De- and reconstructing virulence strategies of fungal plant pathogens

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conVIRgens

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Periodic Reporting for period 4 - conVIRgens (De- and reconstructing virulence strategies of fungal plant pathogens)

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Podsumowanie kontekstu i ogólnych celów projektu

Pathogenic fungi are an increasing threat to plants and animals, causing significant losses in worldwide food production. Prominent examples of fungal pathogens causing devastating crop

damage are soybean rust (Phakopsora pachyrhizi), wheat stem rust (Puccinia graminis f. sp. tritici) and wheat blast (Magnaporthe oryzae. Many important plant diseases are caused by biotrophic pathogens, which colonize and feed from living host tissue. The largest groups of fungal biotrophs are the rust fungi, the powdery mildews, and the smut fungi. The biotrophic virulence strategy requires efficient suppression of immune responses and reprogramming of host metabolism to feed the pathogen. To manipulate the host and promote infection, pathogens secrete large repertoires of virulence proteins, collectively termed “effectors“. Efficient plant immunity can be induced upon recognition of effectors by specific resistance (R) proteins, reflecting the evolutionary arms race between plants and pathogenic microbes.
The smut fungi (Ustilaginomycota) comprise >1200 species, infecting approximately 4000 plant species, including all economically relevant monocot crops. All Ustilaginales smuts are strictly biotrophic parasites, but unlike obligate biotrophs, their life cycle involves a saprophytic phase represented by a haploid yeast stage (sporidia). On host plants, compatible sporidia recognize each other via a pheromone-receptor system and fuse to form dikaryotic filaments; it is these filaments that initiate the pathogenic fungal development.
Genome sequences of seven plant pathogenic Ustilaginales species have been published. All these genomes are small (about 20 Mbp), encode about ~7.000 proteins and show rather low content of repetitive elements and gene duplications. Together, these features reinforce the excellent suitability of these species for functional genetic and genomic approaches.
This project aims to utilize the excellent genetic accessibility of the smut model fungus Ustilago maydis to approach a previously impossible, pioneering enterprise: the synthetic reconstruction of eukaryotic plant pathogens. In a first step, fungal virulence will be deconstructed by consecutive deletion of the U. maydis effector repertoire to generate disarmed mutants. These strains will serve as chassis for subsequent reconstruction of fungal pathogenicity from different sources. A combination of transcriptomics and comparative genomics will help to define synthetic effector modules to reconstruct virulence in the chassis strains.
Dissecting the U. maydis effector repertoire will show how effector modules determine fungal virulence, including those of the previously not accessible obligate biotrophs. conVIRgens will thereby provide fundamentally new insights and novel functional tools towards the understanding of microbial virulence.

Prace wykonane od początku projektu do końca okresu sprawozdawczego oraz najważniejsze dotychczasowe rezultaty

In the first project phase, both the major parts of the project could have been initiated.
Prerequisite of the deconstruction approach is the efficient gene editing in U. maydis using CRISPR/Cas9. In this regard, an optimized protocol has been established for U. maydis, using a modified Cas9 version (Cas9HF). Also, by full genome sequencing of a set of transformants we could demonstrate that gene disruption using this tool does not lead to the generation of off-target mutations, which is an important step for the planned approach. The results have been summarized in a manuscript, which has been recently published (Zuo et al., 2020).
We performed transcriptome analysis of U. maydis infecting different maize lines to elucidate fine-tuned host adaptation. This approach identified maize-line specific activity of U. maydis effectors,

providing novel insight in the co-evolution of the pathogen with its host (Schurack et al., 2021). To further dissect tumor-related effectors and better understand life-style differentiation between U. maydis and its close relative Sporisorium reilianum, comparative transcriptome profiling of both organisms has been performed. This identified a set of effectors which are specifically induced in U. maydis during tumor induction in comparison to their respective one-to-one homologs in S. reilianum. Gene deletion together with CRISPR-Cas9-based gene replacement revealed that both transcriptional regulation and sequence diversification of effector proteins contribute to species-specific functionalization (Zuo et al., 2020).

To reconstruct fungal virulence, two complementary approaches are followed: a) the computational approach which is mainly aiming on comparative genomics-based characterization of effector repertoires, and b) the experimental approach to generate artificial effector gene clusters and to perform gain of function genetic experiments by expressing heterologous effectors in U. maydis.
We have performed de-novo sequencing of smut fungi has been performed using PacBio and Nanopore sequencing. We generated annotated de-novo genomes for three fungal species. In addition, we investigated intraspecific variation by sequencing several Ustilago hordei and Ustilago maydis strains which have been isolated around the globe. Sequencing of Chinese and European U. maydis strains was integrated in an evolutionary analysis of effectors within U. maydis, as well as in comparison with S. reilianum. This approach could identify a link between expression pattern and sequence variation / evolutionary speed of effector genes (Depotter et al., 2020). Complementary to this, we established tools in the barley smut U. hordei to express heterologous virulence factors, which also allows to study effectors of obligate biotrophs pathogens of barley (Ökmen et al., 2021).

We successfully generated annotated genomes for Ustilago striiformis, Ustilago nuda, and Ustilago tritici. Additionally, we explored intraspecific variation by sequencing six Ustilago hordei strains and 14 Ustilago maydis strains collected from various locations worldwide. Our findings revealed that the invasion of transposable elements into smut genomes has led to an increased rate of nucleotide substitution, driving effector evolution. This process likely contributes to host jumps and the adaptation of smut pathogens. Manuscripts detailing these results have been published in 2021 and 2022 (Depotter et al., 2021, 2022).

To achieve a more efficient genetic modification of S. reilianum, we established a CRISPR-Cas9 based transformation protocol for this organism. Here, a ribonucleoprotein (RNP)-mediated CRISPR/Cas9 method for mutagenesis in S. reilianum was developed. We evaluated the efficiency of this approach through both in vitro cleavage assays and in vivo experiments using a GFP-expressing S. reilianum strain. We successfully applied this method to generate frameshift and knock-out mutants in S. reilianum without introducing a resistance marker, instead utilizing an auto-replicating plasmid for selection. The RNP-mediated CRISPR/Cas9 technique demonstrated enhanced mutagenesis efficiency and versatility, being applicable to various types of mutations. Importantly, this approach enables marker-free genome editing in S. reilianum, representing a significant advancement in fungal genetic manipulation techniques (Werner et al., 2024).

Innowacyjność oraz oczekiwany potencjalny wpływ (w tym dotychczasowe znaczenie społeczno-gospodarcze i szersze implikacje społeczne projektu)

Current progress includes methods improvement beyond state of the art at beginning of the project. This includes:
- setup of high fidelity CRISPR-Cas9 mutagenesis in U. maydis
- setup of CRISPR-Cas9- mediated, marker- and scar less gene replacement in U. maydis
- construction of artificial effector gene clusters using modular cloning strategy
- heterologous expression of fungal virulence factors in U. hordei

We have performed:
- comparative transcriptomics of U. maydis infecting different maize lines
- comparative transcriptomics of U. maydis and S. reilianum
- genome sequencing U. maydis and U. hordei field isolates
- Pac-bio de-novo sequencing of three smut species

First key findings were:
- identification of maize-line specific activity of effector genes (Schurack et al., 2021)
- identification of tumor-related effectors in U. maydis in comparison with S. reilianum (Zuo et al., 2020)

Until end of the project, we accomplished the major goals of the project. In particular we:
- dissected the effector repertoire responsible for U. maydis induced formation of plant tumors
- investigated the genetic basis of host jump / host adaptation in smut pathogens
- Explored the role of retrotransposal elements in evolution and host adaptation of smut fungi
- found a new class of effector proteins (transactivator effectors), which are inducing plant tumor formation through transcriptional modulation of host developmental regulators

Key findings made in the project can are summarized in the following publications:
Identification of a new class of effectors that stimulate tumor formation:
Zuo W, Depotter JRL, Stolze SC, Nakagami H, Doehlemann G (2023) A transcriptional activator effector of Ustilago maydis regulates hyperplasia in maize during pathogen-induced tumor formation. Nature Communications 23;14(1):6722.
Retrotransposons driving evolution of smut pathogens:
Depotter JRL, Ökmen B, Ebert MK, Beckers J, Kruse J, Thines M, Doehlemann G (2022) High nucleotide substitution rates associated with retrotransposon proliferation drive dynamic secretome evolution in smut pathogens. Microbiology Spectrum 10(5):e0034922.
Transcriptional regulation and functional diversification determine different pathogen lifestyle in smuts:
Zuo W, Gupta DK, Depotter JRL, Thines M, Doehlemann G. (2021) Cross-species analysis between the maize smut fungi Ustilago maydis and Sporisorium reilianum highlights the role of transcriptional plasticity of effector orthologs for virulence and disease. New Phytologist 232(2):719-733

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