Local Auxin Biosynthesis Mediated by a YUCCA Flavin Monooxygenase Regulates Haustorium Development in the Parasitic Plant Phtheirospermum japonicum

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Parasitic plants in the Orobanchaceae cause serious agricultural problems worldwide. Parasitic plants develop a multicellular infectious organ called a haustorium after recognition of host-released signals. To understand the molecular events associated with host signal perception and haustorium development, we identified differentially regulated genes expressed during early haustorium development in the facultative parasite Phtheirospermum japonicum using a de novo assembled transcriptome and a customized microarray. Among the genes that were upregulated during early haustorium development, we identified YUC3, which encodes a functional YUCCA (YUC) flavin monooxygenase involved in auxin biosynthesis. YUC3 was specifically expressed in the epidermal cells around the host contact site at an early time point in haustorium formation. The spatio-temporal expression patterns of YUC3 coincided with those of the auxin response marker DR5, suggesting generation of auxin response maxima at the haustorium apex. Roots transformed with YUC3 knockdown constructs formed haustoria less frequently than nontransgenic roots. Moreover, ectopic expression of YUC3 at the root epidermal cells induced the formation of haustorium-like structures in transgenic P. japonicum roots. Our results suggest that expression of the auxin biosynthesis gene YUC3 at the epidermal cells near the contact site plays a pivotal role in haustorium formation in the root parasitic plant P. japonicum.

INTRODUCTION

Most plants in the Orobanchaceae have adopted a parasitic lifestyle. Facultative parasites maintain their capability for autotrophic lifestyle, but often parasitize when a host is nearby. In contrast, obligate parasites are not able to complete their lifecycle without a host, at least under natural conditions. Several obligate parasites in the Orobanchaceae, such as witchweeds (Striga) and broomrapes (Orobanche and Phelipanche), are harmful agricultural pests that infect important crops and severely affect host growth and yields by depriving their hosts of water and nutrients. Significant economic losses place the parasitic plant Striga hermonthica as one of the most serious threats to food security (Pennisi, 2010). Fields infested by Striga species affect the agricultural income of 25 countries, causing billions of USD in damage yearly (reviewed in Spallek et al., 2013).

A common characteristic of parasitic plants is the formation of haustoria, i.e., multicellular organs that attach to and penetrate the host tissues. During the early stage of parasitization, the haustorium serves as the organ of penetration, establishing physical connections between parasite and host tissues. During later developmental stages, vascular cells develop in the haustorium, which absorb water and carbohydrates from the host (reviewed in Yoshida and Shirasu, 2012). Host-derived chemicals are able to induce the formation of haustoria in vitro; these chemicals are called haustorium-inducing factors (HIFs) (Lynn and Chang, 1990). Among these HIFs, 2,6-dimethoxy-p-benzoquinone (DMBQ) was originally identified from host root extracts and was shown to activate haustorium formation in parasite roots (Chang and Lynn, 1986; Smith et al., 1990). Although haustoria can be observed in...
different parts of the root, the area between the distal elongation zone and the meristematic root tip is the most sensitive to HIFs (Cui et al., 2016; Baird and Riopel, 1984). Typically, this area becomes swollen and forms a haustorium – 24 h after DMBQ treatment (Albrecht et al., 1999; Ishida et al., 2011).

The morphological changes that occur during haustorium development have been described histologically in several parasitic plant species (Baird and Riopel, 1984; Ishida et al., 2011; Riopel and Musselman, 1979; Jamison and Yoder, 2001; Cui et al., 2016). In the facultative parasite Agalinis purpurea, cortex cells slightly expand radially 6 h after exposure to HIF. By 10 h, epidermal cells start dividing and rapidly differentiate into haustorial hairs. Between 24 and 36 h, the root continues to swell due to cell division and enlargement to form the haustorial structure. Similar morphological events were observed in other facultative parasitic plants in the Orobanchaceae, such as Triphysaria versicolor (Jamison and Yoder, 2001) and Phtheirospermum japonicum (Cui et al., 2016; Ishida et al., 2011), suggesting a conserved mechanism for haustorium induction in this plant family.

The molecular mechanism triggering the initiation of haustorium development is poorly understood. Redox signaling was suggested to play an essential role in this process. In addition to DMBQ, a range of quinones and flavonoids that have certain redox potentials are able to induce haustorium formation in vitro in T. versicolor (Matvienko et al., 2001b; Albrecht et al., 1999). A single electron-transfering quinone-reductase enzyme in T. versicolor (QR1) was found to play an important role in haustorium induction probably, via catalyzing the reduction of DMBQ-related quinones (Bandaranayake et al., 2010). In addition, Pirin, a nuclear protein associated with transcription factor activity, was reported to play regulatory roles in haustorium formation in T. versicolor (Bandaranayake et al., 2012). Accumulating information about the genes expressed in the parasitic Orobanchaceae, including ESTs of S. hermonthica (Yoshida et al., 2010), Phelipanche aegyptiaca, and T. versicolor (http://ppgp.huck.psu.edu) (Yang et al., 2014; Torres et al., 2005), has contributed to our knowledge of the molecular basis of plant parasitism. Recent significant advances in this field include the identification of core parasitism genes through the sequencing of different tissues of three parasitic species in Orobanchaceae (Yang et al., 2014). However, our understanding of early haustorium development is still limited.

Early events in haustorium development involve hormonal accumulation and regulation. Haustorial tissues of the parasite Santalum album accumulate newly synthesized endogenous hormones, associating high auxin-to-cytokinin ratios with haustorium development (Zhang et al., 2012). Indeed, disruption of the auxin concentration gradients, either through the application of auxin transport/auxin activity inhibitors or by providing an excess amount of exogenous auxins, results in a reduction in infection frequency in the nonchlorophilic holoparasite P. aegyptiaca (Bar-Nun et al., 2008). Similar studies in the facultative parasitic plant T. versicolor have shown that disturbing auxin flow either by application of an auxin efflux inhibitor (2,3,5-triiodobenzoic acid) or auxin activity inhibitors (p-chlorophenoxyisobutyric acid) reduces the number of haustorium (Tomilov et al., 2005). Furthermore, T. versicolor roots transformed with ethylene- or auxin-inducible promoter-reporter constructs showed a positive response after DMBQ treatment, suggesting these hormones accumulate near the root tip (Tomilov et al., 2005). Auxin distribution in planta determines the formation patterns of organs such as leaves, flowers, and lateral roots (Reinhardt et al., 2000; Benková et al., 2003). Auxin gradients in tissues are largely modulated by the intercellular auxin transporters PIN proteins and AUX/LAX proteins (reviewed in Adamowski and Friml, 2015; Swarup and Péret, 2012). In addition, de novo biosynthesis of auxin at specific tissues significantly contributes to plant development (Zhao, 2010). The main pathway of auxin biosynthesis involves two steps. The TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS family of amino transferases first converts tryptophan to indole-3-pyruvic acid (Kasahara, 2016). Subsequently, the YUCCA (YUC) family of flavin monooxygenases catalyzes the formation of indole-3-acetic acid (IAA) (Masihuchi et al., 2011; Zhao, 2012). The rate-limiting step for IAA production is regulated by YUC (Zhao et al., 2001). There are 11 YUC gene family members in the Arabidopsis thaliana genome and 14 in rice (Oryza sativa) (Yamamoto et al., 2007; Fujino et al., 2008). Disruption of a single YUC gene in Arabidopsis did not cause an apparent phenotype, whereas triple or quadruple yuc mutants show severe defects in various developmental processes, suggesting that spatially and temporally regulated auxin biosynthesis by multiple YUC genes is essential for plant development (Cheng et al., 2006).

In this study, we examined the facultative parasitic plant P. japonicum to identify differentially regulated genes during the initial stages of haustorium development. Since a transformation system was first established for P. japonicum (Ishida et al., 2011), this plant has served as an excellent model for parasitic plants. Our sequencing and microarray analyses identified 327 differentially expressed genes after DMBQ treatment, providing an overview of expression profile changes during early haustorium development. Among the differentially expressed genes, we focused on a highly upregulated gene, YUC3, encoding a member of the YUCCA flavin monooxygenase family that regulates auxin biosynthesis. Our functional studies indicate that YUC3 is a key regulator of haustorium formation in P. japonicum.

RESULTS

High-Throughput Sequencing and de Novo Assembly of the P. japonicum Root Transcriptome

To prepare for large-scale transcriptome analysis of the facultative parasite P. japonicum, we first tested its host specificity. P. japonicum is an annual herbaceous plant that grows to be 10 to 30 cm tall, with a generation time of 2 to 3 months (Figure 1A). P. japonicum is a generalist that can parasitize a number of species including rice, Arabidopsis, maize (Zea mays), and cowpea (Vigna unguiculata), but not Lotus japonicus (Figure 1; Supplemental Figure 1). We chose rice and Arabidopsis for further experiments because the complete genome sequences and molecular genetic tools are available for both species. We used rice for the de novo transcriptome analysis because P. japonicum haustoria from infected rice are larger than those from infected Arabidopsis, which increases the ease of sampling. Parasites with or without host were incubated in a rhizotron chamber for 4 to 6 weeks (Supplemental Figure 2). Parasites form lateral haustoria with host...
plants during various developmental stages. The newly formed
P. japonicum roots frequently had immature haustoria (Figures 1F
and 1G), in which the connection to the host vascular system was
not yet established (Figure 1G). A haustorium in older roots was
firmly attached to the host tissues (Figures 1H and 1), forming
vascular continuity with the host (Figure 1I). To sequence samples
from distinct parasitic stages, a pool of haustoria at differ-
ent stages was carefully collected. When a haustorium was
tightly attached to the host root, the attached host tissues were
also sampled. As a control, parasite roots without host plants were
similarly sampled.

To perform de novo assembly of the P. japonicum transcriptome,
two distinct sequencing platforms were adopted: the HiSeq
2000 (illumina) and the GS-FLX pyrosequencer with FLX titanium
reagents (454 Life Sciences). Total RNAs were extracted from
autotrophic and parasitic stages of P. japonicum and respective
Illumina libraries were generated. For 454 sequencing, a nor-
malized library was constructed with mRNAs isolated from each
sample and mixed in equal proportions. Approximately 24 million
paired-end reads with a total length of ~4.5 Gb were generated
by the HiSeq 2000, whereas 888,638 reads with an average
length of 333.8 bp and a total length of 296 Mb were generated by
the 454 sequencer (Supplemental Table 1). The reads from
parasitic tissues were mapped against the available full-length
rice transcripts to remove reads derived from host tissues. The
unmapped reads and the reads from the autotrophic stage
libraries were then assembled. The assembly yielded 42 Mb
distributed in 58,137 unigenes (nonredundant sequences;
(P. japonicum haustoria showed similar developmental sequences
in response to DMBQ or host root exudate treatment (Figures 2A
and 2B). The morphological changes in the roots were not ap-
parent until 6 h after treatments, and initiation of haustorial hair
proliferation was recognized at 12 h after treatments. Haustorial
hair proliferation and root swelling became more apparent at 24 h
after treatments (Figures 2A and 2B; Supplemental Movie 1). We
chose eight time points across 48 h (0, 0.5, 1, 3, 6, 12, 24, and 48 h)
for the microarray analysis to focus on the transcriptional changes
that occur prior to morphological changes. We detected 327 dif-
ferentially expressed genes that showed 2-fold higher or lower
expression compared with 0 h samples (Supplemental Data Set 2).
To validate the microarray results, we performed RT-qPCR
analyses of several selected genes. For an internal control to
normalize the expression values, the P. japonicum gene encoding
the RNA binding polyuridine tract binding protein (PTB)
was selected because of its homology to Arabidopsis housekeeping
gene At3g01150 (Czechowski et al., 2005) and its stable
expression levels across all eight time points in the microarray
(Supplemental Data Set 3). Expression profiles of selected genes
generated with RT-qPCR showed similar patterns to those ob-
tained from microarray analysis (Supplemental Figure 3, left
panels), verifying the reproducibility of the microarray data.

Because chemical HIFs and host root exudate can induce the
expression of different sets of genes as reported previously
(Bandaranayake et al., 2010), we investigated the expression
profiles of the DMBQ-induced P. japonicum genes after appli-
cation of rice root exudate (Supplemental Figure 3, right
panels). As shown in Supplemental Figure 4 and Supplemental Movie 1,
rice root exudate initiates haustoria formation in P. japonicum
roots. The tested gene set showed similar gene expression pat-
tterns between DMBQ-treated and host root exudate-treated roots
(Supplemental Figure 3), confirming that the gene expression
profiles in the DMBQ-treated samples most likely reflects gene
expression during early haustorium development.

Functional Classification of P. japonicum Genes with Altered
Expression Patterns in Response to the Haustoria-Inducing
Factor DMBQ

To obtain an overview of the expression profiles of the P. japonicum
transcriptome during haustorium formation, we grouped DMBQ-
regulated genes with similar expression profiles (Olex and Fetrow,
Among the 327 differentially expressed genes, 238 were divided into 38 clusters with two or more members (Supplemental Data Set 2 and Supplemental Figures 5 to 7). For each cluster, the average gene expression values (fold changes against the control sample) was calculated and displayed as a heat map (Figure 2C). The expression of 21 clusters comprising 157 unigenes peaked at very early time points (up to 3 h; Supplemental Figure 5), while the expression of 14 clusters with 73 genes peaked after 3 h (Supplemental Figure 6). Three clusters contained eight genes that were negatively modulated along the time course (Supplemental Figure 7).

Notably, the expression of genes in Cluster 15 peaked at 3 h after DMBQ treatment and showed one of the highest (log₂FC >6) increases in expression among all clusters (Figure 2C; Supplemental Figure 5). This cluster includes homologs of Tv-Pirin and Tv-QR2 (Quinone reductase2), which are expressed in response to DMBQ.
Figure 2. Changes in Root Morphology and Gene Expression in Response to DMBQ Treatment.

(A) Morphological changes in *P. japonicum* roots exposed to 10 μM DMBQ.

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in *T. versicolor* (Bandaranayake et al., 2012; Matvienko et al., 2001b), confirming the conservation of gene expression profiles among different Orobancheaeae species. Interestingly, the closest homolog of *Tv-QR1*, which functions in haustorium formation in *T. versicolor*, was not induced by DMBQ in *P. japonicum* (Supplemental Figure 8).

To investigate functional shifts in the gene population, Gene Ontology (GO) terms were assigned for each gene based on the best BLAST-hit annotations. The overrepresented GO terms were identified in the top five most abundant clusters, which cover 102 (31%) differentially expressed genes (Figures 2D and 2E). Cluster 1 contained 33 early-responsive genes with a peak of expression at 1 h, and the GO terms calmodulin binding and oxidoreductase activity were overrepresented (Figure 2E). Similarly, Clusters 3 and 4 also contained early-responsive genes whose expression peaks at 30 min after DMBQ treatment, followed by a rapid decrease in expression levels (Figure 2D). Although no particular GO term was overrepresented for Cluster 4, the term immune system process was significantly enriched for Cluster 3 (Figure 2E), including genes annotated as WRKY transcription factor family genes; members of this family are often associated with responses to abiotic and biotic stresses (Eulgem et al., 2000). Thus, DMBQ treatment may have provoked stress responses in parasitic plants. In contrast, Cluster 2 contained 24 unigenes whose expression was rapidly repressed after DMBQ treatment. The GO terms cell wall organization and membrane were overrepresented in this cluster (Figure 2E), probably because root elongation was halted upon DMBQ treatment (Riopel and Timko, 1995).

Cluster 5 consisted of late-responsive genes, with high expression levels observed at 6 h and sustained beyond 6 h (Figure 2D). The overrepresented GO terms in this cluster were oxidoreductase activity, peroxidase activity, response to external stimulus, response to wounding, and hormone metabolic pathway (Figure 2E). In particular, for hormone metabolic pathway, we found genes encoding CYTOKININ OXIDASES, which catalyzes the degradation of cytokinin (Bartrina et al., 2011), as well as YUCCA and GH3 (Supplemental Data Set 2), which are involved in auxin metabolism (Kasahara, 2016). In addition, a member of the AUX/IAA transcription factor family was also grouped in Cluster 5 (Figure 2D; Supplemental Data Set 2). Finally, Cluster 17, which included an auxin responsive gene encoding gibberellin 2-β-dioxygenase 2 (GA2ox2) (Yamaguchi, 2008; Frigerio et al., 2006) showed a similar gene expression pattern to that of Cluster 5 (Supplemental Figure 6 and Supplemental Data Set 2). Thus, our expression analysis suggests that auxin may regulate multiple aspects of root cell reprogramming required for haustorium development.

**A YUC Gene Is Upregulated during Haustorium Formation**

Given that a *P. japonicum* homolog of Arabidopsis YUC genes, which encode YUCCA enzymes that catalyze a rate-limiting step in auxin biosynthesis (Mashiguchi et al., 2011), was highly expressed during haustorium formation, we decided to further characterize this gene. First, we searched in the *P. japonicum* transcriptome for homologs of Arabidopsis YUC genes. Four YUC homologs, designated as *P. japonicum* YUC1, YUC2, YUC3, and YUC4, were identified in the *P. japonicum* transcriptome assembly, and their full-length sequences were determined (Supplemental Figure 9). Phylogenetic analysis revealed that *P. japonicum* YUC2, YUC3, and YUC4 are clustered together with Arabidopsis YUC genes that are expressed exclusively in roots (At-YUC5, 7, 9, 8) (Figure 3A). Among these, *Pj-YUC3* was identified as DMBQ-inducible in the microarray analysis and was included as a member of Cluster 5 (CUST_21965_PI426107926 in Supplemental Data Set 2 and Supplemental Figure 6). Consistent with the microarray analysis, the RT-qPCR data show that only YUC3 was highly upregulated at 24 h after host root exudate treatment and at 48 h post-treatment, with expression levels as high as 200-fold those of the mock treatment (Figure 3B). Similarly, the expression of YUC3 was upregulated in haustorial tissues at 1, 3, and 7 d post-infection (dpi). In contrast, three other *Pj-YUC* genes had expression patterns similar to those of the mock-treated samples (Figure 3B) and maintained a constant level of expression after host interaction (Figure 3C).

**YUC3 Encodes a Functional Auxin Biosynthesis Enzyme**

To determine whether *Pj-YUC3* encodes a functional enzyme in the auxin biosynthetic pathway, transgenic *P. japonicum* roots overexpressing this gene under the control of the constitutively expressed ubiquitin promoter were generated with an *Agrobacterium rhizogenes*-based transformation system (Ishida et al., 2011). For comparison, we generated transgenic roots expressing GFP driven by the CaMV 35S promoter (*Pro35S:GFP*) (Figures 4A to 4B). RT-qPCR analysis verified that YUC3-transformed hairy roots had ~70 times higher expression of YUC3 than nontransformed roots (Figure 4D). YUC3-overexpressing roots (*ProUBPjYUC3*) had clear morphological phenotypes, including massive proliferation of lateral roots, short root length, and an increased number of root hairs (Figures 4A to 4C and 4E to 4G), as are often seen in auxin-overproducing plants (Boerjan et al., 1995). To confirm that endogenous auxin accumulated in YUC3-overexpressing roots, we measured the
free IAA levels in the *ProUB*:PjYUC3 and empty vector-transformed roots. The free IAA level was ~3 times higher in YUC3 overexpressing roots than the empty-vector transgenic roots (Figure 4H), indicating that *P. japonicum* YUC3 encodes a functional auxin biosynthesis enzyme.

**YUC3 Expression Localizes to the Haustorium Apex and Haustorial Hairs**

To characterize the roles of YUC3 in haustorium development, the spatio-temporal expression profile of YUC3 was investigated. The promoter region of YUC3 was cloned upstream of the reporter gene GUS and to nucleus-localized VENUS-N7, a modified version of YFP with a nuclear localization sequence, and introduced into *P. japonicum* hairy roots using *A. rhizogenes*. To facilitate the screening of transgenic roots, we modified the vectors by inserting the mRFP driven by the 35S promoter as a visible marker. The RFP-positive roots were selected under fluorescence microscopy and stained for GUS activity or visualized by VENUS-filtered fluorescence. Without any haustorium-inducing treatment, neither VENUS fluorescence (Figure 5A) nor GUS staining (Figure 5B) was detected in the transgenic roots. In contrast, when the transgenic roots were treated with the host root exudate or were infecting host roots, GUS reporter gene expression was visible (Figure 5C). The most pronounced GUS expression was observed within the epidermal and outer cortical

*Figure 3. YUC3 Is Upregulated during Haustorium Development.*

(A) A maximum likelihood phylogenetic tree of Arabidopsis (At) and *P. japonicum* (Pj) YUC proteins. The bootstrap values are shown in percentage on the internal nodes.

(B) and (C) Expression levels of YUC genes measured by RT-qPCR. The values correspond to fold change compared with nontreated roots.

(B) YUC expression in 2-week-old *P. japonicum* root treated with host root exudate for 0.5, 1, 3, 24, and 48 h.

(C) YUC expression in host interaction. Haustorial region was excised after contact with rice roots for 1, 3, and 7 d postinfection (dpi). Values are shown in fold changes compared with the root without host interaction. All data show the average of three biological replicates with at least two technical replicates each. Error bars denote SE. Asterisks indicate significant differences compared with mock treatment (B) and control samples (C) at α = 0.05 by Student’s t test with equal variances.
layers of the haustorium initiation region, as well as in haustorial hairs (Figure 5C). Consistently, using time-lapse fluorescence photographs of ProPj-YUC3 promoter-VENUS-N7 transformed roots coincubated with Arabidopsis roots, the earliest YUC3 promoter activity was detected in the haustorial hairs at 18 hpi (Figure 5D; Supplemental Movies 2 to 4). Initially, the expression activity was distributed evenly around the roots (Supplemental Movie 4). At 25.5 hpi, the fluorescence was detected mainly at the interface between the host and parasitic roots, reaching its peak at 46 hpi. The expression gradually declined from 47 hpi until being only weakly observed at 6 dpi (Figure 5D; Supplemental Movie 4). Confocal microscopy showed ProPjYUC3:3xVENUS-N7 expression in the epidermal cells and outer cortical cells, as well as in the haustorium hairs after 48 h of DMBQ treatment (Figure 5E).

Haustorium Initiation Site Shows Auxin Response

The expression pattern of YUC3 indicates that auxin is biosynthesized de novo at the haustorium apex. Therefore, we investigated whether an auxin response is observed at this site using the pDR5rev:3xVenus-N7 auxin-responsive reporter (Heisler et al., 1999; Ottenschläger et al., 2003; Chaabouni et al., 2009). When Arabidopsis roots were placed near pDR5rev:3xVenus-N7 transgenic P. japonicum roots, the DR5-driven fluorescence signal was first observed at the root hair cells slightly above the haustorium initiation site. At later time points, the fluorescence signal was observed at the epidermal and outer cortical cells at the apex of a growing haustorium, similar to YUC3 promoter expression (Figure 6A; Supplemental Movie 5). At a higher magnification, cell division of epidermal cells including hair cells was observed (Figure 6B). These dividing cells showed DR5 fluorescence signals, suggesting that the high auxin response coincides with cell division. As haustorium development progressed, an auxin response maximum was observed in a haustorium apex that started to invade host root tissue (Figure 6C; Supplemental Movie 6). Similarly, the DMBQ-induced haustorium showed auxin responses at the haustorium apex (Figure 6D).

Endogenous Auxin Levels Increase after DMBQ Treatment at the Haustorium-Forming Sites

Since the auxin-responsive promoter analysis revealed a high auxin response in the haustorium, we investigated the endogenous auxin levels in this organ. P. japonicum roots were
Figure 5. Tissue Localization of YUC3 Expression during Haustorium Development.

(A) Transgenic roots carrying a YUC3 promoter-driven VENUS fluorescent protein gene fused with a nuclear targeting signal. CaMV 35S promoter-driven RFP was inserted as a visual marker (ProPjYUC3:3xVENUS-N7-Pro35S:RFP). Roots without haustorium-inducing treatment were observed under bright-field, RFP, and VENUS fluorescence detecting filter sets.
treated with or without DMBQ for 24 h and the endogenous free IAA levels of tissues within 1 mm from the root tip, where most new haustoria are frequently formed, were measured. Free IAA contents were more than 2-fold higher in DMBQ-treated roots than control roots (Figure 6E). This suggests that IAA levels are elevated at the root segment containing the haustorium forming area after DMBQ treatment, which is consistent with the high levels of DR5 auxin response marker expression at the haustorium.

**The YUC3 Contributes to Haustorium Formation**

To investigate the function of YUC3 in haustorium formation, we knocked down its expression using the RNA interference (RNAi) method. Two target sites for RNAi were selected from the YUC3 cDNA sequence and were inserted into the pHG8YFP vector (Bandaranayake et al., 2010), generating pHG8YUC3-1 and pHG8YUC3-2 (Figure 7A). Efficient and specific silencing of YUC3 was verified by RT-qPCR using specific primer sets for Pj-YUC3 (Supplemental Data Set 4) and its homologs *P. japonicum* YUC1, YUC2, and YUC4 (Figure 7B; Supplemental Figure 11). The knockdown root lines did not show any obvious developmental defects (Supplemental Figures 12A to 12C) or changes in primary root length (Supplemental Figure 12D) and number of lateral roots formed (Supplemental Figure 12E) without chemical treatments or during host absence. However, in the presence of host Arabidopsis roots, the transgenic roots harboring RNAi constructs displayed a significant reduction in host infection (21.5 and 17.8% for pHG8YUC3-1 and pHG8YUC3-2, respectively) compared with the control hairy roots (70.6%; Figure 7C). This indicates that YUC3 positively contributes to host interaction. We further tested the frequency of haustorium induction upon host rice root exudate treatment in the pHG8YUC3-1 transgenic roots. The frequency of haustorium formation was significantly reduced in the RNAi construct-expressing roots compared with control roots (Supplemental Figure 13), suggesting that YUC3 plays an important role in haustorium formation.

**Expression of YUC3 at the Epidermal Cells Induces Haustorium-Like Structure Formation in *P. japonicum* Roots**

To investigate if the expression of YUC3 at the epidermal cells alone is sufficient to induce haustorium formation without host signals, we expressed YUC3 specifically in epidermal cells, using the dexamethasone (DEX)-inducible CRE-lox system (Brocard et al., 1998). In this system, the CRE recombinase fused with the hormone binding domain of glucocorticoid receptor (GR) was placed under the control of the epidermal cell-specific promoter At-PGP4 (Terasaka et al., 2005; Supplemental Figure 14A), and the Pj-YUC3 coding region was driven by the constitutively active At-RPS5a promoter with interruption by 4x terminator sequences sandwiched between two LoxP sequences (Weijers et al., 2001; Supplemental Figures 14B and 14C). Using this construct, YUC3 can be induced in epidermal cells upon DEX treatment due to the release of CRE recombinase in epidermal cells followed by the removal of LoxP-sandwiched terminator sequences inserted between the promoter and the coding regions. The construct was transformed into *P. japonicum* roots, and morphological changes were observed after DEX treatment. Without DEX treatment, no morphological changes in transgenic *P. japonicum* root were observed (Figure 7D). At 24 h after DEX treatment, part of the transgenic root began to swell (Figure 7E), and cell division was observed in the epidermis. This site continued to swell and proliferate and eventually formed a haustorium-like structure (Figure 7F). Furthermore, this structure was often associated with dense and curly hairs, which are similar to haustorial hairs (Figure 7G). Taken together, our results demonstrate that the expression of YUC3 is sufficient to induce a haustorium-like structure in *P. japonicum* roots.

**DISCUSSION**

Parasitic plants in the Orobanchaceae are serious threats to agriculture worldwide. Despite their economic importance, the molecular mechanisms regulating plant parasitism are poorly understood. Here, our combination of de novo transcriptome and customized microarray analysis of the facultative hemiparasite *P. japonicum* provides a comprehensive view of the molecular events during the early stages of haustorium development. Using this information, we showed that the parasite gene Pj-YUC3, encoding a functional auxin biosynthesis enzyme, plays an unequivocal role in haustorium development.

**Comprehensive Survey of Genes Involved in the Early Stages of Haustorium Development in *P. japonicum***

The currently available EST sequences in parasitic plants are still limited to several species; ESTs for three members of the Orobanchaceae family, i.e., *S. hermuthica*, *P. aegyptiaca*, and *T. versicolor* (Yoshida et al., 2010; Yang et al., 2014), the root parasite in Santalales, *S. album* (sandalwood), and the stem parasite *Cuscuta pentagona* (Kim et al., 2014; Ranjan et al., 2014) have...
Figure 6. Time-Lapse Observation of the Expression Pattern of DR5 Auxin-Responsive Promoter during Early Haustorium Development.

(A) to (D) Confocal images of *P. japonicum* roots transformed with *pDR5rev:3xVenus-N7*. Merged images of bright-field and Venus fluorescence (yellow) are shown.

(A) Arrow indicates newly established auxin maximum at haustorium initiation site facing the host root.

(B) Arrows track nucleus of a dividing root hair cell.
been reported. The P. japonicum sequences analyzed in this study provide a valuable addition to parasitic plant gene databases and are suitable for conducting comparative analyses with other parasitic species. Our assembly yielded 57,939 contigs, 64.3% of which have a BLASTx hit with the nr database (Supplemental Table 3). This value is higher than those in other published de novo dicot transcriptome data sets; e.g., 44.7% in the chickpea de novo assembly based solely on 454 reads, 43.3% in tea (Camellia sinensis) unigenes (Shi et al., 2011), and 59.65% in Litchi chinensis (Li et al., 2013). Thus, our de novo assembled transcriptome provides reasonable coverage of P. japonicum genes with an enrichment of transcripts expressed at different stages of haustorium development, thereby representing an excellent resource for mining essential genes during haustorium development.

Our microarray analysis further identified the kinetics of transcriptional changes during early haustorial development processes. We selected a detailed time course, in particular the initial events during haustorium development, with the aim to identify molecular events that correlate with haustorium development. Interestingly, the earliest expression modulation occurs with genes related to calmodulin binding and oxidoreductase activity (Figure 2C), suggesting that calcium and reactive oxygen species (ROS)-related signaling may be triggered in the early stage of development. Indeed, the involvement of ROS in haustorium initiation signaling has been proposed (Kim et al., 1998; Ngo et al., 2013; Matvienko et al., 2001a, 2001b; Honaas et al., 2013; Bandaranayake et al., 2010). The upregulation of WRKY genes in this cluster occurred just prior to visible morphological changes in the roots; thus, these genes may have regulatory functions in haustorium formation. Indeed, it was postulated that ROS generation acts as a signal that triggers haustorium formation in a parasitic plant (Kim et al., 1998; Bandaranayake et al., 2010). The wound response is associated with tissue regeneration processes, including reprogramming of differentiated cells (Ikeuchi et al., 2016). The enrichment of the GO term response to wounding suggests that haustorium formation may occur through reprogramming of root tissues, despite the lack of injury. Proliferation of haustorial hairs and activation of cell division in haustorial tissues indicate that the root cells undergo redifferentiation processes during haustorium formation. The upregulation of the metabolism of plant hormones suggests that auxin plays important roles in haustorium formation.

YUC3 Functions Specifically in Haustorium Formation

An interesting finding from our transcriptome analysis is that the auxin biosynthesis gene YUC3 is transcriptionally upregulated during haustorium development. In Arabidopsis, each of the 11 YUC gene family members has specific and overlapping functions (Zhao, 2008). The coordinated functions of Arabidopsis YUC3, YUC5, YUC7, YUC8, and YUC9 are responsible for root development, and the quintuple mutants have severely disturbed root growth (Chen et al., 2014). In the P. japonicum transcriptome, we identified four YUC sequences; P. japonicum YUC2 and YUC4 are in the same node with root-specific At-YUC genes in the phylogenetic tree. Although Pj-YUC3 is close to the root-specific clade, this gene was placed just outside of this group (Figure 3A). Transgenic P. japonicum roots with silenced YUC3 did not show any defects in root growth (Supplemental Figure 12). However, knocking down YUC3 led to a reduction in haustorium formation (Figure 7C), implying that YUC3 may function specifically during parasitism. A search of the available EST sequences of other parasitic species in the Orobanchaceae family (Yang et al., 2015), including S. hermonthica, P. aegyptiaca, and T. versicolor, revealed Pj-YUC3 homologs (e-value <1e−100 in tBLASTn) in haustorium-induced libraries but not in
prehaustorial or shoot libraries, indicating the conserved function of Pj-YUC3 homologs in this plant family.

**Local Expression of YUC3 Coincides with High Auxin Responses at the Haustorium Apex**

Our promoter-reporter analysis revealed that the expression of YUC3 precedes haustorium formation. YUC3 promoter-driven reporter florescence was observed at the root hairs and epidermal cells at the haustorium initiation sites. Overexpression of YUC3 resulted in more than 2-fold higher auxin accumulation in *P. japonicum* roots compared with control roots, indicating that YUC3 encodes a functional auxin biosynthesis enzyme. Thus, it is likely that cells where YUC3 is expressed perform de novo auxin biosynthesis. Coincidently, the auxin-responsive DR5 promoter construct showed a similar localization pattern. Previous studies have shown that the auxin-responsive *IAA2* promoter was activated in DMBQ-treated roots using a GUS reporter protein, but the resolution was not sufficiently high to specify which cells were responding to auxin (Tomilov et al., 2005). Our DR5-promoter
analysis localized auxin responses in specific cells (i.e., the epidermis and the outer cortex of the haustorium apex as well as the haustorial hairs near the host) in an initiating haustorium, forming auxin response maxima.

Localized auxin responses in particular cells may contribute to the dedifferentiation and differentiation of the cells. Indeed, proliferation of haustorial hairs at the haustorium surface is one of the earliest events in haustorium formation (Cui et al., 2016). Along with the expression of YUC3 and DR5, the haustorial hair cells begin cell division, and one daughter cell becomes an epidermal cell while the other becomes a haustorial hair cell (Supplemental Movie 5). These data suggest that auxin responses correlate with epidermis cell differentiation at the first stage of haustorium formation. Measurement of IAA confirmed that IAA levels are elevated at the root segment containing the haustorium-forming sites after DMBQ treatment. These data indicate that DR5 expression is likely to reflect the accumulation of newly synthesized auxin at the haustorium apex near the host. It is also possible that local de novo production of this hormone could result in the local redistribution of auxin transporters because auxin itself can serve as a signal for relocation of auxin transporters such as PIN proteins (Sauer et al., 2006; Xu, 2006).

Local auxin maxima in organ initiation sites are commonly observed during plant development (Benková et al., 2003; Heisler et al., 2005) and are thought to be sufficient to induce developmental programs in lateral organs (Reinhardt et al., 2003; Dubrovsky et al., 2008). We reconstructed the YUC3 expression pattern in the epidermis using the CRE/lox system aiming to mimic DMBQ treatment. Induction of YUC3 expression at the epidermis cells by DEX treatment led to epidermis cell proliferation and dense hair cell differentiation similar to haustorial hair formation, resulting in the formation of haustorium-like morphology. This observation implies that local YUC3 expression at epidermis cells, to a certain extent, can initiate haustorium development similar to that observed after DMBQ treatment. Taken together, our results demonstrate that localized expression of YUC3 in epidermis cells is necessary and sufficient for priming haustorium formation in P. japonicum. Further studies on the genetic components upstream and downstream of YUC3 will increase our understanding of the mechanism underlying plant parasitism.

METHODS

Plant Materials and Growth Conditions

Phtheirospermum japonicum (Okayama strain) seeds were surface-sterilized and germinated as previously described (Ishida et al., 2011). The outer coats of rice seeds (Oryza sativa cultivar Nipponbare) were mechanically removed, and the seeds were immersed with agitation in 70% (v/v) sodium hypochlorite; Kao Chemicals) for 30 min, and extensively washed with sterile water. The rice seeds were sown in a Petri dish (50 seeds per dish) containing a moistened filter paper (10 to 15 mL water per dish). Arabidopsis thaliana Col-0 seeds were surface sterilized with 10% commercial bleach solution for 5 min and rinsed with sterile water. Sterilized seeds were sown on half-strength MS medium containing 1.0% sucrose and 0.8% agar, and kept at 4°C for 2 d in the dark. Seven- to ten-day-old plants grown vertically at 22°C under long-day conditions (16 h day/8 h night) were used for haustorium induction. One-week-old rice and P. japonicum seeds were separately grown, and 10 rice seedlings and 6 to 10 P. japonicum seedlings were carefully placed in a rhizotron system as previously described (Yoshida and Shirasu, 2009). The rhizotrons were placed vertically at 25°C under a photoperiod of 16 h light/8 h dark with a light intensity of ~701 mol·m⁻²·s⁻¹ illuminated by the daylight-white fluorescence lump (FL40SEX-N-FL; NEC), unless otherwise specified.

Total RNA Extraction

RNA was extracted using a CTAB/LiCl based-protocol as previously reported (Yoshida et al., 2010). Genomic DNA was removed from the RNA samples by treatment with DNase I, Amplification Grade (Life Technologies; Cat. No. 18068-015), followed by a cleanup step with an RNaseasy Mini Spin Column from an RNaseasy Plant Mini Kit (Qiagen; Cat. No. 74904). RNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) using an RNA 600 Nano Kit (Cat. No. 5067-1511). RNA qualities for sequencing and microarray experiments were set at a minimum RNA Integrity Number of 8.0.

RNA Sequencing and de Novo Transcriptome Assembly

P. japonicum and rice seeds were germinated and grown separately for 1 week, and P. japonicum seedlings were transferred to rhizotron chambers without host plants (autotrophic stages) (Supplemental Figure 2, left photograph) or in contact with rice (parasitic stages) (Supplemental Figure 2, right photograph) for 4 to 6 weeks. For the parasitic-stage library, haustoria were carefully excised from P. japonicum roots under a stereoscope and immediately frozen in liquid nitrogen. When haustoria could not be disconnected from rice roots (Figures 1H and 1I), both tissues were collected. RNAs from P. japonicum roots grown with rice (nonparasitizing) were also collected for the autotrophic-stage library.

Twenty micrograms of total RNA was used for constructing the Illumina HiSeq 2000 libraries. Paired-end 90-bp sequences with 200-bp insert lengths were analyzed with the Illumina HiSeq 2000 platform according to the manufacturer’s instructions. The Roche 454 library was constructed using normalized mRNA from tissues with and without host interactions. Approximately 4 μg of mRNA was purified from total RNAs using the Illustra mRNA purification kit (GE Healthcare; Cat. No. 27-9258-01). mRNA normalization was performed using duplicity-specific nucleases, and single-read sequences were obtained with GS FLX titanium chemistry. Library construction and sequencing were performed by Hokkaido Systems Science Company for the 454 sequencer and Riken Genesis for the HiSeq 2000 sequencer.

For the de novo transcriptome assembly, the adapter oligonucleotide sequences from raw Roche 454 reads were trimmed off using CLC Genome Workbench Software 4.9. The trimmed 454 reads and the paired-end Illumina-HiSeq reads from the parasitic stage library were mapped against the annotated cDNA sequences of O. sativa (version 6) using Mosaik, allowing a maximum of four mismatches in a 45-bp overlap (http://bioinformatics.bc.edu/marthlab/Mosaik). The unmapped reads were used for the assembly. The rice sequence-filtered reads and the Illumina reads from the autotrophic stage were assembled using a combination of CLC Genome Workbench Software 4.9 and CAP3 software (Huang and Madan, 1999) to ensure nonredundant sequence assembly. Contigs longer than 300 bp were used for subsequent analyses. BLAST annotation and GO analysis of the translated sequences were analyzed by blast2GO (Conesa et al., 2005). The contigs were then assigned to PlantTribes 2.0 orthogroups (Wall et al., 2008). The expression values were computed through mapping of reads against the assembled contigs using CLC Genome Workbench 4.9 and the RPMK estimation function.
DMBOQ and Rice Root Exudate Treatment

To induce in vitro haustorium formation, *P. japonicum* roots were treated with the haustorium-inducer DMBQ (Sigma-Aldrich; Cat. No. 565032). *P. japonicum* seeds were sown on modified MS medium (1× MS supplemented with 1% [w/v] sucrose, 0.01% [w/v] myo-inositol, 0.06% [w/v] MES, and 0.8% [w/v] agar) in 10 × 14 × 1.5-cm square plates and kept in a vertical position in 25°C under long-day conditions. The bottom halves of the plates were covered with aluminum foil to avoid exposure of the roots to light. After 2 weeks, 7 mL of 10 μM DMBQ solution was dropped onto the *P. japonicum* roots and the plates were incubated horizontally. For microarray analysis, the DMBQ- or mock-treated (0.1% [v/v] DMSO) roots were sampled at 0, 0.5, 1, 3, 6, 12, 24, and 48 h after treatments. Each experiment was repeated three times with 12 to 15 plants per time point.

For the rice root exudate treatment, 50 rice seedlings were soaked in 12 to 15 mL water for 1 week, after which 5 mL of the water was solidified with agar (0.8% [w/v]). In addition, the roots of 1- to 2-week-old rice seedlings were cut into 0.8% [w/v] blocks were placed on the parasite roots. The plates were incubated horizontally at 25°C with the bottom halves of the plates covered with aluminum foil.

Custom Microarray Design, Labeling, and Hybridization

For custom microarray slides, putative coding regions of the assembled contigs were detected by ESTScan2 (Iseli et al., 1999), and 47,817 unigenes with a putative coding region of at least 50 amino acids were selected by ESTScan2. Contigs were detected by ESTScan2 (Iseli et al., 1999), and 47,817 unigenes were selected. The gene-expression data were filtered to select the differentially expressed genes. The RACE method was used to determine the full-length cDNA of *P. japonicum* roots at the parasitic and autotrophic stages. To clone the promoter region and the second exon of *YUC3* genes, the RACE method was used to determine the full-length cDNA of *P. japonicum* roots at the parasitic and autotrophic stages. To clone the promoter region and the second exon of *YUC3* genes, the RACE method was used.

Clustering analysis was performed according to a previous report using SC2ATmd software (Olex and Fetrow, 2011). First, figure of merit analysis was applied to obtain parameters for the accurate clustering algorithm using ED metric for a given input. Next, the filtered DE genes were submitted to consensus clustering with the option of hierarchical clustering (k-means=16, 16 repetitions) with a consensus threshold of 90%. GO terms were assigned for each gene based on the best BLAST-hit annotations, and the enrichment test was performed using the R package GOstat (Beissbarth and Speed, 2004). False discovery rate (FDR) correction was done using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995) with statistical significance of FDR < 0.05.

**RT-qPCR Analysis**

For RT-qPCR analysis, we first identified constitutively expressed genes to be used as normalization controls. We selected homologs of Arabidopsis housekeeping genes (Czechowski et al., 2005) using a local BLAST search (e-value <1e-20) as well as the constitutively expressed genes (in the range of 1.2 fold change) (Supplemental Data Set 3). We identified four *P. japonicum* candidates, and primer sets for each gene were tested for amplification efficiency and specificity for *P. japonicum* by testing with the genomic DNAs of *Lotus japonicus* and rice. A primer pair designed for PTB was used for the internal control in subsequent experiments. The first-strand cDNA was synthesized with a ReverTra Ace-α-kit (Toyobo; code FSK-101), and qPCR was performed with a Thunderbird SYBR qPCR Mix kit (Toyobo; code A4251K). The relative expression was calculated with the standard curve method. All primers used in this study are listed in Supplemental Data Set 4. All experiments were repeated at least three times with at least two technical replications each. For statistical analysis, the t-test was performed across the replicates.

**Cloning of YUC Genes in the *P. japonicum* Transcriptome**

To identify YUC gene homologs in *P. japonicum*, the amino acid sequences of Arabidopsis *YUC* genes (AT4G32540, AT1G04180, AT1G04610, AT1G48910, AT2G33230, AT4G13260, AT4G28720, AT5G25620, and AT5G43890) were aligned against the *Lotus japonicus* and rice. A primer pair designed for *PTB* was used for the internal control in subsequent experiments. The first-strand cDNA was synthesized with a ReverTra Ace-α-kit (Toyobo; code FSK-101), and qPCR was performed with a Thunderbird SYBR qPCR Mix kit (Toyobo; code A4251K). The relative expression was calculated with the standard curve method. All primers used in this study are listed in Supplemental Data Set 4. All experiments were repeated at least three times with at least two technical replications each. For statistical analysis, the t-test was performed across the replicates.

**Phylogenetic Analysis**

Predicted amino acid sequences were aligned using the option Muscle in the CLC genomics workbench (ver. 4.8) program with default arguments. Based on these alignments (Supplemental Files 1 and 2), phylogenetic trees were generated using the UPGMA method, and the reliability of the trees was tested by bootstrap with 1000 resamplings. The topology of the trees was also confirmed by the maximum-likelihood method with the MEGA program (Tamura et al., 2011).

**Plasmid Construction**

For the *Pi2* YUC3 promoter construct, oligonucleotides flanking the putative promoter region and the second exon of YUC3 were designed and used for
PCR amplification using the *P. japonicum* genome as a template. The 4924-bp genomic fragment was cloned, and the sequence was confirmed using an ABI3770 sequencer. The promoter region (3137 bp) was amplified by specific primers (Supplemental Data Set 4). For the GUS and VENUS reporter constructs, the CaMV 35S promoter and mRFP sequences were PCR amplified from the pEYZS-CL vector (Yoshimoto et al., 2004) and inserted within the HindIII site into the linearized R4L1pGWBS32 vector (Nakamura et al., 2009) or into the SacI site of pDRSrev:3×Venues-N7 (Heisler et al., 2005), respectively. pDRsrev:3×Venues-N7 was kindly provided by Elliot Meyerowitz. The YUC3 fragment with attB4 and attB1 recombination sites was cloned into the pDONRG-P4IP1R vector (Oshima et al., 2011) using the Gateway BP reaction and subsequently transferred to the RFP-inserted R4L1pGWBS32 vector through the LR reaction catalyzed by Gateway LR Clonase (Invitrogen). For the VENUS reporter construct, the auxin-specific DR5 promoter from pDRSrev:3×Venues-N7 was replaced with the YUC3 promoter through the multiple fragments cloning strategy using an In-Fusion Cloning Kit (Takara).

For overexpression and silencing constructs, the plasmid pUB-GW-GFP (Maekawa et al., 2008) (http://www.legumebase.brc.miyazaki-u.ac.jp) and the silencing vector pHZ89FP (Bandaranayake et al., 2010) were used, respectively. The YUC3 coding sequence was amplified with the primers listed in Supplemental Data Set 4 and inserted into the pENTR/D-TOPO vector using a TOPO cloning kit (Invitrogen). The resulting clones were verified by sequencing, and the targeted sequence was inserted into the destination vectors through a recombination reaction catalyzed by LR Clonase II. The empty vector was used for the silencing control. Transgenic hairy roots were selected based on the presence of GFP or YFP signals.

For the DEX-inducible CRE-lox system construct, Golden Gate cloning technology was used (Engler et al., 2014). The Arabidopsis PGP4 and RPSSa promoter region and the Pj-YUC3 sequence from the start codon to the 3′ untranslated region were divided into two parts and amplified by PCR from Arabidopsis genomic DNA and *P. japonicum* genomic DNA, respectively. Each fragment was inserted into vector pAGM1311. These sequences were combined into vector pICH41295 for the promoter module (AtPGP4-pro and AIRPSSa-pro) and pICH41308 for the CDS module (pYUC3-CDS). The At-RPSSa promoter sequence was recloned into pAGM1251 using At-RPSSa-pro as a template for the promoter (f) module [At-RPSSa-pro(f)]. The ligand binding domain of human glucocorticoid receptor was PCR-amplified from pSSSPRGR (Iwase et al., 2013) and inserted into pAGM1311. This vector was combined with the synthesized sequence of CRE recombinase and cloned into pICH41308, yielding the CDS module (CRE-GR-CDS). The HSP18.2 terminator sequence was amplified from Arabidopsis genomic DNA and inserted into vector pICH41276 (At-HSP-ter). The 3SS terminator sequences were amplified from pICH41414 (3SS-ter) with the primer including the LoxP sequence and tandemly as assembled into pAGM1311, yielding LoxP-2×Ter-LoxP. To increase the spacer region, two HSP18.2 terminator sequences were inserted between the 3SSs terminator sequences and cloned into vector pICH41276 for the NT1 module, yielding LoxP-4×Ter-LoxP-NT1. AtPGP4-pro and CRE-GR-CDS and 3SS-ter were combined into pICH47732, yielding AtPGP4-CRE-GR. AtRPSSa-pro(f) and LoxP-4×Ter-NT1 and pYUC3-CDS and AHSPT-ter were combined into pICH47781, yielding AtRPSSa-Pro(L)-PjYUC3. These gene cassettes were further combined into pAGM4723, yielding AtPGP4>>PjYUC3, which was used for transformation.

**Transformation of *P. japonicum***

Transformation of *P. japonicum* was performed as previously described by Ishida et al. (2011) with minor modifications. Three-day-old *P. japonicum* seedlings were immersed in a bacterial solution (OD(600) = 1.0) and submitted to ultrasonication using a bath sonicator (Ultrasonic Automatic Washer; AS ONE) for 15 to 25 s. The sonicated seedlings were vacuum infiltrated for 5 min. The seedlings were transferred to cocultivation medium (Gamborg B5 agar medium, 1% [w/v] sucrose, and 450 μM acetosyringone) and kept in the dark at 22°C for 1 to 2 days. After the cocultivation period, the seedlings were transferred to B5 agar medium containing cefotaxime (300 μg/mL). After 3 to 4 weeks, the transformed roots were analyzed. All plasmids, except pDRSrev:3×Venues-N7, contained a visible marker in which a constitutive promoter was fused upstream of a gene encoding fluorescent protein. To identify transgenic roots, the marker protein fluorescence was detected using a Leica M165 FC stereoscope.

**Analysis of Transgenic Roots**

Hairy roots emerging after 3 to 4 weeks were used for haustorium-inducing assays. Fluorescent roots were selected and placed on either host root exudate containing medium or 0.7% agarose together with 10-d-old Arabidopsis seedlings. The number of fluorescent roots that formed at least one haustorium was counted after 24 or 48 h. To verify silencing and overexpression in the transformed roots, RNA was extracted using an Arcturus PicoPure RNA isolation kit (Applied Biosystems Life Technologies) and RT-qPCR was performed. For statistical analysis, at least 30 independent fluorescent roots were analyzed across three to five biological replicates, unless otherwise described. For the DEX-induced CRE-lox system, the hairy roots were treated with 10 μM DEX-containing agar.

**Time-Lapse Photography and Microscopy**

Arabidopsis Col-0 was grown vertically for 10 d on full-strength MS medium supplemented with 1% (w/v) sucrose. Transgenic *P. japonicum* roots were placed carefully near Arabidopsis roots in a Petri dish containing agar 0.7% (w/v). Seedlings and roots were covered with 0.7% (w/v) agarose to avoid dehydration. Time-lapse photographs were automatically taken with a Leica M165 FC stereoscope or by confocal Leica TCS-SP5 II microscope at 30-min intervals. The double staining of the haustorium thin sections and safranin-staining of whole haustorium were performed as previously described (Yoshida and Shirasu, 2009). GUS staining was performed as described (Vitha et al., 1995) and observed under an Olympus BX-50 microscope. Confocal photographs were taken with a Leica TCS-SP5 II confocal microscope.

**Measurement of Endogenous IAA**

For auxin measurements in YUC3-overexpressing roots, root tip tissues (2 mm from the root tip) were carefully excised from pUB:PYUC3- and empty vector-transformed roots. For IAA measurements, a haustorium region (~1 to 2 mm from the root tip) was excised after treatment with DMBQ for 24 h. The root tip region was excluded because high levels of auxin accumulation in root tips may mask the detection of increases in auxin levels in haustoria. All measurements were performed in three biological replicates, and each biological experiment contained 100 excised tissues. Free IAA measurement was performed as previously reported (Okumura et al., 2013) with slight modifications. HPLC was performed with a gradient of 3 to 15% of acetonitrile/0.05% acetic acid over 20 min. Parameters for LC-ESI-MS/MS analysis were modified as follows: fragmentor, 120 V; MS/MS transition for [phenyl-13C6]IAA, 182/136 (m/z). Data were analyzed using Student’s t test, assuming equal variances among three biological replicates.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL libraries under accession number SAMN03271814. MIAME-compliant (minimum information about a microarray experiment) raw microarray data were deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE65723.
Supplemental Data

Supplemental Figure 1. Host specificity of the parasitic plant *P. japonicum*

Supplemental Figure 2. *P. japonicum* grown in a rhizotron with or without rice host plants.

Supplemental Figure 3. RT-qPCR validation of the expression profiles of selected genes.

Supplemental Figure 4. *P. japonicum* roots at 48 h after rice root exudate treatment.

Supplemental Figure 5. Expression profiles of early-responsive genes.

Supplemental Figure 6. Expression profiles of late-responsive genes.

Supplemental Figure 7. Expression profiles of downregulated genes.

Supplemental Figure 8. QR2 rather than QR1 is differentially regulated during parasitism.

Supplemental Figure 9. Alignment of *Pj*-YUC and At-YUC amino acid sequences.

Supplemental Figure 10. Expression pattern of DRS5 promoter in *P. japonicum* roots.

Supplemental Figure 11. Transcript levels of YUCCA homologs in transgenic roots.

Supplemental Figure 12. Morphology of transformed hairy roots.

Supplemental Figure 13. RNAi lines of *P. japonicum* roots targeted to YUC3 show reduced haustorium formation induced by rice root exudates.

Supplemental Figure 14. Expression patterns of At- PGP4 promoter and At-RPSSa promoter in *P. japonicum*.

Supplemental Table 1. Total number of reads obtained in each RNAseq library.

Supplemental Table 2. Investigation of rice contamination in *P. japonicum* unigenes

Supplemental Table 3. Summary of de novo assembly of *P. japonicum* transcriptome.

Supplemental Data Set 1. Annotation and expression values of the *P. japonicum* transcriptome at the autotrophic and parasitic stages.

Supplemental Data Set 2. List of 327 differentially expressed genes divided into 38 clusters in response to DMBQ.

Supplemental Data Set 3. List of genes with unaltered expression levels in microarray samples treated with 10 μM DMBQ.

Supplemental Data Set 4. List of primers.

Supplemental Movie 1. Time-lapse images of haustorium development.

Supplemental Movie 2. Time-lapse images of root harboring ProPjYUC3:3XVenus-N7-Pro3SS::RFP vector during infection of Arabidopsis roots viewed under a bright-field microscopy.

Supplemental Movie 3. Time-lapse images of root transformed with the ProPjYUC3:3XVenus-N7-Pro3SS::RFP vector during infection of Arabidopsis roots shown in Supplemental Movie 2 observed under RFP fluorescence conditions.

Supplemental Movie 4. Time-lapse images of root transformed with the ProPjYUC3:3XVenus-N7-Pro3SS::RFP vector during infection of Arabidopsis roots shown in Supplemental Movie 2 observed under VENUS fluorescence conditions.

Supplemental Movie 5. Confocal time-lapse images of root transformed with the pDR5rev:3xVENUS-N7 vector during infection of Arabidopsis roots.

Supplemental Movie 6. Confocal time-lapse images of root transformed with the pDR5rev:3xVENUS-N7 vector during infection of Arabidopsis roots.

Supplemental File 1. Alignments of predicted amino acid sequences of *Pj*-YUC and At-YUC genes.

Supplemental File 2. Alignments of predicted amino acid sequences of *Pj*-QR and Tv-QR genes

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AUTHOR CONTRIBUTIONS


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Local Auxin Biosynthesis Mediated by a YUCCA Flavin Monooxygenase Regulates Haustorium Development in the Parasitic Plant *Phtheirospermum japonicum*

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