Sugar homeostasis mediated by cell wall invertase GRAIN INCOMPLETE FILLING 1 (GIF1) plays a role in pre-existing and induced defence in rice

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SUMMARY

Sugar metabolism and sugar signalling are not only critical for plant growth and development, but are also important for stress responses. However, how sugar homeostasis is involved in plant defence against pathogen attack in the model crop rice remains largely unknown. In this study, we observed that the grains of *gif1*, a loss-of-function mutant of the cell wall invertase gene *GRAIN INCOMPLETE FILLING 1* (*GIF1*), were hypersusceptible to postharvest fungal pathogens, with decreased levels of sugars and a thinner glume cell wall in comparison with the wild-type. Interestingly, constitutive expression of *GIF1* enhanced resistance to both the rice bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* and the fungal pathogen *Magnaporthe oryzae*. The *GIF1*-overexpressing (GIF1-OE) plants accumulated higher levels of glucose, fructose and sucrose compared with the wild-type plants. More importantly, higher levels of callose were deposited in GIF1-OE plants during pathogen infection. Moreover, the cell wall was much thicker in the infection sites of the GIF1-OE plants when compared with the wild-type plants. We also found that defence-related genes were constitutively activated in the GIF1-OE plants. Taken together, our study reveals that sugar homeostasis mediated by GIF1 plays an important role in constitutive and induced physical and chemical defence.

INTRODUCTION

Plants have developed a sophisticated defence machinery against pathogen attack during the long co-evolution with pathogens, which provides them with both pre-existing, or constitutive, and inducible defence responses. The pre-existing defence mainly consists of physical barriers, such as pectin, cellulose and hemicellulose, lignin and proteins, which affect cell wall integrity during the

defence response against pathogen invasion (Hématy *et al*., 2009; Hückelhoven, 2007; Vorwerk *et al*., 2004). The activation of induced defence depends on a more complicated signalling network through which plants recognize pathogens and activate immune responses (Boller and He, 2009; Chisholm *et al*., 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006). It has been recognized that sugars not only serve as energy resources, but are also required as substrates and signals during plant defence responses (Roitsch and González, 2004; Rolland *et al*., 2006). However, pathogens have also evolved adaptive strategies to suppress host immunity and gain access to sugar supply (Chen L.Q. *et al*., 2010, 2012). Most recently, it has been reported that the disaccharide trehalose is required for the pathogenesis of *Pseudomonas aeruginosa* in *Arabidopsis*, which probably functions to promote the acquisition of nitrogen-containing nutrients, thereby allowing *P. aeruginosa* to replicate in the intercellular spaces in the host (Djonović *et al*., 2013). Therefore, sugar metabolism/homeostasis plays an important role in plant defence responses and pathogen pathogenicity.

The plant cell wall is a matrix of polysaccharides, and provides more than just a physical barrier to invading microbes by playing a crucial role in the sensing or transmission of signals from the external stimuli of pathogen attack to establish basal defence, which is accompanied by various defence responses, such as the production of reactive oxygen species (ROS), activation of pathogenesis-related (PR) proteins, generation of signal molecules (e.g. salicylic acid, jasmonic acid and ethylene), formation of cell wall appositions (CWAs) or papilla, callose deposition and the hypersensitive response (HR) (Hückelhoven, 2007). It has been reported that the polysaccharide composition of the plant cell wall plays an important role in defence to diverse pathogens (Vorwerk *et al*., 2004). Changes in cell wall composition result in altered immune responses to diverse pathogens in *Arabidopsis* (Ellis and Turner, 2001; Ramírez *et al*., 2011; Sánchez-Rodríguez *et al*., 2009; Vogel *et al*., 2002, 2004). Interestingly, impairment of cellulose biosynthesis results in enhanced resistance to different types of pathogen in *Arabidopsis* (Hernández-Blanco *et al*., 2007). The

Arabidopsis agg1agg2 double-mutant and *agb1* mutant plants show decreased xylose levels and increased susceptibility to the necrotrophic fungus *Plectosphaerella cucumerina* (Delgado-Cerezo *et al*., 2012). The overexpression of pectin methylesterase inhibitors restricts fungal infection by *Botrytis cinerea* in *Arabidopsis* (Lionetti *et al*., 2007). Moreover, the hydrolysis of cell wall polysaccharides during pathogen infection may also generate sugar or oligosaccharide signals which stimulate intracellular defence responses (Bolouri-Moghaddam *et al*., 2010; Rolland *et al*., 2006). Thus, cell wall defence, which is associated with polysaccharide composition, is essential in the plant defence machinery.

Cell wall invertases (CW-Invs) catalyse the irreversible hydrolysis of sucrose into glucose and fructose, the hexoses that play important roles in sucrose partitioning, cell differentiation, plant development and responses to biotic and abiotic stresses (Roitsch and González, 2004). The expression of CW-Invs and enzymatic activity are inducible by pathogens (Berger *et al*., 2007; Roitsch and González, 2004). RNA interference (RNAi)-mediated repression of *CW-Inv* results in reduced resistance of transgenic tobacco plants (Essmann *et al*., 2008), and the expression of the yeast invertase in tobacco and *Arabidopsis* leads to the accumulation of sugars and increased defence gene expression and enhanced resistance (Herbers *et al*., 1996; von Schaewen *et al*., 1990). Interestingly, the activity of CW-Inv is strongly dependent on post-translational regulation by proteinaceous invertase inhibitors, whose expression and activity have been shown to be strongly repressed on pathogen infection in *Arabidopsis*, resulting in the release of invertase activity (Bonfig *et al*., 2010). Therefore, CW-Invs and CW-Inv–inhibitor complexes may affect plant defence responses through sugar homeostasis.

As a monocot, rice (*Oryza sativa* L.) has been commonly accepted as a model to study defence responses in cereal crops. Several studies have shown that sugar transportation plays an important role in the rice defence response against pathogens (Chen L.Q. *et al*., 2010, 2012; Chu *et al*., 2006; Yang *et al*., 2006). Recently, D-allose has been found to enhance resistance to rice bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) by up-regulating defence-related genes (Kano *et al*., 2010). However, the role and underlying mechanism(s) of sugar homeostasis in rice defence are not yet fully understood. Previously, we have reported that the rice CW-Inv GRAIN INCOMPLETE FILLING 1 (GIF1) plays an important role in sucrose partitioning during the grain-filling process; sucrose unloaded by GIF1 in the ovular and stylar vascular tissues is critical for the stimulation of starch synthesis in the developing endosperm (Wang E.T. *et al*., 2008, 2010). Here, we report that *gif1* mutant grains with a weakened cell wall barrier are hypersusceptible to postharvest fungal pathogens, whereas constitutive expression of *GIF1* enhances resistance to both bacterial blight and fungal blast with enhanced physical and induced defence responses.

RESULTS

The *gif1* mutant is hypersusceptible to postharvest fungal pathogens

The grains of the rice *gif1* mutant accumulated low levels of sugars with significantly lower CW-Inv activity than that in wild-type plants (Wang E.T. *et al*., 2008). When grown in paddy fields, we found that the *gif1* grains were hypersusceptible to postharvest fungal pathogens (fungi rapidly covered the whole mutant glume surface under humid conditions), whereas the wild-type grains were fully resistant to the pathogens (Fig. 1A,B,D). We isolated the pathogens from the *gif1* glumes and discovered that the majority of the fungi were *Alternaria* sp. and *Rhizopus* sp. (Fig. 1C,E), both postharvest pathogenic fungi in cereals (Narayanasamy, 2006). Thus, we speculated that the loss of *GIF1* function probably disrupts rice basal defence.

Constitutive expression of *GIF1* enhances resistance to bacterial and fungal pathogens

Because *Alternaria* sp. and *Rhizopus* sp. are not established pathogen models in rice, we investigated two established rice pathosystems, rice leaf bacterial blight caused by *Xoo* and fungal blast caused by *Magnaporthe oryzae*, two of the most devastating rice diseases in China. We did not find obvious differences in resistance to *Xoo* and *M. oryzae* between the *gif1* mutant and the wild-type control, because the wild-type control is already highly susceptible to the pathogens (Fig. S1, see Supporting Information). One possibility is that GIF1 functions only in the filling grains (Wang E.T. *et al*., 2008), therefore not affecting the defence responses in leaves where the pathogens initiate infection. However, we could not exclude the possibility that other CINs expressed in leaves might function redundantly in disease resistance against leaf infection by *Xoo* and blast. Therefore, we investigated the expression of other CIN genes in infected leaves, and found that *OsCIN1*, *OsCIN4* and *OsCIN7* were induced in leaves infected by *Xoo* (Fig. S2, see Supporting Information). However, whether the additional CINs contribute to rice disease resistance and redundantly complement the possible GIF1 function in leaves should be confirmed by additional extensive genetic and biochemical studies.

To investigate the role of *GIF1* in rice defence, we generated transgenic plants constitutively expressing *GIF1* (GIF1-OE), in which the expression and activity of CW-Inv was increased significantly in comparison with wild-type plants (Wang E.T. *et al*., 2008, 2010). Two-month-old plants were inoculated with *Xoo* virulent strains PXO99 and DY89031. Surprisingly, GIF1-OE plants showed greatly enhanced resistance to both *Xoo* virulent strains, compared with the wild-type control (Fig. 2A), with a significant decrease in bacterial growth (Fig. 2B,C). Therefore, GIF1 positively
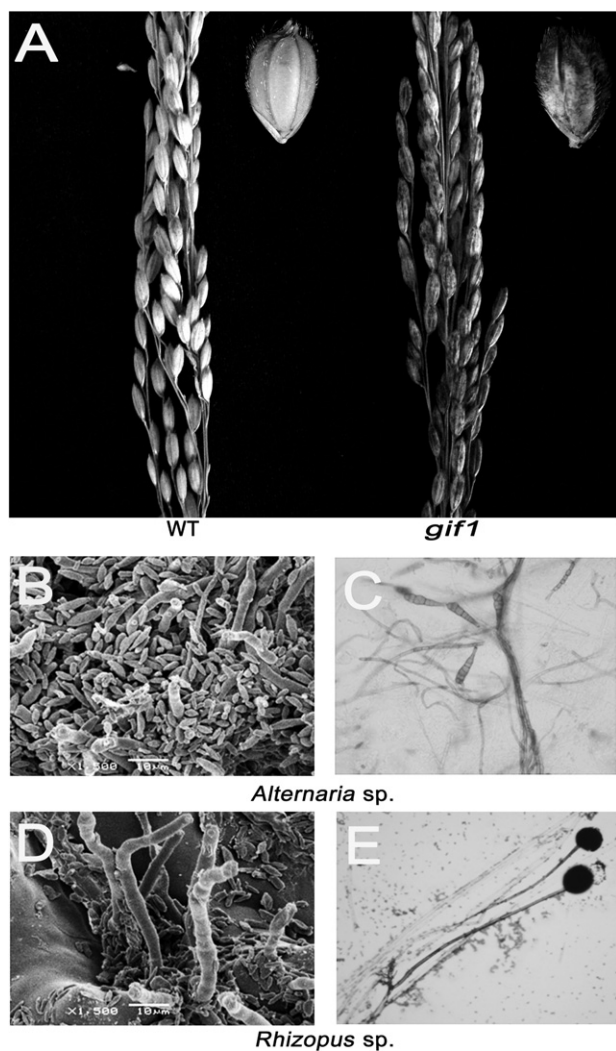
Fig. 1 Hypersusceptibility of *gif1* grains to postharvest fungal pathogens. (A) Panicles of the wild-type (Zhonghua11) (WT) and *gif1* mutant grown in the field under high humidity conditions. Insets are the WT and *gif1* seeds. Note that the surface of the *gif1* seeds is covered by mould, whereas no infection by the pathogen is observed on WT grains. (B, C) Scanning electron microscopy (SEM) images of *Alternaria* sp. on the *gif1* glume surface. Bar, 10 μm. (D, E) SEM images of *Rhizopus* sp. on the *gif1* glume surface. Bar, 10 μm.

regulates bacterial disease resistance. We further assayed disease resistance to rice blast by inoculating 2-week-old seedlings with *M. oryzae* virulent races ZB1 (strain 2000-2) and ZB15 (strain 09-31-1). The GIF1-OE plants were more resistant, with significantly reduced lesion size when compared with the wild-type control (Fig. 3A,B). Disease severity (score) on the GIF1-OE plants averaged 1–2 compared with 6–7 on the wild-type plants. We also investigated fungal penetration and growth *in planta* and quantified infection type frequency as described previously (Nakao *et al*., 2011). The results showed that Type III and IV infections were

decreased significantly and Type I and II infections were increased significantly in GIF1-OE plants compared with the wild-type control at 48 h post-inoculation (hpi) (Fig. 3C). Therefore, we concluded that the overexpression of *GIF1* also increased blast resistance probably through the prevention of the penetration of fungal hyphae and restriction of hyphal growth.

We have shown that *GIF1* and another CW-Inv gene *OsCIN1* constitute a pair of duplicate genes which experience subfunctionalization with different expression patterns and different kinetic parameters of enzymatic activity: *GIF1* transcripts have been detected in roots, elongating internodes, shoots and panicles, but not in leaves. In contrast, *OsCIN1* is expressed strongly in leaves (Wang *et al*., 2010). However, we did not observe any obvious difference in *Xoo* resistance between the *OsCIN1*-overexpressing (OsCIN1-OE) plants and wild-type plants (Fig. S3, see Supporting Information), probably because OsCIN1 has low invertase activity and does not affect rice defence significantly, in addition to unchanged growth when overexpressed (Wang *et al*., 2010), even though it is inducible by *Xoo* (Fig. S2). Therefore, GIF1 probably modulates rice basal defence through its strong activity on sugar metabolism.

GIF1 modulates cell wall physical defence

Because the cell wall mainly consists of carbohydrates, we first postulated that GIF1 might contribute to cell wall-based defence through sugar homeostasis. To explore this possibility, we analysed the cell wall composition and structure of the wild-type, mutant and GIF1-OE plants. We found that the levels of sucrose, glucose and fructose were decreased significantly in the *gif1* glume (Fig. 4A). Interestingly, we found that the cell wall was thinner in the *gif1* mutant than in the wild-type (Fig. 4B), probably resulting from the decreased levels of sugars (Fig. 4A,C). In contrast, we detected higher levels of glucose and fructose in GIF1-OE plant leaves; we also found the accumulation of sucrose in transgenic leaves (Fig. 4C). We did not find changes in sugar levels in apoplastic fluid (Fig. S4, see Supporting Information), suggesting that sugars were mainly accumulated inside the GIF1-OE cell. Similar higher sugar levels were also observed in transgenic tobacco leaves that expressed a yeast invertase (von Schaewen *et al*., 1990). One explanation for the increased sugar accumulation could be that the overexpression of invertase blocks sucrose export from source leaves into the phloem (von Schaewen *et al*., 1990). In support of this postulation, GIF1-OE grains were poorly filled (Wang E.T. *et al*., 2008, 2010). We did not observe significant differences in the cell wall thickness between uninfected GIF1-OE and wild-type plants (Fig. 4D). Interestingly, the cell wall of GIF1-OE plants was much thicker than that of wild-type plants after inoculation with *Xoo* and *M. oryzae* (Fig. 4D). Consequently, levels of xylose and cellulose, which are important cell wall constituents, were increased significantly in GIF1-OE leaf cell walls
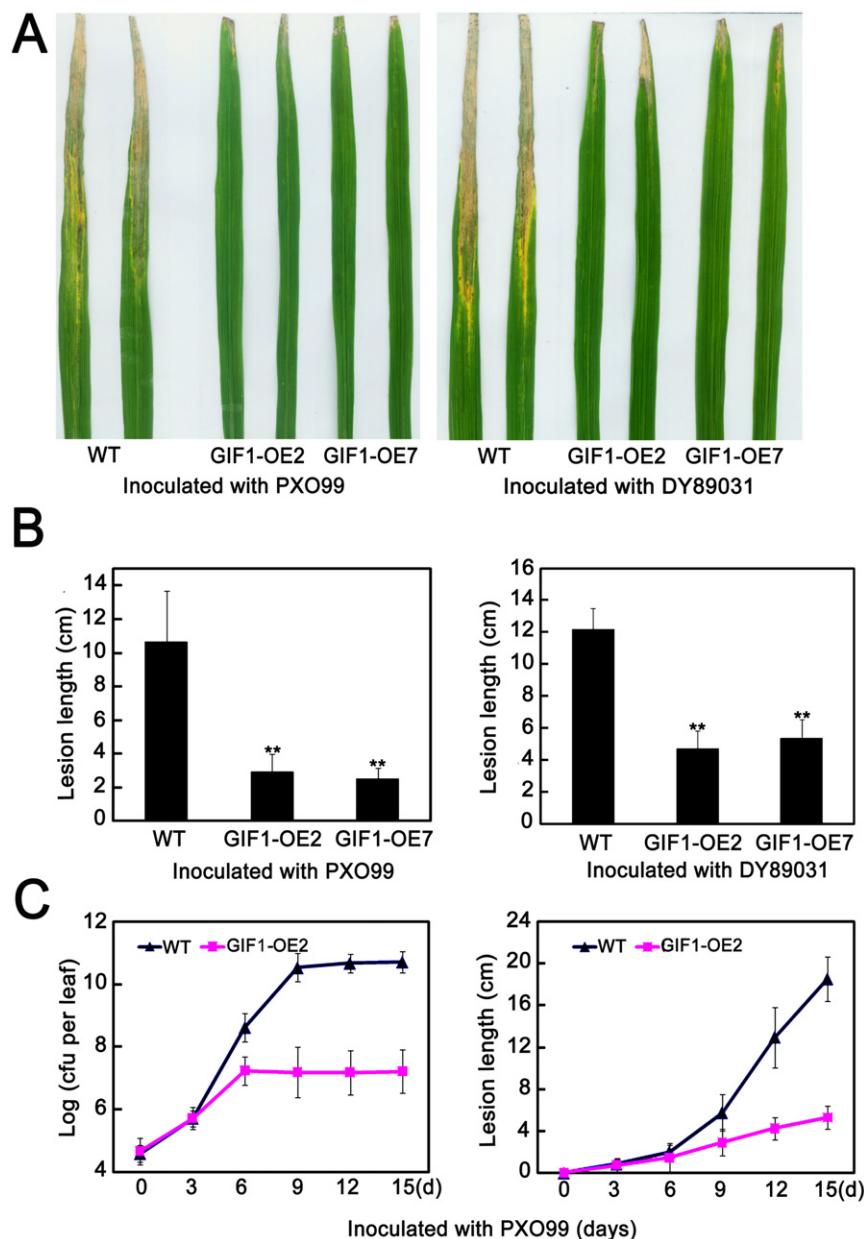
Fig. 2 Enhanced disease resistance to bacterial blight in GRAIN INCOMPLETE FILLING 1-overexpressing (GIF1-OE) plants. (A) Lesions of the representative GIF1-OE lines (GIF1-OE2 and GIF1-OE7) and the wild-type (TP309) (WT) infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) for 2 weeks. (B) The lesion lengths of the GIF1-OE plants inoculated with PXO99 and DY89031 for 2 weeks were decreased significantly. **Significant difference in comparison with the WT control ($P \leq 0.001$). (C) Bacterial growth and corresponding lesion lengths in the GIF1-OE and WT plants infected with PXO99 during a 15-day inoculation assay. The data are given as means ± standard error (SE) ($n = 3$).

compared with the wild-type. Galactose levels were decreased and increased in *gif1* and GIF1-OE, respectively, compared with their corresponding wild-type controls (Fig. 5). These results together indicate that GIF1 mediates rice defence partly through the reinforcement of cell wall-based physical defence.

GIF1 affects callose deposition during pathogen infection

Callose deposition deploys a rapid defence barrier on pathogen attack (Hématy *et al.*, 2009; Hückelhoven, 2007). To assay whether the GIF1-mediated change in sugar homeostasis affects callose deposition during pathogen infection, leaves infected with *Xoo*

were stained with aniline blue at 24 hpi. The results showed that callose deposition was more obvious in infected GIF1-OE cells than in wild-type cells (Fig. 6A). Consistent with this observation, the callose biosynthesis genes, *OsGSL1*, *OsGSL3* and *OsGSL5* (Hao *et al.*, 2008), were more strongly up-regulated in transgenic than in wild-type plants, and reached a peak at 12 hpi (*OsGSL1* and *OsGSL3*) or 48 hpi (*OsGSL5*) in GIF1-OE plants (Fig. 6B). Similar callose deposition and gene induction were also observed in the rice–*M. oryzae* interaction (Fig. S5, see Supporting Information). We also observed similar results in another GIF1-OE line (Fig. S6, see Supporting Information). Therefore, the GIF1-mediated sugar homeostasis regulates callose induction, which probably limits pathogen penetration and proliferation.
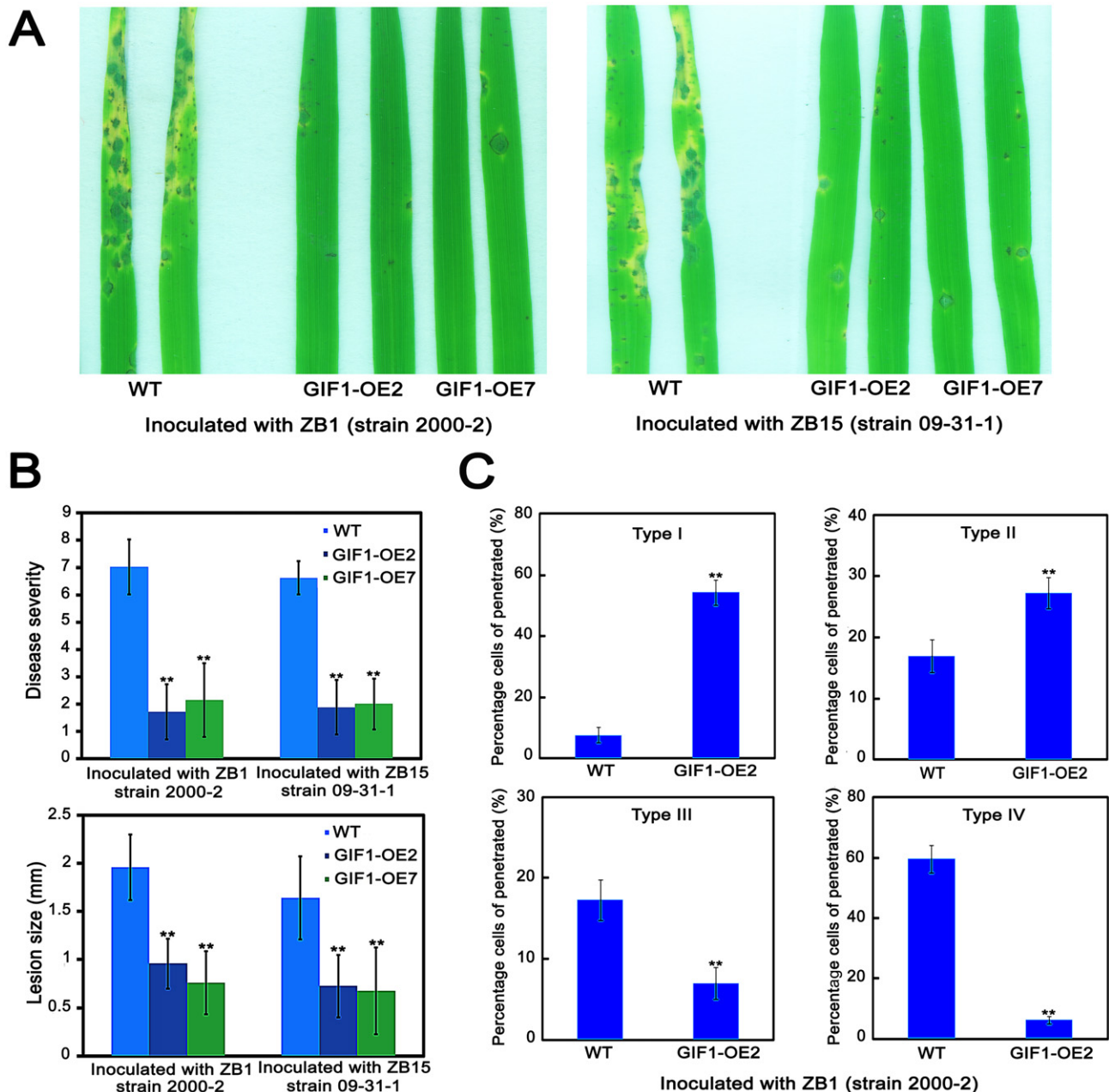
Fig. 3 Enhanced disease resistance to fungal blast in GRAIN INCOMPLETE FILLING 1-overexpressing (GIF1-OE) plants. (A) Blast lesions were less developed in GIF1-OE plants compared with the wild-type (WT) plants. Leaves were spray inoculated with virulent race ZB1 (left) and ZB15 (right), and disease symptoms were recorded at 7 days post-inoculation. (B) Disease severity and lesion size were decreased significantly in GIF1-OE plants relative to the WT. (C) Average infection types in the GIF1-OE and WT plants at 48 h post-inoculation (hpi). Note that Types I and II were increased significantly, whereas Types III and IV were decreased significantly in GIF1-OE. At least 100 lesions from 30 representative diseased leaves of 30 plants were measured. Data presented are the means ± standard error (SE) (n = 3). **Significant difference in comparison with the WT control ($P \leq 0.001$).

GIF1 affects ROS accumulation and the HR

The plant cell wall is associated with defence responses, including ROS accumulation and the HR, when plants counteract pathogen infection (Hückelhoven, 2007). Soluble sugars have been proposed to modulate oxidative stress in plants (Couée et al., 2006), and increased levels of ROS may be a consequence of altered sugar levels in plants (Bolouri-Moghaddam et al., 2010). To determine the involvement of ROS and the HR in the stimulated defence of GIF1-OE plants, we analysed hydrogen
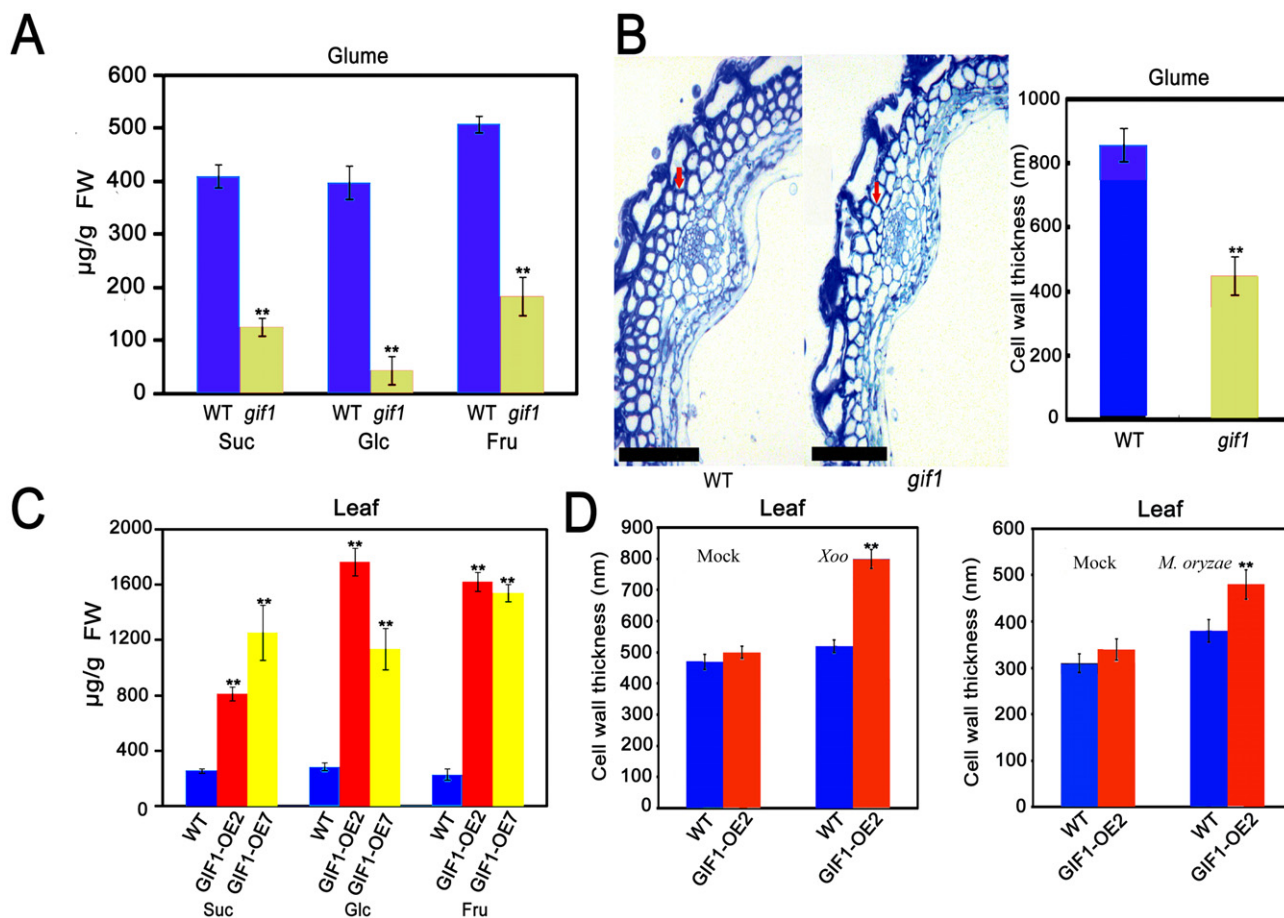
Fig. 4 Comparison of cell wall composition and thickness in GRAIN INCOMPLETE FILLING 1-overexpressing (GIF1-OE), *gif1* mutant and wild-type (WT) plants. (A) Decreased levels of sucrose (Suc), glucose (Glc) and fructose (Fru) in the *gif1* glume compared with the WT control. Data presented are means ± standard error (SE) ($n = 3$) with units of μg/g fresh weight (FW). **Significant difference in comparison with the WT control ($P \leq 0.001$). (B) Cell wall thickness of the *gif1* glume was decreased significantly in comparison with the WT. Red arrows indicate the cell wall. Bar, 50 μm. Glume sections from six individual plants were observed and at least 20 measurements were carried out for each sample. Data presented are the means ± SE ($n = 3$). **Significant difference in comparison with the WT control ($P \leq 0.001$). (C) Suc, Glc and Fru were highly accumulated in the GIF1-OE leaves compared with the WT control. Data presented are the means ± SE ($n = 3$) with units of μg/g FW. **Significant difference in comparison with the WT control ($P \leq 0.001$). (D) Cell wall thickness was increased significantly in GIF1-OE relative to WT plants after infection with PXO99 (left) and ZB1 (right). Leaf sections from six individual plants were observed and at least 20 measurements were carried out for each sample. Data presented are the means ± SE ($n = 3$). **Significant difference in comparison with the WT control ($P \leq 0.001$).

peroxide (H_2O_2) accumulation and the HR in plants infected with bacterial and fungal pathogens. We observed that higher levels of H_2O_2 were accumulated in infected GIF1-OE leaves when compared with the wild-type control (Figs 7A, S6 and S7, see Supporting Information). We also observed stronger HR cell death in GIF1-OE plants than in the wild-type (Figs 7B, S6 and S7). In support of this, the peroxidase genes, *POX8.1* and *POX22.3*, were more strongly induced in GIF1-OE plants after pathogen infection (Figs 7C and S7), which have been suggested to be involved in H_2O_2 production/scavenging in rice (Ning *et al.*, 2010). These results indicate that the GIF1-mediated sugar homeostasis also enhances inducible chemical defence responses in rice.

GIF1 regulates the expression of defence-related genes

It has been shown that sugars regulate the expression of *PR* genes (Rolland *et al.*, 2006). Our previous microarray assay revealed that the expression of some defence-related genes was altered in uninfected *gif1* grains [see the microarray data in the National Center for Biotechnology Information GEO database (http://www.ncbi.nlm.nih.gov/geo/) under accession codes GSE9498 and GSM240994–GSM240999]. Interestingly, the transcript levels of the rice defence-related genes, *PR1a*, *PR1b*, *PR3*, *PR10*, *WRKY45* and *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1* (*NPR1*), were constitutively elevated in GIF1-OE plants when com-
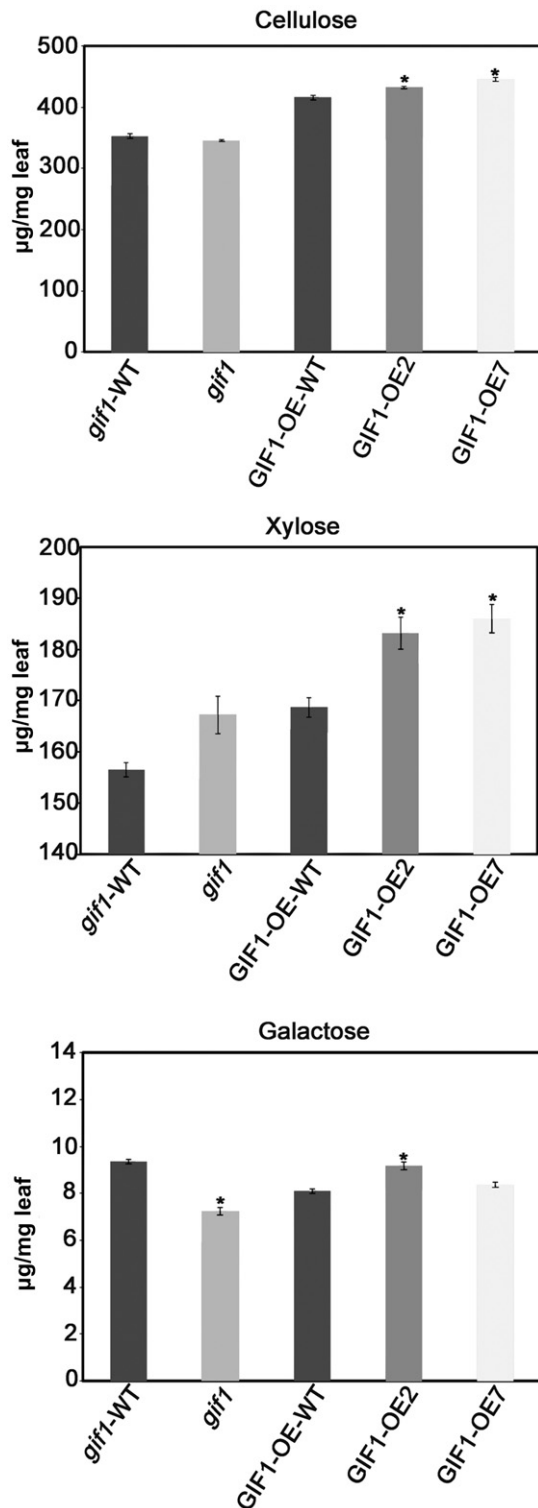
Cellulose

Xylose

Galactose

Fig. 5 Levels of xylose, cellulose and galactose in leaf cell walls of the *gif1* mutant, overexpression lines and wild-types (WT) for *gif1* (gif1-WT) and overexpression line (GIF1-OE-WT). The data are given as means ± standard error (SE) ($n = 3$). *Significant difference in comparison with the WT control ($P \leq 0.01$).

pared with the wild-type control (Fig. 8). We thus conclude that sugar homeostasis mediated by GIF1 activates the rice defence signalling network at the molecular level.

DISCUSSION

Sugars have been recognized as important signalling molecules that affect a variety of physiological responses and, in particular, regulate the expression of genes involved in photosynthesis, sink metabolism and defence responses (Rolland *et al.*, 2006; Smeekens, 2000). In addition, plant defence against pathogens is costly in terms of energy and carbohydrates (Berger *et al.*, 2007). In particular, most plant pathogens actively penetrate the plant cell wall to access intracellular nutrients; whereas plants, in turn, strength the cell wall and secrete antimicrobial components into the cell wall space to stop pathogen invasion. CW-Invs are inducible by pathogen infection, and can increase local respiratory sink activity and are involved in plant defence against various pathogens (Roitsch and González, 2004; Rolland *et al.*, 2006). However the mechanism by which CW-Invs modulate defence remains largely unknown. *Arabidopsis* contains three predicted 'defective invertases' and three genuine CW-Invs in its CW-Inv family (Van den Ende *et al.*, 2009). Our previous study has also shown that OsCIN1 displays low invertase activity (Wang *et al.*, 2010), probably resulting from being a 'defective invertase' (Le Roy *et al.*, 2013), lacking the aspartic acid (Asp)/lysine (Lys) or Asp/arginine (Arg) couple that is needed to catalyse the breakdown of sucrose (Le Roy *et al.*, 2007). Our current experimental evidence indicates that GIF1, as a genuine CW-Inv, plays a role in pre-existing/constitutive and inducible defence responses. The overexpression of *GIF1* strongly increases resistance to both bacterial and fungal diseases.

The crucial role of the plant cell wall in disease resistance has been highlighted by many studies (Hückelhoven, 2007). Cell wall mechanical strength can increase plant resistance to diverse pathogens (Hückelhoven, 2007; Schulze-Lefert, 2004). Our previous study has also reported that the rice E3 ligase OsBBI1 confers broad-spectrum resistance to blast fungus through the enhancement of cell wall defence responses (Li *et al.*, 2011). In addition to forming a mechanical barrier to physical fungal penetration, cell wall reinforcements can also decrease susceptibility to cell wall-degrading enzymes, impede nutrient diffusion to pathogens and possibly restrict the diffusion of toxins (Asselbergh *et al.*, 2007). Interestingly, the cell wall of the *gif1* mutant glume is thinner than that of the wild-type control, a possible explanation of the increased susceptibility to postharvest fungal pathogens (Fig. 1). Cellulose is the main load-bearing polymer and a potentially important factor in cell wall integrity, which mechanically strengthens the plant cell wall, and cellulose-deficient mutant plants are often less resistant to pathogens (Hématy *et al.*, 2009; Hückelhoven, 2007; Vorwerk *et al.*, 2004). Similarly, we also
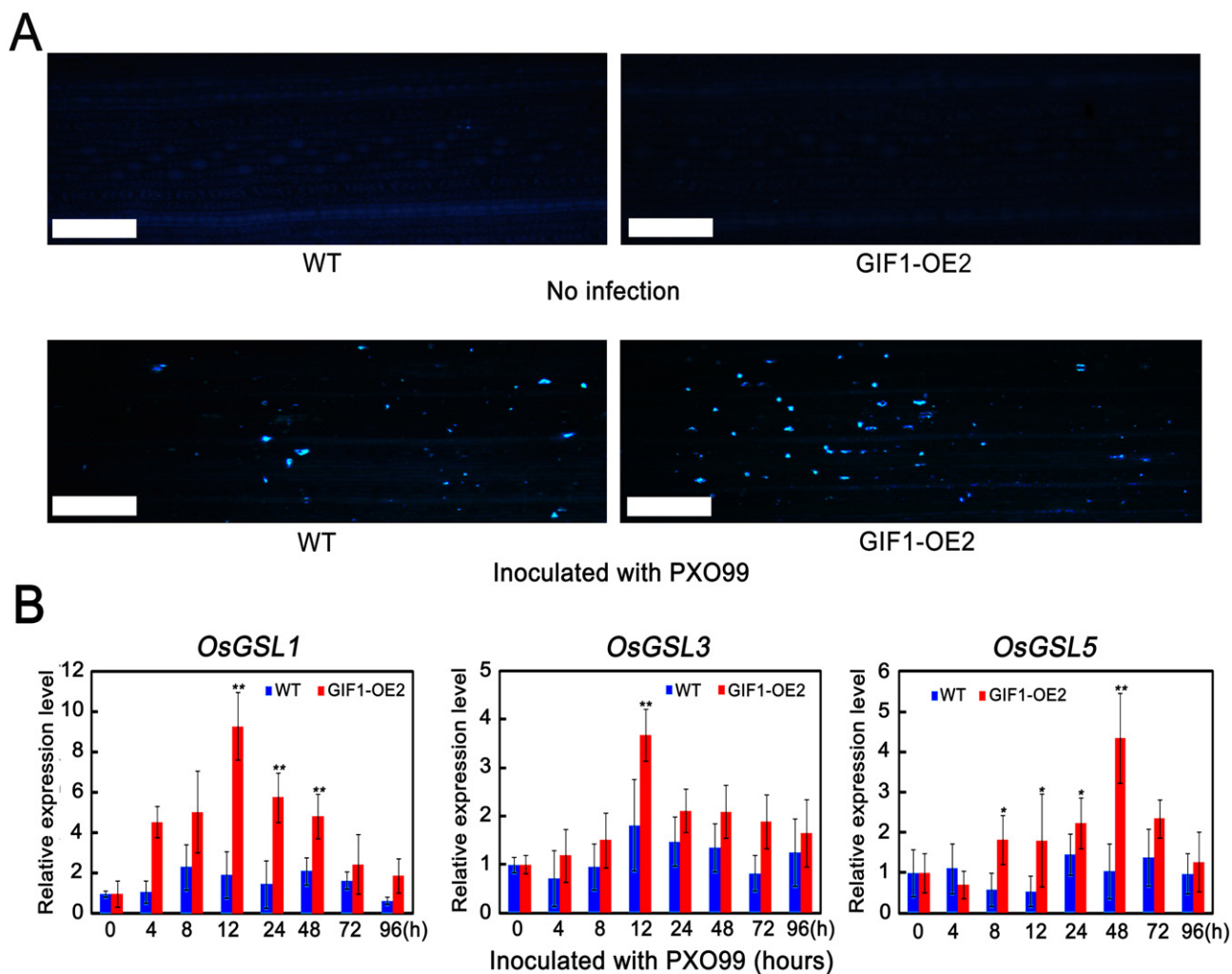
Fig. 6 *GRAIN INCOMPLETE FILLING 1* (*GIF1*) overexpression stimulates callose deposition. (A) Callose deposition was obviously increased in the GIF1-overexpressing (GIF1-OE) cells compared with the wild-type (WT) cells at 24 h post-inoculation (hpi) with PXO99. Bar, 100 μm. (B) Stronger up-regulation of the callose biosynthesis genes *OsGSL1*, *OsGSL3* and *OsGSL5* in GIF1-OE plants after PXO99 infection, in comparison with the WT. The data are given as means ± standard error (SE) (*n* = 3). Significant difference in comparison with the WT control: *$P \leq 0.01$; **$P \leq 0.001$.

observed that the cellulose content was reduced and increased significantly in the *gif1* mutant and GIF1-OE plants, respectively. Moreover, callose deposition is a well-known plant defence reaction requiring a large amount of sugars (Hématy *et al.*, 2009; Hückelhoven, 2007), GIF1 also positively regulates callose deposition during pathogen infection, forming an additional inducible physical barrier against pathogen invasion through cell wall reinforcement.

H_2O_2 has long been recognized as an important signalling molecule leading to plant defence responses. Recent studies have also proposed that sugar-like molecules can counteract oxidative stress by acting as genuine ROS scavengers, and the absence of alkaline/neutral (A/N) invertases has been associated with higher oxidative stress defence gene expression, whereas overexpression of A/N invertases in *Arabidopsis* leaf mesophyll protoplasts down-

regulates ascorbate peroxidase 2 (APX2) (Xiang *et al.*, 2011). In our study, the finding that GIF1 overexpression stimulated H_2O_2 accumulation and activated defence-related genes, including *POX* and *PR* genes, is probably a consequence of the higher hexose levels or hexose/sucrose ratios, suggesting a 'primed' defensive response. We propose that the differential localization of invertases (cell wall, cytosol or organelle) may have a significant effect on ROS generation and/or scavenging, and thus the expression of defence-related genes. Similarly, in transgenic rice plants expressing a fungal-inducible *PR* gene from maize, high concentrations of sucrose acted as a signal to prime defence responses against both bacterial and fungal pathogens (Gómez-Ariza *et al.*, 2007). Similarly, the induction of defence by hexoses generated by increased invertase activity was also observed in *Arabidopsis* (Chou *et al.*, 2000; Fotopoulos *et al.*, 2003), tobacco (Herbers
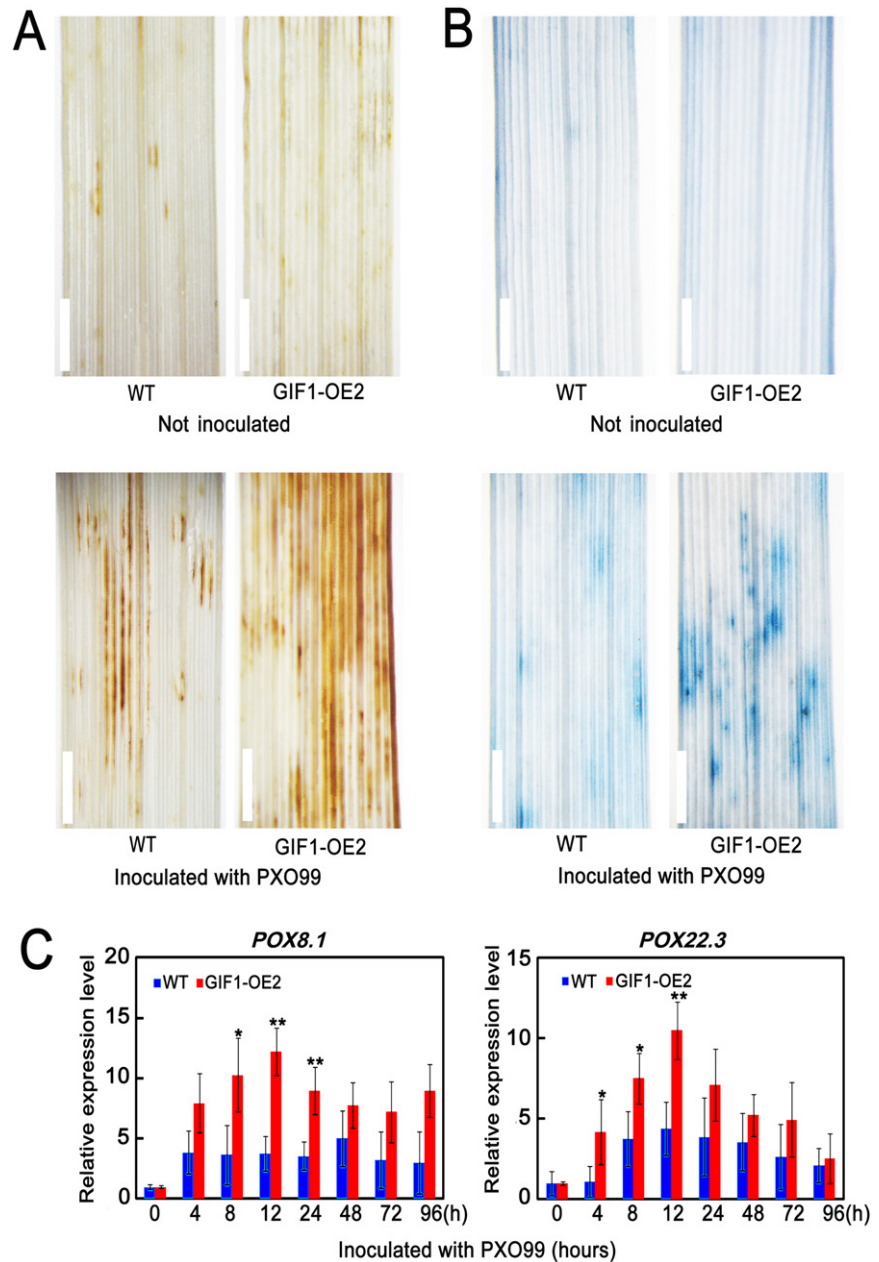
Fig. 7 H_2O_2 accumulation and localized cell death (hypersensitive response, HR) after *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) inoculation. (A) H_2O_2 accumulation was obviously increased in the GRAIN INCOMPLETE FILLING 1-overexpressing (GIF1-OE) leaves compared with the wild-type (WT) control after infection with PXO99 at 24 h post-inoculation (hpi). Bar, 100 μm. (B) Stronger HR cell death occurred in GIF1-OE than in the WT after infection with PXO99 at 24 hpi. Bar, 100 μm. (C) The peroxidase genes *POX8.1* and *POX22.3* were more strongly induced in GIF1-OE plants compared with the WT after inoculation with PXO99. The data are given as means ± standard error (SE) (*n* = 3). Significant difference in comparison with the WT control: *$P \leq 0.01$; **$P \leq 0.001$.

et al., 1996; Scharte *et al*., 2005), wheat (Sutton *et al*., 2007; Wright *et al*., 1995), tomato (Kocal *et al*., 2008) and pepper (Sonnewald *et al*., 2012). Silencing of the tobacco *CW-Inv* impaired basal defence and hypersensitive cell death with significantly reduced sucrose export and apoplastic carbohydrate levels, revealing that CW-Inv is important for the acquisition of carbohydrates during pathogen infection, and the availability of these carbohydrates can ensure successful defence responses (Essmann *et al*., 2008). Therefore, GIF1-mediated sugar homeostasis also plays a role in inducible chemical defence in rice.

It is well known that most plant pathogens actively penetrate the plant cell wall to access intracellular nutrients; whereas plants strengthen the cell wall and secrete antimicrobial components into the cell wall space to stop pathogen invasion. Interestingly, the accumulation of sugars during the later stages of infection might serve as nutrients for invading pathogens, resulting in disease (Seo *et al*., 2007). Moreover, pathogens can induce the sucrose efflux transporter genes *SWEET*s, and the sugar efflux function of SWEET transporters is probably targeted by pathogens for nutritional gain (Chen F. *et al*., 2010).

Sucrose also acts as a signalling molecule in plant immunity to induce defence responses (Bolouri-Moghaddam and Van den Ende, 2012). Interestingly, such 'Sweet Immunity' also affects circadian rhythms in plants (Bolouri-Moghaddam and Van den Ende, 2013a,
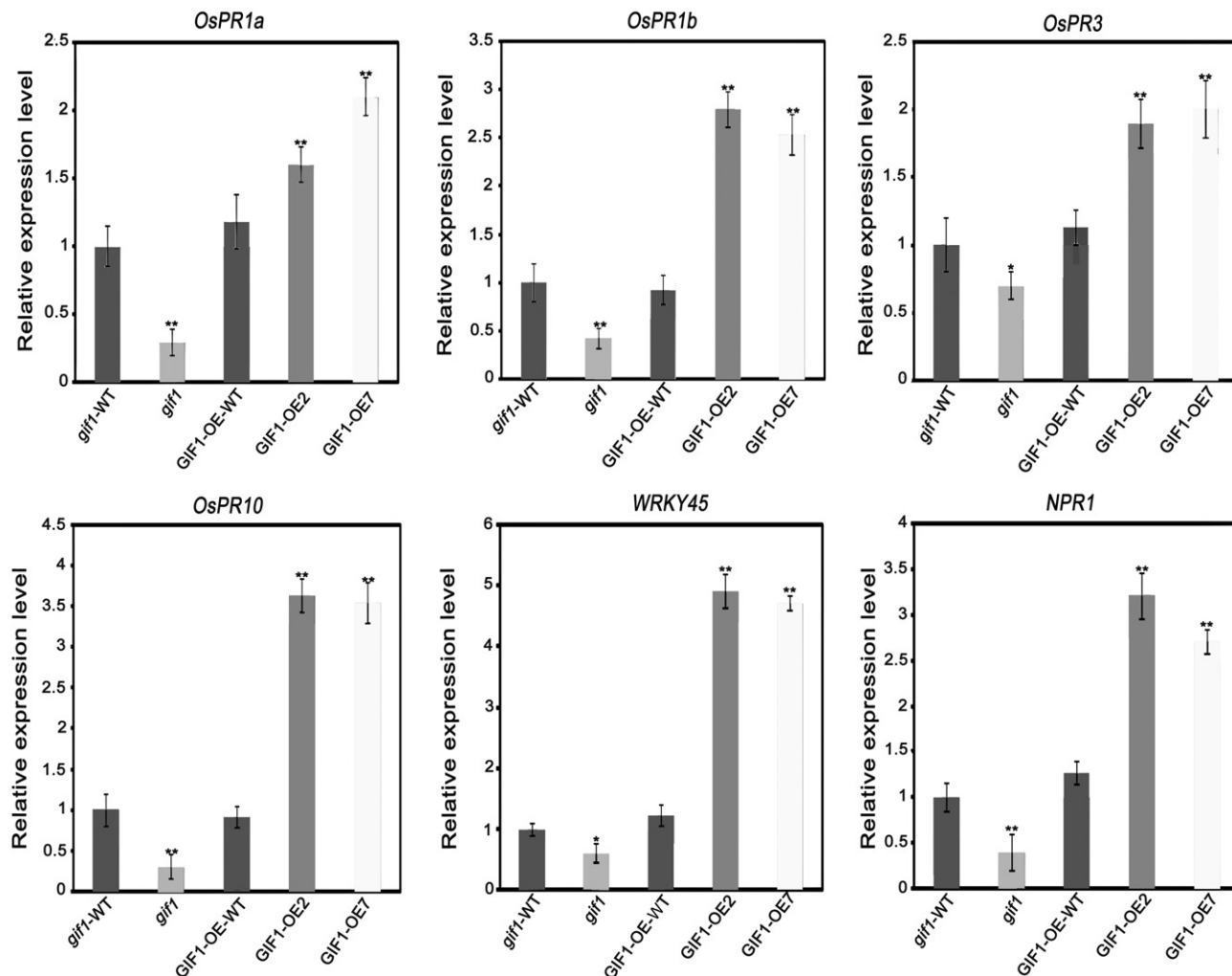
Fig. 8 Constitutive activation of defence-related genes in GRAIN INCOMPLETE FILLING 1-overexpressing (GIF1-OE) plants. The transcript levels of the rice defence-related genes, *PATHOGENESIS-RELATED 1a* (*PR1a*), *PR1b*, *PR3*, *PR10*, *WRKY45* and *NONEXPRESSOR OF PATHOGENESIS-RELATED 1* (*NPR1*), were assayed by quantitative real-time polymerase chain reaction (qRT-PCR) in *gif1*, GIF-OE and wild-type (WT) controls (gif1-WT for *gif1* and GIF1-OE-WT for the overexpression lines). The data are given as means ± standard error (SE) (*n* = 3). Significant differences in comparison with the WT control: *$*P ≤ 0.01$; **$**P ≤ 0.001$.

b). Moreover, in transgenic rice expressing a fungal glucose oxidase, the increased induction of H_2O_2 leads to cell death and enhanced resistance to both bacterial and fungal pathogens (Kachroo *et al.,* 2003). It has also been suggested that the restriction of intercellular transport to the interface of adjacent phloem cells may be an effective mechanism to limit the availability of sucrose in the leaf apoplast to prevent pathogen infection (Chen F. *et al.*, 2012). With this scenario in mind, it would be worth dissecting in more detail the systemic allocation and subcellular partitioning of sugars during plant–pathogen interactions. In addition, further investigation of the involvement and underlying mechanisms of GIF1-associated invertase inhibitors and defence genes in GIF1-modulated defence activation might provide deeper insights into sugar-triggered immunity in plants.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The *gif1* mutant (in the *japonica* Zhonghua 11 background), GIF1- and OsCIN1-OE transgenic lines (in the *japonica* TP309 background) and their respective wild-type controls were used (Wang E.T. *et al.*, 2008, 2010). Rice plants were grown in a glasshouse or growth chamber with a 12-h/12-h day/night cycle at 26–28 °C and 80%–90% humidity.

Identification of postharvest fungal pathogens

The *gif1* seeds infected by fungi in the field were harvested and washed with sterile water, and the glumes were then incubated on potato dextrose agar (PDA) at 28 °C to allow fungal growth. Fungal colonies were isolated

and incubated on PDA at 28 °C for 5–7 days and maintained at 4 °C. The isolated fungi were observed under a microscope (Olympus SZX7, Tokyo, Japan). The pathogenic fungi *Alternaria* sp. and *Rhizopus* sp. were characterized by morphology and re-inoculation of the *gif1* grains in the field during filling.

Pathogen inoculation and disease assay

For *Xoo* resistance assay, 2-month-old rice plants were inoculated with the *Xoo* virulent races P6 (strain PXO99) and K1 (strain DY89031) by the leaf-clipping method; the lesion length was measured after 2 weeks, and bacterial growth was also recorded (Wang Y.L. *et al*., 2008; Yang *et al.*, 2008). For *M. oryzae* resistance, 2-week-old seedlings were spray inoculated with the virulent races ZB1 (strain 2000-2) and ZB15 (strain 09-31-1), and disease severity was evaluated as described by Li *et al*. (2011). The average infection type was recorded for ZB1 after 48 hpi as described by Nakao *et al*. (2011). At least 40 plants of each line were evaluated for infection type.

RNA analysis

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNAs were synthesized from 3 μg of total RNAs using oligo(dT) primer and SuperScript III reverse transcriptase (Invitrogen). Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed on an Eppendorf realplex2 Mastercycler (Eppendorf, Hamburg, Germany) using SYBR Premix Ex Taq (TaKaRa, Tokyo, Japan) and gene-specific primers (Table S1, see Supporting Information). For pathogen induction of *CIN* genes, 2-week-old seedlings of wild-type TP309 were inoculated with PXO99. Leaves were harvested at 0, 6, 24, 28 and 72 h after inoculation. Total RNA was prepared using TRIzol Reagent and the first cDNA strand was synthesized with a PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa). Gene-specific primers (Table S1) were designed and RT-PCR analysis was performed as described previously (Cho *et al*., 2005), using *PBZ1* as a defence activation marker.

Callose deposition analysis

For the detection of callose deposition, 2-week-old seedlings were inoculated with *Xoo* PXO99 and *M. oryzae* ZB1 for 24 h, and the aniline blue method was used to detect callose deposition as described previously (Li *et al*., 2011).

3,3'-Diaminobenzidine (DAB) and trypan blue staining

For the detection of H_2O_2 accumulation in rice, the DAB uptake method was adopted as described previously (Chen F. *et al*., 2010; Li *et al*., 2011). Two-week-old seedlings were inoculated with *Xoo* PXO99 and *M. oryzae* ZB1 for 24 h, and HR cell death was visualized by the trypan blue exclusion assay as described previously (Chen F. *et al*., 2010).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) assays

For SEM observation, seed glumes were fixed in formalin–acetic acid–alcohol (FAA) overnight at 4 °C and observed as described previously in

Lin *et al*. (2012). For TEM assay, leaf pieces (0.5–1.0 cm) were vacuum fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) overnight at 4 °C and visualized as described by Li *et al*. (2011). In addition, leaf pieces after pathogen infection were fixed as depicted in TEM analysis. Serial 150-nm-thick sections were cut using a rotary microtome.

Cell wall composition and sugar content assays

Cell wall composition was analysed according to Zhang *et al*. (2011). Briefly, 1 g glume and flag leaf (fresh weight) were ground into a powder, washed with 70% ethanol and a mixture of chloroform and methanol, and then overnight in sodium acetate buffer. The released sugar and derivatives were assayed. Sugar contents were determined by gas chromatography-mass spectrometry (GC-MS) as described previously (Wang E.T. *et al*., 2008).

Statistical analyses

All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA). Data from three independent assays were analysed using analysis of variance (ANOVA) by least-significant difference test at $P \leq 0.01$.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Resistance of the *gif1* mutant and the wild-type control to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Magnaporthe oryzae*.

Fig. S2 Induction of *OsCIN* genes by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*).

Fig. S3 No difference in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) resistance between *OsCIN1*-overexpressing (OsCIN1-OE) and wild-type plants.

Fig. S4 Sugar concentrations in apoplastic fluid of the wild-type and *GIF1*-overexpressing (*GIF1*-OE) leaves.

Fig. S5 Callose deposition and callose gene expression during *Magnaporthe oryzae* infection.

Fig. S6 Callose deposition, H_2O_2 accumulation and localized cell death during *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Magnaporthe oryzae* infection in GIF1-OE7.

Fig. S7 H_2O_2 accumulation and localized cell death (hypersensitive response, HR) during *Magnaporthe oryzae* infection.

Table S1 Primers used for quantitative real-time polymerase chain reaction (qRT-PCR).