

THE FAMILY OF CHITINASES IN COTTON *G. raimondii*

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doi: [10.15414/jmbfs.2017.6.6.1284-1289](https://doi.org/10.15414/jmbfs.2017.6.6.1284-1289)

ARTICLE INFO

Received 3. 2. 2017
Revised 20. 4. 2017
Accepted 27. 4. 2017
Published 1. 6. 2017

Regular article



ABSTRACT

Chitinases are enzymes widely spread in plants with no endogenous substrate and play significant role to lyse the cell walls of many pathogens. Their role in defense is firmly established. Recently, their functions in plants have been extended to response to abiotic stresses and various developmental plant processes. The gene family of chitinases is well characterized in some model species. Few researches on their involvement in cotton defense against pathogens have been reported. Here we investigated *Gossypium raimondii* genome in the Phytozome database for the presence of homologues. BLAST similarity search, using five chitinase representatives from Arabidopsis belonging to different classes I-V, identified a total of 41 non-redundant chitinase gene sequences in cotton. We analysed them *in silico* using available bioinformatics software and characterized their basic molecular structures. Signal peptides, carbohydrate-binding domains, hinge regions were predicted, molecular weights and expected isoelectric points were calculated. Genes were divided into different chitinase classes based on homology clustering with genes from Arabidopsis. The individual family members were further linked to expression data and/or literary knowledge, which is in relation to cotton rather scarce. Knowledge on chitinase gene family members in cotton provides a basic for further basic- as well as applied cotton research as they are functionally validated.

Keywords: *G. raimondii*, chitinase classes, carbohydrate-binding domain, glycosyl hydrolase domain, chitinase clusters

INTRODUCTION

Plants evolve mechanisms to counter biotic and abiotic stresses. Accumulation of pathogenesis-related (PR) proteins is one of the developed mechanisms highly expressed by plants during the attack by pathogens. Among PR members, chitinases (PR-3) can hydrolyse chitin in the exoskeleton of insects and cell wall of pathogenic fungi, consequently play an important role in the plant defense against those organisms. Chitinases have extensively been studied for their use in different biotechnological programs for plant protection. A number of crops such as tomato, potato, maize, rapeseed, wheat, rice and others have been successfully engineered for resistance either with chitinase alone or in combination with other PR proteins (Cletus *et al.* 2013). In recent 10 years was observed an increasing amount of evidence on the role of these enzymes in plant adaptation to abiotic stresses, including drought (Gregorová *et al.* 2015), toxic metals (Mészáros *et al.* 2014) and even pesticides (Asrorov *et al.* 2013). Besides, chitinases play pivotal role in plant morphogenesis and development with impact on growth and stress tolerance. Therefore, research on chitinases in important crop species is of crucial importance.

Breeding stress-tolerant and high-yield cotton might exploit chitinases and other PR proteins since a couple of researchers proved their importance in cotton defense against aphids and mites. For example, three acidic chitinase isozymes (pI in the range 3.7-4.2) from *G. hirsutum* leaves were gradually elicited by *V. dahliae* for 120 hours (Dubery and Slater, 1997), some of them probably inhibited the germination of conidia (Liu *et al.* 1995). PRs including chitinases likely contribute to restriction of wilting in cotton infected by *Verticillium* (Bu *et al.* 2014). Furthermore, elevated activities of chitinases and other PR proteins resulted in reduced population of aphids in cotton (Rajendran *et al.* 2011), and their inhibition by insecticide treatment resulted in the population growth of spider mites both in greenhouse and field experiments (Szczepaniec *et al.* 2013). On the other hand, chitinases in cotton are believed to play a role in fiber

formation, too (Wiweger *et al.* 2003).

Plant chitinases are structurally well characterized and are divided into several groups. The class I chitinases have an N-terminal cysteine-rich regions involved in chitin-binding (Iseli *et al.* 1993). These regions are separated from the catalytic domain by a short proline-rich variable hinge region and the catalytic domain is often followed by a C-terminal extension which is involved in vacuolar targeting (Neuhaus *et al.* 1991). Class II chitinases have a catalytic domain with a high sequence and structural similarity to those of class I chitinases. However they possess neither the N-terminal cysteine-rich region nor the C-terminal extension. The main structure of class IV chitinases resembles class I chitinases, but is reduced by few deletions along the carbohydrate-binding domain (CBD) and the catalytic region (Passarinho and deVries 2002). The class III chitinases are similar to class V chitinases of plant origin and fungal/bacterial chitinases (Graham and Sticklen, 1994). Class V chitinases have a C-terminal extension for vacuolar targeting and may contain CBD as well (Heitz *et al.* 1994; Ponstein *et al.* 1994). Class V and III chitinases belong to the family 18 of glycosyl hydrolases whereas all other classes belong to family 19. Chitinases of families 19 and 18 do not share sequence similarity, they have completely different 3-D structures and molecular mechanisms (Suzuki *et al.* 1999).

In spite of cotton genome sequence available, the family of chitinases in cotton is low explored. The objective of this work to identify and describe the chitinase genes and their families in *G. raimondii* and link them with corresponding knowledge available in literature.

MATERIAL AND METHODS

Searching the cotton genome for chitinases

The cotton genome in the Phytozome database was searched for chitinases using the BLAST program (Table 1). Five chitinase gene representatives from

Arabidopsis thaliana AT1G56680, AT1G02360, AT3G12500, AT4G19720 and AT5G24090, belonging to classes I-V were used as queries (Passarinho and de Vries, 2002). Redundant sequences were removed at $\geq 98\%$ similarity in amino acid translations. All identified putative chitinases in cotton were back-searched in the NCBI database to prove similarity with chitinases (E value $\leq 10^{-20}$). Subsequently, different databases and softwares (Table 1) were used to predict their structural and molecular characteristics such as molecular weight and putative isoelectric points, the presence of individual protein domains and signal peptides (Table 1). Sequence alignment of chitinases from cotton and *Arabidopsis* was done using ClustalW (Table 1) and evolutionary relationships were observed by viewing Cladogram or Phylogram in the program. The phylogenetic tree was built using software (www.ebi.ac.uk/Tools/msa). Maximum likelihood method was used. Available data on chitinase gene expression in cotton were obtained from the database plex (Table 1).

Table 1 List of used databases and bioinformatic softwares

| | |
|--------------------------------------------------|------------------------------------------------------------------------------------------------------------------|
| Phytozome | http://www.phytozome.jgi.doe.gov/ |
| National Center for Biotechnological Information | http://www.ncbi.nlm.nih.gov/ |
| Molecular Weight / Isoelectrical Point | http://web.expasy.org/compute_pi/ |
| Signal peptide | http://www.cbs.dtu.dk/services/SignalP/ |
| Decrease redundancy | http://web.expasy.org/decrease_redundancy/ |
| ClustalW/Phylogenetic tree | http://www.ebi.ac.uk/Tools/msa/clustalw2/ |
| Expression data/PLEXdb | www.plexdb.org/modules/tools/plexdb_blast.php |
| Arabidopsis Genome database | www.arabidopsis.org |

Protein Extractions and Analysis

We studied total chitinase activity of cotton plant leaves in comparison with other plant materials. For that lyophilized, fully developed mature leaves were collected from *Gossypium hirsutum*, *Malva sylvestris*, *Morus multicaulis*, *Populus*, *Ligustrum vulgare* and *Rumex obtusifolius*, growing in close distance with the same ecological parameters in the locality of Tashkent, Uzbekistan (GPS coordinates: 41° 15' 52.7400" N and 69° 12' 58.5720" E). Tissue material (500 g) was ground with liquid nitrogen using a mortar and pestle. After grinding, proteins were isolated and assayed for chitinase activity as described previously (Reissig et al. 1955). The activity of the enzyme was expressed as mmol of N-acetyl glucoseamine amount hydrolyzed for 2 hours.

RESULTS AND DISCUSSION

Mature cotton leaves contain proteins with chitinolytic activity. This activity is comparable with that measured in leaves of malva (*Malva sylvestris*), white mulberry (*Morus multicaulis*) and poplar (*Populus*), but lower than in wild privet (*Ligustrum vulgare*) or bitter dock (*Rumex obtusifolius*) (Fig. 1).

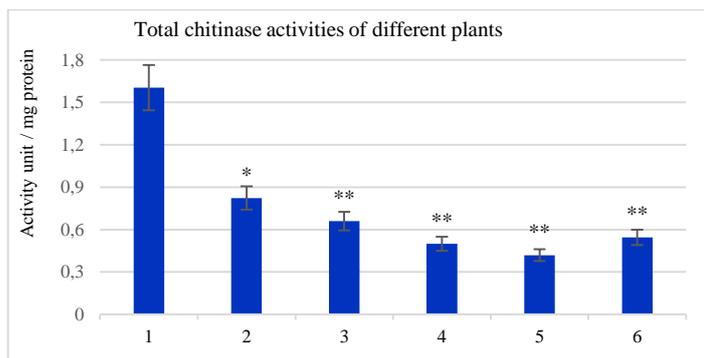


Figure 1 Variability of total chitinase activity in mature leaves of different plant species such as *Rumex obtusifolius* (1), *Ligustrum vulgare* (2), *Populus alba* (3), *Morus multicaulis* (4), *Malva Sylvestris* (5) and *Gossypium hirsutum* (6). (The error bars were calculated based on the four replicates with standard deviation, “*”: significant difference at P < 0.1; “**”: significant difference at P < 0.05)

We have measured the overall activity of chitinases in cotton. The obtained values are, however, hardly indicative since results are from the activities of several individual isoforms of different activity, regulation and function. The activity of chitinases varies among plant species, but also depends on developmental stage and even plant organ (Gregorová et al. 2015). Nevertheless, their total activity in pea was shown to coincide with metal tolerance (Metwally et al. 2005). Moreover, not only absolute activity values but kinetics of individual chitinase isoforms can coincide with plant tolerance to stresses (Mészáros et al. 2013), hence detailed study of corresponding gene family might be very useful for plant improvement.

The measured activities are the results of action of several different isoforms encoded by the members of chitinase family. Here, the genome of *G. raimondii* and *G. hirsutum* were searched with chitinases from *Arabidopsis thaliana* for presence of expressed and putative gene homologues using the BLAST program in Phytozome and NCBI database. This search revealed a total of 43 gene sequences annotated as chitinases, of which 2 were removed as redundant using a software. A total of 41 non-redundant putative chitinases were detected in cotton. Their calculated molecular weights varied in the range of 24.9-49.8 kDa. More than half of them (29 out of 41) were predicted as acidic with the pI lower than 6.5, and only four chitinases were found to have pI in neutral range and 9 chitinases are possibly basic. Clustering with chitinase representatives from *A. thaliana* and the cotton chitinase class VII (Li and Liu 2003) sorted the cotton chitinases into six classes (Figure 2).

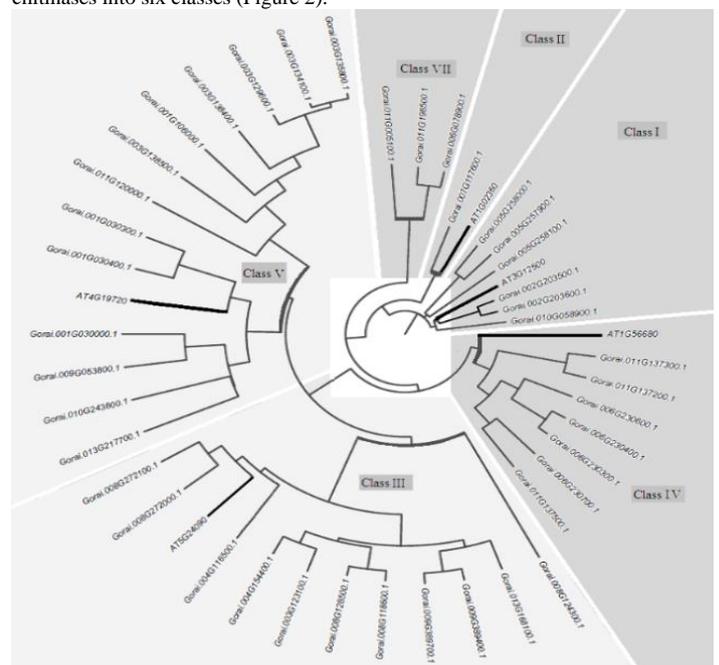


Figure 2 Dendrogram of cotton chitinases based on amino acid sequence alignment with representatives of genes belonging to classes I-V from *A. thaliana*. Representatives of genes from *A. thaliana* are described in bold. In light grey background, genes of Family GH 18 are shown. Genes of Family GH 19 are indicated in dark grey background.

This distribution fits with expected structural data. Translations of 17 identified cotton chitinases contain a Glyco_hydro_19 domain (GH19) (Family 19 chitinases), and 24 chitinases possess a Glyco_hydro_18 domain (GH18) typical for the family 18 chitinases. The family 19 chitinases comprised classes I, II, IV, VII and included 17 putative genes, and the family 18 chitinases possessed 11 genes of class III and 13 of class V chitinases (Figure 2 and 3). A putative signal peptide (SP) is present in 37 chitinase genes (Figure 3). A carbohydrate-binding domain was identified in only some of Family 19 chitinases, namely in six class IV and three class I chitinase genes (Figure 3). Among them, a proline rich, so-called hinge region was found in two out of three class I, and four of six class IV chitinases (Figure 3).

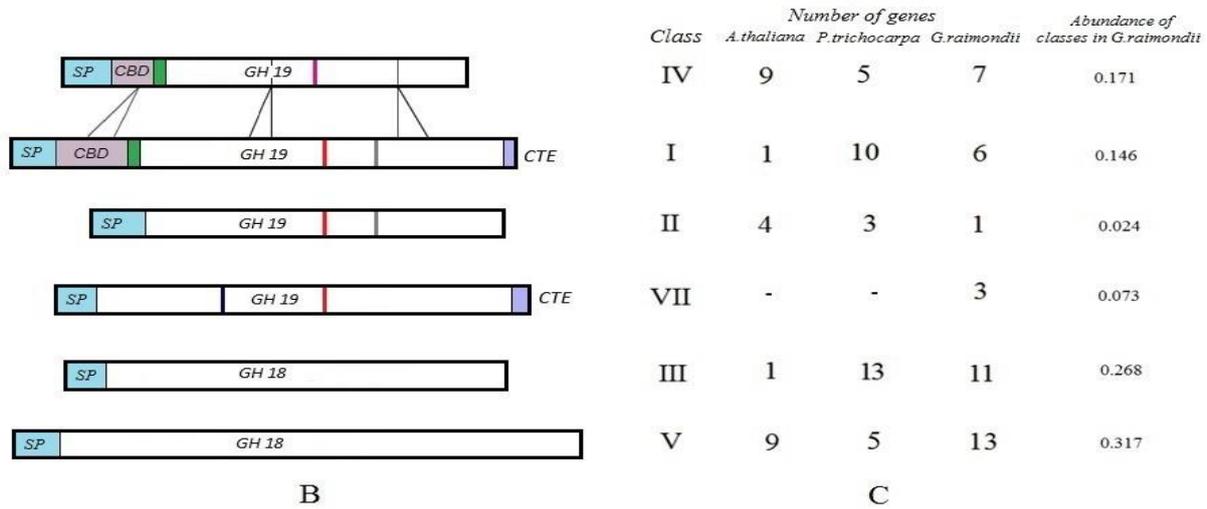


Figure 3 Primary structural maps of chitinase (class I - VII) genes found in cotton (*G. raimondii*) genome and the number of their representatives in different plant species. SP - Signal peptide; CBD - carbohydrate-binding domain; ■ - Hinge region; GH 18 and GH 19 - Glycohydrolase domain 18/19, CTE- C-terminal extension; Δ - Deletions; with red lines YNYG motifs are represented in classes I, II, and VII; dark grey lines in class I and class II chitinases stands for WFWM motifs; CRGP motif in class IV chitinases is noted in pink line; EVAAF motifs are marked with a violet line in class VII chitinases

We identified and characterized to some extent *in silico* a total of 41 chitinase genes in the genome of cotton. This number is higher comparing with 24 genes described in the model plant *A. thaliana* (Passarinho and de-Vries, 2002) or the 37 genes in poplar (*P. trichocarpa*) (Jiang et al. 2013) (Figure 3). Since the number of chitinase genes among dicotyledonous and monocotyledonous plant species is expected to be comparable (Yokoyama and Nishitani 2004), the observed discrepancy is likely because of available rapidly developing bioinformatic tools (Figure 3). Consequently, the currently described gene families in individual species might in reality still be larger and incomplete.

Calculated molecular weights of all encoded cotton chitinases were in the range of 24.9-49.8 kDa which are similar to those found in Arabidopsis 23.3-46.9 kDa (Passarinho and deVries 2002).

Intron-exon structures of cotton chitinases resemble some similarities with respect to enzyme classification (Figure 4). All class I chitinases have 2 introns and 3 exons. All class IV chitinases (except for the Gorai.011G137300) have 1 intron and 2 exons. In contrast, gene structures of Family 18 chitinases were more variable. Seven of eleven class III members lack any intron; 3 genes possess one intron and one gene (Gorai.008G124300) contains 7 introns. Seven of thirteen cotton class V chitinase genes possess one intron. Five of them have a relatively conserved position of intron at the beginning of the gene. The other six class V family members lack any intron.

The chitinase genes in the Phytosome database were assigned to the five typical structural classes, and clustering with chitinases from well-described Arabidopsis confirmed this distribution (Figure 2). In addition to these five typical classes, a class VII chitinase has been described and characterized by Li and Liu (2003). We identified two more chitinases showing high sequence similarity to the class VII chitinase found in the cotton genome (Figure 2 and 4). Typically, these enzymes lack the CBD (Li and Liu 2003). While two of them (Gorai011G005100 and Gorai006G078900) show responsiveness to abiotic stresses (Christianson et al. 2002; Padmalatha et al. 2012; Table 2), the third (Gorai.011G198500) has been suggested to play a role in fiber development (Zhang D et al., 2004).

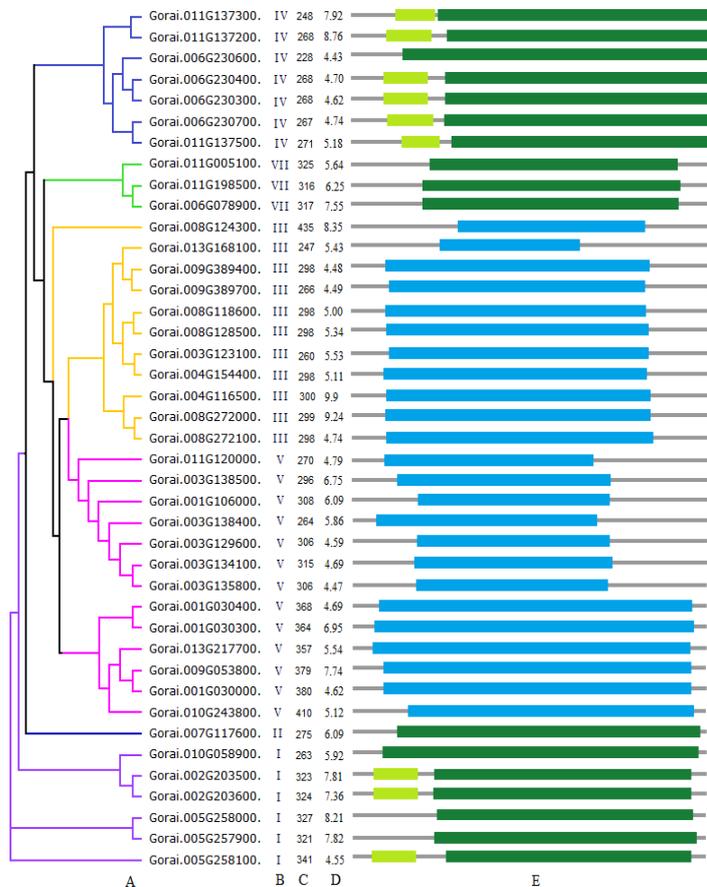


Figure 4 Phylogenetic relationships among *G. raimondii* chitinase genes and their protein structures: A - Phylogenetic tree; B – Chitinase class; C - number of amino acids in the enzyme molecule; D – Putative isoelectric points of enzymes E - Domains: ■ - Glycosyl hydrolase Family 18; ■ - Glycosyl hydrolase Family 19; ■ - Carbohydrate binding domain;

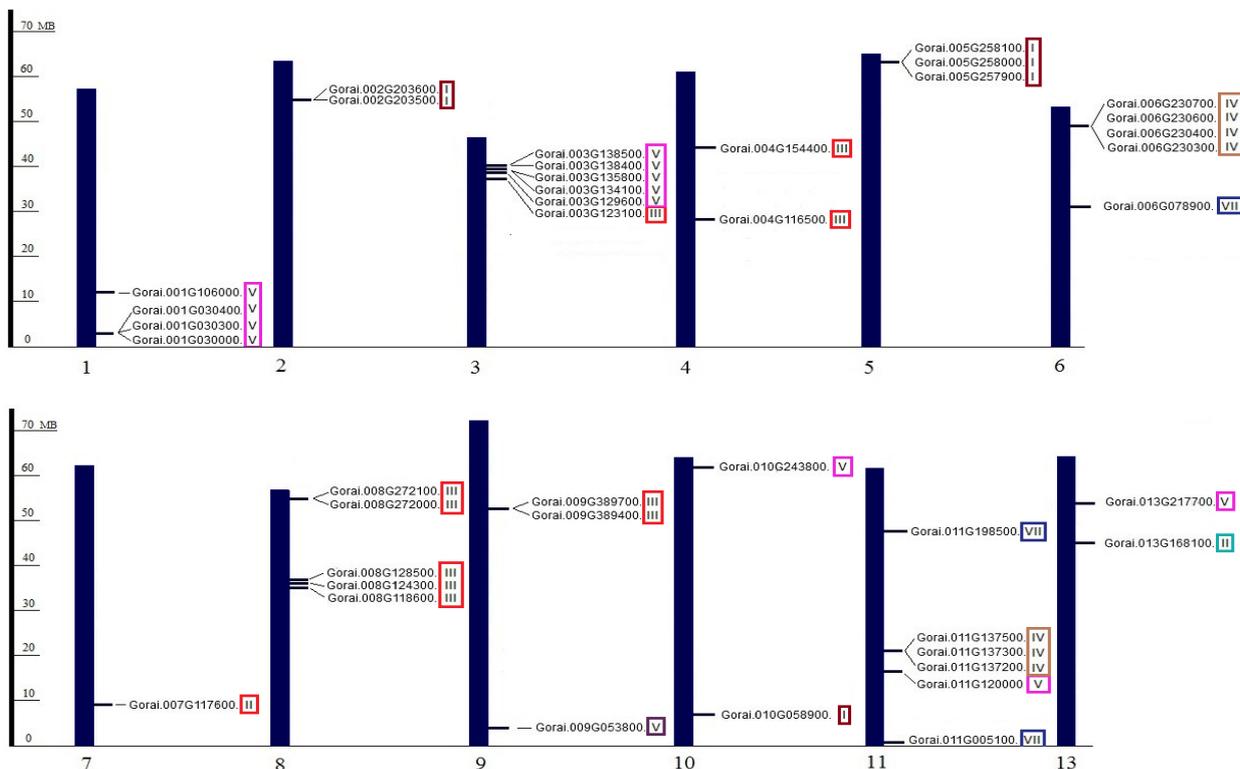


Figure 5 Genomic localization of *G. raimondii* chitinase genes. Transcript names and chitinase classes (in Roman digits) are given in front of localization. Numbers of chromosomes are indicated under the columns. Classes of chitinases are shown in quadrangles.

We retrieved data on the localization of chitinase genes in chromosomes of *G. raimondii* from the Phytozome database (Figure 5). The largest numbers of chitinase genes were located on the chromosomes 3, 11 (6 genes) and 6, 8 (5 genes). Many genes belonging to similar classes are co-localized. Only class III chitinases are located on the chromosomes 4 and 8, class I chitinases are only present on the chromosomes 2 and 5, while only class V chitinases were identified on the chromosome 1. On the other hand, clusters of chitinase genes (small regions of chromosomes where several genes are co-located) were identified on the cotton chromosomes 1, 5, 6 and 11. On the chromosomes 6 and 11 there were two clusters of class IV chitinases, three class I chitinases were clustered on the chromosome 5, and three class V chitinases are clustered on the chromosome 1.

The chitinase genes in cotton are distributed along the almost entire genome, except for the single chromosome 12 (Figure 5). This distribution is relatively even comparing with other species. For instance in *A.thaliana* all the 24 chitinases were found in five chromosomes (Passarinho and de Vries, 2002), while the largest number of chitinases belonged to chromosomes 2 (6 chitinases) and 4 (9 chitinases). The majority (30 of 37) of chitinase genes in *P. trichocarpa* were positioned on the chromosomes 8 of 19 (Jiang et al. 2013). On the other

hand, co-localisation of similar class chitinases in cotton appears as a common feature. Similar pattern was observed in *P. trichocarpa* chitinases, where the class I chitinases were localized on chromosomes 4 and 9, and class V chitinases were located on chromosomes 6 and 18 (Jiang et al. 2013). Similarly, in *A. thaliana* the class IV chitinases were found on chromosome 2 and Class V genes were positioned on chromosome IV (Passarinho and de Vries, 2002). Since expression data are still unavailable for cotton in the Phytozome database, the chitinase genes identified were used as queries for similarity search of the PLEXdb database (Cotton PLEX). Expression data could be retrieved for a total of 8 cotton chitinase genes (Table 2).

Microarray gene expression data were obtained on leaves and roots of experimental cotton plants in the context of flooding (Christianson et al. 2009) and drought conditions (Rodriguez et al. 2014; Padmalatha et al. 2012) (Table 2). A clone with high sequence similarity to the class IV chitinases Gorai.006G230700 was strongly responsive to drought in greenhouse condition (Padmalatha et al. 2012) and significantly lower value was true in field experiments (Rodriguez et al. 2014), however remained unaffected in flood-exposed leaves and roots in a field study (Christianson et al. 2009).

Table 2 Expression data of chitinases in the PLEXdb (www.plexdb.org/modules/tools/plexdb_blast.php)

| Phytozome name | Class | Christianson et al. 2009 | | | | Rodriguez et al. 2014 | | Padmalatha et al. 2012 | |
|--------------------|-------|--------------------------|-------------|-------------|-------------|-------------------------|-------------|------------------------|-------------|
| | | Root | | Leaf | | Leaf (field experiment) | | Leaf (Greenhouse) | |
| | | Control | Flooded | Control | Flooded | Control | Drought | Control | Drought |
| Gorai.005G257900.1 | I | 4.8 | 2.03 | 1.22 | 1.39 | 4.9 | 4.31 | 4.09 | 2.78 |
| Gorai.005G258100.1 | I | 10.05 | 11.82 | 7.98 | 6.29 | 7.03 | 7.49 | 7.4 | 9.0 |
| Gorai.008G272000.1 | III | 11.45 | 11.68 | 5.47 | 5.87 | 7.37 | 7.04 | 8.16 | 8.9 |
| Gorai.008G272100.1 | III | 5.95 | 6.95 | 5.86 | 6.5 | 7.34 | 6.82 | 9.49 | 11.78 |
| Gorai.008G124300.1 | III | 8.42 | 7.58 | 7.02 | 6.68 | 7.16 | 7.88 | 8.85 | 8.76 |
| Gorai.006G230700.1 | IV | 4.29 | 4.92 | 3.35 | 3.1 | 5.48 | 3.95 | 2.72 | 5.07 |
| Gorai.011G005100.1 | VII | 5.98 | 5.73 | 3.85 | 2.43 | 5.84 | 4.01 | 4.9 | 3.97 |
| Gorai.006G078900.1 | VII | 3.54 | 2.27 | 1.62 | 1.26 | 3.44 | 4.16 | 5.17 | 0.8 |

* Genes identified as responsive to given stress types are indicated bold

We detected six class I chitinase gene in cotton (Figure 4). One of them (Gorai.005G257900) has been reported as affected in flooded roots and in drought stressed leaves (Table 1; Padmalatha et al. 2012). The other two class I chitinases (Gorai.010G058900 and Gorai.002G203600) have been described as defensive and inducible by SA and ethylene (Hudspeth et al. 1996; Levorson and Chlan, 1997, respectively). Previously, relatively low number of class I chitinase was identified in Arabidopsis as active in roots and at lower levels in leaves and flowers of aging plants (Passarinho et al. 2002).

A single class II chitinase gene in cotton contradicts the relatively higher number of identified class II chitinases in other plant species (Figure 2 and 3). This class of genes might have evolutionary evolved from class I chitinases and was also described for role during defense (Kirsch et al. 1993; Ponath et al. 2000; Meins et al. 1994). On the contrary, surprisingly high number of class III chitinases was identified in cotton, similarly as in poplar but unlike in *A. thaliana*. The three experimentally studied class III cotton genes are probably involved in the processes other than adaptation to abiotic stresses (Table 2). The single class III

chitinase in *Arabidopsis* was induced by fungi, but not wounding, ethylene or SA (Samac and Shah 1991).

Seven class IV chitinases were identified in cotton. One of them (Gorai.006G230700) was induced in leaves exposed to drought (Table 2, Padmalatha et al. 2012), and another (Gorai.011G137500) was found induced in wilting leaf (Wang et al. 2011). Class IV chitinases in other plant species were proposed to be associated with plant resistance against fungi (Lange et al. 1996; Nielsen et al. 1994), viruses, and also abiotic stresses such as heavy metals and UV irradiation (Margis Pinheiro et al. 1993). However Passarinho and de-Vries (2002) suggested that class IV chitinases are involved in developmental processes rather than in defense.

Thirteen cotton chitinases belong to class V enzymes. Specific function for this rather diverse group of chitinases has not yet been attributed. They are suggested to play a role in plant defense as well as development (Heitz et al. 1994; Melchers et al. 1994). Two class V chitinase genes in cotton together with class II chitinase were significantly upregulated in cotton roots in response to *V. dahliae* and significantly increased following treatment with jasmonic acid and H₂O₂. Authors suggested that these genes may be involved in plant resistance to stress (Xu et al. 2016).

For some of the chitinase classes a typical role has been assigned. Most typically, antifungal activity has been attributed to class I and II chitinases (Neuhaus et al. 1991) by hydrolyzing the cell walls of plant pathogenic fungi (Wargo PM 1975), and/or by releasing elicitors of defense reactions (Hadwiger and Beckman 1980). Furthermore, class I and class II chitinases were linked with antifreeze activity in *Secale cereale* (Pihakaski-Maunsbach et al. 2001; Nakamura et al. 2008), and salt-adaptation in tobacco (*Nicotiana tabacum*) (Yun et al. 1996). The functional differences between individual (cotton) chitinase classes are, however, not clear since chitinases of separate classes can reflect different chitin recognition levels. For example, Sasaki et al. (2006) demonstrated that class I and class III chitinases recognize three contiguous N-acetyl glucose amine residues in different subsites. Furthermore, available expression data indicate that some cotton chitinases of different classes might have overlapping functions. For instance, class I, IV and VII chitinases might be defense-related due to responsiveness to several different abiotic stresses, ethylene or the stress hormone salicylic acid (Chlan and Bourgeois 2001; Nielsen et al. 1994; Li and Liu 2003). In the past decade increasing amount of evidence indicates involvement of chitinases in abiotic stress like metals (Békésiová et al. 2008), cold and spring hardening (Zur et al. 2014), drought (Gregorová et al. 2015). However, these functions have not been attributed to a particular chitinase class (Stintzi et al. 1993). Researches that are more recent prove that chitinases are activated in cotton upon application of commonly used pesticides (Asrorov et al. 2013; Rajendran et al. 2011; Szczepaniak et al. 2013). On the other hand, class IV chitinases have been suggested as markers of embryogenesis (Wiweger et al. 2003). In cotton the chitinase gene corresponding to Gorai.011G198500 has been identified (Li and Liu 2003) as not similar to any of the typical plant chitinase classes I-VI (Neuhaus et al. 1996). This unique chitinase, similar to class I and class II chitinases to only 30%, was defined as belonging into a separate class (class VII chitinase), and is abundant in fibers and in the seedling roots (Li and Liu 2003). More detailed structural and expression analyses suggested that the two closely related cotton genes, GhCTL1 and GhCTL2, can be preliminarily classified as chitin binding lectins. They likely lack hydrolytic activity, while the chitinase activity in fiber is attributable to the expression of authentic chitinases along with putatively non-hydrolytic GhCTL1/GhCTL2 (Zhang et al. 2004). These specific cotton chitinase initiates fiber wall thickening via the deposition of helical cellulose microfibrils in secondary walls (Zhang et al. 2004), but inducibility with salicylic acid and ethylene indicates additional role in defense (Li and Liu 2003; Hudspeth et al. 1996; Levorson and Chlan et al. 1997). The exact mechanism of action of these genes in fiber formation, however, remains to elucidate.

CONCLUSION

In summary, we identified the members of chitinase gene family in cotton using bioinformatics tools. The available genome data enable a more detailed analysis of cotton chitinase genes both *in silico* as well as experimentally. Especially the gene expression data have to be extended in near future to improve our knowledge on the pivotal role of chitinases not only in processes of adaptation to environment, but also in important developmental processes like fiber formation. The obtained results can be a valuable basis for the functional studies of these gene family members in basic- as well as applied cotton research.

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