

Aus dem Institut für Phytopathologie
der Christian-Albrechts-Universität zu Kiel

**Application of CRISPR-Cas9 genome editing systems for improving
oilseed rape (*Brassica napus*) disease resistance against
*Verticillium longisporum***

Dissertation

Zur Erlangung des Doktorgrades

Der Agrar- und Ernährungswissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von

M.Sc. Michael Pröbsting

Kiel, 2019

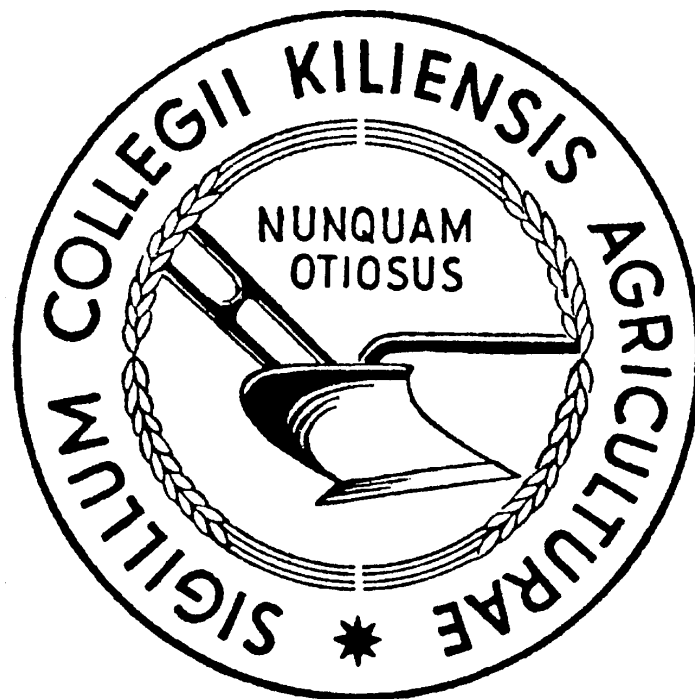
Dekan: Prof. Dr. Dr. Christian Henning

1. Berichterstatter: Prof. Dr. Daguang Cai

2. Berichterstatter: Prof. Dr. Christian Jung

Tag der mündlichen Prüfung: 10.02.2020

Schriftenreihe des Instituts für Phytopathologie
der Christian-Albrechts-Universität zu Kiel; Heft 5, 2020



ISSN: 2197-554X

Gedruckt mit Genehmigung der Agrar- und
Ernährungswissenschaftlichen Fakultät der Christian-Albrechts-
Universität zu Kiel

Table of contents

List of figures	IV
List of tables	IX
List of abbreviations	XI
Abstract	1
Introduction	4
<i>Brassica napus</i>	4
<i>Verticillium longisporum</i>	5
Plant-pathogen interaction	6
a) Plant immune system.....	6
b) Plant-Verticillium interactions	9
Disease management.....	12
Recessive resistance.....	13
Genome-editing for resistance breeding	14
a) Classical non-transgenic approaches	14
b) Genome editing by CRISPR/Cas.....	15
HDR-based genome editing.....	18
Preworks in Arabidopsis and <i>B. napus</i>	21
Aims of the thesis	23
Material	25
Equipment	25
Chemicals.....	26
Enzymes and Kits	27
Laboratory consumables	29
Solutions, buffers and media	30
Software and databases	31
Organisms.....	32
Oligonucleotides.....	32
a) NHEJ cloning	32
b) HDR cloning	34
c) NHEJ genotyping.....	35
d) HDR genotyping.....	35
e) qPCR	36

Methods	37
In silico sequence analysis.....	37
Phylogenetic analysis	37
sgRNA design.....	37
Vector systems for CRISPR/Cas9	37
Assembly of new sgRNAs	38
DNA modifications.....	39
Preparation of competent cells.....	40
Transformation of bacteria	40
Plasmid isolation	41
Agroinfiltration of <i>Nicotiana benthamiana</i> and GFP assay.....	41
Floral Dip <i>Arabidopsis thaliana</i>	42
Hairy roots transformation of <i>Arabidopsis thaliana</i> and <i>Brassica napus</i>	42
Origin of stable transgenic <i>Solanum tuberosum</i> events	43
Total DNA isolation from plant tissue	43
dCAPS and RE-assay	44
Protein extraction from plant tissue	45
SDS-PAGE.....	45
Coomassie staining.....	45
Western Blot with polyclonal Cas9 antibody	45
RNA isolation from plant tissue.....	46
First-strand cDNA synthesis.....	46
Realtime qPCR gene expression analysis	47
Cultivation of <i>Verticillium longisporum</i>	47
Stable transformation of <i>Brassica napus</i> events.....	48
In vivo infection of <i>Brassica napus</i> with <i>Verticillium longisporum</i>	48
In vitro Infection assay of <i>Brassica napus</i> with <i>Verticillium longisporum</i>	48
Disease rating	49
Fungal DNA quantification by qPCR	50
Statistical analyses.....	50
Results	51
Vector systems for NHEJ based approaches	51
In silico analysis and sgRNA design	54
Transformation of our CRISPR/Cas9 constructs.....	59
Screening of CRISPR/Cas-generated mutations <i>CRT1a</i> and <i>HVA22c</i> loci.....	60
Selection of fully mutated events for <i>BnCRT1a</i> and <i>BnHVA22c</i>	62
Infection assays of <i>BnCRT1a</i> and <i>BnHVA22c</i> mutants with <i>V. longisporum</i>	65

Evaluation of grain weight from CRISPR mutants	70
Fungal DNA quantification in petioles of CRISPR mutants	70
Marker gene expression in <i>CRT1a</i> and <i>HVA22c</i> CRISPR mutants	73
Vector systems for HDR based approaches	78
First HDR strategy with multimers	82
Second HDR strategy with viral replicon	82
Discussion	86
The optimised CRISPR/Cas9 vector system proved to be a functional and versatile tool for genome editing in <i>Brassica napus</i>	88
Hairy roots represent a fast and simple <i>in vivo</i> test system for sgRNA functionality and HDR based approaches	90
dCAPS assay screening and inheritance of mutations of CRISPR mutants	92
The Knockout/mutation of <i>CRT1a</i> and <i>HVA22c</i> confers less susceptibility to <i>Brassica napus</i> against <i>Verticillium longisporum</i> infection	93
The involvement of <i>CRT1a</i> and <i>HVA22c</i> in <i>B. napus</i> - <i>V. longisporum</i> interaction remains unclear .	94
Establishment of viral CRISPR expression vectors for HDR application in <i>Brassica napus</i>	97
Application and potential of the CRISPR/Cas9-based genome editing in plant breeding	100
References	102
Supplementary data	127
Danksagung	131
Zusammenfassung	132
Summary	134
Akademischer Lebenslauf	Fehler! Textmarke nicht definiert.

List of figures

- Figure 1: Disease cycle of *Verticillium longisporum* infecting *Brassica napus* (Depotter et al., 2016). Microsclerotia persist in the soil and are responsible and start a new disease cycle (1). Root exudates trigger the germination of microsclerotia and hyphae grow towards the plant (2). The plant is infected through wounds or penetration of epidermal cells of lateral roots. Inside the host, the hyphae grow intercellularly towards the central cylinder to enter the xylem (3). No symptoms can be observed during most parts of cultivation (4). At the end of the growing season, dark unilateral striping develops on the stem and black microsclerotia are formed in the stem cortex (5). Through decomposition of plant residues, microsclerotia are released into the soil (6). 5
- Figure 2: The mechanism of genome editing using CRISPR/Cas9 (Agrotis and Kettler, 2015). 17
- Figure 3: Viral replicons for gene targeting and disarmed viral vector system (Zaidi & Mansoor 2017). The disarmed virus genome without coat and movement protein replaced with Cas9, sgRNA and repair template are cloned in T-DNA. Via *Agrobacterium tumefaciens* mediated transformation the genome is delivered to plants. Inside the plant cell nucleus, the single-stranded viral DNA is converted into the dsDNA replicative form and RNA is transcribed. The Cas9-sgRNA complex binds to the target site where a double-strand break is induced. If homology directed repair is triggered the donor DNA can serve as a repair template. 21
- Figure 4: Comparative RNA sequencing approach for identification of susceptibility factors in the *V. longisporum* - *B. napus* interaction and confirmation through KO mutants of the model system *A. thaliana* and *B. napus*. 22
- Figure 5: Example for sgRNA assembly and cloning scheme for the NHEJ vector 39
- Figure 6: Vector map of BS_01. The vector contained a *B. napus* codon-optimised Cas9 with a C-terminal Mammalian Importin alpha type nucleus localization sequence (NLS) under the control of the CaMV 35S promoter, a sgRNA template driven by an AtU6 promoter within a multiple cloning site and recombination sites for gateway LR reaction. This vector map was created with snap gene viewer. 51
- Figure 7: Confirmation of cassette functionality on the transcriptional and translational level. PCRs with genomic DNA and cDNA of Cas9 transgenic *Solanum tuberosum* plants were conducted with Primer for Cas9. The Western Blot was conducted with a polyclonal antibody for the Cas9 protein.. 52
- Figure 8: Transient expression of Cas9-GFP in epidermal leaf cells of *Nicotiana benthamiana* 48h after inoculation with *A. tumefaciens*. 53
- Figure 9: A Neighbour-Joining phylogenetic tree based on amino acid sequences of AtCrt1a and BnCRT1a as well as AtCrt1b and AtCrt3, which was created using MEGA X software (Kumar et al., 2018). The trees were calculated by applying 1000 bootstraps with the following settings: Maximum Likelihood, Jones-Taylor-Thornton (JTT) model, Uniform Rates, Use all sites, Nearest-Neighbor-Interchange (NNI). 54

- Figure 10: A Neighbour-Joining phylogenetic tree based on amino acid sequences of AtHVA22c and BnHVA22c as well as AtHVA22a, AtHVA22b, AtHVA22d and AtHVA22d which was created using MEGA X software (Kumar et al., 2018). The trees were calculated by applying 1000 bootstraps with the following settings: Maximum Likelihood, Jones-Taylor-Thornton (JTT) model, Uniform Rates, Use all sites, Nearest-Neighbor-Interchange (NNI)..... 55
- Figure 11: Gene structure of candidate genes and localisation of sgRNAs. Homologues of the target genes *CRT1a* and *HVA22c* were identified in *Brassica napus* and the gene structure was manually adjusted due to gene prediction errors. Each target sequence was found in the correct number of loci for the respective gene and organism. One exception is *HVA22c* where 4 copies could be found. 56
- Figure 12: RE-Assay of *HVA22c_2* locus for mutation screening. The sgRNA target locus was amplified from different transgenic hairy roots from *Brassica napus* transformed. PCR products were incubated with restriction enzyme *AsuI* to screen for potential mutations. The wild type locus (WT) was completely digested and the undigested wild type PCR product served as a reference for undigested bands (UD). Several events showed undigested bands suggesting potential mutations in the respective genomes. 59
- Figure 13: dCAPS assay for BnCRT1a and BnHVA22c target loci in *Brassica napus*. PCRs with dCAPS Primer, that introduce RE recognition sequences into PCR products with wild type sequence. UD = Undigested PCR product 60
- Figure 14: Mutations at sgRNA target locus for BnCRT1a and BnHVA22c obtained so far. The sequence data was obtained through sequencing single alleles in T-vectors. For CRT1a 9 WT and 80 PCR products from the target loci and for HVA22c 15 WT and 85 PCR products from the target loci were sequenced. Only T0 events with mutations are listed in this figure..... 61
- Figure 15: Scheme for propagation and selection of CRISPR mutants. The T0 generation was screened with single allele sequencing and dCAPS assay. Four events with high mutation rates were chosen for further propagation. Twenty plants of the T1 generation were screened with dCAPS and three plants per T0 parental event were chosen for further propagation. Three progenies of each T1 event were analysed by dCAPS and 10 plants were used in infection experiments with *Verticillium longisporum*. 62
- Figure 16: dCAPS assay for CRT1a and HVA22c target loci in *Brassica napus*. PCRs with dCAPS Primer, that introduce RE recognition sequences into PCR products with wild type sequence. UD = Undigested PCR product. 63
- Figure 17: dCAPS assay for CRT1a and HVA22c target loci in *Brassica napus* of the T1 progeny. PCRs with dCAPS Primer, that introduce RE recognition sequences into PCR products. UD = Undigested PCR product. 64
- Figure 18: Different infection severities of *Verticillium* stem stripping 21 dpi of the cultivar Mozart. The plants were grown in the greenhouse under long-day conditions. A range of disease severities could be observed with beginning chlorosis (B) to stunted growth and severe chlorosis (D) compared to the non-infected control (A). 65
- Figure 19: Selected CRT1a CRISPR mutants 28 dpi after infection with *Verticillium longisporum* VI4366

Figure 20: Selected HVA22c CRISPR mutants 28 dpi after infection with <i>Verticillium longisporum</i> VI43	67
Figure 21: AUDPC _{norm} of all CRISPR genotypes infected with <i>Verticillium longisporum</i> . The AUDPC values were calculated according to Campbell and Madden (1990) and normalised against the wild type. For each genotype 10 infected and five control plants with one biological replicate were rated weekly until 28dpi. Error bars represent the standard error of the mean for 20 plants of two independent biological replicates and statistics were performed with an ANOVA and multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.....	68
Figure 22: Growth Depression (GD) of all CRISPR mutants infected with <i>Verticillium longisporum</i> . The GD values were calculated through normalising the plant height of infected plants against their control plant height 28 dpi. For each genotype 10 infected and five control plants with one biological replicate were rated 28dpi. Error bars represent the standard error of the mean for 20 plants of two independent biological replicates and statistics were performed with an ANOVA and multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.....	69
Figure 23: Linear model for correlation of AUDPC _{norm} and GD. The model was derived from the AUDPC _{norm} and GD values, which significantly correlated. Genotypes localised in the lower-left corner represent the least susceptible ones.....	69
Figure 24: Seed weight per 1000 seeds normalized to the wild type. Error bars represent the standard error of the mean for 20 plants of two independent biological replicates and statistics were performed with an ANOVA and multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.....	70
Figure 25: Absolute fungal DNA quantification of <i>Brassica napus</i> petioles 28 dpi with <i>Verticillium longisporum</i> . <i>Verticillium</i> specific OLG Primer were used in a qPCR for three samples consisting of petioles of two plants per genotype. Error bars represent the standard error of the mean for 6 plants and statistics were performed with multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.....	71
Figure 26: Induction of salicylic acid marker genes of <i>Brassica napus</i> seedlings at 6 dpi with <i>Verticillium longisporum</i> . Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected C _q values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.....	74
Figure 27: Induction of ethylene marker genes of <i>Brassica napus</i> seedlings at 6 dpi with <i>Verticillium longisporum</i> . Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected C _q values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.	75
Figure 28: Induction of jasmonic acid marker genes of <i>Brassica napus</i> seedlings at 6 dpi with <i>Verticillium longisporum</i> . Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected C _q values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.....	76

- Figure 29: Induction of ER related genes of *Brassica napus* seedlings at 6 dpi with *Verticillium longisporum*. Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected Cq values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test ($*=p\leq 0.05$), comparing WT and CRISPR mutants. 77
- Figure 30: Cloning scheme for the production of template repeats. For the cloning procedure, *BclI* and *BamHI* recognition sequences are added via PCR to the template. After being cut both RE sites have similar overhangs but cannot be cut again if they are ligated to one another which inactivates the RE recognition sequence. If this fragment is ligated into a *BamHI* cut vector- only one side is still cuttable by *BamHI*. This principle was used to clone the fragment multiple times. 78
- Figure 31: HDR approach for the exchange of miRNA binding sites using a repeat based vector for successful HDR. The expression cassette contains sgRNA and Cas9 for the induction of DSB and repair templates with adapter sequences which are targetable by the sgRNA to allow them to be cut out. In the presence of multiple of those repair templates, the HDR should be triggered and seamless exchange of the miRNA binding site should be possible. (Courtesy of Dirk Schenke)..... 79
- Figure 32: Vector map of BS_14. The vector was created by cloning a sequence fragment of the binary vector pGWB401 containing the M13fwd Primer sequence, attR, lacUV5 promoter, Cmr coding sequence, ccdB coding sequence, attR2 and a Nos terminator was cloned into the disarmed genome of the bean yellow dwarf virus (pLSLR) (Baltes et al. 2014). The vector map was created by snap gene viewer..... 80
- Figure 33: HDR approach for the exchange of miRNA binding sites using a vector with a disarmed genome of the Bean yellow dwarf virus for successful HDR. The expression cassette contains sgRNA and Cas9 for the induction of DSB and genes for the viral replication and LIR sites for the processing of the viral genome. Only a single copy of the repair template is cloned into this vector because the template is multiplied by replication of the virus. 80
- Figure 34: PCR based screening for positive HDR events for AGO1 in *A. thaliana*. Target loci the gene were amplified with Primer binding to the wild type sequence and outside the repair template range. With the same samples, a second PCR with Primer binding to the modified sequence and outside the range of the repair template was conducted to have a double confirmation in case of potential positive HDR events..... 82
- Figure 35: PCR for the screening of processed viral genomes. The gDNA was derived from transgenic *Brassica napus* hairy roots transformed with the viral vector. The Primer used for this PCR are localized down and upstream of the LIR and only amplify if the viral genome is processed into its circular form. 83
- Figure 36: RE-Assay and sequencing of undigested bands of *HVA22c_2* locus for mutation screening. The sgRNA target locus was amplified from different transgenic hairy roots from *Brassica napus* transformed with the BS_14 construct. PCR products were incubated with restriction enzyme *AsuI* to screen for potential mutations. The wild type locus (WT) was completely digested and the undigested wild type PCR product served as a reference for undigested bands (UD). Several genotypes showed undigested bands suggesting potential mutations in the respective genomes. Additionally, the undigested bands were cloned into t-vector and sequenced..... 83

Figure 37: RE-Assay of *HVA22c_2* locus for mutation screening in combination with virus persistence testing. The sgRNA target locus was amplified from different transgenic hairy roots from *Brassica napus* transformed with the BS_15 construct. Samples were harvested 5 and 7 weeks after the first roots emerged. PCR products were incubated with restriction enzyme *AsuI* to screen for potential mutations. The wild type locus (WT) was completely digested and the undigested wild type PCR product served as a reference for undigested bands (UD). Several events showed undigested bands suggesting potential mutations in the respective genomes. 84

Figure 38: PCR based screening for positive HDR events for *AGO1* and *TAO1_A* from *B.napus* hairy roots. Target loci for both genes were amplified with Primer binding to the wild type sequence and outside the repair template range. With the same samples, a second PCR with Primer binding to the modified sequence and outside the range of the repair template was conducted to have a double confirmation in case of potential positive HDR events. Unfortunately, no bands could be observed at the correct length of 1 kb for the mutated locus. 85

Figure 39: Model of the compatible *B. napus*- *V. longisporum* interaction. *CRT1a* and *HVA22c* represent the susceptibility factors hijacked by the fungus with effectors (VL1 and VL2) for the successful colonization of the host. *CRT1a* and *HVA22c* may affect a negative regulator in the ethylene (ET) signaling pathway, thus modulating defense responses. In addition to the defense-related influence of those genes, an involvement of *CRT1a* in protein folding and *HVA22c* in vesicular transport can be suggested. In the absence of those genes, these biological processes may not be targetable by the fungus. 97

Figure 40 : Gene structure of candidate genes and localisation of sgRNAs. 127

Figure 41: RE-Assay of *StTXR1* locus for mutation screening. The sgRNA target locus was amplified from different transgenic plants from *Solanum tuberosum* transformed with BS_11. PCR products were incubated with restriction enzyme *AluI* to screen for potential mutations. The wild type locus (WT) was completely digested and the undigested wild type PCR product served as a reference for undigested bands (UD). No events with undigested PCR product could be observed suggesting that no mutations occurred in the respective genomes. 129

List of tables

Table 1: Modification of susceptibility factors and quality traits utilising genome editing tools modified after Scheben et al. 2017	19
Table 2: Equipment used in this thesis.....	25
Table 3: Chemicals used in this thesis.....	26
Table 4: Enzymes and kits used in this thesis.....	27
Table 5: Laboratory consumables used in this thesis.....	29
Table 6: Solutions, buffers and media used in this thesis.....	30
Table 7: Software and databases used in this thesis.....	31
Table 8: Organisms used in this thesis.....	32
Table 9: Oligonucleotides for NHEJ cloning.....	32
Table 10: Oligonucleotides for HDR cloning.....	34
Table 11: Oligonucleotides for NHEJ genotyping.....	35
Table 12: Oligonucleotides for NHEJ genotyping.....	35
Table 13: Oligonucleotides for qPCR.....	36
Table 14: PCR program for sgRNA amplification.....	38
Table 15: PCR program for dCAPS assay	44
Table 16: Disease severity index (Eynck et al., 2007).....	49
Table 17: sgRNA targets for NHEJ and HDR based genome editing obtained from CRISPR RGEN Tools. MM =Mismatches to the original target sequence.....	55
Table 18: Potential off-target sequences of sgRNAs for the respective candidate genes (Only targets up to 3 mismatches were included).* BnaA09g15970D and BnaC01g43040D were excluded from potential off-targets due to possible erroneous sequence data.	57
Table 19: Plasmids cloned in this study for the NHEJ approach with information about promoter, nuclease and sgRNA target.....	58

Table 20: Pheno- and genotypic data for the CRISPR mutants C2E20, C1E22, C4E23 for BnCRT1a as well as H7E8, H15E3, H12E3 for BnHVA22c compared to the wild type and rated for improvement of traits (+, ++, +++) or same characteristics as the wild type (=). Target loci were sequenced with single allele T-vector cloning. Insertions are indicated by green letters, while yellow letters indicate SNPs in WT.	72
Table 21: Plasmids cloned in this study for the NHEJ approach with information about the promoter, nuclease, sgRNA target, repair template amount and backbone.	81
Table 22: CRISPR/Cas9 vector systems designed for stable transformation in <i>Oryza sativa</i> and <i>Arabidopsis thaliana</i> . Modified after Ma et al., 2016.	88
Table 23: CRISPR/Cas9 approaches utilising hairy roots transformation.....	91
Table 24: sgRNA targets for NHEJ and HDR based genome editing obtained from CRISPR RGEN Tools. MM =Mismatches to the original target sequence.....	127
Table 25: Potential off-target sequences of sgRNAs for the respective candidate genes (Only targets up to 3 mismatches were included).	127
Table 26: Plasmids cloned in this study for the NHEJ approach with information about promoter, nuclease and sgRNA target.....	128
Table 27: Sequencing results for <i>A. thaliana</i> CRISPR/Cas mutants for the constructs BS_04 and BS_05	130

List of abbreviations

ABA	Abscisic acid
ACO1	Aconitase 1
AGO1	Argonaute protein 1
AGO10	Argonaute protein 10
APS	Ammoniumperoxodisulfate
AUDPC	Area under disease progress curve
AUX	Auxin
AVR proteins	Avirulence proteins
BAR	Phosphinothricin N-acetyltransferase
BCA	Bio control agent
FLA1	Fascilin-like arabinocalactan protein1
BeYDV	<i>Bean yellow dwarf virus</i>
CaMV	Cauliflower mosaic virus
Cas9	CRISPR-associated protein
ccdb	Cytotoxic protein CcdB
CDS	Coding sequence
CEL I	Endonuclease from cellery
CESA3	Cellulose synthase A catalytic subunit 3
PDS	Phytoene desaturase
CK	Cytokinin
CmR	Canamycin resistance
COI1	Coronatine insensitive 1
CP	Capsid protein
Cpf1	CRISPR-associated protein from <i>Prevotella</i> and <i>Francisella</i> 1
CRISPR	Clustered regulatory interspaced palindromic repeats
CRT1a	Calreticulin 1a
CsLOB1	Promoter
CTAB	Cetyltrimethylammonium bromide
dCAPS	Derived Cleaved Amplified Polymorphic Sequences
DCL1	Dicer like protein 1
DEPC	Diethyl pyrocarbonate
DHPLC	Denaturing high performance liquid chromatography
DMSO	Dimethyl sulfoxide
dpi	Days post infection
DSB	Double strand break
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic Translation Initiation Factor 4E
EMS	Ethyl-methane sulfonate
ERF922	ERF transcription factor
ET	Ethylene
ETI	Effector triggered immunity
ETR2	Ethylene receptor 2
ETS	Effector triggered susceptibility
EWR1	Enhancer of vascular Wilt Resistance 1
FLAG	Flaggelin

GA	Gibberelin
GLS	Glucosinolate
CHI20	Chalcone-flavanone isomerase
FEI2	Leucine-Rich Repeat Receptor Kinase
GS1	Gamma glutamine synthetase
GMO	Genetically modified organism
gRNA	Guide RNA
GT	Genome targeting
HDR	Homology directed repair
HPLC	High performance liquid chromatography
HR	Hypersensitive response
Hva22c	HVA22 homologue C
HYL1	Hyponastic leaves 1
IAA9	Aux/IAA gene
ICS	Isochorismate synthase
InDels	Insertions or deletions
IPTG	Isopropyl β -D-1-thiogalactopyranoside
JA	Jasmonic acid
JA-Ile	Jasmonic acid-isoleucine
JAZ	Jasmonate-zim domain protein
LD	Linkage disequilibrium
LIR	Long intergenic region
LOX3	Lipoxygenase 3
MAMP	Microbial associated molecular pattern
MAS	Marker assisted selection
miRNA	Micro RNA
MLO	Mildew Resistance Locus O
MP	Movement protein
mRNA	Messenger RNA
NBS-LRR	Nucleotide-binding site and leucine rich repeats
NHEJ	Non homologous end joining
NLS	Nuclear localisation signal
OSR	Oilseed rape
PAM	Protospacer adjacent motif
PAMP	Pathogen associated molecular pattern
PDF 1.2	Plant defensin 1.2
PEN	Penetration-resistance genes
PMR6	Pectate lyase-like protein
PR1	Pathogenesis-related protein 1
PR2	Pathogenesis-related protein 2
PRR	Pathogen recognition receptors
PTGS	Post transcriptional gene silencing
PTI	PAMP triggered immunity
QTL	Quantitative trait loci
R genes	Resistance genes
R proteins	Resistance proteins
RE	Restriction enzyme

REP	Replication initiator protein
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species
SA	Salicylic acid
SaCas9	<i>Staphylococcus aureus</i> Cas9
SAG	Salicylic acid glucoside
SDS	Sodium dodecyl sulfate
SE	Serrate
SIR	Short intergenic region
siRNA	Small interfering RNA
sRNA	Small RNA
StCas9	<i>Staphylococcus thermophilus</i> Cas9
SWEET	Sugars will eventually be exported transporter
TAIR	The Arabidopsis Information Resource
TALEN	Transcription activator-like effector nuclease
T-DNA	Transfer DNA
TEMED	Tetramethylethylenedia-mine
TF	Transcription factor
TILLING	Targeting Induced Local Lesions in Genomes
TRIS ultra pure	Tris(hydroxymethyl)aminomethane
TRIS/HCL	Tris(hydroxymethyl)aminomethane/hydrochloric acid
TXR1	Thaxtomin a resistant 1
VE1	Verticillium wilt disease resistance protein
VSP2	Vegetative storage protein 2
WDV	Wheat dwarf virus
WRKY52	WRKY transcription factor
WRKY70	WRKY transcription factor
WT	Wild type
ZFN	Zinc finger nucleases

Abstract

Traditional breeding has strong limitations, e.g. merely relying on a broad genetic pool and is a time-intensive and complex process until a new variety is generated. Advanced breeding strategies and adaption of new techniques become more important to meet the demands of modern agriculture. Site-specific nucleases are promising new tools to enable targeted modification of genomes, which are under normal circumstances not possible, e.g. by using undirected mutagenesis with numerous off-target mutations. CRISPR/Cas9 is the most promising system for targeted mutagenesis in the plant genome. This technology enables the induction of small InDels to cause gene disruption through non-homologous end joining (NHEJ) or in an advanced state the insertion of a repair template for homology-directed repair (HDR). For crops like *Brassica napus* that have a relatively narrow gene pool, this tool offers great opportunities to generate new genetic variance without negative side effects generated for example by undirected random mutagenesis. Of great interest is the application of this tool for resistance breeding via knockout of susceptibility factors to interrupt infection processes leading to the resistance of crops towards a certain pathogen. The soilborne semi biotrophic fungus *Verticillium longisporum* is one of the major fungal pathogens in *Brassica napus* and causes Verticillium stem striping with the progression of the disease leading to premature ripening. This disease can lead to severe yield losses and countermeasures are mainly relying on decreasing the spore concentration in the soil. Genetic resistance resources are very limited and no true resistance has been discovered so far. A method to increase the resistance of a crop towards a certain pathogen is the knockout of plant factors that are not related to the immune system but are required by the pathogen to colonize the host successfully. These so-called susceptibility factors can be disrupted to increase the host's resistance against the pathogen. Genes that were identified by a comparative transcriptomic approach (SSH library) of non-infected and infected *B. napus* plants with *V. longisporum* (VI43) and could potentially act as susceptibility factors were first tested in *Arabidopsis thaliana* knockout mutants. Further experiments to undermine their putative role in the infection process were conducted with OSR mutants of respective genes originating from a TILLING population. To finally validate the role of the candidate genes as a susceptibility factor in this pathosystem and to establish an efficient way to generate durable resistance the genome-editing tool CRISPR/Cas9 was utilized. In this study, we adapted several vector systems containing different nucleases and promoters for *Brassica napus*. The expression of our self-developed, codon-optimized vector system was successfully validated on the transcriptional

and translational level with RT-PCR and Western blot using a polyclonal Cas9 antibody. Protein extracts from transgenic plants carrying the expression cassette were tested positive regarding the Cas9 protein. The potential translocation of the Cas9 protein to the nucleus could be observed by agroinfiltration of *Nicotiana benthamiana* leaves with our Cas9 fused C-terminally to GFP. The Cas9 function itself was validated in the hairy roots of *Brassica napus*. This transformation system was further utilized to serve as an efficient *in vivo* test system to verify sgRNA functionality, which allowed us to obtain results in as fast as three weeks after transformation. To advance genome editing approaches and enable HDR two strategies were developed to overcome the bottleneck of insufficient repair templates after the occurrence of a DSB. The first strategy involved multiple copies of the donor DNA on the T-DNA which hypothetically should be cut out by the Cas9-sgRNA complex. The construct was successfully generated, but no HDR events were detectable in *A. thaliana* with this approach. The second strategy aimed at utilizing viral replicons to overcome the bottleneck of sufficient repair templates to trigger HDR. The disarmed genome of the Bean Yellow Dwarf Virus was combined with our expression cassette to enable efficient use in *Brassica napus* hairy roots for the first time and was able to induce DSBs in the genome. Unfortunately, we could not detect positive HDR events with the system either and this method has to be further refined for the use in *Brassica napus*. For the NHEJ bases approach 22 independent transgenic events for *CRT1a* and 40 for *HVA22c* were generated by Saaten-Union Biotec and characterized. From these, four knockout genotypes for the genes *BnCRT1a* and *BnHVA22c* were selected by dCAPS screening as well as single allele sequencing under the selection premise of high mutation rate. For the functional investigation, the knockout plants were infected with *V. longisporum* under greenhouse conditions in which wild type plants cv. Mozart served as a control. Although the fungus was able to infect all tested plants successfully, a significant reduction in disease severity (AUDPC values) and symptom development, as well as less stunted growth, could be observed in all tested knockout genotypes of both genes compared to the wild type. A linear model with a significant correlation of AUDPC values and stunted growth could be derived which supported the data set regarding a significant reduction of disease severity. To further verify the disease rating data, fungal DNA was quantified by qPCR in petioles of infected plants. Less fungal DNA was detectable in the knockout mutants of both genes compared to the wild type, further supporting a crucial role of *BnCRT1a* and *BnHVA22c* in the plant-fungus interaction and demonstrate that knockout/mutation of the gene interrupted the fungal infection process, consequently leading to less susceptible plants to the fungal infection.

Furthermore, molecular analysis of *CRT1a* mutants revealed no substantial changes in diverse signalling pathways at transcriptional levels as compared with the wild type but with the exception for an elevated level of *ETR2* expression, which is in consent with the finding in *A. thaliana CRT1a* KO mutants. These mutations might imply a fundamental change in the plant physiology and defence status, allowing the plants to react faster to a *V. longisporum* infection. Further molecular characterization is needed to shed light on the resistance mechanism underlying the loss-of-function mutants of both genes.

Introduction

Brassica napus

Brassica napus or commonly referred to as oilseed rape (OSR) is among soybean and sunflower one of the world's most cultivated oilseed crop for vegetable and industrial oil production with 70.91 tons in 2018/2019 worldwide (USDA, 2019). Winter and spring varieties of OSR are both cultivated in Europe. The advantage of OSR is its survivability at low temperatures with reasonable humidity, and therefore it can be grown in temperate zones in contrast to soybean and sunflower. OSR was not of agricultural interest until the breeding of the so-called '00' lines with low erucic acid (<2% in the oil) and glucosinolate (<30 $\mu\text{mol/g}$ in the meal) content in the 1970s (Shahidi, 1990). Without the high content of erucic acid and glucosinolates, OSR became a valuable source for edible vegetable oil, for human nutrition and animal feed containing high amounts of protein. The industrial uses are the production of specialised lubricants and renewable fuels, whereas the last product becomes more and more relevant for reducing CO₂ emissions through sustainable fuel production.

Brassica napus (AACC, $2n=4\times=38$) is a relatively new allopolyploid crop originating from multiple independent inter-specific hybridisations between *Brassica rapa* (AA, $2n=2\times=20$) and *Brassica oleracea* (CC, $2n=2\times=18$) (Prakash and Hinata, 1980; Gupta and Pratap, 2007). The parental species for the ancestral forms of *Brassica oleracea* are located around the Mediterranean Sea and the Middle East for *Brassica rapa* (Song et al., 1988; Warwick & Black 1993; Gomez-Campo, 1999). As a consequence, wild genotypes and relatives are rare and the genetic pool is very limited. Besides, the selection for double zero lines as the primary breeding criterion is another factor, which narrowed the genetic pool. Ultimately, this led to several problems like inferior seed germination (Hatzig et al., 2018) or reduced pathogen resistance (Zhao & Meng, 2003) increasing the lack of genetic resources for resistance breeding.

Modern agricultural systems are following crop rotations with no or little variation and the overall expanding cultivation of OSR is beneficial for pathogens to spread, posing a serious threat to farmers (Coninck et al., 2015). The most economically important fungal pathogens of OSR are Blackleg (*Leptosphaeria maculans*; Zhou et al., 1999), Clubroot (*Plasmodiophora brassicae*; Hwang et al., 2012), Downy Mildew (*Hyaloperonospora parasitica*; Koch and Slusarenko, 1990), Sclerotinia Stem Rot (*Sclerotinia sclerotiorum*; del Río et al., 2007) and Verticillium wilt (*Verticillium longisporum*; Depotter et al., 2016).

Verticillium longisporum

Verticillium longisporum is a hemibiotrophic, soil-borne fungal pathogen and belongs to the family *Plectosphaerellaceae* (Zare et al., 2007), the subclass *Hypocreomycetidae* of the class *Sordariomycetes* in the phylum of *Ascomycota* (Zhang et al., 2006). In the past, Verticillium wilt of *Brassica napus* was assumed to be caused by a strain of *Verticillium dahliae* which was first reported from wilted horseradish (Stark, 1961). It was elevated to the species rank after a detailed evaluation of its pathogenic level and other parameters as well as the fact that this species specifically colonises *Brassicaceae* (Karapapa et al., 1997).

Verticillium stem striping is a monocyclic disease which means only one cycle of the disease with a dormant, parasitic, saprophytic phase and inoculum production occurs during a season (Klosterman et al., 2009) (Fig. 1). Microsclerotia are fungal resting structures and consist of melanised aggregates of enlarged hyphal cells. They germinate in the presence of root exudates and hyphae that grow out of germinating microsclerotia in the soil are following a nutrient gradient to reach the roots of potential host plants (Schreiber and Green, 1963; Huisman, 1982; Olsson and Nordbring-Hertz, 1985).

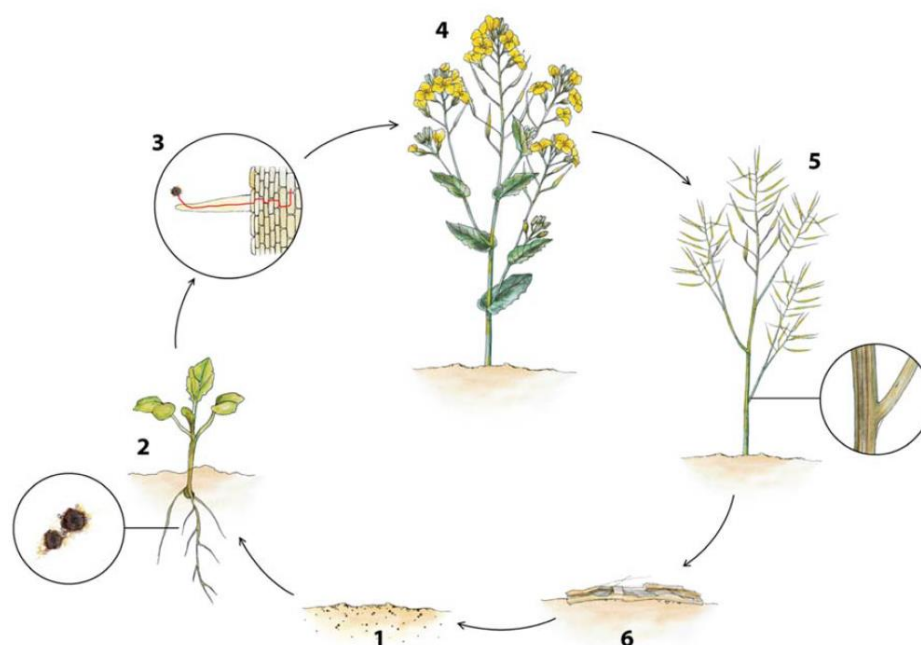


Figure 1: Disease cycle of *Verticillium longisporum* infecting *Brassica napus* (Depotter et al., 2016). Microsclerotia persist in the soil and are responsible and start a new disease cycle (1). Root exudates trigger the germination of microsclerotia and hyphae grow towards the plant (2). The plant is infected through wounds or penetration of epidermal cells of lateral roots. Inside the host, the hyphae grow intercellularly towards the central cylinder to enter the xylem (3). No symptoms can be observed during most parts of cultivation (4). At the end of the growing season, dark unilateral striping develops on the stem and black microsclerotia are formed in the stem cortex (5). Through decomposition of plant residues, microsclerotia are released into the soil (6).

In their parasitic phase, the fungus can directly penetrate the epidermal cells of the root or colonise the plant through natural openings, e.g. wounds to infect the host. Once inside the plant, fungal hyphae grow intra- and intercellular through the root cortex to reach the xylem vessels (Eynck et al., 2007). The vascular system is then colonised by the fungus and the host further infected *via* spore release (Presley et al., 1966). The spores or conidia are transported upwards with the transpiration stream in upper parts of the plant and germinate, forming germ tubes that penetrate adjacent vessel elements starting a new infection cycle (Garas et al., 1986). Near the end of the disease cycle and beginning senescence of the host, the fungus enters a saprophytic phase in which microsclerotia are formed in the dying stem parenchyma to start a new disease cycle (Neumann and Dobinson, 2003).

Symptoms developing during colonisation of the host plant by the fungus do not include wilting in *Brassica napus* but in contrast a premature ripening and chlorosis. That makes it difficult to distinguish between senescence and actual disease symptoms. Yield losses caused by *Verticillium* stem striping are considered to be in the range of 10-50% under field conditions, but this has not been statistically verified (Dunker et al., 2008). Another critical point regarding the diagnosis of this disease is the extended latent phase of the fungus, making it extremely difficult to observe infected plants in the field (Eynck et al., 2007). This can lead to a more severe progression of the disease demanding for preventive measures.

Plant-pathogen interaction

a) Plant immune system

Plants are exposed to numerous biotic stresses from bacterial, fungal or viral pathogens and developed an effective, innate immune system. This immune system detects potential pathogens and initiates, depending on the plant and pathogen interaction, different responses to the invading organism. The nonhost or basal resistance confers resistance against all pathotypes of a pathogen and in all varieties of a plant species (Heath, 2000). Although the nonhost resistance is more durable than the host resistance, it is challenging to implement it in resistance breeding due to the involvement of multiple pathways and networks (Gill et al., 2015). During early phases of the pathogen infection, the basal defence is initiated through recognition of conserved microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) by plant extracellular pathogen recognition receptors (PRRs) (Zipfel, 2008; Dangl et al., 2013) and referred to as PAMP-triggered immunity (PTI). This PTI forms the first line of defence in the multi-layered plant immune system according to the zigzag model introduced by Jones and Dangl (2006). Pathogens can adapt to this first layer of the plant immune system through the evolution and secretion of adapted effectors to avoid the recognition by the

plant PRRs (Zipfel, 2008; Dangl et al., 2013) and/or disrupt defence signalling. This promotes pathogen growth, virulence and leads to the effector-triggered susceptibility (ETS). The next layer of the plant immune system depends on a single or multiple resistance (R) genes. R proteins can recognise those adapted effectors or avirulence (Avr) proteins (Nimchuk et al., 2003). These proteins mostly contain a nucleotide-binding site and leucine-rich repeats (NBS-LRRs). Upon successful direct or indirect recognition of Avr proteins through R proteins the plant induces the effector-triggered immunity (ETI). Indirect interaction is referred to as monitoring effector targets through R proteins and their role as a “guard” protein (DeYoung and Innes, 2006). The triggered defence is associated with a strong hypersensitive response (HR) and leads to local cell death through the generation of reactive oxygen species (ROS) to contain the pathogen. The host, as well as the pathogen, are under natural selection pressure to diversify effectors and R proteins, thus forming multi-layered effector-R gene interactions (Boller and He, 2009). These interactions are the molecular basis for the gene-for-gene resistance (Flor, 1971).

The plant hormone signalling has evolved into a network in which several kinds of phytohormones act antagonistically or enhance one another to change or maintain different physiological states. The most common or relevant phytohormones are auxin (AUX), cytokinin (CK) and gibberellin (GA) which are related to growth and cell differentiation of plant tissue as well as salicylic acid (SA), jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) which are stress-related hormones (Shigenaga and Argueso, 2016). Plant immune responses can roughly be divided into SA or JA dependent while both hormones can trigger defence mechanisms in response to biotrophic and necrotrophic pathogens (Thomma et al., 1998; Spoel et al., 2007). Upon recognition by PRRs or R proteins, pathogens activate signal transduction into the nucleus that initiates one of two SA biosynthesis pathways (Asai et al., 2002; Wang et al., 2009; García et al., 2010). The first pathway is characterised by the activity of phenylalanine ammonia-lyase (PAL) that produces cinnamate, which serves as a substrate for SA synthesis. The second pathway relies on the synthesis of SA from chorismate which is catalysed by isochorismate synthase (ICS) and is strongly connected to pathogen-induced SA accumulation (Chen et al., 2009). Induced SA biosynthesis can be detected through the expression of marker genes involved in SA associated signalling like *PR1* (Thomma et al., 1998). The JA depended defence is induced upon infection with a necrotrophic pathogen or wounding through herbivores (Koo and Howe, 2009). Accumulation of bioactive jasmonic acid-isoleucine (JA-Ile) triggers a signalling cascade that induces the degradation of *JASMONATE-ZIM DOMAIN*

PROTEIN (JAZ) which is dependent on *CORONATINE INSENSITIVE 1 (COI1)* (Thines et al., 2007). JAZ is a nuclear repressor of transcription factors (TF) regulating JA associated marker genes such as *VEGETATIVE STORAGE PROTEIN 2 (VSP2)* and *PLANT DEFENSIN 1.2 (PDF1.2)* (Fernández-Calvo et al., 2011). The phytohormones ET and ABA are involved in defence-related signalling pathways as well but are reduced to fine-tune the crosstalk between SA, which is predominantly involved in defence against biotrophic pathogens, and JA dependent responses, which are related to necrotrophic pathogens (Kusajima et al., 2010; Broekgaarden et al., 2015).

Endogenous noncoding small RNAs (sRNAs) are involved in multiple biological processes. One of them is the modulation of defence responses and plant immunity through post-transcriptional gene silencing (PTGS). sRNAs are divided into micro RNAs (miRNA) and small interfering RNAs (siRNAs). miRNAs are endogenous 20-24 nucleotide small RNAs, involved in the post-transcriptional gene silencing (PTGS) (Iwakawa and Tomari, 2013). They are encoded on specific miRNA genes being transcribed by the RNA-Polymerase II (Xie et al., 2005) and form a double-stranded intermediate due to complementary base pairing after transcription. In the nucleus this primary miRNA is cleaved to a miRNA precursor and processed to small double-stranded miRNAs by multiple cooperating enzymes including DICER LIKE PROTEIN 1 (DCL1), hyponastic leaves 1 (HYL1) and serrate (SE) (Achkar et al., 2016). The processed miRNA is exported to the cytoplasm and one strand is incorporated into ARGONAUTE 1 protein (AGO1) forming the RNA Induced Silencing Complex (RISC). miRNAs guide the RNA-induced silencing complex (RISC) through base pairing to a corresponding mRNA. This negatively regulates the expression of the gene by degrading the mRNA or translational repression. The Influence of endogenous miRNAs on the modulation of the plant immune responses was discovered as a significant and conserved mechanism in plant-pathogen interactions (Shen et al., 2014; Cao et al., 2016). An example of this interaction is the miR2118/482 superfamily which is conserved among different plant species and consisting of miR2118 (*M. truncatula*), miR482 (*Solanaceae*) as well as miR472 (*A. thaliana*) (Fei et al., 2013). Immune receptors were found to be targeted by those miRNAs resulting in constitutive repression of the receptors without an infection of the plant. Upon infection, the miRNA levels decreased resulting in an up-regulation of immune receptors to increase the reception rate of pathogens (Shivaprasad et al., 2012). However, this regulation network can be hijacked by pathogens to prevent recognition and compromise defence mechanisms. This cross-kingdom RNAi can occur in the opposite direction as well as enabling the plant to impede infection (Zhang et al., 2016; Cai et

al., 2018). In conclusion, this adds another layer to plant-pathogen interactions which has to be considered as a potential defence breeding target.

b) Plant-Verticillium interactions

The infection process and colonisation of the host through *Verticillium* can be divided into separate phases. In the initial or prepenetration phase, fungal spores germinate and try to penetrate epidermal root cells of the potential host. In this phase, no defence mechanism was identified and the penetration phase may not play a role in resistance against *Verticillium* (Eynck et al., 2009a). Later stages of the infection are referred to as post penetration, prevascular and vascular stage (Talboys, 1964). The vascular phase seems to be a crucial stage to inhibit systemic fungal growth. Factors influencing resistance in this stage are most likely quantitative rather than qualitative resistance factors and a complete resistance is considered to be unlikely (Eynck et al., 2007; Beckman, 1987). Potential resistance to *Verticillium* is supposed to predominantly rely on physical restriction and chemical inhibition during the vascular stage due to its nature as wilt pathogen (Nicholson and Hammerschmidt, 1992). The phenylpropanoid pathway is likely to play an essential role in defence as increased content of phenolic compounds was measured in resistant genotypes of *Brassica napus* (Eynck et al., 2009a). Those substances serve numerous essential biological functions during plant defences, such as antimicrobial activity and cell wall reinforcement (Tunçel and Nergiz, 1993). In early stages of the colonisation soluble and cell wall-bound phenolics impede the infection by the fungus whereas in later stages de novo formation of lignin and lignin-like polymers suppress the fungal pathogen (Eynck et al., 2009a).

Comprehensive studies in the model plant *Arabidopsis thaliana* have been conducted to characterise this defence response in *Brassicaceae*. Metabolic changes during *Verticillium longisporum* infection were analysed to clarify the potential role of the phenylpropanoid pathway in plant defence against this pathogen (König et al., 2014). The disease caused the accumulation of soluble phenylpropanoids such as sinapoyl glucosides, coniferin, syringin and lignans. Several mutants in the phenylpropanoid pathway were tested regarding their resistance towards a *Verticillium longisporum* infection. The *fah1-2* mutants, which lack the expression of sinapates, showed enhanced susceptibility compared to wild type plants suggesting that sinapoyl esters may play a role in the plant defence. Coniferin overexpressing line *UGT72E2-OE* showed fewer symptoms upon infection confirming its role in plant defence which was then further confirmed in *in vitro* experiments as well as the role of sinpoyl esters (König et al., 2014). It is suggested that secondary metabolites can interfere with the

colonisation of the host through *Verticillium longisporum* (Witzel et al., 2015). Infected tolerant *Arabidopsis thaliana* genotypes showed decreased levels of glucosinolates in leaves, but elevated levels in roots and simultaneously the number of breakdown products decreased. This suggests that tolerant genotype might be more effective in accumulating glucosinolates at infection sites and breakdown products of glucosinolates may interact with fungal targets such as glutathione or tubulin, inhibiting colonisation of the host (Witzel et al., 2015).

The influence and interaction of *V. longisporum* on the host hormone signalling pathways during infection and plant defence mechanisms are not fully understood. However, some approaches have been made to clarify the role of defence-related hormone response of fungal pathogens and *V. longisporum*. The purpose of Abscisic acid (ABA) in fungal infections is not fully explained. In *A. thaliana*, ABA levels increase after infection with *V. longisporum* in leaves and roots (Roos et al., 2014). Infection experiments with *A. thaliana* ABA knockout mutant *nced3* showed an impeded colonisation leading to the suggestion that ABA is required for a successful infection. Infected roots of *B. napus* showed a decreased level of ABA which stands in contrast to the observations in *A. thaliana* leading to the conclusion that in this host a down-regulation of ABA is required for successful infection (Behrens, 2018). Other studies showed that ABA concentrations in the xylem sap are not affected by *V. longisporum* infection which could indicate that downregulation of ABA is only required during the prevascular phase for successful infection (Ratzinger et al., 2009).

Ethylene (ET) is a senescence-related hormone which counteracts to cytokinins and plays a role in the different stages of the infection. The ethylene production, as well as ET-dependent plant defences, are induced by *V. longisporum* in *A. thaliana* (Depotter et al., 2016). Early senescence of the host can provide access to nutrients for the fungal development and is triggered in *A. thaliana* through actively decreased cytokinin levels by *V. longisporum* (Reusche et al., 2013). Analysis of ET-insensitive *A. thaliana* mutants that are impaired in known pathogen response pathways but not in other ET-deficient mutants showed enhanced susceptibility against *V. longisporum*. This indicates the regulation of resistance against *V. longisporum* through an unknown ET-associated pathway (Johansson et al., 2006).

Jasmonic acid (JA) and its marker genes *VSP2* and *PDF1.2* are upregulated in *V. longisporum* infected *A. thaliana* plants. But JA is not likely to play a role or contribute to resistance in *A. thaliana* against *V. longisporum* since mutants with impaired biosynthesis or signalling

pathways have the same resistance/susceptibility as WT plants (Johansson et al., 2006b; Ralhan et al., 2012). A loss of function in the hormone receptor CORONATINE INSENSITIVE1 (COI1) conferred an improved resistance to the respective *A. thaliana* mutant plants. However, the JA-independent *COI1* receptor is needed to fully colonise the host plant for successful infection (Ralhan et al., 2012).

Salicylic acid (SA) or more precise its metabolites salicylic acid glucoside (SAG) are increased after infection with *V. longisporum* in *A. thaliana* and the SA-related marker genes *PR1* and *PR2* are up-regulated. *A. thaliana* SA pathway knockout lines (*eds1-1*, *NahG*, *npr1-3*, *pad4-1* and *sid2-1*) did not show a higher susceptibility to *V. longisporum* excluding SA from an active role in resistance in this system (Johansson et al., 2006; Ralhan et al., 2012). In *B. napus* SA is likely to be involved in susceptibility to *V. longisporum* relying on the correlation of concentrations of SA, SAG and disease severity as well as fungal DNA content in plant tissue (Ratzinger et al., 2009).

On transcriptional level, miRNAs are most likely to play a crucial role in the plant-pathogen interaction of *V. longisporum* and *B. napus* and 62 OSR miRNAs were responsive to an infection either *via* modulation by the fungus or as plant response to infection (Shen et al., 2014). Fourteen genes were identified that were targeted by different miRNAs which are involved in plant immunity. Two representatives of this set of miRNAs are miR168 and miR1885. miR168 targets *AGO1* transcripts which is a key component of PTGS and is downregulated during infection. Further studies showed and proved a functional involvement of *AGO1* in the *V. longisporum* and *A. thaliana* interaction (Shen et al., 2014). Several independent *AGO1* knockout mutants showed fewer symptoms upon *V. longisporum* infection whereas enhanced expression of *AGO1* *via* suppression of miRNA168 led to increased susceptibility. This leads to the conclusion that *AGO1* is hijacked by *V. longisporum* in early stages of the infection to establish a successful infection. miR1885 targets TNL immune receptor transcripts by binding to the TIR domain and was first reported in *B. rapa* to be induced by *TuMV* infection (He et al., 2008). Its role during the infection process remains unclear (Shen et al., 2014).

Disease management

The disease management resources to control *Verticillium* stem striping of *Brassica napus* are very limited to prevent and contain the fungal infection efficiently. Strategies aiming at reducing the potential infection rate by minimising the spore density in the soil are often too labour intensive or detrimental to non-target organisms under field conditions (Depotter et al., 2016). Crop rotation can be used as a possible way to decrease the microsclerotia density in the soil relying on the small host range of *Verticillium longisporum*. The beneficial effects of this strategy are very fragile because certain plants could act as reservoir plants allowing a continuous propagation of the fungus (Johansson et al., 2006). For *Brassica napus*, no long-term crop rotation studies have been conducted to investigate the potential decrease of *Verticillium longisporum* microsclerotia density and effectiveness of this strategy (Depotter et al., 2016). Fungicide application to control this disease is not very practical due to the colonisation of the host through the vascular system. So-called biocontrol agents (BCAs) consisting of beneficial microorganisms that counteract to the pathogen could be a substitute for the lacking fungicide applicability. For example, the endophytic microorganism *Vt305* isolated from cauliflower showed BCA properties under controlled conditions and was able to impede *Verticillium longisporum* infection in cauliflower (Tyvaert et al., 2014). However, the mechanisms leading to a decrease in disease severity remain unclear and its potential under field conditions has to be further evaluated.

Strategies relying on resistance breeding and resistant genotypes are the most favoured defence measures against *Verticillium longisporum*. There are two ways to describe a plant resistance on a genetic level. The first one is qualitative resistance or monogenic resistance that relies on single dominant genes referring to the gene-for-gene hypothesis (Flor, 1971). The interaction between the plant resistance locus and the corresponding pathogen virulence locus is either incompatible (resistant host) or compatible with a susceptible host (Kushalappa et al., 2016). The second one is the quantitative resistance or polygenic resistance that relies on multiple genes. Polygenic resistances are in most cases not as effective as monogenic resistances which confer an absolute resistance to the pathogen but are not as easily overcome by the pathogen due to the lesser selection pressure towards a virulent genotype of the pathogen. The lack of genetic resources in the relatively young species *Brassica napus* and the small genetic basis of current cultivars that dominantly originated from the double zero breeding program (Seyis et al., 2003) increase the challenges of resistance breeding. However, there are a few reports about the identification

of potential resistance genes against *Verticillium longisporum* such as *Ve1* in *Solanum lycopersicum* (Kawchuck et al., 2001; Fradin et al., 2009) and *EWR1* in *Arabidopsis thaliana* (Yadeta et al., 2014) both decreasing disease symptoms. Homologous genes in *Brassica napus* could have similar effects on the fungal infection process, but no studies have been conducted to validate this assumption. In addition to screening for potential resistance genes in model plants, studies to evaluate the plant performance of different infected genotypes of *Brassica napus* and its parental species *B. rapa* and *B. oleracea* have been conducted. Parameters such as fungal spread in the vascular system to the upper plant parts and the area under disease progress were evaluated for disease severity and thus a few genotypes of all three species were identified with partial resistance (Eynck et al., 2009a). This resistance was quantified by increased plant growth, less fungal biomass in upper plant parts and an increased amount of total phenolic compounds in roots and hypocotyls (Eynck et al., 2009a; Eynck et al., 2009b; Knüfer et al., 2017). So far there are only two studies that reported and could directly identify genes which are correlating with increased resistance against *V. longisporum* in *B. napus*. Five quantitative trait loci (QTL) could be identified through a QTL analysis of susceptible and resistant genotypes that significantly correlate with less susceptible genotypes (Rygulla et al., 2008; Obermeier et al., 2013). Three of the loci were located on the C genome of the partially resistant cultivar 'Express 617' representing a potential genetic resource for OSR resistance breeding (Obermeier et al., 2013). The limited of genetic resources for resistance breeding in *Brassica napus* against *Verticillium longisporum* require additional and novel approaches for resistance breeding. One way of generating novel resistant genotypes with higher resistance is the knockout of susceptibility factors which are needed by the pathogen to successfully colonise the host causing recessive resistance.

Recessive resistance

Recessive resistance relies on the loss-of-function of genes which are involved in plant-pathogen interactions. It is divided into negative defence regulators and susceptibility factors. Most studies focused on R genes as a source of resistance against pathogens, but the fast evolution of new pathotypes rapidly overcomes this kind of resistance. In contrast, the knockout of specific genes involved in plant-pathogen interactions provides a more durable source of recessive resistance. *PMR6* was a gene discovered promoting the growth of powdery mildew (Eckardt, 2002) and started research approaches for recessive resistances (Almeida

Engler et al., 2005). One of the first well-characterised examples for negative defence regulators are mutations in the *Mildew Resistance Locus O (MLO)* in barley (Büsches et al., 1997). The transmembrane *MLO* proteins negatively regulate disease resistance linked to the *PEN* genes and prevent in its non-functional state an efficient resistance to powdery mildew (Hardham et al., 2013). Pathogen effectors target plant factors which are not directly related to the immune system but are required for successful colonisation of the host and are termed susceptibility factors. An example of such a susceptibility factor is the *glossy11* mutant of *Zea mays*. In knockout genotypes, very long-chain aldehydes are depleted from surface waxes of leaves acting as insufficient substrates for the germination of spores from the barley powdery mildew (*Blumeria graminis f. sp. hordei*) preventing infection in very early stages of the pathogen's lifecycle (Hansjakob et al., 2011). A recent example of susceptibility factors are the *SWEET* genes encoding putative sugar efflux transporters. *SWEET* genes have a broad spectrum of biological functions that include plant nectar production, seed and pollen development. Bacterial and fungal pathogens induce the expression of certain *SWEET* genes upon infection to exploit the plant and increase the nutritional gain (Chen et al., 2010) and preventing the induction by pathogens deploying genome editing successfully rendered rice more resistant to *Xanthomonas* spp. (Oliva et al., 2019).

Genome-editing for resistance breeding

a) Classical non-transgenic approaches

The improvement of crops has ever since been the goal of breeding programs resulting in higher yields and/or tolerance against biotic as well as abiotic stresses. However, traditional methods like genetic recombination or undirected mutagenesis are very time and labour intensive. This restricts the improvement of important traits in crops to the degree that does not meet the demands of modern agriculture and farmers. Changing climates and more extreme weather conditions are challenges which require a faster adaptation of crops in addition to customer demands. There are several approaches to speed up the breeding process towards improved crop varieties and a non-transgenic one is marker-assisted selection (MAS) (Collard and Mackill, 2008). The principle behind this method is to increase the selection efficiency using the linkage disequilibrium (LD) between molecular markers and quantitative trait loci (QTL). After markers are validated, they represent the desired traits and no phenotypical selection in the breeding process is necessary anymore. Although the marker

technology has advanced polygenic and quantitative-based resistances, these are not entirely suitable for MAS. This method also depends on existing genetic resistance resources.

Undirected mutagenesis has been a valuable tool to create new genetic variation through mutation of genes that serve as susceptibility/compatibility factors. Targeting Induced Local Lesions in Genomes (TILLING) is another non-transgenic approach to detect gene-specific point mutations in ethyl-methane sulfonate (EMS) mutant populations (McCallum et al., 2000). Undirected mutagenesis makes it very difficult and labour intensive to obtain a complete knockout of potential susceptibility factor genes in complex crop genomes, but led for example to the KO of all six wheat MLO alleles thereby increasing resistance to powdery mildew (Acevedo-Garcia et al., 2017). An efficient generation of mutants relies on high throughput screening methods to analyse large mutant populations for the desired mutated locus or loci. For optimal results, it is recommended that only homozygous plants are used for screening because polymorphisms could be interpreted as potential point mutations. The sequence of the target gene is then amplified with sequence-specific Primer from pooled DNA samples and screened using denaturing HPLC (DHPLC) or digestion with CEL I endonuclease from celery (Oleykowski et al., 1998). Both screening methods detect heteroduplexes created by melting and annealing of heteroallelic DNA. The advantage of a CEL I digestion is the possibility to detect the location of the mismatch through denaturing gel electrophoresis, whereas DHPLC requires sequencing. Nonetheless, two major disadvantages are caused by the undirected nature of mutations. Mutations occur in the whole genome and are located in many gene loci leading to unpredictable effects on the physiology and habitus with the possibility to cover potential beneficial effects by the knockout of the target genes. To obtain a “clean” genetic background without off-target mutations, expensive and time-consuming backcrossing is needed. Prerequisite for an increased resistance through a knockout is the mutation of all alleles of the target gene. The undirected nature of mutations makes it difficult to achieve this and to prevent functional redundancy from intact alleles.

b) Genome editing by CRISPR/Cas

Genome editing overcomes the disadvantages or restrictions of natural recombination and undirected mutagenesis, allowing to edit the genome in ways that were not possible before. Technologies such as zinc finger nuclease (ZFN; Carroll, 2011) or transcription activator-like effector nuclease (TALEN; Mahfouz et al., 2011) were the first generation of genome editing tools. A general drawback regarding this first and second generation is high cost and complexity of the protein engineering required for their development and individual use.

Clustered regulatory interspaced palindromic repeats/CRISPR associated protein (CRISPR/Cas) is the latest tool for genome editing and is simple to use, versatile and cost-effective (Doudna and Charpentier, 2014). CRISPR/Cas was derived from the bacterial immune system (Barrangou et al., 2007) and adapted as a molecular biology tool with the addition of artificially generated single guide RNAs (sgRNAs) to target specific regions in the genome. Genomic DNA targets require a protospacer adjacent motif (PAM) and are specified through a 19-22-nucleotide sequence which serves in its complementary form as a guide sequence (Jinek et al., 2013) (Fig.2). The PAM of wild type Cas9 derived from *Streptococcus pyogenes* (SpCas9) is 5'-NGG-3'. The Cas9 protein associates with the sgRNA in the nucleus and is then able to recognise a target sequence complementary to the sgRNA sequence. After successful attachment by Watson-Crick base pairing the enzyme changes into its active conformation cutting both DNA strands three nucleotides upstream of the PAM, resulting in degradation of the DNA at the target locus. In eukaryotes, the occurrence of a double-strand break (DSB) induces host-mediated cellular repair pathways. DSB can either be repaired *via* the non-homologous end joining (NHEJ) pathway or by the homology-directed repair (HDR) pathway (Symington and Gautier, 2011). NHEJ is an error-prone endogenous repair mechanism that frequently leads to insertions or deletions (InDels) in the DNA which causes frame-shifts or disruption of the target gene. HDR is an error-free endogenous repair mechanism which depends on the availability of repair templates and leads to recombination of the damaged locus with a template resulting in an error-free repair. Since the successful application of SpCas9 several modifications and different types of nucleases were added to the genome editing toolkit (Ahmad et al., 2018). One major reason was the restriction of targets to the occurrence of a certain PAM adjacent to them. A way to gain more flexibility in the selection for target was the adaption of different Cas nucleases from different bacteria which have varying PAMs and properties (Esvelt et al., 2013). Examples for SpCas9 orthologs are from *Staphylococcus aureus* (SaCas9) or *Staphylococcus thermophilus* (StCas9) which require the PAMs 5'-NNNNGATT-3' and 5'-NNGGAA-3'.

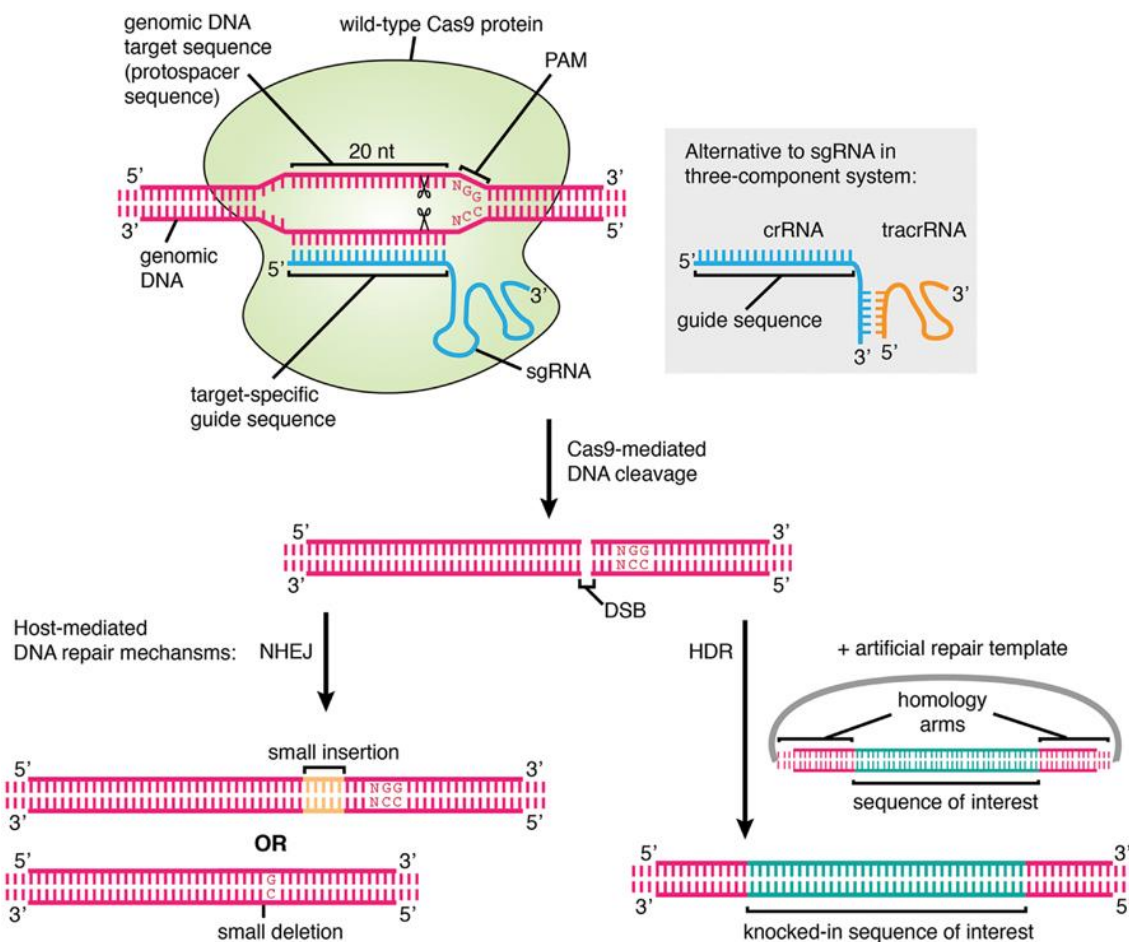


Figure 2: The mechanism of genome editing using CRISPR/Cas9 (Agrotis and Kettler, 2015).

In addition to these Cas9 orthologues other endonucleases like Cpf1, discovered in *Prevotella* and *Francisella* bacteria, were adapted for molecular biological use (Zetsche et al., 2015). In contrast to Cas9, this endonuclease has a thymidine-rich PAM site 5'-TTTN-3' which broadens the number of potential targets even further and does not generate blunt-ended DSBs but 4-5 nucleotide long sticky ends which is supposed to enhance the efficiency of HDR (Zetsche et al., 2015). Another approach to increase the targeting capacity was to engineer the SpCas9 to recognise several PAM sequences (Kleinstiver et al., 2015; Hu et al., 2018). The phage-assisted continuous evolution of SpCas9 generated an evolved PAM SpCas9 variant (xCas9) recognising a wide range of PAMs including NG, GAA, and GAT (Hu et al., 2018). Genome editing approaches with CRISPR/Cas relying on NHEJ has been successfully applied to disrupt susceptibility factors for improving resistance against pathogens in several crop species as well improving traits such as post-harvest life or parthenocarpy (Tab.1). For example, the CRISPR/Cas introduced disruption of the *MLO* allele in *Solanum lycopersicum* to confer increased resistance to powdery mildew (Nekrasov et al., 2017).

HDR-based genome editing

Recent approaches to incorporate CRISPR/Cas9 into resistance breeding mainly rely on the disruption of susceptibility genes to increase the host resistance (Tab.1). However, these S genes are often involved in multiple pathways and their loss of function could lead to potential alteration of plant physiology. Pathogens alter their hosts' gene regulation with miRNAs to achieve a successful infection and colonisation (Fei et al., 2016). An HDR based approach can be used to mutate the miRNA binding sites that are targeted by the pathogen taking advantage of codon degeneracy which allows that the change does not affect the amino acid sequence leaving the target gene function intact. This loss-of-recognition between miRNA and target should lead to an interruption of cross-kingdom RNAi, thus representing a novel strategy to improve plant resistance *via* genome editing avoiding potential negative side effects. However, HDR in plants remains a challenge because NHEJ is the dominant pathway that repairs a DSB (Puchta, 2005). Another problem is the low transformation and regeneration efficiency of many crops, combined with low rates for successful HDR events (Altpeter et al., 2016) demanding for large scale experiments. One approach to enable HDR is to stably transform the CRISPR/Cas9 construct and the repair template simultaneously into the plant (Fauser et al., 2012). While active the endonuclease not only cuts the target locus but also excises repair templates that were integrated into the genome which are then available for HDR.

Such an approach was successfully applied to *A. thaliana* and a site-specific insertion of a repair template was verified (Schiml et al., 2014). Usually, the bottleneck of HDR based approaches is the temporal and local availability of repair templates. This can be overcome by increasing the number of repair templates. In the past, plant viruses were used as simple but efficient vectors and have served multiple purposes, e.g. the production of proteins (Rybicki, 2009). Autonomously replicating virus-based vectors can overcome the template bottleneck and enable an efficient HDR.

Geminiviruses (family *Geminiviridae*) are a large family of viruses which have a broad host range and can infect multiple plants species including important crops like wheat or maize (Rey et al., 2012). The *Bean yellow dwarf virus (BeYDV)* is a member of this family and infects dicot plants. The genome of this virus (2.5-3.0 kb) contains a short intergenic region (SIR), long intergenic region (LIR) and four genes (Gutierrez, 1999). Those genes code for a movement protein (MP), capsid protein (CP) in sense direction and a replication initiator protein (Rep/RepA) in the antisense direction. The cis-elements that are required for replication of

Table 1: Modification of susceptibility factors and quality traits utilising genome editing tools modified after Scheben et al. 2017

	Editing system	Type of edit	Target gene(s)	Target traits	Results	Reference
Citrus (Orange)	CRISPR/Cas9	Promotor disruption	<i>CSLOB1</i> (promoter)	Citrus canker resistance	Citrus canker disease severity was decreased by 83.2–98.3% compared to the wild-type	Peng et al. (2017)
Cucumber	CRISPR/Cas9	Gene disruption	<i>eIF4E</i>	Broad virus resistance	Immunity to cucumber vein yellowing virus infection and resistance to the potyviruses	Chandrasekaran et al. (2016)
					zucchini yellow mosaic virus and papaya ringspot mosaic virus-W	
Grape	CRISPR/Cas9	Gene disruption	<i>WRKY52</i>	Resistance Gray mould (<i>Botrytis cinerea</i>)	Transcription factor involved in response to biotic stress	Wang et al. (2018)
Rice	CRISPR/Cas9	Gene disruption	<i>ERF922</i>	Resistance to Rice blast disease (<i>Magnaporthe oryzae</i>)	Transcription factor implicated in multiple stress	Wang et al. (2016)
Tomato	CRISPR/Cas9	Gene disruption	<i>SIM101</i>	Resistance to powdery mildew	Rapid generation of a transgene-free tomato fully resistant to powdery mildew	Nekrasov et al. (2017)
Tomato	CRISPR/Cas9	Gene disruption	<i>SIIA9</i>	Parthenocarp (leading to seedless fruit)	Fruit development was triggered before fertilisation, generating seedless tomato fruits	Ueta et al. (2017)
Tomato	CRISPR/Cas9	Gene disruption	<i>RIN</i>	Fruit ripening (shelf life)	Delayed and permanently inhibited ripening, possibly without the side effects of conventional	Ito et al. (2015)
Wheat	TALEN and CRISPR/Cas9	Gene disruption	<i>TaMLO-A1, B1</i> and <i>D1</i>	Resistance to powdery mildew	The number of mildew microcolonies formed on the leaves was significantly reduced against the control and no apparent fungal growth was	Wang et al. (2014)

the viral genome are located in the SIR, serving as an origin for C-strand synthesis and containing transcription termination and polyadenylation signals, and LIRs that contain a bi-directional promoter and a stem-loop structure essential for circularization in the hosts' nucleus. Once inside the nucleus of the host cell DNA polymerases are exploited to convert the single-stranded genome to a double-stranded intermediate. This intermediate serves as a template for rolling circle replication that is initiated by the replication initiator protein and transcription of the viral genes. After multiple replication cycles, single-stranded genomes are either converted back to double-stranded genomes or encapsulated (Baltes et al., 2014).

The viral genome of the *BeYDV* was disarmed by removing the movement and coat protein so it could serve as a repair template source (Fig. 3). The removal of those genes was carried out to prevent an encapsulation which would lead to a decrease of templates. It is also kind of a biological safety measurement to inhibit an uncontrolled spread of the modified viral genome (Baltes et al., 2014). The sequence of those proteins can be replaced by the site-specific nuclease (SSN) TALEN or Cas9/sgRNA coding sequences together with the donor template. Embedded into a binary vector, the modified viral genome is delivered *via Agrobacterium tumefaciens* mediated transformation into the host cell where the viral genome begins its rolling circle replication and transcription of the SSN (Fig. 3). Such a disarmed genome was utilised in a study with *Solanum lycopersicum* for the insertion of a 35s promoter upstream of the native gene coding for anthocyanin synthesis to compare HDR frequencies of repair template delivery methods (Cermack et al., 2015). Up to 12-fold higher HDR rates could be achieved using the viral replicons as a template source compared to single repair templates introduced *via* T-DNA into the genome. The *BeYDV* was only applicable in dicot plants referring to its host range and recombinant viral replicons based on the *Wheat dwarf virus (WDV)*. They were generated for HDR in monocot plants and successful HDR events could be produced in wheat, corn and rice (Gil-Humanes et al., 2017; Wang et al., 2017).

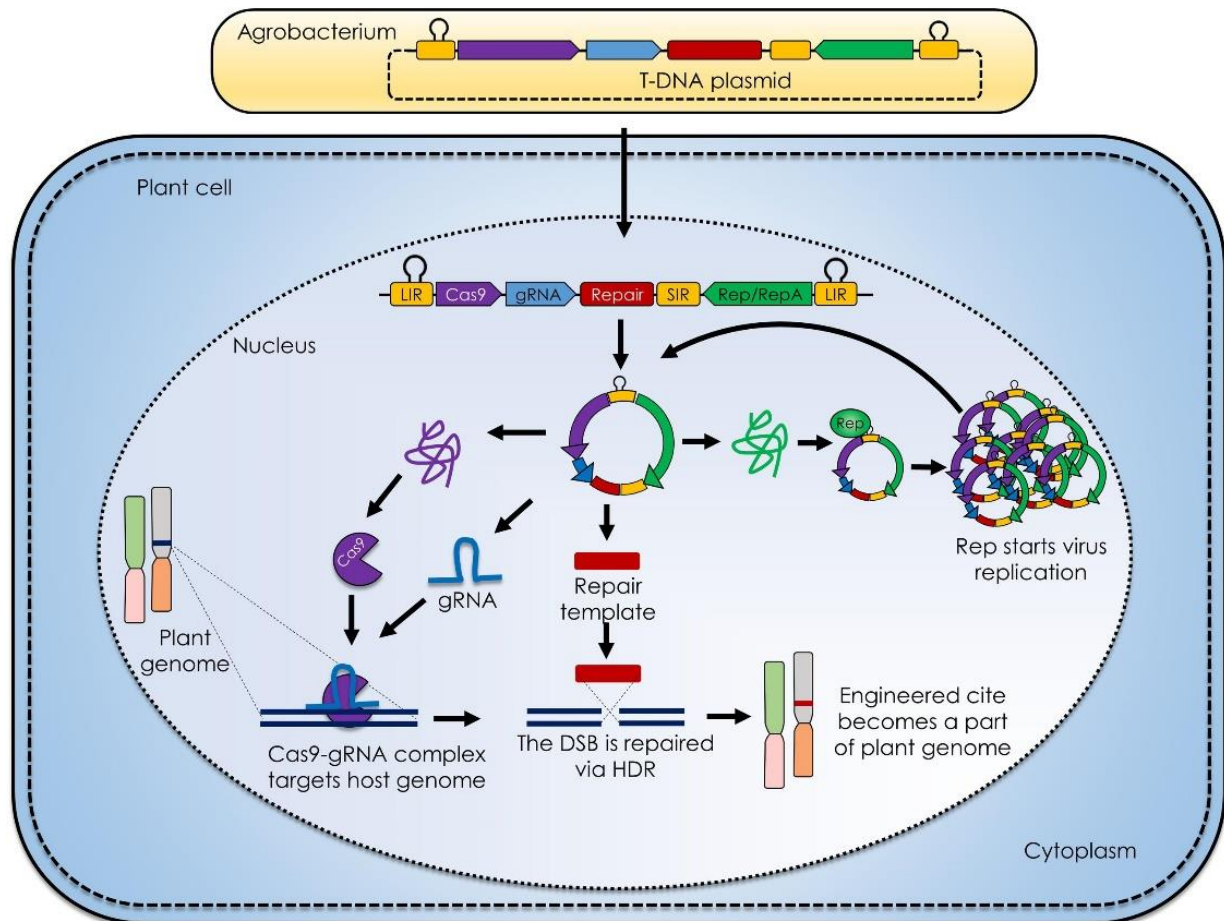


Figure 3: Viral replicons for gene targeting and disarmed viral vector system (Zaidi & Mansoor 2017). The disarmed virus genome without coat and movement protein replaced with Cas9, sgRNA and repair template are cloned in T-DNA. Via *Agrobacterium tumefaciens* mediated transformation the genome is delivered to plants. Inside the plant cell nucleus, the single-stranded viral DNA is converted into the dsDNA replicative form and RNA is transcribed. The Cas9-sgRNA complex binds to the target site where a double-strand break is induced. If homology-directed repair is triggered the donor DNA can serve as a repair template.

Preworks in *Arabidopsis* and *B. napus*

The basis for this current study was a comparative transcriptome analysis of *V. longisporum* infected *B. napus* plants and a non-infected control (Häder, 2013). Three potential candidate genes which showed altered regulation upon infection were tested in the model plant *A. thaliana* by deploying knockout mutants of those genes. The mutants were challenged with *V. longisporum* and compared to the WT. Genes which lead to less susceptibility in their disrupted state were chosen for further studies in *B. napus* (Hossain, 2017). A *Brassica napus* EMS mutant population was screened for mutations in the respective genes and first functional characterisations were conducted (Fig. 4). Mutant genotypes of *B. napus* showed less susceptibility against *V. longisporum* as well, supporting the potential role the candidate genes as susceptibility factors in OSR. However, one drawback of TILLING is that it induces many off-target mutations in the genome, which makes

it challenging to assign the increased resistance to a specific mutation and some plants carrying both a stop codon within the ORF showed different phenotypes. Thus, it is necessary to finally verify the role of the candidate susceptibility genes by applying CRISPR/Cas and thereby generate recessive resistance against *V. longisporum*.

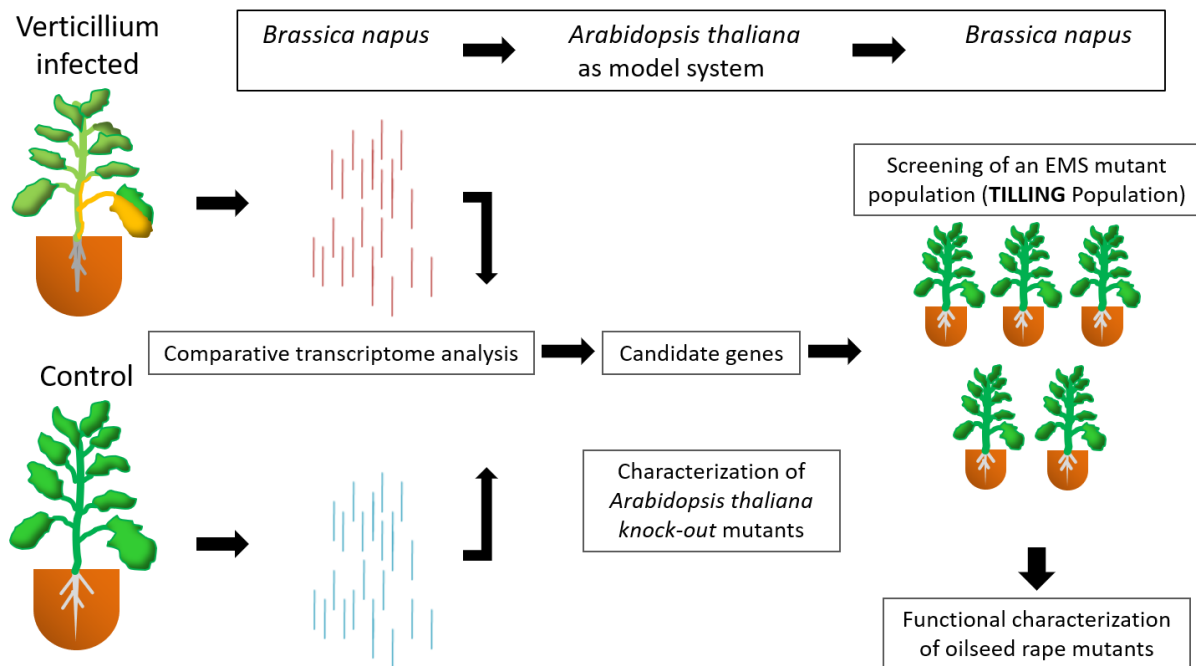


Figure 4: Comparative RNA sequencing approach for identification of susceptibility factors in the *V. longisporum* - *B. napus* interaction and confirmation through KO mutants of the model system *A. thaliana* and *B. napus*.

Besides the KO of potential susceptibility genes, by NHEJ, also an HDR -mediated re-writing of miRNA binding sites in the host genome could result in increased resistance, assuming that pathogens deploy siRNAs to target host resistance factors, a phenomenon recently described as cross-kingdom RNAi (Wang et al., 2016; Cai et al., 2018). Potential miRNA target genes have been identified in transcriptome analysis experiments (Shen et al., 2014) and the generated data can serve now as a starting point to identify candidates for applying HDR in *Brassica napus*.

That CRISPR/Cas generally works also in OSR has meanwhile reported in several publications, though all rely on NHEJ and none describes an HDR approach. The first successful alteration of the *Brassica napus* genome was conducted by targeting two *ALCATRAZ* (*ALC*) homoeologs (Braatz et al., 2017). Those genes are involved in valve margin development and contribute to seed shattering in mature seed pods. Disruption of gene functionality increased the shatter resistance to minimise seed loss during mechanical harvesting.

A more recent study also aimed at the modulation of shatter resistance as well, including the *INDEHISCENT (IND)* genes in addition to the *ALC* genes. Successful disruption of gene functionality for both genes could be achieved in several independent transgenic events and results indicated that *IND* genes play a more critical role in modulation of this trait than the *ALC* gene (Zhai et al., 2019). Other recent approaches in genome editing of *B. napus* are mostly striving to improve important quality traits such as plant architecture for increased yield or oil composition to increase the economic value of this crop (Zhang et al., 2019; Zheng et al., 2019). Approaches directly aiming at an increased disease resistance are not in the main focus, but first attempts have been conducted to implement CRISPR/Cas into resistance breeding strategies for *B. napus* (Sun et al., 2018).

Aims of the thesis

V. longisporum is one of the most important fungal pathogens of *B. napus* and poses an increasing threat to oilseed-rape production, worldwide. Due to the lack of genetic resources, and the limitations concerning the application of fungicides, resistance breeding of oilseed-rape remains a promising strategy for disease management. Recently, we identified two susceptibility genes (*CRT1a* and *HVA22c*) and several microRNAs in the oilseed rape genome which are involved in the oilseed rape-*Verticillium* interactions (Häder, 2013; Shen et al., 2014; Hossain, 2017). The current study aims to generate recessive resistance against the pathogenic fungus *Verticillium longisporum* in oilseed-rape (*Brassica napus*) utilising the genome editing tool CRISPR/Cas9 to knock-out the putative susceptibility factors *CRT1a* and *HVA22c* by NHEJ. This is a common strategy for resistance breeding and should help to increase the resistance of oilseed rape against *Verticillium*. Additionally, an HDR based editing system should be developed for *B. napus*, which allows for an arbitrary modification of a miRNA target sequence without loss of the gene function. This approach will provide us with a unique tool to analyse miRNA functions and to interrupt the plant-pathogen interaction, e.g. by interference with the miRNA-mRNA recognition, consequently leading to an incompatible interaction between the plant and the fungus. The working hypothesis of this study is that we can utilise the genome editing tool CRISPR/Cas to knock out the susceptibility genes *CRT1a* and *HVA22c* in *B. napus* for verification of previous observations conferring less susceptibility to *V. longisporum* infection in *A. thaliana* and OSR. Furthermore, we want to demonstrate that also HDR is feasible in *Brassica napus*, so we developed an efficient and specific expression cassette vector, which can be easily modified according to the demands of the

particular editing experiment. The exchange and modification of the nuclease (Cpf1), as well as the use of single/multiple sgRNAs, is possible with this vector. The generated *B. napus* knockout mutants will be functionally characterized in order to verify the role of our candidate genes without disturbing background off-target mutations, which appeared in the TILLING approach. Finally, the resulting OSR mutants should show less susceptibility to *V. longisporum*. This material will enable us to elucidate this plant-pathogen interaction and also contribute to commercial resistance breeding.

Material

Equipment

Table 2: Equipment used in this thesis.

Device	Model	Company
Autoclave	VX-75	Systec GmbH
Camera	D3000	Nikon GmbH
Centrifuge	mini G	IKA®-Werke GmbH & CO. KG
Centrifuge	5417 R	Eppendorf AG
Centrifuge	Heraeus Multifuge X3R	Thermo Fisher Scientific
Climate chamber	VB0714	Vötsch Industrietechnik GmbH
Drying cabinet	VENTI-Line®	VWR International GmbH
Electroporation Systems	Gene Pulser Xcell™	Bio-Rad Laboratories GmbH
Freezer -80°C	HERAfreeze Basic	Thermo Fisher Scientific
Gel documentation	Gel Doc™ XR+	Bio-Rad Laboratories GmbH
Gel electrophoresis, chamber	Wide Mini-Sub® Cell GT	Bio-Rad Laboratories GmbH
Gel electrophoresis, chamber, vertical	Mini-PROTEAN® Tetra Cell	Bio-Rad Laboratories GmbH
Gel electrophoresis, power supply	PowerPac™ Basic	Bio-Rad Laboratories GmbH
Incubator	Excellent UFE 400-800	Memmert GmbH & Co. KG
Incubator, shaking	CERTOMAT® IS	Sartorius Stedim Biotech GmbH
Magnetic stirrer	Combimag REO	IKA® Works, Inc.
Microscope	Stereo Discovery.V20	Carl Zeiss Microscopy GmbH
Microscope	TCS SP1	Leica Biosystems Nussloch GmbH
Microwave	hNN-E235M	Panasonic
Orbital shaker	GFL 3005	GFL Gesellschaft für Labortechnik mbH
PCR thermocycler	PCR Biometra TOne 96	Analytik Jena AG
pH meter	inoLab pH 720	WTW GmbH
Pipettes	2,5 µl, 20 µl, 200 µl, 1000 µl, 5 ml Research/ Research plus	Eppendorf AG
Realtime PCR System	CFX96 Touch™ Real-Time PCR Detection System	Bio-Rad Laboratories GmbH
Scale	ABJ	KERN & SOHN GmbH
Shaker	Vibramax 100	Heidolph Instruments GmbH & Co.KG
Shaker, overhead	Reax 2	Heidolph Instruments GmbH & Co.KG

Spectrophotometer	NanoVue Plus	GE Healthcare Life Science
Spectrophotometer	UNIKOV 942	Kontron Instruments
Vortex	MS 2	IKA®-Werke GmbH & CO. KG
Water bath	Immersion Circulators Model 1112A	VWR International GmbH
Water bath, shaking	1083	GFL Gesellschaft für Labortechnik mbH
Wet blot, transfer cell	Mini Trans-Blot® Cell	Bio-Rad Laboratories GmbH
Workbench, sterile	HERAsafe™ KS 12	Thermo Fisher Scientific

Chemicals

Table 3: Chemicals used in this thesis.

Chemical	Article number	Company
Acrylamide	1610144	Bio-Rad Laboratories GmbH
Acetic acid	3738.2	Carl Roth GmbH & Co. KG
Agarose	840004	Biozym Scientific GmbH
Agar-Agar	5210.4	Carl Roth GmbH & Co. KG
Ammonium acetate	7869.2	Carl Roth GmbH & Co. KG
Ampicillin	K029.4	Carl Roth GmbH & Co. KG
APS	9592.2	Carl Roth GmbH & Co. KG
Bacto-Agar	214010	OTTO NORDWALD GmbH
β-Mercaptoethanol	4227.1	Carl Roth GmbH & Co. KG
Cefotaxime	C0111.0001	Duchefa Biochemie B.V
Chloroform	3313.2	Carl Roth GmbH & Co. KG
CTAB	9161.2	Carl Roth GmbH & Co. KG
Czapek Dox Broth	C1714.1000	Duchefa Biochemie B.V
D(+)-Sucrose	4321.2	Carl Roth GmbH & Co. KG
Daishin Agar	D1004.1000	Duchefa Biochemie B.V
DEPC	K028.1	Carl Roth GmbH & Co. KG
DMSO	4720.2	Carl Roth GmbH & Co. KG
DNA Gel Loading Dye (6X)	R0611	Thermo Fisher Scientific
DNA oligonucleotides		Eurofins Genomics
dNTP Mix	R0181	Thermo Fisher Scientific
EDTA	8043.2	Carl Roth GmbH & Co. KG
Ethanol	1.00983.2511	Diagonal GmbH & Co. KG
Ethidiumbromide	2218,2	Carl Roth GmbH & Co. KG
GeneRuler 1kb Ladder	SM0311	Thermo Fisher Scientific
GeneRuler 100bp Ladder	SM0242	Thermo Fisher Scientific
Gentamycin	M3121.0001	Genaxxon bioscience GmbH
Glycerol	4043.1	Carl Roth GmbH & Co. KG
HCl hydrochloric acid	P074.2	Carl Roth GmbH & Co. KG
HPLC water	A511.3	Carl Roth GmbH & Co. KG
IPTG	2316.3	Carl Roth GmbH & Co. KG
Isoamylalcohol	8930.1	Carl Roth GmbH & Co. KG
Isopropanol	20842.330DB	VWR International GmbH
Kanamycin	T832.3	Carl Roth GmbH & Co. KG

Lambda DNA	SD0011	Thermo Fisher Scientific
Methanol	4627.5	Carl Roth GmbH & Co. KG
MgCl ₂ magnesium chloride	kk36.2	Carl Roth GmbH & Co. KG
MgSO ₄ magnesium sulfate	P027.3	Carl Roth GmbH & Co. KG
MS basal salts incl. MES and Vitamins	M0255.0050	Duchefa Biochemie B.V
Potato-dextrose broth	CP74.2	Carl Roth GmbH & Co. KG
Phenol	0038.3	Carl Roth GmbH & Co. KG
Potassium acetate	T874.1	Carl Roth GmbH & Co. KG
KOH potassium hydroxide	6751.3	Carl Roth GmbH & Co. KG
Na ₂ HPO ₄ disodium phosphate	X987.2	Carl Roth GmbH & Co. KG
NaCl sodium chloride	3957.2	Carl Roth GmbH & Co. KG
NaOH sodium hydroxide	6771.2	Carl Roth GmbH & Co. KG
Rifampicin	R0146.0005	Duchefa Biochemie B.V
Rotiphorese®-PAGE-Matrixpuffer plus	8996.1	Carl Roth GmbH & Co. KG
Sodium acetate	6773.1	Carl Roth GmbH & Co. KG
Sodium hypochloride	9062.4	Carl Roth GmbH & Co. KG
SDS	4360.2	Carl Roth GmbH & Co. KG
TEMED	2367.3	Carl Roth GmbH & Co. KG
TRIS ultra pure	5429.3	Carl Roth GmbH & Co. KG
TRIS/HCL	9090.3	Carl Roth GmbH & Co. KG
TritonX-100	3051.3	Carl Roth GmbH & Co. KG
Tryptone/Peptone	8952.3	Carl Roth GmbH & Co. KG
Tween20	9127.1	Carl Roth GmbH & Co. KG
VE water	3175.2	Carl Roth GmbH & Co. KG
X-gal	2315.4	Carl Roth GmbH & Co. KG
Yeast extract	A1552.1000	Diagonal GmbH & Co. KG

Enzymes and Kits

Table 4: Enzymes and kits used in this thesis.

Enzyme / Kit	Article number	Company
Alkaline Phosphatase	EF0651	Thermo Fisher Scientific
<i>Apal</i>	ER1411	Thermo Fisher Scientific
<i>BamHI</i>	ER0051	Thermo Fisher Scientific
<i>BclI</i>	ER0722	Thermo Fisher Scientific
<i>Bsp119I</i> , Fast Digest	FD0124	Thermo Fisher Scientific
Cas9 Nuclease, <i>S. pyogenes</i>	M0386S	New England Biolabs GmbH
<i>CsiI</i> , Fast Digest	FD2114	Thermo Fisher Scientific
DNaseI I	EN0521	Thermo Fisher Scientific
Dream Taq Polymerase	EP0704	Thermo Fisher Scientific
<i>Eco72I</i>	ER0361	Thermo Fisher Scientific

<i>EcoRI</i>	ER0271	Thermo Fisher Scientific
<i>EcoRV</i>	ER0301	Thermo Fisher Scientific
Gateway™ BP Clonase™ II Enzyme mix	11789100	Invitrogen AG
Gateway™ LR Clonase™ II Enzyme mix	11791100	Invitrogen AG
GeneJET Plant RNA Purification Kit	K0801	Thermo Fisher Scientific
Guide-it Cas9 polyclonal antibody	632607	Clontech Laboratories
<i>KpnI</i>	ER0522	Thermo Fisher Scientific
Roche Lumi-LightPLUS Western Blotting Kit (Mouse/Rabbit)	12015218001	Sigma-Aldrich
<i>MssI</i>	ER1342	Thermo Fisher Scientific
NEBuilder® HiFi DNA Assembly Cloning Kit	E5520S	New England Biolabs GmbH
<i>NotI</i>	ER0592	Thermo Fischer Scientific
Nucleo Spin Gel and PCR Clean-Up	740609250	Machery und Nagel
NucleoSpin® Plasmid	740588.250	Machery und Nagel
<i>PacI</i>	ER2201	Thermo Fischer Scientific
<i>Pdml</i> , Fast Digest	FD1534	Thermo Fischer Scientific
Pfu Polymerase	EP0571	Thermo Fischer Scientific
Phusion High-Fidelity DNA Polymerase	M0530S	New England Biolabs GmbH
Promega pGEM T-Vector	A3600	Promega GmbH
<i>PstI</i>	ER0611	Thermo Fisher Scientific
qPCRBIO SyGreen Mix Separate-ROX	PB20.14-05	PCR Biosystems Ltd
RevertAid First Strand cDNA Synthesis Kit	K1622	Thermo Fisher Scientific
RNase A	R500-250MG	Sigma-Aldrich
<i>Sall</i>	ER0641	Thermo Fisher Scientific
<i>SfaAI</i>	ER2091	Thermo Fisher Scientific
T4 DNA Ligase	M02025	New England Biolabs GmbH
T7 Endonuclease I	M0302	New England Biolabs GmbH
<i>XhoI</i>	ER0695	Thermo Fisher Scientific

Laboratory consumables

Table 5: Laboratory consumables used in this thesis.

Laboratory consumable	Article number	Company
1/2 Micro Cuvettes	1201	Ratiolab GmbH
Electroporation Cuvettes (0.2 cm)	1652086	Bio-Rad Laboratories GmbH
Erlenmeyer flask (100/500 ml)	391-0260/391-0280	VWR International GmbH
Fuchs-Rosenthal chamber Gaze Filter (mesh 200 µm)	T731.1	Carl Roth GmbH & Co. KG HYDRO-BIOS Apparatebau GmbH
Glas bottles (100/250/500/1000 ml)	X712.1/X713.1/X714.1/X715.1	Carl Roth GmbH & Co. KG
Hard-Shell® 96-Well PCR Plates	HSP-9601	Bio-Rad Laboratories GmbH
Microseal® 'B' PCR Plate Sealing Film	MSB-1001	Bio-Rad Laboratories GmbH
Parafilm	9170002	Erich Eydam KG
Pasteur pipette	4522	Carl Roth GmbH & Co. KG
PCR tube (200 µl)	4ti-0784	4titude® Ltd
Petri dish (Ø120x120x16 mm)	09-231-0000	Nerbe Plus GmbH
Petri dish (Ø92x16 mm)	09-031-0010	Nerbe Plus GmbH
Pipette tips 1000 µl	07-122-0073	Nerbe Plus GmbH
Pipette tips 20 µl	07-361-2005	Nerbe Plus GmbH
Pipette tips 200 µl	07-122-0073	Nerbe Plus GmbH
Pipette tips 5 ml	701183002	Sarstedt AG & Co. KG
Plastic salad dishes	2007147	IGEFA Handelsgesellschaft GmbH & Co. KG
Plastic tubes 0.5 ml	04-332-1051	Nerbe Plus GmbH
Plastic tubes 1.5 ml	72.690.001	Sarstedt AG & Co. KG
Plastic tubes 15 ml	62.554.502	Sarstedt AG & Co. KG
Plastic tubes 2 ml	04-232-1200	Nerbe Plus GmbH
Plastic tubes 5 ml	0030119380	Eppendorf AG
Plastic tubes 50 ml	62.547.004	Sarstedt AG & Co. KG
Sand	0-2 mm	Bauhaus AG
Soil	ED73	Einheitserdewerk Uetersen
Test tubes	9190021	Erich Eydam KG

Solutions, buffers and media

Table 6: Solutions, buffers and media used in this thesis.

Media	Ingredients	Application
LB media	10g/l Tryptone 10g/l NaCl 5g/l Yeast extract 15g/l Bacto Agar pH 7	Cultivation of bacteria
TSS media	10g/l Tryptone 10g/l NaCl 5g/l Yeast extract 10% w/v polyethylene glycol 5% v/v dimethyl sulfoxide 50 mM Mg ² pH 6.5	Cultivation of bacteria
Infiltration media	10mM MES 10mM MgCl ₂ 0.15mM acetosyringone	Transfection of <i>N. benthamiana</i> leaves
Infiltration media	5% w/v sucrose 110 µl/l Silwett	Floral dip transformation <i>A. thaliana</i>
Half MS	MS incl. vitamins and MES 10g/l sucrose 8g/l agarose pH 5.8	Cultivation of plant material <i>in vitro</i>
CTAB isolation buffer	120 mM Tris-HCl 800 mM NaCl 12mM EDTA 2 % v/v CTAB	DNA isolation
Washing buffer 1	76% EtOH 0.2 M Na-acetate	DNA isolation
Washing buffer 2	76% EtOH 0.01 M NH ₄ -acetate	DNA isolation
Protein extraction buffer	25 mM Tris-HCl 5 mM MgCl ₂ 1 mM CaCl ₂ 0.1 % (v/v) Triton x-100 0.05 % (v/v) β-mercaptoethanol 0.5 mM PMSF 5 mM EDTA	Total protein extraction
Sample buffer	9.8 g/l Tris-HCl 20% v/v glycerine 40 g/l SDS 10 ml/l mercaptoethanol 0.1 g/l bromophenol blue	SDS-PAGE
Running buffer	3 g/l Tris ultra-pure 14.4 g/l Glycin 1 g/l SDS	SDS-PAGE

Staining solution	pH 8.6 2.5 g/l Coomassie Brilliant 100 ml/l Glacial acetic acid 900 ml/l MeOH: H ₂ O (1: 1 v/v)	Coomassie staining
Destaining solution	100 ml/l Glacial acetic acid 900 ml/l MeOH: H ₂ O (1: 1 v/v)	Coomassie staining
Transfer buffer	25 mM Tris 192 mM glycine 20% (v/v) MeOH 1% (w/v) SDS	Western blot
TBST	20 mM Tris 150 mM NaCl 0.1% Tween 20 Adjust pH with HCl to pH 7.4– 7.6	Western blot

Software and databases

Table 7: Software and databases used in this thesis.

Software / Database	Source / Company
AxioVision rel. 4.8	Carl Zeiss Microscopy GmbH
Brassica Database	http://brassicadb.org/brad/
Brassica napus Genome Browser	http://www.genoscope.cns.fr/brassicanapus/
CFX Maestro™ for CFX Real-Time PCR	Bio-Rad Laboratories GmbH
Image Lab™ Software	Bio-Rad Laboratories GmbH
LCS Lite (Leica Confocal Software)	Leica Biosystems Nussloch GmbH
MEGA X	https://www.megasoftware.net
Microsoft® Office	Microsoft Corporation
NCBI - BLASTn / Primer-BLAST / CDSEARCH	https://blast.ncbi.nlm.nih.gov/Blast.cgi
R	https://www.r-project.org/
SnapGene Viewer	https://www.snapgene.com
TAIR	http://www.arabidopsis.org/
Unipro UGENE	http://ugene.net/

Organisms

Table 8: Organisms used in this thesis.

Organism	Genotype / Cultivar / Isolate	Source
<i>Escherichia coli</i>	DH5 α	Lab stock
<i>Escherichia coli</i>	K12xB DB3.1	Lab stock
<i>Agrobacterium rhizogenes</i>	AR15834	Lab stock
<i>Agrobacterium tumefaciens</i>	GV3101	Lab stock
<i>Verticillium longisporum</i>	VI43	Dr. E. Diederichsen (FU Berlin, Germany)
<i>Brassica napus</i>	Mozart	NPZ *
<i>Brassica napus</i>	CRISPR <i>CRT1a</i> (cv. Mozart)	SU Biotec **
<i>Brassica napus</i>	CRISPR <i>HVA22c</i> (cv. Mozart)	SU Biotec **
<i>Arabidopsis thaliana</i>	Col-0	Lehle Seeds (Round Rock, USA)

* Norddeutsche Pflanzenzucht Hans-Georg Lembke KG, Holtsee, Germany; ** Saaten-Union Biotec GmbH, Leopoldshöhe, Germany

Oligonucleotides

a) NHEJ cloning

Table 9: Oligonucleotides for NHEJ cloning.

Name	Sequence (from 5' to 3')	Application
M13 fwd	TGTAAAACGACGGCCAGT	Amplification of MCS
M13 rev	CAGGAAACAGCTATGACC	Amplification of MCS
Ubi10 NEB F	gggacagtacctgcaACAGTACCTGCAGGCGACG	Amplification of Ubiquitin promoter for NEBuilder
Ubi10 NEB R	ccatacgcgtgcgatACGCGTGCGATCGCTGTT	Amplification of Ubiquitin promoter for NEBuilder
Ubi10 F	ACTTTGCGTGTAACAACGC	Amplification of Ubiquitin promoter
Ubi10 R	ACGATCAAACCCAGAAAATCGTC	Amplification of Ubiquitin promoter
Cas9 GFP fwd	ggggacaagtttgtacaaaaagcaggctTAATGgataa gaagtactct	Cas9 GFP fusion
Cas9 GFP rev	ggggaccactttgtacaagaagctgggtCcttcttctt cttagcctg	Cas9 GFP fusion
sgRNA F1 Li	AGAAATCTCAAATTCG	sgRNA amplification
sgRNA F1 Schiml	CTTTTTTCTTCTTCTTCGT	sgRNA amplification
sgRNA F2 uni	G + gRNA + GTTTTAGAGCTAGAAATAG	sgRNA amplification
sgRNA R1 uni	gRNA + CAATCACTACTTCG	sgRNA amplification
sgRNA R2 uni	TAATGCCAACTTTGTACAAG	sgRNA amplification

crRNA F1 Li	AGAAATCTCAAAATTCCGGC	crRNA amplification
crRNA F2	gRNA + TTTTTTCTAGACCCAGC	crRNA amplification
crRNA R1	gRNA + ATCTACACTTAGTAGAAATTC	crRNA amplification
crRNA R2	GCAAGCCCCGGGCCCTAATGC	crRNA amplification
sgRNA R1 AtAgo10 Schiml	ccAcggtttctcctccggctacAATCACTACTTCG	sgRNA amplification
sgRNA F2 AtAgo10 Schiml	gtagccggaggagaaaccgTggGTTTTAGAGCTAGAAAT AG	sgRNA amplification
AtAgo10 locus fwd	AGCACCACAATCCAAAAACCG	Target locus amplification
AtAgo10 locus rev	CCTTGGTAGGCAAATCAGCG	Target locus amplification
crRNA F2 StTXR1 Schiml	CCCAGGCATATCGCCAAGCTCTCTTTTTTCTAGACCCA GC	crRNA amplification
crRNA R1 StTXR1 Schiml	GAGAGCTTGGCGATATGCCTGGGATCTACACTTAGTAGA AATTC	crRNA amplification
StTXR1 locus fwd	CTGTGGTAATTGAACCTCAGACC	Target locus amplification
StTXR1 locus rev	CTTCAAGTGCATCAACTAATGGG	Target locus amplification
sgRNA R1 BnCRT1a Schiml	AACTTCATCTCTCTGATTCTCAATCACTACTTCG	sgRNA amplification
sgRNA F2 BnCRT1a Schiml	GAGAATCAGAGAGATGAAGTTGTTTTAGAGCTAGAAATA G	sgRNA amplification
sgRNA R1 BnHVA22c Schiml	TTGGTTGCTCTCGTCTACCAATCACTACTTCG	sgRNA amplification
sgRNA F2 BnHVA22c Schiml	GGTAGACGAGAGCAACCAAGTTTTAGAGCTAGAAATAG	sgRNA amplification
sgRNA R1 BnHVA22c _2 Schiml	CTTCGAACTCATTTTTCTCCACAATCACTACTTCG	sgRNA amplification
sgRNA F2 BnHVA22c _2 Schiml	GTGGAGAAAATGAGTTCGAAGTTTTAGAGCTAGAAATA G	sgRNA amplification

b) HDR cloning

Table 10: Oligonucleotides for HDR cloning

Name	Sequence (from 5' to 3')	Application
F BclI	GCTATGATCAGttccccgagctgcatcaagc	Repeat vector cloning
R BamHI	GAGCGGATCCGAGGTAGCTTGATGCAG	Repeat vector cloning
Vec/Re BclI Restorer F	GAACTAGTTGATCAGTTCCCGAGC	Repeat vector cloning
Re/Vec BamHI R	GCAGCCCGGGGATCCGAGGTAGC	Repeat vector cloning
Circ Test fwd	GCTGGAGAAGAGCATGATAGTGG	Circular test Primer for viral replicons
Circ Test rev	GCATCTTTAACCTTCTTGGAAGG	Circular test Primer for viral replicons
AtAgo1 fwd	TATGGCGATCGCGAAAGAGAAGAACGGATGCTCC	Mutation <i>AtAGO1</i> locus for HDR
Mutated Ago1 rev	CTAgtCgcCtgGtgTaATtcTggCacTGATTGTCTCTGCGGTG	Mutation <i>AtAGO1</i> locus for HDR
Mutated Ago1 fwd	gtGccAgaATtAcaCcaGgcGacTAGTCACCTACTTATCAAGC	Mutation <i>AtAGO1</i> locus for HDR
AtAgo1 rev	TATCGCGATCGCcacaaaactcacATCATAATGGTG	Mutation <i>AtAGO1</i> locus for HDR
Bn_Ago1_fwd	TATGGCGATCGCGAAGGAGGTGAAGGGTCTGG	Mutation <i>BnaAGO1</i> locus for HDR
Bn_Ago1_rev	TATCGCGATCGCACTAACCTTTGTCCCCCAG	Mutation <i>BnaAGO1</i> locus for HDR
Bn_Ago1_mut_fwd	gtGccAgaATtAcaCcaGgcGacTATAACCAACTTATCAAGCGG	Mutation <i>BnaAGO1</i> locus for HDR
Bn_Ago1_mut_rev	TAgtCgcCtgGtgTaATtcTggCacTGATTGTCTCTGCGGCAG	Mutation <i>BnaAGO1</i> locus for HDR
sgRNA BnTao1_A_1 fwd	GGTTGACAACGAGATCAAGCGGTTTTAGAGCTAGAAATAG	sgRNA amplification
sgRNA BnTao1_A_1 rev	CGCTTGATCTCGTTGTCAACCAATCACTACTTCG	sgRNA amplification
sgRNA BnTao1_A_2 fwd	GTTGATCTCGTTGTCAACGAAGTTTTAGAGCTAGAAATAG	sgRNA amplification
sgRNA BnTao1_A_2 rev	TTCGTTGACAACGAGATCAACAATCACTACTTCG	sgRNA amplification
BnTao1 fwd	ATGGCTTCTTCTTCATCTC	Mutation <i>BnTAO1</i> locus for HDR
Bn_Tao1_mut_rev	TATCCACAAACGGAATAATGCCCTTGCTTTTGAGCTCTTTCAG	Mutation <i>BnTAO1</i> locus for HDR
Bn_Tao1_mut_fwd	GGCATTATTCCGTTTGTGGATAACGAGATCAAGCGAAGCGAATC	Mutation <i>BnTAO1</i> locus for HDR

BnTao1 rev	CACCAGCTTGAAGAAGCGTAG	Mutation <i>BnTAO1</i> locus for HDR
------------	-----------------------	--------------------------------------

c) NHEJ genotyping

Table 11: Oligonucleotides for NHEJ genotyping.

Name	Sequence (from 5' to 3')	Application
BnCRT1a locus fwd	GGACAGAAATAGAAAATTGC	Target locus amplification
BnCRT1a locus rev	GCAATCACAACTACTATC	Target locus amplification
BnHVA22c locus fwd	GAAACTGGAGCCTGGAGGG	Target locus amplification
BnHVA22c locus rev	CTCAAATGCGTCAGTGCCG	Target locus amplification
dCaps BnCRT1a fwd	GTTTCCAGCGATGGCGAACTAAAGAACAAC	dCaps analysis <i>BnCRT1a</i>
dCaps BnCRT1a rev	CCTCGAAGATAAACTACTAGC	dCaps analysis <i>BnCRT1a</i>
dCaps BnHVA22c fwd	CTTGCTTTGCTTTTGCTTCTGCAGCCACCTG	dCaps analysis <i>BnHVA22c</i>
dCaps BnHVA22c rev	GCTTTCACCTGATGCATATC	dCaps analysis <i>BnHVA22c</i>

d) HDR genotyping

Table 12: Oligonucleotides for NHEJ genotyping.

Name	Sequence (from 5' to 3')	Application
AtAgo1 wt fwd	GTTCCCGAGCTGCATCAAGCTACC	Screening for HDR events
AtAgo1 mut fwd	CTAgtCgcCtgGtgTaATtcTggCac	Screening for HDR events
AtAgo1 rev	CACAGGGTTTGCCTCTATG	Screening for HDR events
BnAgo1 wt fwd	gttCCCGAGCTGCATCAAGCTACC	Screening for HDR events
BnAgo1 mut fwd	gtGccAgaATtAcaCcaGgcGacTA	Screening for HDR events
BnAgo1 rev	CACGATCAGCATCAGATAAAGG	Screening for HDR events
BnTao1_A wt fwd	GGAATCATACTTTTCGTTGACA	Screening for HDR events
BnTao1_A mut fwd	GGCATTATTCCGTTTGTGGAT	Screening for HDR events
BnTao1_A rev	CAAGATCTCAAGAGACTCATAGCAAGT	Screening for HDR events

e) qPCR

Table 13: Oligonucleotides for qPCR

Name	Sequence (from 5' to 3')	Application
Actin	TCTACAACGAGCTCCGTGTTG	Housekeeping gene
Actin	TGTGAGACACACCATCACCA	Housekeeping gene
CNX1 fwd	GATGAGGAAGATGGTATGTGGG	Marker gene ER
CNX1 rev	GGTCTGTCTAGCTCAAAGTAGTC	Marker gene ER
PP2A fwd	CAATGACGATGACGATGAGGTG	Housekeeping gene
PP2A rev	ATGCTCAACCAAGTCACTCTCC	Housekeeping gene
OLG 70	CAGCGAAACGCGATATGTAG	VL DNA quantification
OLG 71	GGCTTGTAGGGGGTTTAGA	VL DNA quantification
ACO1 fwd	GAGAAGTTTTACCAGTCAGAGATGG	Marker gene ET
ACO1 rev	GGGTATTTAGCCACTTTTGTCC	Marker gene ET
ETR2 fwd	CACTGCCATTGCACCTAGCTCTT	Marker gene ET
ETR2 rev	CAACTCCATTGATCCCAATCTGC	Marker gene ET
LOX3 fwd	GTGAGGAACAAGAACAAGGAG	Marker gene JA
LOX3 rev	CGTTTTTGGATCAAGTTCGGTG	Marker gene JA
PCDB3 fwd	ACACCGTCAAGTCTCACTGCTC	Marker gene ER
PCDB3 rev	TAGCTGGGATCAGAGGCTGAGA	Marker gene ER
PDF1.2 fwd	cagctcacaagtgtatgtgctac	Marker gene JA
PDF1.2 rev	ttacacaaaactaaccaccaaagag	Marker gene JA
PDIL1-1 fwd	GACAGGGTCTTAGCTTCCTTCT	Marker gene ER
PDIL1-1 rev	GGATGATGATGAGAGGAACTTGG	Marker gene ER
PR1 fwd	TCAGGTTGTTTGGAGAAAGTC	Marker gene SA
PR1 rev	CATTAGTAAGGCTTCTCGTTCACA	Marker gene SA
WRKY70 fwd	GGGTAAGAGGATGCTAC	Marker gene SA
WRKY70 rev	CTTGCTTTGTTGCCTTGAC	Marker gene SA

Methods

In silico sequence analysis

All *in silico* analysis and sgRNA designs in this study are based on sequence data from the Tair10 genome database for *A. thaliana* (Berardini et al., 2015) and the Darmor-bzh v4.1 database for *B. napus* (Chalhoub et al., 2014). Annotated genes were obtained from the respective database and the following genes served as a template for sgRNA design: *AtAGO1* (AT1G48410), *AtAGO10* (AT5G43810), *AtCRT1a* (AT1G56340), *AtHVA22c* (AT1G69700). The homologous genes of *AtAGO1*, *AtCRT1a*, and *AtHVA22c* were identified in *B. napus* by blast analysis (Altschul et al., 1990) using the tblastx algorithm and used as a template for sgRNA design.

Phylogenetic analysis

A Neighbour-Joining phylogenetic tree based on amino acid sequences of *CRT1a* and *HVA22c* was created using MEGAX software (Kumar et al., 2018). The trees were calculated by applying 1000 bootstraps with the following settings: Maximum Likelihood, Jones-Taylor-Thornton (JTT) model, Uniform Rates, Use all sites, Nearest-Neighbor-Interchange (NNI).

sgRNA design

sgRNA design was conducted using the web tool Cas-Designer (Bae et al., 2014, Park et al., 2015). The PAM type was adjusted to 5'-NGG-3' (spCas9) or 5'-TTTN-3' (AsCpf1/LbCpf1) depending on the nuclease and the target genome was set to the respective organism. The data output and potential guide sequences were selected with the criteria of one specific hit in the *A. thaliana* and two specific hits in the *B. napus* genome as well as no off-targets with three or more mismatches. Additional criteria were a localization within an exon close to the start of the gene, which was manually verified by alignment of the genomic and coding sequence of the respective gene.

Vector systems for CRISPR/Cas9

Two different vector systems were used in this study to induce mutations for the NHEJ based approach. The first vector (BS_01) contained a *B. napus* codon-optimized Cas9 with a C-terminal Mammalian Importin alpha type nucleus localization sequence (NLS) under the control of the CaMV 35S promoter as well as a sgRNA template driven by an AtU6 promoter within a multiple cloning site flanked by recombination sites for a gateway LR reaction in a pUC57 background. This expression cassette was synthesized by GeneCust (Boynes, France). The recombination sites allow a gateway LR reaction to clone the expression cassette into any binary vector, e.g. pGWB401 (Nakagawa et al., 2007) (Fig. 5).

The second vector (BS_02), kindly provided by Prof. Dr. Ying Miao (Fujian Agriculture and Forestry University, China), contained an *A. thaliana* codon-optimized Cas9 with a C- and N-terminal Mammalian Importin alpha type nucleus localization sequence (NLS) and flagellin tag under the control of the CaMV 35S promoter as well as a *PmeI* restriction enzyme site for blunt end sgRNA insertion in a pCAMBIA background.

For the HDR based approach, the plasmid pLSLR (Baltes et al., 2014) was modified by inserting a sequence fragment amplified *via* PCR with M13 Primer from the binary vector pGWB401 containing the M13fwd Primer sequence, attR, lacUV5 promoter, CmR coding sequence, ccdB coding sequence, attR2 and a Nos terminator into a *Eco72I* restriction enzyme site located on pLSLR. After this modification, this vector could be used in the same way as the binary plasmid pGWB401.

Assembly of new sgRNAs

New sgRNAs were assembled by overlapping PCR, according to Li et al. 2013. The PCR was conducted with Phusion Polymerase (NEB) and the reaction mixture was set up according to the manufacturer with 1 μ l BS_01 (100 ng/ μ l) as template DNA and two Primer containing the guide sequence in sense and anti-sense direction. In the first round, the new guide sequence was introduced by Primer with overhangs containing the new guide sequence and corresponding reverse Primer in independent reactions. For the second PCR 2 μ l of each reaction was used as template DNA and the Primer annealing at the start and end of the sgRNA sequence were used to amplify the complete sgRNA. The PCR rounds to obtain sgRNAs with custom guide sequences were conducted under the following conditions:

Table 14: PCR program for sgRNA amplification

Initial Denaturation	98°C	0:30
30 cycles	98°C	0:10
	58°C	0:20
	72°C	0:20
Final Extension	72°C	5:00
Hold	10°C	∞

The correct amplification of the custom guide sequence was checked by the length of the PCR product on a 1% agarose gel. Amplicons from the first PCR should have a length of 330 bp and 136 bp from the second PCR. After the third PCR round, the assembled sgRNA should have a length of

Preparation of competent cells

Chemically competent *Escherichia coli* cells of DH5 α and K12xB DB3.1 strains were prepared according to Chung et al. (1989) for heat-shock transformation. The stock culture of those strains was streaked out on LB plates with an inoculating loop and kept at 37°C overnight in an incubator. Single colonies were picked and grown in liquid LB to an OD₆₀₀ of 0.3-0.4 and pelleted by centrifugation at 1000g for 10 minutes at 4°C. After centrifugation, all further steps were conducted on ice to provide permanent cooling. The supernatant was discarded and the pellet was resuspended in ice-cold TSS at one-tenth of the original volume. Aliquots of 0.05 ml were frozen in liquid nitrogen and stored at -80°C until use.

Electro competent *Agrobacterium tumefaciens* cells strain Gv3101 or *Agrobacterium rhizogenes* cells strain AR15834 were prepared according to McCormac et al. (1998). The stock culture of those strains was streaked out on LB plates with an inoculation loop and kept at 28°C for two days in an incubator. Single colonies were picked and grown in LB to an OD₆₀₀ of 0.5 and pelleted by centrifugation at 3000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was washed three times in ice-cold 10% v/v glycerol. The final pellet was resuspended at one-tenth of the original volume in 10% v/v glycerol. Aliquots of 0.05 ml were frozen in liquid nitrogen and stored at -80°C until use.

Transformation of bacteria

Heat shock transformation of *E. coli* cells was conducted by thawing aliquots of chemically competent cells on ice and adding 1 μ l of the plasmid (100 ng/ μ l), ligation or gateway reaction to the cells. After 20 minutes of incubation on ice, the mixture was heated to 42°C for 90 seconds in a water bath and placed back on ice. 1 ml liquid LB was added and the cells were incubated for one hour at 37°C on a rotary shaker with 180 rpm. After the incubation time, the cells were centrifuged for 5 minutes at 5000 rpm. The pellet was resuspended in 100 μ l liquid LB and 50 μ l of the bacterial solution was streaked out on LB plates with respective antibiotics. The plates were incubated at 37°C overnight until colonies became visible. Single colonies of transformed cells were checked *via* colony PCR for clones carrying the recombinant plasmid.

Electroporation of *A. tumefaciens* and *A. rhizogenes* was conducted by thawing aliquots of electrocompetent cells on ice and adding 1 μ l of the plasmid (100 ng/ μ l). The mixture was pipetted into electroporation cuvettes (0.2 cm) and placed into the Gene Pulser Xcell (Bio-Rad). Depending on the bacteria, pre-set programs were used for electroporation and the cells were electroporated. 1 ml liquid LB was added after electroporation and the cells were incubated for two hours at 28°C on a rotary shaker with 180 rpm. After the incubation time, the cells were

centrifuged for 5 minutes at 5000 rpm. The pellet was resuspended in 100 µl LB and 50 µl of the bacterial solution was streaked out on LB plates with 50mg/ml rifampicin and respective antibiotic for plasmid selection. The plates were incubated at 28°C for two days until colonies became visible. Single colonies of transformed cells were checked *via* colony PCR for clones carrying the recombinant plasmid.

Plasmid isolation

Single colonies or stock culture was used to inoculate 10 ml of liquid LB with respective antibiotics. The inoculated liquid LB media was incubated overnight at 37°C for *E. coli* cells or two days at 28°C for *A. tumefaciens* / *A. rhizogenes* cells on a rotary shaker with 180 rpm. 1 ml of the saturated liquid culture was centrifuged for 1 minute at 10000g and the supernatant was discarded. The bacterial pellet was used for plasmid isolation with the NucleoSpin® Plasmid (NoLid) kit (Machery-Nagel).

Agroinfiltration of *Nicotiana benthamiana* and GFP assay

Seeds of *Nicotiana benthamiana* were sown on soil (Einheitserde P, Einheitserdewerk Uetersen Werner Tantau GmbH und Co. KG) and grown under long-day conditions (16h light, 8h dark) until the 6-leaf stage with a weekly application of WUXAL (3 ml/l). Stock culture of *A. tumefaciens* strain Gv3101 carrying the desired plasmid and stock culture carrying a plasmid containing the P19 gene, for improved transient expression of the transfected construct, were streaked out on LB media plates with the respective antibiotics. The plates were kept for 1-2 days at 28°C in an incubator until single colonies were visible. Individual colonies were picked to inoculate liquid LB media containing the respective antibiotics and grown overnight at 28°C on a rotary shaker with 180 rpm. The cell suspension was centrifuged at 4500 rpm for 10 minutes and the supernatant was discarded. The pellet was washed 2 times with infiltration media to minimize antibiotic residues and resuspended in one-tenth of the original volume. The OD₆₀₀ was adjusted to 1 and the suspension was incubated for 3 hours at room temperature. Equal volumes of suspension with bacteria carrying the plasmid of interest and the P19 gene were gently mixed in a 50ml falcon tube. The leaves were infiltrated with a 1ml syringe without a needle by pressing the suspension into the leaves from the abaxial side until a wet spot was visible. After 48 hours, infiltrated leaf segments were excised with a razor blade and investigated under the fluorescence microscope for GFP activity in the tissue.

Floral Dip *Arabidopsis thaliana*

Seeds of *Arabidopsis thaliana* cv. Col-0 were sown on soil (Einheitserde P, Einheitserdewerk Uetersen Werner Tantau GmbH und Co. KG) and grown under short-day conditions (8h light, 16h dark) for 5-6 weeks with a weekly application of WUXAL (3 ml/l). After this growth period, the plants were transferred to long-day conditions to induce flowering. When a maximum of closed young flowers had formed, the plants were used for transformation. Stock culture of *A. tumefaciens* strain Gv3101 carrying the desired plasmid was streaked out on LB media plates containing the respective antibiotics. The plates were incubated for 1-2 days at 28°C in an incubator. Single colonies were picked to inoculate liquid LB media containing the respective antibiotics and grown overnight on a rotary shaker at 28°C with 180 rpm. The cell suspension was centrifuged at 4500 rpm for 10 minutes and the supernatant was discarded. The pellet was washed two times with infiltration media to minimize antibiotic residues and resuspended in one-tenth of the original volume. The OD₆₀₀ was adjusted to 1 and the suspension was poured into a beaker. The flowerheads were dipped two times into the bacterial suspension and submerged for 10 seconds under slight agitation. The excess suspension was removed by gently shaking the flowerheads on a paper towel. Inoculated plants were placed on trays, covered with a plastic lid and kept in darkness for two days. After the cocultivation period, plants were placed in the greenhouse under long-day conditions to set seeds with a weekly application of WUXAL (3 ml/l). Ripe yellow pods were harvested and the seeds were separated from the pods. Seeds were surface sterilized in 70% ethanol for 5 minutes, followed by incubation in 5% sodium hypochlorite solution with a drop of Tween 20 for 10 minutes. The seeds were washed three times with sterile water and kept under the clean bench to dry. Sterile seeds were sown on half MS media plates containing the respective antibiotic under short-day conditions to select positive transgenic events. After two weeks selection according to their resistance towards Kanamycin was carried out and plants were transferred to soil. Genomic DNA was isolated and transgene detection was conducted by PCR.

Hairy roots transformation of *Arabidopsis thaliana* and *Brassica napus*

Seeds of *Arabidopsis thaliana* cv. Col-0 or *Brassica napus* cv. Mozart were surface sterilized in 70% ethanol for 5 minutes, followed by incubation in 5% sodium hypochlorite solution with a drop of Tween 20 for 10 minutes. The seeds were washed three times with sterile water and kept under the clean bench to dry. Seeds were sown out on half MS media plates and kept under long-day conditions until the cotyledons were fully expanded. The stock culture of *Agrobacterium rhizogenes* strain AR15834 carrying the desired plasmid was streaked out on LB media plates containing 50 mg/ml rifampicin and the respective antibiotics for plasmid selection. The plates

were kept for 1-2 days at 28°C in an incubator. Single colonies were picked to inoculate liquid LB media containing the respective antibiotics and grown overnight on a rotary shaker at 28°C with 180 rpm. The cell suspension was centrifuged at 4500 rpm for 10 minutes and the supernatant was discarded and LB media was added to resuspend the cells to an OD₆₀₀ of 0.5. The roots of the seedlings were cut with a razor blade and discarded. For *Brassica napus* seedlings the cotyledons were removed. The explants were slightly dipped into the bacterial suspension with the cutting site and incubated for 1 minute. After the inoculation, explants were dried on a sterile paper towel to remove the excess bacterial suspension. Inoculated explants were placed on half MS plates and co-cultivated for two days under long-day conditions. After cocultivation, the explants were placed on half MS with 400 mg/l cefotaxime to remove bacteria and kept under long-day conditions. Roots formed after 1-2 weeks and were separated from the explant at about 2 cm length. Separated roots were placed on plates with half MS and 400 mg/l cefotaxime. Genomic DNA was isolated and a PCR with Primer annealing to the transformation vector was conducted with the isolated DNA.

Origin of stable transgenic *Solanum tuberosum* events

The stable transformation of *Solanum tuberosum* cv. Baltica and Granada was conducted by the company Solana GmbH & Co. KG (Hamburg, Germany). The binary plasmid with Cpf1 as nuclease and sgRNA targeting *StTXR1* was sent to the company after verification through colony PCR and sequencing. Transgenic plants were generated and kept *in vitro* under short-day conditions.

Total DNA isolation from plant tissue

DNA from plant tissue was extracted using the CTAB method (Rogers and Bendich, 1985). An amount of 0.05 g of fresh plant tissue was frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. For extraction 1.25 ml CTAB isolation buffer and 3 µl β-mercaptoethanol were added to the powder and mixed thoroughly. Samples were incubated for 1 hour at 65°C in a water bath and cooled down on the ice after the incubation time. 2 µl of RNase A was added to the samples and incubated for 30 minutes at 37°C in a water bath. After the incubation time, 500 µl chloroform/isoamyl alcohol (24:1) were added and the samples were gently mixed for 10 minutes on an overhead shaker. Samples were centrifuged for 10 minutes at 14000 rpm and the supernatant was transferred into a new 2ml tube. To precipitate the DNA, 700 µl of ice-cold isopropanol was added and the samples were incubated for 30 minutes on ice. The mixture was centrifuged for 10 minutes at 14000 rpm to pellet the DNA. DNA pellets were washed with washing buffer 1 and 2 kept at room temperature for 5 minutes in each washing buffer. The supernatant

was discarded and the pellet dried to remove any traces of ethanol. DNA was eluted in 50-100 μ l ddH₂O and 2 μ l with loading buffer were run on a 1% agarose to check the DNA quality and concentration. Isolated DNA was kept in a freezer at -20°C until use.

dCAPS and RE-assay

For each target locus, individual forward Primer were designed that introduce a restriction enzyme site in the PCR product of wild type locus and corresponding reverse Primer without any modification in sequence. The restriction enzyme that shared the most similarities in comparison to the wild type sequence was chosen and its recognition site implemented into the forward Primer. Restrictions in Primer design were a non-overlapping Primer at the Cas9 cutting site and a minimum forward Primer length of 30-40 bp as well as a maximum amplicon size of 100 bp. PCRs with Dream Taq Polymerase (Thermo Fisher Scientific) and reaction volume of 40 μ l were conducted to amplify the target loci:

Table 15: PCR program for dCAPS assay

Initial Denaturation	95°C	2:00
32 cycles	95°C	0:10
	58°C	0:20
	72°C	0:20
Final Extension	72°C	5:00
Hold	10°C	∞

10 μ l of the PCR were mixed with 2 μ l loading buffer and run on a 1% agarose to check for successful and correct amplification. For digestion of PCR fragments, 10 μ l PCR samples were mixed with 17 μ l ddH₂O, 2 μ l FastDigest Green Buffer (Thermo Fisher Scientific) and 1 μ l FastDigest restriction enzyme (Thermo Fisher Scientific). The mixture was incubated for 1 hour at the optimal reaction temperature for the respective restriction enzyme. After the incubation time, the samples were loaded on a 2% agarose gel and run for 35 minutes at 120 V to evaluate the band pattern. Digested bands indicated that no mutations were present in the PCR products, whereas the undigested band indicated potential mutations.

Protein extraction from plant tissue

For protein extraction, 0.1 g of fresh plant tissue was frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. An amount of 500 μ l protein extraction buffer was added and thoroughly mixed. The sample was incubated for 10 minutes on ice and centrifuged for 10 minutes at 10.000g. After centrifugation, the supernatant was transferred to a fresh tube and stored in a freezer at -20°C until use.

SDS-PAGE

Polyamid gels were prepared with 4x Rotiphorese-PAGE Matrix buffer plus and 30% Acrylamide-/Bisacrylamide solution. The separation gel solution was prepared according to the instructions of the manufacturer, with a total acrylamide concentration of 12 %. The gel solution was poured into the chamber leaving 2 cm of space at the top of the gel tray and isopropanol was carefully added to remove bubbles and prevent the gel from drying. After the separation gel polymerized, the stacking gel solution was prepared according to the instructions of the manufacturer, with a total acrylamide concentration of 5 %. The isopropanol was removed and the stacking gel solution was poured into the gel tray until it was filled. Depending on the number of samples, a 10 or 15-well comb was placed into the chamber.

Protein samples were mixed in a ratio of 1:1 with sample buffer and heated to 100°C for 5 minutes in a water bath. The gel was placed in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell, running buffer was poured in the cell and the combs removed. PageRuler™ Prestained Protein Ladder and samples were carefully loaded in the wells. The gels were run at 100 V until the blue dye line run out of the gel chamber. After the run stacking gels were removed and separation gels were used for Coomassie staining and/or Western blot.

Coomassie staining

For Coomassie staining, the separation gels were placed in staining solution and incubated under slow agitation for one hour. The staining solution was removed by washing the gel in ddH₂O. Unbound Coomassie was removed by incubation in the destaining solution overnight under slow agitation.

Western Blot with polyclonal Cas9 antibody

Gels were blotted *via* wet blot in a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell on a polyvinylidene difluoride (PVDF) membrane. Two pieces of Whatman filter paper (1mm) and the membrane were cut to the size of the gel. The membrane was activated by 100% MeOH, washed two times with ddH₂O and kept in ddH₂O until use. All components were soaked in transfer buffer

for about 15 minutes and the gel sandwich was layered in the following order: fibre pad, filter paper, gel, membrane, filter paper and fibre pad. The gel sandwich was placed into the blot cell and the cell was filled with transfer buffer. Proteins were blotted onto the membrane by applying a current of 10 V/0.1 A and running it overnight at 4°C. After blotting the membrane was washed three times with TBST and incubated for 2 hours in blocking solution (TBST and milk powder). Half of the blocking solution was used for hybridization with the primary Guide-it Cas9 polyclonal antibody from rabbit (Clontech/TaKaRa). The membrane was placed into a plastic bag with a blocking solution containing the primary antibody and the bag was sealed for incubation of 1 hour on an overhead shaker with slow agitation. To remove unbound primary antibodies, the membrane was washed four times with TBST. The Lumi-LightPLUS Western Blotting Kit (Mouse/Rabbit) was used for secondary antibody hybridization. The secondary antibody was added to the other half of the blocking solution and the membrane was incubated in the solution the same way as before. After the incubation time, the membrane was washed four times with TBST and gently dried on a paper towel. Substrates for fluorescence activity were added onto the membrane and incubated for 5 minutes. Excess substrate solution was removed with a paper towel, a sheet of Amersham HyperfilmTM MP (GE Healthcare Ltd.) was exposed to the membrane in a dark chamber and the film was developed.

RNA isolation from plant tissue

For RNA isolation, 0.05 g of plant tissue was frozen in liquid nitrogen and ground to a fine powder with mortar and pestle. RNA was isolated with the GeneJET Plant RNA Purification (Thermo Fisher Scientific Inc.). The resulting RNA-pellet was eluted in 20-50 µl DEPC-treated ddH₂O and stored at -20°C in a freezer until further use. The quality and concentration of the RNA were evaluated by running the samples on a 1% agarose gel.

First-strand cDNA synthesis

For cDNA synthesis, 1 µg of total RNA was incubated with RNase-free DNaseI (Fermentas) for 30 minutes at 37°C in a water bath to remove potential DNA contamination. The DNase I was inactivated by adding 1 µl of 25 mM EDTA solution to the reaction mixture and heating it to 65°C for 10 min in a water bath. The samples were immediately placed on ice. RNA was transcribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) into the first-strand cDNA. For cDNA synthesis 0.1- 5µg total RNA, 1 µl Oligo (dT)₁₈ Primer and nuclease-free water to fill up to a total volume of 12 µl were mixed gently in a reaction tube. 4 µl of the reaction buffer, 1µl RiboLock RNase Inhibitor, 2µl 10 mM dNTP Mix and 1 µl RevertAid M-MuLV RT were added and mixed

gently. The mixture was incubated for one hour at 42°C in a water bath and the reaction was terminated by heating it to 70°C for 5 minutes.

Realtime qPCR gene expression analysis

Expression analysis of genes was conducted by using 2 µl of 1:5 diluted cDNA mixed with 4µl ddH₂O, 4µl Primer and 10 µl qPCR SyGreen Mix Separate ROX (PCR Biosystems Ltd). The PCR was performed on a CFX96 Touch Real-Time PCR Cycler (Bio-Rad). The delta CT Method was used to calculate the relative expression, according to Pfaffl (2001). The two housekeeping genes PP2A (AT1G13320/BnaA06g33370D) and Actin2 (AT3G18780/BnaA10g22340D) were used as reference genes. The Primer efficiency was estimated using a standard curve of a cDNA dilution series and was additionally included in the calculations. The final value for gene expression consists of the mean of three biological replicates with two technical replicates each. Error bars indicate the standard error of the mean and statistics were performed using the Dunnett t-test (*p≤0.05; **p≤0.01; ***p≤0.001).

Cultivation of *Verticillium longisporum*

Verticillium longisporum isolate VI43 (Zeise and Tiedemann, 2001) was kindly provided by Dr. Elke Diederichsen (FU Berlin, Germany) and used to establish a sterile glycerol stock culture. This stock culture was prepared from liquid fungal culture, mixed with glycerol to a final concentration of 22% (v/v) and stored at -80°C in a freezer. To generate new inoculum for infection assays, stock tubes were thawed on ice, centrifuged at 6000g for 8 minutes and the supernatant was discarded. The pellet was dissolved in liquid CDB media with pH 6,8 (Czapek Dox Broth, Duchefa), streaked out on PDA media plates with pH 5,6 (Duchefa) and kept in the dark at RT for two weeks. PDA plates with sufficient fungal growth were sliced in eight pieces and two of them were used to inoculate 200 ml of liquid CDB media. Inoculated liquid cultures were incubated in the dark at 120 rpm on a rotary shaker and RT for three days. The suspension was filtered through sterile gauze (200 µm), centrifuged at 6000g for 8 min and resuspended in a fourth of the original volume. To determine and adjust the conidia concentration a Fuchs-Rosenthal chamber was used to count a 1:10 dilution of the fungal suspension. Conidia of five small squares from three big squares were counted and the concentrations calculated:

$$\text{Mean of conidia per small square} \times \text{dilution factor } 0.0125 \text{ mm}^3 \text{ per small square} \times 1000 = \text{conidia / ml}$$

The concentration of the conidia suspension was adjusted to $2 \cdot 10^6$ conidia/ml with a concentration of 22% (v/v) glycerol and stored at -80°C until use.

Stable transformation of *Brassica napus* events

The stable transformation of *Brassica napus* cv. Mozart was conducted by the company Saaten-Union Biotec GmbH (Leopoldshöhe, Germany). The binary plasmids with sgRNAs targeting *BnCRT1a* and *BnHVA22c* were transformed into *A. tumefaciens* and sent to the company after verification by colony PCR and sequencing. Transgenic plants were generated and transferred to *in vivo* conditions for seed production. In parallel, leaf material was harvested for DNA isolation for genotyping and selection of T0-individuals, which seeds were the basis of experiments in this study.

In vivo infection of *Brassica napus* with *Verticillium longisporum*

Seeds of *Brassica napus* were surface sterilized with 70% ethanol for 5 minutes and a 5% sodium hypochlorite solution with a drop Tween 20 for 10 minutes followed by three times washing with sterile water. Seeds were sown out on sterile filter paper (1mm) saturated with MS media without sucrose and used for inoculation when the cotyledons were fully expanded. *V. longisporum* stock culture was thawed on ice and then centrifuged at 6000g for 10 minutes. The supernatant was discarded and the pellet was dissolved in the same amount of water. Roots of *Brassica napus* seedling were cut with a razor blade to a length of 2 cm and placed in the conidia suspension for 15 minutes under slow agitation. After inoculation plants were grown in soil (Einheitserde P, Einheitserdewerk Uetersen Werner Tantau GmbH und Co. KG) with an initial fertilizer application of WUXAL (3 ml/l) under long-day conditions in the greenhouse.

In vitro Infection assay of *Brassica napus* with *Verticillium longisporum*

Seeds of *Brassica napus* were surface sterilized with 70% ethanol for 5 minutes and a 5% sodium hypochlorite solution with a drop Tween 20 for 10 minutes followed by three times washing with sterile water. The seeds were placed in plastic salad boxes with solid MS media and grown under short-day conditions until the cotyledons were fully expanded. Seedlings were transferred to 120 mm square Petri dishes placing the whole plant horizontally on the media. *V. longisporum* stock culture was thawed on ice and then centrifuged at 6000g for 10 minutes. The supernatant was discarded and the pellet was dissolved in the same amount of liquid CDB media. Roots were inoculated by brushing them with *V. longisporum* conidia suspension and plants were grown under short-day conditions for six days.

Disease rating

Scoring of disease symptoms caused by the *Verticillium* infection was conducted according to Eynck et al. (2007). The plants were scored every 7 days until 28 dpi.

Table 16: Disease severity index (Eynck et al., 2007).

Score	Symptoms
1	no symptoms
2	slight symptoms on the oldest leaf (yellowing, black veins)
3	slight symptoms on the next younger leaves
4	about 50 % of the leaves show symptoms
5	more than 50 % of the leaves show symptoms
6	up to 50 % of the leaves are dead
7	more than 50 % of the leaves are dead
8	only apical meristem is still alive
9	the plant is dead

From disease severity values, the area under the disease progress curve value (AUDPC) was calculated according to Campbell & Madden (1990):

$$\sum_{i=1}^n = \left(\frac{y_i + y_{i+1}}{2} \right) * (t_{i+1} - t_i)$$

y_i = disease score for observation number i ; t_i = days post-inoculation, n = number of observations

Adjusted AUDPC values were calculated to not overestimate the disease severity of each inoculated variant through natural occurring senescence. Therefore, AUDPC values from the mock control were subtracted from the AUDPC values from inoculated plants resulting in the AUDPC_{net} values:

$$AUDPC_{net} = AUDPC (X_{inoc.}) - AUDPC (X_{contr.})$$

The AUDPC_{net} values of each genotype were further normalized against the WT resulting in AUDPC_{norm} values. In addition to the AUDPC values, the plant height was measured at each scoring date as well. The growth depression (GD) compared to the mock control was calculated by dividing the plant height of the infected variant through the mock variant of each genotype:

$$GD = \left(1 - \left(\frac{Mean\ plant\ height_{mock}}{Mean\ plant\ height_{infected}} \right) \right) * 100$$

Fungal DNA quantification by qPCR

Fungal DNA quantification of *Verticillium longisporum* was conducted using the petioles of the first true leaves at 28 dpi. For each genotype three biological replicates containing petioles from 3 different plants were used for quantification. Total genomic DNA was isolated and 2 µl of 1:20 diluted gDNA was mixed with 18 µl qPCR SyGreen Mix Separate ROX (PCR Biosystems Ltd). The PCR was performed on a CFX96 Touch Real-Time PCR Cycler (Bio-Rad) and Primer OLG70/71 (Enyck et al 2007) for *V. longisporum*. The amount of fungal DNA was quantified using samples with known concentrations of fungal DNA to generate a calibration curve.

Statistical analyses

Graphs and plots were created with Microsoft® Office Excel® 2016 and statistical calculations were carried out in R. Simple comparisons of means for only two groups were done using the Dunnett t-test (*= $p \leq 0.05$; **= $p \leq 0.01$; ***= $p \leq 0.001$). Multiple comparisons of means were carried out by an analysis of variance (ANOVA) and a posthoc Tukey test with different letters indicating a significant difference ($p \leq 0.05$).

Results

Vector systems for NHEJ based approaches

A newly constructed vector system was used in this study to induce mutations for the NHEJ based approach in *B. napus* because no optimised constructs were available at the beginning of our experiments. The vector BS_01 contained *B. napus* codon-optimised Cas9 with a C-terminal Mammalian Importin alpha type nucleus localization sequence (NLS) under the control of the CaMV 35S promoter, a sgRNA template driven by an AtU6 promoter within a multiple cloning site and recombination sites for gateway LR reaction. The codon usage was adjusted with the web tool Codon Usage Database with *B. napus* as target organism (<http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=3708>). This expression cassette was synthesised by GeneCust (Boynes, France) and delivered within a pUC57 background (Fig. 6).

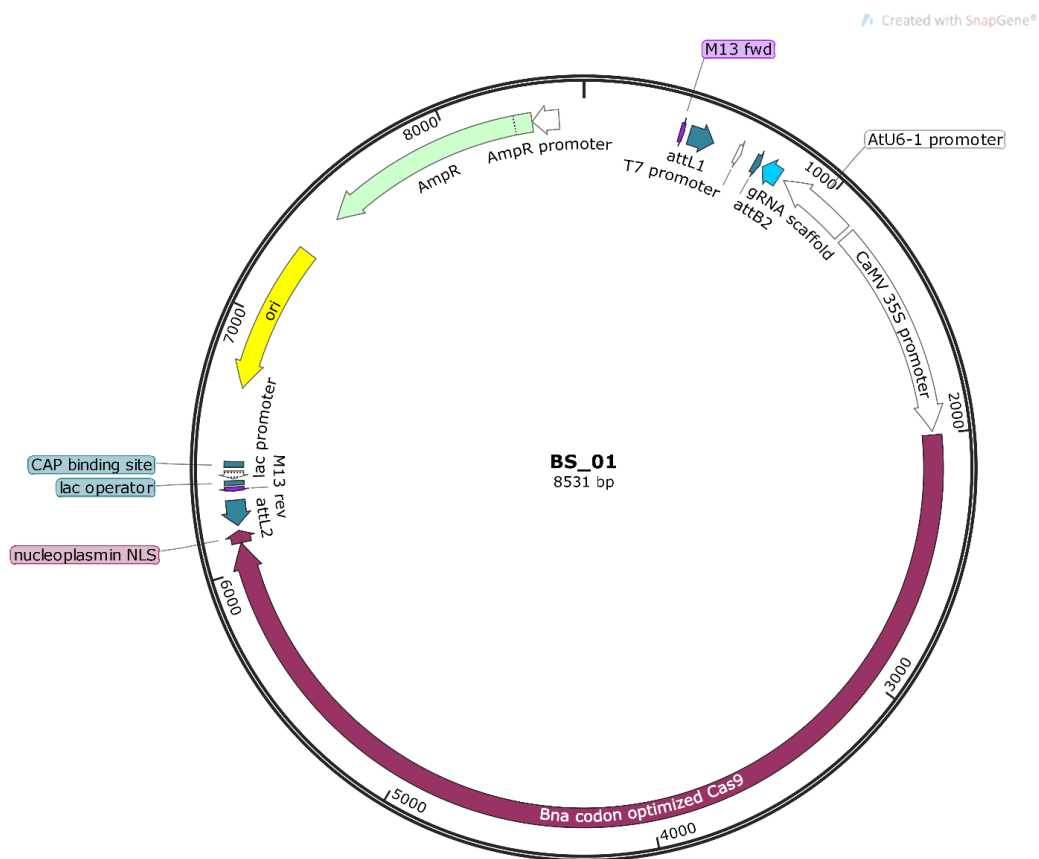


Figure 6: Vector map of BS_01. The vector contained a *B. napus* codon-optimised Cas9 with a C-terminal Mammalian Importin alpha type nucleus localization sequence (NLS) under the control of the CaMV 35S promoter, a sgRNA template driven by an AtU6 promoter within a multiple cloning site and recombination sites for gateway LR reaction. This vector map was created with snap gene viewer.

Successful establishment of this vector system for NHEJ approaches required a thorough functionality testing at the beginning of our experiments. We tested the vector on the transcriptional, translational and functional level. Transgenic *Solanum tuberosum* plants were used

to confirm functionality on transcriptional and translational level. Plants were transformed with the binary plasmid pGWB401 containing Cas9 as nuclease and no sgRNA by the company Solana GmbH & Co. KG (Hamburg, Germany) and plant material was harvested for DNA, RNA and protein extraction. We confirmed the successful transformation with PCR using Cas9 specific Primer (Fig. 7a). RNA was extracted from transgenic events which was transcribed into cDNA and tested positive for Cas9 transcription (Fig. 7b). Total protein was extracted from events used for PCR and RT-PCR, for those events a Western Blot with Guide-it Cas9 Polyclonal Antibody (Clontech) was conducted. The successful translation of the Cas9 protein could be confirmed through a positive signal at the same height as the positive control (Cas9 protein) in the Western Blot (Fig. 7c).

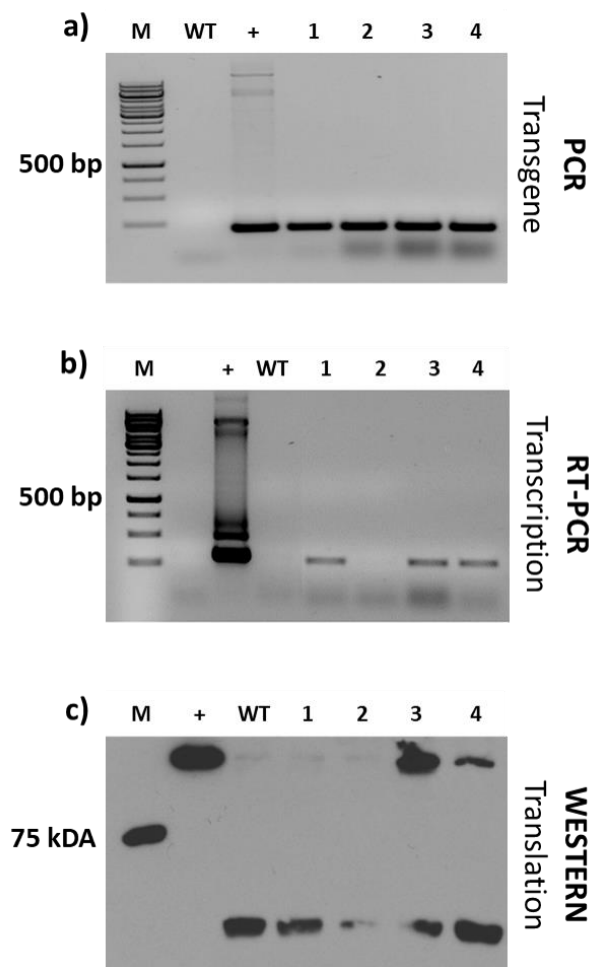


Figure 7: Confirmation of cassette functionality on the transcriptional and translational level. PCRs with genomic DNA and cDNA of Cas9 transgenic *Solanum tuberosum* plants were conducted with Primer for Cas9. The Western Blot was conducted with a polyclonal antibody for the Cas9 protein.

The translocation of the Cas9 protein into the nucleus was further verified to ensure that the NLS is functional. Therefore, transient expression assays in *Nicotiana benthamiana* with our Cas9 fused to GFP were conducted. The CDS of our codon-optimised Cas9 (BS_01) served as a template for a PCR with adapter Primer for a gateway reaction into pGWB403 (Nakagawa et al., 2007). Leaves

from *N. benthamiana* were infiltrated with *Agrobacterium tumefaciens* harbouring the Cas9-GFP fusion plasmid. After 48 hours, epidermal cells of infiltrated leaves were analysed to detect potential GFP signals. GFP signals were concentrated in round cores, leading to the conclusion that the Cas9 protein most likely is transported to the nucleus (Fig. 8).

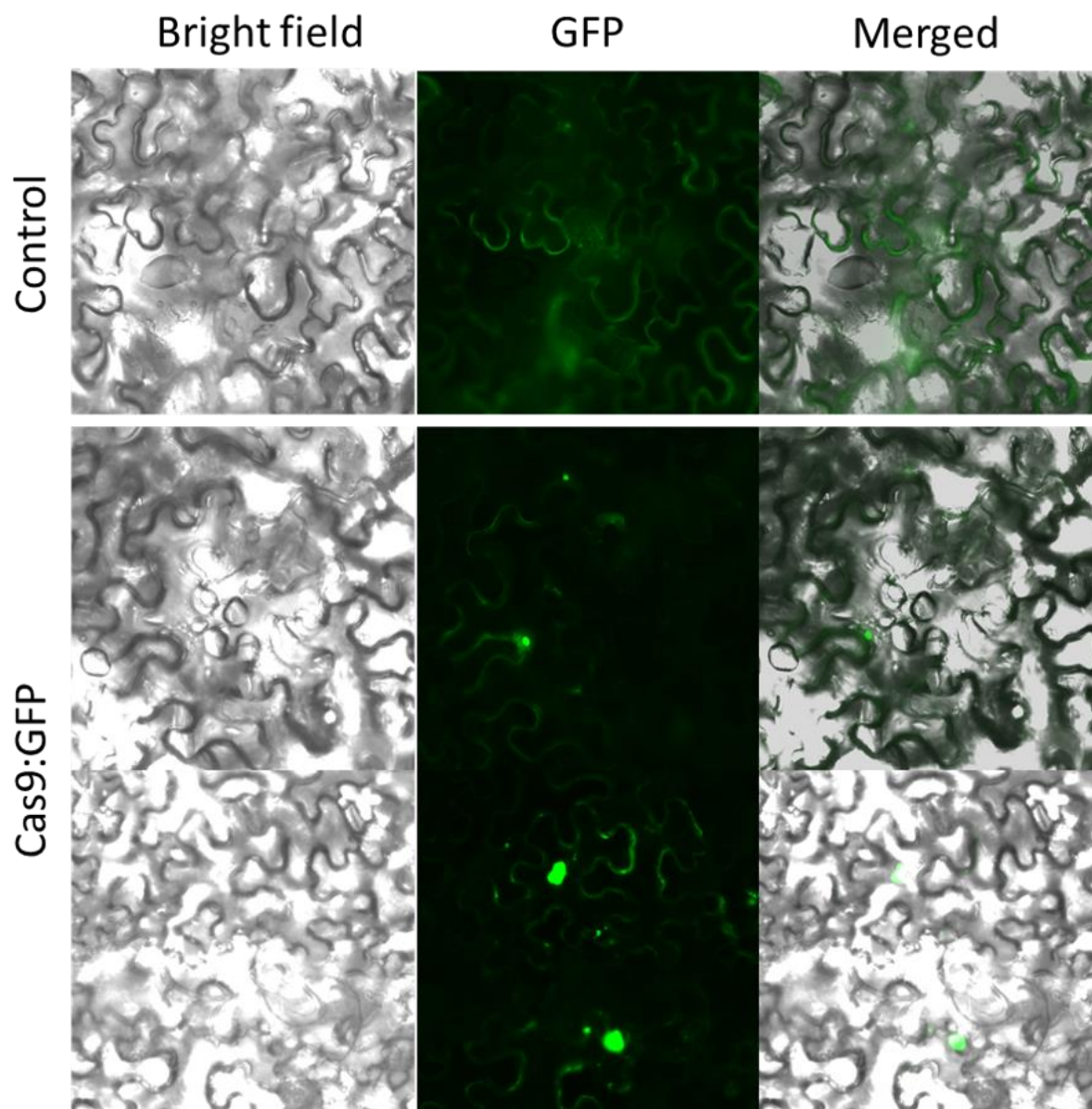


Figure 8: Transient expression of Cas9-GFP in epidermal leaf cells of *Nicotiana benthamiana* 48h after inoculation with *A. tumefaciens*.

In silico analysis and sgRNA design

For the NHEJ based CRISPR approach the genes *AtCRT1a* (AT1G56340), *AtHVA22c*(AT1G69700) and their homologues in *B. napus* *BnCRT1a*, *BnHVA22c* were chosen as targets. To additionally verify putative homologues of *CRT1a* and *HVA22c* in *B. napus* neighbour-joining phylogenetic trees based on amino acid sequences of annotated *A. thaliana* genes and their putative homologues in *B. napus* were generated (Fig. 9 and 10). For *CRT1a* four putative homologues in *B. napus* were closely related to *AtCRT1a* but only the copies BnaA09g15400D and BnaC09g16150D were suggested to be functional orthologs because the other copies were not correctly annotated. A similar pattern identified four putative *HVA22c* *B. napus* homologues being closely related to *AtHVA22c*. For the HDR based approach Genes containing a miRNA binding site targeted by *V. longisporum* were chosen: *AtAgo1* (NC_003070.9), *BnAGO1* (BnaA08g03260D, BnaC08g46720D) and *BnTAO1* (XM_022695834.1). Identification of homologues in *B. napus* was carried out *via* BLAST analysis in *B. napus* with the Darmor-bzh v4.1 database at GENOSCOPE (Chalhoub et al.,2014).

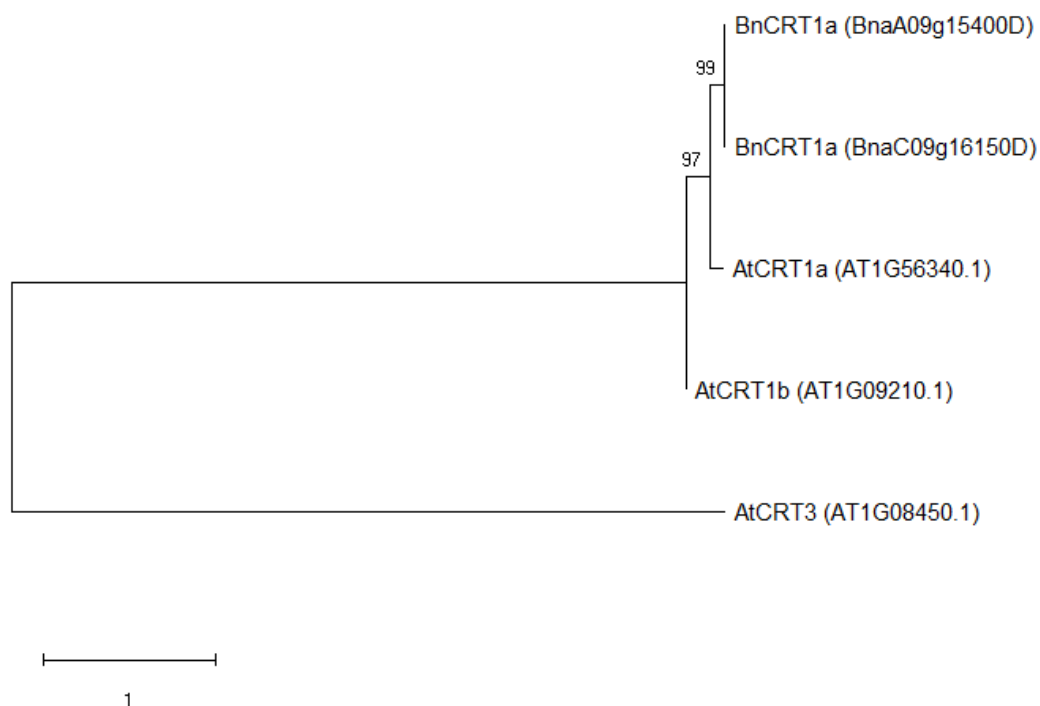


Figure 9: A Neighbour-Joining phylogenetic tree based on amino acid sequences of *AtCrt1a* and *BnCRT1a* as well as *AtCrt1b* and *AtCrt3*, which was created using MEGA X software (Kumar et al., 2018). The trees were calculated by applying 1000 bootstraps with the following settings: Maximum Likelihood, Jones-Taylor-Thornton (JTT) model, Uniform Rates, use all sites, Nearest-Neighbor-Interchange (NNI).

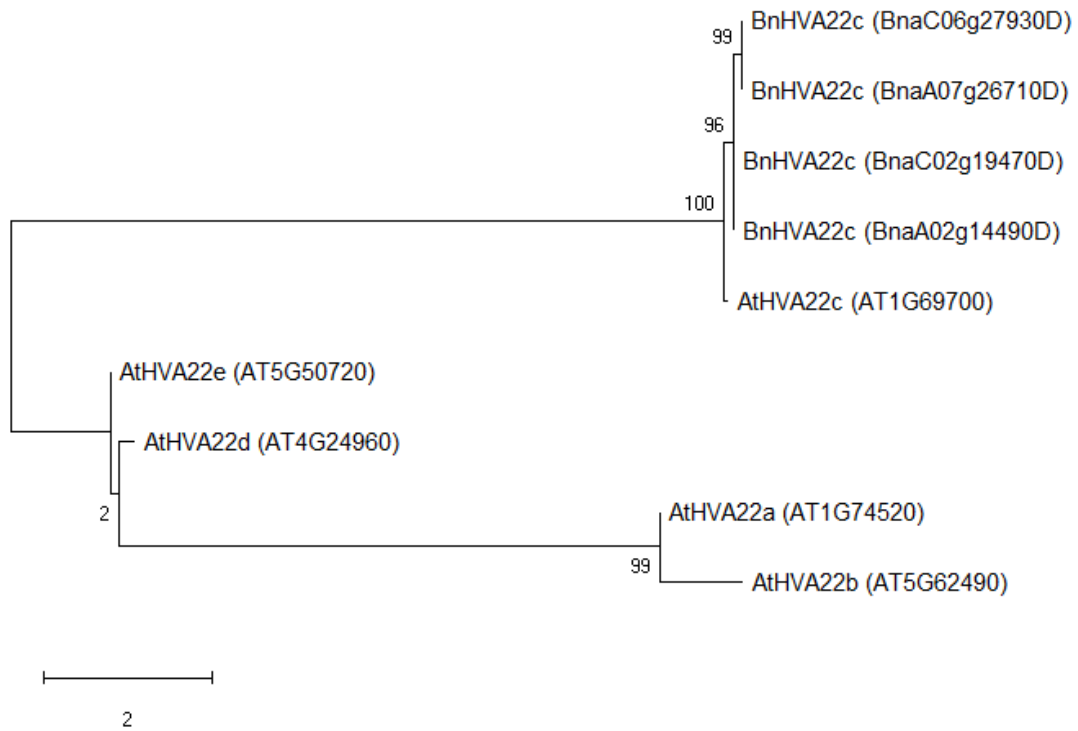


Figure 10: A Neighbour-Joining phylogenetic tree based on amino acid sequences of *AtHVA22c* and *BnHVA22c* as well as *AtHVA22a*, *AtHVA22b*, *AtHVA22d* and *AtHVA22d* which was created using MEGA X software (Kumar et al., 2018). The trees were calculated by applying 1000 bootstraps with the following settings: Maximum Likelihood, Jones-Taylor-Thornton (JTT) model, Uniform Rates, use all sites, Nearest-Neighbor-Interchange (NNI).

The webtool CRISPR RGEN Tools was used for sgRNA design. The threshold for targets that were considered potential off-targets was set to three mismatches. Mismatch sequences were blasted for double confirmation and were not included if the mismatch sequence was not identical to the CRISPR RGEN Tools output. The location of the target sequence in exons was confirmed by a comparison of the genomic and coding sequence. For each target gene, sgRNAs could be designed (Tab. 17).

Table 17: sgRNA targets for NHEJ and HDR based genome editing obtained from CRISPR RGEN Tools. MM =Mismatches to the original target sequence

Target gene	Guide sequence	PAM	Direction	1 MMs	2 MMs	3 MMs
NHEJ						
<i>BnCRT1a</i>	AGAATCAGAGAGATGAAGTT	NGG	-	0	0	5
<i>BnHVA22c_1</i>	GGTAGACGAGAGCAACCAA	TGG	-	0	0	0
<i>BnHVA22c_2</i>	TGGAGAAAATGAGTTCGAAG	AGG	-	0	0	0
HDR						
<i>AtAGO1</i>	GAGGTAGCTTGATGCAGCTC	GGG	+	0	0	1
<i>BnAGO1</i>	GAGGTAGCTTGATGCAGCTC	GGG	+/-	2	0	2
<i>BnTAO1_A_1</i>	GTTGACAACGAGATCAAGCG	AGG	-	0	2	2
<i>BnTAO1_A_2</i>	TTGATCTCGTTGTCACGAA	AGG	+	0	0	1

For the NHEJ based approach, all sgRNAs except *BnCRT1a* had no off-target loci with three or fewer mismatches. One of the major priorities of sgRNA design for the NHEJ based approach was the localisation of the target sequence at the start of the gene to achieve a high impact on the amino acid sequence through a frameshift. For *BnHVA22c* Cas9 suitable target sequences could only be found in the second and third exon. The sgRNAs for the HDR based approach were localised close to the respective miRNA binding sites (Fig. 11). The sgRNAs for the HDR based approach were excluded from the off-target criteria for sgRNA selection due to the limited options of target sequences in or near the miRNA binding site. Primer for each target sequence were designed and the custom sgRNA generated *via* PCR.

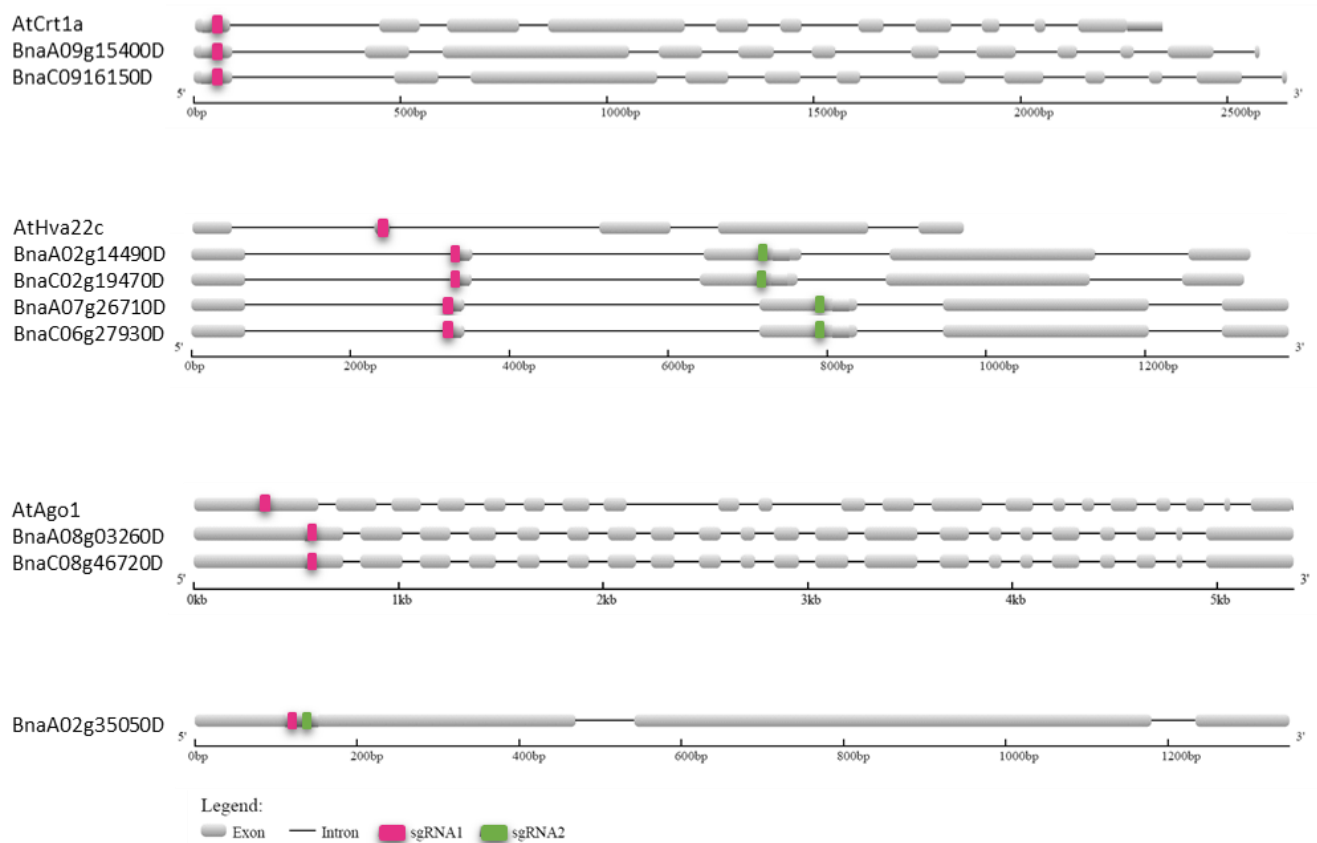


Figure 11: Gene structure of candidate genes and localisation of sgRNAs. Homologues of the target genes *CRT1a* and *HVA22c* were identified in *Brassica napus* and the gene structure was manually adjusted due to gene prediction errors. Each target sequence was found in the correct number of loci for the respective gene and organism. One exception is *HVA22c* where 4 copies could be found.

Table 18: Potential off-target sequences of sgRNAs for the respective candidate genes (Only targets up to 3 mismatches were included). * BnaA09g15970D and BnaC01g43040D were excluded from potential off-targets due to possible erroneous sequence data.

Target	Gene	Chr.	Position	Dir.	MMs
<i>BnCRT1a*</i>					
AGAATCAGAGAGATGAAGTTTGG	<i>CRT1a</i> (BnaA09g15400D)	A09	8960304	-	0
AGAATCAGAGAGATGAAGTTGGG	<i>CRT1a</i> (BnaC09g16150D)	C09	13078276	-	0
AGAATgAGgGAGATGAAGaTAGG	BnaA04g08980D	A04	8042743	-	3
AGAATgAGgGAGATGAAGaTAGG	BnaC04g31230D	C04	33137657	-	3
AGAAagAGAAaAGATGAAGTTAGG	NA	A07	16491979	-	3
AGAAagAGAAaAGATGAAGTTAGG	NA	C06	23806398	-	3
<i>BnHVA22c_1</i>					
GGTAGACGAGAGCAACCAATGG	<i>HVA22c</i> (BnaA02g14490D)	A02	8201222	-	0
GGTAGACGAGAGCAACCAATGG	<i>HVA22c</i> (BnaC02g19470D)	C02	15856625	-	0
GGTAGACGAGAGCAACCAATGG	<i>HVA22c</i> (BnaA07g26710D)	Ann_rnd	30640895	+	0
GGTAGACGAGAGCAACCAATGG	<i>HVA22c</i> (BnaC06g27930D)	C06	29259884	+	0
<i>BnHVA22c_2</i>					
TGGAGAAAATGAGTTCGAAGAGG	<i>HVA22c</i> (BnaA02g14490D)	A02	8201626	-	0
TGGAGAAAATGAGTTCGAAGAGG	<i>HVA22c</i> (BnaC02g19470D)	C02	15857025	-	0
TGGAGAAAATGAGTTCGAAGAGG	<i>HVA22c</i> (BnaA07g26710D)	Ann_rnd	30641377	+	0
TGGAGAAAATGAGTTCGAAGAGG	<i>HVA22c</i> (BnaC06g27930D)	C06	29259399	+	0
<i>BnAgo1</i>					
GAGGTAGCTTGATGCAGCTCGGG	<i>AGO1</i> (BnaA08g03260D)	A08	2681984	-	0
GAGGTAGCTTGATGCAGCTCGGG	<i>AGO1</i> (BnaC08g46720D)	C08_rnd	958508	+	0
aAGGTAGCTTGATGCAGCTCGGG	<i>AGO1</i> like (XM_013816466.2)	A05	12291423	-	1
aAGGTAGCTTGATGCAGCTCGGG	<i>AGO1</i> like (XM_013816466.2)	C05	21121432	+	1
GgGGcAGCTTGtTGCAGCTCAGG	GrpE like (XM_022707193.1)	A03	24797182	-	3
GgGGcAGCTTGtTGCAGCTCAGG	GrpE like (XM_022707193.1)	C07	40828434	+	3
<i>BnTao1_A_1</i>					
GTTGACAACGAGATCAAGCGAGG	<i>TAO1</i> (XM_022695834.1)	A02_rnd	45821	-	0
GTTGACAACGAGATCAAGCGAGG	<i>TAO1</i> (XM_022695834.1)	Cnn_rnd	37713545	-	0
aTTGAtAACGAGATCAAGCGAGG	<i>TAO1</i> like (XM_013883846.2)	A03	14157322	-	2
aTTGAtAACGAGATCAAGCGAGG	<i>TAO1</i> like (XM_013883846.2)	C05	12687684	+	2
aTTGAtgACGAGATCAAGCGAGG	<i>TAO1</i> like (XM_013883846.2)	A09	8678910	+	3
aTTGACAACGAGATCgAGaGAGG	NA	C05	9631109	+	3
<i>BnTao1_A_2</i>					
TTGATCTCGTTGTCAACGAAAGG	<i>TAO1</i> (XM_022695834.1)	A02_rnd	45827	+	0
TTGATCTCGTTGTCAACGAAAGG	<i>TAO1</i> (XM_022695834.1)	Cnn_rnd	37713551	+	0
TTGATCTCGTcaTCAAtGAAAGG	<i>TAO1</i> like (XM_013883846.2)	A09	8678904	-	3

In summary, we cloned several constructs with different nucleases, promoters and sgRNAs for the NHEJ based approach and respective target genes (Tab. 19).

Table 19: Plasmids cloned in this study for the NHEJ approach with information about promoter, nuclease and sgRNA target

Plasmid name	Promoter	Nuclease	sgRNA target	Backbone
BS_01	35s	Cas9 (<i>B. napus</i> codon optimized)	-	<i>pUC57</i>
BS_02	35s	Cas9 (<i>A. thaliana</i> codon optimized)	-	<i>pUC57</i>
BS_07	35s	Cas9 (<i>B. napus</i> codon optimized)	<i>BnCRT1a</i>	<i>pGWB401</i>
BS_09	35s	Cas9 (<i>B. napus</i> codon optimized)	<i>BnHVA22c_1</i>	<i>pGWB401</i>
BS_10	35s	Cas9 (<i>B. napus</i> codon optimized)	<i>BnHVA22c_2</i>	<i>pGWB401</i>

Transformation of our CRISPR/Cas9 constructs

Hairy roots were utilised as another test system and have the advantage of a fast generation, high transformation efficiency and are inducible from a wide range of tissue. For our experiments, we generated hairy roots in *B. napus* from hypocotyl of *in vitro* grown seedlings. Transgenic hairy roots had been generated and gDNA was isolated from positive transgenic events that were confirmed *via* PCR. The *HVA22c_2* target locus was amplified and digested with *AsuII* (Fig. 12). Undigested bands were visible indicating a potential change of the target sequence. Those undigested bands were cloned into T-vector and Sequencing data revealed InDels at the target locus compared to the wild type.

RE *BnHVA22c_2*

WT **CCTCTT | CGAACTCATTTCCTCCA**
 TT | CGAA (AsuII)

SNPs **CCTCTT**A**CGAACTCATTTCCTCCA**
 ~~TT~~CGAA

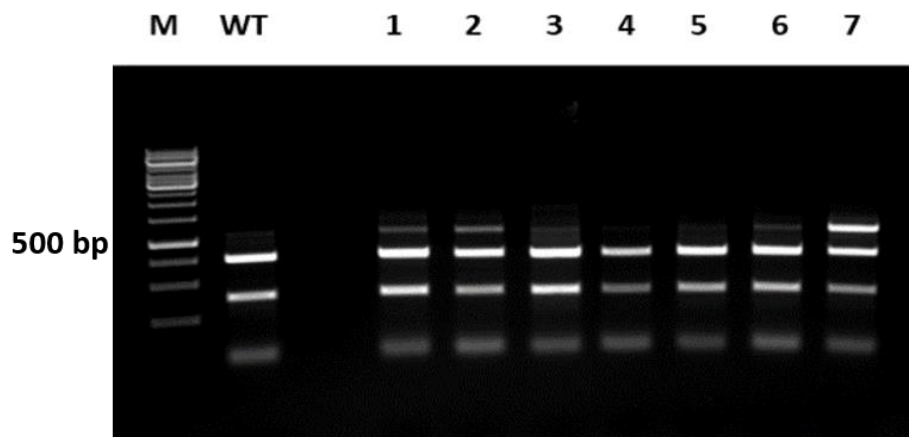


Figure 12: RE-Assay of *HVA22c_2* locus for mutation screening. The sgRNA target locus was amplified from different transgenic hairy roots from *Brassica napus* transformed. PCR products were incubated with restriction enzyme *AsuII* to screen for potential mutations. The wild type locus (WT) was completely digested and the undigested wild type PCR product served as a reference for undigested bands (UD). Several events showed undigested bands suggesting potential mutations in the respective genomes.

Screening of CRISPR/Cas-generated mutations *CRT1a* and *HVA22c* loci

The successful validation of vector functionality allowed us to proceed with stable transformation for targeting both *CRT1a* and *HVA22c* susceptibility factors in *B. napus*. Vector constructs were transformed into *A. tumefaciens* and handed to the company Saaten-Union Biotec GmbH (Leopoldshöhe, Germany) to generate transgenic events in the *B. napus* cv. Mozart background. After the antibiotic selection process, 22 independent events for *CRT1a* and 40 independent events for *HVA22c* could be obtained.

The first screening for mutations at the target loci was conducted *via* dCAPS assay to allow fast identification and semi-quantitative estimation of mutation rates. dCAPS Primers were designed which introduce RE sites in the PCR product of the respective loci from *CRT1a* and *HVA22c*, enabling a RE digestion to screen for mutations with restriction enzyme digestion. The dCAPS assay was conducted for all T0 *CRT1a* and *HVA22c* CRISPR mutants. For confirmation, several undigested bands from the dCAPS assay (Fig. 13) for *CRT1a* and *HVA22c* were cloned into T-vector and sequenced. The sequencing results indicated that the dCAPS Assay was reliably working when bands indicated mutations compared to the wild type locus. After successful validation of the

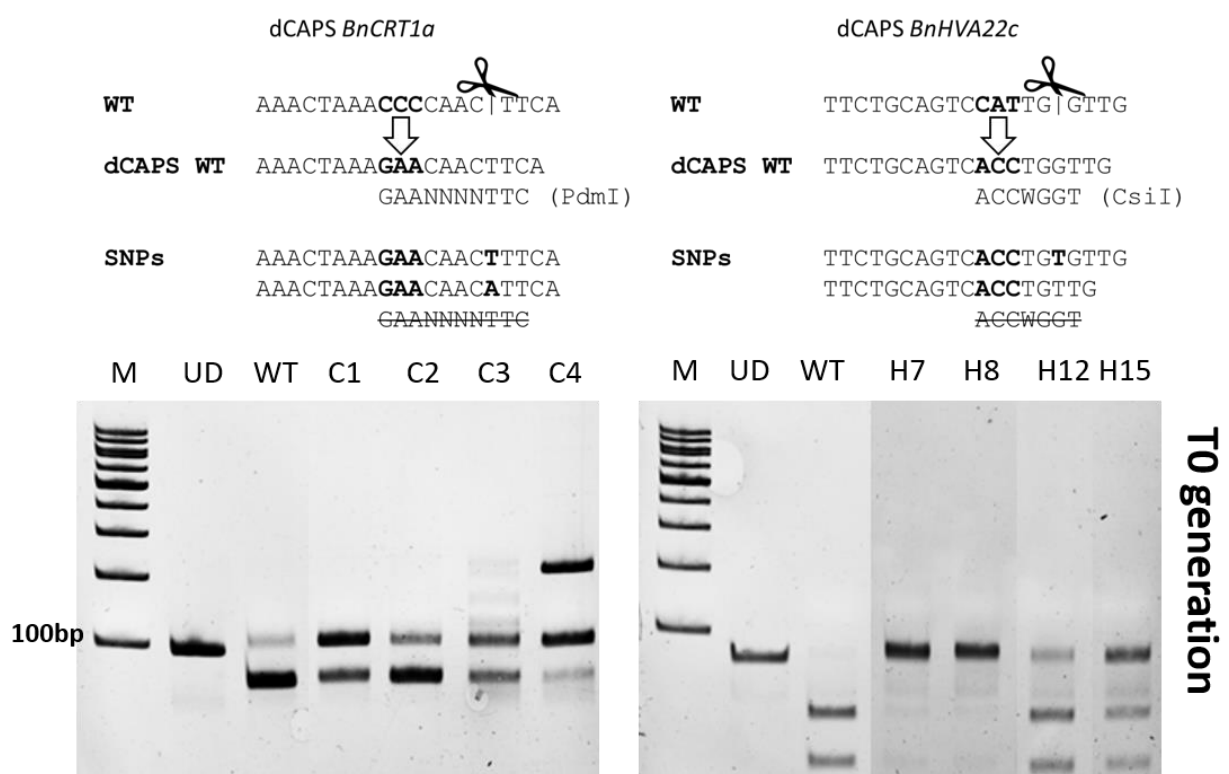


Figure 13: dCAPS assay for *BnCRT1a* and *BnHVA22c* target loci in *Brassica napus*. PCRs with dCAPS Primer, that introduce RE recognition sequences into PCR products with wild type sequence. UD = Undigested PCR product

dCAPS assay the events C1, C2, C3, C4 for *CRT1a* and H7, H8, H12, H15 for *HVA22c* were chosen for cloning of target loci into pGEM T-easy (Promega, USA) for sequencing to gain more precise information about mutations. For *CRT1a* 9 WT and 80 PCR products from 10 independent transgenic events were sequenced and mutations in 5 out of the 10 events could be observed. For *HVA22c* 15 WT and 85 PCR products from 10 independent transgenic events were sequenced and mutations in 6 out of 10 events could be observed (Fig. 14). Unfortunately, we could not conclude whether an event was a homozygous mutation or not because the same mutations in different alleles were not distinguishable with this sequencing method. For instance, for *BnHVA22c* the distinction between the A and C copy of the gene was not possible in the amplified sequence due to a high degree of sequence homology. Therefore, it is possible that the mutation rate of an event was underestimated. Nevertheless, the percentage of independent T0 events carrying expected mutations was estimated to 22,7% for *CRT1a* and 35% for *HVA22c* CRISPR mutants.

<i>BnCRT1a</i> T0 generation		<i>BnHVA22c</i> T0 generation	
	PAM Guide sequence		PAM Guide sequence
WT (A)	TAAAC CCCA ACTTCATCTCTCTGATTCT AT TCG	WT (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
WT (A)	TAAAC CCCA ACTTCATCTCTCTGATTCT AT TCG	WT (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
WT (C)	TAAAC CCCA ACTTCATCTCTCTGATTCT TT TCG	WT (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
WT (C)	TAAAC CCCA ACTTCATCTCTCTGATTCT TT TCG	WT (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C01 (A)	TAAAC CCCA ACT T TCATCTCTCTGATTCT AT TCG	H07 (A/C)	GCAG TCCA TTGG G TTGCTCTCGTCTACCCTCTG
C01 (A)	TAAAC CCCA ACTTCATCTCTCTGATTCT AT TCG	H07 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C01 (C)	-----CATCTCTCTGATTCT TT TCG	H07 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C01 (C)	TAAAC CCCA ACTTCATCTCTCTGATTCT TT TCG	H07 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C02 (A)	TAAAC CCCA ACTTCATCTCTCTGATTCT AT TCG	H08 (A/C)	GCAG TCCA TTG A GTTGCTCTCGTCTACCCTCTG
C02 (A)	TAAAC CCCA ACTTCATCTCTCTGATTCT AT TCG	H08 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C02 (C)	TAAAC CCCA AA--TCATCTCTCTGATTCT TT TCG	H08 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C02 (C)	TAAAC CCCA ACT T TCATCTCTCTGATTCT TT TCG	H08 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C03 (A)	TAAAC CCCA AA---CATCTCTCTGATTCT AT TCG	H12 (A/C)	GCAG TCCA TTG--TGCTCTCGTCTACCCTCTG
C03 (A)	TAAAC CCCA ACT T TCATCTCTCTGATTCT AT TCG	H12 (A/C)	GCAG TCCA TTG T GTTGCTCTCGTCTACCCTCTG
C03 (C)	TAAAC CCCA ACT-CATCTCTCTGATTCT TT TCG	H12 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C03 (C)	TAAAC CCCA ACTTCATCTCTCTGATTCT TT TCG	H12 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG
C04 (A)	TAAAC CCCA AC G TTTCATCTCTCTGATTCT AT TCG	H15 (A/C)	GCAG TCCA TTG---GCTCTCGTCTACCCTCTG
C04 (A)	TAAAC CCCA ACT T TCATCTCTCTGATTCT AT TCG	H15 (A/C)	GCAG TCCA TTG----CTCTCGTCTACCCTCTG
C04 (C)	TAAAC CC ----TTCATCTCTCTGATTCT TT TCG	H15 (A/C)	GCAG TCCA TTG T GTTGCTCTCGTCTACCCTCTG
C04 (C)	TAAAC CCCA AC* T TCATCTCTCTGATTCT TT TCG	H15 (A/C)	GCAG TCCA TTGGTTGCTCTCGTCTACCCTCTG

(A) = BnaA09g15400D
(C) = BnaC09g16150D

(A/C) = BnaA07g26710D
BnaC06g27930D

* = 118 bp Insertion
N = CRISPR mutation N = SNP in WT

Figure 14: Mutations at sgRNA target locus for *BnCRT1a* and *BnHVA22c* obtained so far. The sequence data was obtained through sequencing single alleles in T-vectors. For *CRT1a* 9 WT and 80 PCR products from the target loci and for *HVA22c* 15 WT and 85 PCR products from the target loci were sequenced. Only T0 events with mutations are listed in this figure.

Selection of fully mutated events for *BnCRT1a* and *BnHVA22c*

Because a recessive resistance relies on mutations in all of the targeted loci, we focused on the identification of fully mutated events for *BnCRT1a* and *BnHVA22c* from T2-populations as illustrated in with fully mutated target loci (Fig. 15).

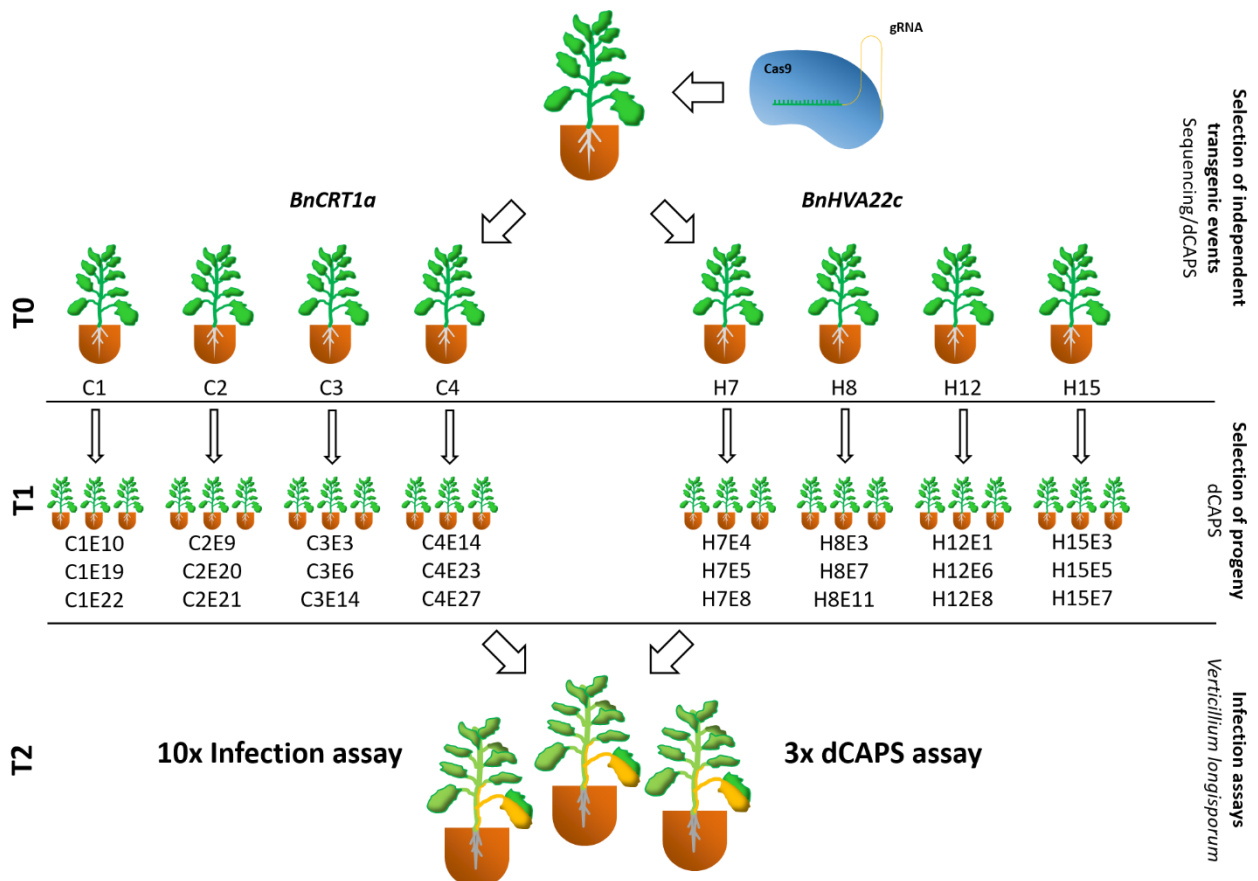


Figure 15: Scheme for propagation and selection of CRISPR mutants. The T0 generation was screened with single allele sequencing and dCAPS assay. Four events with high mutation rates were chosen for further propagation. Twenty plants of the T1 generation were screened with dCAPS and three plants per T0 parental event were chosen for further propagation. Three progenies of each T1 event were analysed by dCAPS and 10 plants were used in infection experiments with *Verticillium longisporum*.

As starting plant material for our experiments, four T0 events with the highest mutation rates for *CRT1a* and *HVA22c* as estimated by the dCAPS assay and validated by sequencing were chosen. The events C1, C2, C3, C4 for *CRT1a* and H7, H8, H12, H15 for *HVA22c* were selected for further investigation. After selfing, twenty plants from the progeny from each T0 event were analysed by dCAPS to select three T1 individuals with the highest mutation rates as estimated by dCAPS assay (Fig. 16). The events include C1E10, C1E19, C1E22, C2E9, C2E20, C2E21, C3E3, C3E6, C3E14, C4E14, C4E23, C4E27 for *CRT1a* and H7E4, H7E5, H7E8, H8E3, H8E7, H8E11, H12E1, H12E6, H12E8, H15E3, H15E5, H15E7 for *HVA22c* (Fig. 16). Subsequently, 10 T2 individuals were subjected to infection assays with *V. longisporum* VI43.

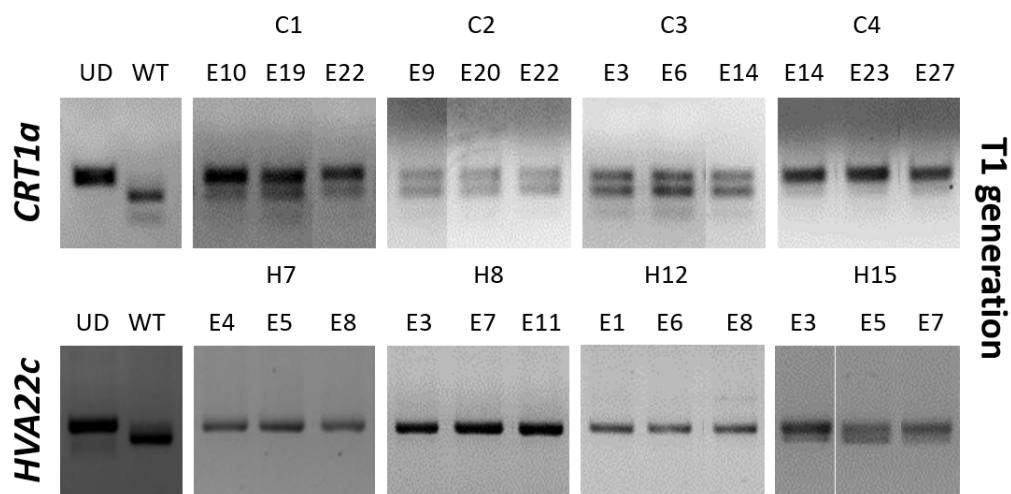


Figure 16: dCAPS assay for *CRT1a* and *HVA22c* target loci in *Brassica napus*. PCRs with dCAPS Primer, that introduce RE recognition sequences into PCR products with wild type sequence. UD = Undigested PCR product.

To ensure the induced mutations are stably inherited in the T2 generation, three T2 plants were randomly selected for dCAPS assay to evaluate and confirm the estimated mutation rate before T2 individuals were used in infection experiments (Fig. 17). Interestingly, all T2 plants showed similar mutation rates compared to their parents, indicating that CRISPR/Cas-induced mutations did not increase over two generations and remained stable.

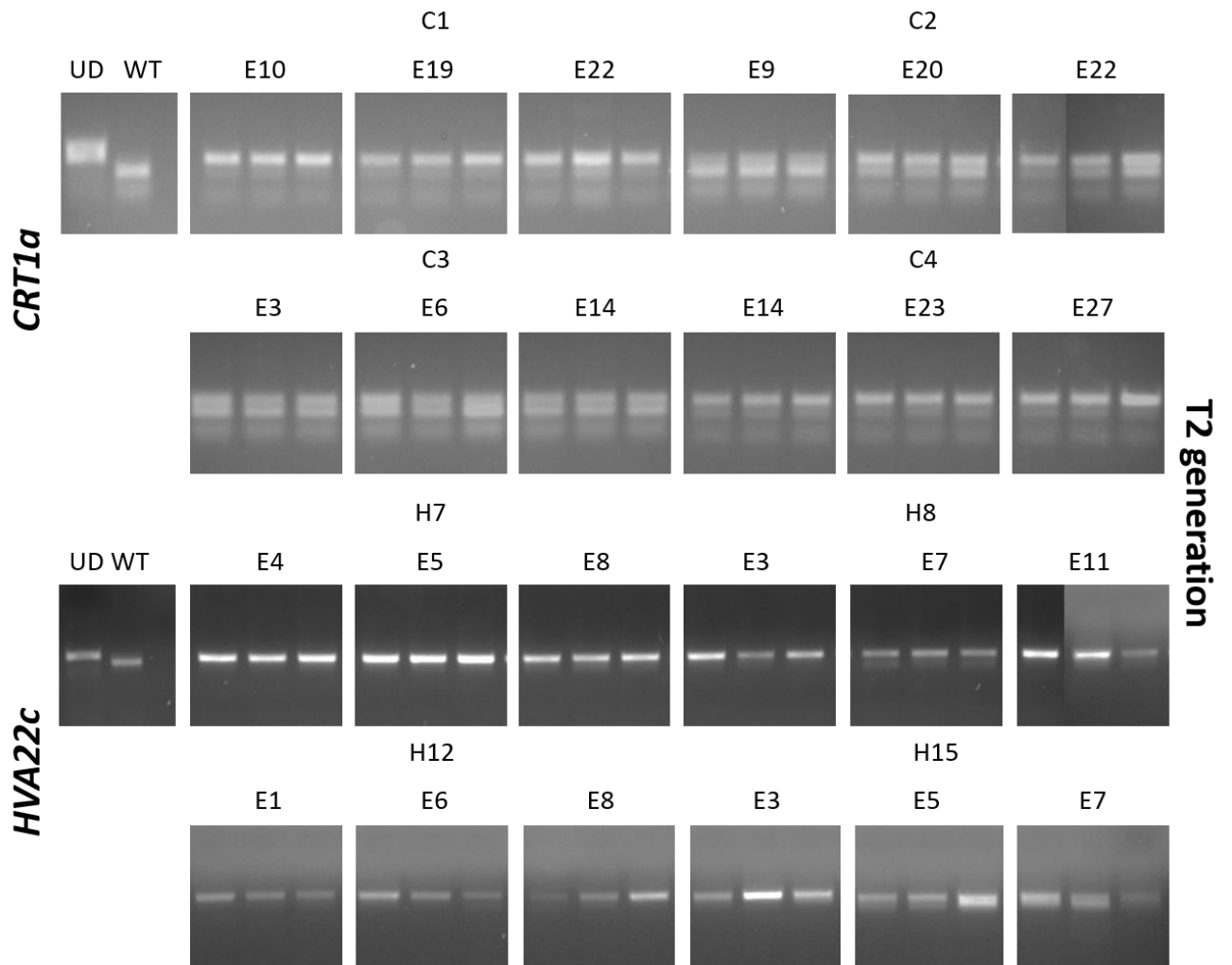


Figure 17: dCAPS assay for *CRT1a* and *HVA22c* target loci in *Brassica napus* of the T1 progeny. PCRs with dCAPS Primer, that introduce RE recognition sequences into PCR products. UD = Undigested PCR product.

Infection assays of *BnCRT1a* and *BnHVA22c* mutants with *V. longisporum*

The cultivar Mozart was first infected with *VI43* to evaluate the infection system under greenhouse conditions. One week after germination seedlings were inoculated *via* root dip and grown under long-day conditions in the greenhouse. At 21 dpi different disease severities could be observed, ranging from beginning chlorosis (Fig. 18a) to visible chlorosis and stunted growth (Fig. 18d).

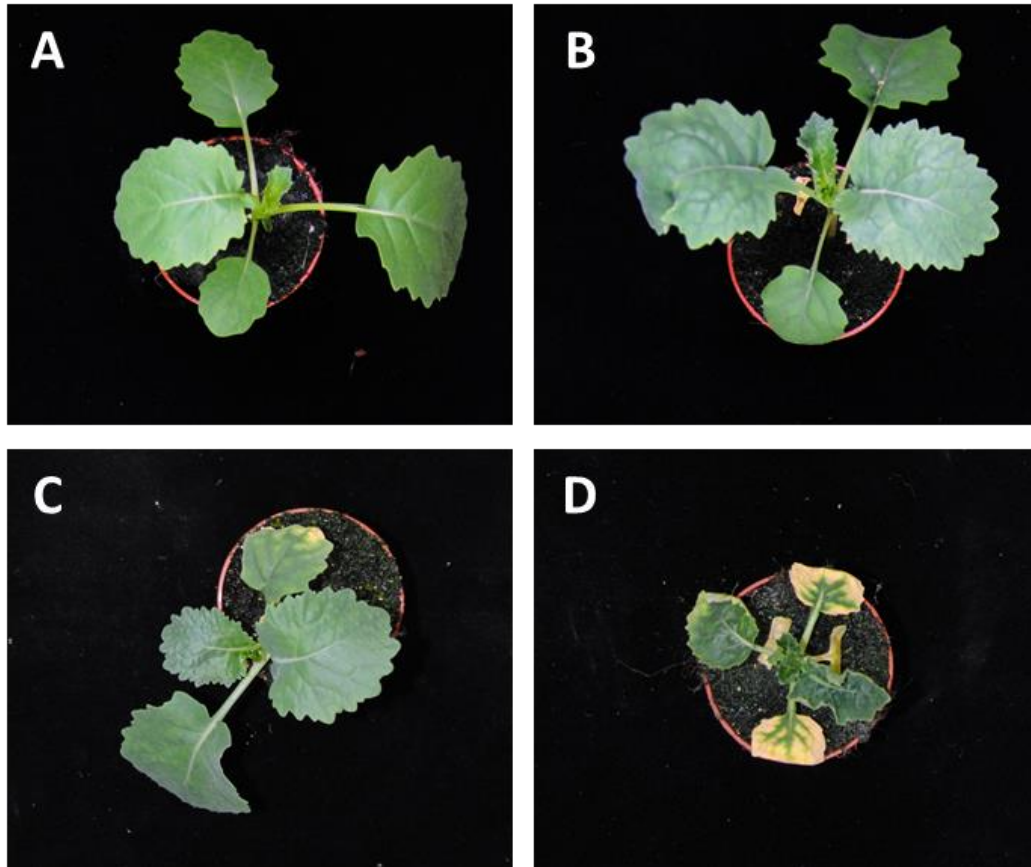


Figure 18: Different infection severities of *Verticillium* stem stripping 21 dpi of the cultivar Mozart. The plants were grown in the greenhouse under long-day conditions. A range of disease severities could be observed with beginning chlorosis (B) to stunted growth and severe chlorosis (D) compared to the non-infected control (A).

The Infection experiment was repeated twice consisting of 10 infected and five mock-treated plants per mutant. Every seven days plant height and disease severity were evaluated. After 28 dpi pictures from three representative plants of each genotype were taken (Fig. 19 and 20). The wild type showed severe stunted growth and chlorosis compared to its non-infected control. Overall the mutants of both genes showed less stunted growth and symptoms than the infected wild type. However, a difference in disease severity between different mutants of *CRT1a* and *HVA22c* was observable. The *CRT1a* genotypes C1E22, C2E9, C2E20 and C4E14 seemed more susceptible to *VI43* infection than the genotypes C3E3, C3E6 and C4E23 compared to their non-infected control. The same range of susceptibility could be observed for *HVA22c* with H7E5 and H7E8 as more

susceptible and H12E1, H12E6, H15E3 and H15E7 as less susceptible genotypes to *Vl43* infection. This range of susceptibility could be correlated with the number of mutated alleles.

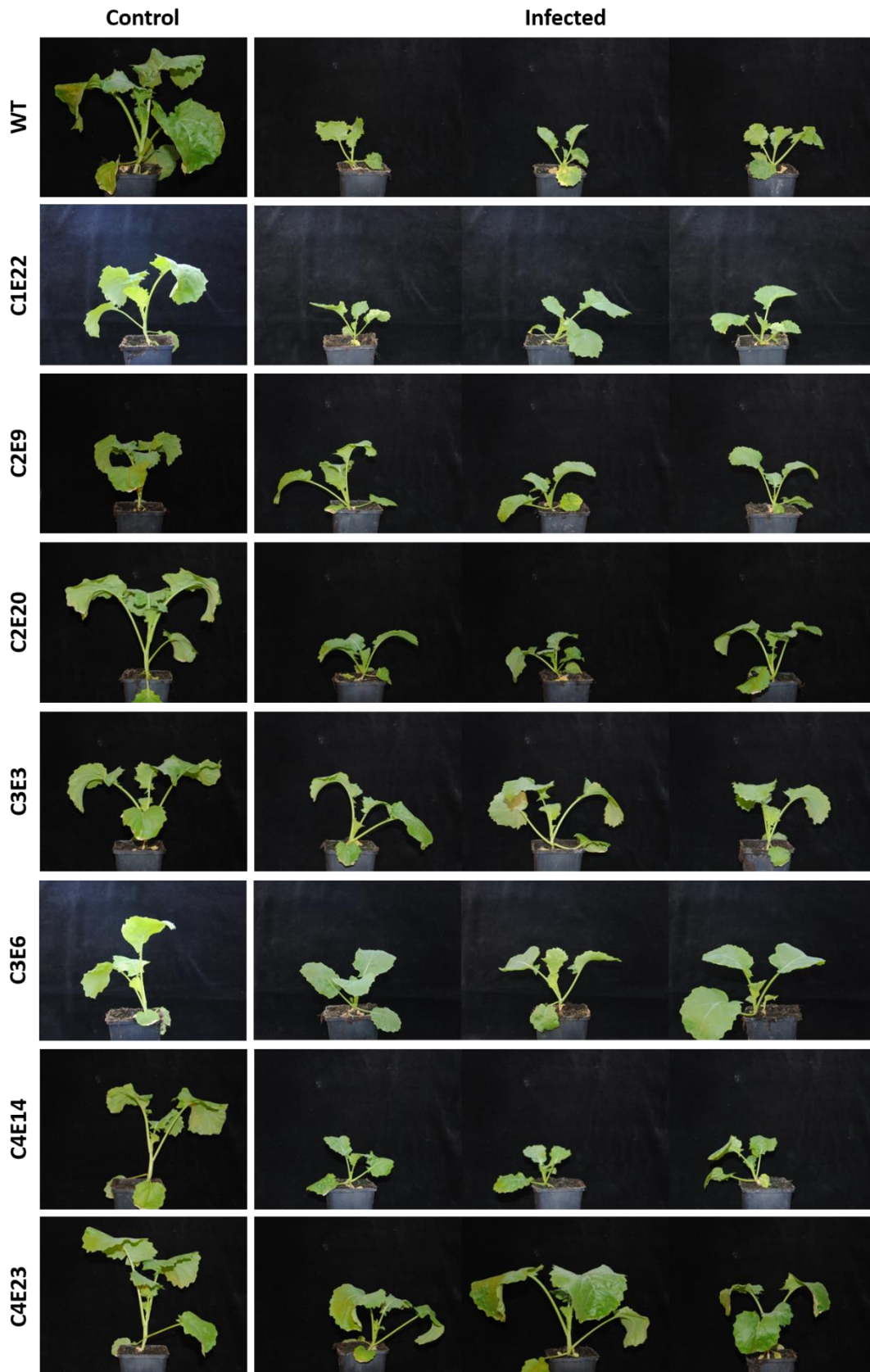


Figure 19: Selected *CRT1a* CRISPR mutants 28 dpi after infection with *Verticillium longisporum Vl43*

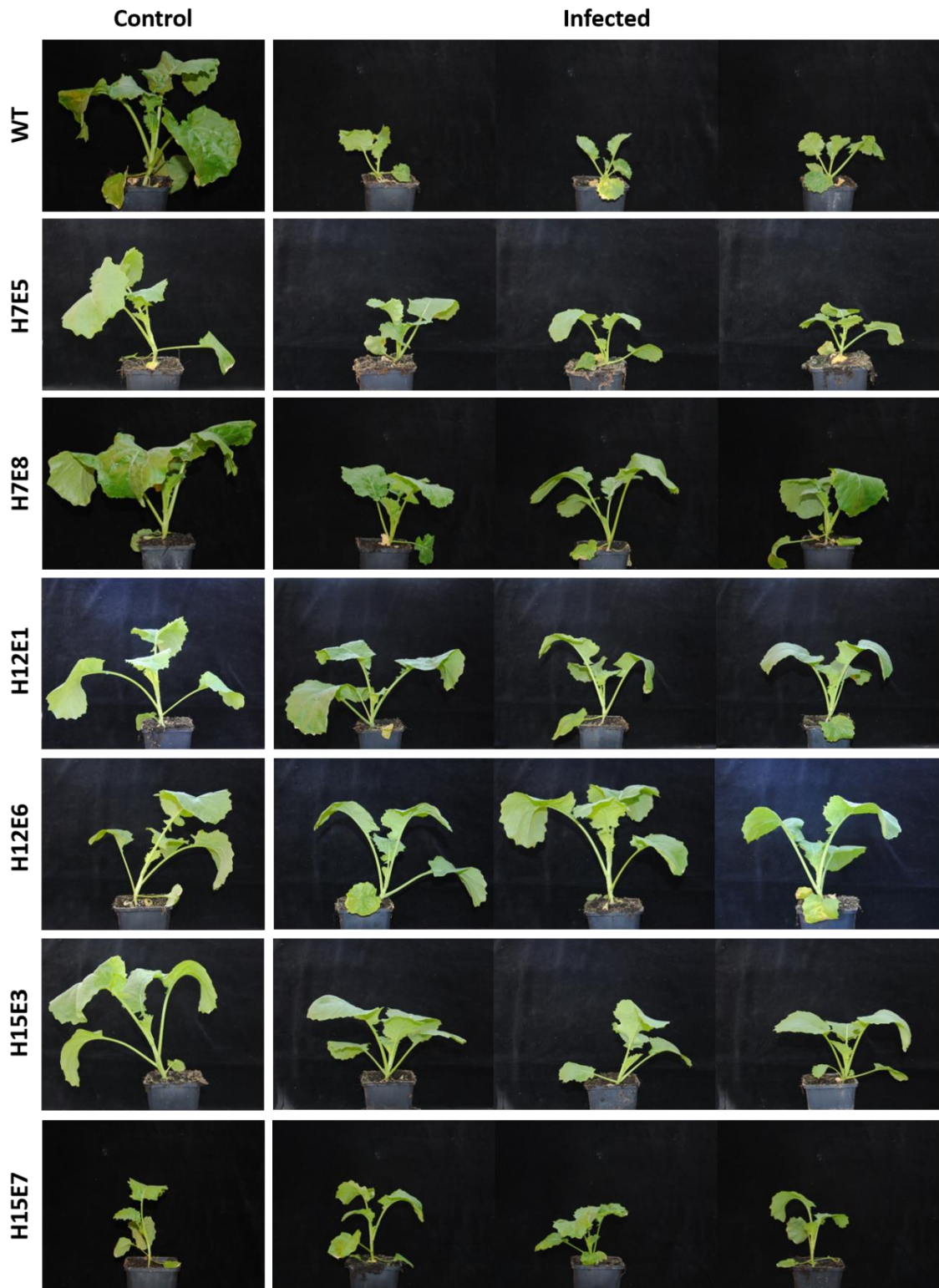


Figure 20: Selected *HVA22c* CRISPR mutants 28 dpi after infection with *Verticillium longisporum* VI43

Also, the AUDPCnorm was calculated for all genotypes to evaluate the disease severity of each genotype (Fig. 21). Disease rating was scored in the greenhouse under long-day conditions with 10 infected and five mock-treated plants for each event. Plants were inoculated *via* root dip and scoring was done every seven days after infection up to 28 dpi. Nearly all CRISPR mutants showed statistically significant lower AUDPCnorm values than the wild type except C2E20, C2E21 and C4E14 indicating a decreased susceptibility of mutants for both genes, which reflects the results of visual scoring at 28dpi.

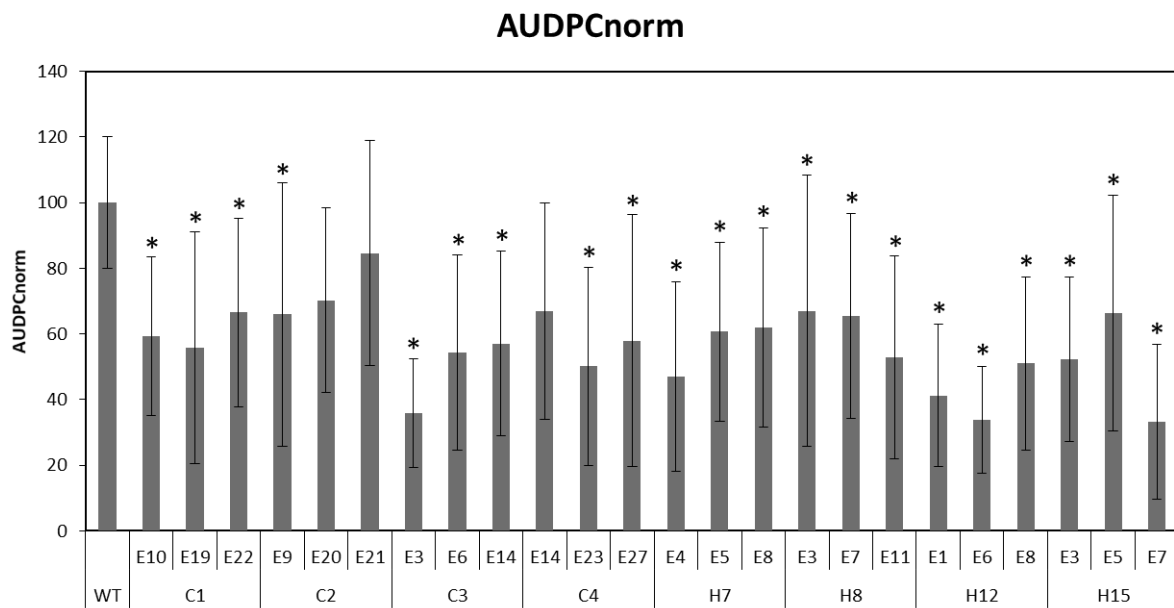


Figure 21: AUDPCnorm of all CRISPR genotypes infected with *Verticillium longisporum*. The AUDPC values were calculated according to Campbell and Madden (1990) and normalised against the wild type. For each genotype 10 infected and five control plants with one biological replicate were rated weekly until 28dpi. Error bars represent the standard error of the mean for 20 plants of two independent biological replicates and statistics were performed with an ANOVA and multiple comparisons using Dunnett's t-test ($*=p\leq 0.05$), comparing WT and CRISPR mutants.

A secondary factor to rate the disease severity is the stunted growth caused by the fungal infection. At the last rating date, the plant height of infected plants was measured and compared to the uninfected control of each event to determine the growth-depression (Fig. 22). Some genotypes showed a statistically significant lower growth depression than the wild type confirming that the knockout of *CRT1a* and *HVA22c* led to less susceptibility. Symptom severity and stunted growth often correlate in the *Verticillium* infection, therefore the AUDPCnorm and GD should be correlated. A linear model was used to determine the correlation between those two parameters (Fig. 23). The two parameters significantly correlated undermining the quality of collected data. This model helped us as well to select a range of genotypes for further experiments evaluating the gene expression of defence and candidate-related marker genes.

Growth depression (GD)

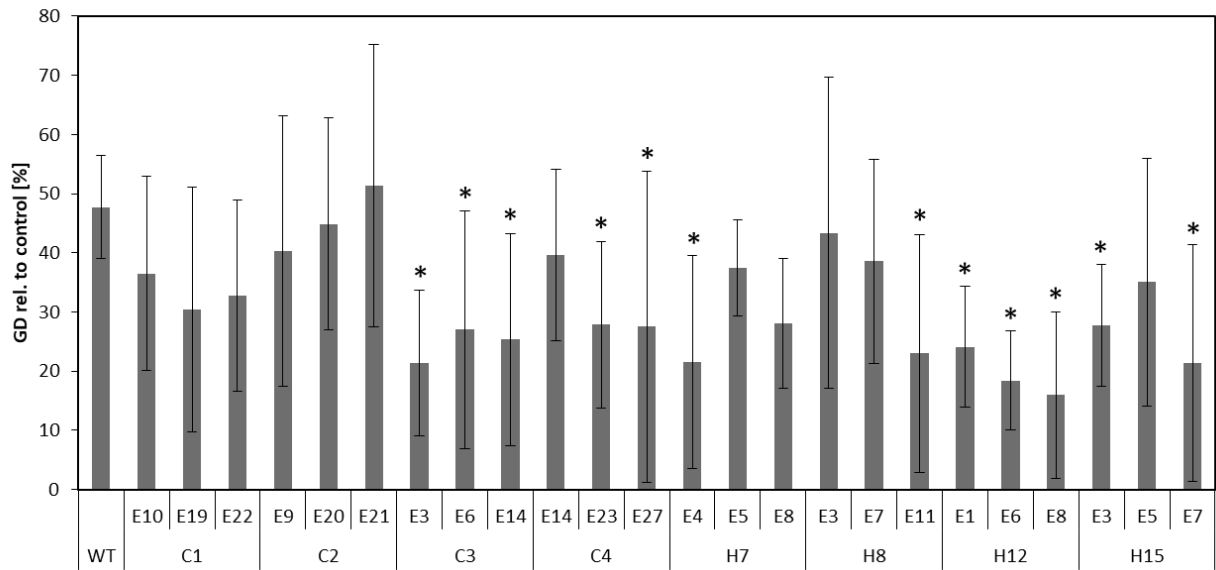


Figure 22: Growth Depression (GD) of all CRISPR mutants infected with *Verticillium longisporum*. The GD values were calculated through normalising the plant height of infected plants against their control plant height 28 dpi. For each genotype 10 infected and five control plants with one biological replicate were rated 28dpi. Error bars represent the standard error of the mean for 20 plants of two independent biological replicates and statistics were performed with an ANOVA and multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.

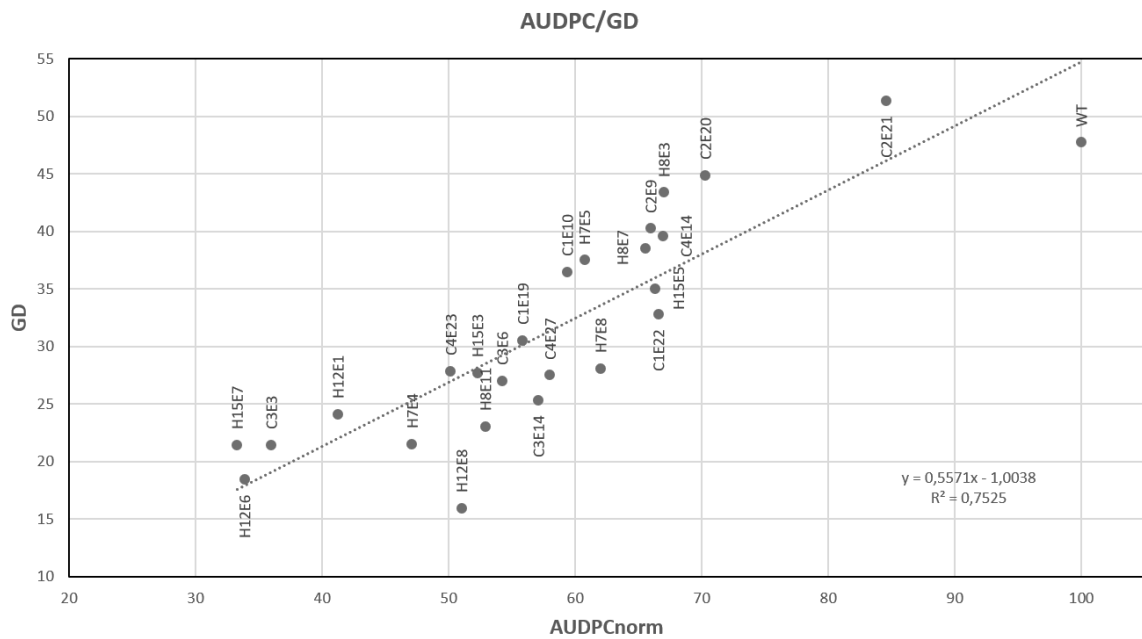


Figure 23: Linear model for correlation of AUDPCnorm and GD. The model was derived from the AUDPCnorm and GD values, which significantly correlated. Genotypes localised in the lower-left corner represent the least susceptible ones.

Evaluation of grain weight from CRISPR mutants

The grain weight was used as a parameter to evaluate potential negative side-effects on yield caused by the knockout of *CRT1a* or *HVA22c*. After the last disease rating, plants were further cultivated until full fruit ripening. The seeds from infected and mock-treated plants were harvested and the weight per 1000 seeds was measured (Fig. 24). Grain weight was measured from 10 infected and five mock-treated plants for each genotype. There was no significant difference between wild type and the mutants of both genes suggesting that there are no negative side effects caused by the mutation of the respective gene.

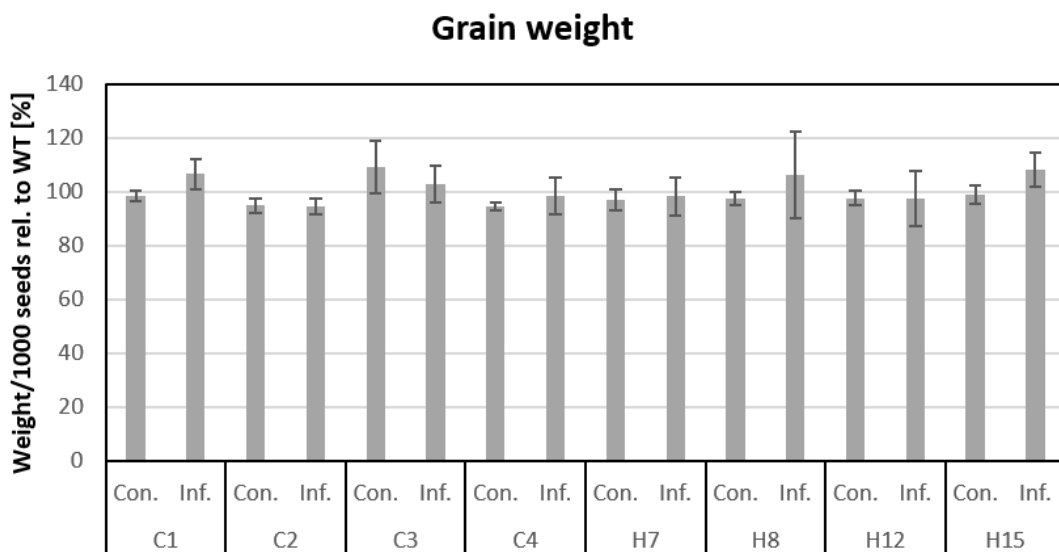


Figure 24: Seed weight per 1000 seeds normalized to the wild type. Error bars represent the standard error of the mean for 20 plants of two independent biological replicates and statistics were performed with an ANOVA and multiple comparisons using Dunnett's t-test ($*=p\leq 0.05$), comparing WT and CRISPR mutants.

The candidate plants for further experiments were selected based on the linear model (Fig. 23). Genotypes like C2E20 which did not perform as well as less susceptible genotypes like C3E3 were chosen for further characterisation. Finally, the genotypes C1E22, C2E9, C2E20, C3E3, C3E6, C4E14, C4E23 for *CRT1a* and H7E5, H7E8, H12E1, H12E6, H15E3, H15E7 for *HVA22c* were chosen for further experiments including quantification of fungal DNA in petioles and marker gene expression analysis.

Fungal DNA quantification in petioles of CRISPR mutants

Fungal DNA quantification was conducted with tissue samples harvested at the final rating date 28 dpi. The petioles of the first two true leaves were harvested and two plants were pooled into one sample to a total of three samples per event. For a first evaluation of the fungal DNA content, PCRs with fungus-specific *OLG* and plant-specific *PP2A* Primer were performed and the PCR products were run on a gel for relative fungal DNA quantification. Differences between the

samples could be observed while the wild type had the strongest bands for *OLG* PCR products. For a precise quantification, a qPCR using *PP2A* as a reference gene and *OLG* Primer for absolute fungal DNA quantification was carried out with selected genotypes (Fig. 25). All tested genotypes showed a lower content of fungal DNA than the wild type which fits the previous observations regarding a less susceptibility of mutant genotypes against *Verticillium longisporum*.

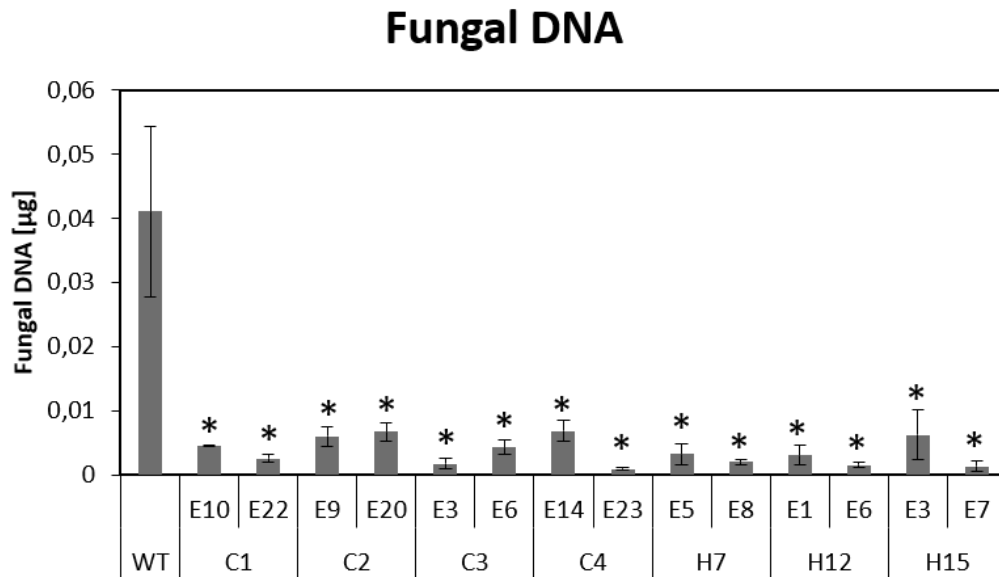










Figure 25: Absolute fungal DNA quantification of *Brassica napus* petioles 28 dpi with *Verticillium longisporum*. *Verticillium* specific *OLG* Primer were used in a qPCR for three samples consisting of petioles of two plants per genotype. Error bars represent the standard error of the mean for 6 plants and statistics were performed with multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.

To ascertain the relationship between pheno- and genotypic data, from each T0 event, three mutants showing varying susceptible degrees to the *V. longisporum* infection were chosen for T-vector sequencing of target loci. An average of 40 clones was sequenced for each genotype and different mutations were observed (Tab. 20). C4E23 and H12E6 represent the least susceptible genotypes with the highest mutation frequencies in the target loci from the three selected mutants, whereas C2E20 and H7E8 represent the most susceptible genotypes with the lowest mutation rate in target loci. This may lead to the conclusion that genotypes with a high mutation rate in the target loci of both genes are less susceptibility against *Vl43* infection and a dose-effect of mutated loci correlating with susceptibility might be the explanation for the range of disease severity among the mutants of *CRT1a* and *HVA22c*.

Table 20: Pheno- and genotypic data for the CRISPR mutants C2E20, C1E22, C4E23 for *BnCRT1a* as well as H7E8, H15E3, H12E3 for *BnHVA22c* compared to the wild type and rated for improvement of traits (+, ++, +++) or same characteristics as the wild type (=). Target loci were sequenced with single allele T-vector cloning. Insertions are indicated by green letters, while yellow letters indicate SNPs in WT.

Phenotype					Genotype
Plants 28 dpi	AUDPC /GD	Grain weight	Fungal DNA	T2 Mutations	
<i>BnCRT1a</i>					
WT					(A) TAAAC CCC AACTTCATCTCTCTGATTCTT A TCG (A) TAAAC CCC AACTTCATCTCTCTGATTCTT A TCG (C) TAAAC CCC AACTTCATCTCTCTGATTCTT T TCG (C) TAAAC CCC AACTTCATCTCTCTGATTCTT T TCG
C2E20		=	=	++	(A) TAAAC CCC AACTTCATCTCTCTGATTCTT A TCG (A) TAAAC CCC AACTTCATCTCTCTGATTCTT A TCG (C) TAAAC CCC AA--TCATCTCTCTGATTCTT T TCG (C) TAAAC CCC AACTTTCATCTCTCTGATTCTT T TCG
C1E22		++	=	+++	(A) TAAAC CCC AA C ATTTCATCTCTCTGATTCTT A TCG (A) TAAAC CCC AA C ATTTCATCTCTCTGATTCTT A TCG (C) -----CATCTCTCTGATTCTT T TCG (C) TAAAC CC ----TTCATCTCTCTGATTCTT T TCG
C4E23		+++	=	+++	(A) TAAAC CCC AA C TTCATCTCTCTGATTCTT A TCG (A) TAAAC CCC AA C TTCATCTCTCTGATTCTT A TCG (C) TAAAC CC ----TTCATCTCTCTGATTCTT T TCG (C) TAAAC CC ----TTCATCTCTCTGATTCTT T TCG
<i>BnHVA22c</i>					
WT					(A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG
H7E8		+	=	+++	(A/C) GCAG TCC ATT G GGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTG---GCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG
H15E3		++	=	+++	(A/C) GCAG TCC ATTG---GCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTG----CTCTCGTCTACCCTCTG (A/C) GCAG TCC ATT G TGGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG
H12E6		+++	=	+++	(A/C) GCAG TCC ATTG--TGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATT G TGGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATT G AGTTGCTCTCGTCTACCCTCTG (A/C) GCAG TCC ATTGGTTGCTCTCGTCTACCCTCTG

Marker gene expression in *CRT1a* and *HVA22c* CRISPR mutants

The resistance mechanisms *CRT1a* and *HVA22c* are involved in remain unclear. Trying to shed light on their function, expression of related hormone marker genes was determined. Seedlings of the genotypes C1E22, C2E20, C4E23 for *CRT1a* and H7E8, H12E6, H15E3 for *HVA22c* were grown *in vitro* and infected with *Verticillium longisporum*. Those mutants were chosen to have a range of from susceptible to less susceptible genotypes (Tab. 20). Six days after inoculation, the roots were harvested with three biological replicates per genotype consisting of root tissue from 6 seedlings. The RNA was isolated and cDNA synthesised. For expression analysis, the genes *PR1* and *WRKY70* were chosen as representatives for the salicylic acid pathway (Fig. 26). The general trend among all genotypes was the induction of those genes in the same strength as the wild type, whereas the genotype H15E3 showed less induction for *PR1* by infection with *V. longisporum*.

For ethylene, the marker genes *ACO1* and *ETR2* were chosen (Fig. 27). Those marker genes were upregulated compared to the control as well as showing the same trend as the salicylic acid marker genes. A difference in expression level between the genotypes was only significant between non-infected control of the wild type and the genotypes C4E23 and H15E3 for *ETR2*, whereas for *ACO1* only H15E3 had a significant difference in expression level to the wild type in the non-infected control.

The jasmonic acid pathway was represented by the marker genes *PDF1.2* and *LOX3* (Fig. 28). Both marker genes showed no general trend of up- or downregulation among the genotypes.

To further investigate the potential function of *CRT1a* and *HVA22c* expression of ER related and genes involved in vesicle transport, which were co-expressed with the candidate genes was evaluated. The genes *CNX1*, *PDCB3* and *PDIL1* were chosen but showed no differential expression compared to the wildtype in infected and non-infected plants (Fig. 29).

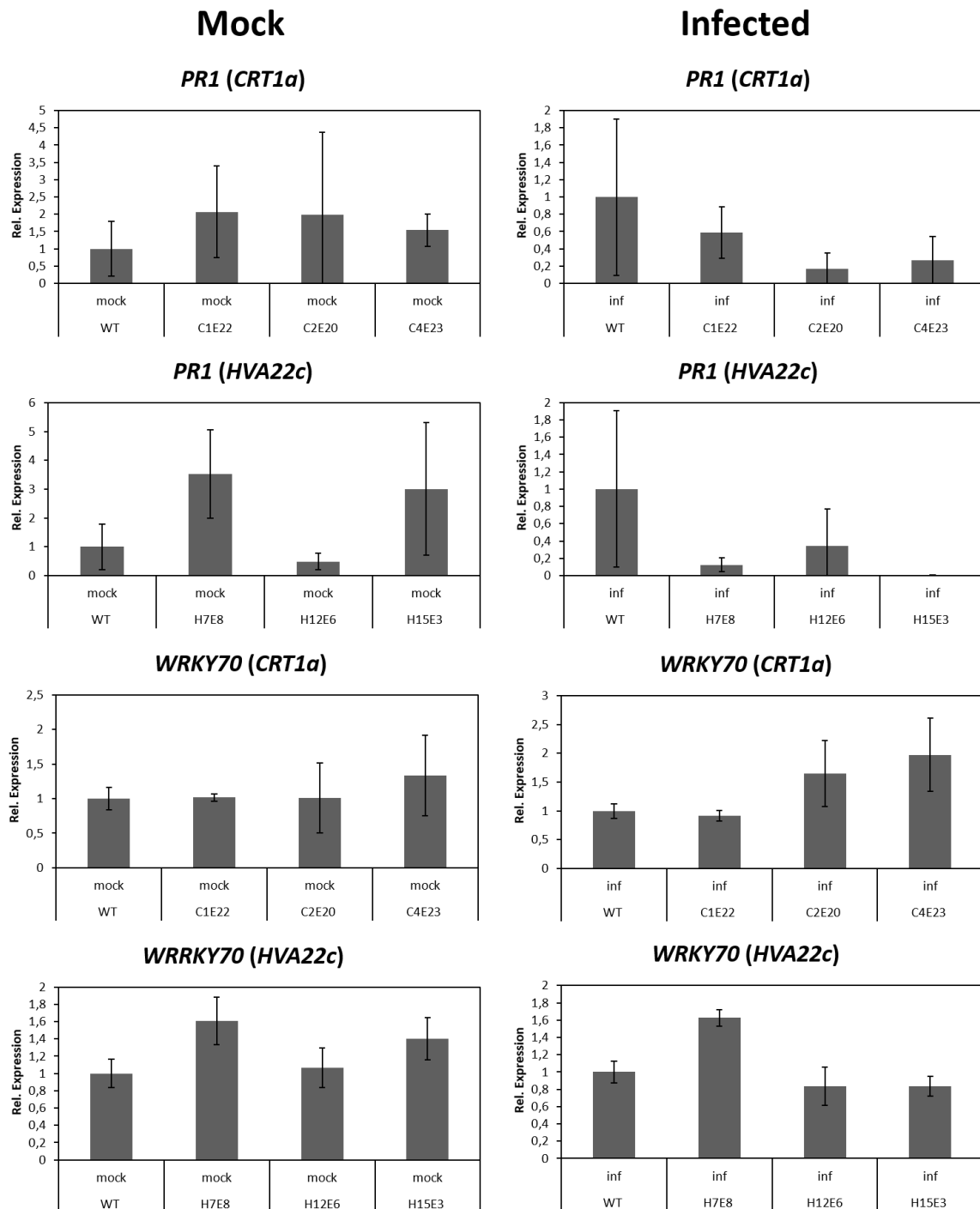


Figure 26: Induction of salicylic acid marker genes of *Brassica napus* seedlings at 6 dpi with *Verticillium longisporum*. Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected Cq values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test ($*=p\leq 0.05$), comparing WT and CRISPR mutants.

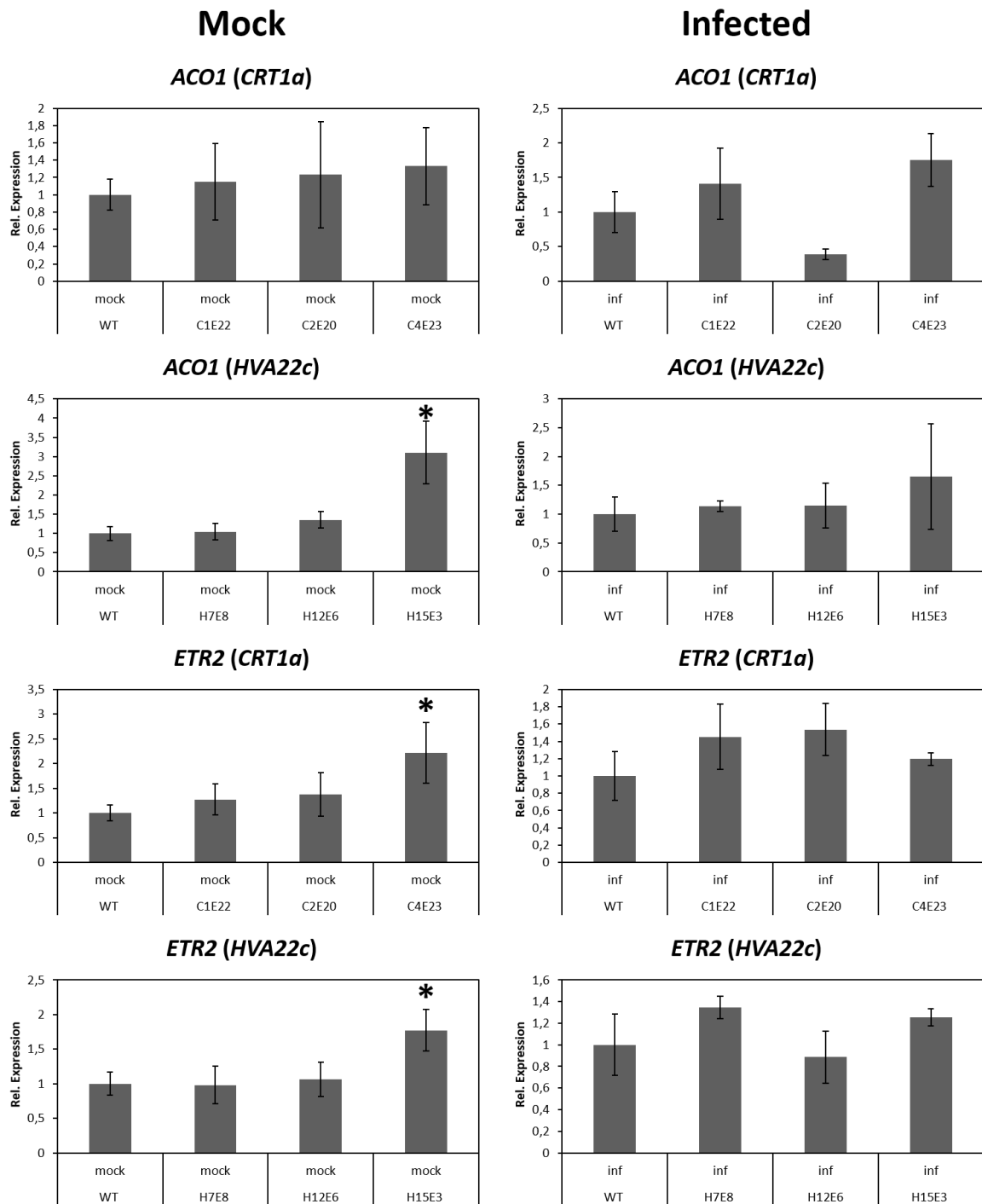


Figure 27: Induction of ethylene marker genes of *Brassica napus* seedlings at 6 dpi with *Verticillium longisporum*. Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected Cq values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.

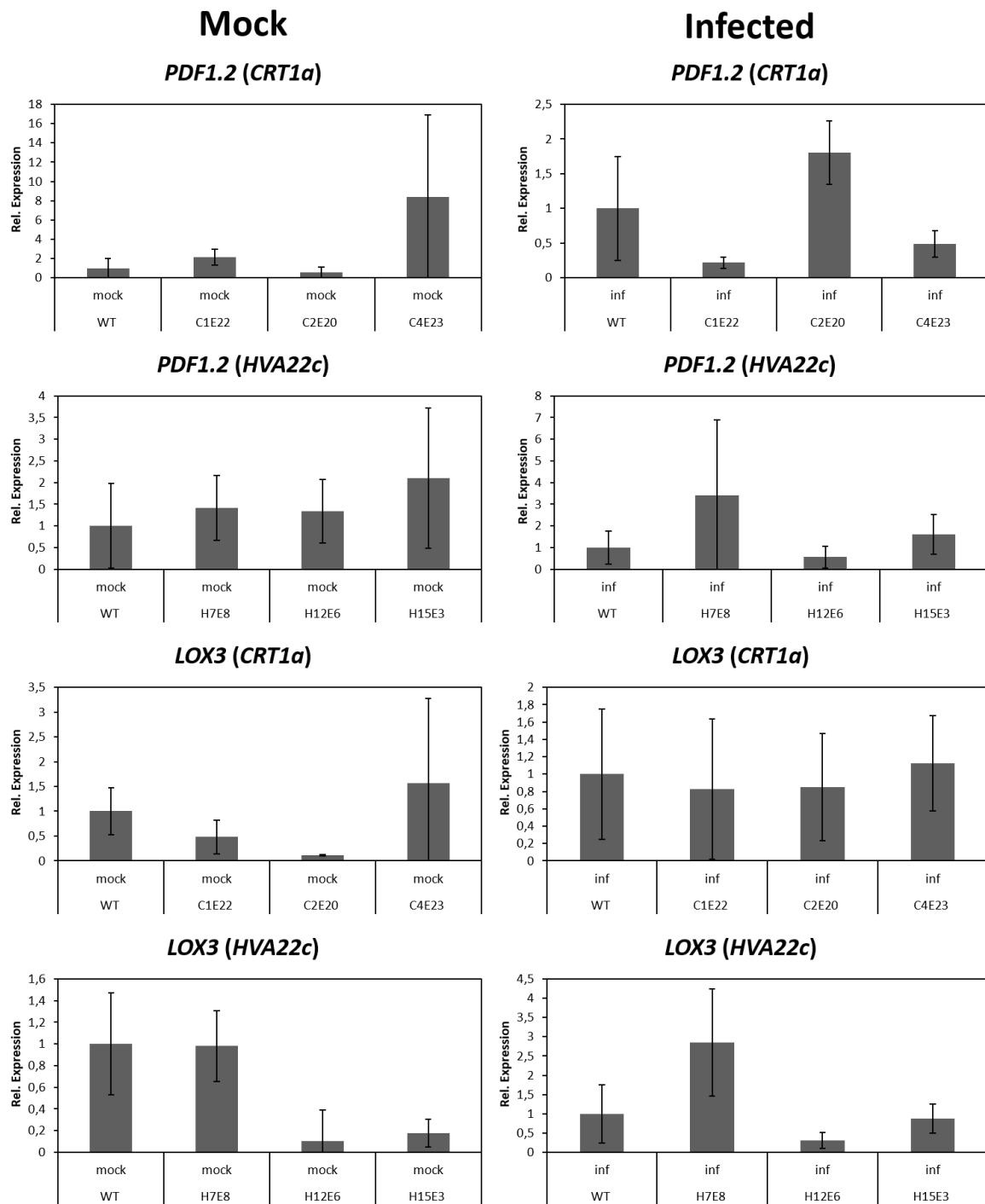


Figure 28: Induction of jasmonic acid marker genes of *Brassica napus* seedlings at 6 dpi with *Verticillium longisporum*. Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected Cq values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test ($*=p\leq 0.05$), comparing WT and CRISPR mutants.

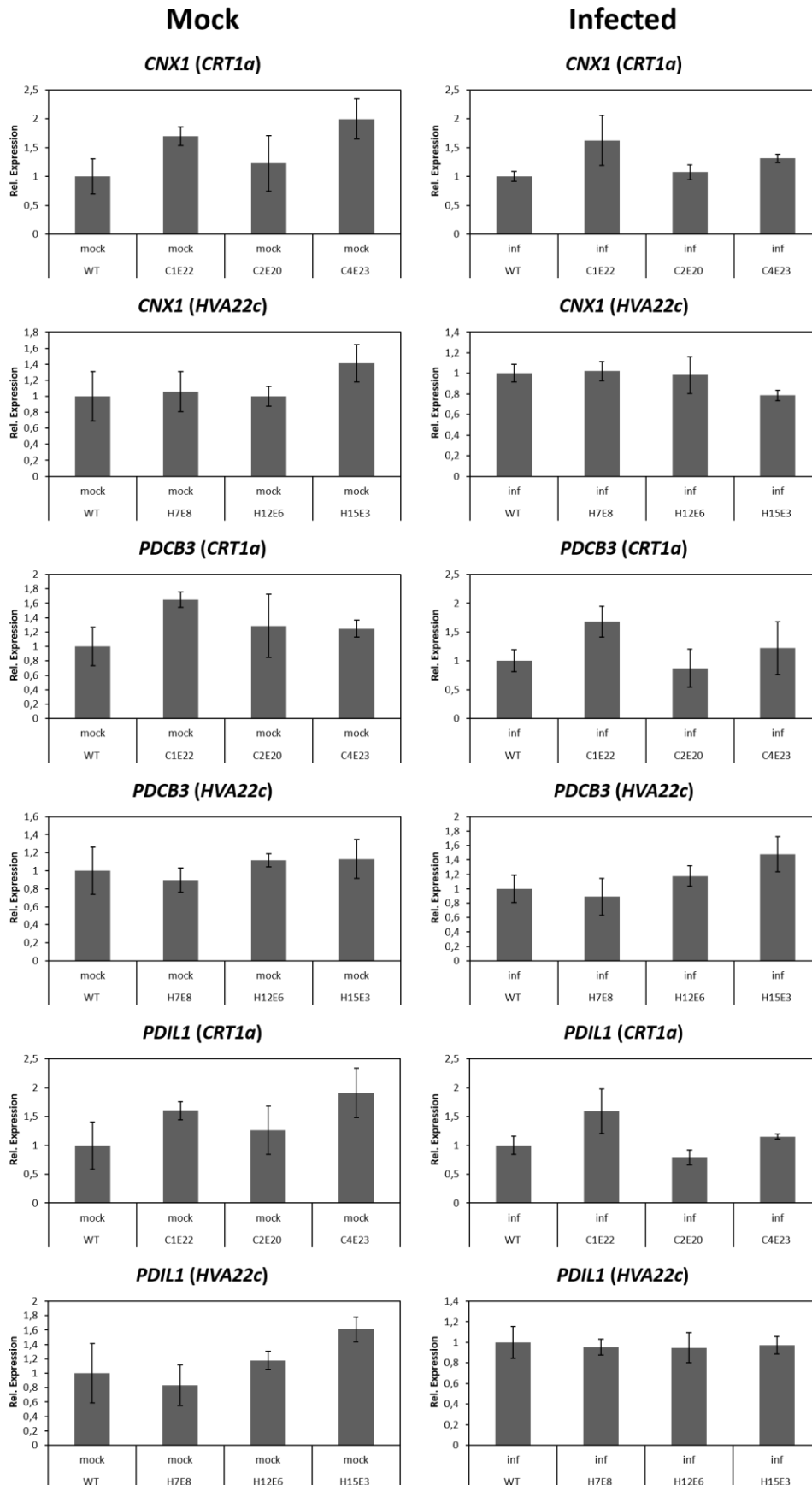


Figure 29: Induction of ER related genes of *Brassica napus* seedlings at 6 dpi with *Verticillium longisporum*. Relative expression normalised to PP2A/Actin2 was calculated with efficiency corrected Cq values. Error bars represent the standard error of the mean for three independent biological replicates and statistics were performed with multiple comparisons using Dunnett's t-test (*= $p \leq 0.05$), comparing WT and CRISPR mutants.

Vector systems for HDR based approaches

The first strategy for a successful application of HDR for genome editing was based on multiple template sequences. Those templates were localised within the T-DNA borders and flanked by adapters with a sequence identical to the target locus (Fig. 31). The template “chain” was produced from a synthesised single copy template for *A. thaliana*. PCR-generated *BclI* and *BamHI* RE-sites were added to the template sequence making it possible to clone it multiple times in the same plasmid. For the first ligation, the template was amplified with *BclI*_F and *BamHI*_R Primer and double digested with *BamHI/BclI*. pBS SK- served as the backbone and was cut with *BamHI*. This step was followed by another single template ligation into the same plasmid after successful cloning of the first template. As soon as two templates were in the same vector, they were amplified with *Vec/Re_BclI*_F Primer and the PCR product was cloned into the double template vector to achieve a quadruple template vector (Fig. 30).

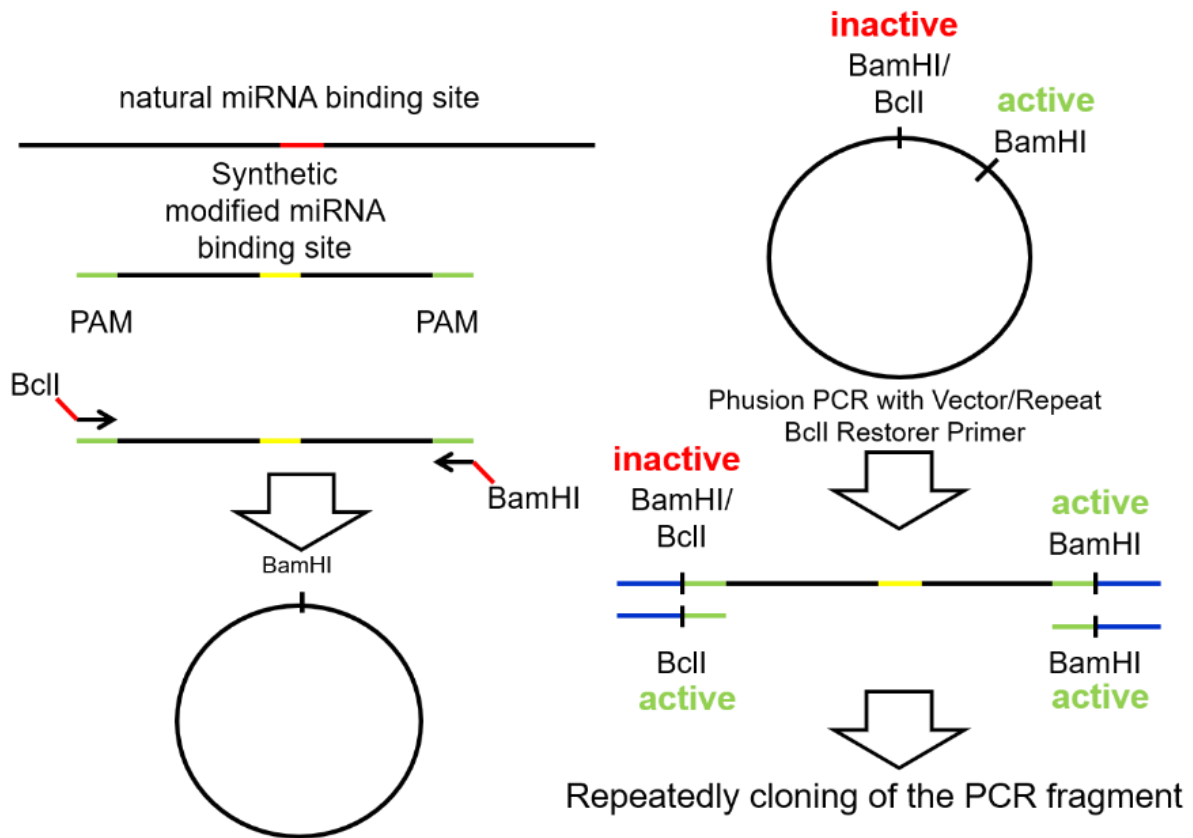


Figure 30: Cloning scheme for the production of template repeats. For the cloning procedure, *BclI* and *BamHI* recognition sequences are added via PCR to the template. After being cut both RE sites have similar overhangs but cannot be cut again if they are ligated to one another which inactivates the RE recognition sequence. If this fragment is ligated into a *BamHI* cut vector-only one side is still cuttable by *BamHI*. This principle was used to clone the fragment multiple times.

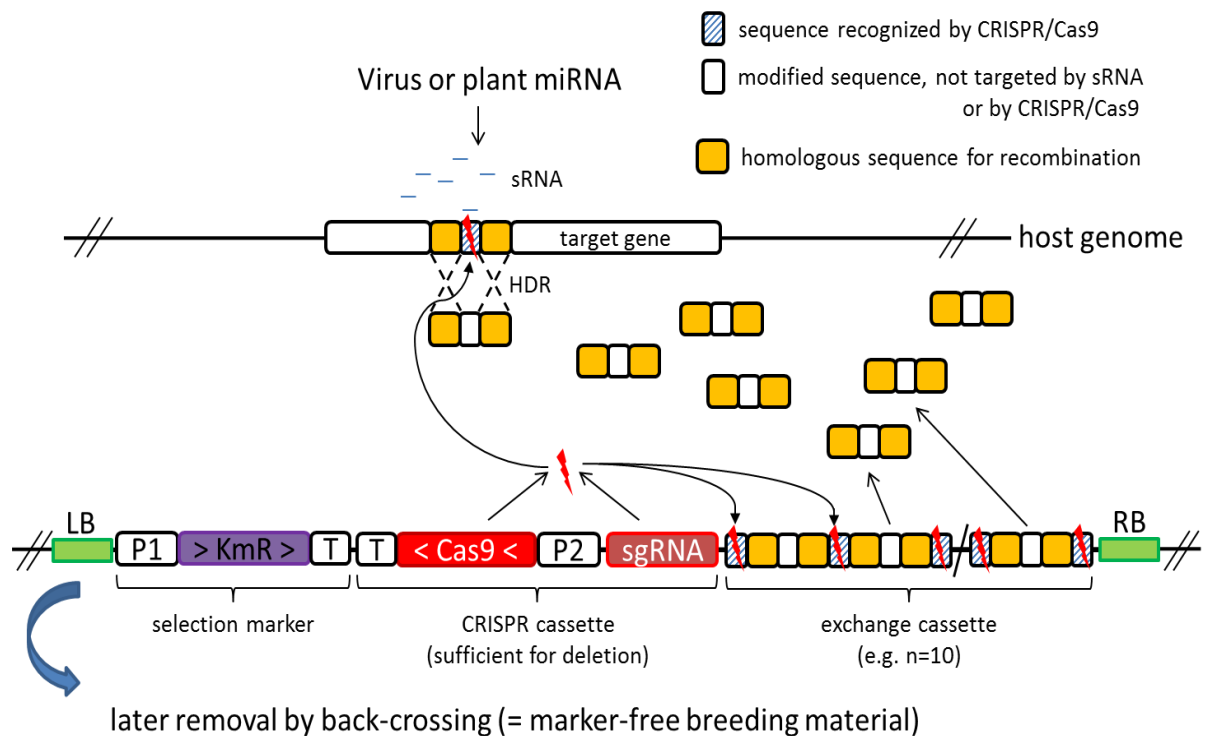


Figure 31: HDR approach for the exchange of miRNA binding sites using a repeat based vector for successful HDR. The expression cassette contains sgRNA and Cas9 for the induction of DSB and repair templates with adapter sequences which are targetable by the sgRNA to allow them to be cut out. In the presence of multiple of those repair templates, the HDR should be triggered and seamless exchange of the miRNA binding site should be possible. (Courtesy of Dirk Schenke).

The second strategy for HDR included a plasmid containing the disarmed genome of the bean yellow dwarf virus (pLSLR) (Baltes et al. 2014) which was modified for our studies (Fig. 32). The vector was cut with *Eco72I* and a fragment from the binary vector pGWB401 containing the M13fwd Primer sequence, attR, lacUV5 promoter, CmR coding sequence, ccdB coding sequence, attR2 and the Nos terminator was cloned into it. This fragment was obtained by double-digestion of the pGWB401 plasmid with *PmeI* and *Pdml*. This vector enables us to use the viral vector in the same way as pGWB401 being compatible with our CRISPR/Cas vectors. After ligation transformation was carried out into *E. coli* strain K12xB DB3.1 because this strain can survive the ccdB toxin and single colonies were identified *via* colony PCR and confirmed by sequencing.

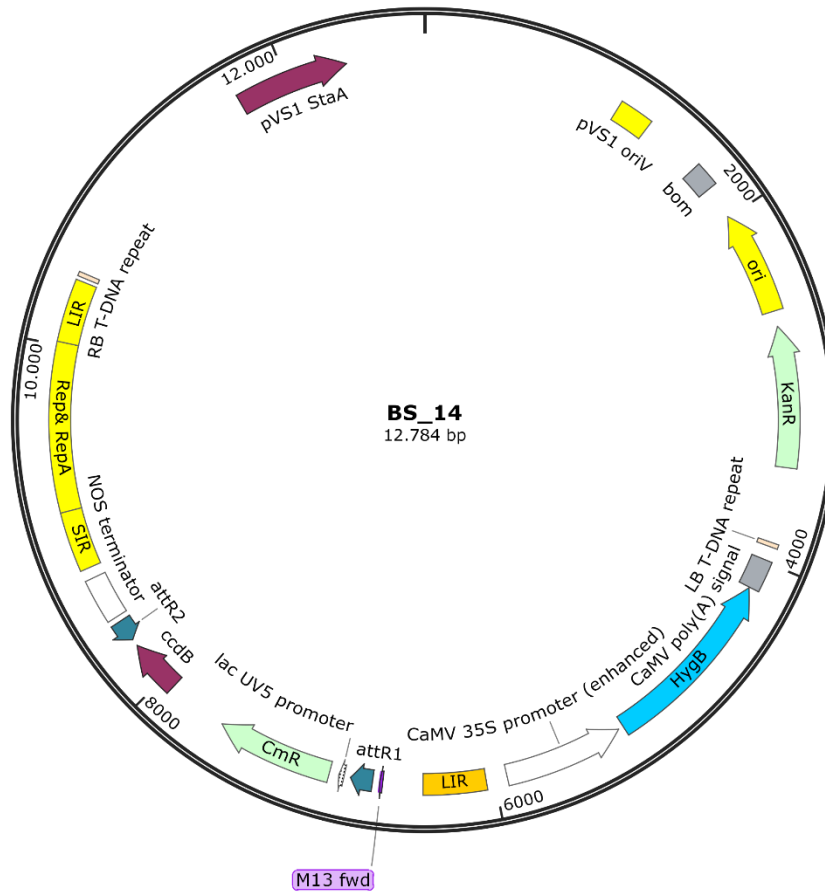


Figure 32: Vector map of BS_14. The vector was created by cloning a sequence fragment of the binary vector pGWB401 containing the M13fwd Primer sequence, attR, lacUV5 promoter, CmR coding sequence, ccdB coding sequence, attR2 and a Nos terminator was cloned into the disarmed genome of the bean yellow dwarf virus (pLSLR) (Baltes et al. 2014). The vector map was created by snap gene viewer.

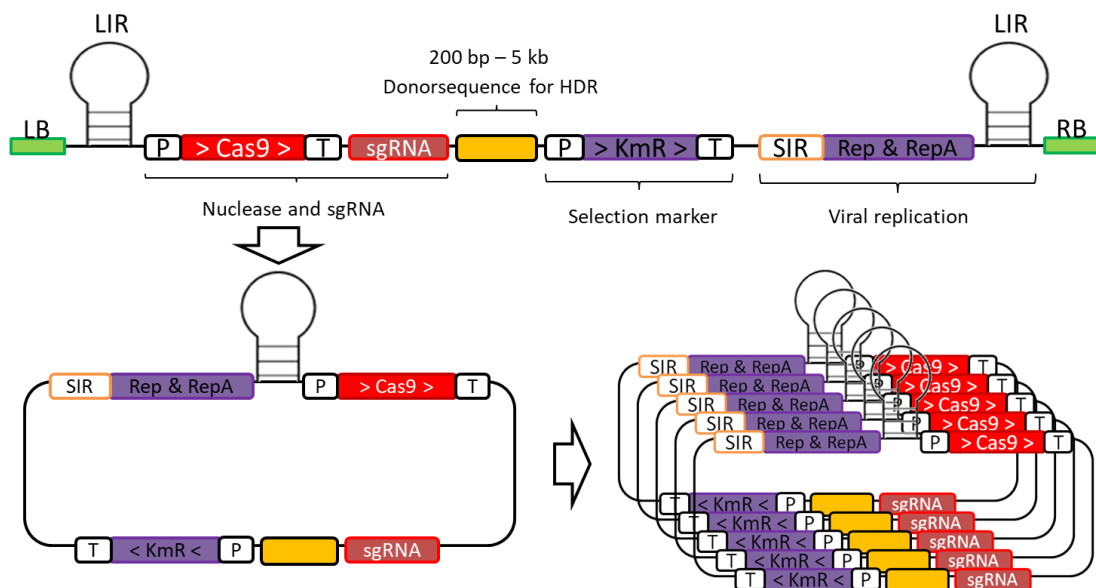


Figure 33: HDR approach for the exchange of miRNA binding sites using a vector with a disarmed genome of the Bean yellow dwarf virus for successful HDR. The expression cassette contains sgRNA and Cas9 for the induction of DSB and genes for the viral replication and LIR sites for the processing of the viral genome. Only a single copy of the repair template is cloned into this vector because the template is multiplied by replication of the virus.

Plasmids from positive colonies were isolated and transformed into the *ccdB* susceptible *E. coli* strain DH5 α to have a confirmation of a functioning *ccdB* gene and correct ligation. No colonies were obtained after this transformation which led to the conclusion that the integration of the fragment from pGWB401 into pLSLR was successful. Plasmids with our Cas9 and sgRNAs for *BnHVA22c_2* and *StCesa3*, which all were confirmed to be functional, were used as entry clone to check whether the modified pLSLR is functional.

In summary, we cloned several constructs with different repair template approaches and sgRNAs for the HDR based approach and respective genes (Tab. 21).

Table 21: Plasmids cloned in this study for the NHEJ approach with information about the promoter, nuclease, sgRNA target, repair template amount and backbone.

Plasmid name	Promoter	Nuclease	sgRNA target	Repair template number	Backbone
BS_13	35s	Cas9 (B. napus codon optimized)	<i>AtAGO1</i>	Multiple	pGWB401
BS_14	35s	Cas9 (B. napus codon optimized)	-	-	Viral replicon
BS_15	35s	Cas9 (B. napus codon optimized)	<i>BnHVA22c_2</i>	-	Viral replicon
BS_17	35s	Cas9 (B. napus codon optimized)	<i>AtAGO1</i>	One	Viral replicon
BS_18	35s	Cas9 (B. napus codon optimized)	<i>BnAGO1</i>	One	Viral replicon
BS_19	35s	Cas9 (B. napus codon optimized)	<i>BnTAO1_A_1</i> & <i>BnTAO1_A_2</i>	One	Viral replicon

First HDR strategy with multimers

To test the functionality for HDR with multiple repeats on the binary plasmid, hairy roots of *Arabidopsis thaliana* were generated with construct BS_13. The construct for this gene was chosen because it served as a selection marker as well. Knockouts of this gene are expected to cause lethal mutations and only positive HDR events should generate viable transgenic events. The DNA of those roots was isolated and screening for a potential HDR event was carried out *via* PCR. A Primer that binds only to the mutated miRNA binding site was used in combination with another binding in the genome outside the template to exclude that the mutated templates caused false-positive signals. No PCR fragments using this Primer combination were detected in confirmed transgenic roots leading to the conclusion that either the transformation method was not suitable or the sgRNA was not functional (Fig. 34).

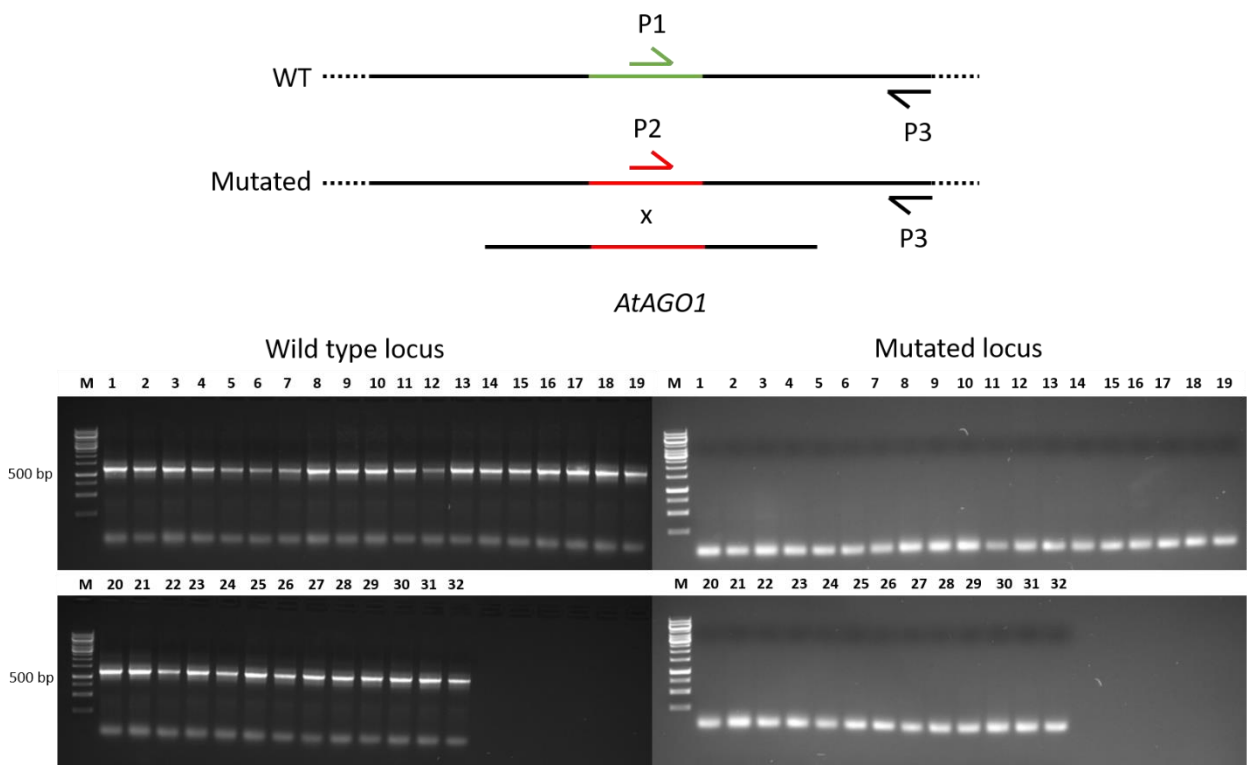


Figure 34: PCR based screening for positive HDR events for *AGO1* in *A. thaliana*. Target loci the gene were amplified with Primer binding to the wild type sequence and outside the repair template range. With the same samples, a second PCR with Primer binding to the modified sequence and outside the range of the repair template was conducted to have a double confirmation in case of potential positive HDR events.

Second HDR strategy with viral replicon

For the viral replicon approach, *B. napus* and *S. tuberosum* were transformed with the respective constructs and sgRNAs. The first step was to confirm whether the viral genome is processed correctly inside the plant cell. A PCR with Primer which only amplify a PCR product from the circular viral genome was conducted from transgenic *B. napus* hairy roots. Several independent transgenic events showed a band which indicated that the viral genome is processed correctly (Fig. 35).

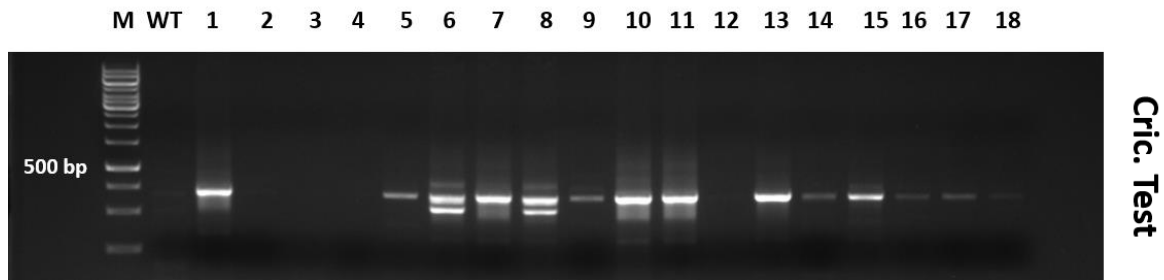


Figure 35: PCR for the screening of processed viral genomes. The gDNA was derived from transgenic *Brassica napus* hairy roots transformed with the viral vector. The Primer used for this PCR are localized down and upstream of the LIR and only amplify if the viral genome is processed into its circular form.

We tested the vector with a sgRNA targeting *BnHVA22c* and loci were amplified from genotypes that were positive for the circularisation test and digested PCR products with *AsuII* to check whether mutations were induced. Several undigested bands could be observed. Undigested bands from this assay were excised from the gel and cloned into t-vector and sequenced for confirmation of a successful mutation (Fig. 36). Mutations in the sgRNA target loci could be confirmed leading to the conclusion that the viral Cas9 construct is functional.

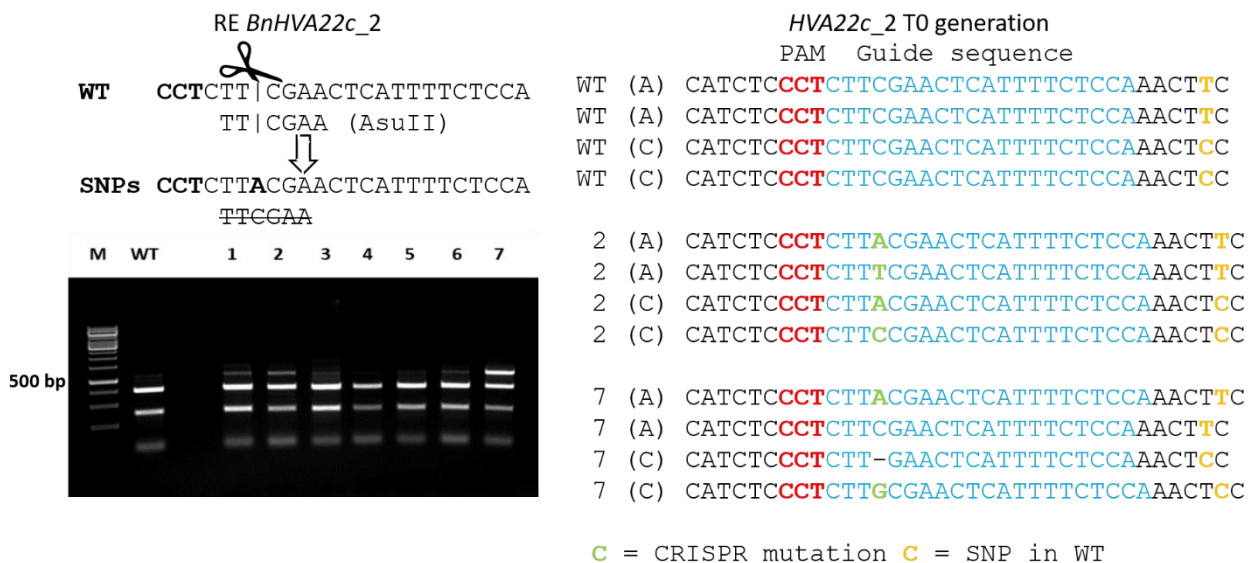


Figure 36: RE-Assay and sequencing of undigested bands of *HVA22c_2* locus for mutation screening. The sgRNA target locus was amplified from different transgenic hairy roots from *Brassica napus* transformed with the BS_14 construct. PCR products were incubated with restriction enzyme *AsuI* to screen for potential mutations. The wild type locus (WT) was completely digested and the undigested wild type PCR product served as a reference for undigested bands (UD). Several genotypes showed undigested bands suggesting potential mutations in the respective genomes. Additionally, the undigested bands were cloned into t-vector and sequenced.

We further investigated the longevity and mutation potential of the viral genome in the plant. Hairy roots were harvested for DNA isolation 5 and 7 weeks after the first roots emerged. Viral genomes were detectable up to 7 weeks but also in 1 of 5 events the viral replicon was already

inactive after 5 weeks. We were not able to observe an increase in mutation rate between both time points (Fig. 37).

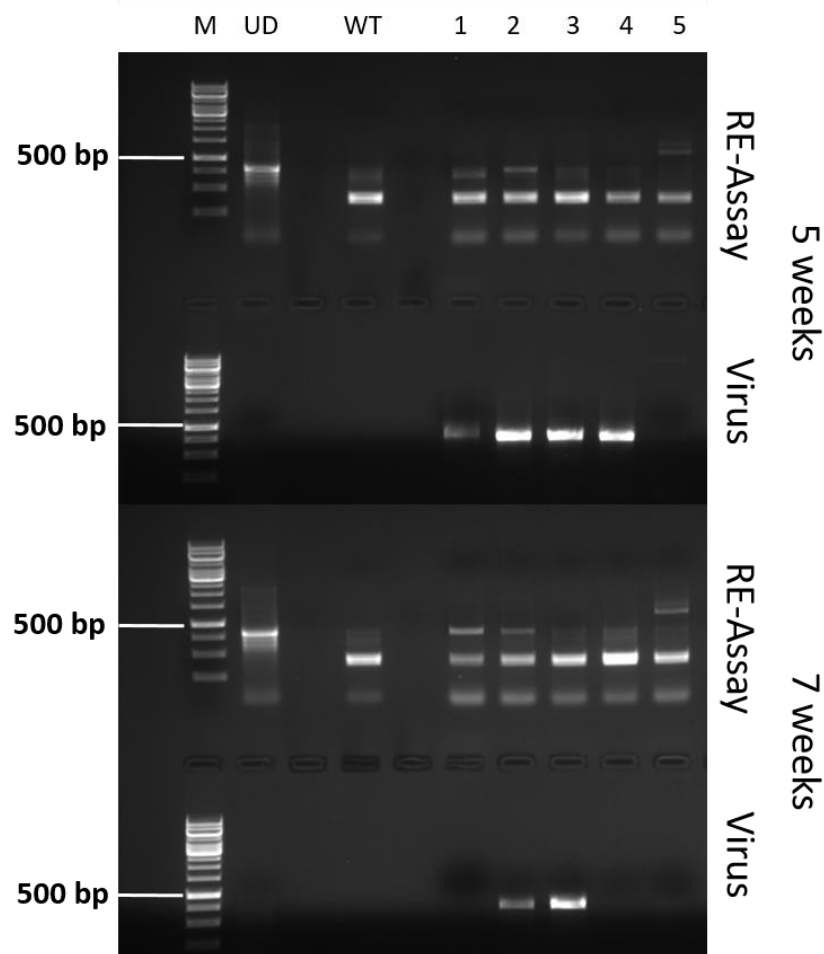


Figure 37: RE-Assay of *HVA22c_2* locus for mutation screening in combination with virus persistence testing. The sgRNA target locus was amplified from different transgenic hairy roots from *Brassica napus* transformed with the BS_15 construct. Samples were harvested 5 and 7 weeks after the first roots emerged. PCR products were incubated with restriction enzyme *AsuI* to screen for potential mutations. The wild type locus (WT) was completely digested and the undigested wild type PCR product served as a reference for undigested bands (UD). Several events showed undigested bands suggesting potential mutations in the respective genomes.

For a final approach to generate positive HDR events the viral vector in combination with the hairy roots transformation system appears to be the most promising option, combining a simple transformation with a high transformation efficiency. The genes *AGO1* and *TAO1* in *B. napus* containing miRNA binding sites which served as targets were chosen for this experiment. Templates for recombination were generated from gDNA and mutated *via* PCR. From 100 hairy roots for each gene, 52 independent *BnAGO1* and 55 independent *BnTAO1* positive events were obtained after transformation of *B. napus* with *A. rhizogenes*. DNA of those roots was isolated and screening for a potential HDR event was conducted *via* PCR as in previous experiments. No PCR fragments with the correct length of 1kb for a positive HDR event were observed for both genes (Fig. 38).

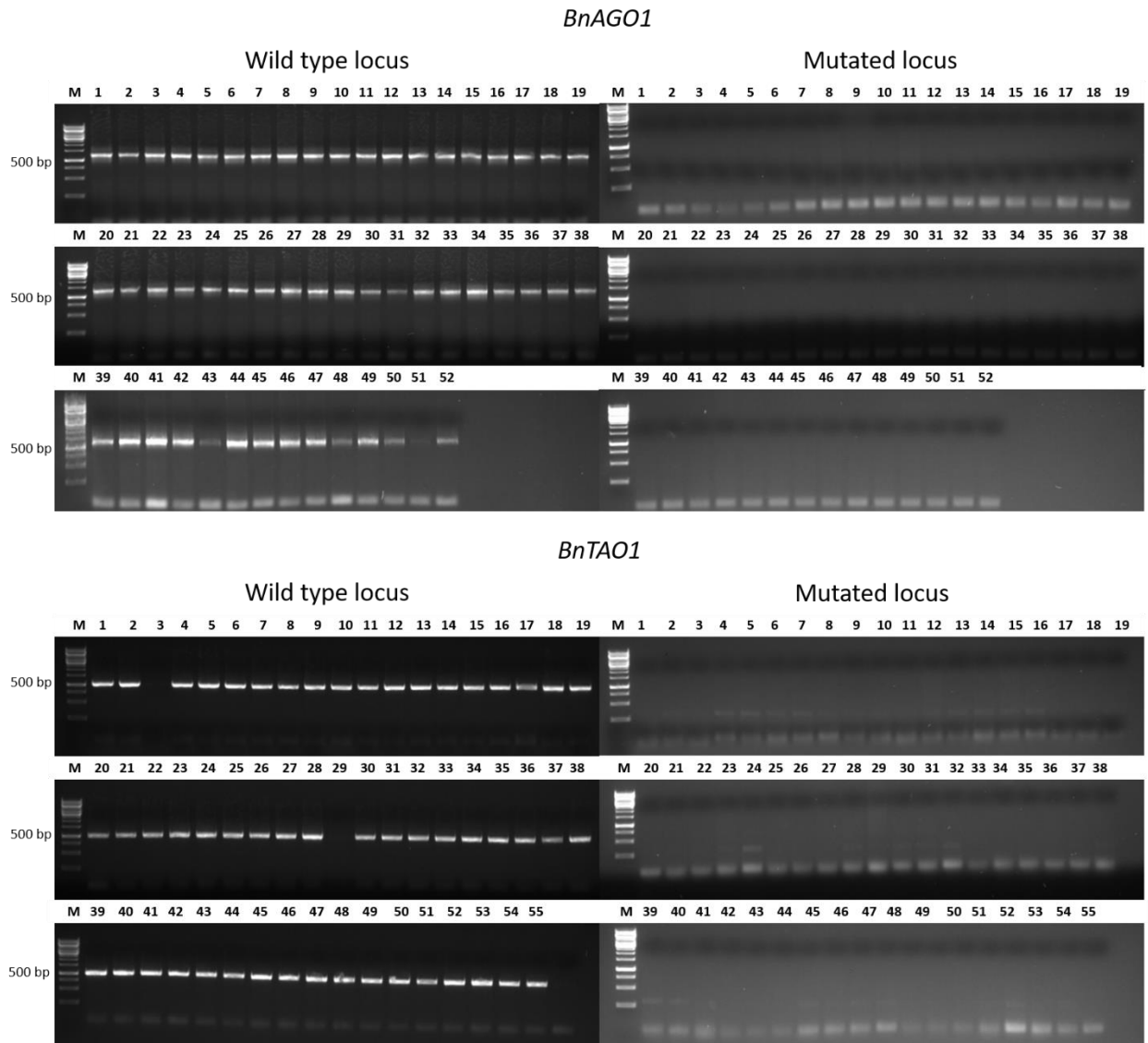


Figure 38: PCR based screening for positive HDR events for *AGO1* and *TAO1_A* from *B.napus* hairy roots. Target loci for both genes were amplified with Primer binding to the wild type sequence and outside the repair template range. With the same samples, a second PCR with Primer binding to the modified sequence and outside the range of the repair template was conducted to have a double confirmation in case of potential positive HDR events. Unfortunately, no bands could be observed at the correct length of 1 kb for the mutated locus.

Discussion

The demands towards modern agriculture to maintain stable yields, increased productivity and flexibility become more and more challenging. Especially considering growing population rates, reduced land availability for agriculture and climate change, new crop varieties are needed for the sustainable food supply chain. In addition to negative abiotic effects, the resistance against pathogens is a major factor to maintain stable productivity under increasingly “pathogen friendly” culture conditions referring to a lack of crop rotation, reduced soil tilling and crops growing in vast monocultures. The genetic crop improvement, not only for quality traits but for resistances against their major pathogens as well, helps to prevent a decrease in productivity. *Brassica napus* has become an important crop species in Europe, Canada and China primarily for vegetable oil production and high protein animal feed (USDA 2019). One major soilborne fungal pathogen targeting *Brassica napus* is *Verticillium longisporum* causing Verticillium stem striping and a premature ripening leading to yield losses up to 50 % (Dunker et al., 2008).

Due to its relatively young age as a species, *B. napus* has a very narrow genetic pool and no effective resistance against *V. longisporum* could be identified so far (Depotter et al., 2016). Some quantitative genetic approaches identified QTL loci that confer resistance but the mechanisms and responsible genes remain unknown (Rygulla et al., 2008; Obermeier et al., 2013). Genetic manipulation of crops by transfer of genes conferring resistance against pathogens has limited public acceptance and is often unstable (Barakate and Stephens, 2017). However, with emerging new technologies for genome editing in the form of programmable nucleases, it is now possible to create a new source of resistance *via* knocking out genes that are required by the pathogen to colonize the host successfully. The public perception of this approach is much more positive and no artificial genetic elements will stay in the crop’s genomes.

Potential susceptibility factors in the *B. napus*-*V. longisporum* interaction were identified by differential gene expression deploying an SSH library (Häder, 2013). From initially 20 selected genes, 15 genes were tested by evaluating the resistance of *A. thaliana* T-DNA insertion lines in the corresponding orthologous gene loci. Two mutants that showed an impeded infection compared to wild type cv. ‘Col-0’, were identified as genotypes with T-DNA insertions in the genes *CRT1a* and *HVA22c*. The knockout of those genes resulted in an impeded infection process and fungal DNA accumulation, leading to the suggestion that those genes might function as susceptibility factors. For confirmation of their role as susceptibility factors in this interaction, a TILLING population of *B. napus* was screened for mutations in the respective loci and infected as

well (Hossain, 2017). Several candidate genotypes with single mutations in the target genes *CRT1a* and *HVA22c* that introduce a stop codon in the coding sequence could be identified. Unfortunately, independent knockout genotypes for both genes did not show consistent results regarding the resistance against infection with *V/43*. This suggested strong side effects caused by the EMS mutational background. To overcome the enormous number of off-target mutations induced by undirected mutagenesis and drastically influencing the resistance in an unpredictable manner, the genes *CRT1a* and *HVA22c* were selected as targets for a specific gene knockout through CRISPR/Cas9. This approach should allow us to confirm their role as susceptibility factors without the negative effect of the mutation background of TILLING mutants. For this directed mutagenesis approach, we generated and established a Cas9 expressions cassette. In an additional approach, we aim to alter the sequence of miRNA binding sites without affecting the gene function *via* HDR. Gene disruptions can have unpredictable side effects with negative influence on desired traits of a crop, especially in regard to susceptibility factors which are often involved in natural biological processes. Potential targets are the genes *AGO1* and *TAO1*, which are targeted by fungal miRNAs in the early stages of the infection of *B. napus* by *V. longisporum*. The colonisation of the plant by the fungus may be impeded without modulation of those genes. Two vector systems based on repair template multimers and a viral genome were established and utilised for HDR.

The optimised CRISPR/Cas9 vector system proved to be a functional and versatile tool for genome editing in *Brassica napus*

First CRISPR/Cas9 vector systems in plants had a relatively low editing efficiency (Li et al., 2013; Nekrasov et al., 2013) and several studies tried to improve the efficiency by modifying components of the respective vector system adapting it to the target organism (Tab. 22).

Table 22: CRISPR/Cas9 vector systems designed for stable transformation in *Oryza sativa* and *Arabidopsis thaliana*. Modified after Ma et al., 2016.

Organism	Cas9 promoter	Cas9 codon optimisation	sgRNA promoter	Multiplex strategy	Editing efficiency	Reference
<i>Oryza sativa</i>	2 × P35S	<i>O. sativa</i>	<i>OsU3</i>	–	8.2%	Shan et al., 2013
	<i>OsUbi</i>	<i>H. sapiens</i>	<i>OsU3</i>	Sequential cloning	45.3%	Zhang et al., 2014
	2 × P35S	plant	<i>AtU6</i>	-	8.6%	Xu et al., 2014
	<i>OsUbi</i>	<i>O. sativa</i>	<i>OsU3</i>	Polycistronic tRNA process system	Up to 100%	Xie et al., 2015
	<i>ZmUbi</i>	<i>O. sativa</i>	<i>OsU3</i> , <i>OsU6a</i> , <i>OsU6b</i> , <i>OsU6c</i>	Golden Gate/Gibson assembly	85.4%	Ma et al., 2015
<i>Arabidopsis thaliana</i>	<i>AtUbi</i>	<i>H. sapiens</i>	<i>AtU6</i>	Sequential cloning	81.2%	Feng et al., 2014
	<i>PcUbi</i>	<i>A. thaliana</i>	<i>AtU6</i>	–	26.7%	Fausser et al., 2014
	2 × P35S	<i>Z. mays</i>	<i>AtU6</i>	Golden Gate/Gibson assembly	82.6%	Xing et al., 2014
	<i>ICU2</i>	<i>O. sativa</i>	<i>AtU6</i>	Sequential cloning	20.6%	Hyun et al., 2015
	2 × P35S	<i>O. sativa</i>	<i>AtU3</i> <i>AtU6</i>	Golden Gate/Gibson assembly	35.6%	Ma et al., 2015
	<i>EC1.2</i>	<i>A. thaliana</i>	<i>AtU6</i>	–	–	Wang et al., 2015
<i>AtUbi</i>	<i>A. thaliana</i>	<i>AtU6</i> , <i>AtU3b</i> , <i>At7SL</i>	Multiple enzymes/sequential cloning	13%–93%	Zhang et al., 2015	

The promoter of sgRNAs and Cas9, alteration of codon usage to the respective target organism and multiplex strategies were adaptations, trying to improve the editing performance. We decided to assemble our system with a *B. napus* codon-optimised Cas9 driven by the 35s promoter, an AtU6 promoter for the sgRNA and sequential cloning as a multiplex strategy. We reasoned our choice for those specifications with the fact that similar systems had on average high editing efficiencies in *A. thaliana* and *O. sativa*. But in summary no definite factor or component was detectable from previous studies that strongly elevated editing efficiencies, even a Cas9 with a codon usage for *H. sapiens* enabled editing frequencies up to 81.2% (Feng et al., 2014) in Arabidopsis.

In initial experiments, transgenic events were generated from *Solanum tuberosum* cv. Baltica as well as Granada and used for functional testing of the vector system. The Cas9 expression and translation could be successfully observed utilising RT-PCRs and Western Blots as well as the translocation of the GFP-Cas9 fusion protein to the nucleus. Experiments to determine the editing capability were carried out in *A. thaliana* cv. Col-0 to test the vector system in a more closely related species to *B. napus*. We could not observe any mutations in the *AtAGO10* loci of transgenic *A. thaliana* events with Sanger sequencing in T0 and T1 generation. Nevertheless, *B. napus* cv. Mozart was transformed with this vector system and we could detect mutations in the target loci for *BnCRT1a* and *BnHVA22c* with mutations in 22.7% in *CRT1a* and 35% in *HVA22c* events. Editing frequencies do not only rely on the specifications of the vector system but secondary factors as well, making it difficult to estimate a precise construct efficiency. One of those factors is the chromatin state of the target locus and genomic region. These target loci can differ in chromatin configuration affecting the binding and thus the editing efficiency of Cas9. DNA methylation does not directly impede binding and cleavage, but nucleosomes can completely block Cas9 access and thereby inhibit genome editing (Verkuijl and Rots, 2019). A potential source for the lack of mutations in Arabidopsis might be the transformation system floral dip combined with our 35s driven Cas9 nuclease. In similar approaches for genome editing in *A. thaliana* floral dip is the standard method to generate transgenic events. However, the 35s promoter is reported to have low efficiency in this system and can lead to chimeric plant because while it is only active from the two-cell stage of the embryo (Wang et al., 2015). A possible solution to overcome this problem was introduced by the same authors which is the exchange of the promoter with an egg cell-specific promoter allowing a higher transcriptional activity in the one-cell stage of embryos.

Another factor could be a non-functional sgRNA which is difficult to exclude because even sgRNA with a high score *in silico* can vary significantly *in vivo* (Verkuijl and Rots, 2019).

In summary, the vector system could induce mutations in *B. napus* with an average mutation rate of 22.7% (*BnCRT1a*) and 35% (*BnHVA22c*). Modifications by exchanging vector components may increase the editing efficiency but external factors, e.g. the chromatin state can have an enormous influence on editing efficiency.

Hairy roots represent a fast and simple *in vivo* test system for sgRNA functionality and HDR based approaches

Testing new vectors and expression cassettes *in vivo* is time-consuming and a stable transformation is needed to efficiently evaluate their function in the respective organism. *Agrobacterium tumefaciens* mediated transformation is often not suitable or very inefficient for crops and requires a lot of handling expertise for successful generation of transgenic events. *Agrobacterium rhizogenes* mediated transformation offers several advantages compared to *A. tumefaciens* e.g. a fast production of transgenic material, simple transformation, and less tissue specificity. In general, hairy roots transformation is used in many studies to produce a large amount of biomass for heterologous protein production which relies on the fast generation of transgenic tissue (Cardon et al. 2019). With regard to genome editing, this transformation system has another valuable application, the assessment of sgRNA functionality *in vivo* in the target crop. Despite many algorithms and design tools available the sgRNA functionality can differ immensely if *in silico* and *in vivo* results are compared (Verkuijl and Rots, 2019) and an *in vivo* test system is providing more reliable information. Establishing hairy roots as a test system for CRISPR with high mutation rates has been conducted successfully in similar approaches (Tab. 23).

We utilised the hairy roots transformation method for *B. napus* as an efficient test system to provide fast results regarding the sgRNA functionality and for the HDR approach. Hypocotyls from *in vitro* grown seedlings of the cultivar Mozart were chosen as starting material, similar to other approaches (Tab. 23), and were transformed with our CRISPR/Cas9 expression cassette. Transgenic roots for DNA extraction could be obtained as fast as two weeks after transformation. Previous studies relied on sterile leaf discs as starting tissue for hairy roots transformation in *B. napus* (Coniglio et al., 2008; Angelini et al. 2011). In our case, hypocotyls were kept as starting tissue due to short cultivation time of seedlings. This system proved to be functional but only induced low mutation frequencies in hairy roots transformed with sgRNA HVA22c_2. In transgenic roots for the HDR based approach, a high percentage of 52% positive transgenic events for *BnAGO1* and 56% for *BnTAO1* could be achieved but unfortunately, no positive recombination events could be

observed. Further experiments have to be conducted to determine the factor that leads to low mutation rates and no positive HDR events. A possible factor could be an insufficient number of independent events or a non-functional sgRNA. To overcome the potential error of a non-functional sgRNA from the start, two sgRNAs targeting the same locus were incorporated into the vector system, which did not lead to positive HDR events either.

Table 23: CRISPR/Cas9 approaches utilising hairy roots transformation

Organism	Target gene	Mutated events (%)	Starting material	Reference
<i>Glycine max</i> L.	<i>Bar</i> , <i>GmFEI2</i> and <i>GmSHR</i>	54	Hypocotyl and cotyledonary node	Cai et al., 2015
<i>Glycine max</i> L.	<i>GFP</i> , <i>MIR genes</i> (<i>miR1514</i> and <i>miR1509</i>)	95	Hypocotyl and cotyledonary node	Jacobs et al., 2015
<i>Glycine max</i> L.	<i>Glyma06g14180</i> , <i>Glyma08g02290</i> and <i>Glyma12g37050</i>	14.7-20.2	Hypocotyl and cotyledonary node	Sun et al., 2015
<i>Glycine max</i> L. and <i>Medicago truncatula</i>	<i>GmGS1</i> and <i>GmCHI20</i> <i>GUS</i> (<i>M. truncatula</i>)	NA	Cotyledons	Michno et al., 2015
<i>Glycine max</i> L.	<i>GmPDS11</i> and <i>GmPDS18</i>	12.5-48.1	Hypocotyl and cotyledonary node	Du et al., 2016
<i>Solanum tuberosum</i>	<i>St16DOX</i>	NA	Shoots	Nakayasu et al., 2018
<i>Brassica carinata</i>	<i>BcFLA1</i>	NA	Seedlings	Kirchner et al., 2017
<i>Cichorium intybus</i> L.	<i>CiPDS</i>	31.25	Leaves	Bernard et al., 2019

In conclusion, hairy roots represent a fast and simple transformation system that applies to nearly all plant species and cultivars. A large number of transgenic independent events can be generated in a relatively short amount of time which makes this transformation system very attractive for sgRNA testing and HDR based approaches.

dCAPS assay screening and inheritance of mutations of CRISPR mutants

Genome editing induces several possible genotypes where some events may be wild type due to either lack of sgRNA as well as Cas9 expression or a lack of efficient target cleavage. sgRNA performing well in *in vitro* cleavage assays have been reported to underperform under *in vivo* conditions suggesting that factors beyond target sequence have an impact on the mutation rate as has been shown in zebrafish (Uusi-Mäkelä et al., 2018). Heterochromatin can negatively influence the mutation rate by affecting Cas9 binding and functioning mainly through nucleosomes completely blocking the Cas9 protein from associating with the DNA (Verkuijl and Rots, 2018). These findings have a high impact taking into consideration that epigenetic factors differ in cell tissues. To genetically characterise CRISPR mutants, which may be homozygous or heterozygous for mutations at the target locus, several methods are available to verify desired edits. Mismatch-cleavage assay or T7 Endonuclease I assay provides a semi-quantitative estimation of the percentage of mutated alleles and is based on the cleavage of DNA heteroduplexes caused by mutations. A disadvantage of this method is the possible insensitivity to SNPs and different efficiencies depending on the kind of mismatch (Guan et al., 2004). If considered and possible to implement during sgRNA design, restriction digestion is another method to detect mutations at the target loci. Mutations in the wild type genomic sequence alter the naturally present restriction enzyme site and undigested bands indicate potential mutations. This principle can be applied as well even if no recognition site is available at the target locus utilising dCAPS assay (Neff et al., 1998). This marker implements a restriction enzyme site in the wild type PCR product through a mutated Primer allowing to estimate the mutation rate by restriction enzyme assay. For final confirmation of potential mutations, sequencing of the target loci has to be carried out with Sanger sequencing, which allows a semi-quantitative assessment of mutation rate, or next-generation sequencing which allows a quantitative evaluation.

Four independent T0 events for *CRT1a* and *HVA22c* with high mutation rates were chosen on the basis of dCAPS assay screening and confirmed by Sanger sequencing. The dCAPS screening and sequencing analysis had similar results but have to be combined to draw a realistic picture of mutated loci from transgenic events if the whole genome is not sequenced. Interestingly, the *HVA22c* CRISPR mutants H7 and H8 showed a high mutation rate in the dCAPS assay but only 1-2 mutation types were identified with Sanger sequencing so far. If those genotypes were only screened with Sanger sequencing, the mutation rate might have been underestimated and the genotypes may not have been selected for further experiments. Ultimately, the *CRT1a* CRISPR mutants C1, C2, C3 and C4 as well as the *HVA22c* CRISPR mutants H7, H8, H12 and H 15 were

chosen for further propagation by self-pollination. Twenty plants of the progeny from each T0 event were screened with dCAPS assay to select three T1 individuals with the highest mutation rates for self-pollination and generation of T2 populations. The mutation rate did not increase significantly and several of the induced mutations were stably inherited from the T0 to the T2 generation. A crucial factor for a high mutation rate seems to be the duration of the callus phase *in vitro* and extending this phase significantly increased the mutation rates in transformed rice tissue (Mikami et al. 2015).

The Knockout/mutation of CRT1a and HVA22c confers less susceptibility to *Brassica napus* against *Verticillium longisporum* infection

Potential candidate genes or susceptibility factors involved in plant-pathogen interaction of fungi and plants have been previously described and already been targeted by CRISPR/Cas9. An example is the increased resistance of grapevine against *Botrytis cinerea* caused by a knockout of the *VvWRKY52* transcription factor (Wang et al., 2018). The resistance of homozygous mutated genotypes and wild type plants against *B. cinerea* was compared and CRISPR mutants showed up to 20% less spreading lesions while the general phenotype did not differ from the wild type, increasing the applicability of this source of resistance. Another example for resistance generation by knockout of susceptibility factors focussing on fungal pathogens was conducted in rice conferring an increased resistance against *Magnaporthe oryzae* by disrupting the *OsERF922* and *OsSEC3A* genes (Wang et al., 2016; Ma et al., 2018). The mechanism behind this resistance originated in a disruption of a putative subunit of a complex involved in exocytosis resulting in high levels of salicylic acid content upregulation of pathogenesis- and SA-related genes. Unfortunately, this resulted in a dwarf phenotype which is an agronomic trade-off often observed in plants with increased pathogen resistance and makes this target less attractive (Ma et al., 2018). An approach to increase fungal resistance against *Sclerotinia sclerotiorum* in *Brassica napus* is the only application of genome editing in OSR to confer resistance against a fungal pathogen so far. *WRKY70* is involved in multiple processes such as leaf senescence (Besseau et al., 2012), BR signalling processes (Chen et al., 2017) and participates in plant immune processes in *A. thaliana* (Zhou et al., 2018). Mutation of the six *BnWRKY70* loci *via* CRISPR/Cas9 increased resistance to *Sclerotinia sclerotiorum* and less severe infection was observed (Sun et al., 2018).

The candidate genes *CRT1a* and *HVA22c* are assumed to play a role in the early stages of the infection with *V. longisporum* in *B. napus* which were identified in earlier experiments (Häder,

2013). Their homologues in *B. napus* were identified by a database search at Genescope revealing four distinct CRT1a, from which only two (BnaA09g15400D and BnaC09g16150D) were taken into consideration because of missing sequence data, and four HVA22c loci. sgRNAs for each candidate gene were designed and transgenic events generated. The T2 populations of selected CRISPR mutants showed less susceptibility against *V. longisporum* VI43 infection under greenhouse conditions compared to the wild type Mozart. The AUDPC, growth depression and fungal DNA content in petioles were measured and showed uniform results suggesting a less susceptibility of CRT1a and HVA22c CRISPR mutants. These findings are in consent with results from previous experiments with T-DNA knockout lines from *A. thaliana* and TILLING mutants from *B. napus*. Interestingly, the combination of mutated and wild type alleles for CRT1a seems to be modulating the resistance strength. For a complete picture of the mutation pattern and potential off-target mutations, selected CRISPR mutants of CRT1a and HVA22c have to be fully sequenced. Sanger sequence as the only sequencing method can never deliver information about all loci simultaneously and only for semi-quantitative detection is possible.

The role of BnCRT1a and HVA22c as susceptibility factors could be proven in these experiments but the exact mutation rate of all alleles has to be further determined. Apparently, even heterozygous mutated genotypes have less susceptibility against VI43 infection and can provide a novel genetic source for resistance breeding.

The involvement of CRT1a and HVA22c in *B. napus*- *V. longisporum* interaction remains unclear
Calreticulins were initially described in rabbit skeletal muscle sarcoplasmic reticulum where they serve as a Ca²⁺ binding protein (Ostwald and MacLennan, 1974). Members of this gene family were first isolated from spinach and tobacco in plants (Menegazzi et al., 1993, Denecke et al., 1995). CRT1a is expressed in all tissues due to its involvement in fundamental biological processes related to protein folding but has a higher expression rate in flowers, root tips and vascular tissue (Christensen et al., 2008). They may have a similar protein function in plants and animals due to their high structural similarities in both kingdoms (Coppolino and Dedhar, 1998). CRTs contain three different domains an N-terminal globular domain with chaperone function, a central proline-rich domain for high-affinity Ca²⁺ binding and a C-terminal acidic domain responsible for Ca²⁺ buffering with a KDEL ER retention signal (Sarwat and Tuteja, 2017). It was shown that AtCRT1a could substitute the function of animal CRTs which strengthens the hypothesis of similar protein function in plants and animals (Christensen et al., 2008). Major functions of this protein in plants

are the Ca²⁺ homeostasis and protein folding in the ER and Golgi apparatus (Borisjuk et al., 1998; Persson et al., 2001; Wyatt et al., 2002). Studies to identify members of the CRT gene family in *A. thaliana* could reveal the existence of three genes *CRT1a*, *CRT1b* and *CRT3* (Coppolino and Dedhar, 1998) and it appears that isoforms have different functions but can compensate each other if single isoforms are not functional for *CRT1a* and *CRT1b* (Christensen et al., 2010; Kim et al., 2013). The role of *CRT1a* as a susceptibility factor could be explained by the potential function of *CRT1a* in protein folding or posttranslational modification. Thus, it can be assumed that a loss of function in CRTs may also result in a misfolding and degradation of other yet unknown susceptibility factors to have an opposite and beneficial effect. Another potential mode of action could be the affection of a negative regulator in the ethylene (ET) signalling pathway by CRTs.

HVA22c was first identified in the aleurone layer of barley (Shen et al., 1993) and is present in eukaryotes such as plants, fungi and human. This gene is expressed in different plant tissues being inducible by abiotic stress (salinity, water deprivation) or ABA and is localised in the cell membrane and plasmodesma (Guo et al., 2008, Fernandez-Calvino et al., 2011). In *A. thaliana* five homologues were identified referred to as *AtHVA22a*, *AtHVA22b*, *AtHVA22c*, *AtHVA22d* and *AtHVA22e*, which under stress conditions or ABA treatment showed differential expression (Chen et al., 2002). In citrus, six members of the HVA family could be identified and the genes *CsHVA22b* and *CsHVA22c* showed a similar expression pattern in all tissues at a lower intensity for *CsHVA22b* indicating that compensating effect by other members of the gene family may be possible (Ferreira et al., 2019). HVA proteins contain casein kinase II phosphorylation sites and two of these are conserved in several homologues (Shen et al., 2001). In aleurone cells of barley, *HVA22* inhibited vesicular trafficking, programmed cell death and negatively regulated vacuolization (Guo et al., 2008). Involvement in vesicular traffic could be proven in citrus by the YOP1/TB2/DP1/HVA22 region in HVA homologues which are associated with the regulation of vesicular traffic (Ferreira et al., 2019). Alteration of the vesicle transport by disruption of *HVA22c* which may be hijacked by the fungus to enhance its nutrient uptake could lead to a decreased nutrient supply for the fungus leading to ineffective colonisation of the host.

To evaluate the involvement of the susceptibility factors *BnCRT1a* and *BnHVA22c* in defence responses and related hormone pathways, *in vitro* grown seedling of knockout mutants for both genes were infected with *V. longisporum*. Gene expression analysis has been conducted with root samples that were harvested at 6 dpi with *VI43*. Two marker genes for ethylene, jasmonic and salicylic acid pathways were normalized to *PP2A* and *Actin* expression. For ethylene, the marker

genes *ACO1* and *ETR2* showed increased expression in infected roots at six dpi. In roots of non-infected *BnCRT1a* CRISPR mutants, with the highest resistance against *V. longisporum* infection, the ET responsive marker gene *ETR2* was upregulated indicative of a potential constitutively activated ET signalling pathway. However, other marker genes of this signalling pathway were not upregulated. This might impede an infection which is in consent with similar fungal pathosystems (Okubara and Paulitz, 2005). The molecular contribution of ET towards plant defence in this interaction could be related to observations that *V. dahliae* degrades the precursor ACC to increase its virulence, while pre-treatments reduced symptoms in *S. lycopersicum* (Tsolakidou et al., 2019). For *HVA22c* CRISPR mutants, an upregulation of the ET marker gene *ETR2* could be observed in non-infected roots of *BnHVA22c* CRISPR mutants in a similar manner to the *BnCRT1a* CRISPR mutants. The salicylic acid marker genes *PR1* and *WRKY70* were upregulated by the infection as well and showed no significant difference in expression strength to the wild type. Marker genes for the jasmonic acid pathway *PDF1.2* and *LOX3* did not vary significantly in their expression profile among the inoculated and control plants either. These findings are in consistence with related experiments in *B. napus* which showed similar expression profiles (Behrens, 2018) and experiments in *A. thaliana* (Kamble et al., 2013). Unfortunately, no new information related to the involvement of *CRT1a* and *HVA22c* in the early stages of infection could be obtained. Therefore, we tested an additional set of potential interaction partner which might help to shed some light on the function. For *CRT1a* such partners are *CNX1* (AT5G61790.1) with similar function to *CRT1a* or protein disulfide isomerase-like (PDIL) genes family especially *PDIL 1-1* (AT1G21750.19) because of their co-regulation in *A. thaliana*. For *HVA22c* the only experimentally confirmed interaction partner is the root hair defective 3 GTP-binding protein (RHD3) (AT3G13870.1) (Schuldiner et al., 2005). Other potential interaction partners are not annotated or just hypothetical interaction partners. Unfortunately, those candidate genes did not vary in their expression profile compared to the wild type revealing no novel interaction partners.

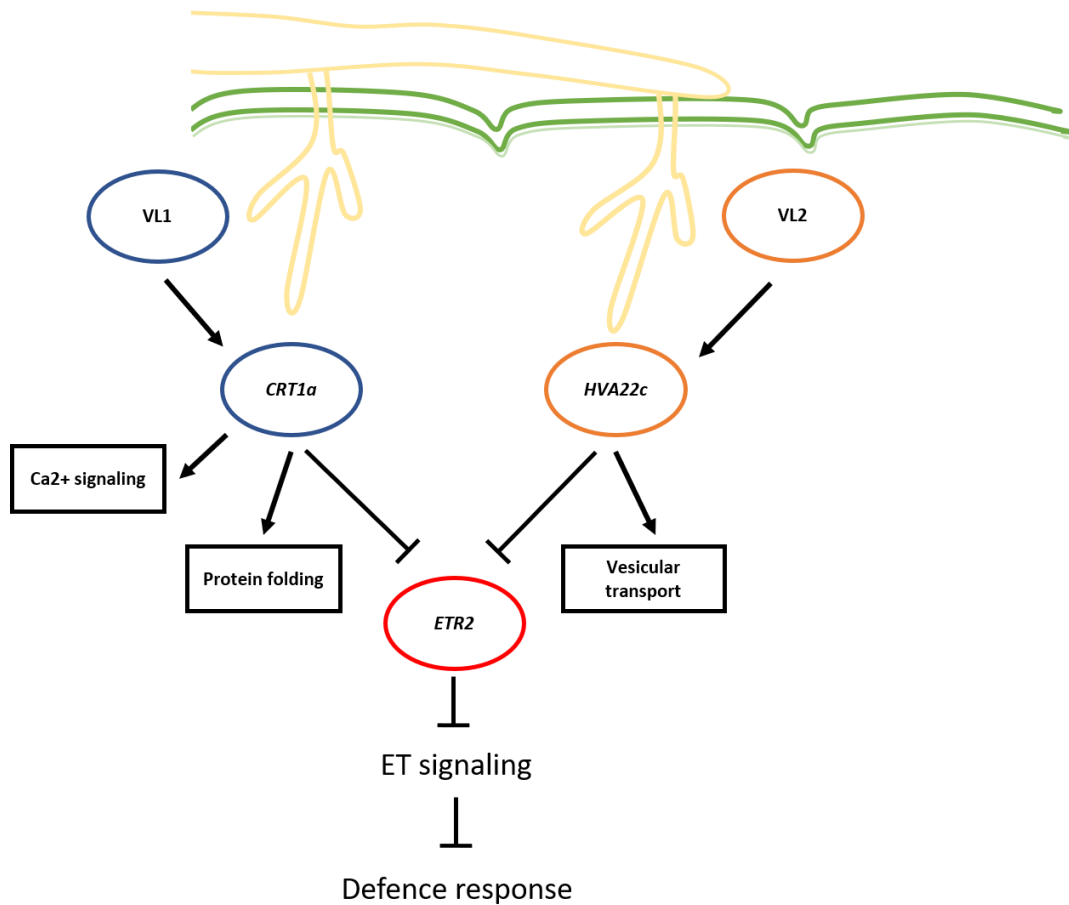


Figure 39: Model of the compatible *B. napus*- *V. longisporum* interaction. *CRT1a* and *HVA22c* represent the susceptibility factors hijacked by the fungus with effectors (VL1 and VL2) for the successful colonization of the host. *CRT1a* and *HVA22c* may affect a negative regulator in the ethylene (ET) signalling pathway, thus modulating defence responses. In addition to the defence-related influence of those genes, an involvement of *CRT1a* in protein folding and *HVA22c* in vesicular transport can be suggested. In the absence of those genes, these biological processes may not be targetable by the fungus.

Establishment of viral CRISPR expression vectors for HDR application in *Brassica napus*

The application of CRISPR/Cas in crops remains challenging due to the complex nature of their genomes and often low transformation efficiency. Even among different tissues, editing efficiency can vary significantly (Char et al., 2017). Highly efficient CRISPR systems are needed to compensate for those negative factors on successful mutation induction. Especially in regard to HDR based approaches, a high mutation rate is required. An additional bottleneck for this repair mechanism is the spatially and temporally availability of repair templates when DSBs are induced (Čermák et al., 2015). For this purpose, the genome of geminiviruses has been modified as an expression vector in dicotyledonous plants (Baltes et al., 2014). This system was first applied in *Nicotiana benthamiana* and has not been tested in *Brassica napus* yet. The utilisation of our Cas9 expression cassette in combination with viral replicons in *B. napus* should allow a higher chance of positive HDR events and may enable seamless genome editing. In the past, the role of protein effectors

that were delivered to the host cells to suppress critical cellular functions and impede a defence response were the primary focus in plant-pathogen interactions (Hilbi et al., 2012; Rafiqi et al., 2012). However, in recent studies, it was demonstrated that sRNAs derived from pathogens can function as effectors as well and a regulation by exogenous miRNAs to manipulate host-pathogen interactions is possible (Weiberg et al., 2013; Wang et al., 2016). Pathogen-derived sRNAs can hijack the host RNAi pathway and suppress plant immunity. This cross-kingdom interaction can also be directed towards the pathogen as a target of exogenous plant miRNAs. In a comprehensive study of the role of RNA silencing during the infection of cotton with the fungal pathogen *V. dahliae* Kleb. two specific miRNAs (miR159 and miR166) were identified, which are exported into the fungal hyphae (Zhang et al., 2016). Those miRNAs were targeting the isotrichodermin C-15 hydroxylase (HiC-15) and Ca²⁺-dependent cysteine protease (Clp-1) mRNAs thereby decreasing the virulence of the fungus.

In previous studies, the miRNAs miR168 and miR1885 were identified that target *BnAGO1* and *TNL immune receptor transcripts (BnTAO1)* (Shen et al., 2014). This results in the transcriptional modulation of host genes in favour of the pathogen. The miRNA binding sites were identified and HDR offers the opportunity to disrupt the interaction of exogenous fungal miRNAs with those genes by altering the nucleotide sequence without changing the protein sequence. This should lead to increased resistance against the pathogen without potential negative side effects that could be caused by a gene knock out. For this approach, two vector systems were utilized to enable HDR. The first system consisted of the codon-optimized Cas9, sgRNA targeting the miRNA binding site and repair template multimers. Those multimers are supposed to be cut out of the vector sequence by the same sgRNA that cuts the target locus being available for homology-directed repair (Schiml et al., 2014). This system was applied for the gene *AGO1* in *A. thaliana* but we could not detect positive HDR events after selection for positive transgenic events. Potential sources of error with this vector system may be an insufficient number of repair templates because only 4 repeats could be inserted into the vector. A higher number of templates would be difficult to achieve due to a complicate cloning procedure. Another factor could be the cut of templates and target locus at the same time to trigger HDR which lead to no positive HDR events.

The second vector system was based on the Cas9 expression cloned into the disarmed viral genome of the Bean Yellow Dwarf Virus (BeYDV) (Baltes et al., 2014). BeYDV based vectors were successfully applied in several crop species like potato (Butler et al., 2016), tomato (Cermák et al., 2015; Dahan-Meier et al., 2018), rice (Wang et al., 2017) and wheat (Gil-Humanes et al., 2017).

One example of those applications is a proof of concept study in which viral replicons were used to insert a 35s promoter upstream of the *ANT1* responsible for anthocyanin production (Cermák et al., 2015). In this study, HDR frequencies 12-fold higher than with standard T-DNA delivery could be achieved. The viral vector was not tested in *Brassicaceae* species at the time the experiments were conducted. In addition, no HDR based approaches have been reported so far and only NHEJ based approaches for gene knockout were conducted in *B. napus* (Braatz et al., 2017; Yang et al., 2017; Sun et al., 2018; Okuzaki et al., 2018; Zhai et al., 2019). sgRNAs targeting miRNA binding sites in the genes *BnAGO1* (miR168) and *BnTAO1* (miR1885a), as well as repair templates harbouring silent mutations at those binding sites, were incorporated into the viral vector. The viral constructs were successfully transformed into *B. napus* hairy roots and processed correctly *in vivo*, indicated by the presence of double-stranded intermediates of the viral genome. Unfortunately, no positive HDR events could be observed *via* PCR screening and this system as well and further optimisation is required.

Although Gemini viral replicons enable high rates of HDR events (rates of up to 25% could be achieved in tomato; Dahan-Meir et al., 2018), selection of positive HDR events remains challenging and a high number of transgenic events is still needed. In this study around 50 independent events for both target genes, *BnAGO1* and *BnTAO1* were generated with hairy roots. This number may have been too low due to the more realistic percentual HDR event numbers about 5% in *Nicotiana tabacum* and *Triticum aestivum* (Baltes et al., 2015; Gil-Humanes et al., 2017). Another factor affecting the HDR efficiency with viral replicons may have been the transformation system. The previously mentioned studies used stable *A. tumefaciens* mediated transformation for the generation of transgenic events. In this study, we used *A. rhizogenes* for the transformation of *B. napus*. In conventional *A. tumefaciens* transformation tissues are cultured on media with hormones promoting callus formation and high cell proliferation. This constant state of high cell activity promotes viral DNA replication and transcriptional activity (Liu et al., 1999). Hairy roots do not have this constant callus state and this may lower the overall transcript and template number of viral replicons. The last factor which may negatively influence the HDR efficiency is the host range of the BeYDV. Although a successful infection of *A. thaliana* was possible with the wild type form of the virus (Liu et al., 1997) modern studies only used its modified genome in experiments with *Solanaceae* species (Baltes et al., 2014; Butler et al., 2015; Cermack et al., 2015). An alternative to the BeYDV system could be a vector system based on the Cabbage Leaf Curl Virus (CaLCuV) which may be better suited for *A. thaliana* and *B. napus* as host.

However, the experiments showed that the virus vector is functional and processed in *B. napus*. Adaptions regarding the transformation system and a change towards an *A. tumefaciens* based approach could improve the efficiency of the system and lead to positive events. In combination with a prolonged callus stage higher HDR rates should be possible.

Application and potential of the CRISPR/Cas9-based genome editing in plant breeding

The application of CRISPR/Cas9 in plant breeding can broaden the gene pool to achieve different goals such as increasing yields, quality improvement or abiotic and biotic stress resistance. The focus of this thesis is on the improvement of biotic stress resistance of OSR caused by the fungal pathogen *Verticillium longisporum*. Applying this approach to increase the resistance of a crop against a certain pathogen requires precise knowledge of plant-pathogen interactions. This information can be obtained e.g. by comparative expression profiling to identify potential candidate genes that are probably involved in the plant-pathogen interaction as susceptibility factors. Those genes that are required by the pathogen to successfully infect the host can then be knocked out to impede an infection. This can simply be achieved by inducing DSBs in the target gene *via* CRISPR/Cas9 and imperfect repair by NHEJ which potentially leads to a frame-shift in the ORF of the target gene resulting in loss of function. This might have potentially negative effects on yield, which cannot be excluded when susceptibility factors are simply knocked-out.

Another approach is the modification of miRNA binding sites in genes targeted by the pathogen. Modified repair templates leaving the gene function intact can be introduced into the target genome *via* HDR and offer a way to avoid potential negative effects on plant physiology or yield. However, HDR-mediated gene editing is very challenging because it requires simultaneous induction of DSBs and the presence of repair templates. A probable negative aspect of this technology are potential off-target mutations caused by the nuclease in loci that have similar nucleotide sequences. In human cells, CRISPR/Cas9 has a high off-target potential (Fu et al., 2013) but this may not be the case in plants which was demonstrated by whole-genome sequencing for off-target mutations (Feng et al. 2014). However, off-target mutations are mostly occurring in paralogues with almost identical sequences (Li et al., 2018). Nevertheless, compared to undirected EMS mutagenesis where an immense amount of off-target mutations is induced, the few potential CRISPR/Cas9 induced off-target mutations are mostly predictable and plant breeders can handle them easily.

The declaration of CRISPR/Cas9 engineered plants as GMOs due to stably transformed intermediate stages, negatively influences the applicability of CRISPR/Cas9 for breeding even if the transgene is crossed out and only the induced mutations are remaining in the genome. This is an issue in countries such as the European Union, which rely on process-based regulation of GMOs (The Council of the European Communities, 1990a, b). The applicability of CRISPR/Cas generated crops massively decreases for farmers due to the cultivation regulations of GMOs. A DNA-free approach to obviate this regulation is the delivery of ribonucleoproteins (RNPs) into the plant cells *via* protoplast transfection (Zhang et al., 2016). Cells and regenerated plants engineered with this technique are not considered transgenic according to the regulation of the European Union that GMOs are characterized by a stable transgenic intermediate state. Modern genome editing approaches offer great opportunities for replacement of single alleles to increase biotic stress resistance or even gene insertions. Genome editing *via* HDR is most attractive but this method still has a very low success rate. Gene replacement via NHEJ is another strategy to insert or replace specific gene fragments in between two introns which might be more applicable for the integration in breeding processes due to a higher success rate. This strategy utilizes a set of sgRNAs targeting introns up- and downstream of the fragment of interest in combination with a donor repair template (Li et al., 2016). Integration of the repair template does not require homologous ends and is incorporated into the target site during NHEJ. With this strategy, alleles can be modified to e.g. generate resistant alleles. CRISPR/Cas offers versatile toolkit and strategies to modify the genome of target organisms to improve or confer new traits and will play an important role in future breeding approaches. We could successfully establish the implementation of this technology in resistance breeding by knocking out the susceptibility factors *CRT1a* and *HVA22c* in *Brassica napus* to increase the resistance against the biotrophic fungal pathogen *Verticillium longisporum*.

References

- Abuamsha, Ruba; Salman, Mazen; Ehlers, Ralf-Udo (2011): Differential resistance of oilseed rape cultivars (*Brassica napus* ssp. *oleifera*) to *Verticillium longisporum* infection is affected by rhizosphere colonisation with antagonistic bacteria, *Serratia plymuthica* and *Pseudomonas chlororaphis*. In: *BioControl* 56 (1), S. 101–112. DOI: 10.1007/s10526-010-9308-8.
- Achkar, Natalia P.; Cambiagno, Damián A.; Manavella, Pablo A. (2016): miRNA Biogenesis. A Dynamic Pathway. In: *Trends in plant science* 21 (12), S. 1034–1044. DOI: 10.1016/j.tplants.2016.09.003.
- Almeida Engler, Janice de; Favery, Bruno; Engler, Gilbert; Abad, Pierre (2005): Loss of susceptibility as an alternative for nematode resistance. In: *Current opinion in biotechnology* 16 (2), S. 112–117. DOI: 10.1016/j.copbio.2005.01.009.
- Altpeter, Fredy; Springer, Nathan M.; Bartley, Laura E.; Blechl, Ann E.; Brutnell, Thomas P.; Citovsky, Vitaly et al. (2016): Advancing Crop Transformation in the Era of Genome Editing. In: *The Plant cell* 28 (7), S. 1510–1520. DOI: 10.1105/tpc.16.00196.
- Altschul, Stephen F.; Gish, Warren; Miller, Webb; Myers, Eugene W.; Lipman, David J. (1990): Basic local alignment search tool. In: *Journal of Molecular Biology* 215 (3), S. 403–410. DOI: 10.1016/S0022-2836(05)80360-2.
- Angelini, Vanina A.; Orejas, Joaquín; Medina, María I.; Agostini, Elizabeth (2011): Scale up of 2,4-dichlorophenol removal from aqueous solutions using *Brassica napus* hairy roots. In: *Journal of hazardous materials* 185 (1), S. 269–274. DOI: 10.1016/j.jhazmat.2010.09.028.
- Anjali S. Iyer-Pascuzzi and Susan R. McCouch: Recessive Resistance Genes and the *Oryza sativa*-*Xanthomonas oryzae* pv. *oryzae* Pathosystem.
- Asai, Tsuneaki; Tena, Guillaume; Plotnikova, Joulia; Willmann, Matthew R.; Chiu, Wan-Ling; Gomez-Gomez, Lourdes et al. (2002): MAP kinase signalling cascade in *Arabidopsis* innate immunity. In: *Nature* 415 (6875), S. 977–983. DOI: 10.1038/415977a.
- Bae, Sangsu; Park, Jeongbin; Kim, Jin-Soo (2014): Cas-OFFinder. A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. In: *Bioinformatics (Oxford, England)* 30 (10), S. 1473–1475. DOI: 10.1093/bioinformatics/btu048.
- Ballvora, Agim; Flath, Kerstin; Lübeck, Jens; Strahwald, Josef; Tacke, Eckhard; Hofferbert, Hans-Reinhard; Gebhardt, Christiane (2011): Multiple alleles for resistance and susceptibility modulate the defense response in the interaction of tetraploid potato (*Solanum tuberosum*) with *Synchytrium endobioticum* pathotypes 1, 2, 6 and 18. In: *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik* 123 (8), S. 1281–1292. DOI: 10.1007/s00122-011-1666-9.

- Baltes, Nicholas J.; Gil-Humanes, Javier; Cermak, Tomas; Atkins, Paul A.; Voytas, Daniel F. (2014): DNA replicons for plant genome engineering. In: *The Plant cell* 26 (1), S. 151–163. DOI: 10.1105/tpc.113.119792.
- Barakate, Abdellah; Stephens, Jennifer (2016): An Overview of CRISPR-Based Tools and Their Improvements. New Opportunities in Understanding Plant-Pathogen Interactions for Better Crop Protection. In: *Frontiers in plant science* 7, S. 765. DOI: 10.3389/fpls.2016.00765.
- Barrangou, Rodolphe; Fremaux, Christophe; Deveau, H el ene; Richards, Melissa; Boyaval, Patrick; Moineau, Sylvain et al. (2007): CRISPR provides acquired resistance against viruses in prokaryotes. In: *Science (New York, N.Y.)* 315 (5819), S. 1709–1712. DOI: 10.1126/science.1138140.
- Beckman, C. H. (1987): The nature of wilt diseases of plants. St. Paul, Minn.: APS Press.
- Behrens, Falk Hubertus (2018): Investigation on the role of miRNAs in regulating plant-*Verticillium longisporum* interactions in oilseed rape (*Brassica napus*) and *Arabidopsis thaliana*. In: Schriftenreihe des Instituts f ur Phytopathologie (Band 3). ISSN: 2197-554X.
- Berardini, Tanya Z.; Reiser, Leonore; Li, Donghui; Mezheritsky, Yarik; Muller, Robert; Strait, Emily; Huala, Eva (2015): The Arabidopsis information resource. Making and mining the "gold standard" annotated reference plant genome. In: *Genesis (New York, N.Y. : 2000)* 53 (8), S. 474–485. DOI: 10.1002/dvg.22877.
- Bergeron, Diane; Boivin, Rodolphe; Baszczynski, Chris L.; Bellemare, Guy (1994): Root-specific expression of a glycine-rich protein gene in *Brassica napus*. In: *Plant Science* 96 (1-2), S. 87–98. DOI: 10.1016/0168-9452(94)90225-9.
- Bernard, Guillaume; Gagneul, David; Alves Dos Santos, Harmony; Etienne, Audrey; Hilbert, Jean-Louis; Rambaud, Caroline (2019): Efficient Genome Editing Using CRISPR/Cas9 Technology in Chicory. In: *International journal of molecular sciences* 20 (5). DOI: 10.3390/ijms20051155.
- Besseau, S ebastien; Li, Jing; Palva, E. Tapio (2012): WRKY54 and WRKY70 co-operate as negative regulators of leaf senescence in *Arabidopsis thaliana*. In: *Journal of experimental botany* 63 (7), S. 2667–2679. DOI: 10.1093/jxb/err450.
- Boller, Thomas; He, Sheng Yang (2009): Innate immunity in plants. An arms race between pattern recognition receptors in plants and effectors in microbial pathogens. In: *Science (New York, N.Y.)* 324 (5928), S. 742–744. DOI: 10.1126/science.1171647.
- Borisjuk, N.; Sitailo, L.; Adler, K.; Malysheva, L.; Tewes, A.; Borisjuk, L.; Manteuffel, R. (1998): Calreticulin expression in plant cells. Developmental regulation, tissue specificity and intracellular distribution. In: *Planta* 206 (4), S. 504–514. DOI: 10.1007/s004250050427.

Braatz, Janina; Harloff, Hans-Joachim; Mascher, Martin; Stein, Nils; Himmelbach, Axel; Jung, Christian (2017): CRISPR-Cas9 Targeted Mutagenesis Leads to Simultaneous Modification of Different Homoeologous Gene Copies in Polyploid Oilseed Rape (*Brassica napus*). In: *Plant physiology* 174 (2), S. 935–942. DOI: 10.1104/pp.17.00426.

Broekgaarden, Colette; Caarls, Lotte; Vos, Irene A.; Pieterse, Corné M. J.; van Wees, Saskia C. M. (2015): Ethylene. Traffic Controller on Hormonal Crossroads to Defense. In: *Plant physiology* 169 (4), S. 2371–2379. DOI: 10.1104/pp.15.01020.

Büschges, Rainer; Hollricher, Karin; Panstruga, Ralph; Simons, Guus; Wolter, Marietta; Frijters, Adrie et al. (1997): The Barley Mlo Gene. A Novel Control Element of Plant Pathogen Resistance. In: *Cell* 88 (5), S. 695–705. DOI: 10.1016/S0092-8674(00)81912-1.

Butler, Nathaniel M.; Atkins, Paul A.; Voytas, Daniel F.; Douches, David S. (2015): Generation and Inheritance of Targeted Mutations in Potato (*Solanum tuberosum* L.) Using the CRISPR/Cas System. In: *PloS one* 10 (12), e0144591. DOI: 10.1371/journal.pone.0144591.

Butler, Nathaniel M.; Baltés, Nicholas J.; Voytas, Daniel F.; Douches, David S. (2016): Geminivirus-Mediated Genome Editing in Potato (*Solanum tuberosum* L.) Using Sequence-Specific Nucleases. In: *Frontiers in plant science* 7, S. 1045. DOI: 10.3389/fpls.2016.01045.

Cai, Chengcheng; Wang, Xiaobo; Liu, Bo; Wu, Jian; Liang, Jianli; Cui, Yinan et al. (2017): Brassica rapa Genome 2.0. A Reference Upgrade through Sequence Re-assembly and Gene Re-annotation. In: *Molecular plant* 10 (4), S. 649–651. DOI: 10.1016/j.molp.2016.11.008.

Cai, Qiang; He, Baoye; Kogel, Karl-Heinz; Jin, Hailing (2018): Cross-kingdom RNA trafficking and environmental RNAi-nature's blueprint for modern crop protection strategies. In: *Current opinion in microbiology* 46, S. 58–64. DOI: 10.1016/j.mib.2018.02.003.

Cai, Yupeng; Chen, Li; Liu, Xiujie; Sun, Shi; Wu, Cunxiang; Jiang, Bingjun et al. (2015): CRISPR/Cas9-Mediated Genome Editing in Soybean Hairy Roots. In: *PloS one* 10 (8), e0136064. DOI: 10.1371/journal.pone.0136064.

Campbell, C. Lee; Madden, Laurence V. (1990): Introduction to plant disease epidemiology. New York: Wiley. Online verfügbar unter <http://www.loc.gov/catdir/enhancements/fy0706/89034349-b.html>.

Cao, Jia-Yi; Xu, You-Ping; Li, Wen; Li, Shuang-Sheng; Rahman, Hafizur; Cai, Xin-Zhong (2016): Genome-Wide Identification of Dicer-Like, Argonaute, and RNA-Dependent RNA Polymerase Gene Families in Brassica Species and Functional Analyses of Their Arabidopsis Homologs in Resistance to *Sclerotinia sclerotiorum*. In: *Frontiers in plant science* 7, S. 1614. DOI: 10.3389/fpls.2016.01614.

- Cao, Jia-Yi; Xu, You-Ping; Zhao, Li; Li, Shuang-Sheng; Cai, Xin-Zhong (2016): Tight regulation of the interaction between *Brassica napus* and *Sclerotinia sclerotiorum* at the microRNA level. In: *Plant molecular biology* 92 (1-2), S. 39–55. DOI: 10.1007/s11103-016-0494-3.
- Cardon, Florian; Pallisse, Roser; Bardor, Muriel; Caron, Aurore; Vanier, Jessica; Ele Ekouna, Jean Pierre et al. (2019): *Brassica rapa* hairy root based expression system leads to the production of highly homogenous and reproducible profiles of recombinant human alpha-L-iduronidase. In: *Plant biotechnology journal* 17 (2), S. 505–516. DOI: 10.1111/pbi.12994.
- Carroll, Dana (2011): Genome engineering with zinc-finger nucleases. In: *Genetics* 188 (4), S. 773–782. DOI: 10.1534/genetics.111.131433.
- Čermák, Tomáš; Baltés, Nicholas J.; Čegan, Radim; Zhang, Yong; Voytas, Daniel F. (2015): High-frequency, precise modification of the tomato genome. In: *Genome biology* 16, S. 232. DOI: 10.1186/s13059-015-0796-9.
- Čermák, Tomáš; Curtin, Shaun J.; Gil-Humanes, Javier; Čegan, Radim; Kono, Thomas J. Y.; Konečná, Eva et al. (2017): A Multipurpose Toolkit to Enable Advanced Genome Engineering in Plants. In: *The Plant cell* 29 (6), S. 1196–1217. DOI: 10.1105/tpc.16.00922.
- Chalhoub, Boulos; Denoeud, France; Liu, Shengyi; Parkin, Isobel A. P.; Tang, Haibao; Wang, Xiyin et al. (2014): Plant genetics. Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. In: *Science (New York, N.Y.)* 345 (6199), S. 950–953. DOI: 10.1126/science.1253435.
- Chandrasekaran, Jeyabharathy; Brumin, Marina; Wolf, Dalia; Leibman, Diana; Klap, Chen; Pearlsman, Mali et al. (2016): Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. In: *Molecular plant pathology* 17 (7), S. 1140–1153. DOI: 10.1111/mpp.12375.
- Char, Si Nian; Neelakandan, Anjanasree K.; Nahampun, Hartinio; Frame, Bronwyn; Main, Marcy; Spalding, Martin H. et al. (2017): An *Agrobacterium*-delivered CRISPR/Cas9 system for high-frequency targeted mutagenesis in maize. In: *Plant biotechnology journal* 15 (2), S. 257–268. DOI: 10.1111/pbi.12611.
- Chen, Ching-Nen; Chu, Chiung-Chih; Zentella, Rodolfo; Pan, Shu-Mei; Ho, Tuan-Hua David (2002): AtHVA22 gene family in *Arabidopsis*. Phylogenetic relationship, ABA and stress regulation, and tissue-specific expression. In: *Plant molecular biology* 49 (6), S. 633–644.
- Chen, Li; Cai, Yupeng; Liu, Xiujie; Guo, Chen; Sun, Shi; Wu, Cunxiang et al. (2018): Soybean hairy roots produced in vitro by *Agrobacterium rhizogenes* -mediated transformation. In: *The Crop Journal* 6 (2), S. 162–171. DOI: 10.1016/j.cj.2017.08.006.

Chen, Li-Qing; Hou, Bi-Huei; Lalonde, Sylvie; Takanaga, Hitomi; Hartung, Mara L.; Qu, Xiao-Qing et al. (2010): Sugar transporters for intercellular exchange and nutrition of pathogens. In: *Nature* 468 (7323), S. 527–532. DOI: 10.1038/nature09606.

Chen, Zhixiang; Zheng, Zuyu; Huang, Junli; Lai, Zhibing; Fan, Baofang (2009): Biosynthesis of salicylic acid in plants. In: *Plant signaling & behavior* 4 (6), S. 493–496. DOI: 10.4161/psb.4.6.8392.

Christensen, Anna; Svensson, Karin; Persson, Staffan; Jung, Joanna; Michalak, Marek; Widell, Susanne; Sommarin, Marianne (2008): Functional characterization of Arabidopsis calreticulin1a. A key alleviator of endoplasmic reticulum stress. In: *Plant & cell physiology* 49 (6), S. 912–924. DOI: 10.1093/pcp/pcn065.

Christensen, Anna; Svensson, Karin; Thelin, Lisa; Zhang, Wenjing; Tintor, Nico; Prins, Daniel et al. (2010): Higher plant calreticulins have acquired specialized functions in Arabidopsis. In: *PloS one* 5 (6), e11342. DOI: 10.1371/journal.pone.0011342.

Chung, C. T.; Niemela, S. L.; Miller, R. H. (1989): One-step preparation of competent Escherichia coli. Transformation and storage of bacterial cells in the same solution. In: *Proceedings of the National Academy of Sciences of the United States of America* 86 (7), S. 2172–2175. DOI: 10.1073/pnas.86.7.2172.

Collard, Bertrand C. Y.; Mackill, David J. (2008): Marker-assisted selection. An approach for precision plant breeding in the twenty-first century. In: *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* 363 (1491), S. 557–572. DOI: 10.1098/rstb.2007.2170.

Coniglio, María S.; Busto, Victor D.; González, Paola S.; Medina, María I.; Milrad, Silvia; Agostini, Elizabeth (2008): Application of Brassica napus hairy root cultures for phenol removal from aqueous solutions. In: *Chemosphere* 72 (7), S. 1035–1042. DOI: 10.1016/j.chemosphere.2008.04.003.

Coninck, Barbara de; Timmermans, Pieter; Vos, Christine; Cammue, Bruno P. A.; Kazan, Kemal (2015): What lies beneath. Belowground defense strategies in plants. In: *Trends in plant science* 20 (2), S. 91–101. DOI: 10.1016/j.tplants.2014.09.007.

Coppolino, Marc G.; Dedhar, Shoukat (1998): Calreticulin. In: *The international journal of biochemistry & cell biology* 30 (5), S. 553–558. DOI: 10.1016/S1357-2725(97)00153-2.

Dahan-Meir, Tal; Filler-Hayut, Shdema; Melamed-Bessudo, Cathy; Bocobza, Samuel; Czosnek, Henryk; Aharoni, Asaph; Levy, Avraham A. (2018): Efficient in planta gene targeting in tomato using geminiviral replicons and the CRISPR/Cas9 system. In: *The Plant journal : for cell and molecular biology* 95 (1), S. 5–16. DOI: 10.1111/tpj.13932.

Dangl, Jeffery L.; Horvath, Diana M.; Staskawicz, Brian J. (2013): Pivoting the plant immune system from dissection to deployment. In: *Science (New York, N.Y.)* 341 (6147), S. 746–751. DOI: 10.1126/science.1236011.

- Del Río, L. E.; Bradley, C. A.; Henson, R. A.; Endres, G. J.; Hanson, B. K.; McKay, K. et al. (2007): Impact of Sclerotinia Stem Rot on Yield of Canola. In: *Plant disease* 91 (2), S. 191–194. DOI: 10.1094/PDIS-91-2-0191.
- Denecke, J.; Carlsson, L. E.; Vidal, S.; Höglund, A. S.; Ek, B.; van Zeijl, M. J. et al. (1995): The tobacco homolog of mammalian calreticulin is present in protein complexes in vivo. In: *The Plant cell* 7 (4), S. 391–406. DOI: 10.1105/tpc.7.4.391.
- Depotter, Jasper R. L.; Deketelaere, Silke; Inderbitzin, Patrik; Tiedemann, Andreas von; Höfte, Monica; Subbarao, Krishna V. et al. (2016): *Verticillium longisporum*, the invisible threat to oilseed rape and other brassicaceous plant hosts. In: *Molecular plant pathology* 17 (7), S. 1004–1016. DOI: 10.1111/mpp.12350.
- DeYoung, Brody J.; Innes, Roger W. (2006): Plant NBS-LRR proteins in pathogen sensing and host defense. In: *Nature immunology* 7 (12), S. 1243–1249. DOI: 10.1038/ni1410.
- Diatchenko, L.; Lau, Y. F.; Campbell, A. P.; Chenchik, A.; Moqadam, F.; Huang, B. et al. (1996): Suppression subtractive hybridization. A method for generating differentially regulated or tissue-specific cDNA probes and libraries. In: *Proceedings of the National Academy of Sciences of the United States of America* 93 (12), S. 6025–6030. DOI: 10.1073/pnas.93.12.6025.
- Doudna, Jennifer A.; Charpentier, Emmanuelle (2014): Genome editing. The new frontier of genome engineering with CRISPR-Cas9. In: *Science (New York, N.Y.)* 346 (6213), S. 1258096. DOI: 10.1126/science.1258096.
- Du, Hongyang; Zeng, Xuanrui; Zhao, Meng; Cui, Xiaopei; Wang, Qing; Yang, Hui et al. (2016): Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. In: *Journal of biotechnology* 217, S. 90–97. DOI: 10.1016/j.jbiotec.2015.11.005.
- Dunker, S.; Keunecke, H.; Steinbach, P.; Tiedemann, A. von (2008): Impact of *Verticillium longisporum* on Yield and Morphology of Winter Oilseed Rape (*Brassica napus*) in Relation to Systemic Spread in the Plant. In: *Journal of Phytopathology* 156 (11-12), S. 698–707. DOI: 10.1111/j.1439-0434.2008.01429.x.
- Eckardt, N. A. (2002): Plant Disease Susceptibility Genes? In: *THE PLANT CELL ONLINE* 14 (9), S. 1983–1986. DOI: 10.1105/tpc.140910.
- Ellendorff, Ursula; Fradin, Emilie F.; Jonge, Ronnie de; Thomma, Bart P. H. J. (2009): RNA silencing is required for Arabidopsis defence against *Verticillium* wilt disease. In: *Journal of experimental botany* 60 (2), S. 591–602. DOI: 10.1093/jxb/ern306.
- Esvelt, Kevin M.; Mali, Prashant; Braff, Jonathan L.; Moosburner, Mark; Yaung, Stephanie J.; Church, George M. (2013): Orthogonal Cas9 proteins for RNA-guided gene regulation and editing. In: *Nature methods* 10 (11), S. 1116–1121. DOI: 10.1038/nmeth.2681.

Eynck, C.; Koopmann, B.; Grunewaldt-Stoecker, G.; Karlovsky, P.; Tiedemann, A. von (2007): Differential interactions of *Verticillium longisporum* and *V. dahliae* with *Brassica napus* detected with molecular and histological techniques. In: *Eur J Plant Pathol* 118 (3), S. 259–274. DOI: 10.1007/s10658-007-9144-6.

Eynck, C.; Koopmann, B.; Karlovsky, P.; Tiedemann, A. von (2009): Internal resistance in winter oilseed rape inhibits systemic spread of the vascular pathogen *Verticillium longisporum*. In: *Phytopathology* 99 (7), S. 802–811. DOI: 10.1094/PHYTO-99-7-0802.

Eynck, C.; Koopmann, B.; Tiedemann, A. von (2009): Identification of *Brassica* accessions with enhanced resistance to *Verticillium longisporum* under controlled and field conditions. In: *J Plant Dis Prot* 116 (2), S. 63–72. DOI: 10.1007/BF03356288.

Fausser, Friedrich; Roth, Nadine; Pacher, Michael; Ilg, Gabriele; Sánchez-Fernández, Rocío; Biesgen, Christian; Puchta, Holger (2012): In planta gene targeting. In: *Proceedings of the National Academy of Sciences of the United States of America* 109 (19), S. 7535–7540. DOI: 10.1073/pnas.1202191109.

Fausser, Friedrich; Schiml, Simon; Puchta, Holger (2014): Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in *Arabidopsis thaliana*. In: *The Plant journal : for cell and molecular biology* 79 (2), S. 348–359. DOI: 10.1111/tpj.12554.

Fei, Qili; Xia, Rui; Meyers, Blake C. (2013): Phased, secondary, small interfering RNAs in posttranscriptional regulatory networks. In: *The Plant cell* 25 (7), S. 2400–2415. DOI: 10.1105/tpc.113.114652.

Fei, Qili; Zhang, Yu; Xia, Rui; Meyers, Blake C. (2016): Small RNAs Add Zing to the Zig-Zag-Zig Model of Plant Defenses. In: *Molecular plant-microbe interactions : MPMI* 29 (3), S. 165–169. DOI: 10.1094/MPMI-09-15-0212-FI.

Feng, Zhengyan; Mao, Yanfei; Xu, Nanfei; Zhang, Botao; Wei, Pengliang; Yang, Dong-Lei et al. (2014): Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*. In: *Proceedings of the National Academy of Sciences of the United States of America* 111 (12), S. 4632–4637. DOI: 10.1073/pnas.1400822111.

Fernandez-Calvino, Lourdes; Faulkner, Christine; Walshaw, John; Saalbach, Gerhard; Bayer, Emmanuelle; Benitez-Alfonso, Yoselin; Maule, Andrew (2011): *Arabidopsis* plasmodesmal proteome. In: *PLoS one* 6 (4), e18880. DOI: 10.1371/journal.pone.0018880.

Fernández-Calvo, Patricia; Chini, Andrea; Fernández-Barbero, Gemma; Chico, José-Manuel; Gimenez-Ibanez, Selena; Geerinck, Jan et al. (2011): The *Arabidopsis* bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. In: *The Plant cell* 23 (2), S. 701–715. DOI: 10.1105/tpc.110.080788.

- Flor, H. H. (1971): Current Status of the Gene-For-Gene Concept. In: *Annu. Rev. Phytopathol.* 9 (1), S. 275–296. DOI: 10.1146/annurev.py.09.090171.001423.
- Fordham-Skelton, A. P.; Skipsey, M.; Eveans, I. M.; Edwards, R.; Gatehouse, J. A. (1999): Higher plant tyrosine-specific protein phosphatases (PTPs) contain novel amino-terminal domains. Expression during embryogenesis. In: *Plant molecular biology* 39 (3), S. 593–605.
- Fradin, Emilie F.; Zhang, Zhao; Juarez Ayala, Juan C.; Castroverde, Christian D. M.; Nazar, Ross N.; Robb, Jane et al. (2009): Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. In: *Plant physiology* 150 (1), S. 320–332. DOI: 10.1104/pp.109.136762.
- Fu, Yanfang; Foden, Jennifer A.; Khayter, Cyd; Maeder, Morgan L.; Reyon, Deepak; Joung, J. Keith; Sander, Jeffrey D. (2013): High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. In: *Nature biotechnology* 31 (9), S. 822–826. DOI: 10.1038/nbt.2623.
- Garas, N.A., Wilhelm, S., Sagen, J.E. (1989): Relationship of cultivar resistance to distribution of Verticillium dahliae in inoculated cotton plants and to growth of single conidia on excised stem segments. In: *Phytopathology* (76), S. 1005–1010.
- García, Ana V.; Blanvillain-Baufumé, Servane; Huibers, Robin P.; Wiermer, Marcel; Li, Guangyong; Gobbato, Enrico et al. (2010): Balanced nuclear and cytoplasmic activities of EDS1 are required for a complete plant innate immune response. In: *PLoS pathogens* 6, e1000970. DOI: 10.1371/journal.ppat.1000970.
- Gil-Humanes, Javier; Wang, Yanpeng; Liang, Zhen; Shan, Qiwei; Ozuna, Carmen V.; Sánchez-León, Susana et al. (2017): High-efficiency gene targeting in hexaploid wheat using DNA replicons and CRISPR/Cas9. In: *The Plant journal : for cell and molecular biology* 89 (6), S. 1251–1262. DOI: 10.1111/tpj.13446.
- Gill, Upinder S.; Lee, Seonghee; Mysore, Kirankumar S. (2015): Host versus nonhost resistance. Distinct wars with similar arsenals. In: *Phytopathology* 105 (5), S. 580–587. DOI: 10.1094/PHYTO-11-14-0298-RVW.
- Gomes Ferreira, Monique Drielle; Araújo Castro, Jacqueline; Santana Silva, Raner José; Micheli, Fabienne (2019): HVA22 from citrus. A small gene family whose some members are involved in plant response to abiotic stress. In: *Plant physiology and biochemistry : PPB* 142, S. 395–404. DOI: 10.1016/j.plaphy.2019.08.003.
- Gómez-Campo, C. (Hg.) (1999): Biology of Brassica coenospecies. ScienceDirect (Online service). 1st ed. Amsterdam, New York: Elsevier (Developments in Plant Genetics and Breeding, 4). Online verfügbar unter <http://site.ebrary.com/lib/alltitles/docDetail.action?docID=10216755>.
- GSL Biotech: SnapGene software. Version. Online verfügbar unter snapgene.com.

Guan, Chudi; Kumar, Sanjay; Kucera, Rebecca; Ewel, Amy (2004): Changing the enzymatic activity of T7 endonuclease by mutations at the beta-bridge site. Alteration of substrate specificity profile and metal ion requirements by mutation distant from the catalytic domain. In: *Biochemistry* 43 (14), S. 4313–4322. DOI: 10.1021/bi036033j.

Guo, Woei-Jiun; Ho, Tuan-Hua; David Ho, Thun-Hua (2008): An abscisic acid-induced protein, HVA22, inhibits gibberellin-mediated programmed cell death in cereal aleurone cells. In: *Plant physiology* 147 (4), S. 1710–1722. DOI: 10.1104/pp.108.120238.

Gupta, Surinder Kumar (2007): Advances in Botanical Research. Rapeseed Breeding. 1. Aufl. s.l.: Elsevier textbooks (Advances in botanical research, v.45). Online verfügbar unter <http://gbv.ebib.com/patron/FullRecord.aspx?p=305506>.

Gutierrez, C. (1999): Geminivirus DNA replication. In: *Cellular and molecular life sciences : CMLS* 56 (3-4), S. 313–329.

Häder, Claudia (2013): Kompatibilitätsfaktoren für die Raps-*Verticillium longisporum*-Interaktion. In: Schriftenreihe des Instituts für Phytopathologie (Band 1). ISSN: 2197-554X.

Hansjakob, A.; Riederer, M.; Hildebrandt, U. (2011): Wax matters. Absence of very-long-chain aldehydes from the leaf cuticular wax of the glossy11 mutant of maize compromises the prepenetration processes of *Blumeria graminis*. In: *Plant Pathology* 60 (6), S. 1151–1161. DOI: 10.1111/j.1365-3059.2011.02467.x.

Hardham, Adrienne R. (2013): Microtubules and biotic interactions. In: *The Plant journal : for cell and molecular biology* 75 (2), S. 278–289. DOI: 10.1111/tpj.12171.

Hatzig, Sarah; Breuer, Frank; Nesi, Nathalie; Ducournau, Sylvie; Wagner, Marie-Helene; Leckband, Gunhild et al. (2018): Hidden Effects of Seed Quality Breeding on Germination in Oilseed Rape (*Brassica napus* L.). In: *Frontiers in plant science* 9, S. 419. DOI: 10.3389/fpls.2018.00419.

He, Xiang-Feng; Fang, Yuan-Yuan; Feng, Lei; Guo, Hui-Shan (2008): Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR-NBS-LRR class R gene-derived novel miRNA in Brassica. In: *FEBS letters* 582 (16), S. 2445–2452. DOI: 10.1016/j.febslet.2008.06.011.

Heath, M. C. (2000): Nonhost resistance and nonspecific plant defenses. In: *Current Opinion in Plant Biology* 3 (4), S. 315–319.

Heim, Marc A.; Jakoby, Marc; Werber, Martin; Martin, Cathie; Weisshaar, Bernd; Bailey, Paul C. (2003): The basic helix-loop-helix transcription factor family in plants. A genome-wide study of protein structure and functional diversity. In: *Molecular biology and evolution* 20 (5), S. 735–747. DOI: 10.1093/molbev/msg088.

Hilbi, Hubert; Haas, Albert (2012): Secretive bacterial pathogens and the secretory pathway. In: *Traffic (Copenhagen, Denmark)* 13 (9), S. 1187–1197. DOI: 10.1111/j.1600-0854.2012.01344.x.

- Hossain, Roxana (2017): Functional characterization of three compatibility factor genes involved in the oilseed rape - *Verticillium longisporum* interaction. Institut für molekulare Phytopathologie, Christian-Albrechts-Universität zu Kiel.
- Hu, Johnny H.; Miller, Shannon M.; Geurts, Maarten H.; Tang, Weixin; Chen, Liwei; Sun, Ning et al. (2018): Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. In: *Nature* 556 (7699), S. 57–63. DOI: 10.1038/nature26155.
- Huisman, O. C. (1982): Interrelations of Root Growth Dynamics to Epidemiology of Root-Invasive Fungi. In: *Annu. Rev. Phytopathol.* 20 (1), S. 303–327. DOI: 10.1146/annurev.py.20.090182.001511.
- Hwang, Sheau-Fang; Strelkov, Stephen E.; Feng, Jie; Gossen, Bruce D.; Howard, Ron J. (2012): *Plasmodiophora brassicae*. A review of an emerging pathogen of the Canadian canola (*Brassica napus*) crop. In: *Molecular plant pathology* 13 (2), S. 105–113. DOI: 10.1111/j.1364-3703.2011.00729.x.
- Hyun, Youbong; Kim, Jungeun; Cho, Seung Woo; Choi, Yeonhee; Kim, Jin-Soo; Coupland, George (2015): Site-directed mutagenesis in *Arabidopsis thaliana* using dividing tissue-targeted RGEN of the CRISPR/Cas system to generate heritable null alleles. In: *Planta* 241 (1), S. 271–284. DOI: 10.1007/s00425-014-2180-5.
- Ito, Yasuhiro; Nishizawa-Yokoi, Ayako; Endo, Masaki; Mikami, Masafumi; Toki, Seiichi (2015): CRISPR/Cas9-mediated mutagenesis of the RIN locus that regulates tomato fruit ripening. In: *Biochemical and biophysical research communications* 467 (1), S. 76–82. DOI: 10.1016/j.bbrc.2015.09.117.
- Iven, Tim; König, Stefanie; Singh, Seema; Braus-Stromeyer, Susanna A.; Bischoff, Matthias; Tietze, Lutz F. et al. (2012): Transcriptional activation and production of tryptophan-derived secondary metabolites in *Arabidopsis* roots contributes to the defense against the fungal vascular pathogen *Verticillium longisporum*. In: *Molecular plant* 5 (6), S. 1389–1402. DOI: 10.1093/mp/sss044.
- Iwakawa, Hiro-oki; Tomari, Yukihide (2013): Molecular insights into microRNA-mediated translational repression in plants. In: *Molecular cell* 52 (4), S. 591–601. DOI: 10.1016/j.molcel.2013.10.033.
- Jacobs, Thomas B.; LaFayette, Peter R.; Schmitz, Robert J.; Parrott, Wayne A. (2015): Targeted genome modifications in soybean with CRISPR/Cas9. In: *BMC biotechnology* 15, S. 16. DOI: 10.1186/s12896-015-0131-2.
- Jez, Joseph M.; Cahoon, Rebecca E.; Chen, Sixue (2004): *Arabidopsis thaliana* glutamate-cysteine ligase. Functional properties, kinetic mechanism, and regulation of activity. In: *J. Biol. Chem.* 279 (32), S. 33463–33470. DOI: 10.1074/jbc.M405127200.
- Jinek, Martin; East, Alexandra; Cheng, Aaron; Lin, Steven; Ma, Enbo; Doudna, Jennifer (2013): RNA-programmed genome editing in human cells. In: *eLife* 2, e00471. DOI: 10.7554/eLife.00471.

Johansson, Anna; Goud, Jan-Kees C.; Dixelius, Christina (2006): Plant Host Range of *Verticillium longisporum* and *Microsclerotia* Density in Swedish Soils. In: *Eur J Plant Pathol* 114 (2), S. 139–149. DOI: 10.1007/s10658-005-2333-2.

Johansson, Anna; Staal, Jens; Dixelius, Christina (2006): Early responses in the *Arabidopsis*-*Verticillium longisporum* pathosystem are dependent on NDR1, JA- and ET-associated signals via cytosolic NPR1 and RFO1. In: *Molecular plant-microbe interactions : MPMI* 19 (9), S. 958–969. DOI: 10.1094/MPMI-19-0958.

Jones, D. T.; Taylor, W. R.; Thornton, J. M. (1992): The rapid generation of mutation data matrices from protein sequences. In: *Computer applications in the biosciences : CABIOS* 8 (3), S. 275–282. DOI: 10.1093/bioinformatics/8.3.275.

Kamble, A.; Koopmann, B.; Tiedemann, A. von (2013): Induced resistance to *Verticillium longisporum* in *Brassica napus* by β -aminobutyric acid. In: *Plant Pathology* 62 (3), S. 552–561. DOI: 10.1111/j.1365-3059.2012.02669.x.

Kang, Hong-Gu; Oh, Chang-Sik; Sato, Masanao; Katagiri, Fumiaki; Glazebrook, Jane; Takahashi, Hideki et al. (2010): Endosome-associated CRT1 functions early in resistance gene-mediated defense signaling in *Arabidopsis* and tobacco. In: *The Plant cell* 22 (3), S. 918–936. DOI: 10.1105/tpc.109.071662.

Karapapa, V. K.; Bainbridge, B. W.; Heale, J. B. (1997): Morphological and molecular characterization of *Verticillium longisporum* comb. nov., pathogenic to oilseed rape. In: *Mycological Research* 101 (11), S. 1281–1294. DOI: 10.1017/S0953756297003985.

Kawchuk, L. M.; Hachey, J.; Lynch, D. R.; Kulcsar, F.; van Rooijen, G.; Waterer, D. R. et al. (2001): Tomato Ve disease resistance genes encode cell surface-like receptors. In: *Proceedings of the National Academy of Sciences of the United States of America* 98 (11), S. 6511–6515. DOI: 10.1073/pnas.091114198.

Kim, Jun Hyeok; Nguyen, Nguyen Hoai; Nguyen, Ngoc Trinh; Hong, Suk-Whan; Lee, Hojoung (2013): Loss of all three calreticulins, CRT1, CRT2 and CRT3, causes enhanced sensitivity to water stress in *Arabidopsis*. In: *Plant cell reports* 32 (12), S. 1843–1853. DOI: 10.1007/s00299-013-1497-z.

Kirchner, Thomas W.; Niehaus, Markus; Debener, Thomas; Schenk, Manfred K.; Herde, Marco (2017): Efficient generation of mutations mediated by CRISPR/Cas9 in the hairy root transformation system of *Brassica carinata*. In: *PLoS one* 12 (9), e0185429. DOI: 10.1371/journal.pone.0185429.

Kleffmann, Torsten; Russenberger, Doris; Zychlinski, Anne von; Christopher, Wayne; Sjölander, Kimmen; Gruissem, Wilhelm; Baginsky, Sacha (2004): The *Arabidopsis thaliana* chloroplast proteome reveals pathway abundance and novel protein functions. In: *Current biology : CB* 14 (5), S. 354–362. DOI: 10.1016/j.cub.2004.02.039.

Kleinstiver, Benjamin P.; Prew, Michelle S.; Tsai, Shengdar Q.; Nguyen, Nhu T.; Topkar, Ved V.; Zheng, Zongli; Joung, J. Keith (2015): Broadening the targeting range of *Staphylococcus aureus* CRISPR-Cas9 by modifying PAM recognition. In: *Nature biotechnology* 33 (12), S. 1293–1298. DOI: 10.1038/nbt.3404.

Kleinstiver, Benjamin P.; Prew, Michelle S.; Tsai, Shengdar Q.; Topkar, Ved V.; Nguyen, Nhu T.; Zheng, Zongli et al. (2015): Engineered CRISPR-Cas9 nucleases with altered PAM specificities. In: *Nature* 523 (7561), S. 481–485. DOI: 10.1038/nature14592.

Klosterman, Steven J.; Atallah, Zahi K.; Vallad, Gary E.; Subbarao, Krishna V. (2009): Diversity, pathogenicity, and management of verticillium species. In: *Annual review of phytopathology* 47, S. 39–62. DOI: 10.1146/annurev-phyto-080508-081748.

Knüfer, Jessica; Lopisso, Daniel Teshome; Koopmann, Birger; Karlovsky, Petr; Tiedemann, Andreas von (2017): Assessment of latent infection with *Verticillium longisporum* in field-grown oilseed rape by qPCR. In: *Eur J Plant Pathol* 147 (4), S. 819–831. DOI: 10.1007/s10658-016-1045-0.

Koch, E.; Slusarenko, A. (1990): Arabidopsis is susceptible to infection by a downy mildew fungus. In: *The Plant cell* 2 (5), S. 437–445. DOI: 10.1105/tpc.2.5.437.

König, Stefanie; Feussner, Kirstin; Kaefer, Alexander; Landesfeind, Manuel; Thurow, Corinna; Karlovsky, Petr et al. (2014): Soluble phenylpropanoids are involved in the defense response of Arabidopsis against *Verticillium longisporum*. In: *The New phytologist* 202 (3), S. 823–837. DOI: 10.1111/nph.12709.

Koo, Abraham J. K.; Howe, Gregg A. (2009): The wound hormone jasmonate. In: *Phytochemistry* 70 (13-14), S. 1571–1580. DOI: 10.1016/j.phytochem.2009.07.018.

Kumar, Sudhir; Stecher, Glen; Li, Michael; Knyaz, Christina; Tamura, Koichiro (2018): MEGA X. Molecular Evolutionary Genetics Analysis across Computing Platforms. In: *Molecular biology and evolution* 35 (6), S. 1547–1549. DOI: 10.1093/molbev/msy096.

Kusajima, Miyuki; Yasuda, Michiko; Kawashima, Akiko; Nojiri, Hideaki; Yamane, Hisakazu; Nakajima, Masami et al. (2010): Suppressive effect of abscisic acid on systemic acquired resistance in tobacco plants. In: *J Gen Plant Pathol* 76 (2), S. 161–167. DOI: 10.1007/s10327-010-0218-5.

Kushalappa, Ajjamada C.; Yogendra, Kalenahalli N.; Karre, Shailesh (2016): Plant Innate Immune Response. Qualitative and Quantitative Resistance. In: *Critical Reviews in Plant Sciences* 35 (1), S. 38–55. DOI: 10.1080/07352689.2016.1148980.

Langner, Thorsten; Kamoun, Sophien; Belhaj, Khaoula (2018): CRISPR Crops. Plant Genome Editing Toward Disease Resistance. In: *Annual review of phytopathology* 56, S. 479–512. DOI: 10.1146/annurev-phyto-080417-050158.

Larkin, R. M.; Hagen, G.; Guilfoyle, T. J. (1999): Arabidopsis thaliana RNA polymerase II subunits related to yeast and human RPB5. In: *Gene* 231 (1-2), S. 41–47. DOI: 10.1016/S0378-1119(99)00090-6.

Li, Chao; Hao, Mengyu; Wang, Wenxiang; Wang, Hui; Chen, Fan; Chu, Wen et al. (2018): An Efficient CRISPR/Cas9 Platform for Rapidly Generating Simultaneous Mutagenesis of Multiple Gene Homoeologs in Allotetraploid Oilseed Rape. In: *Frontiers in plant science* 9, S. 442. DOI: 10.3389/fpls.2018.00442.

Li, Jian-Feng; Norville, Julie E.; Aach, John; McCormack, Matthew; Zhang, Dandan; Bush, Jenifer et al. (2013): Multiplex and homologous recombination-mediated genome editing in Arabidopsis and Nicotiana benthamiana using guide RNA and Cas9. In: *Nature biotechnology* 31 (8), S. 688–691. DOI: 10.1038/nbt.2654.

Li, Jun; Meng, Xiangbing; Zong, Yuan; Chen, Kunling; Zhang, Huawei; Liu, Jinxing et al. (2016): Gene replacements and insertions in rice by intron targeting using CRISPR-Cas9. In: *Nature plants* 2, S. 16139. DOI: 10.1038/nplants.2016.139.

Liebrand, Thomas W. H.; Kombrink, Anja; Zhang, Zhao; Sklenar, Jan; Jones, Alexandra M. E.; Robatzek, Silke et al. (2014): Chaperones of the endoplasmic reticulum are required for Ve1-mediated resistance to Verticillium. In: *Molecular plant pathology* 15 (1), S. 109–117. DOI: 10.1111/mpp.12071.

Liu, L.; van Tonder, T.; Pietersen, G.; Davies, J. W.; Stanley, J. (1997): Molecular characterization of a subgroup I geminivirus from a legume in South Africa. In: *The Journal of general virology* 78 (Pt 8), S. 2113–2117. DOI: 10.1099/0022-1317-78-8-2113.

Liu, Li; Saunders, Keith; Thomas, Carole L.; Davies, Jeffrey W.; Stanley, John (1999): Bean Yellow Dwarf Virus RepA, but Not Rep, Binds to Maize Retinoblastoma Protein, and the Virus Tolerates Mutations in the Consensus Binding Motif. In: *Virology* 256 (2), S. 270–279. DOI: 10.1006/viro.1999.9616.

Longdou, Lu; Wu Jun, Gao; Wang, Jingxue; Hongying, Duan; Ruili, Li (2005): EXPRESSION OF CHITINASE GENE IN TRANSGENIC RAPE PLANTS. In: *Analele Științifice Ale Universității Alexandru Ioan Cuza din Iași, Secțiunea II A : Genetica și Biologie Moleculară* 6.

Ludidi, N. N.; Heazlewood, J. L.; Seoighe, C.; Irving, H. R.; Gehring, C. A. (2002): Expansin-like molecules. Novel functions derived from common domains. In: *Journal of molecular evolution* 54 (5), S. 587–594. DOI: 10.1007/s00239-001-0055-4.

Lynn, K.; Fernandez, A.; Aida, M.; Sedbrook, J.; Tasaka, M.; Masson, P.; Barton, M. K. (1999): The PINHEAD/ZWILLE gene acts pleiotropically in Arabidopsis development and has overlapping functions with the ARGONAUTE1 gene. In: *Development (Cambridge, England)* 126 (3), S. 469–481.

- Ma, Jin; Chen, Jun; Wang, Min; Ren, Yulong; Wang, Shuai; Lei, Cailin et al. (2018): Disruption of OsSEC3A increases the content of salicylic acid and induces plant defense responses in rice. In: *Journal of experimental botany* 69 (5), S. 1051–1064. DOI: 10.1093/jxb/erx458.
- Ma, Xingliang; Zhang, Qunyu; Zhu, Qinlong; Liu, Wei; Chen, Yan; Qiu, Rong et al. (2015): A Robust CRISPR/Cas9 System for Convenient, High-Efficiency Multiplex Genome Editing in Monocot and Dicot Plants. In: *Molecular plant* 8 (8), S. 1274–1284. DOI: 10.1016/j.molp.2015.04.007.
- Ma, Xingliang; Zhu, Qinlong; Chen, Yuanling; Liu, Yao-Guang (2016): CRISPR/Cas9 Platforms for Genome Editing in Plants. Developments and Applications. In: *Molecular plant* 9 (7), S. 961–974. DOI: 10.1016/j.molp.2016.04.009.
- Mahfouz, Magdy M.; Li, Lixin; Shamimuzzaman, Md; Wibowo, Anjar; Fang, Xiaoyun; Zhu, Jian-Kang (2011): De novo-engineered transcription activator-like effector (TALE) hybrid nuclease with novel DNA binding specificity creates double-strand breaks. In: *Proceedings of the National Academy of Sciences of the United States of America* 108 (6), S. 2623–2628. DOI: 10.1073/pnas.1019533108.
- McCallum, C. M.; Comai, L.; Greene, E. A.; Henikoff, S. (2000): Targeting induced local lesions IN genomes (TILLING) for plant functional genomics. In: *Plant physiology* 123 (2), S. 439–442. DOI: 10.1104/pp.123.2.439.
- McCormac, A. C.; Elliott, M. C.; Chen, D. F. (1998): A simple method for the production of highly competent cells of *Agrobacterium* for transformation via electroporation. In: *Molecular biotechnology* 9 (2), S. 155–159. DOI: 10.1007/BF02760816.
- Menegazzi, P.; Guzzo, F.; Baldan, B.; Mariani, P.; Treves, S. (1993): Purification of calreticulin-like protein (s) from spinach leaves. In: *Biochemical and biophysical research communications* 190 (3), S. 1130–1135.
- Michno, Jean-Michel; Wang, Xiaobo; Liu, Junqi; Curtin, Shaun J.; Kono, Thomas Jy; Stupar, Robert M. (2015): CRISPR/Cas mutagenesis of soybean and *Medicago truncatula* using a new web-tool and a modified Cas9 enzyme. In: *GM crops & food* 6 (4), S. 243–252. DOI: 10.1080/21645698.2015.1106063.
- Mikami, Masafumi; Toki, Seiichi; Endo, Masaki (2015): Parameters affecting frequency of CRISPR/Cas9 mediated targeted mutagenesis in rice. In: *Plant cell reports* 34 (10), S. 1807–1815. DOI: 10.1007/s00299-015-1826-5.
- Moghaieb, Reda E.A.; El-Awady, Mohamed A.; Mergawy, Rabab G. El; Youssef, Sawsan S.; El-Sharkawy, Ahmed M. (2006): A reproducible protocol for regeneration and transformation in canola (*Brassica napus* L.). In: *African Journal of Biotechnology* 5 (2), S. 143–148. Online verfügbar unter <https://www.ajol.info/index.php/ajb/article/download/137741/127303>.

Morse, Monique; Pironcheva, Ganka; Gehring, Chris (2004): AtPNP-A is a systemically mobile natriuretic peptide immunoanalogue with a role in Arabidopsis thaliana cell volume regulation. In: *FEBS letters* 556 (1-3), S. 99–103. DOI: 10.1016/S0014-5793(03)01384-X.

Nakagawa, Tsuyoshi; Kurose, Takayuki; Hino, Takeshi; Tanaka, Katsunori; Kawamukai, Makoto; Niwa, Yasuo et al. (2007): Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. In: *Journal of bioscience and bioengineering* 104 (1), S. 34–41. DOI: 10.1263/jbb.104.34.

Nakano, Toshitsugu; Suzuki, Kaoru; Fujimura, Tatsuhito; Shinshi, Hideaki (2006): Genome-wide analysis of the ERF gene family in Arabidopsis and rice. In: *Plant physiology* 140 (2), S. 411–432. DOI: 10.1104/pp.105.073783.

Nakashima, K.; Satoh, R.; Kiyosue, T.; Yamaguchi-Shinozaki, K.; Shinozaki, K. (1998): A gene encoding proline dehydrogenase is not only induced by proline and hypoosmolarity, but is also developmentally regulated in the reproductive organs of Arabidopsis. In: *Plant physiology* 118 (4), S. 1233–1241. DOI: 10.1104/pp.118.4.1233.

Nakayasu, Masaru; Akiyama, Ryota; Lee, Hyoung Jae; Osakabe, Keishi; Osakabe, Yuriko; Watanabe, Bunta et al. (2018): Generation of α -solanine-free hairy roots of potato by CRISPR/Cas9 mediated genome editing of the St16DOX gene. In: *Plant physiology and biochemistry : PPB* 131, S. 70–77. DOI: 10.1016/j.plaphy.2018.04.026.

Navarro, Lionel; Dunoyer, Patrice; Jay, Florence; Arnold, Benedict; Dharmasiri, Nihal; Estelle, Mark et al. (2006): A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. In: *Science (New York, N.Y.)* 312 (5772), S. 436–439. DOI: 10.1126/science.1126088.

Neff, M. M.; Neff, J. D.; Chory, J.; Pepper, A. E. (1998): dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms. Experimental applications in Arabidopsis thaliana genetics. In: *The Plant journal : for cell and molecular biology* 14 (3), S. 387–392.

Nekrasov, Vladimir; Staskawicz, Brian; Weigel, Detlef; Jones, Jonathan D. G.; Kamoun, Sophien (2013): Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease. In: *Nature biotechnology* 31 (8), S. 691–693. DOI: 10.1038/nbt.2655.

Nekrasov, Vladimir; Wang, Congmao; Win, Joe; Lanz, Christa; Weigel, Detlef; Kamoun, Sophien (2017): Rapid generation of a transgene-free powdery mildew resistant tomato by genome deletion. In: *Scientific reports* 7 (1), S. 482. DOI: 10.1038/s41598-017-00578-x.

Neumann, M. J.; Dobinson, K. F. (2003): Sequence tag analysis of gene expression during pathogenic growth and microsclerotia development in the vascular wilt pathogen *Verticillium dahliae*. In: *Fungal Genetics and Biology* 38 (1), S. 54–62. DOI: 10.1016/S1087-1845(02)00507-8.

- Nicholson, R. L.; Hammerschmidt, R. (1992): Phenolic Compounds and Their Role in Disease Resistance. In: *Annu. Rev. Phytopathol.* 30 (1), S. 369–389. DOI: 10.1146/annurev.py.30.090192.002101.
- Nimchuk, Zachary; Eulgem, Thomas; Holt, Ben F.; Dangl, Jeffery L. (2003): Recognition and response in the plant immune system. In: *Annual review of genetics* 37, S. 579–609. DOI: 10.1146/annurev.genet.37.110801.142628.
- Obermeier, Christian; Hossain, Muhammed Ali; Snowdon, Rod; Knüfer, Jessica; Tiedemann, Andreas von; Friedt, Wolfgang (2013): Genetic analysis of phenylpropanoid metabolites associated with resistance against *Verticillium longisporum* in *Brassica napus*. In: *Mol Breeding* 31 (2), S. 347–361. DOI: 10.1007/s11032-012-9794-8.
- Okonechnikov, Konstantin; Golosova, Olga; Fursov, Mikhail (2012): Unipro UGENE. A unified bioinformatics toolkit. In: *Bioinformatics (Oxford, England)* 28 (8), S. 1166–1167. DOI: 10.1093/bioinformatics/bts091.
- Okubara, Patricia A.; Paulitz, Timothy C. (2005): Root Defense Responses to Fungal Pathogens. A Molecular Perspective. In: *Plant Soil* 274 (1-2), S. 215–226. DOI: 10.1007/s11104-004-7328-9.
- Okuzaki, Ayako; Ogawa, Takumi; Koizuka, Chie; Kaneko, Kanako; Inaba, Mizue; Imamura, Jun; Koizuka, Nobuya (2018): CRISPR/Cas9-mediated genome editing of the fatty acid desaturase 2 gene in *Brassica napus*. In: *Plant physiology and biochemistry : PPB* 131, S. 63–69. DOI: 10.1016/j.plaphy.2018.04.025.
- Oleykowski, C. A.; Bronson Mullins, C. R.; Godwin, A. K.; Yeung, A. T. (1998): Mutation detection using a novel plant endonuclease. In: *Nucleic acids research* 26 (20), S. 4597–4602. DOI: 10.1093/nar/26.20.4597.
- Oliva, Ricardo; Ji, Chonghui; Atienza-Grande, Genelou; Huguet-Tapia, José C.; Perez-Quintero, Alvaro; Li, Ting et al. (2019): Broad-spectrum resistance to bacterial blight in rice using genome editing. In: *Nature biotechnology* 37 (11), S. 1344–1350. DOI: 10.1038/s41587-019-0267-z.
- Olsson, Stefan; Nordbring-Hertz, Birgit (1985): Microsclerotial germination of *Verticillium dahliae* as affected by rape rhizosphere. In: *FEMS Microbiology Letters* 31 (5), S. 293–299. DOI: 10.1111/j.1574-6968.1985.tb01162.x.
- Ooka, Hisako; Satoh, Kouji; Doi, Koji; Nagata, Toshifumi; Otomo, Yasuhiro; Murakami, Kazuo et al. (2003): Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. In: *DNA research : an international journal for rapid publication of reports on genes and genomes* 10 (6), S. 239–247. DOI: 10.1093/dnares/10.6.239.
- Ostwald, Thomas J.; MacLennan, David H. (1974): Isolation of a High Affinity Calcium-binding Protein from Sarcoplasmic Reticulum. In: *J. Biol. Chem.* 249 (3), S. 974–979. Online verfügbar unter <http://www.jbc.org/content/249/3/974.full.pdf>.

Park, Jeongbin; Bae, Sangsu; Kim, Jin-Soo (2015): Cas-Designer. A web-based tool for choice of CRISPR-Cas9 target sites. In: *Bioinformatics (Oxford, England)* 31 (24), S. 4014–4016. DOI: 10.1093/bioinformatics/btv537.

Passarinho, P. A.; van Hengel, A. J.; Fransz, P. F.; Vries, S. C. de (2001): Expression pattern of the Arabidopsis thaliana AtEP3/AtchitIV endochitinase gene. In: *Planta* 212 (4), S. 556–567. DOI: 10.1007/s004250000464.

Peng, Aihong; Chen, Shanchun; Lei, Tiangang; Xu, Lanzhen; He, Yongrui; Wu, Liu et al. (2017): Engineering canker-resistant plants through CRISPR/Cas9-targeted editing of the susceptibility gene CsLOB1 promoter in citrus. In: *Plant biotechnology journal* 15 (12), S. 1509–1519. DOI: 10.1111/pbi.12733.

Peng, Z.; Lu, Q.; Verma, D. P. (1996): Reciprocal regulation of delta 1-pyrroline-5-carboxylate synthetase and proline dehydrogenase genes controls proline levels during and after osmotic stress in plants. In: *Molecular & general genetics : MGG* 253 (3), S. 334–341. DOI: 10.1007/pl00008600.

Peremyslov, Valera V.; Mockler, Todd C.; Filichkin, Sergei A.; Fox, Samuel E.; Jaiswal, Pankaj; Makarova, Kira S. et al. (2011): Expression, splicing, and evolution of the myosin gene family in plants. In: *Plant physiology* 155 (3), S. 1191–1204. DOI: 10.1104/pp.110.170720.

Persson, S.; Wyatt, S. E.; Love, J.; Thompson, W. F.; Robertson, D.; Boss, W. F. (2001): The Ca(2+) status of the endoplasmic reticulum is altered by induction of calreticulin expression in transgenic plants. In: *Plant physiology* 126 (3), S. 1092–1104. DOI: 10.1104/pp.126.3.1092.

Pfaffl, M. W. (2001): A new mathematical model for relative quantification in real-time RT-PCR. In: *Nucleic acids research* 29 (9), e45. DOI: 10.1093/nar/29.9.e45.

Pistelli, Laura; Giovannini, Annalisa; Ruffoni, Barbara; Bertoli, Alessandra; Pistelli, Luisa (2010): Hairy root cultures for secondary metabolites production. In: *Advances in experimental medicine and biology* 698, S. 167–184. DOI: 10.1007/978-1-4419-7347-4_13.

Prakash S, Hinata K. (1980): Taxonomy, cytogenetics and origin of crop Brassica, a review. In: *Opera Bot* (55), S. 1–57.

Presley, J.T., Carns, H.R., Taylor, E.E., Schnathorst, W.C. (1966): Movement of conidia of *Verticillium albo-atrum* in cotton plants. In: *Phytopathology* (56), S. 375.

Puchta, Holger (2005): The repair of double-strand breaks in plants. Mechanisms and consequences for genome evolution. In: *Journal of experimental botany* 56 (409), S. 1–14. DOI: 10.1093/jxb/eri025.

Quigley, Françoise; Rosenberg, Joshua M.; Shachar-Hill, Yair; Bohnert, Hans J. (2002): From genome to function. The Arabidopsis aquaporins. In: *Genome biology* 3 (1), RESEARCH0001. DOI: 10.1186/gb-2001-3-1-research0001.

R Core Team (2019): R: A Language and Environment for Statistical Computing. Version. Vienna, Austria: R Foundation for Statistical Computing. Online verfügbar unter <https://www.R-project.org/>.

Rafiqi, Maryam; Ellis, Jeffrey G.; Ludowici, Victoria A.; Hardham, Adrienne R.; Dodds, Peter N. (2012): Challenges and progress towards understanding the role of effectors in plant-fungal interactions. In: *Current Opinion in Plant Biology* 15 (4), S. 477–482. DOI: 10.1016/j.pbi.2012.05.003.

Ralhan, Anjali; Schöttle, Sonja; Thurow, Corinna; Iven, Tim; Feussner, Ivo; Polle, Andrea; Gatz, Christiane (2012): The vascular pathogen *Verticillium longisporum* requires a jasmonic acid-independent COI1 function in roots to elicit disease symptoms in *Arabidopsis* shoots. In: *Plant physiology* 159 (3), S. 1192–1203. DOI: 10.1104/pp.112.198598.

Ratzinger, Astrid; Riediger, Nadine; Tiedemann, Andreas von; Karlovsky, Petr (2009): Salicylic acid and salicylic acid glucoside in xylem sap of *Brassica napus* infected with *Verticillium longisporum*. In: *Journal of plant research* 122 (5), S. 571–579. DOI: 10.1007/s10265-009-0237-5.

Reusche, Michael; Klásková, Jana; Thole, Karin; Truskina, Jekaterina; Novák, Ondřej; Janz, Dennis et al. (2013): Stabilization of cytokinin levels enhances *Arabidopsis* resistance against *Verticillium longisporum*. In: *Molecular plant-microbe interactions : MPMI* 26 (8), S. 850–860. DOI: 10.1094/MPMI-12-12-0287-R.

Rey, Marie E. C.; Ndunguru, Joseph; Berrie, Leigh C.; Paximadis, Maria; Berry, Shaun; Cossa, Nurbibi et al. (2012): Diversity of dicotyledenous-infecting geminiviruses and their associated DNA molecules in southern Africa, including the South-west Indian ocean islands. In: *Viruses* 4 (9), S. 1753–1791. DOI: 10.3390/v4091753.

Rogers, S. O.; Bendich, A. J. (1985): Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. In: *Plant molecular biology* 5 (2), S. 69–76. DOI: 10.1007/BF00020088.

Roos, Jonas; Bejai, Sarosh; Oide, Shinichi; Dixelius, Christina (2014): RabGAP22 is required for defense to the vascular pathogen *Verticillium longisporum* and contributes to stomata immunity. In: *PloS one* 9 (2), e88187. DOI: 10.1371/journal.pone.0088187.

Rybicki, Edward P. (2009): Plant-produced vaccines. Promise and reality. In: *Drug discovery today* 14 (1-2), S. 16–24. DOI: 10.1016/j.drudis.2008.10.002.

Rygulla, W.; Snowdon, R. J.; Friedt, W.; Happstadius, I.; Cheung, W. Y.; Chen, D. (2008): Identification of quantitative trait loci for resistance against *Verticillium longisporum* in oilseed rape (*Brassica napus*). In: *Phytopathology* 98 (2), S. 215–221. DOI: 10.1094/PHYTO-98-2-0215.

Sarwat, Maryam; Tuteja, Narendra (2017): How the ER Stress Protein Calreticulins Differ from Each Other in Plants? In: Khalid Rehman Hakeem, Adeel Malik, Fazilet Vardar-Sukan und Munir Ozturk (Hg.): *Plant Bioinformatics. Decoding the Phyta*. Cham: Springer, S. 403–415.

Scheben, Armin; Wolter, Felix; Batley, Jacqueline; Puchta, Holger; Edwards, David (2017): Towards CRISPR/Cas crops - bringing together genomics and genome editing. In: *The New phytologist* 216 (3), S. 682–698. DOI: 10.1111/nph.14702.

Schimpl, Simon; Fauser, Friedrich; Puchta, Holger (2014): The CRISPR/Cas system can be used as nuclease for in planta gene targeting and as paired nickases for directed mutagenesis in Arabidopsis resulting in heritable progeny. In: *The Plant journal : for cell and molecular biology* 80 (6), S. 1139–1150. DOI: 10.1111/tpj.12704.

Schreiber, L.R., Green, R.J.Jr (1963): Effects of root exudates on germination of conidia and microsclerotia of *Verticillium albo-atrum* inhibited by the soil fungistatic principle. In: *Phytopathology* (53), S. 260–264.

Schuldiner, Maya; Collins, Sean R.; Thompson, Natalie J.; Denic, Vladimir; Bhamidipati, Arunashree; Punna, Thanuja et al. (2005): Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile. In: *Cell* 123 (3), S. 507–519. DOI: 10.1016/j.cell.2005.08.031.

Seyis, F.; Snowdon, R. J.; Luhs, W.; Friedt, W. (2003): Molecular characterization of novel resynthesized rapeseed (*Brassica napus*) lines and analysis of their genetic diversity in comparison with spring rapeseed cultivars*. In: *Plant Breeding* 122 (6), S. 473–478. DOI: 10.1111/j.1439-0523.2003.00859.x.

Shahidi, Fereidoon (Hg.) (1990): *Canola and Rapeseed. Production, Chemistry, Nutrition and Processing Technology*.

Shan, Qiwei; Wang, Yanpeng; Li, Jun; Zhang, Yi; Chen, Kunling; Liang, Zhen et al. (2013): Targeted genome modification of crop plants using a CRISPR-Cas system. In: *Nature biotechnology* 31 (8), S. 686–688. DOI: 10.1038/nbt.2650.

Shen, Dan; Suhrkamp, Ina; Wang, Yu; Liu, Shenyi; Menkhaus, Jan; Verreet, Joseph-Alexander et al. (2014): Identification and characterization of microRNAs in oilseed rape (*Brassica napus*) responsive to infection with the pathogenic fungus *Verticillium longisporum* using *Brassica AA* (*Brassica rapa*) and *CC* (*Brassica oleracea*) as reference genomes. In: *The New phytologist* 204 (3), S. 577–594. DOI: 10.1111/nph.12934.

Shen, Q.; Chen, C. N.; Brands, A.; Pan, S. M.; Ho, T. H. (2001): The stress- and abscisic acid-induced barley gene HVA22. Developmental regulation and homologues in diverse organisms. In: *Plant molecular biology* 45 (3), S. 327–340. DOI: 10.1023/a:1006460231978.

Shen, Q.; Uknes, S. J.; Ho, T. H. (1993): Hormone response complex in a novel abscisic acid and cycloheximide-inducible barley gene. In: *J. Biol. Chem.* 268 (31), S. 23652–23660. Online verfügbar unter <http://www.jbc.org/content/268/31/23652.full.pdf>.

Shigenaga, Alexandra M.; Argueso, Cristiana T. (2016): No hormone to rule them all. Interactions of plant hormones during the responses of plants to pathogens. In: *Seminars in cell & developmental biology* 56, S. 174–189. DOI: 10.1016/j.semcdb.2016.06.005.

Shivaprasad, Padubidri V.; Chen, Ho-Ming; Patel, Kanu; Bond, Donna M.; Santos, Bruno A. C. M.; Baulcombe, David C. (2012): A microRNA superfamily regulates nucleotide binding site-leucine-rich repeats and other mRNAs. In: *The Plant cell* 24 (3), S. 859–874. DOI: 10.1105/tpc.111.095380.

Song, K. M.; Osborn, T. C.; Williams, P. H. (1988): Brassica taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). In: *Theoret. Appl. Genetics* 75 (5), S. 784–794. DOI: 10.1007/BF00265606.

Songstad, D. D.; Petolino, J. F.; Voytas, D. F.; Reichert, N. A. (2017): Genome Editing of Plants. In: *Critical Reviews in Plant Sciences* 36 (1), S. 1–23. DOI: 10.1080/07352689.2017.1281663.

Spoel, Steven H.; Johnson, Jessica S.; Dong, Xinnian (2007): Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. In: *Proceedings of the National Academy of Sciences of the United States of America* 104 (47), S. 18842–18847. DOI: 10.1073/pnas.0708139104.

Sun, Qinfu; Lin, Li; Liu, Dongxiao; Wu, Dewei; Fang, Yujie; Wu, Jian; Wang, Youping (2018): CRISPR/Cas9-Mediated Multiplex Genome Editing of the BnWRKY11 and BnWRKY70 Genes in Brassica napus L. In: *International journal of molecular sciences* 19 (9). DOI: 10.3390/ijms19092716.

Sun, Xianjun; Hu, Zheng; Chen, Rui; Jiang, Qiyang; Song, Guohua; Zhang, Hui; Xi, Yajun (2015): Targeted mutagenesis in soybean using the CRISPR-Cas9 system. In: *Scientific reports* 5, S. 10342. DOI: 10.1038/srep10342.

Symington, Lorraine S.; Gautier, Jean (2011): Double-strand break end resection and repair pathway choice. In: *Annual review of genetics* 45, S. 247–271. DOI: 10.1146/annurev-genet-110410-132435.

Tabata, S.; Kaneko, T.; Nakamura, Y.; Kotani, H.; Kato, T.; Asamizu, E. et al. (2000): Sequence and analysis of chromosome 5 of the plant *Arabidopsis thaliana*. In: *Nature* 408 (6814), S. 823–826. DOI: 10.1038/35048507.

TALBOYS, P. W. (1964): A Concept of the Host–Parasite Relationship in Verticillium Wilt Diseases. In: *Nature* 202 (4930), S. 361–364. DOI: 10.1038/202361a0.

The Council of the European Communities: Council Directive 90/219/EEC of 23 April 1990 on the contained use of genetically modified micro-organisms. In: *Off J* 117, S. 227–247.

The Council of the European Communities: Council Directive 90/220/EEC of 23 April 1990 on the deliberate release into the environment of genetically modified organisms. In: *Off J* 117, S. 15–27.

Theologis, A.; Ecker, J. R.; Palm, C. J.; Federspiel, N. A.; Kaul, S.; White, O. et al. (2000): Sequence and analysis of chromosome 1 of the plant *Arabidopsis thaliana*. In: *Nature* 408 (6814), S. 816–820. DOI: 10.1038/35048500.

Thines, Bryan; Katsir, Leron; Melotto, Maeli; Niu, Yajie; Mandaokar, Ajin; Liu, Guanghui et al. (2007): JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. In: *Nature* 448 (7154), S. 661–665. DOI: 10.1038/nature05960.

Thomma, B. P.; Eggermont, K.; Penninckx, I. A.; Mauch-Mani, B.; Vogelsang, R.; Cammue, B. P.; Broekaert, W. F. (1998): Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. In: *Proceedings of the National Academy of Sciences of the United States of America* 95 (25), S. 15107–15111. DOI: 10.1073/pnas.95.25.15107.

Tsolakidou, Maria-Dimitra; Pantelides, Lakovos S.; Tzima, Aliki K.; Kang, Seogchan; Paplomatas, Epaminondas J.; Tsaltas, Dimitris (2019): Disruption and Overexpression of the Gene Encoding ACC (1-Aminocyclopropane-1-Carboxylic Acid) Deaminase in Soil-Borne Fungal Pathogen *Verticillium dahliae* Revealed the Role of ACC as a Potential Regulator of Virulence and Plant Defense. In: *Molecular plant-microbe interactions : MPMI* 32 (6), S. 639–653. DOI: 10.1094/MPMI-07-18-0203-R.

Tunçel, G.; Nergiz, C. (1993): Antimicrobial effect of some olive phenols in a laboratory medium. In: *Lett Appl Microbiol* 17 (6), S. 300–302. DOI: 10.1111/j.1472-765X.1993.tb01472.x.

Tyvaert, L.; França, S. C.; Debode, J.; Höfte, M. (2014): The endophyte *Verticillium* Vt305 protects cauliflower against *Verticillium* wilt. In: *Journal of applied microbiology* 116 (6), S. 1563–1571. DOI: 10.1111/jam.12481.

Ueta, Risa; Abe, Chihiro; Watanabe, Takahito; Sugano, Shigeo S.; Ishihara, Ryosuke; Ezura, Hiroshi et al. (2017): Rapid breeding of parthenocarpic tomato plants using CRISPR/Cas9. In: *Scientific reports* 7 (1), S. 507. DOI: 10.1038/s41598-017-00501-4.

USDA: Crop Production 2018 Summary. February 2019. ISSN: 1057-7823.

Uusi-Mäkelä, Meri I. E.; Barker, Harlan R.; Bäuerlein, Carina A.; Häkkinen, Tomi; Nykter, Matti; Rämetsä, Mika (2018): Chromatin accessibility is associated with CRISPR-Cas9 efficiency in the zebrafish (*Danio rerio*). In: *PLoS one* 13 (4), e0196238. DOI: 10.1371/journal.pone.0196238.

Verkuijl, Sebald An; Rots, Marianne G. (2019): The influence of eukaryotic chromatin state on CRISPR-Cas9 editing efficiencies. In: *Current opinion in biotechnology* 55, S. 68–73. DOI: 10.1016/j.copbio.2018.07.005.

- Wakao, Setsuko; Benning, Christoph (2005): Genome-wide analysis of glucose-6-phosphate dehydrogenases in Arabidopsis. In: *The Plant journal : for cell and molecular biology* 41 (2), S. 243–256. DOI: 10.1111/j.1365-313X.2004.02293.x.
- Wang, Fujun; Wang, Chunlian; Liu, Piqing; Lei, Cailin; Hao, Wei; Gao, Ying et al. (2016): Enhanced Rice Blast Resistance by CRISPR/Cas9-Targeted Mutagenesis of the ERF Transcription Factor Gene OsERF922. In: *PLoS one* 11 (4), e0154027. DOI: 10.1371/journal.pone.0154027.
- Wang, Lin; Tsuda, Kenichi; Sato, Masanao; Cohen, Jerry D.; Katagiri, Fumiaki; Glazebrook, Jane (2009): Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. In: *PLoS pathogens* 5 (2), e1000301. DOI: 10.1371/journal.ppat.1000301.
- Wang, Ming; Weiberg, Arne; Lin, Feng-Mao; Thomma, Bart P. H. J.; Huang, Hsien-Da; Jin, Hailing (2016): Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. In: *Nature plants* 2, S. 16151. DOI: 10.1038/nplants.2016.151.
- Wang, Mugui; Lu, Yuming; Botella, José Ramón; Mao, Yanfei; Hua, Kai; Zhu, Jian-Kang (2017): Gene Targeting by Homology-Directed Repair in Rice Using a Geminivirus-Based CRISPR/Cas9 System. In: *Molecular plant* 10 (7), S. 1007–1010. DOI: 10.1016/j.molp.2017.03.002.
- Wang, Xianhang; Tu, Mingxing; Wang, Dejun; Liu, Jianwei; Li, Yajuan; Li, Zhi et al. (2018): CRISPR/Cas9-mediated efficient targeted mutagenesis in grape in the first generation. In: *Plant biotechnology journal* 16 (4), S. 844–855. DOI: 10.1111/pbi.12832.
- Wang, Yanpeng; Cheng, Xi; Shan, Qiwei; Zhang, Yi; Liu, Jinxing; Gao, Caixia; Qiu, Jin-Long (2014): Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. In: *Nature biotechnology* 32 (9), S. 947–951. DOI: 10.1038/nbt.2969.
- Wang, Zhi-Ping; Xing, Hui-Li; Dong, Li; Zhang, Hai-Yan; Han, Chun-Yan; Wang, Xue-Chen; Chen, Qi-Jun (2015): Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. In: *Genome biology* 16, S. 144. DOI: 10.1186/s13059-015-0715-0.
- Warwick SI, Black L. D. (1993): Molecular relationships in subtribe Brassicinae (Crucifera, tribe Brassiceae). In: *Can J Botany* (71), S. 906–9018.
- Weiberg, Arne; Wang, Ming; Lin, Feng-Mao; Zhao, Hongwei; Zhang, Zhihong; Kaloshian, Isgouhi et al. (2013): Fungal small RNAs suppress plant immunity by hijacking host RNA interference pathways. In: *Science (New York, N.Y.)* 342 (6154), S. 118–123. DOI: 10.1126/science.1239705.

- Witzel, Katja; Hanschen, Franziska S.; Klopsch, Rebecca; Ruppel, Silke; Schreiner, Monika; Grosch, Rita (2015): Verticillium longisporum infection induces organ-specific glucosinolate degradation in Arabidopsis thaliana. In: *Frontiers in plant science* 6, S. 508. DOI: 10.3389/fpls.2015.00508.
- Wyatt, Sarah E.; Tsou, Pei-Lan; Robertson, Dominique (2002): Expression of the high capacity calcium-binding domain of calreticulin increases bioavailable calcium stores in plants. In: *Transgenic research* 11 (1), S. 1–10.
- Xiang, C.; Werner, B. L.; Christensen, E. M.; Oliver, D. J. (2001): The biological functions of glutathione revisited in arabidopsis transgenic plants with altered glutathione levels. In: *Plant physiology* 126 (2), S. 564–574. DOI: 10.1104/pp.126.2.564.
- Xie, Kabin; Minkenberg, Bastian; Yang, Yinong (2015): Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. In: *Proceedings of the National Academy of Sciences of the United States of America* 112 (11), S. 3570–3575. DOI: 10.1073/pnas.1420294112.
- Xie, Zhixin; Allen, Edwards; Fahlgren, Noah; Calamar, Adam; Givan, Scott A.; Carrington, James C. (2005): Expression of Arabidopsis MIRNA genes. In: *Plant physiology* 138 (4), S. 2145–2154. DOI: 10.1104/pp.105.062943.
- Xing, Hui-Li; Dong, Li; Wang, Zhi-Ping; Zhang, Hai-Yan; Han, Chun-Yan; Liu, Bing et al. (2014): A CRISPR/Cas9 toolkit for multiplex genome editing in plants. In: *BMC plant biology* 14, S. 327. DOI: 10.1186/s12870-014-0327-y.
- Xu, Q.; Fu, H. H.; Gupta, R.; Luan, S. (1998): Molecular characterization of a tyrosine-specific protein phosphatase encoded by a stress-responsive gene in Arabidopsis. In: *The Plant cell* 10 (5), S. 849–857. DOI: 10.1105/tpc.10.5.849.
- Xu, Rongfang; Li, Hao; Qin, Ruiying; Wang, Lu; Li, Li; Wei, Pengcheng; Yang, Jianbo (2014): Gene targeting using the Agrobacterium tumefaciens-mediated CRISPR-Cas system in rice. In: *Rice (New York, N.Y.)* 7 (1), S. 5. DOI: 10.1186/s12284-014-0005-6.
- Yadeta, Koste A.; Valkenburg, Dirk-Jan; Hanemian, Mathieu; Marco, Yves; Thomma, Bart P. H. J. (2014): The Brassicaceae-specific EWR1 gene provides resistance to vascular wilt pathogens. In: *PLoS one* 9 (2), e88230. DOI: 10.1371/journal.pone.0088230.
- Yamada, Kayoko; Lim, Jun; Dale, Joseph M.; Chen, Huaming; Shinn, Paul; Palm, Curtis J. et al. (2003): Empirical analysis of transcriptional activity in the Arabidopsis genome. In: *Science (New York, N.Y.)* 302 (5646), S. 842–846. DOI: 10.1126/science.1088305.

- Yang, Hong; Wu, Jia-Jing; Tang, Ting; Liu, Ke-De; Dai, Cheng (2017): CRISPR/Cas9-mediated genome editing efficiently creates specific mutations at multiple loci using one sgRNA in *Brassica napus*. In: *Scientific reports* 7 (1), S. 7489. DOI: 10.1038/s41598-017-07871-9.
- Zaidi, Syed Shan-E-Ali; Mansoor, Shahid (2017): Viral Vectors for Plant Genome Engineering. In: *Frontiers in plant science* 8, S. 539. DOI: 10.3389/fpls.2017.00539.
- Zapata, Luis; Ding, Jia; Willing, Eva-Maria; Hartwig, Benjamin; Bezdán, Daniela; Jiao, Wen-Biao et al. (2016): Chromosome-level assembly of *Arabidopsis thaliana* Ler reveals the extent of translocation and inversion polymorphisms. In: *Proceedings of the National Academy of Sciences of the United States of America* 113 (28), E4052-60. DOI: 10.1073/pnas.1607532113.
- Zare, R.; Gams, W.; Starink-Willemsse, M.; Summerbell, R. C. (2007): *Gibellulopsis*, a suitable genus for *Verticillium nigrescens*, and *Musciellium*, a new genus for *V. theobromae*. In: *Nova Hedw.* 85 (3), S. 463–489. DOI: 10.1127/0029-5035/2007/0085-0463.
- Zeise, K.; Tiedemann, A. von (2001): Morphological and Physiological Differentiation among Vegetative Compatibility Groups of *Verticillium dahliae* in Relation to *V. longisporum*. In: *Journal of Phytopathology* 149 (7-8), S. 469–475. DOI: 10.1111/j.1439-0434.2001.tb03879.x.
- Zetsche, Bernd; Gootenberg, Jonathan S.; Abudayyeh, Omar O.; Slaymaker, Ian M.; Makarova, Kira S.; Essletzbichler, Patrick et al. (2015): Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. In: *Cell* 163 (3), S. 759–771. DOI: 10.1016/j.cell.2015.09.038.
- Zhai, Yungu; Cai, Shengli; Hu, Limin; Yang, Yang; Amoo, Olalekan; Fan, Chuchuan; Zhou, Yongming (2019): CRISPR/Cas9-mediated genome editing reveals differences in the contribution of INDEHISCENT homologues to pod shatter resistance in *Brassica napus* L. In: *TAG. Theoretical and applied genetics. Theoretische und angewandte Genetik* 132 (7), S. 2111–2123. DOI: 10.1007/s00122-019-03341-0.
- Zhang, Hui; Zhang, Jinshan; Wei, Pengliang; Zhang, Botao; Gou, Feng; Feng, Zhengyan et al. (2014): The CRISPR/Cas9 system produces specific and homozygous targeted gene editing in rice in one generation. In: *Plant biotechnology journal* 12 (6), S. 797–807. DOI: 10.1111/pbi.12200.
- Zhang, Kai; Nie, Liluo; Cheng, Qiqi; Yin, Yongtai; Chen, Kang; Qi, Fuyu et al. (2019): Effective editing for lysophosphatidic acid acyltransferase 2/5 in allotetraploid rapeseed (*Brassica napus* L.) using CRISPR-Cas9 system. In: *Biotechnology for biofuels* 12, S. 225. DOI: 10.1186/s13068-019-1567-8.
- Zhang, Ning; Castlebury, Lisa A.; Miller, Andrew N.; Huhndorf, Sabine M.; Schoch, Conrad L.; Seifert, Keith A. et al. (2006): An overview of the systematics of the Sordariomycetes based on a four-gene phylogeny. In: *Mycologia* 98 (6), S. 1076–1087.

Zhang, Shujuan; Zhang, Rongzhi; Gao, Jie; Gu, Tiantian; Song, Guoqi; Li, Wei et al. (2019): Highly Efficient and Heritable Targeted Mutagenesis in Wheat via the *Agrobacterium tumefaciens*-Mediated CRISPR/Cas9 System. In: *International journal of molecular sciences* 20 (17). DOI: 10.3390/ijms20174257.

Zhang, Tao; Zhao, Yun-Long; Zhao, Jian-Hua; Wang, Sheng; Jin, Yun; Chen, Zhong-Qi et al. (2016): Cotton plants export microRNAs to inhibit virulence gene expression in a fungal pathogen. In: *Nature plants* 2 (10), S. 16153. DOI: 10.1038/nplants.2016.153.

Zhang, Yi; Liang, Zhen; Zong, Yuan; Wang, Yanpeng; Liu, Jinxing; Chen, Kunling et al. (2016): Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. In: *Nature communications* 7, S. 12617. DOI: 10.1038/ncomms12617.

Zhang, Zhengjing; Mao, Yanfei; Ha, Si; Liu, Wenshan; Botella, Jose Ramon; Zhu, Jian-Kang (2016): A multiplex CRISPR/Cas9 platform for fast and efficient editing of multiple genes in *Arabidopsis*. In: *Plant cell reports* 35 (7), S. 1519–1533. DOI: 10.1007/s00299-015-1900-z.

Zhao, J.; Meng, J. (2003): Detection of loci controlling seed glucosinolate content and their association with *Sclerotinia* resistance in *Brassica napus*. In: *Plant Breeding* 122 (1), S. 19–23. DOI: 10.1046/j.1439-0523.2003.00784.x.

Zheng, Ming; Zhang, Liang; Tang, Min; Liu, Jinglin; Liu, Hongfang; Yang, Hongli et al. (2019): Knockout of two *BnaMAX1* homologs by CRISPR/Cas9-targeted mutagenesis improves plant architecture and increases yield in rapeseed (*Brassica napus* L.). In: *Plant biotechnology journal*. DOI: 10.1111/pbi.13228.

Zhou, L.; Hu, Q.; Johansson, A.; Dixelius, C. (2006): *Verticillium longisporum* and *V. dahliae*. Infection and disease in *Brassica napus*. In: *Plant Pathology* 55 (1), S. 137–144. DOI: 10.1111/j.1365-3059.2005.01311.x.

Zhou, Man; Lu, You; Bethke, Gerit; Harrison, Brian T.; Hatsugai, Noriyuki; Katagiri, Fumiaki; Glazebrook, Jane (2018): *WRKY70* prevents axenic activation of plant immunity by direct repression of *SARD1*. In: *The New phytologist* 217 (2), S. 700–712. DOI: 10.1111/nph.14846.

Zhou, Y.; Fitt, B.D.L.; Welham, S. J.; Gladders, P.; Sansford, C. E.; West, J. S. (1999): Effects of Severity and Timing of Stem Canker (*Leptosphaeria maculans*) Symptoms on Yield of Winter Oilseed Rape (*Brassica napus*) in the UK. In: *Eur J Plant Pathol* 105 (7), S. 715–728. DOI: 10.1023/A:1008761219493.

Zipfel, Cyril (2008): Pattern-recognition receptors in plant innate immunity. In: *Current opinion in immunology* 20 (1), S. 10–16. DOI: 10.1016/j.coi.2007.11.003.

Supplementary data

Table 24: sgRNA targets for NHEJ and HDR based genome editing obtained from CRISPR RGEN Tools. MM = Mismatches to the original target sequence.

Target gene	Guide sequence	PAM	Direction	1 MMs	2 MMs	3 MMs
NHEJ						
<i>AtAGO10</i>	TAGCCGGAGGAGAAACCGTGG	TGG	+	0	0	0
<i>BnCRT1a</i>	AGAATCAGAGAGATGAAGTT	NGG	-	0	0	5
<i>BnHVA22c_1</i>	GGTAGACGAGAGCAACCAA	TGG	-	0	0	0
<i>BnHVA22c_2</i>	TGGAGAAAATGAGTTCGAAG	AGG	-	0	0	0
<i>StTXR1</i>	CCCAGGCATATCGCCAAGCTCTC	TTTG	+	0	0	0
<i>StCESA3</i>	GGTCAAGTCTGTCAGATCTG	TGG	+	0	0	0
HDR						
<i>AtAGO1</i>	GAGGTAGCTTGATGCAGCTC	GGG	+	0	0	1
<i>BnAGO1</i>	GAGGTAGCTTGATGCAGCTC	GGG	+/-	2	0	2
<i>BnTAO1_A_1</i>	GTTGACAACGAGATCAAGCG	AGG	-	0	2	2
<i>BnTAO1_A_2</i>	TTGATCTCGTTGTCACGAA	AGG	+	0	0	1

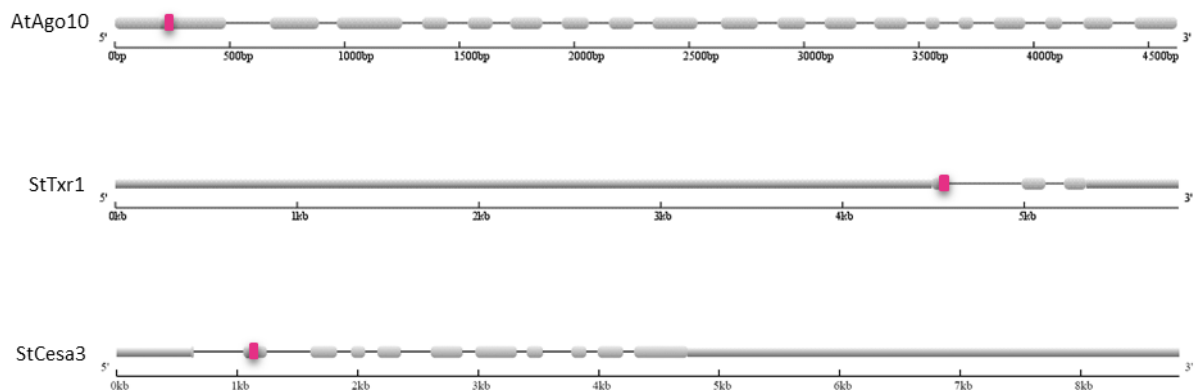


Figure 40 : Gene structure of candidate genes and localisation of sgRNAs.

Table 25: Potential off-target sequences of sgRNAs for the respective candidate genes (Only targets up to 3 mismatches were included).

Target	Gene	Chr.	Position	Dir.	MMs
<i>AtAgo10</i>					
TAGCCGGAGGAGAAACCGTGG	<i>AGO10</i> (AT5G43810)	5	17612132	+	0
<i>StTXR1</i>					
TTTGCCAGGCATATCGCCAAGCTCTC	<i>TXR1</i>	4.03ch06	50458133	+	0
<i>StCesa3</i>					
GGTCAAGTCTGTCAGATCTGTGG	<i>CESA3</i> (NM_001288363.1)	4.03ch01	65772351	+	0

Table 26: Plasmids cloned in this study for the NHEJ approach with information about promoter, nuclease and sgRNA target

Plasmid name	Promoter	Nuclease	sgRNA target	Backbone
BS_03	35s	Cpf1	-	<i>pUC57</i>
BS_04	35s	(<i>A. thaliana</i> codon optimized)	<i>AtAgo10</i>	<i>pGWB401</i>
BS_05	Ubq10	(<i>A. thaliana</i> codon optimized)	<i>AtAgo10</i>	<i>pGWB401</i>
BS_11	35s	Cpf1	<i>StTXR1</i>	<i>pGWB401</i>
BS_12	35s	Cas9 (<i>B. napus</i> codon optimized)	<i>StCesa3</i>	<i>pGWB401</i>

In addition to the original vector with a CaMV 35s promoter, we exchanged the promoter with AtUBQ10 in our expression cassette. The Ubq10 promoter was utilized to decrease the potential amount of chimeric plants in *A. thaliana* since the 35s promoter is only active beginning from the two-cell stage and thus can cause chimeric plants (Wang et al., 2015). The promoter was amplified with Phusion Polymerase from gDNA of *A. thaliana* cv. Col-0 and adapter sequences for a NEBuilder reaction were added in a second PCR. For the NEBuilder reaction, our basic construct (BS_01) was cut with the restriction enzymes *PstI* and *AsiSI* to remove the CaMV 35s promoter from the expression cassette. The NEBuilder assembly reaction was set up according to protocol and transformed into competent *E. coli* strain DH5 α cells *via* heat shock transformation. Colonies were checked with colony PCR for positive clones containing the AtUbi10 promoter. Plasmids from positive clones were isolated and sequenced (IKMB, Kiel). Sequencing data showed that the plasmid was assembled correctly, but the promoter sequence had one SNP compared to the database sequence file. The correct processing of the vector was proven with the previous experiments and as a last step, the protein and sgRNA function were verified in transformation experiments with genotyping of respective loci.

To broaden the range of potential target sites a system based on the nuclease Cpf1 was established as well. For this purpose, a crRNA for the potential susceptibility factor *StTXR1* was derived and the system was tested in *Solanum tuberosum*. The CDS for this nuclease was synthesized and delivered within a pUC57 based vector backbone (Eurofins Genomics) which also served as a template for a PCR adding adapter sequences for a NEBuilder reaction. The original construct was cut with the restriction enzymes *MluI* and *PacI* to remove the CDS for Cas9 and allow the assembly with NEBuilder. Sequencing data showed that the plasmid was assembled correctly and thus could be used in further studies as an addition to the Cas9 expression cassette.

positive transgenic events selected. If plants were positive for the Cas9 PCR fragment a second PCR to amplify the *AtAgo10* locus was performed. The PCR product was cloned into T-vector to enable the sequencing of single alleles.

For each of the two constructs, six independent T0 transgenic plants were chosen and two colonies of each plant were sequenced. Unfortunately, no mutations could be observed in the target loci. Because we could not find any mutations in the T0 generation, we sequenced the progeny to check whether mutations are induced in later generations. Eight individual T1 plants, positive for the Cas9 PCR fragment, were selected and the *AtAgo10* loci amplified and cloned into T-vector. Two colonies of each genotype were sequenced. The progeny showed no mutations at the target loci either (Tab. 27). The lack of mutations at the target loci in *AtAgo10* could have had several reasons which include a non-functional Cas9 or sgRNA and/or an unsuitable transformation system.

Table 27: Sequencing results for *A. thaliana* CRISPR/Cas mutants for the constructs BS_04 and BS_05

T0		
WT	TCGTAGCCGGAGGAGAAACCGTGGTGG AAG	
6X BS_04	TCGTAGCCGGAGGAGAAACCGTGGTGG AAG	No mutation
6X BS_05	TCGTAGCCGGAGGAGAAACCGTGGTGG AAG	No mutation
T1		
16x BS_04	TCGTAGCCGGAGGAGAAACCGTGGTGG AAG	No mutation
16x BS_05	TCGTAGCCGGAGGAGAAACCGTGGTGG AAG	No mutation

Danksagung

Die vorliegende Arbeit entstand während meiner Tätigkeit als wissenschaftlicher Mitarbeiter am Institut für Phytopathologie, Abteilung Molekulare Phytopathologie und Biotechnologie der Christian-Albrechts-Universität zu Kiel.

Ich bin nach meinem Masterstudium in Hannover an dieses Institut gewechselt und wurde von Beginn an mit einem offenen Arbeitsumfeld begrüßt. Ich danke Herrn Prof. Dr. Daguang Cai und Herrn Dr. Dirk Schenke ganz herzlich für die stets hervorragende und kompetente Betreuung, sowie die Forderung und Förderung in fachlicher Hinsicht.

Allen Kolleginnen und Kollegen der Abteilung Molekulare Phytopathologie und Biotechnologie will ich hiermit meinen Dank für die sehr gute Zusammenarbeit und den persönlichen Kontakt aussprechen. Besonders möchte ich mich bei meiner Kollegin Frau Thiele-Belz bedanken, welche mir gerade in der Anfangszeit den Einstieg erleichtert hat und während meiner kompletten Promotion eine wertvolle Hilfe war. Des Weiteren möchte ich mich besonders bei meinen Kollegen Herrn Schemmel und Herrn Bergmann bedanken, die mir immer beratend zur Seite standen.

Daneben bedanke ich mich bei unseren Projektpartnern der NPZ Innovation GmbH und Solana Research GmbH für die gute Kooperation und dem Bundesministerium für Bildung Forschung (BMBF, Grant no. 031B0033C), dem deutschen akademischen Austauschdienst (DAAD) und der Bundesanstalt für Landwirtschaft und Ernährung (BLE) (Grant no. 57317839, 27/16-17-CHN) für die Finanzierung des Projektes.

Zusammenfassung

Die moderne Landwirtschaft verlangt nach innovativen Techniken, um etablierte Strategien in der Zucht voranzutreiben. Traditionelle Ansätze haben ihre Grenzen und basieren vor allem auf der Verfügbarkeit eines breiten genetischen Spektrums und der Auswahl von geeigneten Genotypen zur Verbesserung der gewünschten Eigenschaften. Eines dieser Merkmale ist die Verbesserung der Resistenz gegen biotischen Stress oder Krankheitserreger. Um dieses Ziel bei Kulturpflanzen mit einer schmalen genetischen Basis wie z.B. *Brassica napus* zu erreichen, bedarf es neuer Strategien. Ein Beispiel hierfür ist die Störung von Pflanzenfaktoren, die vom Erreger für die erfolgreiche Kolonisierung des Wirtes benötigt werden (Suszeptibilitätsfaktoren). Die Verticilliumwelke wird durch den bodenbürtigen und hemi-biotrophen Pilz *Verticillium longisporum* verursacht und dieser ist eine der häufigsten Pilzkrankheiten im Rapsanbau. Die Krankheit führt zu einer vorzeitigen Reifung und kann zu erheblichen Ertragseinbußen führen. Die Methoden für das Disease Management sind sehr begrenzt. Gegenmaßnahmen setzen vor allem auf Bodenhygiene oder Präventionsstrategien zur Verringerung der Anzahl der Sporen im Boden. Die genetischen Ressourcen für die Resistenzzüchtung sind limitiert und es wurde bisher keine echte Resistenz entdeckt. Suszeptibilitätsfaktoren in Verbindung mit der Genom-Modifikation basierend auf CRISPR/Cas9 kann als Quelle zur Erzeugung von rezessiver Resistenz genutzt werden. CRISPR/Cas9 ist das vielversprechendste System für die gezielte Mutagenese mit dem Vorteil der einfachen Assemblierung, Verwendung und des Multiplexen. Die Ausschaltung der Zielgene wird durch die Induktion kleiner InDels ermöglicht, die durch non homologous end joining (NHEJ) nach einem Doppelstrangbruch im Zielort verursacht werden. Ein weiterer Ansatz, der durch diese Technologie ermöglicht wird, ist die Einführung einer Reparaturvorlage für homology directed repair (HDR), welche es ermöglicht die Nukleotidsequenz nach Wunsch zu verändern ohne die Genfunktion zu stören. In dieser Studie adaptieren wir mehrere Vektorsysteme, die verschiedene Nukleasen und Promotoren für die Anwendung in *Brassica napus* enthalten. Nach erfolgreicher Etablierung unserer Expressionskassetten liegt der Fokus auf dem Knockout der Kandidatengene *BnCRT1a* und *BnHVA22c*, die an der *V. longisporum* -*B. napus* Interaktion beteiligt sind. Loss-of-function Genotypen für die Gene *BnCRT1a* und *BnHVA22c* wurden generiert und mit *V. longisporum* infiziert. Zusätzlich zu diesem NHEJ-Ansatz sollen miRNA-Bindungsstellen von pilzlichen miRNAs im Wirtsgenom mittels HDR-Ansatzes verändert werden, ohne die Funktion für die Gene *BnAGO1* und *BnTAO1* zu beeinträchtigen. Zu diesem Zweck wurde das virale Genom des *Bean Yellow Dwarf Virus* für die Verwendung in *B. napus* hairy roots angepasst. Zusammenfassend lässt sich sagen, dass alle Loss-of-function Genotypen eine stark reduzierte Ausprägung der Symptome und

Wachstumshemmung zeigten. Diese Ergebnisse unterstreichen unsere Arbeitshypothese, dass *CRT1a* und *HVA22c* die Resistenz gegen *V. longisporum* in ihrem mutierten Zustand hervorrufen können. Die darauffolgenden Experimente beschäftigten sich mit der Funktionsweise dieser Gene in Pflanzen. Leider konnten dabei keine signifikanten Unterschiede im Expressionsprofil der Markergene für molekulare und physiologische Prozesse zwischen den Mutanten und Wildtyp festgestellt werden. Lediglich das Ethylen-Markergen *ETR2* zeigte eine erhöhte Expression in Knockout Genotypen im nicht infizierten Zustand, dies steht in Einklang mit Expressionsdaten aus *A. thaliana*. Auch die Analyse der co-regulierten Gene, welche eine mögliche Rolle in der Proteinfaltung / ER-Stress (für *CRT1a*) oder dem Vesikeltansport / Calloseverschluß der Plasmodesmata (für *HVA22c*) spielen, zeigte keinen Unterschied zwischen Mutanten und dem Wildtyp. Die Vektorsysteme für den HDR-basierten Ansatz wurden ebenfalls erfolgreich implementiert und virale Replicons des *Bean Yellow Dwarf Virus* konnten innerhalb der Zelle nachgewiesen werden. Die miRNA-Bindestellen in den Genen *BnAGO1* und *BnTAO1*, die von *V. longisporum* genutzt werden, waren das Ziel unseres HDR-Ansatzes. Es konnten keine positiven HDR-Ereignisse identifiziert werden, welche einen Sequenzaustausch belegen. Insgesamt zeigte diese Arbeit, dass die Anwendung von CRISPR/Cas in Kombination Suszeptibilitätsfaktoren eine wertvolle Strategie zur Erzeugung der rezessiven Resistenzen gegen Krankheitserreger darstellt. Sie kann in Zukunft besonders für Kulturpflanzen mit limitierten genetischen Ressourcen sinnvoll in bestehende Zuchtmethodik integriert werden.

Summary

Modern agriculture requires innovative techniques to advance established strategies in breeding. Traditional approaches have their limitations and are primarily based on the availability of a broad genetic spectrum and the selection of suitable genotypes to improve the desired characteristics. One of these characteristics is the improvement of resistance to biotic stresses or pathogens. In order to achieve this goal in crops with a narrow genetic base such as *Brassica napus* new strategies are required. An example for this is the disruption of plant factors that are required by the pathogen for a successful colonization of the host (susceptibility factors). Verticillium wilt is caused by the soil-borne and hemi-biotrophic fungus *Verticillium longisporum*, which is one of the most common fungal diseases in rapeseed cultivation. The disease leads to premature ripening and can cause considerable yield losses. The methods for disease management are very limited. Countermeasures are mainly based on soil hygiene or prevention strategies to reduce the number of spores in the soil. The genetic resources for resistance breeding are limited and no real resistance has been discovered so far. Susceptibility factors in combination with genome modification based on CRISPR/Cas9 can be used as a source of recessive resistance. CRISPR/Cas9 is the most promising system for targeted mutagenesis with the advantage of easy assembly and use as well as multiplexing. The knockout of target genes is possible by the induction of small InDels caused by non-homologous end joining (NHEJ) after a double-strand break in the target site. Another approach possible through this technology is the introduction of a repair template for homology directed repair (HDR), which allows the nucleotide sequence to be altered without disrupting the gene function. In this study we adapt several vector systems containing different nucleases and promoters for application in *Brassica napus*. After successful establishment of our expression cassettes, the focus relied on the knockout of the candidate genes *BnCRT1a* and *BnHVA22c*, which are involved in the *V. longisporum* -*B. napus* interaction. Loss-of-function genotypes for the genes *BnCRT1a* and *BnHVA22c* were generated and infected with *V. longisporum*. In addition to this NHEJ approach, miRNA binding sites of fungal miRNAs in the host genome are modified using the HDR approach without affecting the function for the genes *BnAGO1* and *BnTAO1*. For this purpose, the viral genome of the *Bean Yellow Dwarf Virus* was adapted for use in *B. napus* hairy roots. In summary, all loss-of-function genotypes showed a greatly reduced expression of symptoms and growth inhibition. These results support our working hypothesis that *CRT1a* and *HVA22c* may induce resistance to *V. longisporum* in their mutated state. Following experiments focused on the function of these genes in plants. Unfortunately, no significant differences in the expression profile of marker genes for molecular and physiological

processes between mutants and wild type were found. Only the ethylene marker gene *ETR2* showed increased expression in knockout genotypes in the uninfected state, which is consistent with expression data from *A. thaliana*. The analysis of co-regulated genes, which play a possible role in protein folding / ER stress (for *CRT1a*) or vesicle transport / callose closure of plasmodesmata (for *HVA22c*), showed no difference between mutants and the wild type as well. The vector systems for the HDR-based approach were successfully implemented and viral replicons of the *Bean Yellow Dwarf Virus* could be detected within the cell. The miRNA binding sites in the genes *BnAGO1* and *BnTAO1*, which are used by *V. longisporum*, were the target of our HDR approach. No positive HDR events could be identified that would be indicated by a sequence exchange. Overall, this work showed that the use of CRISPR/Cas in combination with susceptibility factors is a valuable strategy for generating recessive resistance to pathogens. In the future, this tool can be integrated into existing breeding methods, especially for crops with limited genetic resources.

In der Schriftenreihe des Instituts für Phytopathologie der Christian-Albrechts-Universität zu Kiel sind bisher erschienen:

- Heft 1: Claudia Häder 2013 Identifizierung und Charakterisierung pflanzlicher Kompatibilitätsfaktoren für die Raps-*Verticillium longisporum*-Interaktion
- Heft 2: Dan Shen 2013 Genome-wide identification and characterization of miRNAs responsive to *Verticillium longisporum* infection in *Brassica napus* by deep sequencing
- Heft 3: Falk Hubertus Behrens 2018 Investigation on the role of miRNAs in regulating plant-*Verticillium longisporum* interactions in oilseed rape (*Brassica napus*) and *Arabidopsis thaliana*
- Heft 4: Samarah Rizvi 2019 Investigation on the role of NAC transcription factors targeted by miR164 in regulating plant-*Verticillium longisporum* interaction