

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 concomitantly target the two *sterol 14 α -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 14 α -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 of azole 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

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(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; Pliengo *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 14 α -demethylation and required for efficient ascospore formation, while *FgCYP51A* is an alternative 14 α -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 sterol 14 α -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 mutational 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 unspecific (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

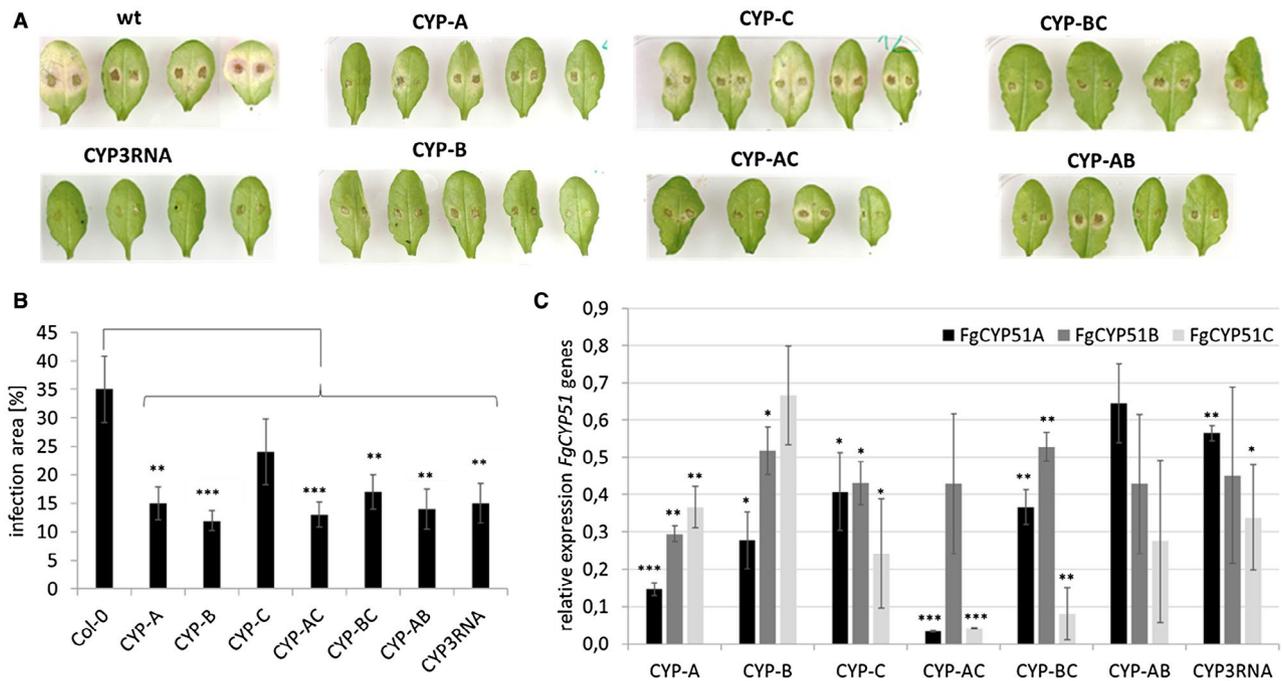


Fig. 1 Host-induced gene silencing in *Fusarium graminearum* upon infection of *Arabidopsis* expressing CYP51-dsRNAs. (A) Fifteen detached rosette leaves of CYP51-dsRNA-expressing *Arabidopsis* plants (T2 generation) were drop-inoculated with 5×10^4 conidia/mL. Infection symptoms were evaluated at 5 days post-inoculation (dpi). (B) Quantification of the visibly infected area at 5 dpi shown as percentage of the total leaf area. Bars represent standard errors (SE) of two independent experiments, each using 15 leaves of ten different plants for each transgenic line. Asterisks indicate statistically significant (** $P < 0.01$; *** $P < 0.001$; Student's *t*-test) differences between CYP51-dsRNA-expressing versus wild-type (wt) genotypes. (C) Gene-specific expression of fungal genes *FgCYP51A*, *FgCYP51B* and *FgCYP51C* was measured by qRT-PCR. Gene expression was first normalized against the fungal reference gene *EF1- α* (FGSG_08811) and subsequently normalized against the Δ -ct of the respective control. cDNA was generated after total RNA extraction from infected leaves at 5 dpi. The reduction in fungal gene expression in infected CYP51-dsRNA-expressing versus wt leaves was statistically significant. Error bars represent standard deviations (SD) of two independent experiments each using 15 leaves of ten different plants for each transgenic line. Asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's *t*-test).

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.

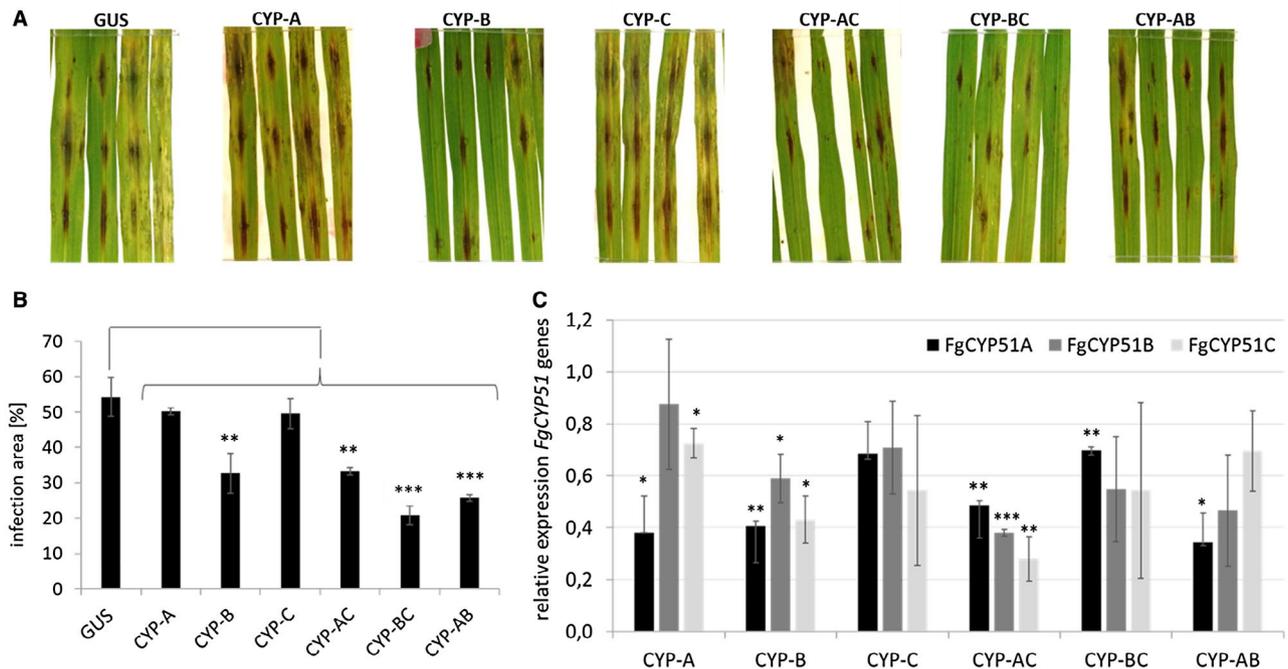


Fig. 2 *Fusarium graminearum* infections and host-induced gene silencing on leaves of barley lines expressing CYP51-dsRNAs. (A) Detached second leaves of 3-week-old barley plants expressing CYP51-dsRNAs and dsRNA derived from the *GUS* gene sequence (*GUS*-dsRNA) were inoculated with 5×10^4 macroconidia/mL. Infection symptoms were assessed at 5 days post-inoculation (dpi). (B) Quantification of the infection area shown as percentage of the total leaf area. Error bars represent SE of two independent experiments, each using ten leaves of ten different plants for each transgenic line. (C) cDNA was generated at 5 dpi after total RNA extraction from infected leaves. Gene-specific expression of *FgCYP51A*, *FgCYP51B* and *FgCYP51C* was measured by qRT-PCR and normalized against the fungal reference gene *EF1- α* and subsequently normalized against the Δ -ct of the respective control. Error bars represent SD of two independent experiments, each using ten leaves of ten different plants for each transgenic line. Asterisks indicate statistical significance (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; Student's *t*-test).

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 *FgCYP51* 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 5×10^4 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

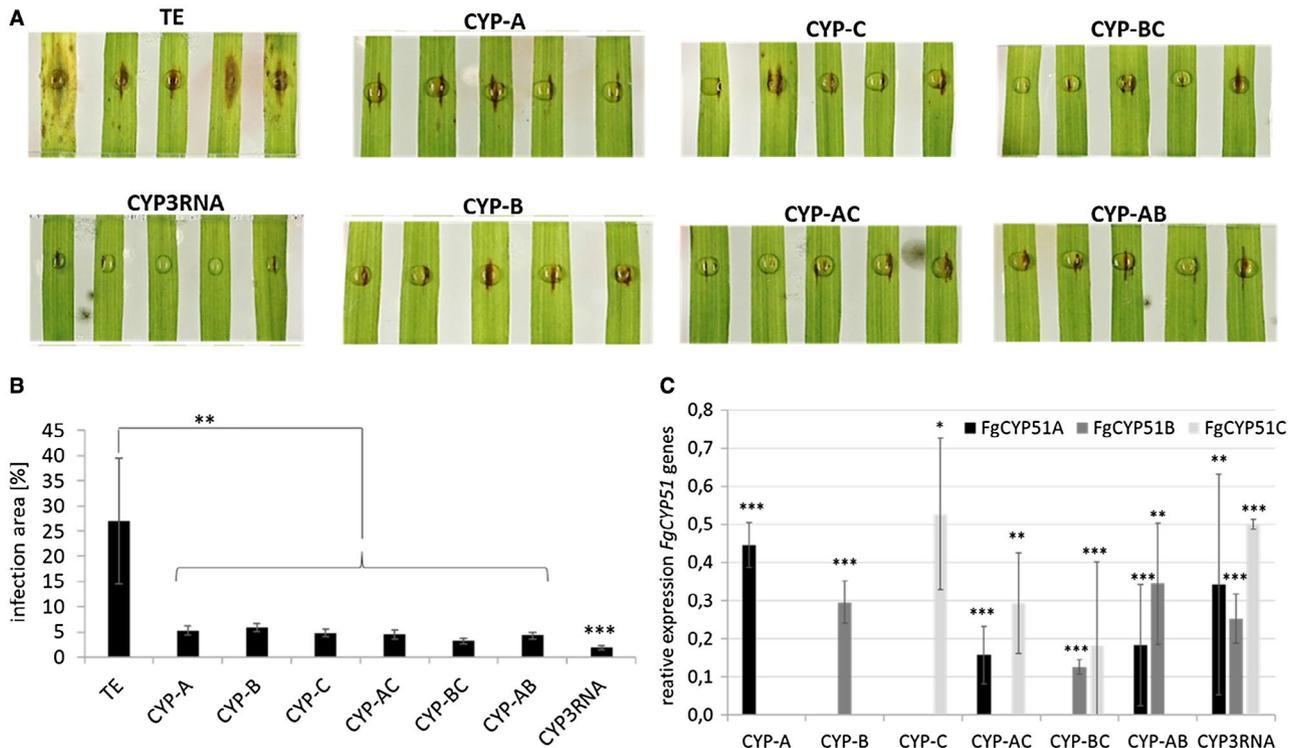


Fig. 3 *Fusarium graminearum* infection symptoms on barley leaves sprayed with CYP51-dsRNAs. (A) Detached leaves of 3-week-old barley plants were sprayed with CYP51-dsRNAs or TE buffer. After 48 h, leaves were drop-inoculated with 5×10^4 conidia/mL and evaluated for infection symptoms at 5 days post-inoculation (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 5×10^4 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).

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/ μ L 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

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 (<i>Arabidopsis</i>)	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.*, 2019; Cai *et al.*, 2018b). While, intuitively, the latter scenario is highly probable, the necrotrophic nature of the fungus, implying strong tissue damage already during early infection stages, makes it difficult to answer this question. Moreover, as both processing and transfer of RNA could also vary from species to species, differences between HIGS efficiencies of CYP51-dsRNAs in barley versus *Arabidopsis* may be explained by variations in the RNAi machinery and/or different pathways of bidirectional RNA transfer (Wang *et al.*, 2016).

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* infection, 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 5×10^4 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 for cDNA synthesis using qScriptTM cDNA kit (Quantabio, Beverly, MA, USA). For qRT-PCR, 10 ng of cDNA 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 $\text{ng}/\mu\text{L}$. 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 $\text{ng}/\mu\text{L}$, 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 5×10^4 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 *Hind*III and *Xma*I 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 $\mu\text{g}/\text{mL}$ glufosinate (Phosphinotricin, Duchefa Biochemie, Haarlem, Netherlands) $1/2 \times \text{MS}$ 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 $2^{-\Delta\Delta\text{Ct}}$ 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.

REFERENCES

- Alexander, P., Brown, C., Arneith, A., Finnigan, J., Moran, D. and Rounsevell, M.D.A. (2017) Losses, inefficiencies and waste in the global food system. *Agric. Syst.* **153**, 190–200.
- Andrade, C.M., Tinoco, M.L.P., Rieth, A.F., Maia, F.C.O. and Aragão, F.J.L. (2016) Host-induced gene silencing in the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*. *Plant Pathol.* **65**, 626–632.
- Baldrich, P., Rutter, B.D., Zandkarimi, H., Podicheti, R., Meyers, B. and Innes, R.W. (2019) Plant extracellular vesicles contain diverse small RNA species and are enriched in 10 to 17-nucleotide 'Tiny' RNAs. *Plant Cell*, **31**, 315–324.
- Becher, R., Hettwer, U., Karlovsky, P., Deising, H.B. and Wirsal, S.G.R. (2010) Adaptation of *Fusarium graminearum* to tebuconazole yielded descendants diverging for levels of fitness, fungicide resistance, virulence, and mycotoxin production. *Phytopathology* **100**, 444–453.
- Birmingham, A., Anderson, E.M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., Marshall, W.S. and Khvorova, A. (2006) 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods*, **3**, 199–204.
- Bechtold, N., Ellis, J. and Pelletier, G. (1993) *In planta* *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *Comptes Rendus de l'Academie des Sciences*, **316**, 1194–1199.
- Bonnet, E., He, Y., Billiau, K. and Van de Peer, Y. (2010) TAPIR, a web server for the prediction of plant microRNA targets, including target mimics. *Bioinformatics*, **26**, 1566–1568.
- Brown, N.A., Evans, J., Mead, A. and Hammond-Kosack, K.E. (2017) A spatial temporal analysis of the *Fusarium graminearum* transcriptome during symptomless and symptomatic wheat infection. *Mol. Plant Pathol.* **8**, 1295–1312.
- Cai, Q., He, B., Kogel, K.H. and Jin, H. (2018a) Cross-kingdom RNA trafficking and environmental RNAi-nature's blueprint for modern crop protection strategies. *Curr. Opin. Microbiol.* **46**, 58–64.
- Cai, Q., Qiao, L., Wang, M., He, B., Lin, F.M., Palmquist, J., Huang, S.D. and Jin, H. (2018b) Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. *Science*, **360**, 1126–1129.
- Chen, W., Kastner, C., Nowara, D., Oliveira-Garcia, E., Rutten, T., Zhao, Y., Deising, H.B., Kumlehn, J. and Schweizer, P. (2016) Host-induced silencing of *Fusarium culmorum* genes protects wheat from infection. *J. Exp. Bot.* **67**, 4979–4991.
- Cheng, W., Song, X.S., Li, H.P., Cao, L.H., Sun, K., Qiu, X.L., Xu, Y.B., Yang, P., Huang, T., Zhang, J.B., Qu, B. and Liao, Y.C. (2015) Host-induced gene silencing of an essential chitin synthase gene confers durable resistance to *Fusarium* head blight and seedling blight in wheat. *Plant Biotechnol. J.* **13**, 1335–1345.
- Dalakouras, A., Wassenegger, M., Dadami, E., Ganopoulos, I., Pappas, M. and Papadopoulou, K.K. (2019) GMO-free RNAi: exogenous application of RNA molecules in plants. *Plant Physiol.* <https://doi.org/10.1104/pp.19.00570>.
- Doll, S. and Danicke, S. (2011) The *Fusarium* toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. *Prev. Vet. Med.* **102**, 132–145.
- Fan, J., Urban, M., Parker, J.E., Brewer, H.C., Kelly, S.L., Hammond-Kosack, K.E., Fraaije, B.A., Liu, X. and Cools, H.J. (2013) Characterization of the sterol 14alpha-demethylases of *Fusarium graminearum* identifies a novel genus-specific CYP51 function. *New Phytol.* **198**, 821–835.
- Fernández-Ortuño, D., Loza-Reyes, E., Atkins, S.L. and Fraaije, B.A. (2010) The *CYP51C* gene, a reliable marker to resolve interspecific phylogenetic relationships within the *Fusarium* species complex and a novel target for species-specific PCR. *Int. J. Food Microbiol.* **144**, 301–309.
- Gaffar, F.Y. and Koch, A. (2019) Catch me if you can! RNA silencing-based improvement of antiviral plant immunity. *Viruses*, **11**, 673.
- Gsaller, F., Hortschansky, P., Furukawa, T., Carr, P.D., Rash, B., Capilla, J., Müller, C., Bracher, F., Bowyer, P., Haas, H., Brakhage, A.A. and Bromley, M.J. (2016) Sterol biosynthesis and azole tolerance is governed by the opposing actions of SrbA and the CCAAT binding complex. *PLoS Pathog.* **12**, e1005775.
- Hu, Z., Parekh, U., Maruta, N., Trusov, Y. and Botella, J.R. (2015) Down-regulation of *Fusarium oxysporum* endogenous genes by host-delivered RNA interference enhances disease resistance. *Front. Chem.* **3**, 1.
- Imani, J., Li, L., Schäfer, P. and Kogel, K.-H. (2011) STARTS—a stable root transformation system for rapid functional analyses of proteins of the monocot model plant barley. *Plant J.* **67**, 726–735.
- Jackson, A.L., Bartz, S.R., Schelzer, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P.S. (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* **21**, 635–637.
- Jansen, C., von Wettstein, D., Schäfer, W., Kogel, K.H., Felk, A. and Maier, F.J. (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted *Fusarium graminearum*. *Proc. Natl. Acad. Sci. USA*. **102**, 16892–16897.
- Kettles, G.J., Hofinger, B.J., Hu, P., Bayon, C., Rudd, J.J., Balmer, D., Courbot, M., Hammond-Kosack, K.E., Scalliet, G. and Kanyuka, K. (2019) sRNA profiling combined with gene function analysis reveals a lack of evidence for cross-kingdom RNAi in the wheat – *Zymoseptoria tritici* pathosystem. *Front. Plant Sci.* **10**, 892.
- Keulemans, W., Bylemans, D. and De Coninck, B. (2019) Farming without plant protection: EPRS European Parliamentary Research Service (PE 634.416; PE 634.416 ISBN: 978-92-846-3993-9 Doi: 10.2861/05433 QA-04-19-225-EN-N; European Union.
- Koch, A. and Kogel, K.-H. (2014) New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. *Plant Biotechnol. J.* **12**, 821–831.
- Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., Linicus, L., Johannsmeier, J., Jelonek, L., Goesmann, A., Cardoza, V., McMillan, J., Mentzel, T. and Kogel, K.H. (2013) Host-induced gene silencing of cytochrome P450 lanosterol C14alpha-demethylase-encoding genes confers strong resistance to *Fusarium* species. *Proc. Natl. Acad. Sci. USA*. **110**, 19324–19329.
- Koch, A., Biedenkopf, D., Furch, A., Weber, L., Rossbach, O., Abdellatef, E., Linicus, L., Johannsmeier, J., Jelonek, L., Goesmann, A., Cardoza, V., McMillan, J., Mentzel, T. and Kogel, K.H. (2016) An RNAi-based control of *Fusarium graminearum* infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathog.* **12**, e1005901.
- Koch, A., Stein, E. and Kogel, K.-H. (2018) RNA-based disease control as a complementary measure to fight *Fusarium* fungi through silencing of the azole target cytochrome P450 lanosterol C-14 α -demethylase. *Eur. J. Plant Pathol.*, **152**, 1003–1010.
- Lazo, G.R., Stein, P.A. and Ludwig, R.A. (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Nat. Biotechnol.* **9**, 963–967.
- Liu, X., Yu, F., Schnabel, G., Wu, J., Wang, Z. and Ma, Z. (2011) Paralogous *cyp51* genes in *Fusarium graminearum* mediate differential sensitivity to sterol demethylation inhibitors. *Fungal Genet. Biol.* **48**, 113–123.
- Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C(T)}$ method. *Methods*, **25**, 402–408.
- Majumdar, R., Rajasekaran, K. and Cary, J.W. (2017) RNA interference (RNAi) as a potential tool for control of mycotoxin contamination in crop plants: concepts and considerations. *Front. Plant Sci.* **8**, 200.
- McLoughlin, A.G., Wytinck, N., Walker, P.L., Girard, I.J., Rashid, K.Y., de Kievit, T., Fernando, W.G.D., Whyard, S. and Belmonte, M.F. (2018) Identification and application of exogenous dsRNA confers plant protection against *Sclerotinia sclerotiorum* and *Botrytis cinerea*. *Sci. Rep.* **8**, 7320.
- Mitter, N., Worrall, E.A., Robinson, K.E., Li, P., Jain, R.G., Taochy, C., Fletcher, S.J., Carroll, B.J., Lu, G.Q. and Xu, Z.P. (2017) Clay nanosheets for topical delivery of RNAi for sustained protection against plant viruses. *Nat. Plants*, **3**, 16207.

- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
- Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumlehn, J. and Schweizer, P. (2010) HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell*, **22**, 3130–3141.
- Oerke, E.-C. and Dehne, H.-W. (2004) Safeguarding production – losses in major crops and the role of crop protection. *Crop Prot.* **23**, 275–285.
- Osborne, L.E. and Stein, J.M. (2007) Epidemiology of Fusarium head blight on small-grain cereals. *Int. J. Food Microbiol.* **119**, 103–108.
- Panwar, V., McCallum, B. and Bakkeren, G. (2013) Endogenous silencing of *Puccinia triticina* pathogenicity genes through *in planta*-expressed sequences leads to the suppression of rust diseases on wheat. *Plant J.* **73**, 521–532.
- Pliego, C., Nowara, D., Bonciani, G., Gheorghe, D.M., Xu, R., Surana, P., Whigham, E., Nettleton, D., Bogdanove, A.J., Wise, R.P., Schweizer, P., Bindschedler, L.V. and Spanu, P.D. (2013) Host-induced gene silencing in barley powdery mildew reveals a class of ribonuclease-like effectors. *Mol. Plant–Microbe Interact.* **26**, 633–642.
- RStudio Team (2016) RStudio: Integrated Development for R. Boston, MA, USA: RStudioInc. <http://www.rstudio.com/>.
- Spolti, P., Del Ponte, E.M., Dong, Y., Cummings, J.A. and Bergstrom, G.C. (2014) Triazole sensitivity in a contemporary population of *Fusarium graminearum* from New York wheat and competitiveness of a tebuconazole-resistant isolate. *Plant Dis.* **98**, 607–613.
- Thakare, D., Zhang, J., Wing, R.A., Cotty, P.J. and Schmidt, M.A. (2017) Aflatoxin-free transgenic maize using host-induced gene silencing. *Sci. Adv.* **3**, e1602382.
- Wang, M., Weiberg, A., Lin, F.M., Thomma, B.P., Huang, H.D. and Jin, H. (2016) Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. *Nature Plants*, **2**, 16151.
- Wickham, H. (2016) ggplot2: Elegant Graphics for Data Analysis. New York, USA: Springer-Verlag.
- Worrall, E.A., Bravo-Cazar, A., Nilon, A.T., Fletcher, S.J., Robinson, K.E., Carr, J.P. and Mitter, N. (2019) Exogenous application of RNAi-inducing double-stranded RNA inhibits aphid-mediated transmission of a plant virus. *Front. Plant Sci.* **10**, 265.
- Yoshida, Y. (1988) Cytochrome P450 of fungi. Primary target for azole antifungal agents. *Curr. Top. Med. Mycol.* **2**, 388–418.
- Zhang, J., Khan, S.A., Heckel, D.G. and Bock, R. (2017) Next-generation insect-resistant plants: RNAi-mediated crop protection. *Trends Biotechnol.* pii: S0167-7799(17)30090-2. <https://doi.org/10.1016/j.tibtech.2017.04.009>.
- Zhu, X., Liu, W., Chu, X., Sun, Q., Tan, C., Yang, Q., Jiao, M., Guo, J. and Kang, Z. (2018) The transcription factor PstSTE12 is required for virulence of *Puccinia striiformis* f. sp. *tritici*. *Mol. Plant Pathol.* **19**, 961–974.
- Zotti, M., Dos Santos, E.A., Cagliari, D., Christiaens, O., Taning, C.N.T. and Smaghe, G. (2018) RNA interference technology in crop protection against arthropod pests, pathogens and nematodes. *Pest Manag. Sci.* **74**, 1239–1250.

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 5×10^4 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 5×10^4 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/ μ L) or TE buffer (mock control), respectively. Forty-eight hours later plants were spray-inoculated with 2×10^4 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 CYP51A_siRNASequence_18, CYP51B_siRNASequence_18, CYP51C_siRNASequence_18.

Table S2 Primers used in this study for generation of CYP51 RNAi silencing constructs and qRT-PCR.