PARASIT Project, Final Report

Principal investigators and institutions:

Dr. Jochen Kumlehn, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK),
Prof. Dr. Holger B. Deising, Martin Luther University Halle-Wittenberg

Funded PhD students: Krishna Mohan Pathi (IPK), Maximilian Groß (MLU)

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PARASIT:

Pathogen resistance achieved by plant-induced silencing of fungicide target genes

Die Entwicklung effektiver neuartiger Methoden ist im Pflanzenschutz unabkömmlich. Dies gilt insbesondere für phytopathogene Pilze, wie das Maispathogen *Colletotrichum graminicola*. Eine innovative Methode ist die Ausstattung von Kulturpflanzen mit Transgenen, deren Produkte doppelsträngige RNAs mit Homologie zu Zielgenen im Pathogen sind. Diese fungieren als hochspezifisches RNA-Interferenz (RNAi)-Signal, sodass es im Laufe der Infektion durch Aufnahme der RNA zu einer Reduzierung der Transkriptmenge im Pathogen kommt. Sind diese Zielgene für das Pathogen essenziell, so wird seine Virulenz beeinträchtigt. Ziel dieses Projektes ist die Ausstattung von Mais mit Transgenkonstrukten, deren Produkte sich spezifisch gegen die Fungizid-Zielgene Succinat-Dehydrogenase und β-Tubulin in *C. graminicola* richten und damit zu *Colletotrichum*-resistentem Mais führen.

I. Project parameters

1. Topic, purpose and state of scientific knowledge

Considering the frequent incidence of fungicide resistance in phytopathogenic fungi, innovative plant protection strategies are required to ensure a sustainable production of crops. Host-induced gene silencing (HIGS), as introduced at the IPK Gatersleben by Nowara *et al.* (2010, The Plant Cell), allows the down-regulation of specific fungal target genes in a process coined RNAi, mediated through double-stranded RNA (dsRNA) being produced by the host plant. Due to its activity on the nucleic acid level, HIGS acts exclusively on either individual or a well-defined group of pathogens. Equipping plants with HIGS constructs homologous to fungal target genes allows the application of this approach as a crop protection strategy. However, as not all tested genes proved suitable for HIGS approaches, a thorough pre-evaluation of candidate HIGS target genes is required. Importantly, screens can be performed genetically and/or chemically. Extended screens of the latter identified inhibitors, several of which have yielded modern

fungicides. Thus, the project has focused on fungicide target genes to genetically evaluate these as RNAi targets and for their suitability in HIGS.

2. Interdisciplinarity of the project

Interdisciplinarity of the project is provided by the cooperation of experts in fungus-plant interaction on the one hand and in plant genetic engineering on the other. Primary plant production and conversion into material that can be directly implemented by plant breeders is an intrinsic principle of the project concept.

3. Novelty of the research

The major innovation of the project relies on the fact that genes encoding fungicide target proteins are used as particularly effective targets for host-induced gene silencing.

4. Application-oriented research

The generation of maize that features resistance to the fungal pathogen *Colletotrichum graminicola* may provide significant benefits in the context of modern plant breeding and industrialized agriculture. The principle of host-induced gene silencing acts solely at the RNA level so that proteinaceous gene products or otherwise active metabolites are neither required nor produced. Consequently, while unprecedented specificity of the effect towards the fungal pathogen is ensured, disadvantageous side effects can largely be ruled out.

5. Relevance of the project for the bioeconomy, the ScienceCampus and the state of Saxony-Anhalt

The major outcome of the project is the generation of maize plants with enhanced resistance to an important fungal pathogen, which is of substantial bioeconomic importance. Since Saxony-Anhalt has a long-standing history, tradition and internationally leading role in plant breeding research, the development of innovative solutions for crop improvement is of high regional relevance.

II. Theory and methods

HIGS describes the occurrence of RNAi in a phytopathogenic fungus, mediated through double-stranded RNA transcribed from (trans)genes of the host plant. These plant-produced transcripts target fungal transcripts that share the same nucleotide sequence and subsequently lead to their degradation. Thereby, translation of the target genes is hampered, which results in reduced fungal virulence.

The HIGS target genes chosen in the frame of the PARASIT project are the *C. graminicola* β-tubulin gene *TUB2* as well as the succinate dehydrogenase encoding genes *SDH1* to *SDH4*. The proteins encoded by those genes serve as targets for two important fungicide classes. Inhibition of TUB2 disturbs fungal nuclear division and vesicle

transport, whereas inhibition of SDH proteins affects the mitochondrial electron transport chain and fungal respiration. To genetically prove their indispensability for the fungus, we performed targeted deletion of these genes. To this end, protoplasts of *C. graminicola* were transformed with expression cassettes containing an antibiotic resistance gene as a selectable marker. Via homologous double cross-over the wild-type gene is replaced by the marker gene. In case the candidate gene is indispensable, mutants are not obtained. In a second approach, the above-mentioned genes are tested for their suitability as HIGS targets by pursuing an RNAi approach in the fungus, taking advantage of the pREDi reporter system in which a *DsRed* small interfering (si)RNA hairpin is expressed (Janus *et al.* 2007, Appl. Environ. Microbiol).

III. Results and perspectives

1. Results

To assess the indispensibility of *TUB2* and *SDH* genes, targeted deletions of their genomic sequences via homologous double cross-over have been performed. Protoplasts of *C. graminicola* were transformed with deletion cassettes containing the *nat1* gene from *Streptomyces noursei* conferring resistance against nourseothricin as a selectable marker, flanked by sequences homologous to the 5'- and 3'-border regions of the wild-type gene (Fig. 1A).

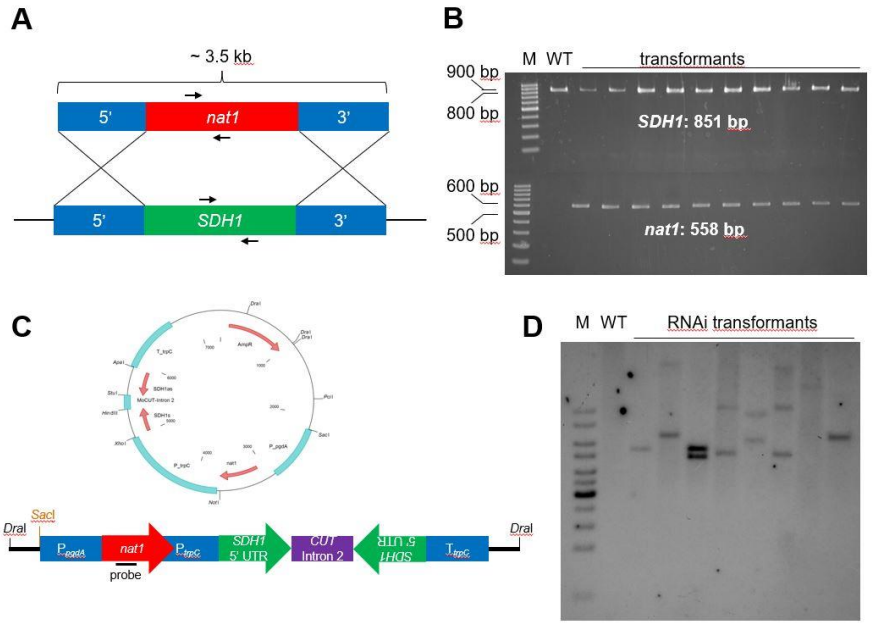

Figure 1: (A) Principle of the homologous double cross-over and construction of the gene replacement cassette carrying an antibiotic resistance gene (*nat1*), exemplified with *SDH1*. Arrows indicate primers used for PCR verification. (B) PCR verification of the ectopic integration of deletion cassettes targeting *SDH1* gene. In transformants both *SDH1* and *nat1* fragments could be amplified, indicating an ectopic integration of the resistance cassette (WT = wild type; M = marker; bp = base pairs). (C) Schematic map of the pREDi vector and the pREDi-derived RNAi construct. 351 bp of *SDH1* target sequence (SDH1-5'UTR) is integrated in sense and antisense orientation, separated by Intron 2 of the *M. oryzae* cutinase gene (*CUT* Intron 2; P_{trpC} = *A. nidulans trpC* promoter; *nat1* = antibiotic resistance gene; *Sac*I = restriction enzyme used for Southern Blot digestion; probe = position of Southern Blot probe). (D) Southern Blot example of RNAi transformants using a probe hybridizing in the *nat1* region of the construct (M = marker; WT = wild type).

Transformations with cassettes targeting *SDH1* and *SDH4* gathered a total of 47 monokaryotic isolates. As expected because of the assumed essentiality of *SDH* genes, only transformants with ectopic integration of the deletion cassette were obtained, as confirmed by PCR (Fig. 1B). For the RNAi approach, we used the RNAi cassette obtained from the pREDi plasmid to generate RNAi constructs targeting *SDH1* and *TUB2*. To avoid off-target effects, *C. graminicola* genes *TUB2 and SDH1-4* were blasted against the maize genome database (http://www.maizegdb.org/). We found homologous sequences at the nucleotide level for *TUB2, SDH1* and *SDH2* with 82, 81 and 85% similarity, respectively. With regards to *SDH3, 4,* we did not find any hits. Designing RNAi vectors against the target region of the fungi is very difficult due to its sequence homology. Therefore, we decided to use less-conserved, i.e. more gene-specific 5' untranslated regions (UTR). Highly specific 5'-untranslated region and starting sequence of the *TUB2* gene (89+11=100 nucleotides) and of *SDH1* (71+46=117 nucleotides) were chosen as target sequences, since these regions showed sufficient sequence diversity from the host (maize). To produce an appropriate amount of siRNA, three repeats of the target sequence were integrated into restriction sites in sense and antisense orientation, respectively. Sense and antisense fragments were separated by the *CUTINASE* intron 2 of *Magnaporthe oryzae* (Fig. 1C). After constructing and sequencing the RNAi cassette, *C. graminicola* protoplasts were transformed and a total of 35 monokaryotic isolates were obtained and confirmed by genomic Southern blots (Fig. 1D). For the construction of maize transformation vectors, full-length *TUB2* and *SDH1* to *SDH4* genes were amplified via PCR from *C. graminicola* and verified by sequencing. To generate hairpin vectors for maize transformation, we used the GATEWAY cloning principle. Artificially synthesized 5'-UTRs were used to create the intermediate vectors pIPKTA38-SDH1 and pIPKTA38-TUB2. The presence of synthesized products in these entry vectors were confirmed by restriction enzyme digestion. The generic RNAi binary vectors IPKb009 and IPKb027 previously developed in the project partner Kumlehn's lab were used to integrate the target gene-specific sequences as inverted repeats interspaced by the wheat *RGA2* intron.

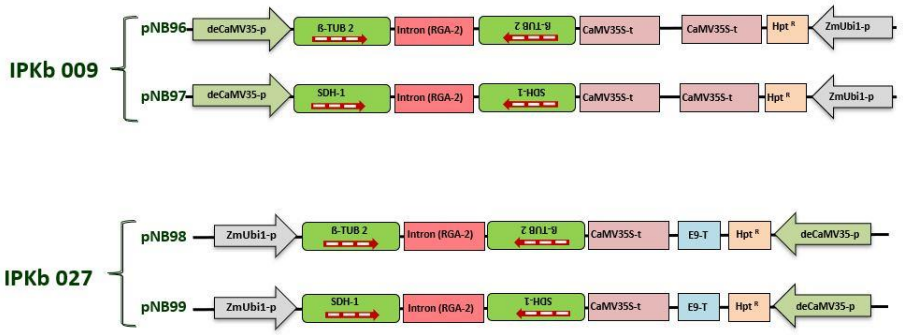

Figure 2: Schematic of the binary RNAi vectors generated for the transformation of maize. pNB96 and 97 are derivatives of pIPKb009 in which a doubled enhanced *CaMV35S* (deCaMV35S) promoter drives the expression of the hairpin construct consisting of 5'-UTR regions of *TUB2* (pNB96) and *SDH1* (pNB97) genes and a *CaMV35S* termination signal (T). The *hygromycin phosphotransferase (HPT)* gene is used as a plant selection marker controlled by the maize *Ubi-1* promoter and *CaMV35S* termination signal (T). pNB98 and 99 are corresponding derivatives of pIPKb027 in which the maize *Ubi-1* promoter drives the hairpin construct.

Hi-II hybrid maize was transformed using the resultant binary vectors according to a standard transformation protocol previously established in project partner Kumlehn's laboratory. To this end, *Agrobacterium* strains carrying the pNB96 to pNB99 transformation vectors (Fig. 2) were used for the inoculation of immature maize embryos followed by co-cultivation. The embryos were then transferred onto callus-inducing medium containing hygromycin for growth suppression of non-transgenic cells and tissues. Selected type II calli were transferred onto regeneration medium. The regenerated shoots were then separated into individual shoots and transferred to rooting medium (Fig. 3).

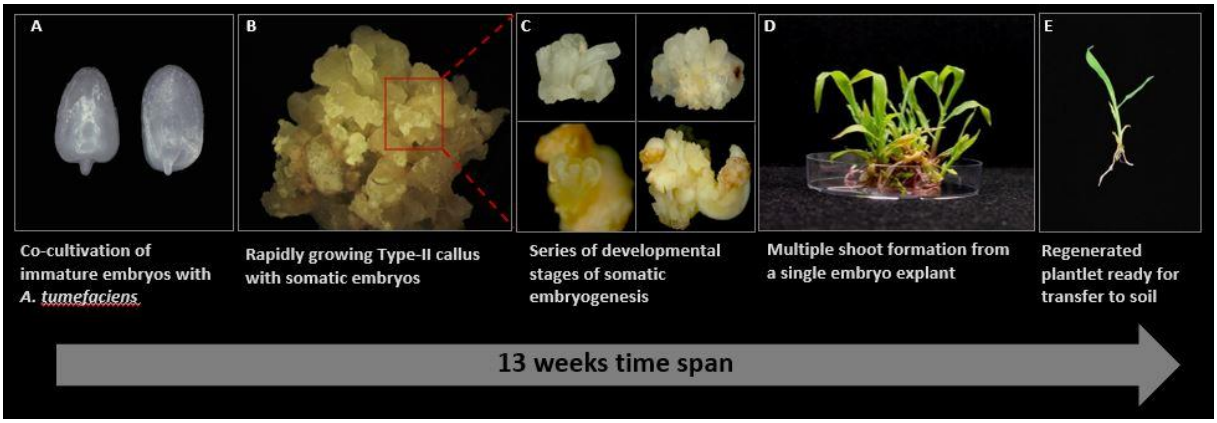

Figure 3: Production of transgenic maize plants via *Agrobacterium*-mediated transformation of immature embryos. (A) Immature embryos used as explants. (B) hygromycin-resistant calli growing on the selection medium (with somatic embryos). (C) Somatic embryos formation. (D) Multiple shoot formation from callus. (E) Plantlet with healthy root.

The complete plantlets were transplanted to plastic pots to grow, followed by planting in a glasshouse. PCR analysis was performed with the isolated genomic DNA of young maize leaves to confirm the presence of T-DNA (Table 1). For the host-induced gene silencing approach, the copy number of the integrated T-DNA is very crucial. The copy number of the transgene may affect transgene expression positively or negatively, and multiple-copy integration may cause transgene silencing.

Table1: Summary of transformation experiments

Vector name	No. of embryos agro-infected	No. of plants produced	Regeneration efficiency (%)	Transformation efficiency (%) (PCR for selectable marker)	Transformation efficiency (%) (PCR for both inverted repeats)
pNB96	107	5	4.6	4.6	4.6
pNB97	145	34	23.4	22.7	6.8
pNB98	87	28	32.1	32.1	11.4
pNB99	220	13	6.5	6.5	6.5

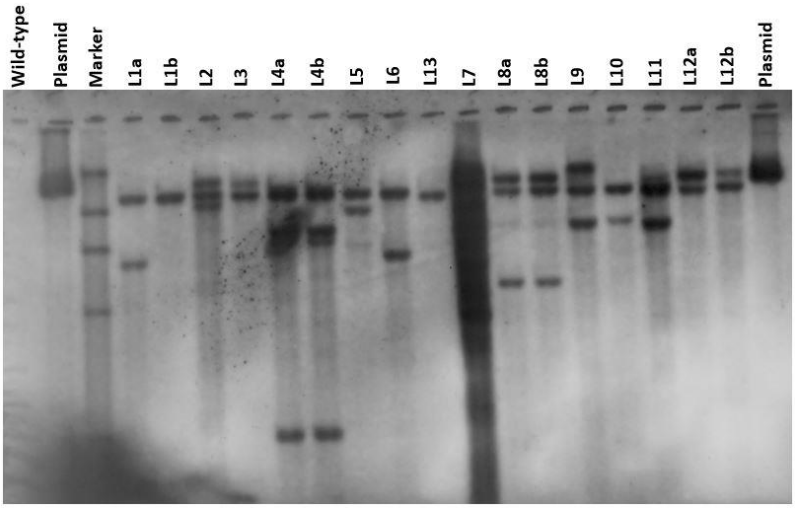

Figure 4: Southern blot analysis of primary transgenic (T0) plants from the transformation experiment with pNB 99. 20 µg genomic DNA each were digested with HindIII and the fragments were separated into 0.8 % (w/v) agarose gel. Hybridization of the specific DNA sequences was performed with an hpt-specific probe. The names of the individual plants are given above the picture. Wild-type used as a negative control, plasmid as a positive control.

To determine the integration and copy number of transgenes in PCR-confirmed T_0 plants, Southern blotting was carried out with a 500 bp fragment of the *hpt* (selectable marker) gene as a probe. To cover a wide range of opportunities, we selected plants with single, double, and multiple copy numbers (Fig. 4). Those plants were self-pollinated to produce homozygous progeny.

Hi-II A x B maize is susceptible to *C. graminicola* infection

Hi-II A x B genetic background was used to generate transgenic maize plants expressing RNAi vectors, since this hybrid has the high potency to produce embryogenic callus, which is an excellent explant source for plant genetic transformation studies. Hi-II is a segregant derived from the hybrid between maize inbreds A188 and B73. Screening experiments conducted in the Deising lab to validate maize accessions for their susceptibility to *C. graminicola* unveiled that B73 is resistance to *C. graminicola* infection. Therefore, it was crucial to determine the infection potency of *C. graminicola* towards Hi-II maize before examining the RNAi-expressing plants. Along with the Hi-II (A x B) hybrid, the cultivars Golden Jubilee (highly susceptible) and Mikado (susceptible) were used for comparison. The virulence of the *C. graminicola* CgM2 wild-type strain towards the different maize lines was assessed by detached leaf assays (Oliveira Garcia and Deising 2013, The Plant Cell). For quantitative PCR (qPCR) assays quantifying fungal biomass, leaf disks containing the infection spot were excised at 4 days post-inoculation (dpi), DNA was extracted and qPCR was performed using primers binding in the *ITS2* region which is highly specific for fungi. Under the assumption that the amount of fungal DNA corresponds with the amount of fungal biomass, the qPCR results allow concluding the infection success of *C. graminicola*. Results confirmed that the susceptibility of the Hi-II (A x B) hybrid towards *C. graminicola* is on a par to that of the standard variety Mikado (Figs. 5 and 6). Consequently, we further used Hi-II (A x B) as a

wild-type control and background to assess the RNAi expressing lines with regard to their resistance towards *C. graminicola*.

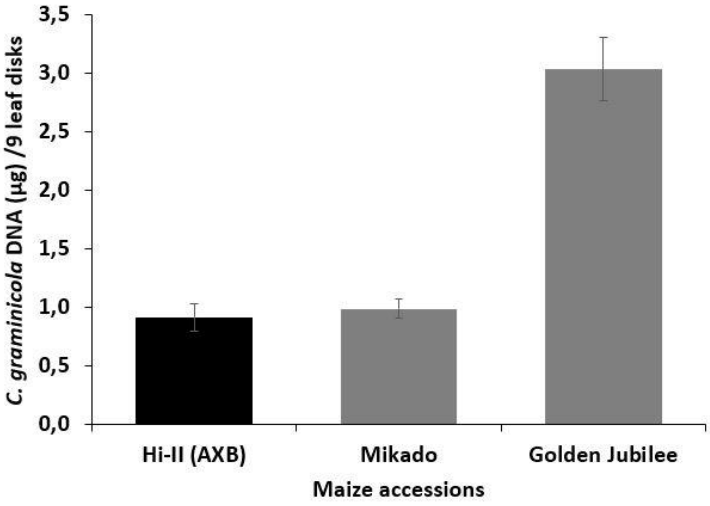

Figure 5: Hi-II susceptibility test towards C. graminicola. Quantification of C. graminicola biomass by using qPCR

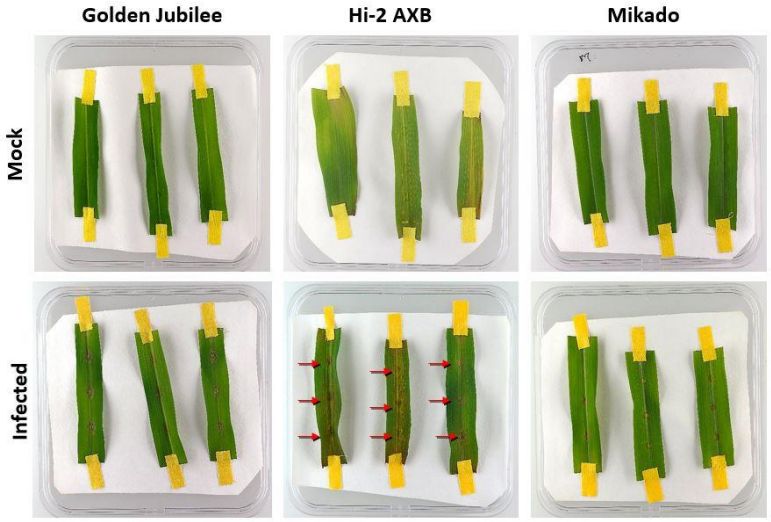

Figure 6: Symptoms on the leaf surface at 4 dpi (red arrows indicated the infection lesions)

HIGS expression constructs confer quantitative Maize resistance towards *C. graminicola*

To assess whether RNAi-expressing maize plants feature resistance to *C. graminicola,* homozygous lines expressing different RNAi constructs were subjected to infection assays with *C. graminicola*. Given the results from the susceptibility test of azygous Hi-II (A x B) used as wild-type control, RNAi line #L25-2 exhibited significantly reduced fungal growth in comparison to the non-transgenic control (Fig. 7, Fig. 8), while further RNAi-expressing lines showed a non-significant tendency of reduced biomass. Beyond the PARASIT project, further generations of the RNAi lines investigated to confirm the stability of the resistance achieved.

Figure 7: Detached leaf assays with *C. graminicola* symptoms occurring at 4 dpi. Transgenic maize lines expressing HIGS constructs show visual quantitative protection against *C. graminicola* in comparison to azygous wild-type.

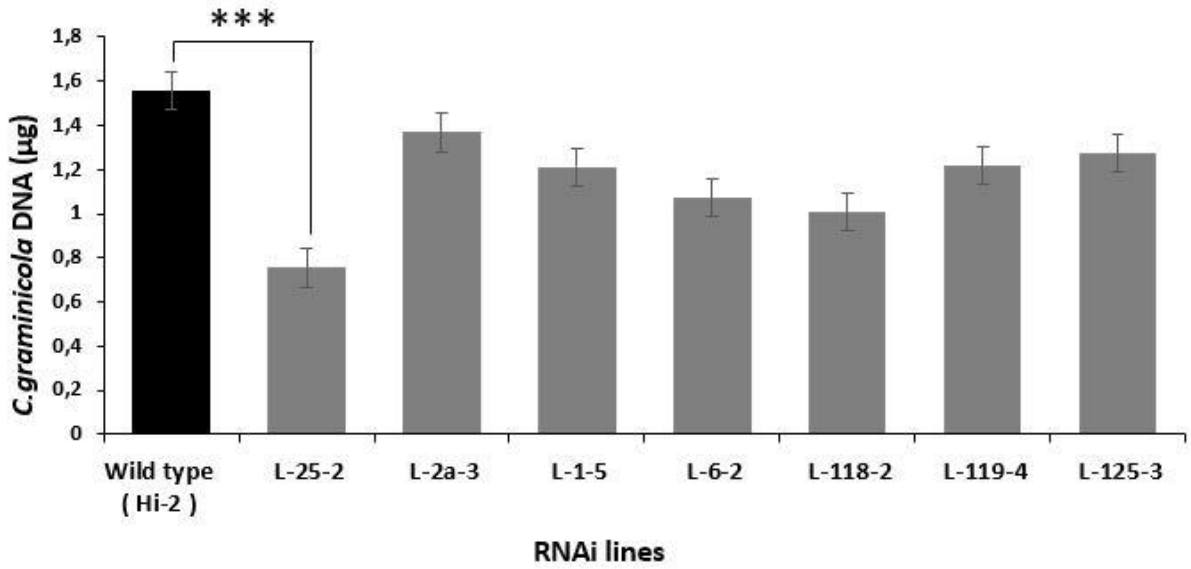

Figure 8: Columns represent means of three independent qPCR experiments. Each pool comprised twelve leaf discs excised from individual leaves carrying a single inoculation site. LINE 2a-3 carries a TUB2, Lines 1-5, 25-2, 6-2 SDH-1 and lines 118-2, 119-4, 125-3 a TUB2 hairpin construct. Three asterisks correspond to significant difference from the wild-type control at $P < 0.001$ (one-way ANOVA with post hoc Tukey HSD).

2. Prospects for further external funding

The principle of silencing essential genes of pathogens or pests can be broadly applied. Therefore, many examples of its utilization and further methodological refinements and variations can be elaborated in future projects. Besides the expression of transgenes by host plants, target gene-specific dsRNAs can be sprayed on plants as new-generation pesticides.

Dissemination of project results

Peer-reviewed publications

Beier S, C Ulpinnis, M Schwalbe, T Münch, R Hoffie, I Koeppel, C Hertig, N Budhagatapalli, S Hiekel, KM Pathi, G Hensel, M Grosse, S Chamas, S Gerasimova, J Kumlehn, U Scholz and Schmutzer (2020) Kmasker plants - a tool for assessing complex sequence space in plant species. The Plant Journal 102, 631–642

Conference talks

Kumlehn J. (2019) Neue Methoden der gezielten genetischen Veränderung von Pflanzen. Wissenschaft für jedermann - Vorträge im Ehrensaal, 30 October 2019, Deutsches Museum, Munich, Germany

Pathi KM, N Budhagatapalli, G Hensel, H Büchner, M Groß, HB Deising, J Kumlehn (2019) Establishment of maize resistance to fungal diseases by host-induced gene silencing and site-directed mutagenesis. Workshop on Molecular Breeding, September 5-6, Geisenheim, Germany

Kumlehn J. (2019) Cas endonuclease technology in cereals - methods and applications. Workshop on genomics and molecular breeding in Triticeae crops. August 27, Xining, China

Kumlehn J. (2019) Targeted genome modification in cereals - methods and applications. 5th International Conference Plant Transformation & Biotechnology, July 4, Vienna, Austria

Kumlehn J. (2019) Targeted genome modification in cereals using Cas endonuclease technology. PlantGen conference, June 25, Novosibirsk, Russia

Pathi KM, N Budhagatapalli, G Hensel, H Büchner, M Groß, HB Deising, J Kumlehn (2019) Establishment of anthracnose disease resistance of maize by Cas endonuclease-mediated mutagenesis and host-induced gene silencing. Plant Science Student Conference, June 18-21, Halle (Saale)/ Germany

Kumlehn J. (2019) Site-directed genome modification in cereals using Cas endonuclease technology. Internat. Congress of the Vavilov Society of Geneticists and Breeders, 18 June 2019, St. Petersburg State University, St. Petersburg, Russia, p81

Kumlehn J (2019) Cas endonuclease technology in cereals - establishment and applications. 6. Quedlinburger Pflanzenzüchtungstage. Julius Kühn Institute, 7 May 2019, Quedlinburg, Germany

Kumlehn J (2019) Cas endonuclease-triggered genome modification in cereals. Ghent University, VIB Center for Plant Systems Biology, 28 March 2019, Ghent, Belgium

Kumlehn J (2019) Cas endonuclease-triggered genome modification in cereals. Institute of Crop Science, National Agriculture and Food Research Organization (NARO), 6 March 2019, Tsukuba, Japan

Kumlehn J (2019) Targeted genome modification in cereals. Plant stress science: What can we do for future agriculture? 35th IPSR International Symposium and 11th Symposium on Plant Stress Sciences, 4 March 2019, Kurashiki, Japan

Kumlehn J (2018) Genetische Veränderungen bei Kulturpflanzen. EURACTIV Symposium, Neue Verfahren der Pflanzenzüchtung: Was kommt als Nächstes? Haus der Bundespressekonferenz, Berlin, 13. Dezember 2018

Kumlehn J (2018) Site-directed genome modification in crop plants. Fachtagung des Nationalen Referenzlabors für gentechnisch veränderte Organismen. Bundesamt für Verbraucherschutz und Lebensmittelsicherheit, Berlin, 6.12.2018

Kumlehn J (2018) Site-directed genome modification in cereals using RNA-guided Cas endonucleases. CiBreed Conference: Plants & Animals – Bridging the Gap in Breeding Research, Göttingen, 10.10.2018

Kumlehn J (2018) Site-directed genome modification in cereals using RNA-guided Cas endonucleases. V. International Plant Science Conference, Fisciano, Italy, 12.09.2018

Kumlehn J (2018) Site-directed genome modification in cereals. Summer School on Plant Genome Editing, TRR175, Berlin, 01.08.2018

Pathi K, Budhagatapalli N, Groß M, Deising H, Kumlehn J: Establishment of anthracnose disease resistance of maize by RNA interference and site-directed mutagenesis. German Society of Plant Biotechnology, Neustadt/Weinstraße, 03-04th September, 2018.

Kumlehn, J.: Zielsequenz-spezifische Genomveränderung bei Kulturpflanzen, Referententagung des Bundesverbandes Deutscher Pflanzenzüchter, Gatersleben, 02.07.-03.07.2018

Pathi KM, N Budhagatapalli, M Groß, HB Deising and J Kumlehn (2018) RNA interference-based strategies for anthracnose disease resistance of maize. Plant Science Students Conference, Gatersleben, June 20

Kumlehn J (2018) Site-directed genome modification in cereal crops: applications and challenges. 7th International Bioeconomy Conference. Halle/Saale, 06.06.2018

Kumlehn (2018) Genetische Veränderungen bei Kulturpflanzen. 20 Jahre Grüne Gentechnik – Risiken und Chancen für eine Landwirtschaft der Zukunft, Vortrag und Panel Diskussion, Leopoldina Halle/Saale, 11.01.2018

Kumlehn J (2017) Was Genome Engineering bei Getreide leisten kann - Ergebnisse und Ausblick. Jahrestagung der Gemeinschaft zur Förderung von Pflanzeninnovation e.V. (GFPi), 07.-09. November, Berlin

Kumlehn J (2017) Site-directed genome modification - Current and future opportunities. Tagung der Deutschen Botanischen Gesellschaft. September 17-21, Kiel, Germany

Kumlehn J (2017) Mutual Benefits of Genome Engineering and Haploid Technology. Workshop: Breeding the future plant, 26. July, Universität Freiburg

Pathi K, Budhagatapalli N, Groß M, Deising H, Kumlehn J: Pathogen resistance achieved by plant-induced silencing of fungicide target genes. Plant Science Student Conference, Leibniz-Institut für Pflanzenbiochemie (IPB), Halle/Saale, 06-09th June, 2017.

Kumlehn J (2017) Genome Engineering in Cereals. 4th PlantGen Conference, 30. May – 3. June, Almaty, Kasachstan

Poster

Pathi K, Budhagatapalli N, Hensel G, Büchner H, Daghma D, Groß M, Deising H, Kumlehn J: Establishment of anthracnose disease resistance of maize by Cas endonuclease-mediated mutagenesis and host-induced gene silencing. IS-MPMI, Glasgow, Scotland, 14-18th July, 2019.

Pathi K, Budhagatapalli N, Büchner H, Daghma D, Groß M, Deising H, Kumlehn J: Establishment of anthracnose disease resistance of maize by Cas endonuclease-mediated mutagenesis and host-induced gene silencing. PLANT 2030 Status Seminar, Potsdam, 13-15th March, 2019.

Pathi K, Budhagatapalli N, Hensel G, Büchner H, Groß M, Deising H, Kumlehn J: Pathogen resistance achieved by plant-induced silencing of fungicide target genes. German Society of Plant Biotechnology, Julius-Kühn-Institut, Braunschweig, 03-04th August, 2017.