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Transcriptome analysis provides new insights into the resistance of pepper to *Phytophthora capsici* infection

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Abstract

Background Phytophthora blight is a highly destructive soil-borne disease caused by *Phytophthora capsici* Leonian, which threatens pepper production. The molecular mechanism of pepper resistance to phytophthora blight is unclear, and the excavation and functional analysis of resistant genes are the bases and prerequisites for phytophthora blight-resistant breeding. We aimed to analyze the expression patterns of key genes in the plant-pathogen interaction metabolic pathway and propose a working model of the pepper defense signal network against *Phytophthora capsici* infection.

Results The 'ZCM334' pepper material used in this study is a high-generation inbred line that is immune to *Phytophthora capsici* and shows no signs of infection after inoculation. Comparative transcriptome analysis of the roots of 'ZCM334' and the susceptible material 'Early Calwonder' revealed significant differences in their gene expression profiles at different stages after inoculation. Most differentially expressed genes were significantly enriched in the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, plant-pathogen interaction, and fatty acid degradation metabolic pathways. Some defense genes and transcription factors significant in pepper resistance to phytophthora blight were identified, including *PR1*, *RPP13*, *FLS2*, *CDPK*, *CML*, *MAPK*, *RLP*, *RLK*, *WRYK*, *ERF*, *MYB*, and *bHLH*, most of which were regulated after inoculation. A working model was constructed for the defense signal network of pepper against *Phytophthora capsici*.

Conclusions These data provide a valuable source of information for improving our understanding of the potential molecular mechanisms by which pepper plants resist infection by *Phytophthora capsici*. The identification of key genes and metabolic pathways provides avenues for further exploring the immune mechanism of 'ZCM334' resistance to phytophthora blight.

Keywords Pepper, *Phytophthora capsici*, RNA-seq, Differentially expressed genes, Regulatory network

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Background

Pepper (*Capsicum annuum* L.) is an annual or perennial plant of the Solanaceae family, belonging to the genus *Capsicum*. It has a unique flavor and is a valuable vegetable crop with high economic and nutritional value worldwide [1, 2]. Phytophthora blight is a devastating soil-borne disease caused by *Phytophthora capsici* Leonian (*P. capsici*). Once this occurs, it can cause reduced production and severe crop failure, resulting in severe economic losses. Since its first discovery in the United States, it has occurred in pepper cultivation areas in multiple countries and regions worldwide, becoming a major disease threatening global pepper production [3, 4].

P. capsici can infect various tissues of pepper plants, including the roots, stems, leaves, flowers, and fruits. The characteristics of phytophthora blight onset vary depending on the host and environment. Phytophthora blight can affect all plant growth stages, and a high-temperature and -humidity environment is more conducive to the spread of *P. capsici* [5]. *P. capsici* primarily spreads through soil and splashing water in the form of micro-zoospores. Owing to accumulated water in fields, it rapidly erts within 1–2 d after summer rainfall. In the presence of sufficient water, *P. capsici* can cause phytophthora blight, affecting the aboveground and underground parts of susceptible hosts [6]. The infection cycle of *P. capsici* begins with the formation of infiltrating hyphae from enclosed zoospores or sporangia. Once entering the susceptible host tissue, the hyphae of *P. capsici* begin to grow and settle on the apoplast while extending protrusions to break through the cell wall barrier and form an intimate interface with the plant cell membrane, conducting extensive signal exchange and maintaining the stage of biotrophic infection [7]. Despite pathogen invasion, the symptoms in plant cells during this early stage are not evident. After the biotrophic stage, *P. capsici* has a unique necrotrophic stage marked by death and tissue collapse, producing sporangia, which initiate a new infection cycle on diffusion [8]. In nature, *P. capsici* is a semi-biotrophic fungus that establishes infection through haustoria structures and intercellular hyphal growth and triggers host cell death within 48 h of successful colonization [9].

When *P. capsici* infects plants, the pathogen secretes and dispatches RxLR effectors to host cells, causing paralysis of the plant host immune system, including endoplasmic reticulum stress-mediated plant immunity [10], pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) [11], and the EDS-PAD4 immune signaling pathway [12]. Additionally, pathogenic effectors can interfere with ethylene biosynthesis [13] and histone acetylation [14]. During the evolution of plant innate immunity, multiple pathogens constantly invade, and plants adopt two defense systems to respond to pathogen attacks, namely the basic defense of recognizing conserved PAMPs through host receptors, known as PTI, and the perception of specific pathogen effectors dependent on plant resistance (R) proteins, known as effector-triggered immunity (ETI) [15]. In the pathological system of resistant host–pathogenic microbe, after identifying pathogen PAMPs, resistant hosts induce PTI, such as mitogen-activated protein kinase (MAPK) activation, Ca^{2+} influx, and reactive oxygen species (ROS) production. Subsequently, plant hormone signaling pathways, such as salicylic acid (SA) or jasmonic acid (JA)/ethylene (ET) signaling pathways, are activated, promoting the biosynthesis of defense-related factors in plants and activating plant disease resistance [16–18]. However, some pathogens transmit secreted proteins (effectors) to inhibit basic defense. Therefore, plants have developed a second immune system called ETI, which stimulates defense responses such as programmed cell death and systemic acquired resistance. SA or JA/ET can induce these defense responses, making plants more resistant to certain biotrophic or semi-biotrophic pathogens [19, 20].

Transcriptome sequencing is crucial for studying gene function and structure, revealing specific biological processes and molecular mechanisms involved in disease occurrence [21]. Transcriptome technology has been applied in some studies of plant disease resistance [22–24]. For example, Fan et al. [23] identified significant differences in the expression profiles between resistant and susceptible varieties of peppers after inoculation with *P. capsici* through RNA-seq, revealing that plant-pathogen interactions and plant hormone signaling pathways have a certain regulatory effect on *P. capsici* infection [23]. Gao et al. conducted RNA-seq on pepper varieties resistant and susceptible to *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) infection at different stages, which revealed potential defense-related genes, such as *PRRs*, *MAPKs*, and calcium signaling regulatory genes that may regulate pepper resistance to *Xcv* [25]. By conducting RNA-seq on peppers inoculated with *Pepper Golden Mosaic Virus* (*PepGMV*), key elements and metabolic pathways related to plant defense mechanisms, such as PR protein, ROS, JA, and ET signaling pathways, were identified [26]. A co-expression analysis indicated that plant hormone signaling transduction, phenylpropanoid biosynthesis, plant-pathogen interaction, and MAPK signaling pathway are crucial in pepper's resistance to powdery mildew infection [24].

'ZCM334' is a high-generation inbred line pepper material immune to physiological race 3 of *P. capsici*. In this study, comparative transcriptome analysis was conducted on the roots of 'ZCM334' and susceptible material 'Early Calwonder' at different stages (0, 12, 24, and

48 h) after inoculation with *P. capsici*. By analyzing the differentilly expressed genes (DEGs) shared by 'ZCM334' and 'Early Calwonder,' as well as the DEGs specifically expressed in 'ZCM334' or 'Early Calwonder,' we identified defense genes, transcription factors, and key metabolic pathways that play important roles in the plant immune system. This provides new insights into the genetic and molecular basis of pepper resistance to phytophthora blight.

Results

Phenotypic characteristics of phytophthora blight immune material 'ZCM334' and susceptible material 'Early Calwonder'

We observed the phenotypes of the higher-generation inbred pepper line 'ZCM334,' which was immune to the physiological race 3 of *P. capsici*, and the susceptible strain 'Early Calwonder.' After 10 d of inoculation with *P. capsici*, the plants of 'Early Calwonder' wilted, with leaves falling off and roots and stems turning black. However, after inoculation with *P. capsici*, all 'ZCM334' plants showed no infection signs, and their growth was not affected by the pathogen infection (Fig. 1). As the infection time of *P. capsici* progressed, the roots of 'Early Calwonder' gradually turned black and moved wards along the stem, while those of 'ZCM334' remained unchanged and fully resisted *P. capsici* infection (Fig. 2). The paraffin section results showed that after infection with *P. capsici*, the lignification degree in the roots of 'Early Calwonder' was higher, with the primary xylem gradually degrading and the secondary phloem and cortical cells gradually shrinking. However, the cells in each layer of the 'ZCM334' roots remained intact without significant changes (Fig. 3).

Quality testing of transcriptome sequencing data

Transcriptome sequencing was performed on the roots of 'Early Calwonder' (E-0 h, E-12 h, E-24 h, and E-48 h) and 'ZCM334' (Z-0 h, Z-12 h, Z-24 h, and Z-48 h) inoculated with *P. capsici* at 0, 12, 24, and 48 h after inoculation. Three biological replicates were performed on each sample gro. Twenty-four root samples were subjected to transcriptome sequencing, resulting in 197.93 Gb of clean data. The clean data of each sample reached 6 Gb, with the Q30 base percentage exceeding 93% and GC content ranging from 42.4% to 42.99% (Table S1).

After comparing the transcriptome data with the pepper reference genome, the alignment rate of the 24 samples ranged from 81.10% to 84.79%, and that of unique clean reads mapped to the reference genome reached 77.25–81.94% (Table S2). Correlation analysis and principal component analysis (PCA) can reduce the complexity of data and aid in deeply exploring the relationships and variation sizes between samples. Sample correlation analysis and PCA revealed a strong correlation between dlicate samples, with high data

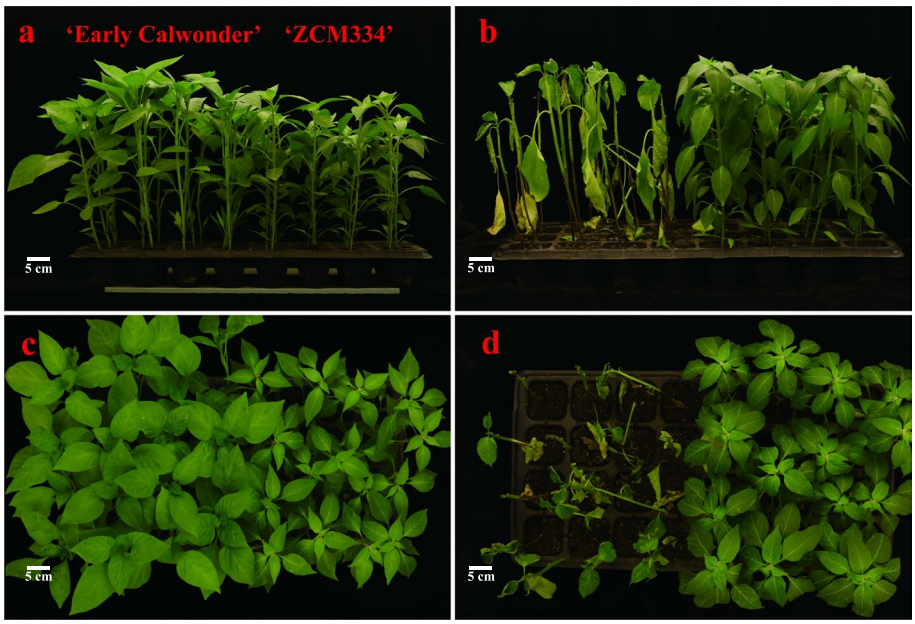

Fig. 1 Phenotypic observation of 'Early Calwonder' and 'ZCM334' before and after inoculation with *Phytophthora capsici*. (**a**) Front view of 'Early Calwonder' (left) and 'ZCM334' (right) before inoculation with *P. capsici*; (**b**) Front view of 'Early Calwonder' (left) and 'ZCM334' (right) inoculated with *P. capsici* for 10 d; (**c**) Top view of 'Early Calwonder' (left) and 'ZCM334' (right) before inoculation with *P. capsici*; (**d**) Top view of 'Early Calwonder' (left) and 'ZCM334' (right) inoculated with *P. capsici* for 10 d

Fig. 2 Root phenotypes of 'Early Calwonder' (left) and 'ZCM334' (right) inoculated with *P. capsici*. Inoculation at 0 h (**a**); 12 h (**b**); 24 h (**c**) and 48 h (**d**)

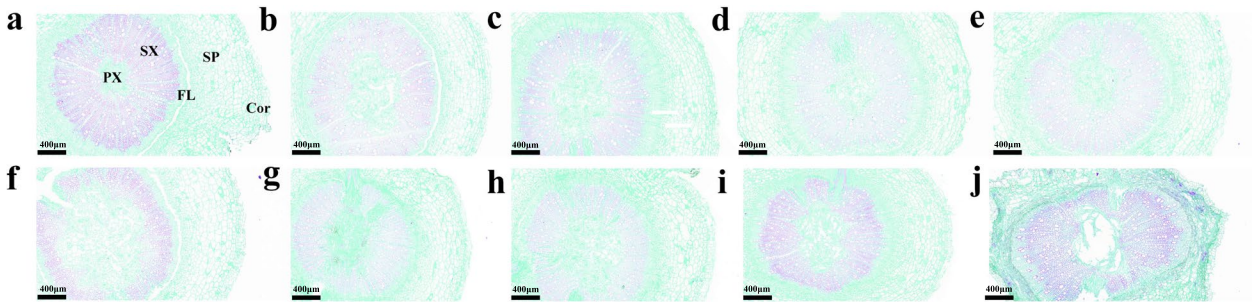

Fig. 3 Cytological observation of 'ZCM334' and 'Early Calwonder' roots at different stages of inoculation with *P. capsici*. (**a–e**) Root paraffin sections of 'ZCM334' inoculated with *P. capsici* at 0 h, 12 h, 24 h, 48 h, and 96 h; (**f–j**) Root paraffin sections of 'Early Calwonder' inoculated with *P. capsici* at 0 h, 12 h, 24 h, 48 h, and 96 h. PX: Primary xylem; FL: Forming layer; SX: Secondary xylem; SP: Secondary phloem; Cor: Cortex

similarity and reliable sequencing results, which can be used for subsequent analysis of DEGs (Fig. S1a, Fig. S1b). In addition, PCA explained the total variation of the sample and the differences in gene expression patterns of 'ZCM334' and 'Early Calwonder' at different stages after inoculation. For PC1, there is a significant separation between the 'ZCM334' and 'Early Calwonder'. On PC2, in 'ZCM334', the time points of 12, 24, and 48 h after inoculation are clustered together, while in 'Early Calwonder', the time points of 24 and 48 h after inoculation are clustered together. This indicates that there is a significant difference in the response of 'ZCM334' and 'Early Calwonder' to *P. capsici* infection (Fig. S1b).

Identification of DEGs in 'Early Calwonder' and 'ZCM334' at different stages after infection with *P. capsici*

To eliminate the interference of genotype and genetic background and further explore genes related to disease resistance and susceptibility, E-0 h and Z-0 h were used as controls to identify DEGs at different stages after inoculation in 'Early Calwonder' and 'ZCM334.' Using | log2 fold change |≥1 and significance false discovery rate (FDR) < 0.05 as the criteria for screening DEGs. A total of 2,284 DEGs were identified between E-0 h and E-12 h, including 1,117 and 1,167 regulated and regulated genes, respectively. Between E-0 h and E-24 h, 4,182 DEGs were identified, including 2,211 and 1,971 regulated and regulated genes, respectively. A total of 5,573 DEGs were

identified between E-0 h and E-48 h, including 3,010 and 2,563 regulated and regulated genes, respectively. A total of 3,952 DEGs were identified between Z-0 h and Z-12 h, of which 1,679 and 2,273 were regulated and regulated, respectively. Similarly, 6,284 DEGs were identified between Z-0 h and Z-24 h, 2,914 of which were regulated, and 3,370 were regulated. Furthermore, 4,028 DEGs were identified between Z-0 h and Z-48 h, with 2,001 regulated and 2,027 regulated (Fig. S2). After inoculation with *P. capsici*, more DEGs were identified in 'ZCM334' than in 'Early Calwonder.' In 'ZCM334' and 'Early Calwonder,' most DEGs were identified 24 and 48 h after inoculation, respectively, indicating differences in the gene expression patterns of resistant and susceptible materials in response to *P. capsici* infection.

Functional enrichment analysis of DEGs

Gene ontology (GO) functional enrichment analysis showed that the GO terms enriched in E-0 h_vs._E-12 h, E-0 h_vs._E-24 h, E-0 h_vs._E-48 h, Z-0 h_vs._Z-12 h, Z-0 h_vs._Z-24 h, and Z-0 h_vs._Z-48 h were similar, with most DEGs enriched in the categories of biological process and molecular function, while the proportion of genes belonging to the category of cellular component was relatively small. In biological processes, these DEGs were mostly enriched in cellular processes, metabolic processes, response to stimulus, biological regulation, and regulation of biological processes. In the molecular function category, DEGs were mostly enriched in binding, catalytic activity, transporter activity, and transcription regulator activity entries. In the cellular composition category, DEGs were only enriched in two entries: protein-containing complex and cellular anatomical entity (Fig. 4a–f).

The Kyoto Encyclopedia of Genes and Genome (KEGG) enrichment analysis showed that most of the DEGs in E-0 h_vs._E-12 h, E-0 h_vs._E-24 h, E-0 h_vs._E-48 h, Z-0 h_vs._Z-12 h, Z-0 h_vs._Z-24 h, and Z-0 h_vs._Z-48 h were significantly enriched in the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, plant hormone signal transduction, starch and sucrose metabolism, alpha-linolenic acid metabolism, metabolic pathways, plant-pathogen interaction, biosynthesis of various alkaloids, flavonoid biosynthesis, and sesquiterpenoid and triterpenoid biosynthesis pathways (Fig. 4g), indicating that the resistance of pepper plants to *P. capsici* is closely related to the synthesis of secondary metabolites.

There were 1,011 DEGs shared by E-0 h_vs._E-12 h, E-0 h_vs._E-24 h, E-0 h_vs._E-48 h (Fig. 5a). These DEGs were mostly significantly enriched in enzyme inhibitor activity (24 genes), response to oxidative stress (27 genes), organic hydroxide compound metabolic process (26 genes), antagonistic activity (19 genes), and ROS metabolic process (18 genes) GO terms (Fig. 5b). In the KEGG enrichment results, these DEGs were primarily significantly enriched in the biosynthesis of secondary metabolites (115 genes), metabolic pathways (165 genes), phenylpropanoid biosynthesis (22 genes), alpha-linolenic acid metabolism (11 genes), pentose and glucuronate

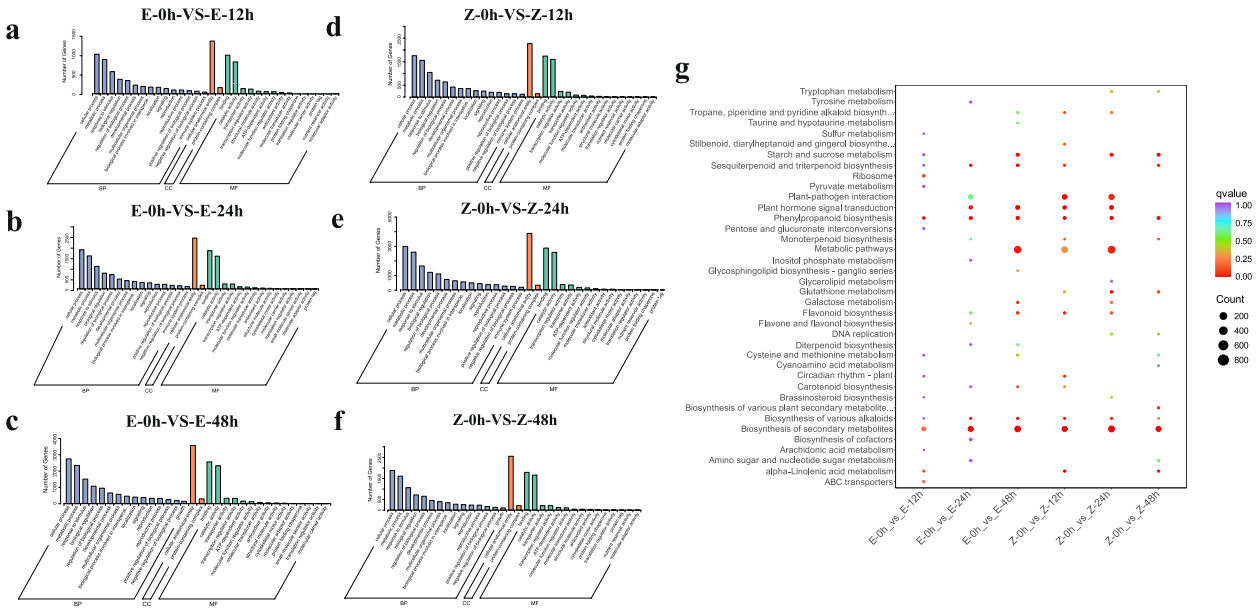

Fig. 4 Gene ontology (GO) classifications (**a–f**) and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway assignments (**g**) for DEGs identified in 'Early Calwonder' and 'ZCM334'

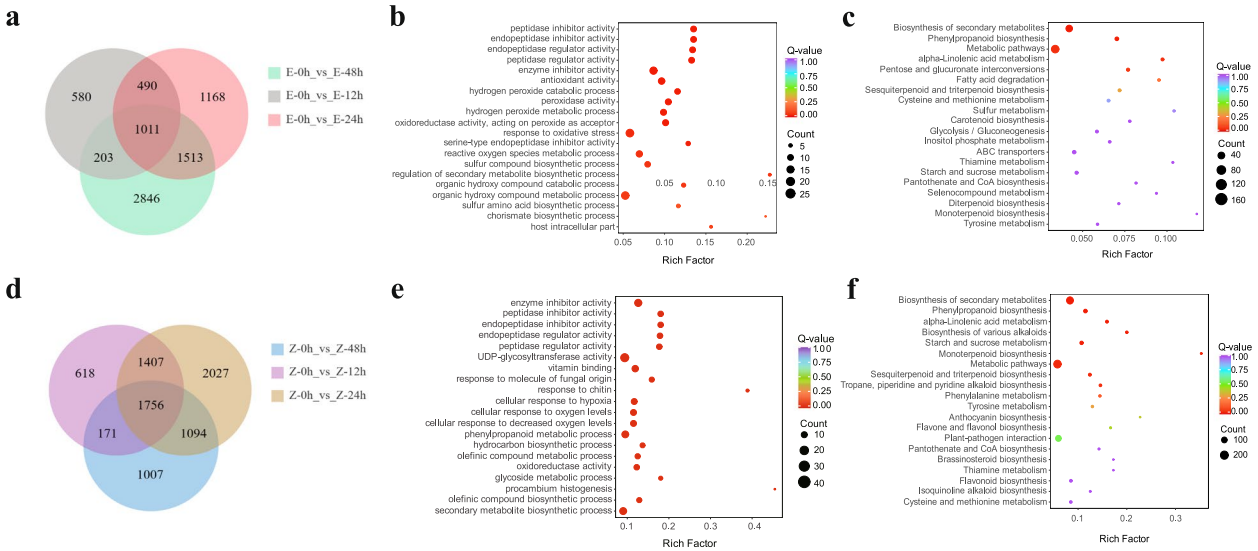

Fig. 5 Functional enrichment analysis of DEGs at different stages after inoculation with *P. capsici* in 'Early Calwonder' and 'ZCM334'. (**a**) Venn diagram of DEGs in 'Early Calwonder' at different stages after inoculation with *P. capsici;* (**b**) GO classifications of DEGs shared by E-0 h_vs._E-12 h, E-0 h_vs._E-24 h, and E-0 h_vs._E-48 h; (**c**) KEGG pathway assignments of DEGs shared by E-0 h_vs._E-12 h, E-0 h_vs._E-24 h, and E-0 h_vs._E-48 h; (**d**) Venn diagram of DEGs in 'ZCM334' at different stages after inoculation with *P. capsici;* (**e**) GO classifications of DEGs shared by Z-0 h_vs._Z-12 h, Z-0 h_vs._Z-24 h, and Z-0 h_vs._Z-48 h; (**f**) KEGG pathway assignments of DEGs shared by Z-0 h_vs._Z-12 h, Z-0 h_vs._Z-24 h, and Z-0 h_vs._Z-48 h

interconversions (14 genes), and fatty acid degradation (8 genes) metabolic pathways (Fig. 5c).

There were 1,756 DEGs shared by Z-0 h_vs._Z-12 h, Z-0 h_vs._Z-24 h, and Z-0 h_vs._Z-48 h (Fig. 5d). These DEGs were mostly significantly enriched in enzyme inhibitor activity (35 genes), UDP-glycosyltransferase activity (46 genes), phenylpropanoid metabolic process (32 genes), secondary metabolite biosynthetic process (33 genes), and vitamin binding (28 genes) GO terms (Fig. 5e). In the KEGG enrichment results, these DEGs were primarily significantly enriched in the biosynthesis of secondary metabolites (228 genes), metabolic pathways (282 genes), plant-pathogen interaction (138 genes), phenylpropanoid biosynthesis (36 genes), and starch and sucrose metabolism (30 genes) metabolic pathways (Fig. 5f). These results indicate that the differences in the DEG-enriched metabolic pathways in 'ZCM334' and 'Early Calwonder' may be the reason for their differences in phytophthora blight resistance.

Functional enrichment analysis of DEGs shared by 'Early Calwonder' and 'ZCM334' at different stages after infection with *P. capsici*

There were 435 DEGs shared by 'Early Calwonder' and 'ZCM334' inoculated with *P. capsici* at 0, 12, 24, and 48 h, which may be crucial regulatory genes in plant response to pathogen infection (Fig. 6a). Most of these DEGs were significantly enriched in the enzyme inhibitor activity, antioxidant activity, response to oxidative stress, peroxidase activity, oxidoreductase activity, and acting on peroxide as acceptor GO terms (Fig. 6b). The KEGG enrichment results indicated that these DEGs were significantly enriched in five metabolic pathways ($P < 0.001$), including biosynthesis of secondary metabolites (including 40 and 26 regulated and regulated DEGs, respectively, after infection with *P. capsici*), phenylpropanoid biosynthesis (including 11 and 4 regulated and regulated DEGs after infection with *P. capsici*), starch and sucrose metabolism (including six each of regulated and regulated DEGs after infection with *P. capsici*), sesquiterpenoid and triterpenoid biosynthesis (including 8 regulated DEGs after infection with *P. capsici*), metabolic pathways (including 55 and 29 regulated and regulated DEGs, respectively, after infection with *P. capsici*) (Fig. 6c).

Among these significantly enriched metabolic pathways, the defense gene information shared by 'Early Calwonder' and 'ZCM334' after infection with *P. capsici* is presented in Table 1. We identified and screened 23 genes related to disease resistance. Most of these DEGs were regulated after inoculation with *P. capsici* in 'Early Calwonder' and 'ZCM334' (Fig. 6d). These genes have an immune response after recognizing the infection of *P. capsici*, playing a crucial defensive role in plant disease resistance. Among these 23 genes related to disease resistance, there were four peroxidases, where *CA06g25780*, *CA08g15610*, and *CA08g15620* were regulated after inoculation with *P. capsici*, and *CA02g18250* was regulated; five cytochrome P450

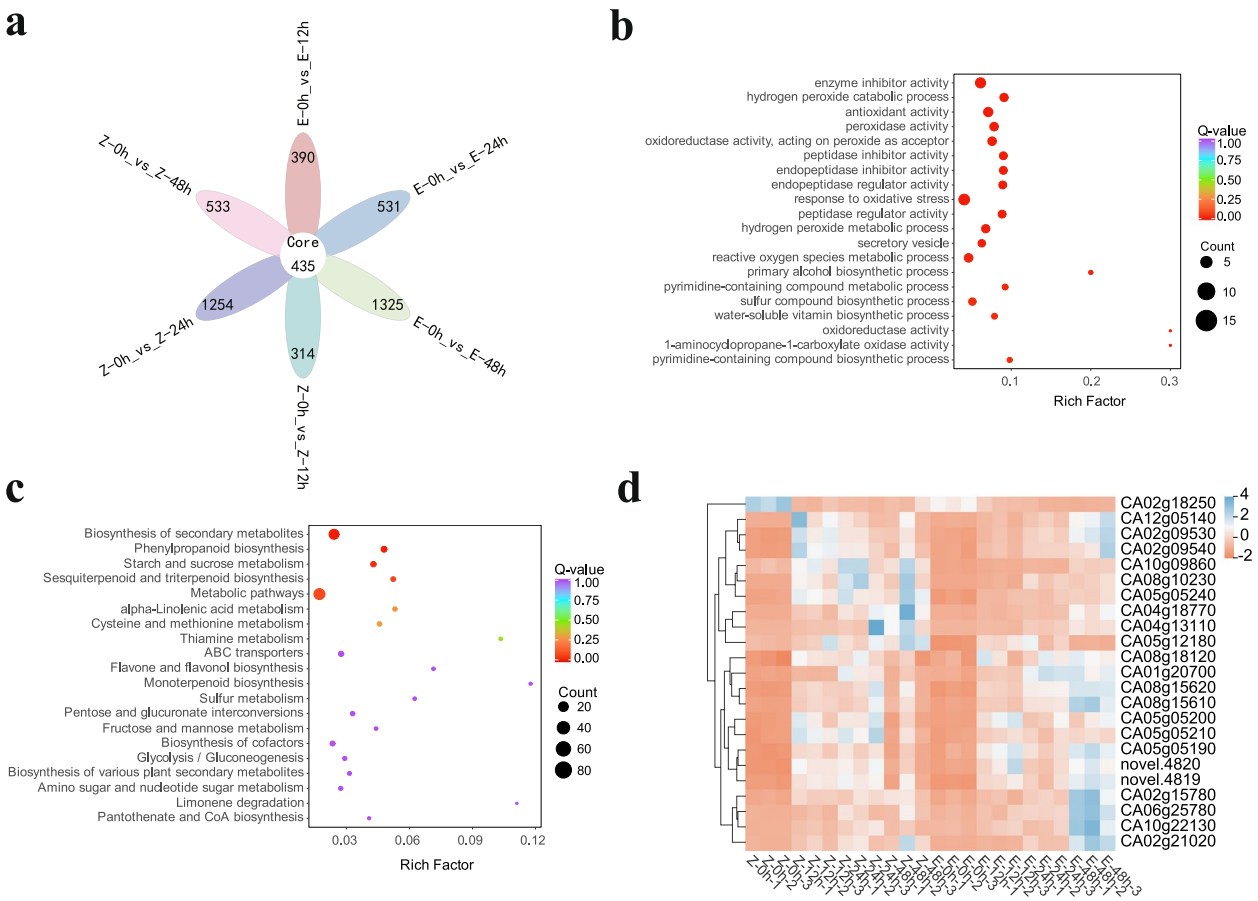

Fig. 6 Functional enrichment analysis of DEGs shared by 'Early Calwonder' and 'ZCM334' at different stages after infection with *P. capsici*. (**a**) Venn diagram of DEGs shared by 'Early Calwonder' and 'ZCM334' at different stages after infection with *P. capsici;* (**b**) GO classifications of DEGs shared by 'Early Calwonder' and 'ZCM334' at different stages after infection with *P. capsici*; (**c**) KEGG pathway assignments of DEGs shared by 'Early Calwonder' and 'ZCM334' at different stages after infection with *P. capsici*; (**d**) Heatmap of expression levels of defense genes shared by 'Early Calwonder' and 'ZCM334' after infection with *P. capsici*

(*CA10g22130*, *CA02g09530*, *CA02g09540*, *CA12g05140*, and *CA08g10230*); six lignin-forming animic peroxidases (*CA05g05190*, *CA05g05200*, *CA05g05210*, *CA05g05240*, *novel.4819*, and *novel.4820*), which were regulated after infection with *P. capsici*; two regulated endochitinases (*CA10g09860* and *CA02g21020*); two regulated polygalacturonases (*CA01g20700* and *CA05g12180*). In addition, we identified polyphenol oxidase *CA02g15780*, alcohol dehydrogenase *CA04g13110*, allene oxide synthase *CA08g18120*, and alpha-amylase *CA04g18770*, which were regulated after infection with *P. capsici* and may be crucial in pathogen resistance.

Functional enrichment analysis of specific DEGs in 'Early Calwonder' and 'ZCM334'

In this study, we identified genes specifically expressed in 'Early Calwonder' and 'ZCM334'. There were 576 genes specifically expressed in 'Early Calwonder,' which were differentially expressed in E-0 h_vs._E-12 h, E-0 h_vs._E-24 h, and E-0 h_vs._E-48 h. There were 1,321 genes specifically expressed in 'ZCM334' and differentially expressed in Z-0 h_vs._Z-12 h, Z-0 h_vs._Z-24 h, and Z-0 h_vs._Z-48 h. Exploring these DEGs provides valuable information for elucidating the mechanism of resistance and susceptibility materials in the pepper disease resistance pathway.

The DEGs specifically expressed in 'Early Calwonder' were mostly significantly enriched in the organic hydroxy compound catabolic process, chorismate biosynthetic process, chorismate metabolic process, host intracellular part, and host intracellular region GO terms (Fig. 7a). Plants may adapt to different environmental conditions by regulating the fatty acid degradation pathways. Pathogen infection may induce the expression of genes related to fatty acid degradation in plants, enhancing their disease resistance [27]. Six DEGs specifically expressed in 'Early Calwonder' were significantly enriched in the fatty acid degradation metabolic pathway ($P < 0.001$) (Fig. 7b).

Table 1 Defense gene information shared by 'Early Calwonder' and 'ZCM334' after infection with *P. capsici*

Gene ID	Functional annotation	log_2 fold-change						After infection with *P. capsici*		KEGG pathway
		Z-0h_vs_Z-12 h	Z-0h_vs_Z-24 h	Z-0h_vs_Z-48 h	E-0h_vs_E-12 h	E-0h_vs_E-24 h	E-0h_vs_E-48 h	In 'ZCM334'	In 'Early Calwonder'	
CA02g18250	Peroxidase	−2.46	−2.51	−1.92	−1.23	−2.02	−2.21	down	down	ko01100, ko01110
CA06g25780	Peroxidase	2.79	4.39	4.32	2.01	3.00	4.96	up	up	ko01100, ko01110
CA08g15610	Peroxidase	2.61	2.96	2.16	2.08	2.82	3.86	up	up	ko01100, ko01110
CA08g15620	Peroxidase	2.34	2.83	2.06	2.21	2.85	3.42	up	up	ko01100, ko01110
CA10g22130	Cytochrome P450	3.22	4.68	3.20	1.85	1.74	4.37	up	up	ko00940
CA02g09530	Cytochrome P450	3.23	2.71	2.28	1.21	1.91	3.03	up	up	ko00909
CA02g09540	Cytochrome P450	3.30	2.79	2.27	1.43	2.05	2.88	up	up	ko00909
CA12g05140	Cytochrome P450	4.79	3.51	3.37	2.13	3.23	5.02	up	up	ko00909
CA08g10230	Cytochrome P450	4.63	5.42	4.87	2.43	4.50	5.23	up	up	ko01100
CA05g05190	Lignin-forming anionic peroxidase	2.40	2.61	1.59	1.90	1.51	2.42	up	up	ko00940, ko01100, ko01110
CA05g05200	Lignin-forming anionic peroxidase	3.08	3.33	1.73	2.81	2.36	2.78	up	up	ko00940, ko01100, ko01110
CA05g05210	Lignin-forming anionic peroxidase	2.24	2.48	1.25	1.35	1.36	1.69	up	up	ko00940, ko01100, ko01110
CA05g05240	Lignin-forming anionic peroxidase	2.54	2.64	2.70	1.94	2.35	2.90	up	up	ko00940, ko01100, ko01110
novel.4819	Lignin-forming anionic peroxidase	2.46	2.72	1.74	2.51	2.31	3.37	up	up	ko00940, ko01100, ko01110
novel.4820	Lignin-forming anionic peroxidase	2.65	2.74	1.66	1.89	1.38	2.28	up	up	ko00940, ko01100, ko01110
CA10g09860	Endochitinase	1.80	2.17	1.85	1.57	2.16	3.62	up	up	ko01100
CA02g21020	Endochitinase	2.42	3.18	3.63	1.29	3.26	5.54	up	up	ko01100
CA02g15780	Polyphenol oxidase	2.33	2.56	2.51	1.15	1.93	3.64	up	up	ko01100, ko01110
CA04g13110	Alcohol dehydrogenase	4.10	6.59	6.46	2.34	4.47	5.57	up	up	ko00071, ko01100, ko01110
CA08g18120	Allene oxide synthase	2.60	2.62	1.68	1.44	1.71	2.04	up	up	ko01100, ko01110
CA04g18770	Alpha-amylase isoform X1	3.47	4.71	5.38	1.92	2.70	4.47	up	up	ko00500, ko01100, ko01110
CA01g20700	Polygalacturonase	2.24	4.03	2.35	1.81	3.31	3.33	up	up	ko01100
CA05g12180	Polygalacturonase	1.05	1.14	1.47	2.15	2.21	1.50	up	up	ko01100

[1] ko01110: Biosynthesis of secondary metabolites, ko01100: Metabolic pathways; ko00940: Phenylpropanoid biosynthesis; ko00071: Fatty acid degradation; ko00500: Starch and sucrose metabolism

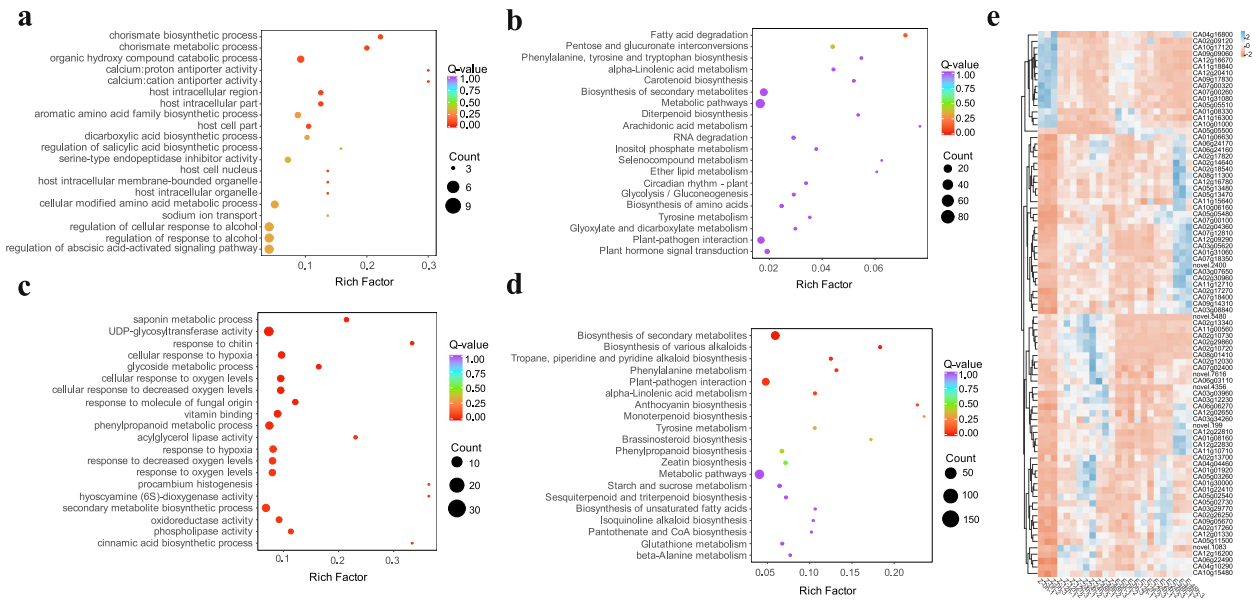

Fig. 7 Functional enrichment analysis of specific DEGs in 'Early Calwonder' and 'ZCM334'. (**a**) GO classifications of specific DEGs in 'Early Calwonder;' (**b**) KEGG pathway assignments of specific DEGs in 'Early Calwonder;' (**c**) GO classifications of specific DEGs in 'ZCM334;' (**d**) KEGG pathway assignments of specific DEGs in 'ZCM334;' (**e**) Heatmap of the expression levels of key genes related to disease resistance in the plant-pathogen interaction metabolic pathway screened from specific DEGs in 'ZCM334.'

Among them, four were significantly regulated in 'Early Calwonder' after *P. capsici* infection, including long-chain acyl-CoA synthetase 1 (*CA01g22440*), two alcohol dehydrogenases (*CA06g10980* and *CA06g10990*), and a glyoxysomal fatty acid beta-oxidation multifunctional protein MFP-a (*CA08g0606060*), and two cytochrome P450 enzymes (*CA08g06550* and *CA08g06560*) were significantly regulated following *P. capsici* infection.

The DEGs specifically expressed in 'ZCM334' were mostly significantly enriched in the UDP-glycosyltransferase activity, phenylpropanoid metabolic process, vitamin binding, and secondary metabolite biosynthetic process GO terms (Fig. 7c). The KEGG enrichment analysis showed that these DEGs were primarily significantly enriched in the metabolic pathways of the biosynthesis of secondary metabolites (including 80 and 82 regulated and regulated, respectively, after *P. capsici* infection), biosynthesis of various alkaloids (including one regulated and 10 regulated after *P. capsici* infection), tropane, piperidine, and pyridine alkaloid biosynthesis (including four and eight regulated and regulated, respectively following *P. capsici* infection), phenylalanine metabolism (including six regulated and four regulated after *P. capsici* infection), plant-pathogen interaction (including 83 and 28 regulated and regulated after *P. capsici* infection) ($P < 0.001$) (Fig. 7d).

In the plant-pathogen interaction pathway, based on gene functional annotation, 85 DEGs specifically expressed in 'ZCM334' related to pathogen infection and disease resistance were identified, most of which were regulated after *P. capsici* infection (Table 2; Fig. 7e). Eleven WRKY transcription factors were identified in the plant-pathogen interaction pathway, which may be crucial in regulating the host's transcriptional immune response to *P. capsici* [28]. Six calcium-binding proteins (CMLs) were differentially expressed after inoculation with *P. capsici* in 'ZCM334,' including four regulated and two regulated genes. Eleven and seven disease and late light resistance proteins, respectively, were screened for specific differential expression in 'ZCM334,' which may play a significant regulatory role in the prevention and control of *P. capsici* in pepper. We identified six receptor-like proteins (RLPs), four cysteine-rich receptor-like protein kinases (CRKs), and five serine/threonine-protein kinases (STPKs), which were regulated after 'ZCM334' infection with *P. capsici*. In addition, five receptor-like protein kinases (RLKs) (including three regulated and two regulated genes), six leucine-rich repeats (LRR) receptor-like serine/threonine-protein kinases (including one regulated and five regulated genes), nine G-type lectin S-receptor-like serine/threonine-protein kinases (GsSRKs) (including eight regulated and one regulated genes), and two regulated receptor-like serine/threonine-protein kinase were identified. In addition, we identified genes and transcription factors with crucial roles in plant immune system PTI and ETI, including tobacco stress-induced gene 1 (TSI1), calcium-dependent protein kinase

Table 2 List of defense genes enriched in plant pathogen interactions specifically expressed in 'ZCM334'

Gene ID	Functional annotation	Z-0h_vs_Z-12 h log_2 fold-change	Z-0h_vs_Z-24 h log_2 fold-change	Z-0h_vs_Z-48 h log_2 fold-change
CA01g01920	WRKY transcription factor 29	1.59	3.30	1.59
CA01g22410	WRKY transcription factor 23	2.12	3.27	1.05
CA02g14640	WRKY transcription factor 7	1.20	1.77	1.21
CA02g18540	WRKY transcription factor 31	1.15	1.26	1.27
CA02g30960	WRKY transcription factor 43	2.93	2.47	2.89
CA10g06160	WRKY transcription factor 29	1.48	1.83	1.08
CA11g12710	WRKY transcription factor 75	2.15	2.34	2.35
CA12g09290	WRKY transcription factor 51	3.09	2.98	3.04
novel.4356	WRKY transcription factor 59	1.71	1.42	1.58
CA03g12230	WRKY transcription factor 70	1.31	1.71	1.47
CA05g11500	WRKY transcription factor 41	1.24	1.90	1.76
CA06g22490	Calcium-binding protein *CML19*	1.86	2.04	1.46
CA10g01000	Calcium-binding protein *CML21*	−1.34	−1.27	−1.09
CA11g15640	Calcium-binding protein *CML19*	1.26	1.32	1.24
novel.2400	Calcium-binding protein *CML19*	1.38	1.18	1.59
CA12g16780	Calcium-binding protein *CML37*	2.20	1.77	1.72
CA09g17830	Calmodulin-like protein 5 (*CML5*)	−1.15	−1.93	−1.56
novel.1083	Disease resistance protein At4g27190-like	3.25	2.83	2.04
CA05g13480	Disease resistance protein RPP13-like	2.51	2.14	2.41
CA02g10720	Disease resistance RPP8-like protein 3	2.05	2.78	1.42
CA01g08330	Disease resistance protein *RGA3*	−2.12	−2.50	−1.43
CA12g16200	Disease resistance protein *Roq1*	2.10	2.25	1.26
CA01g30000	Disease resistance protein *RPM1*	1.49	2.42	1.17
CA02g10730	Disease resistance protein *RPM1*	1.82	2.30	1.12
CA05g05480	Disease resistance protein *RPP13*	2.48	3.09	2.36
CA05g05500	Disease resistance protein *RPP13*	−1.36	−1.15	−1.26
CA05g05510	Disease resistance protein *RPP13*	−1.09	−1.01	−1.21
CA05g13470	Disease resistance protein *RPP13*	2.14	1.57	1.83
CA05g02540	Late blight resistance protein -like protein R1A-10-like	2.22	2.61	1.02
novel.5480	Late blight resistance protein -like protein R1C-3-like	1.29	2.13	1.49
CA08g01410	Late blight resistance protein homolog R1A-10	1.37	1.81	1.68
CA09g14310	Late blight resistance protein homolog R1B-16	1.76	1.83	1.79
CA03g03960	Late blight resistance protein homolog R1B-12	1.10	1.34	1.47
CA10g15480	Late blight resistance protein homolog R1B-23	1.52	1.43	1.24
CA11g16300	Late blight resistance protein homolog R1A-4	−1.75	−2.55	−1.57
CA01g06630	Receptor-like protein (*RLP*)	1.65	1.51	1.82
CA12g02650	Receptor-like protein (*RLP*)	1.18	1.52	1.82
CA06g03110	Receptor-like protein (*RLP*)	1.67	2.54	2.55
CA08g11300	Receptor-like protein (*RLP*)	1.76	1.90	1.51
CA12g22810	Receptor-like protein (*RLP*)	2.16	2.69	2.84
CA12g22830	Receptor-like protein (*RLP*)	1.67	1.73	1.51
CA02g29860	Receptor-like protein kinase (*RLK*)	1.61	2.52	1.40
CA12g16670	Receptor-like protein kinase (*RLK*)	−1.31	−1.50	−1.29
CA10g17120	Receptor-like protein kinase (*RLK*)	−2.27	−3.10	−2.36
CA03g29770	Receptor-like protein kinase (*RLK*)	2.21	2.45	1.21
CA09g05670	Receptor-like protein kinase (*RLK*)	1.16	1.65	1.13
CA02g09120	Inactive leucine-rich repeats receptor-like protein kinase (*LRR-RLK*)	−1.41	−1.86	−1.28
CA03g34260	Cysteine-rich receptor-like protein kinase 10 (*CRK10*)	1.28	1.49	1.36
CA02g17820	Cysteine-rich receptor-like protein kinase 10 (*CRK10*)	1.36	1.67	1.47

Table 2 (continued)

Gene ID	Functional annotation	Z-0h_vs_Z-12 h log_2 fold-change	Z-0h_vs_Z-24 h log_2 fold-change	Z-0h_vs_Z-48 h log_2 fold-change
CA07g02400	Cysteine-rich receptor-like protein kinase 34 (*CRK34*)	1.28	1.93	1.29
CA12g01330	Cysteine-rich receptor-like protein kinase 2 (*CRK2*)	1.02	1.78	1.35
CA05g03260	Receptor-like cytoplasmic kinase 176 (*RLCK176*)	1.38	3.03	1.48
CA01g31060	Pathogenesis-related protein 1 (*PR1*)	3.72	4.01	3.98
CA01g31080	Basic form of pathogenesis-related protein 1	−1.52	−2.24	−2.90
CA07g00320	Basic form of pathogenesis-related protein 1	−1.80	−2.56	−1.92
CA02g04360	Pathogenesis-related genes transcriptional activator *PTI5*	1.50	1.62	1.24
CA03g07650	MYB transcription factor	1.91	1.78	2.71
CA12g20410	Basic Helix-Loop-Helix (*bHLH*) transcription factor	−1.90	−3.42	−1.59
CA03g05620	Ethylene-responsive transcription factor (*ERF*)	6.58	7.01	6.13
novel.199	Tobacco stress-induced gene 1 (*TSI1*)	1.78	1.79	2.97
CA04g10290	Calcium-dependent protein kinase 11 (*CDPK11*)	1.63	2.06	1.13
CA06g06270	Mitogen-activated protein kinase (*MAPK*)	1.14	1.13	1.03
CA06g24160	Serine/threonine-protein kinase (*STPK*)	1.26	1.59	1.72
CA06g24170	Serine/threonine-protein kinase (*STPK*)	1.16	1.43	1.61
CA11g00560	Serine/threonine-protein kinase (*STPK*)	1.25	2.69	1.10
CA11g10710	Serine/threonine-protein kinase (*STPK*)	1.07	1.24	1.09
CA02g26250	Serine/threonine-protein kinase (*STPK*)	1.46	1.85	1.32
CA03g08840	Receptor-like cytosolic serine/threonine-protein kinase	1.34	1.18	1.21
CA11g18840	Receptor-like serine/threonine-protein kinase	−1.46	−1.89	−1.87
CA07g00260	Receptor-like serine/threonine-protein kinase	−1.64	−3.02	−1.94
CA02g12030	Leucine-rich repeats (LRR) receptor-like serine/threonine-protein kinase *FLS2*	1.96	2.88	2.22
CA04g04460	Leucine-rich repeats (LRR) receptor-like serine/threonine-protein kinase	2.17	3.50	1.73
CA05g02730	Leucine-rich repeats (LRR) receptor-like serine/threonine-protein kinase-like	2.33	4.06	1.01
CA09g09060	Leucine-rich repeats (LRR) receptor-like serine/threonine-protein kinase	−1.05	−1.54	−1.56
CA02g13700	Leucine-rich repeats (LRR) receptor-like serine/threonine-protein kinase	1.48	2.51	1.66
CA07g00100	Inactive Leucine-rich repeats (LRR) receptor-like serine/threonine-protein kinase	4.14	4.80	3.89
CA02g13340	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	1.92	2.26	1.37
CA02g17260	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	2.05	2.56	1.61
CA02g17270	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	1.53	1.53	1.37
CA04g16800	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	−3.37	−3.35	−1.57
CA07g12810	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	3.24	3.34	2.49
CA07g18350	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	1.36	1.26	1.10
CA07g18400	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	2.78	2.79	2.53
novel.7616	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	1.24	1.65	1.44
CA01g08160	G-type lectin S-receptor-like serine/threonine-protein kinase (*GsSRK*)	1.71	1.88	1.99

(CDPK), MAPK, pathogenesis-related protein 1 (PR1), pathogenesis-related genes transcriptional activator PTI5, MYB transcription factor, ethylene-responsive transcription factor (ERF), and basic helix-loop-helix (bHLH) transcription factor.

Real-time quantitative PCR (qRT-PCR) of crucial defense genes related to *P. capsici*

To verify the reliability of the RNA-seq data, 10 DEGs were randomly selected for qRT-PCR validation (Fig. 8), including cytochrome P450 (*CA02g09530*), peroxidase (*CA02g18250*), CDPK (*CA04g10290*), CMLs (*CA06g22490*, *CA10g01000*, *CA11g15640*, *CA12g16780,* and *novel. 2400*), and lignin-forming animic peroxidases (*novel.4819* and *novel.4820*). After 'ZCM334' and 'Early Calwonder' were inoculated with *P. capsici*, the expression levels of *CA02g18250* and *CA10g01000* were decreased; those of *CA04g10290* were initially increased before exhibiting a decreasing trend, with the highest expression level 24 h after inoculation; the expression

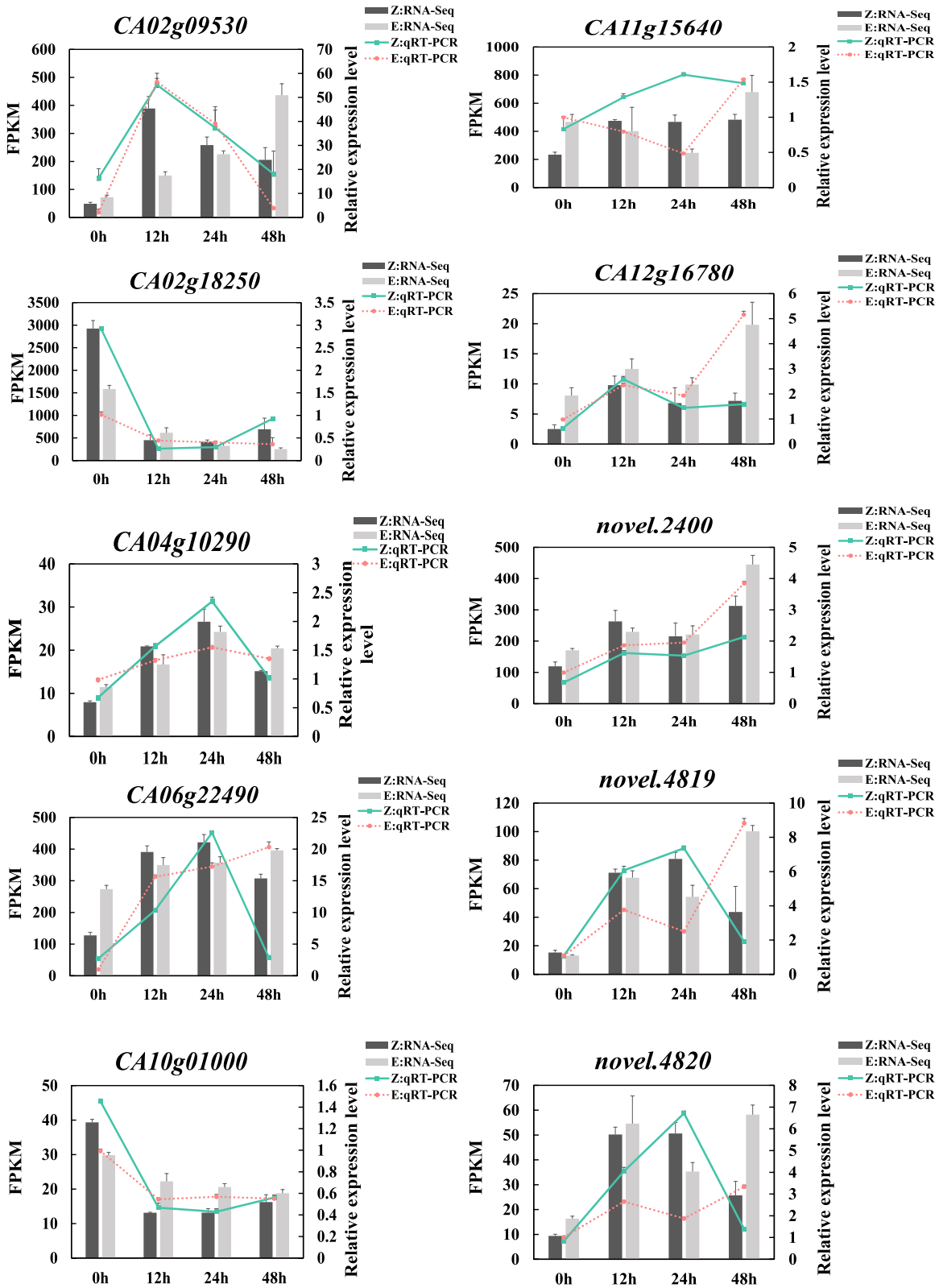

Fig. 8 qRT-PCR expression validation for 10 randomly selected genes. Z: 'ZCM334', E: 'Early Calwonder'

levels of *novel.2400* were gradually increased, peaking at 24 h after inoculation. In 'ZCM334', the expression levels of *CA06g22490*, *CA11g15640*, *novel.4819*, and *novel.4820* were highest 24 h after inoculation; in 'Early Calwonder,' their expression levels were highest 48 h after inoculation. The expression levels of *CA12g16780* were highest at 12 h in 'ZCM334' and 48 h in 'Early Calwonder' after inoculation. qRT-PCR showed that in 'ZCM334', the expression levels of *CA02g09530* were initially increased before showing a decreasing trend, peaking at 12 h after inoculation. In 'Early Calwonder,' the expression levels of *CA02g09530* were increased, peaking at 48 h after inoculation. RNA-seq showed that after *P. capsici* inoculation, the expression levels of *CA02g09530* in 'ZCM334' and 'Early Calwonder' were initially increased before exhibiting a decreasing trend, peaking at 12 h after inoculation. The qRT-PCR expression trends of these genes were consistent with the RNA-seq data, except for *CA02g09530*, indicating that the transcriptome sequencing was accurate, reliable, and can be further analyzed.

Among the DEGs specifically in 'ZCM334' and differentially expressed in Z-0 h_vs._Z-12 h, Z-0 h_vs._Z-24 h, and Z-0 h_vs._Z-48 h, we identified 20 DEGs with vital roles in the defense process of *P. capsici*, including 11 WRKYs, six RLPs, one PR, one MYB, and one MAPK, which are enriched in the metabolic pathway of plant-pathogen interactions (Fig. 9). After *P. capsici* inoculation in 'ZCM334,' the expression levels of nine WRKY transcription factors (*CA01g01920*, *CA01g22410*, *CA02g14640*, *CA02g18540*, *CA02g30960*, *CA10g06160*, *CA11g12710*, *CA03g12230*, and *CA05g11500*), six RLPs (*CA01g06630, CA12g02650, CA06g03110, CA08g11300, CA12g22810* and *CA12g22830*), and MAPK (*CA06g06270*) were initially increased before showing a decreasing trend, with the highest expression levels observed 24 h after inoculation. The expression levels of WRKY transcription factors *CA12g09290* and *novel.4356* were highest 12 h after inoculation. As 'ZCM334' was infected by *P. capsici*, the expression levels of PR *CA01g31060* and MYB transcription factors *CA03g07650* were highest 12 h after inoculation, decreased after 24 h, and increased after 48 h. These results indicate that the expression levels of most genes were highest 12 or 24 h after 'ZCM334' inoculation with *P. capsici*, indicating that these genes may be crucial in response to *P. capsici* infection in 'ZCM334' and plant defense against pathogens.

Discussion

Phytophthora blight is among the primary diseases that harm pepper production. It has a wide incidence range, and the pathogenic bacteria are prone to mutation, leading to the emergence of drug-resistant strains. Owing to the lack of widely applicable pepper resources for phytophthora blight resistance, as well as the complex genetic laws and unclear mechanisms of phytophthora blight resistance, the progress of current breeding

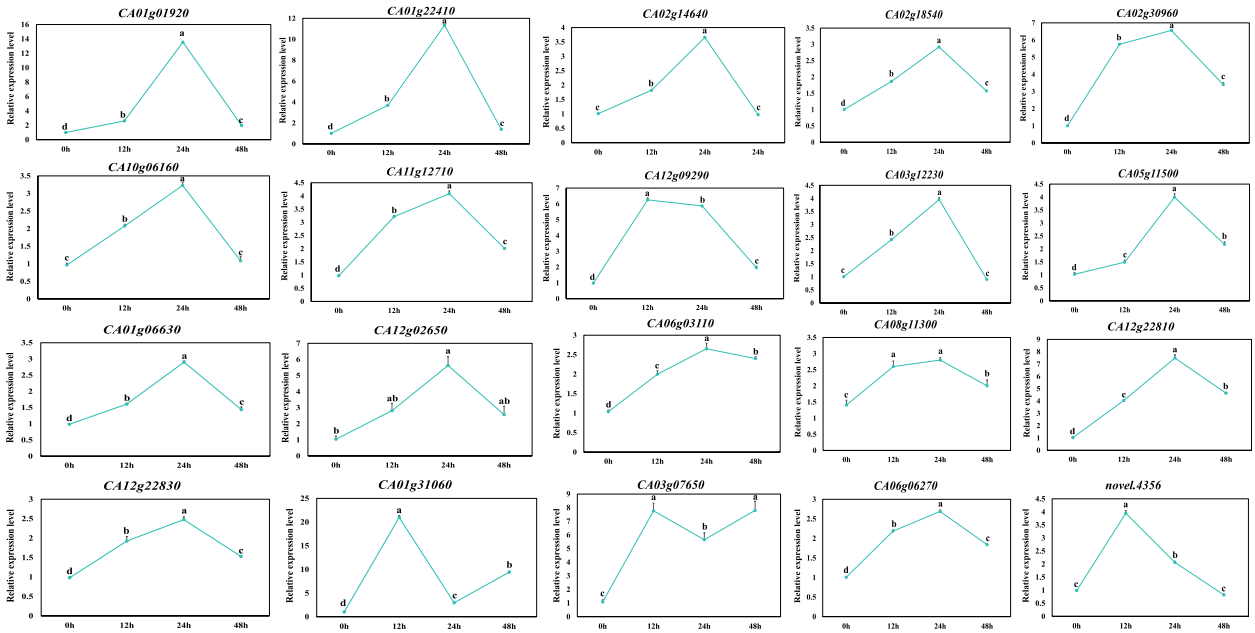

Fig. 9 Analysis of the expression patterns of 20 important defense genes with specific differential expression in 'ZCM334' at different stages after inoculation with *P. capsici*. Different letters indicate significant differences among the different stages after inoculation with *P. capsici* according to the least significant difference (LSD) test at $P < 0.05$

achievements is slow [29, 30]. Planting phytophthora blight immunity varieties is the most economical and effective measure for preventing and controlling phytophthora blight [31, 32]. The lack of immune genes in breeding limits the cultivation of phytophthora blight immunity varieties and the application of molecular breeding techniques for immune phytophthora blight. Therefore, exploring pepper resistance genes to phytophthora blight and studying phytophthora blight resistance mechanisms are currently focused on [33].

In pepper, the symptoms of phytophthora blight are primarily manifested in the roots, where brown or black water stains appear on the roots. With the invasion of pathogenic bacteria, the roots shrink, and the leaves wither, causing the death of the entire plant [34, 35]. Most of the currently reported resistant parents of phytophthora blight are highly resistant and not immune materials [36–39]. The 'ZCM334' variety used in this study is immune to physiological race 3 of *P. capsici*. After inoculation, all 'ZCM334' plants showed no symptoms, and the root cells did not undergo significant changes. It completely resisted the infection of *P. capsici* and is a valuable phytophthora blight immune material with great potential for breeding applications.

We used the roots of 'ZCM334' and the susceptible material 'Early Calwonder' as experimental materials and compared the transcriptome analysis of their roots at different stages before and after inoculation with *P. capsici*. Using 'Early Calwonder' and 'ZCM334' before inoculation with *P. capsici* as controls, the highest number of DEGs (6,284) was identified 24 h after inoculation in 'ZCM334.' In 'Early Calwonder,' the highest number of DEGs (5,573) was identified 48 h after inoculation. This suggests that 'ZCM334' may be more sensitive to *P. capsici* and have a faster response speed.

After inoculation with *P. capsici*, 435 common DEGs were identified in the roots of 'Early Calwonder' and 'ZCM334' at different stages, which were significantly enriched in the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, starch and sucrose metabolism, and sesquiterpenoid and triterpenoid biosynthesis metabolic pathways. Secondary metabolites, as signaling molecules, are crucial in plant disease resistance responses. Some secondary metabolites may act as stream signals, inducing the production of stream antibacterial substances and inhibiting pathogen invasion [40]. Starch and sucrose metabolism may be involved in regulating plant recognition and defense responses against pathogens in the interaction between plants and microorganisms [41]. Most of the DEGs shared by 'Early Calwonder' and 'ZCM334' were regulated after inoculation. From the above significantly enriched metabolic pathways, 23 defense genes related to plant disease resistance were screened, including four peroxidases [42], five cytochrome P450 [43], six lignin-forming animic peroxidases [44], two endochitinases [45], two polygalacturonases [46], one polyphenol oxidase [47], one alcohol dehydrogenase [48], one allene oxide synthase [49] and one alpha-amylase [50]. Except for *CA02g18250*, which was regulated after inoculation, all other genes were regulated in 'Early Calwonder' and 'ZCM334' after inoculation. These genes and their significantly enriched metabolic pathways may play a vital role in plant response to *P. capsici* infection, providing excellent genetic resources for further exploring the molecular mechanisms of plant resistance to phytophthora blight.

The interaction between plants and pathogens stimulates the host defense system of plants to fight against pathogens, inducing signal transmissions and causing plants to produce disease-resistant responses [51]. Among the specifically expressed genes in 'ZCM334', 83 DEGs enriched in the plant-pathogen interaction pathway related to disease resistance were identified. CMLs are essential in plant defense against various pathogens [52, 53]. An increasing number of RLPs have been cloned as immune receptors for plant disease resistance, which may play a crucial role in pathogen recognition [54]. CRKs have functional characteristics in response to pathogen infection, and STPKs can interact with host factors and are involved in the innate immune response of pepper [55, 56]. When plants are attacked by pathogens, RLKs respond in defense against invading pathogens or external signaling molecules on the plasma membrane [57]. Receptor-like serine/threonine-protein kinase and LRR receptor-like serine/threonine-protein kinase are crucial in pathogen recognition, activation of plant defense mechanisms, and signal transduction in developmental regulation [58]. GsSRKs may perceive signals generated by plant pathogen infection and transmit the signals to the nucleus, regulating the expression of resistance-related genes. This signal may be transmitted over long distances and stimulate the immune response of the entire plant [59]. In this study, we identified 11 WRKY transcription factors, 11 disease-resistant proteins, nine GsSRKs, seven late light resistance proteins, six CMLs, six RLPs, six LRR receptor-like serine/threonine-protein kinases, five STPKs, five RLKs, four CRKs, and two receptor-like serine/threonine-protein kinase, which were specifically differentially expressed in 'ZCM334' and may play a significant role in the resistance of 'ZCM334' to *P. capsici* infection.

PAMP is recognized by cell surface pattern recognition receptors (PRRs) that trigger PTI recognition, initiating the first line of defense of host-induced defense responses and enabling plant resistance to most pathogens. We identified some DEGs associated with PTI during the

infection process of *P. capsici*, including *CDPK*, *FLS2*, *CML*, *MAPK*, *WRYK*, *PR1*, and *ERF*, most of which were regulated after inoculation (Fig. 10). CDPK, CML, and PR1 may activate PTI and inhibit *P. capsici* proliferation [23, 60]. FLS2 is related to the recognition of foreign pathogen invasion and the activation of stream resistance genes [61]. In pepper, FLS2 may activate the transcription of disease-resistant genes, such as *WRKY* and *ERF*, initiate MAPK amplification, and activate PTI to resist *P. capsici* infection. The NLR immune receptor recognition effector within the cell activates the second layer of plant immune ETI, which causes faster and stronger plant resistance responses, including PCD, accumulation of ROS, and induction of plant hormone-mediated signaling pathways [62, 63]. To survive pathogen invasion, plants use multiple signaling pathways to activate their resistance response [19]. In this study, we identified that most of the DEGs in 'Early Calwonder' and 'ZCM334' were significantly enriched in the biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, plant hormone signal transduction, starch and sucrose metabolism, plant-pathogen interaction, sesquiterpenoid and triterpenoid biosynthesis, and fatty acid degradation metabolic pathways, which are crucial in plant resistance to pathogen invasion [22, 40]. *P. capsici* effectors may be recognized by NLR-type pathogen resistance proteins, activating ETI and inhibiting the replication and spread of *P. capsici* by promoting the synthesis of secondary metabolites such as plant hormones, phenylpropanoid, starch and sucrose, fatty acid, sesquiterpenoid and triterpenoid (Fig. 10), as also indicated by Zhang et al. [64]. In addition, we identified some DEGs related to plant disease resistance and immunity, such as *RPP13*, *MYB*, *RLP*, *RLK*, *bHLH*, *STPK*, *GsSRK*, *LRR-RLK*, and *CRK*, providing gene resources for further exploration of the regulatory network of *P. capsici* (Fig. 10). Twenty disease-resistant-related genes specifically expressed in 'ZCM334' were selected for qRT-PCR, which revealed that 16 genes had the highest expression levels 24 h after inoculation with *P. capsici* and four had the highest expression levels 12 h after inoculation (Fig. 9). The significantly high expression of these genes may activate the immune mechanism of 'ZCM334' and resist further damage to plants by *P. capsici*.

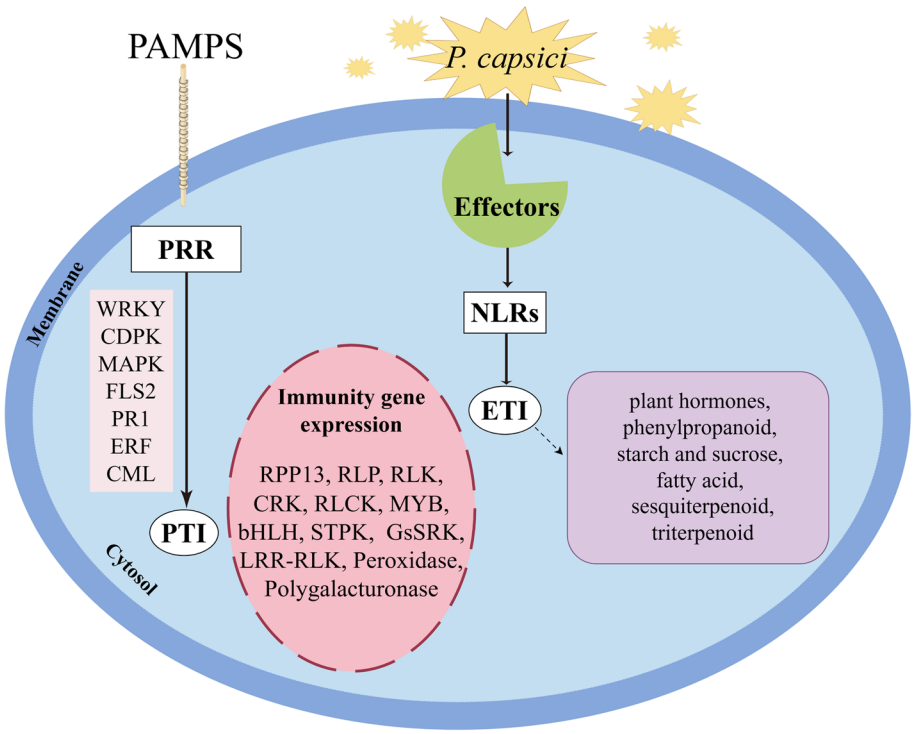

Fig. 10 Working model of pepper defense signal network in response to *P. capsica*. When plant membrane receptors sense *P. capsici* infection, genes such as *CDPK*, *FLS2*, *CML*, *MAPK*, *WRYK*, *PR1*, and *ERF* are expressed, inducing the first layer immunity (PTI) in pepper plants. When the pathogen effector protein breaks through the PTI response, it is bound by NLR-like receptors to stimulate the ETI immune response. Meanwhile, the synthesis of various metabolites such as plant hormone, phenylpropane, fatty acids, sesquiterpenoid, triterpenoid, starch, and sucrose may increase to synergistically protect plants from further pathogen attacks. In addition, the differential expression of some immune-related genes such as *RPP13*, *MYB*, *RLP*, *RLK*, *bHLH*, *STPK*, *GsSRK*, *LRR-RLK*, and *CRK* may play important roles in PTI and ETI. Abbreviations: PRRs, pattern recognition receptors; PTI, pattern-triggered immunity; NLR, nucleotide-binding domain and leucine-rich repeat protein; ETI, effector-triggered immunity

Conclusions

In this study, we analyzed the gene expression profiles of the roots of the immune material 'ZCM334' and the susceptible material 'Early Calwonder' at different stages of *P. capsici* infection. The number of DEGs in 'ZCM334' was higher than that in 'Early Calwonder,' and 'ZCM334' had the highest number of DEGs 24 h after inoculation. We identified some defense genes (*CDPK*, *FLS2*, *CML*, *MAPK*, *PR1*, *RPP13*, *RLP*, *RLK*, *STPK*, *GsSRK*, and *CRK*), transcription factors (*WRYK*, *ERF*, *MYB*, and *bHLH*) and key metabolic pathways (biosynthesis of secondary metabolites, phenylpropanoid biosynthesis, plant hormone signal transduction, starch and sucrose metabolism, and plant-pathogen interaction pathways) that are crucial in the resistance to phytophthora blight. Our future research will focus on functional validation of the important resistance genes identified in this study for key metabolic pathways and understanding the regulatory mechanism of 'ZCM334' in resisting the replication and spread of *P. capsici*.

Materials and methods

Plant materials

In the early stage, we identified and inoculated 'CM334,' a highly resistant material that resists *P. capsici* from the Asian Vegetable World Vegetable Center. After multiple generations of self-pollination, we obtained 'ZCM334,' a high-generation self-pollination line immune to the physiological race 3 of *P. capsici*. 'ZCM334' showed complete immunity against phytophthora blight. In this study, the immune variety 'ZCM334' and the susceptible variety 'Early Calwonder' were used as experimental materials, and their seeds were planted in a sunlight greenhouse of the Liaoning Academy of Agricultural Sciences. After 40 d, 'ZCM334' and 'Early Calwonder' plants were moved to an artificial climate chamber and allowed to grow under 26 °C/16-h light, 23 °C/8-h dark conditions, and 75% relative humidity for subsequent inoculation treatment.

P. capsici inoculation and phenotypic observation

We used *P. capsici* physiological race 3 'ZY14' to infect 'ZCM334' and 'Early Calwonder' plants. 'ZY14' was provided by researcher Liu Changyuan from the Plant Protection Research Institute of the Liaoning Academy of Agricultural Sciences. First, after rejuvenation and propagation of 'ZY14,' it was transferred to V8 medium and cultured at 28 °C for 7 d. After the hyphae were fully grown in the culture dish, the culture medium was cut into small pieces (2 cm^3), which were transferred to sterile water for soaking for 3 d. After spore production, it was placed in a 10 °C incubator for 1 h to promote spore release. After 30 min under 24 °C environmental conditions, the spore concentration was calculated using a blood cell counting plate. The spore concentration of *P. capsici* was diluted with sterile water to 2,000 spores/mL. 'ZCM334' and 'Early Calwonder' plants were inoculated using the root inoculation method, and 5 mL of zoospore suspension was injected into the soil of each plant root using a syringe. After inoculation, the conditions were maintained at an ambient temperature of 25–28 °C and a soil relative humidity of > 90%.

Phenotypic observations were conducted on the 'ZCM334' and 'Early Calwonder' plants at 0, 12, 24, and 48 h after inoculation. According to the procedure detailed by Zhou et al. [65], paraffin section analysis was performed on the transverse section of the main root. The root samples of 'ZCM334' and 'Early Calwonder' were collected at 0, 12, 24, and 48 h after inoculation with *P. capsici*. The root samples were a mixture of main and fibrous roots collected from three plants. These samples were frozen in liquid nitrogen and stored in a refrigerator at −80 °C for subsequent RNA extraction and qRT-PCR. Three biological replicates were performed per gro.

Total RNA extraction, mRNA library construction, and RNA sequencing

Total RNA was extracted from the root samples of 'ZCM334' and 'Early Calwonder' at 0, 12, 24, and 48 h after inoculation with *P. capsici* using SteadyPure Plant RNA Extraction Kit (Accurate Biology, AG21019, Hunan, China). The integrity and quality of total RNA were detected using NanoDrop and Agilent 2100 Bioanalyzer (Thermo Fisher Scientific, MA, USA).

We used ≥ 1ug RNA as the library building material and constructed a library using the NEBNextRUltraT-MRNA Library Prep Kit from I1lumina (NEB, USA). Enrichment of mRNA with polyA tail using Oligo (dT) magnetic beads, followed by random disrtion of mRNA using divalent cations in the NEBFragmentation Buffer. Using fragmented mRNA as a template and random oligonucleotides as primers, the first strand of cDNA was synthesized in the M-MuLV reverse transcriptase system. Subsequently, the RNA strand was degraded using RNaseH, and the second cDNA strand was synthesized using dNTPs as raw material in the DNA polymerase I system. Approximately 200 bp of cDNA that had undergone end repair was screened using AMPureXP beads for PCR amplification. The PCR product was purified using the AMPure XP system to obtain the library.

After preliminary quantification using Qubit2.0 Fluorometer, the library was diluted to 1.5 ng/μL, and the insert size of the library was detected using Agilent 2100 bioanalyzer. The cDNA libraries were sequenced on the Illumina NovaSeq 6000 platform by Metware

Biotechnology Co., Ltd. (Wuhan, China), and 150 bp paired-end reads were generated.

Identification and functional enrichment analysis of DEGs

We filtered the raw data using fastp to obtain clean reads [66], constructed an index using HISAT, and aligned the clean reads to the pepper reference genome (https://solgenomics.net/ftp/genomes/Capsicum_annuum/C.annuum_cvCM334/) [67]. StringTie concatenates transcripts using network flow algorithms and optional de novo assembly to predict new genes [68]. Gene alignment was calculated using featureCounts, and the Fragments Per Kilobase of transcript per Million fragments mapped for each gene was calculated based on its length [69]. Differential expression analysis was conducted between two gros using DESeq2, and the Benjamin–Hochberg method was used to perform multiple tests to correct the hypothesis testing probability (*p*-value) to obtain the FDR [70, 71]. The threshold for screening significantly DEGs is | log2 (fold change) |≥ 1 and FDR < 0.05.

To annotate the DEG functions, we conducted GO term and KEGG pathway enrichment analysis in the GO (http://www.geneontology.org/) and KEGG (http://www.genome.jp/kegg/genes.html) databases, respectively, to obtain information on the biological, biochemical metabolic, and signal transduction pathways of DEG enrichment [72, 73]. Draw the working model of pepper defense signal network response to *P. capsici* using Figdraw software (https://www.figdraw.com/static/index.html#/paint_index_v2).

qRT-PCR and data statistical analysis

To verify the reliability of transcriptome sequencing and explore the expression patterns of DEGs associated with resistance to *P. capsici*, 30 DEGs were selected, and qRT-PCR analysis was performed on the root samples of 'ZCM334' and 'Early Calwonder' at 0, 12, 24, and 48 h after inoculation with *P. capsici*. Total RNA was extracted using the SteadyPure Plant RNA Extraction Kit (Accurate Biology, AG21019, Hunan, China). Reverse transcription was performed using Evo M-MLV RT Premium (Accurate Biology, AG11706, China). Using RNA as a template, Evo M-MLV reverse transcriptase with strong extensibility was used to reverse-transcribe RNA and synthesize cDNA. cDNA was diluted four times for qRT-PCR.

Primers were designed using Primer 5.0 software (Table S3). RT-PCR was performed using the SYBR Green Premium Pro Taq HS qPCR Kit (Accurate Biotechnology, Hunan, China). The fluorescent dye SYBR was added to the PCR solution, and during the extension process of PCR amplification, SYBR ® Green I can chimeric into the double helix small groove region of double-stranded DNA and emit fluorescence. The target genes were qualitatively/quantitatively analyzed by detecting the fluorescence signal value during the reaction process. Each sample was subjected to three biological and three technical replicates. qRT-PCR was performed using Roche LightCycle 480 II (Switzerland) with a reaction procedure of 95 °C for 30 s, 45 cycles of 95 °C for 10 s, and 60 °C for 30 s. The $2^{-\Delta\Delta Ct}$ algorithm and Roche LightCycle 480 II software were used to analyze the relative expression levels of each gene [74].

The SPSS statistical software was used to conduct a statistical analysis of the data [75]. When comparing only two gros, statistical significance was evaluated using T-test. When comparing multiple gros, a one-way analysis of variance was used, followed by the Tukey test to evaluate statistical significance. A *P*-value < 0.05 was considered statistically significant.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-025-11498-w.

Supplementary Material 1.
Supplementary Material 2.
Supplementary Material 3.
Supplementary Material 4.
Supplementary Material 5.

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Not applicable.

Authors' contributions

F.S. and X.W. conducted the experiments and wrote the manuscript. C.Z. and X.L. directed the study, including the experimental design and manuscript revision. M.W. and X.Z. performed data analysis. Z.W. participated in the creation of the plant materials. All authors read and approved the final manuscript.

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Data availability

The raw read sequences were deposited in the Sequence Read Archive Database at the National Center for Biotechnology Information (NCBI) under the BioProject accession No. PRJNA1153702.

Declarations

Ethics approval and consent to participate

The current study complies with relevant institutional, national, and international guidelines and legislation for experimental research and field studies on plants (either cultivated or wild), including the collection of plant material.

Consent for publication

Not applicable.

Competing interests
The authors declare no competing interests.

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