Supplemental Figures and Tables

Figs. S1-S9
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RESEARCH PAPER:
Rare sugar d-allose acts as a triggering molecule of rice defense via ROS generation

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Supplemental figure and table legends

Figure and table legends

Fig. S1. Fisher projections of various monosaccharide structures used in this study.
Four common sugars (D-glucose, D-fructose, D-galactose, and D-mannose), nine rare sugars, and their derivatives (D-glucose 6-phosphate, D-allose 6-phosphate, 6-deoxy D-allose) are shown.

Fig. S2. Effect of D-allose concentration on Xoo growth in liquid culture.
Mean OD600 (± SE) of cultures amended with various D-allose concentrations at selected times during three independent experiments. (E)-2-Hexenal served as the control possessing antimicrobial activity (Gomi et al., 2010).

Fig. S3. OsrbohC overexpression in rice.
(A) Construct of OsrbohC overexpression vector in pBI333-EN4.
(B) Second generation of lines 11 and 28, selected from multiple transgenic rice plants expressing OsrbohC, and WT. No visible phenotype resulted with OsrbohC overexpression.
(C) Excess OsrbohC did not change sensitivity to Xoo with or without 50 mM D-glucose (D-Glc).

Fig. S4. Metabolic pathway of D-allose in Escherichia coli.
Metabolic pathway of D-allose in E. coli described by Kim et al. (1997) was shown.

Fig. S5. E. coli D-allose kinase (AlsK) overexpression in rice.
(A) Construct of AlsK overexpression vector in pBI333-EN4.
(B) Second generation of independent lines 6 and 21, selected from multiple transgenic rice plants expressing AlsK, and WT. No visible phenotype resulted with AlsK
overexpression.

(C) Excess AlsK did not change sensitivity to Xoo with or without 50 mM d-glucose (d-Glc).

(D-E) d-Glucose (D) or d-glucose 6-phosphate (G6P) (E) content in leaves from WT and AlsK-overexpressing lines, relative (± SE, n = 3) to WT, as assessed by HPLC.

(F-K) HPLC detection of ABEE-labeled monosaccharides in extracts from leaves from 24-h d-allose treatment. Each panel on right shows expanded detail of left panel from 1 to 25 min. Absolute value of d-glucose in leaves in panel D was calculated as 2.545 μg·gFW⁻¹. Abbreviations used: d-allose, d-All; d-allose 6-phosphate, A6P; d-glucose, d-Glc; and d-glucose 6-phosphate, G6P.

**Fig. S6.** *E. coli* d-allose 6-phosphate isomerase (AlsI) overexpression in rice.

(A) Construct of AlsI overexpression vector in pBI333-EN4.

(B) Second generation of independent lines 13 and 14 selected from multiple transgenic rice plants expressing AlsI and WT. No visible phenotype resulted with AlsI overexpression.

(C-H) HPLC detection of ABEE-labeled monosaccharides in extracts from leaves treated with d-allose for 24 h. Each panel on right shows expanded detail of left panel from 1 to 25 min.

Abbreviations used: d-allose, d-All; d-allose 6-phosphate, A6P; d-glucose, d-Glc; and d-glucose 6-phosphate, G6P.

**Fig. S7.** Characterization of rice *G6PDHs.*

(A) qRT-PCR analysis of OsG6PDH2-5 gene (accession nos. are in panel B) expression in leaves at 0 to 24 h after treatment with 5 mM d-allose (d-All) or
D-glucose (D-Glc), relative to control (no sugar) (Con) (± SE, n = 4).

(B) Phylogenetic analysis of Os6PDH1–5 genes with Arabidopsis G6PD genes using Clustal W at the DDBJ (http://www.ddbj.nig.ac.jp/) and 1,000 bootstraps. Respective clades were categorized according to those for Arabidopsis G6PD genes (Wakao et al., 2005).

(C) Subcellular localization of GFP-tagged OsG6PDH1 and OsG6PDH2 in plant cells. Epidermal layers of tobacco leaves were bombarded with particles coated with constructs to express GFP alone (upper), OsG6PDH1-GFP (middle), and OsG6PDH2-GFP (lower).

**Fig. S8. Characterization of Tos17 mutants for OsG6PDH1 and its complementation.**

(A) Exon and intron organization of OsG6PDH1 and various Tos17 insertion sites. Solid boxes and lines indicate exons and introns; triangles indicate position of Tos17 insertion. Of 10 lines with Tos17-inserted OsG6PDH1 mutations at the protein-coding region of exon 10 and 11 found in the rice Tos17 mutant database (Fig. S8A) (Miyao et al., 2003), lines NC0350 and NC0573 have morphologically abnormal phenotypes, and the insertion of Tos17 did not match the sites recorded for lines NC0293, NC0320, NC0395, and NC0555. For lines NC0322, NC0695, and NC2550 examined by PCR for Tos17 insertion, no insertion was found at the target site in exon 10 of OsG6PDH1 (data not shown). Among mutant lines, Tos17 was inserted at the target site in exon 11 in NC8489, which we then examined further.

(B) Construct of OsG6PDH1 complementation vector in pBI333-EN4 consists of a 4099-bp DNA fragment with OsG6PDH1 promoter region (2496 bp) connected to entire cDNA (1551 bp) of OsG6PDH1 gene with 42-bp 3′-untranslated region by 10-bp
linker.

Fig. S9. Schematic model of d-allose signal transduction for induction of rice resistance to *Xoo*. Conversion of d-allose to A6P by HXK induces ROS generation by OsrbohC following PR-gene expression, lesion mimic formation, and resistance to *Xoo* with involvement of G6PDH1 in rice.

Table S1. Primers used in this study.

F, forward direction; R, reverse direction. Extra nucleotides attached to introduce restriction sites are underlined.

Table S2. Enzymatic profiles for OsG6PDH1- and OsG6PDH2-recombinant proteins using NADP+ as a kinetic parameter.

Kinetic parameters were determined using G6PDH-coupled assay for G6P (Wakao and Benning, 2005).

Table S3. Property summary for OsG6PDH1- and OsG6PDH2-recombinant proteins.

^aKinetic parameters were determined using G6PDH-coupled assay for A6P with a maximum concentration of 5 mM (Wakao and Benning, 2005). ND: not detected.

^bG6PDH activity with G6P with 10 mM DTT.

Experimental Procedures for supplemental results

Antimicrobial activity analysis

*Xoo* was cultured in YT (0.5% (w/v) Bacto-Yeast Extract (Becton, Dickinson & Co.), 1% (w/v) Bacto-Tryptone (Becton, Dickinson & Co.), pH 6.8) liquid medium
containing 1, 5 or 10 mM of d-allose or 100 μM (E)-2-hexenal. (E)-2-Hexenal was dissolved in DMSO and used as the control possessing antimicrobial activity by the method previously described (Gomi et al., 2010).

**Transient localization assay**

G6PDH1- or G6PDH2-GFP fusion proteins were expressed using a particle gun-mediated DNA delivery to tobacco epidermal cells and imaged using epifluorescence system DP70-SET-A, differential interference contrast optics and an Olympus BX51 microscope (Olympus, Tokyo, Japan). After bombardment, tissues were incubated for 24 h at 24˚C in the dark; ca. 100 cells/construct were examined for GFP localization in at least three independent experiments. All methods are as described by Yamasaki and Akimitsu (2007).

**Phylogenetic tree analysis**

Phylogenetic tree was constructed using both rice (OsG6PDH) and Arabidopsis (G6PD) (Wakao et al., 2008) sequences were used for Clustal W (Thompson et al., 1994) analyses at the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/) using 1,000 bootstraps. Consensus tree was visualized in TREEVIEW (Page, 1996).

**Genotypic determination of homozygote for Tos17 insertion**

Total DNA from leaves of WT and Tos17 mutant lines were isolated using NecleoSpin plant II (MACHEREY-NAGEL, Hoerdtt, France) and manufacturer's instructions. PCR was run using KOD DNA polymerase (TOYOBO, Osaka, Japan) in a thermal cycler with initial PCR activation at 94°C for 2 min followed by 30 cycles of 3-step cycling (denaturation at 98°C for 10 sec annealing at 60°C for 30 sec, and extension at 68°C for 1 min) with gene-specific primers used for PCR (Table S1).
Recombinant G6PDH assays

Activity of recombinant rice G6PDHs was measured spectrophotometrically (340 nm) at 25°C by detecting NADP reduction via G6PDH reaction, which is coupled with G6P production, as described by Wakao and Benning (2005) as described in the main text. To determine \( K_i \) NADPH of G6PDH, activity was examined with 0.01 to 0.1 mM NADPH.

References specific for supplemental information

Page RDM. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* **12**, 357-358.

Thompson JD, Higgins DS, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.

Yamasaki Y, Akimitsu K. 2007. *In situ* localization of gene transcriptions for monoterpene synthesis in irregular parenchymic cells surrounding the secretory cavities in rough lemon (*Citrus jambhiri*). Journal of Plant Physiology **164**, 1436-1448.
Table S1. Primers used in this study.

| Construction of overexpression vectors | Direction | Primer sequences (5' - 3') |
|----------------------------------------|-----------|----------------------------|
| AlsK                                   | F         | GCTCTAGAGCATGCAAAAAACAGCATAACGTC |
|                                        | R         | GGGTACCCTCTATGGGCTTTAGCAGACAGAC |
| AlsI                                   | F         | GCTCTAGAGCATGAAAAAGATTGCATTTGGCTGTG |
|                                        | R         | GGGGTACCCCTCAATTTCCTGCTTGCTTG |
| OsrbohC                                | F         | GCTCTAGAGCTTGTTGGTTGGTGCTAGCTG |
|                                        | R         | GGGTACCCTCCTATGGCTTGCTTG |
| Tos17 analysis by genome PCR            | G6PDH1-F  | F         | CCTTCATTCCTCAAGGCTG |
|                                        | G6PDH1-R  | R         | CCGGCAAAAAACAAAATAGTA |
| Tos17-F                                | F         | ATTGTTAGGTGCAAAGTATGTAAGA |
| Construction of G6PDH1 complementation vectors | OsG6PDH1 promoter region | F | ACATGCATGCATGTCGGTACGGTGGAAGTC |
|                                        | R         | GCTCTAGAGCATGCAAAAAAGCATGCTG |
| OsG6PDH1 translated region             | F         | GCTCTAGAGCATGCAAAAAAGCATGCTG |
|                                        | R         | CCGGCAAAAAACAAAATAGTA |
| Transgenic analysis by RT-PCR           | AlsK      | F         | ATGCAAAAAACAGCATGATAAGC |
|                                        | R         | GTATAATTCCTGGCGGACCTTACATTC |
| AlsI                                   | F         | ATGAAAAAGATTGCATTTGGCTGTG |
|                                        | R         | GTATAATTCCTGGCGGACCTTACATTC |
| OsrbohC                                | F         | GTGATCTTTTCCCGGCCAATC |
|                                        | R         | GTATAATTCCTGGCGGACCTTACATTC |
| OsG6PDH1                               | F         | ATGTCAGGAGGATCTGGTGATTC |
|                                        | R         | CTAGAAATTTTGAAGGTTGGGAG |
| Actin                                  | F         | CCGGCAATCATGAGACCCAC |
|                                        | R         | ACACACAAATCCAAAACAGAG |
| qRT-PCR analysis                       | PBZ1      | F         | GTGGTTGTTTATATGCTGCTTATG |
|                                        | R         | ACTGCCATCTCTTATTACATCCACATG |
| PR1b                                   | F         | AGTGCTCTGATCCGCCAATCC |
|                                        | R         | ATCTGGAACAGAAGAAAGAAAGAGG |
| POX22.3                                | F         | GAGATGCCTGCTTGCTGGAAG |
|                                        | R         | CACACGACCCGATACCTGACCTTG |
| β-1,3-glucanase                        | F         | ACGAGACGGAGGACACTTC |
|                                        | R         | TGATCTCCCTCTAGAGAAGAAGTCTTAC |
| IAI                                    | F         | TCGTGCTATATATCGTTGATGCTG |
|                                        | R         | CCGGCAATCATGAGACCCAC |
| Proteinase inhibitor                   | F         | GTGTTGTTATGCTGCTGATCGT |
|                                        | R         | CACCGGATACCAACATCAAACAC |
| Lipoxigenase                           | F         | CATCTGGTTTAGAGAGTCATC |
|                                        | R         | TACTGGGTTAGCCATTACGATC |
| Chitinase                              | F         | ACGGCAATCCAACACATCAT |
|                                        | R         | GTAGCGCTGGCCAGCAGCAT |
| TLP                                    | F         | GCCCTGCTGCTGCACTCC |
|                                        | R         | CAGGTAACGCTGCGGAGG |
| OsrbohA                                | F         | TGCACTAGCTTATGCAAGTC |
|                                        | R         | GCGCTGCTATGGCTATG |
| OsrbohB                                | F         | ATGTTCCGAGCTGAACAGG |
|                                        | R         | AGTCTGATCCAGCAAAGAAGC |
| OsrbohC                                | F         | CAGGCAGACAGGACATGAG |
|                                        | R         | CCAAGATGATCCAAAAACTC |
| OsrbohD                                | F         | CAAAGCAGCTGCTGCTATTC |
|                                        | R         | TGAGAAGTAGTTGCTAACCAGATG |
| OsG6PDH1                               | F         | CCAATGCAGCTGCTGCTCAG |
|                                        | R         | TCTCAAGATCAACACATACAG |
| OsG6PDH2                               | F         | ATTTTCAGATACACCCGAC |
|                                        | R         | GTAGAAGAGTCATTTATTTC |
| OsG6PDH3                               | F         | CTTTGCATAGCCATTTTCTC |
|                                        | R         | GGCCTGCAAACCTAACATAC |
| OsG6PDH4                               | F         | TGATCGAGGAGCTGCTTAC |
|                                        | R         | AATTTCGCATGAGCAGT |

*Note:* All primers are designed for amplification of specific genomic regions using PCR. The direction (F for forward, R for reverse) and sequences are provided for each primer pair.
Table S2. Enzymatic profiles for OsG6PDH1- and OsG6PDH2-recombinant proteins using NADP⁺ as a kinetic parameter.

| Enzyme     | kcat (s⁻¹) | Km NADP⁺ (M) | kcat/Km NADP⁺ (M⁻¹ s⁻¹) | Ki NADPH (M) |
|------------|------------|--------------|--------------------------|--------------|
| G6PDH1     | 3.04       | 6.49 x 10⁻⁵  | 4.68 x 10⁴               | 4.15 x 10⁻⁵  |
| G6PDH2     | 1.06       | 4.1 x 10⁻⁵   | 2.59 x 10⁴               | 3 x 10⁻⁵     |

Kinetic parameters were determined using G6PDH-coupled assay for G6P (Wakao and Benning, 2005).

Table S3. Property summary for OsG6PDH1- and OsG6PDH2-recombinant proteins.

| Enzyme     | A6Pᵃ | Reductionᵇ | Group       |
|------------|------|------------|-------------|
| G6PDH1     | ND   |Insensitive | Cytosolic   |
| G6PDH2     | ND   |Insensitive | Cytosolic   |

ᵃKinetic parameters were determined using G6PDH-coupled assay for A6P with a maximum concentration of 5 mM (Wakao and Benning, 2005). ND: not detected.
ᵇG6PDH activity with G6P with 10 mM DTT.
Aldose

\[
\begin{align*}
D-\text{glucose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
D-\text{allose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
D-\text{altrose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
D-\text{galactose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
D-\text{mannose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
L-\text{galactose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
L-\text{mannose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
\end{align*}
\]

Ketose

\[
\begin{align*}
D-\text{fructose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
D-\text{psicose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
D-\text{sorbose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
L-\text{fructose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
L-\text{psicose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
L-\text{sorbose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \\
\end{align*}
\]

Sugar derivative

\[
\begin{align*}
D-\text{glucose 6-phosphate} & : \quad \text{C}_6\text{H}_{12}\text{O}_6\text{P}_3 \\
D-\text{allose 6-phosphate} & : \quad \text{C}_6\text{H}_{12}\text{O}_6\text{P}_3 \\
6\text{-deoxy} \quad D-\text{allose} & : \quad \text{C}_6\text{H}_{12}\text{O}_6 \quad \text{CH}_3 \\
\end{align*}
\]
Kano et al Fig. S2
D-allose

\[ \longrightarrow \]

D-allose kinase (AlsK)

D-allose-6-phosphate

\[ \longrightarrow \]

D-allose 6-phosphate isomerase (AlsI)

D-psicose-6-phosphate

\[ \longrightarrow \]

D-allulose 6-phosphate 3-epimerase (AlsE)

D-fructose-6-phosphate

\[ \longrightarrow \]

Glycolysis
Kano et al. Fig. S7

AK073697 (OsG6PDH1)
AK064867 (OsG6PDH3)
AK067510 (OsG6PDH4)
AK101101 (OsG6PDH2)
At5g13110 (G6PD2)
At1g24280 (G6PD3)
At3g27300 (G6PD5)
At5g40760 (G6PD6)
At5g35790 (G6PD1)
At1g09420 (G6PD4)
AK121967 (OsG6PDH5)

B

Cyt
P0
P2
P1

0.1

D

- All
Con
D - Glc

D

- All
Con
D - Glc

Relative expression

C

sGFP(S65T)

G6PDH1-
sGFP(S65T)

G6PDH2-
sGFP(S65T)
