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Parasitic dodder expresses an arsenal of secreted cellulases with multi-substrate specificity during host invasion

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ABSTRACT

Cuscuta campestris is a common and problematic parasitic plant which relies on haustoria to connect to and siphon nutrients from host plants. Glycoside hydrolase family 9 (GH9) cellulases (EC 3.2.1.4) play critical roles in plant cell wall biosynthesis and disassembly, but their roles during *Cuscuta* host invasion remains underexplored. In this study, we identified 22 full-length GH9 cellulase genes in *C. campestris* genome, which encoded fifteen secreted and seven membrane-anchored cellulases that showed distinct phylogenetic relationships. Expression profiles suggested that some of the genes are involved in biosynthesis and remodeling of the parasite's cell wall during haustoriogenesis, while other genes encoding secreted B- and C-type cellulases are tentatively associated with degrading host cell walls during invasion. Transcriptomic data in a host-free system and in the presence of susceptible or partially resistant tomato hosts, showed for especially *GH9B7*, *GH9B11* and *GH9B12* a shift in expression profiles in the presence of hosts, being more highly expressed during host attachment, indicating that *Cuscuta* can tune cellulase expression in response to a host. Functional analyses of recombinant B- and C-type cellulases showed endoglucanase activities over wide pH and temperature conditions, and activities towards multiple cellulose and hemicellulose substrates. These findings improve our understanding of host cell wall disassembly by *Cuscuta,* and cellulase activity towards broad substrate range potentially explain its wide host range. This is the first study to provide a broad biochemical insight into *Cuscuta* GH9 cellulases, which based on our study may have potential applications in industrial bioprocessing.

1. Introduction

Cuscuta spp. (dodders) are obligate stem holoparasites capable of infecting a wide range of host plant species and economically important crops, posing a major threat to agriculture, food security and ecosystems (Parker, 2012; Vurro et al., 2017). Over 200 species belonging to the genus *Cuscuta* have been identified to date, and they are distributed across all the continents (Kaiser et al., 2015). These parasitic species possess no root system and show residual or no photosynthetic activity and therefore rely totally on their infected host plants for nourishment and support to complete their lifecycles (Hartenstein et al., 2023).

Cuscuta parasites infect plants by twining its stem around host stems and developing root-like structures, known as haustoria, to penetrate host tissues and connect to their vascular bundles. During the initial attack, characterized by swelling of the stem (Bawin et al., 2022; Olsen et al., 2016), *Cuscuta* epidermal cells closest to host tissues rapidly elongate and differentiate into secretory trichome-like cells, which secrete cementing substances to attach to the host surface (Vaughn, 2002). Once this contact is firmly established, the prehaustorium develops into a haustorium and penetrates the host stem surface and starts to grow into the host tissues. The advancing haustorium utilizes the tip cells as "searching hyphae" to locate and establish continuous connections to infected host xylem and phloem tissues (Dörr, 1972). The establishment of haustoria and stable vascular connections is the hallmark of a successful infection, allowing water, mineral nutrients and carbohydrates to move from the host to the parasite (Kim and Westwood, 2015; Shahid et al., 2018). This mode of feeding is detrimental to the host plant and may cause severe biomass and yield reductions in important agricultural crops (Lanini and Kogan, 2005; Parker, 2012). Given *Cuscuta* species' wide host range, encompassing both woody and non-woody plants, dodders' strategy for penetrating host tissues with potentially different cell wall composition is intriguing and remains

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largely unknown.

The advancement of the haustorium through host tissues is likely facilitated by mechanical pressure as well as enzymatic degradation of host tissues. Nagar et al. (1984) were the first to show high hydrolytic enzyme activity of pectin esterase, polygalacturonase, xylanase and cellulases in haustorial regions of *Cuscuta reflexa* infecting *Phaseolus vulgaris* and *Solanum nigrum*. They inferred that this activity originated from the parasite and was required for remodeling host cell walls to facilitate haustorial penetration. Further study with *C. reflexa* stems growing on *Solanum lycopersicum* and *Pelargonium zonale* hosts revealed higher cellulase activity in the stems of the parasite than in the host (Johnsen and Krause, 2014). Later, our studies verified that hydrolytic enzymes were indeed secreted by the parasite when we showed an elevated activity of the xyloglucan-modifying enzyme, xyloglucan endotransglucosylase/hydrolase (XTH), at the penetration stage of *C. reflexa* growing on *P. zonale* host (Olsen and Krause, 2017). In recent studies, several genes encoding carbohydrate active enzymes have been identified in *Cuscuta campestris* and expression levels shown to correlate with haustorial invasion (Bawin et al., 2024; Yokoyama et al., 2020). While these enzymes could also be involved in other processes, such as growth of searching hyphae during haustorium penetration, and not directly in remodeling of host cell walls, the work by Johnsen et al. (2015) demonstrated that the one and the same host cell can exhibit different degrees of pectin methylation in cell walls adjoining the haustorium than in cell walls that are not in direct contact with haustorial cells, corroborating direct effects executed by secreted enzymes. Aside from the aforementioned studies, there is limited information about enzymatic modification of host cell walls and functional roles of individual genes/enzymes during *Cuscuta* invasion. This may be attributed to the lack of reliable genetic transformation protocols for *Cuscuta* which limits the use of reverse genetics approaches for analysis of gene function in *Cuscuta*.

Cellulose is the key structural component of plant cell walls forming an eminent barrier which the advancing haustorium must overcome during penetration of host tissues. Plants encode a number of cellulase enzymes, all of which are glycoside hydrolase family 9 (GH9) members (Cantarel et al., 2009; Henrissat, 1991). These cellulases are capable of cellulose degradation by possessing endo-β-(1,4)-glucanase activity and they can be divided into three subfamilies based on sequence domain organization (Lopez-Casado et al., 2008; Urbanowicz et al., 2007a). Subfamily A (GH9A) consists of membrane-anchored proteins, which play a role in cellulose biosynthesis, and deleterious mutations in KORRIGAN (KOR) 1, KOR2 and KOR3 of *Arabidopsis thaliana* have been shown to result in reduced cellulose synthesis and plant growth (Mølhøj et al., 2002). Subfamily B (GH9B) members consist of secreted proteins, which have been reported to remodel endogenous plant cell walls during developmental processes, including fruit softening, organ abscission, aerenchyma formation and cell expansion during plant growth (Abu--Goukh and Bashir, 2003; de Souza et al., 2017; Roberts et al., 2002). Subfamily C (GH9C) are also secreted proteins but contain an additional carbohydrate binding module 49 (CBM49) at the C-terminus of the enzymes (Urbanowicz et al., 2007a). The CBM of GH9C members binds cellulose and makes members of this subfamily potentially more efficient degraders of recalcitrant cellulose in vivo (Lopez-Casado et al., 2008). An in vitro analysis has demonstrated that CBM49 from SlCel9C1, a GH9C enzyme from *S. lycopersicum,* indeed binds crystalline cellulose when expressed as a fusion with the catalytic domain of an endoglucanase from *Thermobifida fusca* (Urbanowicz et al., 2007b). Therefore, it maybe infered that GH9C enzymes are involved in the turn-over of crystalline cellulose in plants.

Implications of cell wall remodeling by GH9 enzymes during parasitism have been reported earlier for some parasitic plants (Honaas et al., 2013; Ranjan et al., 2014; Yang et al., 2015). A secreted GH9B protein, PjGH9B3, has been shown to be involved in xylem bridge formation during invasion of *Arabidopsis* by *Phtheirospermum japonicum* (Kurotani et al., 2020). When expression of *PjGH9B3* was silenced by RNAi, the haustorium still grew but could not reach the host xylem possibly due to reduced enzyme activity that was insufficient to loosen the host cell wall. These results imply that host cell wall remodeling is a key process during plant parasitism and parasitic plants could serve as sources of potent enzymes for biomass degradation. Cellulases are currently the third largest group of industrial enzymes due to their wide array of applications, and their importance is expected to rise due to their key role in 2nd generation biofuel production.

In our recent study, some endoglucanases were detected as among the significantly upregulated genes during haustorium organogenesis in *C. campestris* growing on *Solanum pennellii* and *S. lycopersicum*, which show both differential amount of cellulose in the cell walls and resistance to *Cuscuta* (Bawin et al., 2024), providing a reason to look at the roles and activities of cellulases in more details during parasitic infection. In the current study, we used comparative genomic analysis to identify and classify a complete set of the GH9 cellulase genes in *C. campestris,* based on conserved domain structure. The RNA sequencing analyses during haustorium development and host infection revealed differences in cellulase gene expression that seemed to be partly affected by the interaction with tomato hosts. Further functional studies of eight secreted cellulases associated with haustorium penetration, expressed as recombinant proteins in *Pichia pastoris,* demonstrated endoglucanase activities but also showed differences in their biochemical characteristics and target substrate specificities, which may reflect their specific role during the infection process.

2. Materials and methods

2.1. Plant material

The ecotype of *Cuscuta campestris* used was the same one that was used for the genome sequence analysis published by Vogel et al. (2018) and was originally obtained from the Botanical Garden of the University of Kiel, Germany. The culture was continuously propagated vegetatively on *Pelargonium zonale* as host at the Climate laboratory Holt with 24 h illumination from fluorescent light tubes (photon flux of 200–300 μmol m^{-2} s^{-1}) and a temperature of 21 °C.

2.2. Identification of GH9 genes from C. campestris genome

Protein and nucleotide sequences for GH9 genes encoding endo-β-(1,4)-glucanases (EC 3.2.1.4) were retrieved from *C. campestris* genome (Vogel et al., 2018) in the plaBi database (https://www.plabipd.de/portal/). A Hidden Markov Model (HMM) profile of GH9 cellulases was constructed from *A. thaliana* GH9 protein sequences and used to search a database of *C. campestris* protein sequences using HMMER software (version 3.3.2) with default parameters (Potter et al., 2018). The sequences of the significant hits were then searched against the National Center for Biotechnology Information (NCBI) database using the BLAST search algorithm and the corresponding GenBank accessions retrieved. *A. thaliana* GH9 family protein sequences were retrieved from The Arabidopsis Information Resource (TAIR) database (https://www.arabidopsis.org/).

2.3. Analysis of GH9 protein domain organization and identification of conserved domains

Protein transmembrane domains were predicted using DeepTMHMM v. 2.0 (Hallgren et al., 2022; https://dtu.biolib.com/DeepTMHMM) and CCTop programs. Signal sequences and cleavage sites were predicted using PrediSi (Hiller et al., 2004; http://www.predisi.de/), TargetP-2.0 (Armenteros et al., 2019; https://services.healthtech.dtu.dk/services/TargetP-2.0/) and SignalP-6.0 (https://services.healthtech.dtu.dk/services/SignalP-6.0/). Protein sequences were searched for the presence of GH9 catalytic domains and carbohydrate binding modules (CBMs) using NCBI's Conserved Domain Database (http://www.ncbi.

nlm.nih.gov/Structure/cdd/cdd.shtml). The presence of full-length GH9 catalytic domains was verified by multiple sequence alignments using MEGA X (Kumar et al., 2018). Protein physico-chemical properties were determined using the ProtParam tool (Duvaud et al., 2021).

2.4. Multiple sequence alignment and phylogenetic analysis

A phylogenetic tree was constructed using full-length GH9 family protein sequences from *C. campestris*, *A. thaliana* and *Oryza sativa* as well as previously characterized GH9 proteins from other plant species. Protein sequences were retrieved from GenBank and Phytozome v13.0 database (https://phytozome-next.jgi.doe.gov/), and accession IDs are listed in Supplementary Table S1. Multiple sequence alignments were performed using the MUSCLE program incorporated in MEGA X with default parameters (Kumar et al., 2018). Phylogenetic trees were constructed using Neighbor Joining (NJ) method with 1000 bootstrap replications.

2.5. RNA-seq analysis

Transcriptome libraries of *C. campestris* haustoria during development were generated in earlier studies. Haustorium development was induced in a host-free system (Bawin et al., 2022) as well as separately on two different hosts, partially resistant *S. lycopersicum* and susceptible *S. pennellii* (Bawin et al., 2024). These libraries were mined and analyzed for the expression of GH9 genes at four stages of haustorium development, namely non-infecting (niS), swelling (SWE), attachment (ATT) and penetration (PEN) stages. Gene expression was calculated as Transcripts Per Kilobase Million (TPM) and displayed as heatmaps.

2.6. Heterologous expression of C. campestris GH9 genes in P. pastoris

For functional characterization, coding sequences (CDSs), excluding the native signal sequence and stop codon of eight selected GH9 genes were amplified by PCR from *C. campestris* cDNA using Phusion High-Fidelity DNA Polymerase (Thermo Fischer Scientific, Waltham, MA, USA) with gene-specific primers. The PCR products were separately cloned into EcoRI/SalI or Acc65I/SalI restriction sites in pPICZαA expression vector, which included a N-terminal α-factor secretion signal and C-terminal hexa-histidine (6xHis) tag. Primers used for cloning are shown in Supplementary Table S2. The vector constructs were verified by Sanger sequencing using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and sequencing was performed at the UiT The Arctic University of Norway sequencing facility on a 3130xl Genetic Analyzer (Applied Biosystems).

The constructed expression vectors were linearized with DraI restriction enzyme and transformed into *P. pastoris* SMD expression host by electroporation using GenePulser Xcell™ electroporator (Bio-Rad, Hercules, CA, USA) followed by selection of transformants on YPD plates with 100 μg ml^{-1} zeocin. The *P. pastoris* transformed with the empty vector served as a negative control. Recombinant protein expression in *P. pastoris* was performed following instructions in the EasySelect™ *Pichia* Expression Kit (Thermo Fisher Scientific) with modifications as described in Haeger et al. (2021). Protein expression was induced with 2% methanol added every 24 h. After 5 days of induction of protein expression, the culture media was centrifuged at 10,000 *g* for 15 min at 4 °C and supernatant containing the recombinant protein was stored at 4 °C for cellulase activity assays.

2.7. Identification of secreted cellulases by Western blot

Total proteins of 20 μg were separated on 12% sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) using a Mini-Protean II electrophoresis system (Bio-Rad) at 130 V. The separated proteins were electroblotted for immunodetection onto a 0.2 μm polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad), following the manufacturer's instructions. The membrane was blocked for 1 h using 5% (w/v) non-fat milk dissolved in Tris-buffered saline containing 0.01% Tween 20 (TBST) buffer (pH 7.6). The membrane was then washed three times with TBST buffer and His-tagged proteins were detected using the SuperSignal™ West HisProbe Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Total proteins from the empty vector strain was used as a negative control.

2.8. Cellulase activity assays

Cellulase activity of the recombinant GH9 proteins was determined using 3,5-dinitrosalicylic acid (DNS) method (Ghose, 1987; Miller, 1957) by measuring the amount of reducing sugars released following hydrolysis of a substrate. Briefly, reaction mixtures of 100 μL (20 μg) of crude protein were incubated at optimal pH and temperature containing one of the substrates: 100 μL of 2% carboxymethylcellulose (CMC) (Sigma Aldrich, St. Louis, MO, USA), 2% microcrystalline cellulose Avicel® PH-101 (Sigma Aldrich), 1% phosphoric acid swollen cellulose (PASC) which was generated from microcrystalline cellulose Avicel® PH-101 as described previously (Zhang et al., 2006), 1% glucomannan (GM) (Megazyme, Wicklow, Ireland), 1% xyloglucan (XG) from tamarind seed (Megazyme), 1% xylan (Xyl) from birchwood (Megazyme), or filter paper (FP). Reaction mixtures for activity on FP contained 50 mg of FP suspended in 300 μl of reaction buffer and 100 μl (20 μg) of total protein. Reaction mixtures containing CMC, PASC, GM, XG and Xyl were incubated for 30 min whereas those containing Avicel® and FP were incubated for 2 h. The reactions were terminated with 200 μl DNS and boiling for 15 min. The mixture was quickly cooled, and absorbance measured at 540 nm alongside glucose standards using Epoch microplate reader (BioTek, Winooski, VT, USA). Enzyme activity was calculated as μmol of released glucose equivalent per minute per μg of crude protein (μmol min^{-1}). Protein concentrations were determined according to Bradford (1976), using the Quick Start™ Bradford Protein Assay kit (Bio-Rad) following the manufacturer's protocol. Optimal pH for each enzyme was determined by incubating with 2% CMC at 40 °C in 50 mM buffers, which were sodium citrate for pH 3–6, sodium phosphate for pH 7 and Tris-HCl for pH 8–10. Optimal temperature was determined by incubating reaction mixtures in the optimal buffer at temperatures ranging from 20 to 80 °C. The supernatant from liquid cultures of clones containing an empty vector served as a negative control.

2.9. Statistical analysis

Statistical analysis and determination of differentially expressed GH9 genes by transcriptome analysis were as reported by Bawin et al. (2024).

3. Results

3.1. Cuscuta campestris genome encodes a large group of secreted cellulases

Mining of the *C. campestris* genome revealed 22 full-length GH9 family genes. All the proteins contained eight conserved amino acid regions (I-VIII) with highly conserved residues for GH9 proteins (Fig. 1). Region I, the acidic region III, and hydrophobic regions V and VII are highly conserved in plants (del Campillo, 1999; Perrot et al., 2022). Region II contains the conserved DAGD motif that is common for all plant GH9 proteins and is also present in microbial cellulases, similarly to the hydrophobic region IV (del Campillo, 1999; Perrot et al., 2022). Region VI contains the catalytic histidine (H_{550}) and region VIII the catalytic aspartate (D_{603}) and glutamate (E_{612}), which are important for endoglucanase activity (Tomme et al., 1991, 1992; Velazquez et al., 2023). Sequence analysis further revealed the presence of conserved acidic residues (D or E) in region IV at positions 284 and 288, and in

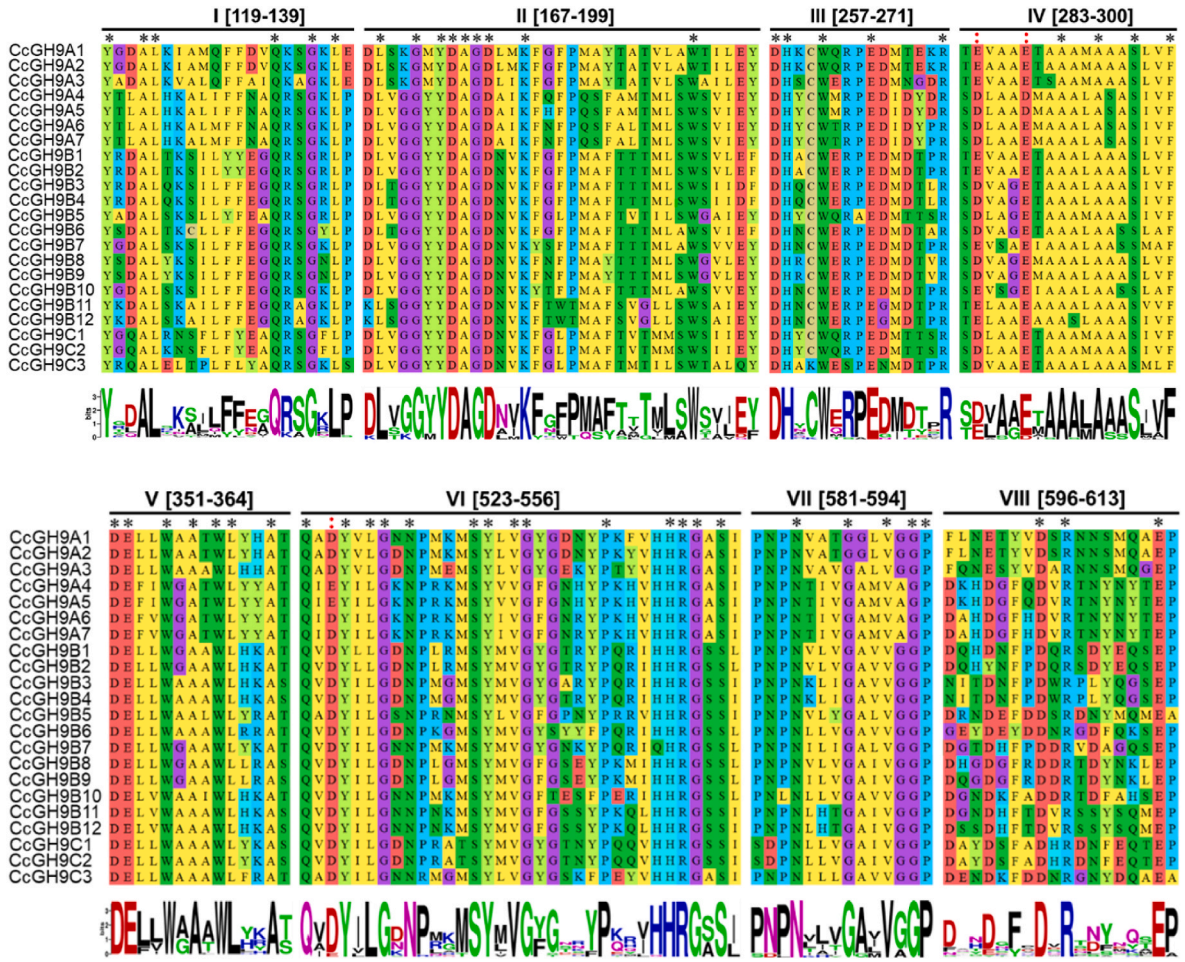

Fig. 1. Conserved regions I-VIII in the catalytic domains of *Cuscuta campestris* GH9 proteins. Numbers in square brackets represent the beginning and end of conserved amino acid blocks in a multiple sequence alignment. Asterisks represent fully conserved residues among GH9 proteins. Red colons show amino acid positions that are occupied by D or E. Sequence logos are shown below the alignment.

region VI at position 525, that are suggestive of a catalytic role as endoglucanase.

C. campestris GH9 cellulases possessed a modular domain architecture with different combinations of transmembrane domain (TMD), signal peptide (SP), GH9 catalytic domain and carbohydrate binding module (CBM) distinguishable in their sequence (Table 1). Seven of the GH9 proteins (CcGH9A1-7) contained TMD and thus were predicted to be membrane-anchored, which is the defining feature for subfamily A, according to the classification suggested by Urbanowicz et al. (2007a). A cleavable N-terminal SP for secretion was found in fifteen proteins, of which three possessed an additional CBM from family 49 (CBM49) after a linker sequence (Li) at C-terminus (CcGH9C1-3), while the remaining twelve secreted proteins were considered as subfamily B members and named CcGH9B1-12 (Table 1).

3.2. Plant GH9 subfamily B is phylogenetically more diverse compared to subfamilies A and C

A phylogenetic tree was constructed based on the full-length GH9 cellulase sequences of *C. campestris* and GH9 sequences from other higher plant species (Supplementary Table S1) to explore their evolutionary and functional relationships. Plant GH9 proteins formed three major groups (Fig. 2). All the seven *C. campestris* GH9 proteins from subfamily A clustered into group 1. The cellulases GH9A4, A5, A6 and A7 clustered in clade GH9A-II with *Arabidopsis* KORRIGAN 1 (KOR1), KOR2, and KOR3. The KOR1 protein has been reported to be co-localized in the plasma membrane with the cellulose synthase (CESA) protein complex and shown to be involved in cellulose biosynthesis (Vain et al., 2014), suggesting that GH9A members in *Cuscuta* may be involved in similar roles.

All the three *C. campestris* subfamily C members were found in group 2. The GH9C3 was placed in GH9C–I, while GH9C1 and GH9C2 clustered in GH9C-II, closely with *A. thaliana* orthologs AtGH9C1 and AtGH9C3, which have been suggested to have a role in root development (Klepikova et al., 2016). While most of the *C. campestris* subfamily B members were found in group 3 (GH9B–III - VI), one member was also found in group 2 (GH9B-II) together with subfamily B members from other species. Several B-type proteins which lack a CBM49 clustered with C-types in group 3, showing that they are more similar to the C-types in their overall sequences than to other subfamily B members in the same species. The tendency of some members of GH9 subfamily B to cluster with C-types has also been observed in grasses by Buchanan et al. (2012). This points to potential limitations of the domain-based classification system for GH9 proteins. Similarly, some B-type GH9 cellulases clustered more closely with A-types in group 1. These B-type GH9 sequences possessed predicted TMD but no clear cleavable signal peptides (Supplementary Table S1) and may be A-types classified as B-types due to inaccuracies of the prediction programs. Nine pairs of *C. campestris* GH9 proteins, GH9A1/A2, GH9A4/A5, GH9A6/A7, GH9B1/B2, GH9B3/B4, GH9B7/B10, GH9B8/B9, GH9B11/B12 and GH9C1/C2, clustered more tightly with each other than orthologs from other species, implying that they could be products of a whole genome duplication event that has reportedly occurred in *C. campestris* (Vogel et al., 2018).

Table 1
Characteristics of *C. campestris* GH9 family genes.

Gene ID	Locus Tag	GenBank Accession	Gene	Genomic sequence (bp)	CDS (bp)	Protein length (aa)	Molecular weight (kDa)	p*I*	GRAVY	Exons	Protein domains	Subfamily
Cc026148.t1	CCAM_LOCUS11192	VFQ69416.1	CcGH9A1	3,689	1,575	524	56.90	6.56	−0.192	6	TMD, GH9	A
Cc021716.t1	CCAM_LOCUS4989	VFQ63213.1	CcGH9A2	4,045	1,575	524	56.72	6.56	−0.214	6	TMD, GH9	A
Cc017680.t1	CCAM_LOCUS45369	VFR03594.1	CcGH9A3	3,492	1,560	519	56.94	6.80	−0.230	6	TMD, GH9	A
Cc002256.t1	CCAM_LOCUS21652	VFQ79876.1	CcGH9A4	4,470	1,851	616	68.98	9.04	−0.382	6	TMD, GH9	A
Cc019547.t1	CCAM_LOCUS1901	VFQ60125.1	CcGH9A5	4,747	1,851	616	68.93	9.04	−0.380	6	TMD, GH9	A
Cc003225.t1	CCAM_LOCUS24181	VFQ82405.1	CcGH9A6	3,909	1,860	619	68.97	8.93	−0.351	6	TMD, GH9	A
Cc027245.t1	CCAM_LOCUS3960	VFQ97833.1	CcGH9A7	3,529	1,857	618	68.69	9.11	−0.330	6	TMD, GH9	A
Cc029981.t1	CCAM_LOCUS15667	VFQ73891.1	CcGH9B1	2,079	1,536	511	56.54	9.14	−0.300	2	SP, GH9	B
Cc018204.t1	CCAM_LOCUS1388	VFQ59612.1	CcGH9B2	1979	1,539	512	56.58	9.19	−0.253	2	SP, GH9	B
Cc001522.t1	CCAM_LOCUS19902	VFQ78126.1	CcGH9B3	7,271	1,503	500	55.31	8.86	−0.192	7	SP, GH9	B
Cc044982.t1	CCAM_LOCUS8291	VFQ66515.1	CcGH9B4	6,494	1,503	500	55.29	8.85	0.189	7	SP, GH9	B
Cc006954.t1	CCAM_LOCUS31235	VFQ89459.1	CcGH9B5	5,190	1,563	520	58.07	5.88	−0.335	4	SP, GH9	B
Cc023880.t1	CCAM_LOCUS7628	VFQ65852.1	CcGH9B6	4,326	1,509	502	55.03	4.94	−0.215	4	SP, GH9	B
Cc002566.t1	CCAM_LOCUS22328	VFQ80552.1	CcGH9B7	6,043	1,473	490	54.13	6.42	−0.284	7	SP, GH9	B
Cc019394.t1	CCAM_LOCUS1750	VFQ59974.1	CcGH9B8	6,809	1,524	507	57.18	8.98	−0.470	6	SP, GH9	B
Cc028500.t1	CCAM_LOCUS13296	VFQ71520.1	CcGH9B9	12,096	1,524	507	57.21	8.99	−0.479	6	SP, GH9	B
Cc016074.t1	CCAM_LOCUS43377	VFR01602.1	CcGH9B10	2,852	1,476	491	54.08	6.13	−0.201	6	TMD, SP, GH9	B
Cc035665.t1	CCAM_LOCUS24624	VFQ82848.1	CcGH9B11	11,147	1,518	505	55.08	6.90	−0.300	8	SP, GH9	B
Cc005807.t1	CCAM_LOCUS29352	VFQ87576.1	CcGH9B12	10,820	1,518	505	55.00	6.90	−0.270	8	SP, GH9	B
Cc045579.t1	CCAM_LOCUS9741	VFQ67965.1	CcGH9C1	5,658	1,872	623	68.98	8.71	−0.325	9	SP, GH9, Li, CBM	C
Cc025057.t1	CCAM_LOCUS9494	VFQ67718.1	CcGH9C2	5,725	1,872	623	69.00	8.87	−0.324	4	SP, GH9, Li, CBM	C
Cc030810.t1	CCAM_LOCUS16790	VFQ75014.1	CcGH9C3	3,408	1,788	595	65.52	5.78	−0.322	4	SP, GH9, Li, CBM	C

CDS, coding sequence; bp, base pair; aa, amino acid; kDa, kilodalton; p*I*, isoelectric point; TMD, transmembrane domain; GH9, glycoside hydrolase family 9 catalytic domain; SP, signal peptide, Li, linker sequence; CBM, carbohydrate binding module.

3.3. B- and C-type cellulases show strong expression in infecting haustorium that is modulated by the host

The expression levels of all the 22 *C. campestris* GH9 genes were analyzed from transcriptome libraries generated from four stages of haustorium development (Fig. 3a) in both the host-free and parasite-host systems (Fig. 3b). Overall, the expression of GH9 genes overlapped across the different stages of haustoriogenesis in all systems. The subfamily A members were constitutively expressed, with none of them showing significant up- or down-regulation during haustoriogenesis or presence of tomato hosts. *GH9A1, A2, A4* and *A5* showed high expression levels already in the non-infecting tissue and thus contributed strongly to the overall cellulase transcript pool (Fig. 3b).

Subfamily B members *GH9B5, B6* and *B10* showed low transcript levels at all stages and systems, while the other members of subfamily B showed more distinct differential expression patterns during haustorium organogenesis (Fig. 3b). The expression of *GH9B1*, *B2*, *B3*, *B4, B8* and *B9* was significantly increased at the SWE stage but only the expression of *GH9B8* and *B9* was gradually increased peaking at the PEN stage, both with and without the presence of host. In the host-free system, expression of *GH9B7, B11* and *B12* was significantly induced at ATT stage and their expression further increased towards PEN stage. However, the expression levels of *GH9B7*, *B11* and *B12* were drastically induced and peaked at the ATT stage followed by decrease at the PEN stage by 1.5-fold in the presence of partially resistant *S. lycopersicum* and 3-fold on susceptible *S. pennellii* host (Fig. 3b), indicating a possible influence of host in modulating expression of these cellulases.

Expression of subfamily C members *GH9C1* and *C2* significantly increased at the SWE stage and expression remained relatively high through the later stages of haustoriogenesis, especially when attached on either of the tomato hosts (Fig. 3b). By contrast, *GH9C3* showed especially strong expression at the PEN stage, suggesting a specific role during infection.

3.4. Recombinant C. campestris GH9B/C enzymes retain cellulase activity over a wide pH and temperature range

To functionally characterize the secreted cellulases associated with host infection, eight *C. campestris* GH9 genes showing high transcript abundance at the PEN stage, namely *GH9C1*, *C2*, *C3*, *B7*, *B8*, *B9*, *B11* and *B12*, were separately cloned into the pPICZαA expression vector (Fig. 4a) and expressed in *Pichia pastoris*. Western blot analysis showed

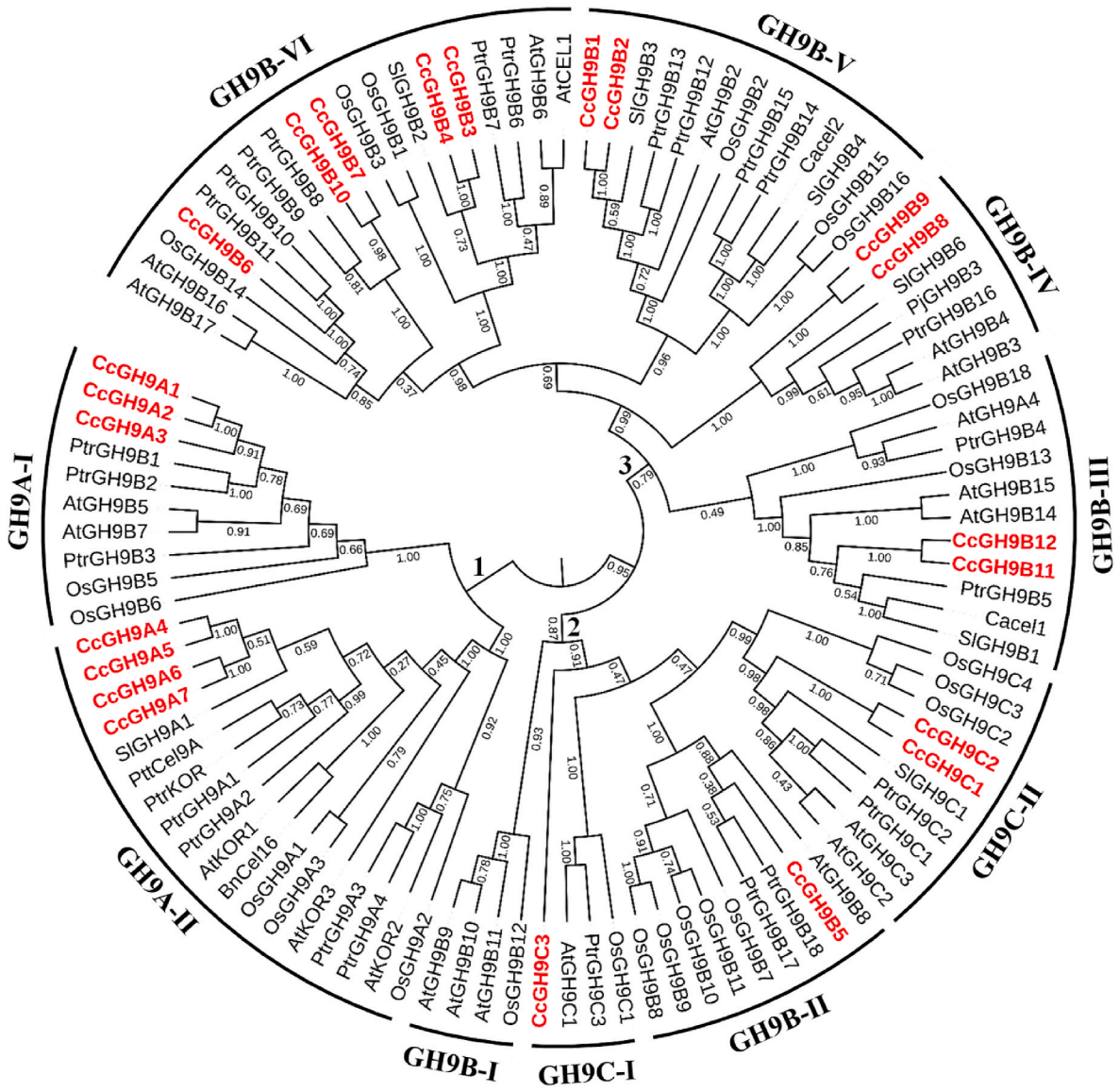

Fig. 2. Phylogenetic relationships between *Cuscuta campestris* GH9 proteins and GH9s from other plant species. The GH9 sequences from *C. campestris* are shown in bold red font. The three major groups 1–3 and the minor clusters GH9A-I–II, GH9B-I–VI, and GH9C-I–II are shown in bold. The tree was constructed in MEGA X program using the Neighbor Joining Method with 1000 bootstrap replications. The numbers inside the tree show bootstrap proportions for each node. A full list of the accession numbers of the sequences is shown in Supplementary Table S1. The first two letters in the protein name show the plant species as follows: Cc, *Cuscuta campestris*; At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Sl, *Solanum lycopersicum*; Ptr, *Populus trichocarpa*; Bn, *Brassica napus*; Ptt, *Populus tremula* × *Populus tremuloides;* Ca, *Capsicum annuum* and Pj, *Phtheirospermum japonicum*.

that the recombinant cellulases were successfully secreted into the culture media (Fig. 4b). In some cases, protein bands that were larger or smaller than the predicted molecular weight sizes were observed, indicating differential post-translation modifications as well as partial protein degradation.

Temperatures from 10 to 80 °C were tested to determine optimal conditions for cellulase activity for the GH9 recombinant proteins using CMC as substrate. All the recombinant proteins showed activity against CMC at all the tested temperatures, indicating that they are able to act on a wide temperature range. The cellulase GH9B11 showed highest activity at 40 °C whereas all others showed an optimal activity at 60 °C (Fig. 5). The activity of each recombinant GH9 was further assayed in buffers at different pH ranging from 3 to 10 (Fig. 6). All the recombinant enzymes showed activity towards CMC at all the tested pH values, but optimal pH varied between enzymes. Recombinant enzymes GH9C1, C2, C3 and B8 showed optimal cellulase activity at pH 9, whereas GH9B7 and B9 were most active at pH 10. By contrast, GH9B11 and B12 showed optimal activity at a slightly acidic pH of 6.

3.5. The C. campestris recombinant GH9B/C enzymes exhibit differential substrate specificities

The activity of each recombinant *C. campestris* GH9 cellulase was tested on different cell wall substrates, including simple, complex as well as branched polysaccharides. The different GH9 cellulases showed noticeable differences in their activity towards the different substrates (Fig. 7). All the enzymes showed activity on microcrystalline cellulose Avicel®, CMC, filter paper (FP), phosphoric acid swollen cellulose (PASC) and xyloglucan (XG), but none showed measurable activity on xylan (Xyl), which is in line with the fact that hemicellulose Xyl cannot be utilized as a substrate by endoglucanases because it is mainly composed of β-1,4-linked xylose in the backbone with α-arabinofuranose or α-glucuronic acids branching. Cellulases GH9C1 and C2 showed very similar substrate specificity patterns, with a preference for CMC,

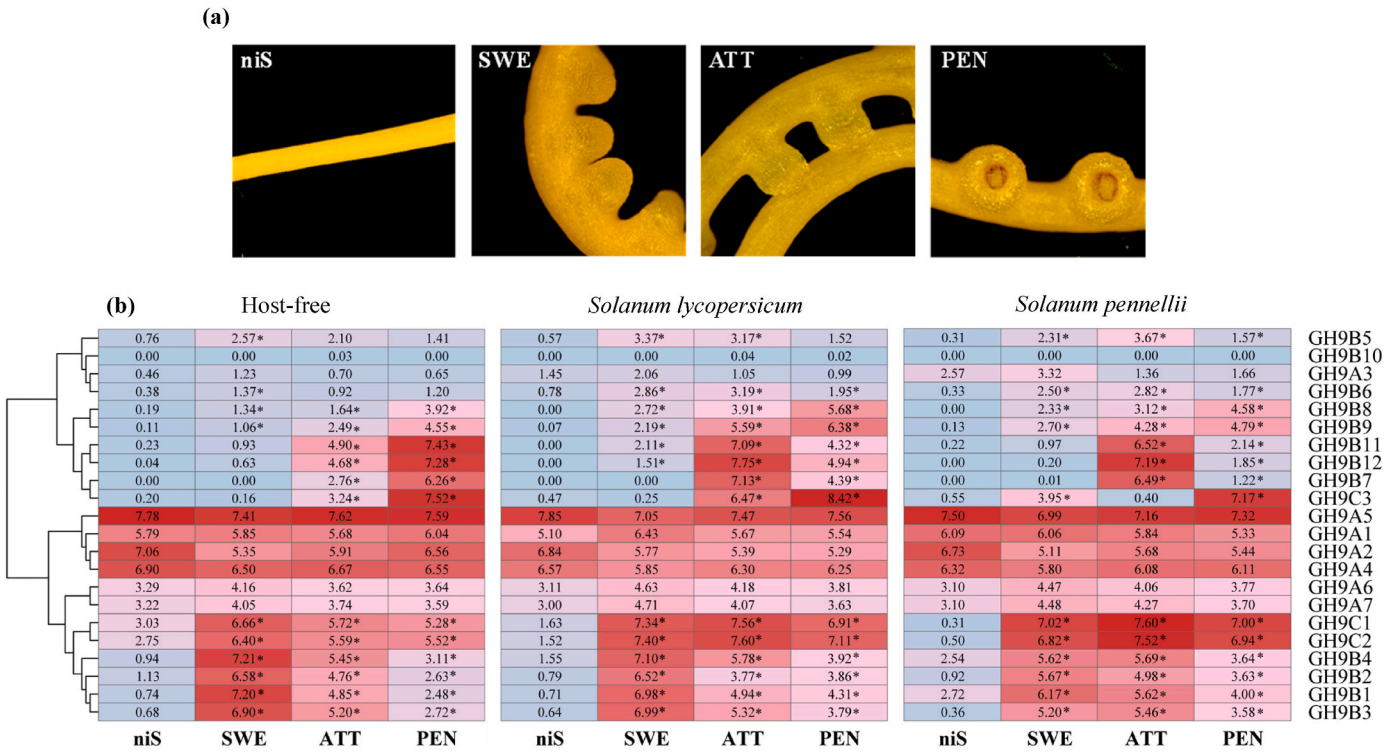

Fig. 3. Expression profiles of *Cuscuta campestris* GH9 genes during haustorium development. (a) Different stages of haustorium development following far-red light induction in a host-free system. (b) Heat maps of expression levels of *C. campestris* GH9 genes based on transcriptome data obtained from haustoria in a host-free system or using either *Solanum lycopersicum* or *Solanum pennellii* as host. The RNA sequencing data was generated from three biological replicates of each infection stage and system. The numbers refer to the $\log_2$ transformed expression values of the corresponding gene. The tree on the left shows the clustering by expression levels. Asterisks (*) show genes that were significantly upregulated relative to the niS stage with fold-change in expression ≥1.50 and $p \leq 0.05$. niS, non-infective stem; SWE, swelling stage; ATT, attachment stage; PEN, penetration stage.

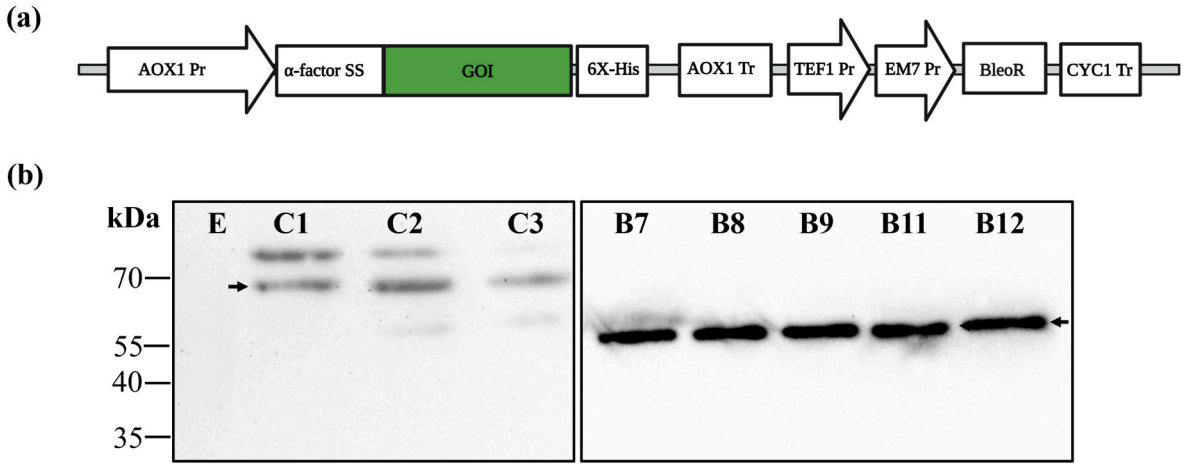

Fig. 4. Heterologous expression of *Cuscuta campestris* GH9 cellulases in *Pichia pastoris*. (a) Schematic presentation of expression vector construct. Gene of interest (GOI) represents coding sequence of *GH9C1*, *C2*, *C3*, *B7*, *B8*, *B9*, *B11* or *B12* without the native signal sequence and stop codon. AOX1 Pr, alcohol oxidase 1 promoter; α-factor SS, alpha-factor secretion signal; 6X-His, hexa-histidine affinity tag; AOX1 Tr, alcohol oxidase 1 transcription terminator; TEF1 Pr, translational elongation factor 1 promoter; EM7 Pr, EM7 promoter; BleoR, bleomycin resistance gene; CYC1 Tr, CYC1 terminator. (b) Analysis of yeast culture media for recombinant GH9 protein secretion by Western Blot. Arrows show the expected sizes of recombinant GH9 C- and B-type cellulases in kilodaltons (kDa). E, empty vector control.

followed by PASC and XG and, to a lesser extent crystalline Avicel® and FP. On the other hand, GH9C3 could utilize Avicel®, PASC, and FP almost as efficiently as CMC and XG, implying that this enzyme is able to degrade insoluble cellulosic substrates as efficiently as the soluble ones. GH9B7, B8 and B9 showed similar pattern of substrate specificities compared to GH9C1 and C2 with the highest preference to CMC. Enzymes GH9B11 and GH9B12 showed similar substrate specificities to each other but, interestingly, both showed a clear preference for hemicellulose glucomannan (GM) over the other substrates. The other GH9 enzymes could not utilize GM as substrate. Notably, GH9B11 and B12, similarly to GH9C3, showed a relatively higher tendency to degrade Avicel® and FP, which are considered to be difficult substrates due to their crystalline nature.

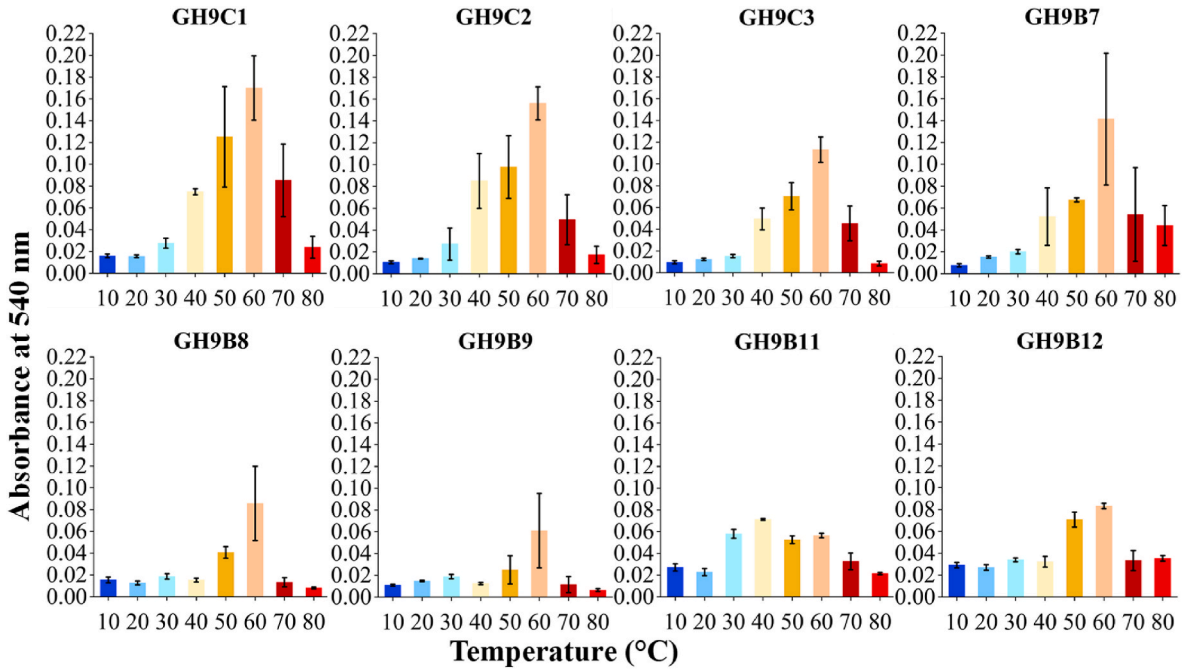

Fig. 5. Optimal temperature for recombinant *Cuscuta campestris* GH9 cellulase activity. Recombinant enzymes were produced in *Pichia pastoris.* The activity of the recombinant enzymes was measured by the DNS method using CMC as a substrate. The reaction mixtures were incubated at temperatures from 10 to 80 °C and level of activities are shown as absorbance at 540 nm. Bars represent means ± SEs of three replicates.

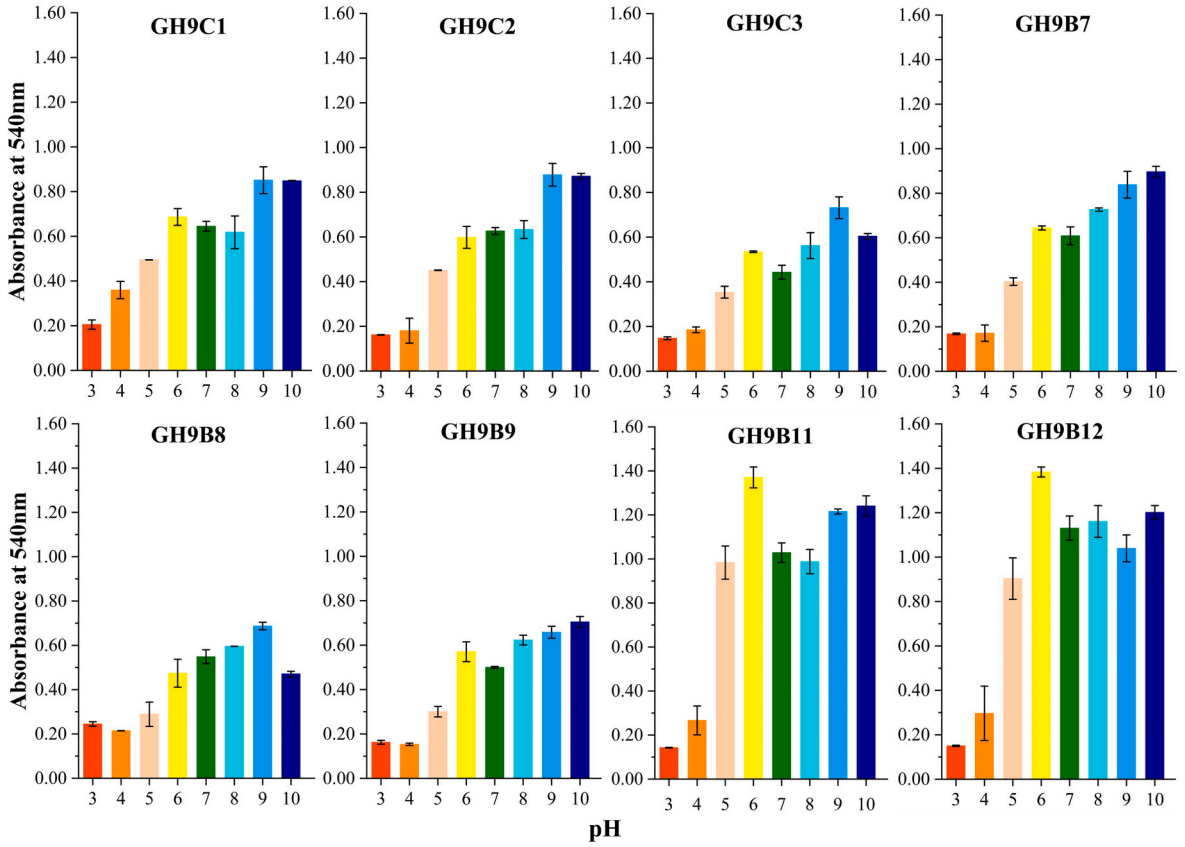

Fig. 6. Optimal pH for recombinant *Cuscuta campestris* GH9 cellulase activity. Recombinant enzymes were produced in *Pichia pastoris.* The activity of each enzyme was tested in varying pH from 3 to 10 and measured by the DNS method using CMC as a substrate. The reaction mixtures were incubated at 40 °C and level of activities are shown as absorbance at 540 nm. Bars represent means ± SEs of three replicates.

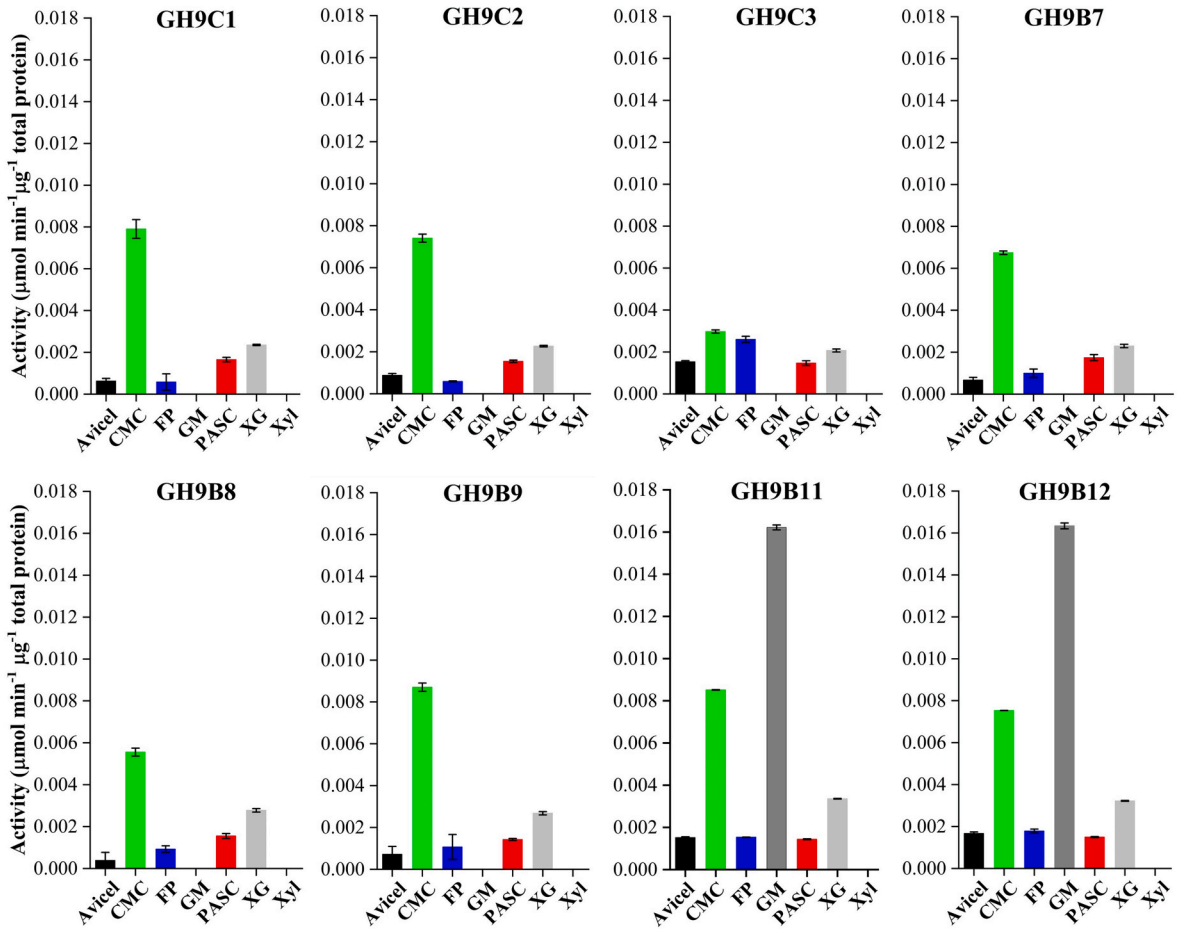

Fig. 7. Activity of recombinant GH9 cellulases on various cellulosic and hemicellulosic substrates. Recombinant enzymes were produced in *Pichia pastoris*. Enzyme activity was measured by the DNS method and reaction mixtures were incubated at the optimal pH and temperature for each enzyme. Enzyme activities are presented as the amount of reducing sugars in micromoles released per minute per microgram of total protein. Bars represent means ± SEs of three replicates. CMC, carboxymethylcellulose; FP, filter paper; GM, glucomannan; PASC, phosphoric acid swollen cellulose; XG, xyloglucan; Xyl, xylan.

4. Discussion

Secreted cell wall modifying enzymes from parasitic plants have been proposed to play important roles in haustorium invasion into host plants (Kurotani et al., 2020; Olsen et al., 2016; Olsen and Krause, 2017; Ranjan et al., 2014). Given the abundance of cellulose in plant cell walls, secreted cellulases can be expected to be involved in host cell wall loosening and degradation during parasitic invasion by *Cuscuta*, but their roles remain unexplored. Therefore, in the current study, we identified and characterized the complete set of *C. campestris* GH9 cellulases and studied their expression in both a host-independent system as well as in the presence of partially resistant *S. lycopersicum* or susceptible *S. pennellii* host, which have recently been shown to display differences in petiole and stem cellulose content (Bawin et al., 2024).

Compared to nonparasitic plants, *Cuscuta* possesses a simpler body plan composed mainly of stem, flowers and root-like haustoria but no roots, fully developed leaves, or fleshy fruits. Hence, it does not carry out many of the processes where secreted cellulases have been implicated in higher plants, such as leaf abscission, fruit softening, root hair growth and aerenchyma formation (Abu-Goukh and Bashir, 2003; de Souza et al., 2017; del Campillo et al., 2012; Roberts et al., 2002). However, our results revealed a total of 22 full-length GH9 genes in the *C. campestris* genome, a number that is comparable to many nonparasitic plants, including *A. thaliana* (24), *Brachypodium distachyon* (24), *Hordeum vulgare* (22), *O. sativa* (24), *Sorghum bicolor* (24) and *Populus* spp. (25) (Buchanan et al., 2012; Du et al., 2015). Phylogenetic analysis clustered *C. campestris* GH9 genes among other GH9 sequences from higher plants and showed that no reduction in specific groups or subfamilies has occurred compared to nonparasitic plants. This suggests that GH9 genes that in normal plants are involved in processes which are absent in *Cuscuta,* may have been repurposed to support its parasitic lifestyle, defined by the formation of haustoria and invasive growth into host plants. Our results show that many of the *C. campestris* GH9 genes which showed strong expression in the haustorium possess orthologs that in *A. thaliana* are strongly induced in roots (Klepikova et al., 2016), adding new members to the growing body of genes that appear to have been neofunctionalized from root to haustorium development (Sun et al., 2018).

Our study showed high transcript levels of the *C. campestris GH9A1*, *A2*, *A4* and *A5* across all the haustorium developmental stages, despite the presence or absence of a tomato host. Orthologous subfamily A proteins KOR1 and KOR2 from *Arabidopsis* have been associated with the biosynthesis of cellulose mircrofibrils (Vain et al., 2014), and the related *Cuscuta* GH9 proteins can have similar roles in *Cuscuta* tissues. In addition, the expression of *C. campestris* cellulases *GH9B1, B2, B3, B4, C1* and *C2* peaked at the SWE stage. These changes at the early stages of haustorium development reflect cell wall dynamics during early haustoriogenesis where biogenesis and disassembly occur side by side. By targeted bond-breakage of polysaccharides in their own cell walls, plants create local wall loosening effects which facilitate cell expansion and anisotropic growth (Chebli and Geitmann, 2017). While earlier studies on cell wall loosening in *Cuscuta* have focused on the remodeling of the interactions between pectin and other cell wall components (Jhu et al., 2021; Vaughn, 2002), the high expression level of *GH9B1, B2, B3,*

B4, C1 and *C2* suggest that some digestion of cellulose takes place at early stages of haustorium development. The GH9 A-type enzymes may instead be involved in meeting the increased demands for cellulose microfibrils associated with rapid cell division and elongation during haustorium development (Vaughn, 2002).

Earlier studies have given indications of involvement of enzymatic degradation during host penetration by parasitic plants (Hozumi et al., 2017; Johnsen et al., 2015; Olsen et al., 2016). Our results show that expression of GH9 family members *B7, B8, B9, B11, B12* and *C3* peaked at ATT or PEN stage, implicating them as possible key secreted cellulases for host cell wall degradation. Earlier, an evidence of secreted B-type GH9 cellulase being critical for host penetration in the root hemi-parasite, *P. japonicum* was given by Kurotani et al. (2020). The *P. japonicum* RNAi mutants of a GH9B cellulase were unable to make vascular connections with their hosts and this phenotype was attributed to insufficient cellulase activity to loosen host cell walls (Kurotani et al., 2020). Furthermore, we observed that *GH9B7, B11* and *B12* expression was highest at the PEN stage in the host-free system, whereas expression peaked earlier, at the ATT stage in the presence of a host, suggesting that *Cuscuta* is able to tune the temporal expression of its cellulases when it entwines and attempts to infect host. While the expression of *GH9B7, B11* and *B12* declined from ATT to the PEN stage in both *Solanum* hosts, this downregulation was more pronounced in *S. pennellii*. This difference could reflect the fact that the stems and petioles of this species contain significantly more cellulose than their counterparts in *S. pennellii* (Bawin et al., 2024). *S. lycopersicum* is also known to be partially resistant and a more difficult host for *Cuscuta* compared to the fully susceptible *S. pennellii* and thus may require stronger and sustained total cellulase activity for successful penetration. On the other hand, though host cell wall degradation is critical for host penetration (Kurotani et al., 2020), excessive and uncontrolled degradation and loosening of host cell walls caused by deregulation of cell wall degrading genes has also been shown to cause haustorium detachment in *C. campestris* (Jhu et al., 2022). Therefore, the dynamic changes in the expression levels of the PEN stage-specific genes may reflect a balance between various cellulase activities needed for successful infection in different hosts.

In the absence of reliable reverse genetics protocols for transformation of *Cuscuta*, we studied the enzymatic activities and substrate specificities of *Cuscuta* cellulases in vitro, by expressing eight PEN stage-related secreted cellulases as recombinant proteins in yeast. All the recombinant *Cuscuta* cellulases showed high enzymatic activity on the non-crystalline cellulose compound CMC as well as moderate activity towards PASC and the XG hemicellulose. In the case of crystalline cellulose compounds, all the cellulases showed endoglucanase activity on Avicel® and FP, but overall GH9B11, B12 and especially GH9C3 showed better ability to utilize these complex substrates. Plant GH9 subfamily C members contain a CBM49 that has been shown to bind and enhance degradation of crystalline cellulose (Lopez-Casado et al., 2008; Urbanowicz et al., 2007b). Therefore, the CBM49 may have contributed to the relatively higher activity of GH9C3 on complex cellulose substrates.

Cellulases GH9B11 and B12 showed a remarkably high activity against GM that could not be detected in other tested cellulases. This finding is interesting since *Cuscuta* possesses dedicated mannanase enzymes (GH5) for degradation of mannans. Bawin et al. (2024) earlier analyzed the cell wall composition of the two tomato hosts. It was found that upon challenge with *C. campestris*, *S. lycopersicum* host significantly fortified its cell walls at the infection site through a buildup of mannans (Bawin et al., 2024). Furthermore, mannanases were also shown to be among the most significantly upregulated genes during haustoriogenesis in *Cuscuta* (Bawin et al., 2024). Thus, mannans being as one of the possible main defense barriers in *Cuscuta*-resistant plants, there may be a need for *Cuscuta* to attack this macromolecule with different enzymes, including endoglucanases. Interestingly, unlike in the host-free system, *GH9B11* and *B12* expression already peaked in ATT stage in the presence of tomato hosts, indicating a need for their upregulation at early stage after recognition of host. When *Cuscuta* was growing on susceptible *S. pennellii*, the expression peak was only transient while on partially resistant *S. lycopersicum,* the expression level was maintained at high level also in PEN stage. How this infection process is orchestrated, and GH9B11 and B12 function is coordinated with the other mannanases is subject to future studies. Overall, our gene expression analysis and the in vitro activity assays suggest that *Cuscuta* secretes a mixture of subfamily B and C cellulases during host penetration, to potentially target the amorphous and crystalline components of cellulose as well as hemicelluloses such as XG and GM in host cell walls. This could have implications for host range in that it allows *Cuscuta* to modulate and fine-tune its cellulase activity to specific needs based on the composition of host cell walls and this could contribute to broad host range of *Cuscuta* (Birchler and Yang, 2022; Koch et al., 2004; Masanga et al., 2022). In the case of *Solanum* hosts, cellulases GH9B7, B11 and B12 seem to have a major role in this process (Fig. 8).

Our in vitro activity assays revealed interesting biochemical properties for *Cuscuta* cellulases. All the recombinant cellulases retained activity over broad temperature (10–80 °C) and pH ranges (3–10). Covering broad pH range is interesting since many cell wall modifying enzymes and proteins are regulated by extracellular pH (Duracho and Cosgrove, 2009; Phyo et al., 2019; Xu and Yu, 2023). It is also known that some fungal pathogens have evolved an elaborate pH sensing system that allows them to fine-tune their secreted virulence proteins to match the pH conditions of their hosts in order to ensure optimal activity and successful infection (Kubicek et al., 2014; Peñalva et al., 2008; Prusky and Yakoby, 2003). It is unknown whether such pH sensing mechanisms exist in parasitic plants, but their similar lifestyles to pathogenic microbes would justify such an assumption. Nevertheless, the wide pH range for different *Cuscuta* cellulases could ensure that individual enzymes maintain some sub-optimal activity level over diverse pH conditions so that their collective cellulase activity remains sufficient to ensure successful infection. Concerning temperature optimum of cellulases, the high optimal temperature conditions for the *Cuscuta* recombinant cellulases are more typical for microbial enzymes (Balasubramanian et al., 2012; Nascimento et al., 2010) than for plant GH9 cellulases (Mølhøj et al., 2001; Yoshida and Komae, 2006). From industry point of view, activity over wide pH and temperature conditions is attractive characteristics as they permit the use of hydrolytic enzymes under various process conditions and minimize the need for precise pH and temperature controls. The broad substrate range further makes *Cuscuta* cellulases attractive for bioconversion applications, including degradation of plant cellulosic biomass for production of biofuels.

5. Conclusion

Our findings provide molecular insights towards understanding the parasitic infection process. Our study suggests that cellulases expressed in *C. campestris* haustorium are not only involved in remodeling the parasite's own cell walls during haustorium organogenesis, but secreted cellulases can also selectively degrade cell wall cellulose and hemicellulose components to facilitate host invasion. The biochemical properties of these cellulases indicate potential usefulness in industrial bioprocessing.

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CRediT authorship contribution statement

Hilary Edema: Writing – original draft, Methodology, Investigation. **Thomas Bawin:** Writing – review & editing, Investigation. **Stian Olsen:** Writing – review & editing, Investigation. **Kirsten Krause:** Writing –

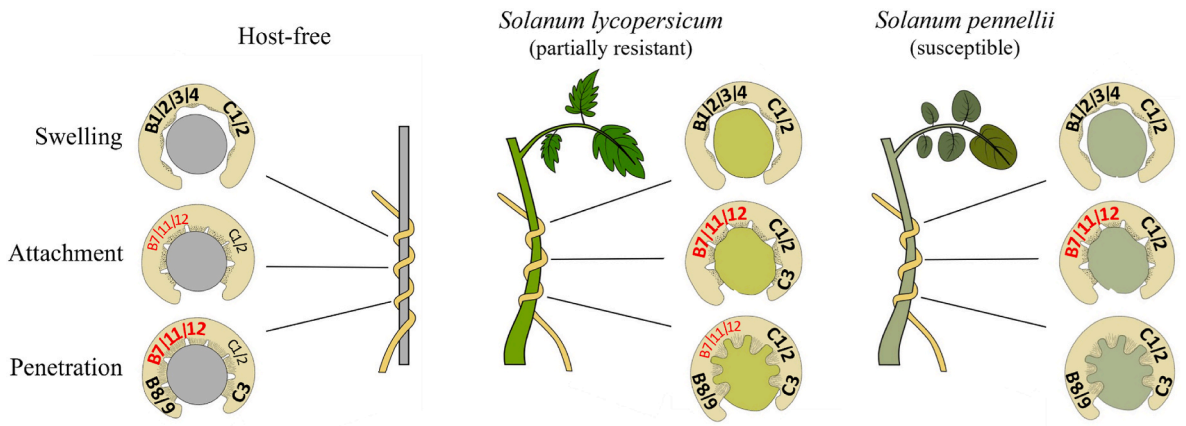

Fig. 8. Induction pattern of the secreted cellulases arsenal during *Cuscuta* haustorium development. The haustorium developmental stages are shown in host-free system as well as on partially resistant *Solanum lycopersicum* and susceptible *Solanum pennellii* hosts. Expressed *Cuscuta* B- and C-type cellulases are shown in the cross-sections of the parasite side. Larger font size indicates higher expression level. Differences in *GH9B7*, *B11* and *B12* expression are highlighted in red.

review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Katja Karppinen:** Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108633.

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