Colletotrichum scovillei, an ascomycete phytopathogenic fungus, is the main causal agent of serious yield losses of economic crops worldwide. The fungus causes anthracnose disease on several fruits, including peppers. However, little is known regarding the underlying molecular mechanisms involved in the development of anthracnose caused by this fungus. In an initial step toward understanding the development of anthracnose on pepper fruits, we retrieved 624 transcription factors (TFs) from the whole genome of C. scovillei and comparatively analyzed the entire repertoire of TFs among phytopathogenic fungi. Evolution and proliferation of members of the homeobox-like superfamily, including homeobox (HOX) TFs that regulate the development of eukaryotic organisms, were demonstrated in the genus Colletotrichum. C. scovillei was found to contain 10 HOX TF genes (CsHOX1 to CsHOX10), which were functionally characterized using deletion mutants of each CsHOX gene. Notably, CsHOX1 was identified as a pathogenicity factor required for the suppression of host defense mechanisms, which represents a new role for HOX TFs in pathogenic fungi. CsHOX2 and CsHOX7 were found to play essential roles in conidiation and appressorium development, respectively, in a stage-specific manner in C. scovillei. Our study provides a molecular basis for understanding the mechanisms associated with the development of anthracnose on fruits caused by C. scovillei, which will aid in the development of novel approaches for disease management.
Pepper (Capsicum annuum L.) is an important vegetable crop cultivated in nearly all countries worldwide (1). The yearly worldwide pepper production has been estimated at approximately 36.8 million tons (2). Peppers have been widely used as a food flavoring agent and as medicine for thousands of years. Peppers are good sources of vitamins and minerals; they contain multiple chemical constituents with pharmaceutical properties (1). A major hindrance in pepper production is anthracnose disease caused by Colletotrichum species, which are ascomycete fungal pathogens. Anthracnose refers to diseases with the typical symptoms of sunken spots or lesions on infected plant tissues (3, 4). The disease causes substantial economic losses by reducing the productivity and quality of fruits in affected plants (5). Several species in the genus Colletotrichum are known to cause anthracnose in pepper plants (6–8). Among them, C. scovillei, which belongs to the Colletotrichum acutatum species complex, is considered a dominant pathogen responsible for serious pepper yield losses in countries in tropical and temperate zones, including Brazil, China, Indonesia, Japan, South Korea, Malaysia, and Thailand (9–15). C. scovillei has also been reported to attack other economically important crops, such as mango and banana (16, 17). Although most anthracnose disease on fruits can cause considerable losses in yield and quality, the molecular mechanism underlying the development of fruit anthracnose has not yet been elucidated, whereas many foliar diseases in a wide range of plants caused by diverse fungal pathogens have been extensively studied at the molecular level (18–24). Therefore, we have initiated a molecular pathosystem study of C. scovillei and pepper fruits to better understand anthracnose disease in fruit.

C. scovillei produces a large number of conidia that serve as a major inoculum. During the disease cycle, conidia adhere to the surfaces of pepper fruits upon hydration; they then produce germ tubes (7, 25). Appressorium, a specialized infection structure, is differentiated at the tip of the germ tube following the recognition of chemical and physical host signals (26). Considering that C. scovillei infects only the fruit, the signals for appressorium development on fruit are different from the signals for appressorium development on other tissues of host plants. Similar to several Colletotrichum species, C. scovillei is a hemibiotroph, which exhibits an early biotrophic phase and a late necrotrophic phase during host-pathogen interactions (6, 26–28). At an early stage of appressorium-mediated penetration of C. scovillei, a unique feature known as a dendroid structure develops in the cuticle layer of pepper fruit; this does not occur in foliar infections involving other Colletotrichum pathogens (4, 29, 30). After colonization of host epidermal cells, the fungus develops the typical sunken anthracnose lesion with mucilaginous acervuli containing a large amount of pinkish conidia, which is important for further infection. Polycyclic infection of the fungus contributes to substantial disease during the growing season, thereby causing serious economic losses. Based on the socioeconomic impact and difficulty involved in disease management of fruit anthracnose in a wide range of crops, we previously elucidate the whole genome of C. scovillei to better understand the molecular mechanisms involved in fruit anthracnose, which have been relatively uncharacterized at the molecular level (8, 31, 32).

Eukaryotic organisms, including fungi, have evolved adaptive genetic responses to a variety of stimuli (33, 34). Transcription factor (TF)-mediated regulation of gene expression plays an important role in controlling crucial aspects of organism survival and development. Gene expression is elaborately orchestrated in a coordinated system of transcription-mediated signaling, in which TFs bind specific sequence elements to initiate or block transcription (35–37). Various TF families are defined by unique DNA-binding motifs (38–40). Although the DNA-binding motifs of TF families are highly conserved across taxa, the members of a particular TF family often have different roles in an organism because of evolutionary divergence (41–43). The availability of genome sequence data has resulted in the identification of a large number of TFs through cross-species comparison, thus enabling TF family-based characterization in functional genomics (44). The homeobox TF family is a prominent TF family associated with fungal development and pathogenicity. Members of this family contain a conserved
60-amino-acid DNA-binding motif known as the homeodomain (45, 46). Since the functional discovery of the homeobox TF family in *Drosophila melanogaster*, which is expressed in an organ-specific manner during development (47, 48), important roles of homeobox TF orthologs have been established in several fungi (33, 49–52). For example, the rice blast fungal pathogen *Magnaporthe oryzae* contains seven homeobox TFs, among which MoHOX2 and MoHOX7 have been identified as stage-specific key regulators of conidiation and appressorium development, respectively (33, 53). Functional roles of homeobox TFs in infection-related development have been demonstrated in *Colletotrichum orbiculare*, a cucumber anthracnose pathogen, which is evolutionarily very distant from *C. scovillei* (54, 55). Unlike MoHOX7, the homolog CoHOx3 was associated with maturation of appressoria in *C. orbiculare*. CoHOx1 was found to be required for pathogenic development, whereas its homolog MoHOX5 was dispensable for pathogenicity. These results reflect the functional divergence of homeobox TF family members, which further increases the genetic complexities of fungal pathogens with different lifestyles, in response to a wide variety of environmental signals. However, the HOX TF family in pepper fruit anthracnose *C. scovillei* has not yet been studied.

To systematically study the functions of HOX genes, we first analyzed whole-genome sequences of *C. scovillei* and isolated 624 putative TFs (based on InterPro annotation), which contain 48 distinct domains. Through detection of the homeobox domain (IPR001356), a total of 10 HOX TFs (CsHOX1 to CsHOX10) were then obtained from putative TFs in *C. scovillei*. These HOX TFs in the homeobox-like domain superfamily (IPR009057) were evolutionarily analyzed in the *Colletotrichum* genus and outgroup species to reveal their origins. To further study the functional roles of CsHOX genes in *C. scovillei*, we then generated deletion mutants for each CsHOX gene, based on homology-dependent gene replacement. Comparative functional analysis of deletion mutants demonstrated that CsHOX genes are associated with stage-specific regulations for disease dissemination and development in *C. scovillei*. Specifically, CsHOX2, CsHOX7, and CsHOX1 were found to be essential for conidiation, appressorium formation, and suppression of the host defense mechanism for anthracnose development on pepper fruits in the *C. scovillei*-host pathosystem, respectively. Our findings provide a useful framework for understanding the development of anthracnose disease on fruits caused by *Colletotrichum* species.

**RESULTS**

**Distribution of TF and homeobox-related proteins in fungal species including Colletotrichum spp.** Our phylogenomic species tree of *Colletotrichum* was consistent with previous phylogenetic trees (56, 57) (see Fig. S1A and Table S1A in the supplemental material). The *C. acutatum* species complex diverged from *Colletotrichum orbiculare*, as previously reported (56). The total number of TFs in *Colletotrichum* species ranged from 337 in *Colletotrichum chlorophyti* to 624 in *C. scovillei* (see Fig. S1A and Table S1B and C). The average number of TFs in this genus was 437.0, which was considerably less than the average number of TFs in the sister taxon *Verticillium* (316.5) but considerably more than the average number of TFs in the distantly related taxon *Fusarium* (985.7). Among the TFs, the average number of genes encoding a protein with a homeobox-like domain was 46.3 in all selected species (see Table S1D). However, *C. scovillei* in the *Colletotrichum* genus and outgroup species, including *Fusarium verticilloides*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*, had significantly higher numbers of genes than average at 206, 77, 73, and 126 genes, respectively. In contrast, the number of HOX genes in the *Colletotrichum* genus consistently ranged from 8 to 11, whereas the number in the outgroup species exhibited considerable variability, from 2 in *Saccharomyces pombe* to 27 in *F. verticilloides* (see Table S1E). These results indicated that plant pathogens possess more TFs and HOX genes than saprotrophs.

In total, 104 different types of structures were observed in proteins with homeobox-like domains in the selected fungal species (see Table S1F). Among them, 13 types were proteins with a homeobox domain, although only eight types were detected in *Colletotrichum* species (see Fig. S1B). Within *Colletotrichum* species, proteins that only
coli etion in Colletotrichum species. Some HOX proteins with $C_{3}H_{2}$-type zinc finger domains also had an HTH CenP-type DNA-binding domain (IPR006600), and were also present in all Colletotrichum species, except Colletotrichum tanacetii. *C. oriculare* contained one of these proteins, with an additional DUF3425 domain at the C-terminal end. A HOX protein with three $C_{3}H_{2}$-type zinc finger domains, NAD(P)-binding domain (IPR036291), and 6-phosphogluconate dehydrogenase-like C-terminal domain (IPR008927) was only present in *Colletotrichum salicis*.

**Evolution and proliferation of the homeodomain superfamily and HOX genes in fungal species, including Colletotrichum spp.** Gain and loss analysis of genes encoding members of the homeodomain superfamily, including the HOX genes, showed that 35 ancestral genes existed in the last common ancestor of the *Colletotrichum* species complex (see Fig. S1C). The last common ancestor of the *C. acutatum* species complex had 37 genes, which remain in *Colletotrichum simmondsii*, *C. salicis*, and *C. orchidophilum*, while 1 and 2 genes were lost in *Colletotrichum nymphaeae* and *Colletotrichum floriniae*, respectively. Overall, the phylogenetic tree of the homeodomain superfamily showed that the superfamily of the selected fungal species was divided into 17 clades (see Fig. S1D and Table S1G). The largest clade had 809 genes (clade 15), while the second-largest clade had 211 genes (clade 10); both contained proteins with homeobox domains. Clades 10 and 15 had 148 and 153 HOX genes, respectively, which formed a subclade within each clade (see Fig. S1D). The subclades of clades 10 and 15 were further divided into 14 and six families, respectively (see Fig. S1E and S1F). Each subclade contained five HOX genes of *C. scovillei*. Although subclade 15 only consisted of HOX genes, subclade 10 contained a highly diverged homeobox-like domain family (family 3). A pair of HOX genes from *Colletotrichum tofieldiae* (KZL74285.1) and *Colletotrichum incanum* (OWH97559.1) (family 2), and a pair of HOX genes from *Colletotrichum gloeosporioides* (EQS7487.1) and *Colletotrichum fructicola* (ELA29112.1) (family 10), each independently formed their own families (see Fig. S1E).

Another notable feature of the tree was that the genes encoding proteins with homeobox-like domains in *C. scovillei* and *S. sclerotiorum* were extensively proliferated in a subclade within clade 15 (see Fig. S1D). This proliferation of genes appeared to be primarily caused by the DNA transposon TcMar-Fot1 in both fungi (see Fig. S2A). Without these duplicated genes, *C. scovillei* only contained 36 homeodomain superfamily genes, similar to its sister species *C. nymphaeae* (see Fig. S1C). Unlike HOX genes and the homeodomain superfamily sparsely scattered in the genome of *C. scovillei*, these genes were located in clusters throughout the genome (see Fig. S2B). This phenomenon may be driven by the activity of a DNA transposon.

As representatives of nuclear localization of HOX TFs in *C. scovillei*, we confirmed nuclear localization of CshOX2 and CshOX7 using transformants expressing the fusion proteins CshHOX2s:GFP and CshHOX7s:GFP, respectively, during fungal development (see Fig. S2C and S2D). HOX proteins of *C. scovillei* have been compared to known HOX proteins of *M. oryzae* and *S. cerevisiae* (33, 58). While *S. cerevisiae* has nine HOX protein-encoding genes, *M. oryzae* has seven; *C. scovillei* has three genes, in addition to the seven present in *M. oryzae* (see Fig. S2A). All HOX proteins in *C. scovillei* were found to be orthologous to those in *M. oryzae*, with the exceptions of CshOX3, CshOX8, CshOX9, and CshOX10. The homeobox domain in CshOX8 with two $C_{3}H_{2}$ zinc finger domains and an HTH CenP-type DNA-binding domain and CshOX9 without any other domains were found to be paralogous to each other (see Fig. S2A). Only two *C. scovillei* proteins, CshOX6 and CshOX7, were found to be orthologous to the HOX genes in *S. cerevisiae* and *M. oryzae*.

**Generation of CshHOX-deletion mutants in C. scovillei.** To investigate the functional roles of CshHOX genes during fungal developments and infection of *C. scovillei*, we generated deletion mutants ($\Delta Cshox1$ to $\Delta Cshox10$) for each CshHOX gene via
TABLE 1 Phenotypic characterization of CsHOX gene deletion mutants in C. scovillei

| Strain       | Growth (mm) | Conidiation (10^4/ml) | Conidial size (μm) | Conidial germination (%) | Appressorium formation (%) |
|--------------|-------------|-----------------------|--------------------|--------------------------|---------------------------|
|              | CMA         | MMA                   | Length             | Width                     |                           |
| Wild-type    | 49.3 ± 0.1A | 44.3 ± 0.5ABC         | 155.0 ± 9.1A       | 3.7 ± 0.5B                | 97.5 ± 1.3A               | 90.0 ± 3.2A               |
| ΔCsHox1c     | 31.8 ± 0.5C | 24.5 ± 0.9B           | 158.0 ± 12.9A      | 3.4 ± 0.3C                | 81.0 ± 4.4D               | 31.0 ± 6.7B               |
| ΔCsHox2c     | 48.5 ± 0.6A | 44.0 ± 0.8ABC         | 148.5 ± 9.8A       | 3.8 ± 0.5B                | 97 ± 1.8AB                | 90.5 ± 4.5A               |
| ΔCsHox2c     | 42.3 ± 0.5D | 32.8 ± 0.9D           | 0.0D               | ND                       | ND                        | ND                        |
| ΔCsHox3c     | 49.0 ± 0.8B | 44.3 ± 1.3ABC         | 150.8 ± 6.4A       | 3.6 ± 0.3B                | 94.8 ± 2.2ABC             | 89.8 ± 5.1A               |
| ΔCsHox3c     | 48.3 ± 0.3B | 44.0 ± 0.8ABC         | 87.8 ± 5.6A        | 4.2 ± 0.3A                | 92.0 ± 3.2ABC             | 88.8 ± 3.9A               |
| ΔCsHox3c     | 48.8 ± 0.9B | 43.8 ± 0.5ABC         | 153.0 ± 13.5A      | 3.7 ± 0.4B                | 96.8 ± 1.2AB              | 89.3 ± 5.5A               |
| ΔCsHox4c     | 44.5 ± 0.6C | 38.0 ± 0.8C           | 150.5 ± 8.8A       | 4.4 ± 0.4B                | 92.8 ± 2.2ABC             | 89.5 ± 4.4A               |
| ΔCsHox5c     | 44.8 ± 0.9B | 44.8 ± 0.5ABC         | 157.5 ± 13.8B      | 3.9 ± 0.4B                | 95.8 ± 2.1ABC             | 89.0 ± 4.0A               |
| ΔCsHox6c     | 48.5 ± 0.6A | 41.5 ± 0.6B           | 151.3 ± 7.0A       | 4.4 ± 0.3A                | 90.5 ± 2.6C               | 89.0 ± 5.0A               |
| ΔCsHox7c     | 48.5 ± 0.6A | 44.5 ± 0.6ABC         | 146.8 ± 4.7A       | 3.6 ± 0.4B                | 95.3 ± 2.2ABC             | 88.8 ± 5.9A               |
| ΔCsHox8c     | 47.3 ± 0.5B | 43.3 ± 0.5CD          | 123.0 ± 6.3B       | 3.7 ± 0.4B                | 95.5 ± 3.4ABC             | 89.0 ± 5.4A               |
| ΔCsHox9c     | 48.8 ± 0.9A | 44.3 ± 0.8ABC         | 150.0 ± 4.4A       | 3.8 ± 0.4B                | 94.5 ± 3.4ABC             | 89.3 ± 5.1A               |
| ΔCsHox9c     | 48.0 ± 0.8B | 42.3 ± 1.3ACD         | 158.1 ± 9.8A       | 3.8 ± 0.3B                | 95.8 ± 2.2ABC             | 85.3 ± 4.3D               |
| ΔCsHox10c    | 48.8 ± 0.9B | 43.8 ± 0.5ABC         | 150.0 ± 7.7A       | 3.8 ± 0.3B                | 95.3 ± 2.5ABC             | 86.0 ± 3.7AD              |
| ΔCsHox10c    | 48.8 ± 0.9B | 43.8 ± 0.5ABC         | 155.5 ± 14.8A      | 3.7 ± 0.4B                | 94.5 ± 2.4ABC             | 88.8 ± 6.2A               |
| ΔCsHox10c    | 49.0 ± 0.8A | 34.8 ± 0.5ACD         | 141.8 ± 12.6A      | 3.7 ± 0.3B                | 95.0 ± 2.2ABC             | 89.4 ± 4.6A               |

aData are presented as means ± the standard deviations of three independent experiments, with three replicates per experiment. Significant differences were estimated using Duncan’s test (P < 0.05). The same superscript capital letters in a column indicate no significant difference. ND, not determined.

bMycolial growth was measured at 6 days postinoculation on CMA and MMA.

bConidial size was determined by measuring the lengths and widths of at least 100 conidia.

bConidial size was determined by measuring the lengths and widths of at least 100 conidia.

bConidial germination was measured at 12 h postinoculation using conidia from 6-day-old oatmeal agar. In each replicate, at least 100 conidia were measured.

bThe percentage of appressorium formation was measured at 16 h postinoculation on a hydrophobic surface using conidia from 6-day-old oatmeal agar. In each replicate, at least 100 conidia were measured.

Homology-dependent targeted gene replacement. The deletion mutants were confirmed by Southern blotting and RT-PCR (see Fig. S3). Compared to the wild-type strain of C. scovillei, the deletion mutants displayed altered phenotypes during fungal developmental and pathogenicity (Table 1; see also Fig. S4A to D). Briefly, the ΔCsHox2, ΔCsHox3, and ΔCsHox6 strains were reduced in conidiation; the ΔCsHox1, ΔCsHox3, ΔCsHox4, and ΔCsHox5 strains produced abnormally morphological conidia; the ΔCsHox1 and ΔCsHox7 strains were impaired in appressorium formation in response to hydrophobic surface; the ΔCsHox1, ΔCsHox7, ΔCsHox8, and ΔCsHox9 strains were defective in pathogenicity; the ΔCsHox2 strain caused normal anthracnose disease through appressorium-like structures developed from tips of mycelia. Moreover, the wild-type strain was not found to produce conidiomata, whereas the ΔCsHox1, ΔCsHox3, ΔCsHox5, and ΔCsHox6 formed conidiomata on artificial medium (see Fig. S4B).

CsHOX genes are important for conidium production. Anthracnose disease of pepper by C. scovillei is quickly spread via conidia in favorable environmental conditions. Therefore, we evaluated the conidiation of CsHOX gene deletion mutants. Quantitative analysis of conidiation showed that ΔCsHox3 and ΔCsHox were significantly defective in conidiation, compared to the wild-type and complemented strains (Table 1). Notably, ΔCsHox2 was completely defective in conidiation, indicating that CsHOX2 is essential for conidiation in C. scovillei. Microscopic investigation revealed that no conidia were observed in ΔCsHox2 with prolonged incubation, while the wild-type and CsHox2c strains developed dense conidiophores bearing conidia in 5 h (Fig. 1A). To further determine whether CsHOX2 is involved in conidiophore formation, lactophenol aniline blue was applied to distinguish conidiophores from aerial hyphae. Microscopic examination indicated that wild-type, ΔCsHox2, and CsHox2c strains developed conidiophores (Fig. 1B). Taken together, these results indicated that CsHOX2 is a specific regulator of conidium formation.
production, and that CsHOX3 and CsHOX6 are quantitatively related to conidium production in C. scovillei.

CsHOX genes are related to the morphology and germination of conidium, and stress response. Proper morphology and germination of conidium are prerequisites for efficient infection in conidium-mediated disease development. In terms of morphology, the \( \Delta \text{Cshox1}, \Delta \text{Cshox3}, \Delta \text{Cshox4}, \) and \( \Delta \text{Cshox5} \) mutants produced abnormal conidia, which were larger than the conidia of the wild-type and complemented strains (Fig. 2A and Table 1). This finding suggested that CsHOX1, CsHOX3, CsHOX4, and CsHOX5 are important for conidial morphology. Conidial germination rates of the \( \Delta \text{Cshox1}, \Delta \text{Cshox3}, \) and \( \Delta \text{Cshox5} \) strains were significantly reduced, indicating that CsHOX1, CsHOX3, and CsHOX5 are involved both in conidial morphology and germination (Table 1). When fungicides (e.g., dimethomorph, oxolinic acid, carbendazim, and fludioxonil) were applied to conidial germination assays, conidial germination of the \( \Delta \text{Cshox1} \) and \( \Delta \text{Cshox4} \) strains was significantly reduced by stress with dimethomorph (1 or 0.1 ppm) and carbendazim (1 ppm), respectively, compared to the germination of the wild-type and corresponding complemented strains (see Fig. S5A). In addition, germ tubes and conidia of the \( \Delta \text{Cshox1} \) strain displayed abnormal swelling following treatment with the chitin synthase inhibitor nikkomycin Z, compared to the wild-type and \( \text{Cshox1c} \) strains (see Fig. S5B). Moreover, we tested whether thermal stress affected the germination of conidia with abnormal morphology. The conidia of \( \Delta \text{Cshox1}, \Delta \text{Cshox3}, \Delta \text{Cshox4}, \) and \( \Delta \text{Cshox5} \) exhibited germination rates that were similar to the rate of the wild-type strain at 20°C, whereas the demonstrated significant inhibition of conidial germination at 32°C (Fig. 2B; see also Fig. S5C). Thus, we tested whether the conidial longevities of the \( \Delta \text{Cshox1}, \Delta \text{Cshox3}, \Delta \text{Cshox4}, \) and \( \Delta \text{Cshox5} \) strains were reduced by heat shock. Phloxine B was used to assess conidial survival. After incubation for 16 h, the ratios of conidial survival of \( \Delta \text{Cshox1}, \Delta \text{Cshox3}, \Delta \text{Cshox4}, \) and \( \Delta \text{Cshox5} \) strains were significantly reduced as the temperature increased from 25°C to
32 or 37°C (Fig. 2C; see also Fig. S5D). These results suggested that CsHOX1, CsHOX3, CsHOX4, and CsHOX5 are involved in conidial morphology, germination, and viability. CsHOX7 is required for appressorium development but not for invasive growth. Appressorium development is a key step in anthracnose disease development in C. scovillei. Strikingly, conidia of the ΔCshox7 strain were completely abolished to produce appressoria, indicating that CsHOX7 is essential for appressorium formation. A few swellings of the germ tube in the ΔCshox7 strain indicated that the ΔCshox7 strain retained the ability to sense the hydrophobic surface (Fig. 3A). Thus, we investigated the effects of exogenous chemical signals including cAMP, cutin monomers, and CaCl2 on the recovery of appressorium development in the ΔCshox7 strain on hydrophobic coverslips. Conidia of the ΔCshox7 strain were unable to form appressoria with the addition of exogenous cutin monomers. However, cAMP (5 mM) induced formation of 24% immature appressoria in the ΔCshox7 strain, in contrast to 87.8% unmelanized appressoria in the wild-type strain (Fig. 3B and C). Compared to 91.5% formation of melanized appressoria by wild-type conidia, 44.5% of ΔCshox7 conidia developed young appressoria with the addition of CaCl2 (0.5 mM) (Fig. 3B and C). These results revealed that exogenous cAMP or CaCl2 partially restored appressorium formation in the ΔCshox7 strain. Treatment with both CaCl2 and cAMP increased the appressorium formation rate of the ΔCshox7 strain to 82.8% and led to the formation of appressoria.
by 92.6% of wild-type conidia (Fig. 3C). Although appressoria of the ΔCshox7 strain induced by treatment with cAMP and CaCl₂ were not melanized, the sizes of appressoria were similar to those in the wild-type and Cshox7c strains (Fig. 3B). These compounds largely induced appressorium formation in the ΔCshox7 strain, suggesting that CsHOX7 is regulated by cAMP and Ca²⁺-dependent signaling pathways.

Compared to the wild-type strain, the ΔCshox7 strain caused full infection of wounded pepper fruit but produced no lesions on unwounded pepper fruit, revealing that the ΔCshox7 strain was unable to penetrate the host surface (Fig. 4A and B). Microscopic observation showed that conidia of the ΔCshox7 strain formed swollen structures at the tips of germ tubes that failed to differentiate into appressoria on the host surface (Fig. 4C). Because exogenous addition of CaCl₂ and cAMP partially restored appressorium formation in the ΔCshox7 strain on hydrophobic coverslips (Fig. 3B and C), we tested whether exogenous CaCl₂ and cAMP contributed to appressorium-mediated penetration of ΔCshox7. Exogenous cAMP failed to restore infection by the ΔCshox7 strain (see Fig. S6A). However, conidia of the ΔCshox7 strain successfully infected unwounded pepper fruits with the addition of exogenous CaCl₂ (Fig. 4D and E). Treatment with both CaCl₂ and cAMP facilitated the formation of lesions by the ΔCshox7 strain on unwounded pepper fruits (Fig. 4D and E). Microscopic observation
showed that the \( \Delta \text{Cshox7} \) strain was partially restored to form dendroid structure and grow invasive hyphae in unwounded pepper fruit with an addition of exogenous CaCl\(_2\) (Fig. 4F and G).

**CsHOX1 gene plays crucial roles in the development of anthracnose on pepper fruits.** The \( \Delta \text{Cshox1} \) strain failed to infect wounded pepper fruit and led to charcoal-colored spots without typical sunken lesions on unwounded pepper fruit, which is not a typical disease symptom (Fig. 5A and B). To figure out the roles of CsHOX1 in development of anthracnose on pepper fruits, we performed microscopic observation and found that the \( \Delta \text{Cshox1} \) strain developed appressorium and penetrated the host surface, in a manner similar to that of \( \text{Cshox1c} \) strains (Fig. 5C and D). Notably, in contrast to the wild-type and \( \text{Cshox1c} \) strains which grew invasive hyphae in host epidermal cells after 4 days, the host cells infected with the \( \Delta \text{Cshox1} \) strain exhibited no invasive growth (Fig. 5C and D). We further investigated the expression
profile of CsHOX1 with qRT-PCR and found that the CsHOX1 gene was highly expressed in infection stage (see Fig. S6B). These findings suggested that CsHOX1 is essential for invasive hypha development during anthracnose development on pepper fruits.

The host cells infected by the ΔCshox1 strain exhibited brown pigmentation, which may reflect host cell defense response (59). Since host defense response is often accompanied by the rapid generation of reactive oxygen species (ROS), we performed the DAB staining experiment and found that pepper fruit cells infected with the ΔCshox1 strain exhibited a high density of H2O2 accumulation in the first challenged epidermal cell, whereas host cells infected with wild-type and Cshox1c strains did not show accumulation of H2O2 (Fig. 5E and F; see also Fig. S6C). To test whether CsHOX1 is involved in the degradation of ROS, we evaluated the mycelial growth rate on CMA containing H2O2. Mycelial growth of the ΔCshox1 strain was significantly inhibited by 5 and 10 mM H2O2, and the ΔCshox1 strain was unable to grow on CMA containing 20 mM H2O2, whereas wild-type and Cshox1c strains could grow on this artificial media (Fig. 6A and B), suggesting that CsHOX1 is required for degrading of ROS. We further evaluated the expression of host defense-related genes and found that CaBPR1, CaPR4C, CaPR10, CaHIR1, CaGLP1, CaPK1, and CaPAL1 were significantly upregulated in pepper...
fruits infected by the $\Delta$Cshox1 strain, compared to those infected by wild-type or Cshox1c strains (Fig. 6C; see also Table S2). This result indicated that CsHOX1 may be involved in suppression of host defense. Consistent with this hypothesis, we investigated the infection of the $\Delta$Cshox1 strain in defense-compromised host plant by inoculating conidial suspensions onto diphenyleneiodonium (DPI; suppressing ROS generation)-treated and heat-killed pepper fruits. The results showed that the invasive growth of the...
The Cshox1 strain was comparable to that of wild-type and Cshox1c strains in heat-killed pepper fruits, and the infection of the Cshox1 strain was partially rescued in DPI-treated pepper fruits (Fig. 6D to G). Taken together, these findings suggested that CsHOX1 is involved in the degradation of ROS and the suppression of host defense.

CsHOX1 regulates the expression of putative virulence-related genes. To further investigate the CsHOX1-dependent genes during anthracnose development in pepper fruits, we performed the RNA-seq to profile the gene expression of host tissues infected by the ΔCshox1 strain versus the wild-type strain. The result showed that 52 genes were significantly downregulated (log2-fold change, <-2; P < 0.05), and 121 genes were significantly upregulated (log2-fold change, >2; P < 0.05) in host tissues infected by the ΔCshox1 strain in reference to the wild-type strain (Fig. 7A; see also Table S3A). To validate the RNA-seq result, we evaluated the 10 upregulated differentially expressed genes (DEGs), 10 downregulated DEGs, and 3 unaffected genes to evaluate their expression with qRT-PCR (Fig. 7B). The expression of 10 selected upregulated genes, encoding...
putative pathogenicity-related secreted proteins, ROS-scavenging enzymes, and plant cell wall-degrading enzymes (see Table S3B), was consistent with that obtained from RNA-seq. The transcripts of 10 selected downregulated genes, involved in stress response and signal transduction (see Table S3B), were also similar to that from RNA-seq. The Gene Ontology (GO) term enrichment test showed that none of the functions in downregulated genes enriched compared to the whole gene sets, but the upregulated genes were enriched with functions involved in many cellular components, bindings, and metabolic processes (see Fig. S6D). The enrichment test between down- and upregulated genes showed genes related to organelles in the cellular component category were enriched in the downregulated genes, and genes with catalytic activity were enriched in upregulated genes (Fig. 7C). This result indicates that CsHOX1 may play an important role in regulation of genes associated with the organelle formation and the catalytic activities. Further analysis of downregulated genes revealed that 13 genes were putative pathogenicity factors, including two ROS-scavenging enzymes (60, 61), five secreted proteins (62), and six plant cell wall-degrading enzymes (see Table S3C) (63). Many genes were upregulated in the ΔCshox1 strain, including regulators such as protein kinases, phosphatases, and transcription factors. The RNA-seq data supported that the CsHOX1 plays a key role in regulating anthracnose development of C. scovillei on pepper fruits.

DISCUSSION

TFs play critical roles in development and pathogenicity in organisms, including fungal pathogens. Essential functions of the evolutionarily conserved homeobox TFs have been demonstrated in several eukaryotes (35, 64). In this study, analysis of TFomes and homeodomain superfamily genes in C. scovillei was extended to the Colletotrichum species complex and selected outgroup species. These analyses revealed that C. scovillei has a unique feature within the Colletotrichum species complex; notably, the fungus exhibited an extremely high number of homeodomain superfamily genes, while HOX genes were evenly distributed among the species complex (Fig. 1A). In terms of InterPro domain relationships, the homeobox-like domain superfamily (IPR009057) is one of the largest gene families in the kingdom Fungi, inclusive of the homeobox domain (IPR001356) in HOX genes (40). This duplication event was observed in both C. scovillei and S. sclerotiorum, an outgroup species (Fig. 1A); this shared feature was attributed to the DNA transposon TcMar-Fot1 (Fig. 2A). While the expansion and enrichment of DNA transposons in the genome of S. sclerotiorum have been reported (65), the relationships of DNA transposons with the duplication of homeodomain superfamily genes are first reported here for both S. sclerotiorum and C. scovillei. Unlike tandem clusters of HOX genes in animals, HOX genes were found to be scattered throughout the genomes of fungi, including C. scovillei (Fig. 2B) (66). However, these homeobox-like genes were revealed to be duplicated multicopy genes located in a tandem manner in the C. scovillei genome (Fig. 2B). This is likely evidence for the recent transposon activity that occurred in C. scovillei after divergence from C. nymphaeae (see Fig. S1A), which presumably occurred approximately 2 million years ago (57, 67). Because transposable elements are the major drivers of fungal genome evolution (68), the association of transposons with homeodomain superfamily genes impacts genome organization and may affect cellular processes related to fungal development and pathogenicity.

Molecular mechanisms that govern anthracnose disease caused by Colletotrichum species on fruits have not yet been characterized. A large number of conidia are reproduced from phialides of conidiophores in many Colletotrichum species (69, 70). However, the genetic basis of the conidiation process remains largely unknown in the Colletotrichum genus. We found that the deletion of CsHOX2 caused a complete defect in the production of conidia in C. scovillei, whereas the ΔCshox2 strain developed normal conidiophores (Fig. 3). Considering that MoHOX2 (orthologous to CsHOX2) is essential for M. oryzae conidiation in a stage-specific manner (33), the development of phialides on top of conidiophores, which are not clearly distinguishable, may be normal in the ΔCshox2 strain. However, HTF1 genes in Fusarium graminearum, F. verticillioides,
and *F. oxysporum* (all orthologous to *CsHOX2*) were revealed to be involved in the formation of clearly differentiated phialides (50). The ΔFghtf1, ΔFvhtf1, and ΔFohtf1 strains alternatively produce macroconidia directly from hyphae at low frequencies, indicating functional differences in *CsHOX2* from orthologous *HTF1* genes in the three *Fusarium* species. Unlike the complete defect of the ΔCshox2 strain in conidium production, it is notable that the deletions of *CsHOX1, CsHOX3, CsHOX4*, and *CsHOX5* resulted in the development of abnormally large conidia (Fig. 4A); in contrast, deletion of *MoHOX4* (orthologous to *CsHOX4*) resulted in the production of abnormally small conidia in *M. oryzae* (33). Notably, 4 of 10 *HOX* genes are associated with conidial size and morphology in *C. scovillei*. Several lines of evidence suggest that the four genes play distinct roles in conidium development of *C. scovillei*. For example, the ΔCshox1 strain produced significantly larger conidia (19.9 ± 2.4 μm) compared to those from the ΔCshox3, ΔCshox4, and ΔCshox5 strains (3.4 ± 0.3 μm, 15.0 ± 1.7 μm, and 4.2 ± 0.5 μm) (Table 1). Abnormal swelling of the conidia and germ tubes was observed in ΔCshox1 alone (not in the ΔCshox3, ΔCshox4, and ΔCshox5 strains), following treatment with nikkomycin Z; this indicated a difference in cell wall integrity (see Fig. S4B). The sensitivity of conidia in the ΔCshox3 strain was much higher following treatment with the fungicide carbendazim, compared to the sensitivities of conidia in the ΔCshox1, ΔCshox4, and ΔCshox5 strains (see Fig. S4A), which support functional differences among the four *CsHOX* genes in *C. scovillei*. The *CsHOX1* gene is a critical regulator of cell wall integrity and conidia morphology, as well as pathogenic development, in *C. scovillei* (Fig. 4A and 7E; see also Fig. S4B), which represents a novel function of homeobox TFs in plant-pathogenic fungi.

The ΔCshox1 strain was not defective in appressorium formation and host penetration on pepper fruits (Fig. 5C and D). The invasive hyphae of the ΔCshox1 strain were unable to grow in the epidermal cells of pepper, following successful penetration into the cuticle layer via appressoria (Fig. 5C). The accumulation of the brown cloud and ROS only in the first epidermal cell challenged by the ΔCshox1 mutant (Fig. 5E and F), and the significant upregulation of host defense-related genes were indicative of the activation of innate host immunity (Fig. 6C; see also Table S2). The increased sensitivity of the ΔCshox1 mutant to H$_2$O$_2$, compared to the wild-type and *Cshox1c* strains, indicates that *CsHOX1*-regulated genes may be related to the modulation of ROS that are encountered in host pepper cells during anthracnose development of *C. scovillei* (Fig. 6A and B). Notably, the ROS-scavenging enzymes CsTRR1 and CsCAT1, orthologous to *M. oryzae* TRR1 and *Sclerotinia sclerotiorum* SCAT1, respectively, were significantly downregulated in gene expression profiles (see Table S3C). Both TRR1 and SCAT1 were reported to play important roles in ROS detoxification and plant infection (60, 61). The significant downregulation of *CsTRR1* and *CsCAT1* in ΔCshox1-infecting host tissues revealed the involvement of *CsHOX1* in ROS detoxification. Moreover, an LysM protein CsELP2, orthologous to *C. higginsianum* ChELP2, were found in the significantly downregulated genes (Fig. 7B). The ChELP2 encodes an extracellular LysM protein, which suppresses chitin-triggered plant immunity (71). The fungal chitin is known to induce host ROS accumulation and defense gene activation (72). This suggests that the *CsHOX1* may be involved in suppression of chitin triggered immunity through transcriptional regulation of CsELP2. When performing pathogenicity assay by using host defense-compromised tissues, the ΔCshox1 could grow invasive hyphae as wild-type strain did on heat-killed host tissues (Fig. 6D and E), while the pathogenicity was partially restored on DPI-treated host (Fig. 6F and G). This result indicated that the *CsHOX1*-regulated genes may be involved in suppression of host defense. In addition, the other four genes encoding secreted proteins (*CsSIX11, CsCRISP1, CsSP1*, and *CsSP2*) were found to be significantly downregulated in ΔCshox1 strain-infected host tissues (Fig. 7B). The *CsSIX11* was predicted to be orthologs to a known effector, SIX11 (*Secretion In Xylem*) of *Fusarium oxysporum*, which was recently demonstrated to be dispensable for fungal virulence, while its role in host defense is still unknown (73). The *CsCRISP1* gene was predicted to encode a cysteine-rich secretory protein. Although the
ortholog of CsCRISP1 was not characterized, the cysteine-rich secretory proteins were reported to be involved in suppression of plant immunity in *Verticillium dahliae* (74). The detailed mechanisms of CsSIX11 and CsCRISP1 await investigation. These findings suggested that CsHOX1 is an essential factor that contributes to anthracnose development by transcriptional regulation of genes involved in the modulation of ROS and host defense (Fig. 7D).

Our study of appressorium-mediated fruit disease development in *C. scovillei* demonstrated that it uses a different strategy, compared to other fungal pathogens, to effectively penetrate the thick cuticle layer (20 to 25 μm) of host cells. For example, *M. oryzae*, a model for foliar disease in rice, generates substantial turgor pressure inside the appressorium to directly reach invading epidermal cells through the host cell wall by means of a strong mechanical force (75). However, *C. scovillei* has evolved a highly sophisticated strategy in which the fungus achieves tiny pin-point entry via the appressorium and then grows in the thick cuticle layer with the formation of dendroid structures within the cuticle layer; it does not use a penetration peg (2 to 4 μm in length) that directly reaches the epidermal cells of the pepper fruit. Therefore, *C. scovillei* generates, possibly, lower turgor pressure inside the appressorium and maintains conidial viability after penetration, in contrast to the autophagic cell death of conidia that occurs following penetration of *M. oryzae* (76). This hypothesis is supported by the observation that unpigmented immature appressoria of *C. scovillei* are able to penetrate the cell wall of pepper fruit and cause anthracnose disease (data not shown).

In this study, we demonstrated the dynamics and proliferation of homeodomain superfamily TFs during the evolution of an ingroup of *Colletotrichum* species and outgroup members. Detailed functional analyses have revealed that members of the homeobox TF family in *C. scovillei* play a key role in fungal development and anthracnose disease on pepper fruit. Our study provides a fundamental basis for understanding the molecular mechanisms involved in conidiation, appressorium development, and anthracnose disease on fruits, which will contribute to the development of novel strategies for use in managing anthracnose disease on many economically important fruits.

**MATERIALS AND METHODS**

Data used in the study and prediction of TFomes and repeat elements. The fungal genomes and proteomes used in this study were downloaded from the National Center for Biotechnology Information (NCBI) database (see Table S1A). TFs were identified from proteomes using InterProScan 5.35-74, based on previously reported fungal TFs (40). Repeat elements were annotated using RepeatMasker 4.0.9 with a fungal repeat library (20170127) from RepBase (77).

**Identification of genes influenced by HOX genes in *C. scovillei***. HOX genes of *C. scovillei*, *M. oryzae*, and *Saccharomyces cerevisiae* and their domains were aligned using MAFFT 7.29 (78); their sequence identities were calculated using the sident program in the trimAl v1.2 package (79). The binding domains of yeast HOX genes (YHP1, YOX1, TOS8, and CUP9) were retrieved from the JASPAR2020 database and used to search potential binding sites in the genome of *C. scovillei* (80). The binding sites were identified by PWMScan using the repeat masked intergenic sequences of the genome (81). Genes within 2 kb downstream of binding sites were collected; level 3 Gene Ontology term functions were visualized using WEGO (20181101) (82).

**Fungal strains and culture conditions.** *C. scovillei* strain KC05, originally isolated from pepper (*C. annuum*) in Gangwon Province, South Korea (32), was used as the wild-type strain. The strain and its transformants were routinely grown on V8 agar medium (80 ml liter\(^{-1}\) V8 juice and 15 g liter\(^{-1}\) agar powder), potato dextrose agar medium (39 g liter\(^{-1}\) powder; Kisan Bio, Seoul, South Korea), or oatmeal agar (50 g liter\(^{-1}\) oatmeal and 15 g liter\(^{-1}\) agar powder) with fluorescent light at 25°C (83). Mycelia for DNA or RNA extraction were cultured in liquid complete medium (CM: 6 g liter\(^{-1}\) yeast extract, 6 g liter\(^{-1}\) Casamino Acids, and 10 g liter\(^{-1}\) sucrose) or on T83 agar medium (200 g liter\(^{-1}\) sucrose, 10 g liter\(^{-1}\) glucose, 3 g liter\(^{-1}\) yeast extract, 3 g liter\(^{-1}\) Casamino Acids, and 8 g liter\(^{-1}\) agar powder).

**Localization of histone H1:DsRed and CsHOX:sGFP fusion protein.** The histone H1:DsRed and CsHOX:sGFP fusion vectors were constructed by overlap cloning method (84, 85). PCR product of histone H1 (1.1 kb) was amplified with paired primers CAP_006988_F/CAP_006988_R (see Table S4), from wild-type genomic DNA. Also, 5.6 kb of DsRed fragment containing HPH gene and promoter of *Neurospora crassa* isocitrate lyase (ICL) encoding gene was amplified with the primer pair pIG-006988_F/pIG-006988_R from pIGPAPA-DsRed (84). The histone H1 fragment and DsRed fragment were combined by using an overlap DNA cloning kit (EpiS Bioitech, Daejeon, South Korea), in which DsRed is under the control of *Neurospora crassa* ICL promoter. The recombinant vector of histone H1:DsRed was transformed into wild-type protoplasts. CsHOX fragments containing 1.5 kb of the promoter region and ORF (open reading frame) minus stop codon of CsHOX2 or CsHOX7 genes was amplified with the paired primers.
Generation of deletion mutants and complemented transformants. The targeted gene deletion vector was constructed based on a modified double-joint PCR (86). Briefly, the 1.5-kb region on upstream and downstream of target gene (Cshox1 to Cshox10) was amplified with the paired primers S5/SR and S3/3R of each gene, respectively (see Table S4). The hygromycin B phosphotransferase (Hph) gene was amplified with the primers HPHF/HPHR and fused with 1.5-kb upstream and downstream regions of each HOX gene by rounds of fusion PCR (87, 88). The fused deletion construct was then amplified in a nested PCR with the paired primers NF/NR. The product from nested PCR was introduced into the wild-type protoplasts by a polyethylene glycol-mediated transformation method as previously described (32). All transformants were first selected by a screening PCR with the paired primers SF/SR and finally confirmed by Southern blotting and RT-PCR. For complementation, the paired primers NF/NR were used to amplify each HOX gene. The PCR product and a 1.5-kb Genomic resistance cassette were then co-introduced into protoplasts of each deletion mutant for Cshox genes. The transformants of complementation were verified by RT-PCR.

Nucleic acid manipulation and gene expression. Fungal genomic DNA was prepared according to a quick and safe extraction method for general screening PCR, or standard extraction method for Southern blot analysis (89, 90). The 500-bp DNA segment used as the Southern blot probe was amplified with the paired primers PF/PR (see Table S4). The genomic DNA was digested with specific restriction enzyme and then probed with that 500-bp DNA segment, which was previously labeled with Biotin-High Prime (Roche, Indianapolis, IN). To perform qRT-PCR and RT-PCR, total RNA was isolated from frozen fungal tissues and infected plant tissues using an Easy-Spin Total RNA extraction kit (iNTRON Biotechnology, Seongnam, South Korea). The complementary DNA (cDNA) was reverse transcribed from 5 μg of total RNA using SuperScript III reverse primer qrtR, and 5 μl of reverse primer qrtF, and 5 μl of reverse primer qrtR, and 5 μl of real-time PCR 2× master mix (Epsis, Daejeon, South Korea). The qRT-PCR experiment was performed with two replicates in three independent experiments using the StepOne real-time PCR system (Applied Biosystems, Foster city, CA). The PCR was set as follows: 95°C for 3 min (1 cycle), followed by 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s (40 cycles). To measure the relative transcript abundance, C_{t} values were normalized to those of β-tubulin of C. scovillei or Actin of C. annum. The normalized fold change of the target gene expression was expressed as 2^{-ΔΔC_{t}}, where −ΔΔC_{t} = ΔC_{t, mutant} − ΔC_{t, control} (91).

Phenotypic characterization of mutants. Vegetative growth of mycelium was evaluated by measuring the diameters of colonies grown on complete medium agar (CMP: 10 g liter\(^{-1}\) sucrose, 6 g liter\(^{-1}\) yeast extract, 6 g liter\(^{-1}\) Casamino Acids, and 15 g liter\(^{-1}\) agar powder) or minimal medium agar (MMA: 0.1 ml liter\(^{-1}\) trace elements, 30 g liter\(^{-1}\) sucrose, 2 g liter\(^{-1}\) NaNO\(_{3}\), 1 g liter\(^{-1}\) KH\(_{2}\)PO\(_{4}\), 0.5 g liter\(^{-1}\) KCl, 0.5 g liter\(^{-1}\) MgSO\(_{4}\), 7H\(_{2}\)O, and 15 g liter\(^{-1}\) agar powder). Conidiation was evaluated by counting conidia collected with 5 ml of distilled water from 7-day-old V8 agar. Lactophenol aniline blue solution was used to distinguish conidiospores from mycelia (33, 92). For conidial germination and appressorium formation, drops of conidial suspension (20 μl) contained 1 μl of cDNA template (25 ng/μl), 1 μl of forward primer qrtF, 1 μl of reverse primer qrtR, and 5 μl of real-time PCR 2× master mix (Epsis, Daejeon, South Korea). The qRT-PCR experiment was performed with two replicates in three independent experiments using the StepOne real-time PCR system (Applied Biosystems, Foster city, CA). The PCR was set as follows: 95°C for 3 min (1 cycle), followed by 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s (40 cycles). To measure the relative transcript abundance, C\(_{t}\) values were normalized to those of β-tubulin of C. scovillei or Actin of C. annum. The normalized fold change of the target gene expression was expressed as 2^{-ΔΔC_{t}}, where −ΔΔC_{t} = ΔC_{t, mutant} − ΔC_{t, control} (91).

Plant infection assays. The wounded plant infection assay was performed by placing a conidial suspension (10\(^{6}\) ml\(^{-1}\)) on wounded healthy pepper fruit, which were then incubated in a humid box for 9 days at 25°C. For the unwounded plant infection assay, the conidial suspension was placed on unwounded healthy pepper fruit, followed by incubation for 6 days at 25°C. To observe fungal invasive hyphae inside pepper cells, a modified trypan blue solution containing 0.05%trypan blue (wt/vol), 30% phenol B (vol/vol), and 30% lactic acid (vol/vol) was applied. Briefly, healthy pepper fruits were inoculated with conidial suspension (5 × 10\(^{6}\) ml\(^{-1}\)) and incubated for 5 days. The thin sections of infected pepper fruits were cut with a razor blade and fixed in a solution containing 10% (vol/vol) acetic acid, 30% (vol/vol) chloroform, and 60% (vol/vol) methanol for at least 16 h. The fixed samples were subsequently dehydrated in ethanol solutions of decreasing concentrations (100% [vol/vol], 70% [vol/vol], and 50% [vol/vol]) for at least 3 h at each concentration. The samples were then stained in modified trypan blue solution and destained with 70% (vol/vol) ethanol. The stained samples were embedded in 30% (vol/vol) glycerol and observed through a microscope. 3,3’-Diaminobenzidine (DAB) was used to stain hydrogen peroxide (H\(_{2}\)O\(_{2}\)) in pepper fruit cells. The thin sections of infected pepper fruits were infiltrated with DAB solution (1 mg ml\(^{-1}\) [pH 7.5]) under vacuum and boiled in 96% (vol/vol) ethanol for 10 min (93).
accumulation of iron (Fe3+) in tissues of pepper fruits was indicated by Perl’s Prussian blue staining according to previously reported methods (94, 95).

RNA-seq and differentially expressed gene analyses. The healthy fruits (green color) of chili pepper (Capsicum annuum) inoculated with 20 μl of distilled water or a conidial suspension (25 × 10^4 conidia/ml) of the wild-type or the ΔCshox1 strain were put in humid plastic boxes and incubated at 25°C for 48 h. The thin segments containing the outer cuticle layer, and epidermal cells were sliced from the infected pepper fruits with a razor blade. Each segment obtained from pepper fruit tissues was observed through a light microscope and confirmed to be infected by the wild-type or ΔCshox1 strain. The confirmed segments were subsequently immersed in the liquid nitrogen. Each RNA sample was extracted from 30 segments using the Easy-Spin total RNA extraction kit (Intron Biotechnology, Seongnam, South Korea) according to the manufacturer’s instructions. The three biological replicates of extracted RNA samples were then sequenced by Illumina HiSeq 2500 (NICEM, Seoul, South Korea). The quality of raw read data from each sample was checked with fastp v0.21.0, including adaptor trimming and low-quality read filtering (96). The trimmed read data were mapped to the reference genome of C. scovillei using HISAT2 v2.2.1 (97), and the read count of reference genes was performed using StringTie2 v2.1.5 (98).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 2.7 MB.
FIG S2, TIF file, 2.2 MB.
FIG S3, TIF file, 2.8 MB.
FIG S4, TIF file, 1.8 MB.
FIG S5, TIF file, 1.5 MB.
FIG S6, TIF file, 1.6 MB.
TABLE S1, XLSX file, 0.2 MB.
TABLE S2, XLSX file, 0.01 MB.
TABLE S3, XLSX file, 0.04 MB.
TABLE S4, XLSX file, 0.02 MB.

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