Inhibition of *OsSWEET11* function in mesophyll cells improves resistance of rice to sheath blight disease

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SUMMARY

Pathogen–host interaction is a complicated process; pathogens mainly infect host plants to acquire nutrients, especially sugars. *Rhizoctonia solani*, the causative agent of sheath blight disease, is a major pathogen of rice. However, it is not known how this pathogen obtains sugar from rice plants. In this study, we found that the rice sugar transporter *OsSWEET11* is involved in the pathogenesis of sheath blight disease. Quantitative real-time polymerase chain reaction (qRT-PCR) and β-D-glucuronidase expression analyses showed that *R. solani* infection significantly enhanced *OsSWEET11* expression in leaves amongst the clade III SWEET members. The analyses of transgenic plants revealed that *Ossweet11* mutants were less susceptible, whereas plants overexpressing *OsSWEET11* were more susceptible, to sheath blight compared with wild-type controls, but the yield of *OsSWEET11* mutants and overexpressors was reduced. SWEETs become active on oligomerization. Split-ubiquitin yeast two-hybrid, bimolecular fluorescence complementation and co-immunoprecipitation assays showed that mutated OsSWEET11 interacted with normal OsSWEET11. In addition, expression of conserved residue mutated AtSWEET1 inhibited normal AtSWEET1 activity. To analyse whether inhibition of OsSWEET11 function in mesophyll cells is related to defence against this disease, mutated OsSWEET11 was expressed under the control of the *Rubisco* promoter, which is specific for green tissues. The resistance of transgenic plants to sheath blight disease, but not other disease, was improved, whereas yield production was not obviously affected. Overall, these results suggest that *R. solani* might acquire sugar from rice leaves by the activation of *OsSWEET11* expression. The plants can be protected from infection by manipulation of the expression of *OsSWEET11* without affecting the crop yield.

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INTRODUCTION

Sheath blight, which is one of the three major diseases of rice, is caused by infection with *Rhizoctonia solani* AGI-1A and is responsible for severe yield losses (Slaton *et al.*, 2003). This disease affects rice throughout its life cycle, from the seedling to heading stage, and causes lesions on leaves, sheaths and even panicles. It causes withering of leaves and sheaths, and reduction in the rate of seed set. At later stages of infection, the entire plant may wither and lodge. Sheath blight can reduce the yield of rice from 8% to 50%, depending on the severity of the disease, stage of the crop at which it was infected by the fungus and overall environmental conditions (Savary *et al.*, 2000).

The use of fungicides remains the mainstay of strategies for the control of sheath blight (Rajesh *et al.*, 2016). However, extensive use of chemicals poses health risks, causes financial strain on farmers and is damaging to the environment. Thus, research aimed at the identification of pathogen-resistant rice and deciphering of the mechanisms underlying pathogen–plant interaction is urgently needed. Previous studies have shown that the overexpression of chitinase and β-1,3-glucanase in rice enhances the resistance to different anastomosis groups of *R. solani* (Mao *et al.*, 2014; Sripriya *et al.*, 2017). However, the overexpression of *OsACS2*, which encodes an enzyme involved in ethylene biosynthesis, increases the endogenous production of ethylene and enhances the resistance to rice sheath blight and rice blast (Helliwell *et al.*, 2013). In addition, the overexpression of *OsPGIP1*, which codes for polygalacturonase-inhibiting protein, significantly improves the resistance to sheath blight in rice (Wang *et al.*, 2015). Recent evidence has suggested that transgenic lines with inducible production of ethylene exhibit resistance to *R. solani*,

without any yield penalty (Helliwell *et al.*, 2013). The overexpression of *OsOSM1*, which encodes a protein belonging to the pathogenesis-related protein 5 family, has been shown to enhance resistance to sheath blight in rice in field trials (Xue *et al.*, 2016). Pyramided transgenic plant lines which co-express both the genes (*OsCHI11* and *AtNPR1*) were found to have more improved performance against sheath blight tolerance than single gene transformants. (Karmakar *et al.*, 2017). However, to date, no gene that confers significant resistance has been identified in rice.

The process through which *R. solani* obtains nutrients, such as sugar required for its proliferation, from plants remains unknown. The elucidation of the process for the acquisition of nutrients by a pathogen is often helpful to devise effective strategies for the generation of pathogen-resistant plants. The recently identified SWEETs constitute a family of sugar transporters (Chen *et al.*, 2010). On average, 20 SWEET genes have been identified to be present in many higher plant species (Chen LQ *et al.*, 2015). In terms of fungi, during *Botrytis cinerea* infection, *AtSWEET4*, *AtSWEET15* and *AtSWEET17* are induced (Ferrari *et al.*, 2007). *Golovinomyces cichoracearum* infection induces several *AtSWEET*s, most prominently *AtSWEET12* (Chen *et al.*, 2010). *OsSWEET11/ Os8N3* is involved in the bacterial blight disease response in rice (Yang *et al.*, 2006). However, the role of *OsSWEET11* in other types of pathogen infections has not been reported. Recent studies have shown that SWEETs are responsible for susceptibility to disease in many plant species; they act as the targets of effector proteins secreted by pathogens during host–microbe interactions. For example, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99[A] can produce the transcription activator-like (TAL) effector, PthXo1, which binds directly to the *OsSWEET11* promoter (Chen *et al.*, 2010; Yang *et al.*, 2006). The TAL effectors are delivered to the cytoplasm of plant cells through the type III secretion system and enter the nucleus to induce the expression of specific SWEET genes, ensuring the delivery of sucrose to the apoplasts of the colonized cells (Chen *et al.*, 2010). For example, *OsSWEET11/Xa13*, *OsSWEET13/Xa25* and *OsSWEET14* have been identified as targets of *Xoo* effectors in rice (Antony *et al.*, 2010; Hutin *et al.*, 2015; Liu *et al.*, 2011; Yang *et al.*, 2006). In cotton, the expression of a sucrose transporter, *GhSWEET10*, can be activated by Avrb6, a TAL effector of *X. citri* ssp. *malvacearum*, during its invasion (Cox *et al.*, 2017).

Despite the progress in the elucidation of plant–pathogen interactions, two critical issues regarding the nutrient acquisition process of pathogens need to be addressed to modify pathogen resistance in rice. The first issue that needs clarification is how *R. solani* acquires nutrients, particularly sugar, from infected plants to support its propagation. The second issue that needs to be resolved is how the balance between the expense on pathogen resistance and that on plant growth and development is maintained.

The phylogenetic analysis of plant SWEET genes has revealed that they can be grouped into four clades, as first defined in *Arabidopsis* (Chen *et al.*, 2010). SWEETs grouped into clades I and II function in the transport of hexoses, whereas those included in clades III and IV are sucrose and fructose transporters, respectively (Chen LQ *et al.*, 2010, 2015). Previous studies have shown that SWEETs play diverse functions in plant development and production, and in the senescence of leaves (Quirino *et al.*, 1999), sugar loading in phloem (Chen *et al.*, 2012), nectar production (Lin *et al.*, 2014), pollen viability (Yang *et al.*, 2006) and grain filling (Ma *et al.*, 2017; Sosso *et al.*, 2015). All of these processes are important for the improvement of the rice yield. Thus, the manipulation of nutrient partitioning in specific tissues is a critical factor that needs to be considered to generate pathogen-resistant rice. However, the role of SWEETs in rice sheath blight has not yet been identified.

In this study, we analysed the *R. solani* AG1-1A-mediated induction of OsSWEET11 (which belongs to clade III) and investigated the resistance of the *OsSWEET11* mutant and overexpression rice lines to sheath blight. We found that *OsSWEET11* has a negative effect on sheath blight resistance. Interestingly, similar to proton–sucrose symporters, which tend to form dimers for normal functioning (Krügel *et al.*, 2008, 2013), SWEETs have been reported to be activated on oligomerization (Xuan *et al.*, 2013). To avoid interference with the essential role of OsSWEET11 during grain filling in rice (Ma *et al.*, 2017), we used the *Rubisco* promoter to express the mutated form of *OsSWEET11* (mSWEET11) which can inhibit OsSWEET11 sugar transport activity by forming a trimer with wild-type OsSWEET11 to inhibit OsSWEET11-mediated sugar transport in leaf cells during pathogen infection. The results showed that the inhibition of OsSWEET11 in leaf cells improved the resistance of transgenic rice to sheath blight without affecting the yield.

RESULTS

Rhizoctonia solani AG1-1A infection induces the expression of *OsSWEET11*

OsSWEET11 and *OsSWEET14* are targets of the type III secretion effector in bacterial blight disease; however, whether *OsSWEET*s are involved in sheath blight disease is not known. To analyse this question, clade III *OsSWEET* gene expression was analysed. The expression levels of *OsSWEET12–OsSWEET15* were not altered by infection (Fig. S1, see Supporting Information). However, the expression of *OsSWEET11* in the leaves and sheaths of mock (medium)- and *R. solani* AG1-1A-infected 1-month-old plants was induced by *R. solani* AG1-1A after 48 h, but not by mock treatment (Fig. 1A). After 72 h of infection, OsSWEET11 was induced five- and three-fold in the leaves and sheaths, respectively (Fig. 1A). The transcriptional activation of OsSWEET11 by *R. solani* AG1-1A infection was further verified using transgenic plants expressing the *pOsSWEET11::GUS* construct (Fig. 1B) (Ma *et al.*, 2017). In the mock treatment, the expression of β-D-glucuronidase (GUS) was barely detectable in both leaves and sheaths. However,

Fig. 1 Expression patterns of OsSWEET11 induced by *Rhizoctonia solani* AG1-1A infection. (A) Expression levels of OsSWEET11 were analysed after 0, 24, 48 and 72 h of *R. solani* infection. Solid potato dextrose agar (PDA) medium was used as a control (Mock), and *R. solani* AG1-1A cultured on the surface of PDA was used for the infection of rice leaves and sheaths. Ten leaves from each time point were sampled for RNA extraction. The experiments were repeated three times, and the data represent the means ± standard error (SE) ($n = 3$). Significant differences at $P < 0.05$ are indicated by different letters. (B) Schematic representation of the plasmid construct used for β-D-glucuronidase (GUS) expression in the transgenic plants, in which the OsSWEET11 promoter drives GUS expression. (C) Analysis of GUS expression in leaf and sheath after 48 h of mock and AG1-1A infection.
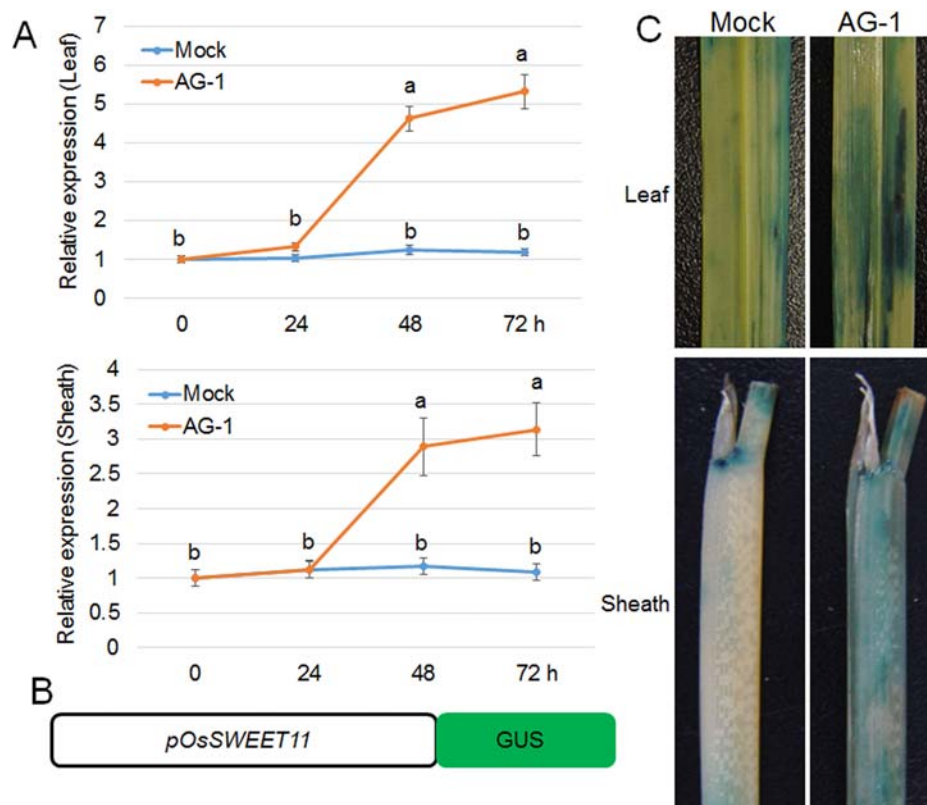
higher GUS staining was observed in the leaves and sheaths after 48 h of *R. solani* AG1-1A infection (Fig. 1C). These results indicate that *R. solani* AG1-1A infection induces the expression of *OsSWEET11*, but not *OsSWEET12–OsSWEET15*.

Expression of *OsSWEET11* is positively correlated with the lesion caused by sheath blight

Because *OsSWEET11* expression was induced by *R. solani* AG1-1A, the causative agent of sheath blight disease, we performed genetic studies on plants in which *OsSWEET11* was knocked out or overexpressed. The response of two *OsSWEET11* knock-out mutants (*Ossweet11-1* and *Ossweet11-2*) and two overexpression (OX) lines (*OX2* and *OX4*) to *R. solani* AG1-1A infection was assessed. Before testing the *R. solani* response, the *OsSWEET11* expression level was examined in four independent *OsSWEET11* OX lines. The data showed that *OsSWEET11* was more highly expressed in OX lines than in wild-type plants (Fig. S2, see Supporting Information). The fungal pathogen was inoculated on detached leaves, and the lesion area was evaluated after 72 h (Fig. 2A). After 72 h, the lesion area with respect to the total leaf area was compared amongst the wild-type, knock-out mutants and OX lines (Fig. 2B); the lesion areas were around 40%, 30% and 60%, respectively (Fig. 2B), indicating that the *Ossweet11* mutants were less susceptible, whereas the OX lines were more

susceptible, to *R. solani* AG1-1A compared with the wild-type plants.

The *OsSWEET11*-silenced lines were insensitive to bacterial blight caused by PXO99[A] (Yang *et al.*, 2006). In addition, the *OsSWEET11* mutants were insensitive, whereas the *OsSWEET11* OX plants were sensitive, to sheath blight disease. Therefore, the response of *OsSWEET11* OX plants to PXO99[A] infection was analysed. The leaves of 2-month-old wild-type and *OsSWEET11* OX plants (OX2 and OX4) were inoculated with PXO99[A]; the lesion length in wild-type plants was around 11 cm, whereas, in OX2 and OX4, it was around 17 and 15 cm, respectively (Fig. S3, see Supporting Information), indicating that *OsSWEET11* OX plants were more susceptible to bacterial blight disease caused by the PXO99[A] strain.

Mutation at conserved residues inhibits the activity of AtSWEET1

Although *OsSWEET11* knock-out mutants showed reduced sheath blight disease index, grain filling and seed set were severely affected (Fig. S4, see Supporting Information) (Ma *et al.*, 2017). In a previous study, the mutation of conserved residues, Y57 and G58, was found to inhibit the glucose transport activity of AtSWEET1, and co-expression of wild-type and mutated AtSWEET1 significantly reduced the glucose transport activity of AtSWEET1 in the EBY4000 yeast strain, which indicates that
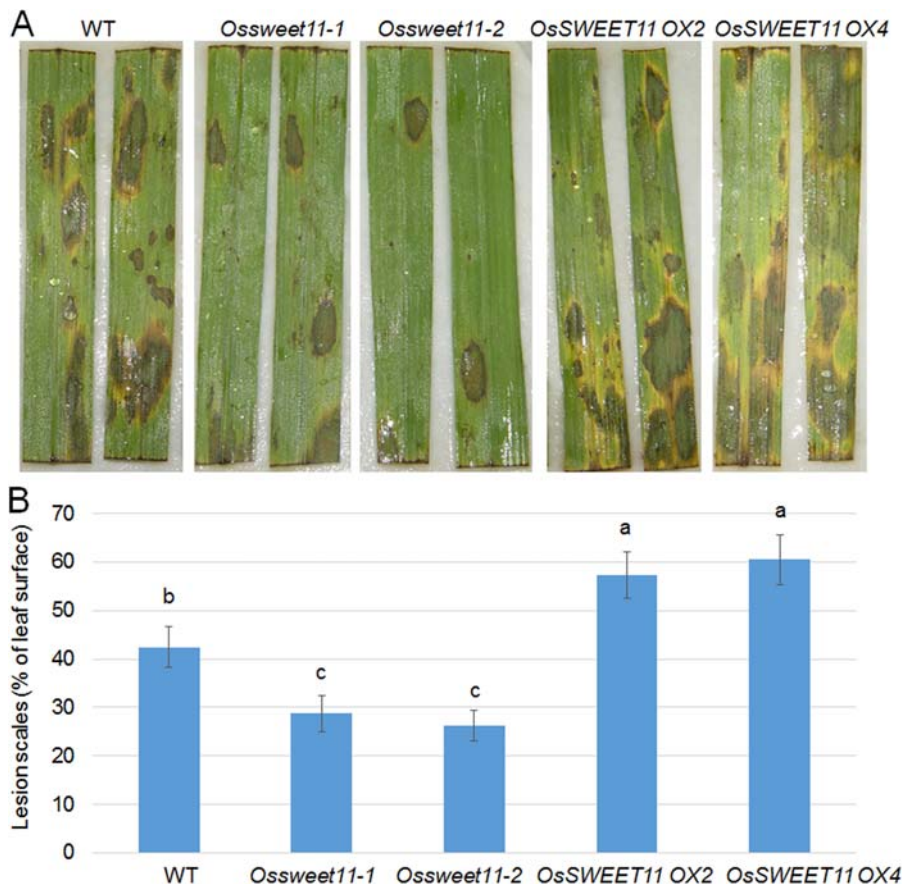
Fig. 2 Response of OsSWEET11 mutants and rice overexpression lines to *Rhizoctonia solani* AG1-1A. (A) Leaves from wild-type (WT) plants, OsSWEET11 mutants (*Ossweet11-1* and *Ossweet11-2*) and OsSWEET11 rice overexpression lines (*OsSWEET11 OX2* and *OsSWEET11 OX4*) were inoculated with *R. solani* AG1-1A and photographed after infection. Six leaves from each line were analysed, and the experiments were repeated three times. (B) The lesion scales were analysed for *R. solani* AG1-1A-infected leaves by determining the lesion area on the leaf surface. Data represent the means ± standard error (SE) ($n > 10$). Significant differences at $P < 0.05$ are indicated by different letters.

the oligomerization of SWEET is necessary for its function (Xuan *et al.*, 2013). Therefore, *mSWEET11* was expressed using a cell type-specific promoter to inhibit OsSWEET11-mediated sugar efflux in the cells, and thus to protect plants from sheath blight disease whilst maintaining normal grain filling. Mutated SWEET11 was generated by collecting and aligning the sequences of AtSWEET1, OsSWEET2b, OsSWEET11 and OsSWEET11 homologues from different model plants (Fig. 3A). The results showed that OsSWEET11 and its homologues contained a relatively longer cytosolic tail compared with that in AtSWEET1 and OsSWEET2b, and many residues were highly conserved amongst these SWEET proteins (Fig. 3A). The SUSY7 yeast strain is able to analyse sucrose transport activity (Lalonde *et al.*, 2003), but OsSWEET11 did not show activity in SUSY7 (Fig. S5, see Supporting Information). Therefore, AtSWEET1 was used to analyse conserved residue function. Five residues (P23, G76, P162, P191 and Q202) of AtSWEET1 with conserved residues (P31, G82, P169, P198 and Q209) in OsSWEET11 were selected for mutation. Empty vector, AtSWEET1 and mutated AtSWEET1 were expressed in the EBY4000 yeast strain deficient in hexose transport, and 2% glucose and maltose were used as carbon sources for the glucose transport activity test of AtSWEET1. The expression of AtSWEET1 and mutated AtSWEET1 in yeast EBY4000 revealed that

AtSWEET1 successfully complemented the growth of yeast cells, whereas the five mutated AtSWEET1 proteins failed to complement the growth in glucose-containing medium (Fig. 3B).

## Mutated OsSWEET11 interacts with wild-type OsSWEET11

Mutated *OsSWEET11* (*mSWEET11*) was constructed by mutating three conserved residues (P31, G82 and P169) amongst the five described above. Whether there was any interaction between mSWEET11 and OsSWEET11 was determined by performing a mating-based split-ubiquitin assay. *OsSWEET11* and *mSWEET11* were cloned into Nub vector pXN25_GW, and *OsSWEET11* was cloned into Cub vector pMETYC_GW; NubWT and NubG were used as positive and negative controls, respectively. The yeast growth assay showed that OsSWEET11 interacted with itself or mSWEET11 (Fig. 4A). Furthermore, a split green fluorescent protein (GFP) system was used to confirm their interaction in tobacco leaves. The open reading frames (ORFs) of *OsSWEET11* and *mSWEET11* were cloned into the pXNGW vector, in which the N-terminal half of the yellow fluorescent protein (YFP) sequence was C-terminally fused to OsSWEET11, whereas the ORF of *mSWEET11* was cloned into the pXCGW vector, in which the C-terminal half
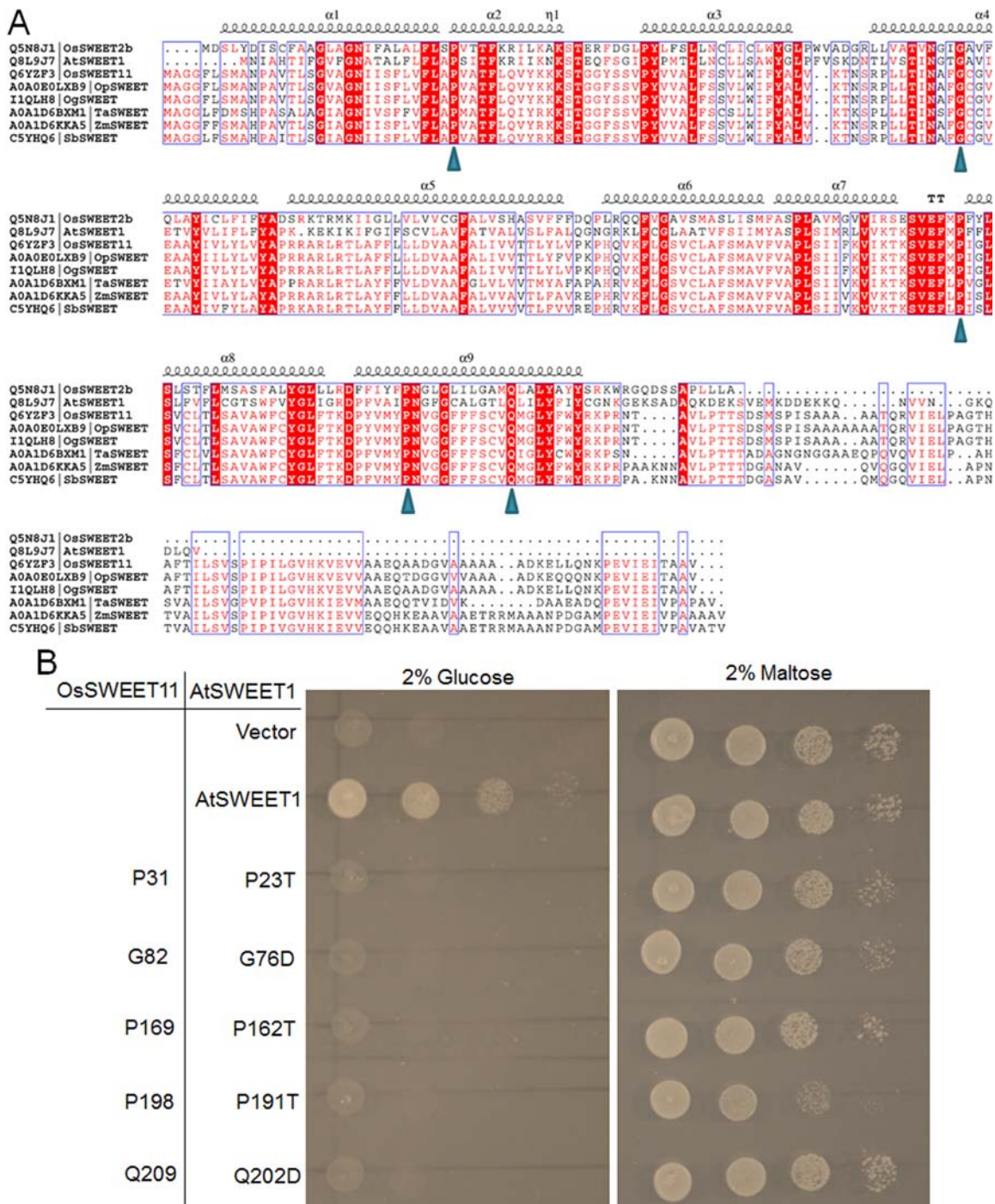
Fig. 3 Alignment of amino acid sequences of the selected SWEETs and functional analysis of the conserved residues in glucose transport. (A) Sequence alignment of OsSWEET2b, AtSWEET1a, OsSWEET11, OpSWEET, OgSWEET, TaSWEET, ZmSWEET and SbSWEET. The conserved residues are shown in red boxes. The triangle indicates the residues tested for glucose transport function. (B) Growth assays of mutant AtSWEET1 proteins expressed in EBY4000 yeast strain were performed on YNB (Yeast Nitrogen Base without Amino Acids) medium containing 2% glucose or maltose. AtSWEET1 mutants carrying P23A, G76D, P162A, P191T and Q202D led to the loss of glucose transport activity. Empty (pDRf1) vector and AtSWEET1 were used as negative and positive controls, respectively. The positions of the conserved residues in OsSWEET11 are shown in the left panel. The yeast cells were grown at 28 °C for 3 days.
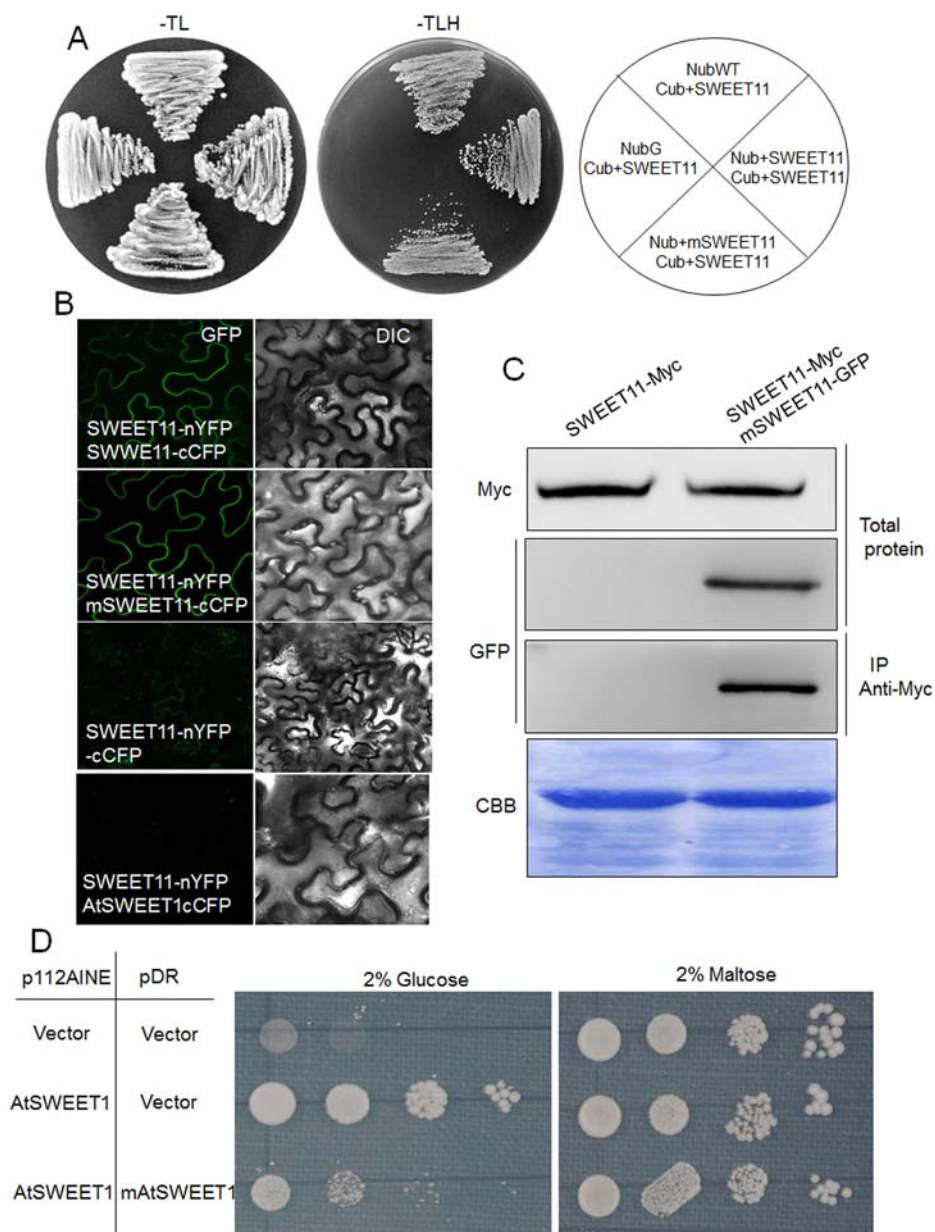
Fig. 4 Assessment of the interaction between wild-type (WT) and mutated OsSWEET11 and inhibition of glucose transport by co-expression of WT and mutant AtSWEET1 proteins in yeast EBY4000. (A) Interaction between OsSWEET11 and mOsSWEET11 was analysed using a split-ubiquitin yeast two-hybrid system. mSWEET11 was cloned into the Nub vector, whereas OsSWEET11 was cloned into the Cub (C-terminal ubiquitin domain driven by methionine-repressible MET25 promoter and fused to the artificial PLV transcription factor) or Nub vector. NubWT (Nub) and NubG (N-terminal ubiquitin domain carrying a glycine mutation) were used as positive and negative controls, respectively. (B) Observation of the yellow fluorescent protein (YFP) fluorescence. Reconstitution of the YFP fluorescence from OsSWEET11-nYFP + OsSWEET11-cCFP or OsSWEET11-nYFP + mOsSWEET11-cCFP (left, fluorescence channel; right, bright field). Co-expression of OsSWEET11-nYFP + cCFP or OsSWEET11-nYFP + AtSWEET1-cCFP was used as negative control. Bars, 20 μm. (C) Co-immunoprecipitation (Co-IP) was performed to analyse the interaction between OsSWEET11 and mOsSWEET11 in tobacco leaves. OsSWEET11-Myc or OsSWEET11-Myc + mOsSWEET11-GFP was transformed into tobacco leaves using *Agrobacterium*-mediated transformation. The total protein and Myc antibody-immunoprecipitated proteins were analysed using Western blot analysis with either Myc or green fluorescent protein (GFP) antibodies. CBB (Coomassie Brilliant Blue) staining was used as a loading control. (D) The p112AINE empty vector or p112AINE-SWEET1 construct was co-transformed with pDRf1 empty vector or pDR-mAtSWEET1 construct into the yeast strain, EBY4000. Growth assays of yeast cells co-expressing WT and mutant AtSWEET1 protein, mAtSWEET1, were performed on YNB (Yeast Nitrogen Base without Amino Acids) medium containing 2% glucose or maltose. The yeast cells were grown at 28 °C for 3 days.
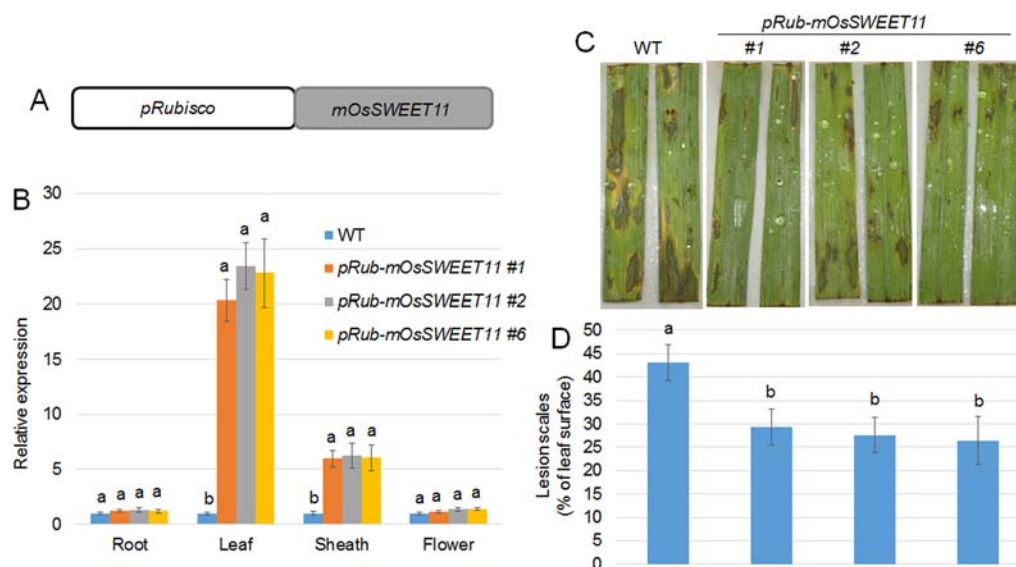
Fig. 5 Expression levels of OsSWEET11 and disease symptoms in the transgenic plants expressing mOsSWEET11 under the control of the *Rubisco* promoter. (A) Schematic diagram showing the construct used for the generation of transgenic plants. The *Rubisco* promoter was used to drive mOsSWEET11, a mutated form of OsSWEET11. (B) The expression levels of OsSWEET11 were analysed in the roots, leaves, sheaths and flowers of wild-type and three independent transgenic plants (pRub-mOsSWEET11 *#1*, *#2* and *#6*) by quantitative real-time polymerase chain reaction (qRT-PCR). The experiments were repeated three times. Significant differences at $P < 0.05$ are indicated by different letters. (C) The leaves of wild-type and three independent transgenic plants (*pRub-mOsSWEET11 #1*, *#2* and *#6*) were infected with *Rhizoctonia solani* AG1-1A and photographed after 3 days of infection. Six leaves from each line were analysed, and the experiments were repeated three times. (D) The lesion scales were analysed for the *R. solani* AG1-1A-infected leaves shown in (C) by determination of the lesion area on the leaf surface. Data represent means $\pm$ standard error (SE) ($n > 10$). Significant differences at $P < 0.05$ are indicated by different letters.

of the cyan fluorescent protein (CFP) sequence was C-terminally fused to mSWEET11. The interaction of the fusion proteins transiently co-expressed in *Nicotiana benthamiana* was checked by observing fluorescence using a confocal microscope. The fusion proteins were found to be localized to the plasma membrane (Fig. 4B). The interaction between OsSWEET11 and mSWEET11 was confirmed by performing a co-immunoprecipitation (Co-IP) assay in a tobacco transient expression system. OsSWEET11-Myc alone or OsSWEET11-Myc together with mSWEET11-GFP was expressed in *N. benthamiana* leaves, and the expression of OsSWEET11-Myc and mSWEET11-GFP was detected using Western blot analysis with anti-Myc or anti-GFP antibodies. Before IP, OsSWEET11-Myc and mSWEET11-GFP were successfully expressed in tobacco leaves, and anti-Myc antibody was used for IP. After IP, the anti-GFP antibody was used to detect the presence of mSWEET11-GFP (Fig. 4C). The results indicated that OsSWEET11-Myc interacted with mSWEET11-GFP. Next, we inferred whether mSWEET11 can affect OsSWEET11 by co-expression of mutated AtSWEET1 (mAtSWEET1; mutation at P23, G76 and P162 which correspond to the residues in mSWEET11) and AtSWEET1 in the EBY4000 yeast strain. AtSWEET1 was driven by a strong ADH promoter, whereas mAtSWEET1 was driven by a strong PMA1 promoter. The results indicated that OsSWEET11 interacts with mSWEET11, and mAtSWEET1 significantly inhibits the glucose transport activity of AtSWEET1 (Fig. 4D).

Expression of *mSWEET11*, driven by the *Rubisco* promoter, in rice mitigates the lesions of sheath blight disease

The photosynthetic production of sugar occurs in mesophyll cells. Rubisco is the major protein expressed during the photosynthesis process; therefore, a 2.0-kb fragment of the *Rubisco* small subunit promoter was used to express *mSWEET11* (Fig. 5A). More than 10 individual transgenic plants were produced. The tissue-specific expression of *OsSWEET11* was analysed in the roots, leaves, sheaths and flowers of wild-type and three independent *pRubisco-mSWEET11* lines (#1, #2 and #6). The results showed that *OsSWEET11* was highly expressed in the leaves and sheaths of the three *pRubisco-mSWEET11* lines, but exhibited similar levels in the roots and flowers of wild-type and *pRubisco-mSWEET11* lines (Fig. 5B). The expression of *OsSWEET11* was around 20–23-fold higher in the leaves, whereas it was around six-fold higher in the sheaths of *pRubisco-mSWEET11* lines compared with the expression in the corresponding tissues of the wild-type plants (Fig. 5B). We also analysed the response of *pRubisco-mSWEET11* lines to *R. solani* AG1-1A infection. After 72 h of infection, the manifestations of the infection on leaves were photographed (Fig. 5C). The results indicated that the lesion area was around 42% in wild-type plants, whereas it was around 25.5–29% in the leaves of *pRubisco-mSWEET11* plants (Fig. 5D). These results indicated
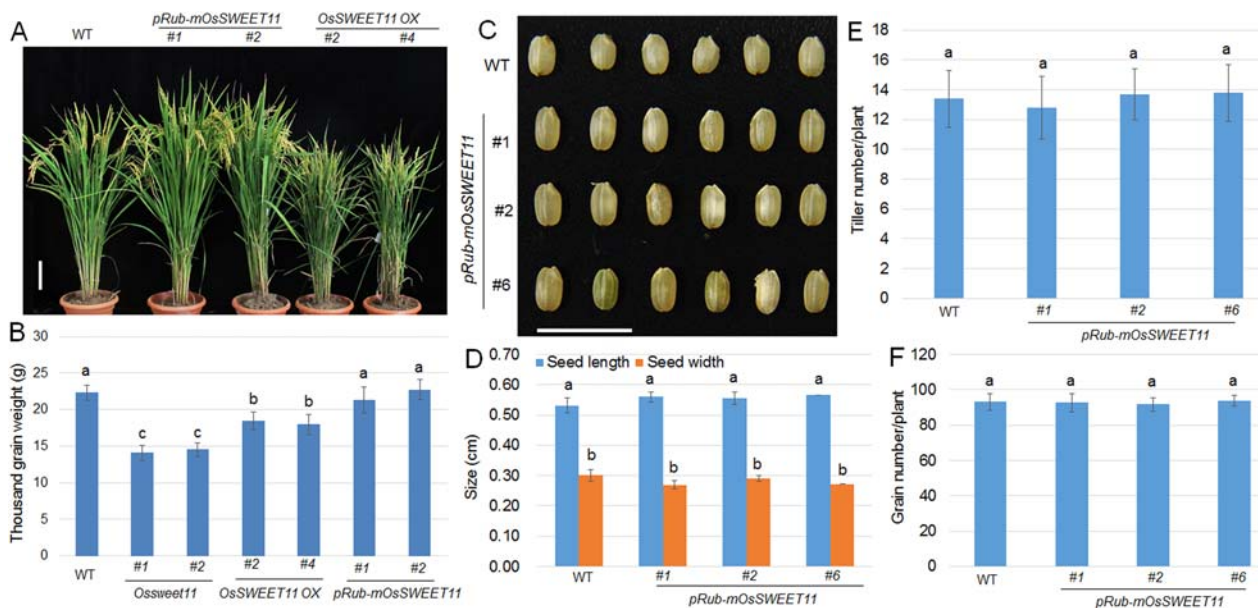
Fig. 6 Morphology and grain weight of wild-type plants, *OsSWEET11* mutants, overexpressors and *pRub-mOsSWEET11* transgenic plants. (A) Four-month-old wild-type plants, *OsSWEET11* overexpressors and *pRub-mOsSWEET11* transgenic plants were photographed. Scale bar, 10 cm. (B) The 1000-grain weight was analysed using the seeds from the different plants. (C) Seeds harvested from wild-type and *pRub-mOsSWEET11* transgenic plants were photographed. Scale bar, 1 cm. (D) The seed length and width in wild-type and *pRub-mOsSWEET11* transgenic plants were analysed. More than 100 seeds were analysed. (E) The tiller number in wild-type and *pRub-mOsSWEET11* transgenic plants was analysed. More than 20 plants were analysed. (F) The grain number in wild-type and *pRub-mOsSWEET11* transgenic plants was analysed. More than 20 plants were analysed. Significant differences at $P < 0.05$ are indicated by different letters.

that *mSWEET11* was highly expressed in the leaves and sheaths of *pRubisco-mSWEET11* transgenic plants, and that the transgenic plants were less susceptible to *R. solani* AG1-1A infection.

Furthermore, the response of *pRubisco-mSWEET11* plants to PXO99[A]-mediated bacterial blight disease was assessed. The leaves of 2-month-old wild-type and *pRubisco-mSWEET11* plants (#*1* and #*2*) were inoculated with PXO99[A]; the lesion lengths in the wild-type plants were around 11 cm, whereas those in the #*1* and #*2* *pRubisco-mSWEET11* plants were around 16 and 17 cm, respectively (Fig. S6, see Supporting Information), indicating that *pRubisco-mSWEET11* plants were susceptible to bacterial blight disease caused by strain PXO99[A]. In addition, *Magnaporthe oryzae* Guy11 was inoculated into *pRubisco-mSWEET11* plants. The *pRubisco-mSWEET11* plants exhibited wild-type-like response to *M. oryzae* (Fig. S7, see Supporting Information).

Transgenic rice plants expressing *mSWEET11* under the control of the *Rubisco* promoter maintain normal yield

The *Ossweet11* mutants were resistant to *R. solani* AG1-1A (Fig. 2), but the grain filling of mutant plants was affected (Fig. S4) (Ma *et al.*, 2017). In the *pRubisco-mSWEET11* transgenic plants, the sugar efflux activity of *OsSWEET11* might be specifically inhibited in the mesophyll cells, and thus provide resistance to *R. solani* AG1-1A when challenged by infection. Therefore, the plant morphology was analysed. The OX lines exhibited a severe dwarf phenotype, whereas the *pRubisco-mSWEET11* plants were similar to the wild-type plants (Fig. 6A). Furthermore, we compared the 1000-grain weight of wild-type, *OsSWEET11* mutant, OX and *pRubisco-mSWEET11* plants. The results showed that *OsSWEET11* mutants had the lowest grain weight, and this value was higher for the OX lines than for the mutants, but was lower than that of the wild-type and *pRubisco-mSWEET11* plants. The *pRubisco-mSWEET11* plants developed normal grains, which were similar to those of the wild-type plants (Fig. 6B). In addition, the seed size, tiller number and grain number of *pRubisco-mSWEET11* plants were similar to those of the wild-type plants (Fig. 6C–F).

DISCUSSION

Rice sheath blight, bacterial blight and blast disease are the three major diseases of rice, which severely affect yields. The defence mechanism of rice against *R. solani* is not yet known. *OsSWEET11* is known to be a target of the type III effector secreted from *Xoo* PXO99[A]. It can be highly induced on PXO99[A] infection and has been shown to be negatively related to resistance to bacterial blight (Yang *et al.*, 2006). In this study, we found that *OsSWEET11* was induced in rice infected with *R. solani* AG1-1A, a major causative agent of rice sheath blight disease. However, other clade III *OsSWEET* genes (*OsSWEET12*–*OsSWEET15*) were not altered by infection with *R. solani* AG1-1A. The transcript levels of the gene

and *pOsSWEET11::GUS* analysis consistently revealed that *R. solani* AG1-1A infection significantly induced the expression of *OsSWEET11* in the leaves and sheaths of rice, suggesting a specific role of *OsSWEET11* in response to sheath blight disease. Based on these results, we conducted further genetic studies using the CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated)-based knock-out mutants and transgenic plants in which the ubiquitin promoter was used to drive the overexpression of *OsSWEET11*. In the *OsSWEET11* OX lines, the transcript levels of *OsSWEET11* were considerably higher than those in the wild-type control. The pathogen infection experiments showed that the *OsSWEET11* knock-out mutants were less susceptible, whereas the *OsSWEET11* overexpressing plants were more susceptible, to sheath blight disease compared with the wild-type plants, suggesting that *OsSWEET11* is a gene responsible for the susceptibility of rice to sheath blight disease.

Ossweet11 mutants were resistant to both sheath blight and bacterial blight disease, and thus *OsSWEET11* may be a potential target for the breeding of resistant rice. However, the *OsSWEET11* mutants exhibited severe defects in grain filling and seed quality (Ma *et al.*, 2017). Previously, TAL effectors of *Xoo* have been used to generate TALENs for editing the *OsSWEET14/Os11N3* promoter region, to which the type III effector binds. The modified plants showed remarkable resistance to bacterial blight disease without causing any change in the normal expression of *OsSWEET14* (Li *et al.*, 2012). In a previous study, we identified that the co-expression of AtSWEET1 with the non-functional mutant form of AtSWEET1 significantly inhibited the glucose transport activity in the yeast EBY4000 strain (Xuan *et al.*, 2013). *OsSWEET11* can be induced by *R. solani* AG1-1A. Therefore, we speculated that the specific expression of the mutated form of OsSWEET11 in certain tissues or cell types could inhibit sucrose transport in the target cells in which *R. solani* AG1-1A hijacks sugar, and without affecting the grain filling process. Before testing this, we selected conserved residues (P23A, G76D, P162A, P191T and Q202D) in AtSWEET1, OsSWEET2b, OsSWEET11 and their homologues from other plants via sequence alignment and evaluated their importance in the glucose transport activity of AtSWEET1, because OsSWEET11 failed to transport sucrose in the SUSY7 yeast strain (Fig. S5). It might be that OsSWEET11 does not localize at the plasma membrane or that OsSWEET11 sucrose transport affinity is much lower than that of AtSUC2. The results showed that the mutation of any one of the five residues abolished the glucose transport activity of AtSWEET1 in the EBY4000 yeast strain. Furthermore, transgenic rice plants expressing mutated OsSWEET11 were generated, with mutations at three positions, P31, G82 and P169; these are conserved positions corresponding to P23, G76D and P162A of AtSWEET1, respectively. Further, split-ubiquitin-based yeast two-hybrid, split GFP and Co-IP assays showed that OsSWEET11 interacts with mSWEET11 in yeast and plants, and

their interaction was observed in the plasma membrane of plant cells. In addition, the co-expression of AtSWEET1 with mutated AtSWEET1 (with mutations at P23, G76D and P162) significantly inhibited the glucose transport activity of AtSWEET1. Our results suggest that the expression of mSWEET11 could very likely inhibit the normal function of OsSWEET11. It would be interesting to perform further experiments to test the activity of OsSWEET11 sucrose transport activity, as well as the inhibition of OsSWEET11, by the co-expression of mSWEET11 in other possible systems.

Mesophyll cells are known to be the main sites of photosynthesis, which leads to the production of sugar. The Rubisco protein is mainly expressed in mesophyll cells. Therefore, the *Rubisco* small subunit promoter was used to express mSWEET11 to determine whether the inhibition of OsSWEET11 function in mesophyll cells can improve the resistance of rice to sheath blight disease, because leaves of rice are vulnerable to this pathogen. Unlike in wild-type plants, the expression level of *OsSWEET11* was higher in the leaves and sheaths, but was similar in the roots and flowers, in *pRub-mSWEET11* plants. In addition, *pRub-mSWEET11* plants exhibited resistance to sheath blight disease, suggesting that the sugar efflux activity of intrinsic *OsSWEET11*, induced by *R. solani* AG1-1A, was counteracted by mSWEET11 expressed in the mesophyll. However, the grain weight of *pRub-mSWEET11* plants was similar to that of wild-type plants, whereas it was affected in all the *OsSWEET11* mutants and overexpressors (Fig. 6). However, whether *OsSWEET11* induced in mesophyll cells by *R. solani* AG1-1A or *Rubisco* promoter activity is restricted to mesophyll cells needs to be evaluated further. *pRub-mSWEET11* plants exhibited susceptibility to bacterial blight disease caused by *Xoo* PXO99[A], suggesting that mSWEET11 expression does not interfere with *Xoo* susceptibility. PXO99[A] strictly relies on the transactivation of *OsSWEET11* by its major TALE PthXo1. PXO99[A] does not transactivate other *SWEET*s which could complement the loss of *OsSWEET11* transactivation (Yang *et al.*, 2006). It could also be induced in the vasculature in which *X. oryzae* is prevalent, or mSWEET11 expression under the *Rubisco* promoter may activate other SWEETs, which may efflux sugar to *X. oryzae*. Nevertheless, *atsweet2* mutants were more susceptible to *Pythium* infection (Chen HY *et al.*, 2015), suggesting a diverse role of SWEETs in the disease response. Expressing of *mSWEET11* under control of the vasculature specific promoter may provide resistant lines against PXO99[A]. *pRub-mSWEET11* plants exhibited a similar response to *M. oryzea* Guy11, suggesting that this mutation is not associated with rice blast disease. Not surprisingly, the plants overexpressing *OsSWEET11* were susceptible to PXO99[A]. To determine whether *OsSWEET11* expression is tightly connected with defence, the *PBZ1* level was analysed in the *OsSWEET11* mutant, overexpressors and *pRubisco-mSWEET11* plants. The data showed that the expression level of *PBZ1* was similar in these lines, indicating that mutation and higher expression of *OsSWEET11* did not alter plant defence in non-inoculated plants (Fig. S8, see Supporting Information).
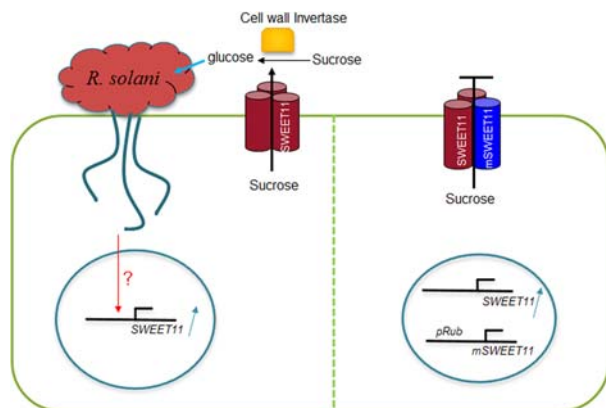
Fig. 7 Schematic representation of the hypothesized function of OsSWEET11 during *Rhizoctonia solani* infection. *Rhizoctonia solani* infection activates the expression of OsSWEET11 via an unknown mechanism to efflux sucrose to the apoplasm, and cell wall invertase catabolizes sucrose to glucose and fructose. Further, glucose can be taken up into *R. solani* cells. The expression of mutated OsSWEET11 via the *Rubisco* promoter may generate a non-functional OsSWEET11 complex. The wild-type (WT) OsSWEET11 induced by *R. solani* may form heterotrimers consisting of WT OsSWEET11 and mutated OsSWEET11, which may be expressed under the *Rubisco* promoter, and the non-functional OsSWEET11 complex may inhibit sugar efflux.

Rhizoctonia solani AG1-1A activates *OsSWEET11* via an unknown mechanism to efflux sugar from the cytosol to the apoplast for its own use. This is a typical strategy of pathogens in microbe–plant interactions. Given the importance of sugar acquisition from the host plant to the pathogen, microbes other than *R. solani* AG1-1A might also take advantage of the sugar efflux transport activity of OsSWEET11, or other SWEETs in rice. Therefore, SWEETs, as sugar efflux transporters, play critical roles during microbe–plant interactions. In this study, the expression of *OsSWEET11* was activated by *Rhizoctonia solani* to efflux sucrose to the apoplasm, which may be further hydrolysed to glucose via cell wall invertase, and taken up by *R. solani* (Fig. 7). More importantly, seed set and grain filling in rice were not significantly affected by this inhibition, and the SWEET11 level was similar in wild-type and *pRubisco-mSWEET11* plants in flower tissue, indicating that the *Rubisco* promoter is not active in flower tissue. Taken together, our findings suggest that the sugar transporter, OsSWEET11, plays an important role in the infection of sheath blight disease, and that manipulation of the expression of susceptible genes may be an efficient way to protect plants from the attack of pathogens without affecting their yield.

EXPERIMENTAL PROCEDURES

Plant growth and pathogen inoculation

Rice plants were grown under glasshouse conditions at Shenyang Agricultural University, Shenyang, China. The daily high and low temperatures of the glasshouse were typically 30 and 23 °C, respectively. The plants were propagated by selfing.

For inoculation of the pathogen *R. solani* AGI-1A, rice plants (Nipponbare) were grown in the glasshouse for 1 month. The second youngest leaf from the main tiller was cut into a 10-cm piece, placed on moistened filter paper and kept in a Petri dish (36 cm × 36 cm × 2.5 cm). For each entry, four leaves were used as one replicate with four replications per entry in a completely randomized design. Colonized potato dextrose agar (PDA) medium (diameter, 7 mm) was excised using a circular cutter from the PDA plate and placed on the abaxial surface of each leaf piece. The leaves were incubated in a chamber with continuous light at 25 °C for 72 h. The filter paper was kept moist with sterile water. After 72 h, the lesion length on each cut leaf piece was measured, and each leaf was visually rated on a scale of 0–9, with '0' for the absence of lesion and '9' for the lesion that covered 90%–100% of the leaf surface. Visual scores of 1–8 represented 10%–80% diseased leaf area (Prasad and Eizenga, 2008).

For PXO99[A] infection, the PXO99[A] strain (Hopkins *et al.*, 1992) was cultured on solid peptone sucrose agar (PSA) medium at 28 °C for 3 days (Yu *et al.*, 2011). After full growth, the bacteria were suspended in distilled (dH$_2$O) to an optical density (OD) of 1.0. The leaves were infected with bacteria after cutting them 1 cm away from the tip using a pair of scissors. For the PXO99[A] infection assay, 2-month-old plants were used, and the lesion length was measured after 2 weeks of infection.

The *M. oryzae* isolate Guy11 was cultivated on oatmeal medium under weak light for 2 weeks to generate spores. For spray inoculation, 3-week-old seedlings were sprayed with 1×10^5/mL spores of *M. oryzae*, as described previously (Ning *et al.*, 2015). The number of typical susceptible lesions in each seedling was counted to evaluate the infection level.

Gene expression constructs and generation of transgenic plants

For the generation of the gene expression construct, a 2.0-kb fragment from the promoter region of *Rubisco* ribulose bisphosphate carboxylase small chain (Os12g17600) was amplified using rice Nipponbare genomic DNA with a primer pair: Rubisco F and R. The polymerase chain reaction (PCR) product was cloned into the pEASY-Blunt vector (Transgen Biotech, Beijing, China). The plasmid containing the promoter sequence and a shuttle vector, pTCK303 (Du *et al.*, 2016), were digested with *Hind* III and *Spe* I, and the promoter fragment was integrated into the pTCK303 vector.

The SWEET mutant plasmids were prepared using overlap extension PCR. Fragments containing three point mutations, *OsSWEET11M-1* (P31T: CCA-ACA), *OsSWEET11M-2* (G82D: GGC-GAC) and *OsSWEET11M-3* (P169T: CCG-ACG), based on the *OsSWEET11* cDNA sequence (XM_015792937), were amplified using the overlap extension PCR method. The PCR products were cloned and sequenced. The sequences of PCR primers are listed in Table S1 (see Supporting Information). The mutated *OsSWEET11* coding sequences were amplified from the plasmids containing the point mutations using the primer pair, 11_303F and 11_303 R, which introduced *Spe* I and *Sac* I sites into the amplified fragments. The PCR products were cloned into the *Rubisco* promoter-containing plasmid at the *Spe* I and *Sac* I sites. The cloning was confirmed by sequencing before the recombinant plasmid was introduced into

Agrobacterium tumefaciens EHA-105 for transformation of rice callus, as described earlier (Hiei *et al.*, 1994).

The *OsSWEET11* OX construct was generated by amplifying the coding sequence of *OsSWEET11* using the primer pair, 11 F and 11 R, and rice *Nipponbare* cDNA as the template. The PCR product was integrated into the pEASY-blunt vector and sequenced. The plasmid was digested with *BamH* I and *Sac* I. The fragment containing the coding sequence of *OsSWEET11* was integrated into pTCK303. The plasmid was confirmed by sequencing before it was introduced into *A. tumefaciens* EHA-105 for transformation of rice callus.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated using an RNeasy Plant Mini Kit (QIAGEN, Duesseldorf, Germany) or TRIzol reagent (Takara, Dalian, Liaoning, China), and was treated with RQ-RNase free DNase (Promega, Madison, WI, USA) for removal of genomic DNA contamination. For cDNA synthesis, reverse transcriptase RNaseH (Toyobo, http://www.toyobo-global.com/) or a GoScript Reverse Transcription Kit was used, according to the manufacturer's instructions (Promega). The products obtained in qRT-PCR were quantified using Illumina Research Quantity software, Illumina Eco 3.0 (Illumina, San Diego, CA, USA), and the values were normalized against the *Ubiquitin* levels in the same samples. The primers used for qRT-PCR are listed in Table S1.

Retrieval of SWEET homologue sequences and their alignment

The sequences of AtSWEET1, OsSWEET2b, OsSWEET11 and OsSWEET11 homologues from different model plants (Table S2, see Supporting Information) were retrieved from the UniProt database (http://www.uniprot.org/). These sequences were analysed by multiple sequence alignment using the CLUSTALW (version 2) program (Larkin *et al.*, 2007).

Assay of sugar transport activity of mutated AtSWEET1 in yeast

The yeast hexose transporter mutant strain, EBY4000 [hxt1–17D::loxP-gal2D::loxP stl1D::loxP agt1D::loxP ydl247wD::loxP yjr160cD::loxP], was used to test the hexose transport activity (Wieczorke *et al.*, 1999). Wild-type and mutant AtSWEET1 were expressed in EBY4000 using the pDRf1-GW vector. The transformants were selected on solid SD–Ura(Synthetic Dropout Medium–Uracil) with 2% maltose as the carbon source at 28 °C for 3 days. Subsequently, the growth of yeast cells on SD medium containing 2% glucose or maltose was monitored. AtSUC2 and OsSWEET11 were expressed in the SUSY7 yeast strain using the pDRf1-GW vector to test sucrose transport activity.

Mating-based split-ubiquitin system

For mating-based split-ubiquitin assays, *OsSWEET11* and mutated *OsSWEET11* (*mSWEET11*) ORFs were cloned into the mating-based split-ubiquitin Nub vectors, pXN22_GW and pXN25_GW, and the Cub vector, pMETYC_GW. The assays were performed as described previously (Lalonde *et al.*, 2010).

Split GFP assay

The nYFP and cCFP sequences were fused to the C-terminal sequences of OsSWEET11 and mutated OsSWEET11 (mSWEET11) in pXNGW and pXCGW vectors, respectively (Xuan *et al.*, 2013). The fusion proteins were introduced into *N. benthamiana* leaves using the *Agrobacterium*-mediated transient expression method (Kim *et al.*, 2009a). The interactions of the co-expressed proteins were monitored by detection of YFP fluorescence under a confocal microscope (SP5; Leica, Solms, Germany). All the constructs were verified by DNA sequencing.

Co-IP and Western blot analyses

OsSWEET11-Myc or mSWEET11-GFP was co-transformed with OsSWEET11-Myc in *N. benthamiana* leaves by *Agrobacterium*-mediated transformation for transient expression. The expressed proteins were extracted and Co-IP assays were performed, as described previously (Kim *et al.*, 2009b). The microsomal fraction was prepared by centrifugation at 20 000 and 100 000 *g* for 1 h. The resulting pellet was resuspended in extraction buffer containing 1% Triton X-100. After centrifugation at 100 000 *g* for 10 min, the solubilized proteins were incubated with anti-Myc antibody bound to protein A beads for 2 h. The beads were washed four times with extraction buffer containing 0.1% Triton X-100 and eluted with sodium dodecylsulfate (SDS) sample buffer.

From each sample, 20 μg of protein was separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto Immobilon-P transfer membrane (MILLIPORE JAPAN, Tokyo, Japan). The membranes were incubated in TBS (tris buffered saline) containing 5% skimmed milk and 0.05% Tween-20 for 60 min and blotted with primary antibodies at 4 °C for 2 h. The anti-Myc (1 : 2000; Abcam, Cambridge, MA, USA) and anti-GFP (1 : 2000; Abcam) antibodies were used as primary antibodies. The membranes were incubated for 1 h with an anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 2000; Cell Signaling Technology Boston, MA, USA). The blots were visualized by chemiluminescence using an ECL Western Blotting Detection System (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

Statistical calculations were performed using Prism 5 (GraphPad, San Diego, CA, USA). All the data are expressed as means $\pm$ standard error (SE). Comparisons between different groups were performed using one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test. $P < 0.05$ was considered to be statistically significant.

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SUPPORTING INFORMATION

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

Table S1 Primer sequences used in this study.
Table S2 Information on SWEET11 homology.

Fig. S1 Expression of *OsSWEET12–OsSWEET15* genes induced by *Rhizoctonia solani* infection. The expression levels of *OsSWEET*s were analysed after 0, 24, 48 and 72 h of *R. solani* infection. Ten leaves were sampled at each time point for RNA extraction. The experiments were repeated three times, and the data represent means ± standard error (SE) ($n = 3$). Significant differences at $P < 0.05$ are indicated by different letters.

Fig. S2 Expression level of *OsSWEET11* in the rice overexpression lines. The expression level of *OsSWEET11* was analysed using RNA extracted from the leaves of 2-week-old wild-type and OsSWEET11 overexpression lines (*OsSWEET11 OX1, OX2, OX4* and *OX5*). The expression level in the wild-type plant was defined as '1', and the relative expression levels are shown. Significant differences at $P < 0.05$ are indicated by different letters.

Fig. S3 Sensitivity of *OsSWEET11* overexpression (OX) plants to PXO99[A]. The leaves of 2-month-old wild-type and *OsSWEET11* OX plants (*#2* and *#4*) were inoculated with PXO99[A]. The lesion length was measured after 2 weeks of infection. Significant differences at $P < 0.05$ are indicated by different letters.

Fig. S4 The phenotypes of wild-type and *ossweet11-1* seeds. The wild-type and *ossweet11-1* mutant seeds were photographed. A comparison of wild-type and *ossweet11-3* mutant seeds shows poor filling in the latter.

Fig. S5 Sucrose transport activity test in the SUSY7 yeast strain. Growth assays of AtSUC2 and OsSWEET11 proteins expressed in the SUSY7 yeast strain were performed on YNB (Yeast Nitrogen Base without Amino Acids) medium containing 2% sucrose or glucose. Empty (pDRf1) vector and AtSUC2 were used as negative and positive controls, respectively. The yeast cells were grown at 28 °C for 3 days.

Fig. S6 Sensitivity of *pRub-mOsSWEET11* plants to PXO99[A] infection. The leaves of 2-month-old wild-type and *pRub-mOsSWEET11* plants (*#1* and *#2*) were inoculated with PXO99[A]. The lesion length was measured after 2 weeks of infection. Significant differences at $P < 0.05$ are indicated by different letters.

Fig. S7 Disease symptoms in plants expressing *OsSWEET11* mutants on infection with *Magnaporthe oryzae*. (A) The leaves of wild-type and two independent transgenic plants (*pRub-mOsSWEET11 #1* and *#2*) were infected with *M. oryzae* Guy11. After 3 days of infection, the leaves were photographed. Ten leaves from each line were analysed, and the experiments were repeated three times. (B) The lesion scales were analysed for the *M. oryzae* Guy11-infected leaves shown in (A) by determination of the lesion area on the leaf surface. The data represent means ± standard error (SE) ($n > 10$). Significant differences at $P < 0.05$ are indicated by different letters.

Fig. S8 Expression levels of *PBZ1* in plants expressing *OsSWEET11* mutants. The levels of *PBZ1* were analysed in the leaves of wild-type plants (WT), *Ossweet11* mutants, *OsSWEET11* overexpressors and *pRub-mOsSWEET11* lines. More than six leaves from each line were used for RNA extraction. Data represent the means ± standard error (SE) ($n = 3$), and the experiments were repeated at least three times. Significant differences at $P < 0.05$ are indicated by different letters.