SIGS vs HIGS: a study on the efficacy of two dsRNA delivery strategies to silence Fusarium FgCYP51 genes in infected host and non-host plants

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SUMMARY
CYP3RNA, a double-stranded (ds)RNA designed to comcomitantly target the two sterol 14α-demethylase genes FgCYP51A and FgCYP51B and the fungal virulence factor FgCYP51C, inhibits the growth of the ascomycete fungus Fusarium graminearum (Fg) in vitro and in planta. Here we compare two different methods (set-ups) of dsRNA delivery, viz. transgene expression (host-induced gene silencing, HIGS) and spray application (spray-induced gene silencing, SIGS), to assess the activity of CYP3RNA and novel dsRNA species designed to target one or two FgCYP51 genes. Using Arabidopsis and barley, we found that dsRNA designed to target two FgCYP51 genes inhibited fungal growth more efficiently than dsRNA targeting a single gene, although both dsRNA species reduced fungal infection. Either dsRNA delivery method reduced fungal growth stronger than anticipated from previous mutational knock-out (KO) strategies, where single gene KO had no significant effect on fungal viability. Consistent with the strong inhibitory effects of the dsRNAs on fungal development in both setups, we detected to a large extent dsRNA-mediated co-silencing of respective non-target FgCYP51 genes. Together, our data further support the valuation that dsRNA applications have an interesting potential for pesticide target validation and gene function studies, apart from their potential for crop protection.

Keywords: CYP51, disease control, Fusarium, Host-induced gene silencing, RNA interference, siRNA, spray-induced gene silencing.

INTRODUCTION
As global population has quadrupled over the last century, one of the biggest challenges of agriculture in the 21st century is how we can achieve global food security that everyone can access. It is estimated that a multitude of different microbial pathogens, viruses, pests and weeds are responsible for plant yield losses on average of more than 30% of global agricultural production (Alexander et al., 2017; Keulemans et al., 2019; Oerke and Dehne, 2004). Mycotoxin contamination of foods and feedstuffs caused by phytopathogenic fungi, such as Fusarium spp., poses an almost intractable problem in agricultural production (Doll and Danicke, 2011). Fusarium species can infect the majority of crop plants, and virtually all cereals such as wheat, barley and maize (Brown et al., 2017; Osborne and Stein, 2007). Current strategies to protect crops from Fusarium infections include fungicide application for both disease control and limitation of mycotoxin accumulation. The most commonly used fungicides are azoles, which target cytochrome P450 sterol 14α-demethylase encoded by CYP51 genes. Inhibition of CYP51 causes depletion of ergosterol, which results in loss of membrane integrity followed by growth inhibition and death of fungal cells (Yoshida, 1988). However, as a consequence of continuous fungicide applications, an increasing rate ofazole insensitivity has been observed in plant pathogenic fungi, including Fusarium species (Becher et al., 2010; Spolti et al., 2014). Filamentous fungi, particularly Ascomycetes, often possess two or more CYP51 paralogues, thus at least partly explaining enhanced tolerance to azole fungicides (Fan et al., 2013; Gsaller et al., 2016). Increasing insensitivity to key pesticides is only one of many production problems, supporting the notion that novel strategies in pathogen and pest control are urgently required.

The use of double-stranded (ds)RNA has promising potential for protecting plants from biotic stress by targeted gene silencing through RNA interference (RNAi) (e.g. Cai et al., 2018a; Dalakouras et al., 2019; Gaffar and Koch, 2019; Koch and Kogel, 2014; Majumdar et al., 2017; Zhang et al., 2017; Zotti et al., 2018). Through a mechanism called host-induced gene silencing (HIGS; Nowara et al., 2010) Arabidopsis thaliana and barley (Hordeum vulgare) plants are more resistant to Fusarium graminearum (Fg) infections when these plants express CYP3RNA, a 791 nucleotide
(nt) long dsRNA targeting all three CYP51 genes (FgCYP51A, FgCYP51B, FgCYP51C) (Koch et al., 2013). Similarly, application of the same dsRNA by spray (SIGS) also protects barley from Fg infection (Koch et al., 2016). While a great number of studies have been published on HIGS-mediated silencing of target genes in pathogenic fungi (e.g. Andrade et al., 2016; Chen et al., 2016; Cheng et al., 2015; Hu et al., 2015; Koch et al., 2013; Panwar et al., 2013; Pliego et al., 2013; Thakare et al., 2017; Zhu et al., 2018; for review see Cai et al., 2018a), targeted gene silencing through exogenously applied dsRNA has been successful in a few additional studies: dsRNA directed against fungal DICER-like (DCL) genes protected various fruits, vegetable and flowers from grey mould caused by Botrytis cinerea infection (Wang et al., 2016). Similarly, both Brassica napus (rapeseed) and Arabidopsis plants were protected by dsRNA sprays against B. cinerea and Sclerotinia sclerotiorum (Mcloughlin et al., 2018). Of note, higher efficacy under field studies directed to control viral diseases was achieved when dsRNA was merged in a composition with non-toxic, degradable, layered double hydroxide (LDH) clay nanosheets (Mitter et al., 2017; Worrall et al., 2019). However, not all fungi are amenable to HIGS or SIGS strategies, as exemplarily shown by the insensitivity of Zymoseptoria tritici to dsRNA that targets essential fungal genes, including ZtCYP51 (Kettles et al., 2019).

The functional diversification of fungal CYP51 genes has not been fully elucidated. It has not been shown whether simultaneous targeting of all three FgCYP51 genes is necessary to reduce Fg infection or whether targeting of a single or two FgCYP51 genes is sufficient. Previous work demonstrated that the mutational deletion of individual FgCYP51 genes can reduce conidiation, but otherwise causes no changes in in vitro morphology, mycelial growth rate or ergosterol content (Liu et al., 2011). A subsequent seminal report showed that FgCYP51B is the major enzyme responsible for sterol 14α-demethylation and required for efficient ascospore formation, while FgCYP51A is an alternative 14α-demethylase that is induced on ergosterol depletion and is responsible for the intrinsic variation in azole sensitivity (Fan et al., 2013). In contrast, FgCYP51C does not encode a 14α-demethylase; homologues are ubiquitous across the genus Fusarium but have not been found in other fungal species (Fernández-Ortuño et al., 2010). Deletion of FgCYP51C results in reduced virulence on host wheat spikes, but not on non-host Arabidopsis floral tissue (Fan et al., 2013).

Here, we comparatively assess the effects of various CYP51-targeting dsRNA constructs (CYP51-dsRNAs) on the development of Fg on barley leaves by employing HIGS and SIGS setups. We show that dsRNA constructs designed to silence concomitantly two paralogous CYP51 genes are highly efficient in inhibiting fungal growth. Moreover, constructs designed to target a single FgCYP51 gene also had detrimental effects on fungal morphology. While the latter finding seemed to contravene mutualistic studies showing that single gene deletion had only minor effects on fungal development (Fan et al., 2013; Liu et al., 2011), further analysis of FgCYP51 expression showed that single and double dsRNA constructs often confer co-suppression in respective non-targeted paralogous FgCYP51 genes.

RESULTS AND DISCUSSION

HIGS by CYP51-dsRNAs targeting single CYP51 genes confers resistance to Fusarium in transgenic Arabidopsis

Previous studies have shown that CYP3RNA, which targets the three FgCYP51 genes, impairs growth of different Fusarium species in vitro (liquid cultures) and in planta (Koch et al., 2013, 2016, 2018), while information on the activity of dsRNA constructs targeting a single or two FgCYP51 genes has been missing. To this end, we assessed the efficacy of single (CYP-A, CYP-B, CYP-C) and double (CYP-AC, CYP-BC, CYP-AB) dsRNA constructs designed to target the same sequence part on FgCYP51A, FgCYP51B and FgCYP51C as the triple construct CYP3RNA (Fig. S1). The respective constructs were expressed under the control of two inverted 35S promoters driving the constitutive production of sense and antisense copies (Fig. S2A). Resistance to Fg was recorded on detached leaves drop-inoculated with 5 × 10⁻⁴ Fg conidia per mL and incubated at room temperature on the laboratory bench. At 5 days post-inoculation (dpi) untransformed Col-0 wild-type (wt) plants showed water-soaked spots with chlorotic and necrotic lesions representing typical symptoms of a successful Fg infection on Arabidopsis leaves (Fig. 1A,B). In clear contrast, and consistent with our earlier work (Koch et al., 2013), CYP3RNA-expressing plants showed strongly reduced infection symptoms at inoculation sites compared to wt. Moreover, plants expressing single or double CYP51-dsRNA constructs equally showed reduced necrotic lesions, though this reduction was only minor and not significant on CYP-C-expressing leaves. Minor activity of CYP-C is consistent with the previous finding that FgCYP51C, in contrast to its function during wheat infections, does not add to virulence of Fg on Arabidopsis (Fan et al., 2013).

Strong resistance in Arabidopsis induced by CYP51-dsRNAs mirrors co-silencing of non-target FgCYP51 genes

To further exclude the possibility that CYP51-dsRNAs have unspecified (antifungal) effects on fungal development rather than inducing gene silencing of corresponding target genes, we measured the expression of the three FgCYP51 genes by qRT-PCR. As anticipated, infection of Arabidopsis plants containing single and double CYP51-dsRNA constructs resulted in down-regulation of the respective fungal targeted gene (Fig. 1C). Notably, all CYP51-dsRNAs also affected the expression
of respective non-target CYP51 genes, suggesting co-silencing effects. For example, CYP-A provoked 80% reduction of FgCYP51A transcripts and also strong reduction of FgCYP51B and FgCYP51C expression by approximately 70% compared to the non-transformed wt. Strong co-silencing of non-target genes by single CYP-dsRNAs may also explain why their effects on fungal virulence are much stronger than single fgcyp51 gene deletions. These deletion mutants showed partly reduced conidiation though normal mycelial morphology in axenic cultures (Liu et al., 2011), while only the double deletion mutants cyp51A/cyp51C and cyp51B/cyp51C reduced growth on potato dextrose agar (though not on SNA agar) (Fan et al., 2013). Of note, co-silencing on the three paralogous FgCYP51 genes is a desired effect of dsRNA-mediated inhibition of ergosterol biosynthesis and fungal virulence when it comes to plant protection. The most efficient construct in terms of overall target gene silencing was CYP-AC, which reduced the transcripts of FgCYP51A and FgCYP51C to less than 10% in comparison to the wt, while non-target gene FgCYP51B transcripts also were reduced by about 50%. Supportive of the finding that FgCYP51C is not required for Fg's virulence on Arabidopsis, the CYP-C construct caused strong down-regulation of FgCYP51C, contrasting its minor effect on fungal infection phenotypes. Overall, these results suggest that resistance in Arabidopsis conferred by various CYP51-dsRNA constructs is mediated to a large extent by co-silencing effects on non-targeted CYP51 genes.

Co-silencing prediction by the TAPIR software

To further explore co-silencing effects, we calculated possible off-targets in CYP51 genes for all tested CYP51-dsRNA constructs. Sequences of the single dsRNA constructs CYP-A, CYP-B and CYP-C were split into k-mers of 21 bases and targeted against the corresponding complementary DNAs (cDNAs) of the three FgCYP51 genes, allowing a maximum of three possible mismatches within the seed region. Based on these parameters, we calculated off-targets for each construct in the other not targeted FgCYP51 genes (Fig. S3). Nearly all of these off-targets in other FgCYP51 genes are in the corresponding region to the targeted region, thus reflecting the strong sequence similarity between them.
Depending on the rather unexplored target specificity of RNAi in fungi, even more relaxed target prediction criteria could be applied that would surely lead to even more predicted off-targets. Supporting this notion, 15 out of 19 bp complementarity between siRNA and target caused off-target effects in mammalian cell culture (Jackson et al., 2003). Additionally, another study reported that bioinformatics prediction of off-targets is still incomplete and that it is likely that a certain number of siRNAs remains undetected due to specificity parameters that are unfounded (Birmingham et al., 2006; Majumdar et al., 2017). We also assessed whether CYP51-dsRNAs would theoretically have additional off-targets in Fg as previously shown for CYP3RNA (Koch et al., 2013). Using the same parameters as for the FgCYP3RNA genes, we found several potential off-targets in the Fg genome, raising the possibility that the virulence of the fungus is additionally affected by down-regulation of these potential targets (Table S1).

### Off-targeting by CYP51-dsRNAs also strengthens HIGS-mediated Fusarium resistance in barley

To further assess the activity of CYP51-dsRNA constructs, we transformed barley cultivar (cv.) Golden Promise with the same CYP51-dsRNA constructs as used for Arabidopsis. Three-week-old detached leaves of transgenic barley were drop-inoculated with $5 \times 10^5$ conidia/mL. At 5 days post-inoculation (dpi), chlorotic and necrotic lesions at inoculation sites were measured. Infection areas on leaves expressing double CYP51-dsRNA constructs were strongly reduced as compared to plants expressing GUS-dsRNA, a non-targeting dsRNA derived from the sequence of the β-glucuronidase (GUS) gene (Figs 2A,B and S4). Moreover, barley leaves expressing the single construct CYP-B also showed strongly reduced infection areas (40%) and leaf necrosis restricted to the inoculation sites, while CYP-A- and CYP-C-expressing plants showed heavy infection symptoms, virtually indistinguishable from the GUS-dsRNA-expressing plants. This result further supports the previous finding that FgCYP51B is required for fungal virulence on cereals and that the additional FgCYP51 genes have only minor roles in the infection process (Fan et al., 2013).

Consistent with their effects on Fg infections, strong co-silencing effects were observed with CYP51-dsRNA double constructs (Fig. 2C): CYP-AC strongly reduced non-target FgCYP51B expression. CYP-BC and CYP-AB co-silenced non-targeted genes FgCYP51A and FgCYP51C, respectively. Moreover, CYP-B strongly reduced FgCYP51B but also non-target genes FgCYP51A and FgCYP51C, further explaining the
strong effect of CYP-B. Similarly, CYP-A strongly silenced FgCYP51A by 60%, but co-silencing of non-target FgCYP51C was weak and co-silencing of non-target FgCYP51B was not significant. Instead, CYP-C effects on the FgCYP51C target and non-target genes overall was minor. Thus, direct silencing and co-silencing effects detected in the FgCYP51 expression analysis fits well the resistance phenotypes observed on leaves. Moreover, the detached leaf assay supports data previously obtained on wheat spikes (Fan et al., 2013), suggesting that this simple and easy setup generates reliable information on gene functions. Overall, differential barley infection phenotypes correlated strongly with FgCYP51 gene expression data. Difference of CYP51-dsRNA activities between the HIGS setups in barley versus Arabidopsis (e.g. equal efficacy of single and double constructs in Arabidopsis) may result from both differences in the RNAi machinery and/or transfer routes of small RNAs from host cells to the interacting pathogen. Furthermore, considering the aspect of host versus non-host plant, the barley immune system might be more affected by pathogen-delivered effector molecules. We also cannot exclude that the uptake/transfer of metabolites including dsRNA or siRNAs is somewhat different in barley versus the non-host plant Arabidopsis.

**Target and off-target effects of CYP51-dsRNAs cause efficient SIGS-mediated resistance to Fusarium**

Next, we tested the activities of CYP51-dsRNAs in SIGS experiments. Consistent with earlier findings (Koch et al., 2016), CYP3RNA reduces fungal development when directly sprayed onto barley leaves prior to inoculation (Fig. S6). To this end, detached barley leaves were sprayed with 20 ng/µL dsRNA and drop-inoculated 48 h later with a suspension of Fg conidia. After 5 dpi, necrotic lesions were visible at the inoculation sites of leaves sprayed with buffer (control). In contrast, CYP51-dsRNAs reduced Fg infection symptoms as revealed by significantly smaller lesions (Fig. 3A). Consistent with earlier results (Koch et al., 2016), spraying the 791-nt long CYP3RNA resulted in a very strong decrease in Fg infection by at least 90%. Overall, infected areas were reduced by CYP51-dsRNAs on average by

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**Fig. 3** *Fusarium graminearum* infection symptoms on barley leaves sprayed with CYP51-dsRNAs. (A) Detached leaves of 3-week-old barley plants were sprayed with CYP51-dsRNAs or TE buffer. After 48 h, leaves were drop-inoculated with 5 × 10^6 conidia/mL and evaluated for infection symptoms at 5 days post-inoculation (dpi). (B) Infection area, shown as percentage of the total leaf area for ten leaves for each dsRNA and the TE control. Error bars indicate SE of two independent experiments. Asterisks indicate statistical significance (***P < 0.01; ****P < 0.001; Student's t-test). (C) Gene-specific expression of FgCYP51A, FgCYP51B and FgCYP51C was measured by qRT-PCR and normalized to fungal EF1-α as reference gene and subsequently normalized against the Δ-ct of the respective control. Detached leaves of 3-week-old barley plants were sprayed with CYP51-dsRNA or TE buffer. After 48 h leaves were drop-inoculated with 5 × 10^6 macroconidia/mL. cDNA was generated at 5 dpi after total RNA extraction from infected leaves. Error bars represent SD of two independent experiments. Asterisks indicate statistical significance (*P < 0.05; **P < 0.01; ***P < 0.001; Student's t-test).
Table 1  Growth inhibition of *Fusarium graminearum* in different RNAi-based silencing setups shown as reduction in % of the infected leaf area

|          | CYP-A | CYP-B | CYP-C | CYP-AC | CYP-BC | CYP-AB | CYP3RNA |
|----------|-------|-------|-------|--------|--------|--------|---------|
| HIGS (Arabidopsis) | 57%   | 66%   | 31%   | 63%    | 51%    | 60%    | 57%     |
| HIGS (barley)      | 7%    | 40%   | 9%    | 39%    | 62%    | 53%    | na      |
| SIGS (barley)      | 80%   | 78%   | 82%   | 83%    | 88%    | 84%    | 93%     |

*controls: HIGS (Arabidopsis) = wild-type Col-0; HIGS (barley) = GUS dsRNA; SIGS (barley) = TE buffer. Values represent means of at least two biological replicates. Statistical significance and SE are indicated in figures (see Figs 1–3). *na, not applicable.

80% compared to the control (Fig. 3B). Expression analysis of *FgCYP51* genes in samples from infected leaves showed target gene silencing in a wide range of always more than 50% (Fig. 3C). Moreover, all CYP51-dsRNAs induced strong co-silencing on *CYP51* genes (Fig. S5). All dsRNAs led to silencing of respective non-targeted *CYP51* genes except for CYP-AB dsRNA, where spray application resulted in up-regulation of *FgCYP51C*. Overall, these data show a strong correlation between resistance phenotypes induced by CYP51-dsRNAs and silenced expression of *CYP51* genes. Notably, under the conditions used here, gene silencing by spray treatment was very efficient (compare HIGS setup Fig. 2 with Fig. 3; Table 1).

Of note, our previous work showed that CYP3RNA was taken up by *Fg* from sprayed leaves and subsequently processed by the fungal RNAi machinery into small RNAs (Koch et al., 2016). Thus, efficient uptake of intact CYP51-dsRNA by fungal infection hyphae could explain their high efficiency in the SIGS setups. Concerning HIGS setups, on the other hand, it is not yet known whether longer dsRNAs such as CYP3RNA are also transferred to the interacting fungus or are processed by the plant’s RNAi machinery into small interfering (si)RNAs in advance of an efficient uptake by the fungus (Baldrich et al., 2016). Therefore, gene silencing by spray treatment was very efficient (compare HIGS setup Fig. 2 with Fig. 3; Table 1).

### Experimental Procedures

#### Fungal material and plant infection

*Fusarium graminearum* strain IFA65 (Department for Agrobiotechnology, Tulln, Austria) was cultured on synthetic nutrient-poor agar (SNA). Preparation of fungal inoculum was performed as described (Jansen et al., 2005). *Arabidopsis thaliana* Col-0 wt and transgenic *Arabidopsis* plants were grown in a climate chamber with 8 h photoperiod at 22 °C with 60% relative humidity. For *Arabidopsis* plants, 15 rosette leaves of each line [transgenic CYP51-dsRNA (T2), wt] were detached and transferred in square Petri plates containing 1% water-agar. Barley cv. Golden Promise and transgenic barley plants were grown in a climate chamber with a 16 h photoperiod at 22 °C with 60% relative humidity. For infection of barley, ten detached second leaves of 2–3-week-old plants were transferred in square Petri plates containing 1% water-agar. Drop-inoculation of *Arabidopsis* and barley leaves was done with 5 and 20 µL, respectively, of a spore suspension of $5 \times 10^4$ conidia/mL. Infection strength was recorded at 5 dpi as infection area by determining the size of chlorotic lesions relative to total leaf area using ImageJ software (https://www.computerbild.de/download/ImageJ-422527.html).

#### Fungal transcript analysis

To assess silencing of the *FgCYP51* genes, mRNA expression analysis was performed using quantitative real-time PCR (qRT-PCR). RNA extraction from the diseased leaves was performed with TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. Freshly extracted mRNA was used as template in the QuantStudio 5 Real-Time PCR system (Applied Biosystems). Amplifications were performed in 7.5 µL of SYBR® green JumpStart Taq ReadyMix (Sigma-Aldrich, St. Louis, MO, USA) with 5 pmol oligonucleotides. Each sample had three technical repetitions. Primers were used for studying expression of *CYP51* genes with reference to *Elongation factor 1-alpha* (*EF1*-a) gene (FGSG_08811) (Table S1). After an initial activation step at 95 °C for 5 min, 40 cycles (95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s) were performed. Ct values were determined with the 7500 Fast software supplied with the instrument. Levels of *CYP51* transcripts were determined via the 2$^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing the amount of target transcript to the amount of the reference transcript *EF1*-a (FGSG_08811).
Single and double CYP51-dsRNAs

CYP-A, CYP-B, CYP-C, CYP-AC, CYP-BC and CYP-AB dsRNA sequences derived from the previously published CYP3RNA dsRNA (Koch et al., 2013). All six constructs were first arranged separately into pGEMT cloning vector (Promega, Madison, WI, USA) (Table S2). The stacked clones were used as template for the synthesis of dsRNA. Synthesis of dsRNA for spray applications was performed using the MEGAscript High Yield Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA) following MEGAscript protocols, primer pairs T7_F and T7_R with a T7 promoter sequence at the 5’ end of both forward and reverse primers were designed for amplification of dsRNA (Table S2). dsRNA was stored at −80 °C.

Spray application of dsRNA on barley leaves

Second leaves of 3-week-old barley cv. Golden Promise were detached and transferred to square Petri plates containing 1% water-agar. The dsRNA was diluted in 500 μL water to a final concentration of 20 ng/μL. For the TE-control, Tris-EDTA (TE) buffer was diluted in 500 μL water corresponding to the amount used for dilution of the dsRNA. Typical RNA concentration after elution was 500 ng/μL, representing a buffer concentration of 400 μM Tris-HCl and 40 μM EDTA in the final dilution. Spraying of the leaves was carried out using a spray flask as described earlier (Koch et al., 2016). Each plate containing ten detached leaves was evenly sprayed with either CYP51-dsRNAs or TE buffer by giving three to four puffs, and subsequently kept at room temperature. Forty-eight hours after spraying, leaves were drop-inoculated with three 20 μL drops of Fg suspension containing 5 × 10⁴ conidia/mL TE. After inoculation, plates were closed and incubated for 5 days at room temperature.

Generation of transgenic Arabidopsis and barley plants

Constructs for plant transformation were obtained by inserting the corresponding CYP51-dsRNA sequences between the HindIII and XmaI restriction sites of p7U10 RNAi for Arabidopsis (DNA Cloning Service, Hamburg, Germany; Fig. S2A) or p6i-Ubi-RNAi2 for barley transformation (Fig. S2B and Table S2). The plasmids were introduced into the Agrobacterium tumefaciens strain AGL1 (Lazo et al., 1991) by electroporation. Transformation of Arabidopsis was performed with the floral dip method as described (Bechtold et al., 1993) and transgenic plants were selected on 7 μg/mL glufosinate (Phosphinotricin, Duchchefa Biochemie, Haarlem, Netherlands) 1/2 × MS medium (Murashige and Skoog, 1962) agar plates. Barley was transformed as described elsewhere (Imani et al., 2011). For barley transformation, inverted ubiquitin promoters from the vector p6i-Ubi-RNAi2 were used (DNA Cloning Service). The transgenic lines that were used in the pathogen assays were previously selected based on expression analysis of partial CYP51 gene fragments in transgenic barley and Arabidopsis. Therefore, RNA was extracted and expression analysis was performed using qRT-PCR as described above. Primers were used for studying transgene expression of CYP51 genes with reference to Arabidopsis and barley Ubiquitin gene (HORVU1Hr1G023660) (Table S2). Levels of CYP51 transcripts were determined via the 2⁻ΔΔCt method (Livak and Schmittgen, 2001) by normalizing the amount of target transcript to the amount of the reference transcript Ubiquitin.

Off-target prediction

The precursor sequences of CYP-A, CYP-B and CYP-C were split into k-mers of 21 bases. These sequences were targeted against the complementary DNA (cDNA) of Fg strain PH-1 (GCA_000240135.3) with TAPIR (Bonnet et al., 2010) using the following settings: score cut-off 6 and minimum free energy ration of 0.55. For each targeted position the number of k-mers was plotted for each construct separately with RStudio (RStudio Team, 2016) and the ggplot2 package (Wickham, 2016). The results were reported as plots (Fig. S3).

Statistical analysis

For statistical analysis, two-tailed Student’s t-test was performed with data gained in plant infection assays and qRT-PCR.

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COMPETING INTERESTS

The authors declare no competing financial and non-financial interests.

AUTHOR CONTRIBUTIONS

K-H.K., A.K. and L.H. wrote the manuscript; K-H.K. and A.K. designed the study; L.H., A.K., J.I. and A.S. conducted the experiments; A.K. and L.H. analysed all data and drafted the figures. B.T.W. and L.J. conducted the bioinformatics analysis and provided bioinformatics support; all authors reviewed the final manuscript.

DATA AVAILABILITY STATEMENT

The genome assembly of the Fg strain PH-1 is openly available from NCBI at www.ncbi.nlm.nih.gov under the accession number GCA_000240135.3.
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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

**Fig. S1** DNA sequences corresponding to CYP51-dsRNAs CYP-A, CYP-B, CYP-C, CYP-AC, CYP-BC, CYP-AB, CYP3RNA and GUS-dsRNA. CYP-dsRNAs derived from sequences of FgCYP51A, FgCYP51B and FgCYP51C of Fusarium graminearum.

**Fig. S2** Schematic representation of RNAi vectors CYP-A, CYP-B, CYP-C, CYP-AC, CYP-BC and CYP-AB used for transformation of Arabidopsis (A) and barley (B).

**Fig. S3** Off-target prediction for single CYP51-dsRNA constructs. Sequences of CYP-A (A), CYP-B (B) and CYP-C (C) were split into 21-mers. These were targeted against the corresponding complementary DNAs (cDNAs) of FgCYP51A, FgCYP51B and FgCYP51C. For each position within the cDNA the number of k-mers that target this cDNA is plotted. As expected, each precursor has a long perfect match on the corresponding CDS. Besides that, one can see potential off-target regions with non-perfect matches.

**Fig. S4** Fusarium graminearum infections on leaves of transgenic barley lines expressing single CYP51-dsRNA constructs CYP-A, CYP-B and CYP-C, and double constructs CYP-AC, CYP-BC and CYP-AB or GUS dsRNA (close-up from Fig. 2A). Detached second leaves of 3-week-old barley plants expressing CYP51-dsRNAs were inoculated with 5 × 10^6 macroconidia/mL. Infection symptoms were assessed at 5 days post-inoculation.

**Fig. S5** Co-silencing of FgCYP51A, FgCYP51B and FgCYP51C by CYP51-dsRNAs as measured by qRT-PCR and normalized to fungal EF1-α as reference gene. Detached leaves of 3-week-old barley plants were sprayed with CYP51-dsRNA or TE buffer. After 48 h leaves were drop-inoculated with 5 × 10^6 conidia/mL. cDNA was generated at 5 days post-inoculation after total RNA extraction from infected leaves. Error bars represent SD of two independent experiments. Asterisks indicate statistical significance. (*P < 0.05; **P < 0.001; Student’s t-test).

**Fig. S6** SiGS-mediated control of Fusarium graminearum on whole barley plants sprayed with CYP3-dsRNA. (a) One-week-old barley plants cv. Golden Promise were sprayed evenly with CYP3-dsRNA (20 ng/µL) or TE buffer (mock control), respectively. Forty-eight hours later plants were spray-inoculated with 2 × 10^6 conidia/mL of F. graminearum and grown for 4 weeks in a growth chamber. Plants were evaluated and categorized according to their infection symptoms: I, without symptoms; II, first leaf infected; III, two leaves infected; IV, more than two leaves infected. (b) Hypocotyls of plants sprayed with CYP3-dsRNA developed less brownish lesions compared to TE-treated control plants.

**Table S1** Primers used in this study for generation of CYP51 RNAi silencing constructs and qRT-PCR.