

RESEARCH PAPER



# Study on the efficiency of dsRNAs with increasing length in RNA-based silencing of the *Fusarium CYP51* genes

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## ABSTRACT

Previously, we have demonstrated that transgenic *Arabidopsis* and barley plants, expressing a 791 nucleotide (nt) dsRNA (CYP3RNA) that targets all three *CYP51* genes (*FgCYP51A*, *FgCYP51B*, *FgCYP51C*) in *Fusarium graminearum* (*Fg*), inhibited fungal infection via a process designated as host-induced gene silencing (HIGS). More recently, we have shown that spray applications of CYP3RNA also protect barley from fungal infection via a process termed spray-induced gene silencing (SIGS). Thus, RNAi technology may have the potential to revolutionize plant protection in agriculture. Therefore, successful field application will require optimization of RNAi design necessary to maximize the efficacy of the RNA silencing construct for making RNAi-based strategies a realistic and sustainable approach in agriculture. Previous studies indicate that silencing is correlated with the number of siRNAs generated from a dsRNA precursor. To prove the hypothesis that silencing efficiency is correlated with the number of siRNAs processed out of the dsRNA precursor, we tested in a HIGS and SIGS approach dsRNA precursors of increasing length ranging from 400 nt to 1500 nt to assess gene silencing efficiency of individual *FgCYP51* genes. Concerning HIGS-mediated disease control, we found that there is no significant correlation between the length of the dsRNA precursor and the reduction of *Fg* infection on CYP51-dsRNA-expressing *Arabidopsis* plants. Importantly and in clear contrast to HIGS, we measured a decrease in SIGS-mediated *Fg* disease resistance that significantly correlates with the length of the dsRNA construct that was sprayed, indicating that the size of the dsRNA interferes with a sufficient uptake of dsRNAs by the fungus.

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## Introduction

Crop plants are challenged by a multitude of different pathogens, insects, animals and weeds that cause many different plant diseases and constitute a constant threat to the food supply. It is estimated that those organisms cause yield losses up to 40% of the global agricultural production, which constitutes extremely high costs for our growing world population [1,2]. Besides the yield losses, mycotoxin contamination of foods and feedstuffs caused by phytopathogenic fungi, such as *Fusarium graminearum* (*Fg*), poses an almost intractable problem in agricultural production [3,4]. Current plant protection strategies rely on fungicide application for both *Fusarium* disease control and limitation of mycotoxin accumulation. The most commonly used fungicides are azoles, which target the cytochrome P450 sterol 14 $\alpha$ -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 [5]. However, as a consequence of continuous fungicide applications, an increasing rate of azole insensitivity was observed in several plant pathogenic fungi, including *Fusarium* species [6–8]. Thus, the development of alternative control strategies

has become one of the biggest challenges for plant pathologists these days.

RNA interference (RNAi) is a mechanism of gene regulation that utilizes small RNAs (sRNAs) to direct the silencing of gene expression at the transcriptional or posttranscriptional level. Over the last decade, RNAi-based gene silencing has emerged as a powerful genetic tool in various fields of applied research, such as agriculture [for a review see: 9; 10, 11, 12, 13, 14]. The mechanism of RNAi-mediated plant protection strategies relies on the expression of the transgene that provokes the formation of a double-stranded (ds)RNA precursor molecule *in planta*. This dsRNA subsequently triggers the plant's RNAi machinery to process small interfering (si)RNAs that in the case of an infection exhibit target gene silencing of a certain pathogen [9].

In a previous study, we demonstrated that transgenic *Arabidopsis* and barley (*Hordeum vulgare*) plants, expressing a 791 nucleotide (nt) dsRNA (CYP3RNA) that targets all three *CYP51* genes (*FgCYP51A*, *FgCYP51B*, *FgCYP51C*) in *Fg*, inhibited fungal infection via a process known as host-induced gene silencing (HIGS) [15,16]. In addition to the generation of RNA silencing signals *in planta*, plants can be protected from pathogens and pests by exogenously applied

RNA biopesticides, known as spray-induced gene silencing (SIGS) [17–20,21–22].

Regardless of how target-specific inhibitory RNAs are applied (i.e. endogenously or exogenously), the use of HIGS and SIGS technologies to control *Fusarium* species has been shown to be a potential alternative to conventional pesticides [15,19,23,24,25–27,28,30–32], supporting the notion that RNAi strategies may improve food safety by controlling the growth of phytopathogenic, mycotoxin-producing fungi [reviewed by 33; 34].

Given the ease of dsRNA design, its high specificity, and applicability to diverse pathogens, the use of target-specific dsRNA as an anti-fungal agent offers an unprecedented potential as a new plant protection strategy. However, successful field application will require optimization of RNAi design necessary to maximize the efficacy of the RNA silencing construct. Recently, we compared the efficiencies of HIGS and SIGS dsRNA delivery strategies to assess the activity of novel dsRNA species that were designed to target one or two *FgCYP51* genes [19]. Using barley as a cereal model, we found that dsRNA constructs targeting two *FgCYP51* genes inhibited fungal growth more efficiently than single constructs, although both types of dsRNAs decreased fungal infections [19]. Based on these findings, we anticipate that constructs that target two genes in parallel were more efficient because the number of effective siRNAs derived from those double constructs is higher.

Therefore, we tested in a HIGS and SIGS approach dsRNA precursors of increasing length ranging from 400 nt to 1500 nt to assess gene silencing efficiency of individual *FgCYP51* genes.

We found that SIGS efficiencies depend on the length of the dsRNA that was sprayed, indicating that the size of the sprayed dsRNA interferes with sufficient uptake mechanisms of the fungus. Interestingly, we found that HIGS-mediated disease resistance was independent of the length of the dsRNA constructs. Our findings suggest that HIGS and SIGS approaches differ concerning their mechanistic basis, thus leading to different silencing efficiencies and disease resistance phenotypes depending on RNAi construct design.

## Results and discussion

### Host-induced gene silencing by CYP51-dsRNAs of different length confers resistance to *Fg* in transgenic *Arabidopsis*

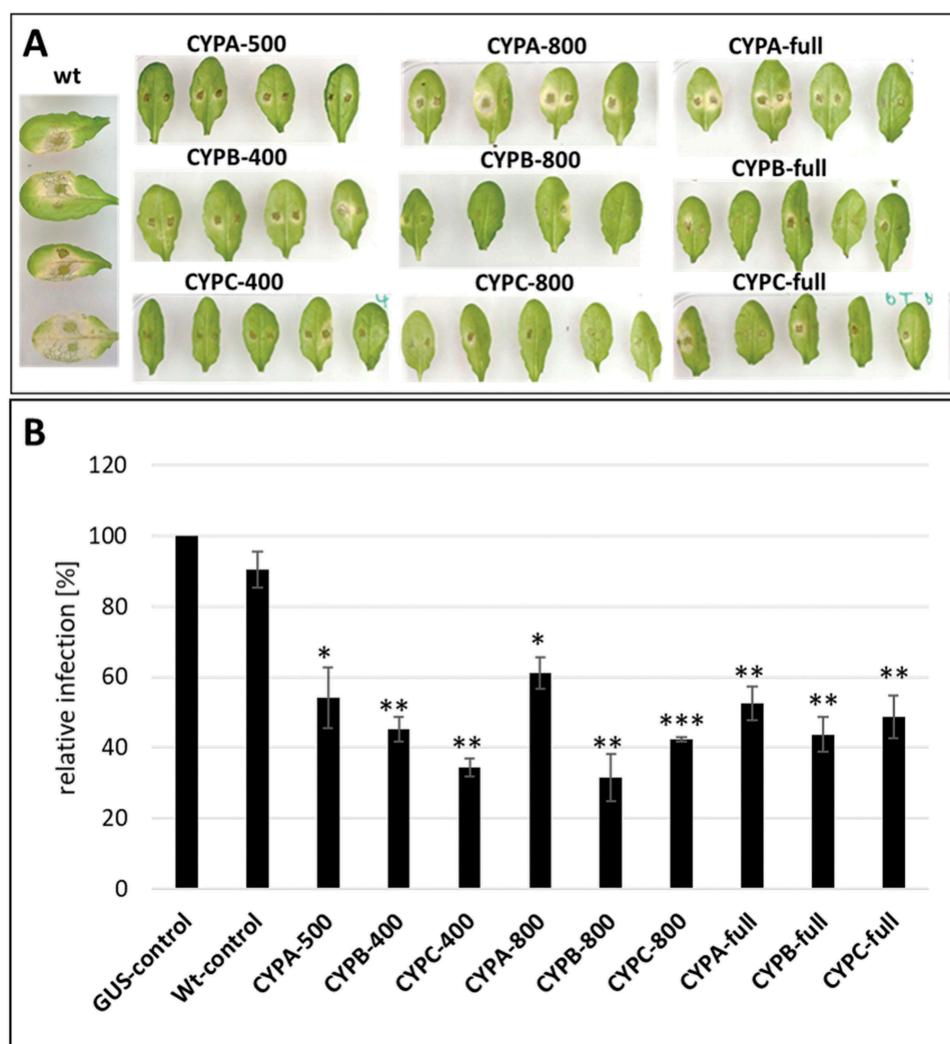
To assess whether silencing efficiency correlates with dsRNA length, dsRNA constructs of 400 nt to 500 nt and 800 nt were generated targeting single *FgCYP51* genes (CYPA-500/800, CYPB-400/800, CYPC-400/800). In addition, the full-length cDNA of each *FgCYP51* gene (CYPA-full, CYPB-full, CYPC-full) was cloned without the start and the stop codon to avoid protein expression. The constructs were inserted into the vector p7U10-RNAi (Fig. S1) and transgenic *Arabidopsis* plants were generated. Resistance to *Fg* was analysed on detached leaves inoculated with  $5 \times 10^{-4}$  *Fg* conidia per ml and incubated at room temperature (RT). At 5 days post-inoculation (dpi) untransformed wild-type (wt) plants showed

water-soaked spots with chlorotic and necrotic lesions representing typical symptoms of a successful *Fg* infection (Fig. 1A). In clear contrast, plants expressing CYP51-dsRNA of different length showed significantly reduced necrotic lesions compared with wt (Fig. 1A). Notably, there were no clear phenotypic differences between 400 nt and 800 nt or full-length constructs (Fig. 1A) and the reduction of the infection area was for nearly all constructs to a similar extent of about 50–60% in comparison to the control (Fig. 1B). The only exceptions were CYPC-400 and CYPB-800, which showed a higher resistance and reduced infection area by 66% and 69%, respectively. Concerning HIGS-mediated disease control, we found that there is no significant correlation between the length of the dsRNA precursor and the reduction of *Fg* infection on CYP51-dsRNA-expressing *Arabidopsis* plants.

Previously, we found that CYP51-dsRNA activity involved co-suppression in the respective non-targeted paralogous *FgCYP51* genes [19]. To analyse whether the observed phenotypes were provoked by co-silencing effects in the non-targeted *CYP51* genes, we measured the transcript levels of *FgCYP51* genes in the infected leaf tissue using quantitative real-time polymerase chain reaction (qRT-PCR). As anticipated, the relative transcript levels of targeted genes *FgCYP51A*, *FgCYP51B*, and *FgCYP51C* were reduced after inoculation of leaves expressing the respective CYP-dsRNA constructs (Fig. 2).

Most interestingly, we found that silencing of *FgCYP51A* was more efficient than for *FgCYP51B* and *FgCYP51C* (Fig. 2). This is consistent with our latest finding, that siRNAs complementary to CYP51A sequence showed the highest abundance among CYP3RNA-transgene-derived siRNAs revealed by RNA-seq analysis (unpublished). Based on this result, we speculate that the amount of siRNAs targeting *FgCYP51A* that reach the fungus is higher, thus, explaining the better gene silencing efficiency. Interestingly, more efficient silencing of *FgCYP51A*, in consequence, did not lead to higher *Fg* disease resistance because CYPA-500 and CYPA-800 showed the lowest resistance by reducing infection areas by only 46% and 39%, respectively (Fig. 1). Furthermore, we found that constructs targeting *FgCYP51B* exhibited higher gene silencing concerning all three *FgCYP51* genes than constructs targeting *FgCYP51A*. This observation is consistent with our off-target prediction that showed the most off-targets for *FgCYP51B* (Fig. 3). Consistent with these findings, we have recently shown that even strong silencing of *FcCYP51A* by almost 90% did not lead to growth inhibition and morphological changes of *Fusarium culmorum* (*Fc*) *in vitro* cultures compared with only 40% silencing of *FcCYP51B*, which resulted in 30% retardation of fungal growth as well as abnormal hyphal phenotypes [30]. These results further support previous reports that have shown that *FgCYP51B* is the most important gene for ergosterol biosynthesis and thus survival of the fungus [19,30,33,35,36].

However, this finding is specific for transgene-derived dsRNAs and their processing by plant Dicers (DCLs) because we did not observe similar results for SIGS. Quite the contrary, as predicted by our off-target analysis, spraying of dsRNA constructs targeting *FgCYP51C* (CYPC-400,



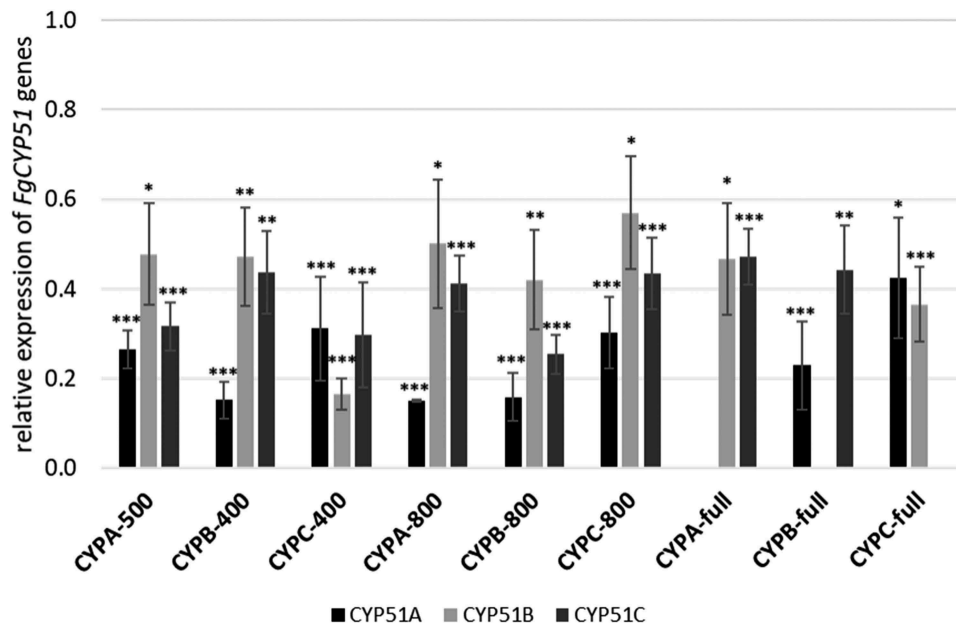
**Figure 1.** Host-induced gene silencing in *Fg* on leaves of transgenic *Arabidopsis* expressing CYP51-dsRNAs of different lengths. (A), 15 detached rosette leaves of CYP51-dsRNA-expressing *Arabidopsis* plants (T2 generation) were drop-inoculated with  $5 \times 10^4$  conidia  $\text{ml}^{-1}$ . Infection symptoms were evaluated at 5 dpi. (B), quantification of the visibly infected area at 5 dpi shown as per cent of the total leaf area. Error bars represent SE of four independent experiments each using 15 leaves of 10 different plants for each transgenic line. Asterisks indicate statistical significance (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's t test).

CYPC-800, and CYPC-full) did not provoke silencing of non-targeted *FgCYP51A* and *FgCYP51B* genes (Fig. 4; see next section).

#### Spray-induced gene silencing efficiency correlates with the length of the sprayed dsRNA

Spraying barley with dsRNA constructs of 200–300 nt in length targeting single *CYP51* genes of *Fg* was superior to HIGS-mediated *Fg* disease control [19]. Encouraged by these findings, we assessed whether 400 nt, 800 nt, as well as full-length CYP51-dsRNAs are also active in spray experiments. Moreover, whether gene silencing efficiency and *Fg* disease resistance can be increased by spraying longer dsRNAs. Towards this, detached barley leaves were sprayed with  $20 \text{ ng } \mu\text{l}^{-1}$  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 TE buffer (control) or GFP-dsRNA (control to exclude unspecific dsRNA effects).

All CYP51-dsRNAs reduced the infection symptoms as revealed by significantly smaller lesions (Fig. 4A). We found the strongest resistance after spraying with 400–500 nt constructs because the infection symptoms were reduced by 82% for CYPA-500, 77% for CYPB-400, and 72% for CYPC-400 (Fig. 4B). Interestingly, infected areas of 800 nt constructs were reduced on average by 65% compared with the control (Fig. 4B), suggesting that the efficiency slightly decreased by spraying 800 nt dsRNA. Recently, we have demonstrated that spraying a 791 nt long non-coding dsRNA (CYP3RNA), which targets the three *CYP51* genes *FgCYP51A*, *FgCYP51B*, and *FgCYP51C* of *Fg*, strongly inhibited fungal growth on barley leaves [18]. Moreover, we have shown that SIGS was mediated by the uptake of the 791 nt dsRNA in the fungus and required the fungal RNAi machinery for the processing of siRNAs [18]. Consistent with these findings, we found that 800 nt CYP51-dsRNAs inhibited *Fg* infection by almost 65% (Table 1). However, 800 nt dsRNAs targeting individual *FgCYP51* genes were less efficient than the 791 nt



**Figure 2.** Silencing of *FgCYP51* genes of infected transgenic *Arabidopsis* leaves. Gene-specific expression of *FgCYP51A*, *FgCYP51B*, and *FgCYP51C* was measured by qRT-PCR and normalized to fungal *EF1- $\alpha$*  (FGSG\_08811) as a reference gene. cDNA was generated after total RNA extraction from infected leaves at 5 dpi. The reduction in *CYP51* gene expression in the *Fg*-inoculated dsRNA-expressing leaves compared with the wt control was statistically significant. Error bars represent SE of four independent experiments each using 15 leaves of 10 different plants for each transgenic line. Asterisks indicate statistical significance (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's *t* test).

CYP3RNA that targets all three *FgCYP51* genes in parallel which led to 93% disease resistance [19]. This difference can be partly explained by co-silencing of *FgCYP51* genes.

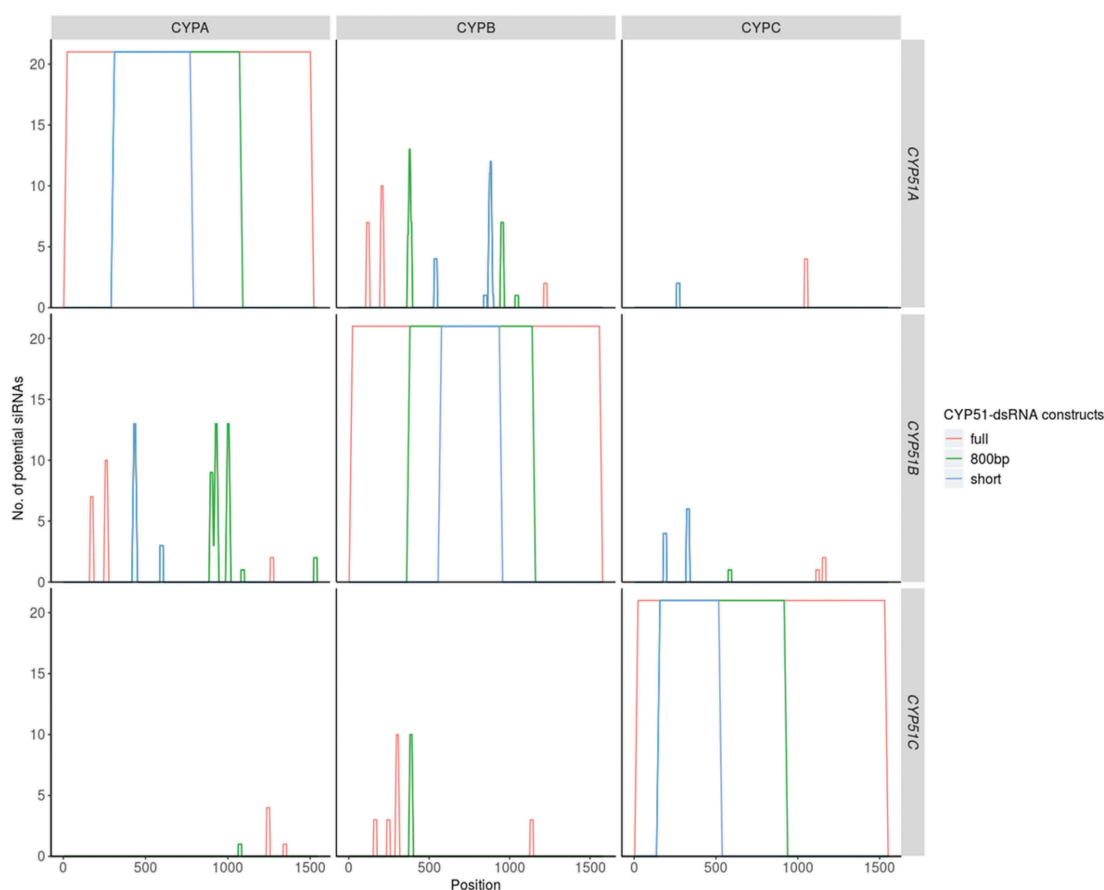
Previously we found that HIGS- as well as SIGS-mediated targeting of individual *FgCYP51* genes resulted in co-silencing of the non-targeted *FgCYP51* genes [19]. To explore co-silencing effects further, we calculated possible off-targets in *FgCYP51* genes for all tested CYP51-dsRNA constructs (Fig. 3; Table 2). Sequences of the different dsRNA constructs were split into k-mers of 21 bases and targeted against the complementary DNAs of the three *FgCYP51* genes, with the plant miRNA target prediction tool TAPIR [37]. Based on the default parameters, we calculated off-targets for all constructs in the respective non-target *FgCYP51* genes (Fig. 3) which is consistent with the observed gene silencing of all three *FgCYP51* genes (Fig. 2; Fig. 5).

Consistent with our previous results, we observed that the number of off-targets increased with the increasing length of the different CYP51-dsRNAs (Fig. 3; Table 2). Thereby, we found that dsRNA constructs targeting *FgCYP51A* and *FgCYP51B* had the highest number of off-target hits in the non-targeted *FgCYP51* genes (Fig. 3). These observations can be explained based on the closer phylogenetic relationship between *FgCYP51A* and *FgCYP51B*, which leads to a higher amount of potential siRNAs targeting both genes (Table 2; Fig. S3). In contrast, *FgCYP51C* as phylogenetically more distant predicts fewer siRNAs complementary to *FgCYP51A* and *FgCYP51B* and vice versa (Table 2; Fig. S3). Thus, phylogenetic relationship correlates with the number of predicted siRNAs causing potential off-target hits. Notably, less stringent prediction criteria increase the predicted off-targeting siRNAs (Table S1; Fig. S4). Thereby, the number of off-targets per construct increased with the length of the

precursor RNA showing a maximum in the full-length constructs, as expected (Table 2). This was regardless of whether *FgCYP51A*, *FgCYP51B*, or *FgCYP51C* was the actual target. Given this correlation, we observed the strongest silencing efficiencies for CYP51A- and CYP51B-dsRNA constructs (Fig. 5). Notably, *FgCYP51C*-derived constructs seem to have fewer off-targets in respective non-target genes than *FgCYP51A*- and *FgCYP51B*-derived constructs, reflected by qRT-PCR results (Fig. 5). Even more important, we observed that CYP51C-400 and CYP51C-800 showed similar *Fg* disease resistance phenotypes compared with CYP51A- and CYP51B-dsRNA constructs (Fig. 5; Table 1). Based on this result, we speculate that silencing of *FgCYP51C* is sufficient to provoke fungal inhibition at least in SIGS. Of note and in clear contrast to the SIGS data, HIGS plants expressing CYP51C-dsRNA showed silencing of the non-targeted genes *FgCYP51A* and *FgCYP51B* although this was not expected by the stringent off-target prediction (Fig. 2; Fig. 4). Together, these findings further prove that the molecular mechanisms underlying the transgene-based (HIGS) or spray-mediated (SIGS) delivery strategy are different, either requiring the plant's or the fungal RNA silencing machinery (Fig. 6), thus leading to different silencing phenotypes.

However, these data were obtained from *in silico* predictions; therefore, their accuracies remain unknown. As such, RNA sequencing (RNA-seq) must be performed to quantify, analyse, and map the HIGS-derived siRNAs to their target genes as well as their dsRNA precursors to prove whether the generation of secondary siRNAs can explain the obtained HIGS results. In addition, RNA-seq of SIGS plants would help to clarify whether the production of siRNAs in SIGS and/or barley plants occurred at all. To prove further whether the longer dsRNAs result in higher numbers of siRNAs, we



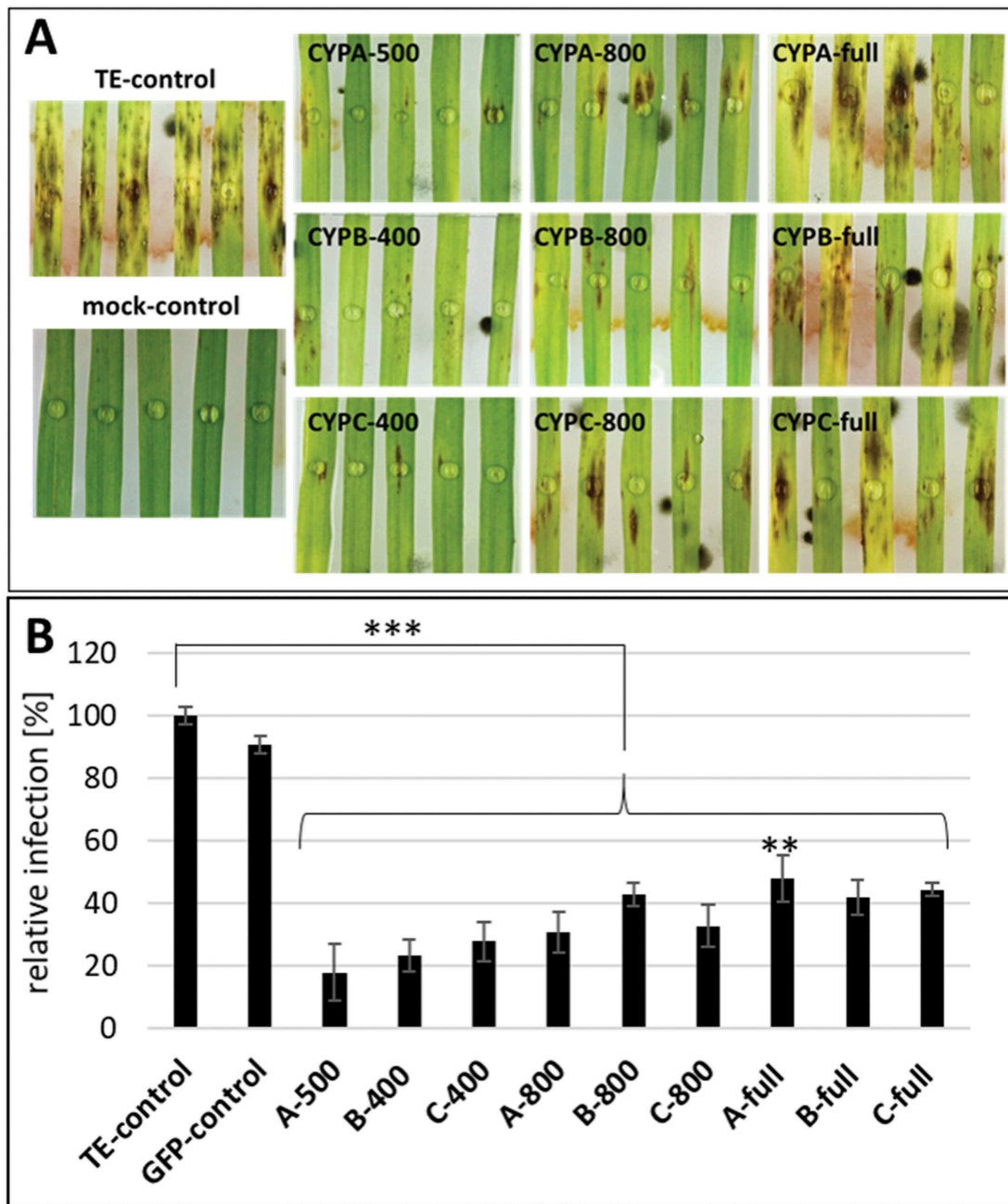


**Figure 3.** Off-target prediction for single CYP-dsRNA constructs with different length. Sequences of CYP51-dsRNAs were split into k-mers of 21 nt. These were targeted against the corresponding complementary DNA sequences (cDNA) of *FgCYP51A*, *FgCYP51B*, and *FgCYP51C*. For each position within the cDNA (x-axis) the k-mers that target the respective gene is plotted. The plot is faceted along the x-axis by the dsRNA constructs and along the y-axis by the target gene. The colours indicate the dsRNA length. While siRNAs deriving from the short dsRNAs (400 bp/500 bp) are shown in blue, logically also the longer dsRNAs produce these siRNAs. Accordingly, the siRNAs deriving from the 800 bp constructs are shown in blue and siRNAs derived exclusively from the full-length dsRNAs are shown in red.

predicted the number of siRNA hits for each CYP51-dsRNA construct using SiFi (<https://sourceforge.net/projects/sifi21>) as a prediction tool. Similar to what we observed for the off-target prediction, we found a strong correlation between the length of the dsRNA precursor and the precursor-derived siRNAs (Table 3). However, these off-target-based co-silencing effects were more obvious for SIGS than for HIGS (compare Fig. 2 with Fig. 5) further supporting our previous finding that SIGS involves the uptake of dsRNA by the fungus and requires the fungal RNAi machinery [18,38]. Consistent with this idea, uptake of unprocessed dsRNAs from leaf tissue and processing by fungal DCLs might be more efficient than application of siRNAs because this would increase the amount of efficient siRNAs in the target organism. Moreover, in contrast to using only one specific siRNA, processing of long dsRNA into many different inhibitory siRNAs by the fungus may reduce the chance of pathogen resistance under field test conditions. Thus, we assume that SIGS can be more efficient than HIGS depending on the lifestyle of the targeted pathogen. Importantly, until now SIGS was demonstrated against necrotrophic fungal pathogens, such as *Fg* and *Botrytis cinerea* [18,22]. However, further research must show whether uptake of SIGS-derived siRNAs by biotrophic pathogens would also increase SIGS-mediated disease resistance.

### **Inhibition of fungal infection negatively correlates with the length of the sprayed dsRNA**

Spraying with full-length CYP51-dsRNA resulted in the lowest decrease in *Fg* infection by at least 50% (Fig. 3B). Thus, we observed a decrease in *Fg* infection that correlates with the length of the sprayed dsRNA (Table 1). However, expression analysis of *FgCYP51* genes in infected leaves showed target gene silencing and an overall strong co-silencing (Fig. 5). All constructs led to downregulation of respective non-targeted CYP51 genes except for constructs targeting *FgCYP51C* (CYPC-400, CYPC-800, and CYPC-full), where upregulation of the non-targeted *FgCYP51A* and *FgCYP51B* genes was measured (Fig. 5). This was consistent with the off-target prediction, where CYPC constructs showed the lowest number of siRNA that match to the *FgCYP51A* and *FgCYP51B* genes (Fig. 3; Table 2). Overall, these data show a strong correlation between resistance phenotypes induced by CYP51-dsRNA constructs and reduced expression of *FgCYP51* genes. Notably, we observed that co-suppression was completely lost when leaves were sprayed with full-length CYP51-dsRNA (Fig. 5). Unfortunately, analysis of target gene silencing was not possible because



**Figure 4.** *Fg* infection of barley leaves that were spray treated with CYP51-dsRNAs of different lengths. (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  $5 \times 10^4$  conidia  $\text{ml}^{-1}$  and evaluated for infection symptoms at 5 dpi. (B), Infection area, shown as per cent of the total leaf area for 10 leaves for each dsRNA and the TE control. Error bars indicate SE of four independent experiments. Asterisks indicate statistical significance (\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's t test).

**Table 1.** Growth inhibition of *Fg* during different RNAi-based silencing setups. Growth inhibition is shown as reduction in % of the infected leaf area in comparison to the control\*.

	CYPA-294	CYPB-220	CYPC-238	CYPA-500	CYPB-400	CYPC-400	CYPA-800	CYPB-800	CYPC-800	CYPA-full	CYPB-full	CYPC-full
SIGS	80%	78%	82%	82%	77%	72%	69%	57%	67%	52%	58%	56%
HIGS	57%	66%	31%	46%	55%	66%	39%	69%	58%	48%	56%	51%

\* (controls: HIGS (*At*) = wt Col-0; SIGS = TE buffer) Values represent means of four biological replicates. Statistical significance and SE is indicated in above shown figures (see Fig.1; Fig.4)

there were no primers available that would not amplify the sprayed RNA as well.

However, the decrease in efficiency from 65% for 800 nt CYP51-dsRNAs to only 50% with the full-length

dsRNAs indicates that the size of the dsRNA interferes with a sufficient uptake of dsRNAs by the fungus. To prove this idea further, we treated *Fg* with full-length CYP51-dsRNAs *in vitro*. We found that there was no

**Table 2.** siRNA prediction of CYP51-dsRNA constructs using TAPIR.

dsRNA-construct	No. Of potential siRNAs		
	Target genes		
	CYP51A	CYP51B	CYP51C
CYP51-500bp	480	16	0
CYP51-800bp	780	54	1
CYP51-full	1498	73	6
CYPB-400bp	17	380	0
CYPB-800bp	38	780	10
CYPB-full	57	1555	29
CYPC-400bp	2	10	380
CYPC-800bp	2	11	780
CYPC-full	6	14	1528

Number of potential siRNAs with 21 nt in length of each dsRNA are shown for each target gene. For the prediction the same criteria as in Fig. 4 were used.

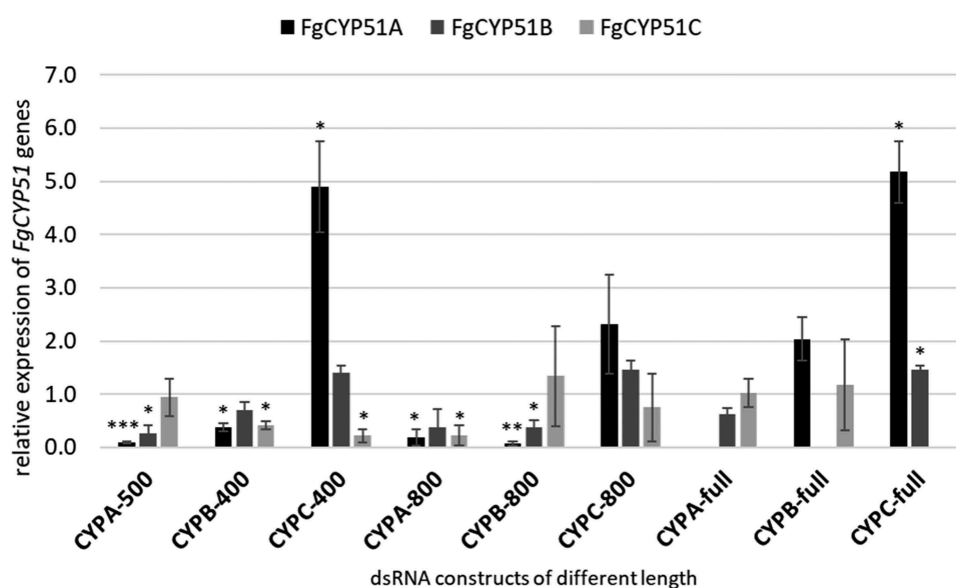
silencing of the non-targeted *FgCYP51* genes, indicating that *Fg* was not able to take up >1500 nt dsRNA from liquid cultures (Fig. S2). Unfortunately, we were not able to analyse target gene silencing directly because there were no primers available that would distinguish between dsRNA that was applied to the medium and silencing of *FgCYP51* target genes. However, because we observed 50% fewer infection symptoms after spraying barley leaves with full-length CYP51-dsRNA (Table 1), we anticipate that there are differences in the mechanism of fungal dsRNA uptake in SIGS compared with *in vitro* culture treatments. In *Aspergillus nidulans* as well as in *Aspergillus fumigatus* uptake of siRNAs resulted in gene silencing [39–41]. In *Candida albicans* a structure-dependent uptake efficiency could be observed because linear nucleic acids were taken up more efficiently than hairpin structures [42].

Congruent with the idea of a different uptake/translocation mechanism, we demonstrated that HIGS, even though 200–500 nt CYP51-dsRNA constructs were less efficient than SIGS, is not limited by the size of the dsRNA precursor the plants were

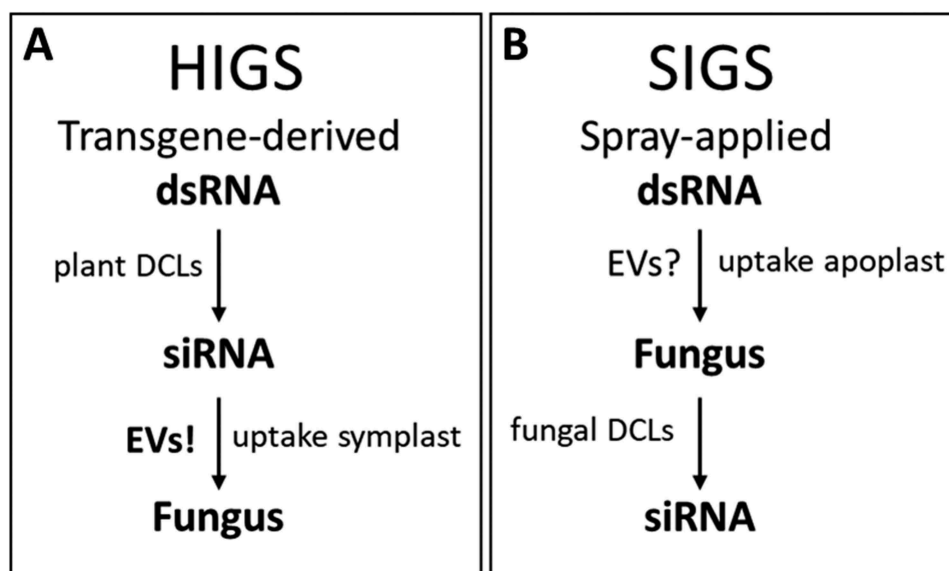
transformed with. Because dsRNAs are expressed *in planta* and subsequently processed by DCLs enzymes, we would expect that increasing the length of the dsRNA results in more siRNAs that were taken up by the fungus (Table 3). Unfortunately, and in contrast to our expectation, the data did not show such a correlation (Table 1). However, even for HIGS, it must be considered that the host produced dsRNA precursor could be taken up and processed by the fungus itself. Consistent with our findings, a size-dependent efficiency of dsRNA-mediated silencing of target genes has been observed in insects [43,44]. Thereby, the most efficient dsRNA length varies hardly among different species whereas most studies show successful gene silencing with dsRNA lengths from 140 to 520 nt [29,45]. In the western corn rootworm, silencing efficiency increased with increasing dsRNA lengths (60–200 nt) whereas a minimum of 60 nt dsRNA is required for successful gene silencing [43]. By targeting the same gene in aphid feeding studies, only dsRNA and not siRNAs were able to achieve gene silencing [46]. In *Leptinotarsa decemlineata* even a 1842 nt dsRNA resulted in mortality in feeding experiments [47,48]. Interestingly, in the same study also shorter dsRNAs of 134 nt and 300 nt conferred gene silencing, suggesting that target gene selection is superior to dsRNA length. Summarizing, our results together with the studies mentioned before suggest that the efficiency of RNA-based disease control depends on the uptake and processing of efficient siRNAs that reach the pathogen/pest. Even more important, the mechanisms underlying uptake and processing of dsRNAs are different for HIGS and SIGS (Fig. 6), thus influencing design criteria of the RNAi trigger compound.

## Conclusion

Plants can be protected from pathogens/pests mediated by lethal RNA silencing signals generated *in planta* [for a review on HIGS, see 9, 10, 11, 12, 13, 14] as well as by



**Figure 5.** Silencing of *FgCYP51* genes of infected spray treated barley leaves. Gene-specific expression of *FgCYP51A*, *FgCYP51B*, and *FgCYP51C* was measured using qRT-PCR and normalized to fungal *EF1-α* as a 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  $5 \times 10^4$  macroconidia  $\text{ml}^{-1}$ . cDNA was generated at 5 dpi after total RNA extraction from infected leaves. Error bars represent SE of two independent experiments. Asterisks indicate statistical significance. (\* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; Student's t test).



**Figure 6.** Molecular mechanisms underlying the transgene-based (HIGS) or spray-mediated (SIGS) delivery strategy. (A), the molecular mechanism of HIGS is based on the plant's silencing machinery. The integration of our findings supports the view that HIGS involves the processing of transgene-derived dsRNA in the plant (plant DCLs) and extracellular vesicle (EV)-mediated delivery into the interacting fungus. (B), the molecular mechanism of SIGS is controlled by the fungal silencing machinery. In summary, our findings support the model that SIGS involves the uptake of sprayed dsRNA by the plant (via stomata) and subsequent uptake of apoplastic dsRNA by the fungus resulting in fungal DCL-dependent production of siRNAs.

**Table 3.** siRNA prediction of CYP51-dsRNA constructs using siFi21. Total and efficient siRNAs of 21 nt were predicted with the siFi21 software for the cDNA of the respective *FgCYP51* target genes *FgCYP51A*, *FgCYP51B* and *FgCYP51C*.

Construct	Total siRNA hits	Efficient siRNA hits
CYPA-500	480	240
CYPA-800	780	390
CYPA-full	1498	740
CYPB-400	380	183
CYPB-800	780	387
CYPB-full	1555	768
CYPC-400	380	186
CYPC-800	780	385
CYPC-full	1528	759

exogenously supplied RNA biopesticides [for a review on SIGS, see 10, 49, 50, 51, 21, 52]. Despite the promising potential of RNA silencing-based disease control the mechanisms underlying HIGS and SIGS technologies are inadequately understood.

Given these knowledge gaps, questions on the design of new and potentially promising dsRNAs arise. Whereas bioinformatics provides a variety of tools for the design, analysis, and evaluation of siRNA agents, little is known about the influence of dsRNA design on its processing and target gene silencing efficiency. Therefore, we attempt to identify the design principles of an optimal dsRNA trigger to improve/guarantee efficacy and specificity of HIGS- and SIGS-based plant protection approaches. By comparing efficiencies of infection reduction of HIGS with SIGS we observed a strong bias between those two approaches (Table 1). For example, CYP51-dsRNA construct CYPC-238 [19, Table 1] exhibited 78% reduction of infection in SIGS compared with only 31% for HIGS (Table 1). Another example is CYPA-500 which showed 82% efficiency in SIGS but only 46% decrease in *Fg* infection in HIGS. In other words, for dsRNA constructs of 200–500 nt, HIGS is around 30% less efficient than SIGS

(under lab conditions) (Table 1). We assume that differences in silencing and disease resistance are due to processing and delivery of dsRNAs rather than depending on the concentration/amount of dsRNA and/or siRNA because we cannot compare the amount of transgene-derived siRNAs generated by plant DCLs with spray-applied dsRNA that were processed by fungal DCLs (Fig. 6). However, we observed that with increasing length of the CYP51-dsRNA construct, differences in efficiencies between HIGS and SIGS approaches became less obvious. If we compared dsRNA constructs of 400 nt length, SIGS was 20% more efficient than HIGS (Table 1). Notably, if we then compared efficiencies of CYP51-dsRNA constructs that were generated out of the full-length sequence (>1500 nt) of the individual *FgCYP51* genes, HIGS and SIGS showed the same level of around 50% reduction of *Fg* infection (Table 1). Even more important and in clear contrast to HIGS, we measured a decrease in SIGS-mediated *Fg* disease resistance that is probably correlated with the length of the dsRNA construct that was sprayed. More explicitly, SIGS-based efficiencies decreased from 80% for 200–500 nt constructs to 65% for 800 nt constructs to 50% for >1500 nt dsRNA constructs.

Taken together, our results strongly support the notion that uptake of dsRNA of 200–500 nt in length and procession of siRNA by the fungus is more efficient in terms of disease control than uptake of siRNA via HIGS. However, little is known about how and which silencing signals (i.e. siRNAs and/or dsRNA precursors) are transferred from plant into fungal cells. Moreover, research into the mechanistic basis underlying HIGS and SIGS technologies is needed to integrate e.g. DCL-processing patterns that might help to define principles for RNAi vector design, producing effective siRNAs [18,23,53].



Therefore, further research must address the question: What is the optimal dsRNA design for uptake, processing, translocation, and silencing efficiency of the RNAi trigger compound?

## Methods

### Construction of CYP51 containing p7U10 RNAi vectors

CYP51-dsRNA constructs CYPA-500/800/full, CYPB-400/800/full, and CYPC-400/800/full were amplified from *Fusarium graminearum* IFA65 cDNA using a gene-specific primer (Tab. S1) and inserted into the HindIII and XmaI restriction sites of p7U10 RNAi (Fig. S1).

### Generation of transgenic *Arabidopsis thaliana* plants

p7U10 plasmids for transformation of *Arabidopsis* were introduced into the *A. tumefaciens* strain AGL1 by electroporation. Transformation of *Arabidopsis* was performed with the floral dip method as described [54] and transgenic plants were selected on ½ MS agar plates containing BASTA (7 µg/ml).

### Plant infection assays and spray application of dsRNA

Fg IFA65 was grown on SNA agar plates at 22°C in an incubator (BINDER). For all leaf inoculation assays, Fg-IFA65 conidia concentration was adjusted to  $5 \times 10^4$  macroconidia ml<sup>-1</sup> in ddH<sub>2</sub>O containing 0.002% Tween-20. After inoculation, plates were stored at RT and infection symptoms were assessed at 5 dpi. To evaluate infection severity, fungal growth was determined by measuring the size of chlorotic and necrotic lesions using the ImageJ software (<https://imagej.nih.gov/ij/index.html>).

For the *Arabidopsis*-*Fusarium* infection, 15 rosette leaves of 10 different 5-wk-old plants of each transgenic line and control plants [Col-0 wild-type (wt)] were detached and transferred in square Petri dishes containing 1% agar. Inoculation of *Arabidopsis* was done by wound inoculation of detached leaves with 5 µl *Fusarium* conidia suspension on each leaf side. Wounding was performed by scratching the leaf surface with a pipette tip. At 5 dpi, leaves were frozen in liquid nitrogen and subjected to RNA extraction and cDNA synthesis.

For spray application, dsRNA was generated using MEGAscript RNAi Kit (Invitrogen) following the manufacturer's instructions. p7U10 plasmids containing CYP51-dsRNA constructs were used as a template. Primer pairs with T7 promoter sequences at the 5'-end of both forward and reverse primers were designed for amplification of dsRNA (Tab. S1). The dsRNA, eluted in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), was diluted in 500 µl water to a final concentration of 20 ng µl<sup>-1</sup>. For the TE control, TE buffer was diluted in 500 µl water corresponding to the amount that has been used for dilution of the dsRNA. Typical RNA concentration after elution was 500 ng µl<sup>-1</sup>, representing a buffer concentration of 400 µM Tris-HCl and 40 µM EDTA in the final dilution. Detached barley leaves were covered with a plastic tray before

spraying, leaving only the upper part (approximately 1 cm) uncovered. After spraying, dishes were kept open until the surface of each leaf was dried. After 48 h, leaves were drop-inoculated as described above.

### Quantification of fungal transcripts by quantitative real-time PCR (qRT-PCR)

Before cDNA synthesis, remaining DNA was digested by DNase I (Thermo Scientific) using RiboLock RNase Inhibitor (Thermo Scientific) for 30 min at 37°C. For cDNA synthesis, 1 µg digested RNA was used. cDNA synthesis was performed using qScript™ cDNA synthesis kit (Quanta). qRT-PCR was performed with freshly synthesized cDNA in the QuantStudio 5 Real-Time PCR system (Applied Biosystems) in 384-well plates using SYBR® green JumpStart Taq ReadyMix (Sigma-Aldrich). For each sample, three replicates were performed, and target transcript levels were determined using a gene-specific primer (Tab. S1) via the  $2^{-\Delta\Delta C_t}$  method [55] by normalizing the amount of target transcript to the amount of reference transcript.

### Bioinformatic off-target analysis

The precursor sequences of CYP51-dsRNAs were split into k-mers of 21 bases. These sequences were targeted against the complementary DNA sequences of *FgCYP51* genes (cDNA) of *Fusarium graminearum* strain PH-1 (GCA\_000240135.3) with TAPIR [37] using the following settings: score cut-off 4 and minimum free energy ratio cut-off 0.7. For each target, the position in the respective cDNA was plotted with RStudio [56] and the ggplot2 package [57].

### Phylogenetic analysis of *FgCYP51* genes

*FgCYP51* genes were aligned with the muscle algorithm with default settings and a maximum likelihood tree was generated using the MEGA7 software [58].

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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## Author Contributions

A.K. and L.H. wrote the manuscript; L.H. and A.K. designed the study; L.H., D.B. and A.S. conducted the experiments; A.K. and L.H. analyzed all data. A.K., L.H. and B.T.W. drafted the figures. B.T.W. and L.J. performed bioinformatics analysis. All authors reviewed the final manuscript.

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