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Expression of pathogenesis-related genes in cotton roots in response to *Verticillium dahliae* PAMP molecules

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Verticillium wilt disease becomes a major threat to many economically important crops. It is unclear whether and how plant immunity takes place during cotton-*Verticillium* interaction due to the lack of marker genes. Taking advantage of cotton (*Gossypium hirsutum*) genome, we discovered pathogenesis-related (*PR*) gene families, which have been widely used as markers of immune responses in plants. To profile the expression of *G. hirsutum PR* genes in the process of plant immunity, we treated cotton roots with two immunogenic peptides, flg22 and nlp20 known as pathogen-associated molecular patterns, as well as three *Verticillium dahliae*-derived peptides, $nlp20^{Vd2}$, $nlp23^{Vd3}$, and $nlp23^{Vd4}$ which are highly identical to nlp20. Quantitative real-time PCR results revealed that 14 *G. hirsutum PR* gene (*GhPR*) families were induced or suppressed independently in response to flg22, nlp20, $nlp20^{Vd2}$, $nlp23^{Vd3}$, and $nlp23^{Vd4}$. Most *GhPR* genes are expressed highest at 3 h post incubation of immunogenic peptides. Compared to flg22 and nlp20, $nlp20^{Vd2}$ is more effective to trigger up-regulated expression of *GhPR* genes. Notably, both $nlp23^{Vd3}$ and $nlp23^{Vd4}$ are able to induce *GhPR* gene up-regulation, although they do not induce necrosis on cotton leaves. Thus, our results provide marker genes and new immunogenic peptides for further investigation of cotton-*V. dahliae* interaction.

Verticillium wilt, *Gossypium hirsutum*, *PR* gene, PAMP, nlp20

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INTRODUCTION

Verticillium dahliae is a soil-borne fungal pathogen that infects over 200 plant species (Klosterman et al., 2009). This fungus can penetrate host plants from root surface, and then colonize the vascular tissues to produce spores (Inderbitzin et al., 2014; Zhao et al., 2014; Zhao et al., 2016; Zhou et al., 2017). Due to the long-term survival in the soil and host "inside" colonization, *V. dahliae* is very difficult to control and becomes major threat to many economically important crops, including cotton, sunflowers, and potato (Inderbitzin et al., 2014). It is unclear how plants respond to *V. dahliae* infection, and how *V. dahliae* overcomes host immunity for successful colonization.

Plant innate immunity triggered by perception of pathogen-associated molecular patterns (PAMPs) is well-known as the first barrier to guard the invasion of pathogens (Jones and

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Dangl, 2006; Li et al., 2016). For instance, bacterial flagellin (flg22) and fungal cell wall component chitin are recognized by plant plasma membrane pattern recognition receptors and induce immune responses (Felix et al., 1999; Liu et al., 2012), which are usually linked with the accumulation of reactive oxygen species (ROS), cell wall cross-linking, the accumulation of secondary metabolites, and the expression of pathogenesis-related (*PR*) genes (Pieterse et al., 2009). Recently, necrosis and ethylene-inducing peptide 1-like proteins (NLP) secreted by bacteria, fungi and oomycetes were reported to show PAMP activity (Böhm et al., 2014). Conserved 20–24 amino acids peptide within Type I NLP is sufficient to induce immune response on *Arabidopsis* leaves by recognition of membrane-bound receptor-like protein RLP23 (Albert et al., 2015).

NLP proteins are also secreted by *V. dahliae* (Santhanam et al., 2013; Zhou et al., 2012). In a cotton-isolated *V. dahliae* strain V592, nine *NLP* genes were cloned and referred to as *VdNLP1* to *VdNLP9* (Zhou et al., 2012). Although only VdNLP1 and VdNLP2 triggered necrosis on cotton leaves, VdNLP1 to VdNLP4 induced expression of *PR* genes, including *PR-1*, *PR-5* and *PR-12* (Zhou et al., 2012).

Plant *PR* genes can be induced by biotic and abiotic stresses (Kothari et al., 2016; Kouzai et al., 2016; Misra et al., 2016), and in many cases are the marker genes responding to the defense-associated phytohormones such as salicylic acid (SA), jasmonate (JA) and ethylene (ET) (Pieterse et al., 2009). *PR* genes have been classified into 17 families (Sels et al., 2008). Typical *PR* family members were discovered and mainly investigated in tobacco (van Loon et al., 2006). The accumulation of the *PR* gene transcripts after pathogen treatment attracted attention of investigators (Antoniw and Pierpoint, 1978; Gao et al., 2006).

Among *PR* gene families, *PR-1* has been frequently used as a marker gene for systemic acquired resistance in many plant species (Lee et al., 2015). *PR-1* and related genes exist as a multigene family in the genomes of tobacco and rice (Cutt et al., 1988; Mitsuhara et al., 2008). Acidic *PR* genes are usually induced by TMV infection and SA treatment, while basic *PR* genes are usually induced by wounding or JA/ET treatments in tobacco (Niki et al., 1998). It is reported that transgenic tobacco and *Arabidopsis* expressing *PR-1* gene confer high level of resistance to bacterial diseases (Li et al., 2011; Rogers and Ausubel, 1997). *PR-2* gene family encodes β-1,3-glucanase. *PR-3*, *PR-4*, *PR-8*, and *PR-11* gene families encode chitinase. *In vitro* evidence showed that β-1,3-glucanase in combination with chitinase has a direct antifungal activity on some phytopathogenic fungi (Mauch et al., 1988). The *PR-5* gene family encodes permatins, osmotins, zeamatins, and thaumatin-like proteins (Agarwal and Agarwal, 2014). *PR-5* is the marker gene of ET signaling pathway in *Brachypodium distachyon* (Kouzai et al., 2016). The transgenic *Arabidopsis* expressing *PR-5* gene of *Ocimum basilicum* confers tolerance to fungal pathogen and abiotic stress (Misra et al., 2016). *PR-6* gene family is up-regulated in responsive to wounding, fungal infection and insect attack (Cordero et al., 1994; Green and Ryan, 1972), and encodes proteinase inhibitors that were reported to inhibit *Botrytis cinerea* proteinases *in vitro* (Dunaevskii et al., 2005). *PR-7* was first characterized in tomato as an endoproteinase (Vera and Conejero, 1988). The typical member of *PR-9* gene family encodes tobacco lignin-forming peroxidase, which catalyzes lignin deposition and strengthens plant cell wall during pathogen attack (Agarwal and Agarwal, 2014; Lagrimini et al., 1987). *PR-10* gene family encodes small proteins with cytosolic localization, which act as RNase, ligand interactor, posttranslational modifiers and are responsive to abiotic and biotic factors (Agarwal and Agarwal, 2014). *PR-12* gene family encodes defensins that inhibit the fungal growth. Transgenic potato overexpressing a defensin peptide confers enhanced resistance to *V. dahliae* (Gao et al., 2006). *PR-12* in particular is often used as a marker for the induction of JA and ET-dependent defense signaling pathways (Lay and Anderson, 2005; van Loon et al., 2006). *PR-13* gene family encodes thionins that have antimicrobial effects causing permeabilization of cell membranes (Agarwal et al., 2013; Sels et al., 2008). *PR-14* gene family encodes lipid transfer proteins that play a role in cutin synthesis, β-oxidation, and plant defense (Agarwal et al., 2013; Sels et al., 2008). PR-15 and PR-16 have oxalate oxidase property and can be induced in barley infected by *Erysiphe graminis* (Agarwal et al., 2013; Sels et al., 2008). *PRp27* is a typical *PR-17* gene family member found in tobacco (Agarwal et al., 2013; Okushima et al., 2000).

It is unknown how many *PR* gene families are present in the cotton genome, and how do they respond to *V. dahliae* PAMP molecules, although a few *Gossypium hirsutum PR* genes are up-regulated during *V. dahliae* infection (Hill et al., 1999; Mcfadden et al., 2001). In this study, the best homologs of *PR* genes were retrieved by taking advantage of updated *G. hirsutum* genome. We determined the expression of *G. hirsutum PR* (*GhPR*) genes in root tissues treated by flg22 (Felix et al., 1999) as well as synthetic peptides derived from VdNLPs that are highly homologous to immunogenic nlp20 peptide (Böhm et al., 2014). The aim of our study is not only to profile *PR* gene expressions during cotton immunity triggered by PAMP molecules, but for further illustrating the molecular mechanism of cotton-*V. dahliae* interaction.

RESULTS

Putative pathogenesis-related (*PR*) gene families in *Gossypium hirsutum* genome

To collect *G. hirsutum PR* genes, reported *PR* gene sequences from tobacco, tomato, barley and *Arabidopsis* (Sels et al., 2008) were collected to search for most identical homologs

in *G. hirsutum* genome (Li et al., 2015). As the result shown in Table 1 and File S1 in Supporting Information, we identified 15 *G. hirsutum PR* genes (*GhPR1* to *GhPR17*) representing 14 *PR* gene families. Each GhPR shows conserved domain identical to reported PR proteins (Figure S1 in Supporting Information). Among these putative *PR* genes, *GhPR2*, *GhPR3*, and *GhPR10* have been reported for gene expression in response to *V. dahliae* infection (Mcfadden et al., 2001). No homologs representing *PR-6*, *PR-12* and *PR-13* gene families can be found in *G. hirsutum* genome. According to the phylogenetic tree made of sequences from reported PR proteins in tobacco, tomato, barley, *Arabidopsis* and cotton (File S1 in Supporting Information), *PR* gene families are well conserved in different plant species, reflecting their conserved function as marker genes for plant immunity (Figure 1).

Identification of nlp20 homologous peptides in VdNLPs

Since *Phytophthora parasitica* peptide nlp20 (named nlp20^{Pp} in this study) showed immunogenic activity (Böhm et al., 2014), the nlp20^{Pp} sequence was used to search for homologous regions in VdNLPs (Zhou et al., 2012). The most identical sequences were obtained from VdNLP2 (nlp20^{Vd2}), VdNLP3 (nlp23^{Vd3}) and VdNLP4 (nlp23^{Vd4}), respectively (Figure 2). Peptides of flg22, nlp20^{Pp}, nlp20^{Vd2}, nlp23^{Vd3}, and nlp23^{Vd4} were synthesized for testing immunogenic activity on cotton roots (Table 2).

Expression of *GhPR* genes in response to flg22 and nlp20^{Pp} peptides

To investigate expression of *GhPR* genes in response to PAMP molecules, we treated cotton roots with 2 μmol L^{-1} of flg22, nlp20^{Pp}, nlp20^{Vd2}, nlp23^{Vd3}, and nlp23^{Vd4}, respectively (Table 2). The cotton root development was not influenced upon PAMP treatment within 4 days (Figure S2 in Supporting Information). Total RNA of treated cotton roots at 3, 6 and 12 hpi and non-treated cotton roots were isolated for quantitative RT-PCR (qRT-PCR) analysis. Specific primers for each *GhPR* gene family were designed for qRT-PCR analysis (File 2 in Supporting Information). Expression of *GhPR* genes was normalized, first to *Ghactin* in each treatment, and then to normalized *PR* genes in non-treated cotton roots (mock) that were arbitrarily designated as 1.

As the result shown in Table 3 and Figure 3, flg22 and nlp20^{Pp} treatments led to up-regulation of most *GhPR* genes

Table 1 *PR* genes in *G. hirsutum*

Name		Homolog to	Description
GhPR1	Gh_A12G0274	*PR-1*	Antifungal
GhPR2	Gh_D05G0709	*PR-2*	Hydrolase activity
GhPR3	Gh_D11G3475	*PR-3*	Chitinase activity
GhPR4	Gh_D13G1816	*PR-4*	Pathogenesis-related protein PR-4A-like
GhPR5	Gh_D12G2247, Gh_A12G2071	*PR-5*	
GhPR6	No hits	*PR-6*	
GhPR7	Gh_A08G1591	*PR-7*	Endopeptidase activity
GhPR8	Gh_D12G2670	*PR-8*	Acidic chitinase
GhPR9	Gh_D09G1208	*PR-9*	Peroxidase activity
GhPR10	AY869698.1, AY704425.1, AY704426.1	*PR-10*	Ribonuclease-like
GhPR11	Gh_A07G0195	*PR-11*	Chitinase activity
GhPR12	No hits	*PR-12*	
GhPR13	No hits	*PR-13*	
GhPR14	Gh_D10G1143	*PR-14*	Lipid binding
GhPR15	Gh_D10G1873	*PR-15*	Manganese ion binding
GhPR16	Gh_D07G1367	*PR-16*	Manganese ion binding
GhPR17	Gh_A13G0387	*PR-17*	

Table 2 Peptide sequences of flg22, nlp20 and nlp20 homologs from *V. dahliae*

Name	Sequence
flg22	QRLSTGSRINSAKDDAAGLQIA
nlp20^{Pp}	AIMYSWYFPKDSPVTGLGHR
nlp20^{Vd2}	AILYAWYMPKDAPSSGLGHR
nlp23^{Vd3}	AIMYSFYMPKDQPIAGNVAGGHR
nlp23^{Vd4}	AIMYEYYFEKDQSVSGSFAGGHR

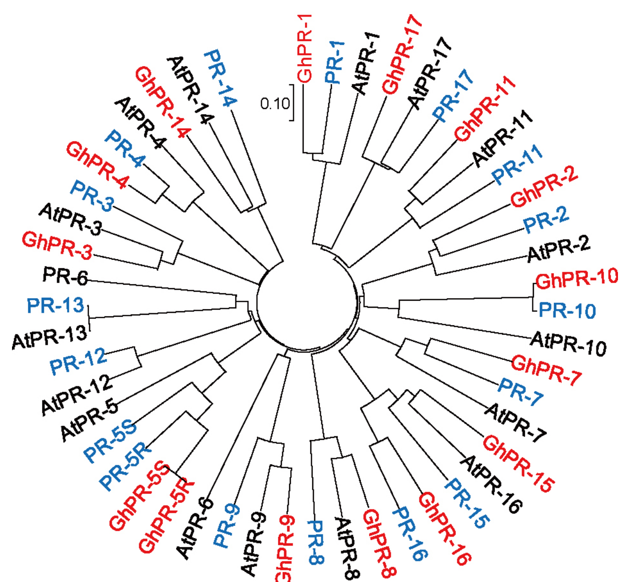

Figure 1 Phylogenetic tree of PR proteins. The full-length sequences of 17 reported PR proteins (PR-1 to PR-17), predicted 16 *Arabidopsis thaliana* PR proteins (AtPR-1 to AtPR-17), and 14 putative *Gossypium hirsutum* PR proteins (GhPR1 to GhPR17). No *PR-15* homologous gene was found in *A. thaliana* genome. No PR-6, PR-12, PR-13 homologous genes were found in *G. hirsutum* genome.

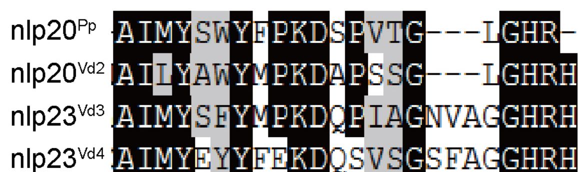

Figure 2 Sequence alignment of nlp20 peptide and homologous peptides from *V. dahliae*. nlp20^{Pp} is 20-aa immunogenic peptide from *Phytophthoraparasitica*. nlp20^{Vd2}, nlp23^{Vd3} and nlp23^{Vd4} are homologous peptides of nlp20^{Pp} from *V. dahliae*. The sequence alignment was done by Bioedit.

in cotton roots at 3 hpi, and some up-regulation of *GhPR*s lasted until 6 or 12 hpi. The extent of *PR* genes up-regulation was generally less than 6 times, and mostly less than 3 times. *GhPR1* is the highest up-regulated gene upon PAMP treatments (approx. 5 times). Both flg22 and nlp20^{Pp} treatments induced *GhPR1* up-regulation at 3 hpi and the induction was maintained to 6 and 12 hpi for nlp20^{Pp} but not for flg22. Other genes also showed up-regulated expression at early time point, such as *GhPR2* and *GhPR4* upon nlp20^{Pp} treatment and *GhPR17* upon flg22 treatment. Interestingly, *GhPR10* was expressed up-regulated upon nlp20 treatments, but down-regulated upon flg22 treatment at 3 hpi.

Expression of *GhPR* genes in response to *V. dahliae*-derived immunogenic peptides

In contrast to bacterial flg22 and oomycete nlp20^{Pp} peptides, VdNLP2-derived peptide nlp20^{Vd2} showed more effective to induce *PR* genes up-regulation on cotton roots (Figure 4). In nlp20^{Vd2}-treated roots, expression of *GhPR1*, *GhPR2* and

Table 3 Transcriptional responses of *PR-genes* to flg22 and nlp20s[a)]

Family	Induced expression				
	flg22	nlp20^{Pp}	nlp20^{Vd2}	nlp23^{Vd3}	nlp23^{Vd4}
PR1	$+^{a, b}$	$+^{a, b, c}$	$++^{a, b}+^{c}$	$+^{b, c}$	$+^{a, b}$
PR2	$+^{a}$	$+^{a, c}$	$++^{a}+^{b, c}$	$++^{a}+^{b}$	$+^{b}$
PR3	…	$+^{c}$	…	$+^{b}$	$+^{a, b}$
PR4	$-^{c}$	$+^{a}-^{c}$	$+^{a, b}$	$+^{a}$	…
PR5	$+^{a, b, c}$	$+^{a, b}$	$+^{a, b}$	$+^{a, b}$	…
PR7	…	$+^{c}$	$+^{a}$	…	$+^{a}$
PR8	…	$+^{a}$	$+^{a, b}$	$+^{a, b}$	$+^{c}$
PR9	$+^{a}$	…	$+^{a}$	$+^{a}$	…
PR10	$-^{a}$	$+^{a}$	$+^{a}$	$+^{a}$	…
PR11	…	…	$+^{b}-^{c}$	…	…
PR14	…	…	$+^{a, c}$	…	$+^{c}$
PR15	…	…	$+^{a, c}$	$+^{a}$	$-^{c}$
PR16	$-^{c}$	$+^{a, b, c}$	$+^{a, b, c}$	$+^{a, b}$	$+^{a}$
PR17	$+^{a, b}$	…	$+^{a, c}$	$+^{a, b}$	…

a) ++, genes induced more than 10-fold compared to mock treatment. +, genes induced more than 2-fold compared to mock treatment. –, genes induced less than 2-fold compared to mock treatment. …, not induced; a, 3 hpi; b, 6 hpi; c, 12 hpi.

GhPR4 genes was highly increased (more than 10 times) at 3 hpi. However, only *GhPR1* maintained high expression to the later time points. Other genes except *GhPR3* were also up-regulated to a moderate level (approx. 4–6 times) at 3 hpi and maintained or declined subsequently, except for *GhPR14* and *GhPR16*. *GhPR16* was up-regulated until at 6 hpi, while *GhPR14* expression declined at 6 hpi but remarkably increased again at 12 hpi.

Interestingly, nlp23^{Vd3}-treatment did not induce *GhPR1* gene up-regulation at 3hpi but induced at 6 hpi up to approximately 5 times. However, nlp23^{Vd3} induced significant *GhPR2* gene up-regulation (approx. 12 times) at 3 hpi. The expression of *GhPR4*, *GhPR10*, *GhPR15* and *GhPR16* were also up-regulated for 5–6 times at 3 hpi and gradually declined at the later time points. Moderately increased expression of *GhPR5*, *GhPR7*, and *GhPR8* (approx. 3–4 times) was maintained to the later time points. It is likely that the less increased expression the longer maintenance for the induction of certain *GhPR* genes upon nlp23^{Vd3}-treatment (Figure 4).

In nlp23^{Vd4}-treated cotton roots, *PR* genes were not expressed as high levels as in nlp20^{Vd2}- or nlp23^{Vd3}-treated roots, although some showed up-regulated expression. The induction level of *GhPRs* was similar to that of the flg22- and/or nlp20^{Pp}-treated roots (Figure 4).

Taken together, all the synthetic peptides can induce the up-regulation of *GhPR* genes. Most *GhPR* genes moderately respond to bacterial flg22 and oomycete nlp20^{Pp} rapidly at 3 hpi. Some *GhPR* genes reached the highest expression level at 6 hpi (Figure 3). Similar to flg22 and nlp20^{Pp} treatment,

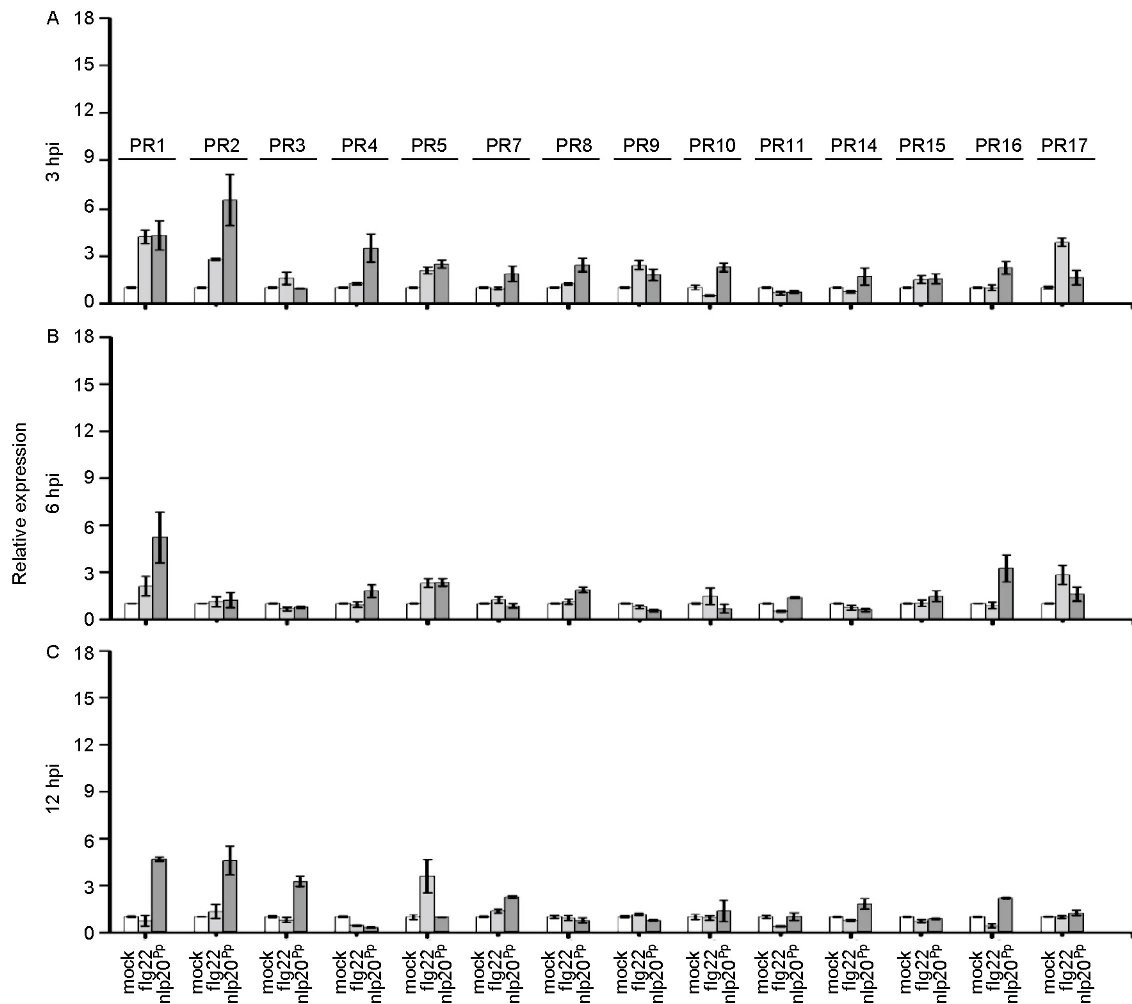

Figure 3 Expression profile of *GhPR* genes in the cotton roots treated by synthesized peptides of flg22 and nlp20^{Pp}. The expression data of all the *GhPR* genes were firstly normalized with endogenous gene *Ghactin*, and the normalized *GhPR* gene expression levels were compared to *GhPR* gene expression levels in mock treatment (0 dpi), which were arbitrarily set to 1. All the experiments have repeated at least three times.

most *GhPR* genes moderately respond to nlp23^{Vd4} (Figure 4), in agreement with the fact that the VdNLP4 is a type II NPP1-domain-containing protein incapable of inducing plant cell death (Zhou et al., 2012). Compared to flg22, nlp20^{Pp} and nlp23^{Vd4}, nlp20^{Vd2} and nlp23^{Vd3} are more effective to trigger *GhPR* gene expressions, while nlp23^{Vd3} is less effective than nlp20^{Vd2}. This is consistent with our previous finding that VdNLP2, rather than VdNLP3 was capable of inducing necrosis-triggered defense responses in cotton leaves, although both VdNLP2 and VdNLP3 were type I NPP1-domain-containing proteins (Zhou et al., 2012).

DISCUSSION

To provide general markers to investigate cotton-*V. dahliae* interaction, we identified 14 *PR* gene families in *G. hirsutum* genome on the basis of significant homology to reported *PR* genes, namely *GhPR1 to 17*. However, no homologs of *PR-6*, *PR-12* and *PR-13* gene families can be found in *G. hirsutum* genome. Previous studies have shown that PR-6 has antimicrobial activity which can be increased synergistically and specifically in combination with thionins (PR-13) (Agarwal et al., 2013; Sels et al., 2008). *PR-12* encodes a plant defensin. Overexpression of an alfalfa *PR-12* gene in potato enhanced resistance of transgenic plant to *V. dahliae* infection (Gao et al., 2006). Missing of these PR gene families might be a reason that *G. hirtusm* is susceptible to *V. dahliae*.

To study the expression of *GhPR* genes in the immune response, we treated cotton roots with two well-known PAMP molecules flg22 and nlp20^{Pp} (Böhm et al., 2014; Felix et al., 1999), as well as three nlp20 derivatives from *V. dahliae* (nlp20^{Vd2}, nlp23^{Vd3}, and nlp23^{Vd4}). This is the first example of a comparative study on the characterization of a set of *GhPR* genes by qRT-PCR. Both flg22 and nlp20^{Pp} induced up-regulated expression of *GhPR* genes as anticipated. However, up-regulated *GhPR* genes induced by flg22 were much less than that induced by nlp20^{Pp}, probably due to the fact that flg22-triggered immunity is very fast and have declined at

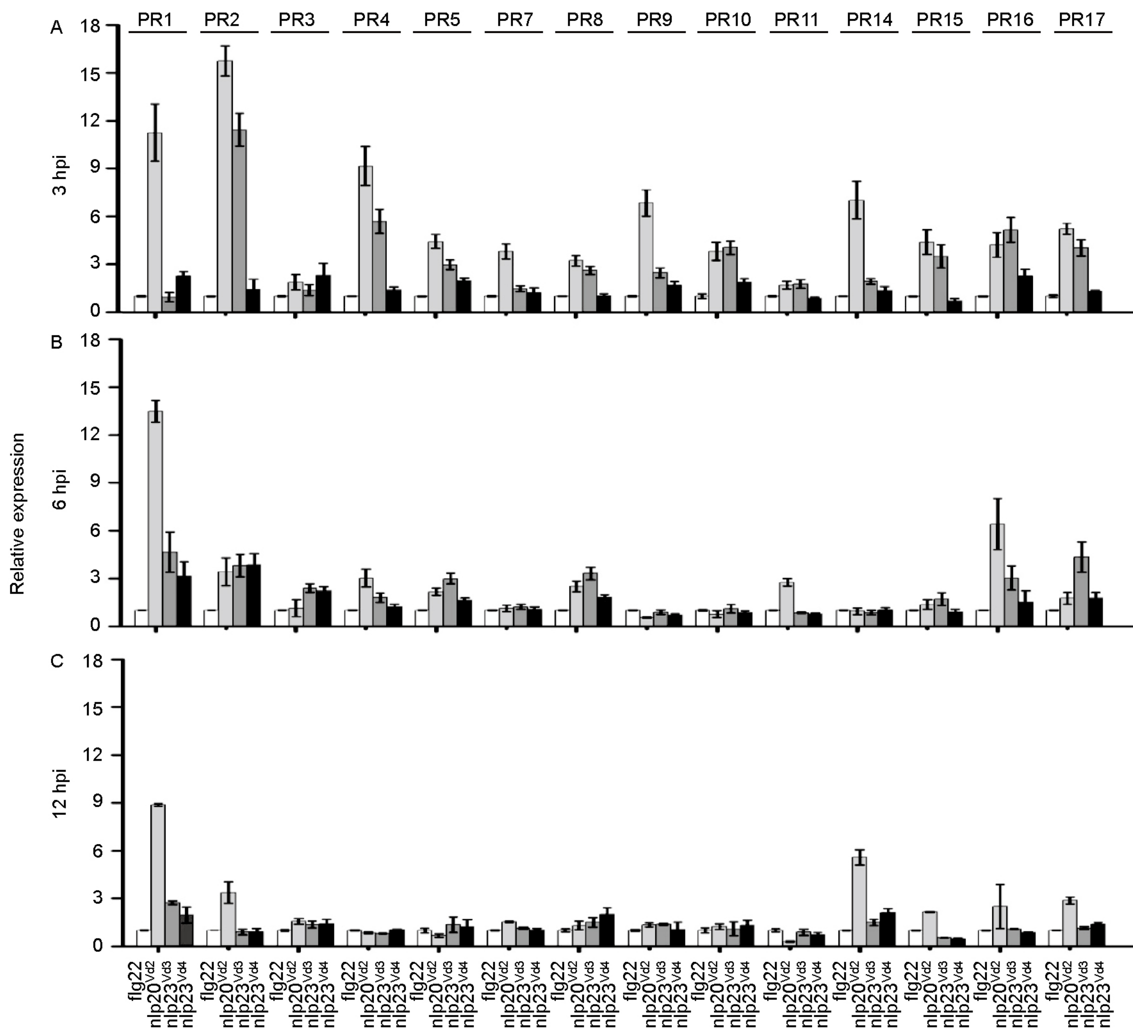

Figure 4 Expression profile of *GhPR* genes in the cotton roots treated by synthesized peptides of nlp20^{Vd2}, nlp23^{Vd3} and nlp23^{Vd4}. The expression data of all the *GhPR* genes were firstly normalized with endogenous gene *Ghactin*, and the normalized *GhPR* gene expression levels were compared to *GhPR* gene expression levels in mock treatment (0 dpi), which were arbitrarily set to 1. All the experiments have repeated at least three times.

3 hpi in cotton roots. Most *GhPR* gene up-regulations were maintained longer by nlp20^{Pp} treatment rather than flg22, suggesting that nlp20^{Pp} is a more suitable immunogenic peptide for study of cotton-*V. dahliae* interaction. Further, both *GhPR1* and *GhPR2* are proper marker genes of cotton immunity since their expression is highly up-regulated and maintained upon nlp20^{Pp} treatment.

Based on the sequence of nlp20^{Pp} peptide we identified homologous peptides from VdNLP proteins (Zhou et al., 2012), which belong to type I and type II NLPs according to the number of cysteine residues (Gijzen and Nürnberger, 2006). Likely, all the type I NLPs can trigger plant immunity because of the conserved immunogenic 20–24 aa-peptide (Böhm et al., 2014; Oome et al., 2014). In *V. dahliae* V592 strain, 9 NLP genes (*VdNLP*) were cloned and functionally characterized, of which *VdNLP1–3* are type I NLPs, whereas *VdNLP4–9* are type II NLPs. Interestingly, the sequences homologous to nlp20^{Pp} peptide can be found in type I NLPs (*VdNLP2* and *VdNLP3*) as well as in type II NLP (*VdNLP4*), namely nlp20^{Vd2}, nlp23^{Vd3}, and nlp23^{Vd4}, respectively (Figure 2). Only *VdNLP2*, but not *VdNLP3* and *VdNLP4* can induce necrotic cell death and trigger plant defense responses in cotton (Zhou et al., 2012). Although the PAMP activity of nlp20^{Vd2}, nlp23^{Vd3}, and nlp23^{Vd4} remains to be further investigated, *GhPR* genes have shown clear up-regulation to these peptides, especially to nlp20^{Vd2} (Figure 4). nlp20^{Vd2} is even more effective to induce up-regulation of *GhPR* genes compared to nlp20^{Pp}, since all the *GhPR* genes were expressed significantly higher upon nlp20^{Vd2} treatment compared to nlp20^{Pp} treatment. Besides *GhPR1* and *GhPR2*, *GhPR4*, *GhPR9* and *GhPR14* showed significant up-regulation (more than 6 times), suggesting that these genes can be markers of nlp20^{Vd2}-triggered immunity.

The expression pattern of *GhPR* genes upon nlp23^{Vd3} treatment is mostly in consistent to that upon nlp20^{Vd2} treatment but with lower expression levels. This is in agreement with our previous conclusion that *VdNLP2*, rather than *VdNLP3* was capable of inducing necrosis-triggered defense responses

in cotton leaves (Zhou et al., 2012), which may also suggest that NLP-triggered necrosis and *PR* gene expression may share different signal transduction pathways, or the necrosis is dependent on the threshold value of *PR* gene expression. nlp23^{Vd4} did not induce significant *GhPR* gene up-regulation at the early time point (3 hpi) in compared to other nlp peptides. *GhPR1* and *GhPR2* genes were induced up-regulated for about 3 times at 6 hpi, which was also slower and less in transcriptional accumulation than upon other nlp peptide treatments. This might be due to the lower identity to peptide sequences of nlp20^{Pp} and nlp20^{Vd2}, and also explain why types II VdNLPs do not induce immune response and necrosis on cotton leaves (Zhou et al., 2012).

MATERIALS AND METHODS

Bioinformatics

In total 17 *PR* gene families have been reported, including *PR-1a*, *PR-2*, *PR-P/Q*, *PR-4a*, *PR-R/S*, *PR-9*, *PR-10*, *PR-11* and *PR-17* from *N. tabacum*, *PR-7* from tomato, and *PR-14*, *PR-15*, and *PR-16* from barley (Sels et al., 2008). These reported sequences of *PR* gene families are used as queries in a BLAST search against the *G. hirsutum* gene database (https://www.cottongen.org/tools/blast). The resulting hits with highest identity to reported *PR* genes were identified as potential upland cotton *PR* genes. Together with PR proteins from *Arabidopsis thaliana*, all the retrieved PR protein sequences were aligned by ClustalX and generated phylogenetic tree by Mega 7. All the sequences are listed in File S1 in Supporting Information.

To search for homologous peptides of nlp20 in *V. dahliae*, the reported nlp20 peptides from *Phytophthora parasitica*, *Pythium aphanidermatum*, *Fusarium oxysporum*, *Botrytis cinerea*, *Bacillus halodurans* and *Bacillus subtilis* were used as queries in a BLAST search against the *V. dahliae* protein database according to NCBI Blastp steps, and the resulting hits with highest identity were identified.

Cotton roots preparation

G. hirsutum susceptible cultivar Xinluzao 16 was used in this experiment. The cotton seeds were sterilized in 4% sodium hypochlorite for 30 min, washed by sterilized water for 5–6 times, and immersed in the water overnight. The seed coat was removed followed by washing with sterilized water for 5–6 times. The washed seeds were air-dried in the flow cabinet before transferred to 1/2 MS solid culture medium (0.22% MS including vitamins, 2% saccharose, 0.3% phytagel, pH=5.8) for germination. The seeds were germinated for 3 days in the greenhouse under the condition of 16 h daylight and 25°C. The cotton seedlings were transferred to a glass beaker (d=3 cm, h=5 cm) containing 20 mL autoclaved double distilled water. The glass beaker containing 6 cotton seedlings was put into a big glass beaker (d=6 cm, h=12 cm), which was covered by a plastic paper and sealed by a rubber band. After additional 3 days' growing, the cotton seedlings can be used for PAMP treatments.

PAMP molecule stocks and cotton root treatments

Peptides of flg22, nlp20, and *V. dahliae*-derived peptides homologous to nlp20 were synthesized (Beijing Protein Innovation, and Beijing SBS Genetech Co., Ltd.) according to published amino acid sequences (Felix et al., 1999; Böhm et al., 2014; Zhou et al., 2012). Each peptide was diluted by sterilized water into 1 mmol L^{-1}stock and stored at −20°C before use. The cotton seedlings were treated by each peptide at 2 μmol L^{-1} concentration by immersing the roots in the peptide solutions. Cotton roots treated by sterilized water were taken as the negative control. Cotton roots in each treatment were collected at 3, 6 and 12 hpi and stored at −80°C for RNA extraction. Each sample was collected three times.

RNA extraction and quantitative reverse transcriptional PCR (qRT-PCR)

Total RNAs were isolated by using an EASYspin Plant RNA Kit (Aidlab, Beijing) according to the manufacturer's instruction. cDNA was synthesized from 3 μg of each total RNA by using GoScriptTM Reverse Transcription System (Promega Corporation, USA), according to the manufacturer's instructions. qRT-PCR is performed using a C1000 Thermal Cycler (Bio-Rad, USA) in combination with the EvaGreen 2× qPCR MasterMix-iCycler (Applied Biological Materials, USA). The primers to detect the *PR* gene transcripts are listed in File S2 in Supporting Information. The program of qRT-PCR is described as follows: an initial 95°C enzyme activation step for 10 min, followed by denaturation for 15 s at 95°C, annealing/extrnsion 60 s at 60°C for 40 cycles. The data were collected using the Bio-Rad CFX Manager software and processed in Microsoft Excel. The transcript levels of target genes were normalized firstly to the transcript levels of the constitutively expressed *Ghactin* gene, and then to *PR* gene transcripts in non-treated cotton roots (negative control) according to the $2^{-\Delta\Delta Ct}$ method.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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SUPPORTING INFORMATION

File S1 PR proteins from At, Gh, Nt.

File S2 Primers for qPCR of *GhPRs* and *actin* genes.

Figure S1 Conserved domains of GhPR proteins.

Figure S2 Cotton roots upon PAMP treatments.

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