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Research

Strigolactone biosynthesis catalyzed by cytochrome P450 and sulfotransferase in sorghum

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Summary

- Root parasitic plants such as *Striga*, *Orobanche*, and *Phelipanche* spp. cause serious damage to crop production world-wide. Deletion of the *Low Germination Stimulant 1* (*LGS1*) gene gives a *Striga*-resistance trait in sorghum (*Sorghum bicolor*). The *LGS1* gene encodes a sulfotransferase-like protein, but its function has not been elucidated.
- Since the profile of strigolactones (SLs) that induce seed germination in root parasitic plants is altered in the *lgs1* mutant, LGS1 is thought to be an SL biosynthetic enzyme. In order to clarify the enzymatic function of LGS1, we looked for candidate SL substrates that accumulate in the *lgs1* mutants and performed *in vivo* and *in vitro* metabolism experiments.
- We found the SL precursor 18-hydroxycarlactonoic acid (18-OH-CLA) is a substrate for LGS1. CYP711A cytochrome P450 enzymes (SbMAX1 proteins) in sorghum produce 18-OH-CLA. When *LGS1* and *SbMAX1* coding sequences were co-expressed in *Nicotiana benthamiana* with the upstream SL biosynthesis genes from sorghum, the canonical SLs 5-deoxystrigol and 4-deoxyorobanchol were produced.
- This finding showed that LGS1 in sorghum uses a sulfo group to catalyze leaving of a hydroxyl group and cyclization of 18-OH-CLA. A similar SL biosynthetic pathway has not been found in other plant species.

Introduction

Striga, *Orobanche*, and *Phelipanche* spp. are root parasitic plants that deprive host plants of nutrients and water. *Striga hermonthica* and *Striga asiatica* are particularly rampant weeds in sub-Saharan Africa that have infested over 50×10^6 ha of farmlands and cause annual losses of $7–10 billion in crop production (Spallek *et al.*, 2013). They pose a growing threat to food security, particularly for smallholder farmers. As obligate parasites, *Striga*, *Orobanche*, and *Phelipanche* spp. must attach to a host root successfully within days of germination to survive. Their seeds have evolved to germinate in the presence of strigolactone (SL) molecules that are exudated from host roots (Cook *et al.*, 1966; Nelson, 2021). Although host plants are disadvantaged by the exudation of SLs, SLs have a beneficial role in the recruitment of symbiotic interactions with arbuscular mycorrhizal (AM) fungi (Akiyama *et al.*, 2005). Furthermore, SL has many roles within plants as a hormone that regulates shoot branching/tillering, root growth, secondary growth, and leaf senescence (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008; Chesterfield *et al.*, 2020).

More than 30 SLs have been isolated from the root exudates of various plants using the seed germination stimulating activity of root parasitic plants as a bioassay to guide purification (Yoneyama, 2020). SLs are biosynthesized from carotenoids, and the canonical structure of SLs consists of a variable tricyclic lactone (ABC rings) connected to a methylbutenolide (D ring) via an enol-ether bridge by *R*-configuration at C-2′ (Fig. 1). SL biosynthetic enzymes have been identified by the increased shoot branching mutants of pea (*Pisum sativum*), *Arabidopsis thaliana*, and rice (*Oryza sativa*) (Beveridge *et al.*, 1996; Booker *et al.*, 2004; Arite *et al.*, 2007). The SL biosynthetic pathway proposed so far is shown in Fig. 1. *all-trans*-β-Carotene is converted to carlactone (CL), a common precursor of SLs, by the carotenoid isomerase DWARF27 (D27), CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7), and CCD8 enzymes (Alder *et al.*, 2012). In *Arabidopsis*, CL is oxidized at C-19 by

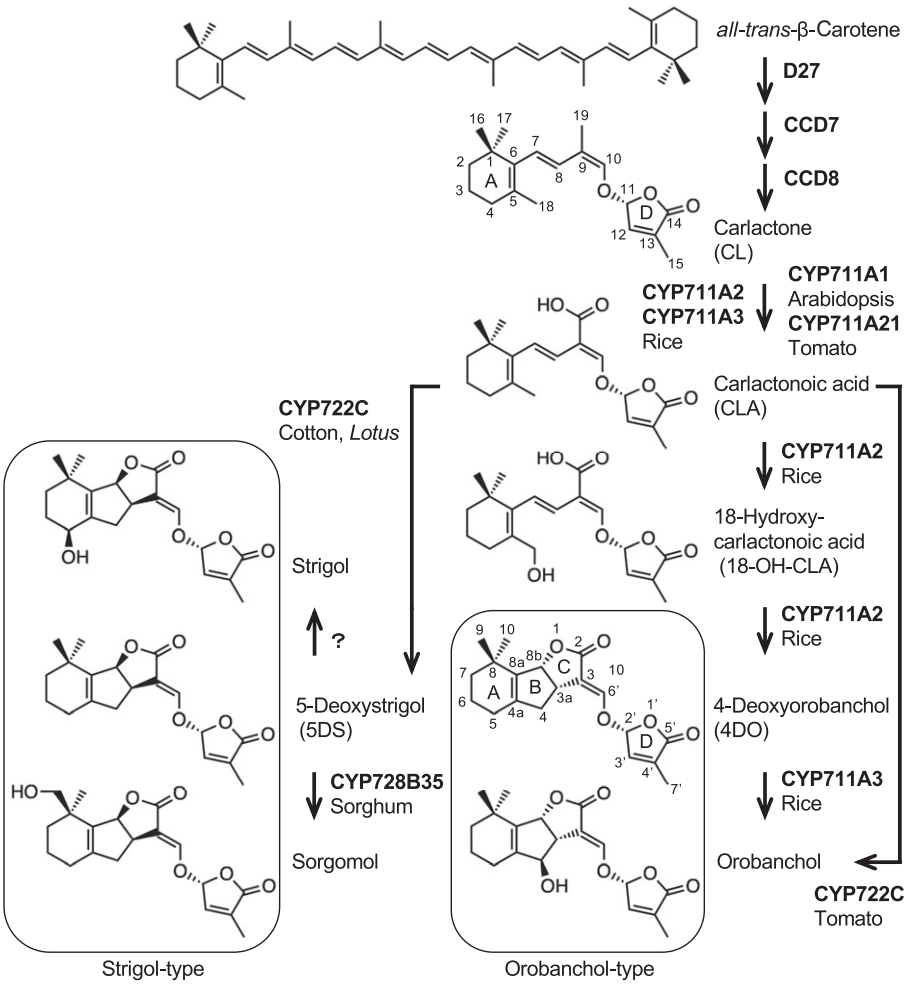

Fig. 1 Biosynthesis of strigolactones from β-carotene. β-Carotene is converted into carlactone (CL) by D27, CCD7, and CCD8. The conversion of CL to carlactonoic acid (CLA) is a conserved function of CYP711A (MAX1) enzymes. CLA is converted to canonical strigol or orobanchol-type strigolactones, which are divided according to the stereochemical difference at the C-ring, by species-specific functions of CYP711A or CYP722C enzymes.

the cytochrome P450 enzyme MORE AXILLARY GROWTH 1 (MAX1, CYP711A1) to form carlactonoic acid (CLA) (Abe *et al.*, 2014). The conversion of CL to CLA is a conserved reaction in MAX1 homologues of various plant species. In rice, however, one of the MAX1 homologues, Os900 (CYP711A2, Os01g0700900), converts CL to the canonical SL 4-deoxyorobanchol (4DO) via CLA and 18-hydroxycarlactonoic acid (18-OH-CLA) (Zhang *et al.*, 2014; Yoneyama *et al.*, 2018). In addition, 4DO is converted to orobanchol by Os1400 (CYP711A3, Os01g0701400) in rice (Zhang *et al.*, 2014). Recently, a variety of noncanonical SLs, methyl ester derivatives of CLA that lack the BC rings, have also been isolated as seed germination stimulants for root parasitic plants (Yoneyama, 2020). Hence, there is diversity in the SL biosynthetic pathway downstream of CLA among plant species.

Canonical SLs can be divided into orobanchol type and strigol type according to the stereochemical configuration at the BC ring junction (Fig. 1) (Xie *et al.*, 2013). Most plants produce SLs in only one of the two configurations, but the physiological significance of this stereoselective synthesis is largely unknown. Tomato (*Solanum lycopersicum*) produces orobanchol, but, unlike the rice pathway, the cytochrome P450 enzyme CYP722C is involved in the biosynthesis of orobanchol from CLA (Wakabayashi *et al.*, 2019). On the other hand, CYP722C in *Lotus japonicus* and cotton (*Gossypium arboreum*) produces 5-deoxystrigol (5DS), a strigol-type SL (Mori *et al.*, 2020; Wakabayashi *et al.*, 2020).

Sorghum (*Sorghum bicolor*) typically produces strigol-type SLs, such as 5DS and sorgomol. 5DS is converted to sorgomol via CYP728B35 in sorghum, but the biosynthetic pathway leading to 5DS itself has not been elucidated (Wakabayashi *et al.*, 2021). It was previously reported that the gene *Low Germination Stimulant 1* (*LGS1*) is defective in many sorghum varieties that show *Striga* resistance and that the predominant SL in these varieties was replaced with orobanchol (Gobena *et al.*, 2017). Orobanchol is weaker than 5DS at stimulating the germination of *Striga* seed, which is a likely reason for the reduced parasitism of *lgs1* mutants by *S. hermonthica* (Mohemed *et al.*, 2018). *LGS1* encodes a protein that is annotated as a sulfotransferase, but its biochemical function has not been characterized. In this study, we found sorghum varieties carrying *lgs1* alleles and analyzed the enzymatic function of LGS1 in the SL biosynthetic pathway. We also investigated the function of MAX1 homologues in sorghum, which act upstream of LGS1. This revealed that SL biosynthesis in sorghum is catalyzed by a cytochrome P450 and a sulfotransferase, a pathway that has not been identified in other plant species.

Materials and Methods

Plant materials

Sorghum seeds of 'Tx430' and 'Ramada' were provided from the Plant Transformation Core Research Facility at the University of Nebraska–Lincoln (Lincoln, NE, USA). Tx lines (Supporting Information Table S1) and Global Core Collection (Table S2) of sorghum were provided from the Genebank Project of National Agriculture and Food Research Organization (Tsukuba, Japan). *Nicotiana benthamiana* seeds were propagated in our laboratory (Yoneyama *et al.*, 2018).

Chemicals

Chemical synthesis for *rac*-18-OH-CLA is described in Methods S1 (Fig. S1). *rac*-CL, 5DS, 2′-*epi*-5DS, 4DO, and 2′-*epi*-4DO were synthesized previously (Umehara *et al.*, 2008; Xie *et al.*, 2013; Abe *et al.*, 2014; Seto *et al.*, 2014).

Strigolactone analysis in root exudates

Five seedlings of 10-d-old sorghum were grown in 10 cm diameter pots filled with vermiculite using tap water under 16 h : 8 h, light (265 μmol m^{-2} s^{-1}, 30°C) : dark (25°C), photoperiod in a growth chamber. Water (200 ml per pot) was poured on the surface of the vermiculite and collected from the bottom of the pot. The collected water was extracted with ethyl acetate. The ethyl acetate phase was dried over anhydrous sodium sulfate and concentrated *in vacuo*. SLs were analyzed by LC–MS/MS (QTRAP 5500; AB Sciex, Framingham, MA, USA) as reported previously (Abe *et al.*, 2014; Yoneyama *et al.*, 2018). Germination assays of *S. hermonthica* and *Orobanche minor* seeds were conducted as reported previously (Yoneyama *et al.*, 2007).

PCR analysis

A leaf tip (*c.* 2 mm in diameter) of 10-d-old sorghum seedling was crushed in 50 μl of dilution buffer (Phire Plant Direct PCR Master Mix; Thermo Fisher Scientific, Waltham, MA, USA) using a pestle homogenizer. A 1 μl volume of the supernatant was used for PCR amplification by KOD FX (Toyobo, Osaka, Japan) using the primers listed in Table S3.

Isolation of complementary DNA clones

Total RNAs were extracted from roots of 10-d-old sorghum seedlings ('Ramada') using an RNeasy Plant Mini Kit (Qiagen) and used to synthesize single-strand complementary DNAs (cDNAs) with a SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific). PCR amplification was performed by PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan) using the primers listed in Table S4. The accession nos. for the sequences cloned in this study are as follows: LC422680 (LGS1, Sobic.005G213600), LC422681 (SbMAX1a/CYP711A31, Sobic.003G269500), LC422682 (SbMAX1b/CYP711A18, Sobic.003G269600), LC422683 (SbMAX1c/CYP711A19, Sobic.004G095500), LC422685 (SbMAX1d/CYP711A13, Sobic.010G170400), LC556201 (SbD27, Sobic.005G168200), LC556202 (SbCCD7, Sobic.006G170300), LC556203 (SbCCD8, Sobic.003G293600), LC556204 (SbCPR1, Sobic.006G245400), and LC556206 (SbCYP722B, Sobic.009G000700).

Heterologous expression in yeast (*Saccharomyces cerevisiae*)

The full-length cDNAs of *SbMAX1*s were cloned into pYeDP60 (Pompon *et al.*, 1996). Heterologous expression of SbMAX1s in yeast was carried out as described previously (Abe *et al.*, 2014; Yoneyama *et al.*, 2018). Microsomes (100 μl) were incubated with 33 μM *rac*-CL and 500 μM NADPH at 28°C for 1 h. The reaction mixtures were extracted with ethyl acetate, dried over anhydrous sodium sulfate, and then subjected to LC–MS/MS analysis.

Heterologous expression in *Escherichia coli*

The full-length cDNA of *LGS1* was cloned into pENTR/D-TOPO (Thermo Fisher Scientific). For constructing an expression vector of *Escherichia coli* β-glucuronidase (GUS)-fused LGS1 protein, the *GUS* gene (LC588893) without stop codon was inserted into the *Not*I site in pENTR/D-TOPO at the N-terminus of *LGS1* in-frame. The full-length *GUS* cDNA was also cloned alone into pENTR/D-TOPO as a negative control. These entry clones were transferred into pET300/NT-DEST for N-terminal 6× His-tagged protein by the Gateway system (Thermo Fisher Scientific). The resulting plasmids were transformed into Rosetta 2 (DE3) pLysS *E. coli* strain (Merck KGaA, Darmstadt, Germany). The transformed *E. coli* was cultured in 150 ml lysogeny broth (LB) medium containing 100 μg ml^{-1} carbenicillin at 37°C, 180 rpm until an optical density at 600 nm (OD_{600}) of 0.5. After preincubation at 25°C for 30 min, 0.1 mM isopropyl β-D-1-thiogalactopyranoside was added and the transformed *E. coli* was cultured at 25°C for 6 h. Cells were harvested by centrifugation at 8000 ***g*** for 5 min and suspended in 15 ml of 20 mM Tris–HCl (pH 7.5). The suspended cells were lysed by sonication (UD-100; Tomy, Tokyo, Japan) and centrifuged at 20 400 ***g*** for 5 min at 4°C. The supernatants were purified using His GraviTrap (Cytiva, Marlborough, MA, USA). The purified protein fraction (14 μg ml^{-1}, 200 μl) was incubated with 15 μM 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and 0.25–20 μM *rac*-18-OH-CLA at 28°C for 1 h. The reaction mixtures were extracted with ethyl acetate, dried over anhydrous sodium sulfate, and then subjected to LC–MS/MS analysis as reported previously (Yoneyama *et al.*, 2018). The kinetic parameters were determined using triplicate samples and calculated using the Michaelis–Menten equation using SIGMAPLOT 14 (Systat Software, San Jose, CA, USA). Chiral-LC–MS/MS analysis was performed on an ultrahigh performance liquid chromatograph (Nexera X2; Shimadzu Corp., Kyoto, Japan) equipped with a chiral column (Chiralpak IC-3, diameter 2.1 mm × 150 mm, 3 μm; Daicel Corp., Tokyo, Japan). The column

temperature was maintained at 0°C using ice-water. The mobile phase consisted of acetonitrile and water, both of which contained 0.05% acetic acid. High-performance liquid chromatography separation was conducted with a linear gradient of 30% (0 min) to 60% acetonitrile (5 min) and then isocratic elution (15 min) at a flow rate of 0.2 ml min^{-1}.

Transient expression in *N. benthamiana*

The full-length cDNAs of SL biosynthetic enzyme genes were cloned into the *Sal*I site of pBYR2HS (Yamamoto *et al.*, 2018) using GeneArt Seamless Cloning and Assembly Enzyme Mix (Thermo Fisher Scientific). Each transformed *Agrobacterium tumefaciens* GV3101 was cultured in LB medium with 50 μg ml^{-1} kanamycin at 28°C, 180 rpm for 16 h. Cells were collected by centrifugation at 2000 ***g*** for 10 min, rinsed with distilled water twice, and then suspended in distilled water at OD_{600} of 0.4. *Nicotiana benthamiana* plants were grown in pots containing a mixture of soil and vermiculite (1 : 1) under 14 h : 10 h, light (100 μmol m^{-2} s^{-1}) : dark, photoperiod at 24°C. The diluted *Agrobacterium* suspensions were mixed and infiltrated into leaves of 5-wk-old *N. benthamiana* plants using a syringe. After 4 d, 1 g of agroinfiltrated leaves were cut into 1 cm × 1 cm squares and extracted in 40 ml of acetone for 2 d at 4°C. The filtrates were evaporated to aqueous residues and 1 ml of distilled water was added. The aqueous residues were extracted twice with 2 ml of ethyl acetate. The ethyl acetate phase was evaporated to dryness, dissolved in 1 ml of 2-propanol, and then loaded onto Bond Elut DEA cartridge column (100 mg; Agilent Technologies, Santa Clara, CA, USA). A neutral fraction containing CL, 5DS, and 4DO was eluted with 2 ml of 2-propanol, and then an acidic fraction containing CLA and 18-OH-CLA was eluted with 3 ml of 1% acetic acid in 2-propanol. The neutral fraction was evaporated to dryness, dissolved in 1 ml of *n*-hexane, and then loaded onto Bond Elut SI cartridge column (100 mg; Agilent Technologies). The column was washed with 10 ml of *n*-hexane : ethyl acetate (9 : 1) and then SLs were eluted with 10 ml of *n*-hexane : ethyl acetate (7 : 3). The eluates were concentrated under nitrogen gas and injected into the LC–MS/MS instrument as described previously (Abe *et al.*, 2014).

Results

Sorghum 'Tx430' produces orobanchol as the dominant strigolactone

The potential to use genome editing for crop improvement has expanded rapidly in recent years. However, the efficiency of transformation is often a bottleneck for genome editing approaches in crops. 'Ramada' and 'Tx430' are inbred sorghum lines that have relatively high transformation efficiency (Raghuwanshi & Birch, 2010; Liu & Godwin, 2012; Sato-Izawa *et al.*, 2018). To explore the possibility of creating sorghum varieties that have improved resistance to parasitism by *Striga*, we first analyzed the SL content in root exudates of 'Ramada' and 'Tx430'. We found that 5DS was the dominant SL, sorgomol was minor, and orobanchol was absent in 'Ramada'. In 'Tx430', the abundance of orobanchol was comparable to 5DS levels in 'Ramada', but 5DS was very low or absent (Figs 2, S2; Table S5). Because 5DS was lacking, we hypothesized that 'Tx430' may already be a *Striga*-resistant variety, although there has been no such report so far (Mohemed *et al.*, 2018). We examined the germination stimulant activity of root exudates of 'Tx430' and 'Ramada' on seeds of *S. hermonthica* and *O. minor*. 'Tx430' root exudates were 1000 times less potent than 'Ramada' root exudates at stimulating *S. hermonthica* germination (Fig. 3). Conversely, 'Tx430' root exudates were much more effective at stimulating *O. minor* germination than 'Ramada' root exudates were. Although sorghum is not a compatible host for *Orobanche* spp., these germination responses reflect the preferences of the two parasitic species for the different types of SLs that are predominant in each sorghum variety (Fernández-Aparicio *et al.*, 2010).

'Tx430' is an *lgs1* mutant

'Tx430' is known to have large inversions on chromosomes 5, 6, and 7 (Deschamps *et al.*, 2018). Therefore, we hypothesized that 'Tx430' has a defect in the *LGS1* gene on chromosome 5, similar to other sorghum varieties that have switched from strigol-type to orobanchol-type SL production (Gobena *et al.*, 2017; Bellis *et al.*, 2020). We tried to amplify the *LGS1* gene and its neighboring genes in the genomic DNA of 'Tx430' and 'Ramada' using PCR. *Sobic.005G213400*, *Sobic.005G213500*, and *Sobic.005G213600* (*LGS1*) could not be amplified in 'Tx430' and were presumably absent, but *Sobic.005G213700*, *Sobic.005G213766*, and *Sobic.005G213832* were present (Fig. S3). This suggests that 'Tx430' has a chromosomal deletion similar to that in the previously reported *Striga*-resistant variety 555 (*lgs1-2*) (Gobena *et al.*, 2017). However, the amplicon for *Sobic.005G213900* in 'Tx430' was also found to be larger than in 'Ramada'. We examined the *LGS1* gene in 18 other 'Tx' lines of sorghum that were bred at Texas A&M University (Table S1). Among these lines, only 'Tx430' had a deletion of the *LGS1* gene.

Next, we analyzed 107 sorghum accessions with a wide geographic distribution from Asia and Africa in the same way (Table S2) (Shehzad *et al.*, 2009). For the African varieties 'E9', 'E1091', and 'E276 FRAMIDA', we only observed nonspecific products in PCR reactions for *LGS1* (Fig. S3). In addition, *Sobic.005G213500*, *Sobic.005G213700*, *Sobic.005G213766*, and *Sobic.005G213832* did not amplify in these three accessions. This suggested they carry a chromosomal deletion similar to that in the *Striga*-resistant variety SRN39 (*lgs1-1*) (Gobena *et al.*, 2017). Supporting the idea that 'E9', 'E1091', and 'E276 FRAMIDA' are *lgs1* mutants, we found these accessions produce orobanchol but little or no 5DS, similar to 'Tx430' (Fig. S4; Table S5).

18-Hydroxycarlactonoic acid is increased in the *lgs1* mutant

The *LGS1* gene encodes a sulfotransferase-like protein (Gobena *et al.*, 2017). Since sulfotransferases generally transfer a sulfo group to a hydroxyl group of their substrates (Hirschmann *et al.*,

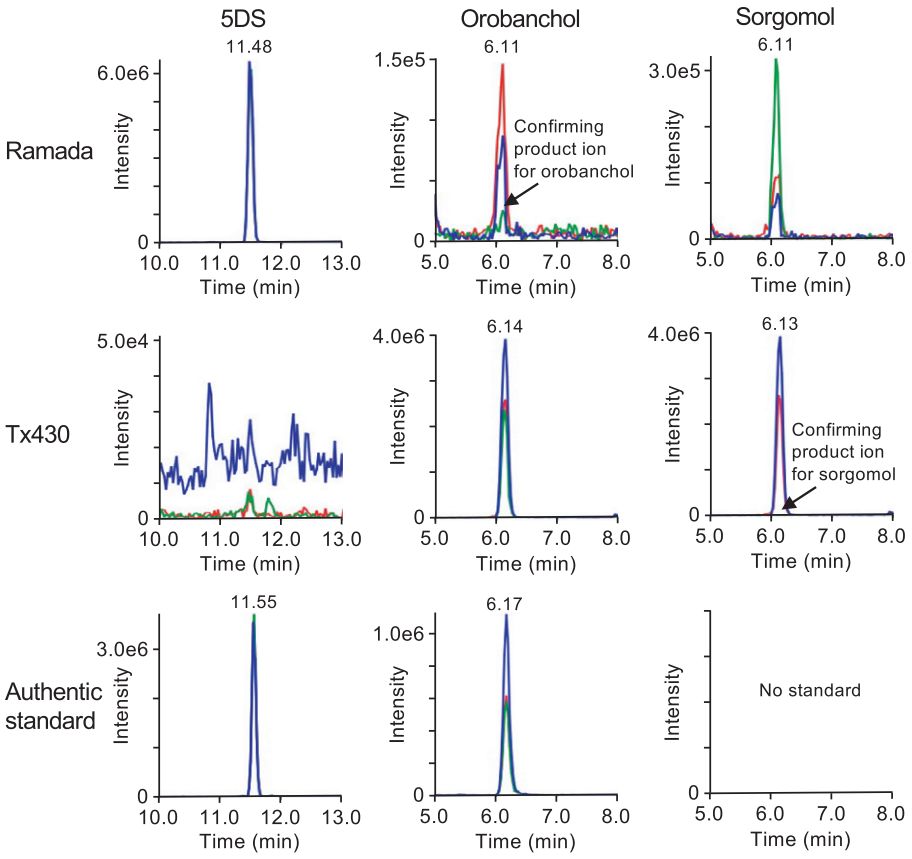

Fig. 2 Detection of 5-deoxystrigol (5DS), orobanchol, and sorgomol in root exudates of *Sorghum bicolor* accessions 'Ramada' and 'Tx430'. Multiple reaction monitoring chromatograms of 5DS (blue, 331.15/97.00; red, 331.15/216.00; green, 331.15/234.00; *m/z* in positive mode), orobanchol (blue, 347.00/233.00; red, 347.00/97.00; green, 347.00/205.00; *m/z* in positive mode), and sorgomol (blue, 347.00/233; red, 347.00/97.00; green, 347.00/317.00; *m/z* in positive mode) by LC–MS/MS are shown. Orobanchol and sorgomol are isomers (Fig. 1), so their product ions are similar, but each has a characteristic ion (green) that can be used to distinguish them.

2014), it seemed possible that the substrates of LGS1 are hydroxylated CL and hydroxylated CLA (Yoneyama *et al.*, 2020). We also noted that 4DO, a stereoisomer of 5DS, is synthesized via 18-OH-CLA in rice (Fig. 1) (Zhang *et al.*, 2014; Yoneyama *et al.*, 2018). This led us to test whether 18-OH-CLA in particular is a substrate of LGS1. We analyzed 18-OH-CLA abundance in the root exudates of 'Ramada', 'Tx430', and 'E1091'. We observed an increase in 18-OH-CLA in the root exudates of the *lgs1* mutants, 'Tx430' and 'E1091', compared with that of wild-type 'Ramada' (Figs 4, S2; Table S5). This was consistent with the idea that 18-OH-CLA is a substrate for LGS1 that overaccumulates in the absence of the enzyme. However, we could not detect endogenous 18-OH-CLA in the roots of wild-type or *lgs1* mutants, which may be due to the instability of 18-OH-CLA. Alternatively, it may be that 18-OH-CLA is used as a precursor for orobanchol biosynthesis in *lgs1* roots.

18-Hydroxycarlactonoic acid is produced by a MAX1 homologue in sorghum

18-OH-CLA has been detected as an intermediate in the conversion of CL to 4DO by Os900, one of the five homologues in rice of the cytochrome P450 CYP711A/MAX1 (Yoneyama *et al.*, 2018). Since sorghum and rice are both members of the grass family (Poaceae), we hypothesized that 18-OH-CLA in sorghum is also produced by a CYP711A enzyme(s). There are four *CYP711A* genes (named *SbMAX1a*, *SbMAX1b*, *SbMAX1c*, and *SbMAX1d*) in the sorghum genome. SbMAX1b is located in the same clade of rice paralogues, Os900, Os1400, and an unfunctional Os1500 (CYP711A4, Os01g071500) (Yoneyama *et al.*, 2018), whereas SbMAX1a is in the next branch, but its orthologue is not in rice (Fig. S5). SbMAX1c and SbMAX1d are putative orthologues of Os1900 (CYP711A5, Os02g0221900) and Os5100 (CYP711A6, Os06g0565100), respectively, which have a weak ability to convert CL to CLA (Yoneyama *et al.*, 2018). In a recent analysis of these sorghum *MAX1* homologues expressed in *E. coli*, SbMAX1a/SbCYP711A31 (Sobic.003G269500) catalyzed the conversion of CL to CLA (Wakabayashi *et al.*, 2021). We expressed the four *SbMAX1* homologues in yeast microsomes, as described previously (Abe *et al.*, 2014; Yoneyama *et al.*, 2018). When yeast microsomal fractions expressing *SbMAX1a* were incubated with *rac*-CL, we detected 18-OH-CLA in addition to CLA (Fig. 5; Table S5). The ratio of CLA and 18-OH-CLA produced by SbMAX1a was about 1 : 100, suggesting that 18-OH-CLA is the major product. Unlike rice Os900, no 4DO was detected. CLA, but not 18-OH-CLA or 4DO, was detected from yeast microsomal fractions expressing *SbMAX1b*, *SbMAX1c*, or *SbMAX1d* after incubation with *rac*-CL (Fig. S6; Table S5).

LGS1 produced 5-deoxystrigol and its isomer *in vivo*

To validate these results, we reconstructed the SL biosynthesis pathway of sorghum with a transient expression system in *N. benthamiana* (Yamamoto *et al.*, 2018). First, sorghum homologues of *D27*

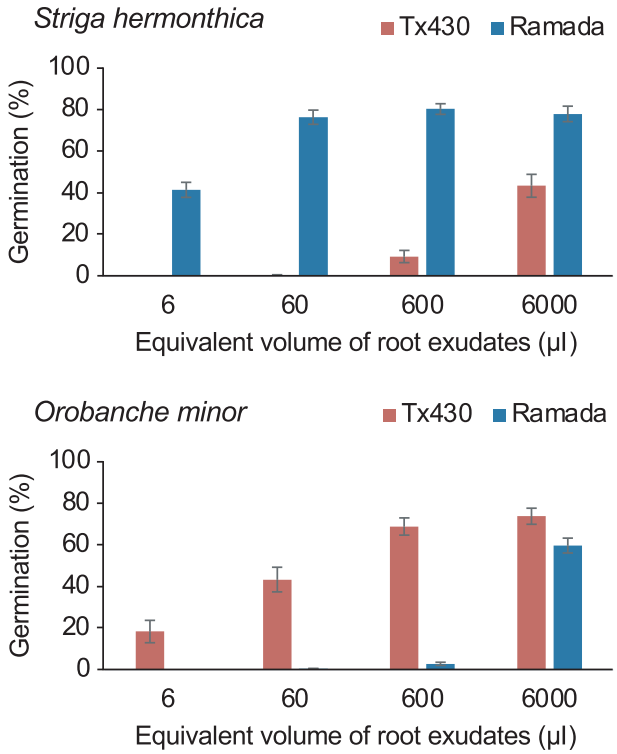

Fig. 3 Germination stimulation activity of root exudates of *Sorghum bicolor* 'Tx430' and 'Ramada' on root parasitic plants. The exudate was extracted and tested for seed germination activity on *Striga hermonthica* and *Orobanche minor*. Data are means ± SE (*c*. 30 seeds per disk, $n = 9$).

(*SbD27*), *CCD7* (*SbCCD7*), and *CCD8* (*SbCCD8*), which are upstream of CL biosynthesis (Fig. 1), were cloned and co-expressed by *Agrobacterium tumefaciens-mediated* infiltration (here, agroinfiltration) in *N. benthamiana* leaves. The SL biosynthetic intermediate CL, which is generated from endogenous β-carotene, was subsequently detected in leaf extracts (Fig. S7; Table S5). When *SbMAX1a* and *SbCPR1*, an NADPH-P450 reductase that donates the electrons required for P450 activity, were co-expressed with *SbD27*, *SbCCD7*, and *SbCCD8*, CLA and 18-OH-CLA were then detected (Fig. 6; Table S5). As with the results using yeast microsomes, no 4DO was detected. When *SbMAX1b*, *SbMAX1c*, or *SbMAX1d* were expressed in the same system instead of *SbMAX1a*, CLA was detected, which had been observed in yeast microsomes (Fig. S8; Table S5). In the case of *SbMAX1b* and *SbMAX1d*, 18-OH-CLA was also detected. When *LGS1* was co-expressed in this system with *SbMAX1a*, a peak that was considered to be 5DS (retention time, Rt 11.53 min) was detected, as well as another peak that seemed to be its isomer, 4DO (Rt 11.37 min) (Fig. 6; Table S5). When *SbMAX1b* or *SbMAX1d* were co-expressed with *LGS1* instead of *SbMAX1a*, a small amount of 5DS and the isomer was detected (Fig. S9; Table S5).

A GUS-fusion protein induces soluble expression of LGS1 in *E. coli*

In the SL pathway reconstruction in *N. benthamiana*, it is possible that an unknown metabolite other than 18-OH-CLA produced *in planta* was used as a substrate by LGS1. Therefore, we

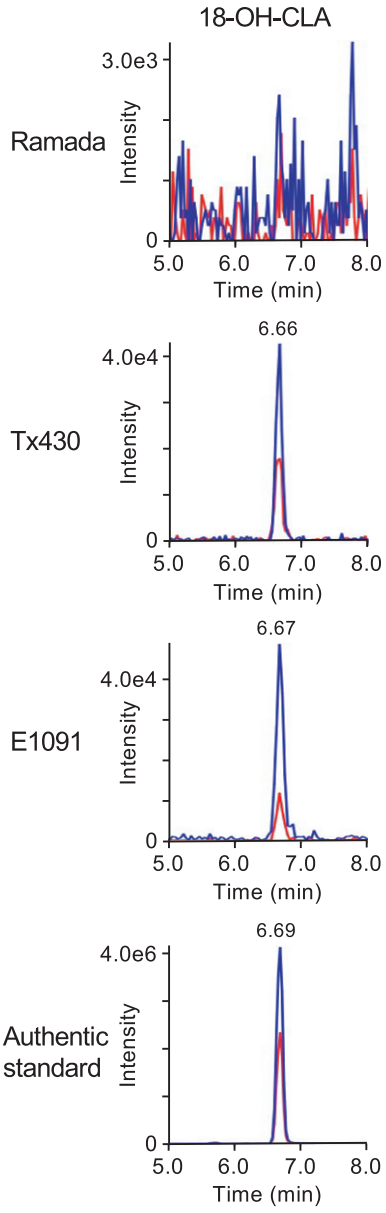

Fig. 4 Detection of 18-hydroxycarlactonoic acid (18-OH-CLA) in root exudates of *Sorghum bicolor* varieties. Multiple reaction monitoring chromatograms of 18-OH-CLA (blue, 347.00/113.00; red, 347.00/69.00; *m/z* in negative mode) by LC–MS/MS are shown.

set out to conduct *in vitro* experiments with a recombinant LGS1 protein and 18-OH-CLA. We first attempted to express a cytosolic LGS1 fusion protein with an N-terminal His-tag in *E. coli*, but it only formed inclusion bodies (Fig. S10). Even with the use of tags that induce solubility, such as *E. coli* maltose-binding protein, and low-temperature induction, LGS1 could not be retained in the soluble fraction at all. This led us to construct a novel vector in which *E. coli* GUS, which is highly expressed in the soluble fraction, was fused to the N-terminus of LGS1. To our delight, the fusion protein expressed successfully in the soluble fraction of *E. coli* (Fig. S10). Although GUS has been used commonly as a genetic reporter protein in plants, there have been no examples of its use as a tag for soluble expression in *E. coli*. Since the

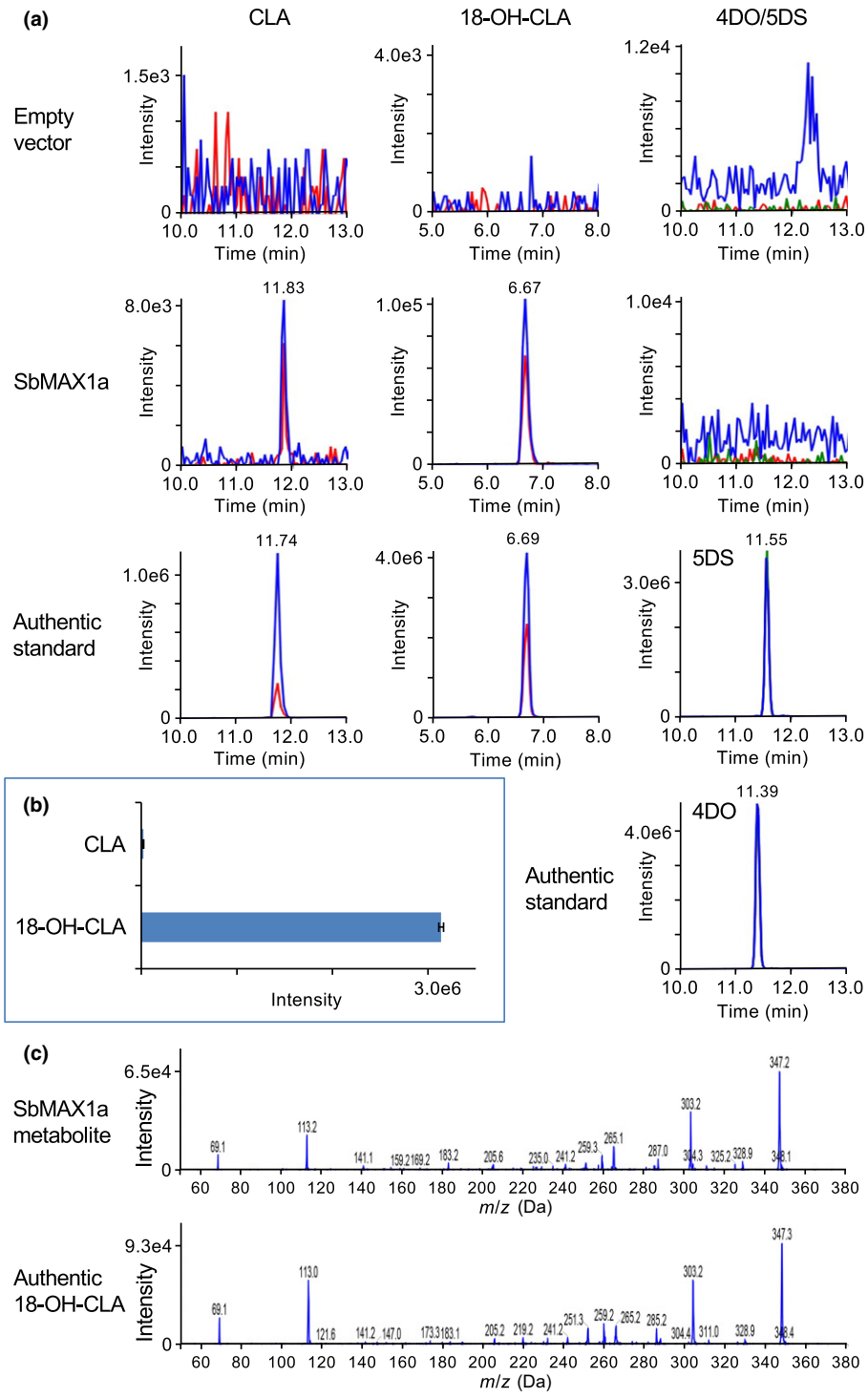

Fig. 5 Carlactone feeding experiments with recombinant SbMAX1a expressed in yeast (*Saccharomyces cerevisiae*) microsomes. Yeast microsomes carrying an empty vector or expressing *SbMAX1a* were prepared. Microsome extracts were analyzed by LC–MS/MS after incubation with *rac*-carlactone (CL). (a) Multiple reaction monitoring chromatograms of carlactonoic acid (CLA) (blue, 331.10/113.00; red, 331.10/69.00; *m/z* in negative mode), 18-hydroxycarlactonoic acid (18-OH-CLA) (blue, 347.00/113.00; red, 347.00/69.00; *m/z* in negative mode), and 4-deoxyorobanchol/5-deoxystrigol (4DO/5DS) (blue, 331.15/97.00; red, 331.15/216.00; green, 331.15/234.00; *m/z* in positive mode) are shown. 4DO and 5DS are diastereomers (Fig. 1) having the same product ions but different retention times (Rts). (b) The levels of metabolites produced from CL by yeast microsomes expressing SbMAX1a. Area intensities of CLA (331.10/113.00) and 18-OH-CLA (347.00/113.00) are shown. Data are means ± SE ($n = 3$). (c) Product ion spectra derived from the precursor ion $[M + H]^+$ (*m/z* 347) of a CL metabolite (Rt 6.69 min) produced by yeast microsomes expressing SbMAX1a and authentic 18-OH-CLA (Rt 6.66 min) are shown.

enzymatic activity of the fused GUS is functional, clones expressing the target protein can be selected using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Fig. S10).

LGS1 produced 5-deoxystrigol and 4-deoxyorobanchol from 18-hydroxycarlactonoic acid *in vitro*

We synthesized *rac*-18-OH-CLA and tested whether it could be converted to SL by the GUS-LGS1 fusion protein. In general, sulfotransferases require PAPS as a sulfo group donor (Hirschmann *et al.*, 2014). In the presence of PAPS, GUS-LGS1 caused a dramatic decrease in 18-OH-CLA. At the same time, 5DS and its isomer, 4DO, were produced in approximately equal amounts, as was observed in the *N. benthamiana* assays (Figs 7, S11; Table S5). In the absence of PAPS, GUS-LGS1 was 100 times less active than in the presence of PAPS. As with this result, GUS alone in the presence of PAPS also produced small amounts of 5DS and 4DO from 18-OH-CLA. The Michaelis constant

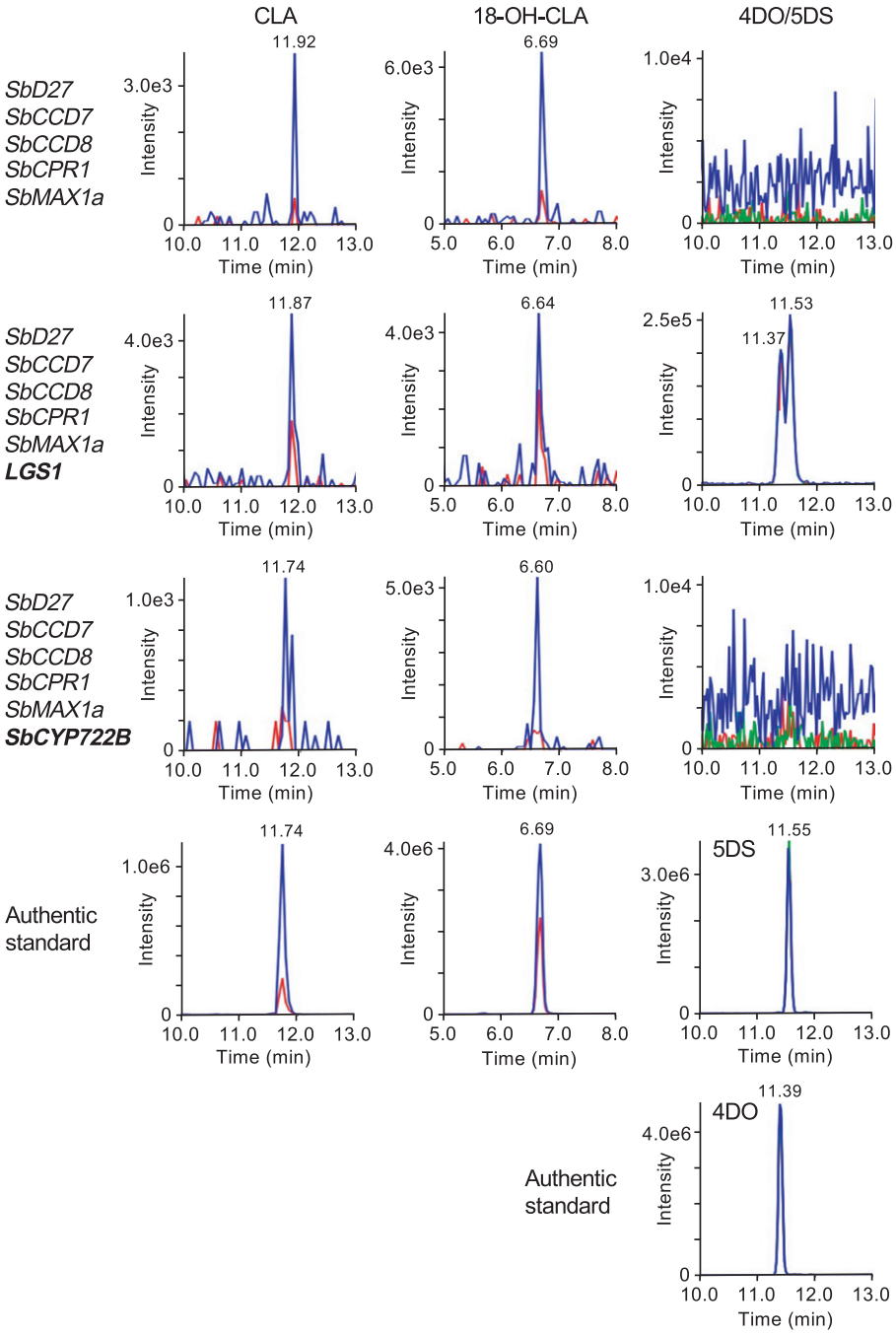

Fig. 6 Strigolactone production by a transient expression using *Nicotiana benthamiana*. *Sorghum bicolor* genes indicated at left were co-expressed in *N. benthamiana* leaves by agroinfiltration. Leaf extracts were analyzed by LC–MS/MS. Multiple reaction monitoring chromatograms of carlactonoic acid (CLA) (blue, 331.10/113.00; red, 331.10/69.00; *m/z* in negative mode), 18-hydroxycarlactonoic acid (18-OH-CLA) (blue, 347.00/113.00; red, 347.00/69.00; *m/z* in negative mode), and 4-deoxyorobanchol/5-deoxystrigol (4DO/5DS) (blue, 331.15/97.00; red, 331.15/216.00; green, 331.15/234.00; *m/z* in positive mode) are shown.

K_m of GUS-LGS1 for *rac*-18-OH-CLA was 6.37 ± 1.27 μM. We analyzed the metabolites on a chiral column to compare their relative stereochemistry with four authentic 5DS stereoisomers. We confirmed that one metabolite produced by GUS-LGS1 was 5DS and the other isomer was 4DO, both of which have a 2′-*R* configuration found in all natural SLs (Fig. 8).

Sorghum CYP722B is not involved in the production of 5-deoxystrigol or orobanchol

The CYP722C family of P450 is involved in the production of 5DS from CLA in *L. japonicus* and cotton (Mori *et al.*, 2020; Wakabayashi *et al.*, 2020). Although there is no CYP722C encoded in the sorghum genome, there is one member of the CYP722B subfamily (SbCYP722B) (Fig. S5). We tested whether SbCYP722B catalyzes 5DS synthesis. We co-expressed *SbCYP722B* with *SbD27*, *SbCCD7*, *SbCCD8*, *SbCPR1*, and different *SbMAX1* homologues in *N. benthamiana*, but no definitive 5DS was detected in any combination (Figs 6, S12; Table S5). It has been reported that CYP722C in tomato produces orobanchol and its diastereomer, *ent*-2′-*epi*-orobanchol, from CLA in an *in vitro* feeding experiment (Wakabayashi *et al.*, 2019). We speculated that SbCYP722B might be responsible for the production of orobanchol in the *lgs1* mutant. However, orobanchol was also

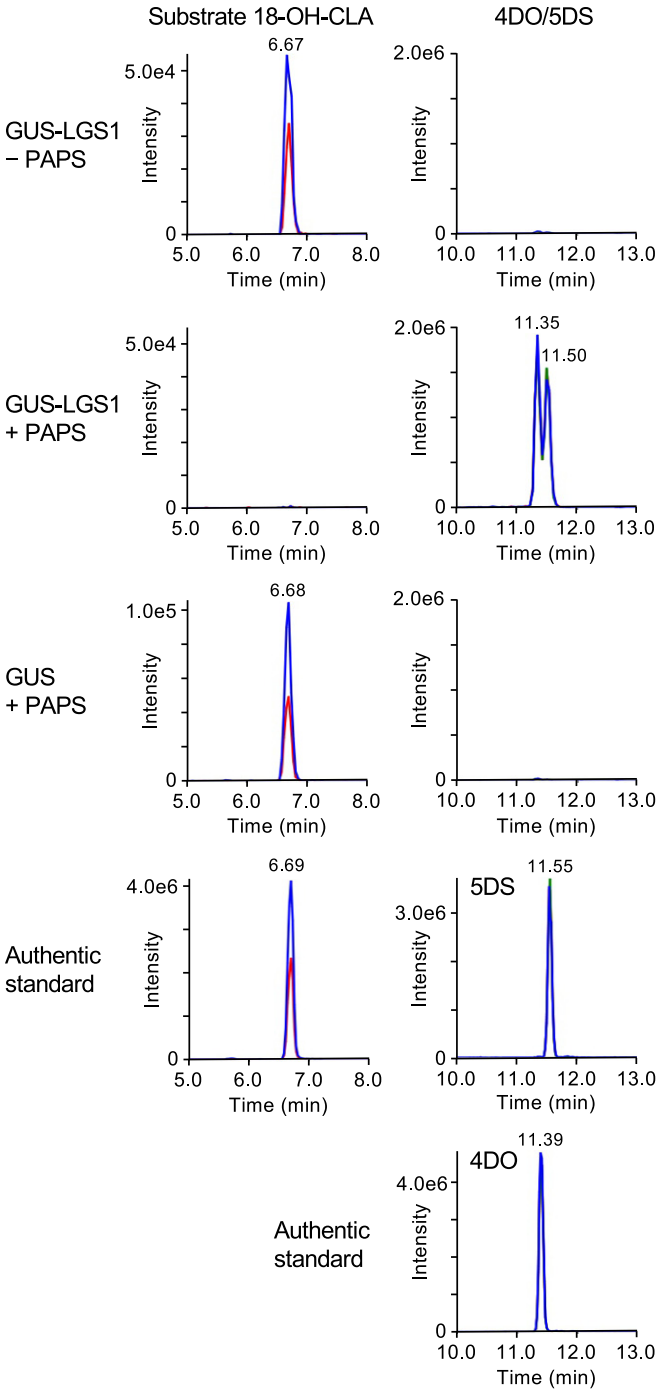

Fig. 7 18-Hydroxycarlactonoic acid (18-OH-CLA) feeding experiments with recombinant Low Germination Stimulant 1 (LGS1) protein expressed in *Escherichia coli*. β-Glucuronidase (GUS) was fused to the N-terminus of LGS1 (GUS-LGS1) to promote soluble expression. GUS was also expressed alone as a negative control. *rac*-18-OH-CLA was incubated with or without 3′-phosphoadenosine-5′-phosphosulfate (PAPS) as a sulfo donor in soluble protein fraction. The reaction mixtures were extracted and subjected to LC–MS/MS analysis after incubation with 18-OH-CLA. Multiple reaction monitoring chromatograms of 18-OH-CLA (blue, 347.00/113.00; red, 347.00/69.00; *m/z* in negative mode) and 4-deoxyorobanchol/5-deoxystrigol (4DO/5DS) (blue, 331.15/97.00; red, 331.15/216.00; green, 331.15/234.00; *m/z* in positive mode) are shown. Product ion spectra of two 18-OH-CLA metabolites produced in the presence of PAPS with recombinant GUS-LGS1 are shown in Fig. S11.

not detected in this *N. benthamiana* transient expression system (Fig. S13).

Discussion

'Tx430' is a particularly useful variety for sorghum improvement because it is a semi-dwarf that has low tannin content in seeds and a relatively high transformation efficiency (Liu & Godwin, 2012; Sato-Izawa *et al.*, 2018). This study revealed that 'Tx430' has a defect in the *LGS1* gene, resulting in a change of its predominant SL from 5DS to orobanchol (Fig. 2). Root exudates of 'Tx430' stimulate *S. hermonthica* germination poorly, suggesting that 'Tx430' will be *Striga* resistant in the field, like other *lgs1* cultivars (Fig. 3). 'Tx430' is an inbred line developed by crossing an African-derived variety at Texas A&M University (Miller, 1984). No other Tx lines examined in this study were found to be deficient in *LGS1*. Among 107 accessions of the Sorghum Global Core Collection, we identified a likely *LGS1* defect in three landraces, all of which were derived from Africa (Fig. S3). It has been reported that the loss of function of *LGS1* is adaptive across smallholder farming systems within a large region in Africa where *Striga* is prevalent (Bellis *et al.*, 2020). In the four orobanchol-producing sorghum varieties found in this study, several other genes surrounding *LGS1* were also absent, making it difficult to determine which gene(s) participate(s) in SL biosynthesis. However, genome editing has demonstrated that *Striga* resistance can result from the loss of *LGS1* alone (Bellis *et al.*, 2020). Defects in the *LGS1* gene in sorghum do not cause SL deficiency, but instead change the SL profile from 5DS to orobanchol. Because orobanchol has weaker germination-stimulating activity on *Striga* seeds than 5DS does, *lgs1* mutants become *Striga* resistant. On the other hand, orobanchol has stronger hyphal branching activity of AM fungi than 5DS does (Akiyama *et al.*, 2010), so AM symbiosis is maintained in *lgs1* mutants (Gobena *et al.*, 2017). These traits suggest that introduction of the *lgs1* mutation may be useful for the generation of *Striga*-resistant varieties in sorghum. Genome editing in the 'Tx430' background could enable beneficial trait stacking in addition to *Striga* resistance.

The loss of *LGS1* changes the SL profile from strigol type (β-configuration of the C-ring) to orobanchol type (α-configuration of the C-ring). This suggests that LGS1 regulates the stereoselective cyclization of SL in sorghum. In this study, we found that the substrate of LGS1 is 18-OH-CLA. Our experiments suggest that 18-OH-CLA is primarily produced by the cytochrome P450 SbMAX1a (Figs 5, 6, S6, S8). Its homologues, SbMAX1b and SbMAX1d, did not produce 18-OH-CLA in a feeding experiment using yeast microsomes (Fig. S6). Furthermore, SbMAX1b and SbMAX1d produced only a small amount of 5DS (Fig. S9) compared with SbMAX1a (Fig. 6) when transiently expressed in *N. benthamiana*. Interestingly, LGS1 generated not only 5DS from 18-OH-CLA *in vivo* and *in vitro*, but also its diastereomer 4DO, indicating that stereoselective conversion did not occur (Figs 6, 8). LGS1 catalyzed the reaction in a sulfo donor-dependent manner. This strongly suggests that LGS1 catalyzes the sulfonation of 18-OH-CLA (Fig. 7), although a sulfate ester

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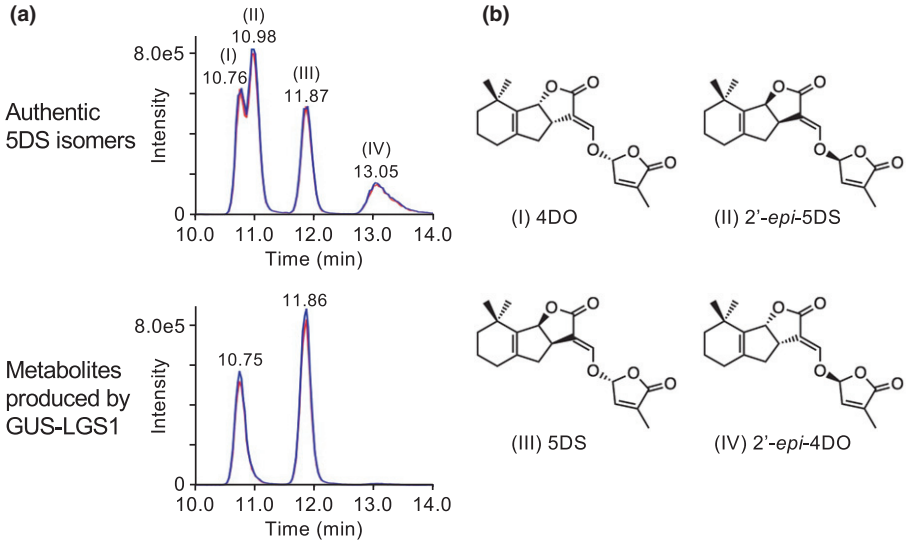

Fig. 8 Chiral-LC–MS/MS analysis of 18-hydroxycarlactonoic acid metabolites produced by recombinant β-Glucuronidase (GUS)-Low Germination Stimulant 1 (LGS1). (a) Multiple reaction monitoring chromatogram of four 5-deoxystrigol (5DS) stereoisomers and the metabolites of GUS-LGS1 (blue, 331.15/97.00; red, 331.15/216.00; green, 331.15/234.00; *m/z* in positive mode) are shown. (b) The chemical structures of 5DS stereoisomers. 4DO, 4-deoxyorobanchol.

of 18-OH-CLA could not be found owing to the lack of synthetic standards. Since 5DS and 4DO are produced in approximately equal amounts in the *in vivo* and *in vitro* experiments of LGS1, the sulfate group may have been eliminated from the sulfate ester of 18-OH-CLA and then artificial nonselective cyclization may have occurred. It is possible that another unknown enzyme(s) is involved in the stereoselective cyclization of the C-ring to the β-configuration and the associated elimination of the sulfate group (Fig. 9). Alternatively, since 4DO is not detected in sorghum, it is possible that 4DO is further converted to unknown SLs or selectively catabolized.

Defects in *LGS1* cause sorghum to produce orobanchol predominantly, but the basis for the biosynthetic bypass to orobanchol has not been determined. In rice, orobanchol is produced by the MAX1 homologues CYP711A2 and CYP711A3 (Zhang *et al.*, 2014), whereas CYP722C is involved in orobanchol biosynthesis in tomato (Wakabayashi *et al.*, 2019) (Fig. 1). It has also been reported that CYP722C is involved in the production of 5DS in *L. japonicus* and cotton (Mori *et al.*, 2020; Wakabayashi *et al.*, 2020). However, neither orobanchol nor 5DS was detected when sorghum *SbMAX1* homologues or *SbCYP722B* were expressed in the *N. benthamiana* expression system (Figs 6, S12, S13). These results imply that there is an unknown enzyme (s) that enables orobanchol production in the absence of *LGS1*.

Although sulfotransferases exist in other plant species, no likely orthologue of *LGS1* has been found in dicotyledons or in even in

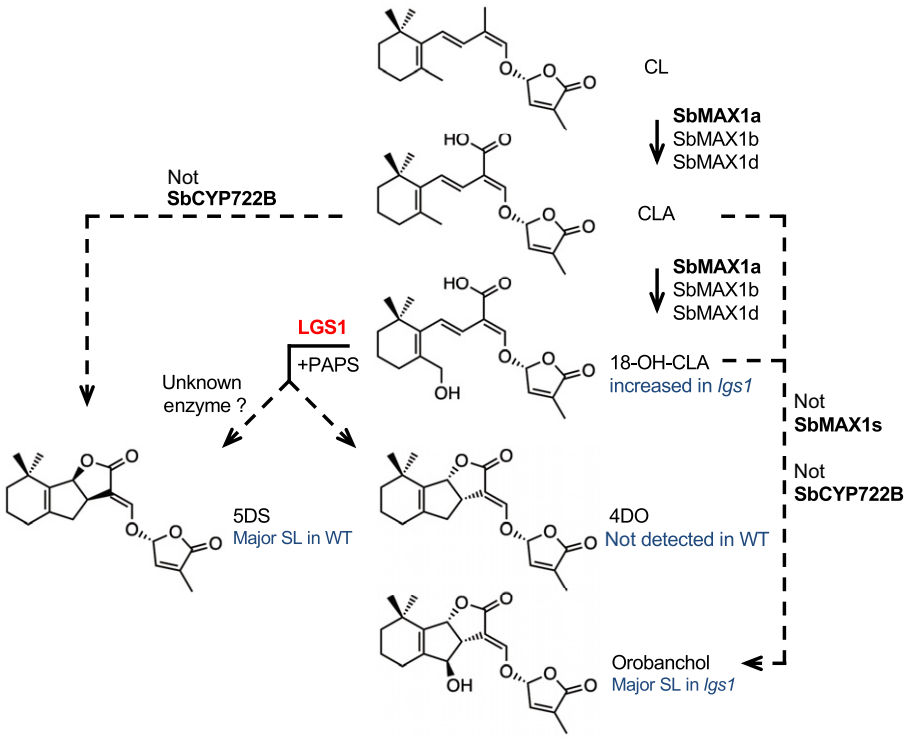

Fig. 9 Proposed pathway for strigolactone (SL) biosynthesis in *Sorghum bicolor*. Carlactone (CL) is converted to 18-hydroxycarlactonoic acid (18-OH-CLA) via carlactonoic acid (CLA) by SbMAX1a. LGS1 (in red) produces 5-deoxystrigol (5DS) and 4-deoxyorobanchol (4DO) from 18-OH-CLA in the presence of a sulfo donor (e.g. 3′-phosphoadenosine-5′-phosphosulfate, PAPS). Stereospecific production of 5DS may be catalyzed by another unknown enzyme(s) in *S. bicolor*. 5DS is not produced by SbCYP722B, unlike CYP722C in *Gossypium arboreum* and *Lotus japonicus*. Orobanchol is not produced by SbMAX1s (CYP711A) or SbCYP722B, unlike Os900 in *Oryza sativa* and CYP722C in *Solanum lycopersicum*. Blue letters indicate the SL production in *S. bicolor* wild-type (WT) and the *lgs1* mutant.

rice or maize, which are also Poaceae. This is another example of the unexpected diversity that has been found among plant species in the SL biosynthesis pathways that act downstream of CLA (Wang *et al.*, 2020). The factors that have driven the evolution of stereoselective SL biosynthesis mechanisms are unknown currently, but they may be a consequence of microbial or parasitic interactions. Regardless, it is apparent that sorghum has recruited a sulfotransferase into the SL biosynthetic pathway to produce 5DS. Among the biosynthetic pathways of phytohormones, it is known that brassinosteroids, jasmonates, and salicylic acid are sulfonated by sulfotransferases (Hirschmann *et al.*, 2014). However, those reactions do not involve dehydration or cyclization like LGS1. Interestingly, sulfotransferases in animals are responsible for the dehydration of carotenoid-derived vitamin A, retinol, using PAPS as co-substrate. Like SLs, the end product, anhydroretinol, is not sulfonated (Vakiani *et al.*, 1998).

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Author contributions

AY, KA, SY, KoichiY, DCN and TN planned and designed the research. AY performed cloning and enzyme assays. NM and KA synthesized *rac*-18-OH-CLA. MK and AY carried out PCR genotyping. AY, XX, and KaoriY carried out LC–MS/MS analysis. AY and KM performed a transient expression. KS-I prepared plant materials. AY, KA, KoichiY and TN analyzed data. AY, KA, DCN and TN wrote the manuscript.

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References

Abe S, Sado A, Tanaka K, Kisugi T, Asami K, Ota S, Kim HI, Yoneyama K, Xie X, Ohnishi T *et al.* 2014. Carlactone is converted to carlactonoic acid by MAX1 in *Arabidopsis* and its methyl ester can directly interact with AtD14 *in vitro*. *Proceedings of the National Academy of Sciences, USA* **111**: 18084–18089.

Akiyama K, Matsuzaki K, Hayashi H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* **435**: 824–827.

Akiyama K, Ogasawara S, Ito S, Hayashi H. 2010. Structural requirements of strigolactones for hyphal branching in AM fungi. *Plant and Cell Physiology* **51**: 1104–1117.

Alder A, Jamil M, Marzorati M, Bruno M, Vermathen M, Bigler P, Ghisla S, Bouwmeester H, Beyer P, Al-Babili S. 2012. The path from β-carotene to carlactone, a strigolactone-like plant hormone. *Science* **335**: 1348–1351.

Arite T, Iwata H, Ohshima K, Maekawa M, Nakajima M, Kojima M, Sakakibara H, Kyozuka J. 2007. *DWARF10*, an *RMS1*/*MAX4*/*DAD1* ortholog, controls lateral bud outgrowth in rice. *The Plant Journal* **51**: 1019–1029.

Bellis ES, Kelly EA, Lorts CM, Gao H, DeLeo VL, Rouhan G, Budden A, Bhaskara GB, Hu Z, Muscarella R *et al.* 2020. Genomics of sorghum local adaptation to a parasitic plant. *Proceedings of the National Academy of Sciences, USA* **117**: 4243–4251.

Beveridge CA, Ross JJ, Murfet IC. 1996. Branching in pea (action of genes *Rms3* and *Rms4*). *Plant Physiology* **110**: 859–865.

Booker J, Auldridge M, Wills S, McCarty D, Klee H, Leyser O. 2004. MAX3/CCD7 is a carotenoid cleavage dioxygenase required for the synthesis of a novel plant signaling molecule. *Current Biology* **14**: 1232–1238.

Chesterfield RJ, Vickers CE, Beveridge CA. 2020. Translation of strigolactones from plant hormone to agriculture: achievements, future perspectives, and challenges. *Trends in Plant Science* **25**: 1087–1106.

Cook CE, Whichard LP, Turner B, Wall ME, Egley GH. 1966. Germination of witchweed (*Striga lutea* Lour.): isolation and properties of a potent stimulant. *Science* **154**: 1189–1190.

Deschamps S, Zhang Y, Llaca V, Ye L, Sanyal A, King M, May G, Lin H. 2018. A chromosome-scale assembly of the sorghum genome using nanopore sequencing and optical mapping. *Nature Communications* **9**: e4844.

Fernández-Aparicio M, Yoneyama K, Rubiales D. 2010. The role of strigolactones in host specificity of *Orobanche* and *Phelipanche* seed germination. *Seed Science Research* **21**: 55–61.

Gobena D, Shimels M, Rich PJ, Ruyter-Spira C, Bouwmeester H, Kanuganti S, Mengiste T, Ejeta G. 2017. Mutation in sorghum *LOW GERMINATION STIMULANT 1* alters strigolactones and causes *Striga* resistance. *Proceedings of the National Academy of Sciences, USA* **114**: 4471–4476.

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun EA, Pillot J-P, Letisse F, Matusova R, Danoun S, Portais J-C *et al.* 2008. Strigolactone inhibition of shoot branching. *Nature* **455**: 189–194.

Hirschmann F, Krause F, Papenbrock J. 2014. The multi-protein family of sulfotransferases in plants: composition, occurrence, substrate specificity, and functions. *Frontiers in Plant Science* **5**: e556.

Liu G, Godwin ID. 2012. Highly efficient sorghum transformation. *Plant Cell Reports* **31**: 999–1007.

Miller FR. 1984. Registration of RTx430 sorghum parental line. *Crop Science* **24**: 1224.

Mohemed N, Charnikhova T, Fradin EF, Rienstra J, Babiker AGT, Bouwmeester HJ. 2018. Genetic variation in *Sorghum bicolor* strigolactones and their role in resistance against *Striga hermonthica*. *Journal of Experimental Botany* **69**: 2415–2430.

Mori N, Nomura T, Akiyama K. 2020. Identification of two oxygenase genes involved in the respective biosynthetic pathways of canonical and non-canonical strigolactones in *Lotus japonicus*. *Planta* **251**: e40.

Nelson DC. 2021. The mechanism of host-induced germination in root parasitic plants. *Plant Physiology* **185**: 1353–1373.

Pompon D, Louerat B, Bronine A, Urban P. 1996. Yeast expression of animal and plant P450s in optimized redox environments. *Methods in Enzymology* **272**: 51–64.

Raghuwanshi A, Birch RG. 2010. Genetic transformation of sweet sorghum. *Plant Cell Reports* **29**: 997–1005.

Sato-Izawa K, Tokue K, Ezura H. 2018. Development of a stable *Agrobacterium*-mediated transformation protocol for *Sorghum bicolor* Tx430. *Plant Biotechnology* **35**: 181–185.

Seto Y, Sado A, Asami K, Hanada A, Umehara M, Akiyama K, Yamaguchi S. 2014. Carlactone is an endogenous biosynthetic precursor for strigolactones. *Proceedings of the National Academy of Sciences, USA* **111**: 1640–1645.

Shehzad T, Okuizumi H, Kawase M, Okuno K. 2009. Development of SSR-based sorghum (*Sorghum bicolor* (L.) Moench) diversity research set of germplasm and its evaluation by morphological traits. *Genetic Resources and Crop Evolution* **56**: 809–827.

Spallek T, Mutuku M, Shirasu K. 2013. The genus *Striga*: a witch profile. *Molecular Plant Pathology* **14**: 861–869.

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K *et al.* 2008. Inhibition of shoot branching by new terpenoid plant hormones. *Nature* **455**: 195–200.

Vakiani E, Luz JG, Buck J. 1998. Substrate specificity and kinetic mechanism of the insect sulfotransferase, retinol dehydratase. *Journal of Biological Chemistry* **273**: 35381–35387.

Wakabayashi T, Hamana M, Mori A, Akiyama R, Ueno K, Osakabe K, Osakabe Y, Suzuki H, Takikawa H, Mizutani M *et al.* 2019. Direct conversion of carlactonoic acid to orobanchol by cytochrome P450 CYP722C in strigolactone biosynthesis. *Science Advances* **5**: eaax9067.

Wakabayashi T, Ishiwa S, Shida K, Motonami N, Suzuki H, Takikawa H, Mizutani M, Sugimoto Y. 2021. Identification and characterization of sorgomol synthase in sorghum strigolactone biosynthesis. *Plant Physiology* **185**: 902–913.

Wakabayashi T, Shida K, Kitano Y, Takikawa H, Mizutani M, Sugimoto Y. 2020. CYP722C from *Gossypium arboreum* catalyzes the conversion of carlactonoic acid to 5-deoxystrigol. *Planta* **251**: e97.

Wang JY, Lin PY, Al-Babili S. 2020. On the biosynthesis and evolution of apocarotenoid plant growth regulators. *Seminars in Cell & Developmental Biology* **109**: 3–11.

Xie X, Yoneyama K, Kisugi T, Uchida K, Ito S, Akiyama K, Hayashi H, Yokota T, Nomura T, Yoneyama K. 2013. Confirming stereochemical structures of strigolactones produced by rice and tobacco. *Molecular Plant* **6**: 153–163.

Yamamoto T, Hoshikawa K, Ezura K, Okazawa R, Fujita S, Takaoka M, Mason HS, Ezura H, Miura K. 2018. Improvement of the transient expression system for production of recombinant proteins in plants. *Scientific Reports* **8**: e4755.

Yoneyama K. 2020. Recent progress in the chemistry and biochemistry of strigolactones. *Journal of Pesticide Science* **45**: 45–53.

Yoneyama K, Akiyama K, Brewer PB, Mori N, Kawano-Kawada M, Haruta S, Nishiwaki H, Yamauchi S, Xie X, Umehara M *et al.* 2020. Hydroxyl carlactone derivatives are predominant strigolactones in Arabidopsis. *Plant Direct* **4**: e00219.

Yoneyama K, Mori N, Sato T, Yoda A, Xie X, Okamoto M, Iwanaga M, Ohnishi T, Nishiwaki H, Asami T *et al.* 2018. Conversion of carlactone to carlactonoic acid is a conserved function of MAX1 homologs in strigolactone biosynthesis. *New Phytologist* **218**: 1522–1533.

Yoneyama K, Yoneyama K, Takeuchi Y, Sekimoto H. 2007. Phosphorus deficiency in red clover promotes exudation of orobanchol, the signal for mycorrhizal symbionts and germination stimulant for root parasites. *Planta* **225**: 1031–1038.

Zhang Y, van Dijk ADJ, Scaffidi A, Flematti GR, Hofmann M, Charnikhova T, Verstappen F, Hepworth JO, van der Krol S, Leyser O *et al.* 2014. Rice cytochrome P450 MAX1 homologs catalyze distinct steps in strigolactone biosynthesis. *Nature Chemical Biology* **10**: 1028–1033.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Synthesis of 18-hydroxycarlactonoic acid (1).

Fig. S2 Ratio of strigolactones in root exudates of *Sorghum bicolor* accessions Ramada and Tx430.

Fig. S3 PCR amplification of the *LGS1* gene and neighboring genes in *Sorghum bicolor* varieties.

Fig. S4 Detection of 5-deoxystrigol and orobanchol in root exudates of Sorghum Global Core Collection.

Fig. S5 Phylogenetic tree of CYP711A (MAX1) and CYP722.

Fig. S6 Carlactone feeding experiments with recombinant SbMAX1 homologs expressed in yeast (*Saccharomyces cerevisiae*) microsomes.

Fig. S7 Carlactone production by a transient expression using *Nicotiana benthamiana*.

Fig. S8 Transient expressions of *Sorghum bicolor* MAX1 homologs using *Nicotiana benthamiana*.

Fig. S9 Transient expressions of *Sorghum bicolor* MAX1 homologs and LGS1 using *Nicotiana benthamiana*.

Fig. S10 Expression of β-glucuronidase (GUS)-fused LGS1 protein in *Escherichia coli*.

Fig. S11 Product ion spectra of 18-hydroxycarlactonoic acid metabolites produced by recombinant GUS-LGS1 protein expressed in *Escherichia coli*.

Fig. S12 Transient expressions of *Sorghum bicolor* MAX1 homologs and SbCYP722B using *N. benthamiana*.

Fig. S13 No detection of orobanchol in transient expression of SbMAX1s and SbCYP722B using *N. benthamiana*.

Methods S1 Chemical synthesis of 18-hydroxycarlactonoic acid (1).

Table S1 Tx lines of *Sorghum bicolor* examined in this study.

Table S2 A global core collection of *Sorghum bicolor* examined in this study.

Table S3 Primer sequences used for genome PCR.

Table S4 Primer sequences used for cloning.

Table S5 Ratio of quantifier/qualifier ion in MRM chromatograms.

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