Ethylene signaling mediates host invasion by parasitic plants

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Parasitic plants form a specialized organ, a haustorium, to invade host tissues and acquire water and nutrients. To understand the molecular mechanism of haustorium development, we performed a forward genetics screening to isolate mutants exhibiting haustorial defects in the model parasitic plant Phtheirospermum japonicum. We isolated two mutants that show prolonged and sometimes aberrant meristematic activity in the haustorium apex, resulting in severe defects on host invasion. Whole-genome sequencing revealed that the two mutants respectively have point mutations in homologs of ETHYLENE RESPONSE 1 (ETR1) and ETHYLENE INSENSITIVE 2 (EIN2), signaling components in response to the gaseous phytohormone ethylene. Application of the ethylene signaling inhibitors also caused similar haustorial defects, indicating that ethylene signaling regulates cell proliferation and differentiation of parasite cells. Genetic disruption of host ethylene production also perturbs parasite invasion. We propose that parasitic plants use ethylene as a signal to invade host roots.

INTRODUCTION

Parasitic plants in the Orobanchaceae family include destructive parasitic weeds, such as Striga spp. and Phelipanche spp., but the molecular basis for their parasitism is largely unexplored. Parasitic plants form haustoria to attach and invade hosts through which parasitic plants acquire nutrients as well as small materials including plant hormones and RNAs (1–3). Formation of haustoria is regulated by host-derived signal molecules. Many Orobanchaceae members initiate haustorium formation in response to cell wall–derived simple quinones or phenolics, such as 2,6-dimethoxy-p-benzoquinone (DMBQ) or syringic acid, collectively termed haustorium-inducing factors (HIFs) (1). In the model Orobanchaceae Phtheirospermum japonicum, HIFs induce the expression of an auxin biosynthesis gene in root epidermal cells at haustorium formation sites, causing cell division and expansion to form a semispherical pre- or early haustorium structure (4). To establish a successful parasitic relationship, a haustorium needs to reach the host roots, and upon host contact, it initiates invasion by differentiating epidermal cells at the apex into slender intrusive cells (movie S1) (5, 6) that function in host intrusion. After the intrusive cells reach host vasculatures, the haustorium develops water-conducting xylem bridges between host and parasite xylems, forming a mature haustorium (5, 7). When exposed to HIFs without a host in close proximity, haustorium formation is induced, but its growth is soon arrested (8). Thus, the fate of haustorium cells is determined by host availability; however, how parasitic plants recognize host availability and how they regulate such a cell fate transition remain unknown.

Ethylene is a gaseous plant hormone contributing important agronomic traits such as fruit ripening, leaf senescence, and root nodulation (9, 10). Ethylene is also broadly implicated in plant interaction with many organisms including insects, bacteria, and virus, conferring plants either resistance or susceptibility, depending on pathogen types (11–13). Ethylene biosynthesis and signaling pathways have been well studied in the model plant Arabidopsis thaliana. The biosynthesis pathway consists of two steps. The first step converts S-adenosyl-l-methionine to 1-aminoacyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). The second step releases ethylene from ACC by ACC oxidase (ACO) (14). Ethylene is perceived by endoplasmic reticulum (ER) membrane–localized ethylene receptors that have similarity to sensor histidine kinases in the bacterial two-component system (15). The Arabidopsis genome contains five ethylene receptors including ETHYLENE RESPONSE 1 (ETR1). In the absence of ethylene, ethylene receptors inhibit the key downstream signaling component ETHYLENE INSENSITIVE 2 (EIN2) by activating CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1). CTR1 phosphorylates the C-terminal domain of EIN2, preventing its nuclear translocation that activates downstream ethylene signaling. Without ethylene, EIN2 is constantly targeted for the ubiquitin proteasome–dependent degradation pathway (15–19). Upon ethylene binding to ethylene receptors, the activity of CTR1 is suppressed, leading to nuclear translocation of the EIN2 C-terminal domain and activation of the downstream signaling cascade via key transcription factors ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE 1 (EIL1) (15–18). In the Orobanchaceae parasitic plant Triphysaria versicolor, it was reported that ethylene production is up-regulated when parasites are exposed to HIFs without a nearby host (20). Pharmacological perturbation of ethylene biosynthesis or signaling in T. versicolor resulted in reduced haustorium initiation, suggesting the involvement of ethylene in haustorium initiation. However, the precise roles of ethylene in parasitic plant and host interaction remain unknown.

In this study, we identified P. japonicum mutants that cannot regulate cell division and cell fate transitions at the haustorium apex

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Cui et al., Sci. Adv. 2020; 6 : eabc2385 28 October 2020 1 of 11
and thus showed defects in host invasion. Genome sequencing revealed that these mutants are impaired in ethylene signaling. Our results suggest that ethylene mediates parasitic plant host recognition for the invasion.

RESULTS AND DISCUSSION
Parasitic plant mutants that show extra haustorium elongation
With the aim of unveiling the genetic programs for haustorium development, we carried out a genetic screening using the facultative root parasitic plant *P. japonicum*. Screening of ethyl methane-sulfonate (EMS)–induced mutant pools identified two mutant lines that had elongated haustoria on a medium containing DMBQ (Fig. 1A) (21). Time-lapse observations showed delayed growth termination of haustoria in both mutants. Wild-type (WT) haustoria stopped its tip growth within 2 days after DMBQ treatment, probably to suppress unnecessary growth in the absence of a host. In contrast, mutant haustoria kept elongating even after 3 days of DMBQ treatment (Fig. 1A), suggesting that the mutants have defects in such a suppression system. Mutant haustoria often produce a single xylem strand in the middle axis (Fig. 1, A and B, and fig. S1). The tracheary elements of mutant xylem strands show spiral patterns observed in the protoxylems of roots or a xylem bridge in a mature haustorium (fig. S1, A to D), but these xylems are not directly connected with root xylems unlike those in the lateral root (arrowheads; Fig. 1B). The cellular structure of the mutant haustoria is also distinctive from those of roots, indicating that elongated mutants’ haustoria are different from roots (fig. S1, E to P). Each mutant was backcrossed (BC) with a WT parental line, and the subsequently obtained BC1F1 and BC1F2 progeny were phenotypically examined. One of two mutant lines (line 1) showed severe germination defects and, therefore, was highly variable in phenotypic segregation ratios in the BC1F2 progeny. Nevertheless, the germinated BC1F1 plants had the elongated haustorium phenotype, indicating a dominant mutation. On the other hand, BC1F2 progeny of the other mutant (line 2) showed almost a 3:1 (211:65) segregation for WT versus the elongated haustorium phenotype, indicating that the phenotype is caused by a recessive mutation in a single gene. Both mutants required at least 1 week of stratification to induce germination, while the WT required 1-day stratification, indicating that both mutants showed a seed germination phenotype.

The mutant phenotype is caused by mutations in ethylene signaling genes
To identify the genes responsible for the phenotype, we sequenced the whole genomes of WT and the mutants and identified single-nucleotide polymorphisms (SNPs) unique to each mutant. The WT reference genome was sequenced and assembled, resulting in a draft genome assembly with an N50 scaffold over 1 million base pairs (Mbp) and an N50 contig over 29 kilo–base pairs (kbp). Gene annotation using the *P. japonicum* transcriptome predicted 30,337 protein encoding genes in the assembly (tables S1 and S2). By Illumina-based resequencing of the pooled mutant genomes, we identified two nonsynonymous SNPs unique to line 2 M5 and BC1F2 progenies (see details in Materials and Methods, table S3, and data S1). One of the SNPs was found in a gene encoding protein phosphatase 2C. Another SNP was found in a gene encoding a homolog of *Arabidopsis EIN2* (16, 22), a key signal transducer of ethylene (11–13). The *P. japonicum* EIN2 homolog (PjEIN2) is a single copy gene in the genome and shares 49% amino acid identity with *Arabidopsis EIN2* (figs. S3 and S4A). The mutation in PjEIN2 causes a stop codon at amino acid 744 by replacing an Arg residue (Fig. 1D and fig. S3), likely resulting in partial removal of the C-terminal half of PjEIN2 that is required for activating ethylene signaling in the nucleus (16).

For the dominant line 1 mutant, we obtained 36 candidates of nonsynonymous SNPs (fig. S5, table S3, and data S2). One of the SNPs occurs in a gene encoding a homolog of *Arabidopsis ETHYLENE RECEPTOR1 (ETR1)* with 80% amino acid identity. The mutation in PjETR1 replaces an Ile residue with Phe within the first of three hydrophobic regions at the N terminus (Fig. 1C) that function in ethylene binding in ETR1 (23). Mutations in these regions in *Arabidopsis ETR1* result in inability of ETR1 to bind ethylene and lead to dominant ethylene-insensitive phenotype (24, 25). Similar to a comparable mutant in *Arabidopsis* (25), the line 1 mutant exhibits dominant phenotype, although there are multiple ethylene receptor–encoding genes in addition to PjETR1 (fig. S4B).

Because both mutants have SNPs in homologs of key signaling genes in the ethylene pathway, we assumed that this phenotype may be caused by defects in ethylene signaling in *P. japonicum*. Consistent with this hypothesis, both mutants were ethylene insensitive for growth inhibition of roots and hypocotyls (fig. S6). Moreover, transformation of a full-length PjEIN2 genomic region restores the haustorium phenotype of the line 2 mutant (Fig. 1E), confirming that haustorium elongation is caused by a loss-of-function mutation in PjEIN2. Therefore, we concluded that the elongated haustorium phenotype is caused by ethylene insensitivity and named these mutants *Pjetr1* and *Pjein2* for lines 1 and 2, respectively (Fig. 1). Elongated haustoria are also induced by treatments with ethylene signaling inhibitors AgNO₃ and 1-methylcyclopropene (1-MCP) (26, 27), mimicking the phenotype of *Pjetr1* and *Pjein2* (Fig. 1, F and G). The *P. japonicum* genome contains 11 ACS-encoding and 4 ACO-encoding genes, each of which belongs to well-conserved subgroups except for type III ACO (fig. S8), indicating the conservation of ethylene biosynthesis pathway. In contrast to ethylene signaling inhibitors, no extra elongation was observed by the treatment of ethylene biosynthesis inhibitors including aminoethoxyvinylglycine (AVG) that inhibits ACSs and aminoisobutyric acid (AIB) that inhibits ACOs (fig. S7). Lack of promotion of haustorium elongation by ethylene biosynthesis inhibitors suggests either that ethylene biosynthesis in *P. japonicum* is not involved in this process or that residual ethylene amounts still produced under inhibitor treatments are sufficient for stopping haustorial elongation. AgNO₃ treatment induced elongated haustoria similarly in *Striga hermonthica*, a close relative of *P. japonicum* (fig. S9). Ethylene biosynthesis genes and receptors are also conserved in *Striga asiatica* (figs. S4 and S8) (28). These results suggest that the function of ethylene signaling in haustorium elongation is conserved in the Orobanchaceae.

Effects of ethylene on haustorium initiation
Defects in ethylene signaling have little effect on haustorial initiation, as neither treatments with AgNO₃ or 1-MCP nor the *Pjetr1* or *Pjein2* mutations affected haustorium number statistical significance, although *Pjein2* produced slightly fewer haustoria, indicating that ethylene signaling is not required for haustorium initiation (Fig. 2, A to C). On the other hand, application of exogenous ethylene strongly reduced the haustorium number and haustorium size only
in the WT (Fig. 2, D to F). Note that this suppressive effect is almost saturated at 0.01 parts per million (ppm) ethylene application, while most of other plants show ethylene response that saturates at several magnitude higher concentration, e.g., inhibition of root elongation in Arabidopsis (29, 30). These suggest that P. japonicum is highly sensitive to ethylene at root transition zone where the haustorium emerges and that ethylene strongly suppresses haustorium initiation in an ethylene signaling–dependent manner. AVG treatment inhibiting production of ethylene precursor ACC significantly reduced haustorium number in P. japonicum WT as well as Pjetr1 and Pjein2 mutants (Fig. 2G), while the same concentration did not affect primary root growth (fig. S10A), indicating that AVG inhibits haustorium initiation independently of PjETR1 and PjEIN2. In T. versicolor, AVG similarly inhibits haustorium induction and

**Fig. 1. Two elongated haustorium mutants in P. japonicum have mutations in ethylene signal transduction genes.** (A) Time-lapse images of haustorium formation after DMBQ treatment in WT and two mutants of P. japonicum, d, days after treatment. (B) Xylem morphology in roots and DMBQ-induced haustoria. Middle panels are magnifications of the left panels. Junctions between the root xylem (RX) and haustorial xylem (HaX) are indicated with open triangles. For comparison, the joint between lateral root xylem (LRX) and primary root xylem (PRX) is shown on the right. (C) Gene structure of PjETR1 and a point mutation (asterisk) in Pjetr1, causing substitution of Ile35 by Phe in the ethylene binding domain indicated by a black line. Sequence alignment of the N-terminal region of etr1, causing substitution of and a point mutation (asterisk) in Pj

**EIN2**

etin2 and a point mutation (asterisk) in Pj

**ein2**

Medicago resulting in a stop codon at Arg 744. The hydrophilic domain

**ETR1**

is highly

**etin2**

is

**etr1**

and a point mutation (asterisk) in Pj

**etin2**

phenotype required for signal activation is highlighted with cyan. Exon (closed boxes) and 5′UTR and 3′UTR (open boxes) are shown. (D) Gene structure of PjEIN2 and a point mutation (asterisk) in Pjein2 resulting in a stop codon at Arg744. The hydrophilic domain required for signal activation is highlighted with cyan. Exon (closed boxes) and 5′UTR and 3′UTR (open boxes) are shown. (E) Complementation of the Pjein2 phenotype by transformation with genomic DNA containing PjEIN2. Red fluorescence is derived from a constitutively expressed DsRED selectable marker. (F) WT haustoria induced by DMBQ with or without ethylene signaling inhibitors (0.1 μM AgNO3, 10 ppm 1-MCP) for 10 days. (G) Average length of the longest haustorium per plant in WT, Pjetr1, and Pjein2 after DMBQ treatment with or without 0.1 μM AgNO3 or 10 ppm 1-MCP for 10 days. Data represent the mean ± SE with n ≥ 14. Asterisks indicate statistically significant differences compared to DMBQ-treated WT (Student’s t test, unpaired, two-tailed, P < 0.01). Scale bars, 200 μm (A, E, and F), 100 μm (B, left), and 50 μm (B, middle and right).
Fig. 2. Roles of ethylene and ethylene signaling on haustorium initiation. (A) Number of haustoria in the WT (n = 24), Pjetr1 (n = 14), and Pjein2 (n = 19) after DMBQ treatment for 1 week. (B and C) Number of WT haustoria after DMBQ treatment for 1 week with or without AgNO₃ (B) or 1-MCP (C). Data represent the mean ± SE. n ≥ 14. (D) WT P. japonicum and the Pjetr1 and Pjein2 mutants were treated with 10 μM DMBQ in the absence or presence of 0.1 ppm ethylene for 7 days. Top panels show macro images of plant roots, and the bottom panels show magnified images of root segments with haustoria (yellow arrowheads). Scale bars, 1 cm (top) and 0.5 cm (bottom). (E) Magnified image of haustorium induced by 10 μM DMBQ in the absence or presence of 0.1 ppm ethylene. (F) Relative number of haustoria in P. japonicum WT, Pjetr1, and Pjein2 after treatment with 10 μM DMBQ for 7 days in the absence or presence of ethylene at the indicated concentrations (ppm). (G) Relative number of haustoria after treatment with 10 μM DMBQ for 7 days in the absence or presence of AVG at the indicated concentrations (μM). Data indicate the relative mean ± SE. Student’s t test, unpaired, two-tailed (*P < 0.01). n ≥ 13 from two independent experiments. Scale bars, 200 μm.
**Fig. 3. Ethylene signaling regulates cell division at the haustorium apex.** (A) Haustorium apices in WT, and the Pjetr1 and Pjein2 mutants after DMBQ treatment. Samples were stained with toluidine blue. (B) Visualization of dividing cells by EdU staining (green) of WT and Pjein2 haustoria after DMBQ treatment. (C) Expression of an auxin response marker (DR5:3×VENUS-NLS, yellow fluorescence) in DMBQ-induced haustoria. (D) Multiple haustorium apices (arrowheads) formed in a haustorium of Pjein2. Left and middle panels indicate haustoria after syringic acid treatment for 10 days. The right panel shows a haustorium induced by placing an *Arabidopsis* root (H) next to a *P. japonicum* root (P) for 4 days. Scale bars, 50 μm (A), 200 μm (B and C), and 500 μm (D).

**Fig. 4. Mutations in ethylene signaling components in parasitic plants cause severe defects in host infection.** (A) Haustoria of *P. japonicum* WT, Pjetr1, and Pjein2 infecting *Arabidopsis* roots for 5 days. Xylem cells are stained by Safranin O. (B) Quantification of the host invasion rate in (A). (C) Cross sections of *Arabidopsis*-infecting *P. japonicum* WT, Pjetr1, and Pjein2 stained by toluidine blue. (D) Haustoria of *P. japonicum* WT, Pjetr1, and Pjein2 infecting rice roots for 5 days. Black, white, and yellow arrowheads indicate invaded, noninvaded, and blocked haustoria, respectively. (E) Quantification of the invasion phenotype of haustoria in (C). Data represent the mean ± SD (*n* ≥ 32) from three independent experiments. Asterisks indicate statistically significant differences compared to WT (Student's *t* test, unpaired, two-tailed, *P* < 0.01). Scale bars, 200 μm.
suboptimal concentration of ACC increases haustorium formation frequency (20). Therefore, ACC as a precursor of ethylene might have a different role from ethylene and is required for haustorium formation. In line with this, ethylene signaling-independent function of ACC is known in several processes including pollen tube attraction, seed viability, and vegetative growth, proposing that ACC itself might act as a hormone (31–33). Alternatively, reduced haustorium initiation may be a consequence of auxin biosynthesis inhibition by AVG. It was reported that AVG inhibits auxin biosynthesis in ethylene-independent manner (34) and local auxin biosynthesis is essential for haustorium initiation (4). Together, these data collectively led us to conclude that while exogenous application of ethylene suppressed haustorium development, ethylene signaling is dispensable during early haustorium initiation but is required for timely termination of haustorium growth in the absence of a host.

**Ethylene signaling negatively regulates cell proliferation and auxin response in haustorial apex**

Apical growth in plants, such as root or shoot growth, is often driven by cell division at the apex wherein the plant hormone auxin plays a key role in maintaining cell division (35). To test whether haustorial apical growth is regulated by similar mechanisms, we observed cell division during haustorium development. A cluster of small cells with darker toluidine blue staining at the haustorium apex was observed up to 2 days after DMBQ treatment in WT, whereas these cells are observed until 3 days in the Pjetr1 and Pjein2 mutants (Fig. 3A). Visualization of cell proliferation by 5-ethyl-2’-deoxyuridine (EdU) incorporation into newly synthesized DNA confirms that haustorium apex cells are proliferated for up to 2 days in WT, whereas cell proliferation activity remains up to 4 days in the Pjein2 mutant (Fig. 3B). In addition, cell proliferation in Pjtein2 occurs in the central parts of haustoria coinciding with haustorial xylem formation, indicating that vascular meristems may develop in mutant haustoria (Fig. 3B). Expression of the auxin-responsive promoter DR5 coincides with haustorial apical cell proliferation, i.e., there was prolonged occurrence of auxin response maxima in Pjtein2 haustorium apices in Pjtein2 compared to WT (Fig. 3C). Failure in refining haustorium apex activity sometimes leads to formation of multiple tips within a single haustorium in Pjtein2 induced by either Arabidopsis roots or syringic acid (Fig. 3D), a precursor of DMBQ. These data suggest that ethylene signaling is necessary to terminate cell proliferation at the haustorium apex via suppression of the auxin response.

**Ethylene signaling is essential for host invasion**

To determine the role of ethylene signaling during host invasion, we performed an infection assay using Arabidopsis and rice (Fig. 4). Most of the haustoria in WT successfully invade host roots at levels of 82 ± 7% and 85 ± 4% against Arabidopsis and rice, respectively (Fig. 4, B and E). However, host invasion levels were significantly reduced in Pjetr1 (compromised 65 to 70%) and, to a greater extent, in Pjtein2 (91 to 98%) (Fig. 4, B and E). Noninvased haustoria of both mutants failed to develop intrusive cells at the apex and kept elongating around the host surface even after direct contact with Arabidopsis (Fig. 4, A, C, and D). In the interaction with rice, most of the Pjetr1 haustoria were arrested at the root surface, resulting in swelling at the haustorial tip without forming intrusive cells (“Blocked” in Fig. 4D), probably due to the thicker roots of rice compared with those of Arabidopsis. However, most haustoria in Pjtein2 passed around the rice roots without stopping apex cell division, as seen in Arabidopsis infection. These observations indicate that ethylene signaling in the parasite is crucial for the haustorium apex cells to differentiate into intrusive cells for host invasion.

**Parasitic plants use host ethylene for invasion**

Next, we examined whether parasite- or host-derived ethylene contributes to invasion. Consistent to the mutant phenotypes, application of the ethylene signaling inhibitor AgNO3 completely suppressed the host invasion (Fig. 5, A and B). Application of ethylene biosynthesis inhibitors AVG and AIB reduced frequency of successful haustorium invasion, suggesting that ethylene production in either the parasite or host is involved in invasion processes (Fig. 5, A and B). Because the same concentration of these inhibitors did not inhibit primary root growth (Fig. S10B), the reduced invasion levels by these inhibitors are not due to toxicity of the treatment. Unlike AgNO3, AVG- and AIB-treated haustoria stopped their elongation at the host root surface (Fig. 5A), suggesting that stoppage of haustorium apex cell division and differentiation to intrusive cells can be uncoupled, although both processes are mediated by ethylene signaling. Invasion by P. japonicum was 35% lower when infecting the Arabidopsis ethylene biosynthesis mutant defective in seven isoforms of ACSs (heptuple) (32) than when infecting WT Arabidopsis. (Fig. 5C). Arabidopsis etr1 and ein2, but not constitutively active ethylene signaling ctr1, were also less invaded (Fig. 5C) to a similar level as the heptuple ACS mutant. These may indicate that the host ethylene signaling is required for parasite invasion. Alternatively, excess amount of ethylene production in hosts may prevent parasite invasion. It was previously shown that ethylene production in Arabidopsis is enhanced in etr1 and ein2 due to the negative feedback

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**Fig. 5. Host ethylene production and signaling contribute to parasite invasion.** (A) Representative images of noninvaed haustoria of WT P. japonicum on WT Arabidopsis in the absence (control) or presence of ethylene biosynthesis (AVG and AIB) and signaling inhibitors (AgNO3) after 3 days of infection. Scale bars, 200 µm. (B) Invasion level of WT P. japonicum haustoria on Arabidopsis in the presence of ethylene biosynthesis and signaling inhibitors after 3 days of infection. Data represent the mean ± SE (n ≥ 14) from three independent experiments. (C) Invasion level of WT P. japonicum haustoria on Arabidopsis WT and the ethylene biosynthesis (heptuple), ethylene overproducing (eto1), ethylene signaling (etr1 and ein2), or constitutively activated ethylene signaling (ctr1) mutants after 3 days of infection (n ≥ 27). AVG, 5 µM; AIB, 1 mM; AgNO3, 0.1 µM. *P < 0.05 and **P < 0.01, Student’s t test, unpaired, two-tailed, compared to control or WT.
regulation of ethylene biosynthesis (36, 37). Ethylene-overproducing mutant eto1 exhibited 15% lower invasion rate than did the WT, although not significantly. The reduced invasion rate might be associated with the negative role of exogenous ethylene on haustorium development as shown in Fig. 2 (D to F). These suggest that very localized ethylene production of host and its perception in parasite could be essential for invasion.

**Haustorium apex cell regulation to archive successful invasion**

Why do parasitic plants regulate haustorium apex growth and cell differentiation using ethylene signaling? We postulate that parasitic plants use this system to reach the root surface of hosts to start invasion at the precise point. Root extracts of *Arabidopsis* and rice occasionally induce long haustoria in *WT P. japonicum* (Fig. 6A). Elongated haustoria are also observed when a *P. japonicum* root is placed on the same agar plate in close proximity to a host, indicating that diffusible signals from the host promote haustorium elongation. These elongated haustoria can eventually infect hosts when they reach hosts (Fig. 6A). Exogenous application of ethylene prevented haustorium elongation induced by host root extracts, indicating that ethylene is a suppressor for haustorium elongation (Fig. 6B and C). Thus, parasitic plants initiate haustorium development when they detect HIFs and maintain haustorial apex growth until they locate the exact position where a host produces ethylene (Fig. 6D).

This study represents the first identification of parasitic plant genes mediating host invasion using forward genetics. Our findings reveal how a parasitic plant uses the ethylene molecule to fine-tune haustorium development and host invasion. Note that in *Striga* genus, which relies on host-derived strigolactones for germination, ethylene production and signaling play important roles in seed germination at downstream of strigolactone signaling (38, 39). Exogenous ethylene application was used for suicidal germination to eradicate *S. asiatica* in the United States invaded in the 1950s (40). The severe germination defects in *Pjetr1* and *Pjein2* mutants confirmed that ethylene is also involved in germination of a parasitic species whose germination does not rely on strigolactones. Together, it appears that parasitic plants have co-opted the ethylene signaling for the parasitism at multiple steps of their life cycle, i.e., germination, haustorium growth termination, and host invasion. These knowledge expand the potential use of ethylene and ethylene inhibitors to control a wider range of parasitic weeds including facultative parasites by manipulating haustorial function.

**MATERIALS AND METHODS**

**Plant materials and growth condition**

*P. japonicum* (Thunb.) Kanitz ecotype Okayama was used as WT (41). The mutants *Pjetr1* and *Pjein2* were isolated from M2 seed pools of EMS-mutagenized *P. japonicum* as previously described.
(21). Arabidopsis ecotype Columbia (Col-0) accession was used as Arabidopsis WT. Arabidopsis heptuple (acs1-1acs2-1acs4-1acs5-2acs6-1acs7-1acs9-1) described previously (32), ein2-1 (42), ctr1-3 (15), and ctr1 (18) mutants were published previously. The heptuple mutant (CS16650) was obtained from the Salk Institute, and ctr1-3 and ctr1 were gifts from Y. Saijo and K. Hiruma at the Nara Institute of Science and Technology. Rice used in this study was Oryza sativa L. subspecies japonica, cv. Koshihikari. S. hermonthica (Del.) Benth seeds were provided by A. G. T. Babiker (Sudan). Surface sterilization of P. japonicum, S. hermonthica, rice, and Arabidopsis seeds was described previously (43). Surface-sterilized Arabidopsis seeds were sown on one-half-strength Murashige and Skoog (MS) medium (Wako Chemicals) with 1% (w/v) sucrose and 0.8% (w/v) agar and incubated at 4°C for 1 to 2 days in the dark before transferring the dishes to 25°C. The WT, Pjetr1, and Pjein2 P. japonicum seeds were sown on one-half-strength MS medium with 1% (w/v) sucrose and 0.8% (w/v) agar and incubated at 4°C for at least 1 week. The P. japonicum seeds were germinated under dark conditions for 3 days at 25°C and grown at 25°C with a photoperiod of 16-hour light (100 μmol m⁻² s⁻¹) and 8-hour dark. Arabidopsis seedlings were grown at 22°C under continuous light conditions. Surface-sterilized rice seeds were germinated and grown on a sterile filter paper in 9-cm-diameter petri dishes filled with 15-ml sterile water at 25°C with a photoperiod of 16-hour light and 8-hour dark. Surface-sterilized S. hermonthica seeds were preconditioned and germinated in the dark at 25°C as described previously (43).

ACC, ethephon, and ethylene treatments
To investigate P. japonicum root responses to ethephon and ACC, the surface-sterilized seeds were directly sown on growth medium supplemented with ethephon (Wako Chemicals) or ACC (Sigma-Aldrich) at the indicated concentrations and grown vertically in the dark for 3 days. For ethylene application, 10-day-old P. japonicum seedlings grown on growth medium in square petri dishes were treated with ethylene gas treatment. Two small holes were made in the lids of the petri dishes containing plants, and the holes and sides of the petri dishes were sealed with surgical tape to ensure air flow. The petri dishes were placed vertically inside a 9-liter plastic container, and ethylene gas (Japan Fine Products) was injected directly into the container. Root tips before and after ethylene treatment were marked, and root growth was observed with a stereomicroscope (Zeiss Stemi 2000-C).

Haustorium induction assay
The haustorium induction procedure and preparation of host root extracts were described previously (43). Briefly, 1-week-old P. japonicum seedlings were transferred to nutrient-free 0.8% (w/v) agar medium for starvation treatment for 3 days. Subsequently, seedlings were transferred to 0.8% (w/v) agar medium containing DMBQ (Sigma-Aldrich) with or without ethylene inhibitors and grown vertically for 7 or 10 days for haustorium induction. Ethylene inhibitors used in this study are as follows: AgNO₃ (Wako Chemicals), AVG (Sigma-Aldrich), AIB (Tokyo Chemical Industry), 2-picolinic acid (Sigma-Aldrich), pyrazinecarboxylic acid (Sigma-Aldrich), pyrazinamide (Sigma-Aldrich), and 1-MCP (AFxRD-0014, AgroFresh). For gas application, seedlings transferred to DMBQ medium were immediately placed inside a 9-liter plastic container followed by injection of ethylene or 1-MCP. In the case of S. hermonthica, germination was induced by 10 nM strigol for 24 hours, and the seedlings were then transferred to a 48-well plate containing water supplemented with DMBQ and ethylene inhibitors and incubated for 48 hours. Haustoria were observed using a stereomicroscope (Zeiss Stemi 2000-C) and a light microscope (Leica DMI3000 B). Haustorium formation and root surface at the opposite side of the haustorium and was measured by ImageJ software (https://imagej.nih.gov/ij/).

Time-lapse movie of host infection
Surface-sterilized rice seeds ( cvs Koshihikari) were germinated on the 0.8% (w/v) agar for 7 days, after which seedlings were transferred individually onto a thin layer of 0.8% (w/v) agar in a square petri dish. A 2-week-old P. japonicum seedling was placed next to each rice seedling, and the P. japonicum root tip was carefully aligned in parallel with the rice root, leaving approximately 0.5- to 1-mm distance between two roots. A cover glass was then placed on the top of the roots. The lid of the petri dish was closed and sealed with vinyl tape. Time-lapse images were taken from the bottom of the petri dish at intervals of 30 min for a period of 3 days under continuous light conditions at 25°C using a Leica M165 FC stereoscope. The images were processed into a movie using ImageJ software.

Xylem staining
For xylem cell staining, haustoria were excised from roots, immersed in 10% (w/v) KOH (Wako Chemicals, Osaka, Japan) for 15 min at 90°C, rinsed three times with water, immersed in 0.1% (w/v) Safranin O (Wako Chemicals, Osaka, Japan) solution for 5 min at 90°C, and rinsed three times with water. The stained haustoria were cleared by soaking samples in clearing solution [chloral hydrate (2.5 g ml⁻¹) (Nacalai Tesque), 33% (v/v) glycerol] from 3 hours to overnight before observation. Safranin O-stained xylem cells were observed with a light microscope (Leica DMI3000 B) and an LSM700 laser-scanning confocal microscope (Carl Zeiss) with excitation and emission wavelength suitable for fluorescent images of red fluorescent protein (RFP).

Host infection
P. japonicum seedlings were grown on growth medium for 7 days and subsequently on nutrient-free 0.8% (w/v) agar for 3 days before infection. Four-day-old Arabidopsis seedlings were carefully placed next to P. japonicum seedlings such that the P. japonicum root tip was touching an Arabidopsis root at 1 cm above the tip to allow efficient infection. One-week-old rice seedlings (cv. Koshihikari) grown vertically on 0.8% (w/v) agar medium were infected by P. japonicum seedlings by placing the root top of P. japonicum next to a rice root. Infection was assayed on 0.8% (w/v) agar medium at 25°C with a light/dark cycle of 16 hours/8 hours. Ethylene inhibitors were added to the 0.8% agar medium during infection. To compare the same-aged haustoria in each experiment, haustoria formed at 1 day after infection were marked by mark pen on the back side of the petri dish and only marked haustoria were subjected to subsequent invasion percentage calculation. Root segments of Arabidopsis and P. japonicum contacting sites were finally excised and stained with Safranin O. Upon staining, intrusive cells showed deep red color and elongated palisade shape at the haustorium tip inside host root, and these characteristics were used as a marker for successful invasion. The invasion level was calculated by dividing the number of invaded haustoria by the total number of host-attached haustoria in each plant and shown as percentages.
Tissue sections and toluidine blue staining
Root segments (about 3 mm) containing haustoria were fixed, embedded in Technovit 7100 (Heraeus Kulzer), and sectioned to a 6- to 8-μm thickness by following a previous report (21). Sections were incubated with 0.01% (w/v) toluidine blue (Sigma-Aldrich) for 2 min, washed once with water, and observed with a light microscope (Leica DMi3000 B).

EdU staining
Haustoria induced by 10 μM DMBQ were incubated in a 10 μM EdU solution (Click-iT EdU Imaging Kits, C10337, Invitrogen) in water for 30 min at 25°C. Tissue fixation and cell permeabilization were performed according to the manufacturer’s protocols. The EdU-stained haustoria were detected with a confocal microscope (Zeiss LSM700) with excitation and emission wavelengths suitable for green fluorescent protein (GFP).

Plasmid construction
For complementation of Pj ein2, genomic DNA of PjeIN2 including the native promoter and the 3′ untranslated region (UTR) with a total length of 10,535 bp was amplified by polymerase chain reaction (PCR) using primers 5′-CTAGAGGA1CCCGGGA-TAATAGTTGAGGGGAGAGTG-3′ and 5′-AAGAATTC-GAGCTCGGACGTCCTATTTGACTTACCAACA-3′, inserted into the binary vector pRedRoot that contains a constitutively expressed UBQ-DsRED construct (44), and linearized by Kpn I using the In-Fusion HD Cloning Kit (Takara Bio). DsRED fluorescence was used as a visible marker for hairy root transformation. The DR5:3xVENUS-NLS construct was provided by E. Meyerowitz and described previously (4).

Hairy root transformation in P. japonicum
All constructed plasmids were transformed into Agrobacterium rhizogenes strain AR1193 or LBA1334. Hairy roots were induced by A. rhizogenes by a previously described protocol (45). Three to 4 weeks after transformation, the hairy roots together with whole plants were transferred to 0.8% (w/v) agar medium for 2 days and subjected to the haustorium induction assay on agar medium containing 10 μM DMBQ. Transformed hairy roots were selected by marker expression.

Construction of the P. japonicum reference sequence
Genomic DNA was extracted from leaves of P. japonicum inbred lines grown on soil for 1 month using the cetyltrimethyl ammonium bromide (CTAB) method as described previously (46). Briefly, the WT reference genome was sequenced with 98.3 times coverage with different insert lengths of Illumina libraries and 10.3 times coverage of PacBio sequencer long reads. The sequencing libraries were prepared using a TruSeq pair-end library kit (Illumina) for insert sizes of 120, 250, and 500 bp and a Nextera mate pair library preparation kit (Illumina) for 5-, 10-, 15-, and 20-kb mate-pair libraries according to the manufacturer’s instruction (table S1). Sequencing reactions were run with 101 or 151 cycles using either HiSeq 2000 or HiSeq 2500 sequencers. Low-quality reads of paired-end sequences were trimmed and filtered using the FASTX toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) with fastq_quality_trimmer function with -t 125 –t 30 options. For mate-pair library sequences, the sequence reads were trimmed to 50 bp length with the fastx_trimmer function, followed by the fastq_quality_trimmer with option -l 45 and –t 30. Quality-filtered reads were assembled with allpaths-lg (version R48894) (47). The PacBio libraries were constructed with DNA template prep kit 2.0 (3 to 10 kb) and sequenced using C2 chemistry with standard or XL polymerase. The sequence reads were error-collected with the program sprai (https://anaconda.org/bioconda/sprai) and gap-filled the allpaths-lg assembled genome using PB jelly (48). Genome annotation was carried out using MAKER-P software (version 2.27) (49). At first, a repeat library was constructed and repeat sequences were masked using LTRharvest (50), MITE-Hunter (51), and Repeat modelar (Smit, AFA, Hubley, R. RepeatModeler Open-1.0. 2008-2015; http://www.repeatmasker.org). Transcriptome data obtained from 1 and 7 days after rice infection in rhizotron systems were mapped to the genome assembly by TopHat2 (version 2.0.13) (52), and the alignments were used as a training dataset for AUGUSTUS (53) and GeneMark-ET (54). Putative gene function was assigned using the Trinotate program (55).

Whole-genome resequencing of the mutant populations
For Pjein2, a single leaf from each of 50 plants of an M5 homozygous mutant line and 50 plants of F2 plants with the elongated haustorium phenotype after one backcross were harvested and combined to make a Pjein2 M pool and a BC1F2 pool, respectively. For Pj etr1, leaves from 25 plants with the elongated haustorium phenotype from M4, M5, BC1F3, and BC1F5 progenies were harvested to make a Pj etr1 M/F pool. These plants are potentially a mixture of homozygous and heterozygous genotypes. Genomic DNA was extracted from each pool using the CTAB method. Paired-end Illumina libraries were prepared using a TruSeq library preparation kit with an average insert size of approximately 550 bp. Sequencing reactions of both ends were run for 251 cycles using HiSeq 2500 sequencers.

Identification of SNPs for Pj etr1 and Pjein2
CLC Genomics Workbench software (version 8) was used for quality filtering, mapping to reference, and detection of SNPs. Four samples of pooled DNA were analyzed, of which the Pj etr1 M/F pool, the Pjein2 M pool, and the Pjein2 BC1F2 pool were prepared as described above. The remaining group was derived from an unrelated mutant, where the DNA was extracted from 50 mutant pools of F2 progeny and designated as an “unrelated mutant pool” (see also figs. S2 and S5) to use for nonspecific background SNP subtraction. Quality of reads was checked by FastQC (56). Reads with a quality score lower than 0.05 and lengths shorter than 50 bp were removed and further trimmed by removing five nucleotides from the 5′ end and 70 nucleotides from the 3′ end, resulting in average read lengths of 175 bp. Paired reads from four groups were mapped to P. japonicum WT reference genome assembly version 1 with parameters of mismatch cost at 2, length fraction at 0.6, and similarity fraction at 0.9. Low-stringency SNPs in each of four groups were called with parameters of minimum coverage 2, minimum count 2, and minimum frequency 10% and used for removing background SNPs across different groups through comparison. High-stringency SNPs in the Pjein2 M pool and the BC1F2M/F pool were called with parameters of required variant probability 90%, minimum frequency 90%, minimum coverage 5, and minimum count 5. The same parameters were applied to high-stringency SNPs in the Pj etr1 M/F pool except for a minimum frequency at 40% because collected plants are potentially mixtures of homozygous and heterozygous plants. Background SNPs (noise) were removed.
by subtracting each of the high-stringency SNPs with low-stringency SNPs appearing in other remaining groups. The resulting SNPs were considered as “unique SNPs” and subsequently annotated onto transcripts in the reference P. japonicum genome to analyze nonsynonymous mutations (nonsynonymous SNPs).

**Phylogenetic tree construction**
The amino acid sequences from each species were obtained from ClustalW (57). Maximum likelihood phylogenetic trees were estimated by RAxML software (58) using GAMMAAUTO model. Bootstrap values were calculated as percentage of 1000 repetitions.

**SUPPLEMENTARY MATERIALS**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/

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Acknowledgments: We thank Y. Yoshimura for technical assistance, M. Kasahara at Tokyo University for helpful advice, AgroFresh for providing 1-MCP, Y. Sajo at the Nara Institute of Science and Technology for providing Arabidopsis ethylene-related mutants, A. G. T. Babiker for providing Striga seeds, K. Mori for providing strigol, and E. Meyerowitz for providing the DR5:3×VENUS-NLS construct. Computations were partially performed on the NIG supercomputer at the ROIS National Institute of Genetics. Funding: This research was partially supported by MEXT KAKENHI grants (nos. 17K1542 to S.C.; 22128008 to T.N.; 24128001 to M.H.; 15H01246, 25711019, 18H02464, and 18H04838 to S.Y.; and 15H05959 and 17H06172 to K.S.), JST PRESTO (IPMU1PR194D to S.Y.), and MEXT/JSPS KAKENHI grant numbers 22150002 and 16H06279 (PAGS). Author contributions: S.C., K.S., and S.Y. conceived the project. S.Y. isolated the original mutants and assembled and annotated the genome. T.N., T.F.S., S.S., and A.T. performed the EdU experiments. S.C. performed the remaining experiments. S.Y. sequenced the transcriptome and conducted the remaining experiments. S.Y. and K.S. wrote and revised the manuscript. All authors read and revised the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: The plant materials used in this study are available from the corresponding author upon reasonable requests. Sequence reads generated in this study have been deposited in DDBJ with accession numbers BMAC01000001–BMAC01010559 and also available at Dryad database (https://doi.org/10.5061/dryad.vt4b8gtpt). Additional data are available from authors upon request.

Submitted 14 April 2020
Accepted 10 September 2020
Published 28 October 2020
10.1126/sciadv.abc2385

Citation: S. Cui, T. Kubota, T. Nishiyama, J. K. Ishida, S. Shigenobu, T. F. Shibata, A. Toyoda, M. Hasebe, K. Shirasu, S. Yoshida, Ethylene signaling mediates host invasion by parasitic plants. Sci. Adv. 6, eabc2385 (2020).