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論　文　名	Epidermal cell-patterning genes of a stem parasitic plant *Cuscuta campestris*, are involved in the holdfast development (茎寄生植物アメリカネナシカズラの表皮細胞パターン形成遺伝子群は付着器の発生に関与する)
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論文要旨

Cuscuta campestris, a stem parasitic plant, is a holoparasite that does not perform photosynthesis and takes up water and nutrient from host plant. Upon contacting 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 host plant. *C. campestris* forms a specialized disc-like adhesive structure called holdfast which helps parasitic stem to stay in contact with the host stem during haustorium penetration. Development of holdfast is always associated with the outgrowth of papillar epidermal cells. The morphology of epidermal cells in the holdfast is very much similar to leaf trichome and root hair of dicotyledonous plants. However, regulatory network underlying the development of holdfast has not been elucidated yet. In this study, we assessed the roles of epidermal cell-patterning genes on the development of holdfast.

Chapter 1: Holdfast cell outgrowth and the expression of epidermal cell-patterning genes occurs in the early stage of attachment of parasitization

Holdfast is a pad-like attachment structure that develops on the stem surface, contacting to host. In this study, the time of initiation of the holdfast developments was determined. Development of the pad-like structure of holdfast and a group of cells on the contacting surface showed outgrowth toward the host surface between 24 and 36 hours after attachment (haa) in our growth condition. The analysis of transcriptome data of parasitic interface between *Cuscuta japonica* and *Glycine max* confirmed that a group of genes that showed high expression levels in the early parasitic stages such as 24 and 48 haa contained epidermal cell-patterning genes of *C. japonica*, such as homologs of *JACKDAW* (*JKD*), *GLABRA2* (*GL2*) and *ROOT HAIR DEFECTIVE 3*(*RHD3*). To examine whether the epidermal cell-patterning genes of *Cuscuta campestris* are involved in the outgrowth of holdfast epidermal cells or not, the expression profiles of the genes in the parasitic interface at 0, 12, 24, 36, and 48 haa was analyzed. *CcJKD* showed a rapid increase after attachment, reached the maximum level at 12 haa, and then decreased. *C. campestris*' homolog of *GLABRA3*(*GL3*), *TRANSPARENT TESTA GLABRA1* (*TTG1*), *WEREWOLF* (*WER*), and *GL2* reached the maximum levels at 24 haa, and then decreased. The expression profile of *C. campestris*' homolog of *CAPRICE* (*CPC*) did not show significant change during the period. These results demonstrated that most of the epidermal cell-patterning genes showed high expression transiently before the onset of the outgrowth of holdfast epidermal cells, except *CcCPC*.

Chapter 2: Interaction pattern of transcription factors encoded by epidermal cell-patterning genes

GL3, TTG1, WER, CPC and *GL2* have been localized to epidermal cells previously whereas *JKD* was localized in quiescent center and cortex. To test whether *C. campestris* homologs show tissue-specific expression patterns consistent with previous results in or not, *in situ* hybridization to localize transcripts of *CcGL2* and *CcJKD* was performed. At 36 haa, when the outgrowth of holdfast epidermal cells was ongoing, unexpectedly *CcGL2* and *CcJKD* were detected in the epidermal cells of the holdfast. These results suggested that *CcJKD* and *CcGL2* were expressed in the same cells in the epidermis of the holdfast when the holdfast epidermal cells exhibit outgrowth. To test whether *CcGL3, CcTTG1, CcWER* and *CcCPC* expressed in parasitic interface are functionally equivalent to those expressed in leaf or root epidermal cells of other plants, the formation of Myb-bHLH-WD40 complex was assessed. By using yeast two hybrid (Y2H) assay, pair-wise interactions between CcGL3 and CcTTG1, CcGL3 and CcCPC, CcTTG1 and CcCPC, and CcWER and CcCPC were detected. CcWER did not interact with CcTTG1. These results indicate that CcCPC-CcGL3-CcTTG1 complexes can be formed in the parasitic interface. The interaction of CcJKD with CcTTG1 and with CcCPC was also confirmed by Y2H assay. In this experimental method CcGL2 interacts with CcTTG1 and with CcCPC. Interaction of CcJKD with CcGL2 and CcGL2 with CcWER were assessed by protein complex immunoprecipitation assay, because CcJKD, CcGL2 and CcWER fused to binding domain exhibited autoactivation of reporter genes. A proof-of-concept experiment by using AD-HA-CcTTG1 and BD-Myc-CcCPC showed that the assay can capture interaction between proteins successfully. Pairs of proteins which are AD-HA-CcGL2 and BD-Myc-CcJKD were co-expressed in the same yeast cell, and AD-HA-CcGL2 by anti-HA-tag mAB-magnetic agarose. BD-Myc-CcJKD were absent in the respective CcGL2-captured fraction.

Chapter 3: *CcJKD* regulates the epidermal cell-patterning genes and holdfast cell outgrowth but does not control establishment of successful parasitization

The unexpected *CcJKD* mRNA expression in the same epidermis of the holdfast with *CcGL2* raises curiosity about their protein interaction profile, done by Y2H and immunoprecipitation assay. The formation of the Myb-bHLH-WD40 complex in the *Cuscuta* holdfast epidermis like *Arabidopsis* root epidermis provides possibility of similar regulatory interaction in root hair trichome formation. On the other hand, the cloned transcription factors' unusual interacting ability opens the possibility of different additional functions of those proteins. Previous transcriptome analyses revealed that *Cuscuta*

campestris genome lacs homologs of *GL1* or *TRIPTYCHON* (*TRY*) are involved in the trichome formation regulatory network. JKD is the upstream regulator of *the Arabidopsis thaliana* root epidermis network, and CcJKD showed few conserved findings and new. It was a logical, sequential approach to find out the role of *CcJKD*. To investigate the role of *CcJKD* expressed in the epidermal cells, *CcJKD* was silenced utilizing a host induced gene silencing (HIGS) approach. Both at 24 and 36 haa, on the second host *A. thaliana*, expression levels of the target *CcJKD* were decreased compared to those parasitized on wild type *N. tabacum*, indicating the silencing effect on the target gene. Expression levels of *CcGL3*, *CcTTG1*, *CcWER*, and *CcGL2* were also decreased except for *CcCPC*. These results suggested that *CcJKD* controls expression of *CcGL3*, *CcTTG1*, *CcWER*, and *CcGL2*, either directly or indirectly, but not *CcCPC*. Cell morphology in the epidermal layer of the holdfast on the second host was observed. Cell length were significantly shorter in *CcJKD*-silenced *C. campestris* than in the non-silenced *C. campestris*, whereas cell width was not significantly different. We further evaluated the effect of *CcJKD*-silencing on the establishment of parasitism by estimating the percentage of parasitic connection. Percentage of the establishment of parasitism by *CcJKD*-silenced *C. campestris* was not significantly different from that by non-silenced *C. campestris*. This indicates that silencing of *CcJKD* represses the processes leading to outgrowth of holdfast epidermal cells, but the extent of repressive effect was not enough to inhibit the establishment of parasitism.

From current experimental model, epidermal cell patterning genes are found to be involved in the holdfast developments in early stage of parasitization. Moreover, *CcJKD* acts as the regulator of this model regulatory network since expression of *CcJKD* in the same epidermal cell with other epidermal cell patterning genes in the early parasitic stage upregulates *CcWER*, *CcTTG1*, *CcGL3* and also activates downstream gene *CcGL2* and results in epidermal cell protrusion towards host. As *CcCPC* expression were not affected by *CcJKD* expression, in the non-parasitic stage relative abundance of CcCPC protein became higher than the abundance and CcJKD and CcWER proteins that leads to the increase the abundance of CcCPC-CcGL3-CcTTG1 complex, which is a repressor complex of *CcGL2* leads to repress outgrowth of holdfast epidermal cells. This model helps partially explaining the regulatory events of holdfast development in the early stage of parasitization and in future the ongoing *CcGL2* silencing method will helps to identify the down stream genes of *CcGL2* which may involve in the establishment of successful parasitization.

審査結果の要旨

寄生植物は、宿主植物から水や養分を吸収して自身が生長と繁殖をする植物である。宿主範囲が広い寄生植物も多く、農業的に重要な作物に大規模な寄生被害をもたらすこともあり、世界的に大きな問題となっている。寄生植物は、水分や養分を吸収するために宿主植物の維管束組織に自身の維管束組織を接続するが、この組織接続に先駆けて宿主植物表面への付着を行わなければならない。付着する際に、寄生植物は宿主植物に接触した部位の表皮に付着器を形成する。付着器は、宿主に接触している部分の細胞の変形、ならびに粘着物質の分泌、という二つの機能によって特徴づけられる。中でも接触部分での細胞の変形に関しては未知な点が多い。同じ特性は寄生植物のみならず蔓植物やよじ登り植物においても見られ、表皮にパッドやフックを持った付着器を形成するが、いずれの植物においても付着器形成の分子機構は明らかにされていない。そこで本研究では、寄生植物の付着器形成段階における表皮形態形成遺伝子群の解析を実施し、付着器に特徴的な表皮細胞形態がどのようにして形成されるかについて解明を試みた。

本研究では茎寄生植物アメリカネナシカズラ (*Cuscuta campestris*)と宿主植物シロイヌナズナ（*Arabidopsis thaliana*）からなる寄生系を用いた。アメリカネナシカズラの付着器では、表皮細胞が宿主植物方向に突出する特徴を持つ。第 1 章ではまず、アメリカネナシカズラがシロイヌナズナに接触した後、24 時間から 36 時間の間に付着器表皮細胞の突出伸長が開始されることを明らかにした。付着器形成開始の前後の時間帯において、モデル植物でトライコームと根毛における表皮細胞パターン形成への関与が知られている遺伝子群の相同遺伝子、すなわち *JACKDAW* (*CcJKD*)、 *GLABRA3* (*CcGL3*)、*TRANSPARENT TESTA GLABRA1* (*CcTTG1*)、*WEREWOLF* (*CcWER*)、*CAPRICE* (*CcCPC*)、*GLABRA2* (*CcGL2*) の発現パターンを調べてみると、*CcJKD* は接触後 12 時間で、*CcGL3*、*CcTTG1*、*CcWER*、*CcGL2* は接触後 24 時間で発現が上昇していた。*CcCPC* だけは発現レベルが変動しなかった。さらに *in situ* ハイブリダイゼーション法により、*CcJKD* と *CcGL2* が付着器表皮細胞で発現していることが明らかにされた。これらのことから、*CcCPC* を除く表皮細胞パターン形成遺伝子群の付着器形成への関与が示唆された。

第２章では、表皮細胞パターン形成遺伝子群がコードするタンパク質間の相互作用が、酵母ツーハイブリッド法と免疫共沈降法を用いて調べられた。この結果、付着器においてCcGL3-CcTTG1-CcCPC 複合体が形成されること、CcTTG1 と CcCPC は直接相互作用をすること、ならびに CcJKD が CcTTG1、CcCPC と相互作用することが明らかにされ、付着器表皮細胞内に表皮細胞パターン形成の中心となる三つの転写因子タンパク質からなる複合体が存在することが示唆された。

第３章では、付着器表皮形態形成において *CcJKD* が他の遺伝子の上流に位置すると仮

定し、Host-induced gene silencing 法で *CcJKD* の発現抑制を試み、その寄生への影響を評価した。*CcJKD* をサイレンシングすると *CcGL3*、*CcTTG1*、*CcWER*、*CcGL2* の発現レベルも低下した。*CcCPC* の発現には影響が見られなかった。よって *CcJKD* は *CcGL3*、*CcTTG1*、*CcWER*、*CcGL2* の上流に位置し、直接または間接的に発現を制御していると考えられた。*CcJKD* のサイレンシングは、付着器表皮細胞の突出伸長を抑制したため、*CcJKD*、*CcGL3*、*CcTTG1*、*CcWER*、*CcGL2* は付着器表皮細胞伸長を正に制御していると考えられた。しかしながら寄生率そのものは *CcJKD* のサイレンシングによって低下しなかったため、突出伸長抑制は寄生抑制のために十分ではないことが示された。

本研究では寄生植物の付着器表皮細胞形態が、表皮細胞パターン形成遺伝子群によって制御されることを初めて明らかにした。表皮細胞パターン形成遺伝子群がコードするタンパク質間の相互作用パターンは、モデル植物のトライコームや根毛内における相互作用パターンと共通しているものの、付着器表皮に特異的な相互作用パターンも認められた。本研究における表皮付着分子機構の成果は、植物生理・分子生物学ならびに植物保護学的な観点から高く評価できる。よって本論文の審査ならびに最終試験の結果と併せて博士（応用生命科学）の学位を授与することを適当と認める。