A pair of effectors encoded on a conditionally dispensable chromosome of *Fusarium oxysporum* suppress host-specific immunity

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Many plant pathogenic fungi contain conditionally dispensable (CD) chromosomes that are associated with virulence, but not growth in vitro. Virulence-associated CD chromosomes carry genes encoding effectors and/or host-specific toxin biosynthesis enzymes that may contribute to determining host specificity. *Fusarium oxysporum* causes devastating diseases of more than 100 plant species. Among a large number of host-specific forms, *F. oxysporum* f. sp. *conglutinans* (*Focn*) can infect Brassicaceae plants including *Arabidopsis* (*Arabidopsis thaliana*) and cabbage. Here we show that *Focn* has multiple CD chromosomes. We identified specific CD chromosomes that are required for virulence on Arabidopsis, cabbage, or both, and describe a pair of effectors encoded on one of the CD chromosomes that is required for suppression of Arabidopsis-specific phytoalexin-based immunity. The effector pair is highly conserved in *F. oxysporum* isolates capable of infecting Arabidopsis, but not of other plants. This study provides insight into how host specificity of *F. oxysporum* may be determined by a pair of effector genes on a transmissible CD chromosome.

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Pathogenic fungi often carry chromosomes that are not necessary for growth in the non-pathogenic state. Analogous to the well-characterized virulence plasmids in bacteria, the number of these dispensable chromosomes in individual isolates can vary. In plant pathogenic fungi, dispensable chromosomes that are associated with virulence are generally referred to as supernumerary, ‘B’, or conditionally dispensable (CD) chromosomes. When pathogenic fungi lack CD chromosomes, they can grow in vitro, but often exhibit attenuated or no virulence. The functions of CD chromosomes in some plant pathogenic fungi are associated with suppression or deactivation of host-specific factors. In Fusarium solani, for example, a CD chromosome carries phytoalexin detoxifying genes. In contrast, the CD chromosomes of Alternaria alternata and Cochliobolus carbonum harbor host-specific toxin genes. Therefore, CD chromosomes can be crucial determinants of host specificity that are defined by phytoalexin activity or by defense against chemicals such as phytoalexins.

Fusarium oxysporum causes devastating diseases of more than 100 plant species, including economically important crops such as tomato, banana, and melon. Individual isolates of F. oxysporum have different host ranges and are classified into formae speciales (ff. spp.) based on the susceptibility of plant species to infection. Although much is known about the genetics and pathology of F. oxysporum, the precise molecular mechanisms of host specificity remain unclear. So far, CD chromosomes have been identified in the tomato-infecting pathogen F. oxysporum f. sp. lycopersici (Fol), and in F. oxysporum f. sp. radicis-cucumerinum (Forc), a cucubit-infesting pathogen. The Fol isolate 4287 and the Forc isolate 016 each contain a single virulence-associated CD chromosome that is transferable to other isolates. Horizontal transfer of the CD chromosomes from Fol4287 or Forc016 converts non-pathogenic F. oxysporum isolates into pathogens of their respective hosts. Part of this phytopathyigenic conversion is often due to the expression of CD-encoded effectors that modulate host immunity against infection, such as Secreted In Xylem (SIX) effectors that are, as their name indicates, secreted into xylem elements during infection. A total of fourteen SIX genes (SIX1 to 14) have been identified from Fol10. The CD chromosome of Fol4287 contains all of the SIX genes except SIX4, which is not present in Fol4287, but is present in certain other Fol isolates. The CD chromosome of Forc016 contains SIX6, SIX9, SIX11, and SIX13 homologs. SIX1, SIX3, SIX5, and SIX6 from F. oxysporum are involved in overcoming resistance in tomato and the SIX6 homolog from Forc016 is crucial for virulence in cucumber. However, their molecular mechanisms as virulence factors are as yet unknown.

Arabidopsis-infecting isolates of F. oxysporum are useful as a model pathosystem. There are at least three ff. spp. that cause disease on Arabidopsis: F. sp. conglutinans, F. sp. matthiolae, and F. sp. raphani. F. oxysporum f. sp. conglutinans (Foc) can also infect other Brassicaceae plants such as cabbage (Brassica oleracea var. capitata). The SIX1 gene is required for full virulence on cabbage in Foc13, but the Focn factor(s) that are required for virulence on Arabidopsis have not been identified. We have previously shown that the Focn isolate Cong:1-1 (FocnCong:1-1) harbors SIX1, SIX4, SIX8, and SIX9 homologs on multiple chromosomes of different sizes. Although these chromosomes are presumed to be conditionally dispensable in Focn, their status as CD chromosomes has not been established.

Here we report, through analyses of chromosome-deficient FocnCong:1-1 strains and through horizontal chromosome transfer, that FocnCong:1-1 has multiple CD chromosomes. Importantly, we identified individual CD chromosomes that are required for virulence on Arabidopsis, cabbage, or both. Furthermore, we identified a pair of effector genes on a CD chromosome that are required for suppression of Arabidopsis-specific phytoalexin-based immunity.

**Results**

**Chromosome-level genome assembly of FocnCong:1-1.** We assembled the FocnCong:1-1 genome sequence into 198 contigs with an N50 of 1.271 Mb. To improve contiguity, we further performed optical mapping using two restriction enzymes. The final assembly consisted of 22 scaffolds (SCs) with an N50 SC length of 4865 kb and a 99.1% complete BUSCO score (Table 1). For gene prediction, we generated transcriptome data from axenic culture and plant infections, resulting in a total of 21,781 genes, among which are eight presumptive effector genes (SIX1, SIX4, SIX8, SIX9, and FOA1-FOA4) that were previously known from Arabidopsis-infecting F. oxysporum, as well as the homologous genes of FOA1 and FOA4, which were named FOA1b and FOA4b, respectively. We did not detect homologs of any other SIX genes. To find unknown effectors, 1467 putative secreted proteins were screened for proteins with an effector-like structure using the EffectorP v1 and/or v2 algorithm. A total of 263 secreted proteins were predicted as effectors by both EffectorP v1 and v2. This prediction did not include FOA1, which is involved in the suppression of pattern-triggered immunity, nor its homolog FOA1b. Therefore, a total of 265 proteins, including FOA1 and FOA1b, were defined as high-confidence effector candidates (Table 1 and Supplementary Data 1).

The F. oxysporum genome is composed of core genomic regions that are conserved among Fusarium species, and additional accessory genomic regions that are conserved in certain isolates. Comparative analysis with the Fol4287 genome as a reference indicated that (i) the FocnCong:1-1 SCs have no homology with known accessory genomic regions in Fol4287 (chr01b; chr02b; chr03; chr06; chr14; chr15), (ii) similarly, there are genomic regions of FocnCong:1-1 that have no homology with Fol4287, and (iii) the non-homologous genomic regions are enriched in transposable elements (TEs) (Fig. 1a). All known effector genes except FOA4 are located in the TE-rich genomic region in FocnCong:1-1 as follows: SIX1 (in SC8), SIX4 (SC9), SIX8 (SC10), SIX9 (SC9), FOA1 (SC5), FOA1b (SC10), FOA2 (SC9), FOA3 (SC3), and FOA4b (SC10) (Supplementary Fig. 1). FOA4 (SC12) may be a pseudogene since it is not expressed either in vitro or in planta (Supplementary Data 1 and 2). TEs are

**Table 1 FocnCong:1-1 genome statistics.**

| Statistics                  | Value                  |
|-----------------------------|------------------------|
| Assembly stats              |                         |
| Assembly size (Mb)          | 68.8 (72.2)<sup>a</sup>|
| No. of scaffolds            | 22                     |
| No. of non-scaffolded contigs | 125                  |
| Max scaffold length (kb)    | 7,006                  |
| NS0 scaffold length (kb)    | 4,865                  |
| GC content (%)              | 48.40                  |
| BUSCO coverage (%)          | 99.1                   |
| Gene models                 |                         |
| Total no. of genes          | 21,781                 |
| Total no. of proteins       | 22,094                 |
| No. of genes encoding secreted proteins | 1,467                  |
| No. of high-confidence effector candidates: | 265<sup>c</sup> |
| Predicted by both EffectorP v1 and v2 | 263       |
| Predicted by either EffectorP v1 or v2 | 193       |

<sup>a</sup>The number in parentheses is total assembly size including non-scaffolded contigs.

<sup>b</sup>FOA1 and FOA1b are added to the list of effector candidates predicted by both EffectorP v1 and v2.

<sup>c</sup>Note that some genes encode identical amino acid.
suspected to be involved in the generation of genomic variations leading to environmental adaptation and, in the case of pathogens, they may have been involved in the acquisition of the ability to infect particular hosts. Therefore, chromosomes containing TE-enriched genomic regions have a high potential to be CD chromosomes.

Recently, a chromosome-level genome assembly of the Arabidopsis-infecting F. oxysporum isolate Fo5176 was reported. The genomes of 4287 and Fo5176 are very similar, sharing from 93.2% to 94.3% of their total scaffold/contig lengths (>95% identity, 10 kb). Synteny analysis revealed that SC16 and SC18 of 4287 correspond to chromosome 14 (chr14) of Fo5176, and (ii) SC10 and SC20 to chr16 (Fig. 1b), indicating that these SCs constitute, or contribute to the respective chromosomes. Due to the observations (i) and (ii) above, we refer to the chromosomes carrying these sequences as chrSC16/SC18 and chrSC10/SC20 in 4287.

Fig. 1 Comparison of whole genome assemblies among Focn Cong:1-1, Fo4287, and Fo5176. Whole genome assemblies were compared between Fo4287 and Focn Cong:1-1 (a) and between Fo5176 and Focn Cong:1-1 (b). Ring A: Circular representation of the pseudomolecules. Red and dark blue indicate dispensable genomic regions in Focn Cong:1-1 and known accessory regions in Fo4287 (chr18; chr2B; chr3; chr6; chr14; chr15), respectively. Light blue indicates the remaining regions. Ring B: Distribution of transposable elements (TEs) in 50 kb windows. Proportions of sequences of respective Focn Cong:1-1 SCs associated with TEs are shown in Supplementary Fig. 1. Ring C: Syntenic regions (>95% identity, 30 kb) between Focn Cong:1-1 HS3, HS4, HS5, and HS6, and (iv) SC9, had been replaced with a hygromycin B resistance gene hph.

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Focn Cong:1-1 has multiple CD chromosomes. To identify CD chromosomes of Focn Cong:1-1, we generated chromosome-deficient strains by treatment with a mitosis inhibitor benomyl. For the parental strain, we utilized the previously generated strain Focn Cong:1-1 ΔSIX4, in which SIX4, located in SC9, had been replaced with a hygromycin B resistance gene (hph) cassette. After benomyl treatment, we obtained six hygromycin B-resistant mutants (HS1 to HS6; Supplementary Figs. 2 and 3). To confirm the loss of dispensable genomic regions, we sequenced the genomes of Focn Cong:1-1 ΔSIX4 and each of the HS mutants. As expected, Focn Cong:1-1 ΔSIX4 maintained SC9 carrying hph, but all of the HS mutants had lost SC9 (Fig. 2a, b). We also found that (i) SC3 was absent in HS2, HS3, and HS4, (ii) SC5 and SC8 were lost only in HS6, (iii) chrSC10/SC20 was missing in HS3, HS4, HS5, and HS6, and (iv) chrSC16/SC18 was lost only in HS4. In addition, duplication of SC2 and part of SC2 and SC17 occurred in HS1, HS5, and HS2, respectively (Fig. 2a).

Among the Focn Cong:1-1 HS mutants, there was no appreciable difference in colony size, but there was a significant difference in conidial formation (Fig. 2c, and Supplementary Figs. 2c and 4). Focn Cong:1-1 HS1 (ΔSIX9) showed no difference in conidial formation or virulence on either Arabidopsis or cabbage compared to the parent strain ΔSIX4, indicating that SC9 is involved in neither conidial formation nor virulence (Fig. 2c, d and Supplementary Fig. 5). Focn Cong:1-1 mutants without SC3 (HS3, HS5, and HS4) had attenuated virulence on Arabidopsis and cabbage, but also had reduced ability to form conidia (Fig. 2c, d and Supplementary Fig. 5), suggesting that SC3 positively regulates conidial formation. To our surprise, loss of chrSC10/SC20 in Focn Cong:1-1 HS5 and HS6 increased conidial formation (Fig. 2c), but reduced virulence on Arabidopsis (Fig. 2d). Interestingly, Focn Cong:1-1 HS6 (ΔSC5/SC8/SC9/chrSC10/SC20) lost virulence on both Arabidopsis and cabbage, whereas HS5 (ΔSC9/chrSC10/SC20) retained virulence on cabbage, but not on Arabidopsis (Fig. 2d). These data indicate that chrSC10/SC20 is required for disease progression on Arabidopsis, but that SC5 and/or SC8 are involved only in causing disease in cabbage. Therefore, SC3, SC5, SC8, and chrSC10/SC20 are CD chromosomes affecting disease levels, with SC3 and chrSC10/SC20 being also associated with conidial formation.

Focn Cong:1-1 CD chromosomes are transferable. We investigated whether Focn Cong:1-1 CD chromosomes are transferable under laboratory conditions, and what their effect might be on virulence. Focn Cong:1-1 HS6 lost multiple virulence-associated CD chromosomes (SC5, SC8, and chrSC10/SC20) along with virulence on both Arabidopsis and cabbage (Fig. 2). Strain HS6

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could therefore be used to determine the effects of chromosome transfer on virulence. A phleomycin-resistant FocnCong:1-1 HS6 strain (HS6-BLE) was generated by introducing the phleomycin resistance gene (ble) (Supplementary Figs. 6a and 7). We co-incubated FocnCong:1-1 HS6-BLE with hygromycin B-resistant FocnCong:1-1 ΔSIX4 as a donor and selected four colonies (HCT1 to HCT4) that were resistant to both phleomycin and hygromycin B. There was no apparent difference in morphology or colony size (i.e., growth rate) in any of the presumptive FocnCong:1-1 recipients (HCT1 to HCT4; Supplementary Fig. 6b). We confirmed chromosome transfer by contour-clamped homogeneous electric field (CHEF) electrophoretic karyotyping as well as PCR (Fig. 3a and Supplementary Figs. 6–8). SIX1 (in SC8) and hph (in SC9) were detected in all FocnCong:1-1 recipients, whereas SIX8 (in SC10) and an SC20 marker (FocnCong_v011766) were detected only in HCT1 (Supplementary Fig. 6a). We did not detect the SC5 marker FocnCong_v011766 in any recipient (Supplementary Fig. 6a). These results indicate that at least SC8, SC9, and chrSC10/SC20 are transferable. Conidial formation of the three FocnCong:1-1 recipients HCT2, HCT3, and HCT4, which acquired SC8 and SC9, was comparable to that of HS6-BLE. In contrast, FocnCong:1-1 HCT1, which received chrSC10/SC20, SC8, and SC9, produced significantly fewer conidia than HS6-BLE, a phenotype similar to the donor ΔSIX4 (Fig. 3b). Because SC8 and SC9 are not involved in conidial formation (Fig. 2c), these results suggest a negative involvement of chrSC10/SC20 in conidial formation. All FocnCong:1-1 recipients (i.e., HCT1 to HCT4) that acquired SC8 and SC9 also regained virulence against cabbage (Fig. 3c and Supplementary Fig. 9). Because SC9 is not involved in virulence (Fig. 2d), we conclude that SC8 is necessary and sufficient for virulence to cabbage. In the case of Arabidopsis, FocnCong:1-1 HCT1 showed higher virulence than the other recipients (HCT2, HCT3, and HCT4; Fig. 3c and Supplementary Fig. 9). Taken together with the pathology results of the chromosome-deficient FocnCong:1-1 mutants (HS1 to HS6; Fig. 2), it is likely that chrSC10/SC20 is required for virulence on Arabidopsis.

**ChrSC10/SC20 is involved in suppression of CYP79B2/CYP79B3-mediated immunity.** A CD chromosome from the Arabidopsis-infecting anthracnose fungus Colletotrichum higginsianum has been reported to be involved in suppression of plant immunity that is dependent on tryptophan (Trp)-derived secondary metabolites. We investigated whether CD chromosomes of FocnCong:1-1 encode products that also suppress specific immunity. For this experiment, we used the Arabidopsis double mutant cyp79b2/cyp79b3 that lacks the ability to synthesize Trp-derived secondary metabolites. Among the chromosome-deficient FocnCong:1-1 mutants (HS2 to HS6) with attenuated virulence to Arabidopsis Col-0 WT, only FocnCong:1-1 HS5 (ASC9/chrSC10/SC20) showed the same level of virulence on...
cyp79b2/cyp79b3 plants as was observed for its parent strain ΔSIX4 (Fig. 4a and Supplementary Fig. 10a, b). These results suggest that chrSC10/SC20 plays a key role in suppressing Trp-derived secondary metabolite-dependent immunity. FocnCong:1-1 WT, ΔSIX4, HS6, HS6-BLE, and HCTs. Asterisks indicate chromosomes on which SIX genes are located. *SIX4, **SIX8, ***SIX10. The table indicates the scaffold (SC) patterns confirmed by PCR as shown in Supplementary Fig. 6a. + and - represent maintained- and lost-SCs, respectively. SIXs located on SCs are shown in parentheses. a Conidial formation in FocnCong:1-1 WT, ΔSIX4, HS6, HS6-BLE, and HCTs. OD600 of conidial suspension was measured from six colonies after 17 days of incubation on potato dextrose agar. Results of three independent experiments were combined and a total of 18 biological replicates are plotted. Boxplots indicate median value, estimated 25th and 75th percentiles, and whiskers represent 1.5 times the interquartile range. Asterisks represent significant differences from FocnCong:1-1 H56-BLE (*p < 0.0001, Welch's t-test). b Virulence of FocnCong:1-1 WT, ΔSIX4, HS6, H56-BLE, and HCTs on Arabidopsis and cabbage. Disease index was scored as described in Methods. Results of at least two independent experiments were combined. n denotes the number of plants investigated. Asterisks represent significant difference from FocnCong:1-1 H56-BLE (**p < 0.001, *p < 0.01 Mann–Whitney U-test). Representative images of Arabidopsis and cabbage at 28 dpi are shown in Supplementary Fig. 9.

### Table: Conidial Formation and Virulence in FocnCong:1-1

| Scaffold | Marker | WT | ΔSIX4 | HS6 | HS6-BLE | HCT1 | HCT2 | HCT3 | HCT4 |
|---------|-------|----|-------|-----|---------|------|------|------|------|
| SC5     | +     | +  | +     | +   | +       |      |      |      |      |
| SC10    | +     | +  | +     | +   | +       |      |      |      |      |
| SC8     | +     | +  | +     | +   | +       |      |      |      |      |
| SC9     | +     | +  | +     | +   | +       |      |      |      |      |
| SC15    | +     | +  | +     | +   | +       |      |      |      |      |
| SC20    | +     | +  | +     | +   | +       |      |      |      |      |
| SC5 (SIX4) | + | +  | +     | +   | +       |      |      |      |      |
| SC10 (SIX8) | + | +  | +     | +   | +       |      |      |      |      |
| SC8 (SIX9) | + | +  | +     | +   | +       |      |      |      |      |
| SC9 (SIX10) | + | + | +     | +   | +       |      |      |      |      |
| SC15 (SIX11) | + | + | +      | +   | +       |      |      |      |      |
| SC20 (SIX12) | + | + | +      | +   | +       |      |      |      |      |

### Figure 3: Effects of chromosome transfer on conidial formation and virulence in FocnCong:1-1 HS6.

- **a**: Electrophoretic karyotype of FocnCong:1-1 WT, ΔSIX4, HS6, H56-BLE, and HCTs. Asterisks indicate chromosomes on which SIX genes are located.
- **b**: Conidial formation in FocnCong:1-1 WT, ΔSIX4, HS6, H56-BLE, and HCTs. OD600 of conidial suspension was measured from six colonies after 17 days of incubation on potato dextrose agar. Results of three independent experiments were combined and a total of 18 biological replicates are plotted. Boxplots indicate median value, estimated 25th and 75th percentiles, and whiskers represent 1.5 times the interquartile range. Asterisks represent significant differences from FocnCong:1-1 H56-BLE (*p < 0.0001, Welch's t-test).
- **c**: Virulence of FocnCong:1-1 WT, ΔSIX4, HS6, H56-BLE, and HCTs on Arabidopsis and cabbage. Disease index was scored as described in Methods. Results of at least two independent experiments were combined. n denotes the number of plants investigated. Asterisks represent significant difference from FocnCong:1-1 H56-BLE (**p < 0.001, *p < 0.01 Mann–Whitney U-test). Representative images of Arabidopsis and cabbage at 28 dpi are shown in Supplementary Fig. 9.

A pair of effectors are involved in virulence on Arabidopsis. Because chrSC10/SC20 is likely to encode effectors that contribute to suppression of Arabidopsis-specific immunity, we searched for genes encoding potential effectors, and found a total of twelve effector candidate genes located on chrSC10/SC20 (Supplementary Data 1). Expression profiling revealed that FocnCong_v001893 (SIX8) and FocnCong_v001894 were highly expressed during infection (Fig. 5a and Supplementary Data 1). Interestingly, SIX8 is adjacent to FocnCong_v001894, with an intergenic distance of 1057 bp on SC10 (Fig. 5b). The intergenic region contains a miniature impala inverted repeat (mimp-IR) sequence, which is related to TE sequences (Fig. 5b and Supplementary Fig. 11). A mimp-IR is also often located in the upstream regions of SIX and other effector candidate genes in Fol, Forc, and the melon-infecting pathogen F. oxysporum f. sp. melonis. To determine whether SIX8 and FocnCong_v001894 are involved in virulence on Arabidopsis, a genome fragment containing the SIX8-FocnCong_v001894 locus was introduced into FocnCong:1-1...
HS5 (ΔSC9/chrSC10/SC20) (Supplementary Figs. 12 and 13), which restored full virulence to FocnCong:1-1 HS5 (Fig. 5c and Supplementary Fig. 14a). In contrast, Arabidopsis WT was resistant to the other FocnCong:1-1 HS5 transformants that contained only SIX8 or FocnCong_v001894 (Fig. 5c and Supplementary Fig. 14a). It should be noted that virulence of knockout mutants that lack the SIX8-FocnCong_v001894 locus in FocnCong:1-1 was significantly lower than for WT (Fig. 5d and Supplementary Figs. 14–16), suggesting that both SIX8 and FocnCong_v001894 are necessary for virulence on Arabidopsis. We therefore designated FocnCong_v001894 as Pair with SIX Eight1 (PSE1).

Genetic and functional conservation of the SIX8 and PSE1 loci.

Next, we investigated whether the SIX8-PSE1 pair is conserved in Arabidopsis-infecting F. oxysporum isolates. Comparative analysis of highly contiguous and available genome assemblies of F. oxysporum isolates (Supplementary Table 1) showed that the SIX8-PSE1 locus is completely conserved in Fo5176 and in the stock-infecting pathogen F. oxysporum f. sp. matthiolae (Fomt) PHW726, which can infect Arabidopsis13,30, but not in isolates that cannot infect Arabidopsis (Fig. 6a, b). For example, the banana-infecting pathogen F. oxysporum f. sp. cubense (Focb) tropical race 4 (TR4), which threatens banana production worldwide, has SIX8 but not PSE1. In the other non-Arabidopsis-infecting isolates, except Folate287, neither SIX8 nor PSE1 is present. Folate287 has multiple copies of SIX8 and its homolog SIX8b12,23, but PSE1 is not present in the published Folate287 genome annotation6. However, we found three loci similar to the SIX8-PSE1 locus in chromosomes 2, 3, and 14 of Folate287. At these loci, adjacent to SIX8, there is a PSE1-like gene (PSL1) differing in the C-terminal 10 amino acids (Fig. 6b and Supplementary Fig. 17). Furthermore, multiple SIX8b loci contain TEs inserted into adjacent PSE1 sequences. For example, a transposase gene was found in the first intron of the PSE1 homologs in two loci of chromosome 3 and another locus in chromosome 6 (Fig. 6b and Supplementary Fig. 18). Similarly, a presumptive transposase was found immediately upstream of the potential-but-unannotated PSE1 homolog in another locus in chromosome 6 (Fig. 6b). Thus, TE insertion seems to have disrupted the PSE1 adjacent to SIX8b in Folate287.

To evaluate if the SIX8-PSE1 locus in FomtPHW726 and the SIX8-PSL1 locus in Folate287 are able to function similarly in FocnCong:1-1, we cloned these loci and transformed them into the FocnCong:1-1 HS5 mutant (ΔSC9/chrSC10/SC20), Supplementary
Figs. 19 and 20). Transformation with the *Fomt* SIX8-PSE1 locus, but not the *Fol* SIX8-PSL1 locus, restored full virulence to *Focn* Cong:1-1 HS5 in Arabidopsis Col-0 WT (Fig. 6c and Supplementary Fig. 21). These results suggest that the SIX8-PSE1 locus is functionally distinct from SIX8-PSL1 and is functionally conserved in Arabidopsis-infecting *F. oxysporum* isolates.

**Discussion**

Here we report the identification of a CD chromosome in *F. oxysporum* that is required for virulence on Arabidopsis. This CD chromosome encodes a pair of effectors (SIX8 and PSE1) that are involved in suppressing Arabidopsis-specific immunity, and are conserved in the other *F. oxysporum* isolates capable of infecting Arabidopsis. The mode of action potentially involves defense against, or suppression of, the phytoalexin camalexin. We also report that another CD chromosome is required for pathogenicity on cabbage. In addition, certain CD chromosomes are involved in conidial formation.

In plant pathogenic fungi, CD chromosomes associated with virulence are usually not involved in vegetative growth. In this sense, SC3 and chrSc10/Sc20 in *Focn* Cong:1-1 are atypical CD chromosomes that affect conidial formation (Fig. 2c). Although the reduced virulence of SC3-deficient *Focn* Cong:1-1 mutants...
virulence on cabbage 14. Consistently,
the CD chromosome chrSC10/SC20-de
chromosome SC8, which harbors a gene,
distinct virulence functions against speci
don cabbage (Fig.2d and Supplementary Fig. 5). This result may be
less virulent on Arabidopsis, but is able to develop severe disease
on Arabidopsis, because the loss of chrSC10/SC20 attenuated virulence of
Focn Cong:1-1 HS5 to WT, but not to the cyp79b2/cyp79b3 double
mutant (Fig. 4a and Supplementary Fig. 10). Thus, we conclude that
chrSC10/SC20 and SC8 are responsible for host-specific virulence on
Arabidopsis and cabbage, respectively.

Focn Cong:1-1 HS6, which
maintains the CD
Focn Cong:1-1 HS5 to WT, but not to the
HS5:empty. (*p < 0.01, Mann–Whitney U-test).
Representative images of Arabidopsis at 28 dpi are shown in Supplementary Fig. 21.

For example, Kidd et al. 32 reported that susceptibility of
cyp79b2/cyp79b3 to F. oxysporum Fol160527 was not different from WT.
Consistent with this report, our study shows that virulence of
Focn Cong:1-1 on cyp79b2/cyp79b3 is comparable to WT (Fig. 4a
and Supplementary Fig. 10). Thus, only the use of CD
chromosome-deficient mutants allowed us to uncover the involve-
ment of CYP79B2/CYP79B3 in resistance to F. oxysporum.
Furthermore, histological analysis suggests that CYP79B2/
CYP79B3-mediated immunity may be associated with inhibition
of root–stem translocation of Focn Cong:1-1 (Fig. 4b). CYP79B2/
CYP79B3 is responsible for synthesis of Trp-derived secondary
metabolites, including sulfur-containing compounds that are
characteristic of the Brassicaceae. 26. These sulfur-containing
antimicrobial compounds differ among Brassicaceae species; for
example, camalexin is produced in Arabidopsis, but not in
cabbage. 28. Our results suggest that Focn Cong:1-1 can overcome
the Arabidopsis-specific immunity conferred by PAD3, a cama-
lexin synthetic gene (Fig. 4d and Supplementary Fig. 10c), when
the CD chromosome chrSC10/SC20 that encodes the paired effec-
tors SIX8 and PSE1 is present. This pair of effectors is highly
conserved in Arabidopsis-infecting F. oxysporum isolates, but not
in other isolates (Fig. 6), thus the presence of a particular CD
chromosome that harbors these effector genes would contribute to
the determination of host specificity.

In this study, Focn Cong:1-1 HSs were generated by treatment
with the mitosis inhibitor benomyl. In the generation process, a
genome rearrangement, but not just a chromosome loss, has
occurred at least in HS1, HS2, and HS5 (Fig. 2a). We also
investigated phenotypes in an additional HS mutant with the
same karyotype as HS5 (HS5L: HS5-like mutant; Supplementary
Figs. 22 and 23). Like HS5, HS5L showed virulence on cyp79b2/
cyp79b3 and pad3 plants, but not on Col-0 WT plants. We cannot

rule out the possibility that these genome rearrangements affect phenotypes. In addition to the results of HS5L, however, the return of HS5 virulence on Arabidopsis in two independent HS5 transformants containing FocnCong1-1 SIX8-PSE1 (Fig. 5c) supports the conclusion that the SIX8–PSE1 pair is required for virulence on Arabidopsis.

We identified SIX8 and PSE1 as a gene pair adjacent but encoded on opposite DNA strands (head-to-head orientation) (Fig. 5b and Supplementary Fig. 11). Head-to-head orientation of effector genes has been documented for other SIX genes in F. oxysporum. For instance, in Fol1, a pair of effector genes SIX3 (also known as AFR2) and SIX5 are also adjacent located in a head-to-head transcriptional orientation2,3,33,34. Both SIX3 and SIX5 are required for not only full virulence in a susceptible host, but also disease resistance in tomato lines containing the resistance gene f-233–35, and the gene products are thus likely to function as a pair. The close head-to-head orientation may ensure coordinated transcription to produce both proteins at similar levels. Such system would be suitable for maintaining tight stoichiometry of two proteins in a complex. Indeed, SIX3 interacts with SIX5 in planta and data in plant cells support the idea that SIX5 movement of SIX333,34. Unlike the SIX3–SIX5 pair, however, we failed to detect direct interaction between SIX8 and PSE1 in a yeast two-hybrid assay (Supplementary Fig. 24). We cannot exclude the possibility that SIX8 indirectly interacts with PSE1, e.g., via host target(s), or the yeast system may not be suitable for detecting interactions of these proteins. Alternatively, SIX8 and PSE1 may act independently. As bioinformatic analysis of SIX8 and PSE1 protein sequences gives no known domain annotations, identification of host targets of SIX8 and PSE1 will be required to clarify functions of the paired effectors. It is also notable that disruption or loss occurs in only PSE1, but not in SIX8, in certain non-Arabidopsis infecting F. oxysporum isolates. Perhaps PSE1, but not SIX8, is recognizable in plants that carry corresponding resistance proteins, leading to its disruption or loss to avoid detection.

In this work we demonstrate that the host range of F. oxysporum depends on CD chromosomes. In this respect, it is interesting that certain isolates, such as Fol4287 and Forc016, have only a single virulence-associated CD chromosome, whereas FocnCong1-1 has multiple CD chromosomes, each of which encodes host-specific effectors. Because the FocnCong1-1 genome is very large (68.8 Mb) compared to most known interesting that certain isolates, such as Fol4287 (59.9 Mb)6 and Forc016 (52.9 Mb)7, FocnCong1-1 is likely to have expanded its host range by acquiring and maintaining additional CD chromosomes. Indeed, Masunaka et al.36 have shown that a field isolate of A. alternata carrying two putative CD chromosomes has a wide host range. In that case, host-specific toxin genes on different chromosomes determine host range36. In the case of F. oxysporum, host specificity can be determined, at least in part, by effectors, as seen in this study. Further functional analyses of the SIX8–PSE1 paired effectors and their derivatives will be needed to dissect out the molecular mechanisms underlying effector-based host specificity in F. oxysporum.

Methods

Fungal strains and plants. Fungal strains used in this study are listed in Supplementary Table 2. For pre-incubation, all strains were incubated on potato dextrose agar (PDA; Nissui Pharmaceutical Co.) at 28°C for 16 h at 28°C. Mycelia germinated from the bud cells were stored at −80°C until RNA isolation.

Arabidopsis (Col-0 wild type, pen2, pad1, cyp82c2, and cyp792c2 mutants36,37) and cabbage (cv. Shikidori and cv. Shosyu; Taki Seed) were cultured in pots containing autoclaved Super Mix A (Sakata Seed) and vermiculite (VS kakou). Arabidopsis was grown at 22°C for 10 h under light and 14 h dark in a growth chamber. Cabbage was grown in a greenhouse.

Bioassays. For evaluation of disease severity, 14-day-old Arabidopsis and cabbage cv. Shikidori roots were injured with a forceps or a plastic peg and then irrigated with 1 ml of FocnCong1-1 bud cell suspension (1 × 107 cells/ml). Inoculated Arabidopsis plants were grown at 28°C for 24 h under light and 14 h dark in a growth chamber. An Arabidopsis disease index was scored at 28 or 29 days post-inoculation (dpi) as: 0, no symptoms; 1, dwarf; 2, yellowing, root cleaving or wilting of one to a few leaves; 3, wilting of a whole plant; 4, dead. A cabbage disease index was also scored at 28 or 29 dpi as: 0, no symptoms; 1, yellowing lower leaves; 2, yellowing lower and upper leaves; 3, whole plant wilting; 4, dead.

For gene expression profiling (Supplementary Data 1 and 2), 20- or 21-day-old Arabidopsis and 17-day-old cabbage cv. Shosyu roots were irrigated with 1 ml of bud cell suspension (1 × 107 cells/ml). At 3 dpi and 10 dpi, infected roots were washed with water to remove soil. The roots were stored at −80°C until RNA isolation.

For observation of colonization of Arabidopsis by FocnCong1-1, roots of 14-day-old Arabidopsis were cut to approximately 1 cm lengths from the border between roots and stems and soaked in bud cell suspension (1 × 107 cells/ml) for 1 min, then transferred to square plates containing soil. At 12 dpi, roots were incubated for 14 days. Arabidopsis cv. Shikidori roots were injured with a forceps or a plastic peg and then irrigated with 1 ml of PDA, bud cells, and hyphae were stored at −80°C until RNA isolation.

Fungal growth assays. FocnCong1-1 strains were grown on PDA for 8 days at 28°C in the dark from a freezer stock. For measurement of colony diameter, mycelia were collected from the growing edges of colonies on sterile plastic straws and placed in the center of fresh PDA plates. After 8 days, colony diameter was measured. For quantification of conidial formation, 17-day-old colonies were soaked in 10 ml of water and scraped with a colony spreader. Conidial suspensions were filtered through a nylon mesh to remove mycelia and conidia were quantified at OD600 with a WPA CO 8000 Cell Density Meter (WPA) or by counting using haemocytometer.

Plasmid construction. Primers used for plasmid construction are listed in Supplementary Table 3. To generate SIX8–PSE1 locus complementation vectors, the FocnCong1-1 SIX8–PSE1, Forc SIX8–PSE1 and Fol SIX8–PSL1 loci were amplified from genomic DNAs of FocnCong1-1, ForcMAFF240332 and Fol4287, respectively, and cloned into pCR®8/GW/TOPO® vector using a pCR®8/GW/TOPO® TA Cloning® Kit (Invitrogen) as described by the manufacturer. To introduce these into loci into FocnCong1-1 HS5, the complementation vector containing each locus was co-transformed with pCSN43 containing an hph cassette37. For transformation vectors of SIX8 or PSE1, an hph cassette was amplified from pCSN337 and assembled with SIX8 or PSE1, which was amplified from the FocnCong1-1 SIX8–PSE1 locus complementation vector as a template, using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs) as recommended by the manufacturer. The assembled fragments were cloned into pCR®8/GW/TOPO®.

To generate the FocnCong1-1 SIX8–PSE1 locus disruption vector, the flanking regions of SIX8 and PSE1 were amplified from the FocnCong1-1 HS5 SIX8–PSE1 locus complementation vector as a template and assembled with an hph cassette using NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs). The assembled fragment was cloned into pCR®8/GW/TOPO®.

Constructs for yeast two-hybrid assays were generated from cDNAs of SIX8 and PSE1 without signal peptide sequences or a stop codon by amplification from cDNA generated from mRNA isolated from FocnCong1-1-infected Arabidopsis. Amplicons were inserted into pENTR®/D-TOPO® (Invitrogen), and then into yeast expression vectors pDEST-DB and pDEST-AD38 using Gateway® LR Clonease® II Enzyme Mix (Invitrogen) as described by the manufacturer.

Protoplast formation and transformation. For creation of GFP-labeled FocnCong1-1 strains (SIX4–GFP, HS2–GFP, and HS5–GFP), HSV transformants (HSV: SIX8–PSE1, HS5–SIX8, HS5–SIX5, HS5–Fomt SIX8–PSE1 and HSV:Forc SIX8–PSE1) and SIX8–PSE1 knockout mutants (SIX8–PSE1), which are listed in Supplementary Table 2, bud cells (4 × 109) were incubated in 80 ml of potato dextrose broth (Difco) at 80 rpm for 16 h at 28°C. Mycelia germinated from the bud cells were collected by centrifugation (1800 g, 10 min) and washed with 1.2 M MgSO4. Mycelial cell walls were digested with 25% 2% (w/v) Driselase (Sigma) and 2% (w/v) Lysing Enzymes (Sigma) in 1.2 M MgSO4 and maintained at 80 rpm for 3 h at 28°C. Protoplasts were collected by filtration with a nylon mesh and centrifugation (1500 g, 10 min), and rinsed twice with 0.7 M NaCl. The protoplasts were resuspended in STC (1.2 M sorbitol, 50 mM CaCl2, 10 mM Tris–HCl pH 7.5) and 10 μl of 10 mM ethylene glycol tetraacetic acid (EGTA) to add 150 μl of the protoplast suspension as previously described39. Transformants were selected and maintained on PDA containing...
hygromycin B (100 μg/ml) or G418 (200 μg/ml) and verified by PCR using primers listed in Supplementary Table 3. Plasmid DNA samples used for transformation are shown in Supplementary Table 4.

**Genome sequencing and assembly.** For PacBio sequencing, genomic DNA of FocnCong:1-1 was isolated using CTAB and 100/G genomic tips (Qiagen) as described in the 100 Fungal genomes project (http://100.fungalgenomes.org). The genome was sequenced on five PacBio RSII cells and assembled by the Hierarchical Genome Assembly Procedure (HGAP) v4 within SMRT Link (v5.1.0). Default values were kept and the expected genome size was set to 70 Mb. For optical mapping, genomic DNA was isolated using a Blood and Cell Culture DNA Isolation Kit (Bionano Genomics) as described by the manufacturer. Genomic DNA was labeled with an NLRs Labeling Kit (Bionano Genomics) with BpQI and BbvCI restriction endonucleases as described by the manufacturer. The labeled DNA was sequenced using a Bionano Irys platform. A library was created using two enzymes (BpQI and BbvCI) (Bionano Solve v3.2) were merged with PacBio sequence assemblies to produce long hybrid scaffolds. Completeness of gene space within the assembly was assessed through the presence of conserved single-copy genes using BUSCO (http://www.repeatmasker.org), TransposonPSI (http://transposonsource.force.net), LTR_retriever56, and LTRPred51 (https://github.com/Hajdkj/LTRpred). Sequences that were longer than 400 bp from TransposonPSI, LTR_retriever, and LTRPred were combined and used as queries for BLASTx against Repbase13 peptide sequences bound in RepeatMasker open-4.0.9.92 (http://www.repeatmasker.org).

Lastly, these sequences were used as queries for BLASTn against each fungal genome. Only sequences with more than five hits (BLASTn E-value cutoff = 1E-5) and/or with a hit to a RepBase peptide (BLASTx E-value cutoff = 1E-5) were retained for further analysis. Sequences from all sources were combined using VSEARCH v2.14.053, using 80% identity as the cutoff threshold. Consensus sequences were obtained using RepeatClassifier (from RepeatModeler open-1.0.11). Known Fusarium repeat sequences registered in Dfam_Consensus-20181026 and RepBase-20181026 were extracted, except for those that were annotated as artefacts, simple repeats, or low complexity sequences. The custom repeat library was created by combining the consensus sequences and known FocnCong:1 repeat sequences, and as used for input for RepeatMasker open-4.0.9-92. The “one code to find them all”54 was used to reconstruct repeat elements.

**Chromosome loss and transfer.** A chromosome loss experiment was performed according to VanEtten et al.22. FocnCong:1-1 ΔSIX4 was incubated in M100 medium (1% glucose, 0.3% KNO3, 6.25% salt solution) with benomyl (1.56, 3.13, or 6.25 μg/ml) at 120 spm for 4 days at 28 °C. The salt solution consists of 0.4% NaCl, 0.4% NaNO3, 0.8% trace elements (0.006% H2BO3, 0.014% MnCl2·4H2O, 0.0844% ZnSO4·7H2O, 0.004% Na2MoO4·2H2O, 0.006% FeCl3·0.4% CuSO4·5H2O, 0.04% MgSO4·H2O). Hyphae were removed with a nylon mesh, and bud cells were centrifuged at 1600 g for 10 min. Supernatants were discarded and a remnant with bud cells was spread on M100 plates containing 2% agar and 0.04% Triton X-100 (Wako), and the inoculated plate was overlaid with an autoclaved filter paper. Plates were incubated at 28 °C for 1 to 3 days, then the filter paper was transferred onto M100 medium containing hygromycin B (100 μg/ml) and incubated at 25 °C overnight. Hygromycin B-sensitive isolates were selected by comparing the plates, and then chromosome loss patterns were verified by PCR (Supplementary Fig. 2) using primers listed in Supplementary Table 3.

**Chromosome transfer experiments.** Chromosome loss patterns were verified by PCR (Supplementary Fig. 2) using primers listed in Supplementary Table 3. Chromosome loss patterns were verified by PCR (Supplementary Fig. 6) using primers listed in Supplementary Table 3.

**Contour-clamped homogeneous electric field (CHEF) gel electrophoresis.** CHEF gel plugs were made by resuspending protoplasts in STE (1 M sorbitol, 25 mM Tris-HCl pH 7.5, 50 mM EDTA). Protoplast concentration was adjusted to 4 × 10^5 cells/ml and added to the same amount of 1.2% low melting agarose gel (Bio-Rad) solution. Protoplast suspensions (2 × 10^5 cells/ml) containing 0.6% low melting agarose gel were added to 50-well dispensable mold plates (Bio-Rad). Plugs were cut into 10 × 10 mm plates, and each plate was submerged in 0.4% low melting agarose with 0.01 M Tris-HCl, 0.05 M EDTA. CHEF gel electrophoresis was done according to Inami et al.38. Briefly, 90-mm-wide CHEF gel blocks were separated on 1% SeaKem Gold Agarose (Lonza) in 0.5×TBE buffer at 4 to 7 °C for 260 h using a CHEF Mapper System (Bio-Rad). Switching buffer was stored at 4 °C delivery. CHEF gel electrophoresis was done according to Inami et al.38.

**Yeast two-hybrid assays.** For yeast two-hybrid assays, bait (pDEST-DB; DB) and prey vectors (pDEST-AD; AD) containing cDNA of SIX4, PSE1 or empty vector controls were transformed into S. cerevisiae Y8930 and Y8800, respectively, with a slight modification of the method described by Lopez and Mukhtar et al.39. Transformants carrying DB and AD were selected with synthetic defined (SD) media (0.67% yeast nitrogen base, 0.5% glucose, 0.01% adenine hemisulfate salt) supplemented with -Leu DO supplement (Clontech) (SD-Leu) and -Tryp DO supplement (Clontech) (SD-Trp), respectively. Yeast transformants were mated in liquid mating medium with SD-Trp, and spotted on SD supplemented with -Leu/-Trp/-His DO supplement (Clontech) (SD-Leu/-Trp/-His) and spotted on SD supplemented with -Leu/-Trp/-His DO supplement (Clontech) (SD-Leu/-Trp/-His). Yeast colonies were observed after 72 h incubation.
5. Edel-Hermann, V. & Lecomte, C. Current status of
4. Tsuge, T. et al. Host-selective toxins produced by the plant pathogenic fungus
7. van Dam, P. et al. A mobile pathogenicity chromosome in
4a, b, d, 5a, c, d and 6c are provided as Supplementary Data 3. Other data are available by
GEO Series accession number GSE157823. The source data underlying Fig. 2c, d, 3b, c,
GenBank under the accession RSAI00000000 (BioProject number PRJNA506492 and
Research Reporting Summary linked to this article.

References

1. Covert, S. F. Supernumerary chromosomes in filamentous fungi. Curr. Genet. 33, 311–319 (1998).
2. Soyer, J. L., Balesdent, M. H., Rouxel, T. & Dean, R. A. To B or not to B: a tale of unorthodox chromosomes. Curr. Opin. Microbiol. 46, 50–57 (2018).
3. Miao, V. P., Covert, S. F. & VanEtten, H. D. A fungal gene for antibiotic resistance on a dispensable ("R") chromosome. Science 254, 1773–1776 (1991).
4. Tsuge, T. et al. Host-selective toxins produced by the plant pathogenic fungus Alternaria alternata. FEMS Microbiol. Rev. 37, 44–66 (2013).
5. Edel-Hermann, V. & Lecomte, C. Current status of Fusarium oxysporum formae specialis and races. Phytopathology 109, 512–530 (2019).
6. Ma, L. J. et al. Comparative genomics reveals mobile pathogenicity chromosomes in Fusarium. Nature 464, 367–373 (2010).
7. van Dam, P. et al. A mobile pathogenicity chromosome in Fusarium oxysporum for infection of multiple cucurbit species. Sci. Rep. 7, 9042 (2017).
8. Vlaarzingerbroek, I., Beerens, B., Schmidt, S. M., Cornelissen, B. J. & Rep, M. Dispensable chromosomes in Fusarium oxysporum f. sp. lycopersici. Mol. Plant Pathol. 17, 1455–1466 (2016).
9. Vlaarzingerbroek, I. et al. Exchange of core chromosomes and horizontal transfer of lineage-specific chromosomes in Fusarium oxysporum. Environ. Microbiol. 18, 3702–3713 (2016).
10. de Sain, M. & Rep, M. The role of pathogen-secreted proteins in fungal vascular wilt diseases. Int. J. Mol. Sci. 16, 23970–23993 (2015).
11. Houterman, P. M. et al. The mixed xylem sap proteome of Arabidopsis leaves (A)}.
12. Schmidt, S. M. et al. Comparative genomics of Fusarium oxysporum f. sp. melonis reveals the secreted protein recognized by the Fom-2 resistance gene in melon. New Phytopathol. 209, 307–318 (2016).
13. Thatcher, L. F., Manners, J. M. & Kazan, K. Fusarium oxysporum hijacks CO1-mediated jasmonate signaling to promote disease development in Arabidopsis. Plant J. 58, 927–939 (2009).
14. Fraser-Smith, S. et al. Sequence variation in the putative effector gene SIX8 facilitates molecular differentiation of Fusarium oxysporum f. sp. cubense. Plant Pathol. 63, 1042–1054 (2014).
15. Kidd, B. N. et al. Auxin signaling and transport promotes susceptibility to the root-infecting fungal pathogen Fusarium oxysporum in Arabidopsis. Mol. Plant Microbe Interact. 24, 733–748 (2011).
16. Ma, L. S. et al. The AVR2-SIX5 gene pair is required to activate I-2-mediated immunity in tomato. New Phytopathol. 208, 507–518 (2015).
17. Cao, L., Blekemolen, M. C., Tintor, N., Cornelissen, B. J. C. & Takken, F. L. W. The Fusarium oxysporum Avr2-Six5 effector pair alters plasmodesmatal exclusion selectivity to facilitate cell-to-cell movement of Avr2. Mol. Plant 11, 691–705 (2018).
18. Houterman, P. M. et al. The effector protein Avr2 of the xylem-colonizing fungus Fusarium oxysporum activates the tomato resistance protein I-2 intracellularly. Plant J. 58, 970–978 (2009).
19. Masunaka, A. et al. An isolate of Alternaria alternata that is pathogenic to both tangerines and rough lemon produces two host-selective toxins, A. alternata Fp-1 and ACR-toxins. Phytopathology 95, 241–247 (2005).
20. Staben, C. et al. Use of a bacterial hygromycin B resistance gene as a dominant selectable marker in Neurospora crassa transformation. Fungal Genet. Rep. 36, Article 22 (1989).
21. Ahmed, H. et al. Network biology discovers pathogen contact points in host protein-protein interactomes. Nat. Commun. 9, 3213 (2018).
22. Watanabe, S. et al. Modelling of Trichoderma atroviride SKT-1, a biocontrol agent against Gibberella fujikuroi. J. Pestic. Sci. 32, 222–228 (2007).
23. Simao, F. A., Waterhouse, R. M., Ioannidis, P., Kriventseva, E. V. & Zdobnov, E. M. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31, 3210–3212 (2015).
24. Waterhouse, R. M. et al. BUSCO applications from quality assessments to gene prediction and phylogenomics, Mol. Biol. Evol. 35, 543–548 (2018).
25. Kurtz, S. et al. Versatile and open software for comparing large genomes. Genome Biol. 5, R12 (2004).
26. Ichihashi, Y., Fukushima, A., Shibata, A. & Shirasu, K. High impact gene discovery: simple strand-specific mRNA library construction and differential regulatory analysis based on gene co-expression network. Methods Mol. Biol. 1830, 163–189 (2018).
27. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 12, 357–360 (2015).
28. Hoff, K. J., Lange, S., Lomsadze, A., Borodovsky, M. & Stanke, M. BRAKER1: unsupervised RNA-seq-based genome annotation with GeneMark-ET and AUGUSTUS. Bioinformatics 33, 767–769 (2016).
29. Bairach, A. & Apweiler, R. The SWISS-PROT protein sequence database and its supplement TREMBL in 2000. Nucleic Acids Res. 28, 45–48 (2000).
30. Almagro Armenteros, J. I. et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat. Biotechnol. 37, 420–423 (2019).
31. Krogh, A., Larsson, B., von Heijne, G. & Sonnhammer, E. L. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567–580 (2001).
Author contributions
Y.A., S.A., P.G., A.T., I.Y., and A.S. conducted experiments. Y.A., S.A., K.K., P.M.H., M.R., K.S., and T.A. conceived and supervised the study. Y.A., S.A., K.S., and T.A. wrote the manuscript. All authors reviewed and approved the manuscript.

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The authors declare no competing interests.

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Author contributions
Y.A., S.A., P.G., A.T., I.Y., and A.S. conducted experiments. Y.A., S.A., K.K., P.M.H., M.R., K.S., and T.A. conceived and supervised the study. Y.A., S.A., K.S., and T.A. wrote the manuscript. All authors reviewed and approved the manuscript.

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