Transcriptomic analysis reveals root metabolic alteration and induction of huanglongbing resistance by sulphonamide antibiotics in huanglongbing-affected citrus plants

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Abstract
Huanglongbing (HLB) is a devastating disease that affects the entire citrus plant, including the root system. Previous studies have shown that sulphonamide antibiotics can suppress titres of the pathogen that causes HLB, 'Candidatus Liberibacter asiaticus' (CLas), and affect root morphology in the plant through unknown mechanisms. To better understand the response of CLas-infected roots to sulphonamide antibiotics, hydroponic cultures of CLas-infected citrus roots were treated with sulfadimethoxine sodium (SDX), indole butyric acid (IBA), or water for 60 days to evaluate root metabolism and resistance against CLas via transcriptomic analysis. This study indicated that SDX and IBA treatments increased active root surface area in HLB-affected citrus, which may be due to root hair and lateral root growth. CLas titres in HLB-affected citrus roots treated with SDX were lower than those of IBA and control treatments. Function categorization indicated that plant hormone signal transduction and plant-pathogen interaction were the most markedly enriched pathways in the HLB-affected citrus root after SDX treatment. The expression of genes involved in biosynthesis of auxin and ethylene, which are related to root hair and lateral root growth, were up-regulated by SDX. Moreover, SDX also induced genes related to the metabolism of jasmonates, brassinosteroids, reactive oxygen species, and secondary metabolites, which are beneficial for resistance against HLB. In conclusion, we propose a model for SDX in regulating metabolic pathways in the root and resistance against CLas in HLB-affected citrus root, which is beneficial for recovering an HLB-affected citrus root system and combating CLas.

Keywords
citrus, huanglongbing
INTRODUCTION

Huanglongbing (HLB), or citrus greening, is a devastating disease that impacts the citrus industry worldwide (Bové, 2006). Florida, a major citrus production area in the United States, has lost over $7 billion in total output in 2005–2014 due to HLB since it was first detected (Spreen et al., 2014). In the USA, HLB is caused by ‘Candidatus Liberibacter asiaticus’ (CLas), a fastidious gram-negative phloem-limited bacterium transmitted by the Asian citrus psyllid, Diaphorina citri (Bové, 2006).

The plant root system serves as a conduit to supply nutrients and water. Increasing the length and diameter of a root within a crop root system enhances the water and nutrient uptake capacity of the crop (Tinker and Nye, 2000). In citrus, a root surface area, which represents the absorption ability of roots, is directly related to canopy size and fruit yield (Morgan et al., 2007). HLB causes loss and degeneration of citrus roots, which results in yield reduction and eventually tree death (Johnson et al., 2014).

Sulphonamide antibiotics are organic sulphur compounds that contain SO$_2$NH$_2$ radicals (the amides of sulphonic acids). It has also been reported that several abiotic stressors, including antibiotic use, heavy metal exposure, hydrogen peroxide, or low phosphate in the rhizosphere, can affect root morphology by altering the normal homeostasis of reactive oxygen species (ROS), antioxidants, auxin, and ethylene (Potters et al., 2009). Sulphonamide antibiotics act as abiotic stressors and can induce growth of the lateral root and root hair in willow plants through hormone regulation (Michelini et al., 2014). However, it is unknown whether sulphonamide antibiotics alter root morphology of HLB-affected citrus or how they affect the citrus root system. In addition, our previous studies have demonstrated that sulphonamide antibiotics, such as sulfadimethoxine sodium (SDX) and sulfathiazole sodium (STZ), are effective against CLas (Zhang et al., 2014). Sulphonamides are structural analogues of p-aminobenzoic acid (PABA), which is a substrate of the enzyme dihydropteroate synthetase required for the synthesis of tetrahydrofolic acid in bacteria and competitively inhibits the incorporation of PABA into folic acid, thereby preventing the synthesis of folic acid (Brain et al., 2008). However, the enzyme dihydropteroate synthase is absent in the CLas genome (Duan et al., 2009). The mechanism of action of sulphonamides against CLas in HLB-affected citrus is still unknown. Sulphonamides have also been identified as plant activators that protect Arabidopsis thaliana from disease by activating the plant immune system (Noutoshi et al., 2012). Whether sulphonamides induce an HLB-affected citrus immune system against CLas remains unclear.

We hypothesized that sulphonamide antibiotics promote the absorption ability of HLB-affected citrus through the regulation of hormones and induction of ROS, and activated resistance against CLas. Therefore, this study sought to observe the morphological response of HLB-affected roots treated with sulphonamide antibiotics as well as elucidate the underlying mechanism regulating this response and defence against CLas via RNA sequencing.

MATERIALS AND METHODS

2.1 | Plant materials

Two-year-old healthy grapefruit (Citrus paradisi) seedlings were graft-inoculated with HLB-affected lemon (Citrus limon) scions and subsequently maintained in potted soil in the greenhouse for further studies. HLB-affected citrus seedlings with typical HLB symptoms were then tested for the presence of CLas using quantitative real-time PCR (qPCR) with CLas-specific primers (HLBas, HLBr, and HLBp) (Li et al., 2006) and kept in an insect-proof greenhouse for further use.

2.2 | HLB-affected citrus root treated with SDX by hydroponic culture

Hydroponic culture was established with Murashige and Skoog medium (MS) (Sigma-Aldrich). Six months after CLas inoculation, the HLB-affected citrus seedlings were selected for hydroponic culture. The soil of HLB-affected citrus seedlings was removed and the seedlings were then transplanted into plastic pots (18 cm diameter ¥ 19.5 cm tall) containing 3 L MS solution amended with 10 mg/L SDX (Sigma-Aldrich) or 10 mg/L indole-3-butyric acid (IBA; Sigma-Aldrich). The previous study indicated that sulphonamide antibiotics act as abiotic stressors and can induce growth of the lateral root and root hair in willow plants through hormone regulation, especially IBA; therefore, IBA was included in this experiment. For untreated controls (CK), no active components (SDX and IBA) were added to the MS solution. Cultures were maintained for 60 days with the MS solution and active components replenished every 6 days. For each active treatment, five replicates were set up. Sixty days after the initial treatment, the morphology of the root was observed and a root sample from each treatment group was collected to determine active root surface area (ARSA), RNA isolation for transcriptome analysis, and DNA isolation for determining CLas titre.

2.3 | ARSA analysis

The determination of ARSA was performed as previously described by Mu et al. (2006). Briefly, a 0.2 mM methylene blue solution was prepared to approximately 10 times the root sample volume and then divided into three beakers. Root samples were washed with distilled water and filter paper was then used to remove the residual water on the surface. The samples were then incubated in each of the three beakers consecutively for 1.5 min. Subsequently, 0.5 ml of solution was collected from each beaker, diluted 20 times with distilled water, and measured on a spectrophotometer at OD$_{660}$. The residual amount of methylene blue from the three beakers was obtained according to a standard curve and the amount of methylene blue uptake by the root
samples in each beaker was determined. The ARSA was calculated by the following equation:

\[
\text{ARSA} \left( m^2 \right) = A_3 \times 1.1 \ m^2
\]

where \( A_3 \) is the amount of methylene blue uptake by the root sample in beaker number 3 and 1.1 \( m^2 \) is the area of methylene blue when present in a single molecular layer.

### 2.4 Genomic DNA extraction and qPCR analysis for CLas titre

The root samples were rinsed three times with sterile water and then cut into 1–2 mm pieces. DNA was extracted from 0.1 g (fresh weight) of root tissue using a DNeasy Plant Mini Kit according to the manufacturer’s protocol (Qiagen). The qPCR was conducted with primer sets (HLBas, HLBr, and HLBp; Li et al., 2006) for CLas using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) in a 20 \( \mu l \) reaction volume consisting of the following reagents: 300 nM (each) target primer (HLBas and HLBr), 150 nM target probe (HLBp), and 1 x TaqMan qPCR Mix (Applied Biosystems) (Li et al., 2006). The amplification protocol was as follows: 95 °C for 20 s followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. All reactions were conducted in triplicate and each run contained one negative (DNA from healthy plant) and one positive (DNA from CLas-infected plant) control. Data were analysed using the ABI 7500 Fast Real-Time PCR System with SDS software.

### 2.5 RNA isolation, cDNA library construction, sequencing, de novo assembly, and gene expression quantification

Total RNA was extracted from washed citrus roots (200 mg) using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. Complete DNA removal was obtained through on-column DNase I treatment using the RNase-Free DNase Set (Qiagen). The quantity and purity of RNA was evaluated using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). To improve reliability and decrease the likelihood of biological error, equal amounts of total RNA from three biological replicates were pooled for Illumina deep RNA sequencing. The quality of the library assembly, adapter sequences and low-quality reads (base quality <20; read length <40 bp) were removed from the raw reads.

The high-quality reads were mapped to the \textit{C. sinensis} reference genome sequence (Xu et al., 2013). Gene expression levels were calculated as reads per kilobase of exon model per million mapped reads (RPKM). The sets of differentially expressed genes (DEGs) were identified using edgeR with a \(|\log_2\text{fold-change}| >1.0\) and a false discovery rate (FDR) ≤0.05. Clustered profiles with \( p \leq 0.05 \) were considered statistically significant. Gene Ontology (GO) annotation was conducted using Blast2GO software (Conesa et al., 2005).

DEGs were enriched in GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases in order to identify changes in biological functions and metabolism pathways.

### 2.6 RT-qPCR analysis for gene expression validation

Quantitative reverse transcription PCR (RT-qPCR) was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) to examine expression patterns of 13 selected DEGs. Specific primers for the DEGs were designed by Primer v. 3.0 (Koressaar and Remm, 2007) and are listed in Table S1. The citrus \textit{GAPDH} gene was used as an internal control to normalize expression levels of the target genes among different samples. The RT-qPCR was conducted with three biological repetitions, and three technical repetitions were run in each biological repetition. All RT-qPCRs were arranged on a 96-well plate. The RT-qPCR was performed in a 10 \( \mu l \) reaction volume that contained 5 \( \mu l \) Thunderbird TM SYBR qPCR Mix (TOYOBO), 1 \( \mu l \) of cDNA, and 1 mM gene-specific primers. To analyse dissociation curve profiles, the following programme was run after the 40 cycles of PCR: 95 °C for 15 s, followed by a constant increase in temperature between 60 and 95 °C. The expression was calculated by \( 2^{-\Delta\Delta Ct} \) and normalized against \textit{GAPDH} expression level.

### 3 Results

#### 3.1 The effect of SDX on HLB-affected citrus root

Sixty days after initial treatment, SDX significantly reduced CLas titres \((C_t = 38.60 \pm 0.62)\) in HLB-affected roots compared to the initial evaluation \((C_t = 25.98 \pm 3.00)\). In contrast, CLas titres were still higher in HLB-affected citrus roots treated with IBA and CK (Figure 1a). Moreover, the ARSA of HLB-affected citrus roots was increased following SDX and IBA treatment and the morphology of HLB-affected citrus roots changed. Lateral root growth increased after SDX and IBA treatments (Figure 1b,c).

#### 3.2 Transcriptome profiles of HLB-affected citrus treated with SDX

The transcriptome changes induced by SDX treatment in HLB-affected citrus root were investigated via RNA-Seq analysis. Between 55,892,034 and 73,540,252 reads per sample were produced, and 88.49% to 88.93% of the reads were uniquely mapped to the reference genome (Table S2).

In this study, 2,063 genes were differentially expressed in HLB-affected citrus root in response to SDX and IBA, and 424 DEGs were shared (Figure 2a). There were 1,606 DEGs observed in response...
to SDX treatment while only 881 DEGs were obtained after IBA treatment. The number of up-regulated genes was lower than the number of down-regulated genes following SDX treatment, whilst the number of up-regulated genes was greater than the number of down-regulated genes after IBA treatment (Figure 2b).

### 3.2.2 Sequence annotation

In GO annotation, 888 and 456 DEGs were identified in SDX and IBA treatments, respectively; these were assigned to at least one term in the GO biological process, cellular component, and molecular function categories. Transcripts from the three categories were further classified into 52 functional subcategories, thus providing an overview of ontology terms (Figure 3a,b). In the biological categories, metabolic processes, cellular processes, and single-organism processes were the most highly represented groups. In the cellular component categories, cell, cell part, organelle, and membrane were prominently represented, while catalytic activity and binding dominated the molecular function categories. We further identified GO terms in the biological process, cellular components, and molecular function, which were over-represented \((p < .01)\) in SDX and IBA treatments (Table S3). These GO terms served as indicators of similar and significantly different biological processes, cellular components, and molecular functions between SDX and IBA treatments.

Several GO terms were enriched in this study. In biological processes, more than five genes, including protein-chromophore linkage, photosynthesis light harvest, defence response, and cysteine biosynthetic process, were enriched in GO terms in both treatments (SDX and IBA). More genes were involved in the integral components of membranes and photosystem I in cellular components under both treatments. In molecular functions, there were more genes involved in chlorophyll binding, carbohydrate binding, and electron carrier activity in both treatments. Some striking differences were found between the two sets of enriched GO terms. In particular, genes related to photosynthetic electron transport in photosystem I, chloroplasts...
**FIGURE 3**: Gene Ontology categories of all genes and differentially expressed genes (DEGs) in huanglongbing-affected roots in response to sulfadimethoxine sodium (SDX) and indole butyric acid (IBA). (a) SDX treatment; (b) IBA treatment. The number and percentage of genes in each subcategory for the three main categories of biological processes, cellular components, and molecular functions are indicated for all genes and DEGs, respectively [Colour figure can be viewed at wileyonlinelibrary.com]
thylakoid membrane, photosystem I reaction centre, plastoglobule, and (iso)eugenol O-methyltransferase activity were most enriched following the SDX treatment.

To identify metabolic pathways in which SDX and IBA were involved and enriched, the KEGG pathway database was used to conduct pathway-based analysis. KEGG analysis assigned the differentially expressed genes to 102 metabolic pathways. Notably, most DEGs of SDX and IBA treatment were annotated to "phenylpropanoid biosynthesis", "photosynthesis", "carbon metabolism", "starch and sucrose metabolism", "photosynthesis - antenna proteins", "biosynthesis of amino acids", "ribosome", "plant hormone signal transduction", "plant-pathogen interaction", "cysteine and methionine metabolism", and "glycolysis/gluconeogenesis" (Table S4).

3.2.3 Plant hormone biosynthesis and signal transduction

Many DEGs involved in auxin biosynthesis and signal transduction were differentially regulated in response to SDX. Tryptophan synthase β chain 2 (Cs2g07700), glutamylerNA(gln) amidotransferase subunit A (Cs8g09350), and auxin-responsive protein SAUR71 (citrus_sinensis_newGene_765) were up-regulated by SDX and auxin-induced protein X10A (orange1.1t04011) and indole-3-acetic acid-induced protein ARG7 (orange1.1t02620) were down-regulated. Moreover, auxin-induced protein X15 (Cs4g12720 and Cs2g08570) was up-regulated by both SDX and IBA, respectively, and auxin response factor 18 (orange1.1t05117) was down-regulated by both SDX and IBA treatment (Table S5).

Several 1-aminocyclopropane-1-carboxylate related genes, which are involved in ethylene metabolism, were induced in response to SDX and IBA (Table S5). In cytokinin metabolism, SDX and IBA both down-regulated cytokinin riboside 5-monophosphate phosphoribohydrolase. SDX up-regulated most DEGs encoding a two-component response regulator, whereas IBA down-regulated these DEGs (Table S5).

SDX up-regulated the abscisic acid-hydroxylase gene (Cs1g24480), whereas IBA treatment down-regulated the abscisic acid receptor PYL9 (orange1.1t00478) (Table S5). In addition, gibberellin 2,4-dioxygenase genes were differentially expressed by SDX, while gibberellin receptor and gibberellin-regulated protein 14 were down-regulated by IBA (Table S5).

3.2.4 Cell wall metabolism

Compared with IBA, DEGs related to cell wall metabolism were enriched by SDX. SDX and IBA up-regulated DEGs encoding endochitinase, probable pectate lyase, laccase, UDP-glucose 6-dehydrogenase, and WAT1-related protein. In response to SDX, mannan endo-1,4β-mannosidase 7 (Cs1g24390), β-glucosidase (Cs7g01340 and Cs2g07740), and inositol oxygenase genes (Citrus_sinensis_newGene_546 and Cs1g16030) were up-regulated (Table S5). Moreover, pectinesterase, polygalacturonase, shikimate O-hydroxycinnamoyltransferase, cellulose synthase 7, and wall-associated receptor kinase were differentially expressed by SDX and IBA.

3.2.5 Reactive oxygen species

SDX up-regulated expression of respiratory burst oxidase homologue protein E gene (Cs4g06920) and primary amine oxidase gene (Cs5g06000), and therefore the expression of putative l-ascorbate peroxidase 6 (Cs7g05450) and squalene epoxidase 1 (Cs2g05900) were down-regulated. Additionally, SDX and IBA up-regulated probable glutathione S-transferase and pyruvate kinase. Peroxidase is a key enzyme for ROS, and we found that peroxidase 10 and peroxidase N were up-regulated by IBA and that peroxidase 4, peroxidase 27, peroxidase 47, and peroxidase 72 were up-regulated by SDX (Table S5).

3.2.6 Secondary metabolism

More DEGs were enriched in secondary metabolism in response to SDX compared to IBA treatment, including: (+)-neomenthol dehydrogenase, 9-cis-epoxycarotenoid dioxygenase NCED1, anthranilate N-methyltransferase, aromatic-α-amino acid decarboxylase, β-amyrin 11-oxidase, β-amyrin synthase, β-carotene isomerase, chalcone synthase 2, cycloartenol synthase, farnesyl pyrophosphate synthase 1, probable 1-deoxy-o-xylulose-5-phosphate synthase, probable 3-β-hydroxysteroid-Δ(8), and probable carotenoid cleavage dioxygenase 4 (Table S5). Caffeic acid 3-O-methyltransferase and cytochrome P450 genes were significantly expressed in HLB-affected citrus root in response to SDX and IBA. Moreover, most genes encoding caffeic acid 3-O-methyltransferase were up-regulated by SDX.

3.2.7 Plant-pathogen interactions

In total, 39 disease resistance protein genes were identified in HLB-affected citrus roots in response to SDX (22 genes) and IBA (19 genes). The disease resistance gene RDL5 was up-regulated and thus RPM1 and At1g12280 were down-regulated by SDX. Several disease resistance protein genes, including At4g27190, RGA2, RPS5, and At5g63020, were differentially expressed in roots treated with SDX and IBA. Of the six heat shock protein genes identified in this study, 6.6 kDa heat shock protein (Cs5g04230) and 17.3 kDa class I heat shock protein (Cs6g07320) were up-regulated by SDX; however, 17.6 kDa class I heat shock protein (orange1.1t05694) was down-regulated.

There were six LRR receptor-like serine/threonine-protein kinases expressed in HLB-affected roots treated with SDX and IBA. SDX down-regulated the LRR receptor-like serine/threonine-protein kinase EFR and FLS2 and up-regulated the LRR receptor-like serine/threonine-protein kinase RCH1. In addition, SDX significantly up-regulated genes encoding calmodulin-binding transcription activator 4 (orange1.1t01821), cyclic nucleotide-gated ion channel 2 (Cs9g18100,
Cs9g18050, Cs6g21840, and Cs6g21850, probable calcium-binding protein CML44 (Cs3g20300), and putative calcium-binding protein CML19 (Cs5g07160) (Table S5).

3.2.8 Transcription factors

There were 39 DEGs with high homology for different kinds of transcription factors (TFs), including the basic helix-loop-helix (bHLH) family (i.e., bHLH3, 25, 93, 113, 126, and 137), ethylene-responsive factor (ERF) family (i.e., ERF1B, 002, 007, 013, 017, 023, 026, 061, 062, 071, 114, and ABR1), myeloblastosis protein (MYB) family (i.e., MYB12, 39, 48, and 86), and others (Table S5). SDX induced all DEGs from the bHLH family. In the ERF family, SDX and IBA up-regulated ERF013 and 071 and down-regulated ERF062 and 114. Under SDX treatment, ERF1B, 2, 7, 017, 026, 061, and ABR1 in HLB-affected citrus roots were up-regulated. Moreover, MYB12, 48, and 86 were up-regulated in HLB-affected roots treated with SDX and MYB39 was up-regulated by IBA (Table S5).

In addition, the TFs, including DIVARICATA, FER-LIKE, HY5, MYC4, ORG2, and UNE10, were up-regulated by SDX. Meanwhile, under SDX and IBA treatment, BEE3, TCP2, and TCP4 were up-regulated, while LUX and UNE10 were down-regulated (Table S5).

3.2.9 Verification of RNA-Seq results with RT-qPCR

To verify our RNA-Seq data, 13 DEGs that were involved in plant hormone metabolism, cell wall metabolism, plant pathogen resistance, and ROS metabolism were selected for RT-qPCR confirmation. All 13 DEGs in the RT-qPCR assay were essentially consistent with their transcript abundance change identified by the RNA-Seq method. The correlation coefficients demonstrated that the gene expression levels of 13 DEGs determined by RT-qPCR were the same as detected by RNA-Seq (Figure 4a,b,c). The pairwise correlation coefficient of SDX and IBA treatment was higher than 0.80 (Figure 4e,f), indicating that the transcriptomic data were reliable.

4 Discussion

In this study, ARSA in HLB-affected citrus roots was increased by SDX and IBA, which may be the result of lateral root and root hair growth. Our results were similar to Micheli et al. (2014) who found that IBA, as well as sulphonamide antibiotics, could affect root morphology in willows (Micheli et al., 2014). However, to date, there has been no report on the mechanism underlying SDX-regulated root metabolism. Based on transcriptomic analysis, this study provided an insight on how SDX might be involved in regulating plant hormone metabolism and inducing ROS in HLB-affected citrus root.

Indole-3-acetic acid (IAA), which is the most abundant naturally occurring auxin in plants, is biosynthesized from tryptophan (Trp) through proposed routes according to their key intermediates, including indole-3-acetamide (IAM) (Woodward and Bartel, 2005). Tryptophan synthase β chain 2 and glutamyl-tRNA(Gln) amidotransferase subunit A genes, which are involved in IAM route, were up-regulated in HLB-affected citrus roots by SDX treatment (Table S5). Tryptophan synthase β chain 2 is responsible for the synthesis of Trp from indole and glutamyl-tRNA (Gln) amidotransferase subunit A is one member of the amidase signature (AS) enzymes, which includes indole-acetamide hydrolase. Indole-acetamide is subsequently oxidatively deaminated by indole-3-acetamide hydrolase, yielding IAA. Therefore, SDX might induce production of IAA by activating Trp metabolism in HLB-affected citrus roots (Figure 5).

Transcription of specific auxin-responsive genes, including small auxin-up RNA (SAUR) and auxin response factors (ARFs), can induce root hair and lateral root growth. SAUR71 plays a role in the regulation of cell expansion as well as root meristem patterning (Spartz et al., 2014). ARFs can function as repressors that regulate the expression of auxin response genes (Hagen and Guilfoyle, 2002). In this study, only the SAUR71 gene was significantly up-regulated in HLB-affected citrus roots in response to SDX (Table S5). ARF18 was down-regulated in HLB-affected citrus roots treated with both SDX and IBA (Table S5). SDX treatment induced expression of SAUR71 and repressed expression of ARF18 to improve HLB-affected citrus root hair and lateral root growth (Figure 5).

The genes 1-aminocyclopropane-1-carboxylate synthase (ACS), 1-aminocyclopropane-1-carboxylate oxidase (ACO), and ethylene-responsive transcription factor (ETF), which are involved in ethylene synthesis, receptor, and signal transduction, respectively, are directly involved in root hair regulation (Rahman et al., 2002). SDX was found to induce ACS1 and ACS5, and IBA also increased the expression of ACO1 (Table S5). In addition, most ETFs (10/13) were induced by SDX, and therefore IBA did not comparatively enhance ETF (2/10) expression (Table S5). Collectively, the data suggest that SDX and IBA regulation of root hair formation might occur through ethylene biosynthesis and signal transduction pathways (Figure 5).

It has been reported that cytokinins have inhibitory effects on lateral root formation (Böttger, 1974). Cytokinin dehydrogenase can catalyse cytokinin degradation and cytokinin riboside 5’-monophosphate phosphoribohydrolase converts inactive cytokinin nucleotides to biologically active free-base forms. SDX up-regulated cytokinin dehydrogenase 7 and both SDA and IBA down-regulated cytokinin riboside 5’-monophosphate phosphoribohydrolase (Table S5), suggesting that inactive cytokinin was much more abundant in HLB-affected citrus root treated with SDX. In addition, type-A Arabidopsis response regulators (ARRs) act as partially redundant negative regulators of cytokinin signalling and Type-B ARRs are transcription factors that act as positive regulators in the two-component cytokinin signalling pathway (To et al., 2004). SDX up-regulated ARR9 and repressed AAR2, which are type-A and type-B ARRs, respectively. Therefore, the abundant inactive cytokinin caused by SDX might have had a negative effect on cytokinin signalling, resulting in a reduction in the inhibitory effect on HLB-affected citrus lateral roots (Figure 5).
Jasmonates (JAs) can positively regulate root hair growth. The bHLH subgroup Ille transcription factor MCY4 is an activator of JA-regulated programmes beneficial for root hair growth (Zhu et al., 2006). SDX induced MCY4 in HLB-affected citrus roots (Table S5). Therefore, SDX regulation of root hair growth involves the JA pathway (Figure 5). In addition, brassinosteroids (BRs) play a key role in root hair formation.
and later root initiation. Cytochrome P450 (CYP724B1) is beneficial for BR synthesis in rice (Tanabe et al., 2005). Thus, P450 (CYP734A1) mediates C-26 hydroxylation of BRs to inactivate the plant hormone (Park et al., 2006). SDX up-regulated the gene encoding CYP724B1, but down-regulated CYP734A1 gene expression (Table S5). The transcription factor Bee3 is a positive regulator of BR signalling, and SDX and IBA induced Bee3 in HLB-affected citrus root (Table S5). Therefore, SDX is a positive regulator of BR synthesis and signalling (Figure 5).

The function of ROS in root hair development has been reported in Arabidopsis (Foreman et al., 2003). Peroxidase (PRX) can produce ROS [hydroxyl radical (•OH)], which is involved in cell wall modification. Kwasniewski et al. (2013) found that accumulation of PRX correlates with root hair initiation in barley. SDX enhanced the expression of (4/6) PRX genes in HLB-affected citrus roots, and PRX genes were also induced by IBA (Table S5). Therefore, the expression of PRX genes enhanced by SDX may induce root hair growth in HLB-affected citrus (Figure 5). Moreover, glutathione homeostasis plays an important role in auxin transport and root development.

Glutathione S-transferase (GST) can convert glutathione from reduced (GSH) to oxidized (GSSG) forms, and GST genes were found to be highly induced by IBA (Wei et al., 2014). In this study, IBA and SDX also significantly induced GST genes in HLB-affected citrus roots (Table S5). Therefore, the enhancement of GST expression in HLB-affected citrus roots treated with SDX may be beneficial for root hair and lateral root growth (Figure 5).

CLas titre were significantly reduced in HLB-affected citrus root in response to SDX, while they remained higher in response to IBA and CK. However, in CK and IBA treatment groups, the CLas titre were much lower at the time of initial treatment compared to 60 days after treatment, which may be due to the collection of new root growth whereby less CLas was present. Several chemical compounds eliminate or suppress plant pathogens by inducing plant resistance (Noutoshi et al., 2012). Jasmonates are associated with the defence response against CLas (Martinelli et al., 2012), and brassinosteroids induce resistance against pathogens and reduce CLas titre in HLB-affected citrus plant (Canales et al., 2016). The results

**FIGURE 4** Quantitative reverse transcription PCR (RT-qPCR) validation of differentially expressed genes (DEGs). (a) Six genes (orange1.1t00416: ACC synthase 1; Cs2g20590: ACC oxidase; orange1.1t00506: ethylene-responsive transcription factor ERF023; Cs1g03260: ethylene-responsive transcription factor 13; orange1.1t05117: auxin response factor 18; Cs9g02750: two-component response regulator ARR2) involved in plant hormones; (b) three genes (Cs7g08820: polygalacturonase; Cs6g22150: UDP-glycosyltransferase 83A1; Cs5g11560: UDP-glucose 6-dehydrogenase 1) involved in cell wall metabolism; (c) three genes (Cs8g03480: pathogenesis-related 1; orange1.1t04592: RP5S; Cs2g23650: calmodulin-like protein) involved in resistance to plant pathogens; (d) one gene (Cs9g10430: glutathione S-transferase) involved in reactive oxygen species; (e,f) Pearson correlation of fold change analysed between RT-qPCR and RNA-Se in sulfadimethoxine sodium (SDX) and indole butyric acid (IBA) treatment, respectively. mRNA abundance was normalized using the housekeeping GADPH gene; relative gene expression levels are represented by the log₂ ratio. RT-qPCR data are presented as mean ± SD (n = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

**FIGURE 5** Overview of major pathways for the effect of sulfadimethoxine sodium (SDX) on huanglongbing-affected citrus roots. The red highlight indicates the pathway is regulated by SDX as well as indole butyric acid (IBA) [Colour figure can be viewed at wileyonlinelibrary.com]
presented here show that SDX increased several genes from the jasmonate and brassinosteroid pathways, which are involved in citrus resistance (Table S5 and Figure 5).

A complex signalling pathway that leads to ROS generation and results in a hypersensitive response (HR) activates the plant defence response to bacterial infection. A veritable symmetry of cytosolic signalling molecules (Ca²⁺) have been suggested to be early HR signalling components. Hetherington and Brownlee (2004) suggested that the cyclic nucleotide-gated ion channel (CNGC) may be involved in Ca²⁺ uptake in plants and plasma membrane Ca²⁺ currents associated with signal transduction cascades. Transient changes in the permeability of the plasma membrane to Ca²⁺ is a common early event in plant defence signalling. The information encoded in transient Ca²⁺ changes is decoded by an array of Ca²⁺-binding proteins giving rise to a cascade of downstream effects (Ranf et al., 2011). In HLB-affected citrus roots, SDX induced expression of DEGs related to CNGCs, calcium-binding protein, and the respiratory burst oxidase homolog protein (Table S5). Therefore, SDX can stimulate the citrus defence response to CLas infection via ROS generation and HR (Figure 5).

Many plant secondary metabolites have antibacterial properties. The expression of several DEGs involved in the formation of secondary metabolites with known antibacterial activity, including (+)-neomenthol dehydrogenase, chalcone synthase, and cycloartenol synthase, were induced in HLB-affected citrus roots in response to SDX (Table S5) (Abe et al., 1993; Choi et al., 2008; Dao et al., 2011). Therefore, SDX-mediated induction of these genes could induce protective effects against CLas (Figure 5).

The results presented here show that SDX increased ARSA in HLB-affected citrus, which may have resulted from the induction of root hair and lateral root growth. Through transcriptomic analysis, we shed light on how SDX regulates HLB-affected citrus root metabolism. In response to SDX, the expression of several genes involved in metabolism of plant hormones (including auxin, ethylene, cytokinin, jasmonic acid, and brassinosteroid) and ROS were enhanced, which may be beneficial for root hair and lateral growth in HLB-affected citrus. SDX can induce plant disease resistance against CLas via regulation of jasmonates, brassinosteroids, ROS, and secondary metabolites. However, the key genes closely related to plant hormones and ROS, and resistance to CLas, must be identified and isolated in future research. We propose a model whereby SDX regulates metabolic pathways for increasing ARSA and resistance against CLas in HLB-affected citrus root. These data provide new insight into how HLB-affected citrus roots respond to sulphonamide antibiotics, and how these may be beneficial for HLB-affected citrus root recovery and CLas defence.

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AUTHOR CONTRIBUTIONS

Conception and design of experiment: C.P., Y.D., and M.Z; performance of experiments: C.Y.; data analysis: M.Z., C.Y., and Y.H; contribution of reagents/materials/analysis tools: C.P. and Y.D; authorship and revision of paper: C.Y., M.Z., V.A., C.P., and Y.D.

DATA AVAILABILITY STATEMENT

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

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REFERENCES

Abe, I., Rohmer, M. and Prestwich, G.D. (1993) Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. Chemical Reviews, 93, 2189–2206.

Böttger, M. (1974) Apical dominance in roots of Pisum sativum L. Planta, 121, 253–261.

Bové, J.M. (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology, 88, 7–37.

Brain, R.A., Ramirez, A.J., Fulton, B.A., Chambliss, C.K. and Brooks, B.W. (2008) Herbicidal effects of sulfamethoxazole in Lemma gibba: using p-amino benzoic acid as a biomarker of effect. Environmental Science and Technology, 42, 8965–8970.

Canales, E., Coll, Y., Hernández, I., Portieles, R., Rodriguez García, M., López, Y. et al. (2016) ‘ Candidatus Liberibacter asiaticus’, causal agent of citrus huanglongbing, is reduced by treatment with brassinosteroids. PLoS ONE, 11, e0146223.

Choi, H.W., Lee, B.G., Kim, N.H., Park, Y., Lim, C.W., Song, H.K. et al. (2008) A role for a menthone reductase in resistance against microbial pathogens in plants. Plant Physiology, 148, 383–401.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M. and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 21, 3674–3676.

Dao, T., Linthorst, H. and Verpoorte, R. (2011) Chalcone synthase and its functions in plant resistance. Phytocchemistry Reviews, 10, 397.

Duan, Y., Zhou, L., Hall, D.G., Li, W., Doddapaneni, H., Lin, H. et al. (2009) Complete genome sequence of citrus huanglongbing bacterium, ‘ Candidatus Liberibacter asiaticus’ obtained through metagenomics. Molecular Plant-Microbe Interactions, 22, 1011–1020.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A. et al. (2003) Reactive oxygen species produced by NAPOX oxidase regulate plant cell growth. Nature, 422, 442–446.

Hagen, G. and Guilfoyle, T. (2002) Auxin-responsive gene expression: genes, promoters and regulatory factors. Plant Molecular Biology, 49, 373–385.

Hetherington, A.M. and Brownlee, C. (2004) The generation of Ca²⁺ signals in plants. Annual Review of Plant Biology, 55, 401–427.

Johnson, E., Wu, J., Bright, D. and Graham, J. (2014) Association of ‘ Candidatus Liberibacter asiaticus’ root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. Plant Pathology, 63, 290–298.

Koressaar, T. and Remm, M. (2007) Enhancements and modifications of Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics, 23, 1289–1291.

Kwasniewski, M., Chwiałkowska, K., Kwasniewska, J., Kusak, J., Siwinski, K. and Szarejko, I. (2013) Accumulation of peroxidase-related reactive oxygen species in trichoblasts correlates with root hair initiation in barley. Journal of Plant Physiology, 170, 185–195.

Li, W., Hartung, J.S. and Levy, L. (2006) Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species
associated with citrus huanglongbing. *Journal of Microbiological Methods*, 66, 104–115.

Martinelli, F., Uratsu, S.L., Albrecht, U., Reagan, R.L., Phu, M.L., Britton, M. *et al.* (2012) Transcriptome profiling of citrus fruit response to huanglongbing disease. *PLoS ONE*, 7, e38039.

Michelini, L., Gallina, G., Capolongo, F. and Ghisi, R. (2014) Accumulation and response of willow plants exposed to environmental relevant sulphonamide concentrations. *International Journal of Phytoremediation*, 16, 947–961.

Morgan, K.T., Obreza, T. and Scholberg, J. (2007) Orange tree fibrous root length distribution in space and time. *Journal of the American Society for Horticultural Science*, 132, 262–269.

Mu, Z., Zhang, S., Zhang, L., Liang, A. and Liang, Z. (2006) Hydraulic conductivity of whole root system is better than hydraulic conductivity of single root in correlation with the leaf water status of maize. *Botanical Studies*, 47, 145–151.

Noutsoshi, Y., Ikeda, M., Saito, T., Osada, H. and Shirasu, K. (2012) Sulphonamides identified as plant immune-priming compounds in high-throughput chemical screening increase disease resistance in *Arabidopsis thaliana*. *Frontiers in Plant Science*, 3, 245.

Park, W., Kim, H.B., Kim, W.T., Park, P.B., An, G. and Choe, S. (2006) Ricebending lamina 2 (bla2) mutants are defective in a cytochrome P450 (CYP734A6) gene predicted to mediate brassinosteroid catabolism. *Journal of Plant Biology*, 49, 469.

Potters, G., Pasternak, T.P., Guizez, Y. and Jansen, M.A. (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. *Plant, Cell and Environment*, 32, 158–169.

Rahman, A., Hosokawa, S., Oono, Y., Amakawa, T., Goto, N. and Tsurumi, S. (2002) Auxin and ethylene response interactions during Arabidopsis root hair development dissected by auxin influx modulators. *Plant Physiology*, 130, 1908–1917.

Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J. and Scheel, D. (2011) Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *The Plant Journal*, 68, 100–113.

Spartz, A.K., Ren, H., Park, M.Y., Grandt, K.N., Lee, S.H., Murphy, A.S. *et al.* (2014) SAUR inhibition of PP2C-D phosphatases activates plasma membrane H+-ATPases to promote cell expansion in *Arabidopsis*. *The Plant Cell*, 26, 2129–2142.

Spreen, T.H., Baldwin, J.P. and Futch, S.H. (2014) An economic assessment of the impact of huanglongbing on citrus tree plantings in Florida. *HortScience*, 49, 1052-1055.

Tanabe, S., Ashikari, M., Fujioka, S., Takatsuto, S., Yoshida, S., Yano, M. *et al.* (2005) A novel cytochrome P450 is implicated in brassinosteroid biosynthesis via the characterization of a rice dwarf mutant, dwarf11, with reduced seed length. *The Plant Cell*, 17, 776–790.

Tinker, P.B. and Nye, P.H. (2000) *Solute Movement in the Rhizosphere*. Oxford: Oxford University Press.

To, J.P., Haberer, G., Ferreira, F.J., Deruëre, J., Mason, M.G., Schaller, E. *et al.* (2004) Type-A Arabidopsis response regulators are partially redundant negative regulators of cytokinin signaling. *The Plant Cell*, 16, 658–671.

Wei, K., Wang, L.Y., Wu, L.Y., Zhang, C.C., Li, H.L., Tan, L.Q. *et al.* (2014) Transcriptome analysis of indole-3-butyric acid-induced adventitious root formation in nodal cuttings of *Camellia sinensis* (L.). *PLoS ONE*, 9, e107201.

Woodward, A.W. and Bartel, B. (2005) Auxin: regulation, action, and interaction. *Annals of Botany*, 95, 707–735.

Xu, Q., Chen, L.L., Ruan, X., Chen, D., Zhu, A., Chen, C. *et al.* (2013) The draft genome of sweet orange (*Citrus sinensis*). *Nature Genetics*, 45, 59.

Zhang, M., Guo, Y., Powell, C.A., Doud, M.S., Yang, C. and Duan, Y. (2014) Effective antibiotics against *Candidatus Liberibacter asiaticus* in HLB-affected citrus plants identified via the graft-based evaluation. *PLoS ONE*, 9, e111032.

Zhu, C., Gan, L., Shen, Z. and Xia, K. (2006) Interactions between jasmonates and ethylene in the regulation of root hair development in *Arabidopsis*. *Journal of Experimental Botany*, 57, 1299–1308.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

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