Changes in gene expression levels and chloroplast anatomy induced by *Leifsonia xyli* subsp. *xyli* in sugarcane

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**ABSTRACT**

Ratoon stunt caused by *Leifsonia xyli* subsp. *xyli* (Lxx) is widely spread across sugarcane planting regions. Here, transcriptomics and cytology were conducted to analyze the changes in gene expression levels and chloroplast ultrastructures after Lxx inoculation. A total of 90.26 Gb clean bases, 156,713 unigenes and 406 differentially expressed genes were obtained. The genes of HSP and ACS were continuously differentially expressed. The photosynthesis-related genes of PsbO, PsbQ, PsaD, Psae, Psaf, and Psah were up-regulated. *PIRH2*, *WRKY*, *NCED*, *AHP* and *GST* were identified as the important specific genes that responded to Lxx inoculation. Lxx inoculation also altered the shape and damaged the membrane of chloroplasts in sugarcane leaves. Sugarcane responds to Lxx infection likely via changing the expression levels of some specific genes and interfered with photosynthesis. A possible molecular regulation network induced by Lxx was speculated. These findings broaden the understanding to the mechanisms of the interaction between sugarcane and Lxx.

**Introduction**

Ratoon stunt is a common disease of sugarcane that is widely spread across the sugarcane planting regions in China, with an incidence between 48.9% and 100% (Li et al. 2014). It causes serious biomass production loss, which reach 80% due to the generalized poor growth of diseased plants (Fu et al. 2016). To date, there have been no effective chemicals to control ratoon stunt, and hot water treatment remains the conventional method to reduce the pathogen titer in sugarcane production although it is not effective for completely removing the pathogen (Carvalho et al. 2016). The external symptoms of sugarcane infected with ratoon stunt are shorter plant height, thinner stalk diameter, and shorter internode length, which are similar to the symptoms of *Lxx* inoculation. A total of 90.26 Gb clean bases, 156,713 unigenes and 406 differentially expressed genes were obtained. The genes of HSP and ACS were continuously differentially expressed. The photosynthesis-related genes of *PsbO*, *PsbQ*, *PsaD*, *PsaE*, *PsaF*, and *PsaH* were up-regulated. *PIRH2*, *WRKY*, *NCED*, *AHP* and *GST* were identified as the important specific genes that responded to Lxx inoculation. Lxx inoculation also altered the shape and damaged the membrane of chloroplasts in sugarcane leaves. Sugarcane responds to Lxx infection likely via changing the expression levels of some specific genes and interfered with photosynthesis. A possible molecular regulation network induced by Lxx was speculated. These findings broaden the understanding to the mechanisms of the interaction between sugarcane and Lxx.

*Sugarcane*; ratoon stunt; transcriptomics; gene expression; ultrastructure

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sugarcane leaves after Lxx infection (Guo et al. 2019), also the defense-related genes PAL, ZFP and NBS-LR are induced to respond to Lxx infection (Zhu et al. 2018). The analysis of the transcriptome and proteome highlights that Lxx accumulation in sugarcane dramatically alters the expressions of proteins and genes involved in control of the cell-cycle, in ABA perception and in synthesis of ethylene and methionine (Cia et al. 2018), and the expressions of genes involved in plant hormone signal transduction, phenylalanine metabolism, phenylpropanoid biosynthesis and starch and sucrose metabolism (Fu et al. 2019). Zhu et al. (2021) considered that the alteration of metabolic pathways such as photosynthesis, phytohormone biosynthesis, phytohormone action-mediated regulation and plant-pathogen interactions were mainly the responses to Lxx infection. The analysis of the metabolomics reveals that inoculation with Lxx results in more relative abundance of amino acids, organic acids, phosphorylated compounds and phenolics, and phenolic substances involved in the resistance of Lxx invasion (de Castro Moretti et al. 2018). Some progress has been made in studying the interaction between sugarcane and Lxx at the physiological and molecular level, especially the development of omics technology gives a further clarification to the interaction between sugarcane and Lxx. Nonetheless, a broader understanding of the mechanisms involved in the interaction with sugarcane and Lxx is remaining necessary.

The objectives of this study were to compare the changes in gene expression levels and ultrastructures in a susceptible variety in response to Lxx inoculation by transcriptomics and cytology, which differ from previous studies in use of different varieties and in investigation of different pathogen inoculation stages. It would give a possible molecular regulation network in sugarcane induced by Lxx at the transcriptions level and improve the understanding of the mechanisms of interaction between sugarcane and Lxx.

Materials and methods

Plant growth and Lxx inoculation

The seedcane of Saccharum officinarum clone Badila (a variety highly susceptible) taken from in vitro cultures were planted in the greenhouse at the Sugarcane Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China. Ten-month-old seedcane plants were collected to obtain stalks. The stalks were cut into 5–7 cm sets with each bearing a single bud. The single bud sets were immersed in hot water at 50°C for 2 h and subsequently inoculated with Lxx. To do this, Lxx culture was obtained using the method of Zhang et al. (2016). 150 μL of 1×10⁸ cfu/ml Lxx suspension (Lxx-inoculated) was applied to each cross section. Inoculation with modified SC liquid medium was used as the control treatment (non-inoculated). Each treatment had 100 sets. All sets were incubated in a sandy bed after accelerating germination at 28°C for 2 days. The uniformly growing plants with three leaves in Lxx-inoculated and the control treatment were transplanted to pots (300 mm in diameter, 350 mm in height). The treatments were designed as 2 inoculation conditions × 2 times × 6 replications. Each replication comprised a pot with three plants to ensure enough samples for collection in the case of unexpected breaking or insect bite on stalks or leaves.

Sample collection

Plant height, stalk diameter, value of Soil Plant Analysis Development (SPAD) and fresh weight were measured before sampling at 60 and 90 days after inoculation (DAI), respectively. At 60 DAI, we randomly picked out three pots from the six pots in each treatment. The three pots were used for selecting three plants and one plant was selected from each pot. All the selected plants had no symptoms of unexpected breaking or insect bite on stalks or leaves. The remaining three pots were used for the sample collection for 90 DAI. 2 cm basal stalks above the ground without leaf sheaths and cortex were sampled from the selected three plants (three biological replicates). The samples were immediately immersed into liquid nitrogen and stored at −80°C until use.

DNA extraction and fluorescence quantitative PCR (qPCR)

Genomic DNA of the collected samples (100 mg) was isolated following the instruction of Ezup Column Bacteria Genomic DNA Purification Kit (Sangon Biotech, Shanghai, China). A primer pair of Lxx12590F1/R1 (F1; GCACTC-GATCTGGAAAAAAGG, and R1, CCGCAGTCTCAGC-CATACC) was used to amplify a 106-bp fragment of Lxx DNA (Carvalho et al. 2016). The standard curve was prepared with Lxx genomic DNA according to the Ct values obtained from fluorescence qPCR after serial dilutions of Lxx DNA. The formula NC = DM/2.63 × 10⁻⁶ was used to calculate the number of Lxx cells according to Carvalho et al. (2016), where NC is the number of Lxx cells and DM is the DNA mass (ng) which was calculated according to Ct values (DM=10⁻^(Ct/2.63)×10⁻⁶). qPCR was performed using the 2× SG Fast qPCR Master Mix (BBI, Roche) in LightCycler480 II (Roche, Switzerland) according to the instruction.

RNA extraction, library preparation and illumina sequencing

Total RNA was isolated from the collected samples with Trizol reagent and genomic DNA was digested with RNase-free DNase. RNA concentration and purity were checked by a NanoDrop (Thermo Scientific, USA). RNA integrity number and quantitation check were performed by Agilent 2100 bioanalyzer (USA), and mRNA was enriched using oligo (dT) beads after the quality check. The mRNA was fragmented randomly by adding fragmentation buffer. The first strand cDNA was synthesized using mRNA template and random hexamers, after which a second strand synthesis buffer (Illumina), dNTPs, RNase H and DNA polymerase I were added to synthesize the second-strand cDNA. After terminal repair and sequencing adapter ligation, the double-stranded cDNA library was constructed by means of size selection (AMPure XP beads) and PCR amplification. The high-quality library was subjected to sequencing by an Illumina HiSeq™ 4000. The sequencing was performed by Beijing Novogene Technology Co., Ltd, China. Raw data
obtained from sequencing were subjected to CASAVA base calling and submitted to the SRA database with the accession number SRP104794.

**Data processing and differentially expressed genes (DEGs) analysis**

Clean data were obtained after removing the reads containing adapter sequences, low-quality sequences with Qphred \( \leq 20 \) was \( >50\% \) and contained unknown bases \( >0.1\% \). The cleaned data were assembled using Trinity (Grabherr et al. 2011) to obtain transcripts, and the longest transcript for each gene was used as a unigene. Unigenes were annotated by searching various protein databases of National Center for Biotechnology (NCBI) including the non-redundant protein (Nr), Protein family ( Pfam), Eukaryotic Ortholog Groups (KO), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO). DEGs were obtained based on the method described by Anders and Huber (2010), and these genes were subjected to KEGG significant enrichment analysis to identify biological functions and metabolic pathways in which these genes participate.

**Real-time quantitative PCR (RT-qPCR) validation for RNA-seq data**

To validate the reliability of the DEGs obtained from Illumina RNA-seq sequencing, five up-regulated or down-regulated genes involved in cysteine and methionine (c59190_g2), phagosome (c62238_g2), glyoxylate and dicarboxylate metabolism (c63190_g1), tryptophan metabolism (c71673_g1), amino sugar and nucleotide sugar metabolism (c72252_g1), were selected for RT-qPCR with three replicates. Primers were designed with software Primer v.5 based on the unigene sequences. The housekeeping gene (GAPDH) was used as an internal reference (Table 1). The first strand cDNAs, obtained from RNA (used for RNA-seq) by using PrimeScript\textsuperscript{TM} RT reagent kit (Takara, Japan), were used as the templates. SYBR Premix Ex Taq II kit (Takara, Japan) was applied for RT-qPCR using a Lightcycler 480 (Roche, Switzerland). There were three RNA samples for each treatment and three reactions for each sample. The reaction contained 100 ng cDNA, 0.8 \( \mu \)L of each primer with 10 \( \mu \)m/\( \mu \)L, and 10 \( \mu \)L of SYBR master mix in a final volume of 20 \( \mu \)L. The reaction conditions were as follows: 95°C, 3 min; 40 cycles of 5 s at 95°C; 60°, 20 s. Distilled water was used as a blank control. Relative fold differences for each sample in each experiment were calculated using the \( \Delta \Delta CT \) method (Livak and Schmittgen 2001).

**Transmission electron microscopy (TEM)**

According to the analysis of KEEG, there were some differential expression genes involved in the metabolic pathway of photosynthesis. Besides, previous studies have illustrated that \( Lxx \) could move from xylem to parenchyma of the leaf cells (Queine et al. 2016) and reduce the net photosynthetic rate of sugarcane leaves (Zhang et al. 2017). Therefore, whether the chloroplasts of sugarcane leaves were affected by \( Lxx \) or not were explored. The samples of leaf+1 in \( Lxx \)-inoculated and non-inoculated sugarcane (10-month age) were cut off, respectively. Then, a leaf segment in central part with \( 0.3 \times 2 \) cm was taken without midrib, sliced into pieces of \( 1 \times 3 \) mm and put into glutaraldehyde with 2.5% to fix for at least 24 h. The method of preparation and observation for TEM followed Zhang et al. (2016). 15 chloroplasts of each treatment were chosen randomly. Imagine J software (National Institutes of Mental Health, USA) was used to measure the length, width and area of chloroplast, and SPSS v15.0 software for windows (SPSS Inc., Chicago) was used to conduct the statistical analysis.

**Results**

**Lxx confirmed infection and accumulation in sugarcane**

\( Lxx \) cell number differed between \( Lxx \)-inoculated plants (9.99 and 36.05 \( Lxx \) cells/100 ng of plant DNA at 60 and 90 DAI, respectively) and the control (0.04 and 3.65 \( Lxx \) cells/100 ng of plant DNA at 60 and 90 DAI, respectively), indicating that \( Lxx \) was successful in infecting sugarcane and accumulating in it (Figure 1).

**Effects of Lxx-infection on plant growth of sugarcane**

At 60 DAI, the SPAD value in \( Lxx \)-inoculated leaves was significantly lower than that in the control. Nevertheless, stalk diameter, plant height and fresh weight in \( Lxx \)-inoculated plants were not significantly different compared with the control. At 90 DAI, plant height and fresh weight in \( Lxx \)-inoculated plants became significantly lower than those in the control (Table 2).

**Global analysis of transcriptomics**

Illumina of RNA-seq of 12 samples yielded 90.26 Gb in total of clean bases. The data from Trinity software assembly contained 299,199 transcripts and 156,713 unigenes. Among them, 175,139 exceeded 500 bp, accounting for 58.54% of all transcripts, and 110,778 exceeded 1.0 kb, accounting for 37.03% of all transcripts. In terms of unigenes, 60,760 exceeded 500 bp, accounting for 38.77% of all unigenes, and 31,776 exceeded 1.0 kb, accounting for 20.28% of all unigenes. The N50 of transcripts and unigenes exceeded 500 bp, accounting for 58.54% of all unigenes (Table 3). The distribution of unigenes length is shown in Figure 2(a).

**Annotation of all unigenes**

Multiple databases were used to perform functional annotations of unigenes. A total of 156,713 unigenes were obtained queried against seven protein databases. The proportion of unigenes successfully annotated from the NT database was 43.73%, whereas the proportion from the NR and KO databases were 40.24% and 13.12%, respectively (Table 4). The analysis of NR annotations showed that 24323 (38.6%) were matched to Sorghum bicolor, 12251 (19.4%) were matched to Zea mays, 4316 (6.8%) were matched to Setaria italica (Figure 2(b)). S. bicolor was closely related to sugarcane, which was being used as reference for the following studies. The sequences with Blast hits were further analyzed to obtain their KOG functions and KEGG pathway annotations.
KOG functional classification of unigenes was grouped into 21 categories based on their involvement in various functions, among these ‘General function prediction only’ presented the largest group (group R, 18.58%). The KEGG classification showed that a total of 19 metabolic pathways were identified and of them, the top three enriched pathways were translation (2498), carbohydrate metabolism (2164), and overview (1807) (Figure 3(a,b)).

### Effects of Lxx-infection on gene expression levels of sugarcane

A total of 406 DEGs (padj < 0.05) were identified through a comparison between Lxx-inoculated and the control samples. There were only two common co-expressed genes (HSP and ACS, 0.5%) differentially expressed at the two time points (Figure 4(a)). Most genes exclusively detected at 60 DAI were down-regulated (28 down-regulated and 9 up-regulated) whereas most identified at 90 DAI were up-regulated (267 up-regulated and 104 down-regulated) (Figure 4(b,c)).

The DEGs at 60 DAI were only enriched in seven metabolic pathways (Figure 5(a), S1and S3 File), whereas there were up to 50 at 90 DAI (Figure 5(b), S2 and S4 File). The metabolic pathways enriched at 60 DAI were all enriched at 90 DAI except for the nitrogen metabolism pathway. There were six up-regulated gene-encoding components of the chloroplast in sugarcane (Damn1978; Zhang et al. 2016), and the area was smaller, and the specific value of width/length was bigger, which illustrated that morphologically the chloroplasts showed as a short oval in the Lxx-infected leaves compared to the long oval shape in the healthy leaves (Figure 7(d)).

### Discussion

Lxx infection reduces plant height of sugarcane due to Lxx cell accumulation and secondary substances plugging the xylem vessels (Damn 1978; Zhang et al. 2016). Lxx increasingly accumulated in the infected cells with plant growth. In this study, plant height showed no difference at the early stage (60 DAI) of sugarcane growth likely because bacterial titer was not high enough in vivo (Quecine et al. 2016; Cia et al. 2018), whereas plant height in Lxx-inoculated treatment at 90 DAI was significantly lower than that in the control, which differed from the result of Cia et al. (2018) with higher Lxx bacterial cells that may be probably because the sugarcane variety Badila used in this study was highly

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Table 1. Primers used for qRT-PCR.

| GeneID | Forward primer | Reverse primer | KOG description |
|--------|----------------|----------------|-----------------|
| c51910_g2 | GGTGGTTCGTCGATTACGTA | TGTGGTGATGTTATGCGA | 1-aminocyclopropane-1-carboxylate synthase, and related proteins |
| c62238_g2 | TACCGACAGTCGACGTCGAA | GTGCTGGTGGTCTGCGATA | Vacuolar H+–ATPase V1 sector, subunit E |
| c63190_g1 | ACACGCCAGCAGCAGTAGC | TTGTCACAGCCTTTGGCAG | Catalase |
| c71673_g1 | GCCTACCACGGATTCATCAT | GACGGGATGCTTCTACGC | Leucine rich repeat proteins, some proteins contain F-box |
| c72252_g1 | TCCCGGACATCGAAGAGCTCA | ACACGCCAGCAGGATGCGTC | UDP-N-acetylglucosaminepyrophosphorylase |

**Validation of DEGs by RT-qPCR**

To validate the RNA-seq reliability, five DEGs were selected for RT-qPCR analysis with three technical replicates, which gave same results to those from Illumina sequencing (Figure 6). RT-qPCR data depicted up/down regulation patterns that were consistent with Illumina sequencing results, suggesting that Illumina data were reliable.

### Effects of Lxx-infection on ultrastructure of chloroplast in sugarcane

Compared to the chloroplasts of non-inoculated leaves, those in Lxx-inoculated leaves were smaller, shorter, and had larger osmium particles and damaged membranes (Figure 7(a,c)). Besides, the measurement results of image J software indicated that the length of chloroplasts in Lxx-inoculated leaves were significantly shorter than that in the control, also the area was smaller, and the specific value of width/length was bigger, which illustrated that morphologically the chloroplasts showed as a short oval in the Lxx-infected leaves compared to the long oval shape in the healthy leaves (Figure 7(d)).
susceptible to Lxx (Guo et al. 2019; Zhu et al. 2021). The lower plant height resulted from shorter stalk length and lower stalk weight at later stage of sugarcane growth (Kazeem and Ikotun 2019). Additionally, hot water treatment (50–52°C) for 30–120 min is the most commonly used method to reduce Lxx titer in sugarcane production. But it is not effective for completely removing the pathogen, which means that some Lxx cells remain in the cane sets and proliferate as the seedcane grows (Carvalho et al. 2016). Thus, Lxx cells were detected in the non-inoculated plants in this study.

Quecine et al. (2016) demonstrated Lxx had a new niche in mesophyll and bundle sheath cells surrounding the vascular system. Our previous studies stated that Lxx inoculation reduced net photosynthetic rate and interfered with the related parameters in sugarcane leaves from 180 to 240 DAI (Zhang et al. 2017). de Marcos Lapaz et al. (2019) observed the leaf ultrastructural changes in sugarcane infected by Lxx and found that xylem diameter and cuticle thickness of the abaxial face were reduced compared to the healthy control. Zhu et al. (2021) stated that RuBP, a gene involved in the key enzyme in photosynthesis was down-regulated in Lxx-inoculated leaves. Chloroplasts are the crucial sites where most photosynthesis takes place. Therefore, the alteration of leaf ultrastructure and the expression of key genes in photosynthesis indicated that Lxx infection affected the photosynthesis of sugarcane.

In this study, the SPAD value in Lxx-inoculated leaves was significantly lower than that in the control at 60 DAI, and the expressions of DEGs associated with photosynthesis at 60 DAI (6 genes) and 90 DAI (1 genes) altered, which provided further evidence that Lxx infection affected the photosynthesis of sugarcane. The DEGs involved in photosynthesis at 60 DAI were PsbO, PsbQ, PsAD, PsaE, PsaG and PsaH. The genes PsbO and PsbQ belong to the subunit genes of photosystem II (PSII), and PsaD, PsaE, PsaG and PsaH are the subunit genes of photosystem I (PSI) (Hallick 1989). Zhu et al. (2021) also found some photosynthesis-related DEGs at 90 DAI, but the specific genes were different from ours, which is probably due to the diverse growing environment and Lxx cell densities. PSI and PSII were identified to be involved in responses to adverse stresses, including drought and salt stresses which could inhibit photosynthesis by limiting diffusion through the stomata and the mesophyll causing expression changes of some photosynthetic genes (Chaves et al. 2009). The RNA-seq technique was also utilized to analyze the DEGs and pathways involved in drought stress in pearl millet, and it was found that the drought response was regulated by the pathways related to photosynthesis (Dudhate et al. 2018). Lehtimäki et al. (2010) found that PsaE2 was up-regulated and PsaD1, PsaD2 and PsaE1 were down-regulated in Arabidopsis after drought stress. PsbO, PsbQ, PsAD, PsaD, PsaG and PsaH in microalgae were up-regulated when subjected to phenol stress at the concentration of 500 mg/L (Zhou et al. 2017). In this study, the genes involved in photosynthesis were all up-regulated, regardless of the time points at 60 or 90 DAI, which was not completely consistent with previous studies (Lehtimäki et al. 2010; Zhou et al. 2017), possibly because different crops have different photosynthetic responses to biotic and abiotic stresses.

Lxx has a number of pseudogenes, including probable non-functional genes involved in cysteine and methionine metabolism. Lxx isolation required the addition of cysteine, suggesting that absorption of this amino acid from the surrounding medium is necessary to synthesize methionine (Monteiro-Vitorello et al. 2004; Zhang et al. 2016). Methionine is converted to S-adenosyl-methionine (AdoMet) by the enzyme AdoMet synthetase. AdoMet serves as a precursor in ethylene biosynthetic pathway. The first committed and generally rate-limiting step in ethylene biosynthesis is the conversion of AdoMet to 1-aminocyclopropane-1-carboxylic

Table 3. Assembly results of sugarcane transcriptomics using Trinity software.

| Length range | Transcript | Unigene |
|--------------|------------|---------|
| 200–500 bp   | 124,060    | 95,953  |
| 500–1k bp    | 64,361     | 28,984  |
| 1k–2k bp     | 61,934     | 18,036  |
| >2k bp       | 48,844     | 13,740  |
| Total number | 299,199    | 156,713 |
| Min length (bp) | 201     | 201    |
| Mean length (bp) | 1089   | 722    |
| Median length (bp) | 656   | 382    |
| Max length (bp) | 15,536  | 15,536 |
| N50 (bp)     | 1875       | 1348   |
| N90 (bp)     | 442        | 279    |
| Total nucleotides | 325,961,619 | 117,833,605 |

Figure 2. Length distribution of transcripts and unigenes (A) and distribution of species aligned by unigenes (B).
acid (ACC) by ACC synthase (ACS) (Yoon and Kieber 2013). One of the results in this study was that ACS gene was down-regulated, which is probably caused by a decrease of Adomet and methionine. The decrease of methionine in the Lxx colonization phase of sugarcane maybe a defensive response of the host to suppress Lxx growth. However, the methionine content in sugarcane needs to be determined to confirm this hypothesis in further study. Cia et al. (2018) found a gene coding for methionine synthase was up-regulated at the very initial stage of Lxx colonization (30 DAI). This was contrary to our hypothesis and the reason probably was that our analyses were conducted at later stages of Lxx colonization.

Table 4. Summary for blast results of unigenes.

| Database       | Number of unigenes | Proportion (%) |
|----------------|--------------------|---------------|
| NR             | 63,065             | 40.24         |
| NT             | 68,517             | 43.73         |
| KO             | 20,575             | 13.12         |
| SwissProt      | 46,138             | 29.44         |
| PFAM           | 48,772             | 31.12         |
| GO             | 53,813             | 34.33         |
| KOG            | 29,052             | 15.98         |
| Annotated in all databases | 9991 | 6.37 |
| Annotated in at least one database | 94,010 | 59.98 |
| Total unigenes | 156,713            | 100           |

Figure 3. Eukaryotic Ortholog Groups (KOG) function classification and Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of unigenes. (A) KOG, (B) KEGG.
colonization (60 and 90 DAI). The relevant ACS genes differentially expressed during the entire growth stage and ACS enzyme is induced by IAA and pathogens (Yamagami et al. 2003; Li et al. 2012). The ACS genes most likely were regulated differently after Lxx colonization. The up-regulation of these genes at the early stage of colonization might be beneficial for Lxx establishment and later growth, while the down-regulation of these genes at the later stage of colonization could suppress Lxx growth.

The genes PIRH2, WRKY, NCED, AHP and GST are associated with the metabolisms of ubiquitin-mediated proteolysis, plant-pathogen interaction and biosynthesis of carotenoid, zeatinand, glutathione, respectively. They are reported involving in plant defense reactions against...
pathogens (Devoto et al. 2003; Tanaka et al. 2004; Citovsky et al. 2009; Fan et al. 2009; Wang et al. 2014; Gullner et al. 2018).

PIRH2 is an E3 ubiquitin ligase targets p53 for ubiquitin-mediated proteolysis. It regulates the turnover and functionality of several proteins involved in cell proliferation and differentiation, cell cycle checkpoints and cell death, and interacts with calmodulin. It is phosphorylated by calmodulin-dependent kinase II (CaMKII). The CaMKII phosphorylation decreases the stability and mediates the ubiquitination of PIRH2 (Duan et al. 2007; Halaby et al. 2013). Cia et al. (2018) found the expression level of calmodulin altered after Lxx infection at 60 DAI. Moreover, Lxx inoculation in this study altered the expression level of PIRH2 and CAML (calmodulin-related gene), which indicated that the metabolic pathways of ubiquitin-mediated proteolysis and plant–pathogen interaction responded to Lxx infection.

WRKY is one of the largest transcription factors in plants and plays an important role in plant responding to biotic stresses. For example, GhWRKY40 gene derived from cotton was induced by the bacteria Ralstonia solanacearum upon infection (Wang et al. 2014); overexpressing of GhWRKY44 induced the expression of several defense-related genes and enhanced the resistance of transgenic tobacco to R. solanacearum and Rhizoctonia solani (Li et al. 2015); overexpression of ScWRKY5 gene cloned from sugarcane

Figure 6. The qRT-PCR results compared with transcriptome results. (A, B) indicated the genes were up-regulated or down-regulated under Lxx-inoculation at 60 DAI and 90 DAI, respectively. ↑ indicated up-regulation, and ↓ indicated down-regulation.

Figure 7. Effects of Lxx-inoculation on ultrastructure of chloroplast. (A) Control (Lxx Non-inoculated leaf), 7000×; (B, C) Lxx-inoculated leaf, 7000×, 10,000×; (D) Results of chloroplasts measurement by ImageJ software. Ch: Chloroplasts; PM: Plasma membrane; OP: Osmium particles; GL: Grana lamella; CM: Chloroplast membrane. * Indicates the mean of each indicator was significantly different between the Lxx-inoculated leaf and the control at p < 0.05 level in T test.
enhanced the resistance of *Nicotiana benthamiana* to the tobacco bacterial pathogen *R. solanacearum* (Wang et al. 2020). In this study, WRKY22/29 in sugarcane was up-regulated after *Lxx* inoculation probably because sugarcane activated the defensive system to restrain the *Lxx* invasion.

NCED is a key enzyme in ABA biosynthesis (Rodrigo et al. 2006). Its overexpression enhances plant resistance to abiotic stress. *LeNCED1* derived from tomato was overexpressed in *Petunia hybrid* (J.D.Hooker) Vilmorin that increased the content of ABA and enhanced drought resistance under water deficit (Estrada-Melo et al. 2015). NCED gene is also involved in plant disease resistance regulation. The expressions of genes, *AtNCED2*, *AtNCED3* and *AtNCED5* in Arabidopsis mutant were induced after inoculating *Pseudomonas syringae* strains, and ABA had massive accumulation (Fan et al. 2009). NCED genes in this study were up-regulated, which was in general agreement with the previous studies that genes related to the metabolism of abscisic acid were up-regulated and the concentration of ABA increased in *Lxx*-inoculated plants (Zhang et al. 2016; Cia et al. 2018).

AHP is involved in the signal transduction pathway in response to cytokinin. Cytokinins have the function in regulation of plant immunity against pathogens (Tanaka et al. 2004). Liu et al. (2020) found that the overexpression of *ARK1* gene led to the up-regulation of AHP and the increase of zeatin content in poplar, which was related to multiple branches. It can be speculated that AHP genes probably related to the tillering of sugarcane when plants suffer *Lxx* infection according to the results of down-regulated AHP genes in this study and *Lxx*-infected sugarcane had less number of tillers (Comstock 2002).

GST participates in antioxidative reactions together with GSH to eliminate ROS and lipid hydroperoxides that accumulate in the infected tissues (Wagner et al. 2002). Previous studies showed that GST genes are markedly induced in the early phase of bacterial, fungal and viral infections and specifically up-regulated by the infections (Gullner et al. 2018). However, some GST genes were up-regulated and some were down-regulated in this study probably because the sampling time happened to GST genes conjugated with glutathione to participate in hormone transport (Gullner et al. 2018).

Overall, a possible molecular regulation network of sugarcane induced by *Lxx* at transcription level was speculated (Figure 8). The network was based on the connection among the pathways as they share common by products, also based on our previous studies that phytohormone and defense enzyme activities were altered in sugarcane in response to the *Lxx* infection (Zhang et al. 2016, 2017) that may increase the tolerance to *Lxx* stress in sugarcane. It gave a further clarification to the mechanism of interaction between *Lxx* and sugarcane.

**Conclusion**

After *Lxx* inoculation, some photosynthesis-related genes (*PsbO*, *PsbQ*, *PsaD*, *PsaE*, *PsaG* and *PsaH*) were up-

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**Figure 8.** DEGs involving in *Lxx*-inoculation on biological pathway of sugarcane. This figure showed only the genes associated with the pathways in transcriptome analysis. Orange boxes showed the metabolic pathways, dark blue box showed the shared product by multiple metabolism pathways, red boxes and red arrows showed the genes up-regulated, green boxes and green arrows showed genes down-regulated and pink and yellow boxes showed the responses of sugarcane to *Lxx* infection. Solid arrows denoted activation, dotted arrows denoted indirect effects, dotted lines with a bar denoted inhibition, and full lines denoted binding. +p indicated phosphorylation process.
regulated. The genes of HSP and ACS continuously differentially expressed at different time points. Also, the genes of PIRH2, WRKY, NCED, AHP and GST were identified as the important specific genes that responded to Lxx inoculation. Lxx inoculation also altered the shape and damaged the membrane of chloroplasts in sugarcane leaves. Thus, sugarcane responds to Lxx infection likely via change the expressions levels of some specific genes and interfered with photosynthesis in sugarcane. A possible molecular regulation network induced by Lxx was speculated.

**Ethics statement**

All the research meets ethical guidelines and adheres to the legal requirements of the study country.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Data availability statement**

All the data supporting the findings of this study are available within the article and its supplementary materials. The raw data have been deposited in NCBI’s database and are accessible through accession number SRP104794 (https://www.ncbi.nlm.nih.gov/sra/?term=SRP104794).

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