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Biochemical analysis of expansin-like proteins from microbes

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ABSTRACT

Expansins cause plant cell wall loosening and are present primarily in the plant kingdom. Gene sequence analysis suggests that expansins are present in several plant-colonizing or plant-pathogenic bacteria and fungi. However, experimental evidence of microbial expansin activity is largely lacking. Here we provide evidence that expansins from three plant pathogenic bacteria and one fungus cause extension of cell walls *in vitro* and weaken filter paper networks, without lytic activity. Since expansins were able to weaken cellulose networks, we tested whether they synergistically enhanced the activity of several cellulases in hydrolysis of cellulose. The microbial expansins did not show such synergism beyond the nonspecific effect of bovine serum albumin. Our results show that the expansins present in several pathogenic microbes have weak wall-loosening activity and we infer a role for these expansins in plant pathogenesis. Additionally, the convenient expression of several expansins in *Escherichia coli* makes a future comparative structure–function analysis among expansins possible in order to understand their activity at the molecular level.

1. Introduction

Primary cell walls provide mechanical support and determine the shape of plant cells and at the same time are able to expand in response to wall-loosening agents, enabling cells to enlarge and thereby plants to grow (Cosgrove, 2005). Expansins comprise a family of plant proteins that mediate plant cell wall loosening and stress relaxation, with multiple roles in plant physiology and development, including cell expansion, fruit softening, organ abscission and pollen tube penetration of the stigma (Cosgrove, 2000; McQueen-Mason, Durachko, & Cosgrove, 1992; Sampedro & Cosgrove, 2005). They lack lytic activity against cell walls and are believed to break non-covalent interactions between cell wall components, allowing polymer slippage (creep) and cell wall loosening, but the molecular details of their action and their specific targets are not yet well defined, in part because of the structural complexity of the cell wall and in part because of the lack of any detectable enzymatic activity by expansins (Cosgrove, 2000; McQueen-Mason & Cosgrove, 1995; Tabuchi, Li, & Cosgrove, 2011). Structurally, canonical expansins consist of two tightly packed domains (D1, D2) with a polysaccharide binding surface spanning the two domains (Yennawar, Li, Dudzinski, Tabuchi, & Cosgrove, 2006). Domain D1 is a double-ψ beta barrel resembling family-45 endoglucanases, but lacks key residues necessary for hydrolytic activity; domain D2 has an immunoglobulin-like beta-sandwhich fold and is the principle determinant of expansin binding to cell wall components (Georgelis, Tabuchi, Nikolaidis, & Cosgrove, 2011; Georgelis, Yennawar, & Cosgrove, 2012).

Looking beyond the plant kingdom, expansin-like proteins are evidently employed by a few, diverse organisms. *Globodera rostochiensis*, a plant-parasitic nematode, contains a functional expansin-like protein, presumably for invading plant roots (Qin et al., 2004) and expansin-like genes or domains are found in the genomes of the slime mold *Dictyostelium discoidium* (which makes cellulose in some life stages) and in some fungi (e.g., 'swollenins') (Bouzarelou, Billini, Roumelioti, & Sophianopoulou, 2008; Darley, Li, Schaap, & McQueen-Mason, 2003; Li et al., 2002; Saloheimo et al., 2002). Recently it was shown by X-ray crystallography that a protein (EXLX1) from the plant-colonizing bacterium *Bacillus subtilis* is structurally homologous to the maize pollen expansin EXPB1 (Kerff et al., 2008). Biomechanical assays showed that BsEXLX1 could induce plant cell wall creep *in vitro*, indicating that it has classical expansin activity, notwithstanding its low sequence identity to EXPB1 (~15%) and its lack of motifs conserved in plant expansins (Kerff et al., 2008). Gene database searches identified other predicted proteins or protein domains with significant sequence similarity to EXLX1 in several plant pathogenic bacteria, providing evidence that expansins may be present in many, though not all, plant pathogens (Kerff et al., 2008; Li et al., 2002).

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This idea is strengthened by tests showing that bacterial mutants lacking expansin genes or genes with expansin modules are crippled in their ability to colonize plant surfaces or to cause symptoms of bacterial wilts (Jahr, Dreier, Meletzus, Bahro, & Eichenlaub, 2000; Kerff et al., 2008; Laine et al., 2000); in addition, ectopic expression of swollenin in the fungus *Trichoderma asperellum* enhanced its ability to colonize cucumber roots (Brotman, Briff, Viterbo, & Chet, 2008). However, the potential cell-wall activities of these expansin-like proteins have not been examined; currently the only bacterial protein demonstrated to have expansin activity is EXLX1 from *B. subtilis* (Georgelis et al., 2011; Kerff et al., 2008).

Here, we tested the ability of three expansin-like proteins or modular domains from plant pathogenic bacteria *Xanthomonas campestris*, *Clavibacter michiganensis* and *Ralstonia solanacearum* and one from the plant pathogenic fungus *Aspergillus niger* to cause loosening (creep) of plant cell walls. We also tested the ability of the proteins to hydrolyze wall polysaccharides and to weaken pure cellulose networks, to gain knowledge into their exact target. Finally, we examined their potential synergistic effects with cellulases for soluble sugar release from cellulose.

2. Material and methods

2.1. Materials

Filter paper #3 (GE Healthcare, Waukesha, WI, USA) was used as cellulose substrate. Dye-coupled cross-linked (AZCL-) polysaccharides were purchased from Megazyme (Bray, Ireland). Wheat coleoptiles (*Triticum aestivum* L. cv Pennmore) were prepared for creep assays as described in Li, Bedinger, Volk, Jones, and Cosgrove (2003). *Gluconacetobacter xylinus* was grown as described in Whitney, Gidley, and McQueen-Mason (2000) and pellicle strips (10 mm × 0.5 mm × 0.5 mm) were prepared for extension assays as described in Georgelis et al. (2011).

2.2. Cloning and expression of microbial proteins in Escherichia coli

A full length protein and an expansin domain (Xantho-FL and Xantho-EXP, respectively) were PCR-amplified from *Xanthomonas campestris* pv. *campestris* str. ATCC 33913 genomic DNA (NCBI reference sequence: NP_638881.1) with primers 5′CAGCATATGCTTGTCGATGCAACAGGTCAGACGCTG3′-5′CAGGCGGCCGCCTACGGAAACTGCACATG3′ and 5′CAGCATATGACGAAGAATGCGAGTGGGAAGAAG3′-5′CAGGCGGCCGCCTACGGAAACTGCACATG3′, respectively. Ralstonia-EXP was PCR-amplified from *Ralstonia solanacearum* str. UW551 genomic DNA (NCBI reference sequence: ZP_00944742.1) with primers 5′CAGCATATGGCCTGGGACAGCACGTTC3′-5′CAGCTCGAGTTACTCGGGAAACTGCAC3′. Clavi-FL, Clavi-GH5, Clavi-GH5-CBM and Clavi-EXP were PCR-amplified from *Clavibacter michiganensis* subsp. *michiganensis* NCPPB 382 plasmid DNA (NCBI reference sequence: YP_001220664.1) with primers 5′GCGATGGCCATGGCGACCGTAGCGGGGCCCGTT3′-5′CCGCTCGAGTCAGTGCACAGGGTAGAA3′, 5′GCGATGGCCATGGCGACCGTAGCGGGGCCCGTT3′-5′CCGCTCGAGCTACAAGATCGGCGCCAGGGCGT3′, 5′GCGATGGCCATGGCGACCGTAGCGGGGCCCGTT3′-5′CCGCTCGAGCTAGGCACGGCCATGCGTCGTG3′ and 5′GCGATGGCCATGACGACGCATGGCCGTGCCACGC3′-5′CCGCTCGAGTCAGTGCACAGGGTAGAA3′, respectively. The cDNA encoding Asper-EXP (NCBI protein accession: EHA21207.1) was synthesized by Life Technologies (Carlsbad, CA, USA) from amino acid sequence (M)ALSSEYSG to TASSNFE(HHHHHH) (a 6 His-tag was added at the C-terminus) and cloned between NcoI and XhoI sites of the pET22b vector (Novagen). Xantho-FL and Xantho-EXP were cloned between the NdeI and NotI sites of the pET22b vector while Ralstonia-EXP was cloned between the NdeI and XhoI of sites of the same vector. All Clavi proteins were cloned between the MscI and XhoI sites of the pET22b vector. A methionine was added at the N-terminus of all proteins. The proteins were expressed in *E. coli* strain BL21 (DE3-pLys). Cells were grown to OD600 = 0.6 at 37 °C and induced with 0.1 mM IPTG for 6 h at room temperature.

2.3. Protein purification

All proteins except for Asper-EXP and Clavi-GH5 were purified as described by Kerff et al. (2008), except 25 mM NaOAc (pH 5.5) was used instead of 50 mM Tris–HCl (pH 8.0). Clavi-GH5 was loaded onto a Q-Sepharose column using 25 mM HEPES pH 7.5 as buffer. Fifty ml of flow-through was collected, added to 25 ml of 3.5 M ammonium sulfate and mixed for 15 min. The mixture was centrifuged at 18,000 × *g* for 15 min and 75 ml of supernatant was mixed with 27.5 ml of 3.5 M ammonium sulfate for 15 min. The mixture was centrifuged at 18,000 × *g* for 15 min and the pellet was resuspended in 2 mL of 25 mM NaOAc (pH 5.5). The sample was loaded onto a Hiprep Sephacryl S-100 column (GE Healthcare) with 25 mM NaOAc (pH 5.5) + 0.15 M NaCl as mobile phase at 0.5 ml/min and fractions containing Clavi-GH5 were collected, concentrated and exchanged into 25 mM NaOAc (pH 5.5) with an Amicon 10-kD filter (EMD Millipore, Billerica, MA). Clavi-FL, Clavi-GH5-CBM and Xaca-FL were also loaded onto Hiprep Sephacryl S-100 after the initial purification steps for additional protein purification, as described for Clavi-GH5. Asper-EXP was purified with Ni-NTA agarose (Qiagen, Valencia, CA, USA) following the instructions in the product manual. All purification steps were done at 4 °C. The purity of 15-μg samples was checked on 12% SDS-PAGE stained with Coomassie Brilliant Blue R-250. For endoglucanase assays, Xantho-EXP and Ralstonia-EXP were further purified by HPLC on a reverse-phase column (Discovery C8, Supelco, Bellefonte, PA, USA) preequilibrated with 10% methanol containing 0.1% trifluoroacetic acid. Bound protein was eluted at 1 ml/min with a linear gradient of methanol at a flow rate of 1 ml/min at 25 °C. We confirmed creep activity of Xantho-EXP and Ralstonia-EXP purified in this way (data not shown).

2.4. Creep assay and paper strength assay

Creep assays of alkali-pretreated wheat coleoptiles and *G. xylinus* pellicle strips and paper strength assays were done as described by Georgelis et al. (2011).

2.5. Protein alignment

Proteins were aligned with ClustalW as implemented by MEGA software (Kumar, Tamura, & Nei, 2004) using default parameters except the BLOSUM substitution matrix. The alignment was visualized by BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html).

2.6. Test for synergism between expansins and cellulases

One disk of filter paper #1 (GE Healthcare) (2.5 mg) was incubated in 250 μl of 25 mM NaOAc (pH 5.5) in the presence of 1 μg of Celluclast 1.5L (CL) (Novozymes, Bagsvaerd, Denmark) or exoglucanase CBHI (Megazyme) or endoglucanase EGI (Cat#: E-CELTR, Megazyme) and 5 μg of expansin or bovine serum album (BSA, a negative control) at 30 °C. The total soluble sugars in the supernatant were measured at 12 and 24 h by phenol/sulfuric acid assay as described by Dubois, Gilles, Hamilton, Rebers, and Smith (1956) and modified by Hanson and Phillips (1981).

2.7. Test for synergism between bacterial expansin EXLX1 and cellulase mix CL

One disk of filter paper #1 (2.5 mg) was incubated in 120 μl of 50 mM citrate buffer (pH 4.8) in the presence of 0.02, 0.04, 0.08 and 0.24 μg of CL and 0.75 μg of expansin or BSA (negative control) at 50 °C. The reducing sugars in the supernatant were measured at 48 h by PAHBAH assay (Lever, 1972).

2.8. Effect of CBM and expansin domain on Clavi-FL cellulase activity

One disk of filter paper #1 (GE Healthcare) (2.5 mg) was incubated in 250 μl of 25 mM NaOAc (pH 5.5) in the presence of 0.6 μM of Clavi-FL or Clavi-GH5 or Clavi-GH5-CBM at 30 °C. The reducing sugars in the supernatant were measured at various times by PAHBAH assay.

3. Results and discussion

3.1. Fungal proteins similar to EXLX1

A BLASTP search of fungal genomic databases was performed with EXLX1 (NCBI accession # O34918) as query. Several candidates were identified (Fig. S1) from which an expansin-like sequence from *A. niger* was selected for further study. The *A. niger* sequence (NCBI accession # EHA21207.1) has a different modular structure than swollenin, which is composed of ~475 amino acids *versus* ~225 for canonical expansins. Swollenin contains a family-1 cellulose-binding module (CBM), a lengthy insertion between the CBM and the expansin-like domain and large gaps in alignments with plant expansins (Saloheimo et al., 2002). Previous work established that swollenin did not cause loosening (creep) of cucumber hypocotyl cell walls (Cosgrove, unpublished results). In contrast, the predicted *A. niger* protein lacks a CBM but does have an N-terminal extension (~140 amino acids) of unknown function which was removed for recombinant protein production, resulting in the protein Asper-EXP. The fungus *A. niger* causes black mold disease in certain fruits and vegetables and several of its plant cell wall degrading enzymes have been studied for potential applications in biofuel production (de Vries, 2003). For these reasons, the expansin domain from *A. niger* was chosen for study over the other fungal expansin-like sequences.

3.2. Expansin-like sequence alignment to EXLX1

The expansin-like proteins or protein domains of *X. campestris* (Xantho-EXP), *C. michiganensis* (Clavi-EXP), *R. solanacearum* (Ralstonia-EXP) and *A. niger* (Asper-EXP) align well with EXLX1 (Fig. 1) and have 66/77%, 25/40%, 58/73%, 23/35% identity/similarity to EXLX1, respectively. EXLX1 residues D71, D82, W125, W126 and Y157 are part of a polysaccharide binding surface spanning domains D1 and D2 (Georgelis et al., 2011; Kerff et al., 2008). These residues, which are important for cell wall loosening (creep) activity (Georgelis et al., 2011), are conserved or changed to amino acids with similar properties in all expansin-like proteins tested here (Fig. 1). The conservation of these sites suggests that these proteins might have wall-loosening activity, despite the low to moderate sequence similarity with EXLX1.

Xantho-EXP and Clavi-EXP (but not Ralstonia-EXP) are C-terminal domains of larger, modular proteins. The N-terminal domain of the full length Xantho-FL (FL: full-length) has sequence similarity to family-5 glycosyl hydrolases (GH-5 endoglucanases) while Clavi-FL contains both a GH5 domain as well as a domain similar to CBMs known to enhance binding of hydrolytic enzymes to cellulose and other saccharides. Although we briefly studied the activity of the N-terminal domains of Xantho-FL and Clavi-FL, we focused mainly of the expansin-like portion of the proteins Xantho-EXP and Clavi-EXP.

3.3. The expansin-like proteins cause plant cell wall creep

Xantho-EXP, Clavi-EXP, Ralstonia-EXP and Asper-EXP were expressed in *E. coli*, purified by various chromatographic procedures (see Section 2), and assessed by SDS-PAGE and various activity assays. The recombinant proteins migrated in the polyacrylamide gel as expected based on their theoretical molecular weight (Fig. 2). All four proteins were able to cause cell wall extension

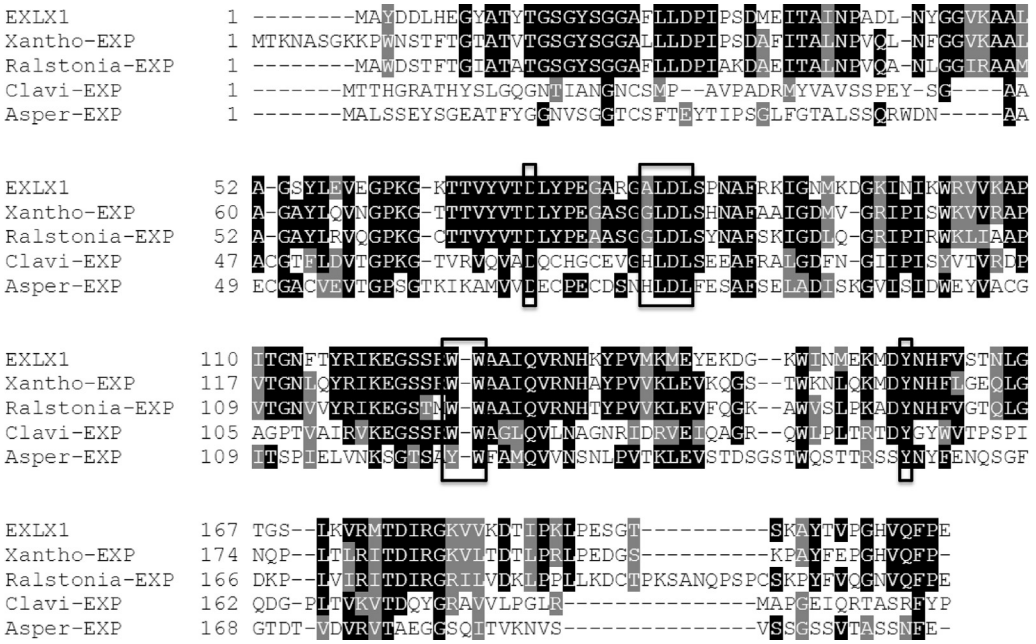

Fig. 1. Protein alignment of EXLX1, Xantho-EXP, Ralstonia-EXP, Clavi-EXP and Asper-EXP. Darker background indicates higher amino acid conservation among the sequences. The boxed amino acids are signature sequences of expansins or amino acid residues important to creep activity in EXLX1.

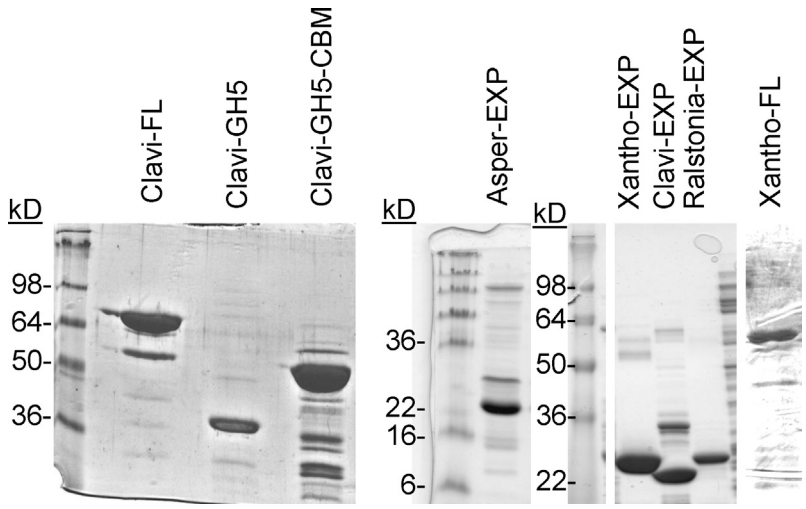

Fig. 2. The purity of proteins was visualized by SDS-PAGE (12%). Ten micrograms of each protein were loaded and stained with Coomassie Brilliant Blue R-250.

in wheat coleoptile specimens (Fig. 3), although the response was relatively small and transient compared with that induced by plant expansins. The wall extension response upon protein application was rapid (1–2 min), which is consistent with previously studied expansins (Kerff et al., 2008; Li et al., 2003; McQueen-Mason et al., 1992) and differs from the delayed extension response caused by family-12 endoglucanases (Park & Cosgrove, 2012). These results show that Xantho-EXP, Clavi-EXP, Ralstonia-EXP and Asper-EXP have wall extension activity characteristic of expansin, although it is weak compared to plant expansins.

We interpret these results as support for the hypothesis that microbial expansins act on plant cell walls to facilitate microbe interactions with plants. Previous work found that a *B. subtilis* mutant knocked out in EXLX1 was severely impaired in its ability to colonize maize roots (Kerff et al., 2008) and that in *C. michiganensis* the modular endoglucanase gene containing Clavi-EXP was a key determinant of *Clavibacter*-induced wilting of tomato seedlings (Gartemann et al., 2003; Jahr et al., 2000; Laine et al., 2000). These results are also important from a technical point because the ability to express a variety of microbial expansins in *E. coli* in active form will facilitate future structure-function analyses to elucidate the role of individual domains and amino acids for wall loosening activity. This is particularly important considering that plant expansins are recalcitrant to expression in heterologous systems, hampering such analyses (Kerff et al., 2008).

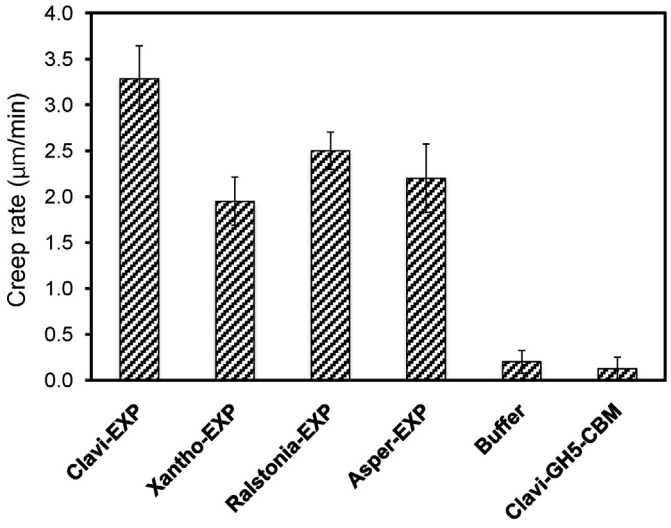

Fig. 3. Extension (creep) rate of alkali-pretreated wheat coleoptile specimens after application of microbial expansins. The rate of extension represents the maximum rate of extension of specimens after application of 100 μg/ml of protein. Application of plain buffer 25 mM NaOAc (pH 5.5) instead of protein was used as a negative control. Error bars indicate standard errors (SE; $4 \leq N \leq 10$).

3.4. Microbial expansins lack lytic activity but loosen pure cellulosic networks

To test for polysaccharide lytic activity, we incubated Xantho-EXP, Clavi-EXP, Ralstonia-EXP and Asper-EXP with dye-coupled cross-linked polysaccharide substrates representative of the major plant cell wall structural polysaccharides. None of the microbial expansins gave appreciable lytic activity (Fig. S2). Partially purified preparations of Xantho-EXP and Ralstonia-EXP showed trace endoglucanase activity, with approximately two orders of magnitude lower specific activity than that of endoglucanase EGI which was used as a positive control. This trace activity evidently arose from contaminating *E. coli* endoglucanases since further purification of Xantho-EXP and Ralstonia-EXP by HPLC completely eliminated their lytic activity (Fig. S2), but not their wall extension activity. These results are similar to those reported previously for EXLX1 and strengthen the conclusion that microbial expansins, like plant expansins, lack wall lytic activity. We also found that the GH5-containing protein from *Clavibacter* (Clavi-FL) possessed appreciable endoglucanase activity (Fig. S2), as expected for an active GH5 enzyme, whereas the GH5-containing Xantho-FL protein had little or no endoglucanase activity.

To test the ability of the microbial expansins to weaken pure cellulose networks, we applied all four expansin proteins individually to pre-washed *G. xylinus* pellicle strips that were clamped under constant force. This material is a tangled mat of cellulose microfibrils whose mechanical behavior is dominated by frictional contacts and entanglements between microfibrils (Whitney, Gothard, Mitchell, & Gidley, 1999). Application of expansins caused the pellicle strip to extend transiently at a faster rate than buffer controls (Fig. 4). Additionally, all the microbial expansins reduced the force required for breakage of filter paper, a network of cellulose fibers (Fig. 5). Collectively, these results indicate that Xantho-EXP, Clavi-EXP, Ralstonia-EXP and Asper-EXP can weaken pure cellulose networks without requiring another hemicellulosic, pectic or protein component of the plant cell wall. Therefore, we conclude that cellulose is a likely target for these expansins.

Similarly, EXLX1, along with the cucumber α-expansins CsEXPA1 and CsEXPA2, have been shown to weaken filter paper

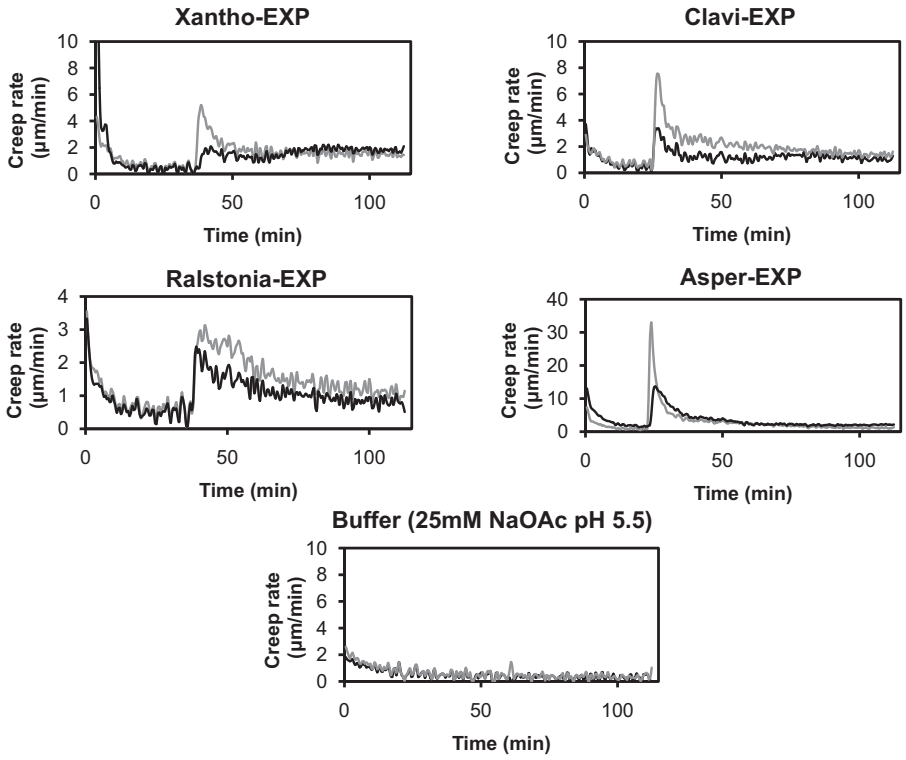

Fig. 4. Extension rate of *G. xylinus* pellicle strips after application of microbial expansins. The rate of extension represents the maximum rate of extension of specimens after application of 100 μg/ml of protein. Application of plain buffer 25 mM NaOAc (pH 5.5) instead of protein was used as a negative control. The graphs show two representative curves for each protein.

(Georgelis et al., 2011; Kim, Lee, Bang, Choi, & Kim, 2009; McQueen-Mason & Cosgrove, 1994), and so we must consider the possibility that cellulose may be the native target of both plant α-expansins and microbial expansins. How likely is it that expansins cause plant cell wall loosening by weakening direct cellulose–cellulose contacts in the plant cell wall? This idea is at odds with most popular models of plant primary cell walls which consider direct contacts between cellulose microfibrils to be absent; these models instead consider microfibrils to be well separated by matrix polysaccharides and linked indirectly by hemicelluloses (e.g., xyloglucan, xylan) configured as tethers (e.g., see Carpita & Gibeaut, 1993). This 'tethered network' model of primary cell wall architecture was recently found to be inconsistent with the results of endoglucanase-induced cell wall loosening (Park & Cosgrove, 2012), where close contacts between microfibrils were inferred. The fact that some expansins can both weaken pure cellulose networks and cause plant cell wall creep suggests the possibility of direct load-bearing contacts between cellulose microfibrils in plant cell walls and these contacts are a site of expansin-induced wall loosening. We consider the extent and significance of direct cellulose–cellulose contacts in primary cell walls to merit further study. A variation of this hypothesis is that expansins may act to dissociate cellulose chains or bundles that entrap hemicelluloses; by freeing such entrapped hemicelluloses, expansins would loosen the cell wall, allowing stress relaxation and consequent cell wall creep. Finally, it is also possible that the expansins studied above may have broad substrate specificity and be able to act on hemicellulose or pectin aggregates, as well as cellulose aggregates. Although we know of no direct support for this idea, the nature of the entropy-driven binding interaction seen in expansin–oligosaccharide crystal complexes suggests the possibility that expansins could interact with a wide range of planar polysaccharides (Georgelis et al., 2012).

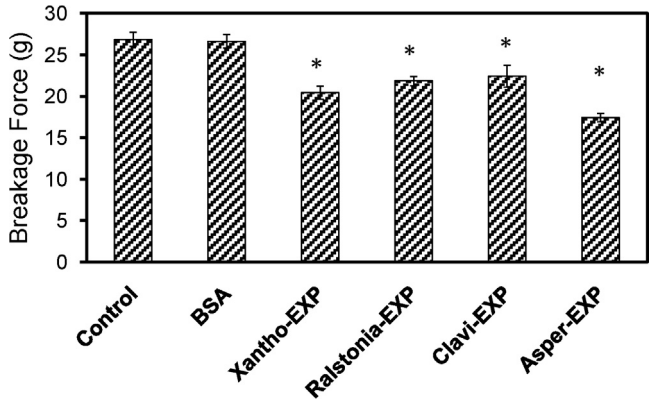

Fig. 5. Filter paper weakening activity of microbial expansins. Proteins were added at 120 μg/mL and an equimolar amount of BSA was used as negative control. NaOAc buffer at pH 5.5 and 25 mM was used as an additional negative control. Error bars indicate standard errors of the mean, $N = 7$. * Denotes $p \leq 0.05$ (Two-tailed t-test) of the tested protein against buffer control.

Despite the fact that microbial expansins can weaken a cellulose network, no detectable soluble sugar was released after incubation of microbial expansins with cellulose (Fig. 6), providing further support for a non-enzymatic action. In contrast, comparable amounts of cellulases released significant amounts of soluble sugars from cellulose.

3.5. Lack of synergism between expansins and cellulases in cellulose degradation

The fact that all expansins tested in our study could modify cellulosic networks prompted us to test whether they might synergistically enhance cellulase hydrolysis of cellulose, potentially for biofuel production. Xantho-EXP, Clavi-EXP and Asper-EXP were mixed with exoglucanase CBHI, endoglucanase EGI or a commercial

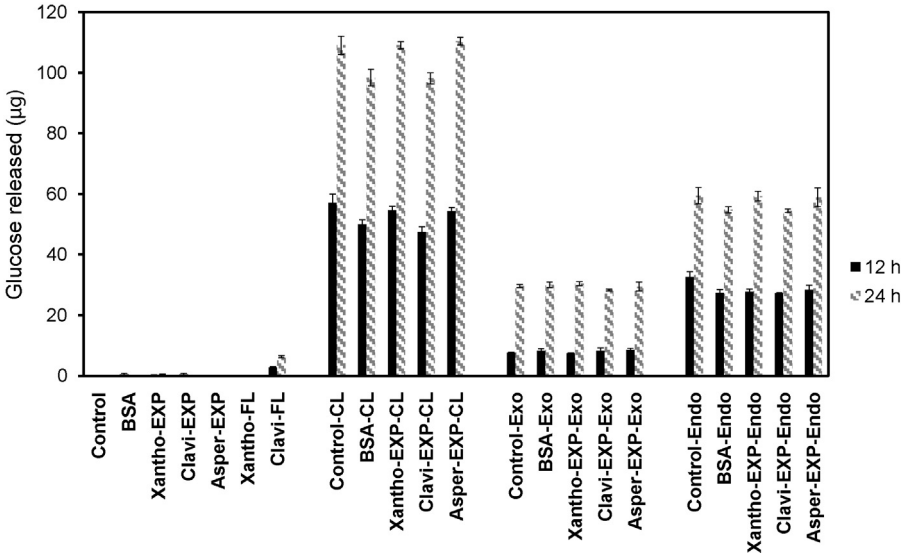

Fig. 6. Test of synergism between microbial expansins and several cellulases. Filter paper disks were incubated in buffer containing expansins alone or cellulases alone or a mixture of an expansin and a cellulase. Exo, CBHI exoglucanase; Endo, EGI endoglucanase; Control, No expansin. The presence of expansin did not increase the sugars released by cellulases. Mean ± SE ($N = 3$).

mixture of cellulolytic enzymes extracted from *Trichoderma reesei* (Celluclast 1.5L or CL). Expansins did not enhance the activity of these cellulases (Fig. 6) despite the fact that they were used at fivefold amounts compared to the cellulases.

Our results contrast with a previous study in which the bacterial expansin EXLX1 was reported to enhance the release of soluble sugars from filter paper by CL (Kim et al., 2009). This study reported that EXLX1 acted synergistically with CL at low CL loadings while the synergism disappeared when higher amounts of CL were used. We mimicked the conditions used in that report and indeed detected some synergism at low CL loadings, but we found the synergism was not specific to EXLX1 but could be seen when an equal amount of BSA was used instead of EXLX1 (Fig. S3). This non-specific type of synergism between EXLX1 and CL was also observed by Kerff et al. (2008) while synergism between BSA and cellulases has been reported for more complicated lignocellulosic material (Yang & Wyman, 2006). The reason that BSA and EXLX1 increase the activity of CL at low CL concentrations is not clear. One possibility is that inclusion of BSA or EXLX1 decreases the non-productive binding of CL cellulases to cellulose, leaving more cellulase molecules available for productive binding. This type of synergism is unlikely to be of use to the biofuel production field since it disappears when cellulase is used at amounts capable of converting the majority of cellulose to soluble sugars (Fig. S3).

Although microbial expansins failed to synergize with cellulases *in vitro*, it is possible that they loosen plant cell walls *in vivo*, increasing wall porosity and exposing new targets for microbial hydrolytic enzymes. Therefore, it is worth expressing microbial expansins in plants to test whether they increase the digestibility of plant cell walls. Alternatively, it is possible that expansins may act synergistically with other classes of cellulases not tested here.

3.6. Functional significance the N-terminal domains of Clavi-FL and Xantho-FL

The N-terminal domain of Clavi-FL, termed Clavi-GH5-CBM, has substantial endoglucanase activity (Fig. S2). Additionally, the Clavi-GH5-CBM protein has cellulase activity, as evidenced by the release of soluble oligosaccharides from filter paper disks (Fig. 7). However, Clavi-GH5-CBM did not cause creep of wheat coleoptile specimens (Fig. 3).

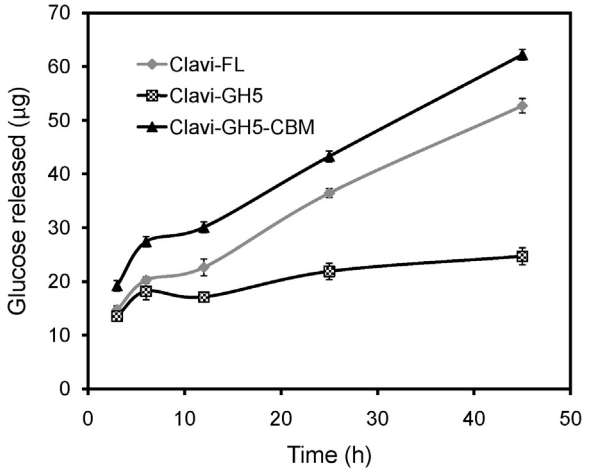

Fig. 7. Cellulase activity of Clavi-FL, Clavi-GH5 and Clavi-GH5-CBM. Filter paper disks were incubated in buffer containing Clavi-FL, Clavi-GH5 and Clavi-GH5-CBM. Each point is an average ± SE of three replicates.

To test the significance of the CBM and the expansin domain of Clavi-FL for cellulase activity, we compared the cellulase activity of Clavi-FL, Clavi-GH5-CBM and Clavi-GH5 expressed in *E. coli* and purified (Fig. 2). Clavi-GH5-CBM lacks the expansin domain while Clavi-GH5 lacks both expansin and CBM domain. The activity of Clavi-FL was slightly lower than that of Clavi-GH5-CBM, indicating that the expansin domain does not have a positive contribution to the cellulase activity of Clavi-FL (Fig. 7), at least under these experimental conditions. In contrast, the inclusion of the CBM domain increased cellulase activity (Fig. 7), consistent with previous reports about the roles of CBM domains in cellulase activity (Bolam et al., 1998; Boraston, Bolam, Gilbert, & Davies, 2004; Herve et al., 2010).

4. Conclusions

Analysis of the phylogenetic distribution of expansins in the rapidly expanding sequence databases has indicated the presence of expansin-like sequences in a variety plant pathogens, but experimental assessment of their potential expansin activity has lagged

significantly. Herein we provide experimental evidence of expansin activity for four such proteins, three from bacteria and one from a fungus. In addition to their wall-loosening activity, the microbial expansins can weaken filter paper and *G. xylinus* pellicles, suggesting cellulose as a potential target of action. However, these expansins – like others tested previously - lack lytic activity for cellulose as well as for the other major polysaccharides of plant cell walls and do not enhance the hydrolytic activity of various fungal cellulases, except in a nonspecific manner. Thus, while these and other published results support the idea that microbial expansins promote interactions of the microbe with the plant by binding or modifying the plant cell wall, the mechanism of such promotion is evidently not by aggressive deconstruction of the cell wall. Moreover, the wall-loosening action of the five microbial expansins tested to date is very modest indeed, so much so that we deem it unlikely to be the primary means by which they exert their large effects on surface colonization and development of symptoms (wilting in the case of *C. michiganensis*). A better understanding of the functions of microbial expansins may lead to new strategies for reducing the negative impacts of plant pathogens or enhancing the effectiveness of biocontrol agents that make use of expansins for colonizing plant surfaces.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2004.08.011.

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