and 16-HO-MeCLAs are potential substrates for LBO

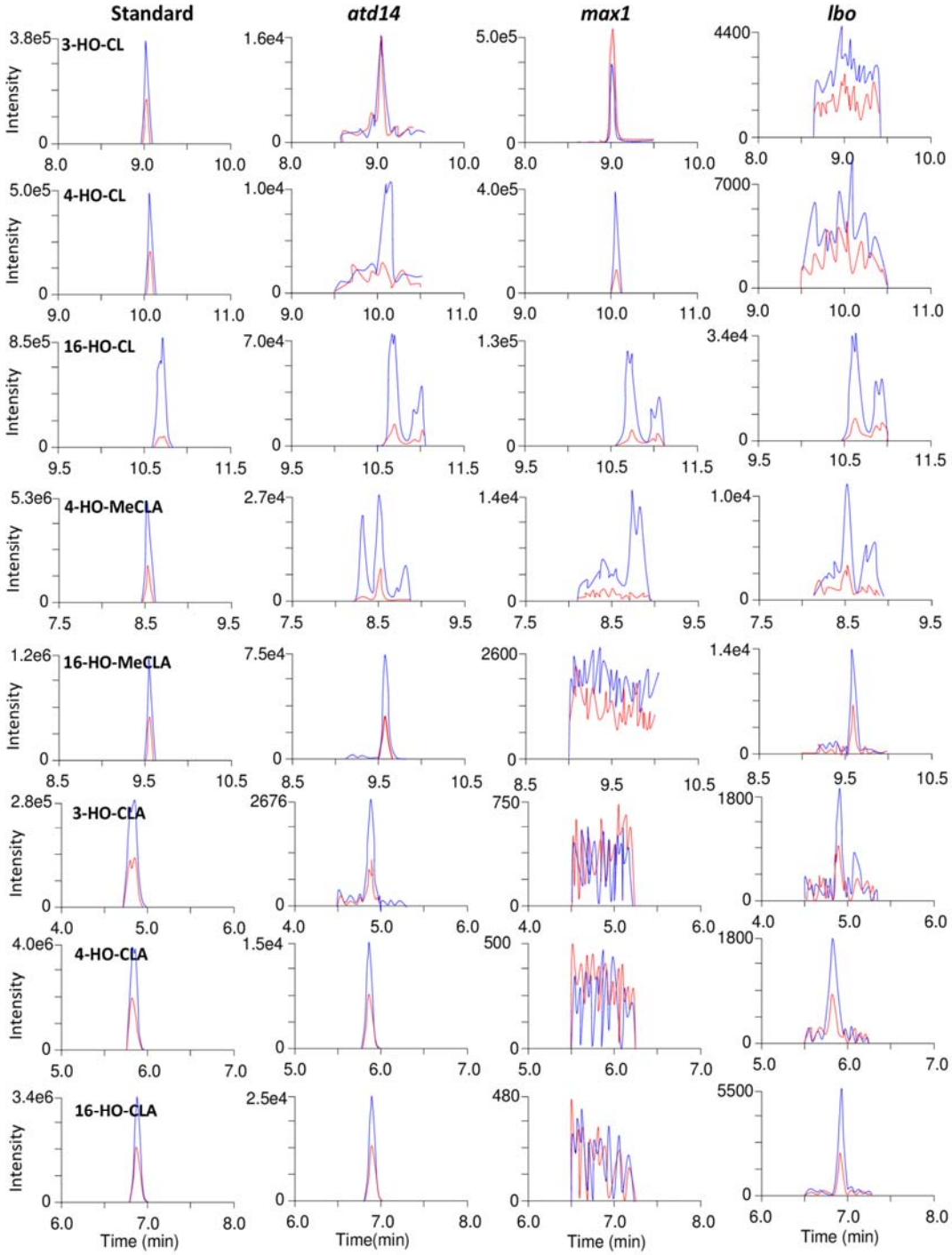

Figure 8. (a) HO-CLs and HO-MeCLAs are detected from ethyl acetate soluble fractions, and (b) HO-CLAs were from acidic fractions in basal parts of shoot tissues of *atd14* mutants, *max1* mutants, and *lbo* mutants. 3-, 4-, and 16-HO-CLs appeared to accumulate in *max1* mutants and 4-, and 16-HO-MeCLA in *lbo* mutants. MRM chromatograms of 3-HO-CL (blue, 301.0/97.0; red, 319.0/205.0; *m/z* in positive mode), 4-HO-CL (blue, 301.0/97.0; red, 301.0/148.0; *m/z* in positive mode), 16-HO-CL (blue, 301.0/97.0; red, 301.0/189.0; *m/z* in positive mode), 4-HO-MeCLA (blue, 345.0/97.0; red, 345.0/216.0; *m/z* in positive mode), 16-HO-MeCLA (blue, 345.0/97.0; red, 363.0/97.0; *m/z* in positive mode), 3-, 4-, and 16-HO-CLA (blue, 347.0/113.0; red, 347.0/69.0; *m/z* in negative mode) are shown.

209

210 In addition to CL, CLA, and MeCLA, 16-HO-CL, 3-, 4-, 16-HO-CLAs, 4- and

16-HO-MeCLAs were found in *lbo* mutants (Fig. 8). MeCLA was found to be a substrate for LBO (Brewer et al., 2016) and therefore these HO-MeCLAs also can be potential substrates for LBO.

Then, these HO-CL derivatives were incubated with recombinant LBO proteins as potential substrates. 4- and 16-HO-MeCLAs, but not other HO-CL derivatives were consumed by LBO. Although we searched for LBO products of 4- and 16-HO-MeCLAs with the D-ring fragment (*m/z* 97) as an indicator by LC-MS/MS, we could not find any candidates for LBO products (Fig. 9). As in the case of MeCLA, the corresponding HO-CLA was detected as a major reaction product.

DISCUSSION

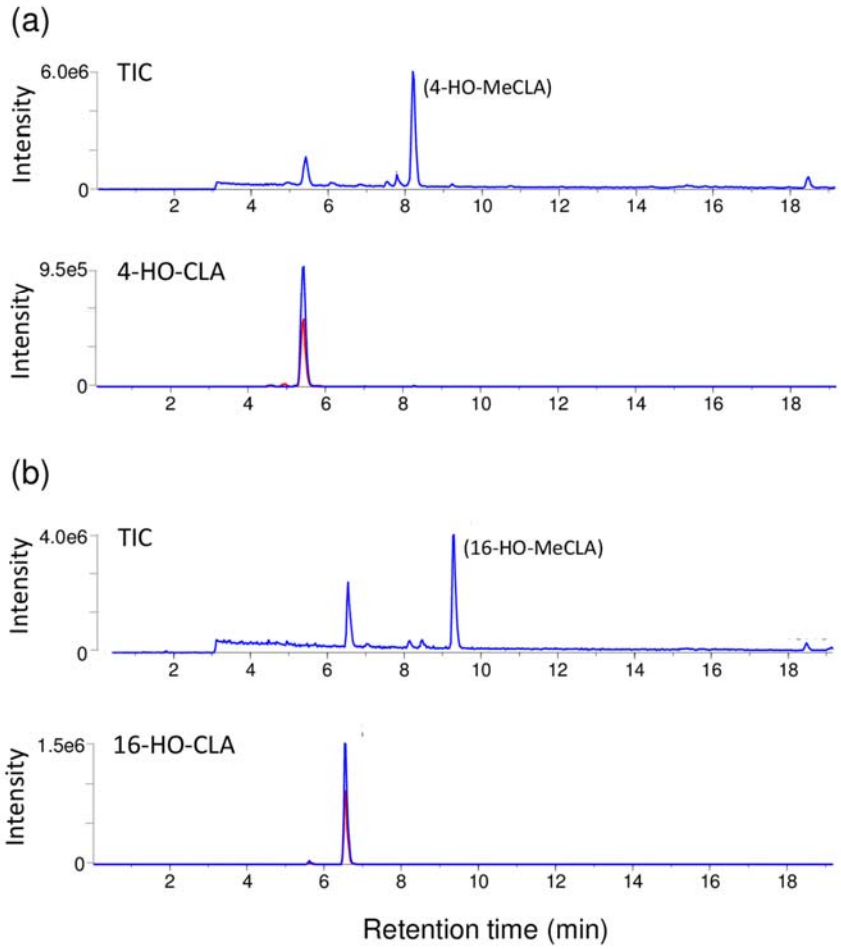

Figure 9. Recombinant LBO proteins convert 4-, and 16-HO-MeCLA mainly into the corresponding HO-CLAs. Each substrate was incubated for 15 min. The extracts were analyzed by LC-MS/MS with the D-ring fragment (*m/z* 97) as an indicator to identify the metabolites from each HO-CLA-fed LBO. Total Ion and MRM chromatograms of HO-CLAs (blue, 347.0/113.0; red, 347.0/69.0; *m/z* in negative mode) are shown.

223

224 The present study demonstrated that the structure of [MeCLA+16] Da is 1'-HO-MeCLA and

225 this LBO metabolite is endogenous in *Arabidopsis* tissues. 1'-HO-MeCLA was also produced

226 by MeCLA-fed maize, tomato and sorghum LBO proteins, suggesting that this conversion of

227 MeCLA into 1'-HO-MeCLA is highly conserved among different seed plant species.

228 Then the question arises whether 1'-HO-MeCLA is a strong shoot branching inhibitor or not.

229 So far, 1'-HO-MeCLA has not been examined for its effect on shoot branching. Unfortunately,
230 at this present time, the synthetic standard for 1'-HO-MeCLA is not available. As mentioned,
231 the yield of 1'-HO-MeCLA by LBO protein reaction is too low to obtain enough for shoot
232 branching assays. Since the substitution of 1'-HO-MeCLA is very unstable and could be
233 readily converted, it is possible that 1'-HO-MeCLA is a precursor for an unknown,
234 downstream shoot branching inhibitor(s) and a subsequent unknown enzyme(s) converts
235 1'-HO-MeCLA into the true shoot branching inhibitor(s). However, we cannot yet find any
236 candidate compounds that are likely to be derived from 1'-HO-MeCLA from *atd14* mutants
237 (data not shown). *LBO* was uncovered from transcriptomics (Brewer et al. 2016) and similar
238 methods recently led to the discovery that CYP722C from cowpea and tomato converts CLA
239 directly to orobanchol (Wakabayashi et al. 2019), and that a 2-oxoglutarate dependent
240 dioxygenase (2-OGD) from a nearby clade to *LBO* is involved in SL biosynthesis in *Lotus*
241 *japonicus* (Mori et al. 2020). We will continue reverse genetic and mass spectrometric
242 approaches to find related SL biosynthetic genes and shoot branching inhibitors. CLA seems
243 to be a key precursor for canonical SLs. We will test how LBO relates to CLA and canonical
244 SLs by identifying *lbo* mutants from plants that produce canonical SLs.
245 Worthy of attention here is that the LBO protein assay produces much more CLA from
246 MeCLA than 1'-HO-MeCLA. Such *O*-demethylations have been reported for 2-OGDs,
247 thebaine 6-*O*-demethylase and codeine *O*-demethylase, catalyzing *O*-demethylation in the
248 final steps of morphine biosynthesis (Hagel and Facchini, 2010). Therefore, we cannot
249 exclude the possibility that the main function of LBO is demethylation of MeCLA and that
250 1'-HO-MeCLA is just an intermediate for demethylation. This is somewhat difficult to
251 reconcile with our previous result that showed that CLA was detected from *lbo* mutants, but
252 not from its wild type (Brewer et al., 2016), indicating that CLA accumulates in *lbo* mutants.
253 As methyltransferase is proposed to be involved in conversion from CLA into MeCLA and

should be functional in *lbo* mutants. Thus, CLA would not be expected to accumulate in *lbo* mutants unless the methyltransferase was somehow downregulated. Identification of the methyltransferase will clarify the meaning of methylation and demethylation in the production of shoot branching inhibitors.

lbo-2 mutants with a point mutation in the predicted catalytic domain display extra shoot branching. The present study demonstrated that recombinant LBO-2 protein is very weak at converting MeCLA into 1'-HO-MeCLA (Fig. 5). In contrast, LBO-3 appears to have normal function in our assay (Fig. 5). The shoot branching phenotype of *lbo-3* mutants with a point mutation elsewhere in the gene is much weaker than *lbo-2*, and only just significantly more than wild type (Brewer et al., 2016). It is possible that the protocol was not sensitive enough to observe subtle defects in reaction efficiency. Alternatively, the LBO-3 mutation may reveal an unknown protein functional or interaction domain at the mutation site, which only affects its bioactivity *in planta*. It may be useful to test LBO and LBO-3 in combination with other SL biosynthesis enzymes as they become discovered.

In addition to 1'-HO-MeCLA, other unstable non-canonical SLs were found in the basal parts of shoot tissues (Fig. 8). So far, identification of SLs has been mainly conducted from root tissues and this is the first report to show that SLs exist in basal parts of shoots of *Arabidopsis*. There were no apparent differences in SL levels between the two tissues when peak areas were compared (Fig. 4). In contrast, levels of SLs are very low or undetectable from shoot of sorghum (Yoneyama et al., 2007) and rice plants (Umehara et al., 2010). *Arabidopsis* could be quite particular in containing the same levels of SLs in the basal part of shoot and root tissues. Perhaps this is because *Arabidopsis* is a non-host of AM fungi. Even though *Arabidopsis* was reported to produce orobanchol (Goldwasser et al., 2008; Kohen et al., 2011), a canonical SL that is widely distributed in plant kingdom (Yoneyama et al., 2008; Yoneyama et al., 2011), we could detect neither orobanchol nor any other known canonical

279 SLs from *Arabidopsis* tissues (data not shown). Non-canonical SLs seem to be predominant
280 in *Arabidopsis* and may not be released into the soil because *Arabidopsis* does not need to
281 attract AM fungi to form a relationship with them. However, there are hints that SLs in
282 *Arabidopsis* may promote interaction with other beneficial soil fungi (Carvalhais et al. 2019).
283 So, there is likely much more to learn on that topic.
284 As summarized in Fig 7, MAX1 oxidizes C-19 methyl group to carboxylic acid not only in
285 CL, but also in HO-CLs in *Arabidopsis* plants. Baz et al. (2018) also detected 3-HO-CL from
286 rice *d14* mutant roots and demonstrated that 9-*cis*-3-HO-β-apo-10'-carotenal-fed to OsCCD8
287 is converted into 3-HO-CL. These results suggest that HO-CLs are also converted by MAX3
288 from HO-carotenal. The *Arabidopsis* MAX1 enzyme has the ability to convert 2-HO-CL and
289 18-HO-CL into respective HO-CLAs. However, these HO-CL derivatives could not be found
290 from *Arabidopsis* plants. It is intriguing why *Arabidopsis* produces such various and
291 particular HO-CL derivatives.
292
293 **CONCLUSION**
294
295 Deciphering the whole SL biosynthetic pathway and characterization of yet unidentified
296 biosynthetic intermediates is essential for devising new strategies to regulate the multiple
297 functions of SLs through manipulation of SL production and exudation, both quantitatively
298 and qualitatively. It should be noted that SL production and exudation vary with plant species
299 (even between cultivars or genotypes of the same plant species), growth conditions, and
300 growth stages. In the present study, we have unveiled the enzymatic functions of LBO and
301 MAX1 and their substrates and products downstream of CL in the SL biosynthetic pathway
302 in *Arabidopsis*. As most seed plant species sequenced so far contain a single *LBO* gene, and
303 the *LBO* gene lineage appears to have been derived deep in plant evolutionary history

(Walker et al., 2019), the biological function of LBO is likely to be highly conserved in the plant kingdom.

Materials and Methods

Plant material

The *lbo-1* and *max1-4* were from our *Arabidopsis* laboratory stocks (Brewer et al., 2016) and the *atd14-2* mutant was obtained from a TILLING project in the Columbia-0 (Col-0) ecotype. To extract total RNAs, tomato (cv Ailisa Craig; Nomura et al., 2005), maize (cv B73; Yoneyama et al., 2018) and sorghum (cv Hybrid; Yoneyama et al., 2008) were used.

Chemicals

3-, 4-, and 18-HO-CLs were synthesized as described previously (Mori et al. 2016; Baz et al. 2018). 2- and 16-HO-CLs were synthesized using the same strategy as the synthesis of 3-and 18-HO-CLs (Baz et al., 2018; Mori et al., 2016). The detailed synthesis will be published elsewhere. 2-, 3-, 4-, 16- and 18-HO-CLA were obtained by MAX1 microsome assay using the corresponding HO-CLs. For this, MAX1 expressed in yeast (*Saccharomyces cerevisiae*) was prepared as described previously (Abe et al., 2014, Yoneyama et al., 2018). 2-, 3-, 4-, 16- and 18-HO-MeCLA were prepared by methylation of the corresponding HO-CLAs with diazomethane.

Synthesis of methyl-d_3 carlactonoate (1'-d_3-MeCLA) (Scheme S1)

(*E*)-4-(2,6,6-Trimethylcyclohex-1-en-1-yl) but-3-enoic acid was synthesized as reported (Abe et al., 2014). To a solution of the C_{13}-carboxylic acid (88.1 mg, 0.42 mmol) in acetone (2 mL), K_2CO_3 (174 mg, 1.26 mmol) and methyl-d_3 iodide (305 mg, 131 μL, 2.1 mmol) were added. The mixture was stirred at room temperature for 21 h under argon. After being concentrated under nitrogen gas flow, the residue was dissolved with ether and water. The organic phase was washed with water and dried over $MgSO_4$. Filtration and evaporation of the solvent afforded C_{13}-carboxylic acid methyl-d_3 ester (82.3 mg, 0.37 mmol, 87%), which was pure enough for the next reaction. Ester condensation of the methyl-d_3 ester (82.3 mg, 0.37 mmol) with ethyl formate (98 mg, 106 μL, 1.32 mmol) by the use of sodium hydride (13.3 mg, 0.56 mmol) in *N,N*-dimethylformamide (1 mL) followed by alkylation with racemic 4-bromo-2-methyl-2-buten-4-olide (99 mg, 55 μL, 0.56 mmol) (Abe et al., 2014) provided 1'-d_3-MeCLA and ethyl carlactonoate (EtCLA, a transesterification product). Purification by silica gel column chromatography (Kieselgel 60, Merck, *n*-hexane-ethyl acetate stepwise) and semi-preparative HPLC (Inertsil SIL-100A, GL Sciences, 5% ethanol in *n*-hexane) gave 1'-d_3-MeCLA (2.5 mg, 0.0072mmol, 1.9%). **1'-d_3-MeCLA**: HR-ESI-TOF-MS *m/z*: 372.1855 $[M+Na]^+$ (calcd. for $C_{20}H_{23}D_3NaO_5^+$, *m/z*: 372.1861).

Synthesis of methyl 18- d_3-carlactonoate (18-d_3-MeCLA) (Scheme S2)

6,6-Dimethyl-2-(methyl-d_3)cyclohex-1-en-1-yl trifluoromethanesulfonate was synthesized as reported (Tanaka et al., 2007). A mixture of the triflate (5.30 g, 19.3 mmol), triethylamine (7.80 g, 10.7 mL, 77.2 mmol), methyl 3-butenoate (3.86 g, 4.11 mL, 38.6 mmol), and bis(triphenylphosphine)palladium(II) dichloride (1.35 g, 1.92 mmol) in *N,N*-dimethylformamide (50 mL) was stirred at 100ºC for 17 h under argon. The reaction mixture was cooled, quenched by pouring into 1 N HCl, and extracted with ether. The

organic phase was washed with brine and water, dried over $MgSO_4$, and concentrated in vacuo. Purification by silica gel column chromatography (Kieselgel 60, Merck, *n*-hexane-ether stepwise) gave crude methyl (*E*)-4-(6,6-dimethyl-2-(methyl-d_3)cyclohex-1-en-1-yl)but-3-enoate (1.31 g, 5.8 mmol, 30%), which was used for the next reaction without further purification. Ester condensation of the deuterium-labeled ester (108 mg, 0.48 mmol) with methyl formate (86.4 mg, 89 μL, 1.44 mmol) by the use of sodium hydride (11.5 mg, 0.48 mmol) in *N*,*N*-dimethylformamide (1 mL) followed by alkylation with racemic 4-bromo-2-methyl-2-buten-4-olide (85 mg, 47 μL, 0.48 mmol) (Abe et al., 2014) provided 18-d_3-MeCLA. Purification by silica gel column chromatography (Kieselgel 60, Merck, *n*-hexane-ethyl acetate stepwise), semi-preparative normal-phase HPLC (Inertsil SIL-100A, GL Sciences, 5% ethanol in *n*-hexane) and semi-preparative reversed-phase HPLC (InertSustain C18, GL Sciences, 85% acetonitrile in water) gave 18-d_3-MeCLA (1.7 mg, 0.0049 mmol, 1.0%). **18-d_3-MeCLA**: HR-ESI-TOF-MS *m/z*: 350.2058 $[M + H]^+$ (calcd. for $C_{20}H_{24}D_3O_5^+$, *m/z*: 350.2041).

Cloning

The primer sequences used are listed in Supporting Information TableS1. Total RNAs were extracted from the shoots and roots of plant materials using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and employed to synthesize single-strand cDNAs by a SuperScript III First-Strand Synthesis System (Invitrogen, Waltham, MA, USA). PCR amplification was performed using PrimeSTAR HS DNA polymerase (TAKARA Bio Inc., Kusatsu, Japan) with/without GC buffer for accurate amplification of GC rich targets. The full-length cDNAs were cloned into the pENTR vector and then transferred to pET300 vector by the Gateway system (Invitrogen). Recombinant plasmid DNA was transferred to

379 *Escherichia coli* strain Rosetta 2(DE3)pLysS (Novagen). At least four colonies for each
380 experiment were sequenced to check for errors in the PCR. Sequence alignment was
381 performed using MAC VECTOR software (Mac Vector Inc., Apex, NC, USA).
382
383 **Heterologous expression in *E. coli***
384
385 Heterologous expression of LBO in *E. coli* was carried out as described previously (Brewer
386 et al., 2016). Briefly, transformed colonies were grown in LB media (0.5% yeast extract, 1%
387 Bacto Tryptone, 1% NaCl) with carbenicilin (100 μg/mL) at 37 ºC in a shaking incubator
388 (180 rpm) until the cell density reached an OD_{600} of 0.5-0.8. After isopropyl
389 -D-1-thiogalactopyranoside (1 mM) was added, transformed *E. coli* were incubated at 20 ºC
390 for 14-16 h. To prepare enzyme fractions, *E. coli* cells were collected by centrifugation of
391 10,000 x *g* for 1 min and suspended in 20 mM phosphate buffer (pH 7.4). The suspend cells
392 were mechanically lysed by using a high-pressure homogenizer (Emulsi Flex B15;
393 AVESTIN) and then, centrifuged at 15,000 x *g* for 5 min at 4ºC.
394
395 **LBO enzyme assays and metabolite extraction**
396
397 Crude protein fraction (5 mL) was incubated with 4 mM 2-oxoglutarate, 0.5 mM iron
398 ascorbate, 5 mM ascorbic acid, and 12.5 μg of test substrates at 27ºC for 20 min, similar to
399 the previous report (Brewer et al., 2016). The reaction mixture was extracted with 5 mL ethyl
400 acetate twice. The ethyl acetate soluble fraction was dried with sodium sulfate and
401 evaporated under nitrogen gas flow at 40ºC with care not to completely dry. Crude extract
402 samples were kept at -20ºC until LC-MS analysis.
403

404 **SL identification in *A. thaliana***

405

406 *Arabidopsis* seeds were sterilized in 1% sodium hypochlorite solution for 10 min and rinsed
407 with sterile water. Seeds were sown on agar (0.5% gellangum with 1/2 Murashige and Skoog
408 medium and 1% sucrose), stratified at 4ºC for 2 days, and grown for 10 days under a
409 photoperiod, 14 h: 10 h, light (150 mol m^{-2} s^{-1}): dark, at room temperature. Then, healthy and
410 uniform seedlings were transplanted on soils [horticultural soil: vermiculite = 1: 2 (v/v)] and
411 further grown until branching phenotype became clear. Basal parts of shoot tissues were
412 harvested, extracted with ethyl acetate for at least 2 days and crude extracts were purified by
413 DEA and silica Sep-pack cartridge as reported previously (Brewer et al., 2016).

414

415 **LC-MS/MS analysis**

416

417 SLs were analyzed by LC-MS/MS as reported previously (Abe et al., 2014). Briefly,
418 LC-MS/MS analysis (MRM, multiple reaction monitoring and PIS, product ion scan) of
419 proton adduct ions was performed with a triple quadruple/linear ion trap instrument
420 (QTRAP5500; AB Sciex, Old Connecticut Path Framingham, MA, USA) with an
421 electrospray source. HPLC separation was performed on a UHPLC (Nexera X2; Shimadzu)
422 equipped with an ODS column (Kinetex C18, 2.1 x 150 mm, 1.7 m; Phenomenex) with a
423 linear gradient of 35% acetonitrile (0 min) to 95% acetonitrile (20 min). The column oven
424 temperature was maintained at 30ºC.

425

426 **Supplemental Data**

427

428 **Supplemental Figure S1**. Detection of CLA from recombinant LBO proteins of various

plants.

Supplemental Figure S2. The number of shoot branching in *max1*, *lbo* and *atd14* mutants.

Supplemental Figure S3. Identification of HO-CLAs from Col-0.

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FIGURE LEGENDS

Figure 1. Proposed strigolactone (SL) biosynthesis pathway in *Arabidopsis thaliana.* An isomerase (AtD27) and two CCD enzymes (MAX3 and MAX4) convert β-carotene into carlactone (CL), an endogenous common precursor for diverse SLs. CL is then oxidized by cytochrome P450 (MAX1) to carlactonoic acid (CLA), which is converted into MeCLA by unknown methyltransferase. The present study showed that 2-oxoglutarate-dependent dioxygenase LBO converts MeCLA into 1'-HO-MeCLA, which is essential for regulating shoot branching.

Figure 2. LBO converted [18-d_3]-MeCLA to [MeCLA+16+3] and [1'-d_3]-MeCLA to [MeCLA+16+2]. To characterize the structure of [MeCLA+16], [18-d_3]-MeCLA (*Middle*)

454 and [1'-d_3] MeCLA (*Light*) were fed as substrates to recombinant LBO proteins and
455 incubated for 15 min. Products were identified by LC-MS/MS (MRM).
456
457 **Figure 3**. Most MeCLAs were converted to CLA. MeCLA was incubated with recombinant
458 LBO proteins for 15 min. The extracts were analyzed by LC-MS/MS (MRM).
459
460 **Figure 4**. 1'-HO-MeCLA was found from *atd14* shoot. Identification of endogenous
461 1'-HO-MeCLA in basal parts of shoot and root tissues was conducted. (a) MRM of
462 chromatograms (363.0/97.0; *m/z* in positive mode) of *atd14* mutants (*Middle*) and *lbo*
463 mutants (*Light*). (b) Product ion spectra derived from endogenous 1'-HO-MeCLA in basal
464 parts of shoot of *atd14* mutants.
465
466 **Figure 5**. Production of 1'-HO-MeCLA is very low in LBO-2. MeCLA was incubated with
467 each recombinant protein for 15 min and extracts were analyzed by LC-MS/MS. MRM
468 chromatograms of 1'-HO-MeCLA (363.0/97.0; *m/z* in positive mode) are shown.
469
470 **Figure 6**. Conversion of MeCLA into 1'-HO-MeCLA is conserved among different plant
471 species. MeCLA was incubated with each recombinant protein for 15 min and extracts were
472 analyzed by LC-MS/MS. MRM chromatograms of 1'-HO-MeCLA (363.0/97.0; *m/z* in
473 positive mode) are shown.
474
475 **Figure 7**. Structures of HO-CLs, HO-CLAs and HO-MeCLAs and a proposed strigolactone
476 biosynthesis pathway in *Arabidopsis*. The present study shows that 3-, 4-, and 16-HO-CL
477 derivatives are predominant and produced through MAX1 and LBO in *Arabidopsis.*
478

Figure 8. (a) HO-CLs and HO-MeCLAs are detected from ethyl acetate soluble fractions, and (b) HO-CLAs were from acidic fractions in basal parts of shoot tissues of *atd14* mutants, *max1* mutants, and *lbo* mutants. 3-, 4-, and 16-HO-CLs appeared to accumulate in *max1* mutants and 4-, and 16-HO-MeCLA in *lbo* mutants. MRM chromatograms of 3-HO-CL (blue, 301.0/97.0; red, 319.0/205.0; *m/z* in positive mode), 4-HO-CL (blue, 301.0/97.0; red, 301.0/148.0; *m/z* in positive mode), 16-HO-CL (blue, 301.0/97.0; red, 301.0/189.0; *m/z* in positive mode), 4-HO-MeCLA (blue, 345.0/97.0; red, 345.0/216.0; *m/z* in positive mode), 16-HO-MeCLA (blue, 345.0/97.0; red, 363.0/97.0; *m/z* in positive mode), 3-, 4-, and 16-HO-CLA (blue, 347.0/113.0; red, 347.0/69.0; *m/z* in negative mode) are shown.

Figure 9. Recombinant LBO proteins convert 4-, and 16-HO-MeCLA mainly into the corresponding HO-CLAs. Each substrate was incubated for 15 min. The extracts were analyzed by LC-MS/MS with the D-ring fragment (*m/z* 97) as an indicator to identify the metabolites from each HO-CLA-fed LBO. Total Ion and MRM chromatograms of HO-CLAs (blue, 347.0/113.0; red, 347.0/69.0; *m/z* in negative mode) are shown.

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