Heterologous Expression of the Constitutive Disease Resistance 2 and 8 Genes from Poncirus trifoliata Restored the Hypersensitive Response and Resistance of Arabidopsis cdr1 Mutant to Bacterial Pathogen Pseudomonas syringae

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Abstract: Huanglongbing (HLB), also known as citrus greening, is the most destructive disease of citrus worldwide. In the United States, this disease is associated with a phloem-restricted bacterium, Candidatus Liberibacter asiaticus. Commercial citrus cultivars are susceptible to HLB, but Poncirus trifoliata, a close relative of Citrus, is highly tolerant of HLB. Isolating P. trifoliata gene(s) controlling its HLB tolerance followed by expressing the gene(s) in citrus is considered a potential cisgenic approach to engineering citrus for tolerance to HLB. Previous gene expression studies indicated that the constitutive disease resistance (CDR) genes in P. trifoliata (PtCDRs) may play a vital role in its HLB tolerance. This study was designed to use Arabidopsis mutants as a model system to confirm the function of PtCDRs in plant disease resistance. PtCDR2 and PtCDR8 were amplified from P. trifoliata cDNA and transferred into the Arabidopsis cdr1 mutant, whose resident CDR1 gene was disrupted by T-DNA insertion. The PtCDR2 and PtCDR8 transgenic Arabidopsis cdr1 mutant restored its hypersensitive response to the bacterial pathogen Pseudomonas syringae pv. tomato strain DC3000 (Pst DC3000) expressing avrRpt2. The defense marker gene PATHOGENESIS RELATED 1 (PR1) expressed at much higher levels in the PtCDR2 or PtCDR8 transgenic cdr1 mutant than in the non-transgenic cdr1 mutant with or without pathogen infection. Multiplication of Pst DC3000 bacteria in Arabidopsis was inhibited by the expression of PtCDR2 and PtCDR8. Our results showed that PtCDR2 and PtCDR8 were functional in Arabidopsis and played a positive role in disease resistance and demonstrated that Arabidopsis mutants can be a useful alternate system for screening Poncirus genes before making the time-consuming effort to transfer them into citrus, a perennial woody plant that is highly recalcitrant for Agrobacterium or biolistic-mediated transformation.

Keywords: Huanglongbing; disease resistance; cisgenics; genetic engineering; Poncirus; Arabidopsis

1. Introduction

Huanglongbing (HLB), also known as citrus greening, is the most destructive disease in citrus worldwide. The typical symptoms of HLB include asymmetrical blotchy yellowing or mottling on leaves and yellowing of leaf veins. As the disease progresses, citrus fruit become lopsided and smaller, and diseased mature fruit remain partially green. Eventually, the infected trees die [1]. HLB is associated with the phloem-restricted gram-negative bacteria Candidatus Liberibacter spp. that can be transmitted.
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by insect vectors or grafting [2]. In the United States (U.S.), the presumptive pathogen of HLB is Candidatus Liberibacter asiaticus (Clas), and the insect vector of Clas is Asian citrus psyllid (ACP) (Diaphorina citri). The outbreak of HLB in Florida has resulted in a 72.2% reduction in citrus juice production and a 20.5% reduction in fresh market fruit in the U.S. from 2007–2008 to 2017–2018 [3]. This disease has been responsible for substantial increases in the costs of citrus grove maintenance and management [4]. The great majority of commercial citrus cultivars are highly susceptible to HLB [5]. The pathogen has not been cultured, and the virulence mechanisms of Clas remain largely unknown. Nevertheless, several reports have shown that Clas encodes effectors to inhibit citrus plant defense and development [6–13].

Management of HLB has been a lofty challenge, although a wide range of management techniques have been tested, ranging from the frequent application of insecticides in an attempt to suppress the psyllid vector, enclosing individual citrus trees in ACP-proof nets to prevent them from contact with infectious psyllids [14] to planting new citrus trees inside ACP-proof screen houses and the application of antibiotics to citrus trees. So far, the primary techniques used by Florida growers for managing HLB under field conditions have been applying insecticides to suppress the population of ACP [15] and modifying nutrient management strategies to reduce HLB symptoms and improve fruit yield and quality [16–19]. These management practices have been extremely costly, making them an unsustainable endeavor. With the citrus production in Florida alone being a $9 billion industry, it is vital to find more effective, economic, and sustainable management strategies to curb the devastation caused by this disease [20].

The development and use of HLB-resistant/tolerant cultivars is considered the best long-term management strategy for this bacterial disease. Toward finding HLB resistance/tolerance, numerous citrus cultivars, close relatives, and distant citrus relatives have been screened under natural disease pressure and ACP presence or by artificial Clas inoculation. Within the genus Citrus, a number of commercial cultivars with HLB tolerance were identified, including ‘LB-9’ Sugar Bello® mandarin, ‘Temple’ tangor, rough lemon, etc. [21–25]. A number of rootstock cultivars resulting from crosses between Citrus and Poncirus showed strong tolerance to HLB [26–28]. Several distant citrus relatives were found having strong resistance to HLB [14,29]. In these evaluations, sweet orange, grapefruit, and most mandarins, citrus of the most important commercial value to the industry, were highly sensitive to HLB. Sweet orange, grapefruit, and mandarin cultivars with strong tolerance, ideally resistance, to HLB are much needed.

To increase citrus tolerance or resistance to HLB, a number of foreign or synthetic genes have been introduced into citrus and have shown some promising results. For example, thionin, belonging to the pathogenesis-related 13 family [30], overexpressed in citrus resulted in increased resistance to HLB and citrus canker [31]. Another antimicrobial peptide, cecropin B, was expressed in citrus phloem and reduced HLB severity in the transgenic citrus [32]. The master regulator gene of plant defense, NPR1 from Arabidopsis, was transferred into citrus, and the transgenic plants showed enhanced resistance to HLB [33]. Although these transgenic approaches can be a powerful tool to develop HLB resistance/tolerance, commercialization and export of transgenic citrus fruit and processed citrus products may encounter marketplace resistance because of negative public perception and anti-GMO sentiments.

To make engineered HLB resistant/tolerant citrus cultivars more acceptable to citrus consumers and producers, cisgenic approaches seem worthy of exploration. Cisgenic approaches have been pursued in a number of crops, and cisgenic apple lines with resistance to several diseases have been developed [34,35]. Consumer surveys indicated that cisgenic products were evidently more acceptable to consumers than transgenic products [36]. Recently, the U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) issued new rules and regulations governing the movement, including environmental release, of certain genetically engineered (GE) organisms (https://www.aphis.usda.gov/brs/ledregister/BRs_2020518.pdf). Under these new rules, GE plants can be exempted from regulation if the genetic modification in the plants introduces a gene known to occur
in the plant’s gene pool. As these new rules and regulations become effective in 2020, citrus cultivars with genetically engineered HLB resistance from cisgenic approaches may become commercialized more readily than before in the U.S. It should be pointed out that at this time, regulatory bodies in other countries have not differentiated cisgenic from transgenic plants.

Numerous efforts have been made to understand the tolerance mechanisms of citrus with regards to HLB. Basal resistance was considered to play an important role in the tolerance of some citrus to HLB, as shown by analyzing the transcriptional profiles of two closely related HLB-tolerant ‘Jackson’ grapefruit-like hybrid trees and HLB-susceptible ‘Marsh’ grapefruit trees [37]. HLB-tolerant rough lemon showed a stronger and faster response to CLas infection at earlier stages than susceptible sweet orange [22]. The constitutive disease resistance 1 (CDR1) has been implicated in HLB tolerance in some studies [28]. This gene was induced in HLB-susceptible ‘Cleopatra’ mandarin, but the basal expression level of CDR1 was much higher in the HLB-tolerant citrus rootstock cultivar ‘US-897’ than in ‘Cleopatra’ mandarin [28]. Constitutive overexpression of CDR1 in Arabidopsis resulted in activation of its defense genes and enhanced plant disease resistance [38]. Arabidopsis CDR1 is an extracellular aspartic protease with the conserved catalytic sequence motifs DTG and DSG [39]. The optimized condition for CDR1 activity is a pH of 6.0–6.5 in a dimerized state [40]. Salicylic acid (SA) was required in CDR1-mediated disease resistance [28]. Expression of the CDR1 gene in rice (OsCDR1) was activated upon treatment with SA [41]. Overexpression of OsCDR1 in Arabidopsis and rice conferred enhanced resistance against bacterial and fungal pathogens [41]. The function of OsCDR1 in disease resistance was found to be dependent on its proteinase activity [41,42].

Poncirus trifoliata is a close relative of Citrus [43] and has been the most important source of disease resistance genes for citrus breeding and genetic improvement. Several studies have shown that P. trifoliata and its hybrids with Citrus are highly tolerant to HLB [14,44]. It was found that the constitutive disease resistance 2 and constitutive disease resistance 8 from P. trifoliata (PtCDR2/PtCDR8) were upregulated upon CLas infection [45]. If these genes could confer citrus resistance/tolerance to HLB, they could be used in a cisgenic approach to produce HLB-resistant/tolerant citrus cultivars that may be more acceptable to citrus consumers and producers. In this study, PtCDR2/PtCDR8 were cloned and transferred into the Arabidopsis cdr1 mutant. PtCDR2/PtCDR8 transgenic Arabidopsis cdr1 lines showed a typical hypersensitive response (HR) to Pseudomonas syringae pv. tomato strain DC3000 (Pst DC3000 expressing avrRpt2 (Pst DC3000 avrRpt2), a model pathogen widely used to test Arabidopsis plants for disease resistance. Expression of PtCDR2 and PtCDR8 in Arabidopsis cdr1 lines inhibited growth of Pst DC3000 avrRpt2. Our results showed that Arabidopsis mutants could be used to screen genes from Poncirus (and Citrus) for their roles in plant disease resistance before extensive studies in Citrus. Using Arabidopsis mutants as an alternate system may help identify candidate genes for engineering citrus for HLB tolerance/resistance.

2. Results

2.1. Cloning and Structure of Constitutive Disease Resistance 2 and 8 from Poncirus trifoliata

PtCDR2 and PtCDR8 were amplified from cDNA synthesized from mRNAs isolated from mature leaves of P. trifoliata. The deduced PtCDR2 and PtCDR8 proteins both contain 428 amino acid residues and share 94.2% identity. PtCDR2 and PtCDR8 were aligned with predicted CDR1 proteins from sweet orange (Citrus sinensis) (CsCDR1 XP_006484735), Clementine mandarin (Citrus clementina) (CcCDR1 XP_006437356), pummelo (Citrus maxima) (Cg6g008160.1), citron (Citrus medica) (Cm260950.1), ‘Mangshan’ mandarin (Citrus reticulata) (MSYJ114420.1), Chinese box orange (Atalantia buxifolia) (sb29852.1), and Arabidopsis (AtCDR1 AY243479). Two conserved domains (DTGS and DSGT) were found in these deduced proteins (Figure 1A). PtCDR2 and PtCDR8 fell into the same clade in the phylogenetic tree (Figure 1B).
T1 transgenic lines were produced. T1 transgenic lines were inoculated with the bacterial pathogen *Pst* in infiltration (DPI) with *Pst* or infection (OD) at 0.0001. The mutant resulting from T-DNA insertion [46] was transformed with *PtCDR2* and *PtCDR8*. To test whether *PtCDR2* and *PtCDR8* can function as *CDR1* in disease resistance, an *Arabidopsis* cdr1 mutant resulting from T-DNA insertion [46] was transformed with *PtCDR2* and *PtCDR8* separately. Multiple T0 transgenic lines were obtained for each gene. These T0 lines were selfed, and multiple T1 transgenic lines were produced. T1 transgenic lines were inoculated with the bacterial pathogen *Pst* DC3000 *avrRpt2* at OD 0.0001. The mutant *Arabidopsis* cdr1 did not show any HR at 3 days post inoculation or infiltration (DPI) with *Pst* DC3000 *avrRpt2*. On the contrary, wild-type *Arabidopsis* (Col-0) showed a typical HR. Twelve *PtCDR2* transgenic *cdr1* mutant lines and fourteen *PtCDR8* transgenic *cdr1* mutant lines showed the same type of HR as the wild-type *Arabidopsis*. For each Poncirus gene, two transgenic *Arabidopsis* lines were selected randomly to record HR on the infiltrated leaves (Figure 2) and to perform subsequent analyses.

2.2. *PtCDR2* and *PtCDR8* Restored the Hypersensitive Response of *Arabidopsis* *cdr1* Mutant to Pathogen

To test whether *PtCDR2* and *PtCDR8* can function as *CDR1* in disease resistance, an *Arabidopsis* *cdr1* mutant resulting from T-DNA insertion [46] was transformed with *PtCDR2* and *PtCDR8* separately. Multiple T0 transgenic lines were obtained for each gene. These T0 lines were selfed, and multiple T1 transgenic lines were produced. T1 transgenic lines were inoculated with the bacterial pathogen *Pst* DC3000 *avrRpt2* at OD 0.0001. The mutant *Arabidopsis* *cdr1* did not show any HR at 3 days post inoculation or infiltration (DPI) with *Pst* DC3000 *avrRpt2*. On the contrary, wild-type *Arabidopsis* (Col-0) showed a typical HR. Twelve *PtCDR2* transgenic *cdr1* mutant lines and fourteen *PtCDR8* transgenic *cdr1* mutant lines showed the same type of HR as the wild-type *Arabidopsis*. For each Poncirus gene, two transgenic *Arabidopsis* lines were selected randomly to record HR on the infiltrated leaves (Figure 2) and to perform subsequent analyses.

![Figure 1](image_url)
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... bacterial count in the inhibition: bacterial counts in these lines were reduced by 8.0- and 15.0-fold, respectively, compared to the bacterial count in the wild-type Arabidopsis, PtdCDR2 transgenic cdr1 lines, and PtdCDR8 transgenic cdr1 lines all showed cell death or HR in the infiltrated leaf area.

2.3. PtdCDR2 and PtdCDR8 Inhibited Pst DC3000 avrRpt2 Growth

At 3 DPI with Pst DC3000 avrRpt2, six leaf disks were randomly collected from each Arabidopsis plant for bacterial counting. On average, the bacterial count in the wild-type Arabidopsis was 6.3-fold less than the bacterial count in the cdr1 mutant (Figure 3). Bacterial counts in PtdCDR8 transgenic lines 5 and 11 (cdr1/PtdCDR8-5 and cdr1/PtdCDR8-11) were similar to the bacterial count in the wild-type Arabidopsis and reduced by 6.86- and 6.67-fold, respectively, compared to the bacterial count in the cdr1 mutant. PtdCDR2 transgenic lines 6 and 9 (cdr1/PtdCDR2-6 and cdr1/PtdCDR2-9) showed stronger bacterial growth inhibition: bacterial counts in these lines were reduced by 8.0- and 15.0-fold, respectively, compared to the bacterial count in the cdr1 mutant (Figure 3).

Figure 2. PtdCDR2 and PtdCDR8 restored hypersensitive response. Wild-type Arabidopsis Col-0, cdr1 mutant, PtdCDR2 transgenic cdr1 line 6/line 9, and PtdCDR8 transgenic cdr1 line 5/line 11 were infiltrated with Pseudomonas syringae pv. tomato strain DC3000 (Pst DC3000) expressing avrRpt2. Cell death or hypersensitive response (HR) was not observed in the infiltrated area of leaves of the cdr1 mutant; wild-type Arabidopsis, PtdCDR2 transgenic cdr1 lines, and PtdCDR8 transgenic cdr1 lines all showed cell death or HR in the infiltrated leaf area.

Figure 3. Bacteria growth assay. Twenty-five-day-old Arabidopsis plants were inoculated with Pseudomonas syringae pv. tomato strain DC3000 (Pst DC3000) expressing avrRpt2 by spraying a bacterial cell suspension. Six leaf disks from each plant were collected randomly at 3 DPI, and bacterial cells were extracted and plated out on a solid selective medium containing two antibiotics. Inoculated plants were wild-type Arabidopsis (Col-0), cdr1 mutant (cdr1), and four transgenic cdr1 lines (cdr1/PtdCDR2-6, cdr1/PtdCDR2-9, cdr1/PtdCDR8-5, and cdr1/PtdCDR8-11)
2.4. PtCDR2 and PtCDR8 Increased the Expression Level of PATHOGENESIS RELATED 1 (PR1) in the cdr1 Mutant

Overexpression of OsCDR1 in Arabidopsis led to the upregulation of PR1 [41], a marker gene in the SA pathway [47]. The PR1 relative expression level was analyzed among different transgenic lines 3 DPI with *Pst* DC3000 *avrRpt2*. Arabidopsis plants in the control were sprayed with a 10 mM MgCl₂ solution containing 0.02% Silwet L-77. PR1 expressed at a lower level in the *cdr1* mutant than in the wild-type Arabidopsis (Col-0) (0.38-fold) without inoculation of the pathogen (Figure 4A). *PR1* expressed slightly higher in transgenic *PtCDR2* lines 6 and 9 (1.66- and 1.47-fold, respectively) but much higher in *PtCDR8* transgenic lines 5 and 11 (8.86- and 10.99-fold, respectively) (Figure 4A). At 3 DPI, *PR1* expression level was much higher in all genotypes, including the wild type, the *cdr1* mutant, and the four transgenic lines (Figure 4B), suggesting that inoculation with *Pst* DC3000 *avrRpt2* induced *PR1* expression in all genotypes. At this time, *PR1* expression in the *cdr1* mutant was still the lowest and lower than its expression in the wild-type *Arabidopsis*. Different levels of *PR1* upregulation were observed in the transgenic lines: *PtCDR2* line 9 showed the greatest *PR1* upregulation, from 1.47 at 0 DPI to 104.24 at 3 DPI, followed by *PtCDR8* lines 5 and 11 (from 8.66 or 10.99 at 0 DPI to 78.75 or 77.22 at 3 DPI), and *PtCDR2* line 6 (from 1.66 at 0 DPI to 43.12 at 3 DPI) (Figure 4B).

![Figure 4: Relative expression of PR1 in wild-type Arabidopsis (Col-0), its cdr1 mutant (cdr1), and four transgenic lines (cdr1/PtCDR2-6, cdr1/PtCDR2-9, cdr1/PtCDR8-5, and cdr1/PtCDR8-11).](image)

3. Discussion

Previous gene expression and genetic mapping studies have indicated that multiple genes could be involved in certain *Poncirus* and *Citrus* cultivars’ resistance/tolerance to HLB [22,28,43,48], but so far very few of these candidate genes have been screened and investigated in depth for their actual roles in HLB resistance/tolerance. The primary impediments leading to the lack of progress in candidate gene screening and gene function confirmation are the existence of substantial difficulties in the production of transgenic citrus plants for these candidate genes, inoculation of the transgenic plants with CLas, and collection of reliable CLas bacterial titers and HLB symptom severity scores from the inoculated transgenic plants. Alternate systems are much needed to circumvent some of these challenges and to screen multiple candidate genes to identify the best ones for use in engineering citrus for HLB resistance. Facing similar difficulties with studying CLas in citrus, plant pathologists have made use of the model plant *Nicotiana benthamiana* to express candidate CLas effector genes and understand their roles in CLas pathogenesis [6]. Recently, *Nicotiana benthamiana* was also experimentally infected with CLas via dodder transmission and used to identify a critical CLas effector LasΔ5315 and determine its role in the development of prominent HLB symptoms, including starch accumulation and leaf chlorosis [49].
Previously, the garden flower periwinkle (Catharanthus roseus) was infected with CLAs, and the infected periwinkle was used to screen antibiotics and various other chemical compounds for controlling or suppressing CLAs in planta [50]. We considered using Nicotiana benthamiana and periwinkle for testing the potential role of PtCDR2 and PtCDR8 in plant disease resistance. Nicotiana benthamiana can be readily transformed by co-cultivation of leaf discs with Agrobacterium, and transgenic lines can be inoculated with CLAs via dodder transmission [49]. However, information was not available regarding Nicotiana benthamiana resident CDR1 or CDR1-like genes, and desired cdr1 mutants were not available. Periwinkle is extremely difficult to transform with Agrobacterium, and no information was available about its resident CDR1 or CDR1-like genes either. This situation prompted us to explore the use of Arabidopsis mutants as an alternate system to test PtCDR2 and PtCDR8 and to determine if they would function in Arabidopsis and play any role in disease resistance.

Our results described above clearly showed that both PtCDR2 and PtCDR8 restored the function of Arabidopsis CDR1 and conferred the cdr1 mutant HR and resistance to the bacterial pathogen Pst DC3000 expressing avrRpt2. Our study indicated that Arabidopsis mutants could serve as a useful proxy for screening candidate genes and confirming their role in disease resistance. Using proper Arabidopsis mutants may offer a number of advantages for screening candidate disease resistance and defense genes, as shown in this study. Foreign genes can be readily introduced into Arabidopsis mutants by simple floral dip procedures. Sufficient numbers of homozygous transgenic lines could be produced within several months by selfing T0 transgenic plants. More importantly, a huge collection of Arabidopsis mutants, including numerous mutants with their resident disease resistance and defense genes disrupted, are readily available for use in both forward and reverse genetic studies [51]. Arabidopsis mutants have especially helped formulate the concept of the plant immune system and reveal various plant disease immunity signaling pathways and key genetic factors in these pathways [52]. This study may represent the first effort in using Arabidopsis mutants to screen Poncirus genes for their potential roles in disease resistance. Our study demonstrated the value of Arabidopsis mutants in such an effort. We believe that these mutants may play even more important roles in future citrus genetic studies toward identifying candidate genes for engineering citrus for HLB resistance when CLAs cultures become available.

Our results showed that PtCDR2 and PtCDR8 induced significant upregulation of PR1 after pathogen inoculation. The induced PR1 upregulation may indicate the potential value of expressing PtCDR2 and PtCDR8 for enhancing HLB resistance in citrus. PR1 is an inducible marker gene for the SA-mediated plant defense system and plays key roles in plant systemic acquired resistance (SAR) to diseases. In a recent study in citrus, the level of PR1 expression in transgenic sweet orange cultivars ‘Hamlin’ and ‘Valencia’ was directly co-related to the Arabidopsis NPR1-mediated resistance to HLB, whereas the expression of the transgene AtNPR1 itself in citrus was not directly co-related to HLB resistance [33]. A similar phenomenon was observed in rice and Arabidopsis transformed with rice CDR1 (OsCDR1). OsCDR1-enhanced disease resistance in transgenic rice and Arabidopsis was also correlated with the induction of PR1 [41]. Based on the observed relationship between PR1 expression and enhanced disease resistance, especially HLB resistance in AtNPR1-transgenic sweet orange, it seems reasonable to speculate that overexpression of PtCDR2 and PtCDR8 may result in similar upregulation of PR1 in transgenic sweet orange and, thus, similarly enhanced HLB resistance. On the other hand, PtCDR2 and PtCDR8 are predicted to be extracellular proteins, like OsCDR1 [41]. This may raise a question: How could PtCDR2 and PtCDR8 as predicted extracellular proteins impart citrus resistance/tolerance to CLAs, a bacterial pathogen residing inside citrus phloem elements? Considering these aspects, we speculate that the functioning of PtCDR2 and PtCDR8 may involve some intriguing pathways or pathway components to activate the plant defense system. It has been hypothesized that OsCDR1 activation can lead to the generation of an endogenous extracellular peptide elicitor, and the released elicitor can rapidly activate basal local and systemic defense responses [40,41]. To answer these questions in citrus, we initiated an effort to introduce these genes into sweet orange and produce transgenic lines. The transgenic sweet orange lines will be inoculated with CLAs to determine their resistance to HLB.
Overexpression of OsCDR1 in rice resulted in enhanced resistance to *Xanthomonas oryzae* and *Magnaporthe oryzae*, the bacterial pathogen of rice blight and the fungal pathogen of rice blast [41]. Overexpression of the same gene in *Arabidopsis* led to increased resistance against infection by bacterial pathogen *Pst* DC3000 and fungal pathogen *Hyaloperonospora arabidopsidis* but not against the necrotrophic pathogen *Alternaria brassicicola* [41]. These previous studies seem to indicate potential of using PtCDR2 and/or PtCDR8 for engineering resistance to multiple pathogens. In citrus, the bacterial pathogen *Xanthomonas citri* ssp. *citri* (*Xcc*) causes citrus canker, a disease that is important and also difficult to control [53]. Natural genetic resistance to *Xcc* is rare in citrus. Transgenic resistance to *Xcc* has been pursued for many years [54,55]. It will be very interesting to find out if PtCDR2 and PtCDR8 transgenic sweet orange will have increased resistance to *Xcc*.

In summary, we have shown that *Arabidopsis* mutants (in this case, the cdr1 mutant) can serve as a useful alternate system for screening *Poncirus* (and *Citrus*) genes for their roles in plant defense and that PtCDR2 and PtCDR8 both are functional genes and play a key role in plant defense responses. These genes may serve as strong candidate genes for engineering citrus for disease resistance, including resistance to HLB, the deadliest bacterial disease of citrus worldwide.

4. Materials and Methods

4.1. Plant Materials

The *Poncirus trifoliata* plants (accession DPI 50-7) were kept in a greenhouse with natural light. *Arabidopsis* plants were grown in a growth room with a 16 h light/8 h dark cycle at 21 °C. The cdr1 mutant (stock number SALK_050514) and wild-type *Arabidopsis* Col-0 (stock number CS70000) were purchased from TAIR.

4.2. Sequence Analysis

Protein sequence alignment and phylogenetic analysis were performed with the software Mega X [56]. The neighbor-joining method was used to generate a phylogenetic tree. The protein sequences of AtCDR1, CsCDR1, and CcCDR1 were retrieved from NCBI [57,58]; other CDRs from *Citrus maxima* (Cg6g008160.1), *Citrus medica* (Cm260950.1), *Citrus reticulata* (MSYJ114420.1), and *Atalantia buxifolia* (sb29852.1) were retrieved from the *Citrus sinensis* annotation project [59].

4.3. Gene Cloning, Construction of Expression Vectors, and Transformation of Arabidopsis cdr1 Mutant

 Mature leaves were collected from *P. trifoliata* plants grown in containers in the greenhouse. Total RNA was extracted from the leaves using the RNeasy plant mini kit (Qiagen, Cat. 74904). RNAs were reverse transcribed to cDNA using the SuperScript™ III First-Strand Synthesis System (Thermofisher Scientific, Cat. 18080051) and oligo(dT). PtCDR2 and PtCDR8 were amplified with Phusion® High-Fidelity DNA Polymerase (NEB, Cat. M0530S) and primers PtCDR F (CCC GGATGGCAACCTTCTTGAGTTGTGC) and PtCDR2 R (GAGCTCCAAAATTTAATTACAGCTTGGTGC) or PtCDR F and PtCDR8 R (GAGCTCGCTTCCCAATTAATTATTGCTTGGTGC). The amplified DNA fragments were purified after treatment with *Taq* polymerase and cloned into the pGEM-T Easy vector (Promega, Cat. A1360). The plasmid containing PtCDR2 or PtCDR8 was digested with *Xma*I and *Sac*I, and then the PtCDR2 and PtCDR8 fragments were cloned into the expression vector pCAMBIA1300-221 [60]. PtCDR2 and PtCDR8 were driven by the CaMV 35S promoter and terminated by the Nos terminator. These expression vectors were then introduced into the *Agrobacterium tumefaciens* strain EHA105 by electroporation. The *Arabidopsis* cdr1 mutant was transformed by floral dip [61]. T0 transgenic seeds were selected on MS plates containing kanamycin (50 mg/L) and hygromycin (20 mg/L). The positive seedlings were transplanted into soil for disease assay.
4.4. Disease Assay

The model pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (Pst DC3000) expressing *avrRpt2* was used in this research. Bacteria were cultured at 28°C on a shaker in the King’s B medium containing two antibiotics, rifampin (20 mg/L) and kanamycin (50 mg/L). When the optical density (OD$_{600}$) of the bacterial culture reached 0.6, bacterial cells were harvested by centrifugation and resuspended in a 10 mM MgCl$_2$ solution. The fifth to seventh rosette leaves of 25-day-old *Arabidopsis* plants were infiltrated with Pst DC3000 *avrRpt2* at an OD$_{600}$ of 0.0001 (5 × 10$^4$ CFU/mL) using a 1 mL needleless syringe. The inoculated leaves were photographed at 3 DPI. For the bacterial growth assay, the 25-day-old *Arabidopsis* plants were sprayed with a Pst DC3000 *avrRpt2* bacterial suspension at an OD$_{600}$ of 0.001 (5 × 10$^5$ CFU/mL) and containing 0.02% Silwet L-77. Six leaf disks were randomly collected from six plants of each genotype with a core borer (6 mm in diameter). The leaf disks were ground, and the ground tissues were diluted serially by an increment of 10 and plated on solid King’s B medium containing rifampin (20 mg/L) and kanamycin (50 mg/L). The plates were incubated at 28 °C in the dark for two days before bacterial colonies were counted.

4.5. Gene Expression Analysis

The rosette leaves of *Arabidopsis* were collected three days after the plants were spray-inoculated with a Pst DC3000 bacterial suspension. RNAs were extracted from the collected *Arabidopsis* leaf samples using an RNeasy Plant Mini kit (Qiagen, Cat. 74904), following the manual exactly. A High-Capacity RNA-to-cDNA™ Kit (ThermoFisher Scientific, Cat. 4387406) was used for reverse transcription according to the manufacturer’s instruction. Quantitative real-time PCR was performed using PowerUp™ SYBR™ Green Master Mix (ThermoFisher Scientific, Cat. A25742) and the QuantStudio 5 real-time PCR system (ThermoFisher Scientific) according to the manufacturer’s specifications. The *Actin 2* gene was used as the internal reference, and it was amplified with a forward primer 5′-GATCTCCAAGGCCGAGTATG-3′ and a reverse primer 5′-CCCCAGCTTTTTAAGCCTTTG-3′. Defense marker gene *PR1* was analyzed with primers PR1F 5′-CTCATACACTCTGGTGGG-3′ and PR1R 5′-ATTGCACGTGTTCGCAGC-3′. The relative expression of *PR1* was calculated using the 2$^{-\Delta\Delta CT}$ method [62].

**Author Contributions:** X.Y. cloned the genes from *Poncirus trifoliata*, constructed expression vectors, produced transgenic *Arabidopsis* plants, conducted disease assays, analyzed gene expression, and drafted the manuscript. B.R. assisted in plant transformation and edited the manuscript. Z.D. supervised the research and critically revised the manuscript. F.G.G.J. secured funding, participated in experimental designing, and critically revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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**Conflicts of Interest:** The authors declare no conflict of interests.

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