Host-induced gene silencing of cytochrome P450 lanosterol C14α-demethylase–encoding genes confers strong resistance to *Fusarium* species

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Head blight, which is caused by mycotoxin-producing fungi of the genus *Fusarium*, is an economically important crop disease. We assessed the potential of host-induced gene silencing targeting the fungal cytochrome P450 lanosterol C-14α-demethylase (*CYP51*) genes, which are essential for ergosterol biosynthesis, to restrict fungal infection. In axenic cultures of *Fusarium graminearum*, in vitro feeding of *CYP3RNA*, a 791-nt double-stranded (ds)RNA complementary to *CYP51A*, *CYP51B*, and *CYP51C*, resulted in growth inhibition [half-maximum growth inhibition (IC_{50}) = 1.2 nM] as well as altered fungal morphology, similar to that observed after treatment with the azole fungicide tebuconazole, for which the CYP51 enzyme is a target. Expression of the same dsRNA in *Arabidopsis* and barley rendered susceptible plants highly resistant to fungal infection. Microscopic analysis revealed that mycelium formation on *CYP3RNA*-expressing leaves was restricted to the inoculation sites, and that inoculated barley caryopses were virtually free of fungal hyphae. This inhibition of fungal growth correlated with *in planta* production of siRNAs corresponding to the targeted *CYP51* sequences, as well as highly efficient silencing of the fungal *CYP51* genes. The high efficiency of fungal inhibition suggests that host-induced gene-silencing targeting of the *CYP51* genes is an alternative to chemical treatments for the control of devastating fungal diseases.

HIGS | *Fusarium* head blight | crop protection | RNA interference | small interfering RNA

The diseases *Fusarium* head blight (FHB) and root rot, caused by pathogenic ascomycete fungi of the genus *Fusarium*, such as *Fusarium graminearum* (*Fg*), are devastating diseases of cereal crops (1). *Fusarium* represents one of the most important cereal killers worldwide, exerting great economic and agronomic impact on global grain production and the grain industry. In addition to considerable yield losses, food quality is detrimentally affected by grain contamination with mycotoxins, which are produced by the fungi during plant infection (2–4). These contaminants represent a serious threat to human and animal health (5).

Plant protection and toxin reduction strategies are presently mediated by chemical treatments, resistance breeding strategies, biological control, and genetic engineering. The latter relies on the use of antifungal transgenes, such as chitinase, defensins, polygalacturonase, and the use of mycotoxin detoxifying enzymes (6). However, the use of antifungal traits has not provided convincing practical solutions in terms of efficiency and reliability under agronomical practice.

Currently, the application of systemic fungicides, such as sterol demethylation inhibitors (DMIs), is essential for controlling *Fusarium* diseases and thereby reaching the attainable production level of modern high-yield cultivars. DMI fungicides, such as tebuconazole, triadimefon, and prochloraz, act as ergosterol biosynthesis inhibitors because of cytochrome P450 lanosterol C-14α-demethylase (CYP51) binding, which subsequently disturbs fungal membrane integrity (7). Because of a shortage of alternative chemicals, DMIs have been used extensively in the field since their discovery in the 1970s. Therefore, it is hardly surprising that reduced sensitivity, or even resistance to DMI fungicides, has begun to develop in many plant pathogenic fungi (8–14). The emergence of DMI-resistant *Fg* isolates over the last few years (15) further underscores the need for alternative control strategies.

RNA interference (RNAi) has emerged as a powerful genetic tool that has both accelerated research in plant biotechnology and facilitated the validation of potentially useful agronomical traits. RNAi is known as a conserved integral part of the gene-regulation processes present in all eukaryotes (16, 17); in plants, it is also named posttranscriptional gene silencing (18). Posttranscriptional gene silencing starts with the initial processing or cleavage of a precursor dsRNA into short 21–25 nucleotide small-interfering RNA (siRNA) or micro RNA (miRNA) duplexes (19) by an RNaseIII-like enzyme called Dicer (20). Double-stranded (ds) siRNAs are incorporated into an RNA-induced silencing complex (RISC) containing an Argonaute (e.g., AGO-1 of *Arabidopsis thaliana*) protein that has a small (s) RNA-binding domain and an endonucleolytic activity for cleavage of target RNAs (21, 22). The activated RISC subsequently unwinds the siRNA in an ATP-dependent reaction, thereby generating an antisense (or guide) strand that targets complementary mRNA transcripts via base-pairing interactions. Subsequent degradation of the targeted mRNA causes inhibition of protein biosynthesis (23–25).

AGRICULTURAL SCIENCES

Significance

We demonstrate that host-induced gene silencing (HIGS) targeting the fungal sterol 14α-demethylase (*CYP51*) genes restricts *Fusarium* infection in plants. *Fusarium* diseases have a significant impact not only on global grain production, but also on food safety because of grain contamination with mycotoxins. We capitalized on the knowledge that demethylation inhibitor fungicides target cytochrome P450 lanosterol C-14α-demethylase. In *Fusarium graminearum* (*Fg*), this enzyme is encoded by three paralogous genes. Transgenic *Arabidopsis* and barley expressing a double-stranded RNA targeting all three *CYP51* genes exhibited complete immunity to *Fg*. Our results provide proof-of-concept that HIGS of the *CYP51* genes is an effective strategy for controlling *Fusarium*, demonstrating that HIGS is a powerful tool, which could revolutionize crop plant protection.

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A variety of studies demonstrating the efficiency of RNAi in vitro have been published (26–30). In addition, *in planta* expression of dsRNAs was recently shown to induce host plant-induced gene silencing (HIGS) in fungal cells. In tobacco, expression of a β-glucuronidase (*GUS*) gene-interfering cassette [hairpin (hp) GUS] specifically silenced transcripts in a GUS-expressing strain of *Fusarium verticillioides* during plant colonization (31). HIGS was also shown in barley expressing a dsRNA targeting the fungal effector gene *Avra10*; the transgenics exhibited reduced powdery mildew incidence on leaves, as seen by reduced numbers of functional haustoria inside epidermal cells (32). In addition, *Agrobacterium tumefaciens*-mediated transient silencing, as well as stable transgenic silencing of the *Puccinia triticina* (leaf rust) pathogenicity genes *mitogen-activated protein kinase* 1 (Pt*MAPK1*), *cyclophilin* (Pt*CYC1*), and *calcineurin* B (Pt*CNB*) partly suppressed the growth of *P. triticina*, as well as *Puccinia graminis* and *Puccinia striiformis*, the causative agents of stem rust and yellow rust, respectively, in wheat (33, 34).

However, there are also setbacks of HIGS approaches: Recently, Yin et al. (35) assessed the possibility to target the two rust fungi *P. striiformis* f. sp. *tritici* (*Pst*) and *P. graminis* f. sp. *tritici* (*Pgt*), respectively. The authors used *Barley Stripe Mosaic Virus* as a virus-induced gene-silencing vector to induce, in wheat leaves, RNAi silencing of several fungal candidate genes, including *PSTha12J12*, which was predicted to be secreted by fungal haustoria; although partial silencing of some fungal genes could be demonstrated, an effect on rust disease development or sporulation have not been found (35). A second study showed that the production of dsRNA sequences in *Arabidopsis* was not sufficient to initiate gene silencing in the oomycete pathogen *Phytophthora parasitica* during plant colonization, when targeting either a GFP transgene or the endogenous *P. parasitica* gene transcript *PnPMA1*. One explanation is the absence of the genetic machinery required for the uptake of silencing signals in the oomycete (36).

The present study was conducted to assess the possibility of controlling devastating *Fusarium* diseases via HIGS of the fungal *CYP51* genes. We demonstrate here that silencing of an azole fungicide target is highly efficient in controlling fungal growth. *F. graminearum* contains three paralogous *CYP51* genes (designated *CYP51A*, *CYP51B*, and *CYP51C*), which mediate differential sensitivity to DMIs (37). The *CYP51A*, *CYP51B*, and *CYP51C* genes, which are 1,574, 1,749, and 1,655 nt in length and encode proteins of 507, 526, and 517 aa, respectively (37), are transcribed both in mycelium and fungal conidia. The *CYP51C* gene is found exclusively in *Fusarium* species, and is ubiquitous across the genus (38), whereas all other species in the subphylum Pezizomycotina with multiple *CYP51* paralogs possess *CYP51A* and *CYP51B* genes, with duplications of *CYP51A* or *CYP51B* generating the third paralog in some species. Phylogenetic studies have revealed that the amino acid sequence of FgCYP51A is 64.8% identical to MgCYP51A of *Magnaporthe grisea*, FgCYP51B is 77.7% identical to NcCYP51 from *Neurospora crassa*, and FgCYP51C shows 80.9% identity to FsCYP51 of *Fusarium solani* (37). Previous work also has demonstrated that the deletion of individual Fg*CYP51* genes can reduce conidiation, but otherwise causes minor or no changes in in vitro morphology, mycelial growth rate, or ergosterol content (37). This finding is consistent with the hypothesis that different Fg*CYP51* genes at least partly complement each other for sterol 14α-demethylase activity, and that regulation of gene expression may at least partially compensate dysfunction of paralogous genes (37). The differential sensitivity of the three Fg*CYP51* genes to DMIs also argues for functional diversification (39). This recent study revealed FgCYP51B as the enzyme primarily responsible for sterol 14α-demethylation and essential for generative ascospore formation, and Fg*CYP51A* encodes an additional sterol 14α-demethylase, which is induced on ergosterol depletion and provides compensation for disrupted FgCYP51B function. In contrast, Fg*CYP51C* is a novel genus-specific *CYP51* gene, which does not encode a sterol 14α-demethylase, but impact indirectly on sterol 14α-demethylation, indicated by the accumulation of sterol intermediates in Fg*CYP51C* mutants. The authors presumed that Fg*CYP51C* is required for full fungal virulence on the host (39).

Here, we show that transgenic *Arabidopsis* and barley expressing a stacked dsRNA, which targets all three Fg*CYP51* genes, exhibited almost complete resistance to infection by *F. graminearum*. These results corroborate that HIGS is a powerful tool that could revolutionize agronomical strategies.

Results

In Vitro Silencing of the *CYP51* Genes Inhibits the Growth of *F. graminearum*. To investigate whether silencing of *CYP51* genes affects *Fg* growth in axenic culture, we generated a 791-nt dsRNA (*CYP3RNA*), which was complementary to all three fungal *CYP51* genes (Fig. S1*A*). Microscopic analysis of *Fg* macroconidia treated with *CYP3RNA* revealed growth inhibition and abnormal branching

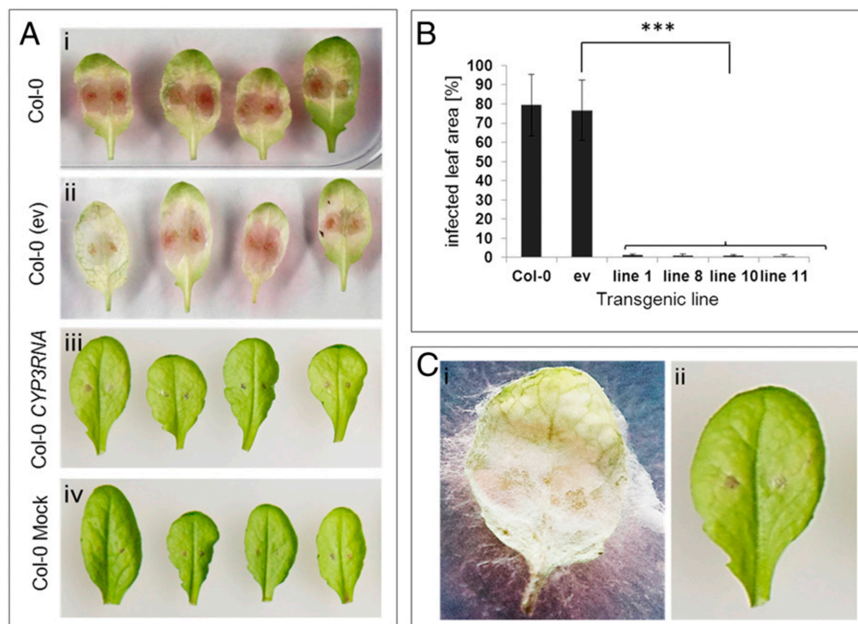

Fig. 1. Infection symptoms on *Arabidopsis* leaves following inoculation with *F. graminearum*. (*A*) Detached leaves of 5-wk-old plants were treated with 5×10^4 macroconidia mL^{-1} and evaluated for necrotic lesions at 3 dpi. (*i*) wild-type (Col-0), (*ii*) Col-0 ev control, (*iii*) Col-0 expressing *CYP3RNA* (representative line L8), and (*iv*) wild-type treated with Tween water (mock). (*B*) Quantification of infected leaf area at 3 dpi; typical infection symptoms are recorded as a percent of the total leaf area. Bars represent mean values ± SDs of three independent experiments, each using 20 leaves collected from 15 different plants of each transgenic line, as well as wild-type and Col-0 ev plants. The reduction in infection symptoms on *CYP3RNA*-expressing leaves compared with the wild-type and Col-0 ev control was statistically significant (***$P < 0.0001$; Student´s *t* test). (*C*) *Fg*-inoculated *Arabidopsis* leaves at 5 dpi. (*i*) The Col-0 ev leaf is heavily infected with *Fg*; (*ii*) Col-0 expressing *CYP3RNA* does not show infection symptoms.

of developing hyphae by 48 h posttreatment (hpt), with increasing concentrations of dsRNA (Fig. S2*A*, *i–iii*). Because CYP51 proteins are the target of azole fungicides, morphological changes of germinating *Fg* macroconidia that were treated with tebuconazole were also monitored. Different concentrations of tebuconazole provoked a phenotype resembling that of the *CYP3RNA* treatment, although inhibition of fungal growth resulted in compact hyphal agglomerates, whereas abnormal branching was less obvious than in *CYP3RNA*-treated samples (Fig. S2*B*, *i–iii*). In comparison, abnormal hyphal branching was not detected in the untreated mycelia (control) (Fig. S2 *A*, *iv* and *B*, *iv*). By 72 hpt, morphological changes of either *CYP3RNA* (Fig. S2*C*, *i*) or tebuconazole (Fig. S2*C*, *ii*) treatment had become more pronounced.

To quantify the inhibitory effects of *CYP3RNA* on fungal growth, the optical density of fungal mycelia was measured (Fig. S2*D*, *i*). Fungal growth was highly reduced at 48 hpt in the concentration range of 4.7–37.5 nM of *CYP3RNA*, and the minimum inhibitory concentration was between 0.3 nM and 0.07 nM *CYP3RNA*. The IC_{50} value was assessed as 1.2 nM *CYP3RNA*. As expected, treatment of *Fg* macroconidia with tebuconazole also inhibited fungal growth (Fig. S2*D*, *i*). These data suggested that *CYP3RNA*, delivered in vitro, silenced the expression of one or more *CYP51* genes in the developing fungus. Moreover, *CYP3RNA* was highly specific for the target *FgCYP51* genes with no sequence off-target in related plant *CYP51* genes (*A. thaliana*, *Hordeum vulgare*), beneficial fungi (*Piriformospora indica*, *Rhizophagus irregularis*), or even human genome, respectively (Table S1). Furthermore, there were no differences in the phenotype of *CYP3RNA* expressing *Arabidopsis* or barley plants, respectively, compared with the control plants.

Expression of *CYP3RNA* in *Arabidopsis* Confers Resistance to *F. graminearum*. Encouraged by the in vitro data, we tested whether expressing *CYP3RNA in planta* could inhibit growth of pathogenic *Fg*. To this end, *Arabidopsis* (*A. thaliana*) Col-0 plants were transformed with either p7U10-*CYP3RNA*, which contains two inverted 35S promoters that drive the constitutive production of sense and antisense copies of *CYP3RNA*, or an empty vector (ev) control (Fig. S1*C*). Resistance to *Fg* was assessed upon inoculating detached leaves of 5-wk-old *Arabidopsis* T2 plants with 5×10^4 *Fg* macroconidia per mL^{-1}. At 3 d postinoculation (dpi), both the untransformed wild-type (Col-0) and the ev control [Col-0 (ev)] showed water-soaked spots with chlorotic or necrotic lesions (Fig. 1*A*, *i* and *ii*); these are typical symptoms of a successful *Fg* infection. In marked contrast, four independent *Arabidopsis* T2 lines (L1, L8, L10, L11), which expressed *CYP3RNA* (Col-0 CYP3RNA), showed no disease symptoms, and their leaves were indistinguishable from those of the noninoculated ev control (Col-0 mock) (Fig. 1*A*, *iii* and *iv*). The percentage of infected leaf area on the four transgenic *CYP3RNA*-expressing lines was 0.9% compared with 77% on the ev control (Fig. 1*B*). Moreover, the *CYP3RNA*-expressing lines remained free of disease symptoms at 5 dpi (Fig. 1*C*, *ii*), but ev plants exhibited substantial symptoms at this time (Fig. 1*C*, *i*).

Fungal hyphae were visualized by microscopy of detached leaves after staining with Trypan blue. On leaves of ev lines, fungal macroconidia germinated and developed mycelia within 3 dpi (Fig. S3 *A* and *C*). Unrestricted fungal development was further indicated by the formation of loose sporodochia formed by branched conidiophores (Fig. S3*C*). In contrast, fungal mycelium formation on *CYP3RNA*-expressing leaves was only visible on the area which was directly inoculated with the fungus; no fungal colonization was detected in the surrounding leaf tissue (Fig. S3*B*). Instead, the fungus rapidly formed macroconidia at the wound sites, indicative of fungal impairment by stress (Fig. S3*D*).

To exclude effects on *Fg* development because of leaf detachment, confocal microscopy of intact *CYP3RNA*-expressing plants was conducted upon inoculation with a *Fg* strain (40), which expressed a GFP. As evidenced by GFP fluorescence, *Fg* effectively colonized leaves of ev lines and developed mycelia within 3 dpi (Fig. S3*E*). In contrast, *Fg* growth on *CYP3RNA*-expressing leaves was restricted to the inoculation site, where stress-induced sporulation could be confirmed (Fig. S3*F*).

Oomycetes are unable to synthesize ergosterol. Consistent with the high specificity of *CYP3RNA* for targeting the ergosterol biosynthesis pathway, growth of the oomycete *Hyaloperonospora arabidopsidis* on *CYP3RNA*-expressing *Arabidopsis* was not impaired (Fig. S4).

***CYP51* Genes Are Strongly Repressed in *Fg*-Infected *CYP3RNA*-Expressing Plant Tissue.** To further confirm that inhibition of *Fg* development was caused by silencing of the *CYP51* genes, quantitative RT-PCR analysis of total RNA from *Fg*-inoculated leaves of *CYP3RNA*-expressing and ev plants was performed at 3 dpi. The relative transcript levels of *CYP51A*, *CYP51B*, and *CYP51C* were

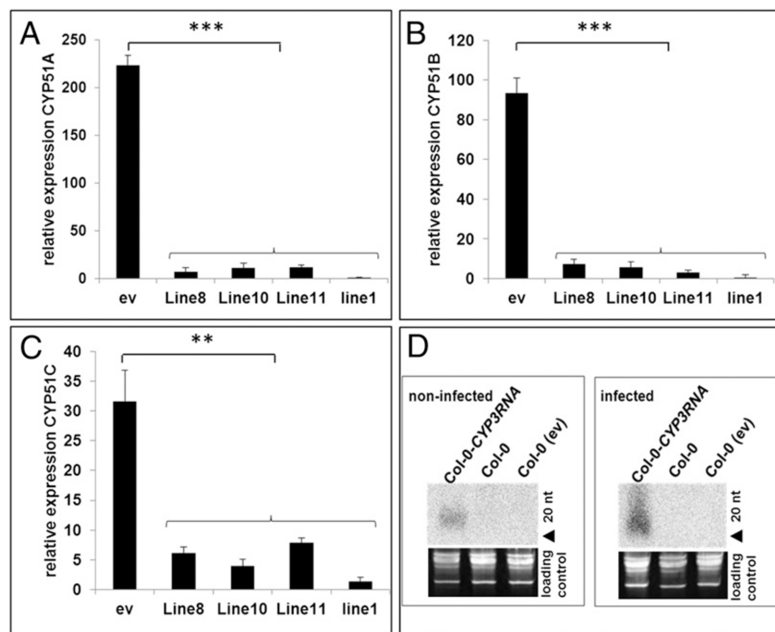

Fig. 2. Abundance of *CYP51* gene transcripts and siRNAs in *Fg*-infected *Arabidopsis* leaves. (*A–C*) Gene-specific analysis of *CYP51* transcripts 3 dpi by quantitative RT-PCR using fungal β-tubulin as the reference gene. (*A*) *CYP51A*, (*B*) *CYP51B*, and (*C*) *CYP51C*. cDNA was generated from total RNA isolated from *Fg*-inoculated leaves collected during the detached leaf assay. Bars represent mean values ± SDs of three independent sample collections. The reduction in *CYP51* gene expression in the *Fg*-inoculated Col-0-*CYP3RNA* leaves compared with the ev control was statistically significant (**$P < 0.001$, ***$P < 0.0001$; Student´s *t* test) (*D*). Detection of low molecular mass siRNA complementary to the *CYP51* genes in pooled leaf tissue from uninfected and *Fg*-infected *Arabidopsis* plants at 3 dpi. Northern blot analysis using ^{32}P-labeled *CYP3RNA* was followed by autoradiography. No signal was detected in the control samples from wild-type and Col-0 ev plants. Ethidium bromide-stained rRNA in the *Lower* panel served as the loading control.

reduced on average by 92%, 89%, and 77%, respectively, in *CYP3RNA*-expressing lines L1, L8, L10, and L11, compared with the ev control (Fig. 2 *A–C*). Whether this reduction in *CYP51* transcripts was associated with the production of corresponding siRNAs was then assessed. Northern analyses using *CYP3RNA* as the probe detected siRNAs corresponding to the targeted *CYP51* sequences in both *Fg*-infected and uninfected leaves from *CYP3RNA*-expressing plants (Fig. 2*D*). In contrast, these siRNAs were not observed in the leaves of wild-type or ev plants. Taken together, these results suggest that expression of *CYP3RNA in planta* effectively silences the three *Fg CYP51* genes, possibly by directing the host plant's silencing machinery to produce the corresponding siRNAs.

Expression of *CYP3RNA* in Barley Confers Resistance to FHB. To further assess the agronomical potential of fungal CYP51 silencing, barley plants of the cultivar Golden Promise were transformed with p6i-*CYP3RNA*, which contains two inverted 35S promoters that drive the constitutive production of sense and antisense copies of *CYP3RNA* (Fig. S1*D*). Quantitative RT-PCR confirmed almost complete silencing of the three *CYP51* genes at 5 dpi in *CYP3RNA*-expressing leaves and roots compared with ev barley (Fig. 3). Silencing efficiency in barley leaves varied between individual lines (Fig. 3*A*). In pooled *CYP3RNA* roots that were rapidly generated in the stable root transformation system (STARTS) (41), all of the three *CYP51* genes showed nearly 100% down-regulation (Fig. 3*B*).

Consistent with strong silencing of *CYP51* genes, detached barley second leaves, expressing *CYP3RNA*, exhibited no (Fig. 4*A*, lines 42, 9, 2, and 7) or weak and locally restricted (lines 3–6, 8, and 14) disease symptoms, respectively, when spot-inoculated

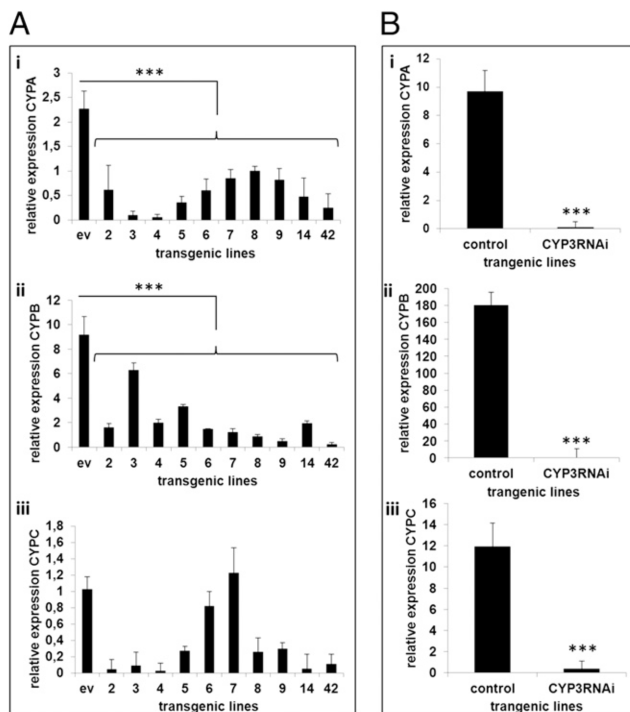

Fig. 3. Quantification of fungal *CYP51* transcripts by quantitative RT-PCR in *Fg*-inoculated barley leaves and roots. (*A*) Transcript abundance of detached leaves at 5 dpi: (*i*) *CYP51A*, (*ii*) *CYP51B*, (*iii*) *CYP51C*. Bars represent mean values ± SDs of two independent experiments. (*B*) Transcript abundance at 3 dpi in roots generated by the STARTS method (41): (*i*) *CYP51A*, (*ii*) *CYP51B*, (*iii*) *CYP51C*. Bars represent mean values ± SDs of three independent experiments. The reduction in *CYP51* gene expression in *Fg*-inoculated Col-0-*CYP3RNA* roots compared with the ev control was statistically significant (***$P < 0.0001$; Student´s *t* test).

with 5×10^4 conidia per mL^{-1} *Fg* macroconidia. In contrast, leaves of wild-type and ev plants were completely overgrown by fungal mycelium.

Next we assessed the effect of *CYP51* silencing on the development of Head Blight symptoms. Ten single caryopses from different ears of each transgenic line were collected during anthesis and inoculated with *Fg* macroconidia. At 4 dpi, caryopses from wild-type and ev plants were heavily infected and colonized, and caryopses from *CYP3RNA*-expressing plants were virtually free of fungal mycelium (Fig. 4*B*). Microscopy of cross-sections confirmed that the inner tissues, such as the endosperm, of wild-type and ev caryopses were heavily infested with *Fg* hyphae, and massive de novo production of macroconidia were found on the surface of necrotic caryopses (Fig. 4*C*, *i* and *ii*). In contrast, virtually no fungal colonization was found on caryopses from *CYP3RNA*-expressing barley plants (Fig. 4*C*, *iii* and *iv*).

Discussion

In this work, we demonstrate that HIGS of essential fungal ergosterol biosynthetic genes is a highly efficient strategy for controlling the growth and development of the phytopathogenic fungus *Fg*. *Fusarium* diseases are devastating both with respect to crop yield and, because of mycotoxin production, yield quality. Despite worldwide efforts to breed *Fusarium* resistance, the most effective strategy for controlling FHB currently involves the application of systemic DMI fungicides. DMIs are the most successful fungicides worldwide, with a market volume of $11.475 million for the year 2010, although other fungicide classes also target CYP51. The extensive use of these chemicals in integrated plant production strategies has led to an increasing incidence of fungi showing resistance to DMI inhibitors (9–14). This resistance is at least partly because of fungi's enhanced capability to detoxify the chemicals.

The *CYP51* genes were selected as potential targets for HIGS based on the previous demonstration that DMI fungicides, such as tebuconazole, function by inhibiting CYP51 activity. Dysfunction of these enzymes leads to a depletion of ergosterol and the accumulation of sterol precursors, including the 14α-demethylated sterols, 4,14-dimethylzymosterol, and 24 methylenedihydrolanosterol, in the plasma membrane and concurrent decrease in CYP51 products [e.g., 14-methylated sterols (42)]. This imbalance alters plasma membrane structure and function, as elevated levels of ergosterol precursors induce permeability changes, membrane leakiness, changes in membrane-bound enzymes, and inhibition of fungal growth. In addition, the 14α-demethylsterols cannot replace ergosterol to stimulate cell proliferation (33–35, 42, 43).

Consistent with the predicted function of the three CYP51 paralogs in ensuring membrane integrity and fungal virulence, targeting their encoding genes for silencing altered *Fg* growth in vitro and inhibited fungal development *in planta*. In vitro treatment with *CYP3RNA* resulted in an increase in hyphal branching and inhibition of fungal growth. This finding also shows that silencing of all three Fg*CYP51* genes is more efficient than depletion of individual *CYP51* genes, which did not cause changes in morphology and mycelial growth rate in an in vitro study (37). Moreover, consistent with the function of CYP51 in the ergosterol biosynthesis pathway we did not find *in planta* suppressive activity of *CYP3RNA* on oomycetes development (Fig. S4).

In transgenic *CYP3RNA*-expressing *Arabidopsis*, fungal growth was restricted to nearly 100%, with a small amount of growth occurring at the wounded area immediately surrounding the inoculation sites. High frequencies of sporulation at these sites also suggest that the fungus is affected in its virulence. Analysis of *CYP51* expression revealed that all three genes were partially silenced in *CYP3RNA*-treated fungus; their expression was suppressed to an even greater extent in *CYP3RNA*-expressing *Arabidopsis* and barley that were colonized by *Fg*. Thus, the altered growth and morphology of the fungus appears to be caused by a reduction in or the complete loss of fungal cytochrome P450 lanosterol C-14α-demethylase expression. The ability of *CYP3RNA*

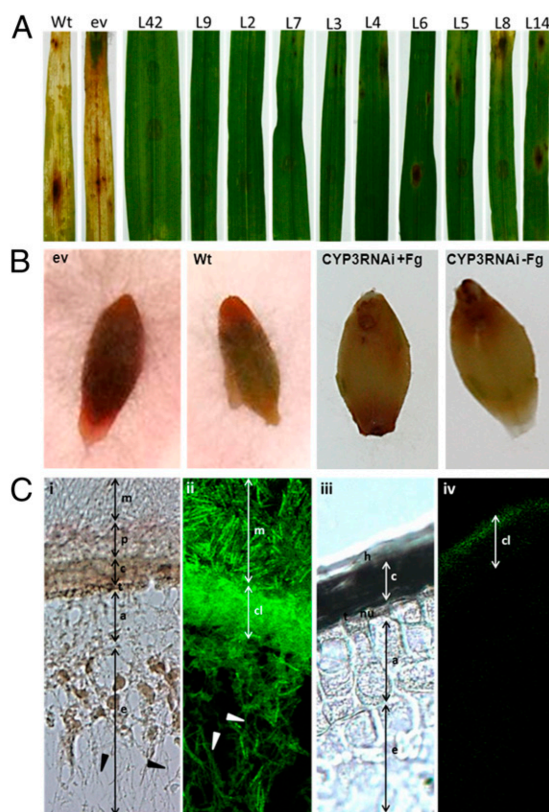

Fig. 4. Infection symptoms on barley leaves and caryopses following inoculation with *F. graminearum*. (*A*) Detached second leaves of 2-wk-old plants were treated with 5×10^4 macroconidia mL^{-1} and evaluated for necrotic lesions at 5 dpi. Wt, wild-type Golden Promise; ev, Golden Promise empty vector control; L2-L9, L14, L42, Golden Promise lines expressing *CYP3RNA*. (*B*) Caryopses were treated with 1.2×10^4 macroconidia per mL^{-1} and evaluated for *Fg* infection at 4 dpi. *CYP3RNA +Fg,* Golden Promise (L2) expressing *CYP3RNA*; *CYP3RNA –Fg*, mock-inoculated (Tween water) Golden Promise expressing *CYP3RNA*. (*C*) Microscopy of *Fg* infection in the epicarp and inner tissue of barley caryopses at 4 dpi: (*i* and *ii*) cross-sections of caryopses from ev plants inoculated with *Fg* (*i*) and with a GFP-expressing *Fg* strain (*ii*); (*iii* and *iv*) cross-sections of caryopses collected from *CYP3RNA* expressing plants treated with *Fg* (*iii*) and with GFP-expressing *Fg* (*iv*). a, aleurone; c, cross cells; cl, cell layers; e, endosperm; m, macroconidia; p, pericarp; t, testa; arrow heads (black and white), infection hyphae.

to silence the *CYP51* genes in germinating macroconidia indicates that the fungus can uptake a 791 nt dsRNA without further delivery support, such as polyethylene glycol, calcium chloride, or electroporation. However, the mechanism of in vitro uptake of RNA is not known. Additionally, the mechanism through which *in planta* expression of a nuclear-integrated *CYP3RNA* construct silences gene expression in plant-colonizing fungi is still a matter of discussion. One can speculate that siRNAs generated by the plant's silencing machinery are transferred and secreted by vesicles. Consistent with this scenario, siRNAs corresponding to the targeted sequences were detected in *CYP3RNA*-expressing *Arabidopsis* independent of fungal infection. However, further studies are required to determine whether *CYP51* gene silencing is mediated by fungal uptake of siRNAs generated by the plant's RNAi machinery, or by their precursor dsRNA.

Taken together, our results demonstrate that HIGS of the fungal *CYP51* genes is an efficient method for inhibiting fungal mycelium formation and plant infection. Our results also demonstrate the potential of RNAi to at least partly supplant application of azole fungicides for control of fungal diseases. Because sequence comparisons suggest that other fungi—such as *M. grisea*, *F. solani*, and *N. crassa*, respectively—contain homologs to the *CYP51* genes, it is possible that this HIGS strategy can be used to control a wide range of fungal pathogens. Concurrently the HIGS technology enables us to generate constructs that are highly specific for the targeted genes, preventing side effects on other (beneficial) microbes and host plants (Table S1). Strikingly, the level of resistance achieved by targeting all three *CYP51* genes for HIGS was substantially greater than that observed in previously published HIGS studies. Taken together, we anticipate that the basic knowledge gained from our studies will open new avenues for developing strategies to prevent devastating plant diseases, including those caused by necrotrophic fungi, such as *F. graminearum*.

Materials and Methods

Construction of *CYP51A*, *CYP51B*, and *CYP51C* Templates and Synthesis of dsRNA. Gene annotations for *F. graminearum CYP51* were obtained from database of the Broad Institute (www.broadinstitute.org). Primers were designed to generate PCR amplicons of 200- to 300-bp length corresponding to exons of selected genes: 294 bp of *CYP51A* (FGSG_04092), 220 bp of *CYP51B* (FGSG_01000), and 238 bp of *CYP51C* (FGSG_11024) (Fig. S1*A*). Genomic template DNA was extracted from *Fg* (40) using DNeasy Plant Mini Kit (Qiagen). All three amplicons were stacked into pGEM-T Easy cloning vector (Promega) (Fig. S1*B*). The stacked clone (*CYP51 B-A-C*) was used as template for the synthesis of dsRNA (*CYP3RNA*) using primers cyp51B (AatII)_F and cyp51C (SacI)_R (Table S2). Synthesis of dsRNA for in vitro studies was performed using BLOCK-iT RNAi TOPO Transcription Kit (Invitrogen) as well as MEGAscript Kit High Yield Transcription Kit (Ambion). Following MEGAscript protocols, primer pairs T7_F and T7_R with T7 promoter sequence at 5′ end of both forward and reverse primers were designed for amplification of dsRNA (Table S2). Synthesized dsRNA was stored at −80 °C.

Plant Infection Assays. For all leaf inoculation assays, *Fg* (44) and *Fg*-GFP (40) conidia concentration was adjusted to 5×10^4 conidia per mL^{-1} (*SI Materials and Methods*). Inoculation of *Arabidopsis* was done by wound inoculation of detached leaves with *Fg*. Twenty rosette leaves of 15 different 5-wk-old plants of each transgenic line (T2) and control plants [empty vector ev and Col-0 wild-type (wt)] were detached and transferred in square Petri plates containing 1% agar. Inoculation was carried out as previously described (44). For assessing the progression of disease symptoms, the lesion size (in centimeters) was measured by 3 dpi from the digital images using the free software ImageJ program (http://rsb.info.nih.gov/ij/index.html). The percentage of leaf area showing infection symptoms relative to the non-inoculated leaf was calculated.

Transgenic barley roots were generated using the STARTS method (41). Roots were inoculated with 1.2×10^4 conidia per mL^{-1} in 0.02% Tween 20 (vol/vol). Inoculated roots were transferred to agar plates. Root samples were harvested at 3 dpi and subjected to RNA isolation, which was used to determine the abundance of fungal CYP51 transcript levels by quantitative RT-PCR.

Leaves of 2-wk-old barley of each transgenic line (T0) and control plants (ev and untransformed Golden Promise wild-type) were detached and transferred in square Petri plates containing 1% agar. Leaves were treated with 5×10^4 conidia mL^{-1} in 0.02% Tween 20 (vol/vol), as described previously (45).

To assess the resistance of *CYP3RNA* transgenic barley plants to FHB, 10 T1 caryopses from different ears of ten individual transgenic lines (T0) were collected. Inoculation was performed as described previously (40).

Interaction studies with *H. arabidopsidis* were performed and analyzed according to ref. 46.

Fungal Transcript Analysis. To assess silencing of the *CYP51* genes in *Fg*, mRNA expression analysis was performed using quantitative real-time RT-PCR (qRT-PCR). For the in vitro assay, 1.2×10^5 *Fg* conidia were grown in 2 mL liquid SNA medium. Next, 162 μg of dsRNA suspended in 200 μL of nuclease-free water (810 $ng \cdot \mu L^{-1}$) was added. Samples were grown at room temperature with shaking (72 h). RNA extraction from the fungal in vitro sample as well as diseased leaves and roots was performed with TRIzol (Invitrogen) following the manufacturer's instructions. Freshly extracted mRNA was used for cDNA synthesis using QuantiTect Reverse-Transcription kit (Qiagen). cDNA was stored at −20 °C. For qRT-PCR, 50 ng of cDNA was used as template in the Applied Biosystems 7500 FAST real-time PCR system. Amplifications were performed in 7.5 μL of SYBER green JumpStart Taq ReadyMix (Sigma-Aldrich) with 0.5 pmol oligonucleotides. Each sample had three repetitions. Primers were used for studying expression

of target *CYP51* genes with reference to β-tubulin gene (FGSG_09530) (Table S2) After an initial activation step at 95 °C for 5 min, 40 cycles (95 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s) were performed. Ct values were determined with the 7500 Fast software supplied with the instrument. Transcript levels of *CYP51* genes were determined via the $2^{-\Delta\Delta Ct}$ method (47) by normalizing the amount of target transcript to the amount of β-tubulin.

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