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Characterization of *Cuscuta campestris* Cell Wall Genes Responsible for the Haustorial Invasion of Host Plants

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Abstract: *Cuscuta* is one of the most widespread genera of parasitic plants that subsist on various types of herbaceous angiosperms. Since their hosts include economically important crops, understanding the molecular basis of parasitism in *Cuscuta* species is critical for crop production. Despite the advances in biology and agriculture, the mechanisms of host plant infection by parasitic plants remain largely unknown. To gain insight into the mechanism of parasitism, we focused on *Cuscuta campestris* genes encoding enzymes involved in the degradation and modification of host cell walls. Analysis of previously published RNA-seq data showed that certain genes encoding pectin methylesterases, polygalacturonases, and cellulase-like enzymes, which can potentially enzymatically weaken cell-to-cell adhesion, were correlated with the haustorial invasion of host tissues. These data confirm that haustorial invasion by *Cuscuta* species is facilitated not only by mechanical action but also by specific biochemical degradation and modification of host cell walls.
Keywords: parasitic plant, haustorium, cell wall, gene family, CAZyme

Introduction

Cuscuta species represent obligate plant stem parasites, characterized by thin stems, almost no roots and leaves, and little or no photosynthetic activity[1)]. The stems of *Cuscuta* plants coil around those of host plants and penetrate into the host tissue using an invasive organ, called a haustorium. The haustorium contains vascular tissue, which connects with the host vascular bundles and draws most of the nutrients from the host plant. Thus, *Cuscuta* species are heterotrophic and potentially harmful to host plants, which encompass a wide range of angiosperms, including economically important crop plants. Therefore, understanding the molecular basis of parasitism by *Cuscuta* species is not only of interest from the biological perspective but also of critical importance from the crop production viewpoint.

A recent significant breakthrough in molecular genetics of *Cuscuta* species has come from the whole-genome sequencing of *Cuscuta australis* and *Cuscuta campestris*, which are phylogenetically closely related to each other. *C. australis* has a relatively smaller genome (272 Mb) and harbors fewer protein-coding genes (19,671)[2)] than *C. campestris* (581-Mb genome, 44,303 protein-coding genes)[3)]. *C. campestris* genome contains a high proportion of gene duplicates and recently proliferated long terminal repeat (LTR) retrotransposons, suggesting a recent genome duplication event in the *C. campestris* lineage[3)]. Notably, *C. campestris* is broadly sympatric in the world and therefore has been studied more intensively than other *Cuscuta* species[1)]. Therefore, considering *C. campestris* as a representative species for the genus *Cuscuta*, we generated genetically identical homozygous lines of *C. campestris* by repeated self-pollination, and sequenced its genome to

establish our own *C. campestris* genome database (unpublished).

To facilitate the investigation of the molecular basis of parasitism and haustorium development in *C. campestris*, we previously developed two experimental procedures. The first procedure entails the microscopic evaluation of live haustorial tissue sectioned vertically relative to the host stem, thus preserving the host–parasite interface. Using this experimental procedure, we demonstrated that *C. campestris* utilizes host-produced ethylene for the proper growth of search hyphae, which finally differentiate into xylem to connect with the host vasculature during haustorium development[4]. The second experimental procedure utilizes rosette leaves detached from *Arabidopsis thaliana* as the host tissue for the analysis of haustorium development *in vitro*. Using this *in vitro* system, we showed that direct contact of the parasitic hyphal cells with the host xylem is required for their differentiation into xylem vessel cells during haustorium development[5].

Within the developing *C. campestris* haustorium, search hyphae elongate to invade the host plant, thus reaching the host vascular bundles. It has been proposed that these invasion processes are facilitated not only by a simple mechanical stimulus but also by the degradation and/or modification of host cell walls[6,7]. It has been shown that the cell wall of the host cell directly in front of the growing tip of search hypha undergoes stretching and thinning to facilitate the entry of the hypha[8]. However, little is known about the mechanisms underlying the biological degradation and modification of host cell walls.

To gain insight into the cell wall changes involved in the regulation of hyphae elongation, we characterized the expression patterns of *C. campestris* cell wall genes during haustorial invasion of the host tissue. The results showed that the expression of genes encoding pectin metabolic enzymes and glucanases were closely correlated to the haustorial invasion event.

Materials and Methods

Data availability

All protein sequence datasets of *Cuscuta campestris* and morning glory (*Ipomoea nil*), a close relative of *C. campestris*, were obtained from the plaBi database (http://plabipd.de/portal/cuscuta-campestris) and *Ipomoea nil* Genome Project database (http://viewer.shigen.info/asagao/index.php), respectively. Additional protein sequence data for the GH9 family of *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), and *Chlamydomonas reinhardtii* were retrieved from Phytozome v13.0 (https://phytozome-next.jgi.doe.gov). The *C. campestris* RNA-seq data, generated previously by our group[5], are available from the DNA Data Bank of Japan Sequenced Read Archive database (https://trace.ddbj.nig.ac.jp/dra/index_e.html/) under the accession number DRA009453.

Bioinformatic analysis

Genes encoding Carbohydrate-Active enZymes (CAZymes) were identified by running HMMER3 scans using the hidden Markov model (HMM) profile downloaded from the dbCAN2 HMMdb (http://bcb.unl.edu/dbCAN2/index.php)[9]. The e-value and coverage cutoffs were 1e-15 and > 0.35, respectively.

Differentially expressed genes (DEGs) were identified using TCC[10] packages in R. Transcript expression levels were expressed as transcripts per million (TPM), and genes with q-value < 0.05 were designated as DEGs. Clustering analysis of DEGs was performed using the MBCluster package in R. Venn diagrams were constructed using the VennDiagram package in R.

To conduct phylogenetic analysis, amino acid sequences of CAZymes were aligned using the DDBJ ClustalW 2.1 online freeware (http://clustalw.ddbj.nig.ac.jp/), and phylogenetic trees were constructed using the neighbor-joining method in MEGAX[11].

Results

Identification and classification of CAZyme-encoding genes in *C. campestris*

Most of the cell wall-related enzymes in plants are commonly designated as CAZymes, which are categorized and listed in the CAZy database (http://www.cazy.org)[12]. Using a protocol of dbCAN[9], we identified all CAZyme-encoding genes in *C. campestris*, including 479 *glycoside hydrolases (GHs)*, 613 *glycosyl transferases (GTs)*, 133 *carbohydrate esterases (CEs)*, and 49 *polysaccharide lyases (PLs)*. The CAZyme-encoding gene composition of *C. campestris* was highly similar to that of I. *nil* (Fig. 1). The results suggest that the genomes of *Cuscuta* species

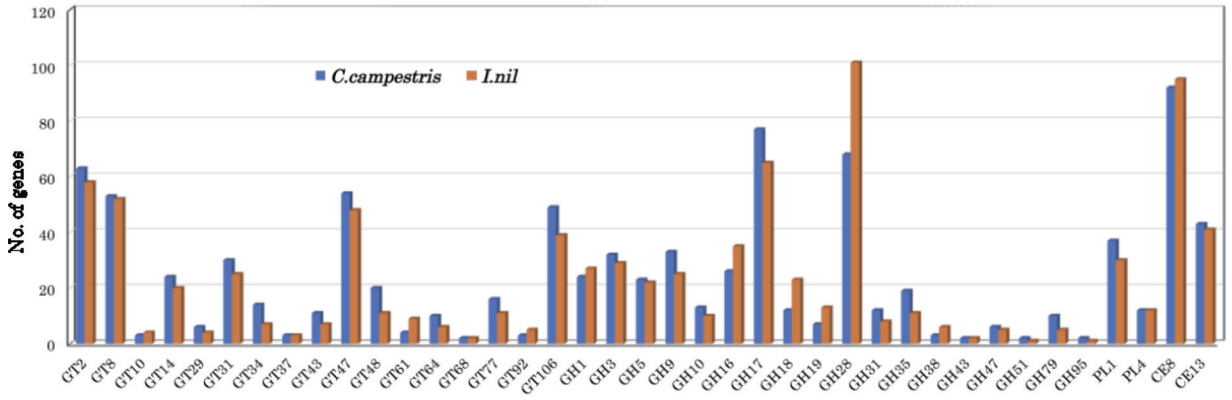

Fig. 1. Numbers of genes in each cell wall-related CAZyme family in *Cuscuta campestris* and *Ipomoea nil*. The cell wall-related families[13] were selected among the CAZyme-encoding gene families classified using dbCAN2.

have not undergone reconstruction of the CAZyme-encoding gene sets, despite the massive changes in its body plan, including haustorium formation.

Expression profiles of CAZyme-encoding genes during haustorial invasion of the host

Next, we focused on CAZyme-encoding genes whose expression profiles were temporarily correlated with the specific stages of haustorial development or host invasion. We previously performed a comprehensive RNA-seq analysis of gene expression patterns in *C. campestris* haustoria at 0, 12, 42, and 54 h after coiling (hac) on an intact Arabidopsis stem[5]. The haustorium invaded the host tissue at 12 hac, and vascular differentiation (involved in secondary cell wall assembly) occurred from 42 to 54 hac. We focused on CAZyme-encoding genes predominantly expressed at 12 hac because the degradation and modification of host cell walls mainly occur during this phase. A heatmap of CAZyme-encoding genes extracted from the previously published RNA-seq data showed differences in the expression patterns of CAZyme-encoding genes among the different developmental stages, generating three clusters (clusters 1, 2, and 3), each containing CAZyme-encoding genes predominantly expressed at 12 hac (Fig. 2). Clusters 1 and 2 included CAZyme-encoding genes (17 *GHs*, two *GTs*, six *CEs*, and two *PLs*) principally expressed at 12 hac, but the expression of these genes was almost undetectable in the epidermal and cortical cells of uninfected (control) *C. campestris* stems (Table 1). Ten of the 17 *GHs* belonged to the *GH28* gene family, which are involved in the degradation of homogalacturonans (HGs). Five of the six

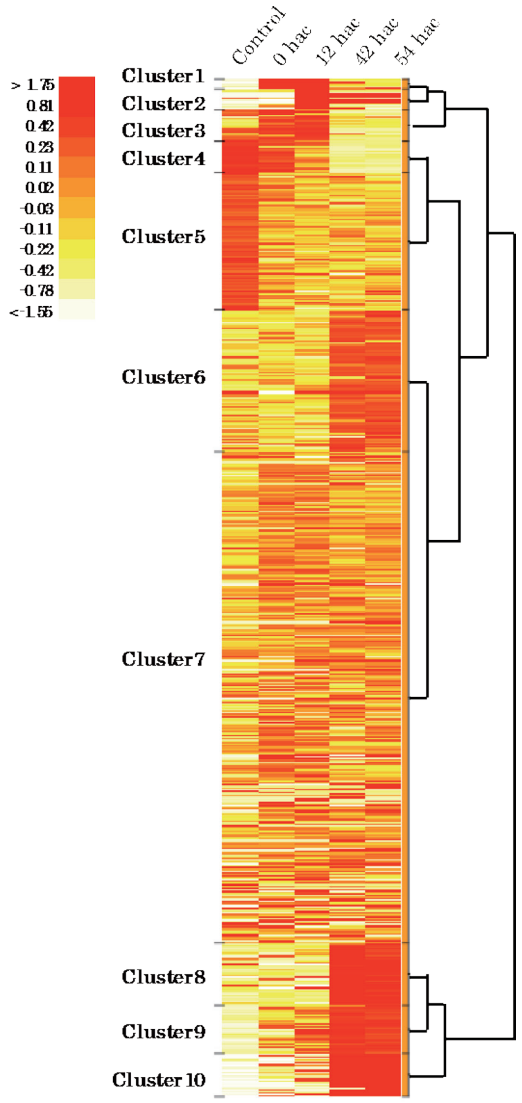

Fig. 2. Hierarchical clustering analysis of CAZyme-encoding genes. The heat map shows the relative expression levels of genes in five samples (haustoria at 0, 12, 42 and 54 hac, and the epidermal and cortical cells of uninfected stems). The color scale is shown on the left. hac, hours after coiling around the host stem.

CEs belonged to the *CE8* gene family, which encode pectin methylesterases (PMEs) that mediate the de-esterification of methyl-esterified HGs, thus leading to HG degradation. Additionally, it is notable that clusters 1 and 2 also included *GH9* family genes, which encode putative glucanases.

Comparison of CAZyme-encoding genes between *in vivo* and *in vitro* experimental systems

We also performed transcriptome analysis of *C. campestris* stems using the *in vitro* haustorium induction system developed previously[5] to identify CAZyme-encoding genes preferentially expressed during haustorium development. In this experiment, 3 cm-long sections of *C. campestris* lateral shoots were prepared, overlaid with a rosette leaf of Arabidopsis, and then weighted with a stack of glass slides for 57 or 87 h; the same set up minus the rosette leaf was used as a control. The pressed lateral shoot samples in contact with the host leaf for 57 and 87 h after infection (hai) were designated as 57 hai (+/+) and 87 hai (+/+), while pressed samples without contact with the host leaf were designated as 57 hai (+/-) and 87 hai (+/-), respectively. Haustoria were induced in all the four pressed samples, i.e. 57 hai (+/-), 57 hai (+/+), 87 hai (+/-), and 87 hai (+/+). Interestingly, DEGs upregulated in the haustoria of these pressed samples included most of the CAZyme-encoding genes that were predominantly expressed at 12 hac. In particular, expression of all CAZyme-encoding genes in clusters 1 and 2 was upregulated in the haustoria that had been induced in vitro by pressing the lateral shoot samples in contact with the host leaf, i.e. in the pressed samples 57 hai (+/+) and 87 hai (+/+) (Fig. 3).

Table 1. CAZyme genes in cluster 1 and 2

Gene ID	Cazy family name	Description
cluster 1		
Cc001522	GH9	endo-ß-1,4-glucanase
Cc044982	GH9	endo-ß-1,4-glucanase
Cc003113	GH28	polygalacturonase
Cc027140	GH28	polygalacturonase
Cc015548	PL1	pectin Lyase
Cc021766	PL1	pectin Lyase
Cc018047	CE8	pectin methylesterase
Cc022203	CE8	pectin methylesterase
Cc030736	CE8	pectin methylesterase
cluster 2		
Cc003068	GT47	ß-1,4-glucuronyltransferase
Cc027096	GT47	ß-1,4-glucuronyltransferase
Cc007143	GH1	exo-ß-1,4-mannosidase
Cc014521	GH9	endo-ß-1,4-glucanase
Cc002566	GH9	endo-ß-1,4-glucanase
Cc005807	GH9	endo-ß-1,4-glucanase
Cc035665	GH9	endo-ß-1,4-glucanase
Cc024138	GH28	polygalacturonase
Cc047633	GH28	polygalacturonase
Cc030703	GH28	polygalacturonase
Cc036187	GH28	polygalacturonase
Cc036210	GH28	polygalacturonase
Cc003083	GH28	polygalacturonase
Cc027112	GH28	polygalacturonase
Cc024920	GH28	polygalacturonase
Cc008607	CE8	pectin methylesterase
Cc002565	CE8	pectin methylesterase
Cc022249	CE13	pectin acetylesterase

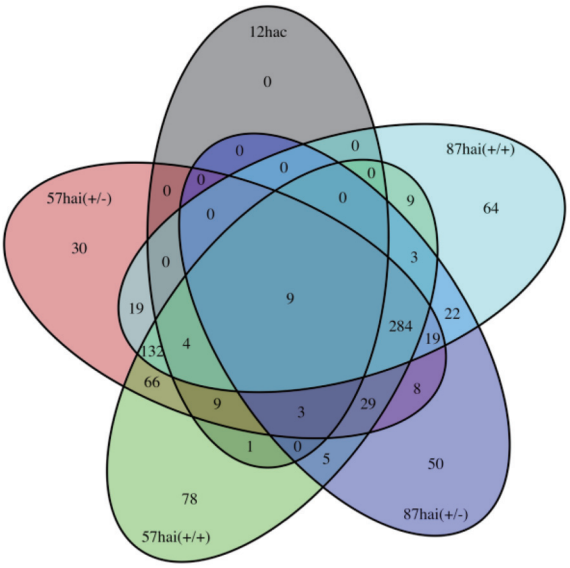

Fig. 3. Venn diagrams of CAZyme-encoding genes identified on the basis of their expresstion patterns. The CAZyme-encoding genes predominantly expressed on an intact Arabidopsis stem at 12 hac, which were classified into clusters 1 and 2, were compared with those upregulated under the four in vitro haustorium induction conditions: 57 hai (+/+) and 87 hai (+/+), genes principally expressed in pressed samples directly in contact with the host leaf for 57 and 87 hai, respectivele; 57 hai (+/-) and 87 hai (+/-), genes principally expressed in pressed samples not in contact with the host leaf for 57 and 87 hai, respectively.

Discussion

In this study, we identified putative CAZyme-encoding genes involved in haustorial invasion, based on gene expression patterns. Many of these genes were classified into the *GH28* and *CE8* families, which are involved in the degradation and modification of HG, a major pectin found in plant cell walls[14]. HG is localized in the middle lamella of the cell wall, particularly in the corners of cell-to-cell junctions, and therefore is thought to play a key role in maintaining cell adhesion within a multicellular structure[15]. The results of this study suggest that degradation and modification of HGs by GH28s and CE8s weak-

ens cell-to-cell connections, which leads to cell separation, thus facilitating haustorial invasion of the host tissue.

The GH9 family is also identified as one of the major CAZyme families involved in haustorial invasion. *GH9* genes are thought to encode endo - 1,4 - β - glucanases that hydrolyze 1,4 - β - glucosidic linkages in glucans. Although their enzymatic function remains unclear, GH9 proteins perform important structural roles in cell adhesion and have been linked to processes such as organ abscission and fruit ripening[16,17]. In tomato, two GH9s, Cel1 and Cel2, have been implicated in tissue abscission and fruit ripening[18]. In this study, we found that the *C. campestris* orthologs of tomato *Cel1* and *Cel2* were principally expressed during haustorial invasion (Fig. 4). In addition to GH9s, CE8s and GH28s are also involved in tissue abscission and fruit ripening[19]. Taken together, these data suggest that the evolutionary acquisition of the degradation and modification process of host cell walls for haustorial invasion may be related to changes in the expression of genes involved in tissue abscission and fruit ripening.

Recently, Kurotani et al.[20] reported that in *Phtheirospermum japonicum* (Orobanchaceae), which is a root parasitic plant, a gene encoding a secreted type of β-1,4-glucanase, classified in the *GH9* family, plays an important role in plant parasitism. Furthermore, a member of the *GH9* family is required for cell-to-cell adhesion in Nicotiana interfamily grafting [21].

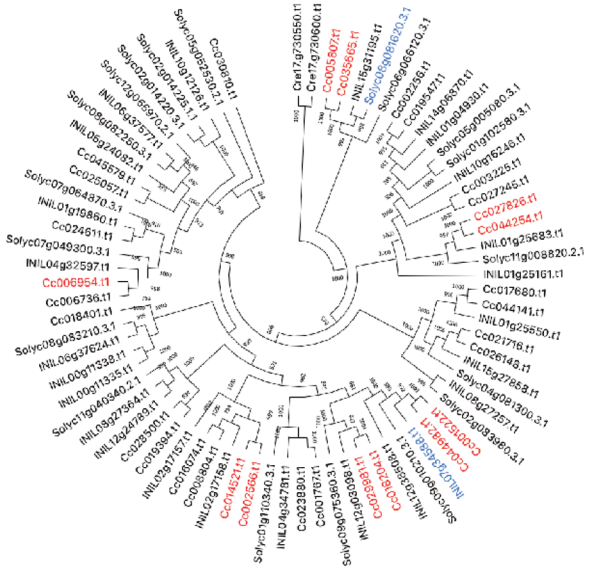

Fig. 4. Phylogenetic analysis of GH9s. The CAZyme-encoding genes predominantly expressed at 12 hac are shown in red. Cel1 (Solyc08g081620.3.1) and Cel2 (Solyc09g010210.3.1) are highlighted in blue. Cc, *C. campestris*; INIL, *I. nil*; Solyc, S. *lycopersicum*; At, *A. thaliana*, Cre, *C. reinhardtiil.*

The results of this study could be interpreted in several different ways. For example, it is possible that CE8s, GH28s, and GH9s are not involved in the degradation and modification of host cell walls but instead contribute to the elongation of search hyphae during haustorium development. Until now, it has been difficult to assign a precise role to each CAZyme-encoding gene during haustorial invasion, mainly because there has been a lack of practical transformation techniques for *Cuscuta* species. One way to overcome this difficulty might be to genetically screen mutants defective in haustorial development and parasitizing capability. Accordingly, we have now begun to mutate a large population of *C. campestris* seeds by ion-beam irradiation, and have obtained mutagenized seeds suitable for the screening of phenotypes defective in various steps of haustorium development. Identification of mutations associated with particular defects in haustorium development in the mutant plants, together with the characterization of haustorial function using the *in vitro* parasitizing system, will help elucidate the function of CAZymes during haustorial development and invasion into host plants.

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