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Lignin Biosynthesis Gene Expression Is Associated with Age-related Resistance of Winter Squash to *Phytophthora capsici*

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Abstract. **The Oomycete plant pathogen, *Phytophthora capsici*, causes root, crown, and fruit rot of winter squash (*Cucurbita moschata*) and limits production. Some *C. moschata* cultivars develop age-related resistance (ARR), whereby fruit develop resistance to *P. capsici* 14 to 21 days postpollination (DPP) because of thickened exocarp; however, wounding negates ARR. We uncovered the genetic mechanisms of ARR of two *C. moschata* cultivars, Chieftain and Dickenson Field, that exhibit ARR at 14 and 21 DPP, respectively, using RNA sequencing. The sequencing was conducted using RNA samples from 'Chieftain' and 'Dickenson Field' fruit at 7, 10, 14, and 21 DPP. A differential expression and subsequent gene set enrichment analysis revealed an overrepresentation of upregulated genes in functional categories relevant to cell wall structure biosynthesis, cell wall modification/organization, transcription regulation, and metabolic processes. A pathway enrichment analysis detected upregulated genes in cutin, suberin monomer, and phenylpropanoid biosynthetic pathways. A further analysis of the expression profile of genes in those pathways revealed upregulation of genes in monolignol biosynthesis and lignin polymerization in the resistant fruit peel. Our findings suggest a shift in gene expression toward the physical strengthening of the cell wall associated with ARR to *P. capsici*. These findings provide candidate genes for developing *Cucurbita* cultivars with resistance to *P. capsici* and improve fruit rot management in *Cucurbita* species.**

Phytophthora capsici is a soilborne Oomycete with a host range exceeding 50 plant species (Tian and Babadoost 2004). This polycyclic pathogen is responsible for significant plant losses when environmental conditions are favorable (Erwin and Ribeiro 1996; Granke et al. 2009; Hausbeck and Lamour 2004). Economically important crops within the Cucurbitaceae (cucurbits), Solanaceae, and Fabaceae families are highly susceptible to *P. capsici* infection (Davidson et al. 2002; Gevens and Hausbeck 2005; Hausbeck and Lamour 2004); however, cucurbits are considered among the most susceptible (Tian and Babadoost 2004). Symptoms of *P. capsici* infection on cucurbits include root and/or crown rot, foliar blight, and fruit rot (Babadoost 2004). Fruit rot threatens cucurbit crops annually, including *Cucurbita* species such as *C. maxima*, *C. moschata*, and *C. pepo* (squash and pumpkin), in Michigan, which is a state that is an important producer of these species (Gevens et al. 2007; Krasnow and Hausbeck 2016; Lamour and Hausbeck 2000; US Department of Agriculture, National Agricultural Statistics Service 2018), and other states (Babadoost 2004; Castro-Rocha et al. 2017). The fruit may become rotted while in the field (Granke et al. 2012; Meyer and Hausbeck 2013) or after harvest (Hausbeck and Lamour 2004), leading to a loss of crop production that may exceed 50% (Babadoost 2000; Meyer and Hausbeck 2013). Protecting squash fruit from *P. capsici* infection is challenging because of the relatively lengthy maturation time during which the fruit are in direct contact with the soil. Although

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host resistance is critical for long-term management (Granke et al. 2012; Quesada-Ocampo and Hausbeck 2010), complete host resistance in commercial squash or pumpkin cultivars is unavailable (Café-Filho et al. 1995). However, age-related resistance (ARR) to *P. capsici* is expressed in the fruit of specific *C. moschata* cultivars (Krasnow and Hausbeck 2016; Meyer and Hausbeck 2013) and other cucurbits (Ando et al. 2009; Gevens et al. 2006) and Solanaceae (Biles et al. 1993) fruit. ARR is associated with resistance to pathogens at specific developmental stages (Stermer and Hammerschmidt 1984; Whalen 2005). Fruit of several cucurbits (Ando et al. 2009; Gevens et al. 2006; Krasnow and Hausbeck 2016; Meyer and Hausbeck 2013) and pepper (*Capsicum annuum*) (Biles et al. 1993) exhibit ARR to *P. capsici* as they mature. However, wounding negates ARR to *P. capsici* (Ando et al. 2015; Biles et al. 1993; Krasnow et al. 2014), suggesting the fruit peel may provide resistance to fruit rot.

The mechanism of ARR in different host–pathogen systems has been investigated (Panter and Jones 2002). ARR can be conferred by preformed or induced defenses (Develey-Rivière and Galiana 2007; González-Lamothe et al. 2009; Panter and Jones 2002), whereby preformed defense is the consequence of structural/physical and/or chemical barriers (Vergne et al. 2010). The plant cell wall serves as a physical barrier that forms an obstacle for the entry of all pathogens, but it can be overcome by pathogen-generated cell wall-degrading enzymes (Bacete et al. 2018; Bellincampi et al. 2014). In addition to the cell wall, the plant surface is covered by the cuticle comprising cutin polymer, which provides another defensive layer against pathogens (Chassot and Métraux 2005). The thickening of bean (*Phaseolus vulgaris*) hypocotyls is correlated with resistance to *Rhizoctonia solani* (Stockwell and Hanchey 1983). Similarly, thickening of the cuticle has been suggested as the mechanism of ARR in pepper fruit (Biles et al. 1993) and *C. moschata* cultivars (Alzohairy et al. 2020) to *P. capsici*. Thickening of the epidermal walls was also observed in cucumber (*Cucumis sativus*) fruit with ARR to *P. capsici* (Ando et al. 2015). Another form of constitutive defense involving a strengthened physical barrier is the formation of lignified xylem vessels in beans that leads to the restriction of lesions spread within the leaves caused by *Colletotrichum lindemuthianum* (Griffey and Leach 1965). In addition, lignin deposition at the cell wall makes it resistant to cell wall-degrading enzymes, as has been observed in the resistance of false flax (*Camelina sativa*) to *Sclerotinia sclerotiorum* (Eynck et al. 2012). However, it remains to be determined whether increased lignin deposition is important for ARR in cucurbits.

Previous studies of fruit ARR in squash and pumpkin against *P. capsici* infection attributed ARR to morphological (Ando et al. 2009) or physiological changes (Meyer and Hausbeck 2013), but not to chemical changes (Alzohairy et al. 2021). The different developmental time points of ARR onset have been observed in cultivars of *C. moschata* (Alzohairy et al. 2020) and other cucurbits (Ando et al. 2009, Gevens et al. 2006), where ARR occurs at variable young fruit ages. The difference in ARR onset has been suggested to be related to the difference in rates of fruit development (Gevens et al. 2006). However, the genetic mechanism of ARR in winter squash and how the difference in ARR is regulated across cultivars are unknown.

Transcriptomic studies have been applied to studying ARR in at least two species. In cucumber, fruit with ARR showed an increase in the level of terpenoid glycosides in resistant maturing fruit compared with the susceptible younger fruit, suggesting a role of constitutive chemical defense (Mansfeld et al. 2017) unlike that detected in winter squash (Alzohairy et al. 2021). In apple (*Malus pumila*), when the molecular mechanisms controlling ARR in leaves to *Venturia inaequalis* was studied, the constitutive upregulation of genes encoding for metallothionein3-like protein, lipoxygenase, lipid transfer protein, and peroxidase 3 and the downregulation of genes encoding for "enhanced disease susceptibility 1 protein" were highly correlated with the observed ARR of aging apple leaves (Gusberti et al. 2013). This study aimed to use transcriptomic studies and differential gene expression analyses of resistant and susceptible fruit stages to assess the molecular mechanisms of ARR in two *C. moschata* cultivars with different ARR onset time points.

Materials and Methods

Plant material. Two *C. moschata* commercial cultivars, Chieftain (butternut winter squash; Rupp Seeds Inc., Waseon, OH, USA) and Dickenson Field (processing pumpkin; Rispers Seeds Inc.), were previously evaluated for ARR (Alzohairy et al. 2020). 'Chieftain' develops ARR at 14 d postpollination (DPP) and 'Dickenson Field' develops ARR at 21 DPP. Planting and fruit harvesting were performed according to Alzohairy et al. (2020). Briefly, seeds were planted on 15 Jun 2015, into 72-cell trays containing soilless peat mixture (Suremix Michigan Grower Products, Inc., Galesburg, MI, USA) and grown for 2 weeks in the research greenhouse at Michigan State University in East Lansing, MI, USA. Thirty seedlings from each cultivar were transplanted on 1 Jul to a field site, previously planted with pumpkin, at the Michigan State University Plant Pathology Farm in Lansing, MI, USA. The soil type was Capac loam, with no known *P. capsici* infestation. Plants were grown on raised plant beds covered with plastic mulch and irrigated twice each week via drip emitters. The length of the plant rows was 30.5 m, with 3.7 m between rows and 61 cm between plants. At anthesis, flowers were hand-pollinated and tagged with the date; then, fruit were harvested at 7, 10, 14, and 21 DPP. Fruit of the same age were harvested on the same day from nine different plants as biological replicates.

RNA extraction. Fruit of 'Chieftain' and 'Dickenson Field' at 7, 10, 14, and 21 DPP were surface-disinfested with 70% ethanol and air-dried on a paper towel. Sterilized fruit were peeled using a vegetable peeler and immediately frozen in liquid nitrogen. Fruit peels were stored at −80 °C until RNA isolation. RNA was extracted from three biological replicates for each cultivar (three individual fruit at each DPP) using E.Z.N.A. total RNA kit (OMEGA BIO-TEK, Norcross, GA, USA) and treated with 2 U/μL Turbo DNase (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) to remove DNA contamination. The RNA concentration was determined using spectrophotometer (Nanodrop ND-1000, Thermo Fisher Scientific). The RNA integrity was checked using a DNA fragment analysis system (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA).

RNA sequencing and RNAseq read processing. RNA library construction and sequencing were performed at the Research Technology Support Facility at Michigan State University, East Lansing, MI, USA. RNA libraries were prepared using a TruSeq Stranded mRNA kit (Illumina, San Diego, CA, USA) following the manufacturer's protocols. RNA sequencing was performed using the Illumina HiSEq 4000 platform with the 2 × 150-bp paired-end format. A total of 24 libraries were divided into two

pools of 12, and each pool was sequenced on two lanes (libraries were bar-coded before pooling). Each lane produced ~30 million reads/sample. Trimmomatic version 0.23 (Bolger et al. 2014) was used for the paired-end reads to remove adaptors and low-quality sequences. The sliding window method was used to scan the reads with four-base wide and cut when the base quality was below a threshold of 2. The minimum read length cutoff was 20 bases. STAR version 2.5.1b (Dobin et al. 2013) was used to map the RNAseq reads to the *C moschata* cv. Rifu reference genome version NEWM01000000 (Sun et al. 2017) from the Cucurbit Genomics Database (Zheng et al. 2019). STAR was used with the default settings using the two-pass mode basic option with intron size 21–6000 nt. In all samples, >88% of the RNAseq reads were mapped to the reference genome. The raw reads, filtered reads, and mapping information are presented in Supplemental Table 1.

Differential expression and clustering analysis. The HTseq-count function in HTseq version 0.6.1 (Anders et al. 2015) was used in the default mode and stranded-reverse for generating read counts. The HTseq-count output was fed into DESeq2 (Love et al. 2014) for the differential expression analysis using the DESeq function. A gene was considered differentially expressed between two RNA-seq samples if the adjusted $P \leq 0.05$ and the log2 fold change ≥ 1. The probability value was adjusted using the Benjamini-Hochberg method (Benjamini and Hochberg 1995). Differentially expressed genes (DEGs) from all comparisons among cultivars were categorized into three main sets, including genes that are upregulated or downregulated among all tested comparisons across cultivars (set 1), genes that are commonly upregulated or downregulated in all comparisons per cultivar (set 2), and genes that are expressed in at least one comparison in both cultivars (set 3). The clustering of expression data was performed using k-means clustering with the Complex Heatmap package in R (Gu et al. 2016), with k = 16. After dividing genes into clusters using the k-means algorithm, hierarchical clustering of the k-means clusters was performed using the complete algorithm based on the Euclidean distance measure to assess which k-means clusters were most similar.

Identification of putative *Arabidopsis thaliana* homologous genes and inference of squash cell well pathway genes. Putative homologous *A. thaliana* genes of *C. moschata* DEGs and non-DEGs were identified using BLASTX (Altschul et al. 1990) by comparing the translated coding sequences of the *C. moschata* DEGs and non-DEGs to the peptide sequence of *A. thaliana* using an E-value threshold of 1e-10. The homologs were identified as reciprocal best matches across species. Because of this criterion, only the best match for each gene was identified. *A. thalianas* pathways related to cell wall structure/composition were downloaded from AraCyc version 15.0 (Mueller et al. 2003). The pathways include cuticular wax biosynthesis, cutin biosynthesis, long-chain fatty acid activation, suberin monomers biosynthesis, esterified suberin biosynthesis, cellulose biosynthesis, homogalacturonan biosynthesis, xylogalacturonan biosynthesis, phenylpropanoid biosynthesis, and xylan biosynthesis.

Functional annotation and pathway enrichment analysis. All DEGs and non-DEGs were functionally annotated by extracting the Gene Ontology (GO) annotations, Interpro, and descriptions from the Cucurbit Genomics database (Zheng et al. 2019). The GO enrichment analysis was performed for the different DEG lists for both cultivars. A pathway enrichment analysis was performed using the *C. moschata* genes homologous to the *A. thaliana* genes in 10 targeted biosynthesis pathways: cuticular wax, cutin, long-chain fatty acid activation, suberin monomers, esterified suberin, cellulose, homogalacturonan, xylan, xylogalacturonan, and phenylpropanoid. Both the GO and pathway enrichment analyses were based on Fisher's exact tests. Probability values were corrected for multiple testing (Benjamini and Hochberg 1995) and reported as q-values. A GO term or pathway was assessed to determine if it contained significantly overrepresented numbers of DEGs by performing Fisher exact tests and calculating log ratios between proportions of genes in a GO term or pathway that are DEGs and proportions of genes in a GO term or pathway that are not DEGs. When the log ratio of a GO term or pathway was positive and the test probability value after multiple testing correction was $P \leq 0.05$, the GO term or pathway was defined as overrepresented. Fisher's exact tests were performed using manual Python scripts, which use the Python package Fisher 0.1.5 (Tang and Pederson 2017). The enrichment analysis and Fisher's exact test scripts can be found on GitHub (GitHub, Inc., San Francisco, CA, USA).

Results

Sequencing and gene expression profiles among cultivars. Our study specifically focused on comparing the changes in gene expression between young susceptible fruit and aging resistant fruit within the same cultivar or between two cultivars that exhibited ARR to *P. capsici*. Therefore, the fruit peel transcriptomes of two cultivars, Chieftain and Dickenson Field, at different developmental time points before and after the development of ARR, as previously identified by Alzohairy et al. (2020), were sequenced. *C. moschata* genes were classified into 16 clusters using the transcriptome of both cultivars at different developmental time points to assess how gene expression patterns correlated with ARR onset (Fig. 1). These clusters were classified into four groups based on the similarity of the gene expression profile during different time points and between cultivars. We anticipated that ARR-associated genes would have two expression patterns. The first pattern was found among genes with a consistent change in expression either by upregulation or by downregulation in both cultivars during at least one resistant time point (groups 1 and 2) (Fig. 1). The second pattern was found among genes whose expression was upregulated or downregulated during at least one resistant stage in only one cultivar (groups 3 and 4) (Fig. 1). Dividing the genes into different clusters facilitated the identification of a group of genes that were likely candidates for ARR (groups 1 and 2) (Fig. 1). Therefore, we performed a differential expression analysis to detect potential ARR-associated genes.

Differential gene expressions of 'Chieftain' and 'Dickenson Field'. In 'Chieftain', ARR develops at 14 DPP and continues through 21 DPP, whereas ARR in 'Dickenson Field' develops at 21 DPP. To identify candidate genes relevant to resistance development at these ages in 'Chieftain', we compared the gene expression of the susceptible fruit peels at 7 and 10 DPP to the resistant ones at 14 DPP and 21 DPP. Similarly, gene expressions of the susceptible fruit peels at 7 DPP, 10 DPP, and 14 DPP were compared with the resistant fruit peels at 21 DPP in 'Dickenson Field'. Candidate genes responsible for resistance against *P. capsici* were significantly upregulated or downregulated at 14 DPP and 21 DPP in 'Chieftain' and at 21 DPP in 'Dickenson Field' compared with their susceptible fruit peel ages (Fig. 2A, Supplemental Table 2).

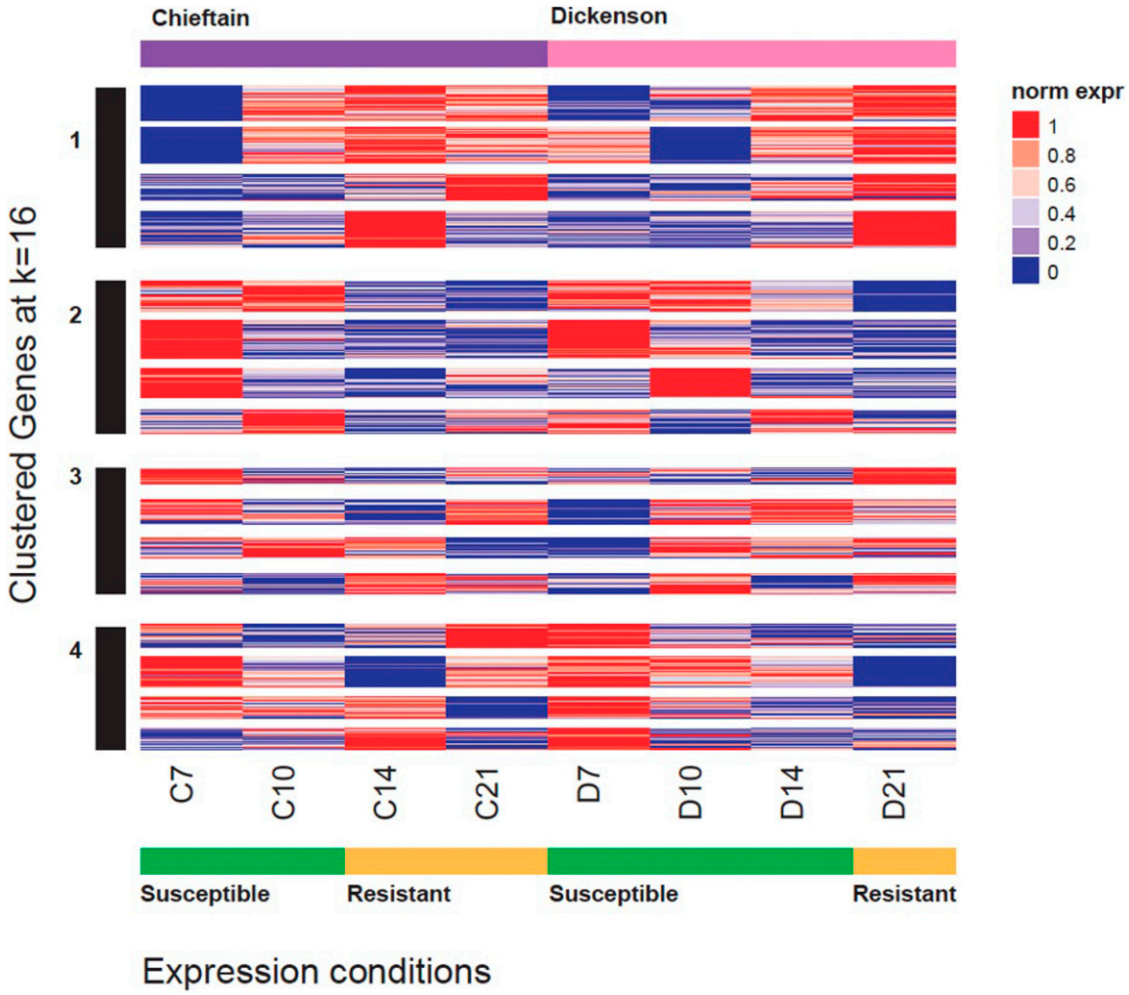

Fig. 1. Expression conditions of *Cucurbita moschata* cultivars Chieftain and Dickenson Field (Dickenson) fruit peel at ages 7, 10, 14, and 21 d postpollination (DPP) and heatmap of k-means clustering of each gene-normalized expression between both Chieftain and Dickenson Field at 7, 10, 14, and 21 DPP. Genes were clustered at k = 16. After dividing into k-means clusters, we performed hierarchal clustering to group together clusters that were most similar. Numbers 1, 2, 3, and 4 denote these hierarchal clusters.

The overlap between the different contrasts of each cultivar for both upregulated (Fig. 2B and D) and downregulated genes (Fig. 2C and E) was used to identify DEGs that were consistently upregulated or downregulated during the resistant stages compared with their susceptible stages. When comparing the resistant stages at 14 DPP and 21 DPP in 'Chieftain' to the susceptible stages at 7 DPP and 10 DPP, DEGs tended to be similarly upregulated or downregulated (Fig. 2A). Thus, there are groups of genes with expression patterns that are correlated with the resistant phenotype. Additionally, fewer DEGs were detected when comparing 14 DPP and 21 DPP to 10 DPP than when comparing 14 DPP and 21 DPP to 7 DPP (Fig. 2B and C), and that difference in DEGs helped to narrow the list of DEGs that are likely candidate genes for ARR. Similarly, in 'Dickenson Field', there was an apparent decrease in the number of DEGs as the fruit gradually transitioned toward resistance that developed at 21 DPP (Fig. 2D and E). From the differential gene expression analysis, we narrowed the DEGs to three sets potentially included in the ARR-associated genes (Fig. 2A). The set 1 genes are likely to include genes associated with resistance when the mechanism of ARR is similar across cultivars. The set 2 genes are likely to be cultivar-specific genes; therefore, we can determine if the mechanism is different between cultivars. The set 3 genes can be related to the resistance mechanism specific to any resistant stage in both cultivars.

Function of upregulated genes in both cultivars. The function of genes upregulated in the resistant fruit peel stages compared with their susceptible stages was investigated to determine which genes are candidates for controlling ARR. This was performed by identifying the GO categories (as the proxy of gene function) enriched in upregulated genes resulting from individual contrasts. In 'Chieftain', we included the resistant stages of 14 DPP and 21 DPP to determine whether there are stage-specific genes that could be related to ARR compared to those that may be constant during both 14 DPP and 21 DPP. First, the function of the upregulated genes detected from the contrast between 14 DPP to 7 DPP and 10 DPP was investigated to detect the functions that were consistently present during the resistant stage 14 DPP and were likely related to ARR. Among 3226 and 1120 upregulated genes in 'Chieftain' resulting from the contrast of the early resistant time point, 14 DPP, compared to susceptible time points, 7 DPP and 10 DPP, they were enriched in 33 and 44 GO terms, respectively (Supplemental Table 3). The most significantly overrepresented GO terms during early resistant time points include those relevant to cell wall structures

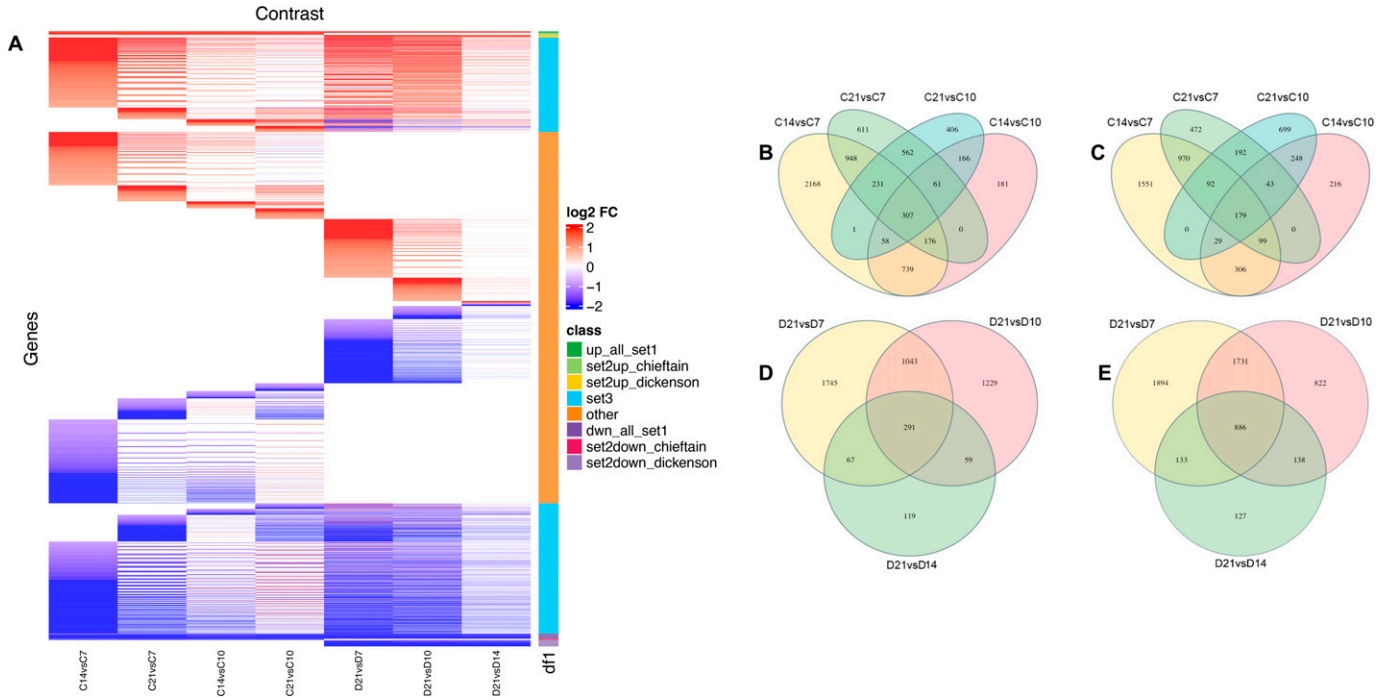

Fig. 2. Differential gene expression analysis of *Cucurbita moschata* cultivars Chieftain and Dickenson Field (Dickenson) fruit at 7, 10, 14, and 21 d postpollination (DPP). The onset of age-related resistance was at 14 DPP and 21 DPP in Chieftain and Dickenson Field, respectively. (**A**) Heatmap of differentially expressed genes with |log2(FC)|>1 (FC = fold change) and adjusted $P < 0.05$. Each column contrasts the resistant and susceptible fruit peel. The set 1 genes include those consistently upregulated or downregulated in both cultivars during the resistant fruit peel ages compared with the susceptible fruit peel ages. The set 2 genes include those commonly upregulated or downregulated during all resistant-susceptible comparisons within each cultivar. The set 3 genes are either upregulated or downregulated during at least one comparison between resistant and susceptible stages of both cultivars. (**B**, **C**) Venn diagrams showing upregulated and downregulated genes in all sets of comparisons of 'Chieftain', respectively. (**D**, **E**) Venn diagrams showing upregulated and downregulated genes in all sets of comparisons of 'Dickenson Field', respectively. Letters "C" and "D" indicate 'Chieftain' and 'Dickenson Field', respectively. Supplemental Table 2 provides the actual log fold change numbers for all differentially expressed genes.

(e.g., lignin biosynthesis process) and phenylpropanoid biosynthesis (e.g., cinnamyl alcohol dehydrogenase activity, sinapyl alcohol dehydrogenase activity, stilbene biosynthetic process, and coumarin biosynthetic process), oxidoreductases (e.g., peroxidase activity), and defense (e.g., defense response to bacterium) (Fig. 3, Supplemental Table 3). This result indicates that a group of genes functioning in cell wall structures and phenylpropanoid biosynthesis is likely related to ARR at 14 DPP fruit in 'Chieftain'.

Next, we studied the function of the upregulated genes detected from the contrast between the late resistant stage (21 DPP) and susceptible stages (7 DPP and 10 DPP) in 'Chieftain'. By defining the function of the upregulated genes in the late resistant stage (21 DPP), the functions related to ARR can be detected by filtering the shared functions with the early resistant stage (14 DPP). Also, comparing the functions detected in the early (14 DPP) and late (21 DPP) resistant stages will define differences in functions that could be related to each resistant time point. Among 2047 and 1482 'Chieftain' upregulated genes when comparing expression levels during the late stage of resistance (21 DPP) to those during susceptible time points (7 DPP and 10 DPP), they were enriched in 38 and 41 GO terms, respectively (Supplemental Table 3). Similar GO terms detected between the early (14 DPP) and late (21 DPP) resistant stages include cell wall structures (e.g., lignin biosynthesis process) and phenylpropanoids biosynthesis (e.g., cinnamyl alcohol dehydrogenase activity, sinapyl alcohol dehydrogenase activity, stilbene biosynthetic process, and coumarin biosynthetic process), oxidoreductases (e.g., peroxidase activity), and defense (e.g., defense response to bacterium) (Fig. 3, Supplemental Table 3). Fewer GO terms were different between 14 DPP and 21 DPP when compared with 7 DPP and 10 DPP, including GO terms relevant to fruit ripening (e.g., xylem development) and sugar hydrolysis (e.g., beta-galactosidase, glycosaminoglycan catabolic process) (Supplemental Table 3). The different GO terms between 14 DPP and 21 DPP did not indicate specific enrichment in functions apparently relevant to resistance (Supplemental Table 3). In contrast, it is apparent that the resistance-associated genes are likely consistently upregulated at both 14 DPP and 21 DPP compared with the susceptible stage (7 DPP and 10 DPP) (Fig. 3, Supplemental Table 3).

In 'Dickenson Field', functions of upregulated genes detected at 21 DPP compared to 7 DPP, 21 DPP compared to 10 DPP, and 21 DPP compared to14 DPP were enriched in 45, 43, and 48 GO terms, respectively (Supplemental Table 3). Overrepresented GO terms most relevant to the resistance, such as lignin biosynthesis process, cinnamyl alcohol dehydrogenase activity, and sinapyl alcohol dehydrogenase activity, stilbene biosynthetic process, and coumarin biosynthetic process, are similar to those detected in 'Chieftain', but contrasts included cell wall structures and phenylpropanoids biosynthesis (Fig. 3). Cultivars' distinct GO terms were not apparently related to resistance (Supplemental Table 3). Taken together, this group of GO terms was consistently enriched in the upregulated genes detected in the resistant stages in both cultivars when contrasted to their susceptible stages, suggesting that the genes involved in ARR are similar across cultivars with differences only in the timing of their upregulation.

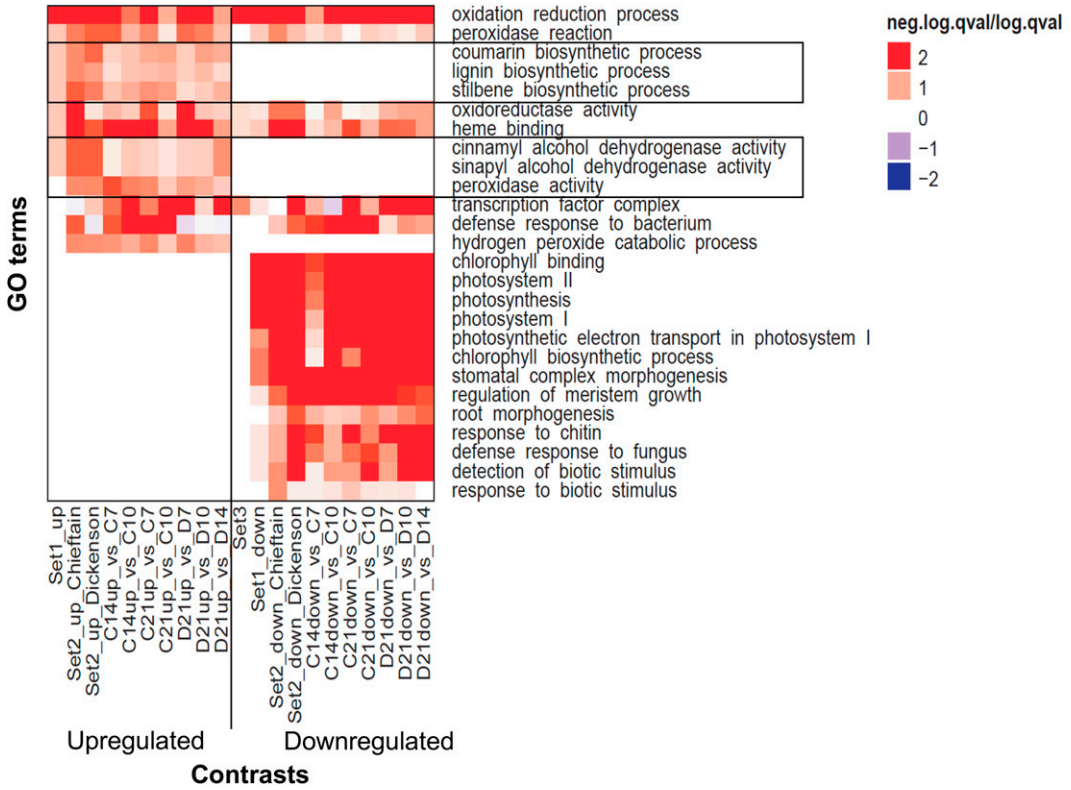

Fig. 3. Heatmap showing selected Gene Ontology (GO) terms that are significantly overrepresented in either upregulated genes or downregulated genes during contrasts between resistant and susceptible peel of fruit at 7, 10, 14, and 21 d postpollination (DPP) in both *Cucurbita moschata* cultivars Chieftain and Dickenson Field. The onset of age-related resistance was at 14 DPP and 21 DPP in 'Chieftain' and 'Dickenson Field', respectively. Letters "C" and "D" indicate 'Chieftain' and 'Dickenson Field', respectively. The value range of the heatmap is shown as the result of Fisher's exact test. If the GO term was overrepresented, then the negative log of the adjusted *P* value (or q-value) was taken; however, if it was underrepresented, then the log of the adjusted *P* value was obtained. Therefore, a positive value ≥1.3 indicates significant overrepresentation and a negative value ≤−1.3 indicates significant underrepresentation. The black rectangles point to functions related to cell wall structures and phenylpropanoid biosynthesis processes that are overrepresented only in the upregulated genes in both cultivars.

Function of downregulated genes in both cultivars. To identify the genes that are downregulated during fruit development and the transition to a resistant state, the function of the detected downregulated genes in the resistant stages in both cultivars using GO was assessed. In 'Chieftain', among the 4628 and 1688 downregulated genes at 14 DPP compared to 7 DPP and at 14 DPP compared to 10 DPP, 127 and 149 GO terms were enriched, respectively. Among the 2896 and 1792 downregulated genes at 21 DPP when compared to 7 DPP and 10 DPP, 152 and 161 GO terms, respectively, were observed (Supplemental Table 4). These GO terms are highly overrepresented in photosynthesis (e.g., chlorophyll-binding, photosystem II, chloroplast thylakoid membrane), cell growth (e.g., regulation of meristem growth, tissue development, regulation of cell size), and cell differentiation (e.g., stomatal complex morphogenesis, root morphogenesis) (Fig. 3, Supplemental Table 4). In 'Dickenson Field', the 4644, 3577, and 1284 downregulated genes during the contrasts of 21 DPP compared to 7 DPP, 10 DPP, and 14 DPP were enriched for 165, 167, and 170 GO terms, respectively (Supplemental Table 4). Shared categories of GO terms among the three different contrasts of 'Dickenson Field' are overrepresented with high significance during photosynthesis (e.g., photosystems I and II, plastid organization, chloroplast thylakoid membrane) and metabolic processes (e.g., sucrose and starch metabolic process) (Fig. 3, Supplemental Table 4). Our findings from the functional annotation of both upregulated and downregulated genes detected during the comparisons of resistant and susceptible fruit ages in both cultivars indicate that the mechanism of ARR is most likely controlled similarly in both cultivars.

Comparison of ARR mechanisms among cultivars. The studied cultivars of *C. moschata* have a different onset of ARR to *P. capsici*, with 'Chieftain' developing resistance at 14 DPP and 'Dickenson Field' developing resistance at 21 DPP. Based on the GO term enrichment analyses, both cultivars have similar enriched terms in upregulated and downregulated genes when comparing the resistant and susceptible stages (Fig. 3). To further determine the extent to which the mechanism of ARR to *P. capsici* is similar across cultivars, DEGs between the two cultivars and among the different developmental stages were examined based on the three sets of genes defined previously (Fig. 2A, Supplemental Table 2).

First, we compared GO terms enriched in set 1 upregulated genes (Set1_up), which are genes that are consistently upregulated in resistant stages in both cultivars, and they were enriched in 38 GO terms (Supplemental Table 3). The overrepresented GO terms included cell wall structures, phenylpropanoid biosynthesis (e.g., lignin biosynthesis process, sinapyl alcohol dehydrogenase activity, cinnamyl alcohol dehydrogenase activity, stilbene biosynthetic process, and coumarin biosynthetic process), and oxidoreductases (e.g., peroxidase activity).

In set 2, upregulated genes in 'Chieftain' (Set2_up_Chieftain) were enriched in 40 GO terms (Supplemental Table 3). The top

10 significantly overrepresented GO terms included the oxidoreductase activity, stilbene biosynthetic process, sinapyl alcohol dehydrogenase activity, and cinnamyl alcohol dehydrogenase activity (Fig. 3, Supplemental Table 3). Other overrepresented GO terms for the lignin biosynthetic process, peroxidase activity, and coumarin biosynthetic process were also detected (Supplemental Table 3). Upregulated genes of set 2 in 'Dickenson Field' (Set2_up_Dickenson) were enriched in 49 GO terms with the highest scores, including sinapyl alcohol dehydrogenase activity, cinnamyl alcohol dehydrogenase activity, lignin biosynthetic process, peroxidase activity, and coumarin biosynthetic process (Supplemental Table 3). We also questioned whether the ARR phenotype is correlated with genes that are expressed (upregulated or downregulated) in both cultivars (set 3) during any comparison. The function of DEGs in set 3 was enriched in 29 overrepresented GO terms but did not seem relevant to ARR such as zinc ion binding, chromatin binding, chloroplast-thylakoid membrane, heme binding, and starch and sucrose metabolic processes (Supplemental Tables 3 and 4).

We also investigated the function of downregulated genes detected in the different sets. Downregulated genes in set 1 (set1_down), which are consistently downregulated in both cultivars during all comparisons, set2_down in 'Chieftain', and set2_down in 'Dickenson Field' were enriched in 114, 154, and 174 GO terms, respectively (Supplemental Table 4). The highly scored GO terms for the three sets involved photosynthesis (e.g., chlorophyll-binding, protein chromophore linkage, photosynthesis, chloroplast thylakoid membrane, and photosystems I and II) (Fig. 3, Supplemental Table 4). According to GO term findings in upregulated and downregulated sets of genes, we questioned which cell wall structures are specifically regulated during development and can be candidates for the winter squash ARR mechanism against *P. capsici*.

Pathway enrichment of cell wall structure-related genes. During the GO enrichment analysis, we detected enrichment of cell wall structures and phenylpropanoid biosynthesis processes in the upregulated genes during the resistant stages in both cultivars. Based on our current findings and previous findings of Alzohairy et al. (2020), who showed evidence of cuticle and epidermal wall thickness increases in the resistant fruit ages, we hypothesized that cell wall structure biosynthesis is related to ARR. To define which cell wall structure may be related to ARR, we assessed whether certain cell wall structure biosynthesis pathways were enriched among upregulated and downregulated genes detected in the peel of resistant fruit ages in both cultivars. To annotate winter squash genes with likely functions in pathways for cell wall structure biosynthesis, we identified squash genes homologous to *A. thaliana* genes involved in 10 cell wall structure biosynthesis pathways. Five pathways were enriched in DEGs during the resistant fruit stages in both cultivars, including cutin, phenylpropanoid, suberin monomers, homogalacturonan, and cellulose biosynthesis (Fig. 4). Cellulose and homogalacturonan biosynthetic pathways were overrepresented in downregulated genes in resistant stages of both cultivars. Although the cutin biosynthetic pathway was overrepresented in both upregulated and downregulated genes in both cultivars, the overrepresentation is higher among downregulated genes in 'Chieftain' (Fig. 4). Suberin monomers and phenylpropanoid biosynthesis pathways were more overrepresented in the upregulated lists of genes for both cultivars than in the downregulated lists of genes (Fig. 4).

To determine which genes are highly associated with the ARR of winter squash fruit peel, the expression of a few upregulated genes from the three pathways (cutin, suberin monomers, and phenylpropanoid) was plotted for both cultivars for all comparisons (Fig. 5A–C). Genes encoding for acyl COA thioesterases and long-chain fatty acid CoA ligase involved in the cutin biosynthesis pathway were upregulated in both cultivars (Fig. 5A). Three upregulated genes were encoding for phenylalanine ammonia-lyase, caffeoyl-CoA *O*-methyltransferase, and cinnamate 4-hydroxylase involved in suberin monomers (Fig. 5B). Three genes encoding for cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductase (CCR), and cytochrome P450 protein (Fig. 5C) associated with monolignols biosynthesis and phenylpropanoid biosynthesis pathways were consistently upregulated during all resistant fruit peel ages in both cultivars when contrasted to their susceptible fruit peel ages. Exceptions occurred with CCR and cytochrome P450 protein, which were not upregulated during the contrast of 14 DPP to 10 DPP or of 10 DPP to 21 DPP in 'Chieftain', respectively.

Discussion

We performed transcriptome profiling of the fruit peel of two *C. moschata* cultivars at developmental stages exhibiting susceptible and resistant phenotypes against *P. capsici*. Furthermore, k-means clustering showed two interesting groups of genes that were expected to include ARR-associated genes (groups 1 and 2) (Fig. 1). We studied the gene expression profiles of resistant and susceptible fruit peel ages in both cultivars to identify the candidate genes associated with ARR. A comparison of the gene expression profile along the cultivar time points and cultivars resulted in consistently upregulated genes (Fig. 2A) with enrichment in function for cell wall structures and phenylpropanoids biosynthesis (Figs. 3 and 4). Several downregulated genes detected in all comparisons between resistant and susceptible fruit peel ages were enriched during photosynthesis and cell growth (Fig. 3). This was expected as the fruit age increased, and the fruit color of both cultivars changed from green to beige. Also, by 14 DPP or 21 DPP in 'Chieftain' and 21 DPP in 'Dickenson Field', the fruit reach full expansion, and cell division is not likely to continue. Although fruit are approaching complete development, other cellular processes, including structural or metabolic, may occur similarly in cucumber (Ando et al. 2015; Mansfeld et al. 2017). Following the complete fruit expansion or elongation stage, cell wall structural changes occur with the deposition of cell wall materials in the secondary cell wall, such as lignin or xylan (Bacete et al. 2018). Deposition of primary cell wall polymers, such as cellulose, hemicellulose, and pectin, is less likely to occur because the primary cell wall has been completed (Bacete et al. 2018).

In different plant systems, ARR can be regulated by preformed or induced resistance mechanisms, and both are regulated by changes in gene expression (González-Lamothe et al. 2009; Panter and Jones 2002). Preformed defense mechanisms include physical barriers such as cell wall strengthening (Juge 2006) or chemical barriers such as the resistance of oat (*Avena sativa*) root against attack by the take-all disease of wheat (*Triticum aestivum*) (Osbourn et al. 1994). The plant cell wall constructs the physical barrier that all pathogens must degrade to infect and colonize the plant (Bacete et al. 2018; Bellincampi et al. 2014). Changes in cell wall-related genes, by upregulation

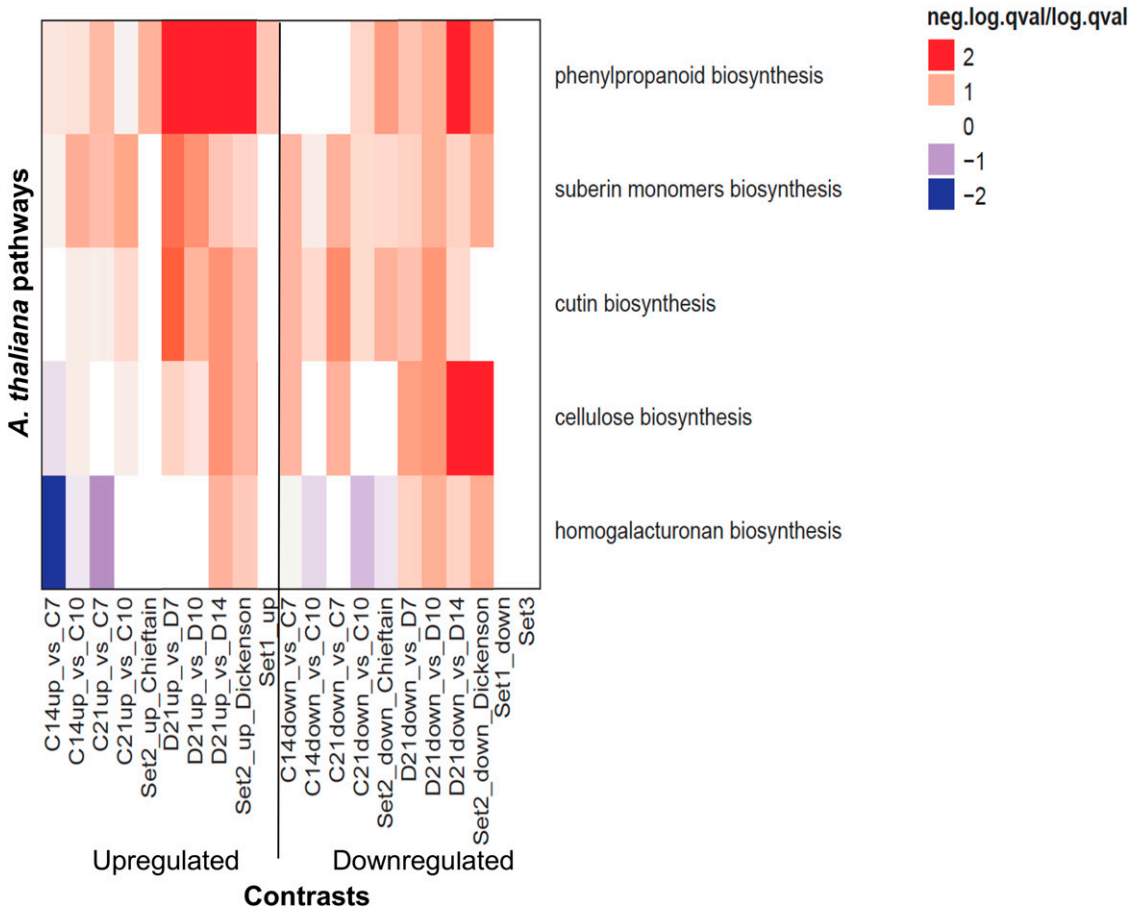

Fig. 4. Heatmap showing the pathway enrichment analysis of hard squash of *Cucurbita moschata* homologous genes in *Arabidopsis thaliana* cell wall structure biosynthetic pathways. Columns show the contrast between resistant and susceptible peel of fruit at 7, 10, 14, and 21 d postpollination (DPP) in both *C. moschata* cultivars Chieftain and Dickenson Field. The onset of age-related resistance is at 14 DPP and 21 DPP in 'Chieftain' and 'Dickenson Field', respectively. Letters "C" and "D" indicate 'Chieftain' and 'Dickenson Field', respectively. The value in the range of the heatmap is shown as the result of Fisher's exact test. If the pathway was overrepresented, then the negative log of the adjusted *P* value (or q-value) was taken; however, if it was underrepresented, then the log of the adjusted *P* value was taken. Therefore a positive value ≥ 1.3 indicates significant overrepresentation and a negative value ≤ -1.3 indicates significant underrepresentation.

or downregulation, significantly affect disease resistance (Bellincampi et al. 2014; Miedes et al. 2014).

Among the upregulated genes detected in our study in all different comparisons within and between the two studied cultivars (Fig. 2A, B, and D), we identified a candidate group of ARR genes that function in monolignol biosynthesis, sinapyl alcohol dehydrogenase activity, cinnamyl alcohol dehydrogenase activity, stilbene biosynthetic process, coumarin biosynthetic process, and peroxidase activity. These genes were upregulated consistently during resistant stages of both cultivars (Fig. 3, Supplemental Table 2), indicating that metabolic changes in the fruit cell wall are targeting the phenylpropanoids biosynthetic pathway because lignin, coumarins, and stilbenes are products of the general phenylpropanoids pathway (Boerjan et al. 2003; Deng and Lu 2017; Vogt 2010). This suggests that physical strengthening of the cell wall increases resistance to *P. capsici.*

Genes encoding for peroxidase enzymes were upregulated during all resistant stages in both cultivars (Fig. 3, Supplemental Table 2). Peroxidases are oxidoreductases involved in several physiological processes during plant development, such as lignin polymerization, fruit ripening, and defense against biotic stress (Passardi et al. 2005), cell wall lignification, and suberization (Quiroga et al. 2000). The last enzymatic step in lignin biosynthesis is catalyzed by peroxidases, which act to oxidize monolignols using H_2O_2 (Higuchi 1985). The biosynthesis and deposition of suberin and lignin polymers in the plant's secondary cell wall are developmentally regulated and strengthen the cell wall to perform the physical barrier function against pathogen attack (Miedes et al. 2014; Pandey et al. 2017).

Sinapyl alcohol dehydrogenase and CAD are enzymes in the phenylpropanoids pathway that catalyze monolignols biosynthesis from phenylalanine (Deng and Lu 2017; Miedes et al. 2014; Vogt 2010). Multiple genes functionally annotated to be involved in the lignin biosynthetic process were detected during the resistant stages of both 'Chieftain' and 'Dickenson Field', and the highest fold change in the resistant fruit peel ages occurred for lignin biosynthetic genes encoding for CCR and CAD (Fig. 5C). In addition, genes encoding for MYB transcription factor regulators of the lignin biosynthesis process were significantly upregulated during several contrasts of both cultivars. A group of MYB proteins has been demonstrated as a positive regulator of cell wall structure biosynthesis, such as lignin (Zhong et al. 2007, 2008; Zhou et al. 2009) and cutin, and consequently control cuticle and epidermis development (Oshima et al. 2013).

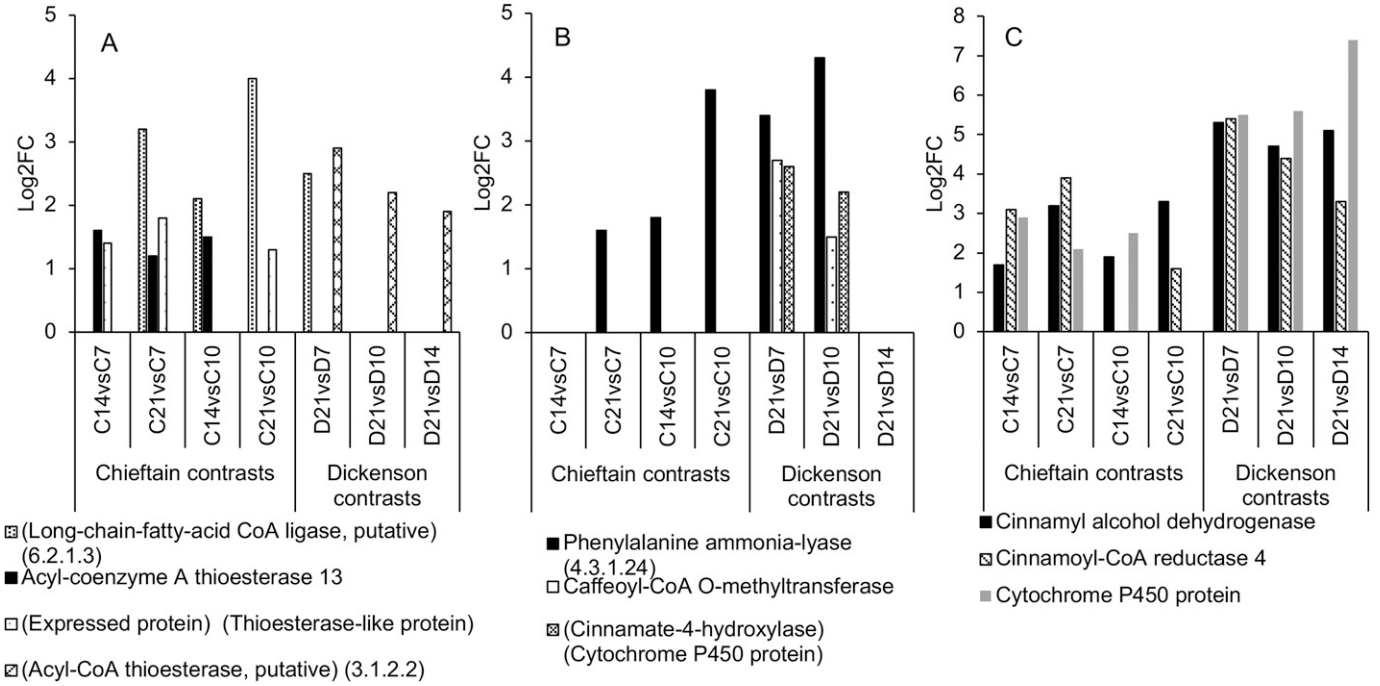

Fig. 5. Expression profile of squash homologous genes that are differentially expressed [$P < 0.05$; log2(fold change) >1] in the different contrasts of both cultivars. (**A**–**C**) Differentially expressed genes involved in cutin, suberin monomers, and phenylpropanoid biosynthetic pathways, respectively.

Our findings suggest that ARR is potentially regulated in *C. moschata* winter squash cultivars through the regulation of cell wall structure biosynthesis. We questioned which cell wall structure is a potential candidate that provides resistance to winter squash fruit against *P. capsici*. The pathway enrichment analysis detected enrichment in cutin, suberin monomers, and phenylpropanoids biosynthesis pathways during at least one resistant stage in both cultivars (Fig. 4). However, enrichment in the phenylpropanoids biosynthesis pathway was consistently detected in upregulated genes during all resistant stages in both cultivars (Fig. 4). Therefore, we examined the expression profile of individual genes involved in the three pathways.

In cutin biosynthesis, thioesterases are essential proteins for the release of de novo free fatty acids required for the biosynthesis process (Lowe 2010). The released fatty acids are attached to CoA by the action of long-chain acyl–CoA synthetases (LACS) (Schnurr et al. 2002). LACS enzymes are the plant's long-chain fatty acid, AMP-dependent synthetase, and ligase family protein (Li et al. 2016). The activation of acyl chains to acyl-CoA by the LACS is an essential step in the biosynthesis of long-chain fatty acids with variable lengths, which are required for cutin and cuticular wax biosynthesis. Cutin and cuticular wax are the components of the plant cuticle and provide a hydrophobic state to the outer plant surface, the cuticle, which acts as a protective barrier against abiotic and biotic stresses (Yeats and Rose 2013). According to our results, the upregulated genes in 'Chieftain' and 'Dickenson Field' were enriched for multiple Acyl COA thioesterases and long-chain fatty acid CoA ligases that are required for the initial steps of cutin biosynthesis (Fig. 5A). Genes encoding for thioesterase in resistant fruit were upregulated at 21 DPP in contrast to those in susceptible fruit at 7 DPP, 10 DPP, and 14 DPP in 'Dickenson Field' and 14 DPP and 21 DPP in contrast to 7 DPP and 10 DPP in 'Chieftain' (Fig. 5A). The LACS gene was only upregulated at 21 DPP in contrast to 7 DPP in 'Dickenson Field' (Fig. 5A). It was upregulated in resistant fruit at 14 DPP and 21 DPP in 'Chieftain' in contrast to 7 DPP and 10 DPP; however, that at 14 DPP was not differentially expressed compared to that at 7 DPP (Fig. 5A). LACS perform a key step in cutin and cuticular wax biosynthesis. The absence of their upregulation in 'Dickenson Field' at 21 DPP when compared with susceptible 10 DPP and 14 DPP (Fig. 5A) might indicate that accumulation of cutin in 'Dickenson' might not be the potential mechanism for ARR as the fruit age increases; however, in 'Chieftain', it might be the potential mechanism for ARR. However, consistently upregulated genes in both cultivars (Set1_up) were not enriched for cutin biosynthesis (Fig. 4).

Suberin is a heteropolymer that consists of both lipid and phenolic polymers. It deposits between the cell wall and plasma membrane, preventing water loss and protecting against pathogen infection (Nawrath and Poirier 2008). Genes involved in the biosynthesis of phenolic and aliphatic monomers required for the assembly and polymerization of suberin have been studied (Havir and Hanson 1970; Le Bouquin et al. 2001; Lee et al. 2013; Lü et al. 2009; Wang-Pruski and Cantal 2004). We detected that genes involved in suberin biosynthesis were upregulated during some contrasts in 'Dickenson Field' but not in 'Chieftain'. Only phenylalanine ammonia-lyase, which catalyzes the first step in the phenylpropanoid pathway, was upregulated during most contrasts between resistant and susceptible fruit ages in both cultivars. No detected upregulated genes were consistently upregulated (Set1_up) in both cultivars in suberin biosynthesis. Therefore, suberin deposition does not seem to be a candidate responsible for ARR. Another compound is causing this thickening, which is potentially the primary cause of ARR to *P. capsici* in hard squash.

The cytochrome P450 monooxygenase gene superfamily encodes for several essential enzymes involved in the phenylpropanoid pathway, such as lyases, transferases, ligases, oxygenases,

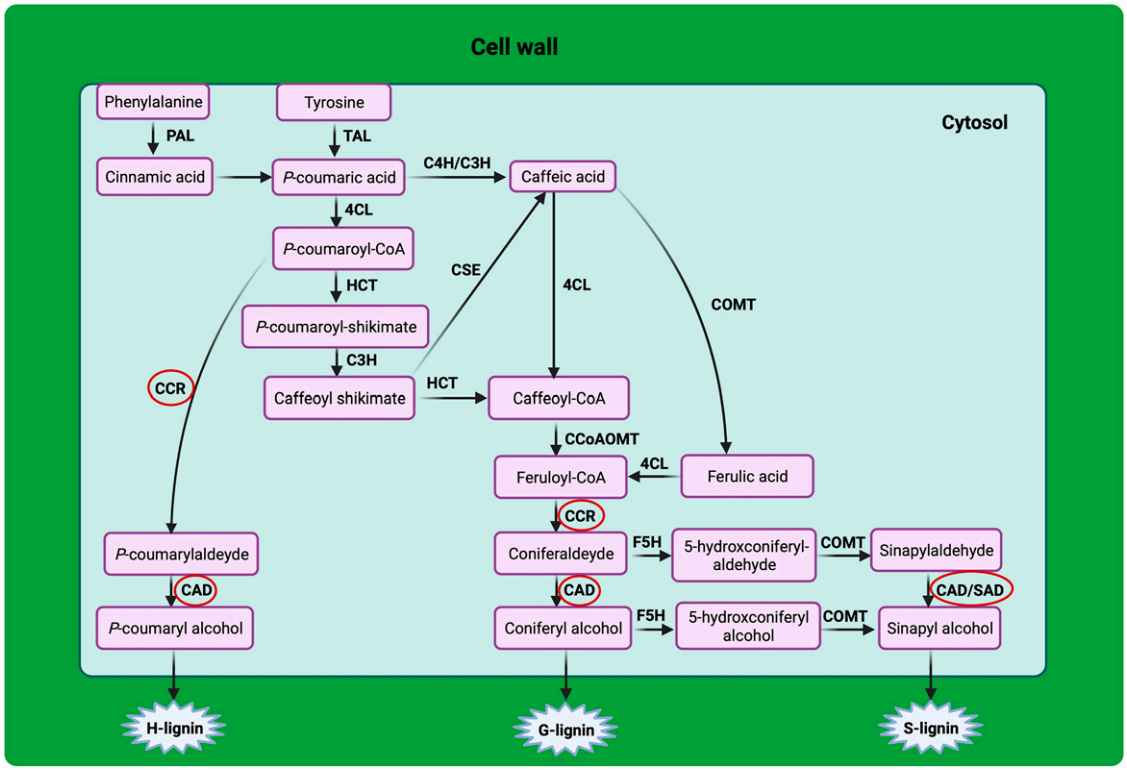

Fig. 6. Lignin biosynthesis pathway in plants: phenylalanine ammonia-lyase (PAL), tyrosine ammonia lyase (TAL), cinnamate 4-hydroxylase (C4H), hydroxycinnamate CoA ligase (C4L), 4-hydroxycinnamoyl CoA shikimate hydroxycinnamoyl transferase (HCT), p-coumaroyl shikimate 3′-hydroxylase (C3H), caffeoyl-CoA 3-O-methyltransferase (CCoAOMT), cinnamoyl CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid O methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), and caffeoyl shikimate esterase (CSE). Enzymes circled in red were consistently upregulated in both cultivars when comparing susceptible and resistant fruit ages in both *Cucurbita moschata* cultivars Chieftain and Dickenson Field. This figure was created with BioRender (Science Suite Inc., Toronto, ON, Canada).

and reductases (Deng and Lu 2017; Ferrer et al. 2008; Gou et al. 2018; Ververidis et al. 2007). Phenylpropanoids are secondary metabolites that include flavonoids, lignin, coumarins, and other phenolic compounds (Mander and Liu 2010). In the phenylpropanoids biosynthesis pathway, phenylalanine is converted through multiple steps to *p*-coumaroyl CoA, which is the precursor for several secondary metabolites, including monolignols, stilbenes, coumarins, flavonoids, and other phenolic compounds (Liu et al. 2015; Mander and Liu 2010; Vogt 2010). The lignin biosynthesis and specific monolignols biosynthesis pathways adapted from Liu et al. (2018) are presented in Fig. 6. The polymerization of the produced monolignols into lignin is catalyzed by peroxidases (Rinaldi et al. 2016; Zhao et al. 2013). Lignin is an insoluble hydrophobic polymer that forms a structural component of the secondary cell wall in plants (Miedes et al. 2014). Lignin provides structural support to the plant cell wall and has multiple functions, including its role as a structural/physical barrier that defends against wounding and pathogen attacks (Bonello et al. 2003; Buendgen et al. 1990; Labeeuw et al. 2015). Cell wall lignification has been known as a disease resistance mechanism in plants (Nicholson and Hammerschmidt 1992; Sattler and Funnell-Harris 2013; Vance et al. 1980). The mechanical strength provided to the plant cell wall by lignin hinders pathogen penetration using appressoria (Bellincampi et al. 2014). Furthermore, the hydrophobic nature of lignin protects against cell wall degradation by the action of the cell wall degrading enzymes produced by plant pathogens (Vance et al. 1980). Cytochrome P450 protein, a key gene superfamily involved in suberin, cutin, and lignin biosynthesis, was consistently upregulated in all the resistant fruit peel ages contrasted with susceptible fruit peel ages in both cultivars except for one contrast of Chieftain (10 DPP vs. 21 DPP). The CAD and CCR genes involved in the phenylpropanoid biosynthesis pathway were consistently upregulated in all the resistant fruit peel ages in both cultivars when contrasted to their susceptible fruit peel ages; however, the expression of CCR did not change during the contrast of 14 DPP to 10 DPP in 'Chieftain' (Fig. 5C). CAD and CCR are the two primary enzymes during the production of the monolignol *p*-coumaryl alcohol and other monolignols incorporated in building the H lignin (Rinaldi et al. 2016). Several studies documented an increase in *p*-coumaryl alcohol or H-enriched lignin in squash, cucumber, and other cucurbit tissues as a response to injury, disease, or elicitors (Hammerschmidt et al. 1985; Robertsen and Svalheim 1990; Stange et al. 1999, 2001; Varbanova et al. 2011).

The results suggest that CAD and CCR genes are potential candidate genes associated with ARR through the production of monolignols and lignin polymerization at the cell wall by the catalysis of peroxidases. This additional lignin deposited in the peel of the resistant aging squash fruit may strengthen the cuticle and epidermal walls to resist pathogens and environmental stresses.

Conclusions

Our results suggest that secondary cell wall structures, cutin, suberin, or lignin, are potential candidates associated with ARR

to *P. capsici* in winter squash. However, the consistency of upregulated expression of genes involved in monolignol biosynthesis in resistant fruit peel in both cultivars suggests a high probability of monolignol biosynthesis and lignin polymerization in cell wall tissues as fruit ages, providing thickening and physical strengthening of the fruit cell wall against pathogen attack. To confirm that lignin is the potential material deposited in the maturing fruit peel cell wall as a constitutive structural defense mechanism against pathogens, further analyses of fruit cell wall tissues must be performed. This study suggests that both *C. moschata* cultivars have a similar mechanism that controls ARR to *P. capsici*. The observed difference in the onset of ARR between cultivars is likely attributable to the difference in their days to maturity because 'Chieftain' matures at 80 d and 'Dickenson Field' matures at 100 d. Although we acknowledge that the differences in days to maturity between the two cultivars may confound the interpretation of our results, we believe that our dataset still contributes valuable insights into the understanding of ARR in winter squash. The observed changes in gene expression are likely influenced by both ARR and general fruit maturation processes.

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