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Research article

Strigolactones positively regulate defense against *Magnaporthe oryzae* in rice (*Oryza sativa*)

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ABSTRACT

This study presents evidence that strigolactones (SLs) promote defense against devastating rice blast fungal pathogen *Magnaporthe oryzae*. Impairment in either SL-biosynthetic *dwarf17* (*d17*) or –signaling (*d14*) led to increased susceptibility towards *M. oryzae*. Comparative transcriptome profiling of the SL-signaling *d14* mutant and WT plants revealed that a large number of defense-associated genes including hydrogen peroxide (H_2O_2)-, ethylene- and cell wall-synthesis-related genes were remarkably suppressed in *d14* with respect to that of WT plants, during *M. oryzae* infection. In addition, various KEGG metabolic pathways related to sugar metabolism were significantly suppressed in the *d14* plants compared to WT, during *M. oryzae* infection. Accordingly, WT plants accumulated increased levels of H_2O_2 and soluble sugar content compared to that of *d17* and *d14* in response to *M. oryzae* infection. Altogether, these results propose that SLs positively regulated rice defense against *M. oryzae* through involvement in the induction of various defense associated genes/pathways.

1. Introduction

Rice (*Oryza sativa* L.) is among the important staple food crops, fulfilling food requirements of about half of the world human population (Dean et al., 2012; Nasir et al., 2018). The global human population is expected to increase to around 9.2 billion at 2050; and, thus to feed this increased population, enhancement in the yield of rice is extremely essential. Unfortunately, rice blast disease caused by the hemibiotrophic fungal pathogen, *Magnaporthe oryzae,* results in approximately 10–30% damage to the rice harvest world-wide per annum, which therefore remains a major threat to food security globally (Dean et al., 2012).

To encounter the attacking pathogen including *M. oryzae*, rice triggers an array of sophisticated basal defense responses, which includes biosynthesis, signaling and cross-communication of various defense-related hormonal pathways, induction of oxidative burst and accumulation of antimicrobial substances (Nasir et al., 2018). Moreover, there is a growing belief that sugar molecules are also important for the establishment of successful defense against phytopathogens (Bolouri and Van, 2012). Besides, studies demonstrated that upon pathogen challenge plants including rice also fortify its cell wall which serves as a physical barrier to inhibit the penetration of the invading pathogens.

In the last two decades, remarkable progress has been made in understanding the role of phytohormones in rice plant defense against *M. oryzae* (Yang et al., 2013; Nasir et al., 2018). It has been demonstrated that phytohormones including ethylene (ET), jasmonic acid (JA) and salicylic acid (SA) are positive regulators for resistance against *M. oryzae* in rice, whereas abscisic acid, auxin and gibberellic acid render rice plants more susceptible to *M. oryzae* infection. As is evident that successful defense to *M. oryzae* in rice is dependent on the activation of ET-, JA- and SA-related pathways, the role of strigolactones (SLs) in regulating defense mechanism against devastating rice pathogens including that of *M. oryzae* remain elusive.

SLs for the first time was identified as a root exudate of cotton (*Gossypium hirsutum* L.) about 60 years ago (Cook et al., 1966) and, it has been found that SLs induce seed germination of parasitic plants (*Orobanche*, *Phelipanche* and *Alectra*) (Xie et al., 2010). Furthermore, years before, it was revealed that SLs as a host-derived signaling molecules are also involved in spore germination, and stimulation of hyphal branching of arbuscular mycorrhizal fungi, as well as in regulation of ectomycorrhizal fungi and gymnosperms interaction, resulting the symbiotic association between host plants and mycorrhizal fungi

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(Akiyama et al., 2005; Herrera-Martínez et al., 2014). Later on, it was found that SLs also serve as plant hormones, and regulate plant developmental and physiological processes, including seed germination, secondary growth, root development, branching and leaf senescence (Brewer et al., 2013). Besides, genetic studies have shown that SLs also positively mediate abiotic stress tolerance such as drought stress, salinity stress and nutrient stress in different plant species (Saeed et al., 2017).

In addition, to the roles of SLs in plant growth, development and abiotic stress resilience, more recently, genetic studies provided ample evidence that SLs also mediate defense against specific bacterial and fungal phytopathogens (Marzec, 2016). For instance, leaves of the tomato (*Solanum lycopersicum*) SL-deficient *ccd8* mutants were highly vulnerable to infection caused by the fungi *Botrytis cinerea* and *Alternaria alternate* (Torres-Vera et al., 2014). Moreover, the *Arabidopsis thaliana* SL-biosynthetic, *more axillary growth1* (*max1*), *max3* (ortholog of *dwarf17, d17* from rice), and *max4* (ortholog of *d10* from rice), and the SL-signaling *max2* (ortholog of *d3* from rice) mutants also showed enhanced susceptibility to the pathogen *Rhodococcus fascians* (Stes et al., 2015). Consistent with this, *A. thaliana max2* mutant plants are also more vulnerable to phytopathogenic bacteria *Pectobacterium carotovorum* and *Pseudomonas syringae* than wild-type (WT) (Piisilä et al., 2015). In addition to tomato and *A. thaliana*, the positive role of SL in immunity also has been confirmed in moss (*Physcomitrella patens*), in such a way that SL-deficient *ccd7* and *ccd8* mutants of moss were more susceptible to *Fusarium oxysporum*, *Sclerotinia sclerotiorum,* and *Irpex* sp. infection than WT (Decker et al., 2017). On the contrary, genetic studies have shown that SL-biosynthetic pathway is not required for regulation of defense against *F. oxysporum* in pea (*Pisum sativum* L.) (Foo et al., 2016).

SLs biosynthetic pathway of rice consists of five main genes, named *d27*, *d17*, *d10*, and two *Arabidopsis max1* orthologs (*Os01g0700900* and *Os01g0701400*). *d17*, *d10*, *d27*, *Os01g0700900* and *Os01g0701400*, coding for enzymes CCD7, CCD8, β-carotene isomerase, carlactone oxidase and orobanchol synthase respectively, function in β-carotene cleavage involving essential catalyzing steps, resulting biosynthesis of SLs (Saeed et al., 2017). In contrast, d14, d3 and d53 are well-known for their regulatory roles in SL-signaling. *d14* and *d3* are coding for receptor proteins namely α/β-hydrolase and F-box, respectively which perceive the presence of SLs and function as activators. On the contrary, d53 coding for a protein which shares predicted features with the class I Clp ATPase proteins and is known for repression of SLs signaling.

The purpose of the current study was to investigate whether SLs function in the regulation of rice defense against *M. oryzae.* To achieve this, we used well-defined rice SL-biosynthetic (*d17*) and -signaling (*d14*) defective mutants and the relevant WT rice plants. Results revealed that rice mutants defective in either SL-biosynthesis or -signaling exhibited increased susceptibility towards *M. oryzae* infection. Comparative transcriptome analysis revealed that the susceptibility was accompanied with the reduced expression of a large number of defense-associated genes including that of cell wall-, ET-, hydrogen peroxide (H_2O_2)-, and sugar/carbon synthesis-related genes. Accordingly, biochemical analysis showed clear reduction in H_2O_2 and soluble sugar contents in *d17* and *d14* mutants compared with that of WT in response to *M. oryzae* infection. Taken together, these results indicated that SLs promote biotic stress tolerance in rice by interacting cell wall-, ET-, H_2O_2- and sugar-associated genes/pathways.

2. Materials and methods

2.1. Seeds and seedling growth

Seeds of rice *d17* and *d14* mutants and WT (*Oryza sativa* L. cv. Shiokari) were originally got from Junko Kyozuka group, Touhoku University, Japan. For getting seedlings, briefly, seeds of *d17*, *d14* and WT were surface sterilized according to the method adapted by Tian et al. (2018). Next, germinating seeds were transplanted into 9 × 9 cm diameter pots (4 seedlings per pot) having 0.5 kg of sterilized soil and placed in the phytochamber. The temperature, photoperiod and relative humidity of the phytochamber were adjusted to 28 °C day/23 night, 16 h light/8 h dark and 70%, respectively. The recorded soil pH was about 6.5. To ensure water and nutrients availability, the plants were watered every other day with Hoagland's nutrient solution containing 75% of standard phosphorous concentration to avoid inhibition of SLs biosynthesis (Liu et al., 2015).

2.2. Pathogen cultivation, infection and disease evaluation

A virulent strain, GUY11 of *M. oryzae* fungus (provided by the Key Laboratory of Biopesticides and Chemical Biology, Fujian Agriculture and Forestry University, China) was grown on an oatmeal agar medium at 26 °C for 10–12 days. In brief, the aerial hyphae were removed by splashing with sterile water and brushing. Next, to induce conidia formation, the plates were irradiated at 24 °C for 3 days under continuous black-blue light (FL15BLB; Toshiba, Osaka, Japan, http://www.toshiba.co.jp). Then the conidia were suspended in 0.01% Tween 20 at a concentration of 1×10^4 conidia ml^{-1}. The inoculation was performed as described by Filipem et al. (2018) with minor modification. Briefly, plants at the five-leaf stage (4 weeks old) were spray-inoculated with spore suspension of GUY11 at 1×10^4 conidia ml^{-1} until runoff occurred. In order to retain high humidity, the inoculated plants were watered and sealed in plastic containers at room temperature. The control (un-infected) plants were sprayed only with 0.01% Tween 20 sterile water. After 24 h, the plants were incubated back in the phytochamber. Leaf samples were collected from fourth leaf at 36 h of *M. oryzae* infection for further analyses. Disease severity on the fourth leaf of each plant was measured at 7 day post inoculation (dpi) via counting susceptible-type lesion number and measuring the size of the three largest susceptible-type lesions (also known as blast lesions) per fourth leaf.

2.3. RNA extraction, generation of cDNA libraries, and RNA-Sequencing

From each treatment, RNA extraction was performed in three biological repeats (five plants per repeat) using TRIzol (Invitrogen) following manufacturer's guidelines. Next, the genomic DNA was removed by using DNase I (Invitrogen, New Jersey, NJ, USA). NanoPhotometer (IMPLEN, CA, USA), Qubit 2.0 Flurometer (Life Technologies, CA, USA) and Agilent Bioanalyser 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) were used for purity, quantitative and integrity assessment of RNA samples, respectively. Then from the RNA samples, cDNA libraries were generated and prepared for Illumina (2 × 120 bp) paired-end sequencing. The sequencing libraries were sequenced by Illumina HiSeq™ 2000 sequencer (Illumina, San Diego, CA, USA) at Novogene Bioinformatics Technology Co., Ltd (Beijing, China). HISAT (v.2.0.4) was used for mapping filtered reads to the reference genome. DESeq was used for calculating gene expression by adopting read count method. The criteria of corrected *p*-value (q-value < 0.05) and [fold change ≥ 1.5] were applied for identification of significantly expressed genes among various comparisons. MapMan software version 3.6.0 (http://mapman.gabipd.org/web/guest) was used for conducting MapMan analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation analysis was performed using KOBAS (v.2.0) and cut-off was adjusted at P-value < 0.05.

2.4. Histochemical detection of H_2O_2 and measurement of soluble sugar content

H_2O_2 accumulation was detected in the fourth leaves of *M. oryzae* treated plants (five biological replicates per treatment) at 36 h dpi by using the DAB (3,3′-diaminobenzidine) staining protocol. In brief, the forth leaves of *M. oryzae* inoculated *d17*, *d14* and WT were collected and dipped into DAB dye (1 mg ml^{-1}; pH 3.8). Then the leaf samples were incubated in light at 28 °C for 8 h. Next, the DAB dye was aspirated off of the leaf samples, and absolute ethanol was added, and then the samples were kept in the water bath (100 °C) and boiled until chlorophyll was removed. Finally the images of the samples were captured for visualization of H_2O_2. Soluble sugar content was determined using three biological repeats. In brief, the soluble sugar content from the fourth leaf of *M. oryzae* infected and non-infected *d17*, *d14* and WT rice plants was extracted at 36 h dpi and determined by using the approach given by Tian et al. (2018).

2.5. Fungal growth and synthetic strigolactone (GR5) application

With the aim to evaluate the possible direct impact of SLs on *M. oryzae* growth, an *in vitro* assay with GR5 (SL analogue) obtained from the RIKEN Plant Science Center, Tsurumi-ku, Yokohama, Japan and *M. oryzae* was performed. In brief, seven millimeter diameter potato dextrose agar (PDA) plugs consisting of fresh growing *M. oryzae* hyphae were transferred to the center of petri-plates containing PDA medium. The effect of four different concentrations of GR5 ranging from 0, 10^{-6}, 10^{-7} to 10^{-8} M were evaluated, all within the physiological concentration range (Torres-Vera et al., 2014). In each petri-plate about four 6 mm diameter Whatman filter paper discs were placed, and about 10 μL of different GR5 concentrations were administered to each disc. Six independent plates were incubated at 24 °C and the resulting colony diameter was recorded in mm at 6 day of inoculation.

2.6. RNA-seq data validation via qRT-PCR

For validation of RNA-sequencing (RNA-seq) data, quantitative reverse transcription PCR (qRT-PCR) was performed on 15 selected genes. Same RNA extracted for the RNA-seq was used to construct cDNA. cDNA synthesis and qRT-PCR analysis were conducted according to procedures described by Liu et al. (2015) with slight modifications. For each treatment, qRT-PCR analysis was carried out in three biological repeats. The $2^{-\Delta\Delta Cq}$ method was used for measurement of the relative expression levels of the selected genes. The gene *O. sativa β-tubulin* was used as housekeeping gene. The primers used in the current study are shown in Table 1.

2.7. Statistical analysis

The resulting data of various experiments were subjected to one-way analysis of variance (ANOVA). All data presented are mean values of each treatment. Differences between two groups were confirmed by *t*-test. To determine statistical significance among multiple comparisons, the Tukey HSD test was applied using the software SPSS statistics version. 22 for Windows. The significant difference between treatments were set as $P < 0.05$.

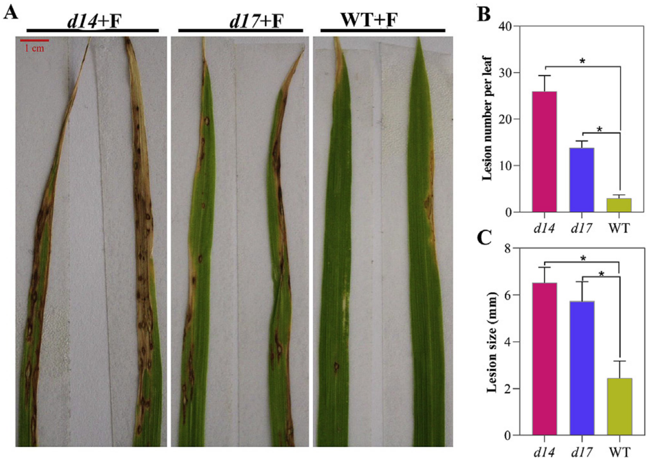

Fig. 1. Phenotypes of *d17*, *d14,* and WT after *Magnaporthe oryzae* infection. (A) Representative photographs showing disease symptoms. Four weeks old plants of strigolactones (SLs)-deficient *dwarf17* (*d17*), -signaling (*d14*) and the corresponding wild-type (WT) rice plants were spray-inoculated with a virulent *M. oryzae* (GUY11) at 1×10^4 mL^{-1} spore suspension. Disease severity was evaluated after seven days of *M. oryzae* challenge by (B) scoring the number susceptible-type disease lesion on the fourth leaves of each plant and (C) measuring the length of the three largest lesions per fourth leaf of the individual plant. Statistical analysis was carried out on the data averaged from five biological repeats. Asterisk symbol represents statistically significant differences ($P < 0.05$) between *d17* and *d14* compared with the WT. The red colored bar in the figure is 1 cm in length. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.8. Data availability

The raw RNA-seq data used in the current study can be accessed via the Sequence Read Archive accession number SRP151248 at the National Center for Biotechnology Information.

3. Results

3.1. SL-deficient and -signaling mutants reveal increased susceptibility to M. oryzae infection

To assess the possible role of SLs in defense against *M. oryzae,* the forth leaves of rice mutants that are defective in either SL biosynthesis

Table 1
List of primers used for validation of RNA-sequencing data.

Gene ID	Forward primer(5'→3′)	Reverse primer(5'→3′)	Amplicon size (bp)
Os12g0168700	TCTGCACTCAAGCCAACACT	CCAACTTCCATTGACTGCGG	156
Os06g0726200	CCGACCGGATTGGGTTCTAC	AGCCATTGTGGGCATTACTGA	217
Os08g0448000	GTAGTTGTCATCACGCGCAC	GAGCGGAAGACGAACTGCTC	132
Os04g0229100	CCAGAAGCAGATGCAGGCTA	ACTCACCGTCCTCTTACCGA	187
Os02g0627100	GTATCCGCTCTACCGGTTCG	GCCTCCACACTCCACTGTTAT	223
Os04g0483500	CGTCCATCAAGAAGGCGTCC	GCGGAGATGAGGAACCACAG	133
Os03g0290300	TTGAGGTTCACCATGCCGTT	CCAGCCAGGATGCAGTTGAT	154
Os01g0360200	CAAACCTGGAGTCGCTGCTG	GGTTCCGGTCGTGCGTCG	120
Os12g0541300	CAGGTTCAAGGACGACGACG	CACCCTCATCCACCCCTCT	95
Os03g0727600	TGAGGAGTGGCTGGAGAAGAAC	GTAGTCCTGGAACAGCGCG	97
Os01g0580500	GTGATCGCGCCGGCGACGGC	GGGGAACCCTGCGTACTAC	257
Os07g0176900	GTCTCCGTCCGCTTCCAC	CAGCCTCTTCAGGTCGTCC	150
Os06g0625700	GATGTGGGAGCAGGTGAAGG	TACACCTTCTCCACGAACAGCA	102
Os02g0143100	TGTCTGTGGGCACTGAGATAAC	CGCTGTTCCGACTCTACCTGA	151
Os03g0377700	GCTGGAATGTGAGGATTGGGCA	GCATAGTCCCACTCCATTCCCT	114
Tubulin	TACCGTGCCCTTACTGTTCC	CGGTGGAATGTCACAGACAC	234

Table 2

Statistics of the RNA-sequencing reads mapped to the reference genes. The four treatments were non-infected wild-type rice (WT), wild-type rice infected with *Magnaporthe oryzae* (WT + F), non-infected rice strigolactone-signaling *dwarf14* mutant (*d14*) and *d14* rice mutant infected *M. oryzae* (*d14* + F).

Sample name	Total raw reads (M)	Total clean bases (G)	Total clean reads (M)	Total mapped reads (M)	Percentage of reads mapped	Average percentage of reads mapped
WT-1	47.216	6.73	44.86	41.703	92.96	93.11
WT-2	43.115	6.08	40.565	37.618	92.74	
WT-3	54.751	7.65	51.024	47.784	93.65	
WT + F-1	44.657	6.45	42.971	40.229	93.62	94.17
WT + F-2	46.696	6.55	43.692	41.264	94.44	
WT + F-3	58.586	8.29	55.274	52.013	94.10	
d14-1	42.699	6.14	40.925	37.862	92.51	92.16
d14-2	47.253	6.74	44.914	41.827	93.13	
d14-3	45.199	6.7	44.675	40.592	90.86	
d14+F-1	41.087	5.88	39.172	36.637	93.53	93.45
d14+F-2	46.192	6.68	44.504	41.505	93.26	
d14+F-3	54.341	7.68	51.213	47.923	93.58	

(*d17*) or signaling (*d14*) and its corresponding WT rice were infected with a hemibiotrophic *M. oryzae* isolate, GUY11. Seven days later, the severity of disease was determined by evaluating the disease lesion size and number (Fig. 1). A significant increase in the susceptible-type lesion number was observed at 7 dpi in *d17*-and *d14*-infected plants compared to WT-infected plants (Fig. 1B). In addition, we also found a significant increase in the lesion size in both *d17*-and *d14*-infected plants relative to WT-infected plants (Fig. 1C). These results clearly indicate that SL-biosynthesis and -signaling pathways are important for positive regulation of defense against *M. oryzae* disease in rice.

3.2. Comparative leaf transcriptome analysis of the SL-signaling d14 and WT plants with or without M. oryzae inoculation

In order to gain insights into genes involved in the downstream pathways influenced by SL-regulated responses against *M. oryzae*, we conducted illumina RNA-sequencing (RNA-seq)-based comparative leaf transcriptome analysis of the *d14* and WT rice plants with or without *M. oryzae* infection. The RNA-seq results are summarized in Table S1. In total, ~571 million raw reads were recovered from the 12 samples (each sample had 41.087–58.586 million raw reads) (Table 2). After quality filtering, a total of ~543 million clean reads were derived with an average of 93.19% that could be mapped to the reference genome of rice. The percentage of clean reads from each sample to be mapped to the rice reference genome ranged from 90.86 to 94.44% (Table 2). As seen in Fig. 2A, the three independent biological repeats were highly grouped, proposing that expression patterns of samples of each group have high similarity and the transcriptome data can be utilized to study the differential expression of transcripts. Cluster and heat-map analyses of the differentially expressed genes (DEGs) based on Fragments Per Kilobase Million values of various comparisons are shown in Fig. 2B. To discriminate between DEGs in each comparison, the cut-off value of [fold change ≥ 1.5] and false discovery rate-corrected *P*-value (*q*-value) of ≤0.05 was applied (Fig. 3A–D; Table S2) (Li et al., 2016). The RNA-seq data were further confirmed by analyzing the expression levels of eight specific genes and seven randomly selected genes using quantitative real-time polymerase chain reaction (qRT-PCR) (Fig. 4; Additional file 2: Table S3). In general, the genes analyzed through qRT-PCR showed almost the same trend in their expression levels as that of RNA-seq (Fig. 4; Additional file 2: Table S3), which further confirms the reliability and accuracy of the RNA-sequencing data. The expression patterns of eight specific genes related ET, H_2O_2 and carbohydrate pathways in various comparisons are shown in Fig. 4.

In total, 7367 genes were identified as DEGs among *d14* and WT comparison, of which 3984 genes were being up-regulated and 3383 were down-regulated in *d14* with respect to WT (Fig. 3A; Tables S2A and E). In addition, we compared the leaf transcriptome data sets of *M. oryzae* infected *d14* (*d14* + F) plants with WT infected (WT + F) at 36 hpi. Of total, 5388 identified DEGs about 2439 and 2949 genes were up- and down-regulated, respectively, in the *d14* + F relative to W + F (Fig. 3B; Tables S2B and F). Furthermore, there were 4476 DEGs (of which 2576 up-regulated and 1900 down-regulated transcripts) in WT infected compared with WT (Fig. 3C; Table S2C and G), and 6677 DEGs (of which 3423 up-regulated and 3254 down-regulated transcripts) in *d14* infected (*d14* + F) compared with non-infected *d14* leaves (Fig. 3D; Tables S2D and H). As shown in Fig. 3E, a large number of transcripts overlapped in various comparisons, while considerable number of transcripts uniquely expressed as well.

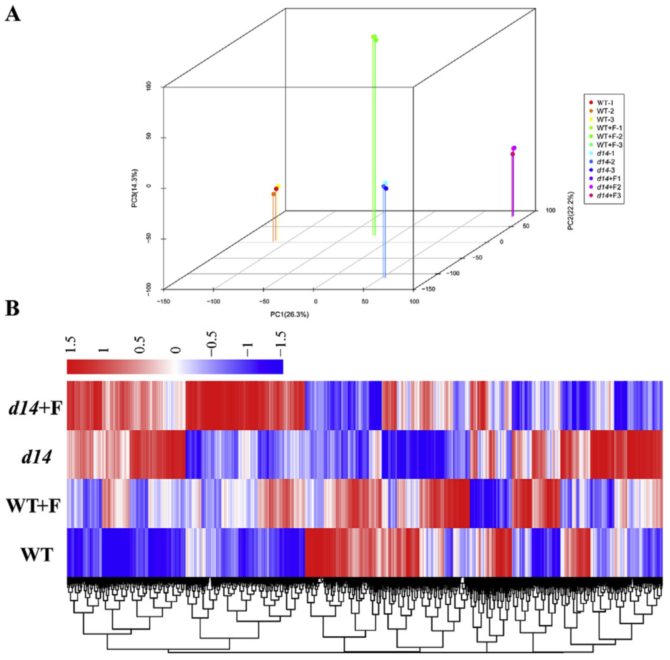

Fig. 2. RNA-seq profiling of *d14* and WT during *Magnaporthe oryzae* infected and non-infected conditions.

(A) The principal component analysis among the biological repeats per treatment. (B) Heat map with hierarchical clustering of genes based on read count values of four treatments. In the color scale, the blue and red color gradients (ranging from −1.5 to 1.5) represent decrease and increase in the gene expression based on read count values, respectively. The color scale in based on the log10(FPKM+1) values. The four treatments were non-infected wild-type rice (WT), wild-type rice plants infected with *Magnaporthe oryzae* (WT + F), non-infected rice strigolactone-signaling *dwarf14* mutant (*d14*) and *d14* rice mutant infected with *M. oryzae* (*d14* + F). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.3. MapMan analysis of biotic stress pathway

In order to obtain a comprehensive functional overview of the SL-mediated defense, we conducted MapMan-based functional annotation analysis of the biotic stress pathway of the DEGs identified in *d14* plants and WT during *M. oryzae* infected and non-infected conditions. First, with the aim to identify SL-inducible and/or downstream genes and to detect functional biotic stress overview/pathway, the resulting DEGs in *d14* vs. WT comparison were annotated using MapMan. Accordingly, MapMan-based analysis of the biotic stress overview showed that a large number of transcripts with reduced expression levels in the *d14* mutant line were related to the plant's defense responses (Fig. 5A). The significant reduction in the expression levels of variety of defense-associated genes in *d14* mutant plants, pointing towards the supportive

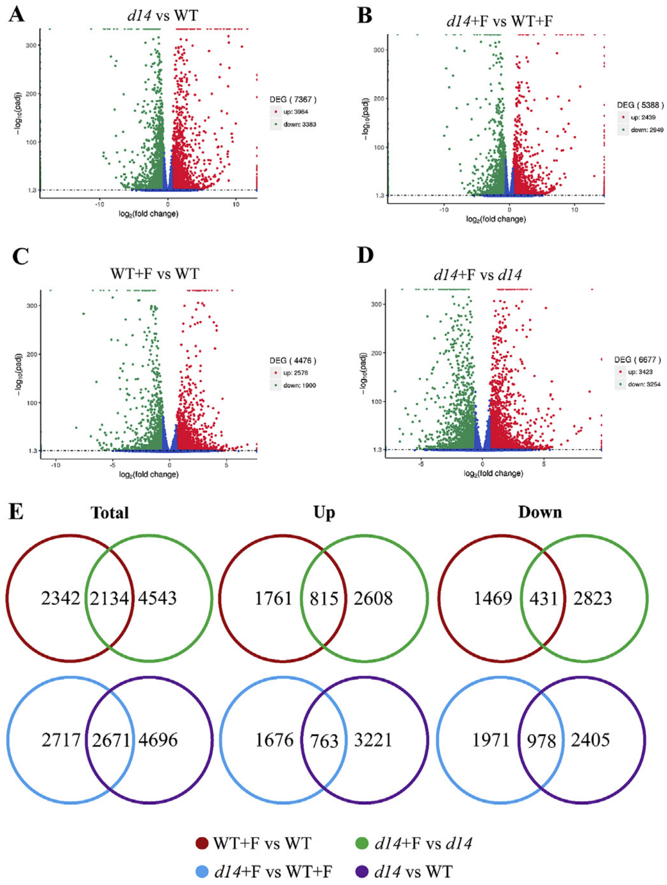

Fig. 3. The differentially expressed gene of the four different comparisons. (A–D) Volcano plots representing significantly up- and down-regulated transcript sets in four different comparisons. (E) Venn analysis of the total, up- and down-regulated gene sets obtained from various comparisons. The four treatments were non-infected wild-type rice (WT), wild-type rice infected with *Magnaporthe oryzae* (WT + F), non-infected rice strigolactone-signaling *dwarf14* mutant (*d14*) and *d14* rice mutant infected with *M. oryzae* (*d14* + F).

role of SLs in regulation of plant defense responses. Thus, to confirm this hypothesis, next we performed MapMan-based biotic stress pathway analysis of the DEGs obtained from *d14* + F vs. WT comparison. Here, in the biotic stress pathway the three gene categories were found to be of specific interest (Fig. 5B).

3.4. Differential expression of cell wall-related genes

First, by analyzing biotic stress overview pathway we found that in

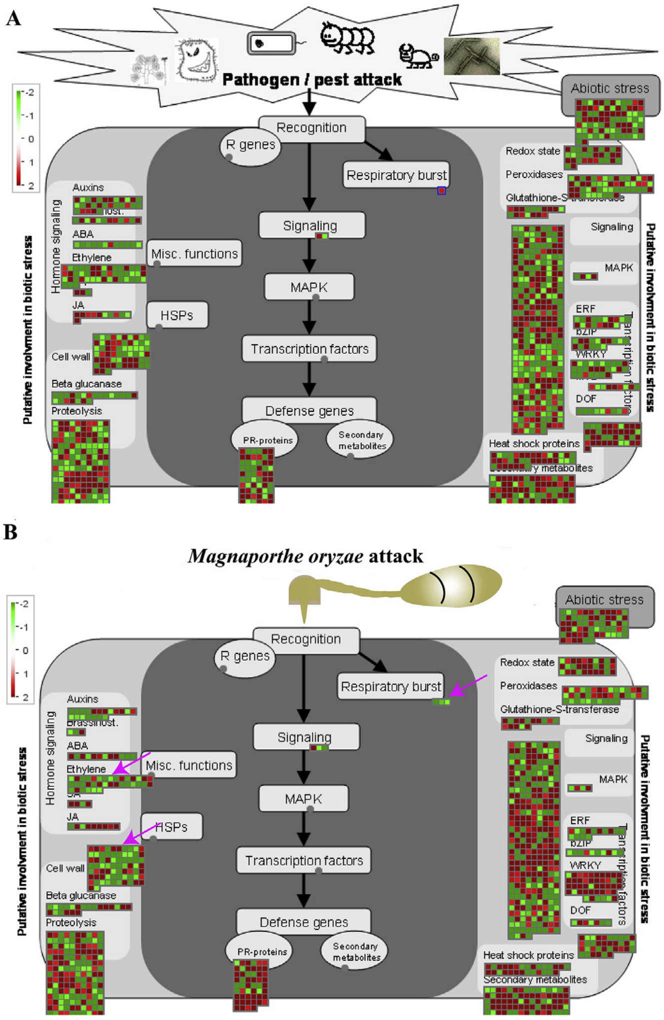

Fig. 5. MapMan analysis of the transcripts related to biotic stress pathway. (A) Biotic stress overview of the differentially expressed genes (DEGs) obtained from *d14* vs. WT comparison. (B) Biotic stress overview of the DEGs obtained from *d14* + F vs. WT + F comparison. Red and green colors show up- and down-regulated transcripts, respectively. The colored boxes in each field indicate fold changes in gene expression. In the color scale, the green and red color gradients (ranging from −2 to 2) represent down- and up-regulation of genes, respectively. The four treatments were non-infected wild-type rice (WT), wild-type rice infected with *Magnaporthe oryzae* (WT + F), non-infected rice strigolactone-signaling *dwarf14* mutant (*d14*) and *d14* rice mutant infected with *M. oryzae* (*d14* + F). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

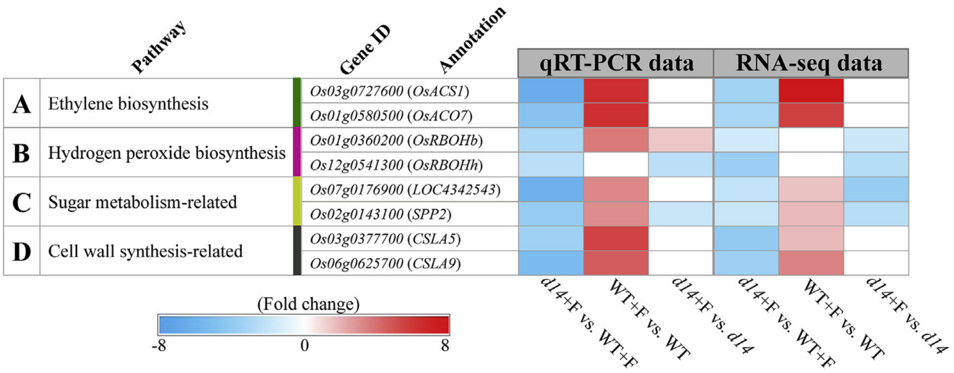

Fig. 4. Heatmap of the qRT-PCR and RNA-sequencing data of the validated genes related to (A) ethylene biosynthesis (B) hydrogen peroxide biosynthesis and (C) sugar/carbohydrate metabolism and (D) cell wall-synthesis The four treatments were non-infected wild-type rice (WT), wild-type rice infected with *Magnaporthe oryzae* (WT + F), non-infected rice strigolactone-signaling *dwarf14* mutant (*d14*) and *d14* rice mutant infected with *M. oryzae* (*d14* + F). In the color scale, the blue and red color gradients (ranging from −8 to 8) represent fold change decrease and increase, in the gene expression, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

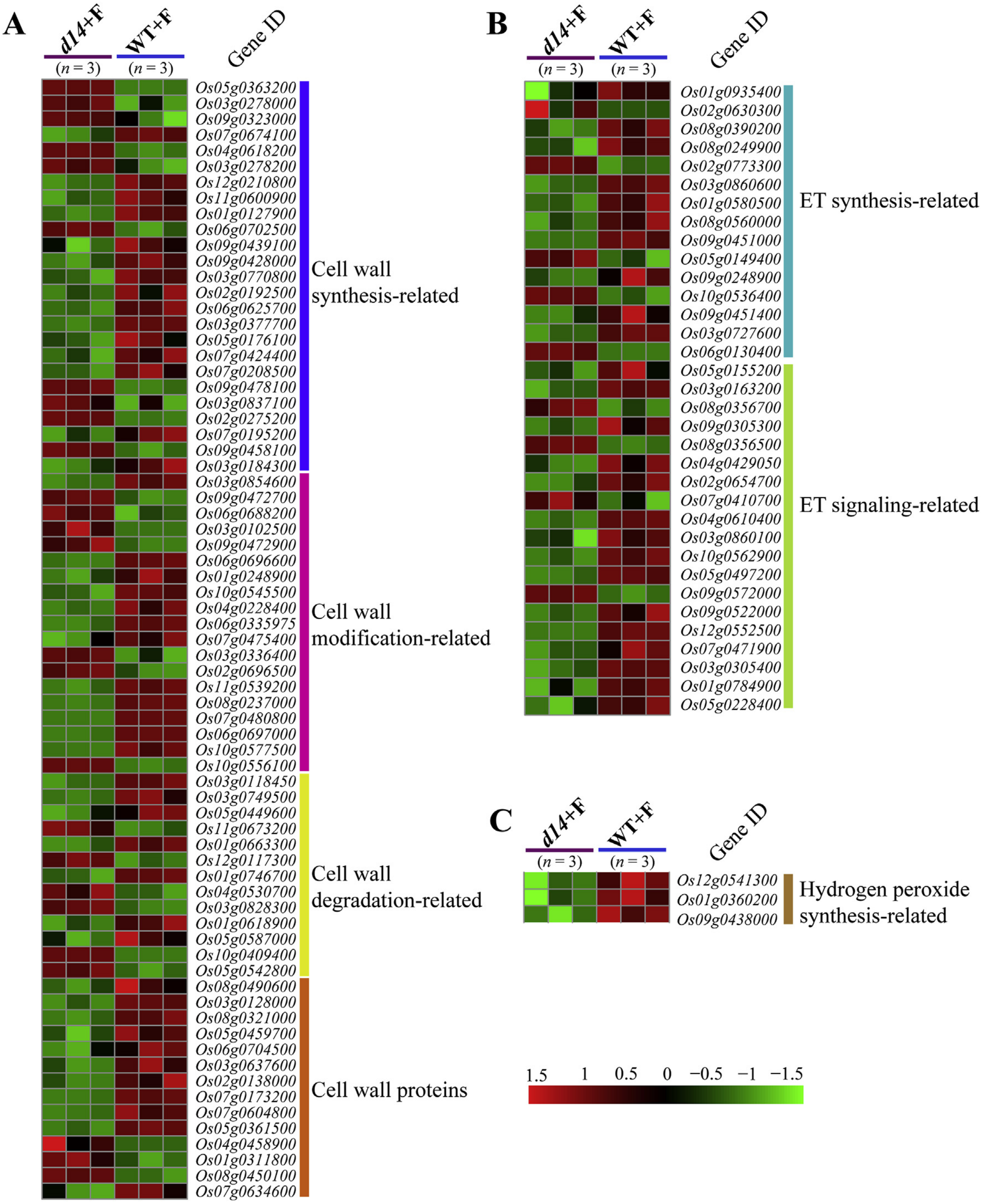

Fig. 6. Heat map of the differentially expressed genes in *d14* + F vs. WT + F comparison that are involved in (A) cell wall synthesis, modification and degradation, (B) ethylene (ET) biosynthesis and signaling, and (C) hydrogen peroxide biosynthesis. In the color scale, the green and red color gradients (ranging from −1.5 to 1.5) represent decrease and increase, respectively, in the abundance of transcripts based on log10(FPKM+1) transformed values in *M. oryzae*-infected strigolactone-signaling (*d14* + F) and *M. oryzae*-infected (WT + F) plants. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the *d14* + F vs. WT + F comparison considerable number of cell wall-related genes were down-regulated in *d14* + F with respect to the WT + F (Fig. 6A; Table S4A). Closer examination revealed that most of the down-regulated genes in *d14* + F pertaining to cell wall biosynthesis and modification (Fig. 6A; Table S4A). In the cell wall synthesis category we observed key DEGs coding enzymes for cellulose (9 down- and 2 up-regulated genes), hemicellulose (3 down- and 2 up-regulated genes) and cell wall precursor synthesis (4 down- and 6 up-regulated genes) (Fig. 6A; Table S4A). Here, the set of down-regulated genes which are likely involved in cellulose synthesis include cellulose synthase family A (*OsCesA1/Os05g0176100*), (*OsCesA3/Os07g0424400*), (*OsCesA8/Os07g0208500*), cellulose synthase-like family A (*OsClsA1/Os02g0192500*, *OsCslA5/Os03g0377700* and *OsCslA9/Os06g0625700*), cellulose synthase-like family C (*OsCslC2/Os09g0428000* and *OsCslC9/Os03g0770800*), and a cellulose synthase-like family E (*OsCslE1/Os09g0478100*). In addition, of the 19 DEGs participating in cell wall modification, 13 have been recorded with lower expression in *d14* + F relative to the WT + F (Fig. 6A; Table

S4A). These included cell wall loosening-related genes such as xyloglucan endo-transglycosylase/hydrolases (*XTHs*) and expansions (*EXPs*). Of these DEGs, nine *XTH* (*Os03g0854600*, *Os06g0696600*, *Os10g0545500*, *Os06g0335975*, *Os11g0539200*, *Os08g0237000*, *Os07g0480800*, *Os06g0697000* and *Os10g0577500*) genes and three *EXP* (*Os01g0248900*, *Os04g0228400* and *Os07g0475400*) genes were appeared to be down-regulated in the *d14* + F compared to WT + F, respectively, while the expression of the one *XTH* (*Os02g0696500*) gene and six *EXP* (*Os09g0472700*, *Os06g0688200*, *Os03g0102500*, *Os09g0472900*, *Os03g0336400* and *Os10g0556100*) genes were induced in the respective comparison (Fig. 5A; Table S4A). As seen in Fig. 6A; Tables S4A and 12 cell wall degradation-related DEGs (7 down- and 6 up-regulated) were also identified in the *d14* + F vs. WT + F comparison. Besides, for genes associated with cell wall protein sub-families including arabinogalactan-proteins (AGPs) and extensins (EXTs) there were 9 down- and 0 up-regulated genes in the *d14* + F with respect to the WT + F (Fig. 6A; Table S4A). For instance, genes (*Os08g0490600/OsFLA4*) and *Os06g0704500* coding for AGP and EXT respectively were down-regulated in *d14* + F (Fig. 6A; Table S4A). Previous studies have shown that AGP and EXT proteins are not only involved in the regulation of cell wall formation and plant developmental processes but also play a defensive role during plant-pathogen interaction (Deepak et al., 2010). Furthermore, we identified 5 pectin methylesterase coding DEGs (2 down- and 3 up-regulated genes) in the *d14* + F vs. WT + F comparison (Fig. 6A; Table S4A), which is thought to be involved in cell wall metabolism. Given the fact that the lower expression of a large number of cell wall biosynthesis- and modification-related genes in *d14* plant compared to WT in response to pathogen infection have been observed (Fig. 6A; Table S4A), proposing that *d14* lines may possess thinner cell wall relative to WT plants. Hence, the cell wall-associated defense might be compromised in the mutant plants.

3.5. Suppression of ET -related genes

It is widely accepted that successful defense of the host plant is largely dependent on phytohormone biosynthesis, signaling and its cross-communication. For instance, during rice-*M. oryzae* interaction, ET serves as critical defense signaling molecule and is well-known to positively regulate basal defense against *M. oryzae* (Nasir et al., 2018). Given the importance of ET in rice defense against *M. oryzae*, special attention has been paid to the ET-related genes. We observed that the set of considerable number of ET-related genes were suppressed in *d14* plants relative to the WT during *M. oryzae* invasion (Fig. 6B; Table S4B). For example, 4 candidate ET-biosynthesis genes, *1-aminocyclopropane-1-carboxylate synthase 1* (*OsACS1/Os03g0727600*), *1-aminocyclopropane-1-carboxylate oxidase 1* (*OsACO1/Os09g0451000*), (*OsACO2/Os09g0451400*), (*OsACO7/Os01g0580500*) and, various ET-signaling-related (especially, ethylene responsive transcription factor genes) genes listed in Table S4B in detail were down-regulated in *d14* + F plants with respect to the WT + F plants (Fig. 6B). Here, our transcriptome results indicate that SLs induce ET-related genes/pathway, especially during biotic stress condition in rice.

3.6. Suppression of hydrogen peroxide synthesis-related genes

Previous studies have shown that upon *M. oryzae* infection host plant triggers the expression of H_2O_2 biosynthesis genes including *respiratory burst oxidase homologB* (*OsRBOHb*), thereby resulting H_2O_2 generation and enhanced resistance to *M. oryzae* (Nasir et al., 2018). Interestingly, we found that the expression levels of three H_2O_2 biosynthesis-related genes, *respiratory burst oxidase homologB* (*OsRBOHb/Os01g0360200*) (*OsRBOHg/09g0438000*) and (*OsRBOHh/Os12g0541300*) were down-regulated in *d14* + F plants compared to the WT + F plants (Fig. 6C; Table S4C). This suggests that SLs function in the positive regulation of H_2O_2, especially during rice blast infection. As mentioned earlier H_2O_2 production promotes disease resistance against the *M. oryzae*, thus impairment in the accumulation of H_2O_2 might be correlated with hypersusceptibility of these mutants toward *M. oryzae* infection.

3.7. Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) pathway analysis

In the next line of observation, to identify the enriched biochemical pathways in the *d14* and WT under *M. oryzae* infected and non-infected conditions, we subjected the DEGs identified in various comparisons to KEGG pathway analysis. The KEGG analysis revealed that among the up-regulated genes of WT + F vs. WT and *d14* + F vs. *d14* comparisons the pathways “biosynthesis of secondary metabolites”, “diterpenoid biosynthesis” “porphyrin and chlorophyll metabolism” and “plant hormone signal transduction” were commonly enriched in the both the respective comparisons (Additional file 2; Table S5). On the contrary, the metabolic pathways such as “cysteine and methionine metabolism”, “carbon fixation in photosynthetic organisms”, “carotenoid biosynthesis”, “pentose phosphate pathway”, “sulfur metabolism”, “metabolic pathway”, “terpenoid backbone biosynthesis”, “fatty acid elongation”, and “glyoxylate and dicarboxylate metabolism”, and “phenylpropanoid biosynthesis”, “plant-pathogen interaction”, “phenylalanine metabolism”, “alpha-Linolenic acid metabolism”, “flavonoid biosynthesis” and “fatty acid degradation” pathways were exclusively enriched in the WT + F vs. WT and *d14* + F vs. *d14* comparisons, respectively (Additional file 2; Table S5). The number of significantly enriched metabolic pathways were higher in the WT + F vs. WT comparison relative to *d14* + F vs. *d14* comparison (Additional file 2: Table S5), suggesting that WT plants may have more complex metabolic defensive responses than mutant lines. Interestingly, the KEGG analysis of down-regulated genes in *d14* + F with respect to the WT + F plants revealed that at least seven highly enriched metabolic pathways were closely related to sugar metabolism which include “carbon metabolism”, “starch and sucrose metabolism”, “pentose phosphate pathway”, “glyoxylate and dicarboxylate metabolism”, “photosynthesis”, “carbon fixation in photosynthetic organisms” and “photosynthesis – antenna proteins” (Fig. 7; Table S6), indicating that these sugar metabolic pathways are regulated by SLs. Notably, a glucose signaling gene (*Os01g0190400/hexokinase 8/HXK8*) was down-regulated in *d14* + F relative to the WT + F (Table S6). Besides, we observed that the expression levels of two sucrose synthase genes (*Os02g0831500/sucrose synthease 6* and *Os04g0309600*) and four fructose-1,6-bisphosphatase coding genes (*Os04g0234600*, *Os01g0866400*, *Os06g0664200* and *Os11g0171300*) were also significantly suppressed in *d14* + F with respect to the WT + F (Table S6). Additionally, ribose 5-phosphate isomerase encoding genes such as; *Os03g0781400*, *Os04g0306400*, *Os07g0176900* and *Os03g0100040* and genes *Os12g0274700*, *Os12g0291400*, *Os12g0291100* and *Os12g0292400*, which are coding for ribulose bisphosphate carboxylase were also remarkably down-regulated in *d14* + F relative to the WT + F (Table S6). It is to be noted that sugar molecules not only serve as typical energy signal molecules to provide energy and structural support to plant cell, but also regulate defense against pathogens including that of *M. oryzae* (Gómez-Ariza et al., 2007; Bolouri and Van, 2012). Thus, based on the results of the KEGG metabolic pathway enrichment analysis, we hypothesized that the hyper-susceptibility of the mutant plants might also be due to the mis-regulation of sugar synthesis/signaling pathways.

3.8. Impaired accumulation of H_2O_2 and soluble sugar content in SL-deficient d17 and SL-signaling d14 mutants during M. oryzae infection

As reported above in our transcriptome data that ROS (H_2O_2) biosynthesis- and sugar-metabolic pathways were significantly down-regulated in the *d14* plants relative to WT after *M. oryzae* treatment (Figs. 6B and 7). Thus it is likely that H_2O_2 and sugar contents were reduced in the *d14* and *d17* mutants, which might be correlated with

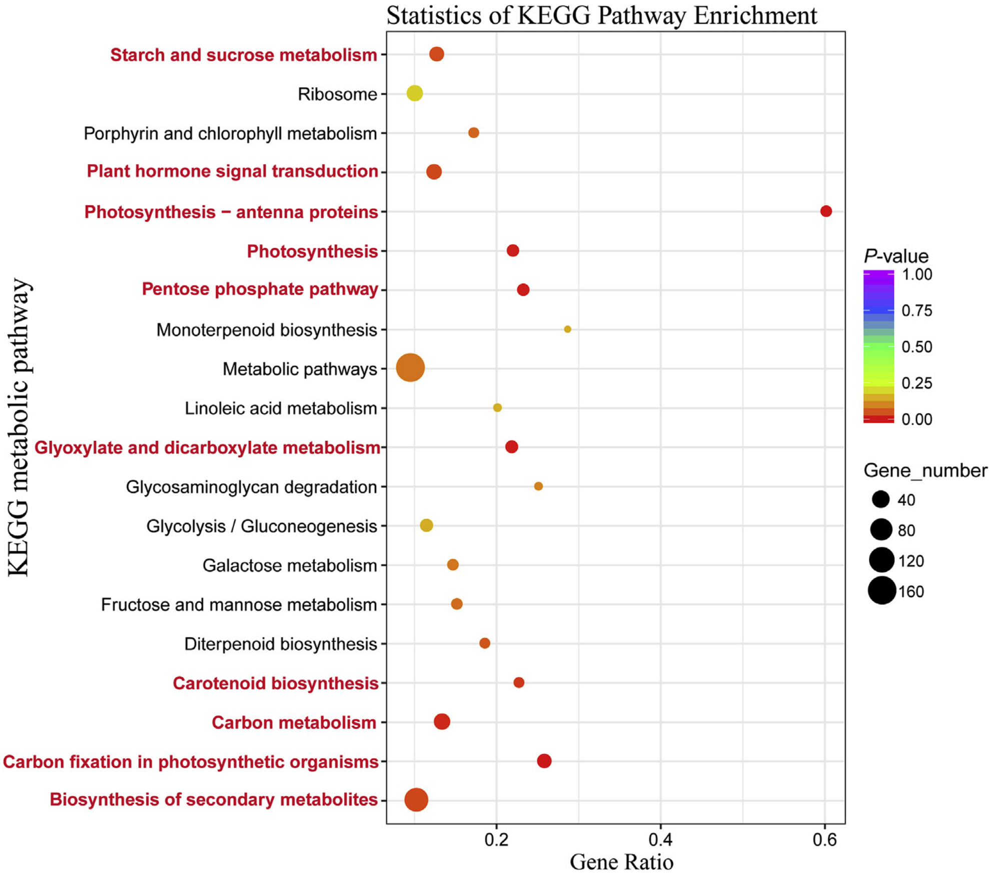

Fig. 7. Highly enriched Kyoto Encyclopedia of Genes and Genomes pathway (KEGG) metabolic pathways of down-regulated genes in *M. oryzae*-infected strigolactone-signaling (*d14* + F) relative to *M. oryzae*-infected (WT + F) obtained from the comparison *d14* + F vs. WT + F.

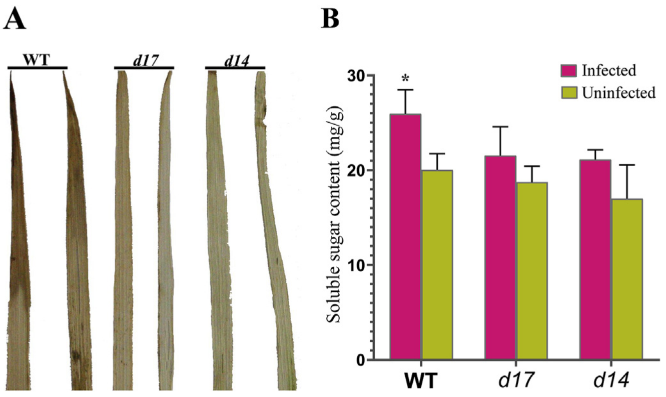

Fig. 8. Accumulation of H_2O_2 and sugar content in *d17*, *d14* and WT rice plants during *Magnaporthe oryzae* infection. (A) Representative photographs depicting hydrogen peroxide (H_2O_2) accumulation in the fourth leaves of four-week-old plants of WT, *d17* and *d14* at 36 h of *M. oryzae* infection. *In-situ* accumulation of H_2O_2 was detected by 3, 3′-diaminobenzidine staining. The six treatments were non-infected wild-type rice (WT), wild-type rice infected with *Magnaporthe oryzae* (WT + F), non-infected rice strigolactone-biosynthetic *dwarf17* mutant (*d17*), rice *d17* mutant infected *M. oryzae* (*d17* + F), non-infected strigolactone rice signaling *dwarf14* mutant (*d14*) and rice *d14* mutant infected *M. oryzae* (*d14* + F). (B) Soluble sugar content in wild-type (WT), strigolactone (SL)-deficient *dwarf17* (*d17*) and SL-signaling *dwarf14* (*d14*) rice during *Magnaporthe oryzae* infected and non-infected conditions at 36 h post inoculation. The error bars show mean standard deviation. *Statistical analysis was carried out on the data averaged from three biological repeats.

hypersusceptibility of the respective mutants toward *M. oryzae*. Thus to valid the RNA-seq data biochemically and to assess whether the increased susceptibility of the *d17* and *d14* plants involve impaired accumulation of H_2O_2 and sugar, we carried out *in situ* histochemical DAB staining, which shows the production of reddish-brown fromazan precipitates (an indicative of H_2O_2 accumulation) and soluble sugar production analysis. In fact, as illustrated in Fig. 8A and B, the levels of DAB (H_2O_2) staining and soluble sugar content were remarkably lower in the leaves of *d17* and *d14* plants relative to WT, during *M. oryzae* infection, respectively. Together with transcriptome data, these results support the notion that SLs are likely positively regulate H_2O_2 and sugar synthesis/signaling, which may function in defense against *M. oryzae.*

3.9. Impact of strigolactone on M. oryzae growth

Previously, it is has been proposed that SL not only promotes hyphal branching and spore germination of arbuscular mycorrhizal fungi, but also affects the development of various fungal pathogens (Dor et al., 2011). Thus, it was of interest to assess the possible direct impact of SL on *M. oryzae* development. To achieve this an *in-vitro* assay with different concentrations of GR5 and *M. oryzae* was performed. As shown in Fig. S1, no direct effect on the *M. oryzae* growth pattern was observed by any of the GR5 concentrations applied. This finding further supported the *in-planta* SL-mediated defense mechanisms, rather than its direct influence on *M. oryzae* growth.

4. Discussion

Rice blast fungus, *M. oryzae* possesses a major threat to global food security, adversely affecting both rice crop yield and quality (Nasir et al., 2018). Given the importance of plant hormones in induction of host defense, tremendous progress has been made in the last two decades on understanding the role of these phytohormones in rice plant defense against *M. oryzae* (Yang et al., 2013; Nasir et al., 2018). In this context, it has been demonstrated that ET, JA and SA promotes disease resistance against *M. oryzae* in rice (Yang et al., 2013). On the other hand, regarding the role of SLs in defense Torres-Vera et al. (2014), noticed that tomato SL-biosynthetic (*CCD8*) mutant lines are more susceptible to infection caused by *B. cinerea* and *A. alternate*, accompanied with marked reduction in JA, SA and ABA content, suggesting that SL-mediates defense against these pathogens by interacting with other defense-associated hormones. Consistent with this, the involvement of SLs in disease resistance against moss (*Physcomitrella patens*), has also been confirmed (Decker et al., 2017). In contrast, it has been confirmed that SL-biosynthetic pathway is not necessary for disease resistance against *F. oxysporum* in pea (*Pisum sativum* L.) (Foo et al., 2016). Although in recent years various reports underpinned the important role of SLs in defense against specific phytopathogens (Marzec, 2016), its possible regulatory role and underlying mechanisms in defense against the most devastating rice blast fungus *M. oryzae* is lacking at present. Thus, we aimed to study the role of SL-biosynthesis and –signaling in rice defense against *M. oryzae* by using transcriptome and biochemical analyses, and reverse-genetics screening using SL-biosynthetic (*d17*) and –signaling (*d14*) mutants of rice.

Here, our study provides evidence that rice SL-biosynthesis and –signaling pathways function in positive regulation of defense to *M. oryzae.* Indeed, *d17* and *d14* mutants exhibited increased susceptibility to *M. oryzae* infection relative to the corresponding WT rice plants (Fig. 1), which clearly indicates that SLs have a positive role in defense against *M. oryzae*. Moreover, to unravel the molecular basis of SL-mediated defense against *M. oryzae*, we conducted a comparative leaf transcriptome profiling of the *d14* and WT rice plants under both infected and non-infected conditions. The results of the leaf transcriptome clearly show that a large number of defense-related genes were suppressed in *d14* + F vs. WT + F comparison (Fig. 5B; Table S3F).

RNA-seq-based transcriptome analysis let to the identification of different downstream genes/pathways through which SLs are likely confer resistance to *M. oryzae.* In this regard, MapMan-based analysis of the biotic stress overview revealed the transcriptional mis-regulation of a large number of cell wall-related genes in the *d14* + F plants compared to WT + F (Fig. 6A; Table S4A). Various cell wall structural proteins such as cellulose, hemicellulos and hydroxyproline-rich glycoproteins (HRGPs) synthesis associated genes, were observed in our study, with significantly low expression in the *d14* plants relative to WT, during *M. oryzae* infection. For example, a large proportion of DEGs pertaining to the cellulose synthesis were down-regulated in *d14* + F vs. WT + F comparison (Fig. 6A; Table S4A). These, include cellulose synthase family A (*OsCesA1/Os05g0176100*), (*OsCesA3/Os07g0424400*), (*OsCesA8/Os07g0208500*), cellulose synthase-like family A (*OsCslA1/Os02g0192500*, *OsCslA5/Os03g0377700* and *OsCslA9/Os06g0625700*), cellulose synthase-like family C (*OsCslC2/Os09g0428000* and *OsCslC9/Os03g0770800*), and a cellulose synthase-like family E (*OsCslE1/Os09g0478100*). Consistent with this, of the 19 DEGs functioning in the modification of cell wall, 13 were found with decreased expression levels in *d14* + F relative to WT + F comparison (Fig. 6A; Table S4A). These mainly consist of cell wall loosening-associated genes such as xyloglucan endo-transglycosylase/hydrolases (*XTHs*) and expansions (*EXPs*). An example of three XTH and three EXP down-regulated genes in *d14* + F with respect to WT + F comparison was *XTH* (*Os03g0854600*, *Os06g0696600*, *Os10g0545500*) and *EXP* (*Os01g0248900*, *Os04g0228400* and *Os07g0475400*) (Fig. 6A; Table S4A). Previous, studies have shown that the XTH and EXP genes are involved in cell wall remodeling (Majewska-Sawka and Nothnagel, 2000; Sharova, 2007). Besides, for genes associated with cell wall proteins arabinogalactan-proteins (AGPs) and extensins (EXTs) which there were 9 down- and 0 up-regulated genes in the *d14* + F compared to WT + F (Fig. 6A; Table S4A). In this regard, genes (*Os08g0490600/OsFLA4*) and *Os06g0704500* coding for AGP and EXT respectively were down-regulated in *d14* + F vs. WT + F comparison as illustrated in (Table S4A) in detail. It is well-known that as a first line of defense response host plant fortifies the cell wall to render the pathogen penetration and/or successful invasion (Malinovsky et al., 2014). Supporting the role of cell wall components such as cellulose in biotic stress tolerance, recently, it has been demonstrated that, upon pathogen attack such as *Blumeria gramis f.* sp. *Hordei* in barley cellulose participates in the formation of papillae (reinforcement of the cell wall at infection sites) (Chowdhury et al., 2014). Besides, reports have shown that hydroxyproline-rich glycoproteins (HRGPs) which includes AGPs and EXTs are not only involved in the regulation of cell wall formation and plant developmental processes but also play a defensive role during plant-pathogen interaction (Deepak et al., 2010). For instance, upon pathogen infection induced HRPGs accumulation have been reported in the resistant pearl millet (*Pennisetum glaucumi*), tobacco (*Nicotiana tabacum*) and sorghum (*Sorghum bicolor*) cultivars (Deepak et al., 2010). Moreover, the increased accumulation of HRPGs have been correlated with the improved defense of these cultivars. Supporting the notion that SLs participate in composition of cell wall components, it has already been confirmed that *A. thaliana* SL-signaling (*max2*) mutants impaired in SL signaling developed thinner cuticle (which is an important part of cell wall) compared with the respective WT (Bu et al., 2014), suggesting that SLs contribute to cuticle (cell wall component) formation as well. Thus, based on transcriptome results we propose that the increased susceptibly of the *d17* and *d14* is probably associated with the alteration in the cell wall structural composition of the respective mutant plants.

Plant hormones including ET are well-known for their regulatory roles in defense against variety of phytopathogens. In this context, the defensive role of ET has already been proved against *M. oryzae*. For example, ET mutants defective in either ET-biosynthesis or signaling render rice plants more susceptible to infection caused by *M. oryzae* (Yang et al., 2016; Nasir et al., 2018). Notably, by analyzing biotic stress overview via MapMan, we observed that a large number of ET-related (including ET-biosynthesis and –signaling genes) genes were remarkably down-regulated in *d14* + F with respect to the WT + F (Fig. 6B; Table S4B). For instance, candidate ET-biosynthesis genes such as; *1-aminocyclopropane-1-carboxylate synthase 1* (*OsACS1/Os03g0727600*), *1-aminocyclopropane-1-carboxylate oxidase 1* (*OsACO1/Os09g0451000*), (*OsACO2/Os09g0451400*), (*OsACO7/Os01g0580500*) and ET-responsive (*OsERF91/Os02g0654700, OsERF77/Os04g0610400, OsERF83, Os03g0860100* and *OsERF96, Os10g0562900*) genes were significantly suppressed in *d14* + F plants with respect to WT + F (Fig. 6B; Table S4B). ET biosynthesis is closely linked with induction of *ACS* gene transcription (Yamamoto et al., 1995; Barry et al., 2000). Notably, a previous report has shown that *OsACS1* and *OsACO7* are among the ET biosynthesis genes induced in response to *M. oryzae,* accompanied with increased ET synthesis in rice (Takayoshi et al., 2006), suggesting that these ET biosynthesis genes are critical for *M. oryzae*-induced accumulation of ET. As illustrated in Fig. 4A, in addition to transcriptome data, qRT-PCR analysis also showed that these two ET biosynthesis-related genes (*OsACS1* and *OsACO7*) were significantly down-regulated in *d14* + F plants compared to WT + F plants. This finding proposes an impact of SLs on ethylene synthesis, which may affect biotic stress tolerance of rice. Supporting the idea that SLs promote ET biosynthesis, two independent studies have already been shown that SLs positively regulate ET biosynthesis, resulting the regulation of root hair growth and seed germination in *A. thaliana* and parasitic weed plant *Striga hermonthica*, respectively (Sugimoto et al., 2003; Kapulnik et al., 2011). In addition, Kapulnik et al. (2011) also showed that application of synthetic SL (GR24) significantly induces the expression of *At-ACS2* in *A. thaliana,* giving idea that SLs act upstream of ET-biosynthesis pathway.

Furthermore, in biotic stress pathways we observed that the expression of three H_2O_2 biosynthesis-related genes (*respiratory burst oxidase homologB* (*OsRBOHb/Os01g0360200*) (*OsRBOHg/09g0438000*) and (*OsRBOHh/Os12g0541300*) were also remarkably suppressed in the *d14* + F plants compared to the WT + F (Fig. 6C; Table S4C). It has been reported that upon infection including *M. oryzae,* rice induces the accumulation H_2O_2 by induction of its biosynthesis genes such as *OsRBOHb* (Yang et al., 2016; Nasir et al., 2018). Here, the suppression of candidate H_2O_2 synthesis *OsRBOHb* gene in *d14* + F plants compared to WT + F plants was not only observed in transcriptome data but its expression was also found to be significantly down-regulated in qRT-PCR data (Fig. 4B). This implies that SLs induce the accumulation of H_2O_2 in response to *M. oryzae* infection. To further investigate this claim, and validate the results of our transcriptome data biochemically as well, we assessed the histochemical H_2O_2 detection in *d17*, *d14* and WT rice plants during *M. oryzae* infection. As illustrated in Fig. 8A, a clear reduction was observed in H_2O_2 intensities in both *d17* and *d14* mutants compared to WT during *M. oryzae* infection. Support for the suggestion that SLs induce H_2O_2 biosynthesis, it is worth noting that a very recent study also has proved that SLs promote H_2O_2 in *A. thaliana* in ABA-independent manner, leading to stomatal closure (Lv et al., 2017). Upon *M. oryzae* challenge, the reduced expression levels of H_2O_2-biosynthesis-related genes (Fig. 6C; Table S4C), supported by the observed reduction in the accumulation of H_2O_2 in *d17* and *d14* relative to WT (Fig. 8A) suggest that SLs promote disease resistance to *M. oryzae* probably by positively regulating H_2O_2 biosynthesis. The outlined reports and our study clearly establish the link between SLs and H_2O_2.

It was observed that the set of down-regulated genes of *d14* + F relative to WT + F were significantly enriched in various KEGG metabolic pathways including carbon and/or sugar metabolism-related pathways (Fig. 7), pointing toward the regulatory role of SLs in sugar biosynthesis and signaling. Closer inspection revealed that two sucrose synthase genes (*Os02g0831500/sucrose synthease 6* and *Os04g0309600*) and four fructose-1,6-bisphosphatase coding genes (*Os04g0234600*, *Os01g0866400*, *Os06g0664200* and *Os11g0171300*) were among the down-regulated genes in *d14* + F with respect to the WT + F (Table S6). Similarly, genes coding for ribose 5-phosphate isomerase (*Os03g0781400*, *Os04g0306400*, *Os07g0176900* and *Os03g0100040*) and ribulose bisphosphate carboxylase (*Os12g0274700*, *Os12g0291400*, *Os12g0291100* and *Os12g0292400*) were also down-regulated in *d14* + F compared with WT + F (Table S6). A glucose signaling gene (*Os01g0190400/hexokinase 8/HXK8*) was also observed to be down-regulated in *d14* + F relative to the WT + F (Table S6). Notably, hexokinase genes are not only involved in glucose sensing but also playing a positive regulatory role in defense against variety of plant pathogens. For instance, it has been shown that introduction of *AtHXK1* and *AtHXK2* in *Nicotiana benthamiana* enhanced disease resistance to *Alternaria brassicicola*, accompanied with induced accumulation of H_2O_2 (Sarowar et al., 2008). In addition, the phosphorylation of d-allose by mitochondrial HXKs increases the production of ROS, thereby leading to improved resistance to *Xanthomonas oryzae* in rice (Kano et al., 2013). As our transcriptome data pointing towards the role of SLs in sugar metabolism and signaling, therefore, we measured soluble sugar content in *d17*, *d14* and WT plants during *M. oryzae* infected and non-infected conditions. Our data reflect that soluble sugar content was significantly reduced in both mutants compared with WT in response to *M. oryzae* infection (Fig. 8B). Supporting the argument that SLs function in the regulation of sugar synthesis, a recent report observed significant reduction in sugar content (glucose and fructose) in both *A. thaliana* SL-biosynthetic (*max1*) and signaling (*max2*) mutants relative to the corresponding WT plants (Li et al., 2016). Li et al. (2016) further confirmed that *A. thaliana* SL-mutants are *sugar insensitive mutants*, proposing that SLs act upstream of sugar signaling. It is worth noting that resistance and/or susceptibility of the host plant is also closely related with alteration in the sugar biosynthesis and signaling pathways (Bolouri and Van, 2012). In this respect, transgenic rice plants overexpressing *PRms* displayed enhanced broad-spectrum resistance to rice pathogens including *M. oryzae*, along with the increased accumulation of sucrose content in leaves (Gómez-Ariza et al., 2007), hinting towards the role of sucrose in rice defense. This notion was further confirmed by the fact that the exogenously administered sucrose also improved disease resistance of rice against *M. oryzae* and other pathogens as well (Gómez-Ariza et al., 2007). In addition to rice, tobacco transgenic plants overexpressing PRms also showed increased resistance to various pathogens, accompanied with improved sucrose content in their leaves (Murillo et al., 2010). Of note, recently published study also showed that the roots of a resistant rice genotype (wild rice) accumulated remarkably higher levels of soluble sugar content compared with the susceptible rice genotype (cultivated rice) in response to *M. oryzae* attack (Tian et al., 2018), further pointing towards the defensive role of soluble sugar molecules against *M. oryzae*. Despite these findings, recently, two independent genetic studies have shown that transgenic rice plants overexpressing either sugar signaling and/or energy sensor genes, *sucrose non-fermenting-1 related protein kinases1a* (*SnRK1a*) or *SnRK1b* significantly enhanced broad-spectrum resistance to *M. oryzae* and *Xanthomonas oryzae pv. oryzae* (Kim et al., 2015; Filipe et al., 2018). These results present ample evidence that endogenous sugar molecules, including sucrose, act as host defense signaling molecules, especially against rice blast infection. Hence, it is likely that the influence of SLs on sugar synthesis/signaling may contribute to the rice ability to confront with the *M. oryzae* stress.

Previous studies indicated that SLs not only induce spores germination and hyphal growth of arbuscular mycorrhizal fungi, but also affect the development of various phytopathogenic fungi *in vitro* (Dor et al., 2011). In the current study, no direct impact of different concentrations of synthetic SL (GR5) on growth of *M. oryzae* was observed (Fig. S1). This result is in line with the recently published reports in which the authors did not observe direct influence of synthetic SL (GR24) on development of *B. cinera* and *P. irregulare* as well (Torres-Vera et al., 2014; Foo et al., 2016). This further indicates that SLs hinder the growth of *M. oryzae* in rice by activating *in-planta* defense mechanisms, rather than its direct impact on *M. oryzae* growth.

In conclusion, our results present evidence that SLs mediate resistance to *M. oryzae* probably by induction various biotic stress-related genes/pathways including cell wall formation-, ET- and H_2O_2-related genes/pathways as well as through involvement in sugar production/signaling. Gain-of-function studies about *d17* and *d14* would better specify the roles of SL-biosynthesis and –signaling in the defense against *M. oryzae*.

Author contributions

CT and FN designed the experiments. FN, LT, CC, SS and LM performed the experiments and analyzed the data. FN, CT and YG wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.06.028.

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