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# Four sugarcane *ScDIR* genes contribute to lignin biosynthesis and disease resistance to *Sporisorium scitamineum*

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## Abstract

Sugarcane (*Saccharum* spp.) is a major sucrose and bioenergy crop in the world. The fungal pathogen *Sporisorium scitamineum* causes sugarcane smut, a devastating disease that destroys stalks and reduces sugar content in sugarcane. This disease can be controlled most effectively by applying smut-resistant sugarcane varieties. Previous studies have shown that *Dirigent* (*DIR*) genes are involved in the synthesis of the lignin precursor pinoresinol, which plays a crucial role in plant resistance to biotic stresses. However, the immune response of the *DIR* homologs in sugarcane (*ScDIR*) has not been reported yet. In this study, we found that the lignin content of smut-resistant sugarcane varieties (ZZ1, ZZ6, and ZZ9) was significantly higher than that of smut-susceptible varieties (GT42, ROC22, and FN41), and the lignin content of sugarcane increased after smut infection. The smut-resistant and smut-susceptible clones derived from the same genetic population (ROC25 × YZ89-7) showed similar patterns. Quantitative real-time PCR assays revealed that among the 64 *DIR* genes in sugarcane, *ScDIR5*, *ScDIR7*, *ScDIR11*, and *ScDIR40* showed elevated expression after *S. scitamineum* infection. In vitro coupling reactions showed that the four corresponding *ScDIR* proteins could mediate the coupling of coniferyl alcohol and its conversion into the lignin precursor pinoresinol. Overexpression of the four *ScDIR* genes in *Nicotiana benthamiana* enhanced disease resistance to the fungal pathogens *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Botrytis cinerea*. Moreover, transgenic sugarcane overexpressing these *ScDIR* genes showed enhanced resistance to smut disease. Taken together, our findings provide evidence that sugarcane *ScDIR* genes can improve the resistance of plants to fungal pathogens and highlight their potentials in sugarcane breeding for disease resistance.

**Keywords** Sugarcane, *Sporisorium scitamineum*, *ScDIR*, Lignin, Disease resistance

## Background

Sugarcane (*Saccharum* spp.), an annual or perennial herb plant, is an important sugar crop and bioenergy in the world, which has been widely planted in over 120 countries, especially in tropical and subtropical areas (Theerawitaya et al. 2020). However, the sugarcane industry suffers severe yield losses due to sugarcane smut disease caused by *Sporisorium scitamineum* (Waller 1970). The teliospores of the fungus germinate on the bud and internode surface of sugarcane and penetrate sugarcane buds for initial invasion. The fungus further infects

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the growing points or meristems (Santiago et al. 2012), resulting in a thin stalk, profuse tillering, small narrow leaves, and the eventual emergence of a whip (Singh et al. 2019).

Moderate heritability was found for smut resistance traits, and the narrow sense heritability ranged from 0.41 to 0.75 in sugarcane (Wu et al. 1988; Chao et al. 1990; Bhuiyan et al. 2021). Smut resistance is a multifactorial process encompassing structural, biochemical, and physiological mechanisms (de Armas et al. 2007). Compared to susceptible cultivars, resistant sugarcane cultivars have more lignified bud scales, more phenolic compounds accumulated in the epidermis, and more trichomes. Cellular defense responses to smut infection include higher pH and higher levels of phenolic compounds, antioxidant enzymes, thiol polyamines, chitinase, glucanase, and pathogenesis-related proteins (Su et al. 2013a).

During biotic and abiotic stresses, plant cells increase their lignin biosynthesis to enhance their resistance. The Dirigent (DIR) protein was first reported in *Forsythia suspensa* and then they are found to be involved in lignin biosynthesis (Davin et al. 1997; Chen et al. 2018). DIR protein guides the coupling of lignin monomers into dimers when biologically active oxidants are present (Dalisay et al. 2015), which allows the stereoselective conversion of coniferyl alcohol into pinoresinol (Li et al. 2017). It was reported that DIR protein could enhance the resistance of *Brassica rapa* to the fungal pathogens *Rhizoctonia solani* and *Leptosphaeria maculans* (Wang and Fristensky 2001).

DIR protein family includes six subclasses, the DIR-a, DIR-b/d, DIR-c, DIR-e, DIR-f, and DIR-g (Ralph et al. 2007); of which, the DIR-e and DIR-b/d subfamilies are involved in lignin biosynthesis (Wu et al. 2009) and the DIR-c subfamily is related to plant disease resistance (Kitur et al. 2010; Song et al. 2014; Weidenbach et al. 2016). In addition, *DIR* genes have been proven to function in many plants to resist biotic and abiotic stresses (Khan et al. 2018), but there is no information for their roles in responding to infections caused by *S. scitamineum* in sugarcane.

We previously found that four of 64 *DIR* genes of sugarcane (*ScDIR*) contributed to drought tolerance (Li et al. 2022). In this study, we showed that these *ScDIR* genes were also involved in smut resistance in sugarcane. We present evidence that (1) the lignin content of the buds was higher in smut-resistant varieties or clones than that in smut-susceptible ones; (2) the expression of *ScDIR* genes was upregulated in response to *S. scitamineum* infection; (3) the prokaryotically expressed *ScDIR* proteins displayed catalytic activity required to synthesize the lignin precursor pinoresinol from coniferyl alcohol; (4) overexpression of *ScDIR* genes in *Nicotiana*

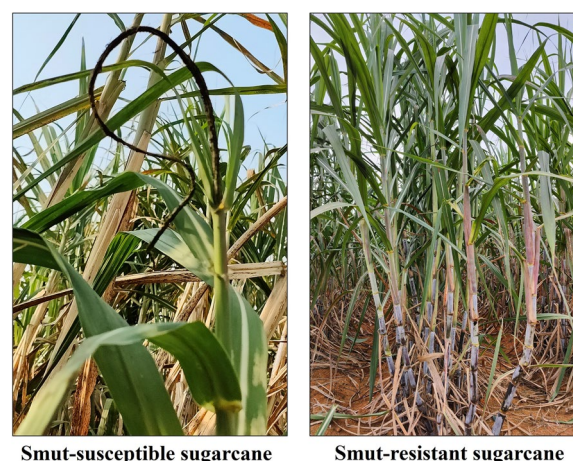
*benthamiana* enhanced disease resistance to three fungal pathogens; and (5) overexpression of *ScDIR* genes in sugarcane significantly enhanced disease resistance to *S. scitamineum* infection. These results demonstrate the feasibility of *ScDIR* genes as a potential resource for smut-resistant sugarcane breeding.

## Results

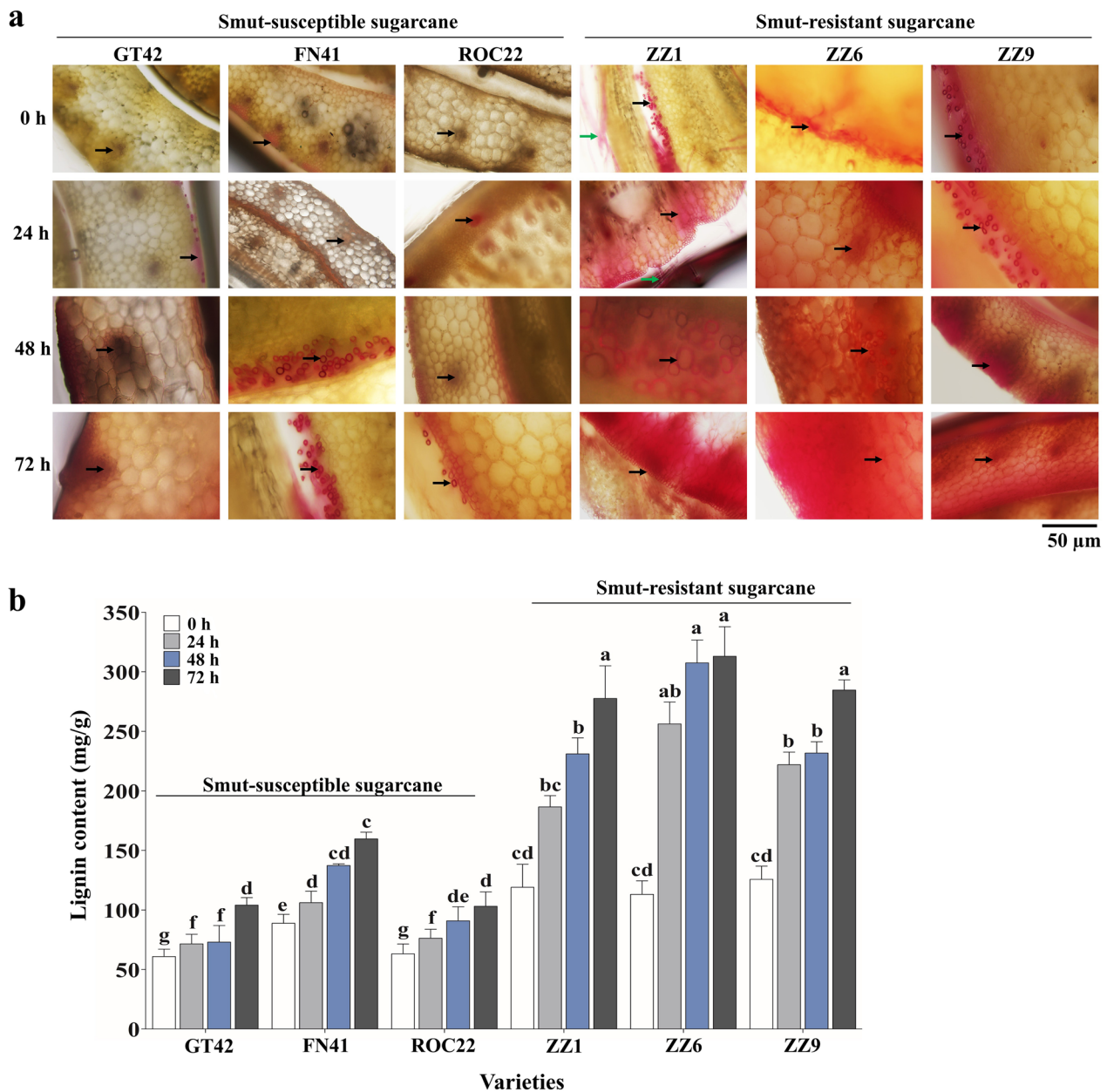
### The lignin content in sugarcane buds differs between smut-resistant and smut-susceptible sugarcane varieties

Six sugarcane varieties (smut-resistant: ZZ1, ZZ6, and ZZ9; smut-susceptible: GT42, FN41, and ROC22) were selected for this study. The natural smut incidences of six main sugarcane varieties in the second ratooning are shown in Additional file 1: Table S1. In the field, it is typical for susceptible sugarcane varieties to form black whip, while resistant varieties do not (Fig. 1). Physically similar sugarcane stems from the six sugarcane varieties were collected and cut into single-bud setts (Additional file 2: Figure S1). After inoculation with *S. scitamineum* for three months, a black whip has been observed in the smut-susceptible sugarcane GT42, whereas no visible symptoms were observed in the smut-resistant sugarcane ZZ9 (Additional file 2: Figure S2).

To investigate whether lignin contents were related to smut resistance, phloroglucinol was used to stain the cross-section of sugarcane bud in smut-resistant and smut-susceptible varieties. As shown in Fig. 2a, lignin accumulated mainly in the silicon cells of the outermost scale buds in smut-resistant plants (ZZ1, ZZ6, and ZZ9) before *S. scitamineum* infection. In contrast, among susceptible varieties (GT42, FN 41, and ROC22), only a



**Fig. 1** The field phenotypes of smut-susceptible and smut-resistant sugarcane varieties at maturity stage. Upon infection with *S. scitamineum*, susceptible sugarcane plants develop black whip-like structures



**Fig. 2** Comparison of lignin contents between smut-resistant and smut-susceptible sugarcane varieties. **a** Observation of lignin in buds of GT42, FN41, ROC22, ZZ1, ZZ6, and ZZ9 after inoculation with *S. scitamineum* at 0, 24, 48, and 72 h. The lignin is stained with red color by phloroglucinol (cross-section of the bud). Black arrows indicate lignin and green arrows indicate trichomes. Scale bars = 50 μm. **b** Quantification of lignin content in buds of different varieties. Six biological replicates were included in each sample. SPSS Software was used to analyze the lignin content of each sugarcane at different time points, and the significance is denoted above each column. Values in the same column with different letters were significantly different ( $p < 0.05$ ), calculated using one-way analysis of variance (ANOVA) followed by multiple Duncan tests

small amount of lignin was observed. At 24, 48, and 72 h post-inoculation with *S. scitamineum*, lignin mainly accumulated in silicon cells and trichomes of the plants. Meanwhile, the accumulation of lignin in smut-resistant varieties was faster than those in smut-susceptible varieties (Fig. 2a).

Similar results were obtained when a lignin detection kit was used. As shown in Fig. 2b, the lignin content of smut-resistant varieties was higher than that of susceptible varieties at all detected time points. Furthermore, smut-resistant varieties exhibited the highest lignin level at 24 h after inoculation, while the lignin accumulation

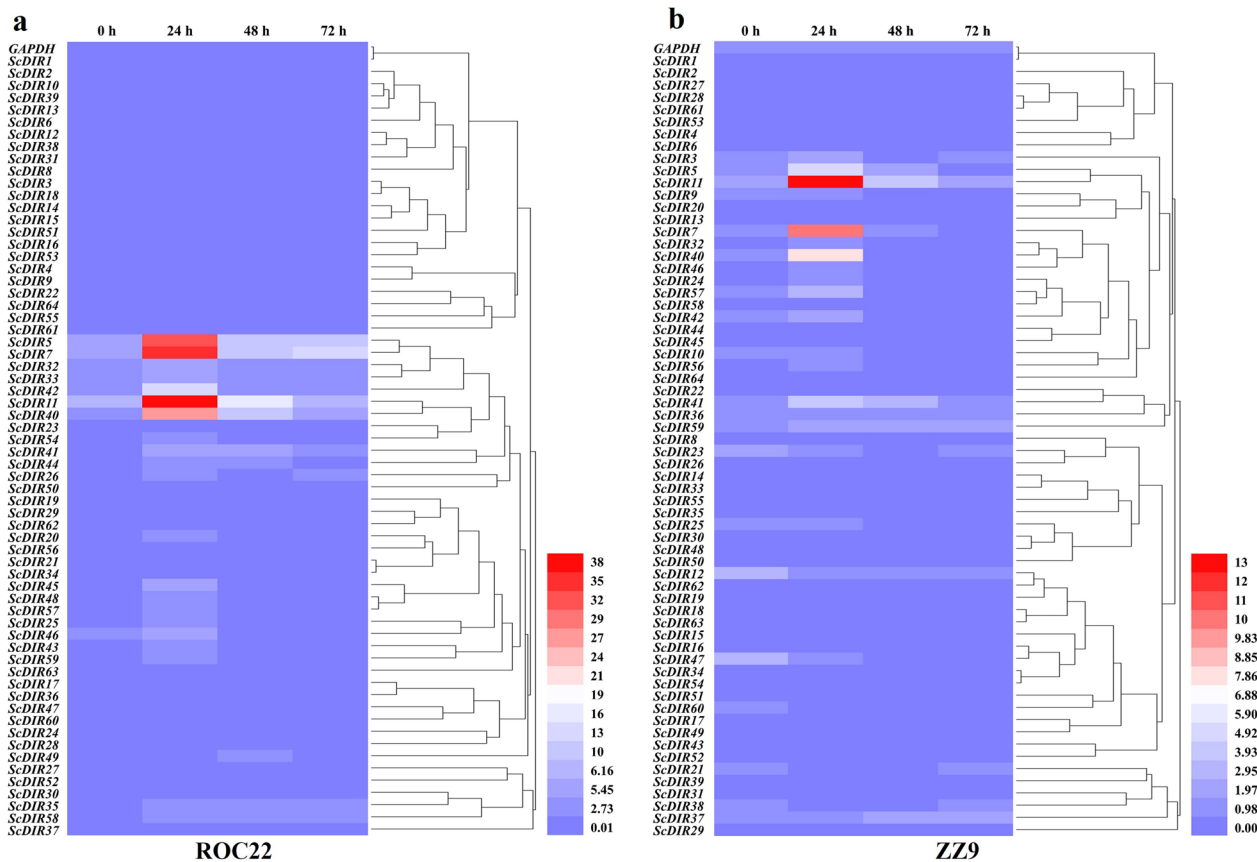
reached the peaks in susceptible varieties at 48 to 72 h post-inoculation.

To verify the above results in more sugarcane materials, we further analyzed the lignin content in five smut-resistant clones and five smut-susceptible clones that are derived from a ROC25 × YZ89-7 F1 generation. The natural smut incidences of ten clones in the second ratooning are shown in Additional file 1: Table S2. Consistent with the abovementioned results, the lignin content of the smut-resistant clones was also consistently higher than that of the smut-susceptible clones (Additional file 2: Figure S3). Based on these observations, we hypothesized that lignin accumulation is related to resistance to *S. scitamineum* infection in sugarcane.

### ScDIR genes are induced in response to *S. scitamineum* infection

Previous studies have shown that DIR proteins are differentially expressed under stress conditions and are located at the plant cell wall (Kim et al. 2012). Thus,

we investigated whether the *ScDIR* genes of sugarcane were also induced during *S. scitamineum* infection. The expression patterns of 64 *ScDIR* genes in the buds of sugarcane ROC22 and ZZ9 were analyzed after *S. scitamineum* inoculation. As shown in Fig. 3a and Additional file 1: Table S3, the expression of 13 *ScDIR* genes derived from ROC22 increased by over two folds during the first 24 h of infection. There was a 4.5-fold increase at the transcript levels for *ScDIR5*, *ScDIR7*, *ScDIR11*, and *ScDIR40* after infection. After 48–72 h of inoculation, the expression of six *ScDIR* genes, including *ScDIR5*, *ScDIR7*, *ScDIR11*, and *ScDIR40*, was still twice folds more than that before inoculation. Furthermore, hierarchical cluster analysis for 64 *ScDIR* genes showed that nine *ScDIR* genes with the highest change of gene expression abundance were clustered at the same level. Because *ScDIR5*, *ScDIR7*, *ScDIR32*, *ScDIR33*, and *ScDIR42* exhibited similar trends of gene expression after inoculation, they were clustered at the same level. Consistent with these findings, 12



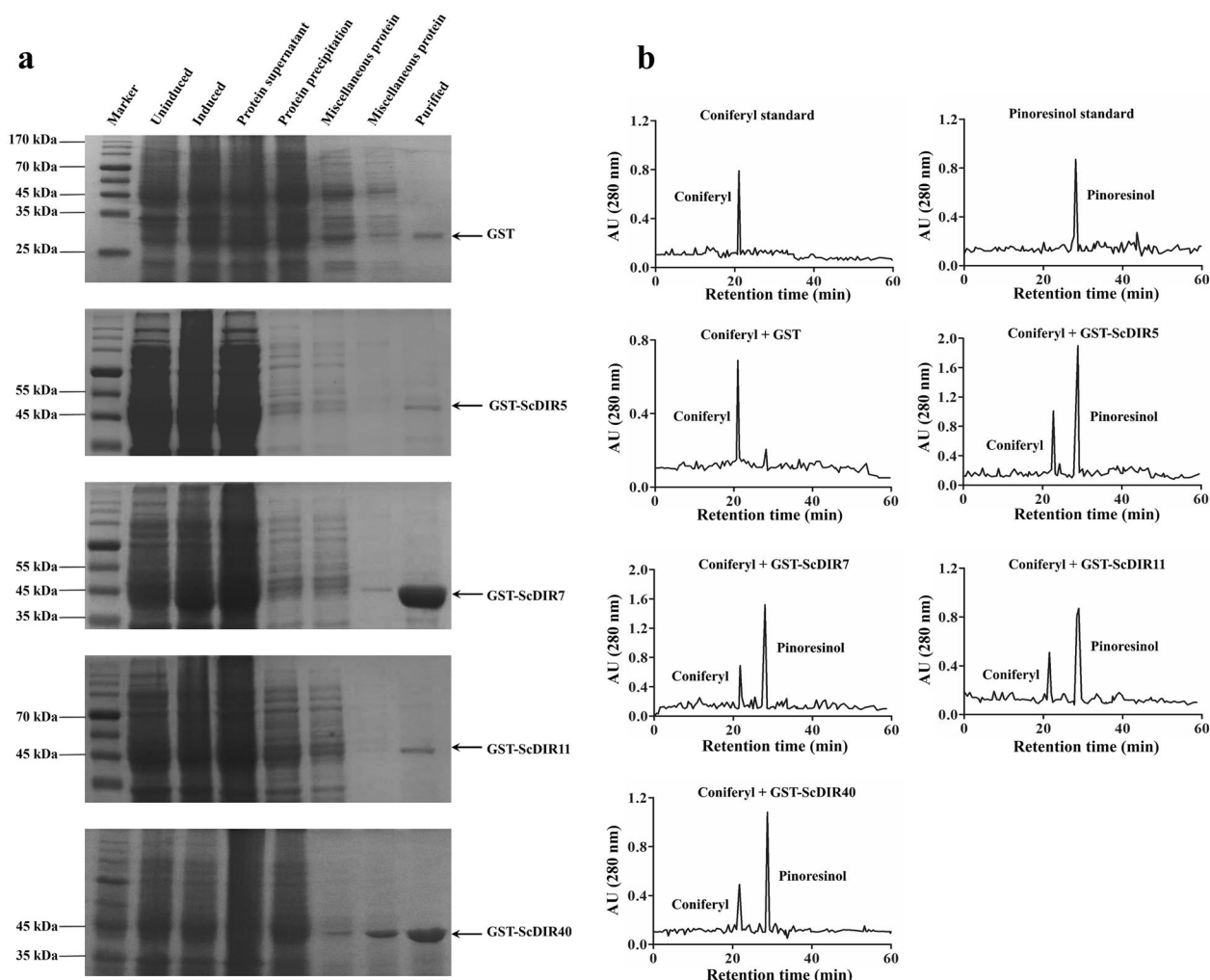
**Fig. 3** Determination of the relative expression of *ScDIR* genes after *S. scitamineum* inoculation. **a** The relative expression abundance of 64 *ScDIR* genes was measured in ROC22 by qRT-PCR after 0, 24, 48, and 72 h of *S. scitamineum* infection. **b** The relative expression abundance of 64 *ScDIR* genes was measured in ZZ9 by qRT-PCR after 0, 24, 48, and 72 h of *S. scitamineum* infection. The relative expression pattern of the gene was generated by Heml 1.0.3.7 software, and hierarchical cluster analysis was carried out. The color code indicates the relative expression. *GAPDH* was used as an internal reference gene. Three biological replications were conducted ( $n = 3$ )



*ScDIR* genes derived from ZZ9 exhibited over two-fold increase in expression at 24 h post-inoculation. The expression of *ScDIR5*, *ScDIR7*, *ScDIR11*, and *ScDIR40* increased significantly (Fig. 3b and Additional file 1: Table S4). The phylogenetic tree analysis revealed a high similarity between *ScDIR11* and *ScDIR5*, *ScDIR7* and *ScDIR33*, and *ScDIR40* and *ScDIR42*, and their three conserved motifs are also at similar positions in the sequences, suggesting a close correlation of gene expression and the sequence similarity in these genes (Additional file 2: Figure S4).

### *ScDIR* proteins mediate pinoresinol formation

To examine whether the four *ScDIR* genes are involved in lignin synthesis, we tested the enzyme activities of *ScDIR5*, *ScDIR7*, *ScDIR11*, and *ScDIR40* in vitro. The four *ScDIR* proteins were recombinantly expressed with an N-terminus GST tag in *Escherichia coli* and were purified using GST-tag purification resin affinity chromatography (Fig. 4a). The results showed that all four *ScDIR* proteins can mediate coniferyl alcohol coupling to form pinoresinol, a lignin precursor, in the presence of ammonium persulfate. *ScDIR5* and *ScDIR7* showed higher enzymatic activity, while *ScDIR11* and *ScDIR40* exhibited lower activity (Fig. 4b and Additional file 1:



**Fig. 4** Recombinant *ScDIR* proteins mediate the synthesis of the lignin precursor pinoresinol. **a** SDS-PAGE analysis of *ScDIR* proteins. Molecular mass marker (lane 1), uninduced total proteins (lane 2), IPTG-induced total proteins (lane 3), supernatant (lane 4), precipitate (lane 5), miscellaneous protein (lanes 6 and 7), and purified protein (lane 8). GST-*ScDIR5* is a recombinant *ScDIR5* with a GST tag. **b** HPLC analysis of the synthesis of pinoresinol from coniferyl alcohol in the presence of recombinant *ScDIR* proteins. Mobile phase A is 10% formic acid + 0.1% TFA and mobile phase B is 15% methanol/acetonitrile (40:60; v/v). Control group contains coniferol alcohol standard and pinoresinol standard, and experimental group contains coniferol + GST, coniferol + GST-*ScDIR5*, coniferol + GST-*ScDIR7*, coniferol + GST-*ScDIR11*, and coniferol + GST-*ScDIR40*. Three biological replicates were performed for each sample

Table S5). These results suggest that the four ScDIR proteins participate in lignin synthesis.

#### Overexpression of ScDIR in *N. benthamiana* enhances their resistance to *S. rolfisii*, *R. solani*, and *B. cinerea*

To investigate the potential functions of the four ScDIR genes in defense against fungal diseases, we generated Agrobacterium-mediated ScDIR-transgenic *N. benthamiana* lines (Additional file 2: Figures S5–S7). Overexpression of ScDIR genes resulted in increased lignin content in *N. benthamiana* leaves, as determined by lignin content assays (Additional file 2: Figure S8). Then, wild-type (WT), empty plasmid transgenic (35S-transgenic) and ScDIR transgenic *N. benthamiana* leaves were inoculated with *Sclerotium rolfisii*, *Rhizoctonia solani*, and *Botrytis cinerea*, respectively. After three days of inoculation, the leaves of all *N. benthamiana* lines showed disease spots, while no disease spots appeared on the leaves in H<sub>2</sub>O-treatment group (Fig. 5a). Quantitative analysis for the *S. rolfisii* inoculated plants showed that the lesion areas on the leaves of the ScDIR-transgenic *N. benthamiana* lines were significantly smaller than those in non-transgenic lines. Particularly, the ScDIR7-transgenic lines expressed the highest resistance levels (Fig. 5b and Additional file 1: Table S6). Consistently, all four ScDIR genes proved effective in enhancing the resistance of transgenic *N. benthamiana* lines upon inoculation with *R. solani* and *B. cinerea*. As a comparison, ScDIR7, ScDIR11, and ScDIR40 exerted a stronger effect than ScDIR5 (Fig. 5c, d). Therefore, these four ScDIR genes contribute positively to disease resistance against fungal pathogen *S. rolfisii*, *R. solani*, and *B. cinerea* in *N. benthamiana*.

#### Overexpression of ScDIR genes in sugarcane enhances resistance to *S. scitamineum*

To explore whether the four ScDIR genes improve the resistance of sugarcane to smut pathogens, the genes were introduced into the smut-susceptible sugarcane variety ROC22 using Agrobacterium-mediated transformation. The verified transgenic sugarcanes were evaluated for the resistance to *S. scitamineum* infection (Additional file 2: Figure S9) and qRT-PCR analysis revealed the elevated expression levels of ScDIRs in the transgenic lines (Fig. 6).

The transgenic sugarcane seedlings were inoculated with two mating compatible haploid strains of *S. scitamineum* (JG35 and JG36) using the root dipping method. The smut symptoms (black whip) firstly appeared in wild-type (WT) sugarcane at 41 days post-inoculation. However, the 35S-transgenic, ScDIR5- and ScDIR40-transgenic sugarcane lines developed whips at the 46 th, 51 th, and 56 th days after inoculation, respectively. In

addition, in ScDIR7- and ScDIR11-transgenic sugarcane lines, the black whip was first observed at 61 days post-inoculation. After 150 days of *S. scitamineum* infection on sugarcane seedlings, the incidences of whip development in WT and 35S-transgenic lines were 37/50 (74.0%) and 36/50 (72.0%), respectively. In comparison, all transgenic sugarcane lines showed a lower disease incidence. The ScDIR5-transgenic lines displayed the highest incidence of 8/28 (28.6%), and the ScDIR7-transgenic lines displayed the lowest incidence with only 4/35 (11.4%). The remaining ScDIR11- and ScDIR40-transgenic lines displayed the incidences of 7/35 (20.0%) and 8/32 (25.0%), respectively (Fig. 7a, b).

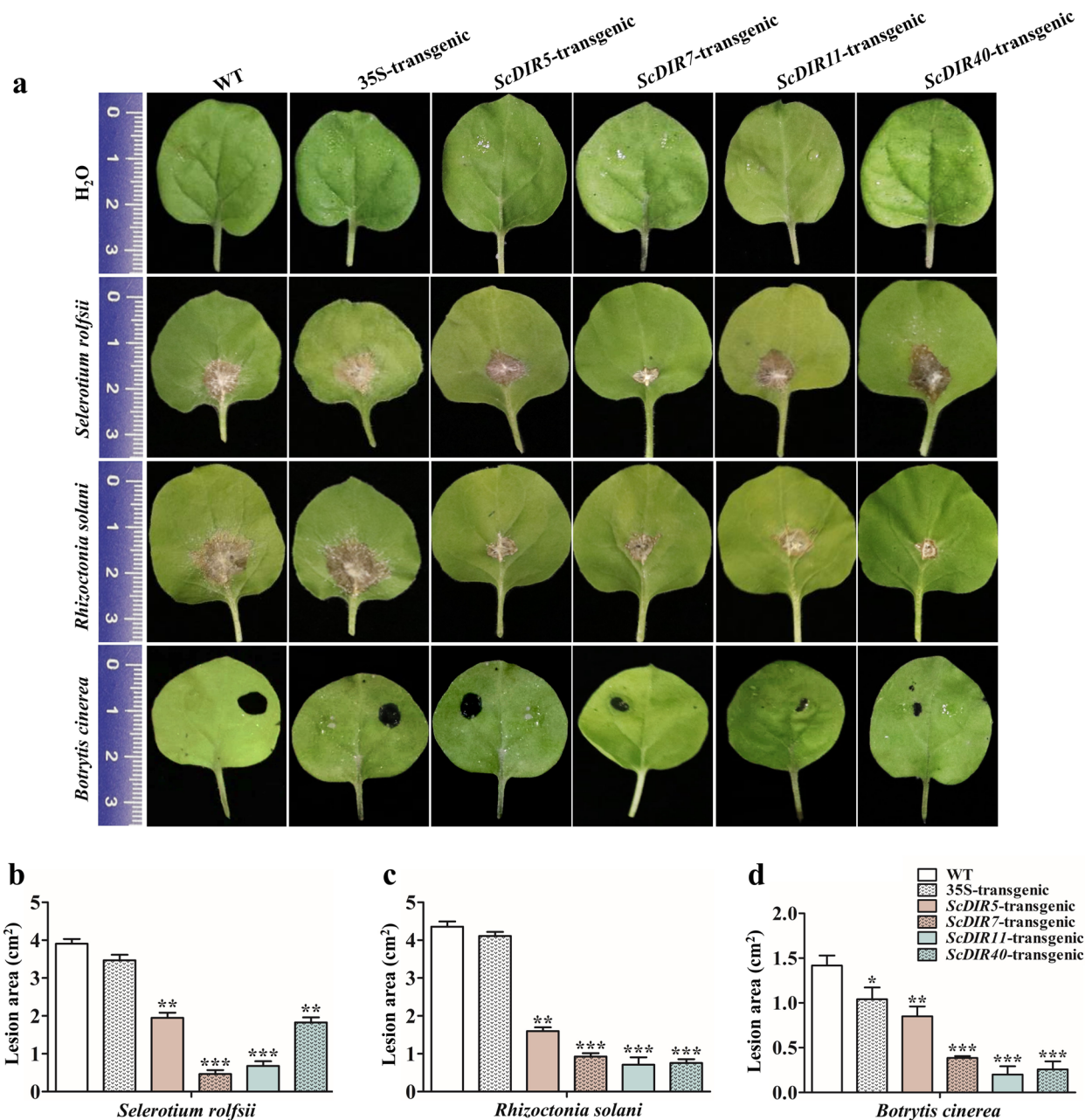
Histopathological examination revealed the presence of fungal hyphae in all sugarcane plants with black whip symptoms, while the reduced fungal hyphae was observed in wild-type and vector control plants than the ScDIRs transgenic lines, which developed less or no whip (Fig. 7c).

By PCR quantification of *bE* gene in *S. scitamineum*, sugarcane that does not have whip indeed contained fungal hyphae, whereas the transgenic sugarcane lines contained less *S. scitamineum* than WT. Furthermore, the *S. scitamineum* content was higher in the sugarcane with a black whip, but there was no significant difference among the transgenic sugarcane lines (Fig. 7d).

To determine whether sugarcane lines overexpressing ScDIR genes had increased lignin content, phloroglucinol staining was used to observe the lignin levels of each sugarcane lines after 150 days of infection. The buds of WT and transgenic sugarcane lines accumulated a large amount of lignin, which further confirmed that lignin accumulation could suppress *S. scitamineum* infection (Fig. 8a). Further quantitative analyses indicated that the lignin content of ScDIR transgenic lines was significantly higher than that of WT which does not have the black whip. However, for plants with the black whip, the lignin content of ScDIR transgenic lines showed no significant difference from that of WT (Fig. 8b). Taken together, overexpression of ScDIR5, ScDIR7, ScDIR11, and ScDIR40 could increase the lignin content in sugarcane, thereby increasing their resistance to *S. scitamineum* infection.

## Discussion

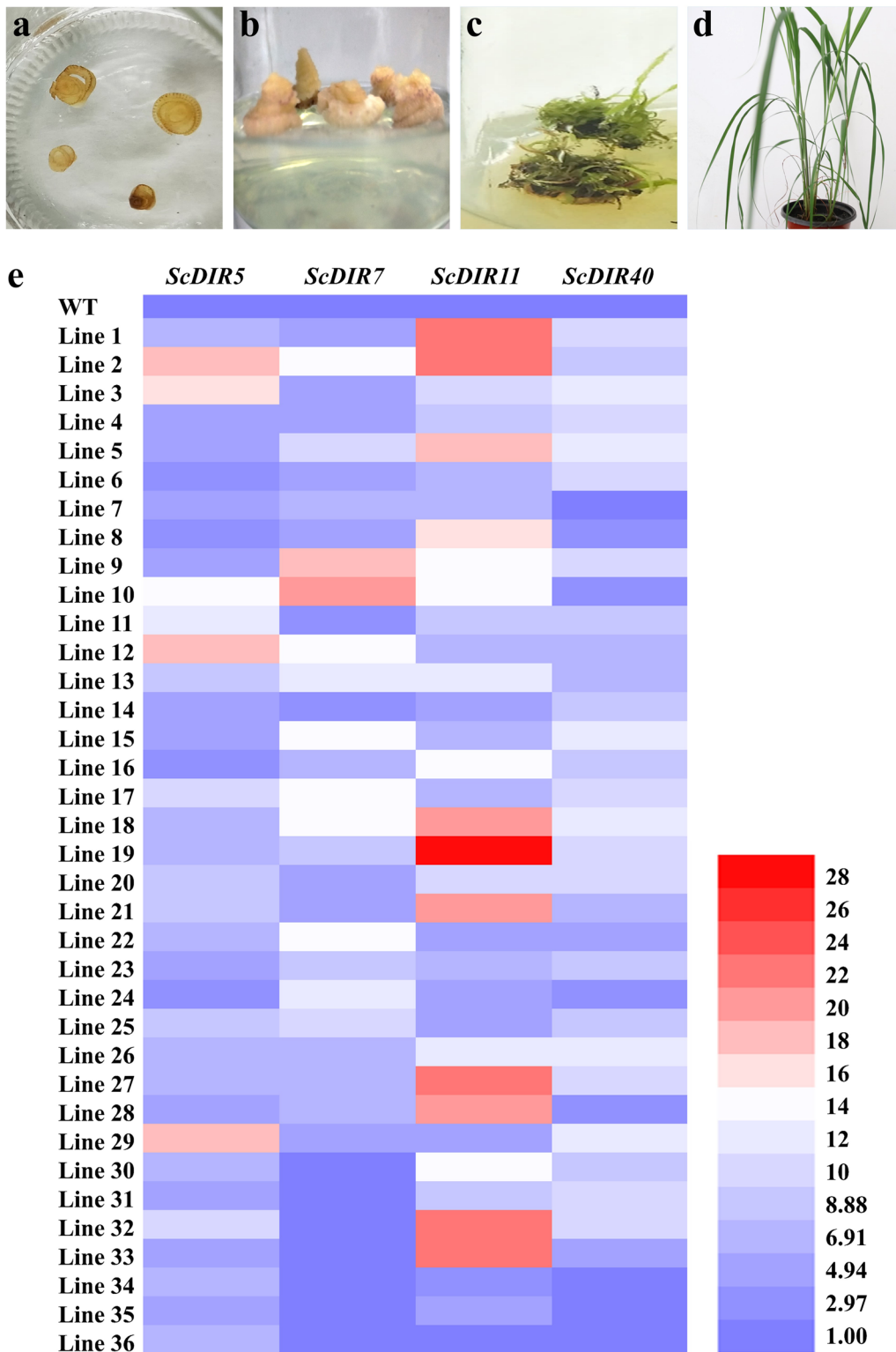
Sugarcane is an important sugar crop, and its disease resistance is closely related to the quality and yield of sucrose. Of all sugarcane diseases, sugarcane smut, caused by the biotrophic fungal pathogen *S. scitamineum*, is the most serious threat to the sugarcane industry (Rajput et al. 2021). The most effective and economical method to control smut disease is to adopt the resistant sugarcane varieties (Que et al.



**Fig. 5** Disease resistance assays in four *ScDIR* transgenic *N. benthamiana* lines. **a** Disease symptoms caused by the indicated fungal strains in transgenic *N. benthamiana* lines that overexpress *ScDIR* genes. Photographs were taken at three days post-inoculation (dpi) and lesion areas were measured. **b** Lesion area in *ScDIR* transgenic lines after *S. rolfsii* infection. **c** Lesion area in *ScDIR* transgenic lines after *R. solani* infection. **d** Lesion area in *ScDIR* transgenic lines after *B. cinerea* infection. Error bars denote the standard deviation calculated from six independent experiments. The asterisk indicates a statistically significant difference from WT (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ; t-test)

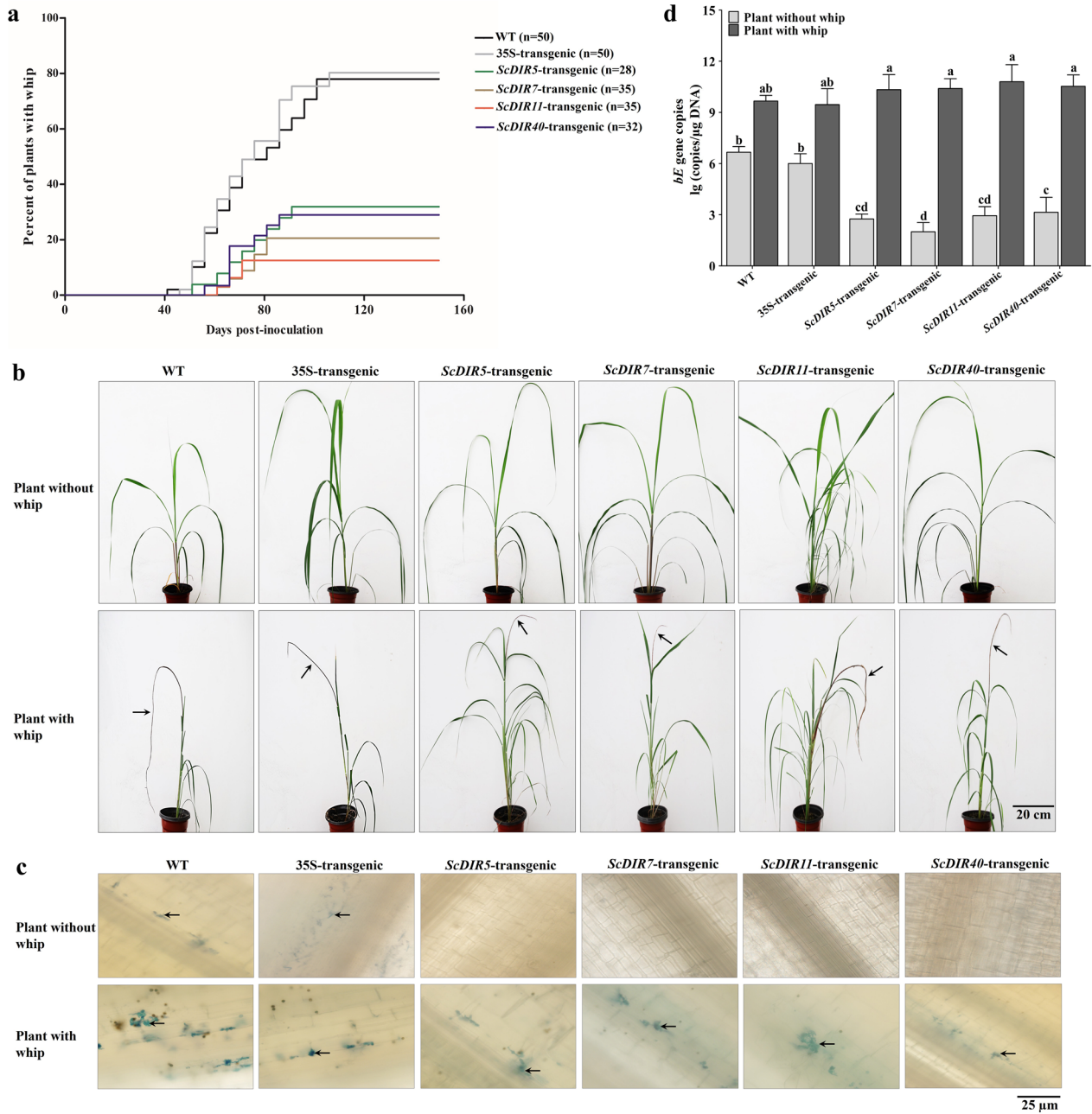
2011). Due to its allopolyploid nature and the complex genetic background, the pollen fertility of most sugarcane clones is low, and it is very difficult to synchronize the flowering of potential parents. Coupled with an extremely low (approximately 1/300,000) recombination rate (Cheng et al. 2022; Wang et al. 2023),

traditional cross-breeding takes approximately 12 years to develop a new sugarcane variety (Wu et al. 2022). Moreover, functional genomic research of sugarcane is far behind that of major crops, such as wheat and rice. Few genes have been identified to confer resistance to smut disease in sugarcane, and no genetically modified



**Fig. 6** Construction and verification of *ScDIR* transgenic sugarcane. **a** *Agrobacterium tumefaciens* cocultured with sugarcane explants. **b** Glufosinate ammonium containing 2.0 mg/L was used to screen transgenic-resistant sugarcane seedlings in the medium. **c** Rooting culture of resistant sugarcane seedlings. **d** The transgenic sugarcane lines. **e** qRT-PCR validation of transgenic sugarcane lines. The relative expression pattern of the gene was generated by Heml 1.0.3.7 software. The color code of the heatmap indicates the expression. *GAPDH* was used as an internal reference gene. Three biological replicates were carried out and each replicate includes three plants



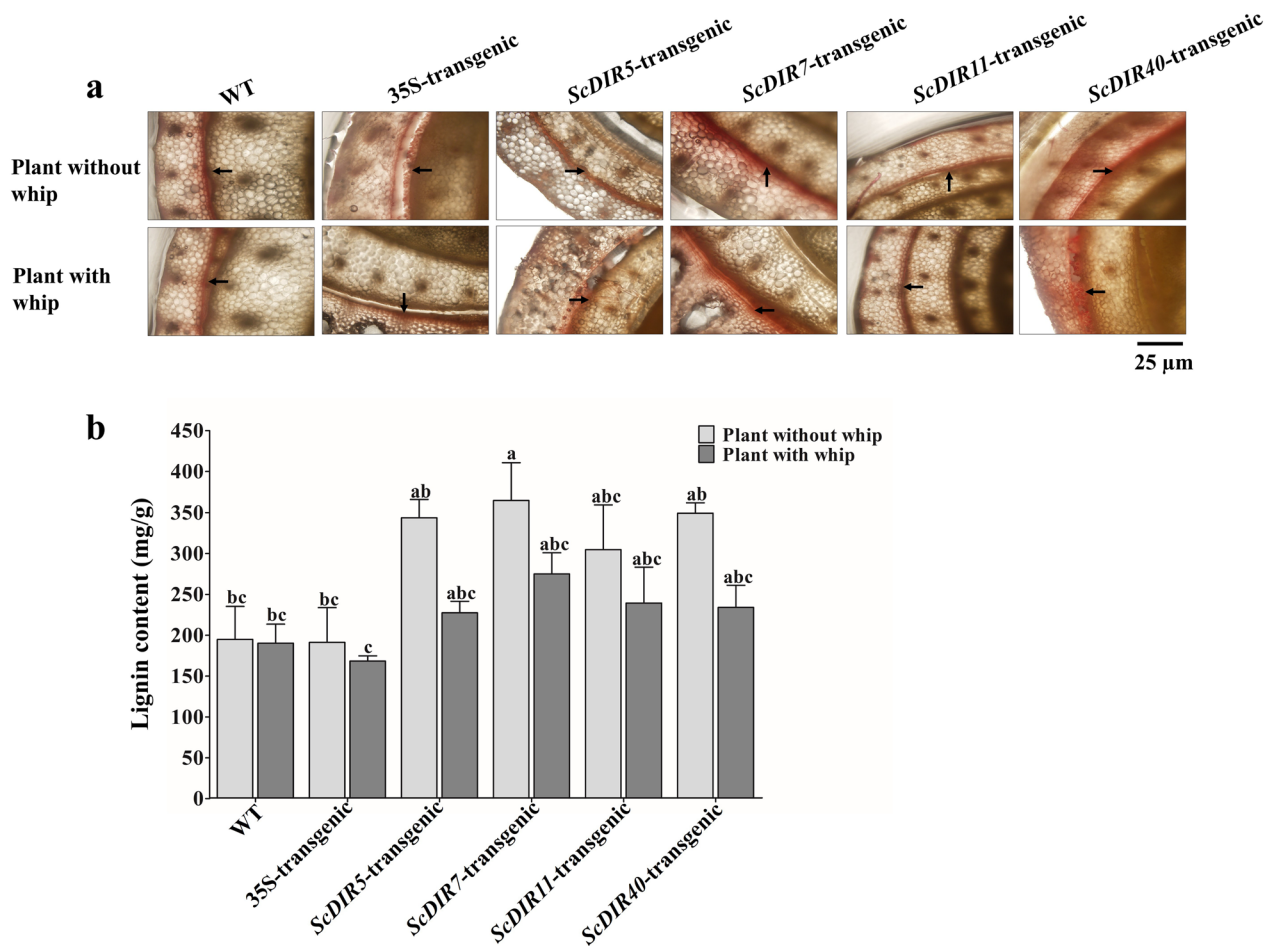


**Fig. 7** Overexpression of *ScDIR* genes in sugarcane enhances resistance to *S. scitamineum*. **a** The progression of whip development in *ScDIR* transgenic lines. Tissue culture-derived plantlets of the *ScDIR* transgenic lines were inoculated with two mating strains of *S. scitamineum* JG35 and JG36, by root dipping method. “n” represents the number of sugarcane-inoculated lines. **b** Symptoms of plantlets inoculated with *S. scitamineum*. Arrows indicate black whips. Scale bars = 20 cm. **c** Histopathology was carried out in the plantlets by dissecting and staining them with trypan blue. Arrows indicate fungal hyphae in vivo. Scale bars = 25 μm. **d** Quantification of *S. scitamineum* *bE* gene copies in *ScDIR* transgenic lines at 150 days post-inoculation. Three biological replications were conducted. Values in the same column with different letters indicate significant difference from the control ( $p < 0.01$ ) using one-way analysis of variance (ANOVA) followed by multiple Duncan tests

sugarcane that is resistant to smut disease has been reported thus far (Maeda et al. 2023).

Plant cell wall composed of wood fibers is the main barrier to protect plants from invasion by pathogenic

organisms (Jones and Dangl 2006). Several studies have shown that *S. scitamineum* infection leads to the accumulation of lignin in sugarcane, which reinforces the defense capacity of sugarcane cell walls (Huang et al.



**Fig. 8** Overexpression of the *ScDIR5*, *ScDIR7*, *ScDIR11*, and *ScDIR40* genes increased the lignin content in sugarcane. **a** Observation of lignin in wild-type sugarcane (WT), sugarcane with empty vector (35S-transgenic), and transgenic sugarcane (*ScDIR5*-transgenic, *ScDIR7*-transgenic, *ScDIR11*-transgenic, and *ScDIR40*-transgenic lines) at 150 days post-inoculation using phloroglucinol staining. Arrows indicate lignin. Scale bars = 25  $\mu$ m. **b** Quantitative analysis of lignin content of different samples. Six biological replicates were included in each sample. SPSS software was used to analyze the lignin content of each sugarcane at different time points, and the significance is denoted above each column. Values in the same column with different letters indicate significant difference ( $p < 0.05$ ) using one-way analysis of variance (ANOVA) followed by multiple Duncan tests

2018; Marques et al. 2018). A similar result was also found in our study. After *S. scitamineum* infection, the lignin content of sugarcane buds increased rapidly in a short time (24–48 h). At the same time, we also found significant differences in the lignin content of smut-resistant and smut-susceptible sugarcane buds (Fig. 2a, b) (Modafar et al. 2000). Additionally, this study provided strong evidence that sugarcane enhances its cell wall defense against *S. scitamineum* invasion by increasing its lignin content. Previous studies have shown that pinorensinol produced by smut-resistant varieties can inhibit the germination and release of teliospores of *S. scitamineum*; while in other species, DIR proteins have been reported to be involved in the synthesis of pinorensinol (Dalisy et al. 2015; Li et al. 2017; Chen et al. 2018), suggesting

that sugarcane smut resistance is related to the catalytic activity of ScDIR proteins.

After the discovery of DIR proteins in 1997, DIR has been reported to control specific chemical bond formation during the polymerization of single-signal alcohols to form lignin polymers, a class of proteins that cause plant lignification and respond to pathogen infection (Cheng et al. 2018). It has been reported that the DIR protein family can be divided into multiple subfamilies (Ralph et al. 2007; Nobile et al. 2017). Of which, the DIR-a subclass is related to lignin synthesis and disease resistance. In contrast, our results showed that *ScDIR13*, a member of the DIR-a subclass of sugarcane, did not respond to *S. scitamineum* infection. Moreover, DIR-b/d is thought to play a role in plant responses to abiotic and

biotic stresses (Wu et al. 2009; Uchida et al. 2017). Consistently, *ScDIR5* and *ScDIR11* belong to this clade and are involved in biotic stress responses. DIR-c proteins are thought to be related to plant disease resistance (Kittur et al. 2010; Ma 2014), and *ScDIR7* and *ScDIR40* in this study can significantly improve the disease resistance in transgenic plants, confirming the functional classification of this clade. Previous studies have identified sugarcane *DIRIGENT* (*SHDIR16*) and *O-methyltransferase* (*SHOMT*) through transcriptional profiling analysis and suggested that they were defense and fiber biosynthesis-related genes that were highly expressed in sugarcane stems (Damaj et al. 2010). In this study, four of the 64 sugarcane *ScDIR* genes, the *ScDIR5*, *ScDIR7*, *ScDIR11*, and *ScDIR40*, could respond rapidly to smut pathogen infection (Fig. 2). Moreover, HPLC assays showed that all four *ScDIR* proteins can mediate coniferyl alcohol coupling to form pinoresinol (Fig. 4). Consistent with this result, some DIR proteins have been found to synthesize lignin precursors in many plants, such as *Arabidopsis*, *Linum usitatissimum*, and *Thuja plicata* (Pickel et al. 2010; Dalisay et al. 2015; Chen et al. 2018). These four *ScDIR* proteins may enhance the defense ability of plants against pathogenic organisms by increasing their lignin content. However, we found that *ScDIR5* exhibited the highest protein coupling activity, and its corresponding transgenic *N. benthamiana* and transgenic sugarcane did not show the strongest disease resistance, which may be attributed to the lower expression level of this gene.

Some studies have shown that transforming the *DIR* gene which confers disease resistance in plants into other plants can enhance the disease resistance of transgenic plants (Ma and Liu 2015). This notion is supported by our current study. Here, we found that the resistance of *ScDIR* transgenic *N. benthamiana* to *S. rolfisii*, *R. solani*, and *B. cinerea* was significantly enhanced (Fig. 5). In addition to the smut disease, *ScDIR* gene also responds to the challenges of other fungi. Therefore, immune response of the *ScDIR* proteins in plants is extensive.

Sugarcane plants overexpressing the four *ScDIR* genes had a significantly reduced incidence of smut with *S. scitamineum* infection (Fig. 7), and their lignin content was much higher than that of control sugarcane (Fig. 8). This result further demonstrates that *ScDIR* genes contribute to the synthesis of lignin and enhance plant disease resistance. However, *S. scitamineum* was detected in both WT and transgenic sugarcane (Fig. 7d), indicating that the accumulation of lignin cannot completely prevent the plants from *S. scitamineum* infection. This is inconsistent with previous studies in which lignin accumulation completely halted *S. scitamineum* invasion (Marques et al. 2018). It is possible that although the lignin accumulation is positively correlated with smut disease resistance,

other mechanisms of resistance may also exist in resistant sugarcane varieties.

Although *DIR* genes have been reported previously to be involved in disease resistance in plants, there are no information about the 64 sugarcane *ScDIR* genes for their roles in smut disease resistance. In this regard, identifying the important smut disease resistant sugarcane *DIR* genes and introducing these genes into sugarcane germplasm are of significant contributions. Moreover, the transgenic sugarcane generated in this study will be evaluated for its agronomic characteristics extensively.

It is well known that both knock-down and knock-up gene modification approaches can be used to digest gene function. However, the commercial sugarcane varieties are interspecific hybrids with a highly polyploid genome ( $2n=100-130; \times=10-13$ ), which typically contains ten or more homo(eo)logs at each locus (Thirugnanasambandam et al. 2018). As a result, it is extremely difficult to knock out a gene in sugarcane. Indeed, there is no successful application of gene knockout technology in sugarcane. Since sugarcane contains a high level of genetic redundancy, it remains challenging to develop effective genome-editing protocols for it (Eid et al. 2021).

The importance of sugar content in sugarcane as an agronomic trait has been widely recognized. Meanwhile, lignin is essential for higher plants as it provides mechanical strength for upright growth, and confers hydrophobicity to vessels that transport water and nutrients. Importantly, it also acts as a physical barrier against pathogens invasion (Eudes et al. 2012). Previous studies have identified a carbon tradeoff where reduced lignin content is associated with increased sugar content in sorghum using multiscale phenotype analysis (Boatwright et al. 2022). However, the correlations between sugar content and lignin content have not been reported in sugarcane. We have determined the sugar content in six sugarcane varieties (smut-resistant: ZZ1, ZZ6, and ZZ9; smut-susceptible: GT42, FN41, and ROC22) during the same growth period. The unpublished data showed that there was no correlation between lignin content and sugar content.

## Conclusions

In conclusion, the relationship between sugarcane resistance and lignin content was identified. The expression of four *ScDIR* genes can be induced in response to *S. scitamineum* infection. Moreover, four *ScDIR* proteins were purified and demonstrated the biochemical function in vitro. Four *ScDIR* genes also improved the resistance to fungal pathogens in transgenic *N. benthamiana* lines by heterologous gene expression. Furthermore, we successfully generated the sugarcane materials with improved smut disease resistance by overexpressing *ScDIR* genes.

The results obtained in this study may contribute to the advancement of sugarcane molecular breeding for disease resistance.

## Methods

### Sugarcane varieties and treatments

Six main sugarcane varieties in Guangxi Province of China (smut-resistant: ZZ1, ZZ6, and ZZ9; smut-susceptible: GT42, FN41, and ROC22) and ten sugarcane clones (smut-resistant: 28-151, 7-136, 1-17, 21-177, and 25-58; smut-susceptible: 9-130, 24-86, 25-55, 27-42, and 28-31) from the F1 population derived from the cross of ROC25 and YZ89-7 were selected for this experiment. All sugarcane varieties and clones were routinely maintained in the Collaborative Innovation Center of the Sugarcane Industry of Guangxi University, Guangxi Province, China. Smut teliospores were isolated from a whip developed in ROC22. Further experiments were conducted using teliospores with a germination rate greater than 90%.

We collected single-node cuttings (30 for each cultivar) from 9-month-old sugarcane intermediate nodes. The buds were washed with water three times, and sterilized in a 0.01% sodium hypochlorite solution (Aladdin Biotech, Shanghai, China) for 15 min, and then rinsed with water. The cuttings were planted in the substrate soil and cultured at 28°C and 80% humidity under long-day conditions (16 h/8 h). Inoculation was performed by placing 20  $\mu$ L of smut teliospore suspension ( $5 \times 10^6$  spore/mL in Tween<sup>®</sup> 20) on the sugarcane buds. The control treatment used water (0.01% Tween<sup>®</sup> 20). After inoculation, sugarcane buds were collected for lignin detection and RNA extraction at different time points (0, 24, 48, and 72 h).

### Detection of lignin content

To observe the lignin in the sugarcane bud, the sugarcane bud materials inoculated with smut teliospores were collected. Then, the cross-section of the bud was cut immediately with a surgical blade and placed in lignin staining solution (phloroglucinol staining method, Qingdao Jisskang Biotech, China) for 30 s. Lignin was then observed with a microscope (Marques et al. 2018). To quantify the lignin in the sugarcane bud, sugarcane buds were dried at 80°C for 2 days, and the lignin content was detected by a lignin detection kit (Solarbio, cat: BC4205, Beijing, China).

### RNA isolation and gene expression analysis

TransZol Plant reagent (TransGen, Beijing, China) was used to extract total RNA from plant materials. The HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) was used for reverse transcription. The mRNA

expression levels were determined by quantitative real-time PCR (qRT-PCR) using a SYBR Green Master Mix Kit (TransGen, China) and Light Cycler 480 II systems (Roche, Shanghai, China). Using the *GAPDH* gene as an internal reference, the expression levels of the *ScDIR* genes were analyzed by the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen 2001) based on three biological replicates. The primers used for the qRT-PCR analysis are listed in Additional file 1: Table S7.

### Heterologous expression and purification of ScDIR proteins

The coding sequences of *ScDIR* genes were PCR amplified from ROC22 bud cDNA and further cloned and inserted into pGEX-KG expression vectors (Beijing Zoman Biotech, Beijing, China). Primer sequences are shown in Additional file 1: Table S7. The resulting recombinant plasmids were named as pGEX-*ScDIR* and then transformed into the *E. coli* strain BL21. For *ScDIR* overexpression, an overnight culture of *E. coli* BL21 ( $\lambda$ DE3)/pGEX-*ScDIR* was transferred into fresh LB medium, and the flasks were shaken at 37°C until  $OD_{600} = 0.5$ . Protein expression was induced using 0.5 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) at 16°C for 12 h. The bacterial cultures were collected and lysed with PBS buffer containing 100  $\mu$ g/mL lysozyme (Ma and Tian 2005; Ma et al. 2013). The GST-*ScDIR* proteins were purified from the supernatant with BeyoGold GST-tag Purification Resin (Beyotime Biotech, Shanghai, China). The BCA assay (Pierce) was used to quantify the purified proteins, followed by SDS-PAGE analysis and stored at -80°C.

### Coupling analysis for ScDIR proteins

The coupling assays were performed based on the methods described by Davin et al. (1997) and Zheng et al. (2011). The reaction mixture consisted of coniferyl alcohol (10  $\mu$ mol/mL), ammonium peroxydisulfate (2  $\mu$ mol/mL), and *ScDIR* proteins (1.5 nmol/mL). After incubation for 3 h at 30°C, the mixture was extracted three times with ethyl acetate. The extract was evaporated to dryness under vacuum, and the residue was redissolved in 50% methanol. A high-performance liquid chromatography (HPLC) analysis of each extract was performed under the following conditions: column temperature, 25°C; flow rate, 1.0 mL/min; chromatogram, 280 nm (Zheng et al., 2011). HPLC analysis was performed on a Waters e2695 system (Milford, MA, USA) equipped with a Symmetry300<sup>™</sup> C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m i.d., Ireland).

### *N. benthamiana* transformation and biotic stress analysis

*N. benthamiana* seeds (provided by the molecular virology and mycology laboratory of Guangxi University)



were sterilized and then were grown for tissue culture. The full-length coding sequence of the *ScDIR* genes was PCR amplified from ROC22 bud cDNA and was inserted into the pCAMBIA3300 vector (Beijing Zoman Biotech, Beijing, China) containing a CaMV 35S promoter. Detailed primer sequences are listed in Additional file 1: Table S7. Using the *Agrobacterium* EHA105-mediated transformation method, the constructed vectors were transformed independently into *N. benthamiana* callus (Lin et al. 1995). Transformed *N. benthamiana* (T0) was harvested and sown on MS medium. T1 and T2 transgenic seedlings were identified by qRT-PCR using *ScDIR* gene primers (Additional file 1: Table S7). The *GAPDH* was used as an internal control. Further analysis was conducted on positive T2 transformants.

To test the resistance of transgenic *N. benthamiana* plants to the pathogens, *S. rolfii*, *R. solani*, and *B. cinerea* were used for the inoculations. Five-leaf stage leaves of T2 transgenic *N. benthamiana* were inoculated with 10  $\mu$ L *B. cinerea* suspension ( $4 \times 10^5$  CFU/mL) using a drop-inoculation method or 3 mm mycelium plugs of *S. rolfii* and *R. solani* (Guo et al. 2004; Ma et al. 2013). The inoculated plant tissue was placed in a 90 mm sterile petri dish containing sterile filter paper soaked in 1 mL of sterile distilled H<sub>2</sub>O, sealed with parafilm, and then was maintained at room temperature for 3 days. *N. benthamiana* leaves inoculated with sterile water using the same method serves as a control (Jerushalmi et al. 2020). The lesion size was measured using ImageJ software (1.8.0) (Schneider et al. 2012).

### Sugarcane transformation and smut stress analysis

The coding sequences of *ScDIR* were inserted into a pCAMBIA3300 vector containing the Ubi promoter to generate the recombinant vector pCAM-Ubi-*ScDIR*. The recombinant vector was electroporated into *Agrobacterium* EHA105 cells and then transferred into sugarcane ROC22 callus by the *Agrobacterium*-mediated method, as described previously (Zhangsun et al. 2007). Transgenic plants were selected for phosphinothricin resistance, and was conferred by the *bar* gene (selectable marker). The presence of the *ScDIR* and *bar* genes was verified in co-transformed sugarcane plants by PCR using the primers listed in Additional file 1: Table S7. The relative expression of *ScDIR* was analyzed by qRT-PCR in the positive transgenic sugarcane lines.

To analyze the resistance of transgenic sugarcane to smut, the *S. scitamineum* JG35 and JG36 mating compatible haploid strains were mixed in a 1:1 ratio and then used to inoculate the 5–7 leaf-stage sugarcane tissue-cultured seedlings using the root dipping method (Lu et al. 2021). Pathogenicity was examined and

documented at 150 days post-inoculation. The hyphae at the growing point of transgenic sugarcane lines were observed by trypan blue staining (Beyotime Biotech, Shanghai, China) (Jerushalmi et al. 2020). To measure the copy number of *S. scitamineum* in sugarcane, the *S. scitamineum* *bE* gene, which is involved in haploid sporidia mating, was used as a target. TaqMan RT-PCR was conducted as described previously (Su et al. 2013b; Liu et al. 2022).

### Abbreviations

DIR	Dirigent
IPTG	Isopropyl-beta-D-thiogalactopyranoside
qRT-PCR	Quantitative real-time PCR
WT	Wild-type

### Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s42483-024-00237-w>.

**Additional file 1: Table S1.** The natural smut incidences of six elite sugarcane varieties in the second ratooning. **Table S2.** The natural smut incidences of ten clones of ROC25  $\times$  YZ89-7 F1 generation in the second ratooning. **Table S3.** The relative expression of *ScDIRs* in ROC22 after *S. scitamineum* inoculation. **Table S4.** The relative expression of *ScDIRs* in ZZ9 after *S. scitamineum* inoculation. **Table S5.** The quantitative data of HPLC result. **Table S6.** The lesion area of transgenic *N. benthamiana* leaf after fungal stress. **Table S7.** Primer sequences used in this study.

**Additional file 2: Figure S1.** Physically similar sugarcane that stems from the six sugarcane varieties were collected and cut into single-bud setts. **Figure S2.** Pathogenicity assays in the smut-susceptible sugarcane GT42 and the smut-resistant sugarcane ZZ9 after *S. scitamineum* infection. **Figure S3.** Comparison of lignin contents between smut-resistant and smut-susceptible sugarcane clones. **Figure S4.** Phylogenetic relationships and architecture of conserved protein motifs in *ScDIR* proteins. **Figure S5.** Construction of transgenic *N. benthamiana* plants overexpressing *ScDIR* genes. **Figure S6.** The T2 transgenic *N. benthamiana* lines were verified by PCR. **Figure S7.** The T2 transgenic *N. benthamiana* lines were verified by qRT-PCR. **Figure S8.** Determination of lignin content in T2 transgenic *N. benthamiana* leaves. **Figure S9.** The transgenic sugarcane lines were verified by PCR.

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### Author contributions

RL conceived and designed the project. XL, ZL, and HW carried out the experiments. ZY, JM, HZ, XD, YS, and BC provided the materials and analyzed the data. RL, BC, and XL wrote the manuscript. All authors read and approved the final manuscript.

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### Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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