CRISPR- mediated Mutation in *Cinnamoyl- CoA Reductase 4* in Allohexaploid Oilseed Crop *Camelina sativa,* Revealed its Pivotal Role in Resistance Against *Sclerotinia sclerotiorum*

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Background: *Sclerotinia sclerotiorum* (*Ss*) is a broad host range necrotrophic ascomycete fungus affecting over 400 plant species. Ss causes stem rot disease on *Camelina sativa* (Cs) an allohexaploid crucifer species that is promoted as a low input crop and industrial oil attributes suitable as biofuel and lubricant. Histochemical and molecular studies has linked resistance to *Ss* in *C. sativa* with the cell wall lignification (Eynck et al., 2012) and reported constitutive expression of *Cinnamoyl-CoA Reductase 4 (CsCCR4)* gene, in the Cs resistant line CN114263. Modern breeding efforts, such as gene editing, are needed to improve commercial lines and to limit the risk of crop loss which would be substantial to producers.
Objectives: To investigate the importance of monolignol biosynthesis and the role of *CsCCR4* in Camelina resistance to *Ss* we generated *CsCCR4* knockout mutants of CN114263 Camelina line using CRISPR/Cas9-mediated gene editing.
Materials and Methods: Thirty T1 plants were produced via floral dip transformation followed by glyphosate spraying that was used in the first step of screening procedures and were confirmed by PCR method. Transgene's T-DNA copy number variation, T-DNA CNV, in T1 and T2 progenitors were determined using digital droplet PCR (ddPCR) and the occurrence of mutation in the three copies of *CsCCR4* homeologues in T1 and T2 generations were scrutinized by drop-off assay technique. To make sure that if the created mutants in T2 plants are real, TOPO TA sequencing flanking the Cas9/ gRNA specific hot point of cleavage for three of them was conducted.
Results: In the T1 generation, 25 plants were confirmed which had between one to nine T-DNA copies in the corresponding Camelina genome. In T2 generation the population were screened for potential mutation in *CsCCR4* gene. Various types of mutations, including insertions and deletions, were demonstrated in three copies of *CsCCR4*. In fact, CRISPR system could have cut one, two or three copies of the gene in events numbered T2-plant 10, T2-plant 15 and T2-plant 19, respectively. The T3-plant 19 which showed mutation in all versions of *CsCCR4* in previous generation had susceptibility to S. *sclerotiorum* invasion and was kept as real *csccr4* mutant material for further investigations of Camelina-Sclerotinia interaction. Mutation in *CsCCR4* had occurred through error-prone none- homologous end joining (NHEJ) nucleus DNA repair pathway. *Ss* challenge on the early flowering T3 generation. The T3 plants with mutation causing premature stop codon at position 217 of CsCCR4 were compromised in their resistance to *Ss* compared to the wildtype resistant control parent CN114263.
Conclusion: Using ddPCR it easily was possible to identify both the T-DNA CNV and occurrence of mutation in *CsCCR4* homeologues in T1 and T2 progenitors. We illustrated that CRISPR/Cas9-mediated mutation is a decent technique that can be utilized to expedite the mutant line development which could assist to figure out the activity of a *CsCCR4* gene in defense responses to the pathogens in *C. sativa* as prospective oilseed crop for biodiesel production.

Keywords: *Camelina sativa, Cinnamoyl- CoA Reductase 4*, CRISPR/Cas9-mediated gene editing, Droplet digital PCR, Drop-off assay mutation detection, Sclerotinia stem rot.

1. Background

Camelina, *Camelina sativa* (L.) Crantz, also called false flax or gold of pleasure an species of the Brassicaceae family is an annual oilseed crop originated from Mediterranean-Central Asia that has been planted since 4000 BCE (1). Because of its low requirement to fertilizers, chemicals, and water as well as its unique oil content and short growing season (less than 100 days), Camelina recently has gain interest as an industrial oil crop Unsaturated fatty acids makes nearly 90% of Camelina total oil. Camelina oil consist of 35% α-linoleic acid (18:3n-3), 15% linoleic acid (18:2n-6), 15% Gonodic acid and 3% erucic acid that comprises 68% of the total fatty acids (2). There has been progress in the genetic improvement of Camelina via classical breeding and biotechnology research during past decades. The most important traits for Camelina breeding are increased seed size/yield, resistance to biotic and abiotic stresses as well as herbicide resistance (3). Moreover, modification in oil content and fatty acid profile based on the desired end use of the oil and decreased level of glucosinolates have been studied (4). Recently, the allohexaploid 756Mb long genome of *C. sativa* in three sub-genomes (2n= 40) has been sequenced (5). In addition, RNA sequencing data has provided an atlas of all developmental transcriptome maps of different tissues (6). Moreover transformation via floral dip method (7), short-term life cycle, 85-100 days of seed to seed, and countless data on its genome and transcriptome (6, 8), has promoted Camelina as an ideal model for oilseed crops on seed oil composition (9). Recently, few studies have repotrted in which the CRISPR/Cas technology has been exploited to target genes in Camelina particularily with the aim of increased total oil and also oil content in the seeds (10-13). While Camelina is resistant to some major *Brassicaceae* diseases like blackleg, (14) and Alternaria black spot *C. sativa* is susceptible to several important crucifers' diseases such as Clubroot, Downy mildew, white rust, Aster Yellows, Sclerotinia stem rot (14, 15). *Ss* the agent of Sclerotinia stem rot in Camelina is a ubiquitous pathogen that infects a broad range of plant species belonging to several families (16). *Ss* produces sclerotia – a black structure that persists for the long term in the soil -which produces air–born spores that easily spread throughout the area which in turn makes its control difficult. During the infection, *Ss* secretes cell wall degrading enzymes such as pectinases, cellulases, glucanases, and glucosidase (16), oxalic acid (OA) which help pathogen to macerate cell wall and absorb the nutrient component from the host. The best strategy to limit the *Ss* invasion would be the cultivation of resistance genotypes however breeding for resistance to Ss is extremely difficult due to the multigene nature of resistance and involvement of many genes with minor effects (17, 18). The first study into the interacction of *Ss* and *Sc* was conducted by Eynck and *et al* that evaluated the role of cell wall lignification and its correlation with resistance against Sclerotinia stem rot (19). This study, compared the level of expression of genes which are involved in monolignol biosynthesis- the precursor of lignin- during the attack of *Ss* in resistant and susceptible Camelina lines, using RT-PCR. It has been showed that the *Cinnamoyl CoA reductase 4 (CsCCR4)* gene with homology to the *At5g58490* was expressed more than 12 times higher in the inoculated stem of resistant line relative compared to the susceptible control. Lignin biosynthesis starts with phenylalanine deamination and continues to the Cinnamoyl CoA esters. The later products are the precursors of a wide range of bioproducts such as coumarins, flavonoids, and lignins with different roles during plant development and responses to environmental signals. Cinnamoyl CoA Reductases (CCRs) by reduction of cinnamoyl CoA into their corresponding cinnamaldehydes are the first enzymes committed of monolignol biosynthesis. Three well-studied monolignols in higher plants are; *ρ*-coumaryl, coniferyl and sinapylalcohol that give rise to hydroxyphenyl (H), guaiacyl (G) and syringyl (s) lignins, respectively. At least two units of these monolignols are needed to form lignin polymer in an oxidative coupling reaction (20). Based on the atlas of Camelina transcriptome, *CsCCR4* gene has three homeologous -Csa02g064110, Csa11g092190 and Csa18g031650- which was expressed in different developmental stages with the exception of Csa02g064110.1 which was not expressed in senescing leaf (**supplementary data 1A**). On the other hand, *CsCCR2* –widely known as defense gene- in resistant line was induced 72 hours after pathogen inoculation in peripheral tissue and was associated with lignin deposition of guaiacyl type. Thus, it is proved that one of the sources of resistance of some Camelina lines to *Ss* is cell wall strengthening via both constitutive and inducible lignification before and during pathogen attack. There are almost 10 *CCR* and *CCR*-like genes in

Camelina, from *CCR1* to *CRR10* which are expressed in a temporal-spatial manner throughout plant development (**supplementary data 1B**).

2. Objectives

To explore the cell wall reinforcement role of the *CsCCR4* gene against *Ss* invasion, our strategy in the first step was to knock out all three homeologous to inhibit their function in monolignol biosynthesis and evaluate its loss-of-function correlation with the level of sensitivity to the pathogen. This was accomplished by using an improved CRISPR/Cas9 mediated gene editing system in a resistant accession CN 114263 of Camelina. As it was expected the *csccr4* mutant plant will be susceptible to *Ss*. To modify simultaneously multiple alleles in different plant species (21) and in Camelina in particular (10-12, 22) the CRISPR/Cas9- mediated gene editing technology has been applied. Here we successfully could have made mutation in highly productive and in a precise manner in three homeologues of *CsCCR4* gene. The *csccr4* mutant will help to expand our understandings about the conundrum of Camelina defense responses to its pathogens. It also will promise an easy and quick procedure to expedite developing new plant material to study resistance mechanisms by reducing of the classical breeding timelines.

3. Materials and Methods

3.1. Plant Materials

A genotype of camelina, with the accession number of CN114263, resistant against *Ss* was provided by Dr. Christina Eynck, AAFC, at Saskatoon Research Center. The seeds were planted – and used for knocking out the gene *CsCCR4* in this study. We furthermore, used susceptible genotype of DH55 - provided by Molecular Pathology Lab, Saskatoon Research Centre- as positive control in this experiment.

3.2. Design and Assembly of GRNA

The genomic sequence of *CsCCR4s*, which corresponds to the At5g58490, were retrieved from camelinadb.ca using nucleotide BLAST search (**supplementary data 2A-B**). Then, a specific 20 nucleotide- single gRNA -sgRNA- along with 3'–NGG5' PAM at the end of its 3' was designed (**Fig.1A**) using CRISPOR (http://crispor.tefor.net) webserver based on the absence of potential off-target sites with less than 4 mismatches

in the Camelina whole genome sequence (CRISPOR). The sgRNA had recognition for three homeologous of *CsCCR4* in conserved sequence in the position of 297-317 from the start codon of its second exon in reverse strand (**Fig. 1B**). Then, the synthesized single strand gRNA was hybridized with its complementary oligonucleotide with ATT and CAAA overhangs in 5' and 3'ends, respectively. Directionally, this molecule was inserted into pAGCAN 34-7 in downstream of the At-U6 promoter by Golden-Gate assembly method (23) between L2 and L4 *att* sites. To assemble the T-DNA in destination vector, pWY452, the sgRNA construct was incorporated into a multisite Gateway cloning reaction together with p454, p457 and p452 which carry *AtoptCas9* was expressed under 2xCaMv35S promoter, *HSP* terminator for *AtoptCas9* and Hygromycin resistance, and *PAT* gene modules, respectively. The map of T-DNA is represented in **supplementary 3**. After 18 hours, 4µL of the reaction was added to One Shot™ TOP10 chemically competent cells (Thermo Fisher Scientific, Cat no; C404003). Few colonies on Spectinomycin-Hygromycin medium were selected to confirm and validate destination construct by restriction digestion and sequencing for transformation into Agrobacterium (AGL1 strain received from Dr. Nickolas Larkan, AAFC-Saskatoon research center) via electroporation (Bio-Rad Gene Pulser X-cell).

3.3. Floral Dip

Twenty 6 inches' pots with 3 seeds in each were sown by CN114263 genotype. After 25 days of the plantation, the plant's apical shoots were trimmed and 10 days later the nearly opened flowers were dipped in Agrobacterium solution (350µL/liter of Silwet-77, 5% Sucrose) with no need to apply vacuum infiltration procedure (7). Then, the pots were lain down on a sterilized wet textile at 28 °C for 24 hours and the next day they were organized on the greenhouse benches to recover plants and to be ready for seed ripening in the pods.

3.4. Screening of T0 Plants

Almost 120gram of seeds (eaquals to 150,000 seeds) from dried plants was harvested seventy-five days after dipping and immediately was germinated on prepared flats and was grown in 16 h days light intensity c. 450 µL.moL. m^{-2} S^{-1} at bench level at 23 °C and 8 hour night at 18 °C in the greenhouse. The T0 screening was
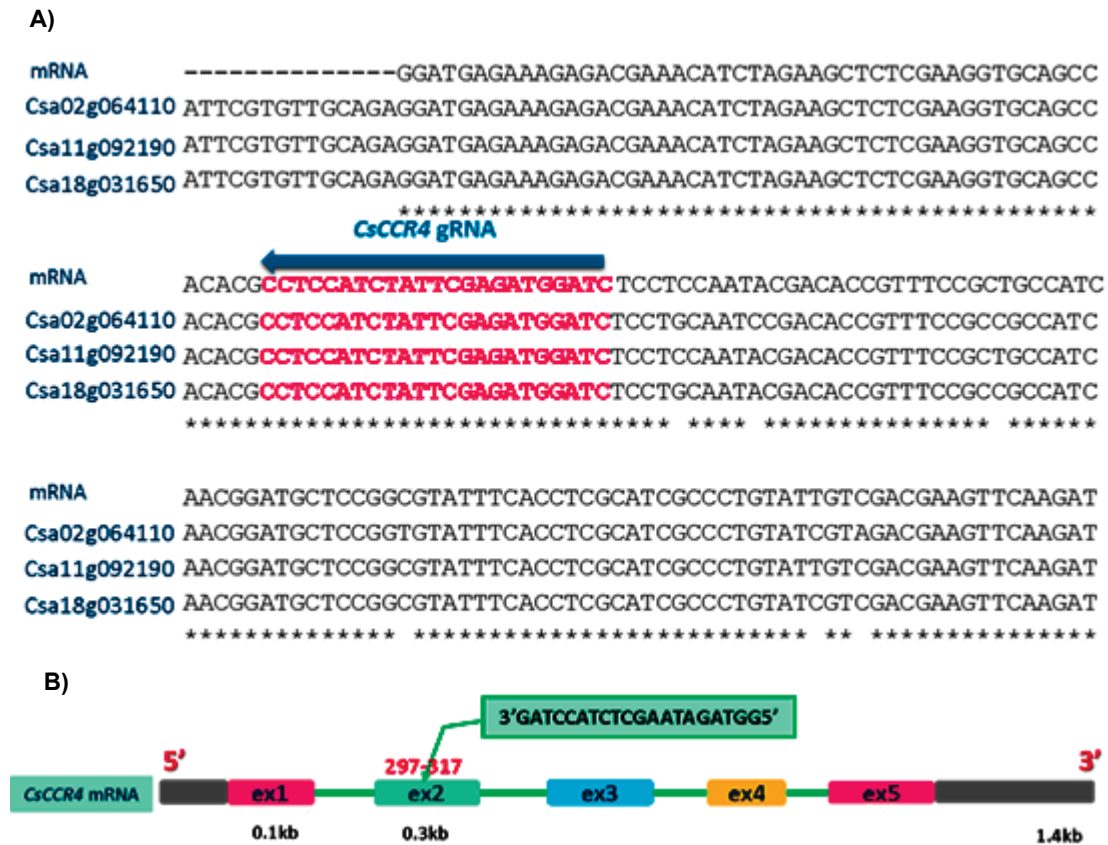
A)

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mRNA        --------------GGATGAGAAAGAGACGAAACATCTAGAAGCTCTCGAAGGTGCAGCC
Csa02g064110 ATTCGTGTTGCAGAGGATGAGAAAGAGACGAAACATCTAGAAGCTCTCGAAGGTGCAGCC
Csa11g092190 ATTCGTGTTGCAGAGGATGAGAAAGAGACGAAACATCTAGAAGCTCTCGAAGGTGCAGCC
Csa18g031650 ATTCGTGTTGCAGAGGATGAGAAAGAGACGAAACATCTAGAAGCTCTCGAAGGTGCAGCC
                          ************************************************
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                                    CsCCR4 gRNA
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mRNA        ACACGCCTCCATCTATTCGAGATGGATCTCCTCCAATACGACACCGTTTCCGCTGCCATC
Csa02g064110 ACACGCCTCCATCTATTCGAGATGGATCTCCTGCAATCCGACACCGTTTCCGCCGCCATC
Csa11g092190 ACACGCCTCCATCTATTCGAGATGGATCTCCTCCAATACGACACCGTTTCCGCTGCCATC
Csa18g031650 ACACGCCTCCATCTATTCGAGATGGATCTCCTGCAATACGACACCGTTTCCGCCGCCATC
            *********************************** **** *************** ******
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mRNA        AACGGATGCTCCGGCGTATTTCACCTCGCATCGCCCTGTATTGTCGACGAAGTTCAAGAT
Csa02g064110 AACGGATGCTCCGGTGTATTTCACCTCGCATCGCCCTGTATCGTAGACGAAGTTCAAGAT
Csa11g092190 AACGGATGCTCCGGCGTATTTCACCTCGCATCGCCCTGTATTGTCGACGAAGTTCAAGAT
Csa18g031650 AACGGATGCTCCGGCGTATTTCACCTCGCATCGCCCTGTATCGTCGACGAAGTTCAAGAT
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B)

Figure 1. A) sequence alignment of three copies of *CsCCR4* genes compared with their consensus mRNA in the middle of exon 2 in the *Camelina sativa* genome. The position and direction of subsequent sgRNA where it binds is shown. The stars at the bottom of the alignment show similar nucleotides and blank spaces are representing SNPs. **B)** Schematic view of gRNA position (highlighted in the green box) nucleotides between 297 and 317 far from start codon located in the second exon of the CsCCR4 gene in reverse strand. Green lines are represented the

performed by the spraying of 1:400 ratio of glyphosate/water in three times 7, 14 and 21 days after sowing. Then, 40-day-old plants were organized in 6 inches pots and labeled for the following experiments. All selected T0 plants were grown and harvested to generate the T1 population.

3.5. DNA Extraction

3.5.1. T-DNA Copy Number Variation (CNV) Using Digital Droplet PCR

In T1 plants and based on ddPCR copy number-CN-analysis, the presence of *AtoptCas9* intron-Cas9-int amplicon- labeled with blue fluorescent probe, FAM, as the target and *CsAls* gene*, acetolactate synthase,* as the internal reference gene, with green fluorescent probe, HEX, were considered to estimate T-DNA

CN inserted in each genome. The fluorescent reads from each well analyzed ddPCR system (QX200™ Droplet Digital™ PCR System) and its corresponding software, Quantasoft version 1.7 as described by the manufacturer's instructions **(supplementary data 6)**. The following formula was used to calculate the T-DNA CN:

T-DNA CN in Camelina genome =

$$\frac{\text{concentration of Cas9}}{\text{concentration of CsAls}} \times \text{the copy number of CsAls as reference}$$

All Primers and probes were designed with PirmerQuest online (25) and ordered from either Integrated DNA Technologies, Inc. (Coralville, IA, USA) idtdna.com or Eurofins Genomics, Toronto, ON, Canada www.eurofinsgenomics.ca/ **(supplementary data 4)**. The ddPCR Supermix for Probes kit 2x

(Bio-Rad Laboratories) was used in all reactions. The actual location of designed primers and probes in both CN analysis and drop-off assay are shown in (**supplementary data 5**).

3.6. Pathogen Challeng on T3 Population

To identify the Camelina mutant lines with susceptibility to *Ss*, the grown stems of T3 population (including 25 T2-TDNA positive events and Contorols) on 65 days old were subjected to the pathogen. To do so, An aggrssive isolate of *Ss* was grown on Potato Dextrose Agar (PDA) at 25°C (26). To reveal susceptibility, three plant replicates with 8mm diameter of PDA culture carrying mycelia were enclosed on the flowering stems. The wild type CN114263 line and an extremely susceptible genotype ,DH55, were used as a negative and positive control in this experiment. Early symptoms of necrosis occurred 48 hours after inoculation. The lesion size on the stems was measured and scored 5 days of postinoculation.

4. Results

4.1. T0 and T1 Screening

Over 30 T0 Camelina of which carried T-DNA were remained after three-time glyphosate spraying. These plants were confirmed by Cas9-int primers PCR analysis as shown in **Figure 2A**. All T0 plants have demonstrated the Cas9-int amplicon. Using the ddPCR copy number variation experiment (**supplementary data 6A**) the CN of T-DNA in each T1 event was estimated. Comparison of T0 PCR results and ddPCR CNV experiments in T1 generation revealed that the transgenic plants numbered T1-18, T1-22, T1-27 and T1-30 did not carry T-DNA in their genome and were removed from the experiment. Whereas, the remained T1 plants were possessed at least one or more T-DNA copy insertion in their relevant Camelina genomes (**Fig. 2B**). In this experiment, a region in the *CsAls* gene of which has one copy on sub-genome 2 in the Camelina genome was amplified as the endogenous reference.

### 4.2. Mutation Detection with Drop off Assay

The T2 lines were screened by ddPCR drop-off assay for occurrence of mutation in *CsCCR4* gene where a fragment around the site which was expected the hot spot cut site was amplified by a pair of primers and a HEX labeled probe to anneal on the target of interest as well as a FAM labeled probe to anneal on a little distant from the potential cut site as reference, was considered (**supplementary data 6B**). It is expected that amplicons where the target probe is attended, HEX, it is banded to the target cut site similar to reference probe, FAM, in wild type plant and non-mutated plants (**Fig. 3A**) resulting in double-positive droplets (droplets with both FAM and HEX signals and these droplets will become orange in the output sheet). Whereas, in case of insertion/deletion (indel) in the cut site in T2 lines, the target probe, HEX, will not bind to its genomic corresponding site resulting in, the drop-off its signal relative to signals from which emitted reference, FAM, probe. Based on this, the T2 generation screening was performed to discern mutant events. According to the literature obtaining six out of six mutation-homozygous mutation- in the allohexaploid genome of Camelina is take placed in a rare manner in T0 and T1 population (10). That is because in Camelina mutation occurred in somatic cells the same as what happens in *Arabidopsis thaliana* (27). Therefore, following the subsequent generations to achieve pure and heritable mutation in all homeologous is the only option ahead (12). The drop-off assay results showed at least one mutation out of 6 in 23 percent of T2 events (including T2-10, T2-16, T2-15, T2-6, T2-23, and T2-19), in the homeologues of *CcCCR4* were occurred (**Fig. 3B-C**). In this case, the Fluorescence intensity plot from wild-type CN114263 line, WT, represents double-positive droplets (totally orange droplets on 2-D plot) for both the FAM and HEX probes. However, the T2 CRISPR-mediated mutant lines display both double-positive droplets and single-positive signals (FAM) stretched across the plots as indicative of mutation in predicted CsCCR4 cut site. The drop-off assay facilitated the calculation of the fraction of mutant alleles in drop-off probe events. These results as the percentage of mutations (observed) have been quantified and along with the expected values of mutations are included in **Figure 3C**.

4.3. TOPO TA Sequencing Results

Three of the potentially T2 Camelina mutants, T2-plant 10, T2-plant 15 and T2-plant 19, detected by the fractional abundance of reference probe events and drop-off probe events of droplets in the output of Quanta Soft were selected to be scrutinized more to determine
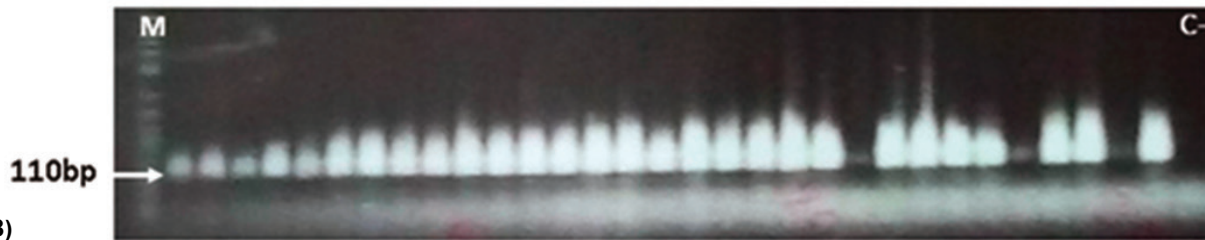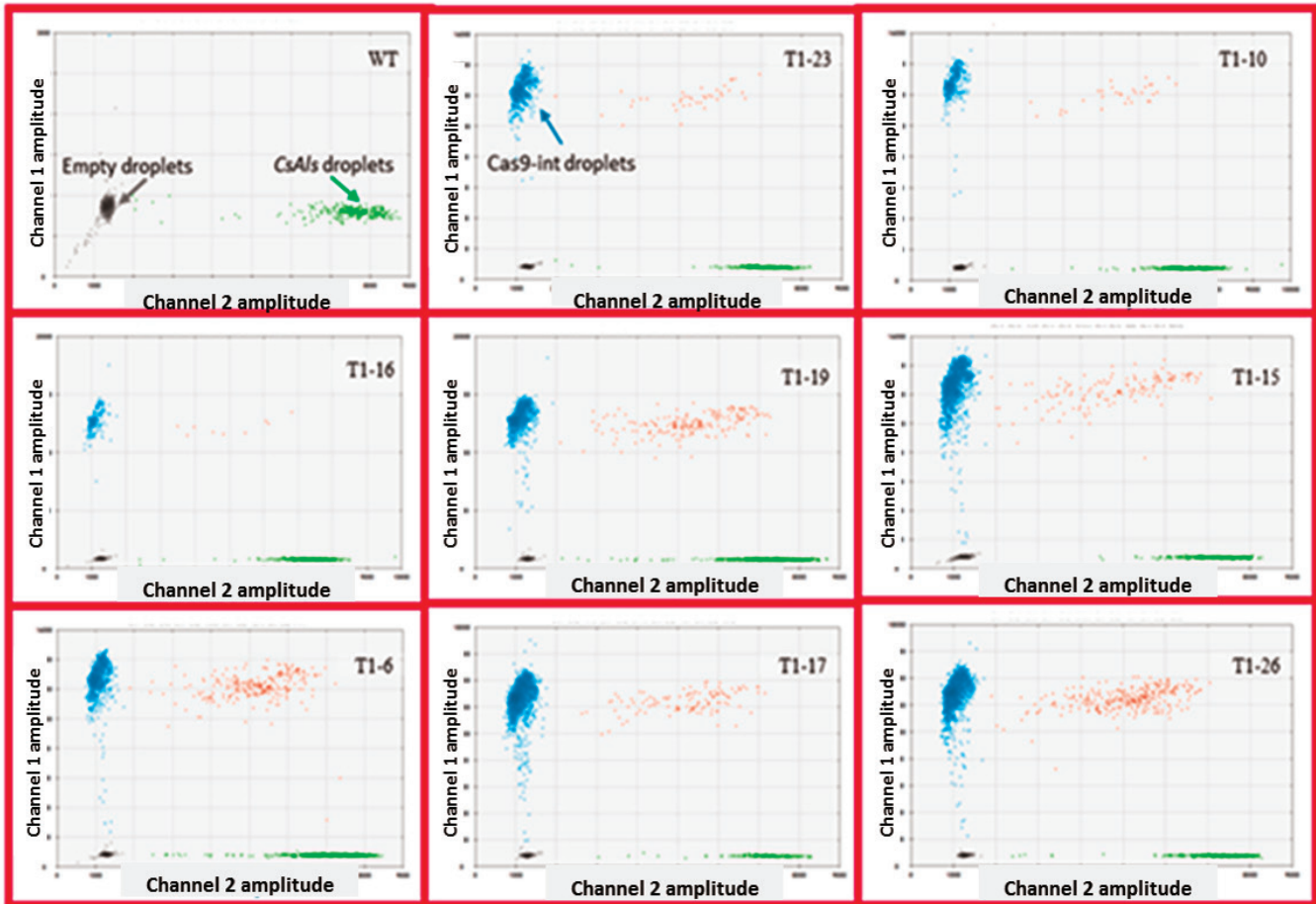
Figure 2. A) Transgenic T1 plants line which carries T-DNA. A fragment of *Atopt-Cas9 intron* (110 base pair) was amplified with specific primers. The templates were genomic DNA extracted from the leaf tissue of T1 transgenic lines. The negative control, C- the right lane, is the wild-type CN114263, M= marker. **B)** Selected sheets of CNV experiment in T1 individual lines. The plants containing T-DNA were validated using ddPCR. To perform this experiment, primers and probes were designed to amplify *CsAls* and *Cas9-int* as reference (HEX) and target (FAM), respectively. The concentration of target droplets divided into concentration reference droplets indicates the T-DNA copy number. Blue droplets, channel 1 amplitude, represent the intensity of FAM binding to Cas9 intron while green droplets, channel 2 amplitude, show the number of HEX probes in which bind to the *CsAls* gene. Empty droplets are in gray. Wild-type Camelina line CN114263, the top left corner of the figure, was used as a template in which only HEX droplets can bind to its target in the *CsAls* gene.

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the type of mutation on the DNA level. The other reason that we select these events was the difference in their T-DNA copy number (please see **supplementary data 6A**). As this plant were representing one, three and six copy of T-DNA respectively, we could figure out the efficiency of the introduced T-DNA in terms of creating mutation in transgenics. In fact, we proved for ourselves that plants with multiple T-DNA insertion in the genome (T2-15) and low insertion T-DNA (T2-10) tented to have decreased edition of the target of interest which here is *CsCCR4*. However, we do not like to generalize this finding over the population of events because we know the T-DNA expression varies in different hosts and other species.
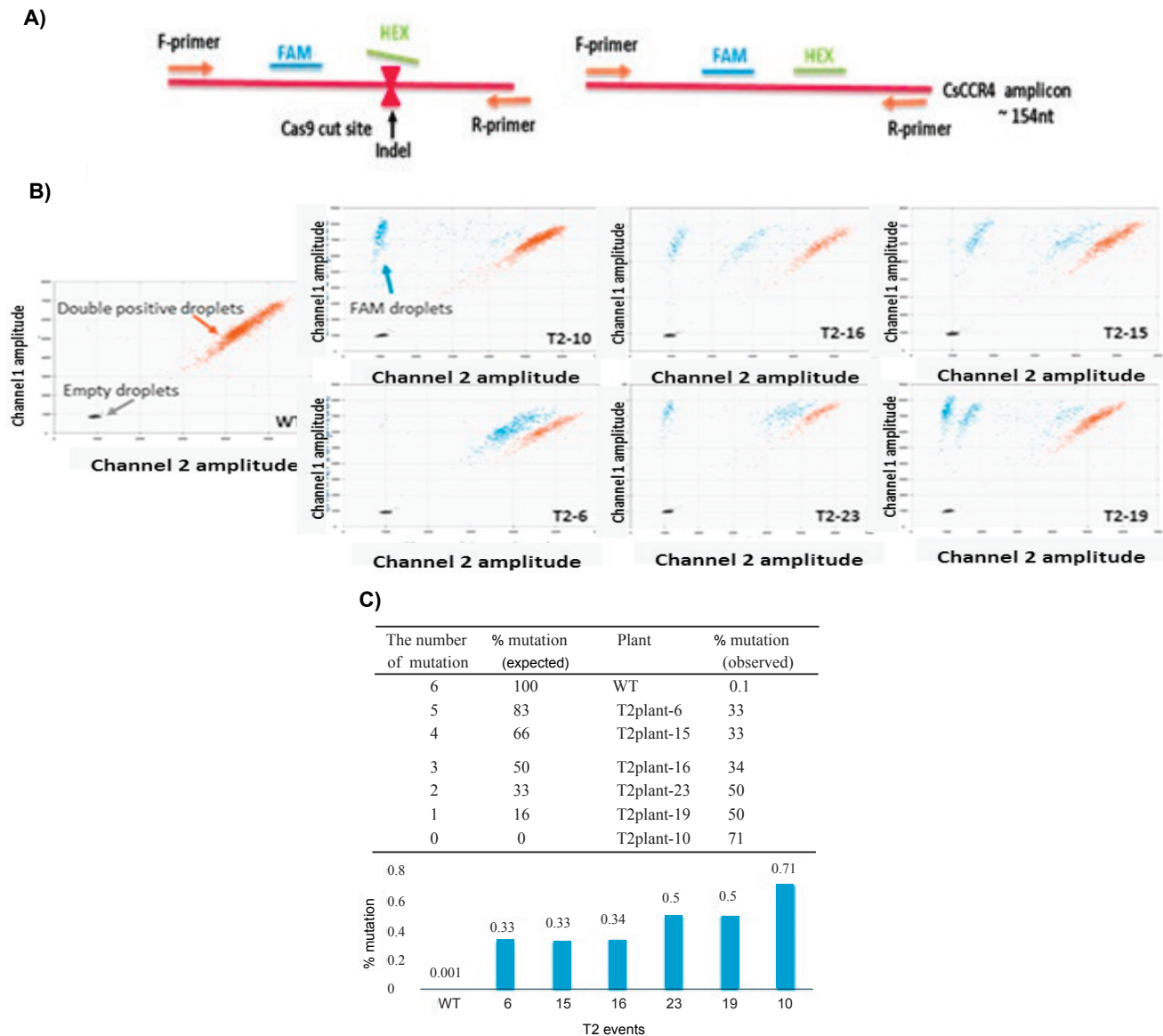
Figure 3. A) Schematic view of primers and probes for drop-off assay experiments. When double-strand breaks occurred during CRISPR/Cas9 mediated editing the probability of insertion/deletion is soared. In the mutant plant, the corresponding probe (Here is HEX) is not able to sit on its target because of base changes that happen in the cut site. In wild type both reference and drop-off probes can anneal to their sequence within the amplicon. **B)** mutation detection of CsCCR4 genes in the T2 generation using a ddPCR drop-off assay. 2-D plots representing double positives (orange droplets where each probe shows its own fluorescent emission) and single-positive (FAM droplets detected by channel 1). Wild type, WT, line in the far left is positive for both reference (FAM) and drop-off (HEX which is absent in channel 2) probe as expected. **C)** Whereas, T2 events plants numbered 6, 10, 15, 16, 19 and 23 display the occurrence of both double-positive and reference probe signals and the absence of target probe as a result of point/oligo mutation on the predicted cut site.

Consequently, a fragment flanked the cut site was amplified and the resulting PCR separately purified and cloned in a TOPO vector using TOPO TA cloning for sequencing Kit (ThermoFisher) for each plant. Approximately, 50 clones on each petri dish plasmid were extracted and sequenced at National Research Council, Saskatoon, SK, Canada (**Fig. 4A** and for more details of sequencing results see **supplementary data 7**).

4.4. In-silico CsCCR4 Protein Knock Out Simulation

CsCCR4 is a Cytosolic protein that has 324 amino acid length, with an Isoelectric point (PI) equal to 5.81, and its molecular weight is 35.5 k. the Aliphatic index of CsCCR4 is 96.3. To interrupt CsCCR4 protein formation, all three versions was targeted by a 20nt sgRNA for CRISPR/Cas9 mediated gene-editing system to cleave the gene in the upstream site. The truncated proteins that are translated in the plant cell as the consequence of targeted indels were mimicked by in-silico point mutation exact the same mutations that observed in the real sequencing results. The alignment of truncated CsCCR4 and their length relative to WT-CsCCR4 are represented in (**Fig. 4B**).

4.5. Evaluation of Stem Rot Symptoms in T3 Events

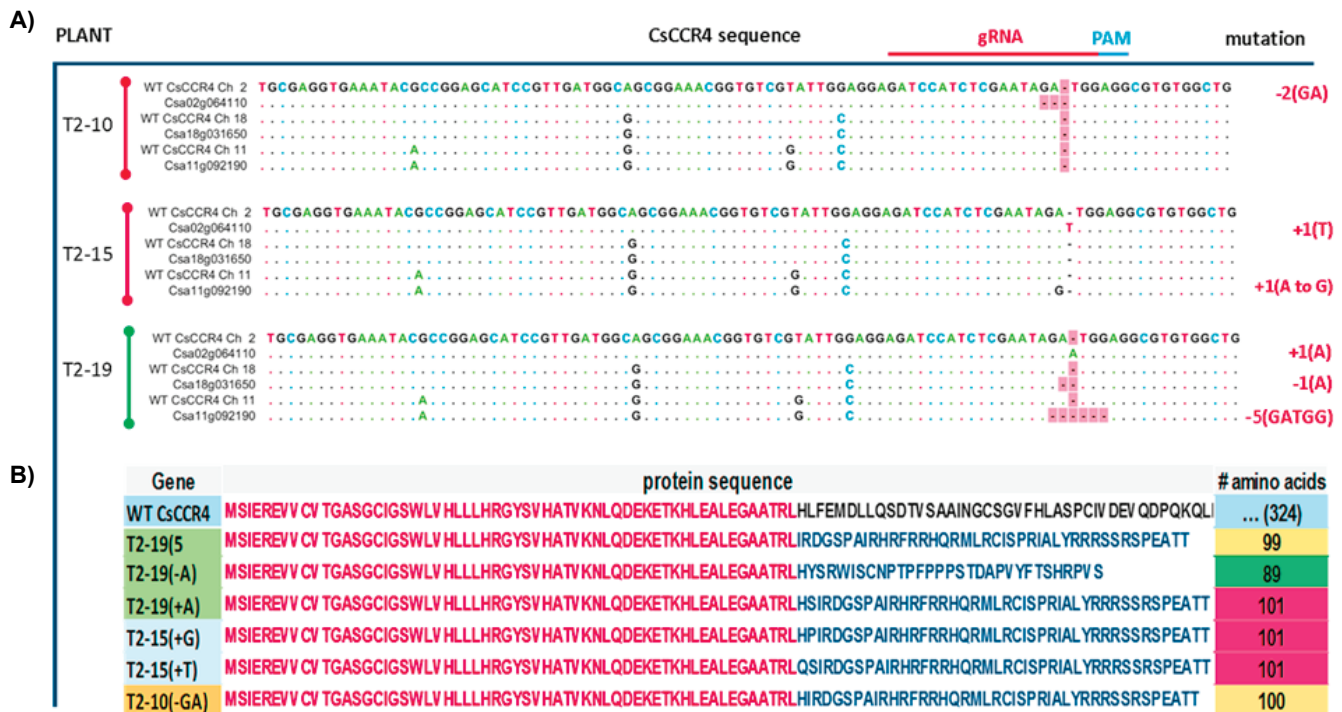T3 plants including T3-6, T3-10, T3-15, T3-16, T3-

Figure 4. A) three T2 sequenced lines containing specific types of mutation. The target sequence is shown by a blue line on top of the figure and PAM is indicated in red. From six plants of which possessed "drop-off" in their 2-D fluorescence intensity plots, three lines were selected and the region surrounded cut site was amplified to be sequenced via TOPO TA cloning for sequencing method. Approximately, 50 clones were sequenced. The number of insertions and deletions, indels, in each line and the content of indels for each target region on *CsCCR4* gene versions is summarized and listed in right. T2 plant number 10 has two nucleotides deletion-GA- before PAM motif on the *CsCCR4* gene located in chromosome 2. T2 plant number 15 has +1 T and A to G replacement on the genes located on chromosome 2 and 11, respectively. T2 plant number 19 has mutation in three versions of *CsCCR4* genes. The SNPs to distinguish the versions of *CsCCR4* genes are represented by letters. The similar nucleotides are indicated by dots. **B)** Translated protein sequence of WT-CsCCR4 with the premature versions obtained by simulation of out-frame mutation based on the sequencing results that was observed in the mutant plants. The type of mutation in each T2- mutant plant are represented in the left of the table with distinct colors. The content of predicted proteins are shown in the middle where the in-frame parts of the proteins are highlighted in red while the out-frame protein sequences are shown in blue. The length of predicted protein in each version are represented in the right.

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19 and T3- 23 as shown in **Figure 5A-B** (and see **supplementary data 8**) had the infected size the same as susceptible line DH55. Whearas, The T3-9, T3-4, T3-3 T3-25, T3-21, T3-20 T3-2, T3-13, T3-14, T3-12 were quite similar to the wild type CN114263. The other evaluated events were shown more or less degrees of susceptibility to *Ss*. According to this results, the T3 population was divided into two susceptible and resistant group. The infection results for T3 plants numbered T3-10, T3-15 and T3-19 that we had their both drop off assay and sequencing data illustated the association between the susceptibility to the pathogen and *CsCCR4* mutation in resistant genotype background.

5. Discussion

Modern molecular genetics technologies like CRISPR/ Cas9 have made the identifying potential resistant gene practical in plant molecular pathology studies, due to its fast, efficient and precise gene manipulation system in plant. In this work, to assess the role of *CsCCR4* gene in susceptibility to *Ss*, we established Camelina *csccr4* mutant that can serve as a model for further plant-pathogen interaction investigations. Three homeologous of *CCR4* genes produce conserved proteins and are highly expressed in almost all tissues and different developmental stages in Camelina and Arabidopsis and other crops. During the *Ss* attacks to Camelina, the expression of *CsCCR4* is upregulated to cease pathogen growth (19). To expand the area for further studies, we deployed a sgRNA and an Arabidopsis codon optimized Cas9 enzyme to enforce double strand break, DSB, at the second exon of *CsCCR4* of the gene using to lead out-frame mutation and producing premature CsCCR4 protein. To recover the knockout events, we screened the transgenic Camelina through the sequential generations. Our filter was herbicide spraying, T-DNA CNV detection, Drop-off assay followed by sequencing in T0, T1 and T2 generations respectively. Using ddPCR drop-off assay technique (28, 29) and TOPO-TA sequencing, we detected three T2 plants possessing mutation (inde1) at the interested cut site in the coding region of the *CsCCR4* gene. One of these, T2- plant 19, had mutation in all three versions but still showed intact genes with no mutation in the TOPO TA sequencing results. Due to the complex nature of Camelina genome, it hinders to gain null mutant in all *CsCCR4* homeologous in T0, T1 and even T2 generations. However, the specific mutations in *CsCCR4* that was

detected by drop-off assay experiment in T2 generation plants are expected to make null mutant, 6 out of 6 mutation, in the following generations because of two reasons: a Camelina is a self-pollinating plant so that the mutant alleles can be replicated and b the activity of gene editing apparatus-DNA containing sgRNA and Cas9, incorporated in the genome would continue to modify the target sequence across the generations unless the researcher cross T-DNA off from the host genome. The content of mutations for T2-plant-19 showed mutations in all three loci of CsCCR4 gene. Overall growth of *csccr4* mutants and their phenotypes were normal same as wild-type. Their length and seed shape and size were not altered compared to wild type in both T2 and T3 generations. Whereas, either mutation or down-regulation in *CCR1* gene, as a structural gene, develops dwarf phenotypes in other species (30, 31). In any level of mutation, the pattern of response to Sclerotinia stem rot relative to wild type was totally changed in all six T3 selected mutants. The mutant plant even in one copy of *CsCCR4* mutation, T3- plant 10, showed significant susceptibility to the Sclerotinia invasion unlike wild type. These results consistent with the role of *CsCCR4* in monolignol biosynthesis where transcript accumulation of *CsCCR4* genes results in increased resistance in Camelina plants. The knockout of *CsCCR4* in Camelina and any other plants has never been reported. Accordingly, providing *CsCCR4* mutant plant is precious material to investigate its effect on the Camelina defense response scenarios to the disease. After infection with *Ss*, lignin content of the cell wall is increased by both in Guaiacyl (G) and Syringyl (S) units' polymerization. G-Lignin accumulation in Camelina is associated with the Cinnamoyl CoA reductase 2 (*CsCCR2*) (19) whose expression is induced by pathogen attack in a broad range of dicot plants (32). Whereas, the *CsCCR4* expression which leads S-Lignin production, is constitutively occurred in resistant line 11 times higher relative to the susceptible line (19). In our study we found that the pattern of resistance against *Ss* was altered in *csccr4* mutant plants and that the susceptibility to this pathogen was significantly enhanced. In this basis, we imply that *CsCCR4* from resistant Camelina lines may potentially be a good source of resistance in classical breeding programs to confer resistance to either Camelina cultivars or by intertribal hybridization into economic elite relatives oilseed crops such as canola and mustard(33) with
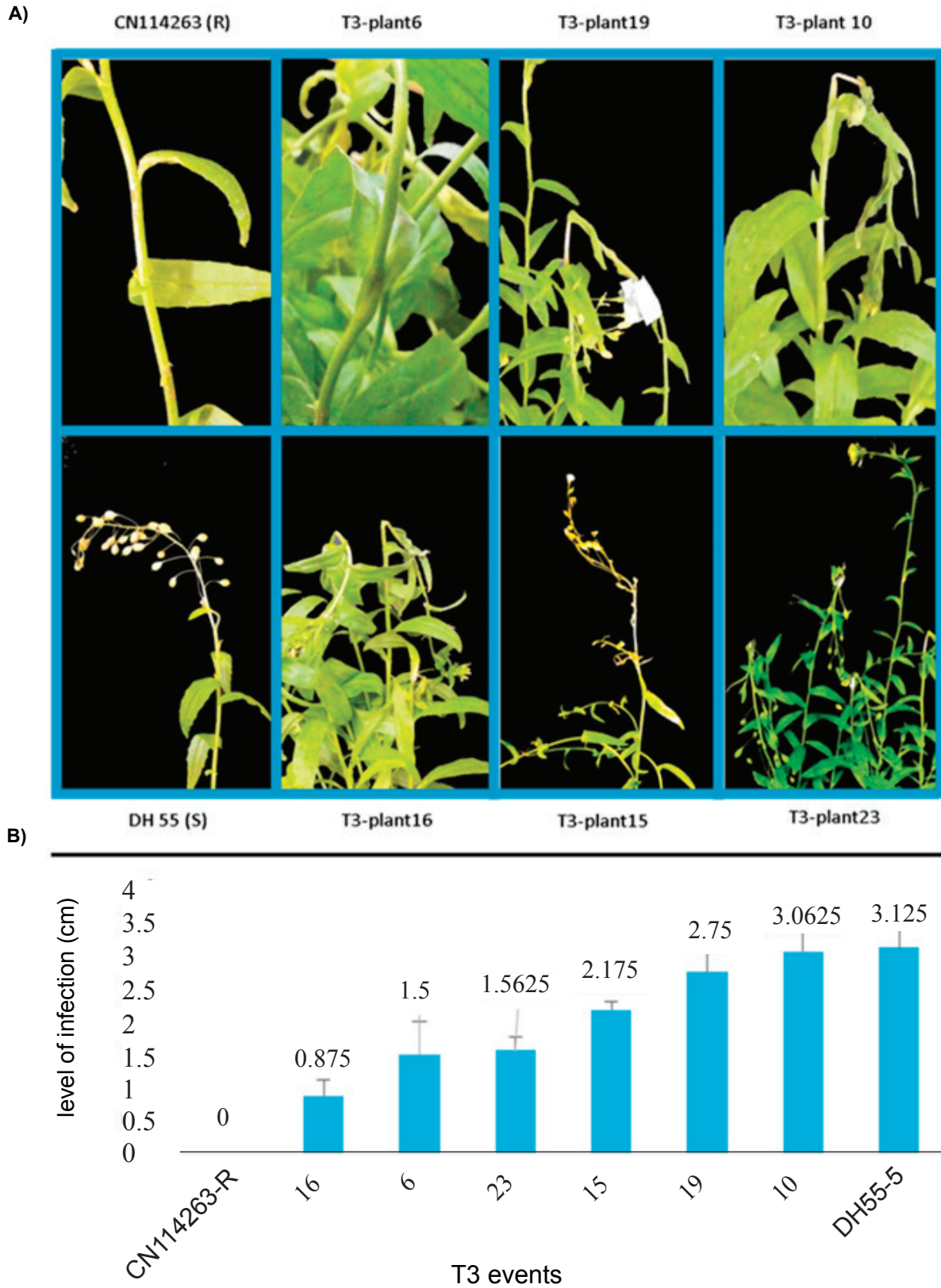
Figure 5. A) Comparison of the T3 mutant plants with susceptible and resistant genotypes as the positive, CN114263, and negative control, DH55, respectively. **B)** infection level (in centimeter) of T3 selected events (T3-16, T3-6, T3-23, T3-15, T319, and T3-10).

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the aim of prevention of crop lose in modern farming. *CsCCR4* produces S-lignin in Camelina (19). S-lignin accumulation induced by pathogen also has been reported in different pathosystems (19, 34, 35). Having only one group of methoxy in its structure, S- lignin is more linear than G-lignin (20) suggesting that it can shield polysaccharide from fungal enzymes. This hypothesis is compatible with mainstream of the literature. Traditionally, lignin deposition is considered to be associated with disease resistance which emphasize that, the reduced cell wall's lignin content will diminish resistance performance by loosening stem fortification in pathogen attacks (36). Lignin with hydrophobic structure would hinder the pathogen access to the nutrient substrates by making a physical barrier in front of fungal penetration. Interestingly, the recent studies in some cases do not support the hypothesis that states more lignin concentration has positive correlation with resistance to the pathogen. Even in soybean (*Glycin max*) the susceptibility to *Ss* was observed in genotypes with high lignin content (37). Moreover, monolignols also can be regulated to flux through to produce chemical compounds other than lignins during pathogen attacks (38). Likewise, there is another speculation about *CsCCR4* enzyme that it can allocate the precursor to accumulate defensive secondary metabolites which have low molecular weight compound relative to lignin from the onset of the pathogen invasion. Soluble derivatives monolignol biosynthesis pathway such as sinapoyl Glucose, coniferyl alcohol and coniferin have been proved that can prevent pathogen with no further lignification. These benefits of *CsCCR4* at the cross-talk of structural and chemical defensive components would therefore suggest dual function in responses against *Ss*. Currently, we do not know how *CsCCR4* gene regulates either in normal condition of growth and development or under biotic stress and how *trans* elements through defense signaling pathways can affect its expression in Camelina. Although the promising results that we achieved, the complexities of resistance mechanism which is conferred by *CsCCR4* gene is remained to be unraveled. To do so, several approaches such as omics platforms during plant-pathogen interaction, double mutants for parallel functional genes or disruption of master transcription factors that interact with *CsCCR4* gene should be incorporated. Therefore, *CsCCR4* mutation perhaps rather than cell wall lignification compromises different types of routes of resistance

against Sclerotinia. Further experiments to proof this assumption is needed and also it is important to follow generations of the validated Camelina mutant events to gain null knockout for *CsCCR4*.

6. Conclusion

The source of resistance in Camelina to *Ss* has been the concern of researchers who have long sought to find an alternative oilseed crop to reduce canola cropping monopoly (39). The aim of this study was to show the *CsCCR4* gene retaliation action against *Ss* during the invasion. We successfully could prove that CRISPR/Cas9-mediated mutation technology is a reliable and easy to use method that can be exploited to accelerate the process of the mutant line development which in this context helped us to figure out the function of a *CsCCR4* gene in defense pathways in *Camelina sativa* with a high level of complexity for manipulation. In this regard, the plant cell wall is thickened via lignification in response to pathogen attack which is an important and complex process that takes place during plant-pathogen interaction in injured stems. Based on the background of our research group we were required to provide appropriate plant material to study these objectives. It is evidenced that, the mentioned lignin deposition is different from regular lignification that occurs in certain cells like vascular cambium and xylem, throughout plant normal development (19, 20, 32, 40). *CCR* genes as pivotal players in the cross-link of several plant phenolic compounds are involved in both developmental growth and several reactions to environmental signals such as salinity (41), UV light circadian rhythm photoperiod reactions and defensive responses against biotic and abiotic stresses. Consistent with the role of *CCR* genes, analysis of the annotated *CCR* orthologues from *CCR1* to *CCR10* in 12 different tissues covering four developmental stages in Camelina, showed that expression of all *CCR* genes is reasonable except *CsCCR2* and *CsCCR10* which poorly expressed throughout all developmental stages and tissues. In addition, *CsCCR4* perpetually is expressed in all tissues and developmental stages. It also upregulates and results in syringyl lignin content in response to necrotrophic pathogen *Ss* both in susceptible and resistant lines. Whereas, *CCR2* which is rarely expressed during developmental stages its transcript accumulation has correlation and causal relation with deposition of guaiacyl monomers in resistant plant 48

hours following pathogen inoculation in inflorescence stems in an inducible manner. The transcript level of *CsCCR4* in the resistant plant is 11 times higher than the *CsCCR4* gene in susceptible lines. It also showed downregulation in susceptible Camelina line 30 times lower than the resistant line under *Ss* attack. Although *CsCCR4* exhibits expression throughout all developmental stages in concert with *CsCCR1* under normal growth, Cs*CCR4* expression in resistant line is increased compared with susceptible lines. Which correspondingly increases Syringyl/Guaiacyl ratio in the resistant line (19). This versatile expression of *CsCCR4* in both defense and developmental lignification suggests the role for *CsCCR4* in coordinating the interplay amidst growth rules and defense dynamics that can react to both signals in the gene regulatory network. However, the mechanism by which *CsCCR4* brings defense and growth routes together is indefinite. The positive correlation between mutation and the size of lesion in several T3 *csccr4* mutants was observed and is consistent with the hypothesis that *CsCCR4* expression is committed in S-lignin synthesis through over-lignification of stem's secondary cell walls in wild type resistant line and perhaps accumulation of defensive secondary metabolites, because we know that monolignols also are produced in non-lignified tissues and over expression of monolignol biosynthesis genes always is not correlated to the lignin accumulation in plants(42), thereby lack of its presence would limit physical defense responses and culminate to increased level of susceptibility to *Ss*. In conclusion, our work has shown that *CsCCR4* mediated resistance is central to the defense responses against *Ss*. This finding also insight into creation of new way for the breeding strategies to improve Camelina with resilience performance in confront with environmental turbulences.

Acknowledgments

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