Temporal expression of defence and susceptibility genes and tospovirus accumulation in capsicum chlorosis virus-infected capsicum

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Abstract
Yolo Wonder (YW) and Warlock (W), two capsicum cultivars that are susceptible to capsicum chlorosis virus (CaCV), were compared in terms of symptom development, tospovirus accumulation, and host gene expression during the first 12 days post infection (dpi). Temporal expression of selected early CaCV-response genes was used to gain insights into plant-virus interactions and to identify potential targets for CaCV control. Symptoms developed faster in YW during the first seven days of infection, while systemic symptoms were similar in both cultivars at 10 and 12 dpi. CaCV accumulation was higher in YW at 7 dpi despite a lower titre at 3 dpi. At 12 dpi, virus accumulation was similar for both cultivars. Symptom development appears to be correlated to virus accumulation over time for both cultivars. Chalcone synthase (CHS), cytochrome P450 (CYP), and tetraspanin 8-like (TSP8) genes followed a similar expression pattern over time in both cultivars. The thionin gene showed increased expression in CaCV-infected plants at 12 dpi. The WRKY40 gene showed significant differential expression at all time points in YW, but only at 12 dpi in W. The strongest correlation of temporal gene expression and virus titre was seen for CYP, TSP8, thionin, and WRKY40. CHS and CYP may be involved in symptom development, and TSP8 may be involved in virus movement. CHS, CYP, and TSP8 may be good targets for future overexpression or silencing studies to clarify their functions during virus infection and, potentially, for control of CaCV in capsicum.

Introduction
Capsicum chlorosis virus (CaCV) is taxonomically classified as a member of the species Capsicum chlorosis orthotospovirus, genus Orthotospovirus, family Tospoviridae, order Bunyavirales [1]. CaCV was first reported in 1999 infecting tomato and capsicum crops in Queensland, Australia [2]. CaCV infects economically important vegetable crops, including tomato in Australia, India, Japan, and Thailand, [2–6], chilli pepper in Australia [2], capsicum in Australia, Greece, and Japan [2, 6, 7], and zucchini and peanut in China [8, 9]. Pineapple as well as ornamental plants and weeds, including Ageratum conyzoides, Emilia sonchifolia, Sonchus oleraceus, and Tagetes minuta, are also hosts of CaCV in Australia [10], as are Rudbeckia spp. in Iran [11], amaryllis (Hippeastrum hybridum Hort), blood lily (Haemanthus multiflorus Martyn), and calla lily (Zantedeschia spp.) in Taiwan [12, 13], and waxflower (Hoya calycina Schlechter) in the United States [14].

Three thrips species have been reported to be CaCV vectors: Ceratothripoides claratris [4], Thrips palmi [15], and Frankliniella schultzei [16]. Like all orthotospoviruses, CaCV has a negative-sense or ambisense genome composed of three single-stranded (ss) RNA segments, named Small (S), Medium (M), and Large (L). The first Australian CaCV isolate to be completely sequenced was QLD-3432 (KM589495, KM589494, and KM589493). Its S segment is composed of 3944 nt and encodes the nucleocapsid (N) protein and a non-structural RNA silencing suppressor (NSs), its M segment of 4846 nt encodes a glycoprotein precursor (Gn/Gc) and a non-structural movement protein (NSm), and its L segment of 8913 nt encodes an RNA-dependent RNA polymerase (L protein) [17]. This isolate has an unusually large intergenic region (1663 nt) in the S segment [17].
All current commercial cultivars of *Capsicum annuum* are susceptible to CaCV, but resistant breeding lines have been obtained recently. These were generated by transferring the resistance trait from *C. chinense* PI290972, followed by several generations of back-crossing [16]. Transcriptome analysis of resistant and susceptible capsicum identified genes that were differentially expressed (DE) in response to CaCV 4 days postinoculation (dpi) [18]. At this early time point, plants of the resistant line showed pronounced necrotic local lesions on the inoculated leaves, indicative of a hypersensitive response, while susceptible capsicum plants did not show any symptoms. Several capsicum genes were found to be up- or downregulated in response to CaCV. In total, there were 2484 DE genes in both resistant and susceptible capsicum infected with CaCV, and of these, 273 were exclusive to the resistant line and 70 were exclusive to the susceptible line [18].

The previous study focused on the genes that were DE in the resistant line to assist in the identification of candidate genes potentially involved in CaCV resistance [18]. Although the functions of the genes were predicted *in silico*, their identity and function in the resistance response to CaCV has not yet been elucidated. Several of the DE genes in susceptible capsicum (cv. Yolo Wonder) are associated with pathogen defence or susceptibility, including chalcone synthase (CHS), cytochrome P450 (CYP), tetraspanin 8-like (TSP8), thionin, and transcription factor WRKY40 [19]. CHS is a key enzyme in the flavonoid biosynthesis pathway; its expression is induced by wounding, pathogens, insect herbivory, and ultraviolet light [20]. Plant P450s produce a diverse set of signalling molecules, growth regulators, plant hormones, and defence-related compounds [21]. TSPs are transmembrane proteins that have crucial and diverse roles in trafficking of vesicles, motility, and differentiation of tissues. They are responsible for maintaining a complex network of signalling molecules and other membrane proteins [22]. Thionins are antimicrobial peptides and have functions in seed germination and packaging of proteins for storage, and they act in signal transduction as secondary messengers [23]. WRKYs are part of a large family of transcription factors that are associated with responses to abiotic and biotic stresses and with various physiological processes [24, 25].

The aim of the present study was to examine the temporal expression of these early plant response genes until the point at which CaCV infection becomes systemic. This will likely identify host genes that may be crucial for virus replication and movement and/or genes that are activated by the plant in defence against CaCV infection. This work provides insights into plant-virus interactions during the first 12 days of CaCV infection and is aimed at identifying potential novel host targets to help control CaCV.

### Materials and methods

#### Gene selection and primer design

Five genes associated with pathogen defence or disease susceptibility that were identified previously at 4 dpi as DE in resistant or susceptible capsicum in response to CaCV infection [18] were selected: chalcone synthase (CHS), cytochrome P450 (CYP), tetraspanin 8-like (TSP8), thionin, and transcription factor WRKY40. PCR primers for those genes (Table 1) were designed using Primer3 [26] in Geneious v.9.1.3 [27] based on the contigs assembled in a previous capsicum transcriptome study [18]. The actin gene was chosen as a reference based on results from the previous study [18].

#### Virus inoculation and sample collection

The capsicum cultivars Yolo Wonder and Warlock were grown in a glasshouse at 25°C with 16 h light/8 h dark conditions. Four-week-old carborundum-dusted plants were rub-inoculated with buffer (mock) or CaCV (isolate QLD-3432). Virus inoculum was prepared by grinding a fresh symptomatic CaCV-infected leaf with a mortar and pestle in 10 mM phosphate buffer (pH 7.6) containing 20 mM sodium sulphite. Twelve plants were used for each cultivar: five were mock-inoculated and seven were virus-inoculated. One leaf disc (1 cm diameter) from mock- and CaCV-inoculated plants was collected on days 3, 7, and 12 post-inoculation (dpi) in a 1.5-mL Eppendorf tube, immediately placed into liquid nitrogen, and subsequently stored at -80°C until RNA extraction. The samples at 3 dpi were

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**Table 1** Sequences of primers used for real-time PCR quantification of gene expression in capsicum RNA extracts

| Primer name | Sequence (5’ to 3’) | Ampli-con size (bp) |
|-------------|---------------------|---------------------|
| Actin_F1 | GCTGGACGTGACCTAACTG | 150 |
| Actin_R1 | GCAGTTCGACGCTGCTTC | 105 |
| CHS_For | GCTTCGACCTCCGTCATAAC | 206 |
| CHS_Rev | GCTCCCTGTGGTTTTCAGC | 196 |
| P450_For | TAGTGAAGTCTCCTGGGACTC | 152 |
| P450_Rev | CTCGCAAAATCCATCTCCCAAATC | |
| TSP8_F1 | GTGAGCCCAAACACACTGGAC | |
| TSP8_R1 | CGACGATGGGAAAGTGAGAC | |
| Thionin_F1 | AGTGCCGACACACCTTTTC | |
| Thionin_R1 | CTGCTGCGAGTCTTTTAGCA | |
| WRKY40_For | AGTGACCGGTTCAAGGAAGAG | 118 |
| WRKY40_Rev | CTGATGAACGTCGAGCAACA | |
collected from the inoculated leaf, while the subsequent samples were taken from the youngest systemic leaf.

**RNA extraction, DNase treatment, and cDNA synthesis**

Total RNA was extracted from leaf discs using an RNAeasy® Plant Mini Kit (QIAGEN), and the RNA concentration was measured using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific). Extracts were treated using a Turbo DNA-free™ Kit (Invitrogen, Thermo Fisher Scientific), and the concentration was measured as above. RNA extracts were diluted to 20 ng/µL for use in real-time PCR as a no-RT control. After DNase treatment, cDNA was synthesized using a SuperScript™ III First-Strand Synthesis System (Invitrogen) and an oligo dT primer. The concentration of cDNA was measured by NanoDrop and adjusted to 20 ng/µL for all samples.

**Quantification of gene expression by real-time PCR**

Real-time PCR was done in a Corbett Rotor-Gene 6000 thermal cycler (QIAGEN) using a SensiFast™ SYBR® No-Rox One-Step Kit (Bioline). Each reaction contained 1.0 µL (20 ng) of cDNA, RNA, or water, 0.8 µL of each primer (forward and reverse at 10 µM), 7.4 µL of nuclease-free water, and 10 µL of SensiFast SYBR® No-Rox mix (2x) in 20 µL. The PCR cycling conditions were 95ºC for 2 minutes, 40 cycles of 95ºC for 5 seconds, 60ºC for 10 seconds, and 72ºC for 12 or 17 seconds, depending on the primers used, and acquiring data at the last step. RNA samples were used as a no-RT control with actin primers, and water was used as a no-template control for all the primers. Two technical replicates and four biological replicates were used for mock and CaCV treatments, respectively. Relative gene expression was calculated as $2^{-ΔΔCt}$. The Shapiro-Wilk normality test followed by an unpaired t-test was done using GraphPad Prism version 8.0.0.

**Symptom assessment and virus titre**

To assess symptom development following CaCV inoculation, photos of the capsicum plants of both cultivars were taken at 3, 5, 7, 10, and 12 dpi, and a symptom severity score was applied, ranging from 0 to 6 (Table 2). Seven CaCV-inoculated biological replicates were used for symptom grading, which were compared to five biological replicates of mock-inoculated plants.

CaCV accumulation was quantified by real-time PCR using a SensiFAST™ SYBR® NO-ROX One-Step Kit (Bioline) with the primers CaCV IR-F (5’-GCTTGTACATTTAGTTATCAGGGTTAG-3’) and CaCV IR-R (5’-CCAATTTGTTGAATGAGCTAACTTTGG-3’), yielding an amplicon of 157 bp. The same RNA samples that were used to quantify capsicum gene expression were used here at a dilution of 1 ng/µL. For the standard curve, a 795-bp fragment of the 5S RNA intergenic region of CaCV-QLD3432 was first amplified by PCR using the primers 5’-CACTCATTGTGGCATGCTGGAA-3’ and 5’-ACACTAAAGCTTGAAGCTAATTGGTA-3’, cloned into pGEM®-T Easy vector (Promega), and sequenced. Then, the plasmid was linearized with Ndel and a standard curve was generated using the One-Step kit, the CaCV IR primer set, and a ten-fold serial dilution series ranging from 10 ng to 0.01 pg of DNA. Cycling conditions were 45ºC for 10 minutes, 95ºC for 2 minutes, and 40 cycles of 95ºC for 5 seconds, 61ºC for 10 seconds, and 72ºC for 5 seconds, acquiring data at the last step. The CaCV S RNA copy number was calculated based on the standard curve. A correlation test for virus titre and gene expression was done for each time point using GraphPad Prism, and correlation over time was calculated using the rmcorr package in R and RStudio [28]. Correlation over time between virus titre and symptom score was also calculated using the rmcorr package.

### Table 2 Symptom grading scale used in this study

| Score | Symptom description                                                                 |
|-------|--------------------------------------------------------------------------------------|
| 0     | No visible symptoms; CaCV-inoculated plants show growth and development similar to that of mock-inoculated plants |
| 1     | Slight yellowing of the inoculated leaf; development similar to that of mock-inoculated plants |
| 2     | Some chlorotic spots in less than 50% of the inoculated leaf area; development similar to that of mock-inoculated plants |
| 3     | Chlorotic spots and/or interveinal chlorosis in more than 50% of the inoculated leaf area; no symptoms on systemic leaves |
| 4     | Chlorotic spots and/or interveinal chlorosis in more than 50% of the inoculated leaf area; yellowing starting to show on leaves right above the inoculated ones |
| 5     | Chlorotic spots, leaf curling and rugose appearance on systemic leaves; reduction in plant development |
| 6     | Very strong curling and rugose appearance, appearance of dark green islands in young systemic leaves; plant stunted |
Results

CaCV symptom development in susceptible capsicum plants

CaCV symptoms were evaluated at 3, 5, 7, 10, and 12 dpi using a scoring system we developed for capsicum cultivars (Table 2, Fig. 1). Gene expression was analyzed for both cultivars at 3, 7, and 12 dpi. Initial symptoms were slight yellowing on inoculated leaves that progressed to chlorotic spots on less than 50% of the inoculated leaf area without systemic symptoms. The next symptom stage was chlorotic spots on more than 50% of the inoculated leaf area without symptoms on upper leaves. The infection then became systemic, with yellowing on leaves above the inoculated ones. Yellowing on systemic leaves progressed to chlorotic spots, curling, and rugose appearance on younger leaves. The final stage was the development of dark-green islands on systemic leaves and plant stunting (Supplementary Fig. S1).

The first visible symptoms started to appear at 3 dpi, with faint yellowing of a small part of the inoculated leaf area in some plants. At 7 dpi, most CaCV-inoculated plants showed mild symptoms on systemic leaves, and symptoms on the inoculated leaf became more pronounced and occupied a larger leaf area. At 10 dpi, clear systemic symptoms were evident, with some curling and rugosity. At 12 dpi, symptoms were stronger overall, and the plants were stunted. Symptom scores were given to five mock-inoculated and seven CaCV-inoculated plants for each cultivar at 3, 5, 7, 10, and 12 dpi. The number of plants for each score is shown in Fig. 1. None of the mock-inoculated plants showed symptoms, and all of the CaCV-inoculated plants showed symptoms. Individual scores for each biological replicate are listed in Supplementary Table S1.

CaCV accumulation at different stages of capsicum infection

CaCV accumulation was measured by RT-qPCR using a standard curve generated with recombinant plasmid DNA. Titration was done for total RNA from the inoculated leaf at 3 dpi and systemic leaves at 7 and 12 dpi, which were the same leaves selected for the gene expression study (Fig. 2).

The CaCV S RNA intergenic region was targeted to quantify virus accumulation in each test plant to gain an understanding of individual disease progression. At 3 dpi, the number of CaCV copies per ng of tissue in the inoculated leaves of four biological replicates was on average 415,726 for Yolo Wonder and 691,905 for Warlock. At 7 dpi, in the first systemic leaf, the average was 615,105 for Yolo Wonder and 188,989 for Warlock, and at 12 dpi, it was 1,883,435 for Yolo Wonder and 1,756,088 for Warlock. The CaCV copy number showed large differences between individual plants (Table 3).

Fig. 1 Symptom scores for seven biological replicates at 3, 5, 7, 10, and 12 dpi with CaCV. Different colours indicate different symptom scores. The number of plants showing each symptom is given inside each bar. See Table 2 for the definition of symptom scores. (A) Cultivar Warlock plants inoculated with CaCV. (B) Cultivar Yolo Wonder plants inoculated with CaCV

Fig. 2 Leaf sample collection diagram for capsicum plants at 3, 7, and 12 dpi. One leaf disc was collected from the inoculated leaf at 3 dpi and the youngest expanded leaf at 7 and 12 dpi. Different leaves were sampled, represented by plants A, B, and C for different biological replicates (Table 3). The number of yellow dots indicates the relative difference in virus titre.
However, overall, the virus titre was higher in Warlock than in Yolo Wonder inoculated leaves at 3 dpi, which was reversed at 7 dpi, when more CaCV had accumulated in systemic leaves of Yolo Wonder. At 12 dpi, both cultivars showed an increase in virus titre compared to 7 dpi, with CaCV accumulating to comparable levels (Fig. 3A and C). Although some differences in virus accumulation could be seen between the two cultivars, these were not statistically significant.

A repeated measures correlation test showed a good correlation between symptom score and virus accumulation over time in Yolo Wonder \((r = 0.80)\), with high statistical significance \((p = 0.00081)\). There was also significant positive correlation in Warlock \((r = 0.62)\), with high statistical significance \((p = 0.023)\), although it was less strong than in Yolo Wonder (Fig. 3B and D).

**Time course of capsicum gene expression in response to CaCV infection**

Several host genes thought to be involved in plant defence responses were DE at 4 dpi in the susceptible cultivar Yolo Wonder in response to CaCV infection [18, 19]. Of those ‘early response’ genes, five were selected to study their temporal expression in the capsicum cultivars Yolo Wonder and Warlock between 3 and 12 dpi. Relative gene expression is shown in Fig. 4, using different scales according to expression of each gene.

The chalcone synthase (CHS) gene was downregulated in CaCV-infected plants compared to mock-infected plants in both cultivars and at all time points (Fig. 4A). CHS gene expression followed the same pattern for mock and CaCV treatments, with increased expression at 7 dpi and reduced expression at 12 dpi. Differential gene expression was statistically significant for Yolo Wonder at all time points when comparing treatments. For Warlock, it was significantly different at 12 dpi only. CHS gene expression and the virus titre in Yolo Wonder were negatively correlated at 3, 7, and 12 dpi individually, although not statistically significant (Table 4).

The cytochrome P450 (CYP) gene was upregulated in CaCV-infected plants compared to mock treatment at all time points. CYP gene expression patterns differed between the cultivars. In Yolo Wonder, mock treatment led to decreased CYP expression over time, while CaCV treatment led to lower gene expression at 7 dpi and higher expression at 12 dpi (Fig. 4B). In Warlock, on the other hand, both treatments showed the lowest expression in systemic leaves at 7 dpi and the highest expression at 3 dpi in CaCV-inoculated leaves. CYP gene expression in Warlock was higher than in Yolo Wonder at 3 and 12 dpi. CYP gene expression in CaCV-infected plants was significantly different from that in mock-treated plants at 3 and 12 dpi for both cultivars. CYP gene expression and the virus titre were positively correlated in Yolo Wonder at 7 dpi and over time. No statistically significant correlation was observed for Warlock (Table 4).

The tetraspanin 8-like (TSP8) gene was also upregulated in CaCV-infected plants, and the temporal expression pattern was similar for both treatments and cultivars, with decreased expression at 7 dpi and increased expression at 12 dpi (Fig. 4C). This gene was significantly upregulated in the presence of CaCV at 3 dpi in Yolo Wonder and at 3 and 12 dpi in Warlock. TSP8 gene expression and the virus titre at 3 dpi were positively correlated in both cultivars, and at 7 dpi in Warlock. A positive correlation over the time frame of the experiment was maintained only for Warlock (Table 4).

Thionin gene expression differed between the cultivars depending on the time point. The common characteristic was that, early in infection in both inoculated and systemic leaves at 3 and 7 dpi, thionin gene expression was low in both mock- and CaCV-inoculated leaves of both cultivars, but there was no significant differential expression between treatments. At 12 dpi, on the other hand, this gene was strongly upregulated in CaCV-infected plants of both cultivars, and the difference between treatments was statistically significant for Yolo Wonder (Fig. 4D). Thionin gene expression and virus titre were positively correlated at 12 dpi for Warlock (Table 4). Over time, there was a strong positive correlation for both cultivars.

| Cultivar      | dpi | Plant 1 | Plant 2 | Plant 3 | Plant 4 | Plant 5 | Plant 6 | Average | SD (% of average) |
|---------------|-----|---------|---------|---------|---------|---------|---------|---------|-------------------|
| Yolo Wonder   | 3   | 143,132 | 406,631 | 415,016 | 757,551 | 424,459 | 347,564 | 415,726 | 47.62             |
|               | 7   | 1,116,446 | 285,286 | 254,314 | 515,136 | 15,904  | 1,503,544 | 615,105 | 93.35             |
|               | 12  | 1,563,974 | 1,264,303 | 1,475,442 | 1,236,621 | 2,359,749 | 3,400,521 | 1,883,435 | 45.04             |
| Sampling type (Fig. 2) |     | A       | B       | B       | C       | A       | B       |         |                   |
| Warlock       | 3   | 782,583 | 1,004,273 | 241,667 | 743,934 | 669,938 | 709,037 | 691,905 | 36.1              |
|               | 7   | 167,151 | 292,901 | 470,620 | 1,831  | 1,370  | 200,059 | 188,989 | 94.93             |
|               | 12  | 1,843,663 | 894,887 | 1,530,212 | 1,125,640 | 2,939,135 | 2,202,991 | 1,756,088 | 42.59             |
| Sampling type (Fig. 2) |     | B       | A       | A       | B       | A       | B       |         |                   |
The WRKY 40 gene was upregulated following CaCV inoculation at all time points for both cultivars, except for Warlock at 7 dpi, where it was downregulated in CaCV-infected plants (Fig. 4E). The highest gene expression was at 12 dpi for both cultivars. This gene was upregulated at all time points in Yolo Wonder and at 12 dpi in Warlock. It is noteworthy that CaCV accumulation in Warlock at 7 dpi was low, coincident with low WRKY40 gene expression, and both measures were increased at 12 dpi (Fig. 4E). There was a positive correlation between WRKY 40 gene expression and virus titre over time in Warlock (Table 4).

**Discussion**

Yolo Wonder and Warlock are two well-known bell pepper capsicum cultivars that are susceptible to CaCV, like all current commercial cultivars. Yolo Wonder (Terranova Seeds) has been widely used for many years, although nowadays it is not commonly grown by farmers in the field. This cultivar is resistant to tobacco mosaic virus (TMV), has been used extensively in research, and was used in a previous transcriptome study by Widana Gamage and colleagues [17, 18].

Fig. 3 (A and C) Virus titre (copy number/ng tissue) over time in capsicum cv. Yolo Wonder and Warlock. (B and D) correlation between symptom score and virus titre over time for six biological replicates (indicated by different colours) of capsicum cvs. Yolo Wonder and Warlock. Correlation coefficient (r) obtained by repeated measures correlation (rmcorr package), calculated using R and RStudio. Stars represent the statistical significance. ns, not significant (P > 0.05); *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001
Gene expression in capsicum chlorosis virus infection

in our research group. Warlock (Seminis®, Bayer), on the other hand, is the market leader for this type of capsicum. It is resistant to potato virus Y (PVY), TMV, and bacterial leaf spot. For these reasons, both cultivars were selected to evaluate potential variation in defence gene expression and symptom development in response to CaCV infection.

CaCV symptoms on a variety of plant hosts can include necrotic and/or chlorotic spots or rings on leaves, leaf distortion and mottling, apical necrosis, plant stunting, and yellow stripes (on calla lily) [2, 7, 13, 29]. Although symptoms caused by CaCV infection in capsicum have been reported, symptom development over time and

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**Fig. 4** Relative gene expression ($2^{-\Delta C_{\text{t}}}$) over time in capsicum cv. Yolo Wonder and Warlock. Mock-inoculated plants are shown in green, CaCV-inoculated plants in red. (A) CHS. (B) CYP. (C) TSP8. (D) Thionin. (E) WRKY40. Statistical significance, calculated by the Holm-Sidak method, is shown as stars. ns, not significant ($P > 0.05$); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$
molecular defence responses have not been studied. There is a gap in our understanding of CaCV symptom development and the correlation between virus titre and host gene expression. The aim of this study was to investigate the progress of CaCV infection in capsicum plants to identify host genes involved in the spread of infection and antiviral defence.

Overall, the two capsicum cultivars tested did not show major differences in temporal symptom development. At 3 dpi, both cultivars showed no symptoms or very mild symptoms. At 5 dpi, most Yolo Wonder plants scored 2 on the grade scale, while Warlock plants were at 1. At 7 dpi, Yolo Wonder plants showed stronger symptoms, with most plants scoring 4, while Warlock plants were at 3. At 10 dpi, both

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Table 4  Correlation (r) between gene expression and virus titre for five genes and two cultivars at 3, 7, and 12 days post-inoculation (dpi) and over time

| Cultivar | dpi | CHS  | CYP  | TSP8  | Thionin  | WRKY40  |
|----------|-----|------|------|-------|----------|---------|
| Yolo Wonder | 3   | -0.6 (ns) | 0.75 (ns) | 0.84 (*) | 0.59 (ns) | 0.16 (ns) |
|           | 7   | -0.56 (ns) | 0.93 (**) | 0.69 (ns) | -0.15 (ns) | 0.8 (ns) |
|           | 12  | -0.58 (ns) | -0.12 (ns) | -0.27 (ns) | 0.8 (ns) | 0.17 (ns) |
| r over time |     | -0.18 (ns) | 0.72 (*) | 0.32 (ns) | 0.87 (*** | 0.56 (ns) |
| Warlock   | 3   | -0.01 (ns) | 0.58 (ns) | 0.91 (*) | 0.00 (ns) | 0.29 (ns) |
|           | 7   | 0.08 (ns) | 0.6 (ns) | 0.92 (**) | 0.49 (ns) | -0.23 (ns) |
|           | 12  | -0.27 (ns) | 0.76 (ns) | 0.11 (ns) | 0.81 (*) | -0.46 (ns) |
| r over time |     | -0.47 (ns) | 0.51 (ns) | 0.65 (*) | 0.89 (****) | 0.62 (*) |

Stars represent the statistical significance. ns, not significant (P > 0.05); *, P ≤ 0.05; ***, P ≤ 0.01; ****, P ≤ 0.001; *****, P ≤ 0.0001
cultivars had similar symptom scores with clear systemic symptoms, and at 12 dpi, all Yolo Wonder and Warlock plants were at the same severe stage of symptom development. Based on these observations, Warlock plants under glasshouse conditions appear to develop CaCV symptoms slightly more slowly in the early phase of infection in the inoculated leaves compared to Yolo Wonder. Once systemic virus spread had occurred, both cultivars showed similar rapid symptom development in the young leaves, with rugosity, curling, and plant stunting.

The first time point at which CaCV accumulation was measured was at 3 dpi. At this time, the inoculated leaves of Warlock plants had a 1.6-fold higher virus titre than the inoculated leaves of Yolo Wonder. This may have been due to differential virus uptake from the inoculum. Even though the same inoculum was used at the same time for all of the experimental plants, mechanical rub-inoculation may lead to different amounts of virus or uneven distribution in each inoculated leaf. This is indicated by the variable virus copy numbers between the biological replicates. Another point to be taken into consideration is that some plants developed faster than others, leading to expanded leaves earlier than in others. Since we collected samples from the youngest fully expanded leaves, the physical distance between sampling sites at 3 dpi and 7 dpi varied between plants (Fig. 2). For some biological replicates, the samples at 3, 7, and 12 dpi were collected from leaves immediately above the previous one, and for others, the samples from 7 dpi were collected from the second-youngest leaf. This appears to have affected the virus titres in those samples, which was reflected in the large standard deviation between individual replicate plants.

For Yolo Wonder, plants 2, 3, and 4 had lower virus titres compared to plant 1, and samples were collected from the second leaf above the inoculated leaf (3 dpi). In Warlock at 7 dpi, samples from plants 1 and 4 were collected from the second leaf above the inoculated leaf, and these also showed lower virus titres compared to plants 2 and 3, lending support to the above hypothesis. Plants 5 and 6 from both cultivars on the other hand, showed opposite patterns.

Overall, in Warlock compared to Yolo Wonder, CaCV accumulated more slowly during the initial phase of infection before 10 dpi, which may suggest initially slower virus replication and/or movement. In a previous study of PVY infecting potato, it was observed that higher initial virus concentration in the inoculum resulted in higher virus accumulation at 5 and 7 dpi and in faster virus accumulation in systemic leaves [30]. Although CaCV-inoculated Warlock plants initially had higher virus titres at 3 dpi, potentially indicating a higher uptake of virus during mechanical inoculation, virus accumulation at 7 dpi and progression of systemic infection were slower than in Yolo Wonder. This progression of infection is opposite to the results of studies with turnip mosaic virus in N. benthamiana and tobacco etch virus in N. tabacum [31, 32]. Our observations suggest that cell-to-cell and systemic movement of CaCV is slower in Warlock than in Yolo Wonder early in infection, while virus replication and movement appear to be faster after 7 dpi, leading to similar virus titres in both cultivars at 12 dpi, when symptoms are evident. Virus titre and symptom scores correlated well in both cultivars, with stronger correlation in Yolo Wonder, since the virus copy number started lower and increased over time, while in Warlock, the virus copy number was higher at 3 dpi than at 7 dpi. During the early phase of infection, CaCV accumulated less in Warlock, and plants had fewer symptoms compared to Yolo Wonder. During the systemic infection phase, virus titres increased to similar levels and symptoms became more severe in both cultivars.

CHS gene expression following CaCV infection was downregulated in both cultivars, with lower expression in Warlock than Yolo Wonder in both mock- and CaCV-infected plants when comparing each time point separately. CHS gene expression in Warlock was only significantly different in CaCV-infected plants when comparing to mock-inoculated plants at 12 dpi, while in Yolo Wonder, the expression of this gene in CaCV-infected plants was significantly lower at all time points. There was no significant correlation between virus titre and CHS gene expression in either cultivar (Table 4). Taken together, these results suggest that Warlock has a different initial response to CaCV regarding CHS expression, taking longer to change the expression in response to virus infection, but the expression is not linked to the virus titre – only to virus infection. CHS gene expression is known to be induced through various stimuli, which can originate from the environment, such as light/UV light, or during plant development [20]. CHS is a key enzyme for the synthesis of flavonoids and isoflavonoid-type phytoalexins that act in response to pathogens. There have been several studies in which CHS expression increased in response to fungal infection [33, 34].

Suppression of CHS gene expression in flax resulted in modification of the cell wall arrangement and morphology as well as stem enlargement [35]. The observed decrease in CHS gene expression from 7 dpi to 12 dpi in CaCV-infected plants could be involved in the rugosity appearance and curling of younger systemic leaves at 10 and 12 dpi. Furthermore, the fact that early CHS gene expression in Warlock is not different between treatments may be responsible for the delayed development of local symptoms in this cultivar when compared to Yolo Wonder. A study of CHS gene expression in soybean seeds in response to soybean mosaic virus and cucumber mosaic virus showed that both viruses led to an increase in CHS mRNA accumulation but suppressed CHS expression through posttranscriptional gene silencing [36]. In our previous study of capsicum gene expression in response to CaCV infection, the CHS gene was upregulated in a CaCV-resistant breeding line when compared to the
susceptible cultivar Yolo Wonder [18]. CHS gene expression was also measured in the early stages of infection (0 to 60 hours) by rice stripe virus (RSV, genus *Tenuivirus*, family *Phenuiviridae*) in resistant and susceptible rice. In that study, CHS gene expression in susceptible rice showed no significant difference between infected and uninfected plants early in infection. However, at 60 h, CHS gene expression in RSV-infected plants was lower than in uninfected plants [37]. The CHS gene expression pattern in that study was therefore similar to that observed in the present CaCV study. Interestingly, both viruses have negative-sense and ambisense genome segments and belong to the order *Bunyavirales*, which may be related to the similar host responses.

CYP is a diverse enzyme superfamily whose members are present in all plants and most other organisms [38]. CYP has distinct functions in plant defence and development, acting in detoxification and biosynthetic pathways [39, 40]. Like CHS, CYP is important for phytoalexin biosynthesis for plant defence, as well as the biosynthesis of other secondary metabolites and hormones [38]. CYP is also known to regulate the jasmonic acid pathway in response to wounding [41]. Capsicum CYP (CaCYP) was upregulated following CaCV infection in this study and differentially expressed at 3 and 12 dpi when compared with mock-inoculated plants. There was a significant positive correlation between virus titre and CYP gene expression over time in Yolo Wonder (Table 4).

*CaCYP1* encodes a cytochrome P450 from *C. annuum L.* Bukanghat that has been shown to be involved in the basal defence response of capsicum to *Xanthomonas axonopodis* [38, 42]. In that study, *CaCYP1* expression was rapidly induced during the first 48 h after infection in a resistant capsicum line. The mock-inoculated line also showed upregulation, but to a lower extent, as shown by northern blot and RT-PCR [42]. In our previous transcriptome study, *CaCYP* was upregulated 8-fold in CaCV-resistant capsicum in response to CaCV infection compared to the susceptible line [18]. The upregulation of *CaCYP* following CaCV infection in the present study supports the conclusion that CYP also plays a role in the defence response to CaCV in susceptible *Capsicum*. In Warlock, the expression of *CaCYP* was higher than in Yolo Wonder at 3 dpi. It is possible that this difference in gene expression is reflected in the slower initial symptom development and virus accumulation in Warlock.

TSPs are transmembrane proteins that participate in various steps of the virus replication cycle [43]. In the present study, TSP gene expression was upregulated at 3 dpi in CaCV-infected Yolo Wonder and at 3 and 12 dpi in Warlock. There was a significant positive correlation between gene expression and virus titre over time in Warlock only, but also at 3 dpi in both cultivars and at 7 dpi in Warlock (Table 4). A study with TSP from *Phaseolus vulgaris* described the localization of TSP on the membrane at the tips of hairs,
function of thionin expression in the resistance response to CaCV, it would be useful to overexpress (in susceptible lines) or silence (in the resistant line) the thionin gene to gauge changes in the response of capsicum to CaCV. Although several studies have shown that enhanced thionin expression was involved in resistance to bacterial and fungal pathogens [56–58], it is unknown if a higher level of expression of this gene would improve the defence against virus infection.

WRKYs are transcription factors that bind to DNA, are involved in physiological and developmental processes, and are expressed in response to various biotic and abiotic stresses. WRKY proteins have essential roles in the kinase signalling network and interact with plant resistance proteins for a rapid immune response [59]. In the present study, WRKY40 was upregulated in CaCV-infected capsicum at all time points in Yolo Wonder, while in Warlock this gene was only differentially expressed at 12 dpi when compared with mock-inoculated plants. There was significant positive correlation between gene expression and virus titre over time in Warlock, but not in Yolo Wonder. In fusarium-resistant chickpea, WRKY40 expression was upregulated, while in susceptible chickpea, this gene was not expressed. Furthermore, a defence response was triggered in response to *Pseudomonas syringae* in *Arabidopsis thaliana* overexpressing WRKY40 [60]. A capsicum WRKY transcription factor (*CaWRKYd*) that belongs to the same subgroup as *AtWRKY40* was reported to play a crucial role during the hypersensitive response to TMV. In plants with silenced *CaWRKYd*, expression of pathogenesis-related (PR) and hypersensitive-response-related genes was suppressed, leading to higher virus accumulation [61]. According to that study, WRKY40 may be involved in negative regulation of PR genes and therefore may have an effect on pathogen susceptibility. Unlike the results of those studies, the expression of WRKY40 in susceptible capsicum cultivars in the present study showed an opposite effect, as Warlock showed a positive correlation over time between gene expression and virus titre. In our previous study, WRKY40 was not differentially expressed in resistant capsicum when compared to susceptible capsicum infected with CaCV, but it was downregulated in CaCV-infected susceptible capsicum when compared to mock-inoculated plants at 4 dpi [19]. Future gene overexpression/silencing experiments may reveal if WRKY40 has a positive or negative regulatory role in the response of capsicum to CaCV infection.

To study the effects of manipulating these early-response genes to potentially induce CaCV resistance in capsicum, strategies such as overexpression, silencing, or gene editing could be used. CRISPR/Cas9 is an efficient gene editing tool used to modify host genes that could be involved in susceptibility or that are crucial for virus replication [62]. Targeting of the eukaryotic translation initiation factor 4E (*eIF4E*) has been shown to confer resistance to cucumber vein yellowing virus, zucchini yellow mosaic virus, and papaya ringspot mosaic virus-W [63]. Further studies to elucidate the interaction of each DE CaCV gene with possible viral genes will be important to understand their potential roles during infection, especially replication and movement. If an essential domain of a host susceptibility gene can be identified, CRISPR/Cas gene editing technologies may be applied to disrupt the compatibility of this virus-host interaction to generate resistant plants that do not support virus infection [64]. The overexpression of a WRKY transcription factor from cotton resulted in enhanced resistance to TMV and cucumber mosaic virus in tobacco plants [65]. The overexpression of plant PR-related genes can also lead to the development of milder symptoms in virus-infected plants, as shown in a study with RSV [66]. Gene silencing studies have also been used to understand gene functions in response to pathogen infection. For example, silencing of *eIF(iso)4E* in plum resulted in accumulation of siRNAs and conferred resistance to plum pox virus [67].

**Conclusions**

In the present study, the progression of visible symptoms in mechanically inoculated, CaCV-susceptible capsicum plants appears to largely correlate with virus accumulation over time. The capsicum early CaCV response genes identified at 4 dpi in a previous report [18] that were further analysed in this study continued to be down- (CHS) or upregulated (CYP and TSP8) during the period of 3-12 dpi in both cultivars, suggesting their importance in the initial and continued host response. Thionin gene expression did not follow this pattern but was initially low and then strongly upregulated at 12 dpi. WRKY 40 was consistently upregulated in Yolo Wonder over time. Increased host gene expression and an elevated virus titre correlated well over time for CYP in Yolo Wonder, TSP8 and WRKY40 in Warlock, and thionin in both cultivars. Future studies may determine if these host proteins interact with viral proteins. CHS appears to be linked to symptom expression, and CYP appears to contribute to the difference between Warlock and Yolo Wonder in initial symptom development. TSP8 may be involved in cell-to-cell or long-distance movement of CaCV through plasmodesmata and extracellular vesicles, respectively. These three genes may represent good target candidates for future studies of host gene editing, overexpression, or silencing to better understand the functions of those genes during virus replication and movement as well as symptom development to help control CaCV.

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Author contributions FYBN conducted all experiments, analysed the data, and wrote the first draft of the manuscript. RGD and FYBN co-designed the experiments. RGD, SMKWG, and NM supervised the research and contributed to data interpretation. All authors edited the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Competing interests RGD is a member of the Editorial Board of Archives of Virology but was not involved in any way in the editorial or review assessment of this manuscript.

Ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

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References

1. Abudurexiti A, Adkins S, Alioto D, Alkhovsky SV, Avišič-Županc T, Ballinger MJ, Bente DA, Beer M, Bergeron É, Blair CD (2019) Taxonomy of the order Bunyavirales: update 2019. Arch Virol 164:1949–1965
2. McMichael L, Persley D, Thomas J (2000) The first record of a serotype IV tospovirus in Australia. Australas Plant Pathol 29:149
3. Kunkalikar S, Poojari S, Rajagopalan P, Zehr UB, Naidu RA, Kankanallu RS (2007) First report of Capsicum chlorosis virus in tomato in India. Plant Health Progr 16:1949–1965
4. Premachandra W, Borgemeister C, Maiss E, Knierim D, Poebling H-M (2005) Ceratothripoides claratris, a new vector of a Capsicum chlorosis virus isolate infecting tomato in Thailand. Phytopathol 95:659–663
5. Knierim D, Blawid R, Maiss E (2006) The complete nucleotide sequence of a capsicum chlorosis virus isolate from Lycopersicum esculentum in Thailand. Arch Virol 151:1761–1782
6. Okuda M (2016) Tospoviruses occurring in and outside Japan. Jpn J Phytopathol 82:169–184
7. Orfanidou C, Boutsika A, Tsiolakis G, Winter S, Katis N, Maligogka VI (2019) Capsicum chlorosis virus: a new viral pathogen of pepper in Greece. Plant Dis 103:379
8. Chen K, Xu Z, Yan L, Wang G (2007) Characterization of a new strain of Capsicum chlorosis virus from peanut (Arachis hypogaea L.) in China. J Phytopathol 155:178–181
9. Sun S, Wang J, Chen S, Zhang S, Zhang D, Liu Y (2018) First Report of capsicum chlorosis orthotospovirus infecting zucchini (Cucurbita pepo L.) in China. Plant Dis 102:2049
10. Sharman M, Thomas J, Tree D, Persley D (2020) Natural range and thrips transmission of capsicum chlorosis virus in Australia. Australas Plant Pathol 49:45–51
11. Bayat H, Hassani-Mehraban A, Safaie N, Shams-bakhsh M (2018) Molecular and biological characterization of an isolate of capsicum chlorosis virus from Iran. J Plant Pathol 100:163–170
12. Chen C, Huang C, Cheng Y, Chen T, Yeh S, Chang C (2009) First report of Capsicum chlorosis virus infecting amaryllis and blood lily in Taiwan. Plant Dis 93:1346
13. Chen C, Huang C, Chen T, Yeh S, Cheng Y, Hsu H, Chang C (2007) First report of Capsicum chlorosis virus causing yellow stripes on calla lilies in Taiwan. Plant Dis 91:1201
14. Melzer M, Shimabukuuro J, Long M, Nelson S, Alvarez A, Borth W, Hu J (2014) First report of Capsicum chlorosis virus infecting waxflower (Hoya calycina Schlechter) in the United States. Plant Dis 98:571
15. Chiaki Y, Kubota K, Tomitaka Y, Usugi T, Sakurai T (2020) Transmission of capsicum chlorosis virus by Thrips palmi (Thysanoptera: Thripidae). App Entomol Zool 55:31–35
16. Persley D, Thomas J, Sharman M (2006) Tospoviruses—an Australian perspective. Australas Plant Pathol 35:161–180
17. Widana Gamage SMK, Persley DM, Higgins CM, Dietzgen RG (2015) First complete genome sequence of a capsicum chlorosis tospovirus isolate from Australia with an unusually large S RNA intergenic region. Arch Virol 160:869–872
18. Widana Gamage SMK, McGrath DJ, Persley DM, Dietzgen RG (2016) Transcriptome analysis of capsicum chlorosis virus-induced hypersensitive resistance response in Bell capsicum. PLoS ONE 11:e0159085
19. Widana Gamage SMK (2017) Thrips-tospovirus-plant molecular interactions: studies on capsicum chlorosis virus. The University of Queensland, PhD thesis https://doi.org/10.14264/uql.2017.894
20. Dao T, Linthorst H, Verpoorte R (2011) Chalcone synthase and its functions in plant resistance. Phytochem Rev 10:397
21. Schuler MA, Werck-Reichhart D (2003) Functional genomics of P450s. Annu Rev Plant Biol 54:629–667
22. Jimenez-Jimenez S, Hashimoto K, Santana O, Aguirre J, Kuchitsu K, Cárdenas L (2019) Emerging roles of tetraspanins in plant inter-cellular and inter-kingdom communication. Plant Signal Behav 14:e1581559
23. Hayes M, Bleakley S (2018) Peptides from plants and their applications. Peptide applications in biomedicine, biotechnology and bioengineering. Elsevier, Amsterdam, pp 603–622
24. Bakshi M, Oelmüller R (2014) WRKY transcription factors: Jack of many trades in plants. Plant Signal Behav 9:e27700
Gene expression in capsicum chlorosis virus infection

25. Phukan UJ, Jeena GS, Shukla RK (2016) WRKY transcription factors: molecular regulation and stress responses in plants. Front Plant Sci 7:760

26. Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. Bioinformatics methods and protocols. Springer, New York, pp 365–386

27. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647–1649

28. Bakdash JZ, Marusich LR (2017) Repeated measures correlation. Front Psychol 8:456

29. Krishnareddy M, Rani RU, Kumar KA, Reddy KM, Pappu H (2008) Capsicum chlorosis virus (genus Tospovirus) infecting chili pepper (Capsicum annuum) in India. Plant Dis 92:1469–1469

30. Pompe-Novak M, Križnik M, Gruden K (2019) The titre of the virus in the inoculum affects the titre of the virus in the host plant and the occurrence of the disease symptoms. Acta Chim Sloven 66:45–49

31. Lafforgue G, Tramou N, Lena SF, Zwart MP (2012) Dynamics of the establishment of systemic potyvirus infection: independent yet cumulative action of primary infection sites. J Virol 86:12912–12922

32. RodriGuez G, Zwart MP, Lena SF (2014) Onset of virus systemic infection in plants is determined by speed of cell-to-cell movement and number of primary infection foci. J R Soc Interface 11:20140555

33. Markunas I, Marczak Ł, Stachowiak J, Stobiecki M (2005) Sucrose-induced lupine defense against Fusarium oxysporum: Sucrose-stimulated accumulation of allene oxide synthase as a defense response of lupine to Fusarium oxysporum. Plant Physiol Biochem 43:363–373

34. Wegulo SN, Yang X-B, Martinson CA, Murphy PA (2005) Effects of wounding and inoculation with Sclerotinia sclerotiorum on isoflavone concentrations in soybean. Can J Plant Sci 85:749–760

35. Zak M, Dzialo M, Richter D, Dymińska L, Matula J, Kotecki A, Hanuza J, Szopa J (2016) Chalcone synthase (CHS) gene suppression in flax leads to changes in wall synthesis and sensing genes, cell wall chemistry and stem morphology parameters. Front Plant Sci 7:894

36. Senda M, Masuta C, Ohnishi S, Goto K, Kasai A, Sano T, Hong J-S, MacFarlane S (2004) Patterning of virus-infected Glycine max seed coat is associated with suppression of endogenous silencing of chalcone synthase genes. Plant Cell 16:807–818

37. Hao Z, Wang L, He Y, Liang J, Tao R (2011) Expression of defense genes and activities of antioxidant enzymes in rice resistance to rice stripe virus and small brown planthopper. Plant Physiol Biochem 49:744–751

38. Xu J, Wang X, Guo W (2015) The cytochrome P450 superfamily: Key players in plant development and defense. J Integr Agric 14:1673–1686

39. Ohkawa H, Imaishi H, Shiota N, Yamada T, Inui H, Ohkawa Y (1998) Molecular mechanisms of herbicide resistance with special emphasis on cytochrome P450 monoxygenases. Plant Biotechnol 15:173–176

40. Li D, Wang Y, Han K (2012) Recent density functional theory model calculations of drug metabolism by cytochrome P450. Coord Chem Rev 256:1137–1150

41. Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J 31:1–12

42. Kim Y-C, Kim S-Y, Paek K-H, Choi D, Park JM (2006) Suppression of CaCYP1, a novel cytochrome P450 gene, compromises the basal pathogen defense response of pepper plants. Biochem Biophys Res Commun 345:638–645

43. Florin L, Lang T (2018) Tetraspanin assemblies in virus infection. Front Immunol 9:1140

44. Jimenez-Jimenez S, Santana O, Lara-Rojas F, Arthikala M-K, Armada E, Hashimoto K, Kuchitsu K, Salgado S, Aguirre J, Quinto C (2019) Differential tetraspanin genes expression and subcellular localization during mutualistic interactions in Phaeosolus vulgaris. PLoS ONE 14:e0219765

45. Widana Gamage SMK, Dietzgen RG (2017) Intracellular localization, interactions and functions of capiscum chlorosis virus proteins. Front Microbiol 8:612

46. Fernandez-Calvino L, Faulkner C, Walshaw J, Saalbach G, Bayer E, Benitez-Antonio Y, Maule A (2011) Arabidopsis plasmodesmal proteome. PLoS ONE 6:e18880

47. Boavida LC, Pin P, Broz M, Becker JD, McCormick S (2013) Arabidopsis tetraspanins are confined to discrete expression domains and cell types in reproductive tissues and form homo- and heterodimers when expressed in yeast. Plant Physiol 163:696–712

48. Reimann R, Kost B, Dettmer J (2017) Tetraspanins in plants. Front Plant Sci 8:545

49. Rutter BD, Innes RW (2018) Extracellular vesicles as key mediators of plant–microbe interactions. Curr Opin Plant Biol 44:16–22

50. Movahed N, Cabanillas DG, Wan J, Vali H, Laliberte J-F, Zheng H (2019) Turnip mosaic virus components are released into the extracellular space by vesicles in infected leaves. Plant Physiol 180:1375–1388

51. Taveira GB, Carvalho AO, Rodrigues R, Trindade FG, Da Cunha M, Gomes VM (2016) Thionin-like peptide from Capsicum annum fruits: mechanism of action and synergism with fluconazole against Candida species. BMC Microbiol 16:12

52. Whitlow M, Teeter M (1985) Energy minimization for tertiary structure prediction of homologous proteins: α1-purothionin and viscotoxin A3 models from crombin. J Biomol Struct Dyn 2:831–848

53. Osorio e Castro V, Vernon LP (1989) Hemolytic activity of thionin from Pyrrylaria pubera nuts and snake venom toxins of Naja naja species: Pyrrylaria thionin and snake venom cardiotocin compete for the same membrane site. Toxicon 27:511–517

54. Bohlmann H, Broekaert W (1994) The role of thionins in plant protection. Crit Rev Plant Sci 13:1–16

55. Mangal M, Srivastava A, Mirajkar SJ, Singh K, Solanki V, Mandal B, Kalia P (2020) Differential expression profiling of defense related genes for Leaf Curl Virus (ChiLCV) in resistant and susceptible genotypes of Chili. Indian J Genet 80:308–317

56. Muramoto N, Tanaka T, Shimamura T, Mitsukawa N, Arimura M, Hara K, Onishi T, Naka M, Nakamura K, Imaeda T (2012) Transgenic sweet potato expressing thionin from barley gives resistance to black rot disease caused by Ceratocystis fimbriata in leaves and to black rot disease caused by Sclerotinia sclerotiorum in storage roots. Plant Cell Rep 31:987–997

57. Carmona MJ, Molina A, Fernández JA, López-Fando JJ, García-Olmedo F (1993) Expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. Plant J 3:457–462

58. Chan Y-L, Prasad V, Chen KH, Liu HY, Han M-T, Cheng C-P (2005) Transgenic tomato plants expressing an Arabidopsis
thionin (Thi2.1) driven by fruit-inactive promoter battle against phytopathogenic attack. Planta 221:386–393
59. Chen X, Li C, Wang H, Guo Z (2019) WRKY transcription factors: evolution, binding, and action. Phytopathol Res 1:1–15
60. Chakraborty J, Ghosh P, Sen S, Das S (2018) Epigenetic and transcriptional control of chickpea WRKY40 promoter activity under Fusarium stress and its heterologous expression in Arabidopsis leads to enhanced resistance against bacterial pathogen. Plant Sci 276:250–267
61. Huh SU, Lee G-J, Kim YJ, Paek K-H (2012) Capsicum annum WRKY transcription factor d (CaWRKYd) regulates hypersensitive response and defense response upon tobacco mosaic virus infection. Plant Sci 197:50–58
62. Mushtaq M, Mukhtar S, Sakina A, Dar AA, Bhat R, Deshmukh R, Molla K, Kundoo AA, Dar MS (2020) Tweaking genome-editing approaches for virus interference in crop plants. Plant Physiol Biochem 147:242–250
63. Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlman M, Sherman A, Arazi T, Gal-On A (2016) Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. Mol Plant Pathol 17:1140–1153
64. Kalinina NO, Khromov A, Love AJ, Taliantsky ME (2020) CRISPR applications in plant virology: virus resistance and beyond. Phytopathol 110:18–28
65. Yu F, Huaxia Y, Lu W, Wu C, Cao X, Guo X (2012) GhWRKY15, a member of the WRKY transcription factor family identified from cotton (Gossypium hirsutum L.), is involved in disease resistance and plant development. BMC Plant Biol 12:1–18
66. Liu Z, Li X, Sun F, Zhou T, Zhou Y (2017) Overexpression of OsCIPK30 enhances plant tolerance to Rice stripe virus. Front Microbiol 8:2322
67. Wang X, Kohalmi SE, Svircev A, Wang A, Sanfaçon H, Tian L (2013) Silencing of the host factor eIF (iso) 4E gene confers plum pox virus resistance in plum. PLoS ONE 8:e50627

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