The rare sugar D-tagatose protects plants from downy mildews and is a safe fungicidal agrochemical

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The rare sugar D-tagatose is a safe natural product used as a commercial food ingredient. Here, we show that D-tagatose controls a wide range of plant diseases and focus on downy mildews to analyze its mode of action. It likely acts directly on the pathogen, rather than as a plant defense activator. Synthesis of mannan and related products of D-mannose metabolism are essential for development of fungi and oomycetes; D-tagatose inhibits the first step of mannose metabolism, the phosphorylation of D-fructose to D-fructose 6-phosphate by fructokinase, and also produces D-tagatose 6-phosphate. D-Tagatose 6-phosphate sequentially inhibits phosphomannose isomerase, causing a reduction in D-glucose 6-phosphate and D-fructose 6-phosphate, common substrates for glycolysis, and in D-mannose 6-phosphate, needed to synthesize mannan and related products. These chain-inhibitory effects on metabolic steps are significant enough to block initial infection and structural development needed for reproduction such as conidiophore and conidiospore formation of downy mildew.

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are sugars are defined by the International Society of Rare Sugars as monosaccharides that are rarely present in nature\(^2\). Saccharides are produced by photosynthesis, and most are stored as polysaccharides in the form of starch, comprising monomers of D-glucose, the most abundant hexose monosaccharide on earth. Among the 34 hexoses are 16 aldoses, 8 ketoses, and 10 polyols; four hexoses (D-glucose, D-fructose, D-mannose, and D-galactose) are profuse in nature, while the other 30 hexoses are rare sugars (Supplementary Figs. 1, 2)\(^2\)\(^-\)\(^2\). In 2002, Izumori presented the Izumoring concept (Supplementary Fig. 2)\(^2\)\(^-\)\(^3\), a blueprint for the enzymatic production of all hexosaccharides possible, rare hexose

**Results**

**D-tagatose effects on plant diseases.** Effective concentrations (w/v) of D-tagatose that were expected to reduce severity to 50% that of the mock treatment (untreated) were examined in either pot or field trials or both against multiple diseases (Table 1). D-Tagatose solution at 0.5–10% (w/v) reduced the severity of symptoms induced by all pathogens tested, and even at 0.5% or 1% (w/v) of D-tagatose significantly reduced the severity of downy mildew and powdery mildew on a wide range of host plants (Table 1). Typical symptoms of cucumber downy mildew and the reduced symptoms by these treatments with D-tagatose are shown in Fig. 1a. These treatments against cucumber downy mildew indicated that the pathogen-infected first true leaf after treatment with 5% (w/v) D-tagatose showed no symptoms, the same as the effect of treatment with agrochemicals such as benzenazole (FBZ), acibenzolar-S-methyl (ASM), or metalaxyl (Fig. 1a). No phytotoxicity was observed on plants treated with D-tagatose, while development of the second true leaf was significantly inhibited on plants treated with D-allose or D-allulose (Fig. 1a).

Diseases on cucumber leaves treated with 1 or 5% D-tagatose or metalaxyl at different timings before or after inoculation with the downy mildew pathogen were compared to verify whether D-tagatose works as a preventive or curative agent (Fig. 1b). The inhibition of disease development by D-tagatose treatment was similar to the inhibition caused by fungicide treatment; no symptoms were observed when either the sugar or fungicide was applied 5 days before inoculation, and 90% or more of symptoms were suppressed when these agents were applied even at 7 days before the inoculation (Fig. 1b). Moreover, no symptoms were observed after either of these treatments was applied within 48 h after inoculation, but D-tagatose treatment at 60 h or later after inoculation and fungicide treatment at 72 h or later after inoculation did not inhibit symptom development completely (Fig. 1b, Curative).

In field trials, grapevine, cucumber, Chinese cabbage, onion, and spinach were then treated with or without 1 or 5% (w/v) D-tagatose, fungicides (cyazofamid FL [CZF], chlorothalonil [CTN] FL, and mancozeb [JMCZ] WP) with inoculation of one of five downy mildew pathogens (detail experimental conditions were shown in Supplementary Table 2). In all cases, the suppression of symptoms by D-tagatose was similar to that by the fungicides (Fig. 1c–g).

Effects of D-tagatose on Arabidopsis downy mildew. In a test of a host-pathogen model system, symptoms on A. thaliana ecotype Colombia-0 (Col-0) caused by H. arabidopsis isolate Noco2 were suppressed by 1% (w/v) D-tagatose (Fig. 2a), indicating its efficacy in this host-pathogen system. To understand the mode of action of D-tagatose using the model system, we assessed the extent of hyphal growth of isolate Noco2 on cotyledons of A. thaliana treated with different concentrations of the rare sugar (Fig. 2b, c). Microscopic observations of hyphal growth of H. arabidopsis isolate Noco2 at 10 days after the inoculation of Arabidopsis seedlings indicated growth was inhibited in leaves treated with 1% (w/v) D-tagatose (Fig. 2b). At 7 days after inoculation, hyphal growth was rated based on hyphal growth efficiency (% of leaf area with hyphae). The percentage of leaves with extensive hyphal growth (+ + + rating) decreased, with a negative correlation to the D-tagatose concentration, and the percentage of leaves with no fungal growth (− rating) increased, with a positive correlation to concentration, with a dose-dependency in both associations (Fig. 2c). After treatment with 2.5 mM D-tagatose, 58.0 ± 1.8% of the cotyledons had 75–100% area (+ + + rating) of their leaves with hyphae similar to that of mock-treated (no-sugar treatment) leaves (57.5 ± 1.8%) (Fig. 2c). Treatment with 25–100 mM D-tagatose lowered the percentage of cotyledons with 75–100% colonization to 9.9 ± 3.1% to 6.7 ± 0.3%, respectively (Fig. 2c).

When the effects of D-tagatose on asexual reproduction were examined, even 2.5 mM of D-tagatose significantly inhibited conidiation on seedlings with four leaves (including the
cotyledons) \((p = 5.29e-9)\), and more than 25 mM D-tagatose almost completely inhibited conidiation (Fig. 2d). D-Tagatose treatment similarly inhibited conidiophore and oospore formation in/on cotyledons (Fig. 2e, f), and formation of conidiophores and oospores (Fig. 2g) was suppressed almost completely by treatment with more than 25 mM D-tagatose. D-Tagatose deoxygenated at C-6 position (50 mM; hereafter “deoxygenated”) (Supplementary Fig. S1), however, did not inhibit conidiophore formation in planta (Fig. 2h). Hyphal length after conidiospores had germinated for 6 h in vitro on medium in microtiter plates was also suppressed by treatment with more than 25 mM D-tagatose, but no further dose response was observed even up to 400 mM (Fig. 2i, j). Treatment with the same concentrations of D-mannitol used as the control to check for a potential osmotic pressure effect did not show any effect even at 400 mM (Fig. 2j).

### Table 1 Effective dose of D-tagatose on various diseases caused by different pathogens on different host plants in pot and field trials.

| Class         | Disease          | Pathogen                      | Host              | Effective Dose (%)* | Reference |
|---------------|------------------|-------------------------------|-------------------|---------------------|-----------|
| Oomycetes     | Downy mildew    | *Plasmopara viticola*         | Grapevine         | 1                   | 3         | 44,50 |
|               |                  | *Pseudoperonospora cubensis*  | Cucumber           | 0.5                 | 0.5       | 44,50 |
|               |                  | *(syn. H. brassicae)*         | Chinese cabbage   | nt.                 | 1         | This study |
|               |                  | *Hyaloperonospora parasitica* | Cabbage           | 1                   | nt.       | 44,50 |
|               |                  | *(syn. H. brassicae)*         |                   |                     |           |         |
|               |                  | *Peronospora destructrix*     |                   |                     |           |         |
|               |                  | *Peronospora farinosa f. sp. spinaciae* |                   |                     |           |         |
|               | Damping off      | *Pythium aphanidermatum*      | Cucumber           | 1                   | nt.       | 44 |
|               | Seedling blight  | *Pythium graminicola*         | Rice              | 1                   | 1         | 44 |
|               | Late blight      | *Phytophthora infestans*      | Tomato            | 10                  | nt.       | 44 |
| Ascomycetes   | Powdery mildew  | *Erysiphe necator*            | Grapevine         | 0.5                 | nt.       | 50 |
|               |                  | *Podosphaera xanthii*         | Cucumber           | 0.5                 | 0.5       | 44,50 |
|               |                  | *Podosphaera leucotrichia*    | Apple             | 0.5                 | nt.       | 50 |
|               |                  | *Podosphaera aphanis*         | Strawberry        | nt.                 | 1         | This study |
|               |                  | *Sphaerotheca fuliginea*      | Eggplant          | 0.5                 | 0.5       | 50 |
|               |                  | *Oidium violae*               | Tomato            | 0.5                 | nt.       | 50 |
|               |                  | *Oidiopsis sicula*            | Pepper            | 0.5                 | 1         | 50 |
|               |                  | *Blumeria graminis f. sp. hordei* | Tomato          | 1                   | nt.       | 50 |
|               |                  | *Botrytis cinerea*            | Pepper            | 5                   | nt.       | 44 |
|               |                  | *Alternaria brassicicola*      | Cabbage           | 5                   | nt.       | This study |
|               |                  | *Colchobolus miyabeanus*      | Rice              | 5                   | nt.       | This study |
|               |                  | *Colletotrichum orbiculare*   | Cucumber          | 5                   | nt.       | This study |
|               |                  | *Pyricularia oryzae* (syn. Magnaporthe oryzae) | Rice         | 5                   | nt.       | 44 |
| Basidiomycetes| Brown rust       | *Puccinia recondita*          | Wheat             | 5                   | 5         | 44 |
|               | Sheath blight    | *Rhizoctonia solani AG-1 IA*  | Rice              | 5                   | nt.       | This study |

*Experiments are described in Supplementary Tables 1 and 2.*

**D-Tagatose acts on pathogen but not host defense activation.**

Toward understanding the mechanism underlying the D-tagatose effects on downy mildew, we analyzed the expression of PR-protein and defense-related genes: peroxidase [abbreviated here as POX], lipoxygenase [LOX], pore-forming toxin-like protein [Hrlf]) and induced defense (4-coumarate-CoA ligase [4CL] and caffeoyl-CoA O-methyltransferase [CCoAMT]) in plants after treatment with D-tagatose at the effective dose of 0.5% (w/v), which suppressed disease severity by more than 50% compared with the mock treatment in both pot and field trials (Table 1) and at 1% (w/v), which gave the same control as the fungicide treatment in a field test against cucumber downy mildew (Figs. 1d and 3a)\(^{24–28}\). Treatment with D-tagatose alone at 1% (w/v) did not significantly induce any of these genes within 48 h (Fig. 3a) or in susceptible cucumber leaves after inoculation with the pathogen without D-tagatose treatment. For all other combinations, gene expression did not change significantly within 48 h, except for minor inductions of LOX at 24 h after inoculation with D-tagatose treatment (Fig. 3a). When the expression of these genes was compared between inoculations with/without D-tagatose treatment, only 4CL was induced at 24 h after inoculation in conjunction with D-tagatose treatment (Fig. 3a).

Since D-allulose and D-allose induced disease tolerance in rice as a plant activator by inducing PR-protein gene expression, which is typically initiated by a transient generation of reactive oxygen species after a hypersensitive reaction or apoptosis-like lesion mimic formation\(^{14,16,17}\), we tested rice for an effect of D-tagatose on the PR-protein genes that were induced by D-allulose and D-allose. Overall expression patterns in scatter plots of microarray data at 2 days after treatment with 0.5 mM of D-allulose\(^{14}\), D-allose\(^{16,17}\), D-glucose\(^{14,16,17}\), or D-tagatose indicated less fluctuation between the induction and reduction of the expression of all genes analyzed after D-tagatose treatment compared with the D-allose\(^{14}\), D-allose\(^{16,17}\), or even D-glucose treatments\(^{14,16,17}\) (Supplementary Fig. 3). Several PR-protein genes that are induced by D-allulose or D-allose in rice were not...
Fig. 1 Effect of D-tagatose on severity of various downy mildews. Typical symptoms of cucumber downy mildew on the first true leaf of untreated cucumber plant and reduced severity after treatment with 1% or 5% (w/v) D-tagatose, D-allulose, or D-allose in pot trials in comparison with probenazole (PBZ), acibenzolar-S-methyl (ASM), and metalaxyl (Scale Bar = 5 cm) (a). The timing of treatments with sugars and agrochemicals in pot trials varied from 1 to 7 days before inoculation with the pathogen (“Preventive”) or from 12 to 72 h after inoculation (“Curative”), and their symptoms were compared using the averages of each severity after 7 days to calculate a relative disease severity against average severity for the mock-treated plants. Data presented are representative of three independent experiments (b). Severity of downy mildews in field trials after treatment with D-tagatose (1% or 5%, w/v) (D-Tag) compared (n = 50 to 163 leaves per group) with a fungicide: cyazofamid FL (CZF) at 94 parts per million (ppm) on grapevine and spinach downy mildews (c, g), chlorothalonil (CTN) FL at 400 ppm for cucumber and Chinese cabbage downy mildews (d, e), and mancozeb (MCZ) WP at 1875 ppm for onion downy mildew (f), or mock treatment (Untreated) (c–g). Disease severity was calculated from the degree of disease index based on the Fungicide Evaluation Manual by the Japan Plant Protection Association for field trials as described in the Methods. Data presented are representative of 3–4 independent experiments. Error bars are SD. Means with different letters differed significantly at p < 0.05 in a Tukey-Kramer multiple comparison test.
induced significantly by D-tagatose (Fig. 3b), while the expression of all these genes was stimulated significantly by D-allulose or D-allose treatment as described previously. Moreover, RNA-sequencing did not show any major up- or downregulation in the expression of PR-proteins, plant hormones (e.g., jasmonic acid, ethylene, and salicylic acid)-related and defense-related genes between D-tagatose-treated and untreated plants of A. thaliana after inoculation with H. arabidopsidis isolate Noco2 (Fig. 3c, Supplementary Fig. 4a). Some genes were up- or downregulated more than 10 times between the inoculated plants that were treated with D-tagatose or untreated (Supplementary Fig. 4b), but no particular trend for any group of genes.
was found to explain the protection conferred by D-tagatose against downy mildew. Several sugar-related genes such as At5g45830 (DELAY OF GERMINATION 1 [DOG1]), At5g09000 (glucosyltransferase), At2g33100 (cellulose synthase-like protein [ATCSLD1]), and At5g30800 (sucrose efflux transporter [SWEET13]) were among the genes that changed more than 10 times compared with those in the untreated *A. thaliana*, but the differences based on q-value were not significant in significance level of 0.05 nor is a direct relation to plant protection apparent for these genes (Supplementary Fig. 4b).

**Effects of D-tagatose on mannose metabolism in the pathogen.** Because conidial germination and germ tube lengths of isolate Noco2 were inhibited by direct treatment of the pathogen with D-tagatose in vitro (Fig. 2i-k), we evaluated the effects of D-tagatose on sugar metabolism in isolate Noco2 in vivo and in vitro. The first enzyme in the sugar metabolic pathway is a monosaccharide kinase that phosphorylates the C-6 position of hexoses, and the target peak indicating phosphorylation of D-tagatose at C-6 (for D-tagatose 6-phosphate) was detected from a reaction mixture of D-tagatose with crude enzyme extracts from conidiospores of the pathogen (Fig. 4a).

Rarer has an enzyme been found to be specific for a rare sugar as the major substrate in nature, but some enzymes involved in sugar metabolism do have a broad substrate specificity that includes rare sugars. Five genes for putative enzymes with a functional annotation for possible monosaccharide phosphorylation—fructokinase (LC500344 equivalent to Hpa812304 of *H. arabidopsidis* isolate Emoy2), glucokinase (LC500564 equivalent to Hpa800730), xylulose kinase (LC500562 equivalent to Hpa801075), ribokinase (LC500561 equivalent to Hpa805763), and galactokinase (equivalent to Hpa809752)—were selected from the genome sequence data for *H. arabidopsidis* isolate Noco2 based on information from the EnsemblProtists database (http://prots.ensembl.org/index.html) and the genomic sequence data for *H. arabidopsidis* isolate Emoy2 (NCBI: txid559515). Heterologous expression products from four of the five putative enzyme-encoding genes in the Noco2 isolate responsible for D-tagatose phosphorylation were expressed in *Escherichia coli* to examine potential phosphorylation of D-tagatose at C-6 (Supplementary Fig. 5). Among these genes, the gene encoding galactokinase was not expressed well in the *E. coli* system and could not be tested for D-tagatose phosphorylation. GST-tagged expression product (58 kDa) from the gene encoding fructokinase (LC500344) had significant kinase activity (Fig. 4b, c), while the other three enzymes did not (Supplementary Fig. 5). Fructokinase (LC500344) phosphorylated both D-fructose and D-tagatose to generate, respectively, D-fructose 6-phosphate and D-tagatose 6-phosphate, with $K_m = 0.197 \pm 0.044 \text{mM}$, $V_{\text{max}} = 3.48 \pm 0.89 \text{µmol mg protein}^{-1} \text{min}^{-1}$ using D-fructose, and $K_m = 52.67 \pm 22.75 \text{mM}$, $V_{\text{max}} = 0.15 \pm 0.04 \text{µmol mg protein}^{-1} \text{min}^{-1}$ using D-tagatose (Supplementary Fig. 6a-d). The optimal temperature for enzyme activity was 35°C, and this enzyme was stable at lower than 40°C (Supplementary Fig. 6e, f). The enzymatic activity was optimal at pH 10.0 to 11.0 and stable above pH 6, indicating that this enzyme was relatively stable in basic conditions (Supplementary Fig. 6g, h). Enzymatic activity was lost after EDTA treatment to chelate ions, and the highest activity was recovered after the addition of Ca$^{2+}$, Fe$^{3+}$, or Mg$^{2+}$ (Supplementary Fig. 6i), respectively.

Since both D-fructose and D-tagatose served as a substrate for the same fructokinase, we tested them together for competitive inhibition of the other and analyzed D-fructose kinase activity by monitoring D-fructose 6-phosphate production. With a $K_i$ for D-fructose of 70.05 ± 5.14 mM, D-tagatose produced less D-fructose 6-phosphate (Fig. 4d and Supplementary Fig. 6j). On the basis of Dixon plot analyses (Fig. 4d), addition of 250 mM D-tagatose was calculated to inhibit the production of D-fructose 6-phosphate by a maximum of 41.0% when combined with 0.5 mM D-fructose and by 13.1% when 50 mM D-tagatose was combined with 0.5 mM D-fructose (Supplementary Fig. 6k).

Because of the competitive inhibitory effect of D-tagatose on fructokinase, we also examined D-tagatose treatment on the next enzyme in D-fructose metabolism; D-fructose is next phosphorylated by fructokinase to D-fructose 6-phosphate, the main energy source for glycolysis. However, D-fructose 6-phosphate can also be converted by phosphomannose isomerase into D-mannose 6-phosphate, the first intermediate in the mannose synthetic pathway. In addition, phosphomannose isomerase can catalyze the interconversion between D-fructose 6-phosphate and D-mannose 6-phosphate, and interestingly, in the process, forms a cis-enediol intermediate, the same intermediate formed by phosphoglucone isomerase. Thus, this enzyme is often a bifunctional phosphomannose/phosphoglucone isomerase mediated by a cis-enediol intermediate (Fig. 5a).

To examine the effect of D-tagatose 6-phosphate on phosphoglucone isomerase, a gene encoding phosphomannose isomerase (LC500563 equivalent to Hpa825767) was selected from the EnsemblProtists database (http://prots.ensembl.org/index.html) based on the genomic sequence data of *H. arabidopsidis* isolate Emoy2 (NCBI: txid559515) and heterologous expression products from this gene in isolate Noco2 were expressed in *E. coli* to assay enzymatic activities. As we expected, His-tagged expression products (45 kDa) from the gene encoding phosphomannose isomerase (LC500563) had significant dual activities, and both D-glucose 6-phosphate and D-mannose 6-phosphate were detected after the addition of D-fructose 6-phosphate (Fig. 5b, c). Because D-tagatose is converted to D-tagatose 6-
phosphate in *H. arabidopsidis* isolate Noco2 by fructokinase, which leads to the inhibition of phosphorylation of D-fructose to D-fructose 6-phosphate, we also examined the effect of D-tagatose 6-phosphate addition on the activity of phosphomannose isomerase; D-tagatose 6-phosphate (12.5–37.5 mM) significantly inhibited the production of D-mannose 6-phosphate by 51.6 (\( p = 8.85 \times 10^{-7}\)) to 60.8% (\( p = 2.47 \times 10^{-7}\)) and D-glucose 6-phosphate by 19.3 (\( p = 3.53 \times 10^{-4}\)) to 38.2% (\( p = 2.25 \times 10^{-6}\)) (Fig. 5d).
Control of crop diseases is essential for maintaining a sufficient yield of agriproducts for food production. Synthetic fungicides are generally used to efficiently control diseases, but natural eco-friendly products to protect crop against diseases are in demand.

In the present study, d-tagatose reduced severity of numerous plant diseases by at least 50% in pot or field trials; as little as 0.5% (w/v) was required against downy mildew and powdery mildew of numerous economically important crops. The effectiveness of d-tagatose against the various diseases tended to differ, and it was more effective against biotrophs than necrotrophs or hemibiotrophs, especially against downy mildews (oomycetes) and powdery mildew (ascomycetes). Interestingly, about 50 mM d-tagatose inhibited symptoms caused by several downy mildews.
D-Tagatose was an effective disease control agent and did not cause any visible phytotoxicity such as leaf growth inhibition, which is caused by D-allose and D-allulose. D-Tagatose had a 7-day residual effect, indicating that applications at 7-day intervals would be practical. Interestingly, D-tagatose also had some curative effect, and no symptoms were observed when D-tagatose was applied at 48 h after the inoculation as well. Although D-tagatose has recently received much attention as an ingredient and sweetener for various foods and beverages because of its sucrose-like taste, with 90–92% less sweetness and less than half the calories (1.5 kcal g⁻¹) of sucrose, its antidiabetic and weight loss potential, association with beneficial increases in HDL, and possible use in managing diabetes/obesity, the fungicidal effects and a mechanism to explain the effects described here were not reported. Thus, we examined the mode of action of D-tagatose to understand the differences in the efficacy of D-tagatose against the different types of diseases.

Our examinations on the mode of action of D-tagatose, starting with a search of gene expression profiles for cucumber, rice and Arabidopsis, showed that D-tagatose likely does not have a direct effect on plants as a plant defense activator; the expression patterns of genes were not altered. The phosphorylation activities of the enzyme for D-tagatose and D-fructose were determined by HPLC analyses. Spectra for authentic compounds in panel c are marked with “Std.”. Dixon plot analysis of the kinetics of fructokinase for D-fructose (0.5–2.5 mM) at different concentrations of D-tagatose (10–250 mM) indicated competitive inhibition by D-tagatose with 1/Vₘₐₓ = 0.321 (Vₘₐₓ = 3.12 µmol mg protein⁻¹ min⁻¹) with Kᵢ for D-fructose of 70.05 ± 5.14 mM. Data presented are representative of four independent experiments. Error bars are SD.

Fig. 4 Competitive inhibition of α-fructose phosphorylation activity of fructokinase from Hyaloperonospora arabidopsis isolate Noco2 by D-tagatose. HPLC profile of reaction products containing crude enzyme extracts from conidiospores and D-tagatose contained D-tagatose phosphorylated at the C-6 position (D-tagatose 6-phosphate, arrows indicate corresponding peaks) (a). A candidate enzyme for the phosphorylation of D-tagatose was identified as a fructokinase (LC500344); the heterologous expression product of fructokinase has a molecular weight of 58 kDa in SDS-PAGE (b). S and P in panel b indicate soluble fraction (S) and purified protein (P), respectively. The phosphorylation activities of the enzyme for D-tagatose and D-fructose were determined by HPLC analyses (c). Spectra for authentic compounds in panel c are marked with “Std.”. Dixon plot analysis of the kinetics of fructokinase for D-fructose (0.5–2.5 mM) at different concentrations of D-tagatose (10–250 mM) indicated competitive inhibition by D-tagatose with 1/Vₘₐₓ = 0.321 (Vₘₐₓ = 3.12 µmol mg protein⁻¹ min⁻¹) with Kᵢ for D-fructose of 70.05 ± 5.14 mM (d). Data presented are representative of four independent experiments. Error bars are SD.

Fig. 5 Competitive inhibition of phosphomannose isomerase from Hyaloperonospora arabidopsis isolate Noco2 for D-fructose 6-phosphate catalysis by D-tagatose 6-phosphate treatment. Phosphomannose isomerase (PMI) often has bifunctional phosphomannose/phosphoglucone isomerase (PGI) activity mediated by a cis-enediol intermediate (a). A candidate enzyme for H. arabidopsis isolate Noco2 was identified as a phosphomannose isomerase (LC500563), and heterologous expression products of the phosphomannose isomerase with a molecular weight of 45 kDa in SDS-PAGE (b). S and P in panel b indicate soluble fraction (S) and purified protein (P), respectively. The enzyme had significant dual activity; both D-glucose 6-phosphate (G6P) and D-mannose 6-phosphate (M6P) were detected on HPLC analysis by adding D-fructose 6-phosphate (F6P) as the substrate (c). The dual activity of the enzyme for F6P was inhibited significantly by addition of D-tagatose 6-phosphate (T6P) (12.5–37.5 mM) for both M6P and G6P production (d). Data presented are representative of three independent experiments. Means (±SD) were compared using a Tukey-Kramer multiple comparison test; different letters above the bars indicate a significant difference (p < 0.05).
which is then converted by D-mannose 6-phosphate isomerase to the model pathosystem, and concentrations of more than 25 mM of D-tagatose on mannose metabolism, which synthesizes energy metabolism and/or mannan synthesis. Thus, we examined only in oomycetes but also ascomycetes and basidiomycetes for evidence, mannose metabolism is thought to be important not in maize seedlings, and its level increased after treatment with 38% of D-glucose 6-phosphate from D-fructose 6-phosphate (Fig. 6). Because the inhibitory effect is not over the Kᵢ (about 70 mM of D-tagatose to fructokinase) and also is not a complete block of the enzymatic reaction, the lack of a dose-dependent inhibition by D-tagatose at 25 mM or higher of (1) hyphal growth in planta (Fig. 2c) or (2) germ tube elongation in vitro (Fig. 2j) are reasonable results. Concentrations of D-tagatose up to the Kᵢ level could reduce the conversion of D-fructose to D-fructose 6-phosphate by competitive inhibition in a dose-dependent manner. However, concentrations of D-tagatose above the Kᵢ would not have any additional inhibitory effect on fructokinase phosphorylation of D-fructose to D-fructose 6-phosphate. We hypothesize that these multiple chain-inhibitory effects on metabolism are probably significant enough to prevent formation of conidiophores and conidia; the fungicidal effects of D-tagatose treatment are similar to those caused by the synthesized fungicides (Fig. 6). These multiple chain-inhibitory effects on plural target sites in metabolic pathways should also be advantageous in reducing the risk of resistance developing in the pathogens. As we described above, these properties and its global approval as safe for foods are highly desirable for the use of D-tagatose as an agrochemical, and we have already developed a methodology and formulation to reduce the effective concentration of D-tagatose by about 6 times without affecting its efficacy43,44.

Interestingly, D-tagatose was found in the root exudates of maize seedlings, and its level increased after treatment with humic acid, which is generated during the microbial decomposition of plant matter45. Twenty sugars including D-tagatose were exuded in larger amounts (4-fold) at the root interface from plants treated with humic acid, and these exudates were predicted to influence microbial population size and community structure46. D-Tagatose was also identified as a component of a gum exudate of the cacao tree (Sterculia setigera)46 and as a component of an oligosaccharide in lichens (Roccella species)47. If the release of D-tagatose as a plant exudate is induced by factors such as humic acid, then we speculate that the secreted D-tagatose might prevent glycosylation and mannan metabolism as a natural biobarrier against microbes around the plants. Although we did not find much involvement of plant defense systems in the D-tagatose-induced resistance to downy mildew, plants might have
alternative defenses against a pathogen weakened by D-tagatose. A lower concentration of D-tagatose was significant enough to prevent not only downy mildew but also powdery mildew. Unlike the case of downy mildew pathogens, typical components of the walls of powdery mildew pathogens are not known. Zeyen et al. reported a suppressive effect by D-mannose on resistance to powdery mildew infection, but there may be different mode of actions of D-tagatose other than what we described for downy mildew and other pathogens. Thus, the mode of action of D-tagatose is neither entirely clear, nor is the role of D-tagatose in nature known. However, we believe our discovery of the effects of the rare sugar D-tagatose opens a gate for new types of agrochemicals to protect various agriproducts.

Methods

Chemicals. Rare sugars and their derivatives (Supplementary Fig. 1) with respective purities of 100% were prepared by the Rare Sugar Production Station, International Institute of Rare Sugar Research and Education, Kagawa University, Japan using methods described previously.[2,3,4]. Common sugars and other reagents described in the Methods section were purchased from Fujifilm-Wako (Tokyo, Japan) unless noted otherwise.

Effects of D-tagatose on diseases in pot and field trials. D-Tagatose was tested against diseases caused by various oomycete, ascomycete, or basidiomycete pathogens (Table 1) to determine the effective dose (percentage in weight per volume, w/v) that reduced disease severity to less than 50% of that caused by the mock treatment (untreated).

Fig. 6 Hypothesis to explain D-tagatose effect on monosaccharide metabolism by fructokinase and phosphomannose isomerase in Hyaloperonospora arabidopsidis, causal agent of Arabidopsis downy mildew. The inhibitory effects caused by D-tagatose are not a simple block of fructokinase (LC500344) activity, but rather a competitive inhibition at the maximum level (Ki), and the phosphorylated product, D-tagatose 6-phosphate (T6P), likely inhibits the metabolic enzyme phosphomannose isomerase (LC500563), which can produce both D-glucose 6-phosphate (G6P) and D-mannose 6-phosphate (M6P) from D-fructose 6-phosphate (F6P). This inhibition leads to a reduction of glycolysis and mannose metabolism in multiple developmental stages of H. arabidopsidis, including the formation of conidiophores, conidia, and oospores, and thus induces disease tolerance, and no symptoms develop.

For pot trials, a control value was calculated based on disease severity after a spray of D-tagatose solution at 0.5–10% (w/v) in distilled water containing New Gramin sticker (3 ml 10 liter−1) (Mitsui Chemicals Agro Inc., Tokyo, Japan) compared to severity without the D-tagatose spray by methods described previously.[43,44,5] (Experimental conditions for the respective host-pathogen combinations for pot trials in Table 1 are summarized in Supplementary Table 1). Typical disease symptoms and less-severe symptoms after other treatments for cucumber downy mildew are shown as examples in Fig. 1a. A solution (5 ml per pot) of 1 or 5% (w/v) D-tagatose, D-allulose, or D-allose was sprayed on the first true leaf of cucumber seedlings (cv. Sagami–Hanshiro; 1- to 1.2-leaf stage) 5 days before inoculation with 5 ml (2 × 10^4 sporangia ml^−1) per pot of downy mildew pathogen (Pseudoperonospora cubensis, FS-1 strain [stock isolate of National Federation of Agricultural Cooperative Associations]). Inoculated plants were incubated at 20 °C in a moist chamber under 100% humidity for 24 h. A solution (5 ml) of 0.01% (w/v) probenazole [PBZ], 0.005% (w/v) acibenzolar-S-methyl [ASM], or 0.03% (w/v) metalaxyl was also sprayed on the first true leaf of cucumber seedlings (cv. Sagami–Hanshiro) 5 days before inoculation with downy mildew pathogen (5 ml, 2 × 10^4 sporangia ml^−1) as described above. These plants and untreated plants without any sugar or agrochemical treatment were incubated for 7 days in a greenhouse at 20 °C. The timing of the treatments with sugars and agrochemicals also varied from 1 to 7 days before inoculation and from 12 to 72 h after inoculation (5 ml, 1 × 10^4 sporangia ml^−1). Symptoms were then assessed after 7 days under the conditions described above. Severity was calculated from a rating 0–3: 0, no symptoms; 0.1, diseased area 3%; 0.3, 10% diseased area; 0.8, 25% diseased area; 1.5, 50% diseased area; 2, 70% or more diseased area (see also Supplementary Table 1) as described previously.[43,44,5] The relative disease severity was calculated against that caused for the untreated plants based on the average (±SD) (N = 3 independent replications), then the data from the respective curative or preventive experiment were analyzed using a Tukey–Kramer multiple comparison test (p < 0.05) in the program JMP 12 (SAS Institute, Cary, NC, USA) (Fig. 1b).
In field trials, the control efficacy of 1 or 5% (w/v) D-tagatose against downy mildew–host combinations (grapevine cv. Kyoho caused by Plasmopara viticola, cucumbers cv. Anciano, and pseudoperonospora cubensis, Chinese cabbage cv. Kikogoro75) was reexamined and compared with that on plants treated or untreated with an appropriate reference fungicide cyazofamid (CFZ; RZ, Ransan, Ishihara Sangyo Kako, Japan) and chlorothalonil (CT, Dainihon Seiyaku, Tokyo, Japan), or mancozeb WP (MCZ, Dithane, Corteva Agriscience, Wilmington, DE, USA) based on the average of disease severity (SD) (N = 50–163 leaves per replication, three independent replications). Since effective concentrations for the respective pathogens were often different against the same species or genotypes, CFZ was used at 94 parts per million (ppm) against grapevine and spinach downy mildew, CTN at 400 ppm against Chinese cabbage and cucumber downy mildew, and MCZ at 1875 ppm against onion downy mildew. All isolates of the pathogens, except those from natural infections, were obtained from standard stocks of the Agrochemical Research Center, Mitsui Chemicals Agro, Inc. and maintained in the laboratory as appropriate.

Inoculation of the pathogens and treatment of D-tagatose or respective agrochemicals were summarized in Supplementary Table 2. Disease severity was calculated from the rating based on the Fungicide Evaluation Manual of Japan Plant Protection Association for field trials31 (also see Supplementary Table 2) as described previously44,50. Briefly, plants grown under a specific Biological Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH) stage52, were sprayed with 1 × 105 conidia ml–1 from natural infections, were obtained from standard stocks of the Agrochemical reexamined and compared with that on plants treated or untreated with an respective fungicides differ against the specific downy mildew, CFZ was used at 94 parts per million (ppm) against grapevine and spinach downy mildew, CTN at 400 ppm against Chinese cabbage and cucumber downy mildew, and MCZ at 1875 ppm against onion downy mildew. All isolates of the pathogens, except those from natural infections, were obtained from standard stocks of the Agrochemical Research Center, Mitsui Chemicals Agro, Inc. and maintained in the laboratory as appropriate.

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The disease severity of the tested plants was scored in accordance with the following criteria 7 days after the inoculation:

- **0**: no lesions
- **1**: lesion < 25% of leaf area
- **2**: lesion ≥ 25% to 74% of leaf area
- **3**: lesion ≥ 75% of leaf area

For comparisons of rice PR-protein gene expression after various sugar treatments based on microarray analyses, an Agilent Rice Oligo Microarray (44K, custom-made; Agilent Technologies, Redwood City, CA, USA) was used with the methods described previously19,20. Briefly, seedlings of two leaf-stage rice plants were cultured in Kimura B broth containing 0.5 mM of D-tagatose, D-allulose14,17, D-allose14,17, D-glucose14,16,17, or no-sugar for 2 days. Total RNA was extracted using Tri Reagent (Sigma, St. Louis, MO, USA). The cDNAs for cucumber genes were prepared with a PrimeScript RT Master mix (Takara). Total RNA was extracted using Tri Reagent (Sigma, St. Louis, MO, USA). The cDNAs for cucumber genes were prepared with a PrimeScript RT Master mix (Takara). Total RNA was extracted using Tri Reagent (Sigma, St. Louis, MO, USA). The cDNAs for cucumber genes were prepared with a PrimeScript RT Master mix (Takara). Total RNA was extracted using Tri Reagent (Sigma, St. Louis, MO, USA). The cDNAs for cucumber genes were prepared with a PrimeScript RT Master mix (Takara). Total RNA was extracted using Tri Reagent (Sigma, St. Louis, MO, USA). The cDNAs for cucumber genes were prepared with a PrimeScript RT Master mix (Takara). Total RNA was extracted using Tri Reagent (Sigma, St. Louis, MO, USA). The cDNAs for cucumber genes were prepared with a PrimeScript RT Master mix (Takara). Total RNA was extracted using Tri Reagent (Sigma, St. Louis, MO, USA). The cDNAs for cucumber genes were prepared with a PrimeScript RT Master mix (Takara).
the instructions, and relative expression of the respective PR-protein genes was calculated with SD values in relation to data from the no-sugar treatment. The relative expression was calculated by comparing the log2 ratio of the reading samples with respective control samples and performed by a comparison test (*p < 0.05) using JMP 12 in Fig. 3b. All microarray data were deposited as data files in the Gene Expression Omnibus Database with accession GSE195955 for v-saloluce, GSE15479 for v-allose16,17 and v-glucose16,17, and GSE136313 for v-tagatose.

For RNA-seq analyses of v-tagatose effects on downy mildew-inoculated Arabidopsis (Fig. 3c, Supplementary Fig. 4), seedlings of A. thaliana ecotype Col-0 grown in soil in plastic pots (7.5 cm diameter) at 22 °C with 16 h light (100–150 µmol m⁻² s⁻¹) and 8 h dark for 10 days were sprayed with or without 100 µM of v-tagatose per plant on the top of the pots (7.5 cm diameter) and kept for a day in the same conditions. After the sugar treatment, Arabidopsis plants were sprayed with 1 × 10⁶ conidia ml⁻¹ of H. arabidopsidis isolate Noco2, then incubated at 18 °C and 90–100% relative humidity with 10 h light (150–200 µmol m⁻² s⁻¹) and 14 h dark for another 6 days. Twenty to thirty biological replicates of plants with conidiospores and conidiophores that were inoculated and treated with or without v-tagatose were ground to a fine powder with a mortar and pestle in liquid nitrogen. Total RNA was extracted using Tri Reagent (Sigma), then purified using a RNeasy Plant Mini Kit (Qiagen). The quality and quantity of the extracted RNA was determined using the Nanodrop Spectrophotometer (ThermoFisher) and Agilent 2100 Bioanalyzer (Agilent Technologies). The A260/ A280 values and the 28 S/18 S ribosomal RNA ratios from each total RNA sample were 2.06 and 1.7 (v-tagatose-treated sample), and 2.13 and 1.6 (mock-treated sample), respectively. The purified total RNA was sent to Hokkaido System Science (Sapporo, Japan) for RNA-seq. The library for RNA-seq analyses was prepared with 5 µg total RNA using the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. The libraries were sequenced using the HiSeq 2000 sequencing platform and TruSeq SBS Kit v3 reagents with 101 cycles. Base-calling and data filtering were performed using CASAVA ver. 1.8.1 software (Illumina), ultimately yielding approximately mapped reads or 122 million (D-tagatose-treated sample) or 84 million (mock sample) bases. The minimum quality scores for the reads were 35 bases for the D-tagatose-treated sample; 91.8% had ≥30 bases and 35.7% (mock sample; 91.18% had ≥30 bases). The adapter sequences were trimmed by Cutadapt (v1.1)53. After preprocessing, the reads were mapped to the A. thaliana genome (Tair10.17) and H. arabidopsidis Emor2 genome (HyAaraEmor2, 2.0) from the Ensemble database (http://ensemblePlantomes.org) using TOPHat (v2.0.13)54. Expression levels of each gene in both v-tagatose-treated and mock-treated samples were quantified by the number of fragments (paired-end reads) mapped to the coding region of each gene with a value of FPKM (fragments per kilobase of exon per million fragments mapped) using Cuffdiff (v2.0.2) program55. Differential expression between v-tagatose-treated and mocked samples was calculated as a log2 ratio of respective FPKM values. Statistical significance (q-value) of the comparison using FPKM values was also calculated using Cuffdiff (v2.0.2). All data, including raw sequence files for samples, were deposited in the Gene Expression Omnibus Database as accession GSE136568.

Selected genes for the differential expression analyses were PR-protein and defense-related genes jasmonic acid (JA) and/or ethylene (ET)-signaling genes (JA/ET [PR-proteins]: PR-3/CHI [At3g12500], PR-4 [At1g04720], PDF1.2a [At5g06860], PDF1.2b [At4g18780], PDF2.2 [At5g06870], JAZ5 [At1g19670], EIN2 [At3g20770], EFL1 [At3g20770], FLS2 [At2g26020], NAM [At2g17120], IRX5 [At5g06950], IRX6 [At5g06950], IRX7 [At5g06950], IRX8/ORE8B [At5g06950], TGA1 [At1g02250], TGA2 [At1g02250], TGA5 [At1g02250], TGA6 [At1g02250], TGA8 [At1g02250], TGA9 [At1g02250], ABEE reagent solution with a borane–thioglycolic acid (TGA) reagent (Genlantis, San Diego, CA, USA) and LtbExpress Competent cells (Oriental Yeast, Tokyo, Japan) were used for initial examination of their D-tagatose phosphorylation activities (Fig. 4b, Supplementary Fig. 4b). For enzyme purifications, 10 µl of the reaction mixture or sugar standard (25 mM D-fructose, D-tagatose, or D-fructose, and 100 ng target enzymes) was incubated for 24 h at 25 °C. For detecting kinase activities using HPLC, ABEE labeling was performed using the method of Yasuno et al.55 with modifications56. Briefly, 10 µl of the reaction mixture or sugar standard (25 mM D-fructose, D-tagatose, D-fructose 6-phosphate, or D-tagatose 6-phosphate) was added to 40 µl of ABEE reagent solution with a borane–pyridine complex (from the kit) and heated at 80 °C. Chloroform and distilled water (200 µl each) were added, the mixture centrifuged at 3000 rpm for 5 min, and the upper layer was used for HPLC analyses (Prominence; Shimazu, Kyoto, Japan) using an Xb-C18 column (4.6 mm ID × 250 mm; Waters, Milford, MA, USA) with a 50-min separation at a flow rate of 1.0 ml min⁻¹ at 30 °C with a running solvent system of 0.2 mM of potassium borate buffer (pH 8.9) /acetaminite (93/7), followed by a 20-min wash with 30% of the buffer and a 10-min wash with the running solvent. The peaks were monitored with a fluorescein detector (RF-10A XL or RF-20A XL, Shimadzu) with emission of 360 nm and excitation of 305 nm or UV detector (UV-VIS Detector SPD-10AV, Shimadzu) at an absorbance of 305 nm.

**Inhibition of monosaccharide phosphorylation activity of fructokinase by v-tagatose.** Activity of fructokinase (10 µg) was determined spectrophotometrically at 340 nm at 25 °C for 5 min by coupling production of ADP to oxidation of NADH via pyruvate kinase (15 U ml⁻¹) (Oriental Yeast, Tokyo, Japan) and lactate dehydrogenase (10 U ml⁻¹) (Oriental Yeast) reactions as described by Miller and Raines56 with 0.5–2.5 mM D-fructose and 10–250 mM v-tagatose in 1 ml reaction mixture containing 30 mM Tris-HCl (pH 7.5), NADH (Sigma-Aldrich, 500 µM), phosphoenolpyruvate (50 mM), and MgCl₂ (8 mM). The same data (mean ± SD of four replications of the reaction) were used for calculations for the kinetic analyses including Dixon plot (Fig. 4d), Lineweaver–Burk plot (Supplementary Fig. 6), and bar graph (Supplementary Fig. 6).

**Characterizations of enzymes in D-fructose 6-phosphate metabolic pathway and inhibition of activity by v-tagatose 6-phosphate.** The cDNA fragment (Supplementary Fig. 5a) of the choline dehydrogenase gene from H. arabidopsidis isolate Noco2 (LCS005653) of H. arabidopsidis isolate Noco2 was subcloned in frame into the pCoLD vector (Takara), and expressed in E. coli SHuffle Express Competent cells (New England Biolabs, MA, USA) according to the manufacturer’s instructions. Primers used for cloning are listed in Supplementary Table 4. The recombinant proteins of the coding region from fructokinase (LCS00344), glucokinase (LCS005646), xylose kinase (LCS00562), or ribokinase (LCS00561) of H. arabidopsidis isolate Noco2 were subcloned in frame into the pEXB1L vector (GE Healthcare) and expressed in SoluBL21 Competent E. coli cells (Genlantis, San Diego, CA, USA) according to the manufacturer’s instructions. Primers used for cloning are listed in Supplementary Table 4. The recombinant proteins of each coding region, generated by the heterologous expression system with 0.4–0.6 mM Isopropl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37 °C were purified using a GSTrap HP column (GE Healthcare) and the manufacturer’s instructions. Images of the recombinant proteins separated using SDS-PAGE with a standard protocol57 were shown in Fig. 4b and Supplementary Fig. 5, and the prepared recombinant proteins were used for initial examination of their v-tagatose phosphorylation activities (Fig. 4c, Supplementary Fig. 5).

For enzymatic characterizations, the recombinant protein of fructokinase (LCS00344) was generated using a mass culture system with 10 liter LB broth containing 100 µg ml⁻¹ carbencillin at 37 °C and 200 rpm for 6 h (preculture), and 25 °C and 200 rpm for 1 day with 0.6 mM IPTG in a jar fermenter TS-M15L (Tokyo Rikakagu, Tokyo, Japan) and purified using a GSTrap HP column (GE Healthcare) as per the instructions and dialyzed against 5 mM Tris-HCl buffer (pH 7.5) for 4 h. The prepared recombinant protein was used to determine phosphorylation activity for a kinetics analysis (Fig. 4d, Supplementary Fig. 6a–d, k), optimal temperature (Supplementary Fig. 6e), thermal stability (Supplementary Fig. 6f), optimal pH (Supplementary Fig. 6g), pH stability (Supplementary Fig. 6b), and optimal cofactor (metal ion) (Supplementary Fig. 6i).
15°C and 200 rpm for 1 h (cold shock induction), and 15°C and 200 rpm for 3 days in Jar Fermenter TS-M15L and puriﬁed using a Hitrap HP column (GE Healthcare). In this step, the manufacturer’s instructions, then dialyzed against 10 mM Tris-HCl buffer (pH 7.4). The recombiant proteins, separated by SDS-PAGE using a standard protocol14, are shown in Fig. 5b.

A reaction mixture (100 μL) containing 100 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 10 μg recombinant protein of phosphomannomerase isomerase (LC500563), and 2.5 mM α-fructose 6-phosphate (F6P) was incubated at 30°C for 6 h (Fig. 5c), or reaction mixtures (100 μL) containing 100 mM Tris-HCl (pH 7.4), 10 mM MgCl2-5 μg recombinant protein with 2.5 mM of F6P and α-tagatose 6-phosphate (T6P) (from 0 to 37.5 mM) were incubated at 30°C for 1 h. Reaction products were labeled and puriﬁed for ABE as described above, and the ABE-labelled sugars (10 μL) were analyzed using an HPLC system (Prominence) with an Xbridge C18 column (4.6 mm ID × 250 mm) and 20-min separation at a ﬂow rate of 1.0 ml min⁻¹ at 30°C with solvent A of the Glyscope solvent set (J-Chemical), followed by a 5-min wash with solvent B from the solvent set and equilibration for 10 min with the running solvent. The peaks were monitored with the ﬂuorescence detector (RF-20A XL) with emission of 360 nm and excitation of 305 nm. Respective data in Fig. 5d are means ± SD of three replications calculated from the peak area on the HPLC spectrum with a calibration curve using standards with known concentrations, and they were compared for signiﬁcant differences using a Tukey–Kramer multiple comparison test (p < 0.05) in JMP 12.

Statistics and reproducibility. All data in this work were collected in multiple and respective data are means ± SD of replications and statistical analyses shown in ﬁgures were performed using a Tukey–Kramer multiple comparison test (p < 0.05) in JMP 12 program (SAS Institute) unless noted otherwise as described above.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All nucleotide sequences were deposited in the DNA Data Bank of Japan (DDBJ) as accession numbers LC500344 (fructokinase), LC500561 (ribokinase), LC500562 (xylose kinase), LC500563 (phosphomannomerase isomerase), and LC500564 (glucokinase).

Microarray and RNA-seq datasets in this work have been deposited in the NCBI Gene Expression Omnibus (GEO) under accession number GSE136313 (microarray) and GSE136568 (RNA-seq). Source data are available as Supplementary Data 1. All other data that support the ﬁndings of this study are available from the corresponding authors upon reasonable request.

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Author contributions

S.M., T.F., T.O., Y.S., K.T., S.T., K.I., K.E., and K.A. designed and conceived the experiments; S.M., T.F., and K.O. performed the experiments, with the first three authors contributing equally; A.Y., K.I., K.G., and K.I. provided rare sugars and critical advice; S.M., T.F., and K.A. wrote, and all authors edited the final draft and revised the manuscript.

Competing interests

T.F., T.O., Y.S., K.T., and K.E. are employed by Mitsui Chemical Agro, Inc. Remaining authors declare no competing interests.

Additional information

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