Characterisation of arabinogalactan endo β 1,4 galactanases from Globodera rostochiensis, Globodera pallida and Rotylenchulus reniformis

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ABSTRACT

Plant parasitic nematodes need to overcome the barrier presented by the plant cell wall in order to invade their host. A variety of plant cell wall degrading enzymes are present in endoparasitic nematodes including enzymes that degrade cellulose (β 1,4 endoglucanases) and various pectin components. We describe the cloning and functional analysis of genes encoding GH53 arabinogalactan endo-1,4-beta-galactosidases from three related plant parasitic nematodes Globodera rostochiensis, Globodera pallida and Rotylenchulus reniformis. Phylogenetic and structural analyses strongly indicate that these genes have been acquired by horizontal gene transfer from bacteria. We show that the genes are expressed at invasive stages of the parasites in the secretory gland cells. We also demonstrate that the enzymes from these species are biochemically active, showing the expected hydrolytic enzymatic activity when galactan was used as a substrate. This work further demonstrates the importance of cell wall degradation to the success of the parasitic process and the extensive role that horizontal gene transfer has played in the evolution of plant parasitism by nematodes.

1. Introduction

The plant cell wall is made up of an interconnected network of proteins and polysaccharides with the most abundant of these being cellulose, hemicellulose, and pectin (e.g. Ref. [1]). This network provides a strong physical barrier due to the properties of the components, including covalent bonding between polysaccharides [2]. Cellulose is the most abundant polysaccharide and consists of large, unbranched strands of β-1,4-linked glucose molecules. Hemicelluloses are also abundant in the cell wall with the most common being xylan, arabinoxylan, and mannan. The hemicelluloses are intertwined with the cellulose fibres via hydrogen bonds and together these are enmeshed within layers of pectin. The composition of plant cell walls may vary among species and tissues, each cell type possessing distinct and dynamic cell wall compositions and organisation [3]. For example, the cell walls of monocots contain relatively low levels of pectic polysaccharides [4].

All plant pathogens, including plant parasitic nematodes, need to break down the plant cell wall in order to gain access to their hosts. The first stage of the life cycle of cyst nematodes requires them to invade host roots and migrate intracellularly (destructively) through the host tissues until they reach the inner cortex layer [5]. The nematode uses a combination of physical disruption of the plant cell wall using the stylet and biochemical degradation of the cell wall components during this migration. Since the initial discovery of genes encoding cellulases (β 1,4 endoglucanases) in cyst nematodes (Smant et al., 1998), a wide range of cell wall degrading and modifying enzymes have been identified from plant-parasitic nematodes [6]. The first plant parasitic nematode genome-wide analysis, of the root-knot nematode Meloidogyne incognita, has revealed that over 60 genes encoding cell wall degrading or modifying proteins from six different families are present, including cellulases, xylanases, polygalacturonases, pectate lyases and arabinases [7]. Likewise, analysis of potato cyst nematode genomes has revealed a similar repertoire of enzymes [8,9]. Phylogenetic analysis of the sequences from a range of root-knot and cyst nematodes suggests that these sequences have been obtained by horizontal gene transfer from a variety of bacterial species [10]. In addition, Bursaphelenchus spp. contain GHF45 cellulases that were very likely to have been acquired by horizontal gene transfer from fungi [11], while Xiphinema index contains GHF12 cellulases most likely acquired from bacteria [12]. This suggests...
that independent horizontal gene transfer events have occurred in each of the clades of nematodes that can parasitise plants. The complement of cell wall degrading enzymes present in plant-parasitic nematodes may vary to reflect the cell wall composition of the host [13] and expression levels of cell wall degrading enzymes can change in response to perceived cues relating to the composition of the plant cell wall in different hosts [14].

Pectin is a key component of the plant cell wall and is composed of three main types of carbohydrates: homogalacturonan (HG), and rhamnogalacturonan I and II (RGI and RGII) with HG being the most abundant. Pectin can be categorised into smooth or hairy regions based on the level of branching sidechains that are present. HG comprises chains of 1,4-linked \(\beta\)-D-galacturonic acid and as there are no branching chains extending from the HG backbone, it is classified as smooth [15]. RGI contains repeating units of \(\alpha\)-rhamnose and \(\alpha\)-galacturonic acid in its backbone. Branching chains such as galactan, arabinan, and arabino-galactan are attached to the \(\alpha\)-rhamnose molecules of the RG backbone [16]. RGII has a backbone of galacturonic acid units (monosaccharides), like HG, but also has branching side chains like RGI. These side chains are often complex and made up of multiple different types of carbohydrates including apiose, fucose, and aceric acid (3-C-carboxy-5-deoxy-\(\gamma\)-L-xylorafuranose) [17]. The presence of branching side chains on RGI and RGII means that these are referred to as hairy regions of pectin.

The breakdown of pectin requires the concerted action of several different degrading enzymes and many of these have been identified in plant-parasitic nematodes. Pectate lyases cleave the glycosidic bond between \(\alpha\)-1,4-polygalacturonic acid units in homogalacturonan through a \(\beta\)-elimination reaction [18]. These enzymes were first described in plant-parasitic nematodes from the potato cyst nematode *Globodera rostochiensis* [19] and have subsequently been identified in many other species including root-knot nematodes and migratory endoparasites (reviewed by [20]). Polygalacturonases, which act on pectate and on other galacturonans by hydrolysing 1, 4-alpha-\(\alpha\)-galactosiduronic linkages, have been identified in root-knot nematodes [21] as well as in a transcriptome dataset for the closely related migratory endoparasitic *Pratylenchus coffeae* [22], although no functional analysis of this predicted protein has been reported. In terms of metabolising pectin side chains, arabinases have been identified in genome sequences for *G. rostochiensis, G. pallida* and *Rotylenchulus reniformis*. We examine spatial and temporal expression patterns and demonstrate that the enzymes from these species are biochemically active.

2. Materials and methods

2.1. Biological material

Populations of *G. pallida* and *G. rostochiensis* were grown on the susceptible potato (*Solanum tuberosum*) cultivar Désirée in a glasshouse. Cysts were extracted using standard protocols and stored at 4 °C for at least 6 months before use. Second stage juveniles (J2) were hatched in tomato root diffusate prepared as previously described [25]. Fixed samples of *Rotylenchulus reniformis* were provided by Dr Catherine Lilley, University of Leeds and *Xanthomonas campestris* pv. *Campestris* (Xcc) wild-type strain 8004 was obtained from Dr. John Maxwell Dow, BIOMERIT Research Centre, Department of Microbiology, University College, Cork, Ireland.

2.2. Cloning

Genes encoding sequences similar to GH53 Arabino-galactan endo-
\(\beta\)-1,4-galactanases were identified from transcriptomic and genomic studies for *G. pallida* [26], *G. rostochiensis* [9] and *R. reniformis* [27]. The coding regions of the putative GH53 genes from the three nematode species (*GROS.g08150* (GrGAL1), *GPLIN.000142900* (GpGAL1), *RrGAL1*) and the bacterial control *Xanthomonas campestris* (*XC.0587* (GaIA_Xc)) were amplified by PCR from cDNA (or gDNA for the *Xanthomonas* control) using the proof-reading KOD Hot Start DNA polymerase (Merck) using primer sets shown in Supplementary Table S1. Messenger RNAs were isolated from nematode material using a Dynabeads mRNA Direct Micro kit (Invitrogen) and treated with RNA DNase (Promega). cDNA was synthesised from approximately 400 ng purified mRNA using the Superscript III system (Invitrogen) with poly(dT) primers following the manufacturer’s instructions. The open reading frame of each of the genes was cloned from the start of the predicted mature peptide to the stop codon (excluding the endogenous signal peptide). PCR products were separated on 1.5% agarose gels, excised, and purified using a QiAquick gel extraction kit (Qiagen) before cloning into the Gateway-compatible TOPO entry vector pcR8/GW/TOPO (Invitrogen), following the manufacturer’s instructions. The cloned genes were sequenced at The James Hutton Institute sequencing facility.

GrGAL1, GpGAL1, RrGAL1 and XC.0587 (GaIA_Xc) were subsequently cloned into the protein expression vector pOPIN_S3C [28]. The pOPIN_S3C vector contains a 6x Histidine (His) tag, a SUMO domain, and a 3C protease cleavage site. The green fluorescent protein (GFP) gene used as negative control, that was previously cloned into the bacterial expression vector pKC026 from a TOPO donor clone [29], was also transferred into the pOPIN_S3C vector. In-fusion cloning into the pOPIN_S3C vector was carried out using the NEBuilder HiFi DNA assembly kit following the manufacturer’s protocol (New England Biolabs). Primer sets for pOPIN_S3C cloning are shown in Supplementary Table S1.

2.3. Phylogenetic analysis and protein structure modelling

BLAST similarity searches were conducted using the tBLASTn function against the non-redundant nucleotide database with the *Globodera* and *Rotylenchulus* GH53s as query sequences [30]. The top 100 results from each of the BLAST searches were combined and filtered to remove duplicates and low confidence hits, resulting in a unique list of 78 sequences. This list was subsequently found to have a very large proportion of very similar sequences from *Xanthomonas* species. The list was therefore manually filtered to remove the majority of these, keeping only the highest percentage identity hits in cases where there were multiple sequences included from one species. Additional fungal sequences were subsequently added to the list, from *Aspergillus aculeatus*, *Aspergillus nidulans*, *Humicola insolens*, and *Thermoactinomyces therma-phiila* species to ensure a diverse phylogenetic representation. These were identified through BLAST searches of the Protein Data Bank (PDB) database. Pairwise alignments of all sequences were created using Muscle and visualised using Jalview [31]. Model selection (LG (general matrix) + F (empirical base frequency) + G4 (rate heterogeneity gamma parameter)) as determined by ModelFinder [52]) and phylogenetic tree inference were carried out using IQ-TREE [33] using default parameters. The phylogenetic tree derived from 1000 bootstrap replicates [34] was then annotated using FigTree v1.4.3.

Predicted structures of the GrGAL1, GpGAL1 and RrGAL1 proteins were produced using a 1-to-1 thread model based on the amino acid sequence of \(\beta\)-1,4-galactanase from *Bacteroides thetaiotaomicron*. The sequence and structure from *B. thetaiotaomicron* were identified using BLAST similarity searches with mask low complexity settings applied. Searches were completed using BLAST and the Research Collaboratory.
3.1. Protein structures

for Structural Bioinformatics Protein Data Bank (RCSB PDB) [35] (www.rcsb.org). The 1-to-1 thread model was achieved using Protein Homology/analogy Recognition Engine V 2.0 (PHYRE2) [36]. Rendered images of predicted protein structures were produced using CCP4 molecular graphics (CCP4mg) (V2.10.10) [37].

2.4. Gene expression profiles

In situ hybridisation was used to determine the spatial expression patterns of the GrGAL1, GpGAL1 and RrGAL1 sequences in J2s of G. rostochiensis, G. pallida and R. reniformis, respectively, using the protocol described in Jones et al. [38]. Normalised gene expression data for various life stages of G. rostochiensis [9], G. pallida [8] and R. reniformis [27] were used to determine temporal expression patterns of each of the genes.

2.5. Protein expression and purification

Ten millilitres of LB media were inoculated with a single colony from GrGAL1, GpGAL1, RrGAL1, GalAXc, or the GFP control in Shuffle Escherichia coli cells (New England Biolabs). Cultures were grown overnight at 37 °C with shaking. One hundred microlitres of the overnight cultures were added to 100 mL of fresh LB media which were incubated at 30 °C with shaking until an OD600 of 0.7 was reached. Cultures were cooled to 18 °C. A sample of each culture was taken and stored as a pre-induction control. Expression of protein in the remaining cultures was induced by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, before incubation overnight at 18 °C with shaking. Cultures were centrifuged at 4000 g for 10 min to pellet the remaining cells. The supernatant was discarded and the pellet was resuspended in 1 mL of ice-cold lysis and wash buffer (50 mM Tris-HCL, 500 mM NaCl, 50 mM glycine, 5% glycerol, 500 mM imidazole, EDTA-free protease inhibitor tablets (Complete Mini; Roche Diagnostics), pH 8.0). Beads were washed in 1 ml lysis and wash buffer, centrifuged at 13000 g for 1 min and then centrifuged twice at 13,000 g for 1 min. The supernatant was transferred to a new Eppendorf tube with 100 μL of Ni-NTA Superflow resin (Qiagen) and incubated for 1 h at room temperature on a rotator. The sample was centrifuged twice at 13,000 g for 1 min and the supernatant was removed. Beads were washed in 1 ml lysis and wash buffer, centrifuged at 13000 g x 1 min before discarding the supernatant. Elution buffer (2.5 mL; 50 mM Tris-HCL, 500 mM NaCl, 50 mM glycine, 5% glycerol, 500 mM imidazole, EDTA-free protease inhibitor tablets, pH 8.0) was added to the beads in a 15 ml Falcon tube, which were then incubated at room temperature for 10 min and then centrifuged twice at 13,000 g for 1 min. The supernatant, containing the purified protein of interest, was stored at −20 °C. Presence and purity of proteins was assessed by Coomassie blue staining following separation on NuPAGE precast gels as previously described [39] (Supplementary Fig. 1).

2.6. Enzyme activity assays

Twenty-five microlitres of each protein solution (GrGAL1, GpGAL1, RrGAL1, GalAXc or the GFP control at a concentration of 500 μg/mL), was incubated with 25 μL of 0.1% (w/v) galactan substrate solution in 50 mM sodium acetate (pH 5) at room temperature for 1 h. A blank sample was also set up containing 25 μL sterile distilled water and 25 μL of galactan solution. Fifty microlitres of DNS reagent (60 mM 3,5-dinitrosalicylic acid, 500 mM NaOH, 150 mM potassium sodium tartrate tetrahydrate) was added to each sample. Samples were boiled for 15 min

Fig. 1. Nematode GH53 sequences. A: Phylogenetic analysis of nematode, bacterial and fungal GH53 sequences. The tree is midpoint re-rooted and based on 1000 bootstraps. Each entry is followed by “-XX” which is the identification number attributed in Supplementary Table S2. Bar, 0.4%, sequence dissimilarity (evolutionary distance). B: Alignment of mature protein sequences GrGAL1 (G. rostochiensis), GpGAL1 (G. pallida), RrGAL1 (R. reniformis) and GalAXc (X. campestris). Catalytic glutamate residue (E) are highlighted in red. C: Predicted structures of nematode GH53 proteins using a 1-to-1 threaded model. Predicted structures shown in the order (left to right) GrGAL1, GpGAL1, RrGAL1, confirmed structure of BTGH53 from Bacteroides thetaiotaomicron (6gp5_A). Catalytic glutamates in the active site are present in all four structures, in very similar positions (highlighted green/red, central).
and cooled on ice for 3 min before adding 200 μL of water. Absorbance readings for each sample were taken at 540 nm using a Promega GloMax multi+ plate reader.

The additional substrates xylan (from beechwood, Sigma), saccharose (VWR chemicals), pectin (from apple, Sigma), arabinogalactan (AG-II) (from Larchwood, Sigma) and polygalacturonic acid (Sigma) were tested using the same protocol as described above. All substrates were used at 0.1% (w/v) solutions in 50 mM sodium acetate pH 5.0. A standard curve was produced using 1 mg/mL (0.1% w/v) galactose (the reducing sugar produced on hydrolysis of galactan polymer). Seven samples were used containing 0.05, 0.2, 0.4, 0.6, 0.8, 1, and 2 mg galactose plus DNS reagent in a total volume of 300 μL. A blank sample containing water and DNS reagent only was also tested.

Data accessibility: All sequence data used in this research are available through previously deposited genome or transcriptome resources for *G. pallida* [8], *G. rostochiensis* [9] and *R. reniformis* [27].

3. Results

3.1. Identification of GH53 sequences

Analysis of the *G. rostochiensis* genome [9] allowed a gene (GROS_g08150) similar to GH53s from a variety of species to be identified. This was subsequently renamed GrGAL1 and the coding sequence used as a query for BLAST similarity searches to identify putative GH53 proteins in related nematodes. The sequence identified in the *G. pallida* genome (GPLIN_000142900) was truncated. A search against the *G. pallida* transcriptome returned two incomplete but overlapping sequences: comp4850_c0_seq1 and comp4850_c0_seq4. These were computationally recapitulated, alongside the partial genomic fragment, and renamed GpGAL1 after successful cloning of the full-length gene. Similarly, two partial sequences were identified in *R. reniformis* (transcripts comp30258_c0_seq1, comp30258_c1_seq1) that were used to generate a putative full-length sequence (RrGAL1) that was confirmed by cloning. GrGAL1 and GpGAL1 shared high sequence percentage identity with a GH53 protein from *Duganella sacchari* (67.11%) (NCBI seqID: WP_011038708.1). *X. campestris* is a Gram-negative bacterial pathogen of Solanaceous plant species such as tomato and peppers (Potnis et al. 2015). GrGAL1 had the highest percentage identity with an arabinogalactan endo-1,4-β-galactosidase from the strain YR242 of a *Roseateles* sp. Bacteria (65.68%) (NCBI seqID: WP_092947600.1). As GH53s are not usually present in animals, a phylogenetic analysis of the nematode sequences and GH53 proteins from bacteria and fungi was undertaken in order to explore the likely origin of these genes (Fig. 1A). This analysis showed that the nematode sequences clustered with the bacterial sequences, while the fungal sequences formed an outgroup. This, coupled with a previous analysis [9], which showed that the *G. rostochiensis* sequence has an extremely high Alien Index score, suggests that like other genes encoding plant cell wall degrading enzymes in plant-parasitic nematodes, the GH53 sequences are present in the nematode genomes as a result of horizontal gene transfer from bacteria. Alignment of the nematode sequences against bacterial GH53 mature protein sequences showed that the key catalytic glutamate (E) residues are conserved across PPN and bacterial species (Fig. 1B).

The predicted protein sequences of GrGAL1, GpGAL1 and RrGAL1 were used for searches against the RCSB PDB to identify similar proteins for which a crystal structure is available. For all three sequences, the highest identity hit (40%ID for Globodera sequences and 39% for the *Rotylenchulus* sequence) was with the β-1,4-galactanase BTGH53 from *Bacteroides thetaiotaomicron* (NCBI seqID: 6GP5_A) [41]. A 1-to-1 thread model for the nematode sequences was produced using the protein structure and sequence alignment with the solved structure from *B. thetaiotaomicron* using Protein Homology/analogy Recognition Engine V 2.0 (PHyre2). The predicted structures suggest that all three nematode sequences follow the same folding pattern: (β/α) barrel. Furthermore, the conserved catalytic glutamates of the GH53 proteins are in the centre of the β-barrel in each structural prediction which matches with the position in bacterial protein structures (Fig. 1C).

3.2. Expression profiles of the GH53 sequences

*In situ* hybridisation was conducted for the genes encoding GrGAL1, GpGAL1 and RrGAL1 to identify localisation of the mRNA transcripts at the J2 life stage of these three species. GrGAL1 and RrGAL1 (Fig. 2A–D) were localised in the subventral gland cells. However, GpGAL1 consistently produced a condensed spherical staining pattern in the region of the oesophageal glands (Fig. 2E–H). This structure is too small to be...
Fig. 3. DNS assay of recombinant nematode GH53 enzymes and positive (GalA_Xc) and negative (GFP) controls against plant cell wall polysaccharide component substrates. A: GrGAL1, GpGAL1, RrGAL1 and GalA_Xc produce detectable reducing sugars as monitored by absorbance at 540 nm while no sugars are released in the GFP sample. B-H: No reducing sugars are produced by any of the recombinant proteins using xylan (B), polygalacturonic acid (C), pectin (D), saccharose (E) or arabinogalactan type II (AGII) as a substrate. Bars represent the mean ± SE for n = 3. G: Image of 96-well plate assay depicting the colour change observed when GrGAL1, GpGAL1, RrGAL1, GalA_Xc, and GFP were tested using different substrates.
either of the pharyngeal gland cells, although it is possible that this structure corresponded to the nucleus of a gland cell. It is unclear why the transcript appears to localise only at the nucleus and not across the full gland cell. ISH negative controls using the appropriate sense primer probes displayed no specific signals with minimal background staining around the cut site (not shown). Analysis of RNASeq data showed that the expression of the GH53 gene was restricted to egg and J2 (G. rostochiensis) or J2 alone (G. pallida) while the RrGAL1 gene was expressed in both J2 and female nematodes (Fig. 2 I).

3.3. Biochemical function of the nematode GH53 proteins

Recombinant proteins were produced from the three nematode GH53 sequences as well as the X. campestris bacterial GH53 protein (GalA_Xc), which was used as a positive control, and GFP, which was used as a negative control. A 3,5-dinitrosalicylic acid (DNS) assay was used to detect reducing sugars released due to the activity of the enzymes on various polysaccharide substrates. The presence of reducing sugars was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that sugars was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicated by a colour change (yellow to red/brown) measurable at 540 nm in a spectrophotometer. It was anticipated that the hydrolytic enzymatic activity of all the GH53 tested here was indicate...
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