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Origin, distribution and 3D-modeling of Gr-EXPB1, an expansin from the potato cyst nematode *Globodera rostochiensis*

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Abstract Southern analysis showed that Gr-EXPB1, a functional expansin from the potato cyst nematode *Globodera rostochiensis*, is member of a multigene family, and EST data suggest expansins to be present in other plant parasitic nematodes as well. Homology modeling predicted that Gr-EXPB1 domain 1 (D1) has a flat β-barrel structure with surface-exposed aromatic rings, whereas the 3D structure of Gr-EXPB1-D2 was remarkably similar to plant expansins. Gr-EXPB1 shows highest sequence similarity to two extracellular proteins from saprophytic soil-inhabiting Actinobacteria, and includes a bacterial type II carbohydrate-binding module. These results support the hypothesis that a number of pathogenicity factors of cyst nematodes is of procaryotic origin and were acquired by horizontal gene transfer.

Keywords: Expansin; Plant parasitic nematode; Horizontal gene transfer; Protein modeling; Cell wall modifying proteins

1. Introduction

The plant cell wall is a complex composite of cellulose microfibrils and cross-linking hemicelluloses, which are embedded in a matrix of pectic polymers and structural glycoproteins. To overcome this structural barrier, a wide range of saprophytic and plant pathogenic bacteria, oomycetes, fungi and nematodes secrete various types of cell wall-degrading enzymes (CWDEs). The compactness of plant cell walls limits the efficiency of CWDEs. Expansins, a relatively diverse protein superfamily that is widespread within the plant kingdom, directly modulate the mechanical properties of the cell wall by weakening non-covalent interactions. These proteins are suggested to open up this compact structure, making it accessible to enzymatic attack [1]. As such, expansins could substantially increase the fitness of CWDE-harboring plant pathogens.

So far, only a few proteins have been identified among plant pathogens and saprophytes that remotely resemble expansins, and for none of these proteins expansin activity was shown (e.g. [2,3]). Recently, we showed the presence of a functional expansin in secretions of infective juveniles of the potato cyst nematode *Globodera rostochiensis* [4]. Here, we show that *Gr-EXPB1* is member of a multigene family, and present in other cyst nematode species as well. Remote homology modeling revealed that the predicted 3D structures of Gr-EXPB1 D2 and plant expansins are similar, even though their primary amino acid sequences show a relatively low level of homology. The striking similarity of Gr-EXPB1 to two proteins from saprophytic soil bacteria and its bacterial type carbohydrate-binding module (CBM) constitute support for the hypothesis that a number of nematode pathogenicity factors has a procaryotic origin. The information given in this paper could facilitate the identification of more expansins outside the plant kingdom, and – more specifically – among other cell wall-degrading pro- and eukaryotes.

2. Materials and methods

2.1. Genomic analysis

Primers designed to anneal to the most extreme 5′- and 3′-ends of the cDNA sequence of *Gr-EXPB1*, gDNA-GrEXPf (GAGCTCCTCTGAAGCAATTC) and gDNA-GrEXPr (AACACTGTATAAACCTTTATGCAATT), were used to amplify the corresponding sequence from genomic DNA isolated from pre-parasitic J2-s as described [5]. For Southern blotting, 5 μg of genomic DNA was isolated from pre-parasitic J2-s as described [6], digested with the restriction enzyme *Eco*RI, separated on an agarose gel and blotted. A DIG-labeled dUTP cDNA probe was synthesized from the *Gr-EXPB1* genomic DNA clone (nt 1524–1932) using the primers D2f (ATGGTTTATTGAAAAATTCGTTG) and D2r (CCTGTCTCGACAAAAGAGTCC).

2.2. Antibody production and immuno-assays

Primers GrExp-pBAD-SPf (AGCTCCTCTGAAGCAATTCTGTGTTTGTTGTGCC) and GrExp-pBADr (AATAGGTGAGCGTACGCCCGTCGCTTTGCC) were used to amplify the coding region of the predicted mature protein (nt 39–848 in *Gr-EXPB1*). The amplified fragment was cloned into the pBAD/Thio-TOPO vector (Invitrogen, Leek, The Netherlands). Hens were immunized with purified recombinant protein, and the resulting chicken IgY antibody was isolated from the eggs as described by [7] and further purified [8]. Western blots of homogenates and immuno-fluorescence microscopy of pre-parasitic J2-s were performed as described previously [9].

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2.3. Cell wall extension assay

Recombinant CBM (residues 26–118) was produced in tobacco using the Gateway-compatible pK2GW7 vector (VIB, Ghent, Belgium) whereas transcription was driven by the 35S CaMV promoter. Both CBM and Gr-EXPB1 were preceded by the original (nematode) signal peptide for secretion. Proteins were extracted from mature leaves by grinding in liquid nitrogen and re-suspending in 50 mM sodium acetate pH 4.5. After centrifugation, the protein concentration of the supernatant was adjusted to approximately 150 μg/ml and used in an extensometer assay [10].

2.4. 3D structure of Gr-Exp1 EXPB1 based on homology modeling

Template identification was performed using 3D-PSSM [11]. Target and template sequences were aligned by using MULTALIN [12], and the output was optimized manually. Based on the CASP4 assessment [13], PHD, PsiPred, Prof and SSPRO [14–17] were used to produce the secondary structure profile of the target. Model building, refinement and analysis were performed using the following Accelrys (Accelrys, San Diego, CA) programs: Insight II, Discover, Homology, Delphy and Affinity.

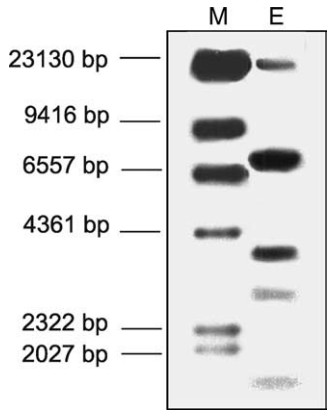

Fig. 1. Southern blot hybridized with a 409-bp genomic probe derived from *Gr-EXPB1*. Lane M is molecular weight marker; Lane E is loaded with 2.5 μg *G. rostochiensis* genomic DNA digested by *Eco*RI.

3. Results

3.1. Analysing cDNA and genomic sequences of Gr-EXPB1

The nematode expansin Gr-EXPB1 contained two distinct domains: domain 1 (D1: residues 26–118) showed significant similarity with carbohydrate-binding module family II (CBM2), and domain 2 (D2: residues 150–271), the actual expansin [4]. Besides being similar to plant expansins and expansin-like proteins, D2 was even more similar to a hypothetical protein from the *Amycolatopsis mediterranei* (AJ319869 – *orfD* [18]; 35% identity, *E*-value = $8.2e^{-12}$) and a predicted open reading frame ORF11 from *Streptomyces lavendulae* (AF127374 [19]; 36% identity, *E*-value = $1.2e^{-10}$). Both are aerial mycelium-forming soil saprophytes belonging to the order Actinomycetales (phylum Actinobacteria). Another remarkable homology was found between Gr-EXPB1 and MAP-1 (*Meloidogyne* avirulence protein) from the root knot nematode *Meloidogyne incognita* (AJ278663 [20]; 35% identity, *E*-value = $2.1e^{-10}$). *orfD*, ORF-11 and *map-1* are predicted to encode extracellular proteins with unknown functions.

To confirm the eukaryotic origin of *Gr-EXPB1*, we investigated its genomic sequence. A single fragment of 2.4 kb (AJ556781) was amplified from genomic DNA of *G. rostochiensis*. Six introns (with length ranging from 57 to 412 bp) were present in the *Gr-EXPB1*. The interspersion of the expansin gene by introns and the presence of a poly-A tail in the corresponding mRNA (AJ311901) exclude a procaryotic origin of *Gr-EXPB1*.

3.2. Multiple expansin-like sequences in cyst nematodes

A Southern blot analysis of *G. rostochiensis* genomic DNA probed with a D2 fragment showed five bands, suggesting the presence of several expansin-related sequences in the genome of *G. rostochiensis* (Fig. 1).

The presence of D2-like sequences in *G. rostochiensis* EST databases confirmed this finding as exemplified by CAC84564 – a hypothetical protein translated from AJ311902 – showing 75% identity with Gr-EXPB1-D2. CAC84564 is preceded by a signal peptide for secretion and lacks a carbohydrate-binding module. Searching soybean cyst nematode EST databases revealed BF014507 (519 bp) with 82% identity to *Gr-EXPB1*. A local alignment of the three nematode sequences with a plant β-expansin and a plant β-expansin-like protein indicated the presence of a series of conserved cysteine residues and an additional number of conserved motifs previously described as signature motifs for α- and β-expansins (Fig. 2) [21].

3.3. Localization of Gr-EXPB1 protein

The polyclonal antibody against Gr-EXPB1 specifically bound to a band of about 28 kDa on Western blots of homogenates of *G. rostochiensis* J2-s (Fig. 3A). This size is in broad agreement with the calculated molecular mass of the predicted protein once post-translational modifications have been allowed for. In a dot-blot experiment, Gr-EXPB1 was shown to be present in concentrated secretions of potato root diffusate-induced secretions from pre-parasitic J2-s (Fig. 3B). Immuno-fluorescence microscopy revealed distinct binding of this antibody to the subventral esophageal glands of pre-parasitic J2-s. Gr-EXPB1 protein was detected in the subventral gland lobe and in the extensions (Fig. 3C). Unlike MAP-1 [20], Gr-EXPB1 was not detected in the amphidial region of infective J2-s.

3.4. Expansin activity assay of CBM

Protein samples extracted from plants transformed with empty vector, *CBM* or *Gr-EXPB1* were analyzed on a SDS–PAGE gel. As compared with samples from empty vector controls, an additional band corresponding to the molecular weight of either CBM or Gr-EXPB1 was detected (Fig. 4). Proteins extracted from transgenic tobacco harboring *Gr-EXPB1* showed a significant increase in expansin activity as compared to the empty vector control (Fig. 5). Gr-EXPB1 consists of an expansin domain and a CBM. CBMs may also induce disruptions of non-covalent interactions between plant cell wall carbohydrate polymers [22]. Therefore, it could be argued that the CBM domain in Gr-EXPB1 is responsible for the cell wall-loosening activity observed in the extensometer assays. However, the activity in transgenic tobacco solely expressing the CBM was not significantly different from the empty vector controls (Fig. 5).

3.5. The 3D structure of Gr-EXPB1 based on remote homology modeling

Domain 1. The xylan-binding domain from *Cellulomonas fimi* xylanase D (PDB code 1xbd) was identified by 3D-PSSM

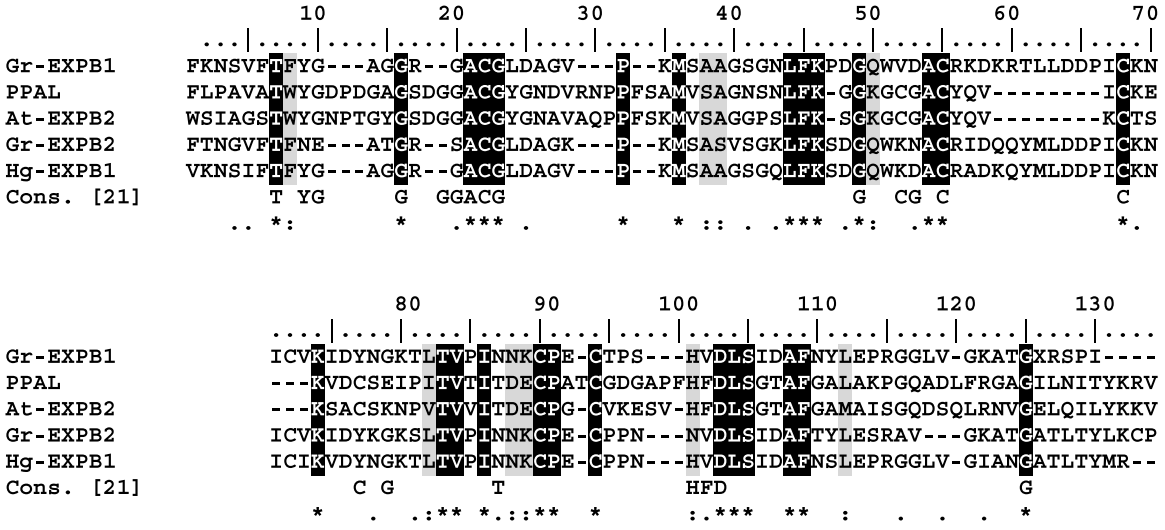

Fig. 2. Alignment of the expansin domain (D2) of Gr-EXPB1 (CAC83611) with domain 1 of PPAL: a pistil-specific β-expansin-like protein from *Nicotiana tabacum* (AAG52887), At-EXPB2: a putative β-expansin from *Arabidopsis thaliana* (Q9SHY6), GrEXPB2: a hypothetical protein translated from *G. rostochiensis* (AJ311902, CAC84564) and Hg-EXPB1: a conceptually translated mRNA sequence from *Heterodera glycines* (BF014507). Cons. [21]: conserved residues between α- and β-expansins as proposed in [21]. * – identical residues in all five proteins (in black boxes); **:** – conserved substitutions (in gray boxes); **.** – semi-conserved substitutions; ↓ – conserved cysteine residues.

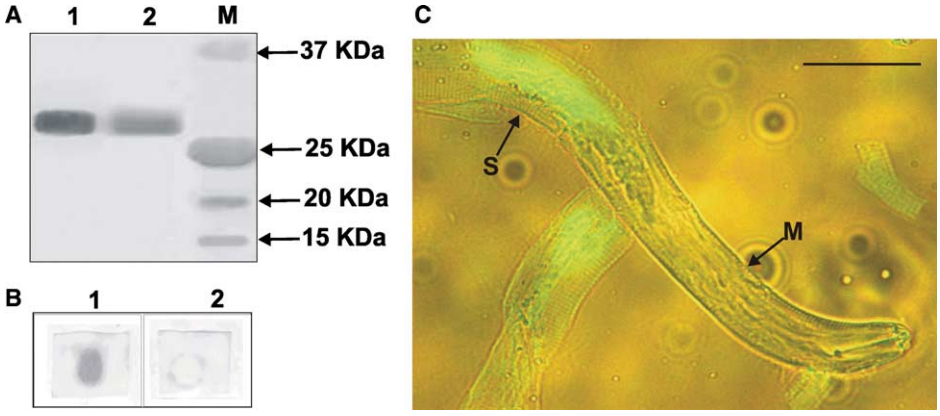

Fig. 3. (A) Detection of Gr-EXPB1 in J2 homogenate on Western blot probed with anti-Gr-EXPB1 IgY antibody (diluted 100 times). 1: Potato root diffusate (PRD)-exposed pre-parasitic J2-s, 2: pre-parasitic J2s, not exposed to PRD; M: molecular weight marker. (B) Dot blot of concentrated natural secretions from nematode J2-s incubated in PRD. Left panel (1) is probed with anti-GR-EXP1 IgY antibody; right panel (2) is probed with pre-immune IgY. (C) Immuno-fluorescence labeling of the subventral gland with IgY antibody against GR-EXPB1. Arrows point at the subventral glands (S) and the metacorpus (M), respectively. Scale bar = 20 μm.

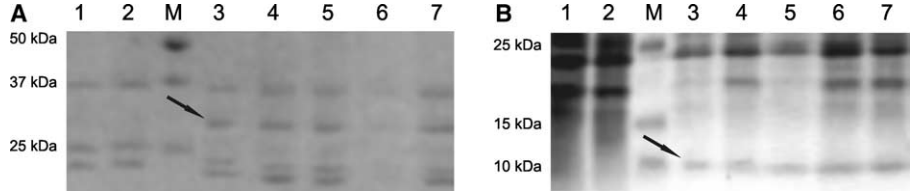

Fig. 4. (A) Coomassie Brilliant Blue-stained SDS–PAGE gel showing expression of Gr-EXPB1 in tobacco plants (lanes 3–7) and two empty vector control lines (lanes 1 and 2). (B) Expression of CBM in tobacco (lanes 3–7) and two empty vector control lines (lanes 1 and 2); M, marker lane; relevant products are indicated with an arrow.

as the closest template for Gr-EXPB1 – D1 (*E*-value < 0.005; 18% identity). The target-template alignment was optimized by incorporating secondary structure information and locking the sequence motifs emerged from a PRODOM database analysis. The consensus secondary structure prediction indicated an all-β pattern. Uncertainties in the alignment due to a 2 aa

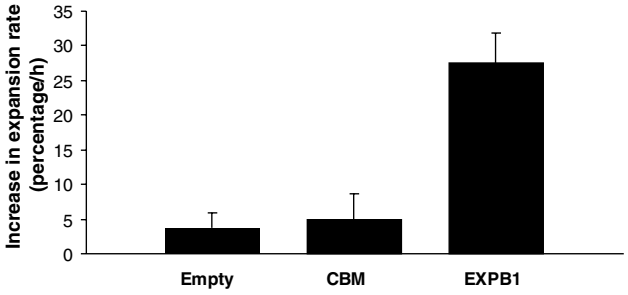

Fig. 5. Effect of tobacco-produced recombinant CBM on the extension rate of wheat coleoptiles. Mature leaf material was collected from two empty vector control lines (Empty), from three independent *CBM*-harboring lines and from four independent *Gr-EXPB1*-lines. For each of the lines, expansin activity was calculated as the difference in extension rates before and after addition of the extract (% increase in length per hour), and activity measurements were done in quadruplicate. Results are given as means with standard errors.

insertion between the first extended β-strand and the first Trp involved in ligand binding prompted us to generate two alternative models (Fig. 6A1 and A2). Both models have the β-barrel structure with aromatic amino acids displayed on the flat surface of CBM2s [23]. Model 1 (Fig. 7, left) preserves a 18.7 Å distance between the two Trp-s involved in ligand-binding – typical for the two crystallized members of PD001333 – by introducing a 3aa loop between the first β-strand and the following β-bridge. This causes an increase in the local flexibility as compared to the other PD001333 structures that show either continuous β-strand or 1 β-bulge in this region (see Fig. 6A3). It is interesting to note that even longer insertions in the region are present in CBMs from the PD029472 family, suggesting that sequence variability in this region does not affect ligand binding. However, no CBM 3D-structures from the PD029472 family are available. Model 2 (Fig. 7, right) eliminates the 3aa loop by allowing the first β-strand to extend all

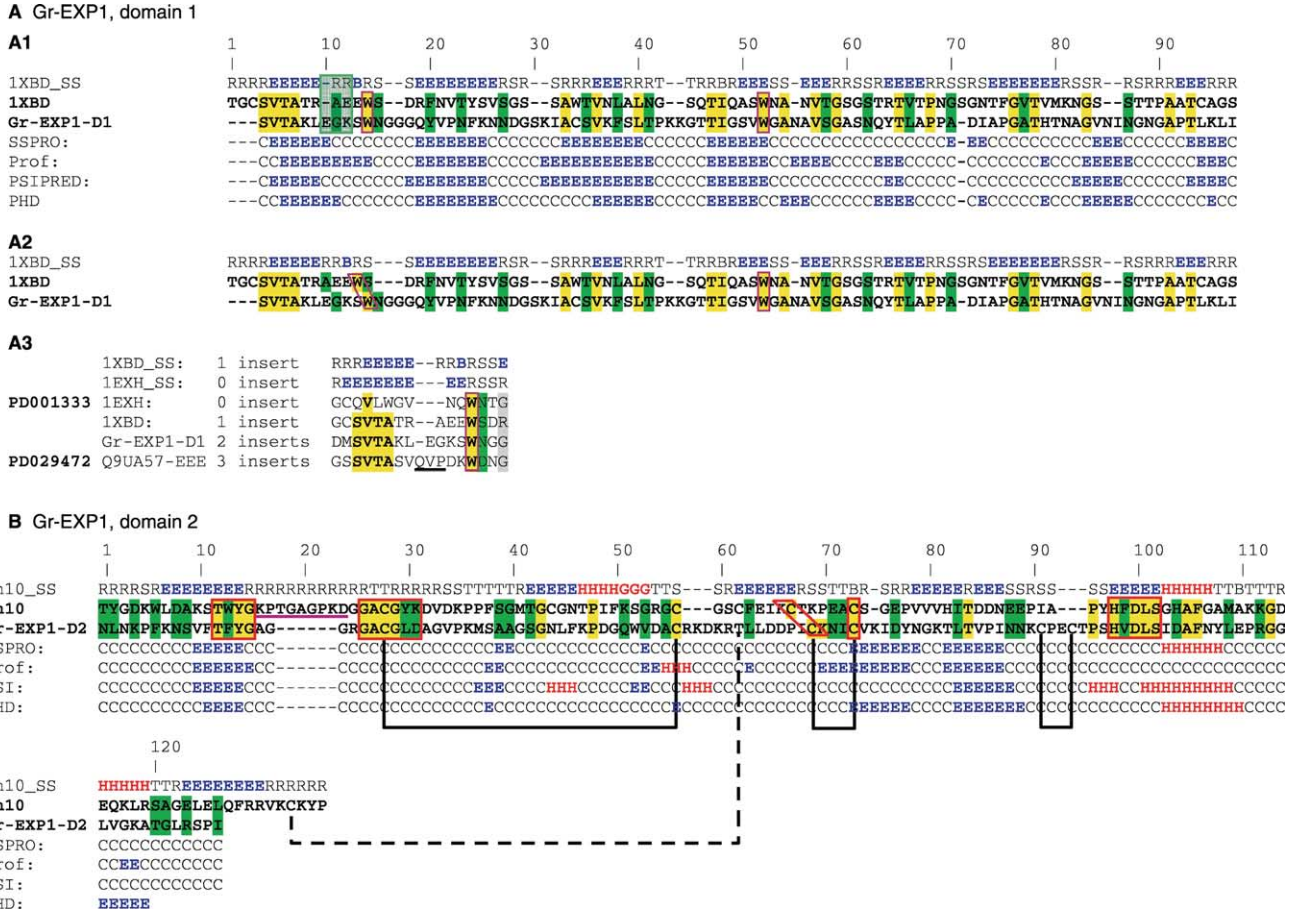

Fig. 6. (A) Target-template sequence alignment of Gr-EXPB1 domain 1 and 1XBD, the xylan-binding domain of *Cellulomonas fimi* exo-1,4-β-glycanase (AAB34464). The 1XBD_SS line shows the secondary structure elements derived from the determined 3D structure of 1XBD. The alignment was refined by incorporation of the secondary structure information of Gr-EXPB1 – domain 1 as predicted by four different methods: SSpro, Prof, PSIPRED and PHD. (A1) Alignment preserving the position of Trp residues (Model 1); (A2) Alignment preserving the length of the loop between the first β-strand and the first β-bridge (Model 2). (A3) Alignment of Gr-EXPB1 – domain 1 with various carbohydrate-binding domains with different insertion lengths between the first β-strand and the first Trp. PD#: PRODOM entries. 1EXH: cellulose-binding domain from *Cellulomonas fimi* (P07986; aa 375–484). Q9UA57: β-1,4-endoglucanase from *Meloidogyne incognita* (aa 406–422). (B) Target-template sequence alignment of Gr-EXPB1 – domain 2 and the N-terminal domain of 1n10, pollen allergen phl p 1 from *Phleum pratense*. The 1n10_SS line shows the secondary structure elements derived from 1n10. Straight brackets represent predicted disulfide bridges. The template disulfide bridge not conserved in the model is indicated by a dotted line. Sequence motifs in expansin family (conserved also in Gr-EXPB1 – domain 2) are outlined in red. Conserved residues are highlighted in yellow, similar residues are highlighted in green. Residues affecting the orientation of Trp side-chain (Arg in 1xbd) are highlighted gray. Trp-s binding the ligand are outlined in purple box. B = β bridge; C = coil; E = β strand; G = 3_{10} helix; H = α helix; S = bend; T = turn; R = random.

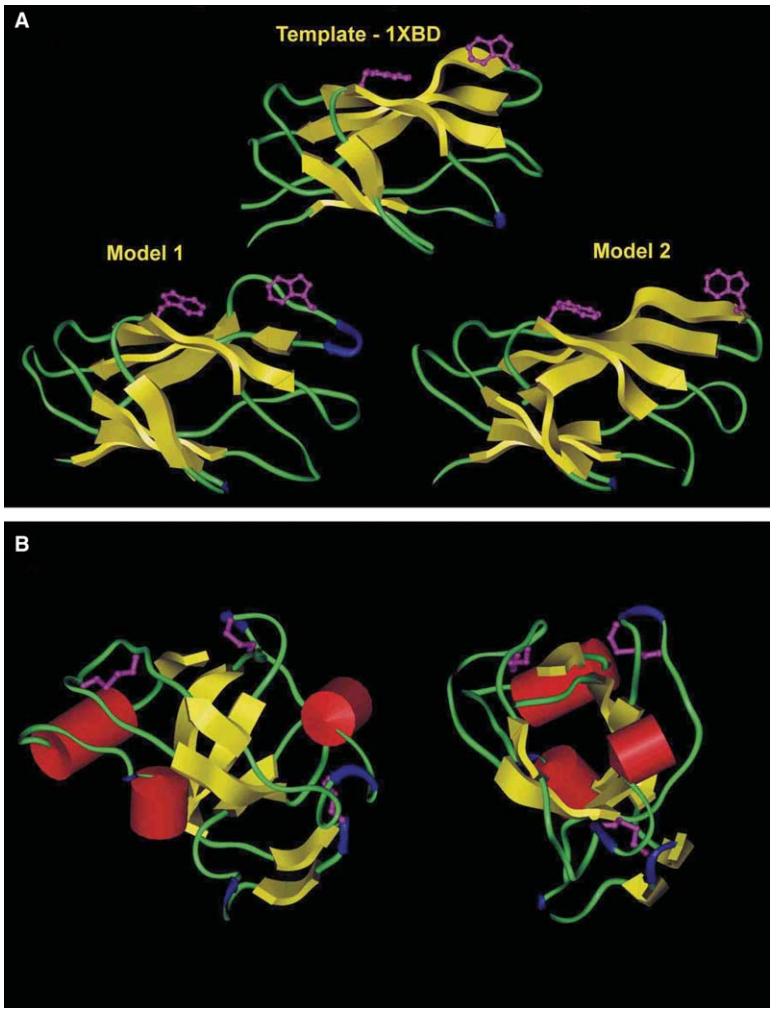

Fig. 7. (A) Ribbon diagrams of the overall fold for template viz. 1XBD (up), Gr-EXPB1 – domain 1, Model 1 (left) and Model 2 (right). The β-strands are colored yellow; the turns are colored blue and random regions are colored green. Two Trp residues involved in glycan binding are shown in magenta, all atoms. (B) Overall fold of Gr-EXPB1 – domain 2. The helices are red cylinders; Cys residues forming disulfide bridges are shown in magenta, all atoms.

along to the first Trp involved in ligand binding. Thus, a more stable local structure is formed, but the distance between the two Trp-s involved in recognition increases by ~5–6 Å, which may result in a different carbohydrate polymer binding specificity.

Domain 2. The N-terminal domain of pollen allergen phl p1 from *Phleum pratense* (PDB 1N10) ranked first in searching for Gr-EXPB1-D2 templates (*E*-value = $3.18e^{-6}$ and 20% identity). Other putative, lower ranked templates such as Barwin lectin (*E*-value = $1.57e^{-1}$ and 14% identity) significantly differ in the arrangement of local patterns. The conserved motifs between plant expansin domain 1 and Gr-EXPB1-D2 are presented in Fig. 6B, shaded in red boxes. Two out of the three disulfide bridges of the template are conserved in the target; the third is not present in Gr-EXPB1-D2. It is replaced by another disulfide bridge that emerges naturally from the sequence to 3D structure alignment confirming that the model is presumably close to the real structure. The core structure of Gr-EXPB1-D2 is very similar to the first domain of plant expansins, and differences are located only in three loops of the protein.

4. Discussion

Gr-EXPB1, a functional expansin from the potato cyst nematode *G. rostochiensis* [4], consists of a bacterial type (II) CBM with an expansin domain that is more similar to two hypothetical proteins from the aerial mycelium-forming soil bacteria *A. mediterranei* and *S. lavendulae* than to plant expansins. It remains to be shown whether these members of the Actinomycetales produce functional expansins, but this finding could point at expansins or expansin-like proteins among procaryotes. Previously, nematode CWDEs such as β-1,4-endoglucanases [24] and polygalacturonases [25] were shown to be remarkably similar to their procaryotic and not to their eukaryotic equivalents. Here, the eukaryotic origin of Gr-EXPB1 is confirmed by the presence of a polyA tail and introns, and our results support the hypothesis that nematode pathogenicity factors related to plant invasion were acquired from bacteria as a result of horizontal gene transfer [26]. Southern blot analysis revealed that Gr-EXPB1 is presumably member of a small gene family, and this result was confirmed by the finding of another expansin-like cDNA

fragment in a potato cyst nematode EST database. Searches in EST databases from other cyst nematode species suggest that the presence of functional expansins is unlikely to be exceptional.

The nematode expansin Gr-EXPB1 showed higher similarity with β-expansins than with β-expansins, and displayed higher cell wall expansion activity on type II primary cell walls [4]. As compared to α-expansins, β-expansins are known to be less effective on type I primary cell walls (typical for dicots and non-grass monocots). Nevertheless, β-expansins have been identified in a range of dicotyledons [21]. So far, the function of β-expansins in dicotyledons is not known. Hosts of potato cyst nematodes invariably have type I primary cell walls, and it is at first sight surprising that potato cyst nematodes produce an expansin with apparently a limited impact on type I cell walls.

Remote homology modeling was used to further characterize the two Gr-EXPB1 domains. D1 belongs to the mainly procaryotic CBM 2 family containing members that bind either crystalline cellulose (CBM2a) or xylan (CBM2b). Carbohydrate binding consists of face-to-face hydrophobic stacking interactions between the surface-exposed aromatic rings in the CBM and the non-polar faces of sugar rings in the polysaccharides. The closest template of Gr-EXP1-D1 is an active xylan-binding domain from *C. fimi* xylanase D [27] and the presence of only two (and not three) Trp-s at the binding site (Trp11 and Trp49) points at a high affinity for xylan [28]. However, the presence of Gly (instead of Arg) after the first Trp (Fig. 5A3) was shown to favor cellulose binding [28]. Further studies are therefore needed to determine the natural ligand and to discriminate between the two models proposed for D1. The similarities between Gr-EXPB1-D2 and the GH45-like domain (SCOP 50685) of plant expansins indicate a comparable mechanism for loosening hydrogen bonds. More study is needed to reveal which part of the structure is crucial for this unique activity and why the two domains of Gr-EXPB1 are swapped as compared to plant expansins.

It is concluded that the presence of a functional expansin in the potato cyst nematode *G. rostochiensis* is unlikely to be exceptional among plant parasitic nematodes. Moreover, the remarkable similarity of two putatively extracellular (both ORFD and ORF11 include a predicted signal peptide for secretion) bacterial proteins with Gr-EXPB1 suggests that production of expansin is not necessarily bound to eukaryotes. It would be interesting to test whether saprophytic soil-bound Actinomycetales use expansins for the degradation of plant material.

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