Genome-Wide Identification and Functional Analysis of Chitinase Gene Family in Grape

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Abstract

Background: Chitinases, the important resistance-related proteins, are crucial hydrolytic enzymes, which attack fungal pathogens by catalyzing the fungal cell wall degradation. As a large gene family, the VvChis have not been systematically analyzed and effectively investigated in grape. Results: In this study, we identified 42 VvChis in grape by searching the conserved domains, and divided them into A, B, C, D and E groups according to pylogenetic relationships, gene structure and conserved domains analysis. Quantitative real-time PCR (qRT-PCR) and publicly microarray data analysis revealed distinct temporal and spatial expression patterns of VvChis in different tissues at various growth stages. The transcriptional level of most genes was high in the root of 'Koyho' and 'Summer Black'. Combining cis-elements in the promoter, GO and KEGG analysis, and prediction of interaction proteins, we revealed the function of Chitinase. After the pathogen infecting the leaves and berries of grape, the expression levels of VvChis in A, B and E groups showed a significant upward trend, of which VvChi5, VvChi25, VvChi11 (leaf) and VvChi16 (fruit) were the most up-regulated. The interaction between Chi-17 and Metallothionein (MTL) was confirmed by yeast two-hybrid system and bimolecular fluorescence complementation (BiFC). In addition, VvChis in GH18 family were up-regulated under MeJA and ETH treatment, in particular to 500 mg·L -1 ETH□50 μmol·L -1 MeJA; The induction of VvChis by low temperature was more significant than that of high temperature; The expression of VvChis was positively correlated with the concentration of NaCl treatment. Conclusion: This study clarified the member composition and expression pattern of VvChi family in grape, initially explored the disease resistance function of VvChi, and analyzed the response of VvChis to hormones (MeJA and ETH) and environmental stress (temperature and NaCl) signals was analyzed, which laid a foundation for constructing the functional regulation network of VvChi in grapes.

Background

Chitin is a linear polymer obtained by N-acetylglucosamine polymerized with β-1,4 glycosidic bonds. Preceded only by cellulose, it is the second most abundant renewable resource on Earth [1]. Chitinase (EC 3.2.1.14) is widely distributed in a variety of biological systems and can hydrolyze substrates containing at least one acetyl-glucosamine residue to low molecular weight β-1,4-N-acetyl-D- Dextran (β-1,4 glycosidic bond). Those containing N-acetyl-glucosamine like chitosan, lipochitooligosaccharide, peptidoglycan, arabinogalactan and glycoprotein, can be used as their hydrolysis substrate [2].

Chitinase belongs to glycosyl hydrolase, mainly composed of members of glycoside hydrolase family 18 (GH18) and family 19 (GH19). GH19 Chts is mainly found in plants, while GH18 Chts is widely distributed in animals, plants and microorganisms. There is no sequence homology between the two families [3]. According to the homology of the protein sequence, the three-dimensional structure, the specificity of the substrate, the mechanism of the catalytic reaction, chitinases are divided into five types in phylogeny, among which I, II and IV belong to the family 19 while III and V belong to the family 18 [4, 5]. There are differences in the amino acid structure of various chitinases. The Class I chitinases consist of a highly conserved major sequence and a Cys-rich N-terminal domain called CBD, which is the main region of

chitin binding. The class II chitinases, with a high degree of sequence similarity, are structurally homologous to class I, but lack a Cys-rich domain. Class I and II chitinases are highly conserved among different species. Class III chitinase has lysozyme activity and is less similar to the I and II chitinase sequences. Class IV chitinase is similar in structure to class I, but partially deleted in the CBD and CR regions (CR Catalytic region). Compared with the class III chitinases, there is a large fragment insertion in the amino acid sequence of the class V chitinases, which makes the difference in the degradation pattern of chitin oligosaccharides [4, 6, 7].

Chitinases are members of the disease-resistant protein group, which are induced strongly when plant cells attacked by pathogen, and chitinase is an important source of plant resistance to fungal pathogens [2, 8]. In B. juncea and tall fescue, fungal infections cause the upregulation of chitinases [9, 10]. Overexpression of the chitinase gene enhances plant resistance to fungal pathogens,which were confirmed in rice [11], wheat [12], carrots [13], litchi [14], cucumber [15], and sugarcane [16], while inhibition of the expression of Chi23, Chi32 or Chi47 in cotton significantly reduced the resistance of plants to jaundice [17]. In addition to defending against pathogen stress, chitinase is also involved in the construction of a symbiotic relationship between plant cells and fungi (eg, mycorrhizal binding) or bacteria (eg, leguminous/rhizobium binding), and many physiological processes such as plant cell division, flower development, early embryo development, seed development, and programmed cell death [1], and the production or degradation of signaling molecules that regulate cell polarity, movement, and division during plant growth and development [18]. Mutations in the Arabidopsis class II chitinase gene can also result in changes in cell shape, ectopic deposition of lignin in the secondary cell wall, and changes in internode and root length of plants [19].

As a worldwide fruit tree, grapes are widely distributed. Class I, Class III, and Class IV chitinases isolated from grapes exhibit activity to inhibit fungal growth [20–24]. The transgenic grape plants overexpressing the wheat chitinase gene showed high resistance to downy mildew [25]. In the grape, although some chitinases have been identified and functionally analyzed, a large part of the family has not yet been identified. The role of grape chitinase in coping with external stress and regulating plant growth remains to be further explored. In the study, we identified all chitinase genes in grapes by functional domain search, and conducted preliminary studies on the functions of various chitinases in grapes by means of homology comparison, gene expression pattern analysis and disease resistance evaluation to lay the foundation for the construction of the grape chitinase functional network.

Results

Genome-Wide Identification of Chitinase Genes and Their Chromosomal Distribution.

We explored VvChis in grape from the whole genome sequence scaffolds of ‘Piont Nior’. HMMER 3.0 and the Pfam protein family databases with the GH18 and GH19 domains were used to search for chitinases in grape genomes. SMAT and INTERPROSCAN programs were used to vertify the predicted genes. As a result, we identified 42 VvChis in grape (Supplementary Table S2). Among them, VvChi1 ~ VvChi25 were

searched out based on GH18 domains, and VvChi26 ~ VvChi42 were searched out based on GH19 domains.

The 42 identified VvChis were found in 9 out of the 19 grapevine chromosomes, as well as in the unplaced contigs chromosome (ChrUn), which contains sequences whose physical position on specific chromosomes have not yet been defined. As shown in Fig. 1, fourteen, seven, seven, four, three, two, one and one chitinase genes located at the chromosomes 5, 11,15, 6, 4, 16, 14 and 3, respectively. Four chitinase genes were also found in ChrUn.

Figures, Tables and Schemes

The phylogenetic relationships of VvChis were further analyzed by constructing an unrooted tree using the maximum likehood method. The results indicated that the 42 VvChis were clustered into five groups, namely A, B, C, D and E (Fig. 2a). These classifications were consistent with the presence of conserved and catalytic domains (Fig. 2b), and exon-intron organizations (Fig. 2c). Twenty-five chitinases belonging to GH 18 family were mainly divided into groups A, B and E, except VvChi24. Seventeen members of the GH 19 family were classified into groups C and D.

The conserved motifs of chitinases were displayed schematically in Fig. 2b. Twenty conserved motifs in the 42 VvChis were analyzed (Figure S1). The motifs 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 were found in the VvChi belonging to GH18 genes family while the motifs 15, 16, 17, 18, 19 and 20 were only detected in those of GH19 family. The type, number, order and motif location of these chitinase were similar within each group, but significantly different from others. The members in group A displayed the same five motif components (motif 8, 10, 12, 13 and 14) ranking in an identical order. The same situation was also found in group C (15, 16, 17, 18, 19 and 20) and 80% in group E (1, 2, 3, 4, 5, 6 and 7). The members in group B homogeneously shared motif 8. VvChis in Group C and D contained the same motifs (15, 16, 17, 18, 19 and 20) but with different number which indicated that these two group had a closer evolutionary and phylogenetic relationship. These findings indicated that the motif compositions of each group in chitinase genes family were relatively conserved. Through the analysis of protein secondary structure (Table S3), all VvChis were with four structures, namely Alpha helix, Extended strand, Beta brige, and Random coil, among which the proportion of Alpha helix and Random coil was the highest, while that of Beta brige was the lowest. The protein secondary structure had no significant disparity in different groups.

From the gene structure of VvChis (Fig. 2c), VvChi34 was longest, and VvChi9 was the shortest. Most genes in the same group generally shared exon-intron organizations with the same number of exons or introns. For example, one or two exons were found in most genes of group A, B and E while at least two exons existed in genes of group C and three in group D. These results showed that the VvChis in GH 19 had more exons than that in GH 18. Overall, the results of the conserved motifs location/components, and exon-intron gene structure of the chitinases in grape were consistent with the phylogenetic classification as well. Moreover, the highly conserved sequences of chitinase family members in the same group indicated that these genes were subject to duplications during their evolution.

Cis-Elements Analysis in the Chitinase Genes Promoters and Functional Prediction of Chitinases

To further clarify the gene function and transcriptional regulation mechanism of VvChis, we amplified the 1500 bp sequence upstream of VvChis and analyzed their cis-elements. These predicted cis-elements related to various process including photoreaction, hormone responsiveness, biotic and abiotic stress responsiveness, and plant development differed among groups and genes (Fig. 3). In the promoter of all genes, the number of elements related to endosperm expression was the largest except those related to light response, indicating that chitinases might had relationship with seeds development.

Compared with other groups, group A had the largest number of cis-element types, but B had the least. In the promoter of group B, there were many elements for gibberellin-responsiveness (GARE), but no for ethylene-responsiveness (ERE). Specifically, would-responsive elements were only found in the promoter of group C and fungal elicitor related elements located in the promoter of all groups of VvChis. Among 42 VvChis, VvChi27 had the largest number of cis-elements. Dehydration, low-temp, salt stresses related elements were only detected in the promoter of VvChi10, while cold- and dehydration-responsiveness related elements were only found in VvChi39, and cell cycle regulation elements only in VvChi10 and VvChi17. Additionally, different elements constitute was also found even in the promoter of the same gene. For example, different from other members in group B, meristem expression and low-temperature responsiveness related elements were only found in the promoter of VvChi21 while abscisic acid responsiveness and drought-inducibility related elements only in VvChi20 and MeJA-responsiveness related elements only in VvChi23.

GO and KEGG analysis indicated that VvChis mainly participate in the biosynthesis of secondary metabolites and amino sugar and nucleotide sugar metabolism (Figure S2a). The biological process of VvChis were all attributed to cell wall macromolecule catabolic process and chitin catabolic process, while molecular function were attributed to chitin binding and chitinase activity(Figure S2b).

As we all know, chitinases play a major role in defense against fungal pathogens by directly attacking divading fungal pathogens. However, the specific regulatory network and effects on grapes is still not clear. Chi31 from group C could interact with PGIP and BG to participate in plant resistance to phytopathogenic fungi (Figure S3). Chi5 from group E, Chi17 from group A, Chi26 from group D, Chi21 from group B, and Chi31 from group C also interacted with sugar metabolism proteins to regulate plant growth, such as HEXO, UGDH, GAD, etc. In addition to the known sugar metabolism and disease-resistant related proteins, Chis could also interact with many undefined functional proteins, and their metabolic network remain to be further explored (Table S4).
Subcellular Localizations of Chitinase Genes

VvChi17, VvChi22, VvChi31, VvChi26 and VvChi5 were cloned and made a fusion protein with GFP, individually. The five VvCHI-GFP fusion proteins (VvChi7-GFP, VvChi22-GFP, VvChi31-GFP, VvChi26-GFP, and VvChi5-GFP) and the GFP control were transformed tobacco leaf epidermal cells. The fluorescent signal in tobacco leaves was detected with confocal laser scanning microscopy. As shown in Fig. 4,

VvChi17 and VvChi5 located on plastids, VvChi22 and VvChi26 located on plasma membranes, and VvChi31 located on nuclear and plasma membranes.

Expression Analysis of Chitinase Genes in Grape

The expression level of twenty-five genes selected from five groups in different grape tissues was detected. There were differences in the expression profiles of VvChis genes in the seed cultivar ‘Kyoho’ and seedless cultivar ‘Summer Black’ (Fig. 5). Overall, the expression level of VvChis in various organs of ‘Kyoho’ was much higher than that of ‘Summer Black’, except tendril. The expression level of most genes in root was the highest and much lower in stem and flowers in ‘Summer Black’. Similarly, the highest expression level of most genes was found in ‘Kyoho’ root and the lowest expression level was found in tendril. It was worth noting that the expression level of most genes in seeds of ‘Kyoho’ was relatively higher compared to other tissues.

The expression level of VvChis from different groups was also different. In these two grapes’ root, the expression level of genes in group A, B, and E was higher than that in group C and D. In the skin, the transcription level of genes in group C and D was high while those in group A, B, and E hardly expressed. Similar to the genes in the skin, the expression of genes in group C and D was also in a high level. In the flowers, only the expression of VvChi31 and VvChi37 were expressed in a high level. In the seeds of ‘Kyoho’, the expression level of genes in group D was significantly higher than those in other groups.

Although the structure of VvChis was similar in the same group, the expression level of these genes was still different. In ‘Summer Black’, the expression level of VvChi22 in group B was higher than that of other members. VvChi16 in group A expressed lowly in root, stem, leaves, flower, flesh and skin, but showed higher expression level in tendril than any other genes. In group C, VvChi33 expressed highly in root, stem, leaves and tendril, while VvChi31 showed the highest expression level in flesh and skin, and VvChi37 highly expressed in flower. In ‘Kyoho’, VvChi12 expressed much higher than other members in group A. In group B, the expression level of VvChi25 and VvChi22 was significantly higher than that of other members. The expression level of VvChi31 was very low in the root, in which VvChi29, VvChi33 and VvChi34 in group C showed a high expression level; Additionally, the expression level of VvChi26 and VvChi27 in leaves was much higher than other genes in group D. VvChi5 and VvChi8 showed the highest expression level in stem, leaves, and flower, while other genes in group E showed the lowest expression level.

To investigate the spatial and temporal expression patterns of VvChis genes in grape, a global transcriptomic atlas comprising 54 tissues in different developmental stages was analyzed. Almost all VvChis genes had the corresponding probes on the NimbleGen array except VvChi8, VvChi25, VvChi27 and VvChi28. As a whole, the expression of VvChis showed the tissue–specific pattern (Fig. 6). Overall, the genes in group A, B and D showed a relatively low expression level, while those in group C and E remained at higher expression levels. The genes in different groups present the different expression patterns. For example, the genes in group C showed higher transcription level in berry pericarp, berry flesh,

berry skins, flower, leaf, root and rachis, while those in group E mainly presented higher expression levels in bud, but lower in berry flesh, rachis and berry skins.

Effect of Botrytis cinerea Treatment on the Expression Pattern of VvChis

The expression level of VvChis was examined after 5 days of inoculation of grape leaves and berries with Botrytis cinerea (Fig. 7a). The results showed that most groups of VvChis were able to respond to pathogen infection (Fig. 7b).

Although the expression level of VvChis in leaves was significantly higher than that in berries, the response of berries to pathogens was much more sensitive than that of leaves. The expression level of these genes in the berries was 3 to 80 times higher than that in leaves. After the pathogen infects the leaves and berries of the grapes, the expression levels of the genes in the A, B and E groups showed a significant upward trend. From the degree of up-regulation, VvChi5 in group E, VvChi25 in group B, and VvChi11 (leaf) and VvChi16 (fruit) in group A were the most up-regulated. VvChi17 in group A, VvChi22 in group B and VvChi5 in group E had the highest expression levels in berries. The expression patterns of genes in group C and group D were different from those in other groups. The expression levels of VvChi37, VvChi29 and VvChi33 in group C increased after infection while VvChi31 and VvChi34 showed a downward trend. The expression levels of VvChi24, VvChi26, VvChi27 and VvChi28 in group D showed a downward trend after infection while the expression of VvChi42 showed an upward trend. Among these berries, the expression level of the VvChi31 in group C and VvChi24 in group D were the highest.
Interaction Vertification between the Chitinase and Metallothionein (MTL) or Mannose-binding lectin (MBL)

According to the phylogenetic analysis, VvChi17 from Vitis vinifera and Herrania umbratica, Theobroma cacao were clustered together with a far kindship to the others (Figure S4).

The vectors pGBKT7-Chi17, pGADT7-MTL and pGADT7-MBL were constructed. Then, the constructed expression vector pGBKT7-Chi17 and pGADT7-MTL or pGADT7-MBL were simultaneously transformed into yeast competent cells Y2H-Gold for interaction verification, individually. The results showed that the pGBKT7-Chi17 and pGADT7-MTL co-transfected yeast cells and the positive control could grow normally and turn blue, while pGBKT7-Chi17 and pGADT7-empty co-transfected and pGADT7-MTL and pGBKT7-empty co-transfected yeast cells had no interaction signal, indicating that the chitinase could interact with MTL. The yeast cells co-transfected with pGBKT7-Chi17 and pGADT7-MBL were able to grow normally but did not turn blue, which proved that pGBKT7-Chi had no interaction with pGADT7-MBL (Fig. 8).

To further verify the interaction between MTL and Chi17, we performed BiFC test. The yellow fluorescence was detected in tobacco epidermal cells only when YNE-MTL and YCE-Chi co-expressed (Fig. 9), further verifying the interaction between Chi17 and MTL.

Effect of VvChis on Disease Resistance of Tomato and Strawberry

To investigate the function of VvChis, we analysis the effects of VvChis overexpression on fruit ripening and disease resistance by transient expression during strawberry and tomato color-changing stage.

The results showed that the overexpression of VvChis could reduce the probability of tomato infection (Fig. 10), among these VvChi17 and VvChi31 were reduced by 42.9% and 64.3% respectively, with the disease index below 30.Considering with various physiological indicators (Table S5, S6), the ability on disease resistance of VvChis was: VvChi31 > VvChi17 > VvChi22 > VvChi5 > VvChi26. Different from tomato, although 5 VvChis can also promote disease resistance in strawberry, VvChi31 has the weakest disease resistance with the disease index 92.8%, and VvChi17 has the strongest ability with infection rate reduced by 78.6%. In conclusion, the function of VvChis in tomato and strawberry was significantly different, and VvChi17 exhibits strong resistance to disease in both two species.

Effects of hormones and environment stress on VvChis

In view of the important effects of ETH and MeJA on grape growth and disease resistance, we studied the effects of ETH and MeJA treatments on the expression pattern of VvChis. The results proved that VvChis can respond to both hormones, showing different expression trends with the species and concentration of hormone (Figure S5, S6). Overall, VvChis is more sensitive to 500 $mg{\cdot}L^{-1}$ ethylene and differs in skin and fruit. The family classification has a great influence on the expression of VvChis: GH18 has an up-regulation trend under both hormone treatment, and more sensitive to ETH 500 $mg{\cdot}L^{-1}$ and MeJA 50 $\mu mol{\cdot}L^{-1}$; GH19 members vary greatly, VvChis of group D were sensitive to ETH 500 $mg{\cdot}L^{-1}$, while group C more sensitive to MeJA 5 $\mu mol{\cdot}L^{-1}$, and others have no obvious features.

The induction of chitinase by low temperature is more sensitive than high temperature (Figure S7). The reaction of flesh and skin were not synchronized to the temperature, and flesh was more sensitive. The response to temperature was different between different groups: Group A was higher under high temperature; Group B caused significant up-regulation in skin at 37 °C and − 20 °C; Group C were up-regulated at 4 °C in skin; VvChis in group D were significantly affected by temperature; Group E were higher in skin at higher temperature except VvChi5. The enzyme activity assay showed that the temperature had significant effects on SOD, POD, CHI, APX and β-1,3 GA, which were generally higher at 37 °C. Different from other enzymes, CHI had the highest activity at -20 °C.

VvChis showed an up-regulation trend after treatment with different concentrations of NaCl, and more sensitive to high concentrations (Figure S8). On the other hand, NaCl treatment increased the activity of five enzymes during short time, and then slowly decreased, which had little effect on SOD and β-1, 3 GA, and obvious effects on POD, APX and CHI. Among these, CHI activity is consistent with POD, but different from β-1,3 GA.

Discussion

Characteristics and Expression Patterns of Chitinases in Grape

The number of chitinase genes changes as the plant evolves. 47, 49, 92, and 116 chitinase genes were identified from four sequenced cotton varieties [16]. In our study, 42 VvChis family members were identified in the grape population, two fewer members than that in rice, and much higher than 25 in the model plant Arabidopsis [33]. Through homology analysis, grapes are closely related to spinach and melon. According to the homology of the protein sequence, the three-dimensional structure, the specificity of the substrate and the catalytic reaction mechanism, the predecessors divided the chitinase into five categories [5]. In this study, we screened all the gene sequences containing the chitinase-related binding sites and active sites in the grapes, and constructed them according to the amino acid sequence. All genes were also classified into five types. By comparing the previous classification with ours, we found that Group A, Group C, Group D, and Group E correspond to the Class III, Class IV, Class I, and Class V chitinases in the traditional classification, respectively. Class B proteins, with one short chitinase active site, do not have the characteristics of the previous classification. It is worth noting that the typical class II chitinase gene was not found in our study. Class II chitinase is mainly found in fungi [34]. From an evolutionary perspective, the class I and class IV chitinase genes are all derived from class II genes. The presence of five classes of chitinase has been found in cotton, Arabidopsis and other species [17, 35]. The lack of class II chitinase genes in grapes may be related to evolutionary differences between species.

The expression of the gene encoding chitinase exhibits multiple patterns in the grape, which can be influenced by developmental stages, tissues and cell types. There are few studies on chitinase in grapes. Derckel et al. [20] detected chitinase activity in roots, stems, leaves, berries and other organs of grapes, and the activity in berries was the highest. Robert et al. [36] found that the expression of class I chitinases was not detected in grape berries, and the enzyme was only highly expressed in leaves. Bézier et al. [37] found that the expression level of class IV genes was high in grape berries, lower in flowers, and almost undetectable in leaves, roots and seeds [38]. Bézier et al. [37] also did not detect the expression of the class III gene VvCH3 in berries. However, the above studies were limited to a single gene in a certain class. In this study, we analyzed the expression patterns of all types of chitinase genes in grape berries. Overall, except for individual genes, the expression level of genes in groups C and E (i.e. class IV and class V) was higher than those in groups A, B, and D. From the berries setting period to the maturation stage, the expression level of chitinase genes in grape berries is increasing, which is similar to the findings of Robinson et al. [38], indicating the accumulation of sugar and chitin in grape fruit. The increase in enzyme activity is consistent. The expression of the chitinase-encoding gene in the different tissues of the seeded grape 'Koyoho' and the seedless grape 'Summer Black' was different, and most of these genes were highly expressed in the roots, which was different from the the data in the gene chip of Pinot Noir, indicating that the expression of the chitinase gene in different grape varieties may be species-specific.

The expression level of the chitinase gene in seeds was also high, which may be related to the function of chitinase to promote seed germination and embryo development [1, 39]. Class I and IV chitinase genes were more responsive to abiotic stresses [40], where class I genes were the most sensitive to drought, chilling, and salt damage [41–42]. The chitinases of groups C and D belong to class IV and class I chitinase, respectively, and their expression levels in grape skin were higher, indicating that chitinase in the skin may be more responsive to external stress.

Functional Analysis of Grape Chitinases

Previous studies have shown that chitinases are involved in the regulation of plant growth and disease resistance [1]. Plant seeds can accumulate chitinases without any stimulation [43]. We analyzed the cis-acting elements in the promoter of chitinase genes and found that the endosperm development-related elements were the most abundant, suggesting that the chitinases in the grape may be involved in seed development. In the promoter of the grape chitinase genes, we also found a number of functional elements involved in hormone response, fungal induction and abiotic stress response. In Arabidopsis, tobacco, potato and other species [40, 44–46], the expression of chitinase-related genes can be affected by biotic and abiotic stresses. The presence of related elements in the grape indicated that the chitinases in the grape may be involved in the response of the plant to biotic and abiotic stress signals.

When the pathogen infects the plant, the expression level of the chitinase genes is affected. Different classes of chitinases in different species can respond to pathogen infections, such as Class I in the hedgehog [47], Casuarina [48] and cotton [49],Class II in barley [50], class III in sugar cane [16], oil palm [51], IV in spruce [52] and Class V chitinase in ginger [53]. In this study, after gray mold infected the grape berries and leaves, the chitinase genes showed a similar expression pattern. Except VvChi31, VvChi34, VvChi24 and VvChi26, the expression of other genes was significantly up-regulated, indicating that most of the chitinase genes in the grape can respond to gray mold stress, and various chitinases in grapes may have functional similarity. We found a close relationship between VvChi expression and plant immunity by transient expression of VvChis in tomato and strawberry, which was same with Collinge et al. [4], While VvChis have significant differences in response to biotic stress in both climacteric and non-respiratory climacteric fruit. Overexpressing VvChi31 of Group C (Class IV) in tomato showed the strongest resistance to Botrytis cinerea, while its resistance in strawberry was the weakest. The causes of large differences may be related to the different mechanisms of fruit ripening, so chitinases of different structural classes play different roles. This indicates that we should fully consider the developmental characteristics and traits of fruit when conducting disease resistant gene screening and molecular breeding.

The results of the GO and KEGG databases indicated that the chitinases played a major role by participating in the synthesis and metabolic pathways of amino acids, ribose and secondary metabolites, and interacted with proteins that were resistant to disease and glucose metabolism to regulate plant growth and development. Metallothioneins(MTs)or metallothionein-like proteins had various function in plants. MTs played significant roles in particular stages of development and BnMT4 was demonstrated to regulate germination processes in Brassica napus L. [31]. Tobacco metallothionein-like proteins are related to plant disease resistance, and play an important role in wounded and pathogen-stressed plants [30]. In this study, we confirmed that chitinase in grapes can bind to metallothionein by yeast two-hybrid system and BiFC test, indicating that chitinases may participate in the regulation mechanism of grape disease resistance and growth by interaction with metallothionein.

VvChis and External Environment

Previous studies have confirmed the ability of VvChis to respond to mechanical damage, ozone, ultraviolet light, cold, heavy metals, salinity, drought, hormones, etc.[54, 55];. But it's only for one or several genes that does not fully reflect the expression characteristics of VvChis. In this study, we analyzed the response of different classes of VvChis by exogenous phytohormone MeJA and ETH, as well as temperature and NaCl abiotic stress treatment.

Plants respond to pathogens by activating basal resistance to prevent disease. This process is controlled by a complex signal transduction network, which mainly involves SA, JA, ETH signal transduction pathways and their complex interactions [56]. JA and ETH signal transduction pathway interact each other [57]. Previous studies have shown that ETH, JA and SA have an effect on the expression of some chitinase genes [58, 59, 55], not specific to different categories, nor the concentration of hormones. In this study, we investigated the effects of different concentrations of JA and ETH on the expression of VvChis in grape. The results showed that VvChis of group A, B and E were up-regulated under treatment. All VvChis of group A, B, D and E had the most sensitive response to 500 $mg \cdot L^{-1}$ ETH, and group A, B and E responded to 50 $\mu mol \cdot L^{-1}$ MeJA, while Group C responded sensitively to 5 $\mu mol \cdot L^{-1}$ MeJA.

Induction of resistance to pathogens in postharvest fruits by abiotic measures has become a potential method for reducing the use of synthetic fungicides [57]. Studies have shown that chitinase was induced by abiotic treatment [46]. But previous studies have focused on the expression of one or several specific VvChis under stress, such as NaCl [45], mechanical damage [20], osmotic stress [60], drought and chilling damage [41], etc.. In this study, we synthesized the expression of all VvChis. VvChis in group A and B were more consistently affected by abiotic stresses. The expression of genes in group C and D was particularly low in flesh, which mean VvChis of groups C and D may be weaker function in flesh at this stage. The induction of VvChis by low temperature is more obvious than high temperature. The effect of concentrations of NaCl on the expression of VvChis was positively correlated, which confirm that Class I response to chilling injury [41], Class I, II and V response to salt stress [45, 60].

Conclusions

We identified 42 VvChis from GH18 and GH19 families in the grapevine and these members were divided into A, B, C, D and E groups according to phylogenetic relationships, gene structure and conserved domains analysis. Among them, Group A, C, D, and E correspond to the Class III, Class IV, Class I, and Class V chitinases in the traditional classification, and class II chitinase gene was not found in our study. The 42 identified VvChis were found to be distributed in 9 out of the 19 grapevine chromosomes.

The relative expression level of VvChis varied in different tissues at different developmental stages, while that in various tissues of 'Kyoho' was much higher than that of 'Summer Black', except tendril. VvChis can response to biotic stress and the expression level of VvChis in A, B and E groups showed a significant upward trend after the pathogen infecting the leaves and berries of grape. By yeast two-hybrid system and bimolecular fluorescence complementation (BiFC), we confirmed the interaction between VvChi-17 and Metallothionein (MTL). At last, we studied the effects of MeJA, ETH, NaCl and temperature on

VvChis, confirming the significant differences on stress response between and within groups. These findings could lay the theoretical foundation for the function study of Chis and the further construction of grape disease resistance mechanism.

Methods

Plant Materials and Treatments

Different Tissues of Grape in Development

Potted 2-years-old grape varieties ‘Kyoho’ and ‘Summer Black’ seedlings were grown in a greenhouse at 25℃, a relative humidity of 80%, and the materials were provided by the Jiangsu Vocational College of Agriculture and Forestry grape farm, Jurong, China (our partners, we have a long-term relationship). All samples were frozen in liquid nitrogen immediately and stored at -80℃.
Leaves and Berries Used for Pathogen infection
Leaves and berries of ‘Kyoho’ were inoculated with Botrytis cinerea. The samples were collected 5 days after the inoculation, frozen immediately in liquid nitrogen upon collection and stored at -80℃ for RNA extraction.

Botrytis cinerea was preserved in the laboratory, transferred to a PDA plate and cultured at 28 °C. After the spores were produced, the bacteria were picked up into sterile water and filtered with double gauze to obtain a conidium suspension and diluted to 10^5 /mL^{-1}.
Genome-Wide Identification and Annotation of Chitinase Genes

In the present study, we used HMMER 3.0 and Pfam protein family databases (http://pfam.xfam.org) with the Glyco-hydro-18 (PF00704) and Glyco-hydro-19 (PF00182) domains to search for chitinases in the Grape Genome Database (CRIBI. Available online: http://genomes.cribi.unipd.it/grape/, Version 2.1.). The predicted genes were further confirmed the existence of the conserved domains of Glyco-hydro-18 or Glyco-hydro-19 using SMAT (Available online: http://smart. embl-heidelberg.de/smart/set_mode.cgi?GENOMIC = 1) and INTERPROSCAN (Available online: http://www.ebi.ac.uk/interpro/search/sequence-search) programs. Number of amino acids, theoretical Mw, theoretical pI, aliphatic index, grand average of hydropathicity and secondary structure of protein were analyzed by Ex-PaSy (http://expasy.org).
Chromosomal Distribution, Phylogenetic Trees Construction, and Structural Analysis

Chromosomal distribution of VvChis were determined by MapInspect software according to the CRIBI database. Phylogenetic trees were constructed using the Maximum Likelihood (ML) method with MEGA 6 (Sudhir Kumar,Arizona State University, USA). The reliability of the obtained trees was tested using bootstrap with 1000 replicates.

We compared gene structures and motifs to identify relationships in the structural evolution of VvChis. Exon and intron structures were determined by Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/)based on coding sequence (CDS) and the correspondent full-length gene

sequences in NCBI. The conserved motifs were constructed in the MEME program (http://meme-suite.org/tools/meme) using full-length amino acid sequences, setting the motif number as 20 with E-values ≤ $1e^{-30}$.

Promoter Analysis, GO and KEGG Analysis, and Interaction Protein Prediction

The 1500 bp upstream of VvChis was used to perform cis-elements analysis in PlantCARE (http://bioinformatics. psb.ugent.be/webtools/plantcare/html/). GO and KEGG analysis were carried out using gene sequence on the platform of Nuohe Zhiyuan company (https://magic.novogene.com/public/customer/main#/home). The interaction protein analysis and prediction were determined in the online software STRING (https://string-db.org).

Microarray and Transcriptome Data Acquisition and Analysis

The transcriptome data in the Gene Expression Omnibus under the series entry GSE36128 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lfcrxesyciqgsjoan dacc = GSE36128) were downloaded. The expression of VvChis in the 54 tissues of grapevine was analyzed [26].

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (qRT-PCR)

Total RNAs were extracted using a CTAB method according to Wang et al. [27]. The cDNA was synthesized from 4 μg of DNA-free RNA using a Revert AidTM First-Stand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). The cDNA was diluted at 1:10 for qRT-PCR.

The qRT-PCR reaction comprised 5 μL of SYBR Premix Ex Taq™ (Takara, Japan), 0.3 μL of each primer (10 μM), 2 μL of cDNA, and 2.4 μL of RNase-free water in a total volume of 10 μL. The reaction started with a preliminary step of 95 °C for 30 s followed by 35 cycles of 95 °C for 5 s and 58 °C for 35 s. Relative gene expression was calculated using the $2^{-\triangle\triangle Ct}$ method [28].

Subcellular Localization Testing

Full-length coding sequences of VvChi5, VvChi17, VvChi22, VvChi26 and VvChi31 were cloned from the cDNA of ‘Kyoho’ grape. The cloned sequence was then transferred into the pCAMBIA1302 vector to produce the plasmids VvChi5-GFP, VvChi17-GFP, VvChi22-GFP, VvChi26-GFP, and VvChi31-GFP, which were then transfected into Agrobacterium (strain EHA105).

Transient expression of VvChis in tobacco were carried out according to Kou et al. [29]. Plants were incubated for 3–4 days at 20–25 °C and images were acquired using a Zeiss Axio LSM 700 inverted confocal laser scanning microscope (CLSM). All transient expression assays were repeated at least three times. Primers used for cloning genes and constructing vectors are listed in Table S1-1.

Yeast Two-Hybrid Testing

We previously identified chitinase in grape xylem sap (data not shown), and only identified the chitin protein VvChi17, so we explored the function of this protein. Mannose-binding lectin (MBL) and Metallothionein (MTL) were multifunctional proteins [30–31] and were also identified in xylem sap. This study determined the binding ability of VvChi17 protein to MBL and MTL for the further function study.

Full-length coding sequences of VvChi17, VvMBL and VvMTL were cloned and digested using restriction enzyme as shown in Table S1-2. VvChi17 was fused into pGBKT7 vector to produce the plasmids pGBKT7-Chi, and VvMBL, VvMTL were fused into pGADT7 vector to produce the plasmids pGADT7-MTL and pGADT7-MBL.

pGBKT7-Chi and pGADT7-MTL, pGBKT7-Chi and pGADT7-MBL, were simultaneously transformed into yeast competent cells Y2H-Gold for interaction verification. pGBKT7-Chi and pGADT7-empty□pGADT7-MBL and pGBKT7-empty, pGADT7-MTL and pGBKT7-empty were imultaneously transformed as self-activation control, while positive control was carried concurrently. The products were coated on two-deficent (SD/-Trp/-Leu) medium. Then the yeast grown on two-deficent medium were picked to the YPDA liquid medium, and shaked to turbid at 28 °C, 2 μL were taken to the four-deficent (SD/-Ade/-Leu/-Trp/-His) medium.

Bimolecular Fluorescence Complementation (BiFC)

BiFC was performed according to Waadt et el. [32]. Full-length coding sequences of VvChi17 and VvMTL were cloned and digested using restriction enzymes as shown in Table S1-3. Then Chi was fused to C-terminal YFP to obtain 35S: YCE-Chi, and MTL with N-terminal YFP to form 35S: YNE-MTL. YNE-MTL × YCE, YNE × YCE-Chi, YNE-MTL × YCE-Chi were imultaneously transformed to N. benthamiana, cultivating at 28 °C for 3–4 days. The bimolecular fluorescence was also detected under CLSM.

Transient expression of VvChis in tomato and strawberry

The constructed vectors VvChi5-GFP, VvChi17-GFP, VvChi22-GFP, VvChi26-GFP, and VvChi31-GFP were transfected into Agrobacterium (strain EHA105), then infected tomato and strawberry during color-changing period, with pCAMBIA-1302 as CK. After 3 days, the incidence and disease index were observed and counted, and the lesion diameter, TSS, weight loss rate were determined. The enzyme activities of SOD, POD, CAT, APX and MDA were determined by using enzyme activity test kit (Solarbio), and the contents of Vc and lignin were determined. The enzyme activities of chitinase and β-1,3 GA were tested by ELISA kit (LYYE BIOTECHNOLOGY). The tomato and strawberry also sampled from Jiangsu Vocational College of Agriculture and Forestry farm, Jurong.

Response to hormones and environment of VvChis

Hormone and salt signal response: Prepare ETH solution (200 $mg \cdot L^{-1}$, 500 $mg \cdot L^{-1}$, 2000 $mg \cdot L^{-1}$), MeJA solution (5 $\mu mol \cdot L^{-1}$, 50 $\mu mol \cdot L^{-1}$, 100 $\mu mol \cdot L^{-1}$), and NaCl solution (20 $mmol \cdot L^{-1}$, 60 $mmol \cdot L^{-1}$, 100 $mmol \cdot L^{-1}$) respectively. The grape fruit was immersed in solution, and distilled water was used as a control. Each treatment was repeated three groups, and 10 fruits were repeated in each group. Samples were taken at 1 h, 12 h, 24 h, and 48 h after treatment. Temperature signal response: Grape fruits were placed at 4 °C, 25 °C, 37 °C, -20 °C, and room temperature was setted as control. Samples were taken at 5 d, 10 d, 15 d and 20 d, respectively. The skin and flesh were separated when sampling, and stored at -80 °C after liquid nitrogen treatment for subsequent RNA extraction and enzyme activity determination.

Statistical Analysis

Statistical analysis of variance (ANOVA) was performed using SPSS statistics 17.0 (SPSS Inc, Chicago, ILL, USA). Heml 1.0.3.7 software and Origin Pro 9 (Origin Inc., Northampton, MA, USA) were used to produce figures.

Abbreviations

Chi:chitinase; qRT-PCR:quantitative real-time PCR; GO:gene ontology; KEGG:kyoto encyclopedia of genes and genomes; MTL:metallothionein; BiFC:bimolecular fluorescence complementation; MeJA:methyl jasmonate; ETH:ethylene; GH:glycoside hydrolase; CBD:Cys-rich N-terminal domain.

Declarations

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Availability of data and materials

The datasets supporting the results presented in this manuscript are included within the article (and its additional files).

Authors' Contributions:

Conceptualization, HJ and JF; Data curation, XZ; Formal analysis, XZ; Funding acquisition, JF; Investigation, TZ and ZL; Methodology, TZ and KZ; Project administration, JF; Resources, KZ; Software, KZ; Supervision, HJ; Validation, SJ and TD; Visualization, SJ; Writing – original draft, TZ; Writing – review & editing, KZ and LG. All of the authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interest:

The authors declare that they have no competing interest.

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Supplementary Files Legend

Supplementary Figures

Figure S1 The sequence of conserved motifs in VvChis

Figure S2 The GO and KEGG analysis of *VvChis*

Figure S3 The interaction protein prediction of *VvChis.*

Figure S4 Phylogenetic analysis of *VvChi17* in different species

Figure S5 Expression analysis of grape *VvChis* in response to ETH (Fig.A, B, C, D, and E represent the gene expression level of five groups, respectively).

Figure S6 Expression analysis of grape *VvChis* in response to MeJA (Fig.A, B, C, D, and E represent the gene expression level of five groups, respectively).

Figure S7 Expression analysis of grape *VvChis* in response to temperature (Fig.A, B, C, D, and E represent the gene expression level of five groups, respectively).

Figure S8 Expression analysis of grape *VvChis* in response to NaCl (Fig.A, B, C, D, and E represent the gene expression level of five groups, respectively).

Supplementary Tables

Table S1 Information on chintinase genes

Table S2 Analysis of protein secondary structure

Table S3 Sentences of primers. S3-1 Primers used for subcellular localization; S3-2 Primers used for Yeast Two-Hybrid analysis; S3-3 Primers used for BiFC analysis; S3-4 Primers used for the expression detection of tissues.

Table S4 Prediction functional partners of CHIs and their annotation.

Table S5 Effect of overexpression of *VvChis* on disease resistance index after inoculate *B. cinerea* in color-changing cherry tomato

Table S6 Effect of overexpression of *VvChis* on disease resistance index after inoculate *B. cinerea* in color-changing strawberry

Figures

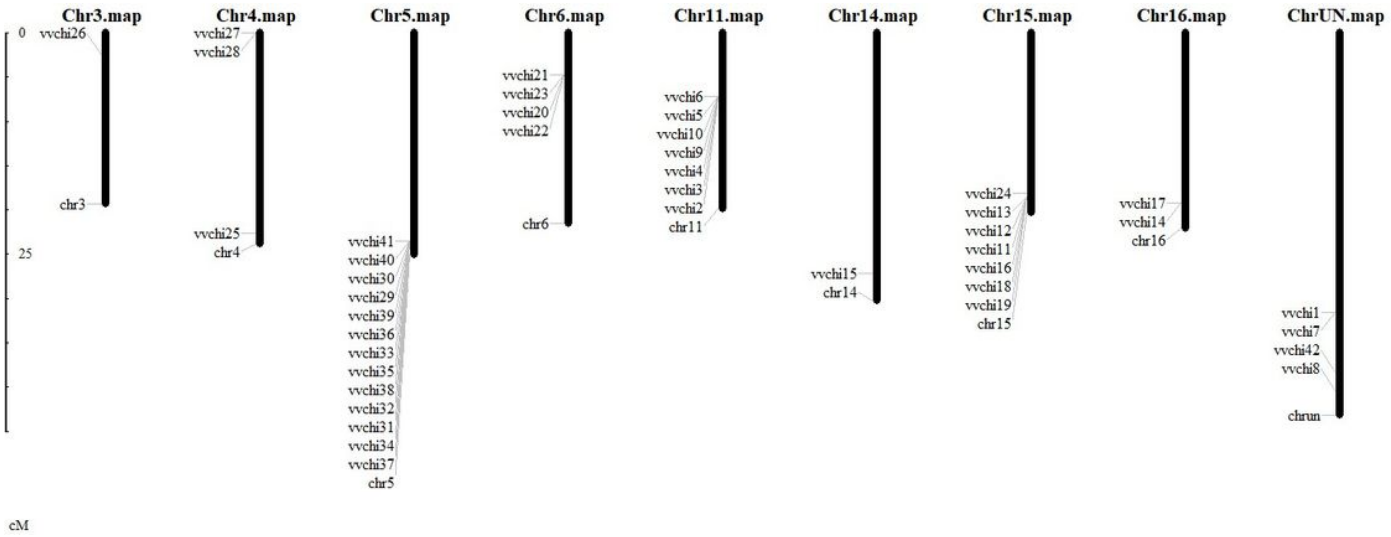

Figure 1

Chromosomal location of 42 VvChis.

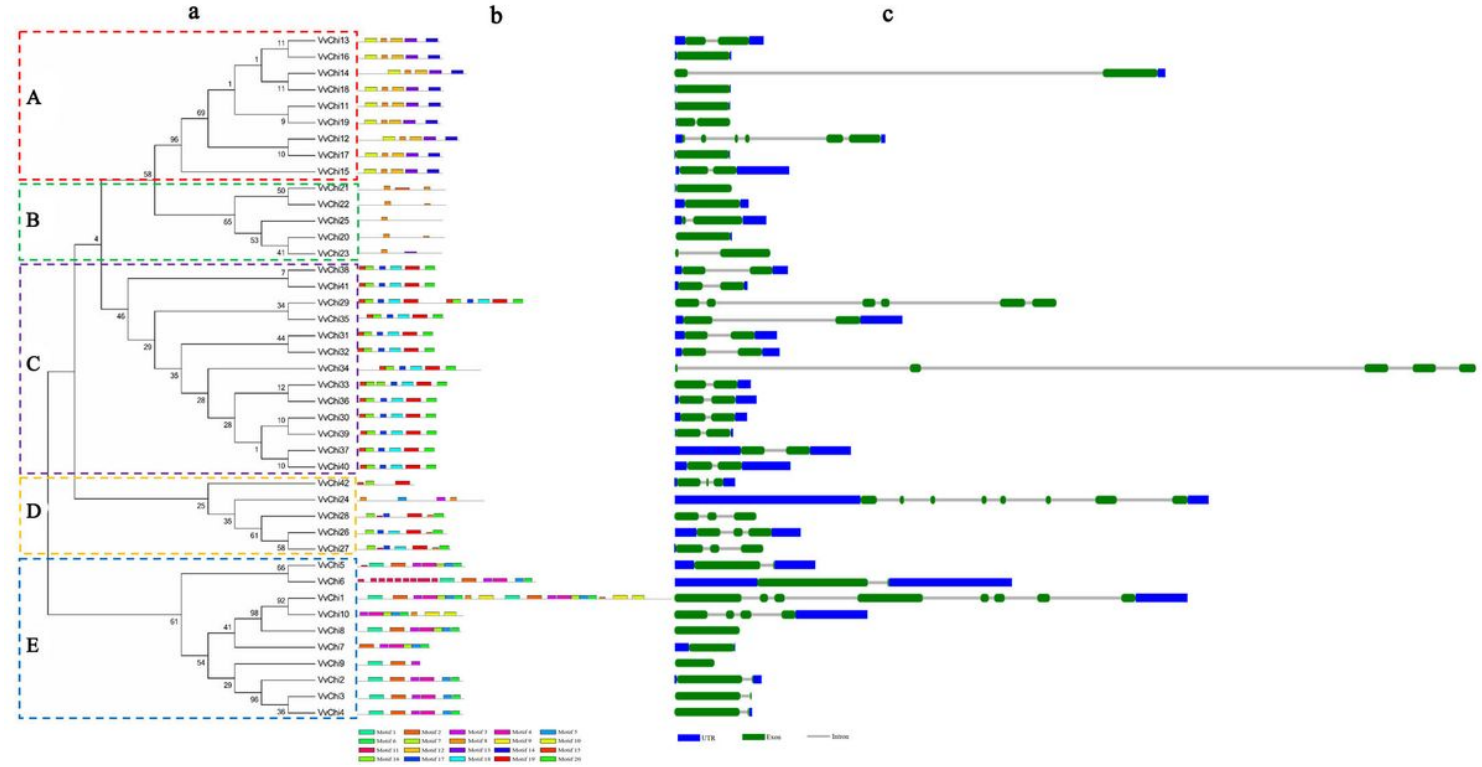

Figure 2

Phylogenetic classification and structural analysis of chitinase genes in Vitis vinifera. (a) The phylogenetic tree (axis numbers means the relative divergence time). (b) MEME analysis of the conserved motifs of chitinase (motif number was set as 20). The amino acid sequence of each motif was present in Figure S1. (c) The gene structure (exon-intron organization) of the chitinase genes.

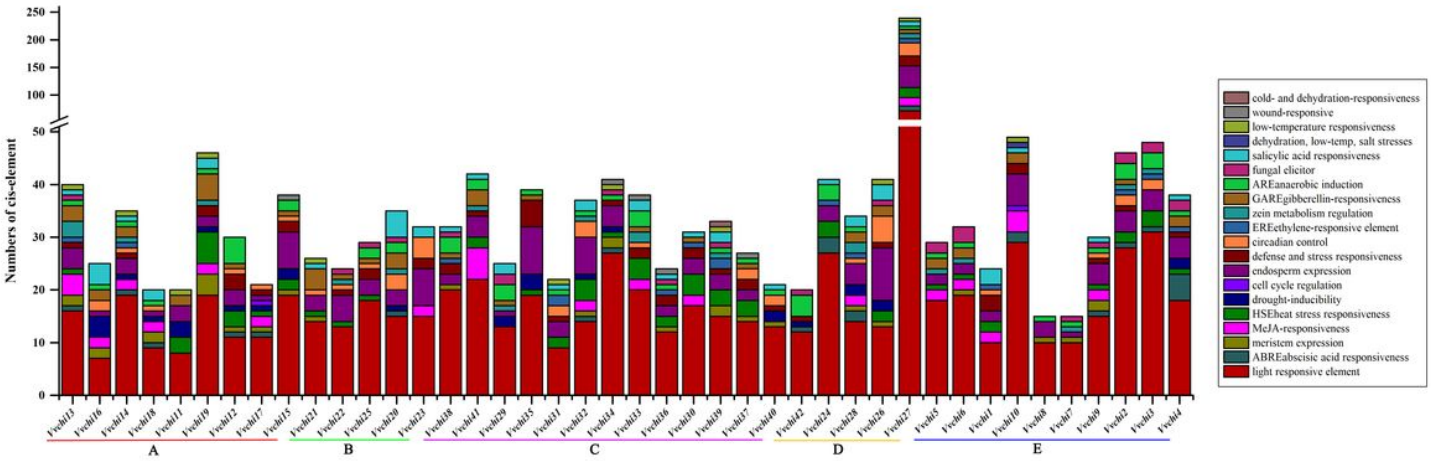

Figure 3

Cis-elements in the promoter of chitinase genes that are related to stress responses and plant development.

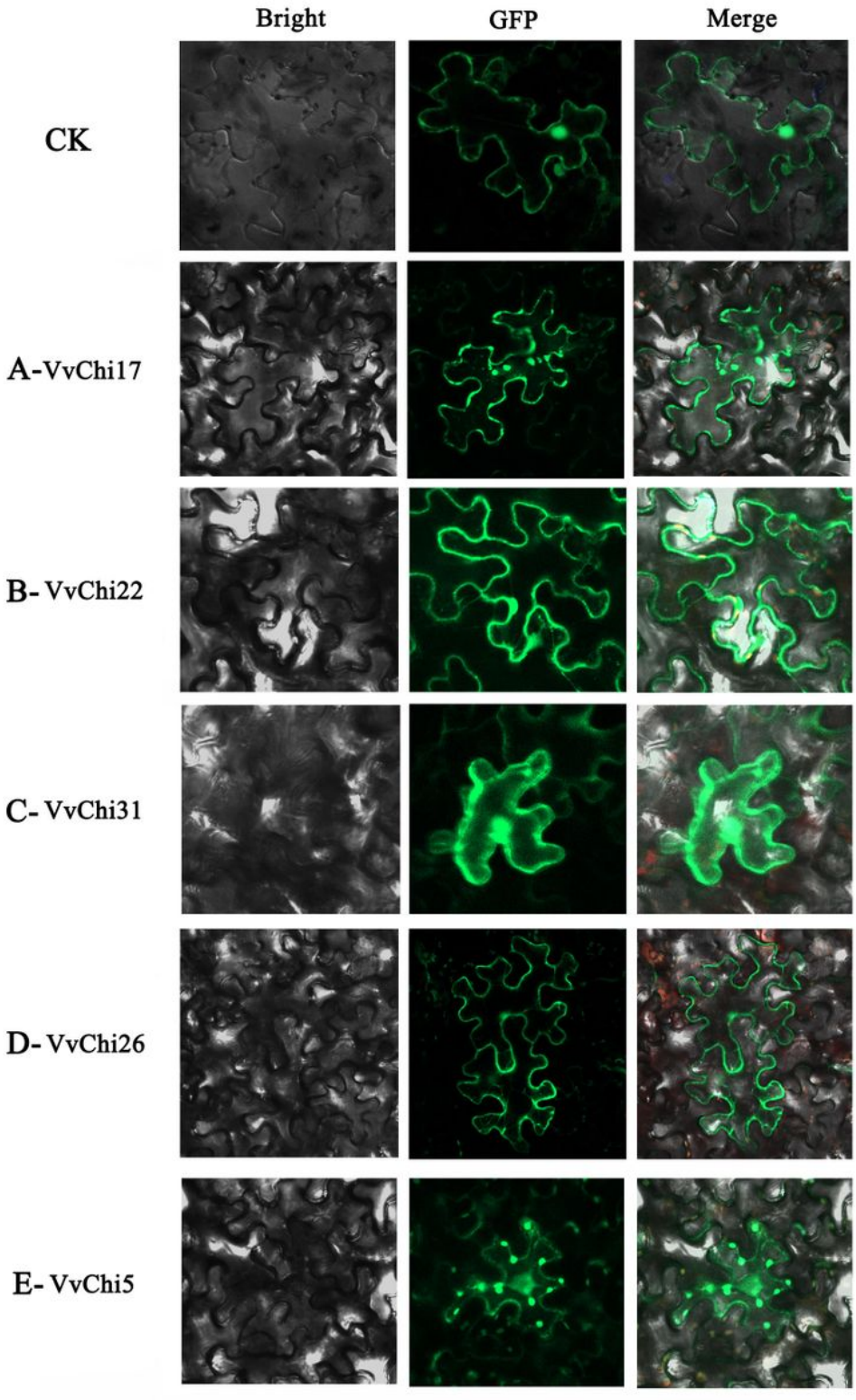

Figure 4

Subcellular localizations of five VvChis. Merged pictures include the green fluorescence channel and the bright channel.

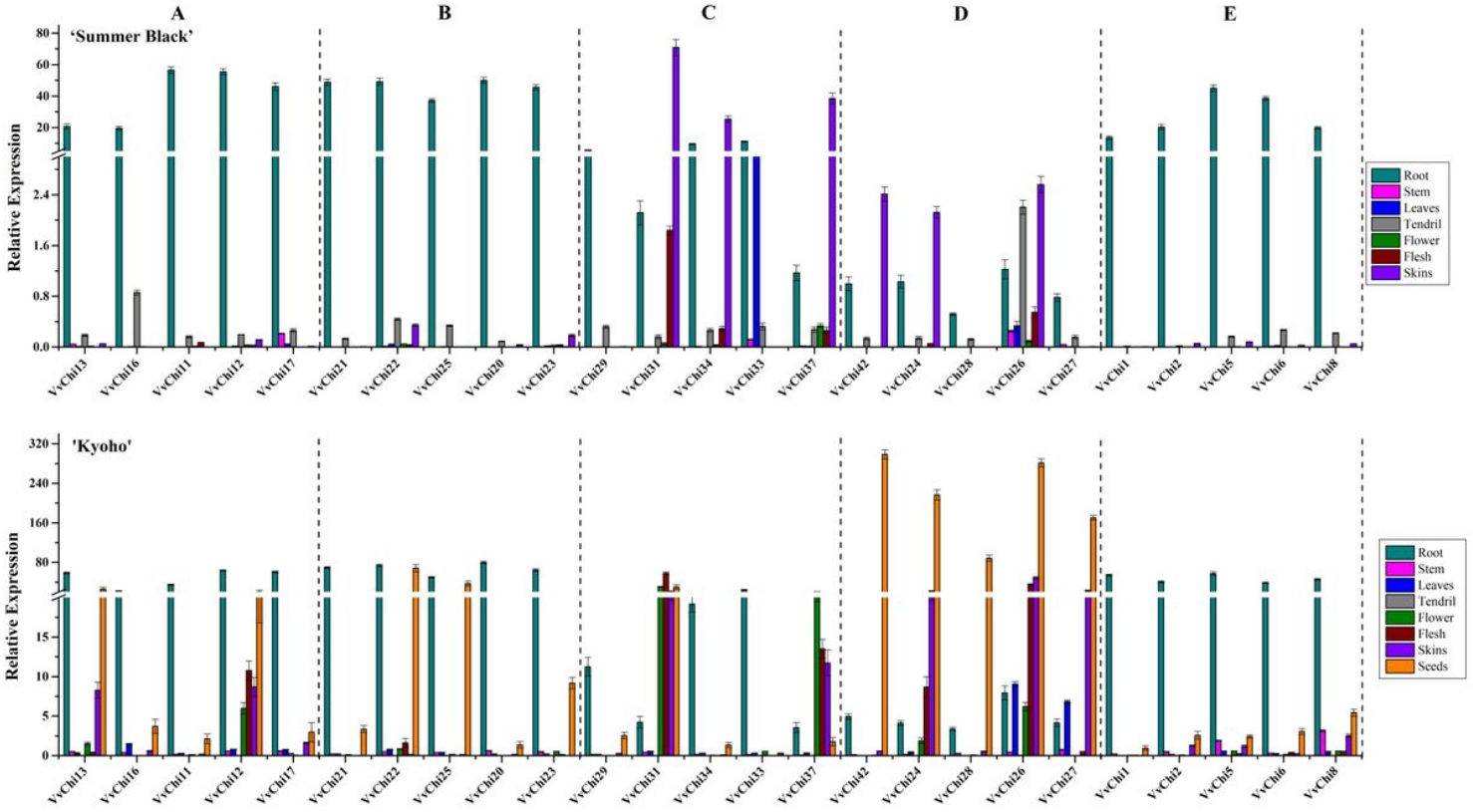

Figure 5

Relative expression levels of VvChis in different organs of Sunmmer Black and Kyoho. Values were normalized against the expression data of VvChis and are given as means ± standard error among three biological replicates.

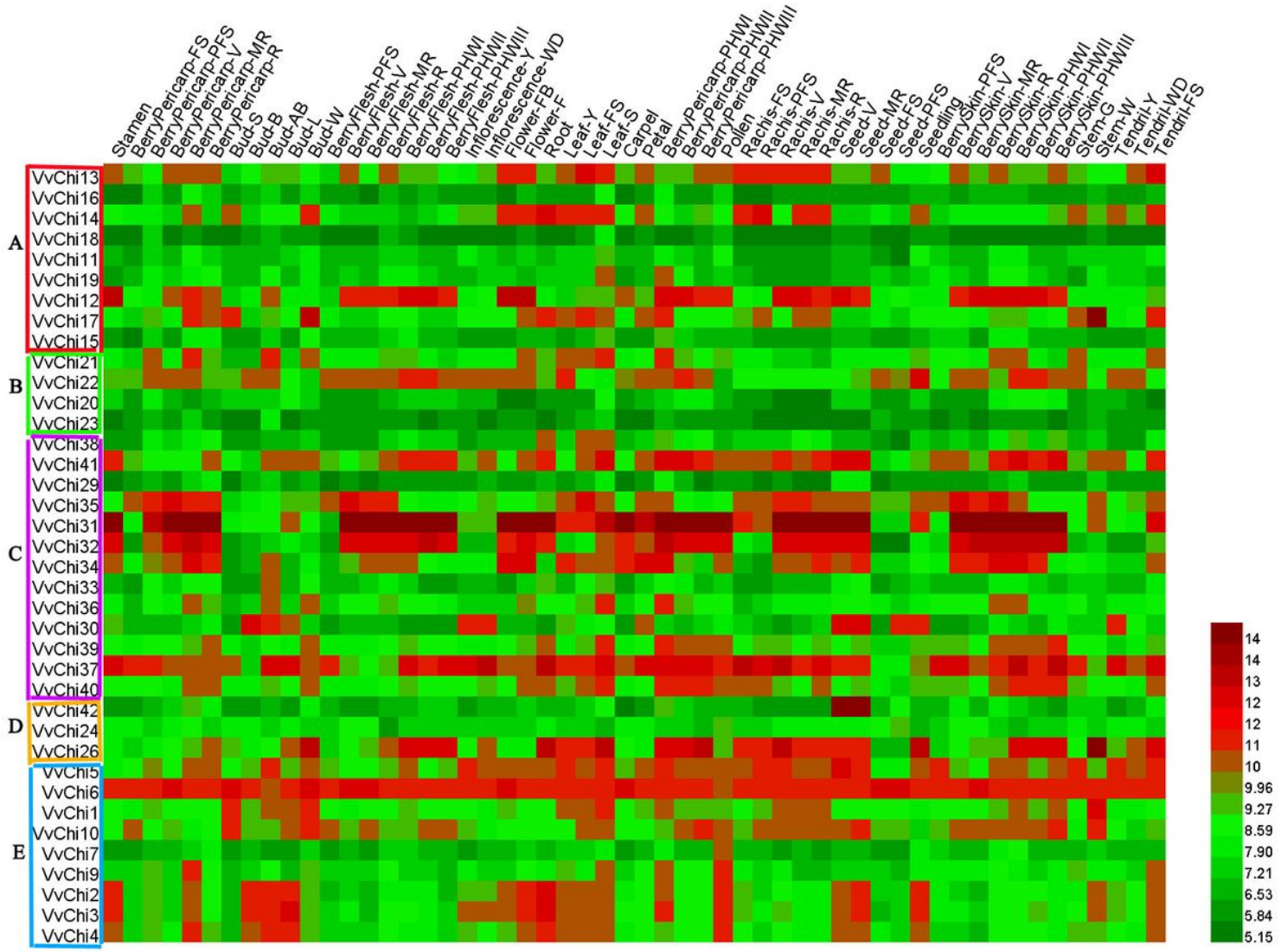

Figure 6

Expression profiles of grapevine VvChis in different tissues at different developmental stages. Red and green boxes indicate high and low expression levels, respectively. -AB: after-burst; -B: burst; -F: flowering; -FB: flowering begins; -FS: fruit set; -G: green; -L: latent bud; -MR: mid-ripening; -PFS: post-fruit set; -PHWI: post-harvest withering I (1st month); -PHWII: post-harvest withering II (2nd month); -PHWIII: post-harvest withering III (3rd month); -R: ripening; -S: swell; -V: véraison; -W: woody/ winter; -WD: well developed; -Y: young.

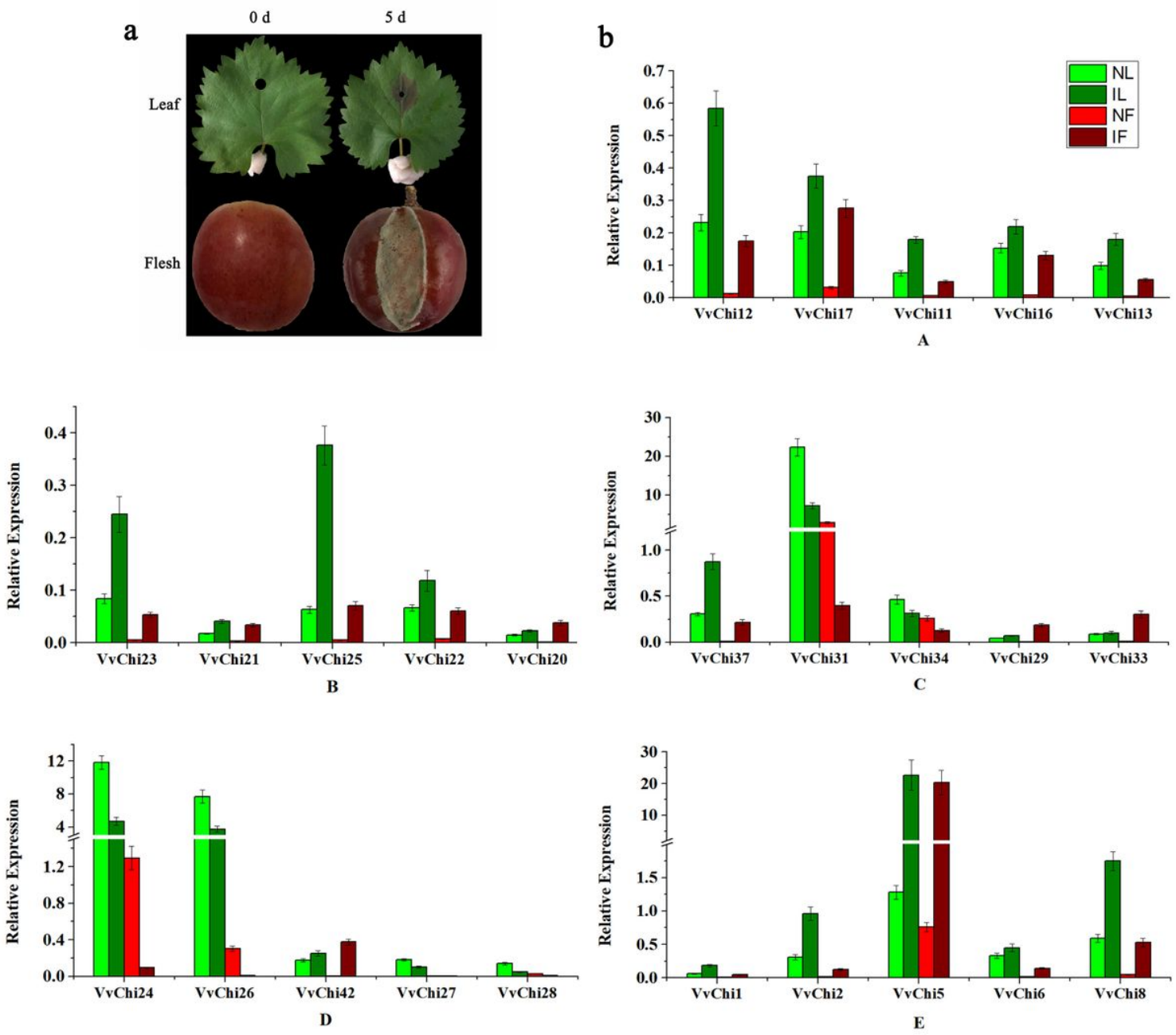

Figure 7

VvChis responsed to biotic stress. (a) Leave and berry state under different treatments after inoculation of Botrytis cinerea; (b) Gene expression level of VvChis under different treatments after inoculation of Botrytis cinerea

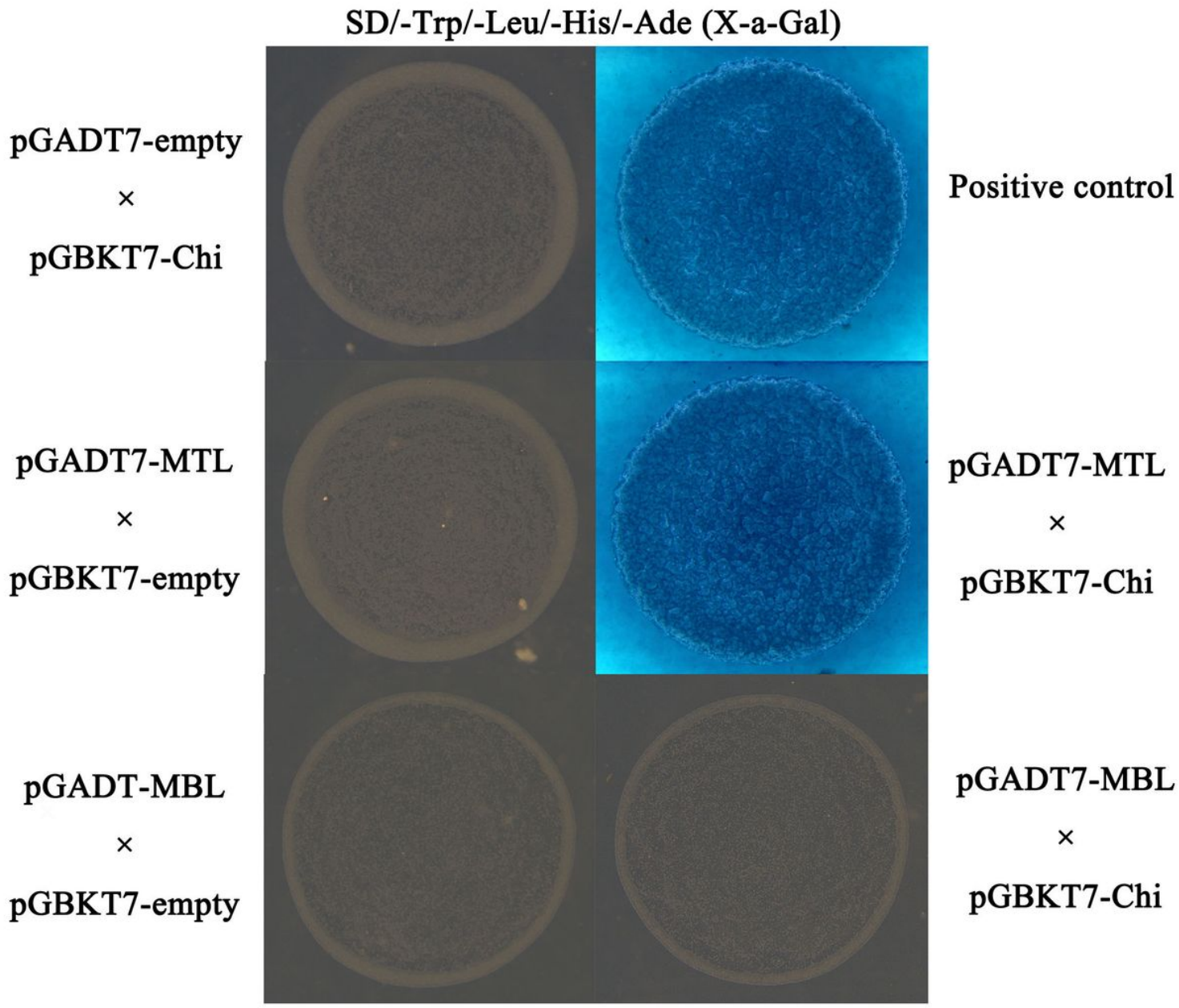

Figure 8

The interaction between Chi and MTL, MBL proteins by yeast two-hybrid

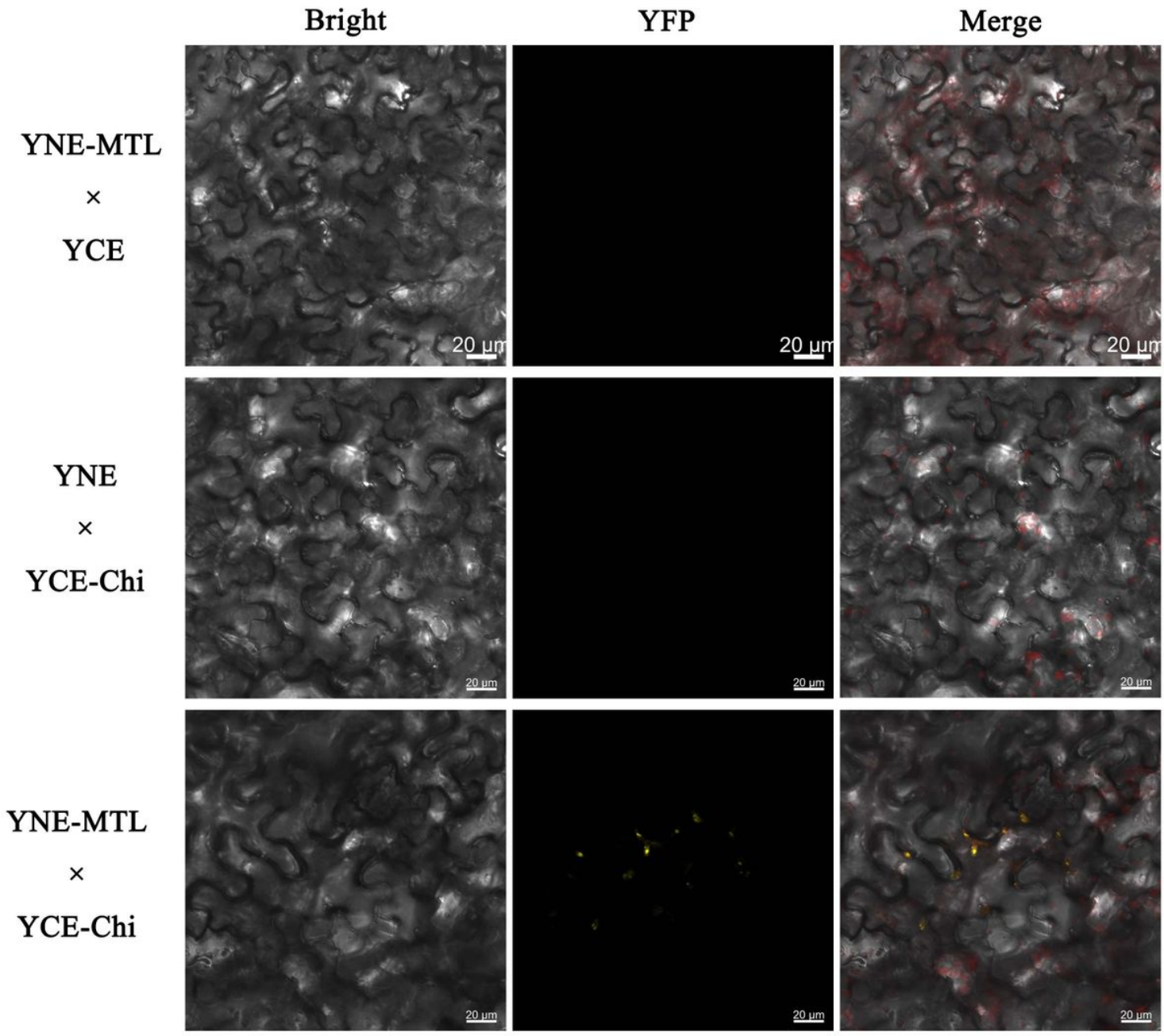

Figure 9

BiFC visualization of the Chi and MTL interaction in transiently co-expressed in N. benthamiana leaf. Note: Bright indicates bright field; YFP indicates fluorescence of YFP; Merged is digital merge of bright field and fluorescent images.

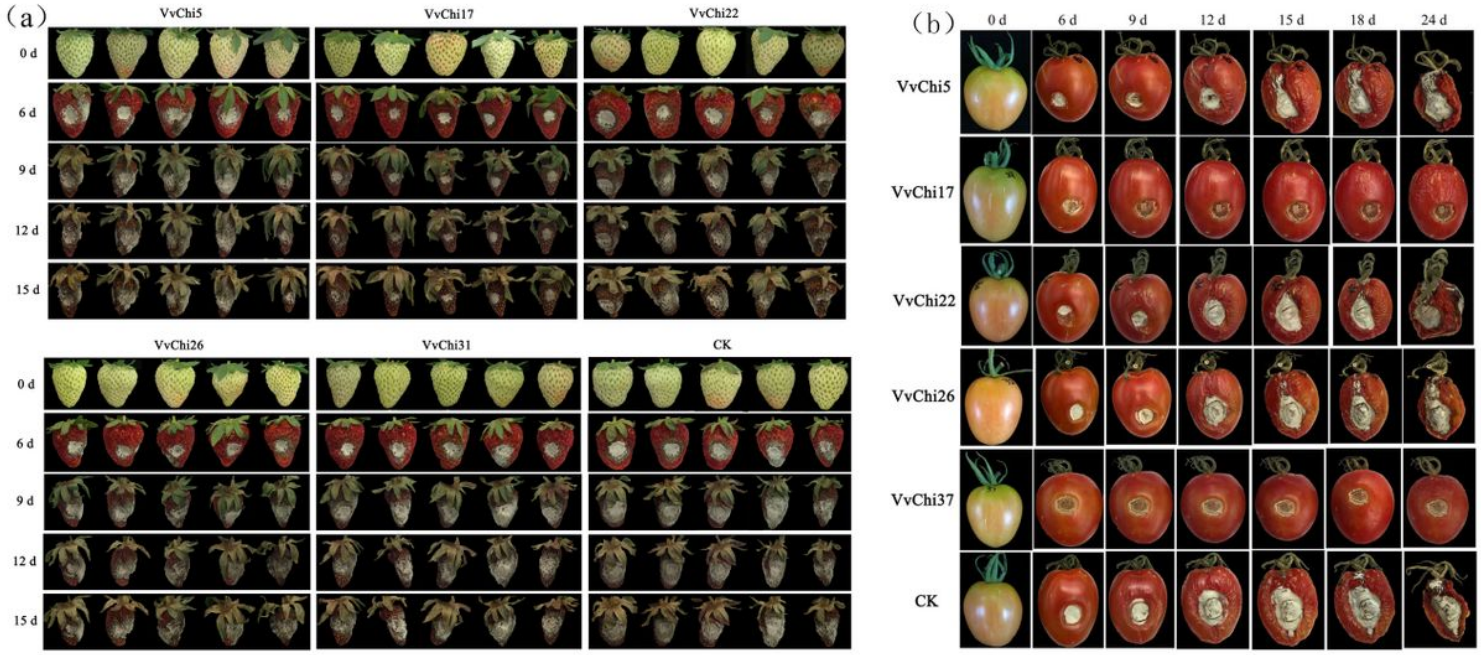

Figure 10

Effects of overexpression of VvChis on disease resistance of color-changing strawberry (a) and tomato (b) fruits.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS8.jpg
- TableS5.xlsx
- TableS6.xlsx
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