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Mechanisms of resistance to powdery mildew in cucumber

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Summary. *Podosphaera xanthii* causes powdery mildew of cucumber, and is associated with significant yield and quality losses. Development of resistant or tolerant varieties is the most effective and eco-friendly strategy for powdery mildew management. An important host resistance mechanism is based on the recognition of conserved resistance genes, resulting in durable resistance. To determine powdery mildew resistance mechanisms in cucumber, total RNAs were isolated from the powdery mildew resistant cultivar Meltem, the tolerant line VT18, and the susceptible local variety Camlica. Expression levels of nine genes in these plants were analysed by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The host reactions were assessed using microscope observations of stained specimens. *Serine/threonine* (*STN7*), *transcription factor* (*WRKY22*), *serine/threonine-protein kinase* (*D6PKL1*), and *serine/threonine receptor kinase* (*NFP*) genes were induced, as positive regulators in defence mechanisms against powdery mildew. *Polygalacturonase Inhibitor (PGIP*) did not express after *P. xanthii* inoculation of Camlica, resulting in susceptibility. After inoculation, *callose synthase (CALLOSE)* and *cinnamyl alcohol dehydrogenase* (*CAD*) gene expression levels were increased in resistant Meltem, but Hypersensitive Reaction (HR) and ROS formation were only linked in the tolerant VT18. Powdery mildew development was less in Meltem than in VT18, indicating that cell wall thickening and HR play separate roles in resistance to this disease.

Keywords. Signaling pathway genes, *Podosphaera xanthii.*

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is an economically important vegetable crop belonging to *Cucumis* genus and *Cucurbitaceae* family. The world's annual cucumber production is concentrated in Asia, where Turkey ranks second in production with 1.9 million tons of produce after China contributing about million tons of cucumber production in 2019 (FAO, 2019). Cucumber plants with broader leaves contain a high-water content resulting in their susceptibility to diseases and pests that damage green parts reducing yields. Powdery mildew is caused by the obligate biotrophic pathogen *Podosphaera xanthii*, which is the most common fungal pathogen of cucurbits (Zitter *et al.*, 1996). The most effective and eco-friendly strategy for disease management is the development of resistant host varieties. It is important to under-

stand cucumber genetic and molecular defence mechanisms to develop resistant varieties to powdery mildew.

Characterization of powdery mildew resistant genotypes began with cucumber lines Porto Riko 37 (Kooistra, 1968), PI 197087 (Barnes, 1961), PI 279465 (Fugieda and Akiya, 1962), PI 200815 and PI 200818 (Kooistra, 1968). Six temperature-dependent quantitative trait loci (QTLs) have been identified for resistance to the disease, with a population established using susceptible Santou and resistant PI 197088-1 cucumber lines (Sakata *et al*., 2006). The QTLs *pm5.1*, *pm5.2*, *pm5.3*, and *pm6.1* have been defined using F_2 and F_3 populations of line K8 (Zhang *et al*., 2011), and six QTLs have been mapped on chromosome 4 and *pm5.1* and *pm5.2* major QTLs on chromosome 5 also using F_2 and F_3 populations of line WI2757 (He *et al*., 2013). A total of nine different QTLs were identified for powdery mildew resistance on Recombinant Inbred Lines (RILs) derived from CS-PMR1 × Santou (Fukino *et al*., 2013). Among these, the loci *pm3.1*, *pm5.1*, *pm5.2,* and *pm5.3* were confirmed, and associated with the powdery mildew resistance. Additionally, four different QTLs were found in a RIL population from the PI197088 (resistant) × 'Coolgreen' (susceptible) (Wang *et al*., 2018).

Many genes have been identified for genetic control of powdery mildew resistance in different cucumber cultivars, but the identified QTLs in these studies are inconsistent and uncertain (Chen *et al*., 2020), and the molecular mechanisms underlying powdery mildew resistance are still not clearly understood (Nie *et al*., 2015a). Research on the mechanism of resistance to powdery mildew in cucumber has revealed the host defences at the molecular level (Liu *et al*., 2008). Resistance is associated with cell wall thickening (Nie *et al*., 2015b), reactive oxygen species (ROS) and programmed cell death (PCD) with the expression of pathogen defence proteins (Xu *et al*., 2019). Resistance to powdery mildew in cucumber is genetically complex, cucumber plants can contain many genes and have signal transduction pathways for resistance (Chen *et al*., 2020).

In the present study, responses to powdery mildew in susceptible, tolerant, and resistant cucumber plants were analyzed. For investigation of cucumber-*P. xanthii* interactions, *WRKY*22 transcription factor, *D6PKL*1, *NFP*, and *STN*7 receptors were selected from the Plant-Pathogen Interaction pathway associated with fungal effectors in the Kyoto Encyclopedia of Genes and Genomes (KEGG) Database. Other genes in this study were selected from different plant homologous genes that provide resistance against powdery mildew. After inoculations, target gene expression levels were compared in resistant, tolerant, and susceptible cucumber plants. Additionally, 3, 3'-diaminobenzidine (DAB, Sigma Germany) and trypan blue staining were performed after inoculation to visualize hypersensitive reaction (HR) and ROS accumulation.

MATERIALS AND METHODS

Plant material and Podosphaera xanthii *inoculation*

Previously described powdery mildew resistant *Cucumis sativus* 'Meltem' (resistance score 0.66), tolerant line VT18 (score 1.00), and susceptible local variety Camlica (score 2.75) were used (Yuceson *et al*., 2020). The resistance of these phenotypes was previously scored on a 0 to 4 scale, where 0 is resistant, and 4 is susceptible (Adam and Somerville, 1996). Six seeds from each variety (Meltem, VT18, or Camlica) were germinated and planted separately in pots containing a 1:1 mixture of sterile soil and perlite. Two plants from each variety were inoculated with the *P. xanthii*; and non-inoculated control plants were also used for RT-PCR analyses. The other two inoculated plants of each variety were also assessed visually and microscopically. Also, the remaining plants from each group were used as healthy plants for comparisons with disease symptoms. The *P. xanthii* conidia used for inoculations were collected from infected leaves, and suspended in water 10^6 conidia mL^{-1} (Zhang *et al*., 2021). Plants of the different varieties with first true leaves were inoculated by spraying with conidium suspensions. The experiment was replicated three times.

Trypan Blue, DAB staining and microscope observations

Following the procedures of Thordal-Christensen *et al*. (1997), DAB staining was carried out to determine HR and ROS, and Trypan Blue staining was used to observe of *P. xanthii* structures after inoculation of plant leaves. Inoculated leaves were harvested at 7 dpi; each leaf from each plant group was boiled in 96% ethanol to remove chlorophyll. Approximately 100 µL of trypan blue solution (250 mg L^{-1}) was spread on each microscope slide, and the leaf preparations were viewed with a light microscope (Leica DM 500).

RT-PCR primers

The *Clathrin adaptor complex subunit* (*CACS*) gene and its specific primers (Miao *et al*., 2019) have been used as a housekeeping control gene. For *CACS* prim-

ers, annealing temperature was optimized for RT-PCR. *WRKY*22 transcription factor, *D6PKL*, *NFP*, and *STN7* receptors were selected from Plant-Pathogen Interaction pathway in KEGG Database associated with fungal pathogens. The *PGI*, *CALLOSE*, *GLYK*, and *CAD* genes were selected because homologs of the genes play a role in powdery mildew resistance in different plants. Sequence information of genes were downloaded from Phytozome (https://phytozome-next.jgi.doe.gov). Gene-specific primers were designed using Primer-BLAST (NCBI) software (Table 1).

RNA Isolation and RT-PCR amplification

A 100 mg sample of leaf tissue was collected from the un-inoculated plants before inoculation (0 dpi). At l and 2 dpi, leaves were harvested separately from each host variety (Meltem, VT18, and Camlica). The samples were quickly crushed in liquid nitrogen with a pre-chilled mortar and pestle. Total RNAs were isolated using the GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific), using the manufacturer's protocol. The isolated RNA was quantified with a spectrophotometer, and Verso 1-Step RT-PCR Kit ReddyMix (Thermo Fisher Scientific) was used for RT-PCR amplification. The reaction mixtures were each prepared using 0.25 µL of enzyme mix, 7.5 µL PCR Reddy mix, 7.5 µL distilled nuclease-free water, 0.75 µl RT-enhancer, 1 µL each of forward and reverse *CACS* primers (10 µM), and 20 ng RNA. The RT-PCR has performed with cDNA synthesis at 50°C for 15 min, RT inactivation at 95°C for 2 min and 36 cycles of denaturation at 95°C for 45 sec, annealing at 56°C for 50 sec, and extension at 72°C for 50 sec.

Relative expression assay

After RT-PCR amplification, the amplified products were run on 2% agarose gel using electrophoresis, and were visualized by the gel imaging system, and photographed. The gel electrophoresis analysis was performed by scanning densitometry in GelAnalyzer 19.1 (Lazar *et al*., 2010). The raw data were obtained from band intensity, and the data value was calibrated as an expression unit for calculating relative expression (RE) level. After the calibrations for individual genes, the RE values were calculated using the following equation (Martiansyah *et al*., 2018):

RE = expression unit of target genes/expression unit of *CACS*.

The RE value was accepted as 1 for the housekeeping *CACS* gene, and if the target gene was not amplified in any band, the target gene RE value was accepted as 0.

Table 1. Genes and their primers, associated with host resistance that were used in RT-PCR analyses.

Gene and accession number	Forward and reverse sequence 5'-3'	Product size (bp)
Clathrin adaptor complex subunit CACS (Cucsa.313280)	TGGGAAGATTCTTATGAAGTGC CTCGTCAAATTTACACATTGGT	171
WRKY22 transcription factor WRKY22 (Cucsa.106960)	TAGCCTCTCCGATCCCGAAG CTTCCCCATCTGTCACACCT	218
Thioredoxin F1 THXF1 (Cucsa.356300)	TCGAAGAGTGGCTGCTTCAG GGGTGTACATGTCGAGCACA	211
Polygalacturonase Inhibitor PG1 (Cucsa.038100)	CAATCCCACCCTTTGTGGGT GGTTGGCAAGAGAGCTAGGG	243
Cinnamyl alcohol dehydrogenase CAD (Cucsa.219440)	CACATGGGGGTGAAATTGGC GGATGACCATGGGAGAGACG	289
Serine/threonine-protein kinase STN7 (Cucsa.033920)	AATCCCTGGTGCCATTCTGG GCTATTAGCACAAGCACGCC	310
Callose synthase CALLOSE (Cucsa.249900)	ATAGAGCTACCGGTCGTGGT TGGTTGCAGGCACACCAATA	318
Glycerol kinase GLYK (Cucsa.105620)	CGAGAAATTGGCATCGCAGG CCAAGGGCAGTTGTCTCGAT	363
Serine/threonine receptor kinase NFP (Cucsa.101780)	GGGCAGCTTCTGTTCATTCC AAACCAACACCCAAAGCCAC	509
Serine/threonine-protein kinase D6PKL1 (Cucsa.083190)	TTGCAGCATGGGGTCCTTAG CAGGTAGAAACCTGGGGCTG	631

RESULTS AND DISCUSSION

Inoculations and symptom development

Typical powdery mildew symptoms appeared on cotyledons and leaf surfaces of the susceptible variety Camlica. Each plant was covered by conidia and white mycelia (Figure 1a). Symptoms of the disease were not common in the tolerant line VT18 compared to Camlica (Figure 1 b). The disease symptoms were not observed on the resistant Meltem (Figure 1 c). The inoculated plants developed the same powdery mildew symptoms as previously reported by Yuceson *et al.* (2020).

Microscope observations and hypersensitive response (HR)

After inoculations, HR-associated superoxide accumulated only in the tolerant line VT18 (Figure 2, d e and f). However, powdery mildew growth and sporulation continued on the host leaf surfaces. The HR was not observed in resistant Meltem plants after inoculations (Figure 2, g, h and i). Typical powdery mildew symptoms appeared on Camlica cotyledons and leaves, which were covered with powdery mildew (Figure 1 b). The disease symptoms were not common on the tolerant line VT18 (Figure 1 f), and powdery mildew was not observed on plants of the resistant variety Meltem (Fig-

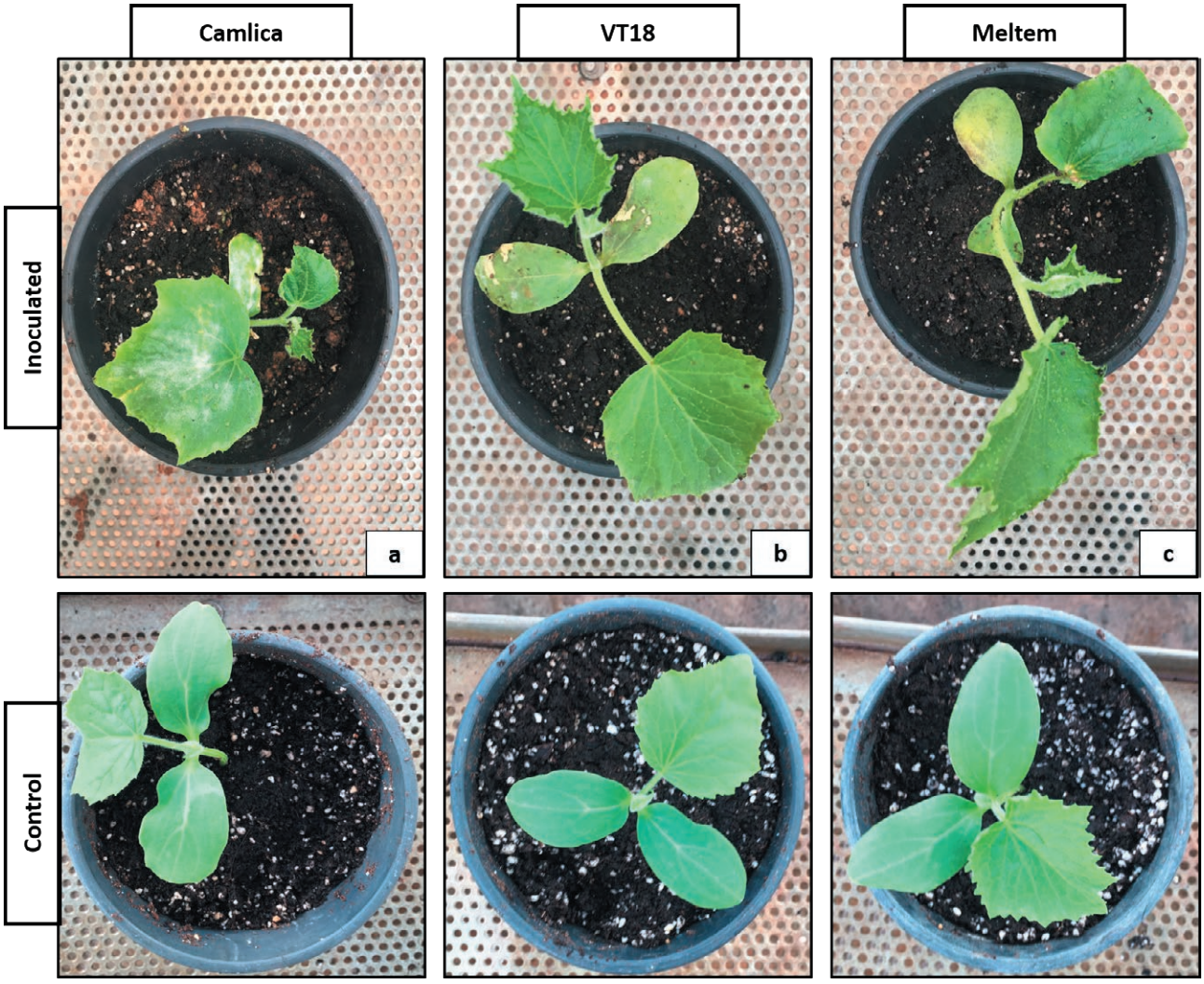

Figure 1. Phenotypes of powdery mildew for inoculated and un-inoculated (Control) cucumber plants of three varieties at 15 days post inoculation.

ure 1 j). These inoculation results were similar to those previously reported by Yüceson *et al.* (2020). The microscope observations with DAB and trypan blue staining showed no association with HR (Figure 2, g, h and i).

RT-PCR and relative expression

The housekeeping *CACS* gene was similarly expressed at the different inoculation time points (0, 1 and 2 dpi) in Meltem, VT18, and Camlica. However, *PGI*, *CALLOSE*, *ThxF1*, *STN7*, *GLYK*, *WRKY22*, *D6PKL1*, *NFP,* and *CAD* genes accumulated in different amounts at these time points for the three host groups (Figure 3). The relative expression levels of the *CACS* gene were obtained after RT-PCR analysis using the GelAnalyzer program (Figure 4).

Transcription factors play regulatory roles in response to different stress conditions (Gao et al., 2020). The *WRKY22* gene has been identified as one of the transcription factors in KEGG database, which affect plant-pathogen interactions. The *WRKY22* showed increased gene expression levels in resistant Meltem plants at 1 dpi (Figure 4 a), but levels decreased after 2 dpi. In the susceptible Camlica plants, the *WRKY22* gene was not expressed (Figure 4g), indicating that *WRKY22* is a candidate gene in the regulation of defense signalling pathways in early stages to powdery mildew infections.

In cucumber plants, the *Thioredoxin* (*ThxF1*) gene regulates accumulation of ROS and controls changing ROS levels, where accumulation of *ThxF1* was previously reported in powdery mildew resistant and susceptible plants (Xu *et al*., 2019). In the present study, the expression level of the *ThxF10* was very high in non-inoculated plants (Figure 4, a, d and g). However, expression of *ThxF10* was not detected in tolerant VT18 plants after 2 dpi (Figure 4, e and f).

The *Polygalacturonase* (*PG1*) gene is a fungus pathogenicity factor allowing decomposition of plant cell wall polygalacturone at early stages of fungal penetration. Conversely, cucumber plants employ *cell wall-binding polygalacturonase inhibitory* (*PGIP*) to limit fungal PG1 enzyme activity (De Lorenzo and Ferrari, 2002). There was no *PGIP* expression in the susceptible Camlica plants at 1 dpi (Figure 4, g and h), but resistant Meltem and tolerant VT18 showed a greater expression at 1 dpi (Figure 4, a and d).

Cinnamyl alcohol dehydrogenase (*CAD*) is synthesized in plants for physical fortification of cell walls

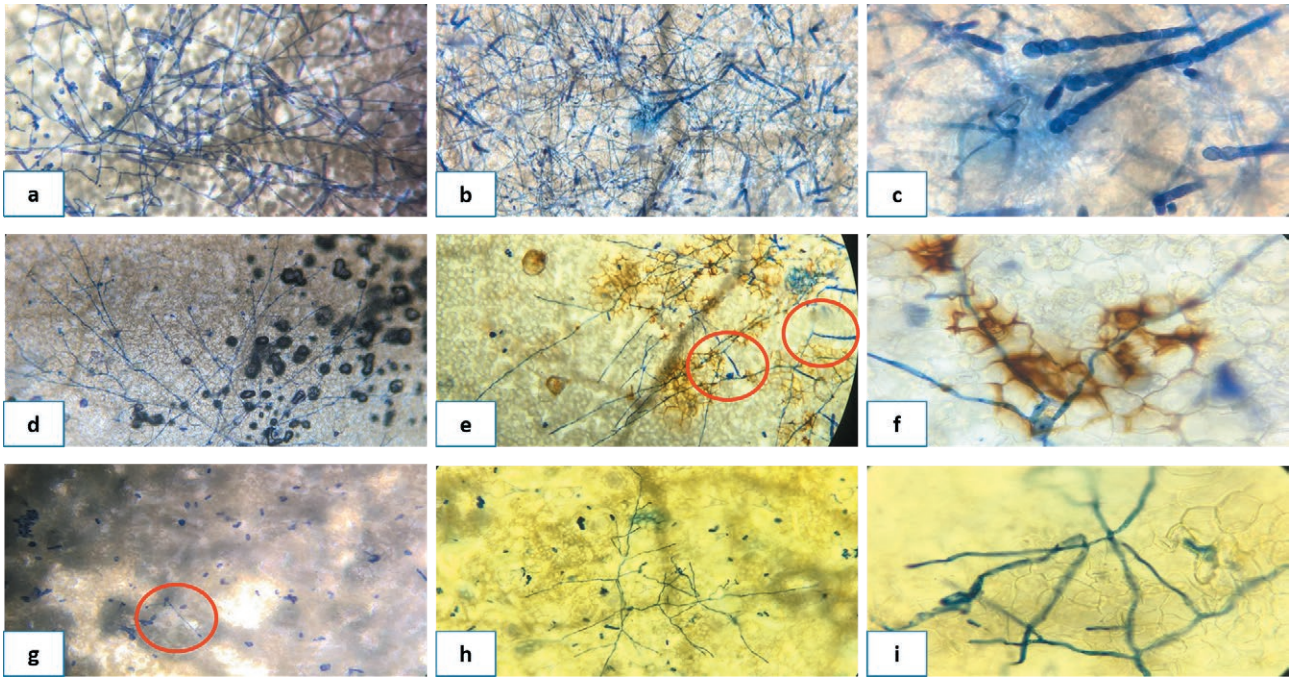

Figure 2. Micrographs of powdery mildew inoculated cucumber leaves with trypan blue and 3,3'-diaminobenzidine (DAB) staining. a, b and c: Growth of mycelia, conidiophores and conidia on Camlica (susceptible) leaf surfaces. d, e and f: Accumulation of superoxide and conidium germination tubes revealed after DAB and trypan blue staining in VT18. Superoxide production was visible as brown-reddish colour in the plant cells. Although conidia germinated and penetrated on the leaf surfaces of resistant Meltem (g, h and j), no new conidiophores or conidia developed, and no superoxide production was associated with conidia (red circle).

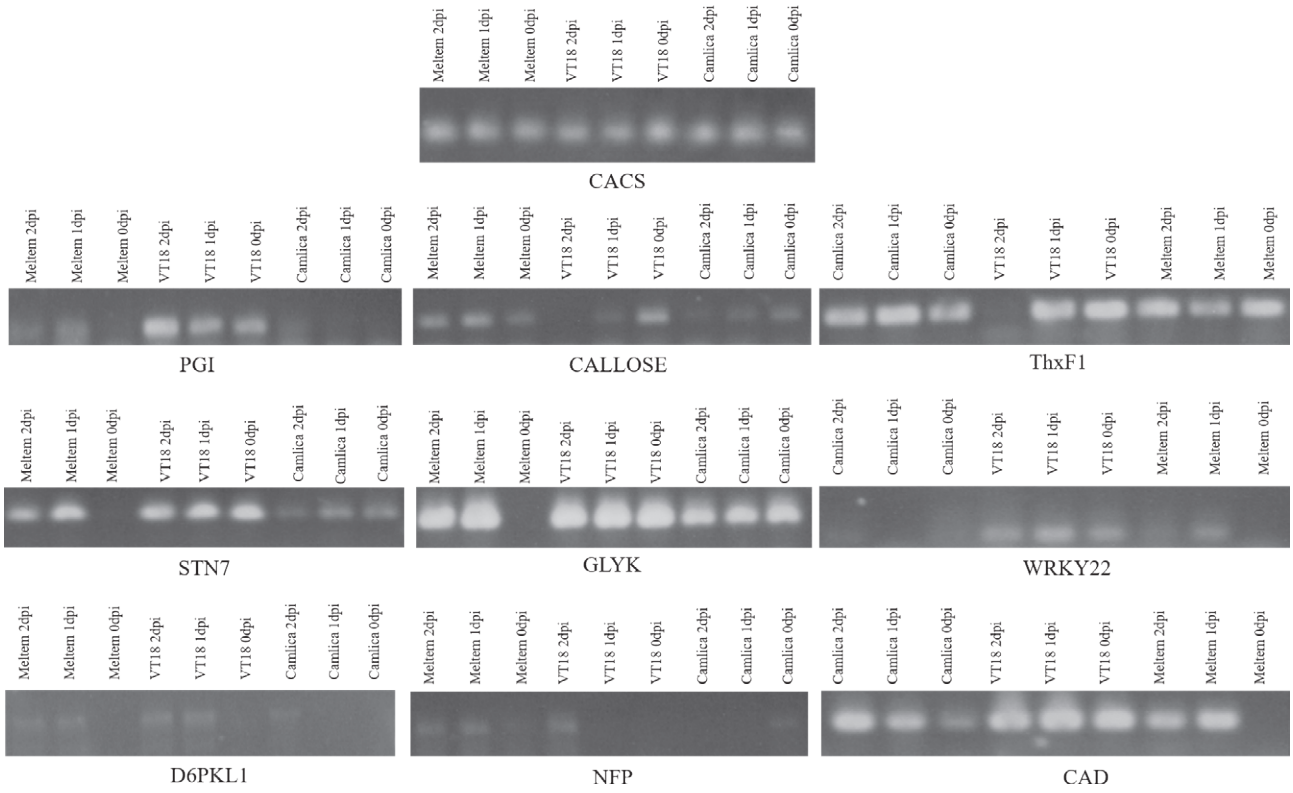

Figure 3. Semi-quantitative RT-PCR analysis results for powdery mildew resistant Meltem, tolerant VT18 and susceptible Camlica cucumber varieties, at 0, 1 or 2 dpi. CACS: *Clathrin adapter complex subunit gene* used as housekeeping gene, PG1: *Polygalacturonase Inhibitor* gene, CALLOSE: *Callose synthase* gene, ThxF1: *Thioredoxin F1* gene, STN7: *Serine/threonine-protein kinase* gene, GLYK: *Glycerol kinase* gene, WRKY22: *WRKY22 transcription factor* gene, D6PKL1: *Serine/threonine-protein kinase* gene, NFP: *Serine/threonine receptor kinase* gene, and CAD: *Cinnamyl alcohol dehydrogenase* gene.

and preventing pathogen penetration. *CAD* has been involved in the synthesis of monolignol, a chemical precursor for lignin biosynthesis (Kim *et al*., 2004). This gene also plays a crucial role in defense mechanisms against bacterial and fungal pathogens (Rong et al., 2016). *CAD* was highly induced in resistant Meltem plants at 1 dpi (Figure 4 a, b), but induction of this gene was reduced in tolerant VT18 at 1 dpi (Figure 4, d and e). The *CAD* expression levels increased in susceptible Camlica (Figure 4, g and h) after powdery mildew inoculations, and expression of this gene was high in susceptible Camlica, tolerant VT18, and resistant Meltem plants after inoculation (Figure 4). Expression of *CAD* in susceptible Camlica, and tolerant VT18 plants showed that this gene did not play a role of in cell wall thickening. However, induction of *CAD* after the inoculation of Meltem could be induced by different genes in other pathways for cell wall thickening, and result in powdery mildew resistance.

Another important gene is *CALLOSE,* usually accumulating during plant growth and development, and in response to different stress conditions. The callose protein plays an essential role in defense against plant pathogens. It is involved in cell wall synthesis and thickening, acting as a physical barrier to slow and prevent pathogen penetration. *CALLOSE* accumulation provides resistance in *Arabidopsis* against powdery mildew (Nauman *et al*., 2013). As in *Arabidopsis*, powdery mildew resistant Meltem plants produced more *CALLOSE* in the inoculated plants (Figure 4, a and b) compared to the susceptible/tolerant plants (Figure 4, d, e, g and h). This indicates that callose expression is an important resistance response in resistant/tolerant cucumber plants.

Serine/Threonine (*STN7*) is another important gene involved in plant-pathogen interactions. Increased expression of *STN7* was measured in resistant Meltem plants at 1 and 2 dpi (Figure 4, a and b) compared to susceptible Camlica plants (Figure 4, g, h and i). There was no difference in the expression of *STN7* between 0 dpi and post-inoculation in VT18 (Figure 4, d, e and f). However, high expression of *STN7* was found in VT18 plants. Thus, *STN7* in resistant Meltem and tolerant

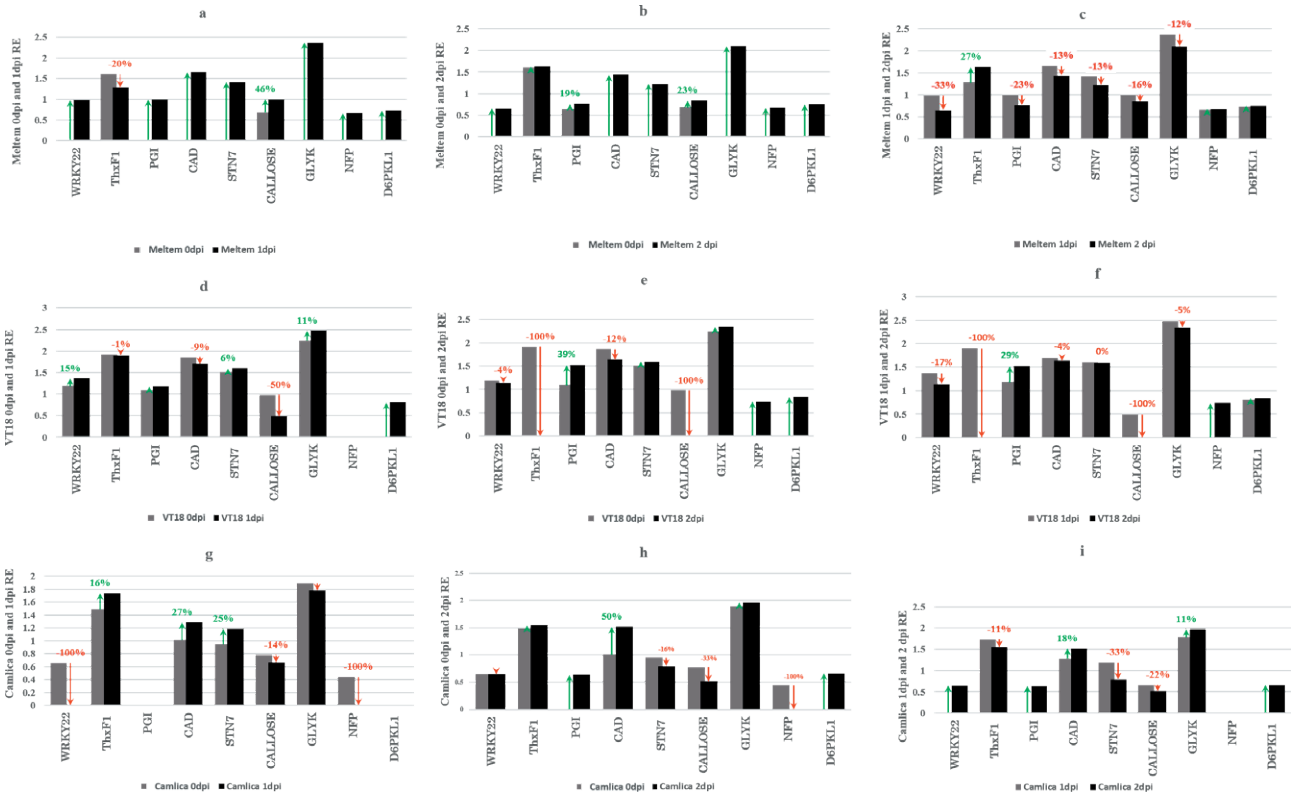

Figure 4. Relative expression values for nine genes, obtained from RT-PCR results using the GelAnalyzer 19.1 program.

VT18 plants indicated that this gene may control resistance in these plants (Figure 4, a and d).

Glycerol kinase (*GLYK*) converts glycerol to glycerol-3-phosphate, and controls resistance in *Arabidopsis thaliana* ecotypes to *Pseudomonas syringae* (Kang *et al.*, 2003). Additionally, glycerol applications on wheat plants induced resistance to the wheat powdery mildew pathogen *Blumeria graminis* f. sp. *tritici* (Li *et al.*, 2020). In the present study, *GLYK* was highly induced in resistant Meltem plants after powdery mildew inoculations (Figure 4, a and b). Expression of *GLYK* was similar at 0 dpi and in inoculated tolerant VT18 and susceptible Camlica plants at 1 dpi (Figure 4, d, e, g and h). These results indicate that *GLYK* could be involved in the functional differences in resistance of the three host groups.

The other key receptor in the KEGG pathway database involved in plant-pathogen interactions is *serine/threonine receptor-like kinase* (*NFP*). This gene was at a greater level in resistant Meltem and tolerant VT18 plants at 1 dpi (Figure 4, a, b, d, and e) than in Camlica. The susceptible plants did not accumulate *NFP* at 1 dpi (Figure 4, g and h). This indicates that *NFP* may have a specific receptor for powdery mildew recognition in resistant plants.

Expressions of the *Serine/Threonine-protein kinase* (*D6PKL1*) gene was increased in Meltem and VT18 plants at 1 dpi (Figure 4, a, b, d, and e). However, there was also a little expression of *D6PKL1* in susceptible Camlica plants. Although expression of this gene increased in Camlica at 2 dpi (Figure 4 h), it was slightly lower than in resistant and tolerant plants. This indicates that *D6PKL1* was a receptor at the penetration stage of *P. xanthii*.

In the present study, powdery mildew resistant Meltem, tolerant VT18, and susceptible Camlica plants were used to provide understanding of the cucumber resistance mechanisms to *P. xanthii*. DAB and trypan blue staining clearly showed that resistance in VT18 plants depends on HR, but this resistance is not associated with HR in resistant Meltem. Instead, the RT-PCR results showed that cell wall thickening associated with *Callose* played an essential role in resistance in Meltem. The study also demonstrated that *PG1*, *NFP*, *STN7* and *D6PKL1* are involved with varying levels of expressions in resistant Meltem, tolerant VT18, and susceptible Camlica plants. *PGI* did not express in susceptible Camlica, indicating that *PGI* expression is crucial for limiting powdery mildew. The cucumber receptors *NFP*, *STN7*,

and *D6PKL*1 can recognize powdery mildew. After recognition, *PGIP* is triggered and restricted pathogen penetration. *PGIP* is essential for host resistance. When this gene was not triggered, powdery mildew developed rapidly, as occurred in susceptible Camlica. Two different host defence mechanisms against powdery mildew were identified; HR and cell-wall thickening. The HR restricted powdery mildew in VT18. However, cell-wall thickening associated *Callose* and *CAD* trigged by the pathogen and highly was expressed in resistant Meltem. Microscope observations showed that *P. xanthii* could not develop conidia although there was no HR in resistant Meltem.

Understanding the different powdery mildew resistance mechanisms in cucumber is important for development of resistant cultivars. Development of new resistant cultivars is difficult because the resistance is complex and polygenic. The present study also showed that cell wall thickening is a more effective defence mechanism in cucumber against *P. xanthii* than HR. Although HR is generally known as a dominant pathogen resistance mechanism, different defence mechanisms could provide resistance against powdery mildew pathogens. Studies are continuing to focus on these HR mechanisms, with the aim of using alternative mechanisms for development of new powdery mildew resistant cucumber cultivars.

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