Epidermal cell-patterning genes of the stem parasitic plant *Cuscuta campestris* are involved in the development of holdfasts

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Abstract *Cuscuta campestris*, a stem parasitic plant, commences its parasitic behavior by forming a specialized disk-like adhesive structure called a holdfast, which facilitates tight adhesion to the stem surface of the host plant. The morphology of epidermal cells in the holdfast is similar to that of the leaf trichome and root hairs of dicotyledonous plants. However, the regulatory network underlying the development of the holdfast has not been elucidated to date. In this study, we assessed the roles of epidermal cell-patterning genes in the development of a holdfast. Epidermal cell-patterning genes of *C. campestris*, including CcWER, CcGL3, CcTTG1, CcGL2, and CcJKD, were expressed slightly before the initiation of the outgrowth of stem epidermal cells. CcJKD-silencing repressed CcJKD, CcWER, CcGL3, CcTTG1, CcGL2; therefore, CcJKD is an upstream regulator of other epidermal cell-patterning genes. Unlike other genes, CcCPC was not upregulated after attachment to the host, and was not repressed by CcJKD-silencing. Protein interaction assays demonstrated that CcJKD interacted with CcTTG1 and CcCPC. Furthermore, CcJKD-silencing repressed the outgrowth of holdfast epidermal cells. Therefore, *C. campestris* invokes epidermal cell-patterning genes for the outgrowth of holdfast epidermal cells, and their regulatory mechanism is different from those for leaf trichome or root hairs.

Key words: attachment, *Cuscuta campestris*, epidermal cell-patterning genes, holdfast, outgrowth.

Introduction

*Cuscuta campestris*, a stem parasitic plant, is a holoparasite that does not perform photosynthesis and takes up water and nutrients from the host plant. On contact with the host stem surface, *C. campestris* coils around the stem and initiates parasitic processes. The parasitic processes begin with tight adhesion to the stem surface of the host plant (Heide-Jørgensen 2008; Vaughn 2002). *C. campestris* forms a specialized disk-like adhesive structure called a holdfast, which helps the parasitic stem stay in contact with the host stem during haustorium penetration (Lee 2007). The development of a holdfast is always associated with the outgrowth of papillar epidermal cells. The morphology of epidermal cells in the holdfast is very similar to that of the leaf trichome and root hairs of dicotyledonous plants. Based on morphological observations using scanning electron microscopy, a group of elongated epidermal cells in the proximal area of the host have been referred to as “unicellular secretory type trichomes” (Vaughn 2002).

The genetic network involved in the morphogenesis and patterning of epidermal cells, including the leaf trichome and root hairs of the model plant *Arabidopsis thaliana* has been studied extensively. In *A. thaliana*, morphogenesis of the trichomes is activated by an activator trimeric protein complex consisting of GLABRA1 (GL1), which is a Myb-domain protein; GLABRA3 (GL3)/ENHANCER OF GLABRA3 (EGL3), which are basic helix-loop-helix (bHLH) proteins; and TRANSPARENT TESTA GLABRA1 (TTG1), which is a WD40-repeat-containing protein (Ishida et al. 2001). The trimeric complex then promotes the transcription of trichome-activator genes, including GLABRA2 (GL2), which encodes a homeodomain-leucine zipper (HD-Zip) protein and of TRANSPARENT TESTA GLABRA2 (TTG2), which encodes a WRKY transcription factor (Ishida et al. 2001).
Holdfast development needs epidermal cell-patterning genes and CcGL2 localization of CcJKD transcripts using in situ expression profiling during the early attachment stages, downstream transcription factor, Myb-bHLH-WD40-complex, including CcWER. C. campestris holdfasts. We selected epidermal cell-patterning genes in the development of C. campestris holdfasts. Results of gene expression profiling during the early attachment stages, localization of CcGL2 and CcJKD transcripts using in situ hybridization, and protein–protein interaction analysis suggested the involvement of CcJKD in the outgrowth of the holdfast epidermal cells. CcJKD-silencing demonstrated the role of CcJKD in the regulation of the other epidermal cell-patterning genes and the outgrowth of holdfast epidermal cells.

**Materials and methods**

**Plant materials and growth conditions**

*Nicotiana tabacum* 'Xanthi' and *Arabidopsis thaliana* ecotype Columbia (Col-0) were used as wild type host plants. Seeds were germinated on Murashige and Skoog medium (Murashige and Skoog 1962) in a growth cabinet at 22°C and grown as described previously (Hozumi et al. 2017). Mutant lines were germinated under appropriate antibiotic selection. *C. campestris* was grown and parasitized, as described previously (Hozumi et al. 2017). The interface region containing both *C. campestris* and *A. thaliana* was harvested at 0, 12, 24, and 48 h after attachment (haa).

**RNA extraction**

Total RNA samples were prepared from the parasitized *C. campestris* stem tissues, which were peeled off from the parasitic interface region and immediately frozen in liquid nitrogen. A RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used to isolate the total RNA from the parasitic interface region frozen samples. The extracted total RNA was then treated with a TURBO DNA-free™ Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

**cDNA cloning**

Gene-specific primers were designed based on the sequences of RNA-seq contigs of *C. campestris* (cucam_0.32.annot.cds.fasta, https://www.plabipd.de/project_cuscuta2/start.ep, Vogel et al. 2018). Partial cDNAs of CcJKD (Cc015901.t1), CcWER (Cc023188.t1), CcGL3 (Cc022187.t1), CcTTG1 (Cc046705.t1), CcCPC (Cc000579.t1) and CcGL2 (Cc003411.t1) were cloned with RT-PCR using total RNA obtained from the parasitic interface region frozen samples. The extracted total RNA was then treated with a TURBO DNA-free™ Kit (Thermo Fisher Scientific). Accession numbers of target genes. PCR amplification was performed using DNA polymerase, KOD-Plus Neo (Toyobo), and a My Cycler™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) or a T100™ Thermal Cycler (Bio-Rad). PCR products were extracted from the agarose gel using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and cloned into a pCR®-Blunt II-Topo® vector using a Zero Blunt TOPO® PCR Cloning Kit (Thermo Fisher Scientific). Accession numbers of
cDNA sequences are as follows. CcGL3, LC586082; CcTTG1, LC586083; CcWER, LC586084; CcCPC, LC586085; CcGL2, LC586086; CcJKD, LC586087.

**Quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

The primers used in qRT-PCR are shown in Supplementary Table S1. qRT-PCR of CcJKD, CcWER, CcGL3, CcTTG1, CcCPC, and CcGL2 was performed using oligo(dT) primer and ReverTra Ace®-α-(Toyobo) in cDNA synthesis. Fast SYBR® Green Master Mix (Thermo Fisher Scientific) and the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) were used to perform real-time PCR. Expression levels were normalized to the C. campestris gene encoding the ribosomal small subunit protein CcRPS18 (Cc020048.t1 in cucam_0.32.annot.cds.fasta, https://www.plabipd.de/project_cuscuta2/start.ep; Vogel et al. 2018).

**In situ hybridization**

RNA probes for in situ hybridization were produced using CcGL2 and CcJKD partial sequences cloned in pCR®-Blunt II-Topo® vector. Probe synthesis and hybridization were performed according to a previously published protocol (Shimizu et al. 2018).

**Yeast two-hybrid (Y2H) assay**

Yeast two-hybrid assay was performed using the Matchmaker™ Gold Yeast Two Hybrid System (Clontech, Mountain View, CA, USA). The coding sequences of CcJKD, CcWER, CcGL3, CcTTG1, CcCPC, and CcGL2 were amplified and fused to both pGBK T7 DNA-BD and pGAD T7 AD cloning vectors (Clontech). The primers used to amplify full-length cDNAs are shown in Supplementary Table S1. Autoactivation of yeast containing the bait was tested using selective medium SD/-Trp/-Leu (Clontech) and aureobasidin α containing the bait was tested using selective medium SD/-His/-Phe (Sucitech). The yeast strain Y2H Gold (Clontech) expressing the pairs of pGBKT7 DNA-BD and pGADT7 AD cloning vectors were transformed according to a previously published protocol (Shimizu et al. 2018).

**Protein complex immunoprecipitation**

Yeast strain Y2H Gold (Clontech) expressing the pairs of proteins, HA-tagged (HA-) epidermal cell-patterning proteins and Myc-tagged (Myc-) epidermal cell-patterning proteins under the ADH1 promoters (Ammerer 1983; GenBank accession number Z25479; Ruohonen et al. 1991) was grown in liquid selective media (SD/-Trp/-Leu, Clontech) overnight as a preculture. YPDA broth (Clontech) was inoculated with the preculture, and the initial optical density (OD) 600 of YPDA culture was 0.2. The yeast culture was incubated at 30°C, 250 rpm for 3–5 h until OD600 reaches to 0.4–0.6. The yeast cells were centrifuged for 5 min at 4,000×g. Cells were washed with cold, sterile distilled H2O. The yeast cell pellets were frozen and stored until lysis at −80°C. An equal volume of acid-washed glass beads (425–600 μm; Merck KGaA, Darmstadt, Germany), 10 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20, 1× proteinase inhibitor cocktail for general use (product number 04080, Nacalai Tesque)) in every 10 mg of the wet weight of yeast cell was added to frozen yeast pellets. The cells were lysed using a Tissue Lyser LT (Qiagen) for 7 min at its highest speed. Yeast lysates were clarified by centrifugation at 16,000×g for 10 min at 4°C. Lysis was repeated by adding the same lysis buffer volume each time as previously to the pellets, and the lysates were combined. The total protein concentration was measured using a Pierce™ 660 nm protein assay reagent (Thermo Fisher Scientific). For protein complex immunoprecipitation, anti-HA-tag mAB-magnetic agarose (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was used. A total of 20 μl of anti-HA-tag mAB-magnetic agarose beads, which were pre-equilibrated three times with lysis buffer for 30 s, was mixed with 1 ml of cell lysate containing 1 mg of total protein and incubated at 4°C with rotation overnight. After incubation, the cell lysates were separated from the magnetic agarose using a magnetic rack, and the magnetic agarose beads were washed four times with 1 ml of cold lysis buffer for 1 min each. The magnetic agarose beads were then resuspended in 40 μl of Laemmli sample buffer and boiled for 3 min at 96°C. The supernatant was subjected to SDS-PAGE. Electrophoresis was performed using a 1 mm-thick 10% (w/v) polyacrylamide gel. A total of 20 μl of the boiled sample was loaded in each lane. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (ATTO, Tokyo, Japan) at 30 V overnight using a Mini Trans-Blot® Cell (Bio-Rad). The PVDF membranes were blocked with 5% (w/v) skimmed milk (in phosphate buffered saline (PBS), pH 7.2) for 1 h at 25°C, and incubated with anti-HA-tag mAB-HRP-DirecT or anti-Myc-tag mAB-HRP-DirecT (Medical & Biological Laboratories Co., Ltd.), for 1 h at 25°C. The membranes were washed three times for 5 min in PBS, 0.1% (v/v) Tween-20. Finally, the membranes were incubated with Chemi-Lumi One L (Nacalai Tesque, Kyoto, Japan) for 1 min. Images were taken using LuminoGraph I (ATTO).

**Artificial microRNA (amiRNA) synthesis**

A synthetic DNA fragments for the amiRNA construct targeting CcJKD was designed using WMD3-Web MicroRNA Designer (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) according to the WMD3-Web’s protocol (Schwab et al. 2006), and clone into pRS300 (plasmid number 22846; Addgene, https://www.addgene.org/). The primers used for synthesizing the amiRNA construct are shown in Supplementary Table S1. The amiRNA fragment was inserted into SacI-HF (New England Biolabs, Ipswitch, MA, U.S.A.) and SalI-HF (New England Biolabs) treated binary vector pB121 (Accession number AF485783) using Ligation High ver. 2 (Toyobo) to express the amiRNA precursor under the cauliflower mosaic virus (CaMV) 35S promoter. The vector plasmid containing a sequence to express amiRNA was transformed into the Agrobacterium strain GV3101.
Holdfast development needs epidermal cell-patterning genes

Transformed of N. tabacum

Agrobacterium GV3101 harboring the amiRNA expression construct was grown at 37°C by shaking at 180 rpm until the culture reach to OD600 to 1.0. The leaf disks were prepared by cutting young leaves of N. tabacum grown at 25°C in sterile containers into 1 cm squares. Leaf disks infected with Agrobacterium culture were co-cultured on solid Murashige Skoog (MS)-medium containing 1 mgl−1 6-benzylaminopurine (BAP) (Nacalai Tesque) and 0.1 mg l−1 indole-3-acetic acid (IAA) (Nacalai Tesque) at 25°C in the dark for one day. Subsequent co-culturing on fresh solid MS-medium containing 1 mg l−1 BAP, 0.1 mg l−1 IAA, 100 mg l−1 kanamycin (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 50 mg l−1 meropenem (Sumitomo Dainippon Pharma, Osaka, Japan) at 25°C in the light for 25–30 days was performed for callus and shoot regeneration. The shoots (2–3 cm tall) were transferred to MS-medium containing 100 mg l−1 kanamycin and 50 mg l−1 meropenem (Sumitomo Dainippon Pharma, Osaka, Japan) at 25°C in the dark for one day. Subsequent co-culturing on fresh solid MS-medium containing 1 mg l−1 BAP, 0.1 mg l−1 IAA, 100 mg l−1 kanamycin (FUJIFILM Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 50 mg l−1 meropenem (Sumitomo Dainippon Pharma, Osaka, Japan) at 25°C in the light for 25–30 days was performed for callus and shoot regeneration. The shoots (2–3 cm tall) were transferred to MS-medium containing 100 mg l−1 kanamycin and 50 mg l−1 meropenem for root regeneration. After root regeneration, the transgenic plants were transferred to soil (Sukoyaka-ZERO-1N (Dosaka EM Co., Ltd., Kyoto, Japan) into 400 µm-thick sections. The sections were then stained with 0.25% (w/v) toluidine blue O (Waldeck GmbH & Co KG, Münster, Germany) at room temperature for 10 s, washed with water, and observed under a BX53 upright microscope (Olympus, Tokyo, Japan). A tangent line touching the boundary between the first and the second layers was drawn to measure the cell length, and a rectangle bounding the cell was made based on this tangent line. The height of the rectangle was measured as the cell height and the width as the cell width using ImageJ software (https://imagej.nih.gov/ij; Schneider et al. 2012).

Host induced gene silencing (HIGS)

C. campestris was parasitized to the transgenic N. tabacum (first host), which expressed amiRNA targeting CcJKD. After successful parasitization, C. campestris formed lateral branches. These lateral branches were allowed to grow for approximately 1 week. Wild type A. thaliana was parasitized by the lateral branches of C. campestris without excising from the first host. C. campestris stems from the interface region occurred at 24 and 36 haa. N. tabacum leaves and C. campestris stems approximately 5 cm from the parasitic interface were harvested to check for miRNA production.

Stem-loop PCR

The presence of miRNA generated from amiRNA was confirmed using stem-loop PCR (Marcial-Quino et al. 2016). The primers used for the stem-loop PCR are shown in Supplementary Table S1.

Results

Outgrowth of holdfast epidermal cells during the early attachment stage

By re-analyzing the previously published transcriptome data of the parasitic interface between Cuscuta japonica and Glycine max (Ikeue et al. 2015), we found a group of genes showing high expression levels during the early parasitic stages, i.e., 24 and 48 haa, that contained epidermal cell-patterning genes of C. japonica, including homologs of JKD, GL2, and RHD3 (Supplementary Figure S1). We hypothesized that epidermal cell-patterning genes were involved in forming holdfasts, which was tested using another Cuscuta species, C. campestris, because its entire genome has been sequenced (Vogel et al. 2018). We first obtained cDNAs of genes encoding constituents of the trimeric Myb-BHLH-WD40-complex, including CcWER, CcCPC, CcGL3, and CcTTG1, based on sequence similarity. We also obtained upstream and downstream factors of the

Figure 1. Appearance of a holdfast and the epidermal cells of the Cuscuta campestris stem. All panels show paraffin-embedded sections stained with toluidine blue. (A) Longitudinal section (with respect to the C. campestris stem) of parasitic interface tissue at 36 h after attachment (haa). At, Arabidopsis thaliana; Cc, Cuscuta campestris; hf, holdfast; ph, prehaustorium. Scale bar, 500 µm. (B) Longitudinal section of the stem epidermis of C. campestris at 24 haa. ep, epidermal cells. Scale bar, 20 µm. The ep did not initiate outgrowth. (C) Longitudinal section of the stem epidermis of C. campestris at 36 haa. ep, epidermal cells. Scale bar, 20 µm. The ep were outgrowing. Holdfast development began between 24 and 36 haa.

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trimeric Myb-bHLH-WD40-dependent regulatory pathway, with \( CcJKD \) as an upstream factor and \( CcGL2 \) as a downstream factor. Despite our intensive search, homologs of \( GL1 \) or \( TRIPTYCHON (TRY) \) were not found in the \( C. campestris \) genome.

A holdfast is a pad-like attachment structure that develops on the stem surface in contact with the host (Figure 1A). We first determined the time at which the development of the holdfast initiated. At 24 haa, the stem surface cells contacting the host stem did not show outgrowth (Figure 1B). At 36 haa, the development of the pad-like structure of the holdfast was evident, and a group of cells on the contacting surface showed outgrowth toward the host surface (Figure 1C). Thus, we concluded that holdfast development was initiated between 24 and 36 haa under our growth conditions. Nearly all the holdfast epidermal cells contacting the host stem showed outgrowth and did not show the alternately arranged positional pattern that is seen in the leaf trichome and root hairs.

Expression of epidermal cell-patterning genes coincided with the onset of outgrowth of the holdfast epidermal cells

To examine whether the epidermal cell-patterning genes of \( C. campestris \) were involved in the outgrowth of the holdfast epidermal cells, we investigated the expression profiles of the genes in the parasitic interface at 0, 12, 24, 36, and 48 haa. \( CcJKD \) showed a rapid increase after attachment and reached its maximum level at 12 haa, and then decreased (Figure 2A). \( CcGL3, CcTTG1, CcWER, \) and \( CcGL2 \) reached maximum levels at 24 haa, and then decreased (Figure 2B–D, F). The expression profile of \( CcCPC \) was different from those of the other genes, which did not show significant changes during the period (Figure 2E). These results demonstrated that most of the epidermal cell-patterning genes showed high expression transiently before the onset of the outgrowth of the holdfast epidermal cells, except for \( CcCPC \).

\( CcJKD \) and \( CcGL2 \) were expressed in the holdfast epidermal cells

To test whether \( C. campestris \) homologs showed tissue-specific expression patterns consistent with these previous results, we performed in situ hybridization to localize the mRNAs of \( CcGL2 \) and \( CcJKD \). At 24 haa, before the onset of outgrowth of the holdfast epidermal cells, \( CcGL2 \) was detected in the stem epidermal cells on the host-contacting side (Figure 3A, B). At 36 haa, when the outgrowth of the holdfast epidermal cells was ongoing, \( CcGL2 \) was detected in the holdfast epidermal cells (Figure 3C, D). At 24 haa, \( CcJKD \) was detected in the stem cortex (Figure 3E, F). At 36 haa, \( CcJKD \) was detected in the holdfast epidermal cells, but not in the cortex (Figure 3G, H). Therefore, although mRNAs of \( CcGL2 \) and \( CcJKD \) were localized in different tissues before the onset of the outgrowth, they were localized in the same epidermal cells during the outgrowth of the holdfast epidermal cells.

![Figure 2. Expression profiles of epidermal cell-patterning genes during the early parasitic stage.](image-url)
Holdfast development needs epidermal cell-patterning genes

CcJKD protein interacted with CcTTG1 and CcCPC

The results of in situ hybridization suggested that CcJKD protein was present in holdfast epidermal cells with CcGL2, which tempted us to examine protein–protein interaction between CcJKD and CcGL2. We tested the interaction of CcJKD with CcGL2 using a protein complex immunoprecipitation assay. We co-expressed AD-HA-CcGL2 and BD-Myc-CcJKD in the same yeast cell and captured AD-HA-CcGL2 to determine whether BD-Myc-CcJKD co-precipitated with HA-CcGL2. BD-Myc-CcJKD was absent in the CcGL2-captured fraction (Supplementary Figure S2), suggesting that CcJKD did not interact with CcGL2. We next tested the interaction of CcJKD protein with other epidermal cell-patterning proteins using yeast two-hybrid (Y2H) assay. Y2H successfully detected the positive-control interaction between CcGL3 and CcTTG1 as expected from the previous study using the homologous proteins of *A. thaliana* (Payne et al. 2000). Pair-wise interactions of CcJKD with CcTTG1 and CcCPC were detected (Figure 4). We have to mention that several pair-wise interactions between *C. campestris* epidermal cell-patterning protein remained elusive, because CcJKD, CcGL2, CcWER fused to the binding domain exhibited autoactivation of reporter genes in Y2H assay.

**Silencing of CcJKD repressed the expression of other epidermal cell-patterning genes except for CcCPC**

To investigate the role of CcJKD expressed in the epidermal cells, we attempted to silence CcJKD using host-induced gene silencing (HIGS) approach (Nowara et al. 2010). Previously, this approach successfully silenced the *Cuscuta* gene expressed in haustorium (Alakonya et al. 2012). We first established an *N. tabacum* transformant that produced an amiRNA trigger targeting CcJKD under the CaMV 35S promoter. To the *N. tabacum* plant, *C. campestris* established an initial parasitic connection, which allowed the transfer of miRNA to *C. campestris* (Figure 5A). To test the effect of silencing, *C. campestris* stem that was elongated from the initial parasitic connections was parasitized to the inflorescent stems of *A. thaliana* (Figure 5A). The miRNA production from the amiRNA construct was confirmed using stem-loop PCR (Marcial-Quino et al. 2016). In *N. tabacum* leaves and the elongating stem of *C. campestris*, PCR products of 75 bp indicated the presence of a 21-mer miRNA produced from the amiRNA transgene (Figure 5B). At both 24 and 36 haa on the second host *A. thaliana*, the expression levels of the target CcJKD were lower than those parasitized on the wild type *N. tabacum* (Figure 5C, D), indicating a silencing effect on the target gene. Expression levels of CcGL3, CcTTG1, CcWER, and CcGL2 also decreased (Figure 5C, D). However, the expression level of CcCPC was not significantly affected (Figure 5C, D). These results suggested that CcJKD controlled the expression of CcGL3, CcTTG1, CcWER, and CcGL2, either directly or indirectly, but not CcCPC.
Silencing of CcJKD repressed the outgrowth of the holdfast epidermal cells, but did not decrease the frequency of parasitization

To estimate the effect of CcJKD-silencing on holdfast formation, we observed cell morphology in the epidermal layer of the holdfast on the second host. C. campestris was peeled off from the host stem at 24 haa and grown another 12 h without a host to release from the compression caused by coiling. The length and width of the holdfast epidermal cells were measured using image analysis software. The cell length was significantly shorter in the CcJKD-silenced C. campestris than in the non-silenced C. campestris (Figure 6A). However, the cell width was not significantly different (Figure 6B). We further evaluated the effect of CcJKD-silencing on the establishment of parasitism by estimating the percentage of establishment of parasitic connections, which was shown as further elongation of the stem. The percentage of parasitism of the CcJKD-silenced C. campestris was not significantly different from that of the non-silenced C. campestris (Figure 6C). Therefore, the silencing of CcJKD repressed the processes leading to the outgrowth of the holdfast epidermal cells, but the extent of the repressive effect was not enough to inhibit the establishment of parasitism.

Discussion

In the present study, we analyzed the roles of epidermal cell-patterning genes of C. campestris in the formation of parasitic connections.
Holdfast development needs epidermal cell-patterning genes

of a holdfast. We prioritized *C. campestris* homologs of the epidermal cell-patterning genes based on sequence similarity to *A. thaliana*. Homologs of *JKD, GL3, TTG1, WER, CPC*, and *GL2* were found; however, homologs of *GL1* and *TRIPTYCHON (TRY)* were not found. The TRY protein, a single-repeat R3 Myb protein, which is known to interact with GL3, disrupts the interaction between GL3 and GL1 and then negatively regulates trichome initiation (Esch et al. 2003; Schellmann and Hulskamp 2005). Thus, the functional role of canonical GL1 and TRY in trichome development is substituted by other Myb-proteins during holdfast development in *C. campestris*. Alternatively, *C. campestris* lacks functionally equivalent genes of *GL1* and TRY, which is consistent with the absence of trichomes on the stem surface (Figure 1A, Supplementary Figure S3).

As the first step toward elucidating the regulatory mechanism of the initiation of holdfast development, we tested whether epidermal cell-patterning genes of *C. campestris* were associated with the outgrowth of holdfast epidermal cells. Upregulation of the expression of epidermal cell-patterning genes slightly preceded the outgrowth of the holdfast epidermal cells (Figure 2A–D, F), suggesting that epidermal cell-patterning genes of *C. campestris* might be involved during the initiation of the outgrowth of holdfast epidermal cells. To provide further support for the involvement of epidermal cell-patterning genes in the outgrowth of holdfast epidermal cells, localization of *CcGL2* and *CcJKD* mRNAs were examined. At 24 haa, *CcGL2* was localized in the stem epidermis, while *CcJKD* was localized in the stem cortex before the onset of the outgrowth (Figure 3A–D). At 36 haa, during the outgrowth, both *CcGL2* and *CcJKD* were localized in the holdfast epidermal cells (Figure 3E–H). The change of the tissues in which *CcJKD* mRNA was localized could be explained by some putative mechanisms, including alternative use of tissue specific *cis*-elements, chromatin remodeling, and movement of mRNA from the cortex to the epidermis. Cell-to-cell movement of *A. thaliana* *JKD* mRNA was shown in the previous grafting study (Thieme et al. 2015). However, experimental proof of these models was left to future study. Although localization of mRNAs of *CcWER, CcCPC, CcGL3*, and *CcTTG1* were not experimentally demonstrated in this study, they are likely localized in the epidermal cells as shown for the homologs of *A. thaliana* (Bernhardt et al. 2005; Rerie et al. 1994; Ryu et al. 2005; Tominaga-Wada et al. 2017; Wada et al. 2002; Zhao et al. 2008).

Gene expression profiles and localization of mRNAs raised two questions. First, does *CcJKD* have a regulatory relationship with other epidermal cell-patterning genes? Second, does *CcGL2* activate or repress the outgrowth of holdfast epidermal cells? To answer the first question, silencing of *CcJKD* was performed using the HIGS approach (Figure 5A). Silencing of *CcJKD* repressed the expression of *CcGL3*, *CcTTG1*, *CcWER*, and *CcGL2* (Figure 5C, D). Therefore, *CcJKD* was responsible for the transcriptional regulation of *CcGL3*, *CcTTG1*, *CcWER*, and *CcGL2*, although we did not clarify whether the regulation was direct or indirect. To answer the second question, morphological changes in the holdfast epidermal cells were analyzed using *CcJKD*-silenced *C. campestris* in which *CcGL2* expression was repressed. The cell length of the papillar-shaped holdfast epidermal cells became shorter in *CcJKD*-silenced *C. campestris*, indicating that cell outgrowth was repressed (Figure 6A). Cell outgrowth should be controlled by genes in the downstream of *GL2*, as shown in the trichome and root hairs (Masucci and Schiefelbein 1994; Yi et al. 2010); therefore, *CcGL2* is an activator of holdfast outgrowth.

*CcCPC* showed nearly constant expression levels during the holdfast initiation stage (Figure 2E) and was not affected by *CcJKD*-silencing (Figure 5C, D); therefore, it is likely that relatively constant expression of *CcCPC* lead to constitutive formation of CPC-GL3-TTG1 complex in *C. campestris* stem epidermis, as reported for their homologous proteins in *A. thaliana* root epidermal cells, which represses the expression of *GL2* (Figure 7; Song et al. 2011; Wada et al. 2002). This hypothesis is consistent with the absence of trichomes on the *C. campestris* stem surface (Supplementary Figure S3). When *C. campestris* attaches to host stem, *CcJKD* expression is induced, which directly or indirectly upregulated the expression of *CcWER, CcGL3*, and *CcTTG1* (Figure 5C, D). Upregulation of these genes subsequently promoted the formation of WER-GL3-TTG1 complex, as reported for their homologous proteins in *A. thaliana*, and probably activates *GL2* expression (Figure 7; Ryu et al. 2005; Song et al. 2011).

![Figure 7. Model of the regulatory network of epidermal cell-patterning genes in holdfast development. Solid open arrow, activation of transcription; blunt-end arrow, repression of transcription; solid line, protein–protein interaction demonstrated in this study; broken line, protein–protein interaction demonstrated in Arabidopsis thaliana.](image)
Presence of CcJKD mRNA in the holdfast epidermal cells suggested the co-localization and the occurrence of protein–protein interaction of CcJKD with the other epidermal cell-patterning proteins. We hypothesize that interactions of CcJKD with CcTTG1 and CcCPC (Figure 4) could have repressive effect on the formation of CPC–GL3–TTG1 complex, which consequently upregulates CcGL2. It is also likely that CcJKD is present in the same cells with CcWER. In the A. thaliana roots, JKD expressed in the cortex negatively controls the function of WER protein by non-cell-autonomous signaling via the SCRAMBLED receptor protein (Hassan et al. 2010). In C. campestris, unlike A. thaliana, CcJKD might regulates CcWER in a cell-autonomous manner.

CcJKD-silencing repressed CcGL2 and also repressed outgrowth of holfast epidermal cells (Figure 6A) but did not inhibit the parasitism (Figure 6C), suggesting that production of molecules necessary for parasitism, e.g., secreted adhesive molecules, was not repressed by CcJKD-silencing. To understand the direct and indirect consequences of CcGL2 activation, research regarding the establishment of silencing trigger tobacco plants that target CcGL2 is ongoing. Testing whether CcGL2 repression in the CcJKD-silenced C. campestris is related to the direct binding of the CcJKD protein to the CcGL2 promoter is also underway. Identification of regulatory network of holfasts, which are unique to stem parasitic plants, will provide new insights into the diverse roles of epidermal cell-patterning genes to establish various attachment structures.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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