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The Agronomic Potential of Gene Silencing Applications

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REQUIREMENT FOR NOVEL CROP PROTECTION STRATEGIES

Present-day efficient plant production strategies still require the usage of chemical pesticides. While modern pesticides are largely safe, there are still several issues that demand further optimization of their use including i. the emergence of compound resistance due to wrong handling of pesticide use, ii. soil/water pollutions (e.g. excessive use of copper in organic farming), and iii. largely inefficient pesticide activities against certain ear and root diseases. In consequence there is a need for novel crop protection compounds and strategies.

More than half of the world's harvested area is allotted to cereals such as rice, maize and wheat (ca. 2.3 billion tons in 2010; FAO 2013). Diseases of cereal crops such as Fusarium head blight (FHB) and Fusarium seedling blight (FSB), caused by necrotrophic fungi of the genus Fusarium, exert a particularly great economic and agronomic impact on global grain production and the grain industry (Bai et *al.* 2004; Broekaert et *al.* 2015). Food safety can be compromised by contamination of agricultural products with mycotoxins, which are produced by Fusarium fungi (Jansen et *al.* 2005; Merhej et *al.* 2011) and represent a serious threat to human and animal health. One of the predominant mycotoxins, the trichothecene Deoxynivalenol (DON) has a high acute toxicity (LD_{50} = 43 mg kg^{-1} body weight, [mouse oral]) that is much higher than that of a modern fungicide such as strobilurins (LD_{50} = >5000 mg kg^{-1} body weight) and azoles (LD_{50} = between 600 to >2000 mg kg^{-1} body weight). Currently, the major strategies to control Fusarium diseases include resistance breeding, crop rotation, and plowing, along with the application of demethylation inhibitor (DMI) fungicides (Kazan et *al.* 2012). These chemicals are the most successful fungicides worldwide, with a market volume of $11,475 million in the year 2010. DMI fungicides, such as tebuconazole, fenbuconazole, and propiconazole inhibit ergosterol biosynthesis by binding to cytochrome P450 lanosterol C-14 α-demethylase (CYP51), thereby disrupting fungal membrane integrity (Krämer et *al.* 2012). However, heavy reliance on DMI fungicides since their discovery in the mid-1970s holds a risk of the emergence of DMI-tolerant strains of plant pathogens (Gsaller et *al.* 2016). Greater compound tolerance at least partly is because of fungal enhanced capability to detoxify the chemicals. Even worse, the quantitative nature of FHB and FSB resistance and the lack of true resistance (R) genes does not allow straightforward breeding programs (Jansen

et *al.* 2005). Together these problems reveal Fusarium species as most problematic cereal pathogens worldwide.

We have been exploring the potential of double-stranded (ds)RNA as a ecofriendly compound to control pests and diseases. We refer here to case studies on a cereal disease caused by the necrotrophic fungal acomycete *Fusarium graminearum* that were originally published in Koch et *al.* (2013, 2016).

RNA-BASED PLANT PROTECTION STRATEGIES IN AGRICULTURE

Exogenous dsRNA triggers suppression of gene activity in a homology-dependent manner (Fire et *al.* 1998). Since this discovery and the identification of small RNAs (sRNAs) as a new class of regulatory molecules (Hamilton & Baulcombe 1999) that functions via RNA interference (RNAi), our understanding of the essential cellular function of gene silencing has increased considerably (Vaucheret & Fagard 2001; Castel & Martienssen 2013). Mobile RNA silencing signals are capable of translocating from the host to its interactors, and vice versa (Tomilov et *al.* 2008; for reviews see Baulcombe 2015; Knip et *al.* 2014; Koch & Kogel 2014; Wang et *al.* 2016). Consistent with this, a recent work showed the significant role that small RNAs may play in the communication between plants and a pathogenic fungus (Weiberg et *al.* 2013). Exploiting this mechanism in plants has a strong potential for agricultural applications. Encouragingly, transgenic expression of inhibitory dsRNAs in the appropriate host plant resulted in protection from predation or infection by targeted gene silencing (Price & Gatehouse 2008; Novara et *al.* 2010; Koch et *al.* 2013; Cheng et *al.* 2014; Ghag et *al.* 2014)

Transgenic Strategies to Control Pathogens by Non-Coding RNAs

In Arabidopsis (*Arabidopsis thaliana*) and barley (*Hordeum vulgare*), transgenic expression of *CYP3*-dsRNA, a 791 nt long dsRNA targeting the three fungal *Cytochrome P450 lanosterol C-14α-demethylase* genes *CYP51A*, *CYP51B*, and *CYP51C*, mediates plant resistance to infection with *Fusarium graminearum* (Koch et *al.* 2013). Antifungal RNA delivery by transgenic plants resulting in silencing of target genes of interacting pathogens/pests was termed Host-Induced Gene Silencing (HIGS, Nowara et *al.* 2010). The general mechanism of HIGS is shown in Fig. 1. Silencing of essential ergosterol biosynthesis genes is a highly efficient strategy for controlling growth and development of the phytopathogenic Fusarium fungus. Initially, *CYP51* genes were selected as potential HIGS targets because of the previous demonstration that CYP51 enzymes are well known targets for DMI fungicides, whose inhibition leads to fungal arrest and disease control. Specifically, dysfunction of CYP51 enzymes results in a depletion of ergosterol and the accumulation of sterol precursors, including the 14α-demethylated sterols, 4,14-dimethylzymosterol, and 24 methylen-edihydrolanosterol, in the plasma membrane and concurrent decrease in CYP51 products [e.g., 14-methylated sterols (Ghannoum & Rice 1999)]. This imbalance alters plasma membrane structure and function, as elevated levels of ergosterol precursors induce permeability changes,

membrane leakiness, changes in membrane-bound enzymes, and inhibition of fungal growth. In addition, the 14α-demethylsterols cannot replace ergosterol to stimulate cell proliferation (Liu et *al.* 2011; Fernández-Ortuño et *al.* 2010; Fan et *al.* 2013). Consistent with the predicted function of the three *F. graminearum* CYP51 paralogs in ensuring membrane integrity and fungal virulence, silencing of their encoding genes altered fungal growth and development. Treatment of fungal axenic cultures with *CYP3*-dsRNA resulted in an increase in hyphal branching and inhibition of hyphal growth (Koch et *al.* 2013, 2016).

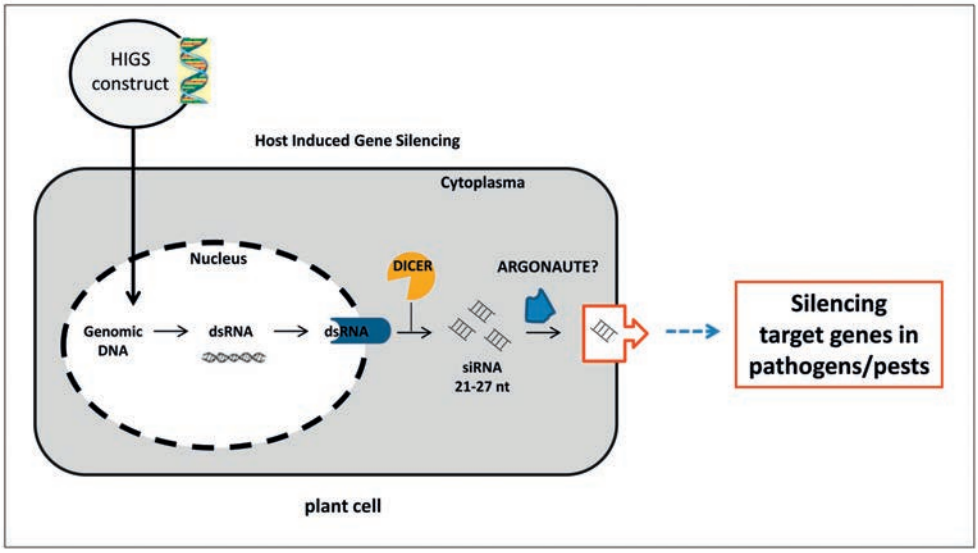

Figure 1. Host-Induced Gene Silencing (HIGS) for the control of microbial pathogens and pests on plants. A DNA construct containing inverted promoters is introduced into the plant by agrotransformation (Imani et *al.* 2011). The sequence is transcribed into two complementary RNA strands giving rise to a double-stranded (ds)RNA. The dsRNA is exported from the nucleus and processed by RNase III enzymes, called Dicer-like, into small interfering (si)RNAs. The siRNAs interact with proteins of the ARGONAUTE family of RNase III enzymes and/or are exported from cytoplasm to apoplast and the invading pathogen/pest. In the target pathogen/pest the siRNA is channeled into the RNA interference machinery probably interacting with the pathogen's/pest's ARGONAUTE to eventually cleave the target mRNA and kill the pathogen/pest.

In transgenic Arabidopsis expressing *CYP3*-dsRNA, fungal development was restricted to nearly 100%, with a small amount of growth occurring at the wounded area immediately surrounding the inoculation sites (Fig. 2). Analysis of *CYP51* expression at infection sites showed that all three fungal genes were partially silenced. Thus, the altered growth and morphology of the fungus appears to be triggered by a reduction in fungal *Cytochrome P450 lanosterol C-14α-demethylase* expression (Koch et *al.* 2013).

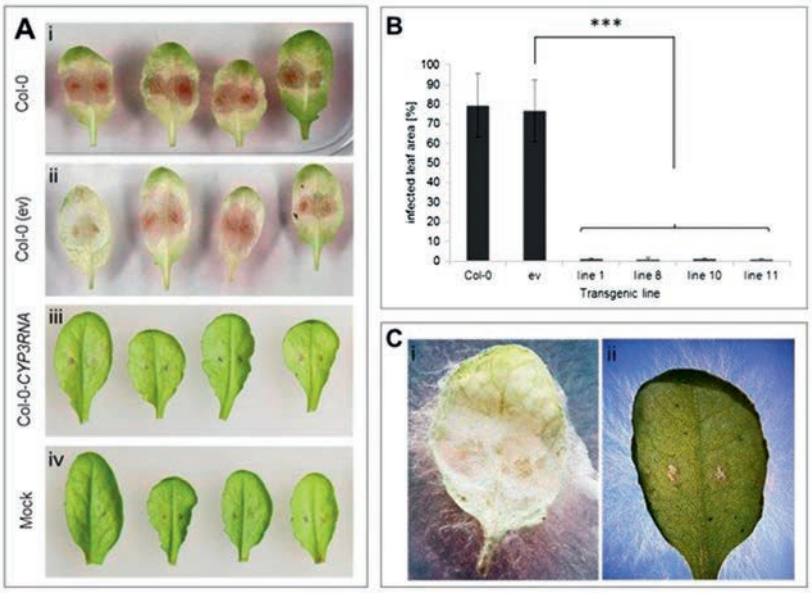

Figure 2. Transgenic Arabidopsis plants expressing CYP3-dsRNA are highly resistant (immune) to *Fusarium graminearum* infection. (A) Detached leaves of 5-week-old plants were treated with 5 × 104 macroconidia mL-1 and evaluated for necrotic lesions at 3 dpi. (i) wild-type (Col-0), (ii) Col-0 empty vector (ev) control, (iii) Col-0 expressing CYP3-dsRNA (representative line L8), and (iv) wild-type treated with Tween water (mock). (B) Quantification of infected leaf area at 3 dpi; typical infection symptoms are recorded as a percent of the total leaf area. Bars represent mean values ± SDs of three independent experiments, each using 20 leaves collected from 15 different plants of each transgenic line, as well as wild-type and Col-0 ev plants. The reduction in infection symptoms on CYP3-dsRNA-expressing leaves compared with the wild-type and Col-0 ev control was statistically significant (***P < 0.0001; Student´s t test). (C) Arabidopsis leaves infected with *Fusarium graminearum* at 5 dpi. (i) The Col-0 ev leaf is heavily infected; (ii) Col-0 expressing CYP3-dsRNA does not show infection symptoms. (Image modified after Koch et *al.* 2016)

The exact mechanism through which *in planta* expression of a nuclear-integrated *CYP3*-dsRNA construct silences gene expression in plant-colonizing fungi is still unresolved. One can speculate that siRNAs generated by the plant's silencing machinery from the long *CYP3*-dsRNA are transferred and secreted via vesicles. Consistent with this scenario, small interfering 21 to 24 nt siRNAs corresponding to the targeted sequences were detected in *CYP3*-dsRNA-expressing Arabidopsis independent of fungal infection. However, further studies are required to elucidate whether *CYP51* silencing is mediated by fungal uptake of siRNAs generated by the plant's RNAi machinery, or by uptake of the unprocessed precursor *CYP3*-dsRNA.

Mechanistic Considerations

Elucidating the molecular mechanisms of HIGS is a key for successful future implementation. RNA silencing has been described in many organisms as post-transcriptional gene silencing (PTGS), RNA interference (RNAi), and quelling, in plants, animals, and fungi, respectively. These silencing pathways are involved in transposon silencing, viral defense, DNA elimination, heterochromatin formation, and post-transcriptional repression of genes. The critical steps of these pathways include production of sRNAs of 19–27 nucleotide (nt) from structured or dsRNA by DICER enzymes, followed by loading into ARGONAUTE-containing complexes to form RNA-induced silencing complexes (RISC) that guide the cleavage of target transcripts.

Gene annotation of the *F. graminearum* genome (http://www.broadinstitute.org) predicted genes coding for two ARGONAUTE-like proteins, two DICER-like proteins, and five RNA-dependent RNA Polymerases (RDR; Chen et *al.* 2015). Consistent with these findings, RNAseq analysis of axenically grown *Fusarium graminearum*, treated with *CYP3*-dsRNA, showed high numbers of reads of *CYP3*-dsRNA-derived siRNAs, together showing that the fungus possesses a functional gene silencing system, which is a prerequisite for disease control by HIGS.

Open Questions

In mammalian cells, perception of certain dsRNAs via toll-like receptors triggers an inflammation response (Gantier and Williams 2007; Karpala et *al.* 2005). In contrast, expression of *CYP3*-dsRNA in barley or Arabidopsis, respectively, did not trigger an innate immune response (Koch et *al.* 2013; Koch et *al.* 2016). This result showed that HIGS-mediated diseases resistance does not rely on activation of canonical defense pathways. That the plant immune system is not triggered by dsRNA also suggests that efficient HIGS does not impose relevant fitness costs, and so may not negatively affect yield performance under field conditions. Further research is required to establish rules for optimal dsRNA structures, including dsRNA lengths, combinatorial order of gene fragments, target sites in a given gene target, and the number of genes targeted by one dsRNA.

Aside from this, many more questions must be addressed in the future to eventually judge the agronomical potential of RNA-based plant protection strategies, including the stability of the silencing construct under field conditions. More research on RNA uptake by the target pathogen/pest is also required. Another yet open issue is the risk that microbial strains may become insensitive to a given RNA product. Such scenario could probably be resolved by using dsRNA that target different regions in one gene or different genes. Most importantly, a commercial dsRNA should be designed not to have off-target effects in other organisms that might be relevant in the respective agroecosystem, including beneficial fungi and bacteria.

Together, the use of target-specific inhibitory dsRNA to mediate protection against pathogens and pests potentially is an alternative to conventional chemicals because dsRNAs are *i.* highly specific and solely depending on their nucleotide sequence and *ii.* can be developed against an

unlimited range of pathogens provided that the RNAi machinery is in place. Given that dsRNAs accumulate in the plant phloem, sucking insects also can be efficiently controlled by HIGS (Abdellatef et *al.* 2014; Eamens et *al.* 2008).

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