A rhomboid-like protease gene from an interspecies translocation confers resistance to cyst nematodes

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Summary

- Plant-parasitic nematodes are severe pests in crop production worldwide. Chemical control of nematodes has been continuously reduced in recent decades owing to environmental and health concerns. Therefore, breeding nematode-resistant crops is an important aim if we are to secure harvests. The beet cyst nematode impairs root development and causes severe losses in sugar beet production. The only sources for resistance are distantly related wild species of the genus Patellifolia. Nematode resistance had been introduced into the beet genome via translocations from P. procumbens.
- We sequenced three translocations and identified the translocation breakpoints. By comparative sequence analysis of three translocations, we localized the resistance gene Hs4 within a region c. 230 kb in size. A candidate gene was characterized by CRISPR-Cas-mediated knockout and overexpression in susceptible roots.
- The gene encodes a rhomboid-like protease, which is predicted to be bound to the endoplasmic reticulum. Gene knockout resulted in complete loss of resistance, while overexpression caused resistance.
- The data confirm that the Hs4 gene alone protects against the pest. Thus, it constitutes a previously unknown mechanism of plants to combat parasitic nematodes. Its function in a nonrelated species suggests that the gene can confer resistance in crop species from different plant families.

Introduction

Plant-parasitic nematodes are major pests in agriculture. Global economic losses by plant-parasitic nematodes are estimated to be around US$157 billion annually (Abad et al., 2008). Damage is highest in narrow crop rotations and perennial crops. Cyst nematodes invade the root as juveniles, where they develop into males and females. The males leave the root after a few days, whereas females stay in the root for the rest of their life. Larvae induce a unique feeding site within the root vascular cylinder. A syncytium is formed after cell walls of neighboring procambial or cambial cells have been dissolved and protoplasts have been fused (Holtmann et al., 2000). Nematodes penetrate the syncytium via a stylet. A feeding tube forms at the end of the stylet associated with the endoplasmic reticulum (ER) of the syncytium (Sobczak et al., 1999). Small molecules are secreted into the syncytium, resulting in transcriptional and metabolic reprogramming (Mitchum et al., 2007; Jin et al., 2011; Ali et al., 2013; Holbein et al., 2016). After a male has fertilized the female, it completes its life cycle within 4–5 wk and turns into a solid cyst, filled with hundreds of eggs that can survive in the soil for 10 yr or more.

Sugar beet (Beta vulgaris ssp. vulgaris) is a biennial dicotyledonous plant and a member of the family Amaranthaceae (Yang et al., 2015). Sugar beet is the second most important sugar crop after sugarcane and supplies c. 20% of the world’s sugar (Finkenstadt, 2014). At a small but progressive scale, sugar beet is also used in energy production (Rodriguez et al., 2010; Maung & Gustafson, 2011). The sugar beet genome consists of 18 chromosomes with proper chromosome nomenclature (Schondelmaier & Jung, 1997). Its estimated genome size is 758 Mb (Arumuganathan & Earle, 1991), with a repeat content of 63% (Dohm et al., 2014). The first assembled sugar beet genome of the doubled haploid line KWS2320 comprised 567 Mb with 27 421 protein-coding genes (Dohm et al., 2014).

The beet cyst nematode (BCN, Heterodera schachtii Schm.) is the most important sugar beet pest (Supporting Information Fig. S1). Sugar beet and its close relatives are highly susceptible to the BCN, which causes whiskered roots and early wilting after mild drought stress, resulting in severe yield loss. Two distant relatives of the genus Patellifolia (P. procumbens and P. patellaris) are fully resistant to the BCN through complete suppression of nematode development (Savitsky, 1973). Therefore, they are the only source of resistance in the genus Beta (Viglierchio, 1960). In P. procumbens, resistance genes are located on chromosomes 1, 7 and 8 (Lange et al., 1993). Several attempts have been made to transfer resistance genes to sugar beet (Coons, 1975; Savitsky,
resistance was observed (Wenthin 2003). Therefore, we reasoned after transformation to different beet genotypes, only incomplete other plant disease resistance genes (Cai et al. 2018). It encodes an NBS–LRR type R-protein receptors (Li et al., 2014). The R-genes from soybean encode proteins with different functions. Rhg4 encodes a serine hydroxymethyltransferase, whereas the Rhg1 locus houses three genes encoding an amino acid transporter, an a-SNAP protein, and a wound-inducible domain protein (Cook et al., 2012; Liu et al., 2012). The first nematode resistance gene Hs1prr-1 had been cloned from a wild beet/sugar beet translocation line. It encodes an NBS–LRR protein with homology to other plant disease resistance genes (Cai et al., 1997). However, after transformation to different beet genotypes, only incomplete resistance was observed (Wenthin 2003). Therefore, we reasoned that a second gene, Hs4 (formerly referred to as Hs1–2), is located on the wild beet translocation, which works together or independently of Hs1prr-1.

Here, we describe the identification and functional analysis of the Hs4 gene. We identified the nucleotide position where the translocation is linked to the beet chromosome. We narrowed it down to a translocation region, which is only present in resistant lines but absent from susceptible translocation lines. A candidate gene from this region was functionally characterized by CRISPR-Cas9-mediated gene knockout and overexpression in susceptible roots. Infection tests gave compelling evidence for its function as a gene conferring complete resistance to cyst nematodes.

Materials and Methods

Plant material

The plant material used in this study is described in Table S1. The dominant allele of the bolting gene B was combined with nematode resistance to accelerate generation cycles. The resistance-carrying translocations from TR520 and NEMATA are derived from line A906001 (Kleine et al., 1997) while the resistant line TR363 has been selected independently (Brandes et al., 1987). All lines carry P. procumbens translocations attached to the same position at the end of chromosome 9 (Heller et al., 1996). The resistant hybrid variety NEMATA and the susceptible line 093161 were used for CRISPR-Cas and overexpression experiments.

Gamma irradiation of sugar beet translocation lines

The annual allele b was introduced into the TR520 addition line to shorten the generation cycles. The biennial nematode-resistant translocation line 940043 was crossed with the self-compatible line 930190 (annual, susceptible). Five F1 plants (940081) were self-pollinated, giving rise to the F2 population 950631 (Table S1; Fig. S2). A total of 2000 seeds of this population were γ-irradiated with a Co-60 source (IAEA, Seibersdorf, Vienna) with 200 and 400 Gy, respectively. The germination rates did not differ much between 200 Gy (140%) and 400 Gy treatments (136%). Retarded growth could only be observed during the first 3 wk in the 400 Gy treatments. Plants were grown in the field. As expected, the population segregated for early bolting in a 3:1 manner (Table S2). F3 seeds were harvested from 593 early-flowering plants (BB, Bb) after bag isolation (only 400 Gy experiment). A total of 451 F3 families (15 plants/family) were tested for nematode resistance in the glasshouse; 226 families were segregating for nematode resistance. The segregation for resistance confirmed the formerly known non Mendelian transmission rate (unpublished data) (Table S3). The fraction of nonsegregating resistant families was expected to be 25% (observed: 6%). The segregation ratios demonstrated that only 50% rather than 75% of the progeny were resistant.

Nematode resistance tests

Beet cyst nematodes were propagated in the glasshouse under nonsterile conditions on susceptible sugar beet plants. Fully developed brown cysts were harvested from the roots onto 50 μm sieves. A 3 mM ZnCl₂ solution was used to stimulate the hatching of juveniles in the dark. Nematodes were examined under a binocular microscope. Only suspensions with >90% mobile nematodes were taken as inoculum. For in vitro tests, nematodes were surface-sterilized by soaking them in 0.05% HgCl₂ solution for 30 s, and were then washed four times with sterile water and resuspended in 0.2% (w/v) Gelrite (Duchefa Biochemie BV, Haarlem, the Netherlands). In all, 250 sterile nematodes were used to inoculate the hairy roots (Sijmons et al., 1991).

For glasshouse resistance tests, plants were grown in 20 ml tubes filled with sterile sand (grain size 0.1–1.5 mm), sterilized at 80°C for 3 h. Six hundred freshly hatched second-stage juveniles (J2 larvae) were added to each plant with a syringe (Löptien, 1984). At 4 wk after infection, plants were harvested and washed, and roots were examined under a binocular microscope. Root samples were collected 3, 6, 9 and 12 d post-inoculation (dpi), with three biological replicates per sample.

Nucleic acid extraction and PCR conditions

For marker analyses, DNA was isolated from freeze-dried leaves of 4-wk-old sugar beet plants using a magnetic bead kit (MagAttract 96 DNA Plant Core Kit; Qiagen). For whole-genome sequencing (WGS), PCR experiments and genotyping of CRISPR-Cas mutagenized and overexpression hairy roots, DNA was isolated from 2–3 g of leaves or hairy root tissues using the
Fig. 1 Comparative sequence analysis of wild beet translocations from two resistant and two susceptible beet lines (*Beta vulgaris* spp. *vulgaris*). (a) Schematic representation of the chromosome translocation attached to the end of sugar beet chromosome 9. (b) Physical map of the *Patellifolia procumbens* translocation from the resistant line TR520 based on whole-genome sequencing (WGS) and published molecular markers compared with translocation lines TR363 (resistant) and TR659 (susceptible). The black bar depicts the sugar beet chromosome 9, the red bar shows the *P. procumbens* translocation, and gray bars depict the sequences present in resistant but absent from susceptible lines ('critical region'). Inverted triangles represent positions of molecular markers used for mutant screening. (c) Locations of predicted gene models in the regions absent from TR659. ORF1 is located at the distal end (blue color). (d) Schematic representation of the translocation breakpoint in TR520. The primer binding sites used to amplify this region are indicated by arrows. (e) Genotyping the translocation breakpoint with two flanking markers (primer combination H208/H203). A 567 bp fragment encompassing the translocation breakpoint is amplified only from TR520 and TR363. Fragments in TR520 and TR363 are different in size. (f) Genotyping resistant and susceptible translocation plants with three molecular markers (see b). Translocation (TR520)-specific PCR fragments are depicted by arrows. Nonspecific fragments were sequenced and did not map to the translocation.
CTAB extraction method (Rogers & Bendich, 1985). Leaves and hairy root tissues were freeze-dried for 48 h and stored at room temperature until further use.

RNA was isolated from root and leaf tissues and hairy roots (overexpression experiments). Root samples were harvested from inoculated plants at 3, 6 and 9 dpi along with samples from non-inoculated plants. Roots were briefly washed with water to remove sand. Samples from red fluorescent protein (RFP)-positive hairy roots were collected before infection tests to measure relative gene expression. Leaves, roots and hairy roots were shock-frozen in liquid nitrogen and stored at −70°C until further use. Frozen tissues were homogenized in four cycles for root tissues and two cycles for leaf tissues (2 min each cycle) using a Geno/Grinder® (SPEX SamplePrep, Metuchen, NJ, USA) according to the manufacturer’s instructions. Samples were submerged into liquid nitrogen after each cycle to prevent them from thawing. RNA isolation and DNase treatment were carried out according to the PeqGold Total RNA Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany). The RNA quality was checked by agarose gel electrophoresis and a NanoDrop2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Primer sequences and annealing temperatures are listed in Table S4.

For the RNA sequencing experiment, the root material of inoculated TR520 plants was harvested at 1 and 3 dpi, and total RNA was isolated. The RNA sequencing library was constructed using the Illumina TruSeq RNA sample preparation kit and subsequently sequenced (2 × 100 bp) on an Illumina HiSeq 2000 sequencer (Illumina, Inc., San Diego, CA, USA) at the sequencing unit of the Institute of Clinical Molecular Biology (IKMB), Kiel, Germany.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was carried out using a Platinum™ SYBR™ Green qPCR SuperMix (ThermoFisher Scientific). Expression levels were calculated with the comparative ΔΔCt method (Livak & Schmittgen, 2001). Primers, hs4_F, and hs4_R, used for gene expression analysis, are listed in Table S4. BeGAPDH was used as an endogenous control to normalize gene expression levels.

Molecular marker analysis
Each family of the mutant-derived F3 population (Fig. S2) segregating for nematode resistance was analyzed with molecular markers. Of each family, three resistant plants and one susceptible plant were chosen for marker analysis. Plants were genotyped with four markers to detect larger deletions within the translocation. Three marker sequences are specific for P. procumbens (1832, 3133, and 3136) while the ubiquitin marker served as a positive control for the sugar beet genome (Fig. 1h) (Schulte et al., 2006).

Whole-genome sequence analysis
Sequencing libraries were prepared with leaf DNA of the translocation lines TR520, TR659, and TR363 using the Illumina TruSeq DNA sample preparation kit. Each library was sequenced (paired-end, 2 × 100 bp) on two lanes of the Illumina HiSeq 2000 sequencer (Illumina, Inc., San Diego, CA, USA) at the sequencing unit of the Institute of Clinical Molecular Biology (IKMB, Kiel, Germany). Also, a TR520 mate-pair library (2 × 100 bp) with 5 kb distance was sequenced with the same Illumina device (Table S5, S6). SOAPdenovo2 (v.2.04-r241) was used to generate two de novo assemblies, with k-mer size 91 and 61, of the translocation line TR520, using the paired-end read libraries and the mate-pair read library. Reads for TR363, TR659, KWS2320 (NCBI: SRR869754) (Dohm et al., 2014) and P. procumbens were mapped to TR520 de novo assemblies using bwa-mem with default settings (v.0.7.13) (Li & Durbin, 2010). Mapping files were processed with SAMtools, using default settings (Li et al., 2009). Three strategies were used to find translocation-specific sequences. First, to find the initial set of translocation-specific sequences, using local BLASTN (word_size = 30), scaffolds were screened for the presence of previously published translocation-specific molecular markers, in addition to bacterial artificial chromosome and yeast artificial chromosome sequences (Schulte et al., 2006). Second, using blastn (word_size = 30), further screening of scaffolds was done to detect repetitive sequences specific to the P. procumbens genome (Schmidt & Heslop-Harrison, 1996; Dechyeva et al., 2003). Third, mapping coverage of P. procumbens and KWS2320 WGS reads was used to decide if particular scaffolds belong to the translocation. Mapping-depth coverage was calculated for all the mapped lines using genocompare (Quinlan & Hall, 2010). Using an all to all local BLASTN search, in addition to manual visualization of mate-pair read mapping in Integrated Genomic Viewer (IGV) (v.2.8.7) (Thorvaldsdóttir et al., 2012), scaffolds from both assemblies were then arranged, joined, or combined to get the consensus sequence. rnaSPAdes from the SPAdes package (v.3.12.0) was used to de novo assemble RNA sequences. Structural annotation was performed with the MAKER (v.3.01.02-beta) genome annotation pipeline (blast_type = ncbi+)(Holt & Yandell, 2011), using de novo assembled RNA sequencing reads as transcript evidence, and published protein sequences as protein evidence (Dohm et al., 2014).

In-silico analysis of the candidate gene
The subcellular localization of the Hs4 protein was predicted by DEEPLOC-1.0 (http://www.cbs.dtu.dk/services/DeepLoc-1.0/) (Almagro Armenteros et al., 2017). Sequence similarity of Hs4 to entries from the peptidases database MEROPS and UniProt database was assessed using the online BLASTP service of EMBL-EBI and UniProt BLASTP service, respectively, using default settings (The UniProt Consortium, 2018; Madeira et al., 2019). Sequence alignments were generated using the CLUSTAL OMEGA webservice (Madeira et al., 2019).

Vector construction and plant transformation
For targeted (CRISPR-Cas) mutagenesis, two vectors, pChimera (Fausser et al., 2014) and p201G (Jacobs et al., 2015), were used. We selected two 20 bp target sequences (T1 and T2) located
within the first exon of the *Hs4* gene and next to a 5'-NGG PAM site. For cloning into the pChimera vector, DNA oligonucleotides of the designed target sequences with overhangs of the *Bbhl* restriction site were synthesized (Eurofins Genomics, Ebersberg, Germany). The p201G vector (Addgene, Watertown, MA, USA) contains the GFP-encoding gene under the transcriptional control of the enhanced CaMV35S promoter as a selectable marker, and the *Cas9* gene controlled by another CaMV35S promoter. A promoter-single guide RNA (sgRNA) cassette was cloned into the I-*Ppol* restriction site. The AtU6-26(P)-sgRNA cassette was amplified from the pChimera vector and subcloned into the p201G vector via the I-*Ppol* restriction sites (Fig. S3a).

For overexpression of the *Hs4* gene, the vector pBin35SRed was used (Pidkowich et al., 2007) (Fig. S3b). pBin35SRed carries the gene encoding the RFP (DsRed) as a selectable marker under the transcriptional control of the cassava vein mosaic virus (CsVMV) promoter. The coding sequence of the *Hs4* gene was amplified by PCR using primers F_OEX and R_OEX with *Xba*I and EcoRI restriction sites (Table S4). The PCR product (665 bp) and the pBin35SRed vector were digested with *Eco*RI and *Xba*I. Then, the digested PCR product was ligated into the pBin35SRed plasmid (Fig. S3b). The recombinant plasmids obtained (p201G-Cas9-gRNA and pBin35SRed-Hs4) were transformed into *A. rhizogenes* using a heat-shock approach (Quandt, 1993).

**Hairy root transformation**

*A. rhizogenes* (strain ARqua1)-mediated transformation was carried out to produce hairy roots from NEMATA and the susceptible line 093161 leaf stalks (Table S7). Seeds of NEMATA and 093161 were surface-sterilized for 20 min in 5% sodium hypochlorite containing 0.02% Tween-20, followed by washing with sterile distilled water. Surface-sterilized seeds were germinated and grown on hormone-free 1/2B5 medium under photoperiodic conditions of 16 h : 8 h, light: dark at 22°C. According to our standard protocol, Agrobacterium transformation and hairy root production were carried out (Kifle et al., 1999). In brief, 1–2 cm leaf stalks were cut and cocultivated with *A. rhizogenes* and placed on B5 plates where they were kept in a climate chamber in the dark at 22°C for 48 h. Afterwards, the leaf stalks were placed onto B5 plates with cefotaxime (400 mg L⁻¹) and kept at 20°C under long-day conditions until hairy roots were observed. Hairy roots 1–2 cm in size were cut from the leaf stalks and placed on fresh B5 plates containing cefotaxime (100 mg L⁻¹) (Duchefa Biochemie BV).

**Microscopic studies**

Transgenic hairy roots from the overexpression and knockout experiments displayed the green and red fluorescence marker genes. Whole roots were examined under a fluorescence stereomicroscope (Nikon SMZ25; Nikon Instruments Europe BV, Amsterdam, the Netherlands) using the NIS-ELEMENTS BR microscope imaging software (Nikon). Further processing of the raw images was performed using the Fiji image processing package (Schindelin et al., 2012).

**Statistical methods**

Two-tailed unpaired *t*-tests were performed using R (R Core Team, 2013), and graphs were plotted using GGPLOT2 (Wickham, 2011) in RSTUDIO (RStudio Team, 2020).

**Results**

A single breakpoint marks the proximal end of the TR520 translocation

We sequenced one resistant plant of the population TR520, which was segregating for the *P. procumbens* translocation, and performed *a de novo* assembly. *In situ* hybridization of extended chromatin fibers had demonstrated that the translocation constitutes the very end of chromosome 9 (Desel et al., 2001). First, we aimed to precisely position the translocation breakpoint, which was expected to be a sharp borderline between wild beet- and sugar beet-specific sequences. Translocation-specific sequences were found by mapping WGS reads from *P. procumbens* and the susceptible line KWS2320 to the assembled TR520 genome in combination with translocation-specific molecular markers (Schulte et al., 2006) and *P. procumbens*-specific repetitive sequences (Schmidt & Heslop-Harrison, 1996; Dechyeva et al., 2003). Translocation-specific scaffolds were joined manually by inspecting mate-pair library mapping data resulting in two super scaffolds harboring 228 gene models. The translocation-specific superscaffold2 was linked to scaffold C5128660, which shares a 100% sequence similarity to the sugar beet reference genome (Dohm et al., 2014). Therefore, we concluded that the genomic region joining these two scaffolds harbors the translocation breakpoint. We further characterized the breakpoint sequence in the TR520 translocation by amplifying and sequencing the genomic region between the two scaffolds (Fig. 1d). A second translocation line (TR363) was used as a positive control. Resistant plants carrying the translocation showed the expected fragment, while a susceptible beet control (093161) and the wild beet (950056) were lacking it (Fig. 1e). This indicates a translocation hotspot because both translocation breakpoints are precisely at the same position on chromosome 9, thus confirming earlier mapping studies (Heller et al., 1996). The minor size difference between TR520 and TR363 amplicons indicates a short insertion/deletion polymorphism between both lines. To confirm the breakpoint’s exact position, we mapped TR520 WGS reads to the beet reference genome. Within scaffold Bvchr9_un.sca002, the mapping coverage of TR520 dropped to almost half of the original coverage, thus confirming the translocation breakpoint at this position on chromosome 9 (Fig. S4). A drop in coverage by nearly half also indicated that the TR520 translocation is present in a hemizygous state. The identified breakpoint marks the proximal end (towards the centromere) of the translocation. The translocation-specific sequence scaffolds sum up to 3.13 Mbp, which exceeds earlier estimations by 113% (Schulte et al., 2006).
Identifying truncated translocations by irradiation mutant mapping

Owing to the complete absence of chromosome pairing between wild beet and sugar beet chromatin, fine mapping of the resistance gene was impossible. Therefore, we produced translocation mutants by γ-irradiation (Fig. S2). Seeds of a segregating F₂ population were γ-irradiated with 200 and 400 Gy. For mutant identification, four plants of each F₃ family (a total of 974 plants) segregating for resistance were genotyped with three molecular markers (3133, 1832 and 3136) (Fig. 1b,f). All but two families displayed the expected genotypes. All markers were present in resistant but absent from susceptible plants lacking the translocation. However, in two F₃ families, susceptible plants carried marker loci from the translocation. Their offspring (TR320 and TR659) were uniformly susceptible. They had lost the resistance locus and one (TR659) or two (TR320, data not shown) additional marker loci, which points at major deletions within the translocation (Fig. 1b). Genotyping with more translocation-specific markers revealed that TR320 had lost most of its translocation (data not shown). Therefore, this line was excluded from further studies. Because the TR659 deletion was relatively small, this line proved valuable for further delimiting the resistance bearing region.

Identifying a critical region housing the Hs₄ gene

We sequenced TR659 and the second independent translocation line, TR363, which is also fully resistant and carries a translocation from P. procumbens chromosome 1 (Brandes et al., 1987). Based on read mapping coverage depth of WGS reads from TR659 and TR363 mapped to TR520, we identified a region of c. 660 kbp absent in TR659 (Fig. S5). Within this region, we identified 42 gene models (Fig. 1c). We expected that the resistance gene lies within a ‘critical region’ of c. 230 kbp present in resistant lines (TR520 and TR363) but absent from the susceptible line (TR659). We manually screened for the presence or absence of each gene in TR520, TR363 and TR659 using read mapping data on IGV. Using this approach, we could identify 33 gene models present in TR520 and TR363 but absent from TR659.

A candidate gene encoding a rhomboid protease gene

We sequenced an mRNA library from TR520 roots at 1 and 3 dpi to annotate expressed sequences from the critical region. We could identify 33 gene models (open reading frames, ORFs) within the critical regions, of which 19 were expressed (Table S8). We hypothesized that Hs₄ shares sequence homology to other plant nematode or disease resistance genes. Therefore, we aligned genes from the critical region to the UniProt database to identify homologs involved in plant disease resistance. However, none of the gene models was similar to any published resistance gene. One sequence named ‘ORF1’ attracted our interest because the encoded polypeptide was similar (60%) to two rhomboid proteases (A0A0J8FQU9 and A0A0K9RQE5) from sugar beet and spinach (Spinacia oleracea) (Fig. S6).

Moreover, the genome of the nematode-trapping fungus Pochonia chlamydosporia houses rhomboid proteases to degrade and penetrate nematode egg-shells (Larriba et al., 2014). The genomic sequence of ORF1 is 5664 bp in size, consisting of five exons and five introns (Fig. 2a). The translated region encodes a polypeptide of 210 amino acids. Using the DeepLoc webserver (Almagro Armenteros et al., 2017), the most probable cellular localization of the ORF1 protein is in the membrane of the ER (Fig. 2b), which is supported by its 16-amino-acid leader sequence for post-translational transport into the ER (Fig. 2c). Sequence alignment with its closest homolog from sugar beet (A0A0J8FQU9) revealed significant differences. The beet polypeptide has an additional 102 amino acids at its N-terminus, lacking the leader sequence (Fig. 2c). Therefore, it is unlikely to be integrated into the ER membrane. Then, we performed a BLASTP analysis of the ORF1 polypeptide sequence against the MEROPS database (Rawlings et al., 2017), followed by multiple sequence alignment and construction of a phylogenetic tree using Clustal Omega (Figs S6, S7). The Hs₄ protein differs from all other sequences by two insertions of nine and two amino acids at positions 103 and 147 (Fig. S8), respectively. None of the proteases showing similarity to ORF1 have been reported to be involved in plant–pathogen interactions.

We analyzed the expression of ORF1 in the resistant translocation line TR520 and found expression in roots and leaves (Fig. S9). Expression was also studied in roots 3–12 dpi, where a collapse of the initial feeding cell had been observed (Holtmann et al., 2000). Despite a slight increase in transcript abundance at 9 dpi, no significant difference was observed between infected and noninfected roots, indicating that transcriptional activity is not altered upon nematode infection (Fig. 3).

ORF1 functional analysis by overexpression and knockout experiments

We applied CRISPR-Cas mutagenesis to knockvout ORF1 in the resistant variety NEMATA, which carries the same translocation as TR520. We chose two sgRNA target sites, T1 and T2, within the first exon (Fig. 4). Potential off-targets were searched using the BLASTN approach for the shorter query sequence. No potential off-target sites were detected. After A. rhizogenes transformation with the vector plasmid p201G (Fig. S3a), we obtained 184 independent hairy root clones. Of these, 66 carried the CRISPR-Cas T-DNA, as they were positive for GFP, which was used as a reporter gene (Fig. 5a). After sequencing the target sites from 66 clones, we found four mutant alleles (hs₄_1-4) with deletions between five and nine nucleotides and another mutant where an additional nucleotide had been inserted (Fig. 4). Clone hs₄_1 has a 9 bp in-frame deletion, suggesting no effect on protein function. Then, we performed resistance tests with H. schachtii J2-larvae using nontransgenic hairy roots from NEMATA and the susceptible sugar beet (seed code: 093161) as controls. The roots of the knockout clones were highly susceptible because large numbers of J4 females and cysts filled with eggs could be observed. By contrast, the NEMATA roots were devoid of developing nematodes (Fig. 5b,c).
Interestingly, clone hs4_1 carrying an in-frame mutation was as resistant as the control, demonstrating that a loss of three amino acids did not alter protein function.

The next step was to transform ORF1 into the roots of the susceptible sugar beet line with transcription under the control of the constitutive 35S promotor (Fig. S3b). The DsRed gene under the control of the CsVMV promoter served as a reporter gene. In total, 11 DsRed-expressing roots were observed (Fig. 6a). After infection with H. schachtii, strikingly different infection rates were observed, ranging from high susceptibility to complete resistance (Fig. 6b). Because expression rates of the transgenes can vary widely between different transgenic genotypes, we measured the relative expression of ORF1. All but one root clone was expressing ORF1. Notably, infection rates were negatively correlated with the expression levels of the candidate gene (Fig. 6b). Clones highly expressing ORF1 were completely resistant (Fig. 6c), while clones with low expression were moderately susceptible. Clone OEX3, where no ORF1 transcript could be detected, was highly susceptible.

Discussion

These results collectively confirmed our hypothesis that ORF1 is the long-sought Hs4 gene conveying complete resistance to the BCN. The Hs4 gene constitutes a novel mechanism of plant resistance to nematodes. Previously reported nematode resistance genes encode proteins that are important in recognizing and transducing pathogen signals (Goverse & Smant, 2018). Thus they are part of signal transduction pathways, like many other disease resistance genes we already know about (Zhou & Zhang, 2020).

Hs4 encodes a protease of the rhomboid family known for its high substrate specificity (Urban & Freeman, 2003). It has been reported that proteases are involved in resistance to various plant pathogens (Hou et al., 2018; Thomas & van der Hoorn, 2018; Salguero-Linares & Coll, 2019). In Arabidopsis, the serine protease SBT3.3 (Subtilase 3.3), a subtilase member of the S8 family, regulates defense priming. Arabidopsis ib3.3 mutants are impaired in priming both gene expression and signaling activity and were hyper-susceptible to both Pseudomonas syringae and the
oomycete *Hyaloperonospora arabidopsidis* (Meyer *et al.*, 2016). The apoplastic papain-like cysteine protease Cathepsin B (CathB) acts as a positive regulator of the hypersensitive response in Arabidopsis (Gilroy *et al.*, 2007). CDR1 (Constitutive Disease Resistance-1), another apoplastic protease in Arabidopsis, has also been shown to be implicated in local and systemic defense signaling. Activation tagging of the *CDR1* gene resulted in the constitutive expression of pathogenesis-related (PR) genes in a salicylic acid-dependent manner and enhanced resistance to multiple *P. syringae* strains. By contrast, mutations in the active sites of the protease resulted in reduced expression of PR genes. These results indicated that *CDR1* generates an extracellular mobile signal capable of inducing defense responses (Xia *et al.*, 2004). The rice (*Oryza sativa*) *CDR1* (*OsCDR1*) gene after overexpression in *A. thaliana* conferred a similar systemic defense, which indicated that the *CDR1* function might be conserved between species (Prasad *et al.*, 2009). *AtCEP1* is a specific papain-like cysteine endopeptidase belonging to the C1A family. A C-terminal ‘KDEL’ sequence sequesters the protease within ER-derived compartments. It has been shown that the expression of *AtCEP1* was induced upon the infection with the fungus *Erysiphe cruciferarum*. Programmed cell death (PCD) was reported upon penetration by fungal haustoria. By contrast, Arabidopsis *cep1* mutants showed hypersusceptibility to *E. cruciferarum* with characteristically reduced PCD (Höwing *et al.*, 2018). While the role of these proteases in response to pathogen attack was demonstrated, their substrates have not been identified as yet.

The function of plant proteases upon attack by plant-parasitic nematodes has been demonstrated in tomato and Arabidopsis. In tomato, the *Cf-2* gene confers resistance to *Cladosporium fulvum*. To activate *Cf-2*-mediated resistance, an effector molecule secreted by the fungus interacts with the extracellular papain-like cysteine protease Rcr3 (Rooney *et al.*, 2005). Later, it was reported that an effector Gr-VAP1 of the cyst nematode *Globodera rostochiensis* also interacts with Rcr3 (Lozano-Torres *et al.*, 2012). Gr-VAP1 belongs to a class of secreted proteins found in all plant-parasitic nematodes. It is expressed in the esophageal glands, and it shares no sequence homology with the...
fungal effector. In a yeast two-hybrid analysis, Gr-VAP1 was found to bind to Rcr3, which led to the conclusion that the extra-cellularly secreted cysteine protease Rcr3 from tomato triggers a hypersensitive reaction upon attack by a fungus and by G. rostochiensis. It is not clear how Gr-VAP1 can bind to an apoplastic protein if nematode elicitors from the esophageal glands are secreted into the host cell’s cytoplasm via its feeding tube. However, in this study, nematode infection rates have not been analyzed. Another study highlighted the role of vacuolar proteases. The vacuole can store inactive proteases released upon pathogen attack (Buono et al., 2019). In A. thaliana, a BCN effector molecule targets a vacuolar papain-like cysteine protease released into the apoplast under stress conditions (Pogorelko et al., 2019). It was suggested that after binding to the effector molecule, the protease is directed from the vacuole to the nucleus and cytoplasm, which could impair its function as a resistance molecule. However, there is no genetic evidence for its role as a nematode resistance gene.

The Hs4 gene could offer new opportunities to control sedentary nematodes in other species because rhomboid proteases preserve their functional activity against their substrates from different organisms and do not require cofactors to catalyze intramembrane enzymatic reactions (Urban & Wolfe, 2005). Moreover, the Hs4 function was preserved entirely in a distantly related species (sugar beet), suggesting that Hs4 or functionally related rhomboid protease genes can be introduced into elite crop genotypes as single Mendelian genes. However, the choice of the right promotor is a critical point because the expression of the Hs4 gene in shoots under the control of the 35S promotor resulted in lethality (data not shown). Therefore, we propose transforming plants with the Hs4 gene under the control of a promotor responding to nematode infection such as the Hs1 promotor (Thurau et al., 2003).

The function of Hs4 as an ER-linked protease could complement earlier cytological studies. It is known that the BCN produces a feeding tube right in front of its stylet through which it takes up nutrients and secretes effector molecules into the cell. The feeding tube is attached to the cisternae of the ER (Sobczak & Golinowski, 2011). Feeding tubes surrounded by tubular ER were found in root-knot nematode (Meloidogyne incognita)-induced giant cells in rose balsam (Impatiens balsamina L.). Cisternal ER and mitochondria were located around the tubular ER (Miyashita & Koga, 2017). This study revealed that membrane systems from earlier studies are fine tubular structures connected to the outer surface of the feeding tube and bundles of tubular ER. Syncytia of resistant sugar beet carrying the same
translocation as TR520 contained rough ER compared with susceptible plants where smooth ER prevailed (Holtmann et al., 2000). Moreover, syncytia from resistant plants displayed aggregations of the endomembrane system, which composed most ER at later stages. It is tempting to speculate that these structures could result from the activity of the Hs4 gene, which might be acting as a suicide gene, directly or indirectly, disrupting the initial feeding cell soon after the nematode has initiated its feeding structure. As a next step, the Hs4 protein’s predicted position should be verified by in vivo experiments, for example, after transformation with a Hs4-RFP fusion construct.

A post-translational modification is supported by the fact that Hs4 is constitutively expressed at low rates and not transcriptionally responding to the nematode attack. Therefore, we hypothesize that the nematode, directly or indirectly, elicits the protease activity, which then destroys the ER and, subsequently, the feeding cell, which stops further development of the nematode as a result of the lack of nutrients. In the future, yeast two-hybrid experiments could identify H. schachtii effector proteins binding to the Hs4 protease. The fungus P. chlamydosporia uses rhomboid and other proteases to degrade and penetrate the eggshell of H. schachtii and other nematodes (Larriba et al., 2014). Interestingly, this is an endophyte that is colonizing plant roots. It will be a challenging hypothesis if, during evolution, similar functions to respond to effector molecules elicited by the beet cyst nematode have evolved in plants and fungi.

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**Author contributions**

AK designed and performed the experiments, produced the data, and was involved in writing the manuscript. H-JH, JL and BD produced the data, SM supervised the work, and CJ supervised the work and wrote the manuscript.

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**Data availability**

The raw RNA-seq data generated during this study were deposited in the NCBI Sequence Read Archive (SRA) database under the accession code PRJNA673875. Source data are provided with this paper. The authors declare that any other supporting data are available from the corresponding author upon request.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Resistant and susceptible accessions used in this study and beet cyst nematodes developing on sugar beet roots.

**Fig. S2** Pedigree of the mutant translocation lines TR320 and TR659 obtained after γ-irradiation of 1000 F2 seeds derived from the resistant line TR520.

**Fig. S3** Plasmid vectors used for CRISPR-Cas9 targeted mutagenesis and *Hs4* overexpression.

**Fig. S4** Visualization of the breakpoint using the Integrative Genomics Viewer.

**Fig. S5** Identification of the critical regions.

**Fig. S6** Comparison between ORF1 and its closest rhomboid homologs from other eukaryotes.

**Fig. S7** Phylogenetic analysis of the *Hs4* protein and related rhomboid proteases.

**Fig. S8** Multiple sequence alignment of the *Hs4* protein sequence with top hits from the MEROPS database showing an extra nine amino acids unique for *Hs4*.

**Fig. S9** Expression of *Hs4* in the resistant translocation line TR520 in leaf and root tissues.

**Table S1** Plant material used in this study.

**Table S2** Results of the γ-irradiation experiment with the sugar beet F2 population 950631.

**Table S3** Results of the F3 genotyping experiment to detect new translocation mutants.

**Table S4** Primer sequences and PCR conditions.

**Table S5** Summary statistics of whole genome/transcriptome sequencing of resistant and susceptible translocation lines and the wild beet *P. procvmbens*.

**Table S6** Assembly statistics for the TR520 translocation line.

**Table S7** Results from hairy root transformation experiments.

**Table S8** Genes localized within the critical region and have transcript evidence from RNA sequencing.

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