

# Xyloglucan endotransglycosylase/hydrolase genes from a susceptible and resistant jute species show opposite expression pattern following *Macrophomina phaseolina* infection

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Two of the most widely and intensively cultivated jute species, *Corchorus capsularis* and *Corchorus olitorius*, suffer severely from a stem rot disease caused by the fungus *Macrophomina phaseolina*. Wild jute species, *C. trilocularis*, shows resistance to this pathogenic fungus. In this study, the technique of differential display was applied to identify genes which are differentially expressed, under both infected and un-infected conditions, between *C. trilocularis* and *C. olitorius* var O-72. Two xyloglucan endotransglycosylase/hydrolase (XTH) genes designated CoXTH1 (from *Corchorus olitorius*) and CtXTH1 (from *C. trilocularis*) were identified from each of the two species which show different expression patterns upon fungal infection. A steady rise in the expression of CtXTH1 in response to infection was observed by quantitative real time PCR whereas the expression of CoXTH1 was found to be downregulated. Full length sequences of these two genes were determined using primer based gene walking and RACE PCR. This study confirms the involvement of XTH in molecular interactions between *M. phaseolina* and jute. However, it remains to be explored whether XTH is an essential component of the signaling pathway involved in plant-fungal interaction.

## Introduction

Both *Corchorus capsularis* and *Corchorus olitorius* suffer severely from stem rot and charcoal rot, caused by a pathogen *Macrophomina phaseolina*.<sup>1</sup> Other than jute this phytopathogenic fungus infects more than 500 cultivated and wild plant species and has a wide geographic distribution.<sup>2,3</sup> The pathogen can cause infection and damage at all stages of plant growth, from seedling emergence right up to maturation. *M. phaseolina* survives as microsclerotia in the soil and on infected plant debris that serves as the primary source of inoculum.<sup>4</sup> It disrupts nutrient and water transport to the upper parts of the plant as it affects the vascular system of the roots and basal internodes leading to wilting, premature death and reduced yield. It also causes root rot, basal stem rot and seedling blight.<sup>4,5</sup> Since the yield of jute is severely reduced, development of a variety that shows resistance to *M. phaseolina* will be of great importance to the countries growing this economically important fiber crop.

Wild jute species, *C. trilocularis* shows resistance to *M. phaseolina*,<sup>6</sup> while *C. olitorius* var O-72, a widely cultivated variety,

is susceptible to it. We have studied differential gene expression between these two species under both infected and uninfected conditions at different time intervals using a technique called mRNA differential display<sup>7</sup> and have identified two differentially regulated xyloglucan endotransglycosylase/hydrolase genes (CtXTH1 in *C. trilocularis* and CoXTH1 in *C. olitorius*) which show difference in their expression patterns upon fungal infection.

Because of their inherent resistance capacity plants have overcome numerous challenges from different pathogens. Before encountering the intracellular defense, a pathogen has to face the cell wall, which has as an important role in plant defense. Upon attack, various defense related polymers are synthesized to reinforce the cell wall at the sites, where the pathogens attempt penetration.<sup>8</sup> XTHs are one of the major players in this process as they cause rearrangement of the cellulose/xyloglucan architecture in the cell wall. The enzyme catalyzes transfer of a segment of one xyloglucan molecule allowing for molecular grafting between the polysaccharide molecules that subsequently change both the cell wall plasticity and architecture.<sup>9–11</sup> There is

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evidence that one of the key interactions in the primary wall of dicotyledons takes place between cellulose and the hemicellulose xyloglucan, which together typically comprise about two thirds of the dry wall mass. Xyloglucan binds non-covalently to cellulose, coats and cross-links adjacent cellulose microfibrils and the resulting extensive xyloglucan-cellulose network is thought to act as the principal tension-bearing structure in the cell wall.<sup>12-14</sup> Our current understanding suggests that, either the breakage of xyloglucan cross-links or their detachment from cellulose microfibrils is necessary for microfibrils to move apart to ensure cell wall expansion.<sup>15,16</sup> Several homologs of XTH have been identified in different plant species. They constitute a large multi-gene family designated as xyloglucan-related proteins (XRP).<sup>16,17</sup> Although the key function of XTH is cell wall modification, the existence of many different isozymes and their different temporal and spatial expression indicates additional roles other than cell wall growth and elongation.

## Results

**Identification of differentially expressed XTH gene and its phylogenetic analyses.** In order to study the interaction between jute (*Corchorus spp*) and *M. phaseolina* at the molecular level, we inoculated jute seedlings with *M. phaseolina* sclerotia. *C. olitorius*, the susceptible species, started showing diseased symptoms such as browning of the root within 24 h of infection. To confirm the sclerotia infection, we examined the root tissue sections under a light microscope. Sclerotial attachment was observed in the samples collected 15 and 24 h after inoculation, in both the species (figure not shown). However, two days after incubation, the fungus showed signs of growth and colonization in *C. trilocularis* roots (Fig. 1). Germination of the hyphae together with its penetration (Fig. 1B), formation of sclerotia in root tissues (Fig. 1C) and conidia formation and local destruction of host tissue (Fig. 1D) could be seen under the microscope. The inoculation process we followed in the laboratory is perhaps different from the actual infection process in the field; nevertheless this method can be an effective pathosystem to study molecular interactions between jute and *Macrophomina*.

The transcript profiles of infected and uninfected *C. olitorius* (var: O-72) and *C. trilocularis* seedlings were compared by mRNA differential display. Total RNA from these plants were collected and their quality and quantity were determined. Quality of isolated RNA was checked by electrophoresis (data not shown). In order to check the reproducibility of the method used, two separate mRNA differential display RT-PCR were performed and, almost equal number of bands was produced (Table S1) both the times. Some of the differentially expressed genes were amplified in all the three samples ( $S_0$ ,  $S_{15}$  and  $S_{24}$ ) from susceptible plants but none from the resistant ones. The opposite was also observed. In other cases, amplifications were found only in samples after a certain period of infection. Therefore, both constitutive and induced patterns of differential gene expression can be studied following the technique of differential display. In our study, we focused on one particular differentially expressed gene (starting with a cDNA fragment of

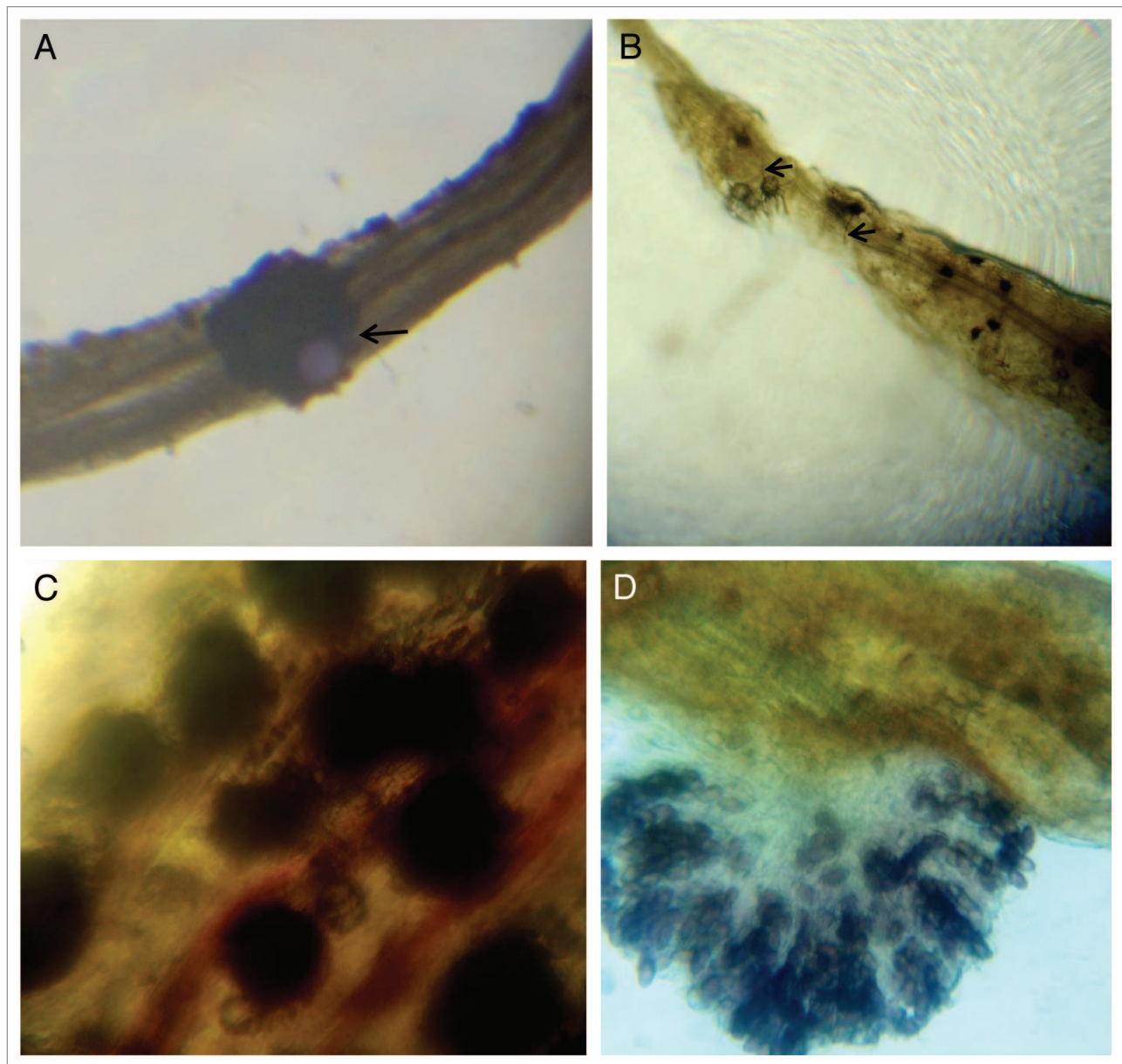
246bp from *C. trilocularis*). The amplified product of this gene was recovered from the gel, sub-cloned and sequenced. BLASTn and BLASTx analyses indicated the sequence to be a putative XTH gene.

The full length sequences of *CtXTH1* and *CoXTH1* were determined using primer-based gene walking and conventional 3' and 5' RACE. PCR of genomic DNA helped to identify the introns. Both the genes were found to have three introns and the coding region was 873 bp long (Fig. 2), having a similarity of 92% (BLASTn between two sequences). SignalP<sup>18</sup> analysis shows that for both the proteins, first 26 amino acids act as signal peptide to guide the same to the cell wall. Rose et al.,<sup>14</sup> classified Arabidopsis XTHs into three major groups. Phylogenetic analysis using 33 Arabidopsis XTHs have classified *CtXTH1* and *CoXTH1* with group I genes. As shown in Figure 3, they are placed in the same clade with seven of the Arabidopsis group I genes (*AtXTH 10*, *AtXTH 5*, *AtXTH4*, *AtXTH 8*, *AtXTH 9*, *AtXTH 7* and *AtXTH 6*). Michailidis and coworkers<sup>19</sup> classified the rest of the group I genes (*AtXTH 1*, *AtXTH 2*, *AtXTH 3* and *AtXTH 11*) as ancestral group and they are grouped in a different clade.

The conserved motif, DEIDFELFG, described as the catalytic site<sup>17</sup> appears in *CtXTH1* and in *CoXTH1* as DELDFELFG, whereas, the sequence of the motif for the seven Arabidopsis group I genes is found to be DE(I/F/L)DFELFG in the multiple sequence alignment (Fig. 4). In addition, the motif DWATRGG that ensures the hydrophobic stacking interactions with glucose unit is also present.<sup>20</sup> The 3D model of *CtXTH1* (Fig. 5) constructed using CPHmodels-3.0 server and represented using Swiss-Pdb Viewer<sup>21</sup> shows  $\beta$ -jellyroll-type structure typical of other GH16 family enzymes.<sup>22</sup>

***CtXTH1* and *CoXTH1* are members of a large gene family.** To determine the copy number of XTHs in *C. olitorius* and *C. trilocularis*, gDNA from both the plant species was digested with EcoRI and BamHI as these restriction enzymes do not have a recognition site within *CtXTH1* and *CoXTH1* genes. Digested gDNA was hybridized with cDNA derived probes at low stringency. For both *CtXTH1* and *CoXTH1*, more than one band was detected implying that they are members of a large gene family (Fig. 6).

***CtXTH1* and *CoXTH1* shows different expression profile upon *M. phaseolina* infection.** To estimate the expression patterns of these two genes, expression analysis was performed using semi-quantitative RT-PCR and real-time qPCR with a pair of primers which amplify the same region in both genes. Initially, we expected the expression of XTH in the sensitive plant (*CoXTH1* in *C. olitorius*) would be higher after fungal infection and the XTH from the tolerant plant (*CtXTH1* in *C. trilocularis*) should show a different pattern. To our surprise, the results indicated that *CtXTH1* expression is upregulated following fungal infection whereas *CoXTH1* shows downregulation (Fig. 7 and 8). *CoXTH1* expression was found to decrease at the initial time point (15 h) compared with the uninfected sample ( $S_0$  expression level) followed by a slight increase at 24 hrs. However, the initial expression level of *CtXTH1* (untreated *C. trilocularis* seedlings,  $T_0$ ) was much higher compared with *CoXTH1* and the highest

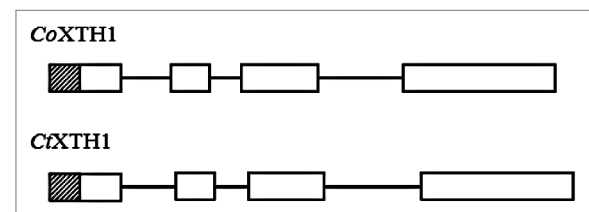


**Figure 1.** *M. phaseolina* infected seedlings after two days of infection. (A) *C. trilocularis* seedlings, sclerotia attached to the root (B) Both intra and intercellular Hyphal growth in root and subsequently growth of sclerotia in *C. olitorius* seedlings (C) New sclerotia (dark spots) observed in infected *C. olitorius* seedlings (D) Conidia formation and destruction of the host tissue (in *C. olitorius*).

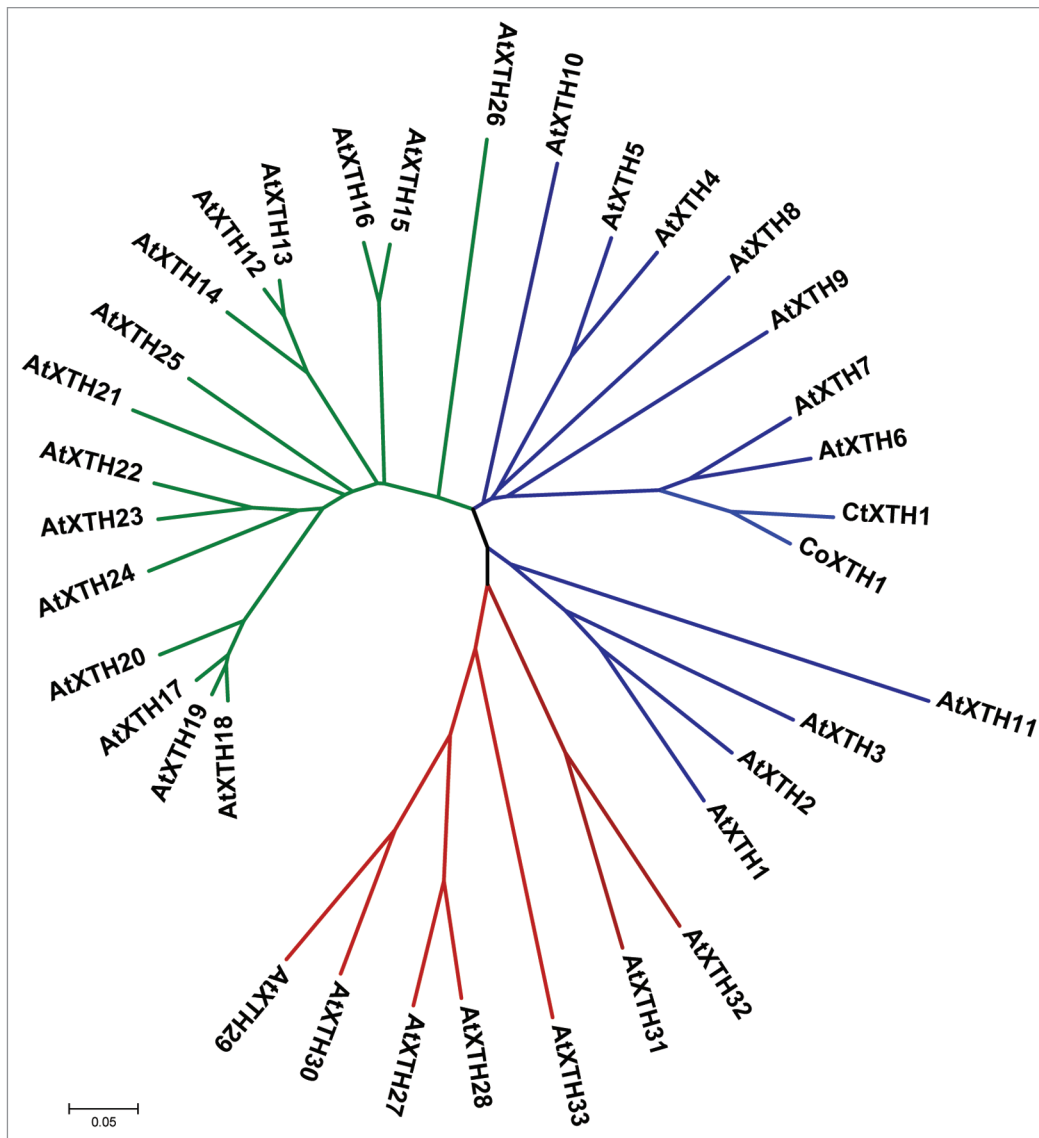
level of *CtXTH1* expression was observed after 24 h of infection (Fig. 8).

## Discussion

Till date, little is known about the molecular mechanisms behind the pathogenicity of *M. phaseolina*. One way to comprehend these molecular interactions is to look at the gene expression pattern(s). It is possible that there is a certain correlation between the pathogenicity of the fungus, its ability to invade the host and the different gene expression profiles of the tolerant and sensitive plant species. Both constitutive and induced gene expression, especially at the early stages of fungal infection, could therefore explain



**Figure 2.** Schematic diagram of *CtXTH1* and *CoXTH1* gene structure, the white boxes represent exons, lines introns and the shaded boxes are the putative signal peptide sequences.



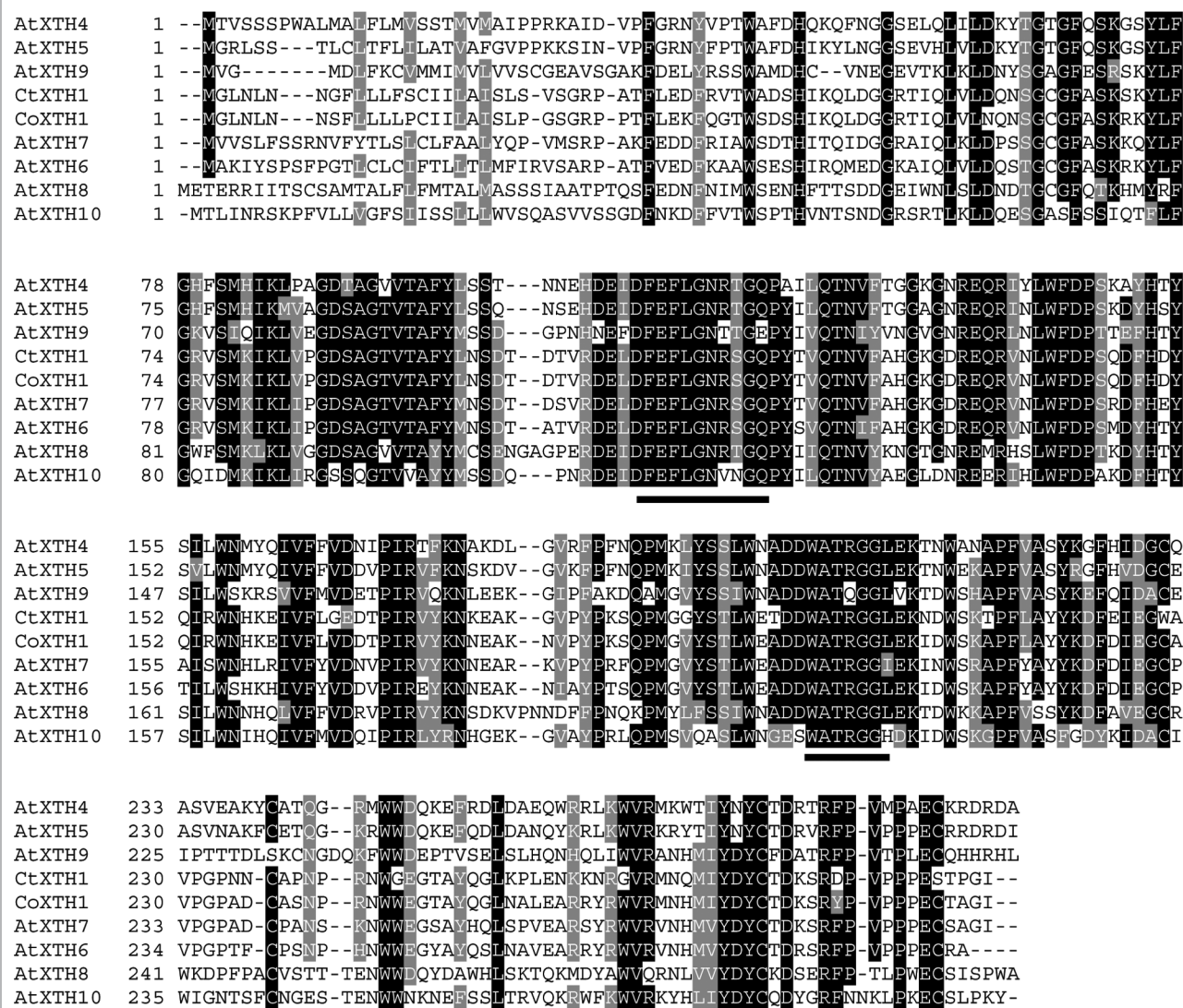
**Figure 3.** Phylogenetic relationship of CoXTH1 and CtXTH1 with Arabidopsis XTH genes. The tree was generated by MEGA 4.1 using neighbor-joining method and  $p$ -distance correction. The Arabidopsis genes from Group 1, 2 and 3 are shown in blue, green and red, respectively.

the response of the host to the invading pathogen. It could elucidate, albeit partially, the level of tolerance or sensitivity against the pathogen and the physiological responses displayed by the host. In line with this reasoning, this study aimed to look at the gene expression pattern of a tolerant and sensitive species using a technique called differential display.<sup>23</sup>

We have identified two orthologs, one from *C. olitorius* and another from *C. trilocularis* which show differential expression upon fungal infection. Both the genes share a high homology with XTH genes of Arabidopsis group 1 subfamily (Fig. 3) and the intron-exon patterns are typical of previously annotated XTH genes. To the best of our knowledge, *CtXTH1* is the only gene from *C. trilocularis* that has been completely sequenced and characterized (GenBank accession: GU809232).

Due to its association with many physiological and developmental processes, XTH gene family is perhaps one of the few

plant gene families that have been extensively studied. In every species, this family has expanded and through evolutionary changes, each member of the gene family within a species has attained the capability of different spatial and temporal expression. Prior studies have shown that duplication has been the primary mediator of XTH family expansion in Arabidopsis<sup>24</sup> and it will not be surprising, if like other plants the same is also found true for jute. Although there are numerous studies on XTH genes, relationship between their expression pattern and function is still a paradox. Previous studies have shown that 33 Arabidopsis XTH genes exhibit different organ specific expression pattern and respond differently to different sets of plant hormones.<sup>24-26</sup> Generally, XTHs are thought to play two distinct roles<sup>27,28</sup> which are necessary for cell wall expansion. One is “integrational” transglycosylation and the other is “restructuring” transglycosylation. The “integrational” transglycosylation is

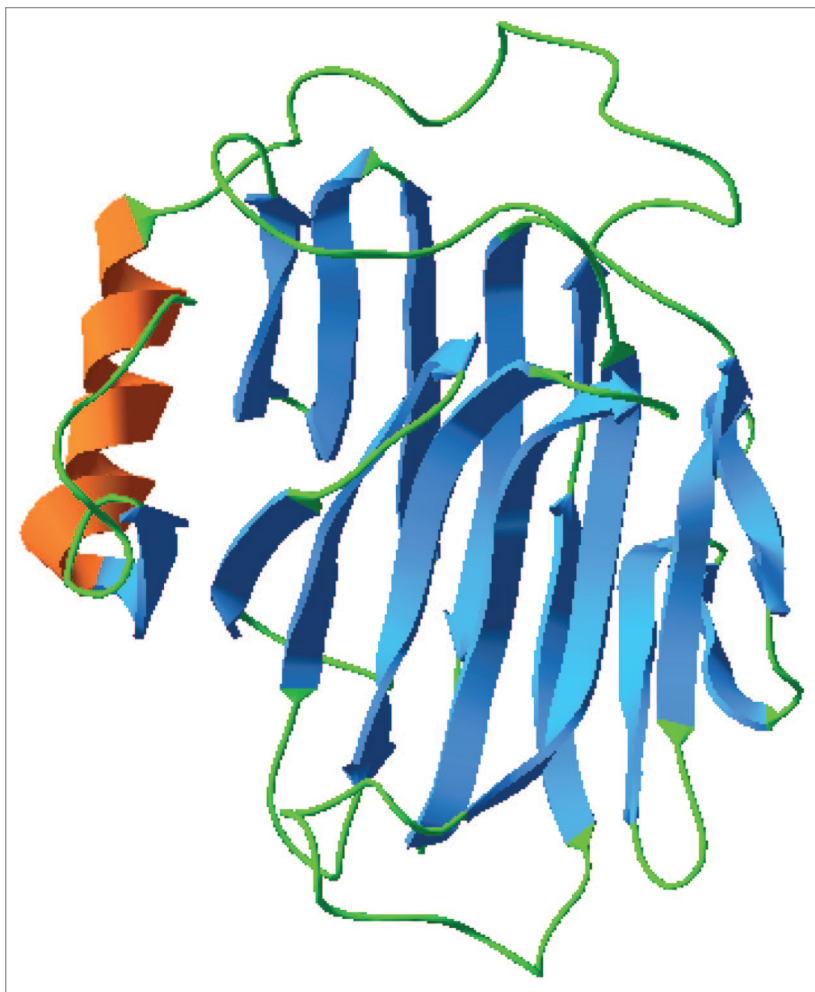


**Figure 4.** Alignment of the amino acid sequences of seven Arabidopsis group I XTH genes and CtXTH1 and CoXTH1, constructed using ClustalW program and drawn with the Boxshade program. The DELDFELFG and DWATRGG motifs are underlined.

a wall strengthening function of XTH where a newly synthesized and secreted xyloglucan chain is grafted to existing wall bound xyloglucan.<sup>29</sup> On the other hand, ‘restructuring’ type of transglycosylation leads to cutting and joining of xyloglucan chains bound previously to the cell wall.<sup>25</sup> This activity is of particular importance as it causes wall-strengthening. Takeda and coworkers<sup>30</sup> have shown that addition of xyloglucan to the excised pea stems causes it to become stiffer.<sup>30</sup>

Previous physiological studies have indicated that compared with its high yielding counterpart *C. oleritorious*, *C. trilocularis* is relatively sturdy and more adaptive.<sup>6</sup> It is possible that the high XTH expression in *C. trilocularis* is one of the major players in causing the cell wall stiffness that eventually acts against fungal invasion. Additionally, regulation of XTH expression can be modulated by hormonal response and the physical organization of the genes in a genome.<sup>31,32</sup> Earlier studies have also shown that XTH genes demonstrate a broad range of responses

to environmental stresses, both biotic and abiotic.<sup>17,33-35</sup> XTH expression is also correlated with cell elongation, as observed in Arabidopsis shoots<sup>17,36</sup> and root tips.<sup>37</sup> Albert and coworkers<sup>25</sup> found the tomato XTH (*LeXTH1*) expression level to increase upon parasitic attack however mechanical wounding was found to have no effect. Another group, Maldonado-Mendoza et al.,<sup>31</sup> found XTH activity to be induced systemically in mycorrhizal roots. In our case, even though both the enzymes were found to show high homology at the sequence level, but the difference in their expression pattern was possibly due to a distinct pattern of their regulation. This could be the outcome of (i) a difference in the accumulation and fixation of mutations between the two species, or (ii) a hormonal difference in the control of the regulon of *CoXTH1* and *CtXTH1*. However, we did not find any positive Darwinian selection between the two genes when we analyzed the substitution pattern. The codon based Z test also indicated neutrality for most (91.29%) of the pair wise comparisons of



**Figure 5.** 3D model of CtXTH1 constructed using CPHmodels-3.0 server and represented using Swiss-Pdb Viewer showing typical  $\beta$ -jellyroll-type structure.

Arabidopsis XTH genes (data not shown). So, the different regulatory pattern of XTH genes may have arisen from gene duplication and evolutionary diversification that ensured their tissue and hormone specific (spatial and temporal) expression patterns. It is evident from the current study that *CoXTH1* and *CtXTH1* genes in *C. olitorius* and *C. trilobularis* are members of large gene families (Fig. 6) and it is possible that due to evolutionary diversification, most of the members of this family are controlled by different regulatory circuits. If this was to be true, then the members of this gene family will exhibit different expression patterns under the same biotic or abiotic stress/stimuli. This is corroborated by studies in other plants<sup>17,19</sup> and it remains to be explored whether in jute other members of this gene family show a similar trend upon infection by *M. phaseolina*.

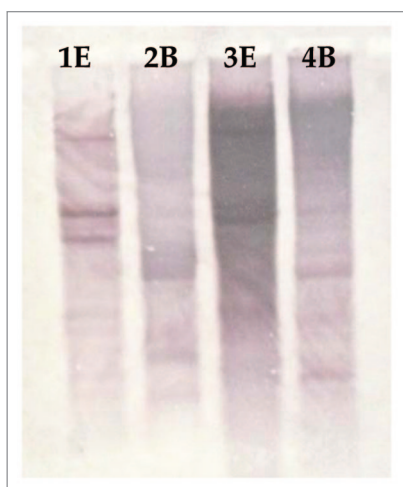
We speculate that when *C. trilobularis* comes in contact with *M. phaseolina*, the expression of *CtXTH1* increases with time, but its ortholog, *CoXTH1* could possibly be under a different regulatory circuit and shows an opposite expression pattern. We cannot substantiate a differential regulation between the two genes as there are no studies on the hormonal regulation of jute XTH. Nevertheless, this study shows that two closely

related XTH genes, with high homology in their sequences, show completely different expression patterns between fungus susceptible and resistant jute species, and it is highly possible that they are one of the major signaling components of jute *M. phaseolina* interaction.

## Materials and Methods

**Fungal isolates and plant materials.** *M. phaseolina* was cultured in PDA medium as described previously.<sup>38</sup> Seeds from both the susceptible (*C. olitorius*) and the resistant (*C. trilobularis*) plants were germinated on Petri dishes. These seedlings were allowed to grow on water only (without any growth medium) for three days in the absence of light. After the three days, two sets of seedlings from both species were sprayed with fungal suspension according to a protocol described earlier.<sup>39,40</sup> A set of control (uninfected) seedlings from both species were collected during the first collection of infected seedlings. One set of infected seedlings from each species were collected at intervals of 15 and 24 h, from the time of fungal spray. These seedlings were washed with distilled water, snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further use.

**RNA isolation and differential display.** Total RNA was isolated from 1.0 g frozen tissue (seedlings) using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration was measured spectrophotometrically. For differential display, first strand cDNA was prepared from all six samples using three anchored primers T<sub>12</sub>A, T<sub>12</sub>G and T<sub>12</sub>C. Three  $\mu\text{g}$  of total RNA was used in each 20  $\mu\text{l}$  reverse transcription (RT) reaction using SuperScriptIII RT (200 units/ $\mu\text{l}$ ) (Invitrogen). One  $\mu\text{l}$  of anchored primers (50  $\mu\text{M}$ ), 5  $\mu\text{l}$  of RNA solution, 4  $\mu\text{l}$  of 2.0 mM dNTPs mixture and 3  $\mu\text{l}$  of RNase-free water were mixed in a thin walled PCR tube and heated to  $65^{\circ}\text{C}$  for 5 min and then, incubated on ice. Then 4  $\mu\text{l}$  5  $\times$  RT-buffer (Invitrogen), 1  $\mu\text{l}$  0.1 M DTT, 1  $\mu\text{l}$  RNaseOUT (Invitrogen) and 1  $\mu\text{l}$  SuperScriptIII RT (200 units/ $\mu\text{l}$ ) (Invitrogen) were added in the tube and the mixture was incubated at  $25^{\circ}\text{C}$  for 5 min followed by 60 min at  $50^{\circ}\text{C}$  and heating at  $70^{\circ}\text{C}$  for 15 min. 0.5  $\mu\text{l}$  of *E. coli* RNaseH (Invitrogen) was added and the mixture was further incubated at  $37^{\circ}\text{C}$  for 20 min. The use of three different anchored primers gave rise to three different samples for each species. One 11-mer arbitrary primer was used as 5' primer in different combinations with each of the anchored primers for PCR using a thermal cycler (Eppendorf Mastercycler Personal). The PCR conditions used are as following: 1 cycle at  $94^{\circ}\text{C}$  for 3 min and 40 cycles at  $94^{\circ}\text{C}$  for 40 sec,  $40^{\circ}\text{C}$  for 50 sec and  $72^{\circ}\text{C}$  for 2 min followed by a final extension at  $72^{\circ}\text{C}$  for 5 min. Ten  $\mu\text{l}$  of each reaction was loaded onto 10% polyacrylamide gel and electrophoresis was performed for 3 h at 70 V. Following separation,

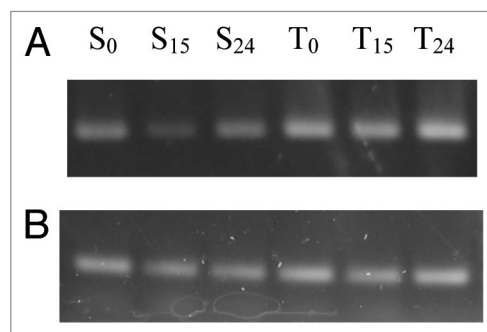


**Figure 6.** Southern hybridization analysis of genomic DNA from *C. olitorius* (Lane **1E** and **2B**) and *C. trilocularis* (Lane **3E** and **4B**) digested with *EcoRI* (**E**) and *BamHI* (**B**).

the amplified products were stained with silver nitrate using a method described earlier.<sup>41</sup> For a list of primers used in this article, please see **Table 2**.

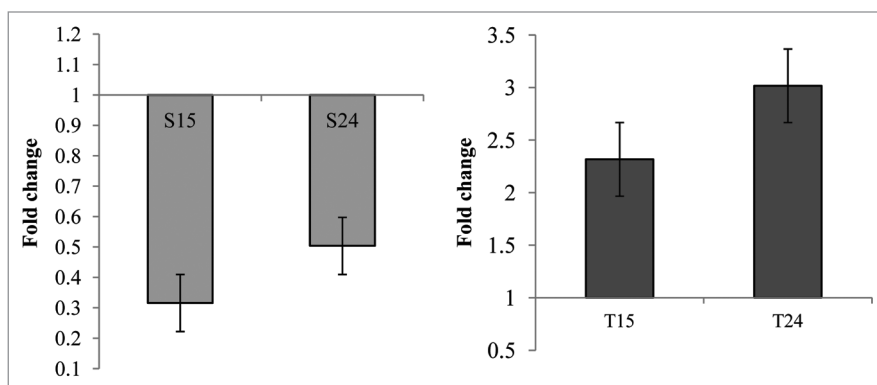
**Differentially expressed cDNA of interest.** The differentially expressed band was excised and incubated overnight in 2X volume (~100 $\mu$ L) of elution buffer (0.5M EDTA pH 8.0, 1M ammonium acetate) at 37°C with gentle shaking. The cDNA was ethanol precipitated and re-amplified using the arbitrary and the anchored primer (the primer pair that amplified the differentially expressed gene) and cloned into the pCR8/GW/TOPO TA Cloning vector (Invitrogen) following supplier's protocol and propagated in *E. coli* (DH5 $\alpha$ ) cells in LB medium. The cloned fragment was sequenced using M13 forward and M13 reverse primers.

**Primer based gene walking and rapid amplification of cDNA ends (RACE).** BLASTn and BLASTx search indicated the sequence to have high homology with an enzyme xyloglucan endotransglycosylase/hydrolase. A downstream degenerate primer XTHD2Rev was designed and checked by PCR (35 cycles, 95°C for 50 sec, 60°C for 40 sec, and 72°C for 1 min) in combination with gene specific primer XTHGF using genomic DNA as template. To explore the 3' end of the differentially expressed gene, 3' RACE was performed using the 3' RACE system (Invitrogen) as per supplier's instructions. cDNA was synthesized using adaptor primer AP. PCR of the cDNA was performed using gene specific forward primer XTH3'F and AUAP for 35 cycles (35 cycles, 95°C for 40 sec, 60°C for 30 sec, and 72°C for 1 min). For 5' RACE, two gene specific primers were custom synthesized (GSP and GSP2). The first strand cDNA was prepared from mRNA using the primer GSP.



**Figure 7. (A)** Semi-quantitative Reverse Transcription-PCR expression analysis of three *C. olitorius* samples (S0, S15 and S24) and three *C. trilocularis* samples (T0, T15 and T24) using XTHG'F and XTH 3'R primers. **(B)** The housekeeping gene  $\beta$ -actin was used to verify the quantitation.

For purification and the other subsequent steps, recommendations of the manufacturer were followed (5' RACE system, Invitrogen). PCR of target cDNA was performed with GSP and AAP primers for 35 cycles (95°C for 1 min, 60°C for 40 sec, and 72°C for 1 min). Nested PCR of the first round PCR product was performed using GSP2 and AUAP for 40 cycles (95°C for 1 min, 60°C for 40 sec, and 72°C for 1 min). Another gene specific primer (XTH 3'R) was designed from the 3' end and PCR amplification from the genomic DNA of *C. trilocularis* using XTH 3'F and XTH 3'R (35 cycles, 95°C for 1 min, 58°C for 40 sec, and 72°C for 50 sec) produced a 632bp product which led to identification of an intron. Interestingly, this same pair of primers yielded a PCR product of similar size from *C. olitorius* gDNA. Both the bands were sequenced and homology search for amplified *C. olitorius* gDNA band showed that it was also an XTH (*CoXTH1*) and the aforementioned approaches were also applied to determine its sequence. PCR amplification of gDNA of *C. olitorius* and *C. trilocularis* with XTH5'F and XTHGR primers (35 cycles, 95°C for 1 min, 59°C for 40 sec,



**Figure 8.** Real-time PCR analysis of **(A)** *CoXTH1* and **(B)** *CtXTH1* genes to see the change in expression level by *M. phaseolina*. Samples were infected and collected after 15 (S15 and T15) and 24 (S24 and T24) hours of incubation. The mean fold changes of expression (of two duplicate assays) using RNA samples from infected seedlings shows the downregulation of *CoXTH1* and upregulation of *CtXTH1* followed by fungal infection.

**Table 1.** Different samples for differential gene expression study

Species	Sample	Age of seedling + time before RNA isolation
<i>C. olitorius</i>	S <sub>0</sub>	3 d uninfected + 15 h of uninfected growth
	S <sub>15</sub>	3 d uninfected + 15 h of infected growth
	S <sub>24</sub>	3 d uninfected + 24 h of infected growth
<i>C. trilocularis</i>	T <sub>0</sub>	3 d uninfected + 15 h of uninfected growth
	T <sub>15</sub>	3 d uninfected + 15 h of infected growth
	T <sub>24</sub>	3 d uninfected + 24 h of infected growth

**Table 2.** Primers used in this study

Primer	Sequence
T <sub>12</sub> A	5'-TTT TTT TTT TTT A-3'
T <sub>12</sub> G	5'-TTT TTT TTT TTT G-3'
T <sub>12</sub> C	5'-TTT TTT TTT TTT C-3'
Arbitrary primer	5'-CTG GCG TGT CA-3'
XTHD2Rev	5'-CCC AMC KRT AWC KTY KAG CTT C-3'
XTHGF	5'-GTC CAA ACA AAC GTG TTC GC-3'
Adaptor primer (AP)	5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3'
XTH 3'F	5'-GCA GCC GAC ACT TGT ATG GT-3'
AUAP	5'-GGC CAC GCG TCG ACT AGT AC-3'
GSP	5'-CCT GTC ACC CTT TCC AT-3'
GSP2	5'-TGC CCA CTT CGG TTT CCC AAG A-3'
AAP	5'-GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG-3'
XTH 3'R	5'-GGA TTT GTC GGT GCA ATA AT-3'
XTH 5'F	5'-TTT GTA ATT CAA AAC ACG ACA ATG GGT-3'
XTHGR	5'-CCT TAT GGT TCC ATC TAA TTT GG-3'

and 72°C for 1 min) helped to identify the introns of *CtXTH1* and *CoXTH1*.

**Southern blot analysis.** 15 µg of *C. trilocularis* and *C. olitorius* gDNA were digested with EcoRI (Invitrogen) and BamHI (Invitrogen) and transferred to a positively charged nylon membrane (Amersham Hybond™-N) according to manufacturer's protocol. *CtXTH1* and *CoXTH1* sequences were amplified using XTH5'F and XTHGR primers and labeled with digoxigenin-dUTP following manufacturer's protocol (DIG nucleic acid detection and labeling kit, Roche). XTH specific sequences

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blotted on nylon membrane were detected following an immunologic assay according to manufacturer's protocol.

**Semiquantitative reverse-transcription-PCR.** The quantity and quality of six RNA samples (Table 1) were measured both spectrophotometrically and by agarose gel electrophoresis. First strand cDNA was synthesized using XTH 3'R primer. PCR amplification of the cDNA was performed for 30 cycles using primers XTH GF and XTH 3'R following the amplification conditions mentioned earlier. β-actin was used as a normalization control for reverse transcription polymerase chain reaction (RT-PCR).

**Real-time PCR analysis.** First strand cDNA synthesis (for six samples, two replicates each) was performed with 3 µg of total RNA using XTH 3'R primer and Superscript III reverse transcriptase (Invitrogen) following the manufacturer's recommendations. Real time quantifications were performed in Roche LightCycler® 480 Real-Time PCR System using LightCycler® 480 SYBR Green I Master kit (Roche) in a 20 µL PCR volume containing 10 µL of 2X LightCycler® 480 SYBR Green I Master (contains FastStart Taq DNA Polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and MgCl<sub>2</sub>), 1.5 µL cDNA and 0.15 µM of each of the gene specific and β-actin gene primers (internal control). The thermal cycling parameters included a pre-incubation at 95°C for 5 min and 35 cycles of amplification (95°C for 40 sec, 58°C for 40 sec, and 72°C for 40 sec). Following the amplification, a three stage cycle was used for the determination of the melting curve (95°C for 0 sec, 70°C for 1 min and 95°C for 0 sec at continuous acquisition mode). The data was analyzed using the 2<sup>-ΔΔC<sub>t</sub></sup> method.<sup>42,43</sup>

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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## Supplemental Material

Supplemental material may be downloaded here:  
[www.landesbioscience.com/journals/cib/article/21422/](http://www.landesbioscience.com/journals/cib/article/21422/)



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