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*Article*

# Membrane Localized Gbtmem214s Participate in Modulating Cotton Resistance to Verticillium Wilt

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**Abstract:** Verticillium wilt (VW) is a soil borne fungal diseases caused by *Verticillium dahliae* Kleb, and lead to serious damage to cotton production annually in the world. In our previous study, a *transmembrane protein 214 protein* (*TMEM214*) gene associated with VW resistance was map-based cloned from *Gossypium barbadense* (*G. barbadense*). TMEM214 proteins are a kind of transmembrane protein, but their function in plants is rarely studied. To reveal the function of *TMEM214s* in VW resistance, all six *TMEM214s* were cloned from *G. barbadense* in this study. These genes were named as *GbTMEM214-1*, *GbTMEM214-4* and *GbTMEM214-7* according to their location on the chromosomes, and the encoded proteins are all located on cell membrane. *TMEM214* genes were all induced by *Verticillium dahliae* inoculation and showed significant differences between resistant and susceptible varieties, but the expression patterns of *GbTMEM214s* under different hormone treatments were significantly different. Virus-induced gene silencing analysis showed the resistance to VW of *GbTMEM214s*-silenced lines decreased significantly, which further proves the important role of *GbTMEM214s* in the resistance to *Verticillium dahliae*. Our study provides an insight into the involvement of *GbTMEM214s* in VW resistance, which was helpful to better understand the disease resistance mechanism of plants.

**Keywords:** Verticillium wilt; cotton; transmembrane protein; resistance; plant immunity

## 1. Introduction

Cotton production is seriously affected by Verticillium wilt (VW), and the breeding of resistant varieties has been a major problem faced by breeders. The main reason for this dilemma is the lack of immunity in cultivated varieties or high resistance to VW. With the improvement of biotechnology, transgenic technology is an important way to breed VW resistant varieties, and the core of this technology is to clone important VW resistant genes. In our previous study, a VW resistance-related transmembrane protein gene, named as *GbTMEM*, was cloned from *Gossypium barbadense* (*G. barbadense*) by map-based cloning [1]. As the result showed, *GbTMEM* was highly expressed in *G. barbadense* when infected by VW, and its silent expression would reduce disease resistance.

The resistance mechanism of cotton to VW is a very complex process, and the research progress is delayed by the lack of cotton germplasm resources immune to VW. With the development of biotechnology, various omics and high-throughput sequencing technologies have been applied to study the resistance mechanism of cotton to VW, and a large number of valuable data was obtained [2,3]. In addition, Virus-Induced Gene Silencing (VIGS) technology has been widely used in the study of cotton resistance mechanism.

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The function of resistance genes can be confirmed by specific silencing and observation of phenotypic changes [4,5]. These methods provide important reference for screening candidate resistance genes and play an important role in elucidating the regulatory network of cotton resistance.

The resistance mechanism of cotton to VW is a very complex biological process, involving a variety of substances and signaling pathways. Terpenoid aldehydes and phenylpropanoids, as well as reactive oxygen species, salicylic acid, jasmonic acid, ethylene, brassinosteroids and other signaling pathways are involved in cotton resistance to VW [4,6-10]. In the process of exploring the resistance mechanism of plants to VW, a large number of genes related to VW resistance were cloned. *Ve1* in tomato is one of the well-known resistance genes to VW. But studies have shown that this gene is not involved in the resistance of cotton to VW, which indicates the different resistance mechanisms between cotton and tomato [11]. Some *Ve* genes were also cloned in cotton, and the transgenic plants showed a certain degree of resistance to VW. However, further exploration of its resistance mechanism has not been reported (Zhang et al., 2011; Zhang et al., 2012). In addition to *Ve* genes, a large number of genes related to VW resistance were cloned from cotton genome, including *GbRLK*, *GhPAO*, *Gbvdr*, *GbTLP1*, *GbERF1-like*, *GhSAMDC*, *GbNRX1*, *GhLMM* and so on [10,12-17]. In addition, some exogenous genes can also improve the resistance of cotton to VW, such as *Hpa1Xoo*, *p35*, *NaD1*, *GAFPs* and *Hcm1* [18-21]. In this study, we found that a new class of genes, TMEMs, also play an important role in resistance of cotton to VW.

Transmembrane proteins are a kind of special proteins located in the biofilm, which mainly undertake the function of intracellular and extracellular environmental information and material exchange, and are indispensable in biological activities. In the process of plant disease resistance, transmembrane proteins can recognize and accept pathogen signals, activate intracellular reactions, transmit extracellular signals to the intracellular, and induce defense responses. A large number of resistance-related transmembrane protein genes have been cloned in plants, such as *Xa21*, *Pi-d2*, *FLS2*, *Ve1*, and *PigmR* [22-25]. But the resistance function of *TMEMs* in plants has never been reported. A series of studies have shown that the TMEM protein family in animals is involved in intercellular signal transduction, immune-related diseases and tumor development [26,27]. TMEMs have been proved to be participated in many physiological processes, such as the formation of plasma membrane ion channels, activation of signal transduction pathways, mediating apoptosis and autophagy [27]. However, the related research is still in its infancy, and the functions of these proteins have not been fully revealed. In this study, other members of the TMEM gene family in *G. barbadense* were cloned. VIGS and expression analysis showed that these genes were also involved in resistance of cotton to VW.

## 2. Results

### *2.1. Identification and phylogenetic analysis of GbTMEM214s*

In our previous study, a VW resistant gene, *GbTMEM214*, on chromosome D4 of *G. barbadense* was identified by QTL mapping [1]. The gene was located on the chromosome D4, and its resistance function was verified by qRT and VIGS experiments. Two other genes in *TMEM214* gene family were cloned from chromosome D1 and D7 in this study. The three genes were respectively named as *GbTMEM214-1*, *GbTMEM214-4* and *GbTMEM214-7* according to the chromosomes they are located on.

A comparison of gene structures revealed differences among the homologs of Gb*TMEM214s* (Figure 1). The structures of *GbTMEM214-1* homologous genes in A- and D- genomes of *G. hirsutum* and *G. barbadense* were same. The homologous genes of *GbTMEM214-4* were different in the first exon of 3'UTR region, and similar differences existed among homologous genes of *GbTMEM214-7*. Proteins encoded by *TMEM214* ho-

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mologous genes in *G. barbadense* were further subjected to phylogenetic tree analysis together with homologous genes in other plants. It was shown that the genetic relationship of TMEM214 homologous proteins between A- and D- genomes was very close (Figure 2). Among TMEM214s in cotton, GbTMEM214-4 was closer to EOY01031 and EOY01030 in cocoa, while GbTMEM214-7 was closer to EOY03934.

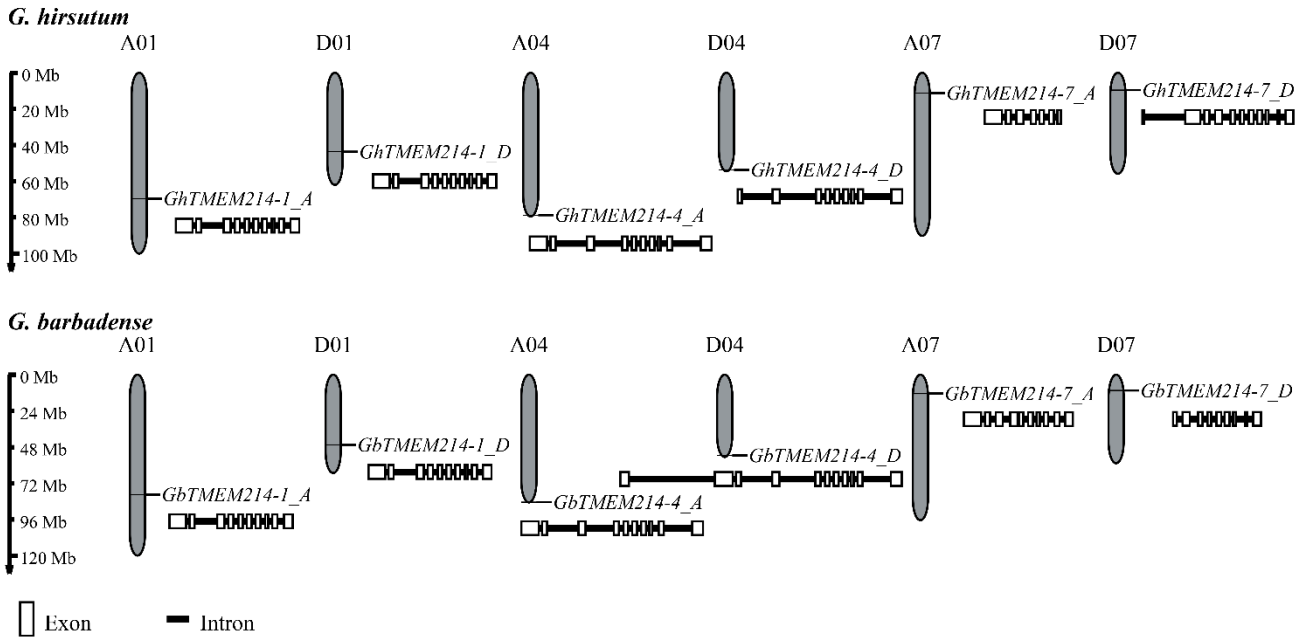


**Figure 1.** The position of *TMEM214* genes in chromosomes of *G. barbadense* and *G. hirsutum*. The *TMEM214* genes in cotton were predicted with HMM and HMMER 3.0 software with HMM seed file of TMEM214 (PF10151). *GbTMEM214s and GhTMEM214s* were named according to their localization on the chromosome of *G. barbadense* and *G. hirsutum* respectively. The gene structure was analyzed with GSDS 2.0 (http://gsds.cbi.pku.edu.cn/). The exons were boxed and the intron were lined.

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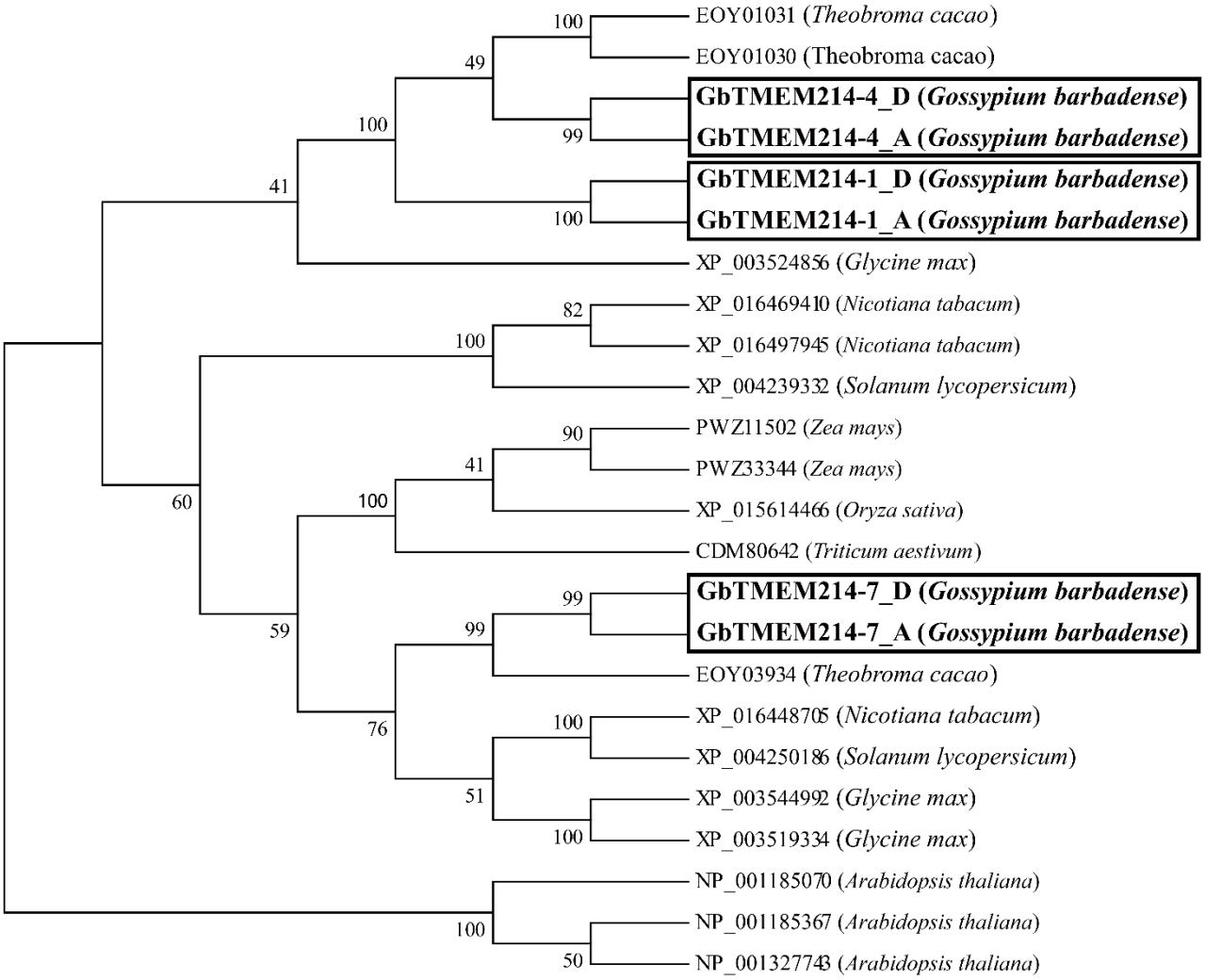


**Figure 2.** The phylogenetic trees of TMEM214 proteins in plants. MEGA 5.05 was applied to construct the phylogenetic trees of TMEM214 proteins. GbTMEM214s from *G. barbadense* were fontly bolded and marked by box.

### *2.2. Protein structure and subcellular localization of GbTMEM214s*

To understand *TMEM214s* in cotton, bioinformatic and molecular biologic experiments were carried out to analyze the characteristics of GbTMEM214s. As shown in the results, the homologous proteins of GbTMEM214s in A- and D- genomes are highly similar in secondary and 3D structures (Figure 3). Protein domain analysis showed that all six GbTMEM214s contained a TMEM214 domain of different sizes (Figure 3a). The TMEM214 domain of GbTMEM214-7 was significantly shorter than other GbTMEM214 proteins. At N-terminal of protein sequence, GbTMEM214-4 and GbTMEM214-7 contained one and two low complexity regions, respectively. Although the three GbTMEM214s were classified in TMEM214 protein family, the 3D modeling results showed the wide difference among the 3D model structures (Figure 3b). Although no transmembrane region was found in the protein sequences and the 3D structures were quite different, the three proteins were co-localized with PIP2A on the cell membrane as subcellular localization results showed (Figure 4).

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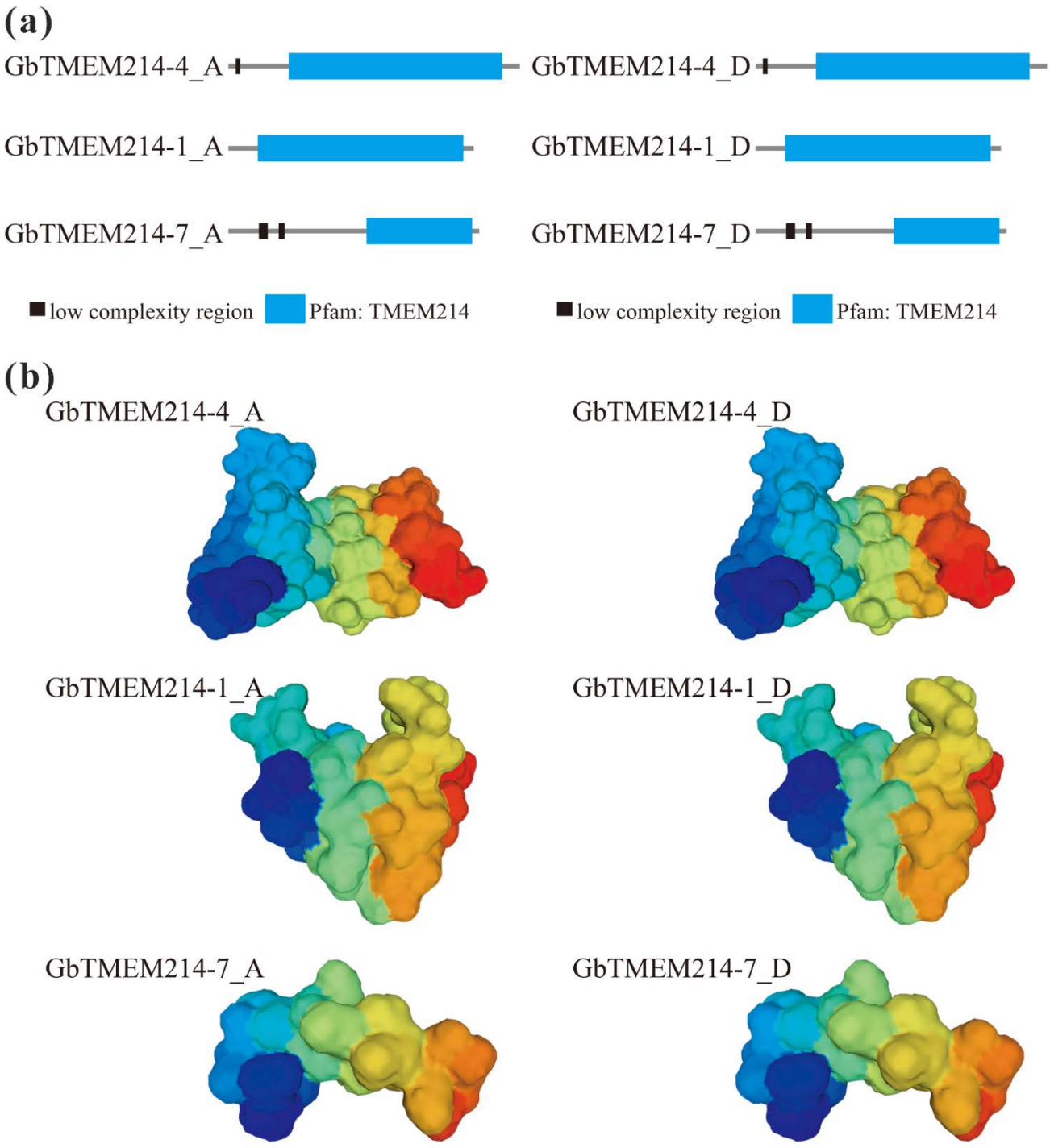


**Figure 3.** The protein structure of GbTMEM214s. (a) Protein sequence of GbTMEM214s in D-genome were analyzed with Simple Modular Architecture Research Tool (SMART, http://smart.embl-heidelberg.de/). Low complexity region and TMEM214 domain were represented by black and blue boxes, respectively. (b) 3D structural models of GbTMEM214s D-genome.

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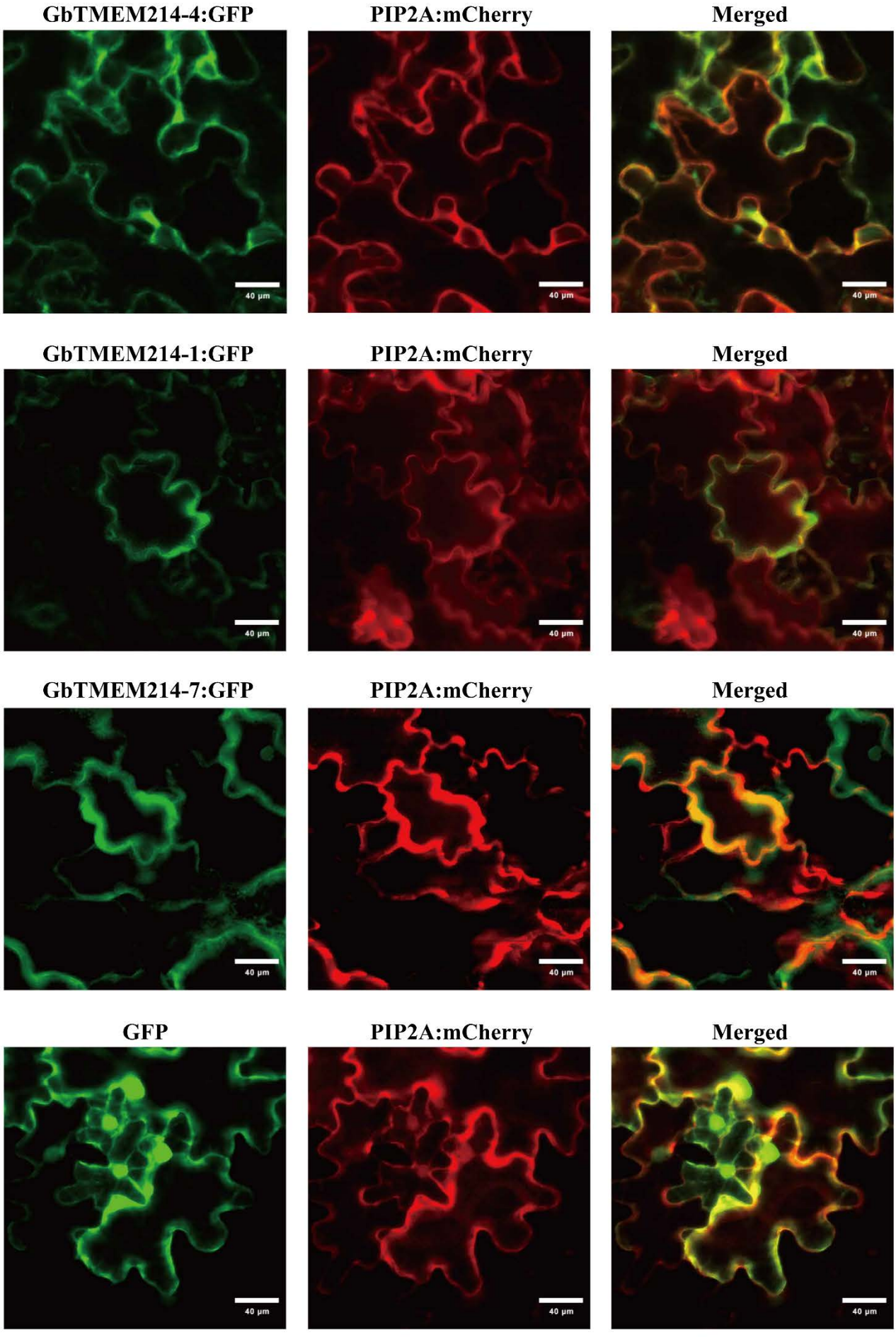

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**Figure 4.** Subcellular localization of GbTMEM214s. Subcellular localization of GbTMEM214s-GFP in tobacco leave. The constitutive GFP serves as a control. AtPIP2A-mCherry was used as a membrane localization marker. The green signal of GFP was fused with the red signal of membrane marker to determine the localization of protein. Bars=40 μm.

*2.3. Verticillium dahliae induced expression analysis of TMEM214s in cotton*

In order to find out the differential expression of *TMEM214s* in susceptible and resistant cotton varieties infected by *Verticillium dahliae* (*V. dahliae*), qRT-PCR was applied to examine the expression of homologs in TM-1 and H7124. (Figure 5). As shown in result, all three *TMEM214s* were induced by *V. dahliae* inoculation and involved in the response to *V. dahliae*. The expression level of *GbTMEM214-4* in H7124 was significantly higher than *GhTMEM214-4* in TM-1 at 24 h and 48 h after inoculation, reaching 5.57-fold and 19.09-fold, respectively. However, the expression of *GbTMEM214-1* and *GbTMEM214-7* in H7124 was lower than their homologous genes in TM-1, *GhTMEM214-1* and *GhTMEM214-7*, from 48 h to 144 h after inoculation, and the expression of *GhTMEM214-7* was significantly increased by 6.00, 1.85 and 3.16-fold, respectively. Overall, the expression of *TMEM214-4* in resistant variety was significantly higher than that in susceptible variety, while the expression of *TMEM214-1* and *TMEM214-7* in resistant and susceptible varieties was not significantly different.

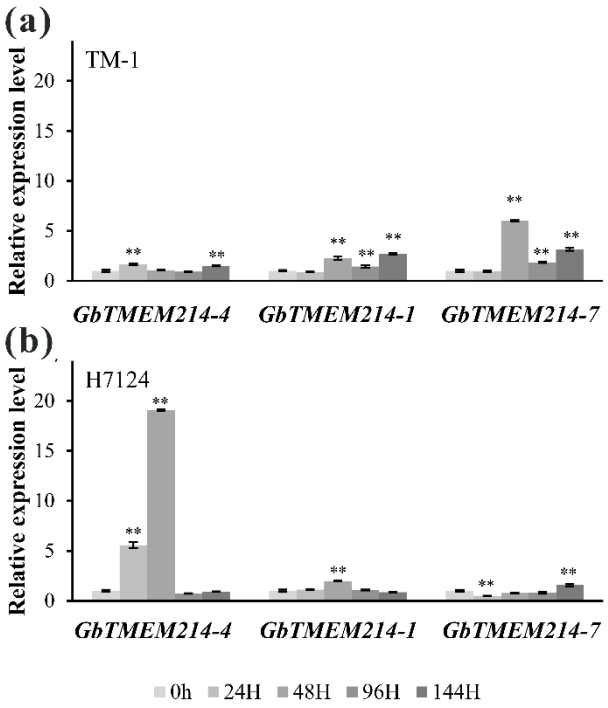


**Figure 5.** The relative expression levels of *TMEM214* genes under the challenge of *V. dahliae*. (a) *GhTMEM214* genes in susceptible variety TM-1; (b) *GbTMEM214* genes in resistant variety H7124. The qRT-PCR was applied to analyze the expression of *TMEM214 genes*. "*", "**" represent significant differences relative to each control and *P*-value<0.05 or *P*-value<0.01, based on student's *t*-test. Each value was the mean ±SD of three biological determinations.

*2.4. Phytohormone induced expression analysis of GbTMEM214s*

Under phytohormone induction, the expression patterns of *GbTMEM214s* were quite different. Following treatment, *GbTMEM214-4* was induced by Jasmonic acid (JA), salicylic acid (SA), gibberellin (GA) and ethylene (ET), and reduced by indole-3-acetic acid (IAA) and Zeatin. *GbTMEM214-1* was induced by JA, SA, abscisic acid (ABA), GA, Zeatin and ET, and reduced by IAA and brassinosteroids (BR). *GbTMEM214-7* was induced by

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JA, SA, IAA, GA, and ET, and reduced by ABA, BR and Zeatin (Figure 6). Under ET treatment, *GbTMEM214-4* was highest up-regulated with a maximum of 8.42-fold at 10 h, but the expressions of *GbTMEM214-1* and *GbTMEM214-7* were not so obvious. Among the eight hormones, *GbTMEM214-1* was highest up-regulated by JA with a maximum of 5.48-fold at 1 h, and *GbTMEM214-7* was highest up-regulated by IAA with a maximum of 4.00-fold at 1 h. Three *GbTMEM214s* were not induced by BR, and the expression levels even decreased at 12 h after treatment.

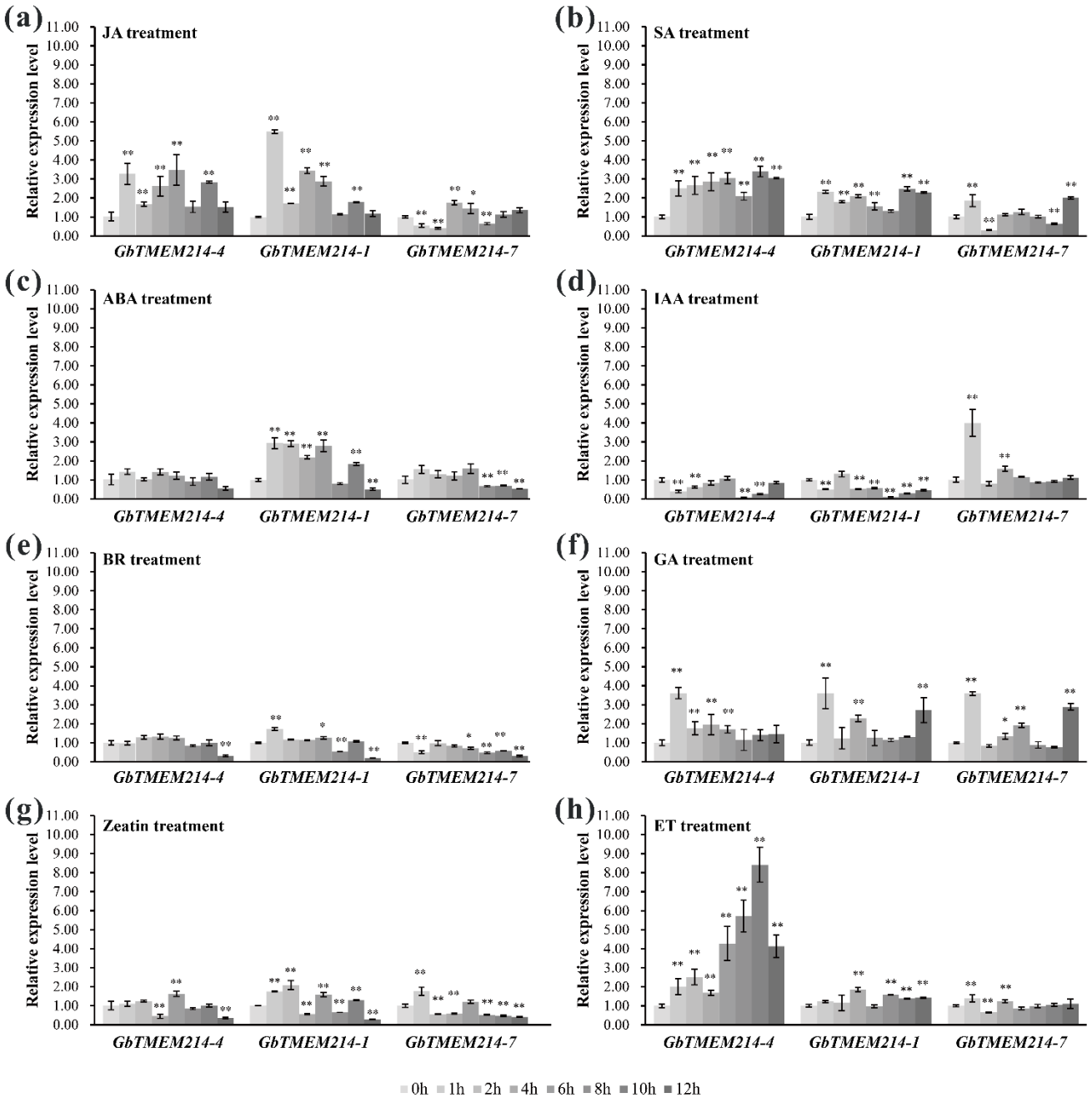


**Figure 6.** The relative expression levels of *GbTMEM214* genes with the treatment of Phytohormone. (a) Jasmonic acid (JA) treatment; (b) Salicylic acid (SA) treatment; (c) Abscisic acid (ABA) treatment; (d) Indole-3-acetic acid (IAA) treatment; (e) Bbrassinosteroid (BR) treatment; (f) Gibberellin (GA) treatment; (g) Zeatin treatment; (h) Ethylene (ET) treatment. The qRT-PCR was applied to analyze the expression of *TMEM214 genes*. "*", "**" represent significant differences relative to each control and *P*-value<0.05 or *P*-value<0.01, based on student's *t*-test. Each value was the mean ±SD of three biological determinations.

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*2.5. Resistance function analysis of GbTMEM214s*

To verify the resistance function, *GbTMEM214s* was silenced by VIGS to define loss-function in response to the pathogen. Two weeks after VIGS infiltration, the positive control, TRV:*GbCLA*, showed obvious photobleaching phenotype, and the expression of *GbTMEM214s* in silenced plants was significantly down-regulated (Figure 7a, 7c). After inoculation with V. dahliae, all three *GbTMEM214s*-silenced lines exhibited more wilting, etiolated and even abscission of leaves than mock (Figure 7b). The disease index was calculate 25 days after inoculation, which reached 78.8%, 70.8% and 75.0% respectively for *GbTMEM214-4*, *GbTMEM214-1* and *GbTMEM214-7* deficient lines, compared with 39.3% for Hai7124 and 41.7% for the mock treatment line (Figure 7d). The increased susceptibility of silenced lines indicated the important role of *GbTMEM214s* in cotton resistance to *V. dahliae*.

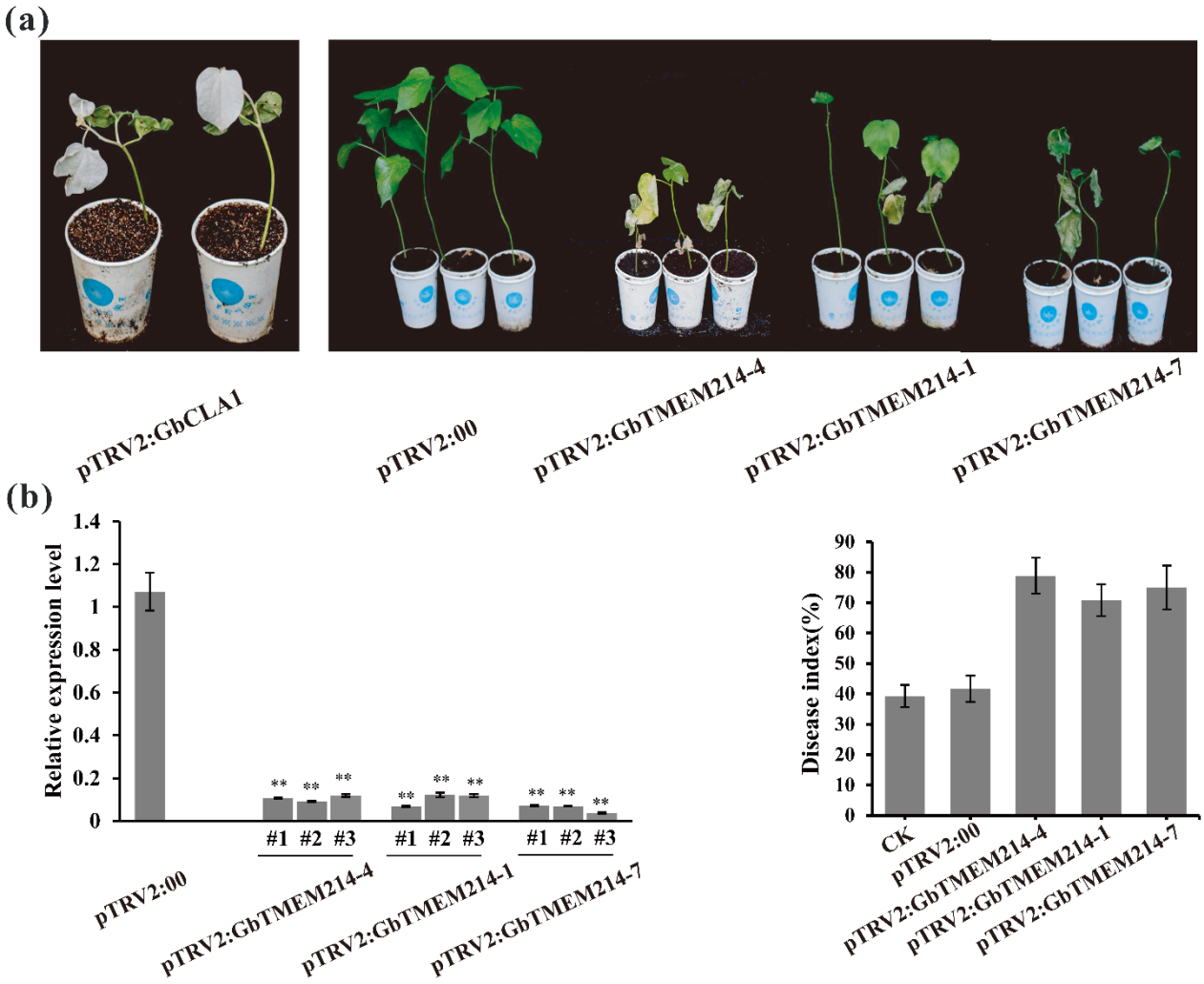


**Figure 7.** Resistance function analysis of the *GbTMEM214* genes by VIGS. (a) The cotton gene *GhCLA1* was used as a positive control, and VIGS of *GhCLA1* results in a phenotype of white leaves; (b) The phenotypes of H7124 under infection by *V. dahliae* after VIGS with *Agrobacterium* carrying *pTRV2:GbTMEM214s* and *pTRV2:00*, and the photos were taken at 42 days after *V. dahliae* inoculation; (c) qRT-PCR analysis of the expression levels of *GbTMEM214s* in the silenced lines; (d) The disease index of plants with silenced *GbTMEM214s*. The results were evaluated at 28 d after *V. dahliae* inoculation, with three replications containing at least 20 plants each. "*", "**" represent significant differences relative to each control and *P*-value<0.05 or *P*-value<0.01, based on student's *t*-test. Each value was the mean ±SD of three biological determinations.

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## 3. Discussion

Transmembrane proteins are a class of proteins with unique structure, which are ubiquitous in various animal and plant cells. According to plant genome data, 20 % to 30 % of the proteins have transmembrane domains, indicating that these proteins play a very important and extensive role [28].

Based on a previously cloned disease resistance gene, *GbTMEM214*, the function of its homologous gene in *G. barbadense* against VW was analyzed in this study. The results showed that these genes played an important role in cotton disease resistance. Plant immune system is composed of cell surface and intracellular immunity [29]. In cell surface immunity, immune receptors sense common signatures of pathogens outside the host cell via extracellular domains (ECDs) and initiate cellular responses to resist infection via intracellular kinase domains (KDs) [30]. Membrane-localized receptor-like kinases (RLKs) and receptor-like proteins (RLPs) are two major components of cell-surface immunity to detect signatures of infection [31]. RLKs contain a variable extracellular domain mediating pathogen recognition, a single-pass transmembrane domain, and an intracellular KD transducing signal to downstream immune pathways [32]. Whereas RLPs exhibit a similar overall structure to RLKs, but only contain a short intracellular tail, lack kinase domain, and require co-receptor to transduce signals [33,34]. Cell-surface immune receptors, also known as pattern recognition receptors (PRRs), monitor the extracellular environment for pathogen invasion patterns, including microbial-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs) [35,36]. In the process from sensing patterns to immune responses, co-recepters are required to transduce immune signals [37,38]. In this study, GbTMEM214s were found to be located on the cell membrane of plants, and they were considered to play an important role in cell surface immunity. Based on the analysis of the sequence and structure of the three GbTMEM214 proteins, they were obviously different in the secondary and 3D structures. Therefore, the members of this protein family were speculated to play different roles in the resistance of cotton to VW, but the specific mechanism remained to be further studied.

*TMEMs* belong to a large gene family containing TMEM domain, but their functions have been rarely studied and even not reported in plants. According to existing functional studies, it has been found that *TMEMs* in animals are related to intracellular signal transduction, immune-related diseases, and tumorigenesis, but the function of most genes in this family is still unclear [39]. The immune system of plants is similar to the innate immune system of animals [40]. But as plants lack an adaptive immune system, they rely solely on natural immunity against pathogens. As reported, several TMEM proteins in animal were found to be involved in immune related diseases. TMEM9B can activate NF-κB pathway induced apoptosis, and act as an important factor in TNF-activated MAPK signaling pathway [41,42]. TMEM176 is related to transplantation immunity, and its overexpression can inhibit rejection after transplantation [43]. TMEM214 in animal, which belongs to the same subfamily as GbTMEM214 in this study, mediates endoplasmic reticulum stress-induced Caspase 4 activation and apoptosis [44]. Unlike GbTMEM214s, which have only one TMEM domain, the animal TMEM214 protein contains two transmembrane domains at its C terminus and a large at N terminus, and these domains are essential for the function of TMEM214. Nevertheless, current understanding of TMEM in animals is still not sufficient, and plant immunity is quite different from animal, which limit the implication of the functional mechanism of GbTMEM214 from animal studies. Our study provides an important insight into the involvement of *GbTMEM214s* in plant disease resistance, but the molecular mechanism remains to be revealed through further experiments.

## 4. Materials and Methods

### *4.1. The bioinformatics analysis of TMEM214 superfamily*

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The genome data were downloaded at CottonFGD (https://cottonfgd.org/). The TMEM214 proteins in cotton were predicted with Hidden Markov Model (HMM) and HMMER 3.0 software. The HMM seed file of TMEM214 (PF10151) were obtained from Pfam database (http://pfam.sanger.ac.uk/). The protein sequences of TMEM214 in other plants were obtained from NCBI (https://www.ncbi.nlm.nih.gov/). The sequence alignment of TMEM214 proteins were performed with ClastalX 1.83 software. The result was visualized with GENDOC software. The maximum likelihood method in MEGA 5.05 software was applied to build the phylogenetic trees of *TMEM214* genes. The ORFs were predicted with the Fgenesh subroutine in MolQuest 2.3.3 software. The gene structure was analyzed with GSDS 2.0 (http://gsds.cbi.pku.edu.cn/).

*4.2. Protein structure analysis of TMEM214s*

Domains in TMEM214 proteins were identified using the Simple Simple Modular Architecture Research Tool (SMART, http://smart.embl.de) following the instruction. Homology modelling of protein structures was applied to construct the 3D structural models using SWISS-MODEL web server (https://swissmodel.expasy.org/).

*4.3. Plant materials and treatments*

VW resistant variety Hai7124 and susceptible variety TM-1 were used in this study. Cotton seedlings were grown in greenhouse at 28 °C during the day/night period of 16 h/8 h for 2 weeks. The *V. dahliae* strains were cultured on potato dextrose agar medium (PAD) at 24 °C for 5 days, in Czapek's medium at 25 °C for 5 days, and then adjusted to $1\times10^7$ conidia/mL with deionized water for inoculation. The seedlings were inoculated with *V. dahliae* by dip-inoculation method, and the root samples were harvested at 0, 24, 48, 96 and 144 h, respectively.

*4.4. RNA isolation and expression pattern analysis*

Total RNA was extracted from root samples using (Omega Bio-tek) according to the manufacturer's instructions, and cDNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). Quantitative PCR primers of *GbTMEMs* and *histone 3* (AF024716) were designed using Beacon Designer 7.0 software or following the previous studies (Table S1), in which *histone 3* was used as reference gene. Quantitative PCR was performed using TB Green qPCR Master Mix (TaKaRa) on ABI QuantStudio 5 PCR System.

*4.5. Cloning of the GbTMEM214s in Hai7124*

Gene-specific primers of *GbTMEMs* were designed using Primer Premier 5 software (Table S1). Standard PCR reactions were performed using Ex Taq Hot Start Version DNA Polymerase (TaKaRa) to amplify the *GbTMEMs* with complete ORFs. The final product was cloned into pMD19-T Vectors (TaKaRa) and transformed into *E.coli* strain DH5$\alpha$.

*4.6. VIGS experiments*

VIGS was performed using the binary pTRV1 and pTRV2 vectors to silence *GbTMEMs*. TRV2: *GbCYP72A* and TRV2: *GbCLA1* vectors were constructed by inserting the 3'UTR region specific sequences into pTRV2, and subsequently transformed into *Agrobacterium tumefaciens* (*A. tumefaciens*) strain GV3101. The *A. tumefaciens* cultures were grown overnight at 28 °C on solid LB medium (50 μg/ml kanamycin, 25 μg/ml gentamicin), and then inoculated into liquid LB medium (50 μg/ml kanamycin, 25 μg/ml gentamicin, 10 mM MES and 20 μM acetosyringone). The cultures were grown overnight in 28 °C shaker. Cells were harvested and re-suspended in infiltration medium (10 mM MgCl2, 10 mM MES and 200 μM acetosyringone). The cell suspensions were adjusted to an O.D. of 2.0 and incubated at room temperature for 3 h. The *A. tumefaciens* cells containing pTRV1 and pTRV2 vectors were mixed at a ratio of 1:1 and infiltrated into two fully expanded cotyledons of 2-week-old cotton plants. Thirty plants per VIGS treatment were infected with *V. dahliae* as previously descripted [1].

*4.7. Subcellular localization of GbTMEMs*

The ORFs of *GbTMEMs* were inserted into pBin-GFP4 vector to express GbTMEMs-GFP fusion protein. The vector expressing AtPIP2A-mCherry fusion protein was used as positive control [45]. The vectors were transformed into *A. tumefaciens* strain GV3101, and the *A. tumefaciens* cells containing each vector were mixed at a ratio of 1:1 and infiltrated into *Nicotiana benthamiana* (*N. benthamiana*) leaves to transiently co-express fusion proteins following previous study [46]. Visualization of fluorescence signals was observed using confocal laser scanning microscope (Zeiss, EVO-LS10) 3 d after infiltration.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Primer pairs used in this study.

**Author Contributions:** Conceptualization, S.X. and J.Z.; methodology, J.X.; laboratory investigation, J.Z., J.X. and Y.W.; bioinformatics analysis, L.Z. and Z.X.; data curation, Q.G., P.X. and J.C.; field investigation, J.L., C.D., X.W. and J.W.; Plant material cultivation, N.A. and G.F.; writing—original draft preparation, S.X. and J.X.; writing—review and editing, S.X., J.X. and J.Z. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Not applicable.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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