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Hybrid Assembly of Whole Genome Shotgun Sequences of Two Sugar Beet (*Beta vulgaris* L.) Translocation Lines Carrying the Beet Cyst Nematode Resistance Gene *Hs1-2* and Functional Analysis of Candidate Genes

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Abbreviations

AFLP	Amplified fragment length polymorphism
APAF-1	Apoptotic protease-activating factor-1
ATP	Adenosintriphosphate
AVR	Avirulence
BAC	Bacterial artificial chromosome
BAK1	BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1
BC*n*	*nth* backcross
BCN	Beet cyst nematode
BES	BAC-end sequences
BGI	Beijing Genome Institute
bHLH	Basic helix-loop-helix
BIK1	BOTRITYS INDUCED KINASE 1
BKK1	BAK1-LIKE1
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BV	*Beta vulgaris*
BWA	Burrows-Wheeler Aligner
bZIP	Basic leucine-zipper
CaMV	Cauliflower mosaic virus
CAPS	Cleaved amplified polymorphic site
CAU	Christian Albrechts University
CC	Coiled-coil
CCD	Charge-coupled device
cDNA	Complementary DNA
CDS	Coding sequence
CED-4	*Caenorhabditis elegans* death-4
clp	Caseinolytic protease

cM	CentiMorgan
CMS	Cytoplasmic male sterility
Cry	Cytolytic δ-endotoxin
CTR1	CONSTITUTIVE TRIPLE RESPONSE1
CTAB	Cety trimethylammonium bromide
CUL1	Cullin1
DAMP	Danger-associated molecular pattern
DEPC	Diethylpyrocarbonat
DH	Doubled haploid
DNA	Deoxyribonucleic acid
ds	Double-strand
DSB	Double-strand breaks
dpi	Days post inoculation
E. coli	Escherichia coli
EDR1	ENHANCED DISEASE RESISTANCE 1
e.g.	*Exempli gratia*
EMBL	European Molecular Biology Laboratory
EMS	Ethyl methane sulfonate
EST	Expressed sequence tags
et al.	*et alii*
ETI	Effector triggered immunity
EU	European Union
ExPASy	Expert Protein Analysis System
FISH	Fluorescence *in situ* hybridisation
FLS2	Flagellin sensing 2
FRET	Fluorescent resonant energy transfer
GABI	Genomanalyse im Biologischen System Pflanze
GabiPD	Genomanalyse im biologischen System Pflanze, Primärdatenbank

Gbp	Giga base pair
gDNA	Genomic deoxyribonucleic acid
GHF5	Glycosyl hydrolase family 5
GISH	Genomic *in situ* hybridisation
GLPL	Glycin Leucin Prolin Leucin
GM	Genetically modified
GSL	Glucosinolate
GSP	Gene specific primers
GSS	Genomic survey sequences
GUS	β-Glucuronidase
HPRT	Hypoxanthine phosphoribosyltransferase
HR	Hypersensitive response
Icd2	Isocitrate dehydrogenase
ICMB	Institute of Clinical Molecular Biology, Kiel, Germany
IGH	Immunoglobulin heavy chain
Indel	Insertion/Deletion
ISR	Induced systemic resistance
IRGSP	International Rice Genome Sequencing Project
J	Juvenile
JA	Jasmonic acid
kbp	Kilo base pair
LB	Lysogeny broth
LCM	Laser capture microdissection
LD	Linkage disequilibrium
LG	Linkage group
LRR	Leucine rich repeat
LysM	Lysine-motifs
µg	Microgram

µl	Microlitre
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAP3K	Mitogen activated protein kinase kinase kinase
MAS	Marker assisted selection
MCR	Major chromosomal rearrangements
Mbp	Mega base pair
ml	Millilitre
min	Minute
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NB-ARC	Nucleotide-binding adaptor shared by APAF-1, R proteins, and CED-4
NBS	Nucleotide-binding site
NCBI	National Center for Biotechnology Information
ng	Nanogram
NGS	Next-generation sequencing
NHEJ	Non-homologous end-joining
NIL	Near isogenic lines
nt	Nucleotides
NTP	Nucleotide triphosphates
OD	Optical density
ORF	Open reading frame
PAC	Bacteriophage P1-derived artificial chromosome
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PD	Petri dishes
PFGE	Pulsed-field gel-electrophoresis

PI	Proteinase inhibitors
PIP	Plasma membrane intrinsic protein
PLACE	Plant Cis Acting Regulatory Elements
Pm21	Powdery mildew 21
PR	Pathogenesis related
PRR	Pattern recognition receptor
Pto	*Pseudomonas syringae* pathovar *tomato*
PTI	PAMP triggered immunity
QTL	Quantitative trait locus
R	Resistance
RGA	Resistance gene analogues
RIL	Recombinant inbred lines
RKN	Root knot nematodes
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA	Ribonucleic acid
RNAi	RNA interference
RPS4	RESISTANCE TO PSEUDOMONAS SYRINGAE 4
RT-PCR	Reverse-transcriptase polymerase chain reaction
RTq-PCR	Real-time quantitative polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SCF	SKP1-CUL1-F-box complex
SCN	Soybean cyst nematode
SERK	SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE
SHMT	Serine hydroxymethyltransferase
SKP1	S-phase kinase-associated protein 1
SNAP	α-synaptosomal-associated protein

SNP	Single nucleotide polymorphism
SOAP	Short oligonucleotide alignment program
SSH	Suppression subtractive hybridization
SSR	Single sequence repeat
TAC	Transformation competent artificial chromosome
TB-RAM	Terrabyte random access memory
TIR	Toll-interleukin-1 receptor
v/v	Volume per volume
WGS	Whole genome shotgun
w/v	Weight per volume
YAC	Yeast artificial chromosome

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1 General Introduction

1.1 Recent Advances in Plant Genomics

Plant breeding focuses on direct needs of farmers and consumers, such as yield, disease resistance, agronomic performance, and product quality. Genetic variation is essential in plant breeding and requires the understanding of genetic architecture for agronomic traits. In recent years, DNA sequencing experienced a major leap forward with the advent of a new technology based on the principle of sequencing by synthesis (see 1.1.1.1) (Ansorge, 2009). This cutting-edge next generation sequencing technology now enables researchers of all molecular biological disciplines to access scientific problems in a new way and to discover genes and their expression at a never-known speed and resolution. Additionally, high throughput and dense genotyping shifted from traditional quantitative trait locus (QTL) mapping to association or linkage disequilibrium (LD) mapping (see 1.3.1). Physical mapping strategies including libraries, e.g. of bacterial artificial chromosomes (BAC) or fosmids (see 1.3.2), are used to establish maps of genomes without a considerable amount of polymorphism. Consequently, analysis of the genome structure, comparison with other genomes, and positional cloning of genes is conceivable. Molecular biological disciplines are now being redefined by an increasing number of reference genomes due to the rapid development in sequencing technologies (Barabaschi et al., 2012; Fleury et al., 2010).

1.1.1 Development of Sequencing Technologies

Frederic Sanger originally developed the first applicable method for DNA sequencing in 1977 (Sanger and Coulson, 1975; Sanger et al., 1977), while the chemical degradation method was established in parallel by Maxam and Gilbert in 1977 (Maxam and Gilbert, 1977). Because of lower technically complexity, fewer amounts of toxic chemicals, and a higher efficiency, Sangers technique became the method of choice. Sanger's Nobel prize awarded chain-termination method bases on a single-strand DNA template, DNA primers, DNA polymerase, and two different types of nucleotide triphosphates (NTPs) (Pettersson et al., 2009).

Simultaneously, the first automated sequencers had been produced in the laboratories of Leroy Hood at the California Institute of Technologie, commercialised by Applied Biosystems (ABI Model 370, Applied Biosystems, Foster City, USA) and Wilhelm Ansorge at the European Molecular Biology Laboratory (EMBL), commercialised by Pharmacia-Amersham (Ansorge et al., 1986; Ansorge et al., 1987; Smith et al., 1986).

Sanger's sequencing method results in maximum read lengths of 1,000-1,200 bases (Schadt et al., 2010; Zhang et al., 2011) after significant improvements, such as the development of fluorescent dye terminators of different colors (Smith et al., 1986), the usage of capillary gel electrophoresis (Luckey et al., 1990), and the sequencing-by-synthesis method (Hyman, 1988). Sequencing-by-synthesis is defined as the detection of every single nucleotide directly after incorporation into the growing DNA strand in a polymerase reaction. Wilhelm Ansorge and others extended the initial version, which uses sequences of one end of each fragment, while sequencing the human hypoxanthine phosphoribosyltransferase (HPRT) by including sequences of both ends (paired-end sequencing). That provides the advantage of obtaining linkage information (Edwards et al., 1990). During the assembly procedure, reads are clustered into long contiguous sequences (contigs), and these contigs were further assembled into scaffolds. Contigs deliver a multiple sequence alignment of reads including the consensus sequence, whereas scaffolds provide the contig order and orientation as well as the size of gaps between the contigs (Miller et al., 2010).

Stephen Anderson (1981) introduced "shotgun" sequencing, which is defined as sequencing of randomly fractured DNA via enzymes, basing on Sanger technology (Anderson, 1981; Sanger et al.,

1982). Another method had been explored by Deininger (1983) who used sonication for DNA fragmentation. Shotgun sequencing can be divided into hierarchical shotgun sequencing and whole genome shotgun (Scheibye-Alsing et al., 2009; Waterston et al., 2002):

- Whole genome shotgun sequencing implies that the DNA is directly broken into individual random fragments and cloned into sequencing vectors. Subsequently, the genome is assembled as a whole.
- The hierarchical shotgun sequencing also referred to as 'map-based', 'BAC-based' or 'clone-by-clone' approach deals with the development and organization of large insert clones that cover a genome in a minimal tiling path. Each clone is shotgun sequenced and the genome sequence can be determined by merging the sequences of the BACs to contigs.

These two different strategies had been explored during the Human Genome Project (HGP), which formally started in October 1990, with the aim to discover all estimated 20,000- 25,000 human genes and the determination of the 3 billion bases that compose human DNA in a five year plan (starting 1990- finishing 2003) (Human Genome Project, 2012). The HGP used the hierarchical shotgun approach, whereas the biotechnology company Celera Genomics, founded by Craig Venter in 1998, decided to use the whole genome shotgun approach (Venter et al., 2001; Weber and Myers, 1997). The limitations of the Sanger sequencing technique became apparent, as only a low number of samples could be analyzed in parallel and there was a need for gels in order to separate the fluorescently-labeled fragments. These limitations and the goals of the Human Genome Project triggered the development of higher throughput sequencing techniques (Ansorge, 2009). The team of H. Kambara developed a high-throughput capillary array DNA sequencer in the Hitachi laboratories, Japan (Hitachi, 2012). ABI (Applied Biosystems, Foster City, USA) and Amersham (acquisition in 2004 by General Electric Healthcare, Buckinghamshire, UK) commercialised automated sequencing with a parallel 384 capillary system. With these improvements, Sanger's technique made sequencing of the human genome possible (Ansorge, 2009).

1.1.1.1 Second and Third Generation Sequencing Technologies

The European Molecular Biology Laboratory (EMBL) presented the first patent application for DNA sequencing without gels and with detection of reversible dye terminators via CCD camera (charge-coupled device) (Ansorge and EMBL, 1991). Parts of the described principles are similar to the next generation techniques used today. An important improvement by the new technologies is the renunciation of vector cloning prior to sequencing (Scheibye-Alsing et al., 2009). The novel massively parallel DNA sequencing systems have substantially reduced the costs per base compared to Sanger sequencing and provide high speed and throughput. Compared to traditional sequencing, the obtained sequence length is reduced to short reads in the range of 35-500 bases (Ansorge, 2009; Shendure and Ji, 2008). Reads are defined as sequences of single letter base calls including a numeric quality value (QV). Prominent features of the new strategies for sequencing can be summarized as follows (Deschamps and Campbell, 2010; Mitra and Church, 1999; Mitra et al., 2003; Shendure and Ji, 2008):

- Random fragmentation of the DNA via nebulization or sonication
- Universal adapter ligation to both ends of the DNA fragments
- Immobilization of the adapter-linked fragments to arrays
- Amplification of the fragments to generate PCR colonies
- Cyclic-array-sequencing: sequencing of dense arrays of DNA fragments by repeating cycles of enzymatic manipulation and image-based data collection

'Second generation sequencing' is used in reference to the delineation above. These technical features had been recently realized in different commercial sequencing platforms, such as (i) 454 GenomeSequencer FLX (Roche Applied Science, Mannheim, Germany), (ii) Illumina Genome Analyzer and HiSeq 2000 (Illumina, San Diego, USA), (iii) Applied Biosystems SOLiD™ Sequencer

(Applied Biosystems, Foster City, USA), (iv) HeliScope (Helicos BioSciences, Cambridge,USA), and (v) Ion Torrent (Life Technologies, Guilford, USA). In the following, the Illumina/ Solexa Genome Analyser platform is described in detail, as this technique has been used within this thesis.

The Illumina/Solexa Genome Analyzer is the most widely used system today and came to the market as the second sequencing platform in 2006 (Metzker, 2010). Single-stranded adapter-ligated DNA fragments hybridize to a solid support that is densely coated with adapters and complementary adapters. Binding of DNA fragments with their free ends to the complementary adapter creates the Illumina specific 'bridges'. The adapters act as primers for the following amplification to generate clusters of 1000 copies of single-stranded DNA fragments, termed DNA 'polonies'. Sequencing-by-synthesis is performed with reversible terminator nucleotides differently labeled for the four DNA bases. A specific DNA polymerase enzyme incorporating them is directly supplied onto the surface. A CCD camera detects the position on the support surface and the incorporation into the DNA strand via fluorescent dyes. Several wash -and scan cycles are required to simultaneously obtain the sequence of at least 40 million polonies (Ansorge, 2009; Deschamps and Campbell, 2010; Turcatti et al., 2008).

To date, the Illumina HiSeq 2000 produces single reads of 2 x 100 bp (paired-end reads). Paired-end reads are generated by sequencing with a forward and a reverse primer on a 400-500 bp fragment. In one run, about 200 Gbp of reads can be produced and the raw base accuracy is > 99.5% (Zhang et al., 2011). The limiting factor of the Illumina sequencing system is the read length which is dependent of multiple factors, such as incomplete cleavage of fluorescent labels or terminating moieties. Substitutions are the dominant error types (Shendure and Ji, 2008).

The HeliScope (Helicos BioSciences Corporation, Cambridge, USA) instrument is the first commercially available sequencing platform close to the boundaries of third generation sequencing approaches because it bases on single-molecule sequencing (SMS) technology, which had been proposed by Keller and co-workers in 1989 (Jett et al., 1989). To date, in SMS technique, a highly sensitive fluorescence detection system is used for the incorporation of single DNA molecules via sequencing by synthesis (Gupta, 2008; Magi et al., 2010). The present third generation sequencing technologies, promising higher throughputs and longer reads, are based on improvements of the SMS method. One approach, termed single-molecule real-time sequencing (SMRT™), developed by Pacific Biosciences (Menlo Park, USA), directly determines the incorporation of fluorescently labeled nucleotides by single DNA polymerase molecules in real time (Eid et al., 2009). As the phosphate chain is cleaved the fluorescence is emitted and the nucleotide is incorporated (Deschamps and Campbell, 2010; Schadt et al., 2010; Zhang et al., 2011). A promising approach to third generation sequencing is based on fluorescent resonant energy transfer (FRET) developed by VisiGen Biotechnologies (Houston, USA) (Ansorge, 2009). Some companies use Nano technologies for single-molecule sequencing (Oxford Nanopore Technologies, Oxford, UK; Electronic Biosciences, San Diego, USA; Roche Applied Science, Mannheim, Germany) (Branton et al., 2008; Iqbal et al., 2007). Before Sanger sequencing had been established, electron microscopy was the first attempt to sequence DNA and was re-evaluated by LightSpeed Genomics (Santa Clara, USA), ZS Genetics (North Reading, USA), and Halcyon Molecular (Redwood City, USA) (Zhang et al., 2011).

1.1.1.2 Impact on Plant Genomics

The genome of the model species *Arabidopsis thaliana* had been the first plant genome sequenced (Arabidopsis Genome Initiative, 2000) and is today the best investigated and assembled genome in terms of completeness. Currently, the assembled size is 119,146,348 bp sequence of an estimated total size of 125 Mbp (The Arabidopsis Information Resource TAIR, 2012). Rice (*Oryza sativa* ssp. *japonica* Nipponbare) has been the second plant genome sequenced (International Rice Genome Sequencing Project, 2005) and, to date, nearly reached a quality level similar to the *A. thaliana* genome. Table 1 is modified according to Feuillet (2011) and lists the current status of assembled genomes of economically important crops and the model species *A. thaliana*.

Table 1: Current status of genome sequencing projects of economically important crops (yearly production larger than 130 million tons) and the model species *A. thaliana* (Feuillet et al., 2011)

Species name	Size of genome Mbp	Sequencing strategy	Sequence assembly size Mbp (%)	Number of genes	References
Arabidopsis thaliana	125	Sanger: BAC[a], Phage, TAC[b] libraries	119.146 (95.3)	33,602	(Arabidopsis Genome Initiative, 2000); (The Arabidopsis Information Network TAIR, 2010)
Oryza sativa	430	Sanger: BAC, PAC[c] libraries	372.080 (95.6)	55,986	(International Rice Genome Sequencing Project, 2005; Rice Genome Annotation Project, 2011)
Zea mays	2500	WGS[d] BAC, Fosmids	2,150 (93.5)	32,690	(Schnable et al., 2009); (Maize Genome Database, 2012)
Triticum aestivum	17,000	BAC, WGS of chromosomes, 454 GS FLX	NA[e]	320,000[f]	(Kumar et al., 2011; Paux et al., 2006)
Hordeum vulgare	5,100	BAC, WGS of chromosomes Illumina, 454 GS FLX	NA	30,670	(Mayer et al., 2011)
Glycine max	1,115	WGS ABI3730XL, BAC	950 (85.2)	66,430	(Schmutz et al., 2010)
Solanum tuberosum	844	BAC, WGS Illumina GAII, 454 GS FLX	725 (85.9)	39,031	(The Potato Genome Sequencing Consortium, 2011)
Cassava	760	WGS, Roche 454	533 (70.1)	34,151	(Prochnik et al., 2012)
Elaeis guineensis	1,800	WGS, Sanger	1,688 (93.8)	NA	(Zieler et al., 2010)

[a]BAC = bacterial artificial chromosome; [b]TAC= transformation competent artificial chromosome; [c]PAC= bacteriophage P1-derived artificial chromosome; [d]WGS = whole genome shotgun; [e]NA = No information available; [f]Estimated number of genes.

Improved assembly algorithms enabled WGS sequencing for recent plant genome sequencing projects in which genomic DNA is the basis for producing sequencing libraries (Feuillet et al., 2011; Jaillon et al., 2007; Mayer et al., 2011; Paterson et al., 2010; Prochnik et al., 2012; Schmutz et al., 2010; Schnable et al., 2009; Tuskan et al., 2006). A reduction of sequencing costs over 10,000 times during the past ten years supports a routine practice of DNA sequencing (Collins, 2010).

Nevertheless, a genome sequence is still in demand for many organisms, as they are phylogenetically too distant from a sequenced relative to benefit from re-sequencing strategies. These re-sequencing approaches map short reads against a reference sequence, usually to a complete genome sequence. The quality and relatedness of the reference sequence has a great impact on the quality of the re-sequenced genome (Imelfort and Edwards, 2009). Benefits of re-sequencing projects are detection of single-nucleotide polymorphisms (SNPs) or other genetic or structural variations between individuals (Cao et al., 2011; Lam et al., 2010; Schnable et al., 2009).

In contrast to re-sequencing, the strategy of *de novo* sequencing is defined as the assembly of short reads without a reference genome. Sequencing and *de novo* assembly of larger genomes becomes more difficult as the content of repetitive sequences increases. Schnable et al. (2009) analyzed the maize genome and showed, that apart from 32,000 genes, 85% of the genome consists of hundreds of transposable element (TE) families, whereas only 10% of TEs were reported for the *A. thaliana*

genome. To create high-quality *de novo* assemblies and increase the probability of capturing the maximum information, sequencing data from different sequencing systems are mixed. An example is the sequencing of the rice genome. The International Rice Genome Sequencing Project, IRGSP, (International Rice Genome Sequencing Project, 2005) started in 1998 to sequence *O. sativa* ssp. *Japonica* Nipponbare by a hierarchical clone-by-clone approach. To date, the assembled sequence covers 95.6% of the estimated 430 Mbp rice genome (Rice Genome Annotation Project, 2011). At the same time, three other groups produced draft sequences of rice. Monsanto produced a draft of ~ 259 Mbp of Nipponbare by a BAC sequencing approach (Barry, 2001), whereas the Beijing Genome Institute (BGI) (Yu et al., 2002), and Syngenta (Goff et al., 2002) used whole-genome strategies to present sequences of two genomes, the *O. sativa* ssp. *japonica* Nipponbare and the *O. sativa* ssp. *indica* line 93-11 genome. The WGS assemblies suffer from misassemblies because of the challenge to place repeats correctly. However, an overview of the genome structure is provided and the integration into the IRGSP analysis contributed to a highly accurate map-based sequence (Feuillet et al., 2011; International Rice Genome Sequencing Project, 2005).

For years, the impact of improved sequencing technologies on plant genome research increases since years in different ways. One example is the different number of determined quantitative trait loci (QTL) from the sequenced crop rice and wheat, whose genome sequencing project had been started much later in 2005 (International Wheat Genome Sequencing Consortium, 2012). Since the release of the draft genome sequence of rice in 2000, the number of cloned QTLs has risen from two in 2000 to 18 in 2007, which correlates with the increasing completion of the genome assembly, whereas the number of cloned QTLs in wheat stagnates (Feuillet et al., 2011), as there currently is no wheat genome sequence available. Identification of (QTL) for disease resistance, flowering time, and abiotic stress were accelerated by the release of high quality genome sequences for rice and *A. thaliana* (Century et al., 2008; Yamamoto et al., 2009; Zhang et al., 2004). Map-based cloning of QTLs involved in agronomically important traits had been the first outcome of a molecular marker analysis based on the rice genome sequence (Yamamoto et al., 2009). A sequenced genome provides the advantage of much faster results in projects that would take eight to ten years in the absence of a genome sequence (Feuillet et al., 2011). *In silico* analysis of genome sequences yields advantages in speed and costs for gene identification, for example by using sequences with functional or structural orthologous for closely related or model species (Nelson et al., 2007).

Furthermore, NGS technologies are also used for whole-transcriptome sequencing. The transcriptome is the entire set of transcripts of a cell, a plant organ or whole plants for a definite developmental stage or physiological circumstance. Previously, the knowledge of the transcriptome had been limited by gene prediction and analysis of expressed sequence tags (EST). The recently developed RNA-Seq method for transcriptome profiling uses deep-sequencing technologies (Wang et al., 2009). The basis for the RNA-Seq technology is the assumption that the coverage depth of a sequence is related to the expression level of the corresponding gene of interest. RNA-Seq is a complex next generation application. Compared to microarrays, the complete detection of gene expression using RNA-Seq provides a better quantitative and qualitative insight and allows the detection of expression levels, differential splicing, allele-specific expression, and micro RNAs (miRNA). Recently, the discovery of the evolution of meiosis and their transcriptional complexity in *A. thaliana* has been achieved using RNA-Seq (Chen et al., 2010a; Yang et al., 2011).

Conclusively, a single genome does not reflect the total genomic complement of a species. In consequence, the pan-genome model recently introduced for plants by Morgante (2007) becomes more and more important. The pan-genome is defined as the total gene repertoire in a given species. It includes the core genomic characteristics equal to all individuals and separately non-shared DNA elements that can be individual or population specific.

1.2 The Sugar Beet Crop

Sugar beet, *Beta vulgaris* L. ssp. *vulgaris*, is a biennial dicotyledonous plant that produces a large taproot with a sugar content of 15 – 20% (Märländer et al., 2011) and a leaf rosette during its first growing season. In the second year, the nutrients are used for the formation of reproductive organs induced by long day conditions after exposure to low temperatures, called vernalization. Sugar beet is the only source for sugar production in Europe and next to sugar cane (*Saccharum officianarum* L.) the largest source from which sucrose can be economically produced (Biancardi et al., 2010; Joersbo, 2007). The cultivation of sugar beet is distributed among 38 countries worldwide and in Germany ~389,000 ha sugar beets were cultivated in 2011 (Statista, 2012; Zucker Statistik, 2011). Furthermore, the importance of sugar beet for bioenergy production is growing. In 2011, ~26.000 ha were cultivated for the production of bioethanol, and ~3,800 ha are cultivated for biogas production (Kleffmann, 2011).

Taxonomically, sugar beet belongs to the family of the *Amaranthaceae*, the subfamily *Chenopodiaceae* and therein to the genus *Beta*. The progenitor of all domesticated beet species is the wild sea beet *B. vulgaris* ssp. *maritima* which could be confirmed by molecular data, e.g. DNA fingerprinting using RFLP analysis (Jung et al., 1993; Letschert et al., 1994). Kadereit (2006), proposed a new taxonomy of the genus *Beta* which became official (Germplasm Resources Information Network, USDA) in 2009. Cultivated beets are included into section Beta, which forms the primary gene pool. The secondary gene pool is composed of the section Corollinea. The species *B. nana*, which is endemic to Greece and the only member of the section Nanae was included to the section Corollinea (Kadereit et al., 2006). The genus *Patellifolia* belongs to the tertiary gene pool. Letschert (1994) described each section as more difficult to hybridize with *B. vulgaris*.

The cultivation of sugar beet started about 200 years ago. Andreas Sigismund Marggraf had been the first who extracted sucrose from sugar beet in 1747. His scholar Franz Carl Achard started with mass selection on fodder beet which led to the first sugar beet variety “weiße schlesische Rübe” (white Silesian beet) (Biancardi, 2005). The discovery of cytoplasmic male sterility (CMS) and genetic monogermity were two milestones in sugar beet breeding (Owen, 1945; Savitsky, 1952). Today, the main breeding work concerns an increase of beet yield and the extractable sugar yield per hectare and year. Of main importance for root yield in sugar beet, a strong selection against premature bolting and therefore the control of bolting and flowering are important breeding objectives. Further breeding objectives are winter hardiness, tolerance to cold, heat, and drought as well as resistance to biotic stress (Biancardi et al., 2010; Kirchhoff et al., 2012; Ober et al., 2004). Important yield reducing diseases and pathogens of the sugar beet crop are the fungi *Rhizoctonia solani*, *Cercospora beticola*, *Erisyphe betae*, and *Ramularia beticola*, the rhizomania virus (beet nectrotic yellow vein virus) and the beet cyst nematode (BCN) *Heterodera schachtii* (Biancardi et al., 2010)

1.2.1 Organization of the *Beta* Genome

The DNA content of *B. vulgaris* had been estimated to be 714 to 758 Mbp per haploid genome (n = x = 9), and the nine chromosomes are morphologically similar in the mitotic metaphase. The chromosomes have an average length of 2.5µm and can be divided by the location of their centromere into 7 metacentric chromosomes and 2 sub-metacentric chromosomes (Arumuganathan and Earle, 1991; Bennett and Smith, 1976; Bosemark, 1969; Bosemark and Bormotov, 1971; Nakamura et al., 1992). On chromosome 1, a terminal constriction carries the major cluster of 18S-5.8S-25S ribosomal RNA genes. The 5S ribosomal RNA genes have been genetically and cytologically located near the centromere on chromosome 4 (Schmidt et al., 1994; Schondelmaier et al., 1997).

The analysis of trisomics is a basic cytogenetic tool Schondelmaier and Jung (1997) used the primary trisomic series of (1964) to standardize the chromosome nomenclature by defining molecular, isozyme, and morphological markers. Schmidt and Heslop-Harrison (1998) presented the chromosome

structure after determining a defined structure for each chromosome, including functional centromeres and telomeres, and families of highly repetitive sequences adjacent to centromeric and telomeric regions.

Flavell (1974) suggested a total of 63% highly repetitive DNA sequences in the sugar beet genome. This fraction consists of numerous families of short (140 to 160 nt) repeating units present at high copy numbers of 10^5 - 10^6 copies per genome (Schmidt and Heslop-Harrison, 1996) and various classes of transposable element-like sequences (Schmidt et al., 1995; Staginnus et al., 2001). Numerous dispersed and tandemly repeated sequences have been localized along chromosomes by the use of different techniques, such as fluorescence *in situ* hybridization (FISH), Southern blotting, pulsed-field gel electrophoresis of large restriction fragments and sequencing of PCR products (Dechyeva and Schmidt, 2006; Dechyeva et al., 2003; Gindullis et al., 2001b; Jacobs et al., 2004; Schmidt et al., 1991; Schmidt et al., 1995; Schmidt et al., 1998; Staginnus et al., 2001). An advantage for the characterization of interspecific hybrids is the high diversity of repetitive sequences among the *Beta* genomes, especially between sections (Desel et al., 2002). Telomeric DNA of *A. thaliana* ($TTTAGGG_n$ repeats) had been cloned and analyzed (Richards and Ausubel, 1988). This Arabidopsis-type telomere repeat is present in most angiosperms (McKnight and Shippen, 2004) as well as in sugar beet (Schmidt, 1998).

1.3 Genomic Research in Sugar Beet

1.3.1 Genetic Mapping

A classical mapping effort with morphological markers in sugar beet had been the investigation of the inheritance of two color genes. Gene *R* confers a red phenotype, and gene *Y* confers a yellow root phenotype, located within a linkage distance of 7.5cM (Keller, 1936). Abegg (1936) showed a linkage of this group with the bolting gene *B* in a genetic distance of 15cM from *R*, resulting in a linkage group of *Y-R-B*. Recently, the highly expressed cytochrome P450 (*CYP76AD1*) gene has been identified by transcriptome analysis using next-generation sequencing and had been mapped to the *R* locus. In 7.5cM distance, a MYB type transcription factor has been determined and mapped to the *Y* locus, verifying the classical mapping data (Hatlestad, 2012). Wagner (1992) discovered a rough linkage of ca. 27 cM between the monogerm seed locus *m* and the stem fasciation character *fas*. The examination of only a limited number of morphological markers in sugar beet is due to recessive alleles, which are hidden in open pollinated populations (McGrath et al., 2007a). Isozyme markers are generally independent from environmental conditions, and therefore investigated for linkage to morphological markers in sugar beet. Analysis of the trisomic series, based on Butterfass (1964) resulted in the identification of five isozyme loci located on four chromosomes. For example, an association between isocitrate dehydrogenase (*Icd2*) and the color locus *R* had been revealed (Smed et al., 1989; Wagner et al., 1992; Abe et al., 1992; Pillen et al., 1993).

The applications of molecular markers range from improvement of plant varieties by marker-assisted selection and localization of genes for the construction of genetic maps. The first genetic map introduced by Pillen (1993) included 2 morphological markers, 7 isozyme markers, and 168 DNA based restriction fragment length polymorphism (RFLP) markers. The development of randomly amplified polymorphic DNA (RAPD) markers, amplified fragment length polymorphisms (AFLP), simple sequence repeats (SSR), and single nucleotide polymorphisms (SNP) increased the marker density. Several genetic maps were established with the amount of markers ranging from 85 to 524. The genetic distance over all nine linkage groups varies between 526.3 cM and 1057 cM (Barzen et al., 1992; Barzen et al., 1995; Hallden et al., 1996; Hansen et al., 1999; Laurent et al., 2007; McGrath et al., 2007b; Nilsson et al., 1997; Pillen et al., 1993; Pillen et al., 1992; Rae et al., 2000; Schneider et al., 2007; Schondelmaier et al., 1996; Schumacher et al., 1997; Uphoff and Wricke, 1995). The most frequently used marker types in plant breeding context are microsatellites and SSR markers. Schneider et al. (2007) presented a genetic map based on SNP markers of 524 loci covering 664.3 cM, McGrath

et al. (2007b) used 308 AFLP and 23 SSR markers spanning 526.3 cM, and Laurent et al. (2007) constructed a map that contains 284 EST-based SSR markers and spans over 555 cM. Recently, Lange et al. (2010) investigated a novel approach for high-throughput development of genetic markers. Using a 15 K Agilent micro array that contained 146,554 custom made oligonucleotides of sugar beet, their approach resulted in mapping of 511 dominant markers: 392 BAC-end sequences and 119 EST based markers.

The development of linkage disequilibrium (LD) mapping had been a milestone for genetic mapping. Association analysis, also referred to as LD mapping or association mapping, is a population-based study to identify trait-marker relationships based on LD (Flint-Garcia et al., 2003). Association mapping helped to identify quantitative traits of economic importance in *B. vulgaris*, such as sugar content and beet yield (Stich et al., 2008a; Stich et al., 2008b). Würschum et al. (2011) employed an association mapping approach using a very large population of 924 elite sugar beets and 677 SNP markers, covering the entire genome in order to analyze the genetic architecture of six agronomical important traits.

Breeding programs in general strongly select against annual behavior resulting in a reduction of sugar yield. Therefore, the cultivated sugar beets are grown as biennials. In *B. vulgaris*, early bolting without a requirement for vernalization (annuality) is under the control of a single dominant Mendelian factor termed *B*, also referred to as the 'bolting gene' (Abegg, 1936). The bolting gene had been genetically assigned to chromosome 2by RFLP- and high resolution AFLP-mapping (Boudry et al., 1994; El-Mezawy et al., 2002). To map the bolting locus *B*, the first genome-wide association study in plants had been investigated in *B. vulgaris ssp. maritima* (Hansen et al., 2001). Bulked segregant analysis and BAC library screening identified sequence-based markers which either flank the *B* locus or completely co-segregate with *B* (Gaafar et al., 2005). Two recent studies determined previously unknown loci that further contribute to annual bolting in beets beyond the *B* gene. One locus, termed *B2*, could be mapped to chromosome 9and is suggested to act epistatically to *B* (Abou-Elwafa et al., 2011; Büttner et al., 2010). Furthermore, Abou-Elwafa et al. (2011) showed that the *B4* locus acts independently to *B* and mapped it in a genetic distance of 11cM to *B* on chromosome 2.

Weber et al. (2000) identified QTLs involved in traits related to sugar production, but most of the analyzed loci were unstable between populations and environments. ESTs predicted to function in carbohydrate and nitrogen metabolism map to seven QTLs (Schneider et al., 2002). Until today, four QTLs were detected being stable in different environments: root and corrected sugar yield on chromosome 4, sucrose content on chromosome 9, and potassium level on chromosome 2 (Biancardi et al., 2010).

The rhizomania disease causes sugar yield losses of up to 80%. It is triggered by the beet necrotic yellow vein virus (BNYVV), which is inoculated into sugar beet roots by the soil-borne fungus *Polymyxa betae* (McGrann et al., 2009). Three sources of resistance exist, with one of them, identified in a commercial hybrid of Holly Hybrids, Tracy CA, being regarded as the basis for resistant sugar beet hybrid varieties dominating the market for the last 15 years. The ’Holly–resistance‘ is qualitative and conditioned by a single dominant gene called *Rz1* (Lewellen et al., 1987; Scholten et al., 1996). Lewellen (1995) identified a second resistance locus, designated as *Rz2*, in the WB42 accession, during screening trials with a sea beet (*B. vulgaris* ssp. *maritima*) collection (Scholten et al., 1999; Scholten et al., 1997). Based on a combined use of AFLP, SNP, and RAPD markers, QTL analysis in sugar beet identified five loci for rhizomania resistance up to now: *Rz1*, *Rz2*, *Rz3*, *Rz4*, and *Rz5* (Gidner et al., 2005; Grimmer et al., 2008; Grimmer et al., 2007; Scholten et al., 1999). According to the work of Lein et al. (2007), who mapped four resistance gene analogues (RGAs) as molecular markers, the five major genes for rhizomania resistance seem to reside at two distinct loci on chromosome 3 (Pavli et al., 2011). Research to fine-map and finally clone the *Rz2* locus is in progress at the Plant Breeding Institute in Kiel (Plant Breeding Institute Kiel, 2012).

1.3.2 Physical Mapping and Genomic Libraries

For the purpose of gene discovery and positional cloning, physical maps of the target regions are essential. Several types of physical maps exist based on different genotypes and cloning vectors. Yeast artificial chromosomes (YAC) were the first cloning vectors used in the sugar beet libraries of Eyers et al. (1992), Klein-Lankhorst et al. (1994), and Kleine et al. (1995) (Table 2). Kleine et al. (1995) established a YAC library of the sugar beet translocation line A906001 for the investigation of nematode resistance derived from the wild relative *Patellifolia procumbens.* Using this library, a YAC could be identified bearing the nematode resistance gene $Hs1^{pro-1}$ which could be cloned by Cai et al. (1997).

Due to the instability of the YAC vectors, BACs had been introduced, and several libraries were established with the purpose of cloning flowering genes and bolting genes, nematode resistance genes, apomixes genes, and CMS restorer genes (Fang et al., 2004; Gindullis et al., 2001a; Hagihara et al., 2005a; Hohmann et al., 2003; McGrath et al., 2004; Schulte et al., 2006). Hohmann et al. (2003) constructed a physical map of the bolting gene region of a BAC library containing 57,600 clones with an average insert size of 116 kbp. In order to clone the *Rf1* gene that controls fertility restoration in cases of cytoplasmic male sterility caused by the Owen cytoplasm, Hagihara et al. (2005a; 2005b) screened a BAC library of 32,180 clones with an average insert size of 97.8 kbp with five AFLP markers closely linked to *Rf1*. This resulted in the identification of eight BACs clustered to two contigs (Hagihara et al., 2005a; Hagihara et al., 2005b)

Table 2: Overview of published genomic libraries of *B. vulgaris*

Cloning vector	Genotype	Number of clones	Average insert size	Genome coverage	Reference
YAC	Ar+	15,000	140	2.7	Eyers et al. (1992)
YAC	AN5-203b	20,000	140	3.2	Klein-Lankhorst et al. (1994)
YAC	A906001	13,000	130	2.2	Kleine et al. (1995)
BAC	Pro1	50,304	125	8.0	Gindullis et al. (2001a)
BAC	KWS2320	57,600	116	8.8	Hohmann et al. (2003)
BAC	US H20	36,864	120	5.7	McGrath et al. (2004)
BAC	M14	49,920	127	7.5	Fang et al. (2004)
BAC	NK198	32,180	98	3.4	Hagihara et al.(2005a)
BAC	TR520	61,056	97	6.8	Schulte et al. (2006)
Fosmid	KWS2320	115,200	39	5.7	Lange et al. (2008)
BAC	PAT2	36,096	120	5.6	Jacobs et al. (2009)

Jacobs et al. (2009) generated a BAC library of a monosomic addition line PAT2 in order to investigate the large-scale organization of the centromere. Lange et al. (2008) established a fosmid library of the DH line KWS2320 as a part of the GABI BEETSEQ-project (Genomanalyse im Biologischen System Pflanze GABI, 2012) for sequencing of the sugar beet genome. Recently, Dohm et al. (2012) presented a physical map of *B. vulgaris* that includes 535 contigs, anchored to the chromosomes, 8,361 probes, and 22,815 BACs. By gene-order comparison of the physical map to rosid species, the authors could identify syntenic regions involving 1,400-2,700 genes in the sequenced genomes of *A. thaliana*, poplar, grapevine, and cocoa (Dohm et al., 2012). Paesold et al. (2012) published a reference FISH karyotype for *B. vulgaris*. They used a set of 18 physically anchored BACs to discriminate all nine chromosomes by FISH. In order to find markers for the identification of homoeologous chromosomes of wild *Beta* species *(B. vulgaris* ssp. *maritima*, *B. patula*, *B. Corolliflora*, *P. procumbens*) FISH experiments revealed a conserved position of a BAC marker on chromosome 1 homoeologous in all four wild species. Because of a different distribution of repetitive elements on the chromosomes a multicolor FISH procedure that allows the identification of all nine sugar beet chromosomes in one hybridazation step with a pool of satellite DNA probes was established (Paesold et al., 2012).

1.3.3 Proteomics and Transcriptomics in Sugar Beet

Proteomics is a rather recent research area and its objective is the investigation of the entity of all proteins isolated from tissues, individual cells, or cell compartments, at individual time points, at different stages of development or under specific conditions (Samaj and Thelen, 2007). Proteomic research in sugar beet includes studies with regard to drought stress, salt stress, and the development of biomarkers for seed vigor (Catusse et al., 2008; Catusse et al., 2011; Hajheidari et al., 2005; Wakeel et al., 2011).

Transcriptomics or expression profiling is also proven to be a valuable tool for studying the beet genome. Cai et al. (1997) generated a cDNA library of sugar beet roots of the nematode resistant translocation line A906001 to clone the resistance gene $Hs1^{pro\text{-}1}$. Herwig et al. (2002) published cDNA

libraries of leaves, storage roots, developing roots, and inflorescences. Of these ESTs, 315 were mapped in the genetic map of Schneider et al. (2007). For the identification of candidate genes related to root development and carbohydrate metabolism, macroarrays from two cDNA collections of sugar beet were generated (Bellin et al., 2007; Bellin et al., 2002). Concerning disease resistance studies, Takenaka et al. (2006) used a PCR-based cDNA library subtraction and identified five highly expressed genes in response to *Pytium oligandrum* infection. Furthermore, changes of gene expression profiles between compatible and incompatible interactions of sugar beet and the soil-borne fungus *Rhizoctonia solani* were explored using cDNA-AFLP (Nagendran and McGrath, 2006). Menkhaus (2006) created a suppression subtractive hybridization (SSH) library for the BCN (*H. schachtii*) resistant sugar beet translocation line (line 940043) subtracted by the susceptible sugar beet (line 93161p) to identify the genes involved in resistance reactions towards the nematode. Differential screening of a sugar beet cDNA library performed with anther tissues of various stages had been investigated by Matsuhira et al. (2007). Dry mature and germinating seeds under different environments were used to generate 2,784 ESTs, representing 2,251 'unigenes'. The analyses revealed different expression pattern of 157 genes in response to stress (Pestsova et al., 2008). Microarrays offer the advantage of simultaneous detection and amplification of multiple targets.

1.3.4 Sequence Resources for Sugar Beet

After publication of the *A. thaliana* mitochondrial genome (Unseld et al., 1997), the mitochondrial genome of sugar beet had been sequenced. It sums up to 368,799 bp, with a GC content of 43.9%, and it encodes 59 recognizable genes (Kubo et al., 2000). The *B. vulgaris* chloroplast sequence of 149, 696 bp had been submitted to GenBank in 2007 (EF534108).

Since 2004 the sugar beet genome mapping and sequencing consortium (Genomanalyse im Biologischen System Pflanze GABI, 2012) started working with the aim to generate a *de novo* assembly of the sugar beet genome. As sugar beet is not closely related to any eudicot plant species with existing or upcoming genome sequence, essential new information on plant genome evolution will be provided. Several sequence resources for assembly of the sugar beet genome are presently employed: whole genome shotgun single read data (454, Roche); 1 kbp, 3 kbp, and 20 kbp paired end data (454, Roche); 2.5 kbp and 4.5 kbp paired end data (Illumina/Solexa); transcript data for mRNA and small RNA (454 Roche, Illumina/Solexa); and end sequences from fosmids and BACs (Sanger sequencing). Annotations already yielded approximately 28,000 gene models (Genomanalyse im Biologischen System Pflanze GABI, 2012; Weisshaar et al., 2011). Recently, a pre-publication draft of the sugar beet genome sequence became publicly available (The Sugar Beet Resource, 2012). This RefBeet-0.9 version consists of 590 Mbp, including 82,305 assembled sequences. Furthermore, the sugar beet wild relative *P. procumbens* had been sequenced using the Illumina platform and the reads had been *de novo* assembled using SOAP (Li et al., 2008a; H. Himmelbauer, personal communication).

Several databases contain and save sequence information of different species. There are three main databases listed in Table 3 storing sugar beet specific sequences: NCBI (National Center for Biotechnology Information (NCBI)), TIGR (The Institute for Genomic Research TIGR, 2012), and the GABI primary databse (Genomanalyse im Biologischen System Pflanze Primärdatenbank GABIPD, 2012). Each database contains only a fraction of all publicly available sugar beet sequences, and some sequences are present in all databases.

Table 3: Sugar beet sequence types in the NCBI, TIGR, and GABI databases

Database	No. of sequences	Type of sequences
NCBI	30,234	EST
	67,235	Genome survey sequences
	2,970	Proteins
	2,735	Nucleotides
TIGR	25,834	ESTs
GABI Primary Database	33,316	BAC ends
	2,894	EST
	11	Cosmid
	200,511	Fosmid

1.4 Plant Response to Pathogens

Plants are acting as hosts for many pathogens and are involved in a daily struggle against them. They have evolved two strategies to counteract pathogens: PAMP triggered immunity (PTI) and effector triggered immunity (ETI). PTI, formerly known as basal resistance, is an ancient and conserved defense mechanism that is based on expression of pattern recognition receptors (PRR) on the plant side to recognize microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Chisholm et al., 2006). Pre- and post-invasive responses, elicited by M/PAMPs, belong to this immunity system. Macroscopic symptoms are usually not associated with PTI (Dodds and Rathjen, 2010; Göhre and Robatzek, 2008; Postel and Kemmerling, 2009).

M/PAMPs are defined as being essential to microorganisms, structurally conserved through whole classes of microbes, and not present in the host. The best studied bacterial PAMP peptide, bacterial flagellin flg22, is recognized by the first identified PRR, the flagellin receptor FLS2. Other bacterial PAMPs are lipopolysaccharides and peptidoglycans, whereas chitin belongs to the fungal PAMPs. Glycan is present in nematodes secretory proteins and act as PAMPs in the animal's pathosystem, but until now, no epitope had been identified acting as a PAMP in plant innate immune response. Danger-associated molecular patterns (DAMPs) are endogenous signal molecules encoded by the host and released in case of plant damage, e.g. by pathogen invasion (Jones et al., 2011; Perry and Wharton, 2011).

PRRs are commonly located on the plasma membrane, but they can also be found localized on endosomal compartments or in the cytoplasm (Nürnberger et al., 2004). Stimulation of PRRs leads to the activation of signaling cascades involving Ca^{2+} fluxes and mitogen activated protein kinases (MAPKs), which in turn give raise to defense reactions. These reactions include the production of reactive oxygen species (ROS), medium alkalinization, deposition of callose at the cell walls, or expression of pathogenesis related (PR) proteins (Göhre and Robatzek, 2008; van Loon et al., 2006). PRRs can be grouped into two classes: receptor-like kinases (RLKs), carrying a serine/threonine kinase domain, and receptor-like proteins (RLPs), which have an intracellular cytoplasmic tail. Characteristic for PRRs are leucine-rich-repeats (LRRs) or lysine-motifs (LysM) as extracellular domains. In *A. thaliana*, 610 members of the receptor-like kinase family are present whereas the receptor-like protein gene family consists of only 57 members (Wang et al., 2008).

Successful pathogens that endured the first layer of defense, inject effector molecules, also called avirulence (Avr) proteins suppressing defense responses during perception, signaling or defense action. On the host side, R-genes encode proteins, which mostly consist of a nucleotide-binding site (NBS) and an LRR motif for effector detection (DeYoung and Innes, 2006; Göhre and Robatzek, 2008). This plant defense response, elicited by effector recognition, is the second mechanism of

pathogen detection and induces effector-triggered immunity (ETI), classically known as gene-for-gene resistance introduced by Flor (1942). ETI relies on co-evolutionary dynamics between the plant and the pathogen, which is quite different from PTI. Additionally, ETI response often involves a form of programmed cell death (hypersensitive response, HR) and is qualitatively stronger and faster than PTI (Dodds and Rathjen, 2010).

Bacterial effectors are injected through the bacterial type II or type III secretion system, while fungi and oomycetes release their effectors via exocytosis. Suppression of PTI-induced host genes is a target of those effector molecules. An example is the expression of *NOH1*, which is required for basal resistance against *Pseudomonas* bacteria upon recognition of flagellin. Subsequently, virulent pathogens elicit effector molecules as HopS1, HopC1 or AvrPto in order to preclude the transcriptional induction of *NOH1* and suppress PTI (Göhre and Robatzek, 2008; Li et al., 2005).

1.4.1 Plant–Nematode Interaction

Plant parasitic nematodes comprise about 15% of the phylum Nematoda and are responsible for global agricultural losses of $ 157 billion annually (Abad et al., 2008). All agriculturally important crops, such as soybean, rice, maize, wheat, potato, tomato, and beet, are hosts for one or more species of nematodes. The largest and economically most significant groups of plant parasitic nematodes are comprised in the order Tylenchida as the sedentary endoparasitic nematodes of the genera *Meloidogyne*, *Heterodera* and *Globodera* as well as the migratory endoparasitic nematodes of the genus *Pratylenchus* (Abad and Williamson, 2010; Fuller et al., 2008).

The sophisticated relationship of host and pathogen starts by root diffusates from the host via indirect stimulation of the hatch of invasive J2 larvae of the egg. Root knot and cyst nematodes penetrate the plant directly behind the root cap. Invasion and migration is hindered by physical barriers of the cell wall, consisting of a cellulose framework surrounded by a cross-linked matrix of hemicellulose and pectin. Nematodes are capable to secrete a wide repertoire of cell wall degrading enzymes produced in their pharyngeal glands. Cellulases or endo-1,4-ß-glucanases are able to hydrolyse the glycosidic bonds by degrading cellulose (Davis et al., 2011). Most of the studied cellulases in sedentary nematodes belong to the glycosyl hydrolase family 5 (GHF5) (De Ley and Blaxter, 2002). Hemicellulose can be degraded either by specific endoxylanases as identified in *Meloidogyne* or by the GHF5 endoglucanases (Abad et al., 2008; Gao et al., 2004; Mitreva-Dautova et al., 2006). Pectin decomposition by phytopathogenic nematodes is due to two types of depolymerases: pectate lyase and polygalacturonase (de Boer et al., 2002; Doyle and Lambert, 2002; Jaubert et al., 2002; Popeijus et al., 2000). Especially in cyst nematodes, an arabinogalactan endo-1,4-ß-galactosidase had been identified and is assumed to hydrolyse ß-1,4-galactan into pectin (Vanholme et al., 2009).

Cyst nematode J2 larvae migrate intracellularly and initiate the formation of a feeding site called syncytium (Grundler et al., 1998). The root knot nematode (RKN) J2 larvae migrate intercellularly into the vascular cylinder and become sessile within the cortical tissue in the zone of differentiation for initiating their feeding sites, called giant cells (Wyss et al., 1992).

1.4.1.1 Plant Resistance Genes against Plant Parasitic Nematodes

Incompatible interactions occur through recognition of effectors, delivered into the host cells, and an immediate activation of resistance response, mediated by R-genes. Until now, eleven major plant R-genes conferring resistance against nematodes (further referred to as nematode R-genes) have been cloned, and several others have been mapped. All cloned nematode R-genes have a leucine rich repeat (LRR) region, which is typically involved in protein-protein interactions. The LRR motif may play a role in pathogen recognition and signal transduction. Nine out of eleven genes have a NBS motif in common, which binds or hydrolyses adenosintriphosphate (ATP). Conformational changes lead to signaling in response to elicitor recognition. The N-termini of plant NBS-LRR proteins can be divided

into subgroups of coiled-coil (CC) or toll-interleucin receptors (TIR). NBS proteins are present in all angiosperms analyzed to date but differences exist between monocot and dicot species. More than half of the NBS-encoding genes evaluated in *A. thaliana* encode TIR-domains. This subgroup appears to be absent in cereal species (Abad and Williamson, 2010; Meyers et al., 2003; Monosi et al., 2004; Zhou et al., 2004).

Table 4: List of published plant parasitic nematode resistance genes.

Gene	Species of Origin	Nematode	Protein Structure	Reference
Hs1^{pro-1}	*Patellifolia procumbens*	*Heterodera schachtii*	N-terminal extra-cellular LRR[a], TM[b]	Cai et al. (1997)
Mi-1.2	*Solanum peruvianum*	*Meloidogyne incognita, M. javanica, M. arenaria*	CC[c]-NBS[d]- LRR	Milligan et al. (1998); Vos et al. (1998)
Gpa2	*Solanum tuberosum* ssp. *Andigena*	*Globodera pallida*	CC-NBS-LRR	van der Vossen et al. (2000)
Hero	*Solanum pimpinellifolium*	*G. pallida, G. rostochiensis*	CC-NBS-LRR	Ernst et al. (2002)
Rhg4	*Glycine max*	*H. glycines*	TIR[e]-NBS-LRR; SHMT[f]	Lightfoot and Meksem, (2002); Liu et al. (2012b)
Gro1-4	*Solanum spegazzinii*	*G. rostochiensis*	TIR-NBS-LRR	Paal et al. (2004)
rhg1	*Glycine max*	*H. glycines*	TM-LRR; CNV[g]	Ruben et al. (2006), Cook et al. (2012)
Mi-9	*Solanum arcanum*	*M. incognita*	CC-NBS- LRR	Jablonska et al. (2007)
CaMi	*Capsicum annuum*	*M. incognita*	CC-NBS- LRR	Chen et al. (2007)
XiR1	*Vitis arizonica*	*Xiphinema index*	non-TIR-NBS-LRR	Hwang et al. (2010)
Ma	*Prunus cerasifera*	*M. incognita, M. arenaria, M. javanica*	TIR-NBS-LRR	Claverie et al. (2011)

[a]'LRR' leucine-rich-repeat; [b]'TM' transmembrane-domain; [c]'CC' coiled-coil; [d]'NBS' nucleotide binding site; [e]'TIR' toll-interleukin-receptor; [f]serine hydroxymethyltransferase; [g]CNV=copy number variants of a 31 kbp fragment (details see 4.6)

The best-known and -analyzed nematode resistance gene is *Mi-1.2* from tomato, encoding for a protein, which belongs to the largest class of R-proteins with a central NBS domain and C-terminal LRR domains. The *Mi* gene had been isolated by positional cloning and was introduced into cultivated tomatoes from the wild relative *S. peruvianum*. This gene does not only confer resistance to three species of the RKN, but also to the white fly *Bemisia tabaci* (Nombela et al., 2003), the potato aphid *Macrosiphum euphorbia* (Rossi et al., 1998), and to the tomato psyllid *Bactericerca cockerelli* (Casteel et al., 2006). A hypersensitive response is triggered before the feeding site is significantly initiated. If soil temperatures exceed 28°C the resistance reaction mediated by *Mi-1.2* is inactive, whereas *Mi-9*,

ensures resistance at soil temperatures of up to 32°C (Ammati et al., 1986). The *Mi-9* gene is a homolog of *Mi-1.2* and was mapped to the short arm of chromosome 6 in the tomato wild relative *S. arcanum* (formerly *S. peruvianum*) in close proximity to *Mi-1.2*. *Mi-9* confers resistance to *Mi-1.2* avirulent forms of *M. arenaria, M. incognita,* and *M. javanica* but not to *Mi-1.2* virulent nematodes (Ammiraju et al., 2003; Jablonska et al., 2007; Veremis and Roberts, 2000)

The first cloned cyst nematode resistance gene was obtained from the wild beet *P. procumbens*. The $Hs1^{pro-1}$ gene had been positionally cloned from a translocation of *P. procumbens* chromosome 1 attached to the end of chromosome 9 of *B. vulgaris* (A906001). The resulting protein is 282 amino acids (aa) in size and contains a putative N-terminal extracellular LRR-region, and a transmembrane domain (Cai et al., 1997). The $Hs1^{pro-1}$ gene does not have obvious similarities to other cloned resistance genes, since the majority of cloned R-genes encode proteins that carry a NBS-domain (Abad and Williamson, 2010). $Hs1^{pro-1}$ is a single-copy gene on the translocation and conferred complete resistance to *H. schachtii* in a complementation study using sugar beet hairy roots. After six weeks of inoculation, stagnating females had been observed in transgenic hairy root clones similar to the females' phenotype on the resistant line A906001 (Cai et al., 1997). In transformed sugar beet plants, only partial resistance had been determined (D. Cai and C. Jung, unpublished data).

The cyst nematode resistance gene *Gpa2* confers resistance in potato against G*lobodera pallida*. It had been positionally cloned by van der Vossen et al. (2000), and its protein shares an overall identity of > 88% to *Rx1*, which mediates resistance against potato virus X (van der Voort et al., 1999). The sequence divergence between the two genes is mainly concentrated in the LRR-region and the C-terminal domain, whereas the effector domains are structurally more conserved. Common structures in virus and nematode resistance cascades are assumed (Danchin, 2011; van der Vossen et al., 2000). Paal et al. (2004) cloned the R-gene *Gro1-4*, which confers resistance to *G. rostochiensis* in potato using a candidate gene approach. *Gro1-4* is a member of a gene family comprising nine homologs and belongs to the second NBS-LRR subfamily because of its N-terminal TIR domain. With a sequence identity of 38%, the *Gro1-4* gene is more closely related to the *Nicotiana benthamiana N*-gene providing resistance to the tobacco mosaic virus than to any other nematode resistance gene (Paal et al., 2004).

Not only potatoes, but also tomatoes are susceptible to potato cyst nematodes. In this regard, Ernst et al. (2002) identified the *Hero* gene in tomato by map-based cloning on chromosome TIV. The gene is effective against the potato cyst nematodes *G. pallida* and *G. rostochiensis*. The functional analysis had been performed by complementation of two susceptible tomato lines. Sequence analysis revealed that *Hero* is a member of a gene family of 14 highly homologous genes, clustered in a 118 kbp region. Upon an active resistance reaction in tomato mediated by *Hero*, an abnormal development of the nematode´s syncytia is followed by a hypersensitive response of the surrounding plant cells, resulting in an isolation of the syncytium (Ernst et al., 2002; Sobczak et al., 2005).

In soybean, resistance to cyst nematodes is conferred by a combination of recessive (*rhg*) and dominant (*Rhg*) genes. Analyses of two major QTLs (*rhg1* and *Rhg4*) in different germplasm indicate the effect of additional genes affecting resistance and epistatic inheritance. Receptor-like kinase sequences were patented as candidate genes for both loci but to date, functional evidence is missing (Jones et al., 2011; Kandoth et al., 2011; Wu et al., 2009). Recently, Afzal et al. (2012) inferred that the *rhg1* locus might be a multi-gene resistance locus. Three genes located within the co-segregating marker-bound region of *rhg1* were identified with sequence similarities to a receptor-like kinase, a laccase and a predicted sodium/ hydrogen antiporter (Ruben et al., 2006). Recently, Cook et al. (2012) identified one copy of a 31-kbp fragment, carrying genes encoding an amino acid transporter, an α-synaptosomal-associated protein, and a wound-inducible domain 12 protein, per haploid genome in susceptible soybean varieties, and detected 10 tandem copies in the resistant *rhg1-b* haplotype (see 4.6). At the *Rhg4* locus, a leucine-rich repeat receptor-like kinase (LRR-RLK) was identified by Liu et al. (2011).Using targeted induced local lesions in genomes (TILLING), a mutant containing a

nonsense mutation in the LRR domain of the candidate resistance protein was identified but the SCN-resistant phenotype of the mutant was not altered. Hence, the authors conclude that resistance in soybean to *H. glycines* is independent of the LRR-RLK at the *Rhg4* locus (Liu et al., 2011). However, recently, Liu et al. (2012b) reported about the positional cloning of *Rhg 4* gene from recombinant inbred lines (RIL). They determined the expression of a serine hydroxymethyltransferase (*SHMT*) within syncytia and verified the proposed function by transformation of a susceptible RIL with a 5.1 kbp genomic fragment of *SHMT* including the gene specific promoter, and by the application of RNAi in hairy roots. Moreover, they suggest a role for *SHMT* to trigger the hypersensitive-response-like programmed cell death of developing feeding sites (Liu et al., 2012b).

In different plant species, several other loci are described to be responsible for resistance to cyst nematodes, especially of the genus *Heterodera*. In wheat and barley, *H. avenae* is the most widely distributed cyst nematode and causes severe yield losses. The genes *Cre1* and *Cre8* originate from hexaploid wheat, whereas *Cre2*, *Cre3*, *Cre4*, *Cre5*, *Cre6*, and *Cre7* originate from Aegilops species (Delibes et al., 1993; Eastwood et al., 1994; Eastwood et al., 1991; Jahier et al., 2001; Ogbonnaya et al., 2001; Romero et al., 1998; Williams, 2003; Williams et al., 1994). These genes are mapped and confer partial resistance to *H. avenae* depending on the nematode pathotype. Introgression into cultivated wheat had been possible via chromosome addition or substitution lines. For example, the *Cre3* locus had been identified by a candidate gene approach using NBS-LRR coding sequences and is efficient against the Australian pathotype of *H. avenae* in reducing the number of cysts. In barley, the *Ha2* locus confers resistance to *H. avenae* and is collinear to the *Cre3*/*Cre1* locus in wheat (Kretschmer et al., 1997). *Ha4* is an additional resistance locus which maps to chromosome 5H of the barley genome (Barr et al., 1998). Furthermore, *H. sacchari* also belongs to the cyst nematodes and harms sugarcane and rice. In rice, resistance has been found in the African rice species *O. glaberrima* (Lorieux et al., 2003).

An important resource for the identification of genes for nematode resistance activated in plants is comparative expression analyses between infected and uninfected plants. Klink et al. (2007) used laser capture microdissection (LCM) and comparative microarray expression analysis to determine gene expression in syncytial cells isolated from susceptible and resistant soybean roots infected by the soybean cyst nematode (SCN; *H. glycines*). They identified both differentially and equally expressed genes in syncytial cells and in the entire roots both in compatible and incompatible reactions. In a subsequent study, Klink et al. (2009) analyzed the gene expression in the second phase of syncytium development. In a resistant reaction the syncytium development fails and in a susceptible, compatible interaction, the syncytium is established. They identified lipoxygenase-9 and lipoxygenase-4 as the most highly induced genes in the compatible reactions. Additionally, the analysis uncovered genes of the phenylpropanoid pathway, e.g. phenylalanine ammonia lyase, chalcone isomerase, isoflavone reductase, cinnamoyl-CoA reductase, and caffeic acid O-methyltransferase. The presence of induced levels of these genes implies the importance of jasmonic acid and phenylpropanoid signalling pathways. Mazarei et al. (2011) analyzed the expression of two genetically related soybean lines, one susceptible and one resistant to SCN race 2. Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) verified the results of microarray analysis. Among the transcripts to which functions could be allocated, genes playing a role in metabolism, cell wall modification, signal transduction, transcription, and defense, were identified (Mazarei et al., 2011).

1.4.1.2 Novel Strategies for the Control of Plant-Parasitic Nematodes

Resistance genes represent an effective resource for nematode control. However, they are often not available for a particular host, and attempts to transfer cloned resistance genes to new hosts showed limited success, as the unsuccessful transfer of the tomato gene *Hero* to potato shows (Sobczak et al., 2005). Consequently, other strategies had been investigated, as the expression of transgenic proteins that are harmful to the nematodes. In this regard, expression of transgenic proteinase inhibitors (PI) is the most widely used approach. Plant proteins with toxic effects exist, which reduce nematode growth

and fertility. The effect of cysteine PIs, defined as cystatins, had been analyzed in tomato hairy roots and *A. thaliana* resulting in the reduced development of the females. Analysis of sugar beet hairy roots expressing sweet potato serine PI sporamin showed inhibited growth and development of *H. schachtii* females (Cai et al., 2003). Genes encoding cytolytic δ-endotoxin (Cry-toxin) proteins from *Bacillus thuringiensis* had been found to exhibit toxicity against nematodes, first demonstrated for the free-living nematode *Caenorhabditis elegans* (Borgonie et al., 1996). Results from successful expression experiments in tomato roots suggested this approach to be rather promising with regard to plant-parasitic nematode control (Li et al., 2008b).

RNA interference (RNAi) can trigger the silencing of target genes by mRNA degradation. Fire and Mello et al. (1998) first described the promising technique in *C. elegans* and had been awarded the Nobel Prize in physiology or medicine in 2006. To date, more than twenty studies of successful RNAi in root knot and cyst nematodes have been published (Jones et al., 2011). In the case of *H. schachtii*, Vanholme et al. (2007) identified a pectate lyase secreted by this nematode in the migratory stage. Post-transcriptional gene silencing against the gene by soaking the nematodes for 24 h in double stranded RNA solution resulted in a reduction of the infectiveness.

Patel et al. (2008) used host-derived RNAi to silence the expression of the Hs4F01 parasitism gene within the nematode. *A. thaliana* plants homozygous for the Hs4F01-RNAi construct had been inoculated with approximately 50-60 J2 larvae of *H. schachtii*. Three weeks post-inoculation, the number of developed females decreased significantly compared to the control plants (Patel et al., 2008). Recently, tobacco plants expressing RNAi constructs of different *M. incognita* parasitism genes, *HSP90*, isocitrate lyase, and three proteinase genes, resulted in a reduction in egg number per root gram 45 days after nematode infection of 28 – 37%, 67 – 76%, and 30 – 40%, respectively (Grossi-de-Sa et al., 2012).

1.4.2 The Beet Cyst Nematode *Heterodera schachtii*

The BCN *H. schachtii* Schmidt is a widespread pest in sugar beet over all cultivating areas in the world: North America, Europe, the Middle East and Australia. *H. schachtii* belongs to the sedentary endoparasitic nematodes, and its life cycle consists of seven stages: an egg stage, four larval stages (J1, J2, J3, and J4), and two adult stages. The endurance to complete their life cycle varies depending on environmental conditions though the typical time is about 30 days and can be reduced in warmer climates (Turner and Rowe, 2006). The hatching process displays the end of dormancy. Specific hatching stimuli turned out by the host root system and an increase in soil temperature induce the hatch of motile J2 larvae. Upon leaving the cyst, the infective J2 larvae invade the root usually directly behind the growing root tip. The larvae use thrusts of their stylet and a set of cell wall degrading enzymes, like cellulases or endo-1,4-ß-glucanases, which are able to hydrolyze the glycosidic bonds by degrading cellulose. In cyst nematodes, an arabinogalactan endo-1,4-ß-galactosidase was identified and is assumed to hydrolyze ß-1,4-galactan in pectin (Vanholme et al., 2009).

Intracellular migration to the pericycle and induction of a specific feeding site, called syncytium, are the next steps in the nematode's life cycle. *H. schachtii* J2 larvae prefer procambial cells as an initial syncytial cell and select pericycle cells if the induction fails (Sobczak et al., 1997). The release of nematode secretions directly into the cytoplasm is followed by widening of the plasmodesmata. Cell wall breakdown occurs between the initial feeding site cell and adjacent cells upon protoplast fusion, forming a syncytium. They may incorporate several hundred cells so that a multinuclear cell complex with high metabolic activity is developed. The number of phloem elements increases around syncytia, suggesting a high nutrient transfer to the feeding structures (Hofmann et al., 2007; Hoth et al., 2005).

Up to this point, there is no difference between male and female larvae. However, the J3 males have longer ingestion periods, although total food consumption is less compared to female larvae. Syncytia formed by male nematodes are less extensive than those of females. The female remains sedentary

after the molt to J4 and the shape of its body becomes spherical. During their molt to the J4 stage, the male nematodes become vermiform again and towards their last molt, they leave the root for copulation. Fertilized eggs (J1) are produced in the female converting to a protective cyst of the dead body (Wyss, 1992).

Symptoms on the host plants upon compatible reactions of the BCN vary depending on developmental stage of the host, season, and soil temperature. Infested plants are usually stunted, the beets are retarded, and a strong growth of the rootlets can be observed (Agrios, 2005).

Resistance to the BCN in *Patellifolia* had been observed for the first time in 1937 (Golden, 1959; Hijner, 1952; Savitsky, 1975). The incompatible reactions have been studied on the cellular level. Hatching stimulance of wild beet and sugar beet has been studied by Holtmann et al. (2000) and both showed comparable levels of volume and efficiency. *H. schachtii* infective J2 larvae are able to invade the roots of *Patellifolia* species. Furthermore, migration to the vascular cylinder to induce their feeding site is feasible for a small portion of nematodes. The syncytia develop abnormally in comparison to the compatible reaction, causing the death of J2/J3 larvae (Holtmann et al., 2000). Occasionally, females develop a transparent appearance due to the absence of eggs. Thus, the nematodes are not able to complete their life cycle (Cai et al., 1997).

Oil radish (*Raphanus sativus* L. var. *oleiferus*) shows complete resistance and is therefore often used as a catch crop to reduce the number of cysts in infested soils. Resistant plants stimulate the hatching of the larvae, which invade the roots, but prevent them to fulfill their life cycle. Budhan et al. (2009) identified a major QTL defined as $Hs1^{Rph}$, that explains 46.4% of the phenotypic variability.

Investigations of nematode resistance present in the genus *Patellifolia* revealed the existence of four *H. schachtii* pathotypes. The pathotype Schach0 is most widely distributed and of economic importance, because it infects susceptible plants from oil radish (*R. sativus* ssp. *oleiformes*), white mustard (*Sinapis alba*), and sugar beet. Sugar beet cultivars carrying the resistance originating of *P. procumbens* chromosome 1 are resistant to Schach0 but are susceptible to pathotype Schach1. This pathotype is able to break the resistance derived from *P. procumbens* chromosome 1 but cannot affect plants with resistance originated from *P. procumbens* chromosome 7. Another pathotype called Schach2 is virulent only to resistance coming from chromosome 7, whereas Schach1.2 is able to overcome the resistance from chromosome 1 and 7 of *P. procumbens* (Lange et al., 1993a; Müller, 1992; Müller, 1998a; Müller, 1998b).

1.4.3 Transfer of Nematode Resistance from *Patellifolia* species to *Beta vulgaris*

P. procumbens and *P. patellaris* belong to the tertiary gene pool of the genus *Beta* and bear resistance against *H. schachtii*. Transferring this resistance to cultivated sugar beets is a laborious work and had only been possible by interspecific hybridization and subsequent backcrosses (Coons, 1975; Heijbroek et al., 1983; Löptien, 1984b; Savitsky, 1975; Speckmann and de Bock, 1982). The first hybrids produced from interspecific crosses suffered from necrosis and generally died at the seedling stage. Additionally, when using diploid *B. vulgaris* as seed parents, the hybrids had been completely sterile. The solution to this problem had been crossings of a tetraploid *B. vulgaris* as seed parent and a diploid *P. procumbens* producing a triploid offspring (2n = 3x = 18+9). However, the disadvantages associated with wild beets crossings is the introduction of several deleterious traits, such as poor growth, beet development, and annual behavior that cause the need for back-crossing. Because of that, the triploid offspring had been backcrossed with diploid sugar beets. The selected plants with one additional extra chromosome, called monosomic addition lines (2n = 18+1), showed the same resistance reaction as the wild beet (Jung and Wricke, 1987; Löptien, 1984a; Savitsky, 1975; Tsuchiya and Nakamura, 1976).

Isozyme analysis of monosomic addition lines identified three chromosomes of *P. procumbens* bearing resistance genes, whereas in *P. patellaris* one chromosome could be determined (Jung et al., 1986; van

Geyt et al., 1988). The genes had been designed according to the chromosome on which they are located. The *Hs1*$^{pro-1}$ gene is located on chromosome 1, *Hs2*$^{pro-7}$ on chromosome 7 and *Hs3*$^{web-8}$ on chromosome 8 of *P. procumbens* (Jung et al., 1986; Kleine et al., 1998; Lange et al., 1993b). In the genome of *P. patellaris* the *Hs*$^{pat-1}$ gene is located on chromosome 1 (Kleine et al., 1998; Yu, 1984). The hypothesis of a second nematode resistance gene (*Hs1-2*) being present on chromosome 1 of *P. procumbens* emerged after evaluation of translocation lines, as described in the following.

Table 5: Nematode resistance genes identified on different wild beet chromosomes of *Patellifolia* species.

Resistance gene	***Patellifolia* species**	**Chromosome**	**Reference**
Hs1^{pro-1}	*P. procumbens*	1	Cai et al. (1997)
Hs1-2	*P. procumbens*	1	Capistrano (2009)
Hs2^{pro-7}	*P. procumbens*	7	Lange et al. (1993b)
Hs3^{web-8}	*P. procumbens*	8	Kleine et al. (1998)
Hs^{pat-1}	*P. patellaris*	1	Kleine et al. (1998); Yu (1984)

Commercial hybrids were introduced to the market with resistance to the BCN derived from *P. procumbens*. The first cultivar licensed was ‘Nematop’ in 1998 (Table 6). Though sugar beet hybrids carrying the *P. procumbens* translocation show a significant yield penalty, breeders have re-evaluated the partial resistance to BCN from *B. vulgaris* ssp. *maritima* of the secondary gene pool of the genus *Beta* (Chen et al., 2008a; Heijbroek, 1977; Panella and Lewellen, 2007). Since the year 1998, four resistant cultivars became available (Table 6). The resistant cultivars show a significant decrease in net sugar yield compared to beets bearing BCN tolerance from *B. maritima*. The cultivation of resistant sugar beets is reasonable as the nematode population in infected fields can be reduced to 60 – 70%. The first cultivar with BCN tolerance, ‘Pauletta’, came to the market in 2005. In the absence of nematodes the BCN tolerant cultivars also show a similar yield to normal sugar beet cultivars (Heinrichs, 2011).

Table 6: Sugar beet cultivars licensed in Germany with resistance or tolerance against *H. schachtii* (Bundessortenamt, 2012)

Cultivar	Year of release	Tolerant/ resistant	Net sugar yield [a](BZE)	Breeder company
Brix	2012	Tolerant	8	Strube GmbH & Co. KG
Finola KWS	2012	Tolerant	9	KWS SAAT AG
Kleist	2012	Tolerant	8	Strube GmbH & Co. KG
Kepler	2011	Tolerant	7	Strube GmbH & Co. KG
Kristallina KWS	2011	Tolerant	8	KWS SAAT AG
Hella	2010	Tolerant	7	Syngenta Crop Protection AG
Kühn	2010	Tolerant	7	Strube GmbH & Co. KG
Nemata	2010	Resistant	4	Syngenta Crop Protection AG
Adrianna KWS	2009	Tolerant	7	KWS SAAT AG
Belladonna KWS	2009	Tolerant	8	KWS SAAT AG
Corvetta KWS	2009	Tolerant	7	KWS SAAT AG
Theresa KWS	2008	Tolerant	7	KWS SAAT AG
Mauricia	2007	Tolerant	6	Syngenta Crop Protection AG
Sanetta	2007	Resistant	3	KWS SAAT AG
Annalisa	2006	Tolerant	[b]NA	KWS SAAT AG
Pauletta	2005	Tolerant	6	KWS SAAT AG
Paulina	2000	Resistant	2	KWS SAAT AG
Nematop	1998	Resistant	2	Syngenta Crop Protection AG

[a]BZE= bereinigter Zuckerertrag = net sugar yield; 1 lowest level to 9 highest level according to Bundessortenamt (2012).
[b] NA =No information available.

1.4.3.1 Nematode Resistant Translocation Lines of *Beta vulgaris*

Among the offspring of monosomic addition lines, plants were identified that carry only a fragment of the wild beet (2n = 18+ fragment) or that possess a translocation derived from a wild beet chromosome. This is due to interspecific crosses and non-homologous recombination between sugar beet and wild beet chromosomes (Jung and Wricke, 1987). Savitsky et al. (1975) described the first diploid *B. vulgaris* genotype with resistance to *H. schachtii* originating from *P. procumbens*. The formation of trivalents, which could be observed in 2 to 5% of the plants, resulted in rare crossing-over events and the generation of translocation lines (De Jong et al., 1985).

Analysis of growth type and isozyme pattern helped to identify three translocation lines. Two lines carry a translocation of *P. procumbens* chromosome 1: A906001 (seed number: 940043) and TR363

(former name: Pro4; seed number: 930363), and one line that carries a translocation derived from *P. procumbens* chromosome 7, defined as Pro3 (seed number 930153). All three translocation lines are resistant against the BCN. Pro3 houses the *Hs2*$^{pro-7}$ gene (Jung and Wricke, 1987). The translocation line A906001 carries two nematode resistance genes, *Hs1*$^{pro-1}$ and *Hs1-2*, whereas TR363 only bears the *Hs1-2* gene. Heller et al. (1996) genetically mapped the location of four translocation lines to the end of chromosome 9 of the sugar beet.

1.4.3.2 Physical Mapping of the Translocation

Due to the lack of recombination between the sugar beet chromosomes and the introgressed wild beet chromosomes, genetic mapping within the translocation is impossible. Therefore, positional cloning of the resistance gene started by physical mapping of the whole translocation. Jung et al. (1992) developed a plasmid library of the fragment addition line Pro1 (Brandes et al., 1987; Jung et al., 1990). Screening the library with *P. procumbens* derived genomic DNA probes resulted in the identification of the marker pRK643 (CAU1757). This marker is present in three copies on the translocation line A906001, but absent from translocation line TR363. The RAPD marker X2.1 had been identified to be closely linked to the nematode resistance gene and had been localized on both translocation lines A906001 and TR363, derived from chromosome 1 of *P. procumbens*, suggesting an overlapping region between both translocation lines (Kleine et al., 1998; Salentijn et al., 1995).

In order to estimate the sizes of the translocations, pulsed field gel electrophoresis (PFGE) combined with Southern blot analysis had been carried out with digested genomic DNA of A906001 and TR363. The size of the A906001 translocation had been estimated as 1,500 kbp via hybridization, using translocation specific probes. Fragment sizes differ between A906001 and TR363, which was determined using the random amplified polymorphic DNA (RAPD) marker X2.1 as probe. This suggests various translocation sizes between A906001 and TR363 (Kleine et al., 1998). Restriction with *Bss*HII, *Mlu*I, and *Sal*I, and subsequent hybridization using the single copy-probe 14b and a probe of YAC 128R revealed an overlapping region between the translocation lines A906001 and TR363, which had been estimated to be 350 kbp (Schulte et al., 2006).

Kleine et al. (1995) established the first physical map using YACs from the translocation line A906001. Screening of the YAC library with the *P. procumbens* specific probes pRK643 and X2.1 resulted in a total of seven YACs belonging to three contigs. Moreover, chromosome walking with two YAC-ends identified two new clones. The identification of the *Hs1*$^{pro-1}$ gene goes back to the screening of a cDNA library established from roots of A906001 with YAC120 and the selection of two cDNA clones, one of which had been identified as the *Hs1*$^{pro-1}$ gene (Cai et al., 1997; Kleine et al., 1995).

Thus, there is evidence for a second nematode resistance gene on the translocation lines A906001 and TR363. Schulte et al. (2006) established a BAC library of the translocation line 000520. This line had been generated by a cross of A906001 with the sugar beet line 930190, resulting in the F1 population 940081. The translocation line 000520 is one of the progenies out of the fourth backcross (BC4) between 940081 and 930190, and it is hemizygous for the translocation. In this thesis, the translocation line 000520 is designated as TR520. As a result of Schulte's physical map analysis, a minimal tiling path of five BACs covering 580 kbp, including 524 kbp shared between the translocation lines TR520 and TR363 and the translocation breakpoint had been presented. Sequence analysis showed that among 133 open reading frames (ORFs), 12 ORFs displayed homology to genes involved in biotic stress resistance reactions or to transcription factors (Schulte et al., 2006).

Refinement of the physical map by Jäger (2007) showed that the translocation breakpoint had not yet been covered. Capistrano (2009) generated a complete revision of the physical map. Finally, after identification of 24 new BACs, a third generation physical map could be established with a minimum tiling path of 17 BACs grouped to 3 contigs, and a coverage of 1,417 kbp of the translocation. Eleven BACs had been sequenced. The analysis of 950,769 bp displayed 11.09% repetitive elements. A total

of 128 sequences with homology to ESTs and genomic sequences showing an average sequence similarity of 66.39% to *B. vulgaris* were identified.

One milestone to narrow down the location of the *Hs1-2* gene had been the development of two translocation lines, which are both susceptible to *H. schachtii*. Gamma irradiation (400 Gy) using seeds of the line 950631, which is essentially the same as A906001, lead to the generation of the two translocation lines termed TR320 and TR659 (H. Harloff, personal communication). Marker analysis using sequences derived by the physically mapped BACs revealed a loss of 766 kbp in the case of the susceptible translocation line TR320, corresponding to BAC contig 1 and 2 of the third generation physical map. The translocation line TR659 shows a deletion of 376 kbp, equivalent to the BACs 149P7, 64A10 and 7C2 of BAC contig 1. It is susceptible in spite of the presence of the $Hs1^{pro-1}$ gene. Altogether, the estimated size of the susceptible translocations TR320 and TR659 are about 734 kbp and 1,124 kbp, respectively (Capistrano, 2009).

1.4.4 Candidate Genes Involved in the Interaction between *Beta vulgaris* and *Heterodera schachtii*

According to the analysis of two susceptible (TR320 and TR659) and two resistant (TR520 and TR363) translocation lines, the location of the nematode resistance gene has been narrowed down to a genomic region present on both resistant translocation lines and absent from both susceptible translocation lines. Capistrano (2009) selected the two overlapping regions between both resistant translocation lines on BAC contig 1 in order to identify candidate sequences for the *Hs1-2* gene. Two BACs are located within these regions. Sequence analysis using the gene prediction programs GENSCAN (GENSCAN, 2009) and FGENESH (Softberry FGENESH, 2007) revealed 22 ORFs on BAC3L6 and 16 ORFs on BAC7C2. No sequences with significant homology to known nematode resistance genes had been identified. The sequences ORF401, with homology to a protease and ORF 702 showing homology to an AVR elicitor response protein and to a ß-1,3-galactosyltransferase (Table 7) had been selected for further analysis. AVR genes and also galactosyltransferases are known to be involved in resistance reactions (Langlois-Meurinne et al., 2005; Schaff et al., 2007). Proteases are involved in many cellular processes such as degradation of proteins and cell growth control. Additionally, proteases appear to play a role in pathogen recognition and induction of defense response (van der Hoorn and Jones, 2004). RT-PCR using inoculated and non-inoculated root material from resistant translocation lines and susceptible controls revealed a constitutive expression of ORF 702 only in the resistant translocation line. The candidate gene ORF 401 is neither expressed in resistant translocation lines nor in susceptible sugar beet lines and therefore ORF 401 has been excluded as a candidate sequence. Determination of the exon-intron structure, cloning, sequence analysis and functional analysis of ORF 702 are objectives of this thesis and are detailed in Research Chapter 2.

Table 7: List of proposed candidate sequences for the *Hs1-2* gene. Their presence (X) or absence (-) on resistant lines (TR520, TR363), susceptible lines (TR659, TR320) and the *B. vulgaris* (Bv) and *P. procumbens* (Pp) genome is shown. Furthermore, the result of functional analyses with sugar beet hairy roots and *A. thaliana* is listed.

Candidate sequence	TR520	TR363	TR659	TR320	Pp	Bv	Sequence description	Functional analysis	Reference
ORF 702	X	X	-	-	X	-	ß-1,3-Galactosyltrans-ferase; Avr9 elicitor response like protein	This work	Capistrano (2009)
BpPIP1	X	-	X	X	X	-	Aquaporin; [a]NPA; membrane embedded helices	[b]Sign. reduced no. of cysts	Menkhaus (2011)
cZR-3	X	X	[c]NA	NA	X	X	[d]NBS-[e]LRR	Sign. reduced no. of cysts	Tian (2003); Knecht (Knecht, 2010)
cZR-7	X	X	NA	NA	X	X	NBS-LRR	Sign. reduced no. of cysts	Tian (2003); (Knecht, 2010)
BvGLP-1	-	-	-	-	-	X	Germin-like protein; Oxalate oxidase	Sign. reduced no. of cysts	(Knecht, 2010)

[a]NPA = asparagine-proline-alanine; [b]Sign. Reduced no. of cysts = significantly reduced number of cysts; [c]NA = No information available; [d]NBS = nucleotide binding site; [e]LRR = leucine-rich-repeat;

It is generally noted that NBS-LRR disease resistance genes are involved in both elicitor recognition and the activation of downstream signaling pathways leading to resistance response (Moffett et al., 2002). Thus, using degenerate primers designed on these motifs had been an approach for the identification of resistance gene analogues (RGA) for nematode resistance genes located on a wild beet translocation. For this, five highly conserved motifs of the NBS domain had been chosen: P-loop, kinase-2, kinase-3a, a hydrophobic domain (Glycin-Leucin-Prolin-Leucin, GLPL) and TIR/non-TIR consensus sequences. The four cloned nematode resistance genes *Cre3*, *Mi*, *Gpa2*, *Hero* and the tomato *Prf*-gene that confers resistance against *Pseudomonas syringae* had been used for amino acid alignment analyses which resulted in the identification of highly conserved regions for each of the five motifs. Amongst 12 expressed RGAs, four full-length sequences had been determined that show NBS-LRR motifs (cZR-1, cZR-3, cZR-7, cZR-9) (Tian et al., 2004). Species-specific primers had been designed according to SNP-analysis between *P. procumbens* and *B. vulgaris*. Primers derived from cZR-3 and cZR-7 amplified with genomic DNA of *P. procumbens*, and the translocation lines TR520, and TR363. No fragment with genomic DNA of *B. vulgaris* had been determined. In contrast, primers designed for cZR-1 and cZR-9 amplified with genomic DNA of the translocation lines TR520 and TR363 and with genomic DNA of sugar beet, but not with *P. procumbens* (Tian, 2003).

Knecht (2010) suggested a *B. vulgaris* germin-like protein, termed as *BvGLP-1*, that encodes for an oxalate oxidase to be involved in the $Hs1^{pro-1}$ -mediated nematode resistance response. The *BvGLP-1* gene was identified by cDNA-AFLP and transcript profiling using the ATH1 Gene chip array (Affimatrix, Santa Clara, CA, USA). *BvGLP-1* encodes for a polypeptide of 208 aa. Expression

analyses in sugar beet hairy roots and *A. thaliana* plants showed significantly reduction of nematode infection.

Menkhaus (2011) identified a gene with high sequence similarity to plant aquaporins, which can be divided into seven subfamilies. The gene belongs to the subfamily of plasma membrane intrinsic proteins (PIP) and was denoted as *BpPIP1*. Its coding sequence is 1,071 bp (285 aa) in size. Transcript analysis of *BpPIP1* revealed constitutive expression in the resistant translocation line TR520 and up-regulation upon nematode infection. Functional analysis was done by overexpression of *BpPIP1* in *A. thaliana* plants and sugar beet hairy roots and a reduction of developed females was determined (Menkhaus, 2011).

1.5 Origin and Features of Chromosomal Translocations

Chromosome rearrangements include deletions, duplications, and inversions of segments and translocations. A translocation is a change in the arrangement of genetic material, altering the location of a chromosome segment. Translocations generate novel chromosomes without normal pairing partners and place genes in a new linkage. Chromosome translocations can result in a balanced or unbalanced manner dependent on loss or duplication of genetic material (Madan, 2012). Two types of translocations exist and the most common form is reciprocal, involving the exchange of two terminal segments between two non-homologous chromosomes (O'Connor, 2008). The American biologist William R. B. Robertson (1916) first described a translocation in grasshoppers. In these Robertsonian translocations (or whole arm translocations) the long arms (q) of two acrocentric chromosomes associate at a single centromere (O'Connor, 2008).

Translocations occur spontaneously or are artificially induced by ionizing radiation, mutagens or by transposons. The first example for a successful X-ray-induced translocation of agronomic importance is the transfer of brown rust (*Puccinia triticina*) resistance from *Aegilops umbellulata* to bread wheat (Sears, 1956). Moreover, a sugar beet translocation line ‘Pro4’ conferring cyst nematode (*H. schachtii*) resistance transferred from *P. procumbens* had been developed after irradiation of an addition line (Jung and Wricke, 1987). Barbara McClintock´s analyses in the 1940s showed that a maize *activator* (*Ac*) element induces the transposition of the non-autonomous *dissociation* (*Ds*) element. Via cytogenetic methods she determined that transposition of the *Ds* element is associated with major chromosomal rearrangements (MCR), including deletions, duplications, inversions, and reciprocal translocation (McClintock, 1950). However, the underlying mechanism has not been identified yet. Recent studies of Zhang (2009) identified the formation of 19 MCR, 17 reciprocal translocations and, 2 large inversions in maize lines containing pairs of closely linked *Ac* transposable elements. Because the breakpoints of the 19 MCRs are specified by *Ac* termini and typical 8-bp target site duplications, a generation of MCRs by precise transposition reactions involving *Ac* termini of two closely linked elements has been suggested (Zhang et al., 2009).

The development of chromosome translocations is also possible by DNA repair mechanisms following upon DNA damage. DNA damage results in double-strand breaks (DSB) where both strands of the double helix are not linked to each other. Spontaneous DSBs may originate from endogenous damage by reactive oxygen species produced from normal metabolic by-products or by replication errors of DNA polymerase (Waterworth et al., 2011). In *in vitro* dividing mammalian cells, 5 – 10% of the cells seem to have at least one chromosome break at any time. DSBs have to be repaired before genomes can be replicated (Lieber, 2010a; Puchta, 2005). Two major DSB repair mechanisms exist: homology-dependent, termed ‘homologous recombination’, and homology-independent ‘non-homologous end-joining’ (NHEJ). Homologous recombination proceeds during late S- or G_2- phase (interphase) of the cell cycle when the sister chromatids are in close vicinity. Restoration of the original sequence is the typical result of homologous recombination, which requires identical or nearly identical sequences as templates but has the potential to copy allelic genes or lead to crossing over. The major and highly conserved pathway NHEJ, which functions throughout the complete cell cycle, rejoins DNA ends by

terminal micro-homology but without the requirement of homologous chromosomes. The process is much better understood in mammals; nonetheless there are three enzymatic steps whose activities are required for repair of DSBs by the NHEJ pathway: (1) nucleases for removal of damaged DNA, (2) polymerases for random addition of nucleotides with the benefit of generating terminal microhomology, and (3) ligases for rebuilding of the phosphodiester backbone (Lieber, 2010b; Lieber and Wilson, 2010).

Both DSB repair pathways are potentially mutagenic. Derivative chromosomes can be generated by joining of homologous and non-homologous chromosomes. Multi-processing steps prior to DNA ligation are required to have compatible termini because DSBs of damaged DNA differ from those induced by endonuclease activities by missing 5' phosphates and 3' hydroxyl groups at the ends required for ligation. Because of several enzymatic steps and imprecise repair mechanisms by nucleases, which remove nucleotides or polymerases that randomly add nucleotides and therefore may generate a micro-homology between two DNS ends, the NHEJ repair pathway causes deletions, insertions, and translocations(Lieber, 2008; Lieber et al., 2010). In *Saccaromyces cerevisiae*, Yu and Gabriel (2004) demonstrated that NHEJ-mediated reciprocal translocations can be created as a consequence of DSB repair. In human research, the group of Michael R. Lieber (Keck School of Medicine University of Southern California, 2012) focuses on proteins involved in the NHEJ pathway and how pathologic gene rearrangements function in the immune system, in cancer, and aging. In plants, NHEJ products have been studied in tobacco by using extra-chromosomal rejoining assays, and an association of deletions ranging from a few bases up to 1 kbp was identified with NHEJ (Gorbunova and Levy, 1997). The use of rare-cutting restriction endonucleases like I-*Sce*I for the induction of site-specific genomic DSBs improves the research in the field of DSB repair mechanisms and Puchta et al. (1993) has been the first to report about the application of I-*Sce*I restriction in tobacco (Puchta, 2005). In *A. thaliana*, the research focuses on collections of T-DNA insertion lines as T-DNA insertions are commonly related with local DNA disturbances including deletions, duplications, addition of filler sequence, and chromosomal translocations (Clark and Krysan, 2010; O'Malley and Ecker, 2010).

1.5.1 The Role of Chromosome Translocations in Humans and Plants

Translocations in humans were first detected by the German cancer geneticist Theodor Boveri in the late nineteenth and early twentieth centuries as novel chromosomes (Balmain, 2001). Numerous disorders, such as mental retardation, infertility and cancer are linked to translocations involving human chromosomes. The balanced reciprocal translocation between chromosomes 8 and 14, termed as t(8;14), was described in patients with Burkitt's lymphoma. In this translocation, the *MYC* proto-oncogene from chromosome 8, which encodes for a transcription factor and regulates the expression of about 15% of all human genes (Gearhart et al., 2007), is placed under the control of the constitutively expressed immunoglobulin heavy chain (*IGH*) promoter on chromosome 14. This leads to an overexpression of *MYC* target genes, many of which play a role in cell proliferation, often resulting in carcinosis (Dalla-Favera et al., 1982). The Robertsonian translocation between chromosomes 14 and 21 generates the derivative chromosome der(14;21) which is observed in patients with familial Down syndrome. These patients have 46 chromosomes including two normal copies of chromosome 21 and a Robertsonian translocation with chromosome 21 (Hanna et al., 1981).

In plants, translocations can be differentiated into (i) species-specific intragenomic, chromosome rearrangements – as it is the case for polyploid wheat, rye, cultivated oat and others (Gale and Devos, 1998; Jellen et al., 1994; Liu et al., 1992; Naranjo and Lacadena, 1982) – and (ii) intergenomic chromosome rearrangements. It is suggested that species with a polyploid nature may tolerate more genome rearrangements as loss of chromosome fragments, chromosome arms, and whole chromosomes (Lu et al., 2005; Pacher et al., 2007). The most frequent intragenomic re-organisation in wheat is the 5B-7B translocation. This can be explained by a high adaptive value, since chromosomes 5B and 7B bear some important genes for controlling plant growth and development, such as

vernalization response (*Vrn1*, *Vrn2*, *Vrn3*), frost resistance (*Fr1*), resistance to *Septoria* leaf blotch (*Stb1*, *Stb8*), and others (Schlegel, 1996). In wheat, several intergenomic translocation lines exist that bear the advantage of transferring resistance to numerous pathogens. The Robertsonian translocation involving one arm of chromosome 1R from rye and the long arm of chromosome 1B from wheat have been successfully incorporated in many hexaploid wheat cultivars with the benefit of resistance to leaf stem and stripe rusts (*Lr26,Sr31, Yr9*) as well as to powdery mildew (*Pm8*). Recently, Zou et al. (2011) identified a wheat-barley 2A/2H translocation line that houses the *Isa* gene, which has an effect on improved grain hardness, protein composition, and starch quality. A novel Robertsonian translocation event was analyzed by Qi et al. (2011) between bread wheat and *Dasypyrumvillosum* transferring the stem rust resistance gene *Sr52*. In sugar beet, the introgression of a reciprocal translocation from *P. procumbens*, belonging to the tertiary gene pool of the genus *Beta*, confers resistance against the *H. schachtii* (Cai et al., 1997).

1.5.2 Microscopic and Molecular Analysis of Chromosome Translocation

The karyotype describes the number and the appearance of chromosomes in the nucleus and staining is one method to visualize it. G-banding is defined as staining of partially trypsin restricted chromosomes with Giemsa (Giemsa, 1904), which is specific for DNA phosphate groups resulting in dark-stained heterochromatic (AT-rich) and light-stained euchromatic (GC-rich) regions. C-banding is determined as staining of constitutive heterochromatin which can be found at centromeres (Gill et al., 1991). Several research groups used these techniques to identify unusual banding patterns on chromosomes as translocations (Forsstrom et al., 2002; Qi et al., 2008; Qi et al., 2011).

The field of molecular cytogenetics involves *in situ* hybridization analysis, which had first been reported by Gall and Pardue (1969). Genomic *in situ* hybridization (GISH) is characterized by using total genomic DNA as a probe, whereas fluorescence *in situ* hybridization (FISH) uses chemical coupling of fluorochromes and small DNA probes to hybridize with denatured target DNA (Friebe et al., 2005). In a nematode resistant sugar beet line, FISH was used to identify the chromosomal location of the *P. procumbens* translocation (Desel et al., 2001). Several modifications of FISH exist. Using fibre FISH, the chromosomes were stretched in interphase on a slide, rather than being coiled as in conventional FISH (Heng et al., 1992). Spectral karyotyping (SKY), defined as multicolor staining, was used to analyze human chromosome translocations by Abdel-Rahman et al. (2001). Recently, Shah et al. (2011) improved the technique for fluorescent *in situ* hybridization analysis by a special preparation on metaphase chromosomes.

Marthe and Künzel (1994) used Giemsa (1904) banding patterns of somatic metaphase chromosomes to locate breakpoints in barley. The application of this method is limited since most of the Giemsa stained bands are situated close to the centromere. In rye, translocation breakpoints involving chromosome 1R had been localized by staining of large telomeric C-bands only from chromosome 1R (Catarino et al., 2006). Bhat et al. (2007) reported about mapping of translocation breakpoints in the wheat-rye translocation 1B1R by array hybridization. In 2010, a paired-end sequencing strategy had been applied to the human genome. Flow sorting of chromosomes had been used in advance to the sequencing process and the reads were aligned to reference sequences of the corresponding chromosomes. The selection of reads mapping to both chromosomes unraveled the breakpoint-spanning region (Chen et al., 2010b). Information of typical characteristics on translocation breakpoints in plants are rare, but Molnar et al. (2011) analyzed the chromosomal distribution of microsatellite clusters in relation to the intergenomic translocations in the allotetraploids *Aegilops biuncialis* and *A. geniculata.* They mapped translocation breakpoints to SSR-rich chromosomal regions, suggesting that microsatellite sequences may facilitate the formation of chromosomal rearrangements. In human, the analysis of disease associated balanced chromosome rearrangements revealed repeats of the MER1 (meiotic recombination 1) and MER2 type, and short interspersed elements (SINE) in the breakpoint region.

Furthermore, deletions of 3 bp and 18 bp as well as a 9 bp insertion had been identified (Chen et al., 2008b). Additionally, Gajecka (2008) identified additions of nucleotides, deletions, and duplications at the breakpoints in reciprocal translocations in human but no breakpoint specific sequence motifs were determined. Initially, breakpoints had been identified by "array-painting"(Howarth et al., 2007). Isolating chromosomes by flow cytometry and subsequent hybridization to custom oligonucleotide arrays lead to the identification of a 46 kbp duplication at a reciprocal translocation breakpoint of chromosome 4 and 6, t(4;6) of human breast cancer cell lines present on both translocation products. The duplications were confirmed via FISH, PCR on isolated chromosomes, and subsequent cloning of breakpoints. Moreover, on the reciprocal translocation between chromosomes 12 and 22 a duplication of 199 kbp on chromosome 12 and a duplication of 156 kbp on chromosome 22 were determined by Howarth et al. (2011).

1.6 Objectives and Hypothesis

The hypothesis of this work is that the nematode resistance gene *Hs1-2* against the sugar beet cyst nematode *H. schachtii* is located on the overlapping region shared only by resistant sugar beet translocation lines TR520 and TR363. Both carry a translocation attached to the end of chromosome 9 derived from wild beet relative *P. procumbens*. The objectives of this work are as follows:

1. Cloning and functional analysis of the *Hs1-2* candidate sequence ORF 702
2. Sequencing of the translocation lines TR520 and TR363 by whole genome shotgun sequencing
3. Identification of the translocation breakpoint of the translocation line TR520
4. Sequence comparison of the translocations TR520 and TR363 for the identification of overlapping regions on the *P. procumbens* translocation
5. Sequence analysis of the shared *P. procumbens* sequences between the translocation lines TR520 and TR363 for the identification of candidate genes for *Hs1-2* and
6. Expression analysis of *Hs1-2* candidate genes

2 Functional Analysis of ORF 702 as a Candidate for the Beet Cyst Nematode Resistance Gene *Hs1-2*

2.1 Introduction

In order to identify the nematode resistance gene *Hs1-2*, which is located on a wild beet translocation of *Patellifolia procumbens* attached to chromosome 9 of *Beta vulgaris* and mediates resistance to *Heterodera schachtii*, Schulte et al. (2006) generated a BAC-based physical map of the *P. procumbens* translocation of the line TR520. After Capistrano (2009) refined the map, it contained 17 BACs grouped to three contigs. Comparative marker analysis of two resistant translocation lines, TR520 and TR363, and two susceptible lines lead to the definition of sections on the physical map. A BAC contig can be formed by one or more sections. The only two sections present on the resistant lines TR520 and TR363 and absent from the susceptible lines TR659 and TR320, named A with 102 kb and C with 81 kb in size, correspond to BACs 7C2 and 3L6, respectively (Figure 1). Among 13 predicted open reading frames (ORF) in this region, ORF 702 was selected for further analysis and proposed to be a candidate sequence for the *Hs1-2* gene.

When I started my work, the following data were available from the doctoral thesis of Capistrano (2009):

The location of ORF 702 was determined to be on BAC 7C2, which belongs to BAC contig 1 in section A on the BAC based physical map Capistrano (2009) (see Figure 1).This section is shared by the translocation lines TR520 and TR363. The exon-intron structure of ORF 702, determined by the software GENSCAN (GENSCAN, 2009), resulted in seven exons and six introns according to a total length of 9,664 bp. The predicted exon sizes sum up to 1,113 bp, with an expected polypeptide of 370 amino acids (aa). Sequence similarity analysis to other proteins from known organisms was performed using the non-redundant protein database of the NCBI platform (National Center for Biotechnology Information NCBI, 2009). The overall sequence of ORF 702 shows 33% similarity to an Avr9 elicitor response protein from *A. thaliana*. A transmembrane domain was predicted between 39 aa and 61 aa using the TMHMM server for prediction of transmembrane helices (TMHMM Server, 2012). Additionally, a galactosyltransferase domain was predicted between amino acids 121 and 316 according to the Conserved Domain Database of NCBI (National Center for Biotechnology Information (NCBI), 2012). Expression analyses using cDNA of nematode infected and non-infected roots of resistant translocation lines resulted in a similar expression levels in both probes.

In summary, the following lines of evidence indicated that ORF 702 could have a function as the nematode resistance gene *Hs1-2*. First, the gene is located on a region shared between the resistant translocation lines TR520 and TR363 and absent from the susceptible translocation lines TR659 and TR320. Second, the gene is expressed in roots of resistant sugar beet translocation lines. Third, the sequence homology to an AVR elicitor response protein and a galactosyltransferase suggests an involvement of ORF 702 in nematode resistance. AVR genes are known to be involved in the gene-for-gene resistance reaction and the involvement of glycosylation during plant-pathogen interaction has already been suggested by earlier studies (Langlois-Meurinne et al., 2005; Sanseverino et al., 2010; Schaff et al., 2007)

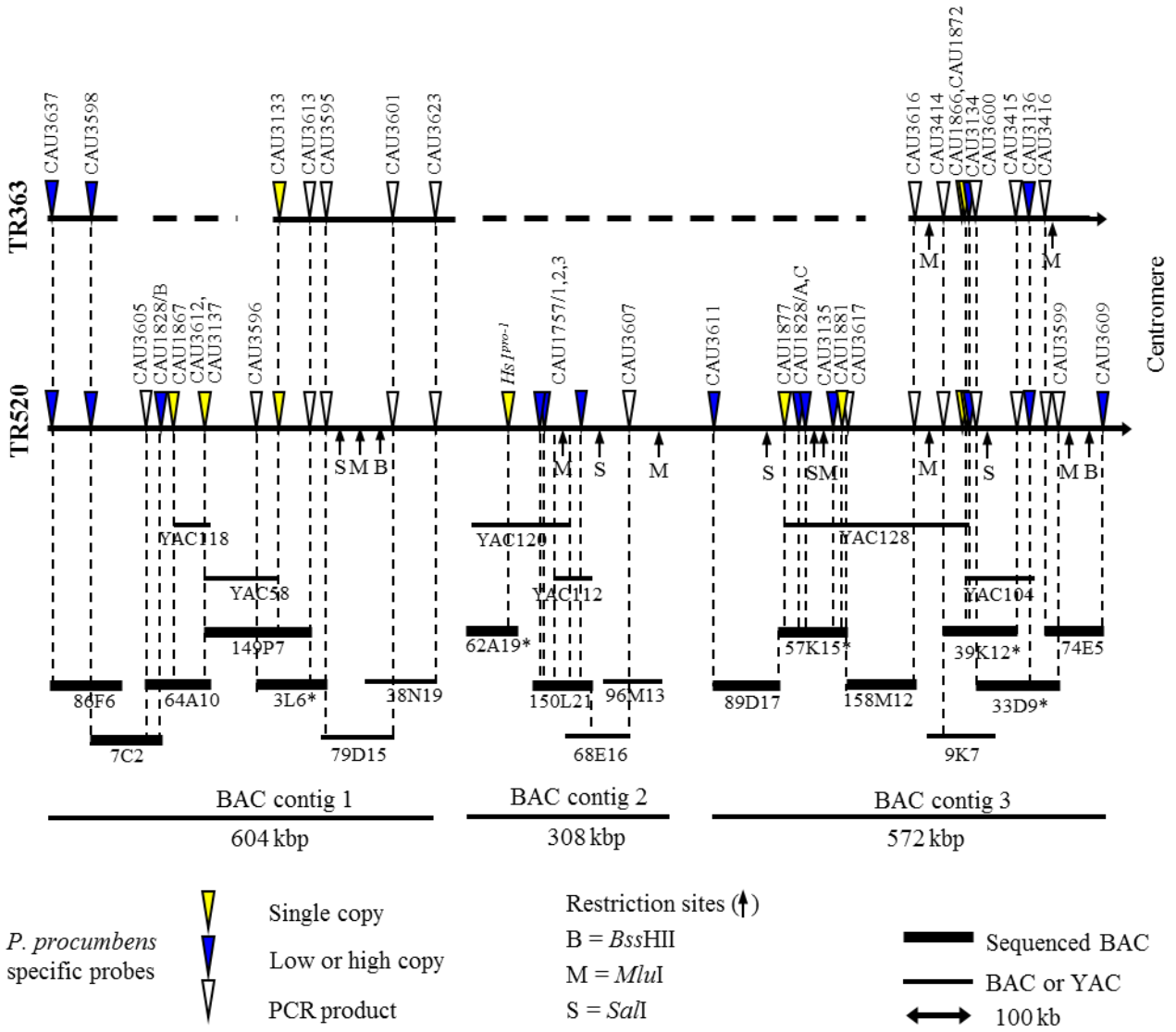

Figure 1: A BAC based physical map of the *P. procumbens* translocation of line TR520 modified after Capistrano (2009). The two *P. procumbens* translocations of the lines TR520 and TR363 are shown. Probes are indicated by a triangle: yellow for single copy markers, blue for low or high copy markers and white for PCR products. YAC = Yeast Artificial Chromosome. BAC = Bacterial Artificial Chromosome. The restriction sites are from B = *Bss*HII, M = *Mlu*I and S = *Sal*I. The map only shows the minimal tiling path of BACs *: BAC present on the second-generation physical map by Schulte et al. (2006).

The purpose of this work package was the functional analysis of the candidate sequence ORF 702. Therefore, the sugar beet hairy root system, based on *Agrobacterium rhizogenes*-mediated transformation, was used. *A. rhizogenes* bacteria transfer T-DNA segments from their root-inducing (Ri) plasmids into the genome of infected plant cells. Following expression of the root locus (rol) genes, auxin and cytokinin biosynthesis result in the production of adventitious roots, defined as hairy root (Skaracis and Pavli, 2010). Additionally, the model plant *A. thaliana*, which is a host of the beet cyst nematode, was used on *A. tumefaciens*-mediated transformation and *in vitro* nematode resistance tests.

2.2 Material and Methods

2.2.1 Gene Structure Analysis of ORF 702 using Rapid Amplification of cDNA Ends (RACE®)

The full-length sequence of ORF 702 was identified using the GeneRacer® Kit (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. A total of four gene specific primers (GSPs) for the 5'-UTR and three GSPs for the 3'-UTR, and four nested primers were designed to amplify the cDNA (Table 8).

Table 8: Primers used for rapid amplification of cDNA ends (RACE) analysis of ORF 702

Gene specific primers	
Primers 5'UTR	Sequence
G844	5' CTGGACGCGACCACAGCGGAAGA 3'
G872	5'GGCGATCAAGTGAATTCCCCCCATT 3'
G874	5'CGTTTTGTATGGAGGGCTAGCATGA 3'
G881	5' CCTGTTATTCCTGGACGTAGCCAAT 3'
Primers 3'UTR	Sequence
G842	5' CACACACTTCCCGCCCTCCCTTA 3'
G876	5' ATGGCTGAACTCAACCAGGAAGTGAA 3'
G882	5' GCTTCATTGGCTACGTCCAGGAATAA 3'
Nested gene specific primers	
Nested primers 5'UTR	Sequence
G845	5' ACAGCGGAAGATGGGAGTGGATTGG 3'
G875	5' GGCTAAACCAGTAGCTTGCTCCAATC 3'
Nested primers 3' UTR	Sequence
G843	5' GCCCTCCCTTACCCTTAATCGTCTTT 3'
G877	5' AGATCATGCTAGCCCTCCATACAA 3'
Vector specific primers	
Sp6_G500	5' AGCTATGACCATGATTACGCC 3'
T7_G501	5' TAATACGACTCACTATAGGG 3'

To obtain specific RACE products, touchdown PCR was performed (see Table 9). The touchdown PCR products were used as templates to perform nested PCR with one amplification step at 65 °C and 30 cycles with the nested primers.

Table 9: Touchdown PCR program used in RACE analysis of ORF 702

PCR step	°C	Time (seconds)	cycle
Denature	94	120	1
Denature	94	30	5
Amplification	72	80	
Denature	94	30	7
Amplification	68	60	
Denature	94	30	
Amplification	66	30	25
Elongation	72	60	
Elongation	72	600	1

Following the amplification, PCR products were separated on a 1.2% agarose gel and purified with the NucleoSeq Kit (Macherey and Nagel, Düren, Germany). For sequencing, the fragments were cloned into the pGEM-T vector (Promega, Madison, USA) and transformed into one-shot competent *E. coli* cells (Invitrogen, Karlsruhe, Germany). To select positive clones, colony PCR with SP6 (G500) and T7 (G501) primers as well as PCR with gene specific primers were performed (see Table 8). Of any PCR amplification, six independent clones were sequenced. Colony PCR was performed using the following protocol: 10 min at 94 °C, 35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 64 °C, and elongation for 1 min at 72 °C, and a final elongation step for 10 min at 72 °C before cooling the samples at 8 °C.

2.2.2 Sequence Analyses

Sequence analysis was performed using the NCBI platform in order to identify vector contamination and sequence homologies. The sequence alignments between the predicted sequence of ORF 702 and the sequenced RACE clones were conducted with DNAStar Lasergene SeqMan Pro 7.2.1 (DNASTAR, Madison, WI, USA). For translation into protein sequence and analyses of the six possible reading frames, the tool ExPASy (Expert Protein Analysis System, 2012), a proteomics server of the Swiss Institute of Bioinformatics (SIB), was used. Analysis of the ORF 702 promoter region was performed using the web-based promoter analysis program PLACE (Higo et al., 1999; Plant Cis Acting Regulatory Elements, 2012). Sequence alignments were generated using the multiple alignment tool CLUSTALW2 (European Bioinformatics Institute, 2012).

2.2.3 RNA Isolation and cDNA Synthesis

Total RNA was extracted using the TRIZOL® method (Invitrogen, Karlsruhe). Plant material (0.2 g) was grinded in liquid nitrogen. 1.5 ml TRIZOL was added and the solution was mixed vigorously. After 5 min. of incubation at room temperature, 0.2 ml chloroform was added and vortexed strongly, followed by centrifugation at 12,000 x *g* at 4 °C for 15 min. The supernatant was transferred to a new tube containing 0.75 ml isopropanol and then shaken at room temperature for 10 min. The RNA pellet was precipitated by centrifugation at 12,000 x *g* at 4 °C for 10 min. Subsequently, two washing steps were applied in order to purify RNA, using 80% ethanol followed by air drying and dissolving in 40 µl DEPC water. RNA from *A. thaliana* plants was generated using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany).

The cDNA synthesis was performed using the Superscript II First-Strand Synthesis System (Invitrogen, Karlsruhe) according to the manufacturer's protocol. 1 µg total RNA was used as template for cDNA synthesis.

2.2.4 Plasmid Construction and Transformation into *Agrobacterium* Strains

The strategy for cloning the CDS of ORF 702 into a binary vector bases on the application of primers with an integrated *Xho*I restriction site, which amplify the whole length of the CDS of ORF 702. The amplified fragment length is 1,253 bp. PCR was performed using the following protocol: 3 min at 94 °C, 25-35 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 64 °C, and elongation for 1 min at 72 °C, and a final elongation step for 10 min at 72 °C before cooling the samples at 8 °C.

Primer G901: 5' GAACTCGAGGATCATCATCATCTCATCACTCCTC 3'

Primer G902: 5' CATCTCGAGTAATGTAAAAGCCGCATTTGAACTG 3'

The PCR was performed with cDNA of inoculated roots of the resistant genotype A906001 (seed code: 940043), and 8 µl of the sample were separated via agarose gel electrophoreses in order to check the amplification of the appropriate DNA fragments. Fragments showing the expected length of 1,253 bp were ligated into the pGEM-T vector (Promega, Madison, USA) and transformed into One-Shot competent *E. coli* cells (Invitrogen, Karlsruhe, Germany). To confirm the exact sequence, colony-PCR with Sp6 and T7 primers was performed, and the amplified fragments as well as the plasmids were sequenced. Clone CAU3724 was selected for further analysis.

Subsequent to the identification of the right sequence of ORF 702 within one of the plasmids by Sanger sequencing, the corresponding insert was sub-cloned into the binary vector pAM194 using the integrated *Xho*I restriction sites. After restriction and cloning, the insert is 1,245 bp in size (CAU3725). The sequence is under the control of the constitutive 35S promoter, a 35S terminator, and the reporter gene GUS (KWS Saat AG, Einbeck, Germany) (Kifle et al., 1999). In order to confirm the correct orientation of the ligated cDNA into the binary vector, PCR amplification was performed using one sequence specific primer and one vector specific primer, following sequencing of the amplified products. The construct for transformation of the candidate sequence ORF 702 is defined as 35S::ORF702::GUS (CAU3725), and as a control the empty vector pAM194 was transformed and is defined as 35S::GUS.

Following plasmid isolation and sequencing, the construct was transformed into competent cells of *Agrobacterium rhizogenes* strain Ri15834 and *A. tumefaciens* strain GV2260 by electroporation using the Gene Pulser II (Bio-rad, Munich, Germany) with the following conditions: 200 R, 2.5 kV, 25 µF.

2.2.5 Sugar Beet Hairy Root Transformation

Leaf stalks of the susceptible sugar beet line 930176 and the resistant translocation line 930363 were used for sugar beet hairy root transformation. Leaf stalks were sterilized by submergence in 5% (w/v) $CaCl_2O_2$ for 10 min, followed by treatment with 70% (v/v) ethanol for 5 min. After two washing steps with sterile water, the sterilized leaf stalks were cut into 2 cm pieces and incubated for 5 min with *A. rhizogenes* solution. The soaked explants were dried on sterilized Whatman filter paper before cultivation on solid ½ B5 Gamborg agar medium. After two days of co-cultivation in the dark at 24 °C, explants were transferred to new ½ B5 Gamborg agar medium containing 400 µg/ml cefotaxime and grown in a plant growth chamber (24 °C, 16/8 h light/dark photoperiod). Single hairy roots comprising 1 cm in size were cut and sub-cultured on ½ B5 medium containing 150 µg/ml cefotaxime and grown under the same conditions. In the following, two different types of hairy roots will be distinguished: (i) 'dependent hairy roots' are defined as roots grown on one and the same transformed sugar beet leaf stalk, and share the same number at the beginning (e.g. 24_1, 24_2). (ii). 'Independent hairy roots' originate from different transformed leaf stalks, and are named with different numbers at the beginning (e.g. 23_1, 25_1).

2.2.6 *Arabidopsis thaliana* Transformation

Transgenic *A. thaliana* plants were generated by using the floral dip transformation protocol (Clough and Bent, 1998). Seeds of the *A. thaliana* C24 ecotype were grown for three to four weeks (24 °C, 16/8 h light/dark) until flowering. Developing floral tissues of the T_0 generation were dipped into the transformed *A. rhizogenes* inoculation medium for 2 min. T_1 seeds were harvested and stored at 4 °C. The seeds were surface sterilized by washing in 70% ethanol for 1 min and incubated in sterilization solution containing 2.625% NaCl and 0.05% Tween for further 5 min, followed by three washing steps with sterile H_2O each for 30 sec. Sterile seeds were plated on 0.2 KNOP agar plates containing 50 µg/ml kanamycin. Selected vital T_1 plants were transferred and isolated for growing in single pots, selfed, and T_2 seeds were harvested.

2.2.7 *In vitro* Nematode Resistance Tests

The nematode resistance test was performed *in vitro* according to the protocol described by Sijmons (1991). Sugar beet hairy roots of 1 cm length were transferred to Petri dishes containing 0.2x KNOP medium supplemented with Daishin agar and T_2 seeds as well as unmodified seeds of ecotype C24 as controls were sown in 6-well plates. After 14 to 16 days of growing in the climate chamber (22 °C, 16/8h light/dark photoperiod) each *A. thaliana* plant was inoculated with 100 sterilized *H. schachtii* Schach0 J2-larvae. The hairy roots were inoculated after 10-14 days of growth (22 °C; dark) with 200 sterilized *H. schachtii* Schach0 J2 larvae. Nematodes on the inoculated plants and clones were evaluated by counting the cysts under the stereomicroscope 28 days after inoculation.

Table 10: Experimental design of the nematode resistance tests using the sugar beet hairy root system and transformed *A. thaliana* T_2 plants. Five different experiments (A-E) were performed for the sugar beet hairy root clones and two tests with *A. thaliana* T_2 plants. The inoculation was performed with *H. schachtii* pathotype Schach0 J2 larvae. This pathotype is able to break the resistance originated of chromosome 1 of *P. procumbens*. The inoculum contained 200 J2 larvae for each sugar beet hairy root clone and 100 J2 larvaefor each *A. thaliana* T_2 plant. Two sterilization solutions were used: 0.05% $HgCl_2$ sterilization, and $HgCl_2$+, which includes antibiotics (Streptomycin sulphate (0.2 mg/ml), Cefotaxime (0.25 mg/ml) and Miconazole (0.025 mg/ml) (see 2.2.7.1).

Experiment	Inoculum	Start	End	Propagation	Hatch	Sterilisation
Sugar beet hairy root experiments						
A	Schach0	10.11.2009	15.12.2009	*in vitro*	sterile	$HgCl_2$
B	Schach0	18.12.2009	21.01.2010	*in vitro*	sterile	$HgCl_2$
C	Schach0	26.04.2010	29.05.2010	green house	non-sterile	$HgCl_2$+
D	Schach0	21.06.2010	27.07.2010	green house	non-sterile	$HgCl_2$+
E	Schach0	16.07.2010	22.08.2010	green house	non-sterile	$HgCl_2$+
A. thaliana T_2 nematode experiments						
1	Schach0	02.11.2010	21.12.2010	green house	non-sterile	$HgCl_2$+
2	Schach0	05.01.2011	27.02.2011	green house	non-sterile	$HgCl_2$+

Regarding the *H. schachtii* pathotype Schach1, we were able to propagate the larvae on the resistant translocation line 940043, and sterilized and inoculated them in the same way as explained for Schach0 but all hairy root plates were contaminated with bacteria and fungi even after three independent experiments. Therefore, the inoculation experiments were stopped. The larvae of the Schach1.2 pathotype did not produce enough cysts on the resistant translocation line needed for the experiments. An explanation for this might be the age of the cysts that were stored in the soil at the Julius Kühn Institute (JKI) for more than 10 years (personnel communication B. Niere, JKI, Braunschweig, Germany).

2.2.7.1 Propagation and Sterilization of the *Heterodera schachtii* Larvae

The nematode pathotype *H. schachtii* Schach0 was propagated on *in vitro* stock cultures of mustard (*Sinapis alba* cv. Albatros) roots grown on 0.2x KNOP medium, supplemented with 2% (w/v) sucrose and 0.8% (w/v) Daishin agar under sterile conditions, and on *B. vulgaris* (seed code: 930176) in the greenhouse under non-sterile conditions.

Fully developed cysts were harvested from the roots with 50 µm gauze. Soaking the cysts in 3 mM $ZnCl_2$ for eight to ten days stimulated hatching of juveniles. The larvae were harvested with 10 µm gauze, surface-sterilized with 0.05% $HgCl_2$ solution for 30 seconds, washed four times in sterile water, resuspended in 0.2% (w/v) Gelrite (Duchefa, Harlem, Netherlands), and used directly for inoculation experiments. Another sterilization step was performed, for non-sterile J2 larvae, including incubation for 10 min in a solution containing streptomycin sulfate (0.2 mg/ml), cefotaxime (0.25 mg/ml), and miconazole (0.025 mg/ml).

2.2.8 Expression Analysis

The expression of ORF 702 was evaluated by qualitative reverse transcriptase-PCR (RT-PCR) on cDNA of the hairy root clones, and *A. thaliana* plants. RNA isolation and cDNA synthesis is described in chapter 2.2.3. For RT-PCR amplification, 0.5 µl of the first-strand reaction was used with the following standard PCR-program: 3 min at 94 °C, followed by 32 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C and elongation for 1 min at 72 °C, and finally 10 min elongation at 72 °C. Primers were specifically designed for exon regions (Table 11).

Table 11: Primers used for expression analysis of ORF 702 in resistant and susceptible plants, in sugar beet hairy root clones and in transgenic *A. thaliana* plants.

Primer	Sequence	Region	Size (bp)
H50 H51	for 5’ CTAGCTGCACCACTAACTGC 3’ Rev 5’ CGTTAAGAGCTGGAAGCACC 3’	GAPDH	218
G498 G499	for 5’GGGCATTCCTCCTGACCA 3’ rev 5’ GCTTACCGGCAAAGATGAG 3’	Ubiquitin	500
G842 G879	for 5’ TGTATGGAGGGCTAGCATGA 3’ rev 5’ GGAGGGCTAGCATGATCTTCTTCAAC 3’	ORF 702 exon 1- exon 2	419
G945 G890	for 5’ CCTCATCTGGTGGAAGCGTTGG 3’ rev 5’ ATCACCTGGGAAAAGAATGCC 3’	ORF 702 exon 6- exon 7	204

2.2.8.1 Histochemical GUS Assays

The histochemical GUS test was performed with *in vitro* cultured sugar beet hairy roots and *A. thaliana* leaves. In the test, the plant material was incubated overnight at 37 °C in 10 ml X-Gluc solution containing 50 mM Na_3PO_4 buffer (pH 7.0), 0.2 mg/ ml X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide), and 20 µl Triton. The solution was removed, and the plant material was washed several times with 70% ethanol. Blue staining was evaluated using a stereomicroscope (Stemi SV 11, Zeiss, Germany).

2.3 Results

2.3.1 Defining the Gene Structure of ORF 702 using RACE®

According to the predictions of the *in silico* sequence analysis of Capistrano (2009), the exon-intron structure was ascertained via RACE (Table 12). Comparative sequence analysis of the cloned RACE products and the genomic sequence of ORF 702 obtained from BAC 7C2 revealed the exon-intron structure of the candidate sequence ORF 702. Subsequently, the translation of the exon sequences into the six different reading frames using ExPASy verified the correct structure. In the exactly translated exon sequence, only one stop codon at position 370 aa (1,108-1,110 bp) within the last exon was present. Seven exons and six introns were determined, displaying an open reading frame of 10,210 bp. The coding sequence (CDS) sums up to 1,110 bp and can be translated to the correct amino acid level in reading frame +1, which leads to a polypeptide of 370 aa.

Table 12: Results of the RACE analyses to determine the gene structure of ORF 702. Sizes are listed for ORF 702 exons, introns, 3'- and 5'- untranslated region (UTR), the total genomic length and the coding sequence (CDS) from the start codon in exon 1 to the stop codon in exon 7.

Exon	Size bp[a]	Intron	Size bp
1	417	**1**	3,258
2	155	**2**	85
3	81	**3**	247
4	103	**4**	97
5	111	**5**	3,114
6	146	**6**	1,753
7	97	-	-
5' UTR	-186	**3' UTR**	+360
Genomic length bp			10,210 bp
CDS bp (aa[b])			1,110 (370)

[a]bp: base pair; [b]aa: amino acid

Figure 2A details the exon-intron structure of the candidate sequence ORF 702. The exons are highlighted in grey and are separated by three large inserts of ~1 kb to ~3 kb in size and three smaller inserts (Table 12). Figure 2B shows the exact position of the start codon in exon 1 and the stop codon in exon 7 and, in addition, the position of primers that were designed with integrated *Xho*I restriction sites for cloning into the binary vector pAM194. After ligation to pAM194, the insert size sums up to 1,245 bp (CAU3725).

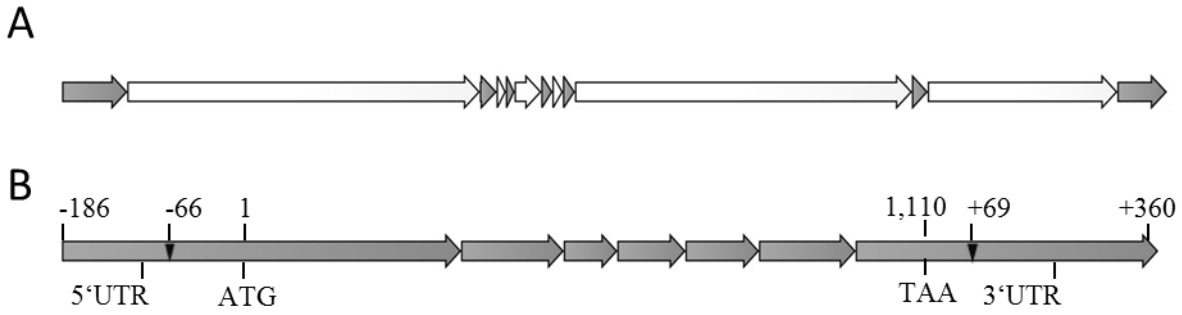

Figure 2: (A) Graphical overview for the exon-intron structure of ORF 702. Exons are highlighted in grey and introns in white. **(B)** Coding sequence of ORF 702 composed of 7 exons with a total length of 1,110 bp, start codon ATG and stop codon TAA, -186 bp 5'untranslated region (UTR) and +360 bp 3'UTR are shown. ▼: Primer combination G901G902 with integrated *Xho*I restriction site used for cloning and therefore resulting in an insert size of 1,245 bp ORF 702 in the binary vector pAM194. The numbers on the sequence are given in base pair (bp).

In total, 440 clones were picked and after PCR amplification and gel separation, 17 clones with the expected insert size of 1,245 bp were sequenced. Clone 63 could be identified to bear the correct sequence and will be further referred to as ORF 702 (CAU3725). Moreover, one clone with a splice variation was identified, which will be described in the following.

2.3.1.1 Splice Variation of ORF 702

With regard to the selection of a clone bearing the correct sequence of ORF 702, one of the 17 sequenced clones was identified possessing a splice variation. Clone 6 has an additional 23 bp at the 5' end of exon 7 and will further be referred to as ORF 702v2 (CAU3726). Analysis of the translated protein sequence displayed a premature stop codon and therefore a shorter open reading frame consisting of 358 aa. Sequence analysis on the amino acid level between clone 63 and clone 6 shows a homology between the first 337 aa (Supplementary Figure 1). Comparing the amino acid sequence of ORF 702 and ORF 702v2 to β-1,3-galactosyltransferase of *Vitis vinifera* (XP_002279252.1) and *A. thaliana* (NP_180179.2) results in 257 identical amino acids with *V. vinifera* and 230 with *A. thaliana* of the ORF 702 protein, whereas the splice variation ORF 702v2 shows 239,and 212 identical amino acids respectively (see Suppl. Figure 1 and Table 13).

The results of the protein sequence analysis of ORF 702 and its splice variation ORF 702v2 to the NCBI non-redundant protein database is shown in Table 13. The e-value is defined as the number of hits that would be expected to occur by chance when searching a sequence database of particular size. The e-values, as well as the identity thresholds for the sequence homology between ORF 702 and the β-1,3-galactosyltransferases of *V. vinifera* and *A. thaliana,* indicate a higher sequence homology than for the splice variation ORF 702v2. Additionally, the bit score values show a qualitative better alignment for ORF 702 than for ORF 702v2. The weak sequence homology to an Avr9 elicitor response protein known from *A. thaliana* (AAG51626) and *Oryza sativa* (BAD25162) is additionally more significant in ORF 702 than in the alignment to ORF 702v2.

Table 13: Results of the protein sequence analysis of ORF 702 and the splice variant ORF 702v2 using the non-redundant protein database on the NCBI platform. Both sequences show homology to β-1,3-galactosyltransferases of *V. vinifera* and *A. thaliana* and to an Avr9 elicitor response protein of *A. thaliana* and *O. sativa*. The identity on aminoacid level, e-value, and bit score, which indicates the quality of alignments, is listed for alignments with ORF 702 and ORF 702v2.

Sequence	Sequence description	Accession number	E-value	Identity	Bit Score
ORF702	β-1,3-galactosyltransferase [*V. vinifera*]	XP002279252	0.0	257 aa[a] (69%)	530
	β-1,3-galactosyltransferase [*A. thaliana*]	NP180179	4e-161	230 aa (62%)	467
ORF702	Avr9 elicitor response protein [*A. thaliana*]	AAG51626	6e-41	93 aa (25%)	157
	Avr9 elicitor response protein [*O sativa*]	BAD25162	9e-41	116 aa (31%)	157
ORF702v2	β-1,3-galactosyltransferase [*V. vinifera*]	XP002279252	8e-170	239 aa (64%)	488
	β-1,3-galactosyltransferase [*A thaliana*]	NP180179	1e-144	212aa (57%)	424
ORF702v2	Avr9 elicitor response protein [*A. thaliana*]	AAG51626	1e-37	79 aa (22%)	148
	Avr9 elicitor response protein [*O. sativa*]	BAD25162	1e-37	81 aa (23%)	148

[a]aa: amino acid

The fact that the protein sequence of ORF 702 is more conserved to known galactosyltransferases and to Avr9 elicitor response proteins lead me to the decision for a further functional analysis of ORF 702.

2.3.1.2 Promoter Analysis

Sequence analysis of the endogenous promoter of ORF 702 was performed using 3,000 bp of the 5' upstream region of ORF 702 (2.2.2). In Table 14, a number of selected *cis*-elements are listed that are involved in root specific expression or related to defense response. The transcriptional start site indicated by a TATA box was identified to be 122 bp upstream of the start codon. All regulatory elements that were identified are listed in Suppl. Table 1

Table 14: Overview of selected root and disease specific regulatory elements present in the 3 kbp promotor region of ORF 702 that were identified using the web-based program PLACE (Higo et al., 1999; Plant Cis Acting Regulatory Elements, 2012). Name of regulatory element, sequence, copy number on + and – strand and functions are given. All regulatory elements identified are listed in Suppl. Table 1.

Regulatory element	Sequence	Number of copies[a]	Function	Reference
NODCON 1GM	AAAGAT	+1, -1	Root nodule expression	Sandal et al. (1987)
NODCON 2GM	CTCC	+4, -3	Root nodule expression	Sandal et al. (1987)
ROOT MOTIF	ATATT	+7, -13	Root specific expression	Elmayan and Tepfer (1995)
W-box	TGAC	+13, -5	Binding site of WRKY transcription factors in pathogenesis related genes	Eulgem et al. (2000)
MYB1	GTTAGTT	-1	Defense-related gene expression	Chakravarthy et al. (2003)
TATA Box	TATTAAT	+1, -1	Transcriptional initiation	Grace et al. (2004)

[a]copy number of the positive strand (+) and the negative strand (-)

Three putative root specific *cis*-elements were identified in the promoter region: two copies of AAAGAT, seven copies of CTCC (Sandal et al., 1987), and 20 copies of ATATT (Elmayan and Tepfer, 1995). Furthermore, 18 copies of the W-box regulatory element TGAC, which act as binding site for members of the WRKY transcription factor family (Eulgem et al., 2000), and one copy of a MYB1 regulatory element (GTTAGTT), which is involved in defense related gene expression, were determined (Chakravarthy et al., 2003).

2.3.2 Functional Analysis of ORF 702 as a *HS1-2* Gene Candidate

2.3.2.1 Functional Analysis in Sugar Beet Hairy Roots

The aim of this experiment was the constitutive expression of ORF 702 in sugar beet hairy roots and *A. thaliana*, as R-genes are often expressed constitutively in plants (Gu et al., 2005). The coding sequence of ORF 702 was under the control of the constitutive 35S promoter, a 35S terminator and the reporter gene GUS in the binary vector pAM194 (Kifle et al., 1999; KWS Saat AG, Einbeck, Germany) (2.2.4). The *A. rhizogenes* strain Ri15834 was used to transform different sugar beet accessions (2.2.5).

The sugar beet accession 930176 was chosen for hairy root transformation with ORF 702 because of its susceptible phenotype (2.2.5). It was transformed with the constructs 35S::ORF702::GUS, 35S::ORF702v2::GUS, and 35S::GUS. To generate hairy root clones that serve as controls, the

susceptible line 930176 and the resistant line 930363 were inoculated with untransformed *A. tumefaciens* to induce hairy root growth. The number of transformants ranged from 2 to 57, and the number of independent hairy roots/transformation event ranged from 2 to 16 (Table 15).

Sugar beet hairy root clones were grown on ½ B5 medium containing 150 µg/ml cefotaxime for 21 days (2.2.5). They were numbered in the following way e.g. 1, 2, 3, 4. Then they were further cultivated by cutting into 2-4 cm pieces. Those sub-clones were numbered in the following way e.g. 1_1, 2_1, 2_2, 2_3, 3_1.

Table 15: *A. rhizogenes* mediated transformation of two sugar beet accessions with two constructs. The obtained hairy root clones, the number of transformants, and the number of independent transformants are given. Transformants were identified by histochemical GUS expression tests (2.2.8.1). Independent clones are defined as hairy roots from different leaf stalks. In the lower part of this table the numbers of clones analyzed in the nematode resistance experiments are given. The number of Petri dishes (PD) tested (biological replications) for each construct is listed. Clones obtained by transformation of the susceptible genotype (930176) with the empty vector construct 35S::GUS and with untransformed *A. rhizogenes* serve as a susceptible control and clones obtained by transformation of the resistant genotype (930363) with *A. rhizogenes* serve as a resistant control

Sugar beet accession		**930176**	**930176**	**930176**	**930363**
construct		**35S::ORF702::GUS**	**35S::GUS**		
Total number of hairy root clones		103	18	3	2
Number of GUS positive transformants		57	8	-	-
Number of independent GUS positive transformants		16	3	3[a]	2[a]
Experiments					
A	clones	5	2	1	0
	PD	25	9	6	0
B	clones	6	1	2	0
	PD	28	5	8	0
C	clones	12	3	0	2
	PD	44	12	0	7
D	clones	11	2	1	2
	PD	46	12	5	8
E	clones	12	3	1	2
	PD	54	10	3	11

'a': Untransformed controls, GUS positive clones cannot be selected.

Altogether, 16 different transformation events were tested for nematode resistance by inoculation with 35S::ORF702::GUS. The number of sub-clones tested ranged from 3.3 to 5.5 per transformation event. After 3 - 4 weeks, cysts were counted under the stereomicroscope (Suppl. Table 2).

Table 16: Means of *H. schachtii* J4 females developed on hairy root clones transformed with 35S::ORF702::GUS, the empty vector construct 35S::GUS or incubated with untransformed *A. rhizogenes*. The two-sided student's t-test was performed using the R-software package (R Foundation, 2012). No significant differences between clones within one experiment are indicated with equal letters, whereas different letters mark significant differences between clones. Equal letters indicate non-significant differences between clones, whereas significant differences between groups are marked by different letters within each experiment. Mean values of developed J4 females, standard deviation (STD) and number of Petri dishes (n) analyzed for the different clones for each nematode *in vitro* experiment of sugar beet hairy roots are given. The resistant control 930363 was transformed later, therefore hairy root clones were analyzed in experiment C, D, and E.

Experiment	35S::ORF702::GUS		930363		35S::GUS		930176	
	mean±STD	n	mean±STD	n	mean±STD	n	mean±STD	n
A	5.5 ± 4.7^{a}	25	-	-	9.9 ± 6^{ab}	9	16.5 ± 5.7^{b}	6
B	8.2 ± 5.4^{c}	28	-	-	13.8 ± 3.2^{cd}	5	17.25 ± 7.9^{d}	8
C	3.1 ± 4^{e}	44	2.1 ± 2.5^{e}	7	10.4 ± 5.5^{f}	12	-	-
D	3.5 ± 4^{g}	46	2.5 ± 4.7^{g}	8	4.8 ± 3^{gh}	12	10 ± 3^{h}	5
E	4.2 ± 3.5^{i}	54	3.1 ± 2.9^{i}	11	8.3 ± 3^{j}	10	9 ± 1^{j}	3
Total	4.4 ± 4.4^{k}	197	2.8 ± 3.7^{k}	26	8.8 ± 5.2^{km}	48	14.01 ± 6.5^{m}	21

As a result, no significant differences were found between 35S::ORF702::GUS clones and the resistant control root clones, and the two groups of the susceptible control clones. However, differences between the control clones 35S::GUS and the 35S::ORF702::GUS clones were also non-significant (see Figure 3).

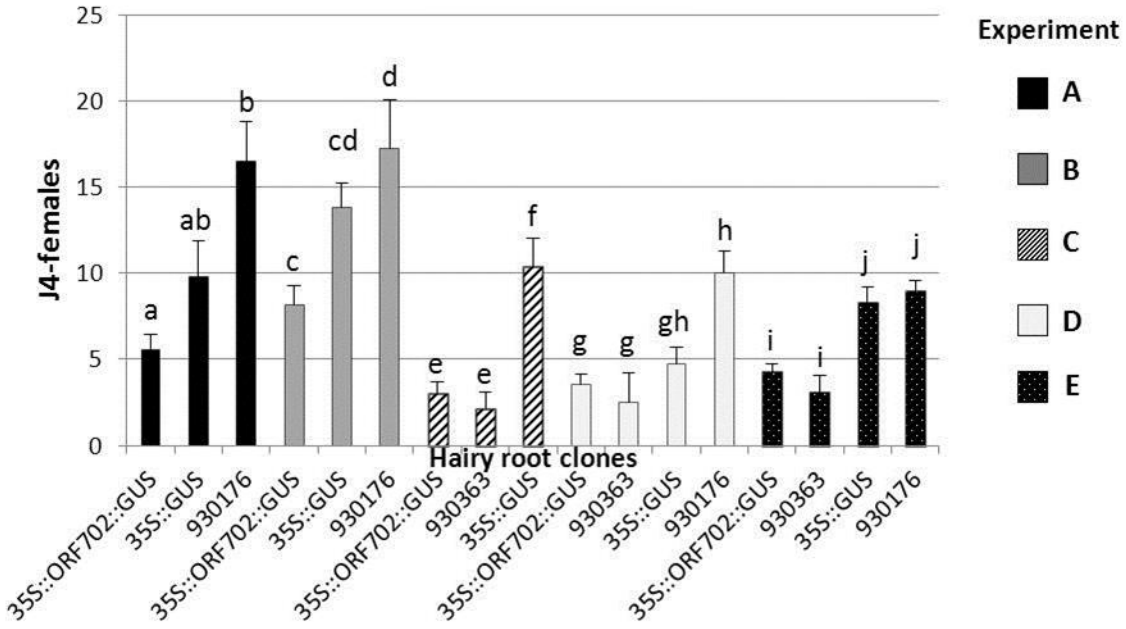

Figure 3: Results of five *in vitro* nematode resistance experiments with sugar beet hairy root clones. One bar denotes the mean number of developed J4 females of all experiments in which the clone was tested (Table 16). The different constructs are shown below the x-axis. '35S': promotor; 'ORF 702': candidate sequence for *Hs1-2*; 'GUS': reporter gene (beta-glucuronidase), marked as 35S::GUS and hairy root clones of the susceptible line 930176 and the resistant line 930363 obtained by inoculation with *A. rhizogenes*. A student's t-test was performed with the R software package (R Foundation, 2012). Different letters indicate significant differences.

The lowest number of J4 females was found in clone 63_1 with an average of one, whereas the highest number of developed J4 females was determined in clone 32 with an average of 10.25 J4 females of 35S::ORF702::GUS hairy roots. The number of developed J4 females for the 35S::GUS susceptible control ranged from 9.6 to 23 in clone 61 and 27, whereas the number of established J4 females for the susceptible untransformed control ranged from 5.4 to 12.9 in clone 60 and 22_1, respectively.

Regarding the resistant control hairy root clones, the number of developed J4 females ranged from 0.4 to 6.1 in clone 40 and 41, respectively.

The number of J4 females between experiments ranged from 8.2 to 3.1 (transgenic roots), from 13.8 to 4.8 (susceptible control roots, 35S::GUS) and from 17.25 to 9 (930176). On the resistant control roots, the number of J4 females between experiments ranged from 3.1 to 2.1.

Differences in the amount of J4 females between transgenic and susceptible control roots were between 4.8 and 11 (930176) and between 4.1 and 7.2 (35S::GUS). The number of J4 females between transgenic and the resistant control roots differed from 1 to 1.1.

Light-microscopic analysis of inoculated hairy root plates was repeated weekly. The following parameters were investigated: presence of male larvae, development of J4 females to cysts, and lesions that occur during root penetration of the infective J2 larvae. The developing females were analyzed for abnormalities in growth behavior and shape. 28 days post inoculation (dpi), the number of J4 females was determined (Figure 4).

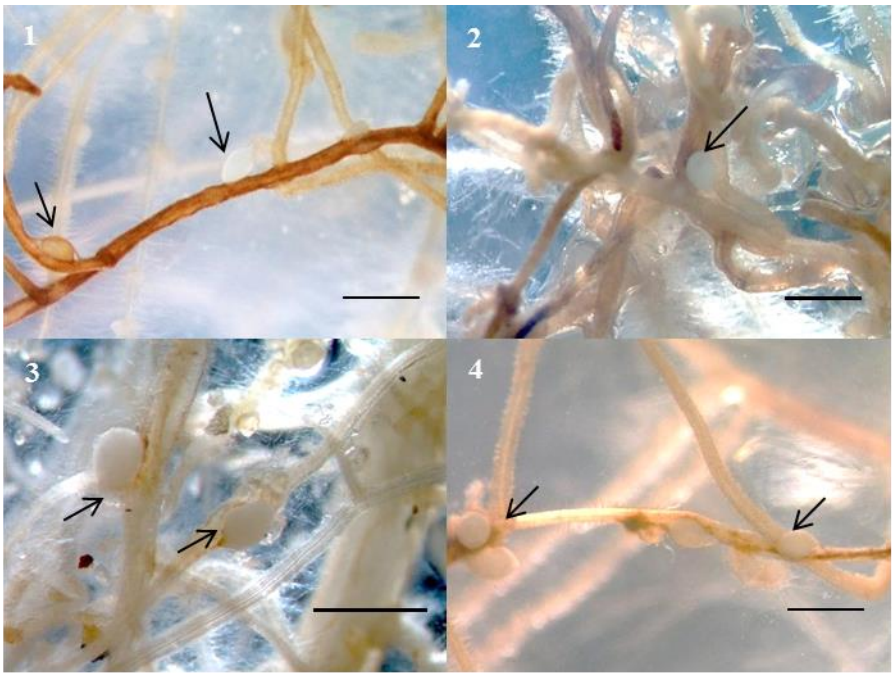

Figure 4: Microscopic analysis of sugar beet hairy roots 28 days after inoculation (dpi) with 200 *H. schachtii* J2 larvae. The developed J4 females were counted. (1) Susceptible line 930176 transformed with the construct 35S::ORF702::GUS. (2) Resistant control 930363. (3) Susceptible line 930176 transformed with the empty vector construct 35S::GUS. (4) Susceptible control 930176. The bar equals 350µm. Arrows depict females.

No remarkable differences in growth habit or appearance of the developed females between clones of the susceptible control and clones transformed with 35S::ORF702::GUS could be observed (Figure 4). Although slight differences in root growth between all clones and within sub-clones were visible such as poor growth or growth retardation, no effect of the transgene on nematode development could be found.

2.3.2.1.1 Expression Analysis of Transformed Sugar Beet Hairy Roots

In this experiment, the qualitative expression of the candidate gene ORF 702 in hairy root clones was measured by RT-PCR analysis. Primer pairs specifically targeting ORF 702 (G842G879 for exon 1

and exon 2, and G945G890 for exon 6 and exon 7) were used. PCR fragments were analyzed by gel electrophoresis using 1% agarose gels. As a result, 24 fragments with the expected size of 419 bp and 204 bp respectively were found in all 22 root clones transformed both with the construct 35S::ORF702::GUS and in the two root clones obtained from the resistant control clones. As expected, no expression in the root clones transformed with 35S::GUS was detected (Figure 5).

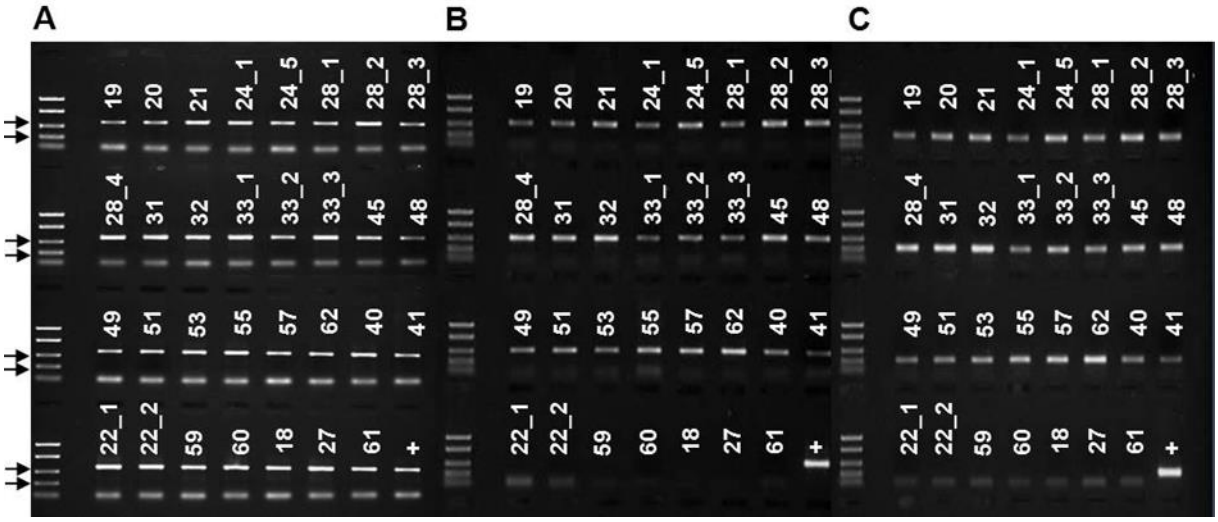

Figure 5: Expression analyses of ORF 702 using hairy root cDNA from the nematode resistance tests. The numbers above the lanes indicate the hairy root clone tested. The expression of the sugar beet ubiquitin gene served as a control. Primers were designed from the exon regions of ORF 702. **(A):** PCR products after ubiquitin amplification (primer G498G499), expected fragment size 500 bp; '+' genomic DNA of *B. vulgaris* as positive control. **(B):** PCR products after exon1-2 amplification of ORF 702 (primers G842G879), expected fragment size 419 bp; '+' plasmid DNA of pAM194::35S::ORF702::GUS as positive control. **(C):** PCR products after amplification of exon 6-7 with the primer combination G945G890 exon 6-7; fragment size 204 bp; '+' plasmid DNA of pAM194::35S::ORF702 as positive control. FastRuler™ Low Range DNA Ladder was used as a marker. The upper and lower arrows indicate 400 bp and 200 bp, respectively. Fragments were separated in 1**%** agarose gels.

Furthermore, expression of the GUS gene was determined. GUS expression was used to select transgenic root clones transformed with the construct 35S::ORF702::GUS and 35S::GUS. Short pieces (2 cm) of the developing hairy root clones (sub-clones) from the nematode resistance tests were used for GUS staining.

Slight differences in the GUS staining pattern between and within the sub-clones were observed. The different expression observed for the GUS reporter gene might also indicate a different expression of the candidate gene ORF 702. However, RT-PCR with ORF 702 specific primers (Figure 5) showed no remarkable differences in expression levels, and no correlation to the amount of developed J4 females was observed.

2.3.2.2 Functional Analysis by Transformation of *Arabidopsis thaliana*

2.3.2.2.1 *Arabidopsis thaliana* Transformation Experiments

Plasmids used for *Agrobacterium*-mediated transformation consist of the binary vector pAM194 and the construct 35S::ORF702::GUS (2.2.4). The empty or recombinant *A. tumefaciens* strain GV2260 was used for transformation of the *A. thaliana* ecotype C24. As the transformation method, the Floral Dip protocol according to Clough and Bent (1998) was performed on 100 *A. thaliana* T_0 plants. To select transgenic T_1 plants, 100,000 T_0 seeds were sown on growth media that contained kanamycin, and vital green plantlets were selected for further analysis via GUS staining. Table 17 showed the results of the Floral Dip transformation of the *A. thaliana* ecotype C24. In total, 65 kanamycin resistant and GUS positive T_1 plants were identified (transformation efficiency 0.065%).

Table 17: Results of the *A. thaliana* Floral Dip transformation experiments with the construct 35S::ORF702::GUS.

Candidate sequence	Promoter	Selectable marker	Binary vector	No. of T_1 seeds tested	No. of T_1 plants selected	Efficiency rate (%)
ORF 702	35S	kanamycin	pAM194	100	65	0.065

Transformed T_1 plants were propagated *in vitro* by selfing, and the seeds of 29 T_2 families were harvested. Among the 29 T_2 families, 24 T_2 families could be selected with a minimum amount of ≥36 seeds as required for further analysis in the *in vitro* nematode resistance test.

2.3.2.2.2 Nematode Resistance Test with *Arabidopsis thaliana*

The *in vitro* nematode resistance test was divided into two experiments as shown in Table 18. A total of 24 T_2 families were selected for nematode inoculation experiments. In experiment 1, a total number of 15 T_2 families were tested. In experiment 2, two T_2 families of experiment 1 were also included. Thus, the number of tested T_2 families sums up to 11. In total, 36 seeds of each T_2 family were sown on 0.2x KNOP media in six 6-well plates and inoculated with 100 sterilized J2 larvae per plant (see also 2.2.7). Besides that, untransformed plants of the *A. thaliana* ecotype C24 were grown as controls (Table 18).

Table 18: *In vitro* nematode resistance test (experiment 1 and 2) with transgenic 35S::ORF702::GUS *A. thaliana* T_2 families and untransformed C24 plants as control. The test was splitted into two experiments. Each plant was inoculated with 100 sterilized *H. schachtii* J2 larvae (2.2.7)

Experiment	T_2 families	Inoculum	Number of T_2 plants inoculated	Number of C24 control plants inoculated
1	15	100 J2 larvae	183	16
2	11	100 J2 larvae	171	31

During the experiments, leaf material of all T_2 plants was harvested for GUS staining. The T_2 families segregated into transgenic (GUS positive) and non-transgenic plants. A χ^2 -test was performed with phenotypic data to test deviation from 3:1 segregation as expected for a single copy introgression of the transgene.

To reveal significant differences in the amount of developed cysts between GUS positive and the GUS negative plants, a student's t-test was performed for each segregating T_2 family, including the non-transformed C24 plants as a control. This test was done with the software R (R Foundation, 2012) and a critical p-value of 0.05 (Table 19).

Table 19: Results of the nematode *in vitro* tests conducted with transgenic *A. thaliana* T_2 families. The calculation of the germination rate refers to 36 seeds sown for each T_2 family. A χ^2 -test was performed using the GUS-data (expected segregation ratio 3:1, critical value 3.84). Results of the statistical analysis using the student's t-test are given in p-values (critical value $p<0.05$).

***A. thaliana* family**	**Germination rate (%)**	**GUS+ (n)**	**GUS- (n)**	**χ^2 - test**	**Mean of cysts on GUS+**	**Mean of cysts on GUS-**	**p-value GUS+/ GUS-**	**p-value GUS+/C24**
Experiment 1								
T2.4	27.7	9	1	1.20	9.89	5.00	0.362	0.978
T2.5	30.5	7	4	0.75	6.29	6.75	0.820	0.039
T2.6	27.7	9	1	1.20	7.89	9.00	0.836	0.260
T2.7	50.0	13	5	0.07	11.25	11.00	-	-
T2.12	44.5	16	0	5.30[a]	4.00	-	-	0.003[b]
T2.13	36.1	12	1	2.07	4.50	3.00	0.601	0.000[b]
T2.14	13.9	3	2	2.07	6.30	10.50	0.539	0.147
T2.17	25.0	8	1	1.20	13.13	13.00	0.986	0.143
T2.18	50.0	17	1	3.60	7.51	9.00	0.751	0.106
T2.19	16.7	6	0	2.00	8.84	-	-	0.624
T2.26	22.2	8	0	2.50	10.25	-	-	0.858
T2.27	41.7	12	3	0.20	9.42	8.76	0.741	0.715
T2.29	33.3	12	0	4.00[a]	11.67	-	-	0.249
T2.30	44.4	15	1	3.00	9.87	10.00	0.972	0.959
T2.31	44.4	16	0	5.30[a]	13.11	-	-	0.080
C24[c]	44.4	-	16	-	-	9.94	-	-
Experiment 2								
T2.2	30.6	8	3	0.03	8.37	9.66	0.729	0.179
T2.15	72.2	26	0	8.66[a]	12.92	-	-	0.101
T2.16	66.6	19	5	0.22	14.12	16.60	0.281	0.014
T2.19	36.1	13	0	4.33[a]	11.69	-	-	0.559
T2.20	30.6	9	2	0.27	9.44	12.50	0.301	0.102
T2.22	47.2	17	0	5.66[a]	9.23	-	-	0.239
T2.26	52.7	14	5	0.02	13.14	16.20	0.301	0.102
T2.32	50.0	10	8	3.60[a]	9.70	9.87	0.921	0.490
T2.33	25.0	8	1	0.93	12.25	15.00	0.637	0.410
T2.35	47.2	14	3	0.49	14.93	13.00	0.494	0.005[b]
T2.36	16.7	6	0	2.00	7.50	-	-	0.114
C24[c]	57.4	0	31	-	-	10.74	-	-

'-' no value available; [a]calculated value exceeds the critical value; [b]significant to $p<0.05$;[c]untransformed control plant C24

The inoculated number of plants varied depending on the germination rate, which ranged from 13.9% to 72.2% (T_2) and from 44.4% to 57.4% (C24 control). The number of developed J4 females ranged from 4 to 14.9 (GUS positive T_2 plants) and from 3 to 16.6 (GUS negative T_2 plants). The number of J4 females in the C24 control ranged from 9.9 to 10.7. The lowest number of J4 females was found in T2.12 with a mean of 3 per plant, whereas the highest number of J4 females was detected in the T2.16 family with a value of 16.6.

As a result, no significant differences in the amount of established cysts in both experiments could be observed between the GUS positive and the GUS negative T_2 plants (Table 19 and Figure 6). Additionally, the mean values of developed cysts of the GUS positive plants were calculated with regard to a significant difference to the mean values of the 31 C24 control plants. Only T2.15 showed a significant difference in the amount of established cysts compared to the C24 control plants (Table 19).

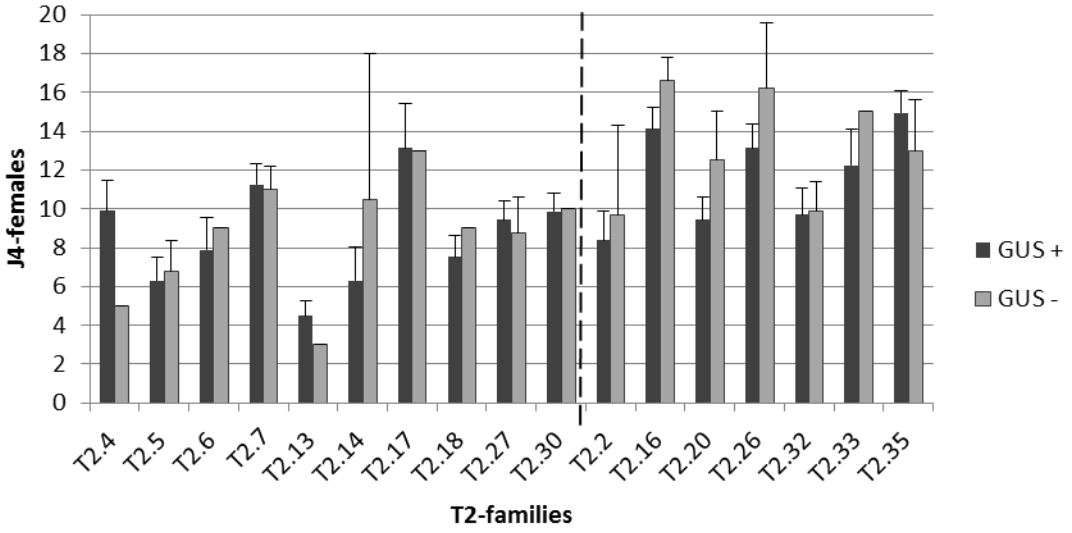

Figure 6: Results of two nematode resistance experiments with transgenic *A. thaliana*. Mean values of evolved J4 females on *A. thaliana* T_2 plants transformed with the construct 35S::ORF702::GUS displayed for the GUS positive (GUS+ black) and the GUS negative (GUS- grey) plants for each segregating T_2 family tested in experiment 1 (T2.4 –T2.30; left part of the diagram) and experiment 2 (T2.2- T2.35; right part of the diagram). The dashed line separates T_2 families tested in experiment 1 and experiment 2 (Table 17).

Observations of the inoculated plants under the light microscope were repeated weekly in order to determine modifications in root growth or nematode development in both experiments. When comparing the root and plant growth of the *A. thaliana* control plants, the segregating T_2 plants, transgenic 35S::ORF702::GUS plants, and GUS negative T_2 plants, no differences were observed (see Figure 7). Moreover, invasion of larvae into the roots, cyst development, and number of males showed no difference between controls and T_2 plants.

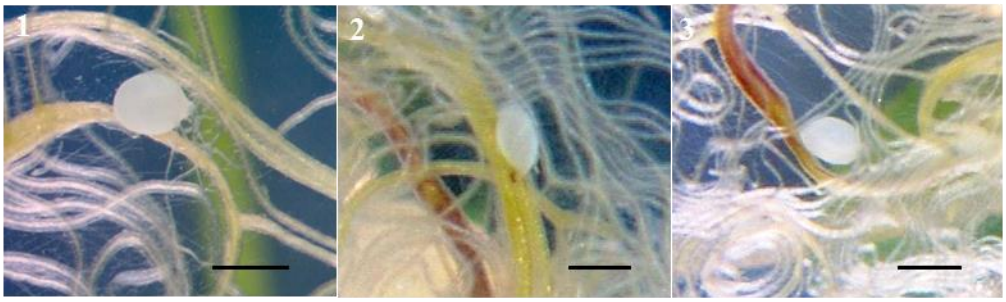

Figure 7: Light-microscopic analysis of in vitro cultured *A. thaliana* T_2 plants 28 days post inoculation with 100 J2 larvae of *H. schachtii*. (1) J4 female on roots of a 35S::ORF702::GUS T_2 plant. (2) J4 female on roots of a GUS-negative T_2 plant. (3) J4 female on roots of a C24 control plant. Bars equal 200 µm.

2.3.2.2.3 Expression Analysis with Transformed *Arabidopsis thaliana* Plants

To select segregating T_2 families, a histochemical GUS test was performed using leaf material of *A. thaliana* plants (2.2.8.1). Slight variances in the GUS staining pattern between the samples were visible. The different expression observed for the GUS reporter gene might also indicate different expression of the candidate gene ORF 702. However, no relation between the different intensities of the GUS staining and the number of developed J4 females could be observed.

Furthermore, the qualitative expression of the candidate gene ORF 702 in transformed *A. thaliana* plants was measured by RT-PCR analysis. Primer pairs specifically targeting ORF 702 (G842G879 for

exons 1 and 2, and G945G890 for exons 6 and 7) were used. PCR fragments were analyzed by gel electrophoresis using 1% agarose gels. As a result, fragments with the expected size of 419 bp for exons 1 and 2, and 204 bp for exons 6 and 7, were detected in all 29 selected *A. thaliana* plants transformed with the construct 35S::ORF702::GUS. As expected, no expression was detected in the non-transformed wild type plants C24 (Figure 8).

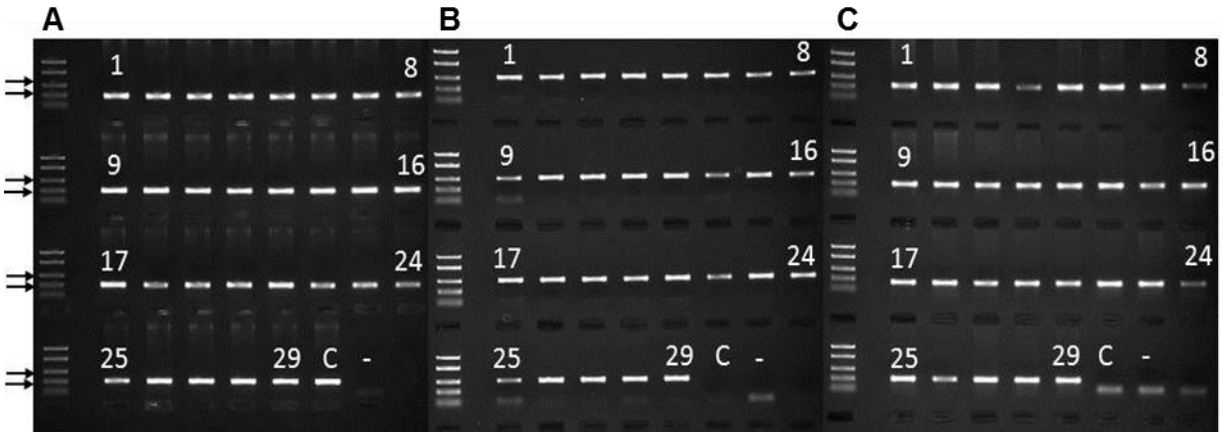

Figure 8: Expression analysis of ORF 702 using cDNA of 29 *A. thaliana* T_2 families (1-8: T2.2, T2.4, T2.5, T2.6, T2.7, T2.8, T2.11, T2.12; 9-16: T2.13, T2.14, T2.15, T2.16, T2.17, T2.18, T2.19, T2.20; 17-24: T2.21, T2.22, T2.23, T2.26, T2.27, T2.28, T2.29, T2.30; 25-29: T2.31, T2.32, T2.33, T2.35, T2.36).'C': cDNA of the untransformed *A. thaliana* wild type C24 used as a control. '-': water control. Primers specifically designed for exon regions of ORF 702 and the housekeeping gene GAPDH of *A. thaliana* were used. 1: GAPDH (H50H51), fragment size 218 bp. 2: Primers G842G879 exon1-2 of ORF 702, fragment size 419 bp. 3: Primers G945G890 exon 6-7 ORF 702, fragment size 204 bp. FastRuler™ Low Range DNA Ladder was used as a marker. The upper and lower arrows indicate 400 bp and 200 bp, respectively. Fragments were separated on 1**%** agarose gels.

2.4 Discussion

In the present study, functional and expression analyses were performed for ORF 702, which was suggested as a candidate sequence for *Hs1-2*, a putative nematode resistance gene mediated by translocation of a genomic region from a wild beet relative *P. procumbens* to *B. vulgaris*. ORF 702 was further analyzed because of its position being present on the resistant translocation lines TR520 and TR363, and absent from the susceptible translocation lines TR659 and TR320. Moreover, ORF 702 showed sequence homology to an Avr9 elicitor response-like protein (GenBank: CAB36540), and a galactosyltransferase family protein from *A. thaliana* (GenBank: NP_180179). An important and well-characterized perception mechanism is based on resistance (R) genes in plants whose products confer recognition of cognate avirulence (Avr) proteins in pathogens (Sanseverino et al., 2010). Furthermore, the involvement of glycosylation during plant pathogen interaction has been suggested by (Langlois-Meurinne et al., 2005). Analyses of Schaff et al. (2007) showed that a single glycosyltransferase gene was significantly up-regulated within the root transcriptome of the resistant (*Mi*+) compared to the susceptible (*Mi*–) tomato cultivars during RKN infections.

For the sequence analysis of the endogenous promoter of ORF 702, a 3 kbp region upstream of the start codon was chosen as known promoters sizes of other sugar beet genes are around 3kbp in size, such as the promoter of the gene Bv*cab*I2 (3,049 bp) and the endogenous promoter of the nematode resistance gene $Hs1^{pro-1}$ (3,082 bp) (Stahl et al., 2004; Thurau et al., 2003). Due to similar expression levels of ORF 702 in roots of inoculated and not-inoculated plants (Capistrano, 2009), it was suggested that ORF 702 acts independently of endogenous promoter induction. Nevertheless, the promoter sequence of ORF 702 was analyzed for the presence of *cis*-regulatory elements, which revealed specific root expression motifs. This leads to the assumption that ORF 702 could be specifically expressed in roots. R-genes are often expressed constitutively in uninfected plants (Gu et al., 2005) and, therefore, the strong constitutive CaMV 35S promoter was chosen also to ensure expression of the transgene in roots.

Regarding the functional analysis in sugar beet hairy roots, two different kinds of susceptible controls were used. The susceptible sugar beet line 930176 was transformed with the empty vector construct 35S::GUS to serve as transformed susceptible control. In total, four different clones were generated and tested with an average of 13.25 replications each. Additionally, hairy roots of the susceptible sugar beet line 930176 were induced by inoculation with unmodified *A. rhizogenes* bacteria. Three different clones were obtained and tested with an average of 7.3 replications per clone. Likewise, the resistant sugar beet translocation line 930363 was transformed to induce hairy roots as resistant controls. Therefore, two clones were generated during the experiments and tested with an average of 13.5 replications each.

The resistance tests were divided in experiments A, B, C, D, and, E. Notably, more females developed on hairy root clones in experiments A and B compared to C, D, and E. This may be due to the change of the nematode propagation methods: in experiment A and B, the larvae were propagated sterile *in vitro* on *Sinapis alba*, whereas the larvae used for inoculation in experiment C, D, and E were propagated and hatched in a non-sterile way (Table 10). This change was required because the increasing number of J2 larvae needed for inoculation of the rising number of generated hairy root clones could not be produced in a sufficient amount *in vitro*. Consequently, a reduced vitality of non-sterile produced larvae is suggested to be due to an extreme change from their natural habitat to *in vitro* culture media. In experiment A, I determined that 4.95% of the inoculated J2 larvae developed to J4 females, and in experiment B, 6.9% of the J2 larvae evolved to J4 females. These observations are comparable to the analysis of sugar beet hairy root *in vitro* nematode resistance tests of other research groups using sterile propagated J2 larvae. Kifle et al. (1999) presented an improved transformation protocol for sugar beet hairy roots facilitating co-inoculation of *A. tumefaciens* and *A. rhizogenes*. Three weeks after inoculation, about 10% of the inoculated J2 larvae developed to females on the control clones. Cai et al. (2003) analyzed the potential role of the sporamin gene *SpTI-1* in *H. schachtii*

parasitism. They inoculated sugar beet hairy root clones with 300 J2 larvae and determined that four weeks later 7.6% of the inoculated larvae established to J4 females on control clones.

Based on the assumption that ORF 702 confers resistance against *H. schachtii* to host plants, I expected a significantly lower amount of established J4 females compared to susceptible control clones and *A. thaliana* plants after constitutively expressing ORF 702 in sugar beet hairy root clones and *A. thaliana* plants. Figure 9 shows an overview of the expected conditions regarding the amount of established J4 females between the tested sugar beet hairy root clones.

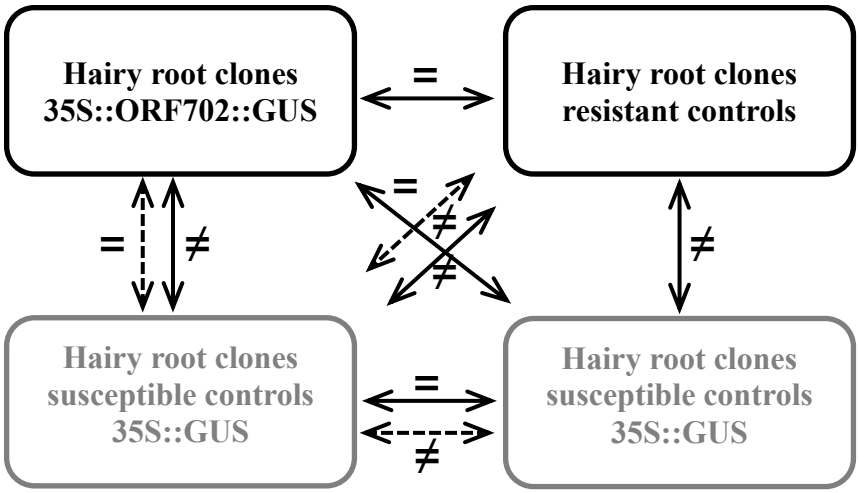

Figure 9: Expected conditions versus observed results regarding the established amount of J4 females between analyzed root clones. The expected resistant clones are defined in black boxes and the estimated susceptible control clones are highlighted in gray boxes. Continuous arrows indicate the expectations and dashed arrows show the observed results and are present only if differences exist between expected and observed values. '=': no significant differences or equal amount of established J4 females; '≠': significant differences or different amount of established J4 females.

The two groups with an expected resistance reaction were, on the one hand, the hairy root clones expressing the 35S::ORF702 construct and, on the other hand, the induced hairy roots of the resistant translocation line 930363 indicated in black in Figure 9. Consequently, an approximately equal amount of J4 females was projected in both groups. Analogously, the expectation was to find non-significant difference in the amount of evolved females between both susceptible control groups, consisting of hairy roots of the susceptible line 930176 bearing the empty vector 35S::GUS and hairy roots induced by unmodified *A. rhizogenes* of the susceptible line 930176, as indicated in gray in Figure 9. As the resistant and susceptible groups differ in the amount of established J4 females mediated by the constitutive expression of ORF 702 as a candidate gene for resistance, significant differences were expected between those.

Figure 9 also graphically summarizes the expectations versus the observed results in regard to the amount of established J4 females between the different hairy root groups. Continuous arrows indicate the expected conditions, whereas dashed arrows indicate the observed settings. As expected, the resistant control clones and the susceptible control clones induced by transformation with unmodified *A. rhizogenes* showed a significant difference in the amount of developed J4 females. Moreover, the transgenic 35S::ORF702::GUS clones showed a comparable amount of J4 females to the resistant control clones, being well in accordance with the expectations for this experiment.

Unexpectedly, an equal amount of females was observed between the transgenic clones 35S::ORF702::GUS and the susceptible 35S::GUS control clones in three out of five experiments. Furthermore and contrary to the experimental hypothesis, no significant differences between

susceptible 35S::GUS control clones and resistant control clones were observed in at least one experiment and significant differences could be determined between the two susceptible control groups which also were not expected.

Different research groups already described a low or non-expression of the gene of interest in expression studies. Guivarc'h et al. (1999) established hairy root clones from carrot root discs and observed clones carrying the reporter gene *gus* that failed to express it or showed a very irregular expression. Further analysis showed that the *gus* gene was methylated and, therefore, its expression was decreased. In addition, clones hypothesized to exhibit continuous expression showed different levels of expression related to the copy number of the gene. In transgenic regenerates of tobacco, negative relationships between *gus* insertion numbers and GUS activity have already been reported and were interpreted to be the result of co-suppression effects involving silencing of homologous transgene loci (Hobbs et al., 1990). Borgio (2009) explained co-suppression as gene silencing mediated by a sense transgene. Smigocki (2009) observed differences in expression levels of the *NaPI* gene integrated to transgenic sugar beet hairy roots and discussed the possibility of a co-suppression via overexpression. In my work, the sugar beet hairy root clones used for the functional analysis of ORF 702 were GUS stained before each *in vitro* nematode resistance test was started, and the expression of ORF 702 in the different clones was analyzed by RT-PCR. In this way, expression of ORF 702 was verified.

Although my experiments indicate a tendency towards a lower amount of evolved J4 females on the transgenic 35S::ORF702::GUS root clones compared to the susceptible control, no significant differences in the amount of established J4 females between the expected resistant and the expected susceptible clones could be identified over all experiments. Thus, these results do not confirm a function of ORF 702 in nematode resistance in sugar beet. In order to verify these results, the *A. thaliana* model system for functional analysis of candidate sequences was adopted.

Sijmons et al. (1991) established the role of *A. thaliana* in plant-nematode interaction. They published a protocol for infection of *A. thaliana* roots by several plant-parasitic nematodes, including *H. schachtii*. Since then, a lot of studies were published about BCN interactions using *A. thaliana* as a host. In order to analyze the nematodes feeding site establishment, Grundler et al. (1998) investigated formation changes of the plant cell walls, such as the deposition of callose-like layers, during syncytium differentiation. Recent analyzes of cell walls of syncytial cells revealed presence of cellulose and hemicelluloses xyloglucan and absence of galactan, which suggests a high flexibility of syncytial cells (Davies et al., 2012; Urwin and Davies, 2012). Juergensen et al. (2003) determined the expression of membrane-bound sugar transport proteins in syncytia and identified AtSUC2 as the first disaccharide carrier activated by pathogens. The number of phloem elements increased around syncytia suggesting a highly facilitated nutrient transfer into the feeding sites (Hofmann and Grundler, 2007; Hoth et al., 2005). The transcriptome of induced syncytia was investigated using the Affymetrix Arabidopsis GeneChip ATH1and resulted in 18.4% up-regulated, and 15.8% lower expressed genes compared to control roots. In accordance to that, a clear transcriptionally difference of syncytia compared to other root tissues was determined (Szakasits et al., 2009). Recently, Siddique et al (2011) investigated the syncytium specific expression of the promoter of the *A. thaliana* defensin gene *Pdf2.1* by infection with *H. schachtii*. This promoter turned out to be an interesting candidate for syncytium-specific expression of genes.

Baum et al. (2000) developed an *in vitro* screening protocol for the analysis of *A. thaliana* mutants with altered susceptibility to the BCN. Screening of 5,200 M_2 plants revealed two mutants, *rhd1-4* (root hair defective) and *asc1* (altered susceptibility to cyst nematodes). The hypersusceptible *rhd1-4* mutant was further analyzed by Wubben et al. (2001). They discovered that *A. thaliana* mutants that overproduce ethylene are hypersusceptible, and mutants that are ethylene-insensitive are less susceptible to *H. schachtii*. In this respect, Wubben et al. (2008), investigated *A. thaliana* mutants with altered salicylic acid (SA) biosynthesis and showed an increase in susceptibility in SA deficient

mutants. This indicates a suppression of SA signaling in roots necessary for cyst nematode parasitism. Hewezi et al. (2008a) investigated the role of regulatory RNA by using DICER-like (*dcl*) and RNA-dependent RNA polymerase (*rdr*) mutants of *A. thaliana*. Decreased susceptibility in the examined mutants suggests a role of small RNAs in gene regulation during plant-nematode interaction.

Nematode parasitism genes are expressed in the esophageal gland cells and encode effector proteins that are secreted into the host to support the nematodes parasitism of the plant. In RNA interference (RNAi) assays transgenic *A. thaliana* plants express double-stranded (ds) RNA of potential nematode parasitism genes. Patel et al. (2008) performed so-called host-derived RNAi in order to investigate the role of successful parasitism derived by the *H. schachtii* genes *Hssyv46*, *Hs4e02*, and *Hs5d08*. Only *Hssyv46* showed significant differences in reduction of the number of established females compared to the control lines. Sindhu et al. (2009) analyzed four potential *H. schachtii* parasitism genes using RNAi assays. Although no complete resistance was determined, a decrease of 23% to 64% developing females in different RNAi lines could be observed. Patel et al. (2010) and Hewezi et al. (2010) analyzed nematode effector proteins which lead to the identification of Hs4F01 and 10A06. The *Hs4F01* protein interacts with an oxidoreductase and 10A06 with a Spermidine Synthase2 (SPDS2), as shown in yeast two-hybrid assays. Recently, Lee et al. (2011) discovered the *H. schachtii* effector protein Hs19C07 that interacts with the *A. thaliana* auxin influx transporter LAX3. Hs19C07 is proposed to increase LAX3-mediated auxin influx. The authors suggest this as a mechanism stimulating cell wall hydrolysis for syncytium establishment of cyst nematodes.

For confirmation of the actual predicted function of ORF 702 in resistance to *H. schachtii* and for verification of the sugar beet hairy root results, 24 transgenic *A. thaliana* T_2 families were analyzed *in vitro*. Following GUS staining and RT-PCR analysis, 17 segregating T_2 families could be selected and inoculated with infective J2 larvae for *in vitro* nematode resistance tests. On average, 10.3 J4 females (10.3%) developed on each GUS positive T_2 plant, whereas 11.05 (11.05%) J4 females were observed on GUS negative plants. A total number of 47 *A. thaliana* C24 unmodified wild type plants was tested and the average amount of J4 females evolved per plant accounted for 10.5 (10.5%). The equal amount of evolved females confirms a similar fitness of the larvae in both experiments and optimal conditions for development during the experiments. These data are within a range of developed J4 females in *in vitro* nematode resistance tests of other research groups using *A. thaliana* wildtype C24 plants and *H. schachtii*. Hewezi et al. (2008b), investigating the role of a cellulose binding protein (CBP) from *H. schachtii* in nematode parasitism, observed 15 (6%) developed J4 females on inoculated *A. thaliana* C24 wild type plants. A number of 14 (23%) developed J4 females on *A. thaliana* C24 wild type plants were determined by Patel et al. (2010) during analysis of the nematode parasitism gene *Hs4F01* that encodes for an annexin like effector. While analyzing the nematode effector protein 10A06 by transformation of *A. thaliana* and subsequent inoculation with *H. schachtii* larvae, 10 (4%) J4 females on average were observed at *A. thaliana* C24 wild type plants (Hewezi et al., 2010). The amount of inoculated J2 larvae ranged from 60 to 250 per plant (Hewezi et al., 2008b; Hewezi et al., 2010; Patel et al., 2010).

If ORF 702 is involved in nematode resistance, a significantly lower amount of established J4 females in transgenic plants compared to the non-transgenic plants within each segregating T_2 family is expected. However, in all 17 tested segregating T_2 families no significant differences between the numbers of evolved J4 females in the GUS negative compared to the GUS positive plants were detected. Furthermore, *A. thaliana* C24 unmodified wild type plants were additionally analyzed in nematode resistance tests and no significant differences were detected compared to the GUS negative plants of the segregating T_2 families, as expected. In summary, *A. thaliana* C24 wild type plants, GUS positive T_2 plants, and GUS negative T_2 plants showed an equal range of established J4 females and, consequently, no significant differences could be detected.

Recently, Liu et al. (2012) reported about the positional cloning of *Rhg 4* gene from recombinant inbred lines (RIL). They determined the expression of a serine hydroxymethyltransferase (*SHMT*)

within syncytia and verified the proposed function by transformation of a susceptible recombinant inbred line (RIL) with a 5.1 kbp genomic fragment of *SHMT* including the gene-specific promoter, and by the application of RNAi in hairy roots. However, my data suggest that ORF 702, also coding for a transferase, is not involved in plant resistance response to *H. schachtii*. Therefore, different strategies must now be discussed concerning further options and strategies for identification of the *Hs1-2* nematode resistance gene. A necessary prerequisite to investigate the translocation line *in silico* is the availability of the complete genome sequence of *B. vulgaris* and *P. procumbens*. Due to repetitive sequences, further chromosome walking with BAC ends to extend the sequence coverage of the physical map is impossible. Also, fine mapping of the gene is impossible because no recombination occurs within the translocation. The application of new sequencing methods (Next Generation Sequencing, NGS) dramatically accelerates forward genetics (see chapter 3). In times of fast innovations in biotechnology, a NGS approach following an assembly of the translocation region should result in the identification of so far unknown sequences specific for the wild beet translocation, which, after detailed analysis, may also fulfill the requirements for a candidate region for the resistance gene.

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3 Re-Sequencing and Hybrid Assembly of Two Nematode-Resistant *Beta vulgaris* Translocation Lines

3.1 Introduction

Sugar beet (*Beta vulgaris* ssp. *vulgaris*) is a biennial dicotyledonous plant that produces during its first growing season a leaf rosette and a large taproot with a sugar content of 15 –20%. In the second year, the nutrients are used for the formation of reproductive organs induced by long day conditions after exposure to low temperatures, which is called vernalization (Märländer et al., 2011). As the only source for sucrose production in temperate climates, the cultivation of sugar beet is distributed among 38 countries worldwide (Zucker Statistik, 2011). In Germany, 389,000 ha were cultivated in 2011 (Statista, 2012).

The genome size of *B. vulgaris* has been estimated to be 758 Mbp per haploid genome (n = x = 9) (Arumuganathan and Earle, 1991). Genetic and physical maps of a genome are essential tools for structural, functional, and applied genomics. A genetic map allows the detection of quantitative trait loci (QTLs) and facilitates marker-assisted selection (MAS) for the improvement of plant varieties. Using different types of molecular markers, such as single-sequence-repeat (SSR), single-nucleotide-polymorphism (SNP), or expressed-sequence-tags (EST) markers, several genetic maps of sugar beet have been developed (Barzen et al., 1992; Barzen et al., 1995; Hallden et al., 1996; Hansen et al., 1999; Laurent et al., 2007; McGrath et al., 2007; Nilsson et al., 1997; Pillen et al., 1993; Pillen et al., 1992; Rae et al., 2000; Schneider et al., 2007; Schondelmaier et al., 1996; Schumacher et al., 1997; Uphoff and Wricke, 1995). Lange et al. (2010) recently investigated a novel approach for high-throughput development of genetic markers. Using a 15 K Agilent micro array, which contained 146,554 custom-made oligonucleotides of sugar beet, resulted in the mapping of 511 dominant markers. For the purpose of gene discovery and positional cloning, physical maps of the target regions are indispensable. Several types of physical maps based on different genotypes and cloning vectors exist, such as yeast artificial chromosomes (YAC) (Eyers et al., 1992; Klein-Lankhorst et al., 1994; Kleine et al., 1995) and bacterial artificial chromosomes (BAC) (Fang et al., 2004; Gindullis et al., 2001a; Hagihara et al., 2005; Hohmann et al., 2003; Jacobs et al., 2009; McGrath et al., 2004; Schulte et al., 2006). Lange et al. (2008) established a fosmid library of the DH line KWS2320 as a part of the GABI BeetSeq project (Genomanalyse im Biologischen System Pflanze GABI, 2012). Recently, Dohm et al. (2012) presented a physical map of *B. vulgaris* that includes 535 contigs anchored to the chromosomes, 8,361 probes, and 22,815 BACs. By comparing the gene-order of the physical map with rosid species, the authors could identify syntenic regions involving 1,400-2,700 genes in the sequenced genomes of *Arabidopsis*, poplar, grapevine, and cocoa (Dohm et al., 2012). Menzel et al. (2012) performed a genome-wide analysis of repetitive elements, which lead to the identification of specific DNA transposons for species of the genus *Beta*.

The obligate biotrophic beet cyst nematode (BCN) *Heterodera schachtii* is one of the most important pathogens of sugar beet. This sedentary endoparasite is widespread in sugar beet cultivating areas all over the world, and its presence leads to a severe decrease in root yield and sugar content (Biancardi et al., 2010; Jones et al., 2011). Resistance against the BCN has been determined in wild relatives of the cultivated sugar beet, belonging to the genus *Patellifolia*, and it has been transferred to sugar beet by interspecific crosses (Jung and Wricke, 1987). Resistant, diploid sugar beet plants have been selected that carry a translocation of the wild beet. The line Pro3 carries a translocation from *P. procumbens* chromosome 7 that includes the resistance gene $Hs2^{pro\text{-}7}$. The translocation line TR520 has been generated by a cross of the resistant homozygous translocation line A906001 with the sugar beet line 930190, resulting in the F1 population 940081. The translocation line TR520 is one of the progenies out of the fourth backcross (BC4) between 940081 and 930190, and it is hemizygous for the translocation. TR520 and TR363 (formerly Pro 4) bear monogenic resistance derived from chromosome 1 of *P. procumbens* (Jung and Wricke, 1987; Kleine et al., 1995). The wild beet

translocation of the resistant line TR520 has been extensively studied for more than 20 years and has been estimated to be ~1,500 kb in size. A first nematode resistance gene *Hs1*$^{pro-1}$ has been positionally cloned (Cai et al., 1997). The existence of a second nematode resistance gene is suggested as the resistant line TR363 shares homologous sequences with TR520 but does not carry the gene *Hs1*$^{pro-1}$.

In recent years, DNA sequencing experienced a major leap forward with the advent of a new technology based on the principle of sequencing by synthesis (Ansorge, 2009). This cutting-edge next generation sequencing technology now enables researchers of all molecular biological disciplines to approach scientific problems in a new way and to discover genes and their expression at a previously unknown and resolution.

Since 2004, the sugar beet genome mapping and sequencing consortium (Genomanalyse im Biologischen System Pflanze GABI, 2012) has been working with the aim to generate a *de novo* assembly of the sugar beet genome. As the sugar beet is not closely related to any eudicot plant species with existing or upcoming genome sequences, essential new information on plant genome evolution will be provided by this research. Several sequence resources for assembly of the sugar beet genome are presently employed, such as whole genome shotgun (WGS) sequences, as well as sequences from fosmids, and BACs obtained by Sanger sequencing (Dohm et al., 2011). Preliminary annotations already yielded approximately 28,000 gene models (Weisshaar et al., 2011). Recently, the pre-publication draft RefBeet-0.9 of the sugar beet genome sequence became publicly available and consists of 590 Mbp (The Sugar Beet Resource, 2012). Moreover, a draft sequence assembly of *P. procumbens* was generated using the Illumina HiSeq 2000 sequencer (H. Himmelbauer, personal communication).

As of this writing, the following resources are available. A BAC library of the translocation line TR520 had been established by Schulte et al. (2006). After refinement of the physical map by Capistrano (2009), 17 BAC clones were assembled to three BAC contigs, which resulted in 962,626 bp translocation-specific sequence, but four gaps remain uncovered. Recently, two susceptible translocation lines were identified by gamma irradiation using seeds of the translocation TR520 (H. Harloff, unpublished data). They carry partly homologous sequences of the translocation TR520 (Capistrano, 2009). We assume that a resistance gene, named *Hs1-2*, must be located on the overlapping regions between the resistant lines whereas it must be absent from the susceptible ones.

In order to fill the remaining gaps on the physical map of the nematode resistant translocation TR520, we performed WGS sequencing of the two diploid sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) translocation lines, TR520 and TR363. The quality and relatedness of the reference sequence has great impact on the quality of the re-sequenced genome (Imelfort and Edwards, 2009). Consequently, we used two different strategies to assemble the short reads as only draft reference genome assemblies were available for both *B. vulgaris* (RefBeet-0.4 at the beginning of this project, later RefBeet-0.9) as well as for *P. procumbens*.

3.2 Material and Methods

3.2.1 Plant Material

Two translocation lines carrying translocations from chromosome 1 of *P. procumbens* were used for WGS sequencing. The translocation of the line TR520 was estimated to be ~ 1.5 Mbp in size. We sequenced the line A906001 (seed code 940043), which carries essentially the same translocation as the line TR520 in a homozygous form. The line TR363 (seed code 9300363) is hemizygous for the *P. procumbens* translocation and estimated to have a translocation of different size (Kleine et al., 1998). Physical mapping and marker analysis by Schulte et al. (2006) and Capistrano (2009) suggest that the two lines share ~ 700 kbp of the *P. procumbens* translocation. Both carry the nematode resistance gene *Hs1-2* and both are resistant to the BCN *H. schachtii*.

Two susceptible translocation lines, TR659 and TR320, were identified after a marker based screening of 578 M1 offspring of 400 gy gamma irradiated seeds of the line 950631, which is essentially the same as the resistant translocation line TR520. Both lines had lost most of the translocation and show a susceptible phenotype (personal communication, H. Harloff).

Table 20: Plant material used for WGS sequencing

Seed code	Botanical name	Other code	Phenotype[a]	Reference
930363	*B. vulgaris*	TR363[b]	Resistant	Jung and Wricke (1987)
000520	*B. vulgaris*	TR520[b]	Resistant	Schulte et al. (2006)
940043	*B. vulgaris*	A906001	Resistant	Jung und Wricke (1987)
070659	*B. vulgaris*	TR659[b]	Susceptible	Capistrano (2009)
071320	*B. vulgaris*	TR320[b]	Susceptible	Capistrano (2009)
930176	*B. vulgaris*	93161p	Susceptible	Dieckmann Seeds[c]
090012	*B. vulgaris*	KWS2320	Susceptible	KWS[d]
960051	*P. procumbens*	35335	Resistant	FAL[e]; Kleine et al. (1998)
100001	*P. procumbens*	BETA 951	Resistant	IPK[f]

[a]Phenotype: resistant or susceptible against *H. schachtii*; [b]translocation line [c]Dieckmann Seeds, Nienstädt, Germany; [d]KWS SAAT AG, Einbeck, Germany; of 940043; [e]FAL=Research center for agriculture (Bundesforschungsanstalt für Landwirtschaft), Braunschweig, Germany, disintegrated in 2008; [f]IPK = Leibniz Institute of Plant Genetics and Crop Plant Research., Gatersleben, Germany.

3.2.2 Primer Sequences

Table 21: Primer sequences, amplicon sizes, and primer locations.

Primer	Sequence	Amplicon size	Location
H42	5’ TCGCCATCACCGTCGAATACC 3’	266 bp	ORF 803
H43	5’ GATGGTCAGATTCATCATCATTTC 3’		
H44	5’ GATTGGCATTTTCATATCCTCG 3’	1,128 bp	ORF 801
H45	5’ CAAATGATCCCTCCCCCAATATG 3’		
H46	5’ CTCTTCACCACCTTCATCGAGTCG 3’	667 bp	ORF 802
H154	5’ TCCCGTTTCTTCTGTCCATC 3’		
H48	5’ CACTTCGCACAGCTGATGAACT 3’	482 bp	ORF 804
H49	5’ GCAACTGTCGCCCAGATAATCAG 3’		
H54	5’ CTCTCGTTTCGTACGAACCGCTAC 3’	273 bp	ORF 806
H55	5’CTAGAACGGCTTCGCGGG 3’		
H59	5’ GACTAGTGAGCACTGCCTTGTCG 3’	286 bp	super contig 1
H60	5’ GATAAGCAGGACACTTCCATGAGC 3’		
H61	5’ ATAGCATCATAACCAAGCCAATTA 3’	330 bp	
H62	5’ GTTTCTGCAGTCAAAGTTGAGATTC 3’		
H65	5’ TGGATAACCATGGAGCTTGTAAC 3’	631bp	super scaffold 1
H66	5’ TGCTACTGAGATTTGGAATGATC 3’		
H67	5’ ACAAGTAAGCTATCGTCAGTGG 3’	413 bp	
H68	5’ ACATGACTCATGCCTTCATGCG 3’		
H69	5’ GTGATGGTAGGTGTCTATGCTGAC 3’	595 bp	super scaffold 13
H70	5’ GCTCTTGAGCTGAGTTTTCTCTC 3’		
H71	5’ CATGTTAACTCACCCTTGACTCAC 3’	599 bp	
H72	5’ CTATCCCAACAACCCAATAGAAAC 3’		
H73	5’ CATGAATAAGCATACCTGCTGAAC 3’	435 bp	super scaffold 2
H74	5’ AGCAAATGTGATCCCTCTCTATTC 3’		
H75	5’ ACTTGCGGAGTAGAAAACTGAAC 3’	487 bp	
H76	5’ TCCTGGACATGGTATATTGTTACG 3’		
H77	5’ GTGGTTGATGGCATAAAACTACAC 3’	426 bp	super scaffold 3
H78	5’ TATCCGTCTTCAAGCACATTACAC 3’		
H79	5’ GAGGGGAGTCAGTAGGAAGACG 3’	435 bp	
H80	5’ GTCTCCCCCTTTTCAGAAGTCTAC 3’		
H81	5’ GACGCAGACTAGAGGAAAGAAAAC 3’	434 bp	super scaffold 4
H82	5’ GTGAACAGTTAACACGGAGACAAC 3’		
H83	5’ AAACCAGCTCCATGACTTAGTACC 3’	458 bp	
H84	5’ CACATTGTTACTTCATAGGGCTTG 3’		
H85	5’ CTAAGACTTGGTTGAGAATACACCAC 3’	422 bp	super scaffold 5
H86	5’ TAAAGATGTTCCTCAAGCTTACCC 3’		
H87	5’ CTGGTTACTAGTGGTTGTGGAGTG 3’	451 bp	
H88	5’ ATGGAGCGGTGGTTAGTAAGTAAG 3’		
H89	5’ CAAATAGTCGATACATCTGCCTTG 3’	445 bp	super scaffold 6
H90	5’ TGGATACTTGAGCTGAGAAAAGTG 3’		
H91	5’ AGATGCCAAGAAAGTAACTCAG 3’	471 bp	
H92	5’ GATTGTGATGAGACATTTAGTCC 3’		
H93	5’ ATCTAGGGAGTGTTGCCTTATTTG 3’	419 bp	super scaffold 7
H94	5’ AGTGGATGAGGATAAGGTATCTGC 3’		
H95	5’ AAACAGACGACTTTATTGGAGACC 3’	401 bp	super scaffold 8
H96	5’ CTAGCAAGTGTTTTGCCAGTCTTAG 3’		

H97	5’ GTGTTATGTAGCTTGGGTTCTGTG 3’	440 bp	super scaffold 9
H98	5’ TTCAAGCTCTAGAAGCTTTGTGGG 3’		
H99	5’ CTAATATGGAGAGTGCAGATGTGG 3’	488 bp	super scaffold 10
H100	5’ ACTACTAACCCACTTCACCCAGAG 3’		
H101	5’ GATGTGTAACTCACGAAAACAAGC 3’	458 bp	super scaffold 11
H102	5’ CAAGCATACTGACCTGTTATGACC 3’		
H103	5’ TGACTCAAAATCACTCTCTTGCTC 3’	437 bp	
H104	5’ CCATTTCAACTCAGACGTAACAAC 3’		
H105	5’ GGTGTTAATGTTTGTGAGCTTGAC 3’	476 bp	
H106	5’ TGATCACTAGCTCTGGTTACAAGC 3’		
H107	5’ CTCAAGTCTTCTCCACCTTTCTTC 3’	401 bp	super scaffold 12
H108	5’ AGGATAACAGAGACACCCAAATGTG 3’		
H111	5’ GTATCGCAAGCCACTCCAACTG 3’	811 bp	ORF 810
H112	5’ TCAAGTGCCCACAAGTTGAAC 3’		
H113	5’ ATGAAGTGGCCAGCATTAGC 3’	711 bp	ORF 807
H114	5’ GTGACTCGAGGTCTCCTTCG 3’		
H119	5’ GAGACAGCAGTCCAAGCATAG 3’	606 bp	ORF 808
H120	5’ AAGGTTACATGTGGGTGATG 3’		
H121	5’ TGTCATCAATTCTGCGATCTG 3’	476 bp	ORF 809
H122	5’ TGAAAAACAAGGGGAACTGC 3’		
H123	5’ TCCTGATGCTGCTCACACTC 3’	534 bp	ORF 805
H124	5’ ACAGGACGGCAATTTTCTTG 3’		
H129	5’ AGTTGATGTGGGGAATGAGG 3’	307 bp	ORF 811
H130	5’ CTTTCGTTTCTTCGCAGGAC 3’		
H132	5’ TTATTTCTTCAGCATTAGC 3’	-	ORF 803
H133	5’ GCTGTTAGAACCGATCCAAG 3’	357 bp	ORF 803
H134	5’ GAAATTGAATCTATGACCCTC 3’		
H172	5’ CTAGCTGCACCACTAACTGC 3’	218 bp	*B. vulgaris* GAPDH
H173	5’ CGTTAAGAGCTGGAAGCACC 3’		

3.2.3 Sequences and Clone Libraries

Draft sequence assemblies (RefBeet-0.4; RefBeet-0.9) of the *B. vulgaris* (090012) and *P. procumbens* (100001) (Table 20) genome kindly provided by H. Himmelbauer (Centre for Genomic Regulation, Barcelona, Spain) were used. The assembly size of the *B. vulgaris* RefBeet-0.4 is 967 Mbp, includes 35,475 sequences, and has a N50 size of 358 kbp. Regarding the RefBeet-0.9, the assembly size is 590 Mbp and includes 82,305 sequences. The N50 size is 759 kbp. The assembly size of *P. procumbens* adds up to 641 Mbp, with 94,485 sequences, and a N50 size of 38 kbp.

Schulte et al. (2006) generated a BAC library of the translocation line TR520. Currently, 17 BACs have been physically mapped on the translocation. They have been grouped into three contigs which are estimated to cover 1,417 Mbp of the *P. procumbens* translocation of the line TR520 (see Figure 1). Eleven BACs and several BAC ends had been sequenced using Sanger technology and sum up to 962,625 bp (Capistrano, 2009; Schulte et al., 2006). With PCR-based marker analysis of the BACs, the overlapping region between the translocation lines TR520 and the TR363 was estimated to be 689 kbp (Capistrano 2009).

3.2.4 Whole Genome Shotgun (WGS) Sequencing

The two nematode resistant translocation lines TR520 and TR363 were used for sequencing on the Illumina HiSeq2000 platform and subsequently, two hybrid assembly strategies were applied. Figure 10 shows the workflow.

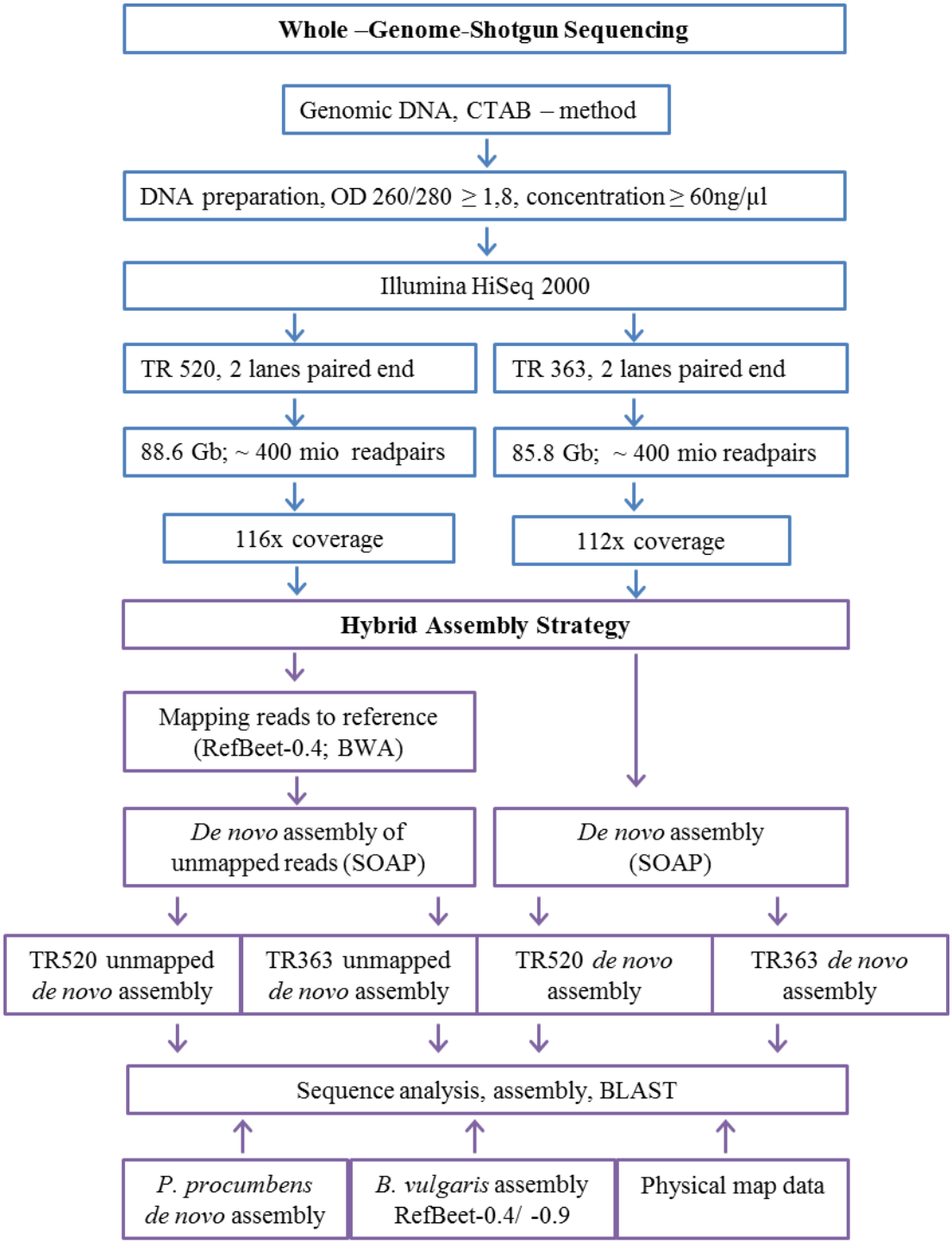

Figure 10: Flowchart of the whole genome shotgun (WGS) sequencing approach of two nematode resistant translocation lines TR520and TR363. In the upper part of the figure, the DNA preparation and sequencing features are shown. The following hybrid assembly strategy was used to identify translocation specific sequences and is shown in the lower part of the flowchart. Two different assembly strategies were used and two assemblies for each line were generated. Sequence analysis was performed by using the four translocation genome assemblies and four reference assemblies of, *P. procumbens*, *B. vulgaris* RefBeet-0.4 and RefBeet-0.9, and the physically mapped sequences (physical map data: BACs and YACs).

3.2.4.1 Genomic DNA Preparation for WGS Sequencing

Genomic leaf DNA of the lines TR520 and TR363 was isolated using the CTAB protocol according to Rogers and Bendich (1985). The requirements for sequencing on the Illumina HiSeq 2000 platform for DNA are $OD_{260/280}$ >1.8, and a concentration of ≥ 60 ng/µl (measured on a Nanodrop spectrophotometer) in a total volume of 20 µl.

3.2.4.2 Sequencing Library Preparation for WGS Sequencing

Genomic leaf DNA (see 3.2.4.1) of the translocation lines TR520 and TR363 (Table 20) was used for sequencing-library preparation using the Illumina TruSeq DNA sample preparation kit. Each library was sequenced (paired-end, 2x100 bp, 600 bp fragment size) on two lanes of the Illumina HiSeq 2000 platform (Illumina, Inc., San Diego, CA, USA) at the sequencing unit of the Institute of Clinical Molecular Biology (ICMB), Kiel, Germany.

3.2.5 Inoculation of Plants with Nematodes

Plants were grown in the greenhouse for two weeks, transferred to quartz sand, and three days later inoculated with 500 *H. schachtii* J2-larvae. Roots were sampled from inoculated plants one and three days after inoculation, and were subsequently frozen in liquid nitrogen.

3.2.6 RNA Isolation and cDNA Synthesis

In vitro expression of predicted candidate genes was done by qualitative RT-PCR with cDNA of inoculated root material (3.2.5) of *B. vulgaris* (930176), *P. procumbens* (950056), and the translocation lines TR520, TR363, TR659, and TR320. Sequencing of mRNA was done by isolating total RNA of inoculated root material of the line TR520 (Table 20). Total RNA was extracted using the Qiagen RNeasy Mini Kit according to the manufacturer's recommendations. Each RNA sample was quantified on the Nanodrop spectrophotometer (ThermoFisher Scientific Inc., Waltham, MA, USA). One µg of RNA was transcribed to cDNA using the Fermentas First Strand cDNA synthesis kit. RT-PCR amplification of the housekeeping gene GAPDH of *B. vulgaris* was used to verify cDNA quality (Suppl. Figure 2 A and B).

3.2.7 Sequencing of Root RNA

Root material of inoculated TR520 (940043) plants (see 3.2.5) was harvested one and three days post inoculation (dpi), total RNA was isolated (see 3.2.6) and separated on agarose gels (Supl. Figure 3). A RNA sequencing library was constructed using the Illumina TruSeq RNA sample preparation kit and subsequently sequenced (2x100 bp) on the Illumina HiSeq 2000 system at the sequencing unit of the Institute of Clinical Molecular Biology (ICMB).

3.2.8 Bioinformatical Analysis

De novo genome assembly of short reads was conducted using the Short-Oligonucleotide-Alignment Program (Li et al., 2008) with K-mer size set to 41 on a 1.5 TB-RAM compute node. Mapping of short reads to draft reference assemblies was performed using the Burrows-Wheeler Aligner (BWA) (Burrows and Wheeler, 1994) with the following parameters: bwa aln -n 2 -q 15 -l 5000 -t 6; bwa sample -a 400. The Samtools package (Li et al., 2009a) was used to format .bam files and to filter for unmapped sequencing reads. For *de novo* transcriptome assembly the Trinity package (Grabherr et al., 2011) was used with the following parameters: --seqType fq --JM 100G. The assembled scaffolds and contigs were analyzed via the CLC bio Genomics Workbench 5.1 (CLC bio, Aarhus, Denmark).

3.2.8.1 Assembly Strategies

One of the strategies to separate the translocation specific read pairs is based on mapping the total number of reads against the draft genome assembly of *B. vulgaris* (RefBeet-0.4) using the Burrows-Wheeler-Aligner (BWA) (3.2.8) (Burrows and Wheeler, 1994). The unmapped reads were extracted and assembled by SOAPdenovo (3.2.8) (Li et al., 2008; Li et al., 2009b). The output datasets are denoted in the following as 'TR520 unmapped *de novo*' and 'TR363 unmapped *de novo*'.

Using the second strategy, all short reads were directly assembled *de novo* by SOAPdenovo (Li et al., 2008; Li et al., 2009b). The output datasets were denoted as 'TR520 *de novo*' and 'TR363 *de novo*'.

3.2.8.2 Sequence Analyses

The reference assemblies of RefBeet-0.4, RefBeet-0.9, *P. procumbens*, and the new generated assemblies of the translocation lines TR520 and TR363 (3.2.8.1) were used to create a BLAST database of each via the CLC bio Genomics Workbench. Additionally a BLAST database was generated from the BAC-, BAC-end, and YAC-end sequences. In order to identify new translocation specific sequences, the datasets 'TR520 *de novo*' and 'TR363 unmapped *de novo*' were mapped to both the *P. procumbens* reference assembly and to the physical map data using local nucleotide BLAST (blastn) of the CLC bio Genomics Workbench. The parameters used were: low complexity filter, expect = 10, word size = 11, match 1, mismatch -3, gap costs, existence 5, and extension 2. The resulting datasets were separated by (1) bit score >1400, (2) e-value smallest to largest, and (3) identity threshold largest to smallest.

I determined promoter-specific regulatory elements by using the signal scan search of the program PLACE (Plant Cis Acting Regulatory Elements, 2012) with default settings (Higo et al., 1999).

3.2.8.3 Open Reading Frame (ORF) and Conserved Domain Analyses

To identify putative coding regions using the *A. thaliana* matrix, *ab initio* ORF-analysis was performed using the programs FGENESH (Softberry FGENESH, 2007) or GENSCAN (GENSCAN, 2009). ORFs predicted by either program were analyzed by protein BLAST using the NCBI database of non-redundant proteins and default settings (Altschul et al., 1997; National Center for Biotechnology Information (NCBI), 2012).

The presence of conserved domains was identified using the Conserved Domain Database of NCBI (National Center for Biotechnology Information (NCBI), 2012) with the following settings: Search strategy – global & local (merged), and E-value <1.0. The TMHMM Server version 2.0 (TMHMM Server, 2012) was used for the prediction of transmembrane helices in proteins.

3.2.8.4 mRNA Analysis

The multi FASTA file of the mRNA assembly was transformed to a BLAST database. All sequences anchored and not anchored to the physical map were blasted (blastn) against this database. The resulting output was filtered by score > 100. Significant aligned sequences of the mRNA dataset were extracted and analyzed by tblastx using the non-redundant protein database and default settings on the NCBI platform.

3.3 Results

3.3.1 Whole Genome Shotgun Sequencing and Assembly of the Short Reads

The paired-end sequencing of the translocation lines TR520 and TR363 using the Illumina HiSeq 2000 platform resulted in 88.6 Gbp and 85.5 Gbp high quality 100 bp-reads, respectively. This represents 116.6 (TR520) and 112.5-fold (TR363) coverage of the sugar beet translocation genome (758 Mbp + ~ 2 Mbp translocation).

Sequence reads generated in WGS sequencing are assembled into contiguous sequences and are denoted as contigs. Contigs are organized into scaffolds on the basis of linking information provided by read pairs. Therefore, scaffolds provide the contig order and orientation as well as the size of gaps between the contigs (Green, 2001). The term 'super scaffold' is used for assembled contigs and scaffolds of different genome assemblies of *P. procumbens* and of the translocation lines TR520 and TR363 of the WGS data. 'Super contigs' are the largest types of sequence assemblies on the physical map and incorporate BACs and super scaffolds of the WGS sequencing whereas the contigs of the physical map presented by Capistrano (2009) are named 'BAC contigs' in the following.

The aim of the project was to select sequence reads from the *P. procumbens* translocation. Two different assembly strategies were designed to identify translocation-specific read pairs (Figure 10). One strategy is based on mapping the total number of reads to the draft genome sequence of *B. vulgaris* (RefBeet-0.4) using the Burrows-Wheeler-Aligner (BWA), allowing two mismatches (Burrows and Wheeler, 1994) (3.2.8). We expected most of the sugar beet reads to map to the draft reference assembly. The actual result was that 68.7% (TR520) and 70.6% (TR363) reads could be mapped to the sugar beet reference genome. Thus, the fractions of unmapped reads were 546 million (TR520) and 506 million (TR363). These data were provided for the *de novo* assembly (3.2.8) of the unmapped reads, which was performed using the genome assembler SOAPdenovo. SOAPdenovo is based on the de Bruijn graph algorithm (de Bruijn, 1946), and had been specifically developed for application in next-generation short-read sequencing (Li et al., 2008; Li et al., 2009b). The output of the *de novo* assembly of the unmapped reads resulted in a total of 475,034 (TR520) and 663,544 (TR363) sequences and a total assembled length of 523 Mbp (TR520) and 595 Mbp (TR363) (Table 22).

The second strategy started directly with the *de novo* assembly of the total reads of both WGS sequencing experiments. We decided to implement this strategy in order to maintain the translocation-specific reads that may map to the sugar beet reference sequence in the first strategy because of high homology on the short read level. The settings for the *de novo* assembly using SOAPdenovo were the same as for the first strategy (see 3.2.8). As a result, 560,797 (TR520) and 817,526 (TR363) sequences were received with a total assembly size of 260 Mbp (TR520) and 325 Mbp (TR363) (Table 22).

We expected the output of the *de novo* assemblies to be a mixture of different sequences: those originating from *P. procumbens* equal to translocation-specific sequences and those belonging to the *B. vulgaris* genome but missing in the draft reference assembly.

Table 22: Output of different assembly strategies. The number of contigs displays all sequences that could not be integrated into scaffolds. A contig is defined as a contiguous sequence without gaps. A scaffold is composed of contigs including gaps.

Assembly	Total no.[a] of sequences	No. of contigs	No. of scaffolds	Size of longest scaffold (bp[b])	N50[c] (bp)	Mbp[d]
TR520 *de novo*	560,797	398,030	162,767	173,388	5,468	523
TR520 unmapped *de novo*	475,634	335,847	139,787	46,684	1,298	260
TR363 *de novo*	817,526	610,635	206,891	1,095,955	4,330	595
TR363 unmapped *de novo*	663,544	514,754	148,790	1,095,502	1,138	325

[a]no: number;[b]bp: basepair; [c]N50 is the size of the smallest scaffold in a subset of the largest scaffolds that adduce half of the total assembly size; [d]Mbp =megabasepair

N50 is not comparable between different assemblies and is defined as the smallest sequence in a subset of the fewest sequences whose assembled length adds up to 50% of the whole assembly (Miller et al., 2010).

The maximum sizes of the scaffolds of the translocation line TR520 assemblies are smaller than the scaffold sizes of the TR363 assemblies (Table 22). Differences in the assembly quality may be due to DNA quality, pre-sequencing processes as library preparation and PCR amplification, and during the sequencing process itself (Glenn, 2011; Hoeijmakers et al., 2011).

Table 23 shows the distribution of the sequence (scaffolds and contigs) sizes >1,000 bp obtained by the different assembly strategies of the translocation lines TR520 and TR363 after a first evaluation of the four assemblies. The share of sequences >1,000 bp is 13.7% for the 'TR520 *de novo'* and 16.1% for the 'TR520 unmapped *de novo'* assembly. The amount of sequences >1,000 bp adds up to 12.9% for the 'TR363 *de novo*' assembly and to 10.3% for the 'TR363 unmapped *de novo*' assembly.

Table 23: Evaluation of the four different assemblies of the translocation lines TR520 and TR363 regarding the distribution of sequence length > 1,000 bp. Assembly strategies are explained in 3.2.8.1.

Assembly	TR363 *de novo*		TR363 unmapped *de novo*		TR520 *de novo*		TR520 unmapped *de novo*	
range	**seq[a]**	**%**	**seq**	**%**	**seq**	**%**	**seq**	**%**
1,001-5,000	82,930	78.29	63,727	93.42	65,030	71.92	60,659	92.84
5,001-10,000	13,622	12.86	3,397	4.98	14,586	16.13	4,182	6.4
10,001-25,000	8,067	7.62	780	1.14	9451	10.45	485	0.74
25,001-35,000	806	0.71	113	0.17	931	1.03	6	0.01
35,001-50,000	301	0,28	68	0.1	341	0.4	2	0.003
50,001-75,000	109	0.1	57	0.1	79	0.09		
75,001-100,000	33	0.03	32	0.05	5	0.005		
100,001-200,000	29	0.03	25	0.04	1	0.001		
200,001-500,000	12	0.01	14	0.02				
500,001-1,000,000	2	0.002	2	0.003				
>1,000,000	2	0.002	2	0.003				
total	105,913	100	68,217	100	90,424	100	65,334	100

[a]Seq: number of sequences includes scaffolds and contigs

In summary, the assembly data of the 'TR363 unmapped *de novo*' and 'TR363 *de novo*' shows a broader range of sequence length and includes larger scaffolds and contigs compared to the translocation line TR520 assemblies. Of the two TR520 assemblies, the 'TR520 unmapped *de novo*' dataset has the lower amount of large sequences (> 50,000 bp). Therefore I decided to mainly use the 'TR520 *de novo*' and 'TR363 unmapped *de novo*' assemblies for further analyses.

3.3.2 Identification of New Translocation Specific Sequences Anchored to the Physical Map

The physical map of TR520 is composed of three BAC contigs (see Figure 1) (Capistrano, 2009). One aim was to select translocation-specific sequences anchored to BACs or YACs to extend the physical map. We performed scaffold walking in order to identify new sequences linked to the physical map in the following way: BLAST output datasets (see 3.2.8.2) were screened for significant alignments of the translocation lines TR520 and TR363 to BACs or YACs and significant alignments to *P. procumbens*. The identified sequences, scaffolds or contigs, were extended by BLAST analyses against the datasets 'TR520 unmapped *de novo*', 'TR520 *de novo'*, 'TR363 unmapped *de novo*', 'TR363 *de novo'*, and *P. procumbens* in order to identify new scaffolds that overlap on the proximal (centromeric) or distal (telomeric) end. In this way super scaffolds could be generated. By assembling the scaffolds and contigs of *P. procumbens* as well as the translocation lines TR520 and TR363 to the present BAC- and BAC/YAC-ends, additional sequence information anchored to BAC contig 1, BAC contig 2, and BAC contig 3 could be obtained (Supplementary Table 3-6). Consequently, super contigs were generated by incorporation of super scaffolds to the BAC contigs.

BAC contig 1 had been predicted to cover ~ 604 kbp. Prior to this work 362,095 bp were available from sequenced BACs and BAC-ends (Figure 1) (Capistrano, 2009). BAC 86F6 and BAC 113H20 were identified (data not shown) at the distal end of BAC contig 1. BAC 86F6 was Sanger sequenced and 113,221 bp were obtained. It overlaps 47,030 bp with BAC 7C2, meaning 66,190 bp were additionally provided (Suppl. Seq. information). Distal to BAC 86F6, BAC 113H20 was identified and the T7 end was sequenced (655 bp). Therefore the number of BAC and YAC sequences of BAC contig 1 sum up to 428,940 bp. After analysis of the new assembled sequences BAC contig 1 could be extended by 88,820 bp of the WGS data on the distal side. This sequence is exclusively located on the translocation line TR520 and absent from the translocation line TR363. On the proximal side, 164,795 bp were identified, that are shared between the *P. procumbens* translocations of the lines TR520 and TR363 with a sequence similarity between 86 – 100% (Suppl. Table 3 and 4). BAC contig 1 incorporates now 253,615 bp WGS sequences and in the following I will refer to that as super contig 1. Up to now, the total sequence of super contig 1 sums up to 682,555 bp (Table 24; Figure 11)

BAC contig 2 had been suggested to be ~ 308 kbp in size. Prior to this work, 76,716 bp sequence information from BACs and YACs were available (Figure 1) (Capistrano, 2009). On the proximal side of BAC contig 2, two new BACs, 142D13 and 56F10, were identified by chromosome walking with BAC-end 96M13Sp6 (data not shown). Therefore, additional sequence information of the sequenced BAC-ends was provided: 142D13 Sp6 (582 bp), 142D13 T7 (676 bp), 56F10 Sp6 (800 bp), and 56F10 T7 (810 bp) (Suppl. Seq. information). The lengths of BAC and BAC-end sequences of BAC contig 2 add up to 79,584 bp. The sequence analysis of the WGS data resulted in 119,052 bp new sequences derived from the translocation line TR520. A new region of 44,415 bp shared between the *P. procumbens* translocations of the lines TR520 and TR363 was identified as part of this scaffold (Figure 11). Additionally, 16,213 bp were found to be located only on the translocation line TR363 (Suppl. Table 3 and 4). Thus, the sequence information of BAC contig 2 was extended by incorporating 179,680 bp of the WGS sequences and therefore adds up to 259,264 bp (Table 24).

BAC contig 3 had been suggested to be ~572 kbp in size. Prior to this work 523,813 bp BAC and YAC sequences were available (Capistrano, 2009) (Figure 1). Sequencing extended this scaffold by 59,599 bp originating from the translocation line TR520 (Suppl. Table 3 and 4). As a result, BAC contig 3 incorporates now 59,599 bp WGS sequences and adds up 583,412 bp (Table 24).

Figure 11 (shown on next page): A sequence-based physical map of the *P. procumbens* translocation of line TR520. The map was aligned with molecular markers (CAU numbers) derived from bacterial artificial chromosome (BAC) and yeast artificial chromosome (YAC) ends, and scaffold sequences obtained by WGS sequencing (see Figure 1). A minimal tiling path of YACs and BACs, and the scaffolds from WGS sequencing are integrated into this map. 'Sections' denote translocation specific sequences present or absent on different translocation lines identified by marker analysis. The term 'super scaffold' (SS) is used for assembled contigs and scaffolds of different genome assemblies of *P. procumbens* and of the translocation lines TR520 and TR363 of the WGS data. 'Super contigs' are the largest types of sequence assemblies on the physical map and incorporate BACs and super scaffolds of the WGS sequencing whereas the contigs of the physical map presented by Capistrano (2009) are named 'BAC contigs' in the following (compare Figure 1). Scaffold mapping is shown in Figure 14. Regions in common between both resistant translocations and absent from the susceptible translocation are highlighted in yellow. BAC 149P7 carries a B. vulgaris insertion which is indicated in green (see chapter 3.3.9). Regions in common between both resistant translocations and absent from the susceptible translocation are highlighted in yellow. The identified 16.2 kbp region located only on TR363 is not indicated in this map (see Table 24).

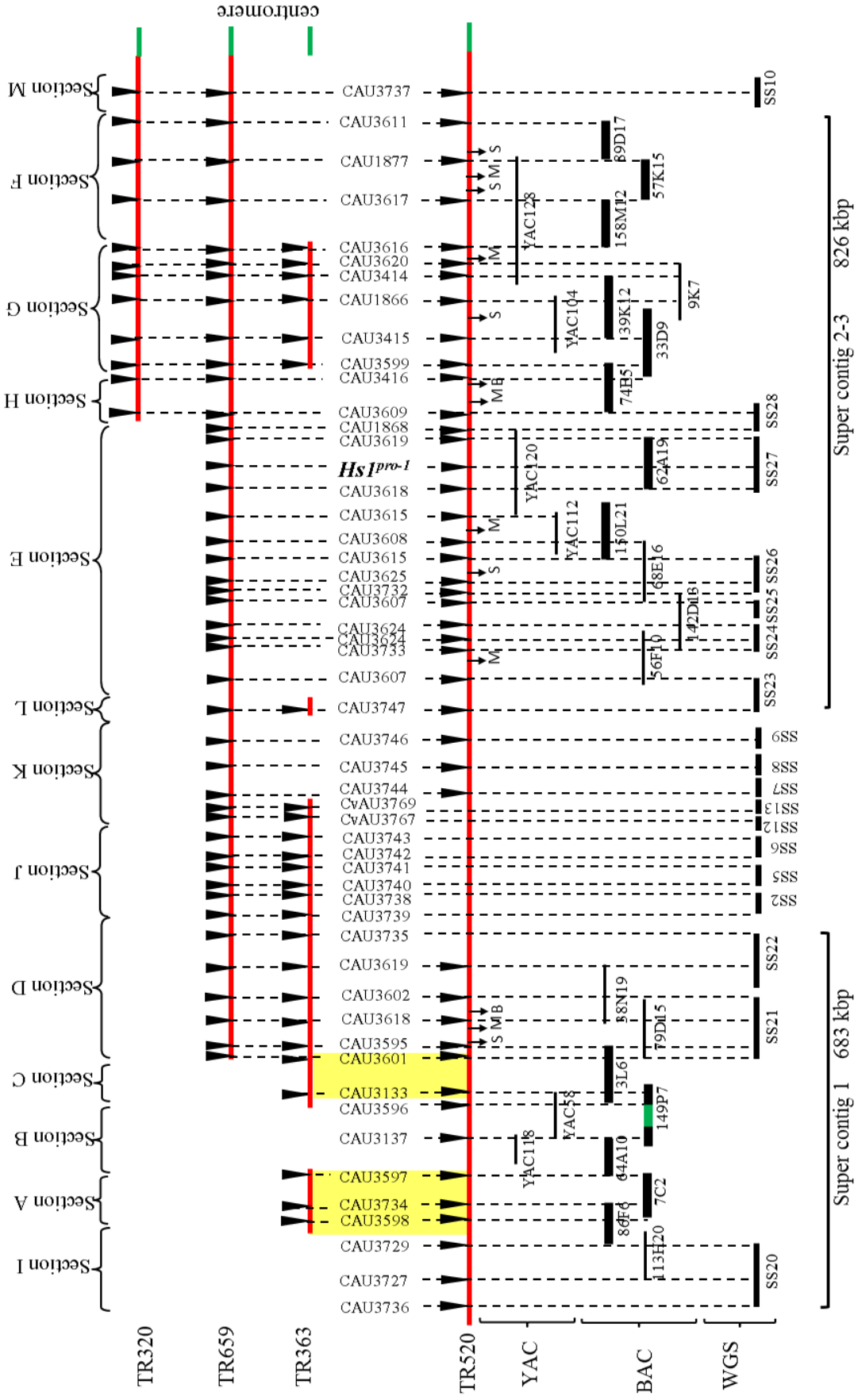

Figure 11: A sequence-based physical map of the *P. procumbens* translocation of line TR520. (For details, see previous page.)

In summary, the amount of BAC and YAC sequences sum up to 1,032.3 kbp (BAC contig 1: 428,940 bp + BAC contig 2: 79,584 bp + BAC contig 3: 523,813 bp) (Table 24). The number of new sequences identified in the assemblies of the translocation lines TR520 and TR363, that were incorporated into BAC contig 1, 2, and 3 sum up to 492,894 bp (incorporated into BAC contig 1: 253,615 bp + BAC contig 2: 179,680 bp + BAC contig 3: 59,599 bp). New sequences identified in the scaffolds and contigs of the assembly data exclusively located on the translocation line TR520 add up to 267,471 bp (incorporated into BAC contig 1: 88,820 bp + BAC contig 2: 119,052 bp + BAC contig 3: 59,599 bp). New sequences identified in the assembly data shared by the translocation lines TR520 and TR363 sum up to 209,210 bp (incorporated into BAC contig 1: 164,795 bp + BAC contig 2: 44,415 bp). New sequences identified in the assembly data exclusively located on the translocation line TR363 sum up to 16,213 bp (Table 24).

Table 24: Results from WGS sequencing of two translocation lines (TR520 and TR363) to increase the previously identified BAC contigs on the *P. procumbens* translocation from nematode resistant translocation lines TR520 and TR363. Total number of kilo base pairs (kbp) is the summarized amount of sequence information.

Sequences	**BAC contig 1** (kbp[a])	**BAC contig 2** (kbp)	**BAC contig 3** (kbp)	**Total** (kbp)
New sequences[b] only on TR520	88.8	119.1	59.6	267.5
New sequences only on TR363	-	16.2	-	16.2
New sequences overlapping between TR520 and TR363 (kbp)	164.8	44.4	-	209.2
Total of new sequences (kbp)	253.6	179.7	59.6	492.8
Previously available BAC and YAC sequences[c] (kbp)	428.9	79.6	523.8	1,032.3
Total of new and previously available sequences (kbp)	682.5	259.3	583.4	**1,525.2**

[a] kbp: kilo basepair; [b]New sequences describe the size of scaffolds and contigs in kbp identified in the assemblies of the translocation lines TR520 and TR363. [c]BAC and YAC sequences correspond to the sequence information determined by Capistrano (2009) prior to this work, and extended in this work.

During the analysis of the WGS scaffolds anchored to the physical map, the *P. procumbens* scaffold 6340 (12,371 bp) was identified, which enabled physical linkage of YAC-end 120R (BAC contig 2) located on scaffold 27215 of the 'TR520 *de novo*' assembly and BAC-end 74E5T7 (BAC contig 3) located on scaffold 42352 of the 'TR520 *de novo*' assembly. This new sequence linker connecting the BAC contigs is 319 bp in size and present on *P. procumbens* scaffold 6340. It originates exclusively from the translocation line TR520 while it is absent from the line TR363. In this way the BAC contig 2 and 3 could be linked and incorporate 239 kbp WGS sequences. From now on I will refer to that as super contig 2-3 with a total size of 842,995 bp (259,264 bp + 583,412 bp + 319 bp) (Figure 12).

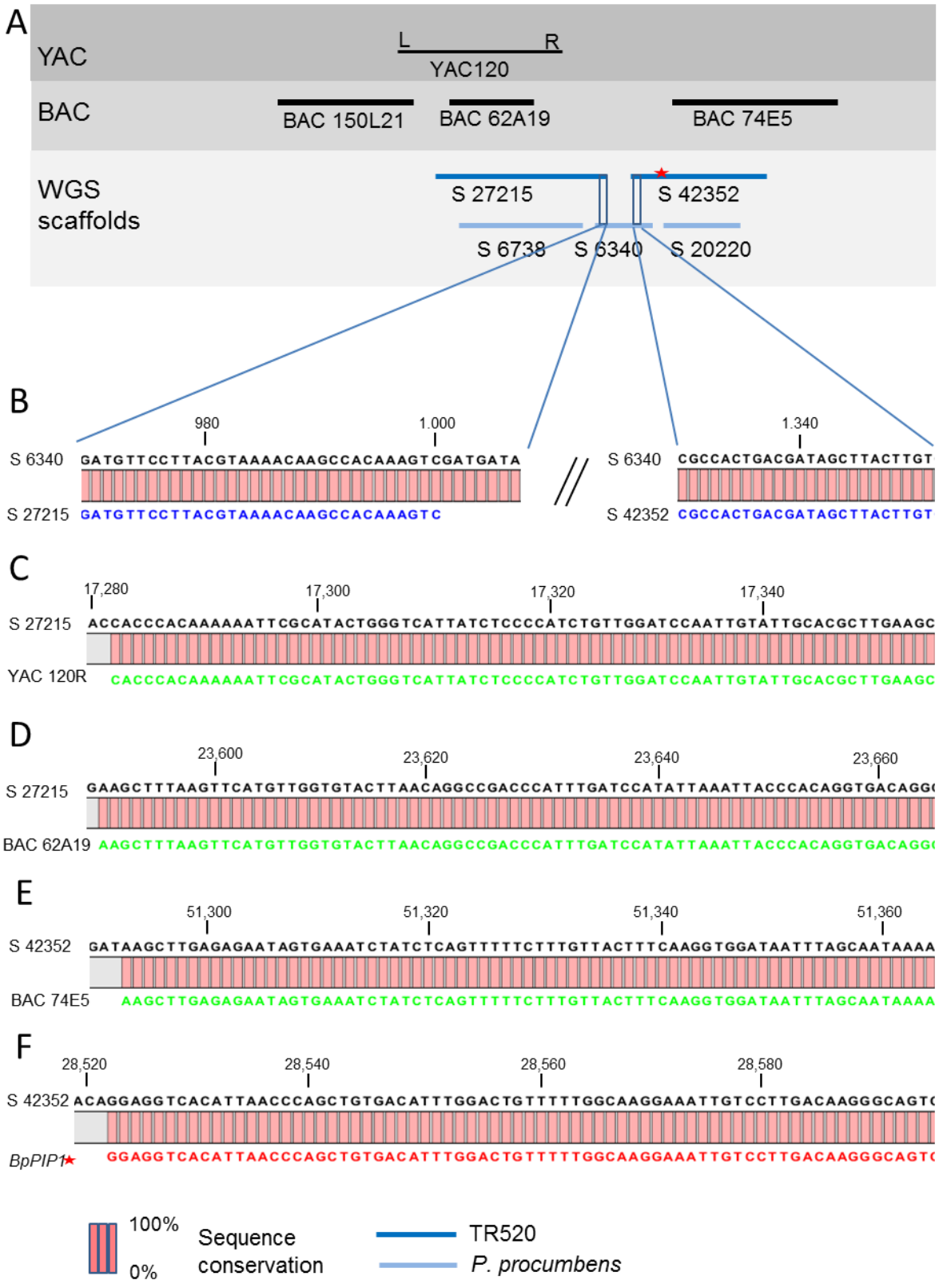

Figure 12: This figure gives a graphical overview about how BAC contig 2 and BAC contig 3 are linked. **(A)** The upper part of this figure shows an overview of the sequence linker (319 bp) of *P. procumbens* scaffold 6340 overlapping with 'TR520 *de novo*' scaffold 27252 (bearing YAC-end 120 R and BAC 62A19) and 'TR520 *de novo*' scaffold 42352 (overlapping BAC 74E5 and the gene *BpPIP1* (see 3.3.8). In this way, BAC contig 2 and BAC contig 3 could be linked and orientated on the physical map. Prior to this work, BAC 74E5 was linked to BAC contig 3 and BAC 62A19 to BAC contig 2 on the BAC-based physical map (Figure 1). **(B)** In the lower part of the figure, apertures of the sequence alignments are shown. The sequence comparison is shown as a graph. A scale is given in base pair. Scaffold 6340 (*P. procumbens*) overlaps with the scaffold 27215 and 42352 of the assembly 'TR520 *de novo*'. **(C)** Scaffold 27215 of the 'TR520 *de novo'*

assembly overlaps with YAC end 120 R. **(D)** Scaffold 27215 of the 'TR520 *de novo'* assembly overlaps with BAC 62A19. **(E)** Scaffold 42352 of the 'TR520 *de novo'* assembly overlaps with BAC 74E5. **(F)** Scaffold 42352 of the 'TR520 *de novo'* assembly overlaps with the gene *BpPIP1* (see 3.3.8) (Menkhaus 2011). Different colors in the alignments highlight the different sequence types: blue and black = scaffolds; green = BAC- and YAC sequences; red = genes. The complete information about the sequence alignments can be found in Suppl. Tables 5, 6, and 26.)

3.3.3 Identification of New Translocation Specific Sequences Not Anchored to the Physical Map

There are two different groups of not anchored translocation-specific sequences. The first group is formed by sequences where no anchor marker present on the physical map could be identified. The second group is formed by sequences that are exclusively present in the translocation line TR363. To identify translocation-specific sequences that are not anchored to the physical map, BLAST analyses were performed with the 'TR520 *de novo*' and the 'TR363 unmapped *de novo*' datasets against the *P. procumbens* genome assembly and the physical map BLAST database using the CLC Bio Genomics Workbench (3.2.8.2). All sequences of the translocation lines TR520 and TR363 that were significantly aligned to *P. procumbens* and absent in the BLAST output data of alignments to the physical map sequences were separated. Subsequently, the datasets were analyzed in order to select *P. procumbens*-specific sequences that were significantly aligned to the data of the translocation lines TR520 and TR363. This resulted in the identification of 22 *P. procumbens* scaffolds (see Supplementary Table 7).

These 22 *P. procumbens* scaffolds were extended by scaffold walking as explained in 3.3.2, resulting in 13 super scaffolds (denoted as super scaffold1 – super scaffold 13), which integrate shared sequences of scaffolds and contigs of the *P. procumbens* assembly, the 'TR520 *de novo*', and the 'TR363 unmapped *de novo*' assembly (Figure 14; see Suppl. Tables 8-20 for BLAST results). As an example, the new super scaffold 2 that is not anchored to BACs and YACs of the physical map is graphically detailed in Figure 13.

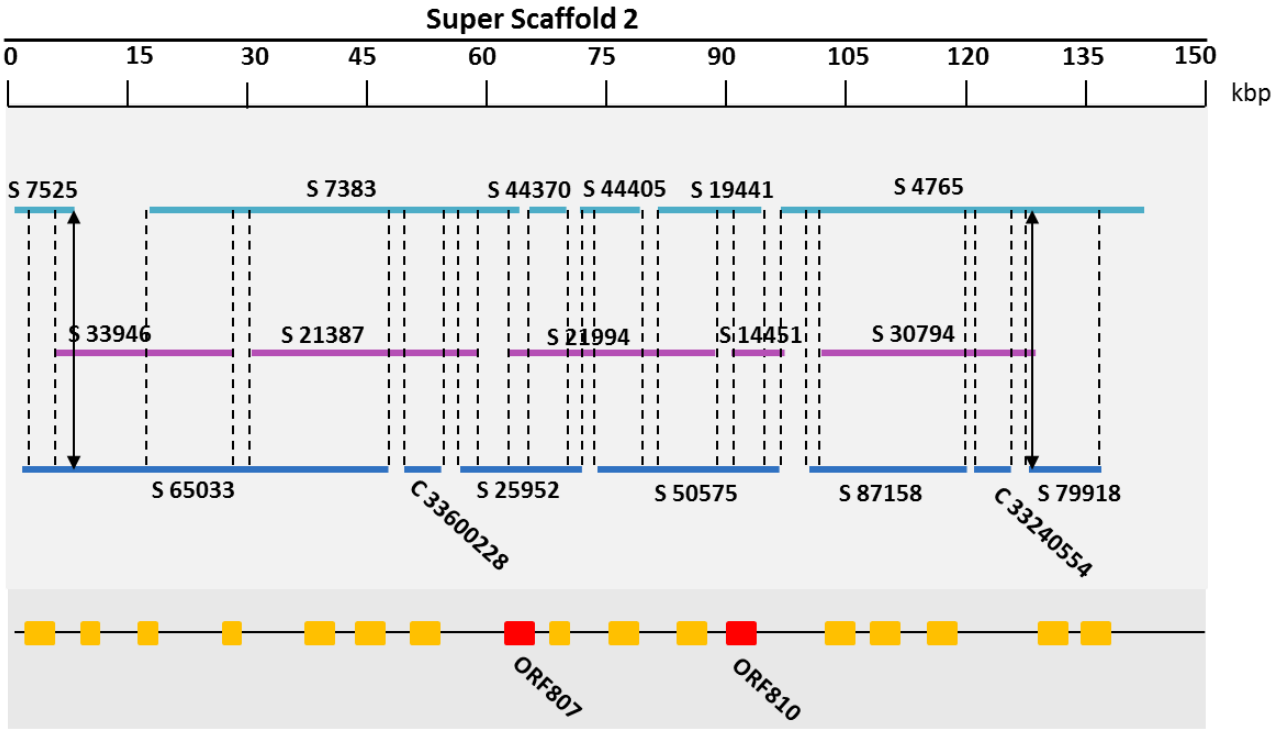

Figure 13: Graphical overview of super scaffold 2 that is not anchored to the physical map. A scale is given in kilo base pairs (kbp). This super scaffold integrates scaffolds (S) and contigs (C) of the 'TR520 *de novo'* (dark blue), 'TR363 unmapped *de novo'* (purple) and of the *P. procumbens* (P.p.) (light blue) assembly. At least two sequences overlap at any

given point in super scaffold 2. In the lower part, the location of the predicted ORFs (yellow boxes) is shown and the two selected ORFs 807 and 810 are highlighted in red. Detailed information about the alignments is listed in Suppl. Table 7 and further information about the ORF prediction on super scaffold 2 can be found in Suppl. Table 19.

The number of assembled super scaffolds that integrate the 22 *P. procumbens* scaffolds is lower as scaffold walking sometimes resulted in the integration of more than one previously identified *P. procumbens* scaffold. Table 25 shows the result of the scaffold walking experiments.

Table 25: 13 super scaffolds (super scaffold 1- super scaffold 13) were identified and assembled by scaffold walking. They integrate a number of overlapping scaffolds/contigs of the 'TR520 *de novo*'-, the 'TR363 unmapped *de novo*', and the *P. procumbens* genome assembly. The number of homologous sequences between the translocation lines TR520 and TR363 is shown (Suppl. Tables 6-18).

Super scaffold name	*P. procumbens* no. of scaffolds/ contigs	Size (bp[a])	TR520 no. of scaffolds/ contigs	Size (bp)	TR363 no. of scaffolds/ contigs	Size (bp)	Homologous sequences TR520/ TR363 (bp)
1	4	107,429	4	26,719	2	109,576	19,868
2	6	136,506	7	124,116	6	119,915	103,001
3	1	54,426	6	50,357	6	44,567	34,015
4	7	114,952	1	4,016	2	122,134	2,889
5	7	173,102	2	90,656	2	59,953	14,380
6	5	251,960	4	112,662	1	73,600	57,589
7	1	80,745	5	75,496	-	-	-
8	7	32,457	1	34,447	-	-	-
9	3	21,524	2	27,810	-	-	-
10	6	204,206	4	78,655	-	-	-
11	3	97,463	3	48,801	2	94,289	39,933
12	1	9,674	1	8,906	1	12,157	6,890
13	2	67,035	1	34,284	5	60,758	29,321
Total	**53**	**1,351,479**	**41**	**716,925**	**27**	**696,949**	**307,886**

[a]bp: basepair

Super scaffold 2 integrates the largest amount of sequences (124.1 kbp, 7 scaffolds) of the 'TR520 *de novo*' assembly, whereas super scaffold 4 integrates 122.1 kbp (2 scaffolds) of the 'TR363 unmapped *de novo*' assembly. The largest amount of *P. procumbens* specific sequences sums up to 252 kbp (5 scaffolds) and was integrated into super scaffold 6. During the scaffold walking analysis, a number of TR520 translocation line specific scaffolds (super scaffold 7 – super scaffold 10) were identified, adding up to an amount of 216.4 kpb. These sequences are present in the genome assembly of *P. procumbens* but absent from the translocation line TR363. The amount of overlapping sequences between the lines TR520 and TR363 was calculated by adjusting the BLAST output dataset to alignments with a bit score >250. The size of the homologous sequences (denoted as 'hit length' in the BLAST output data) between the translocation lines TR520 and TR363 is 307,886 bp (see Suppl.Tables 8-13; 18-20).

A total of 716.9 kbp of the line TR520 not anchored to the physical map were identified. The number of sequences for the *P. procumbens* translocation of the line TR363 adds up to 696.9 kbp. New sequences shared between the translocation lines TR520 and TR363 add up to 307.9 kbp. The identified scaffolds and contigs selected of the *P. procumbens* genome assembly add up to 1351.5 kbp.

In total, the sequences identified for the *P. procumbens* translocation of the line TR520 add up to 2,226 kbp (1,509 kbp + 717 kbp) (see Table 24 and Table 25.)

For the first time sequences exclusively located on the *P. procumbens* translocation of the line TR363 were selected. A total of 16.9 kbp (Table 24) anchored to the physical map and 389 kbp (Table 25) not anchored were identified. In addition, the BAC sequences could be compared to the assemblies of TR363 whereas in previous works only short sequences mostly of BAC ends were used to investigate the sequence similarity to the *P. procumbens* translocation of TR363 (Capistrano, 2009; Schulte, 2006). The comparative sequence analysis revealed 282,937 bp with high sequence similarity and low e-values to TR363 (Suppl. Table 3-4). This result has to be regarded as a preliminary result since the assemblies of the line TR363 contain gaps. Moreover, a total of 209 kbp (Table 24) anchored and 308 kbp (Table 25) not anchored to the physical map shared between the lines TR520 and TR363 were identified in this work. In summary 1,206 kbp could be identified for the *P. procumbens* translocation of the line TR363.

3.3.4 An Integrated Map of the TR520 Translocation

The existing physical map (Capistrano, 2009) was extended by identification of new sequences anchored to BAC contig 1, BAC contig 2, and BAC contig 3. After connecting BAC contig 2 and 3 to one super contig which is now termed super contig 2-3, the physical map now consists of two super contigs, super contig 2-3 and super contig 1 (Figure 11, Figure 1). Additionally, the new non-anchored translocation-specific super scaffolds were integrated into the physical map after mapping to the translocations lines as described in the following.

To physically map the new sequences, primers were designed from the proximal and distal ends of each super scaffold. PCR was performed with genomic DNA of all four translocation lines, as well as *P. procumbens* (35335) and *B. vulgaris* (930176) (see Figure 14).

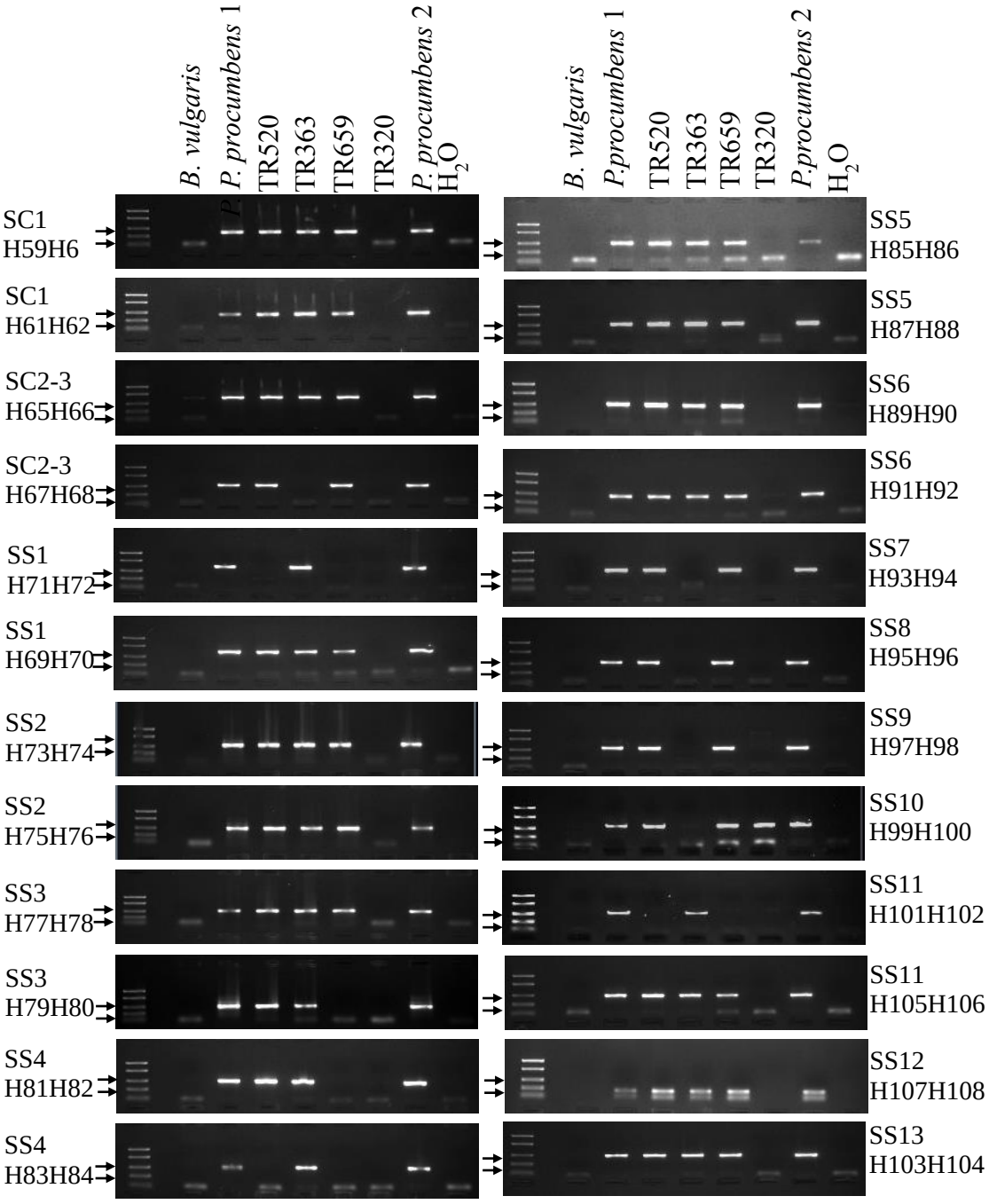

Figure 14: Mapping of sequence super scaffolds by PCR. Primers were derived from proximal and distal sequences of the scaffolds (as an example see Figure 13; Table 21). Genomic DNA of *B. vulgaris* (seed code 930176), *P. procumbens* 1 (seed code 35335), *P. procumbens* 2 (seed code 100001) and the translocation lines TR520, TR363, TR659, and TR320 were used as templates. The upper arrow indicates the 400 bp and the lower arrow indicates the 200 bp fragment of the low range DNA ladder. Fragments were separated in 1.5**%** agarose gels. 'SC'= super contig; 'SS'= super scaffold.

Table 26: PCR with genomic DNA from *B. vulgaris* (930176), *P. procumbens* (35335 and 100001), and the translocation lines TR520, TR363, TR659, and TR320 in order to map the new sequences assembled to super scaffolds or integrated into the BAC contigs (super contigs) to the physical map (super scaffold and super contig map positions see Figure 11). Primers were derived from the distal and/or proximal side of the sequence (Table 21).

Sequence	Primer	*B. vulgaris*	*P. procumbens*	TR520	TR363	TR659	TR320
Super contig 1	H59H60	-[a]	x[b]	x	x	x	-
	H61H62	-	x	x	x	x	-
Super contig 2-3	H65H66	-	x	x	x	x	-
	H67H68	-	x	x	-	x	-
Super scaffold 1	H69H70	-	x	x	x	x	-
	H71H72	-	x	-	x	-	-
Super scaffold 2	H73H74	-	x	x	x	x	-
	H75H76	-	x	x	x	x	-
Super scaffold 3	H77H78	-	x	x	x	x	
	H79H80	-	x	x	x	-	-
Super scaffold 4	H81H82	-	x	x	x	-	-
	H83H84	-	x	-	x	-	-
Super scaffold 5	H85H86	-	x	x	x	x	-
	H87H88	-	x	x	x	x	-
Super scaffold 6	H89H90	-	x	x	x	x	-
	H91H92	-	x	x	x	x	-
Super scaffold 7	H93H94	-	x	x	-	x	-
Super scaffold 8	H95H96	-	x	x	-	x	-
Super scaffold 9	H97H98	-	x	x	-	x	-
Super scaffold 10	H99H100	-	x	x	-	x	x
Super scaffold 11	H101H102	-	x	-	x	-	-
	H105H106	-	x	x	x	x	-
Super scaffold 12	H107H108	-	x	x	x	x	-
Super scaffold 13	H103H104	-	x	x	x	x	-

[a]X: indicates presence of amplicons with expected size; [b]-: indicates no amplicons.

As a result of the PCR experiments I was able to confirm that all sequences are derived from the *P. procumbens* genome, because no amplicon was obtained with genomic DNA of sugar beet (Figure 14, Table 26). Figure 11 shows the integration of super scaffolds to the physical map. As explained at the beginning of chapter 3.3.1, scaffolds are composed of contiguous sequences (contigs) with gaps in between and provide order and orientation of the contigs. On the physical map super scaffolds are composed of *P. procumbens* sequences (scaffolds and contigs) and sequences (scaffolds and contigs) derived from the new assemblies of the translocation lines TR520 and TR363. Super contig 1 and super contig 2-3 integrate BACs, YACs and the newly identified and assembled super scaffolds that could be anchored to the physical map. Sections define regions that are shared by different translocation lines according to the comparative analysis of the resistant and susceptible translocation lines by Capistrano (2009). Thus, a super contig or a super scaffold can integrate one or more sections. The existing sections (section A – section H) on the physical map (Capistrano, 2009) were extended by the new scaffolds and in this way new sections were established (section I - section M) (Figure 11). The presented physical map consists of a minimum tiling path of two super contigs (super contig 1 and

super contig 2-3) and new super scaffolds that could be mapped to the physical map but that are not anchored to any BAC or YAC (Table 26).

Section I (BAC 86F6 T7, BAC-end 113H20 T7 and super scaffold 20) is located at the distal (telomeric) side of super contig 1 on the physical map and represents a region that is present on *P. procumbens* and the translocation line TR520. It is absent from the translocation lines TR363, TR659, and TR320. Super scaffold 2, super scaffold 5, super scaffold 6, super scaffold 12 and super scaffold 13 map with their ends to *P. procumbens*, and the translocation lines TR520, TR363, and TR659. They were placed to section J because in this section the translocation lines TR520, TR363 and TR659 share homologous sequences. On the physical map section J is located proximally (centromeric) to super contig 1 and is not covered by BACs. The sequences of these 5 scaffolds sum up to 370,625 bp (TR520 sequences; Table 25). Section K represents three scaffolds (super scaffold 7, super scaffold 8, and super scaffold 9) that map to *P. procumbens*, and to the translocation lines TR520, and TR659. They were placed distally to super contig 2-3. The total size of these scaffolds adds up to 137,753 bp. In accordance to this, a total of 508 kbp new sequences were identified. These super scaffolds are suggested to be located between super contig 1 and super contig 2-3. However, the scaffold orientation, and order in sections J and section K as well as the gap sizes between them, remains unknown.

New sequences present on *P. procumbens*, and the translocation lines TR520, TR363, and TR659 were identified and anchored to BAC 56F10 on the distal side of super contig 2-3 on the physical map. This region is denoted as section L. Super scaffold 10 maps to *P. procumbens* and the translocation lines TR520, TR659, and TR320, and was placed to section M in the very proximal region of the physical map next to super contig 2-3.

The following super scaffolds could not be integrated into the physical map because they were mapped to regions that are not yet determined. These sequences must be further analyzed by an assembly with long-mate pair sequences. In the cases of super scaffold 11 one end was determined to be present on *P. procumbens* and the translocation lines TR520, TR363, and TR659, whereas the other end is present only on *P. procumbens* and the translocation line TR363. Super scaffold 3 was mapped with one end to *P. procumbens* and the lines TR520, TR363, and TR659 and with the other end to *P. procumbens*, and the resistant translocation lines TR520 and TR363. Super scaffold 4 was also mapped to a region present on *P. procumbens* and the resistant lines TR520 and TR363, but in contrast to super scaffold 3 the other end of super scaffold 4 is present only on *P. procumbens* and the translocation line TR363. These sequences are suggested to be located within a region that is not homologous between the resistant translocation lines TR520 and TR363. The reason for this might be different origins of the TR520 and TR363 translocation lines.

3.3.5 Open Reading Frame Prediction and Annotation

Even though Capistrano (2009) analyzed 962,624 bp of BAC and YAC sequences, I decided to analyze all TR520 translocation sequences that are anchored to the physical map including super contig 1 and super contig 2-3 (Suppl. Table 3-6 and 22). The reason for this is that either most of the sequences are exclusively located on the translocation line TR520 or they are shared with high sequence similarity to the translocation line TR363, and BACs and YACs. In addition, the databases on NCBI (National Center for Biotechnology Information (NCBI), 2012) were updated frequently, so that more information is available since Capistrano's (2009) analyses (Suppl. Tables 3-6). The ORF prediction and the exon-intron structure was determined using the ORF prediction web-based tools FGENESH (Softberry FGENESH, 2007) or GENSCAN (GENSCAN, 2009), as explained in 3.2.8.3. Sequence similarities to known proteins from other organisms were identified using the tool protein BLAST with the non-redundant protein database on the NCBI platform.

In respect of the anchored sequences of super contig 1, 667,171 bp were analyzed and 69 ORFs were predicted. Sequence similarity analysis to known proteins revealed (Suppl. Table 20) no significant similarities for 21 ORFs. Another 21 ORFs showed significant similarity to unclassified proteins. A number of 8 predicted ORFs were detected to have significant similarities to retroelements and transposons. Therefore, the number of predicted ORFs showing significant similarities to known proteins from other organisms is 19.

All identified sequences of the 'TR520 *de novo* assembly' (813,921 bp) of super contig 2-3 were analyzed for the presence of ORFs. A total of 117 ORFs were predicted (3.2.8.3) and analyzed for similarity to known genes using the NCBI non-redundant protein database. No significant similarities to known genes from other organisms were observed for 35 ORFs, 30 ORFs showed similarities to unclassified proteins, and 14 ORFs were detected to have significant similarity to retroelements or transposons. Significant similarities to proteins with known functions from other organisms were identified for 38 predicted ORFs.

Since a large part of the non-anchored super scaffolds is shared between the lines TR363 and TR520 and to avoid an overestimation of the identified ORFs, I performed the ORF identification either with TR363 or TR520 scaffolds, always choosing the largest scaffolds of a super scaffold for the analysis. The following sequences of the super scaffolds were searched for ORFs: 109,576 bp of super scaffold 1, 124,116 bp of super scaffold 2, 50,357 bp of super scaffold 3, 122,134 bp of super scaffold 4, 90,656 bp of super scaffold 5, 122,662 bp of super scaffold 6, 75,496 bp of super scaffold 7, 34,447 bp of super scaffold 8, 27,810 bp of super scaffold 9, 78,655 bp of super scaffold 10, 94,289 bp of super scaffold 11, 12,157 bp of super scaffold 12, and 60,758 bp of super scaffold 13 (Table 25). In total 993,113 bp non-anchored sequences were analyzed. ORF prediction of the non-anchored sequences resulted in 134 ORFs. Significant similarities to known genes from other organisms were identified by BLAST (blastp) using the non-redundant protein database on the NCBI platform (3.2.8.3). A number of 44 ORFs out of the predicted 134 ORFs, showed no significant similarity to known proteins from other organisms in the non-redundant protein database of NCBI. Sequence similarity to unclassified hypothetical proteins was identified in 25 predicted ORFs, and a number of 20 ORFs showed significant similarity to retroelements or transposons. For 45 predicted ORFs, significant similarities to proteins from other organisms were determined (All data in Suppl. Table 21).

Table 27: Results of the open reading fame (ORF) prediction of all sequences identified to be anchored and non-anchored to the physical map. Sequence similarities to known proteins from other organisms were determined by BLASTp analysis using the non-redundant protein database on the NCBI platform. The number and percentage of ORFs with significant similarities (e-value > 0) to unclassified proteins, retroelements, and known proteins are given.

Sequences	**Anchored sequences**		**Non-anchored sequences**	**Total**
	Super contig 1	**Super contig 2-3**		
Sequences analyzed (bp)	667,171	813,921	993,113	2,474,205
ORFs with no significant similarity (%)*	21 (30.4)	35 (30.0)	44 (32.8)	100 (31.1)
ORFs with similarity to unclassified proteins (%)	21 (30.4)	30 (25.6)	25 (18.7)	76 (23.6)
ORFs with similarity to retroelements (%)	8 (11.6)	14 (11.9)	20 (14.9)	42 (13.0)
ORFs with similarity to known proteins (%)	19 (27.5)	38 (32.5)	45 (33.6)	103 (32.0)
Total number of ORFs	69	117	134	320

*percentage of specified ORFs per column

In summary, 320 ORFs were analyzed to identify candidate sequences for the *Hs1-2* gene. No significant similarities to known proteins from other organisms were determined for 31% of all ORFs.

Significant similarities to unclassified proteins were determined for 23.6% of the ORFs. ORFs with similarity to retroelements and transposons were determined for 13% of the ORFs, and 32% of the ORFs showed significant similarity to proteins with known functions from other organisms. Eleven ORFs (ORF 801 - ORF 811) were selected for further analysis in accordance to their position on the physical map and with sequence similarity to known genes involved in plant stress-response

3.3.6 Candidate Gene Identification

Eleven ORFs were selected as resistance gene candidates for *Hs1-2* due to their homology to genes known to be involved in plant response to nematodes or plant stress response (Table 28). The searching criteria were as follows: plants have evolved two systems to defend themselves against pathogens: the pathogen-associated molecular patterns (PAMP) triggered immunity (PTI) and the effector-triggered immunity (ETI). Pattern recognition receptors (PRRs) play a major role on the plant side, and can be grouped into two classes: receptor-like kinases (RLKs), carrying a serine/threonine kinase domain, and receptor-like proteins (RLPs), which have an intracellular cytoplasmic tail. During ETI, resistance genes (R-genes) encode proteins that mostly consist of a nucleotide-binding site (NBS) and an LRR motif for effector detection (Göhre and Robatzek, 2008). Many of the cloned nematode resistance genes belong to the group of NBS-LRR genes, such as *Mi-1.2*, *Gpa2*, *Gro1-4*, or *Ma* (Claverie et al., 2011; Milligan et al., 1998; Paal et al., 2004; van der Vossen et al., 2000; Vos et al., 1998). Additionally, plants have evolved mechanisms of systemic immunity called systemic acquired resistance (SAR) in which phenolic signaling molecules like salicylic acid (SA) are required. Plants express pathogenesis-related (PR) proteins as it is the case for the *A. thaliana NPR1* gene (Cao et al., 1997). Another form of systemic resistance has been referred to as induced systemic resistance (ISR) which is independent of SA but requires the operation of the plant growth hormones jasmonic acid (JA) and ethylene (Feys and Parker, 2000).

The first criteria for the selection of candidate sequences for the *Hs1-2* gene was the sequence similarity to genes known to be involved in stress response as explained above. Regarding the hypothesis that the *Hs1-2* gene must be located on sequences only shared by the *P. procumbens* translocation of the resistant lines TR520 and TR363, the location of the ORFs was the second criterion. Nevertheless, ORFs that fulfill the first criterion but not the second one were selected from the sections D and J (Figure 11). These sections are shared by the lines TR520, TR363, and TR659. ORFs present in these sections were selected for further analyses because the susceptible translocation lines TR659 and TR320 were generated by irradiation and thus, a possibility of mutations within genes exist.

The ORFs mentioned in this chapter are from newly assembled super scaffolds both anchored and non-anchored to the physical map (super contig 1 and super contig 2-3, and super scaffold 1 - super scaffold 13) (Suppl. Table 21 and 22). Their numbers start with an ‘8’. The mapping of the ORFs to the translocation lines TR520, TR363, TR659 and TR320 is shown in Table 29. The exon/intron structure was determined by transferring selected non-anchored sequences of the TR520 and TR363 assemblies to the web-based tools FGENESH (Softberry FGENESH, 2007) or GENSCAN (GENSCAN, 2009) (3.2.8.3). The exon/intron structure of the selected ORFs is shown in Supplementary Figure 4A.

Table 28: NCBI protein BLAST (National Center for Biotechnology Information (NCBI), 2012) search for open reading frame sequences (ORF) predicted by FGENESH (Softberry FGENESH, 2007) or GENSCAN (GENSCAN, 2009) (3.2.8.3), which display homology to known genes that are involved in plant pathogen resistance. Identity on amino acid (aa) level, query region of homology (aa) and coverage in percent (%) is given.

ORF	Amino Acids (aa)	Location	Query region aa (%)	Accession No.	Description	E-value	Identity (aa)
801	351	super contig 1	119-347 (65)	XP_003555013.1	Leucine rich repeat receptor-like serine/threonine-protein kinase [*Glycine max*]	2e-107	71%
802	556	super contig 1	3-446 (77)	XP_002267025.1	phosphatidylinositol 4-kinase [*Glycine max*]	0	72%
803	1,131	super contig 1	909-1,070 (15)	CAB87658.1	MAP3K[a] delta-1 protein kinase [*Arabidopsis thaliana*]	5e-81	83%
804	147	super contig 1	1-67 (45)	NP_196746.2	alpha motif and leucine zipper containing kinase (AZK) [*Arabidopsis thaliana*]	8e-29	75%
805	349	super scaffold 5	1-175 (50)	NP_179268.2	basic leucine zipper (bZIP) domain-containing protein [*Arabidopsis thaliana*] transcription factor	7e-44	51%
806	1,154	super scaffold 4	1-1,146 (99)	XP_002526758.1	Disease resistance protein RGA[b]2, putative [*Ricinus communis*]	0	39%
807	556	super scaffold 2	3-533 (95)	XP_002280747.1	receptor-like protein kinase At1g67000-like [*Vitis vinifera*]	5e-141	45%
808	165	super scaffold 13	41-153 (68)	XP_003550743.1	spermidine synthase-like [*Glycine max*]	7e-42	61%
809	386	super scaffold 6	35-101 (17)	AAC35489.1	clp[c] protease [*Arabidopsis thaliana*]	7e-29	87%
810	316	super scaffold 2	8-202 (62)	NP_567422.1	F-box/LRR[d]-repeat protein [*Arabidopsis thaliana*]	6e-11	30%
811	348	super scaffold 5	146-343 (56)	XP_002279064.2	ATP[e]-dependent zinc metalloprotease [*Vitis vinifera*]	8e-69	59%

[a]mitogen activated protein kinase kinase kinase; [b]resistance gene analog; [c]caseinolytic protease; [d]leucine-rich repeat; [e]adenosin triphosphate;

3.3.6.1 Expression Analysis of Selected Candidate Sequences Detected after WGS Sequencing

Eleven ORFs shown in Table 28 were selected due to their sequence similarity to known proteins involved in plant stress response. Primers were designed based on exon regions of the ORFs (Table 21 and Suppl. Figure 4A). Selected ORFs were mapped to the physical map by PCR experiments using genomic DNA of *B. vulgaris*, *P. procumbens*, and the translocation lines TR520, TR363, TR659, and TR320 (Table 29; Figure 15A).

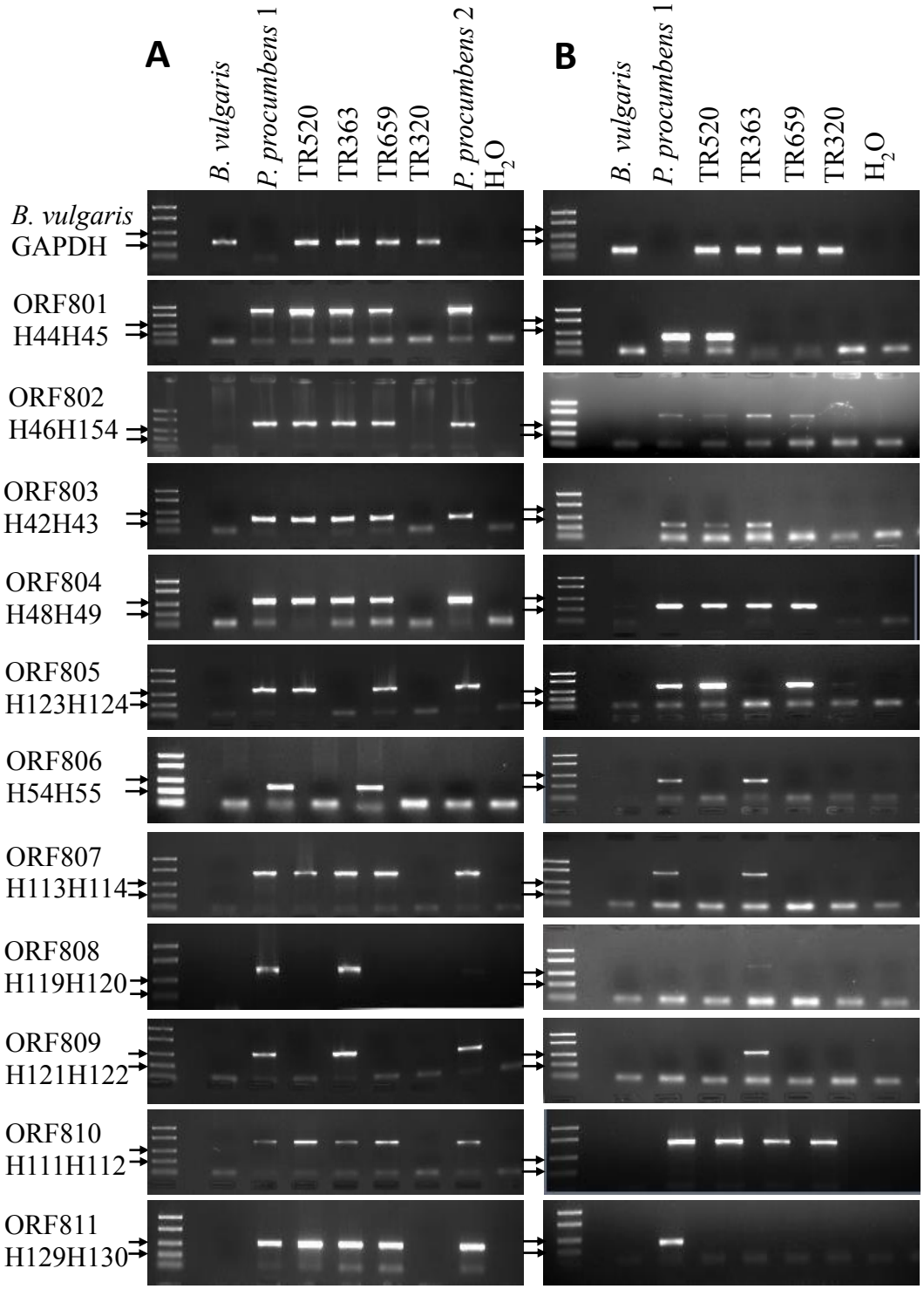

Figure 15: (A) Mapping of the selected ORFs by PCR. Primers were derived from predicted exon sequences of the ORFs. Genomic DNA of *B. vulgaris* (seed code 930176), *P. procumbens* 1 (seed code 35335), *P. procumbens* 2 (seed code 100001) and the translocation lines TR520, TR363, TR659, and TR320 was used as a template. **(B)** Expression analysis of the selected ORFs by RT-PCR. cDNA of inoculated root material of *B. vulgaris* (seed code 930176), *P. procumbens* 1 (seed code 35335), and the translocation lines TR520, TR363, TR659, and TR320 were used as template. The ORFs are denoted as ORF 801 - ORF 811. BvGAPDH (Glyceradehyd-3-phosphate-dehydrogenase) is a housekeeping gene of *B. vulgaris*. The upper arrow indicates the 400 bp and the lower arrow indicates the 200 bp fragment of the low range DNA ladder. Fragments were separated on 1.5**%** agarose gels.

The transcriptional activities were analyzed by RT-PCR amplification using cDNA of inoculated root material of the translocation lines (3.2.5; Figure 15B). Table 29 summarizes the results of the PCR analysis using cDNA and genomic DNA.

Table 29: PCR analyses of the predicted open reading frames (ORF). ORFs were mapped to the physical maps by amplification of genomic DNA (gDNA). Expression of the ORFs was determined by RT-PCR using cDNA of inoculated root material of *B. vulgaris*, TR659 (susceptible), TR320 (susceptible), *P. procumbens*, TR520 (resistant), and TR363 (resistant). Primer sequences are given in Table 21.

ORF	Primer combination	DNA	*B. vulgaris*	*P. procumbens*	TR520	TR363	TR659	TR320
801	H44H45	gDNA	-[a]	X[b]	X	X	X	-
		cDNA	-	X	X	-	-	-
802	H46H154	gDNA	-	X	X	X	X	-
		cDNA	-	X	X	X	X	-
803	H42H43	gDNA	-	X	X	X	X	-
		cDNA	-	X	X	X	-	-
804	H48H49	gDNA	-	X	X	X	X	-
		cDNA	-	X	X	X	X	-
805	H123H124	gDNA	-	X	X	X	X	-
		cDNA	-	X	X	-	X	-
806	H54H55	gDNA	-	X	-	X	-	-
		cDNA	-	X	-	X	-	-
807	H113H114	gDNA	-	X	X	X	X	-
		cDNA	-	X	-	X	-	-
808	H119H120	gDNA	-	X	-	X	-	-
		cDNA	-	-	-	X	-	-
809	H121H122	gDNA	-	X	-	X	-	-
		cDNA	-	-	-	X	-	-
810	H111H112	gDNA	-	X	X	X	X	-
		cDNA	-	X	X	X	X	-
811	H129H130	gDNA	-	X	X	X	X	-
		cDNA	-	X	-	-	-	-

[a]X: Amplicon of the expected size observed; [b]-: No amplicon determined.

All eleven primer combinations yielded fragments of the expected sizes with genomic DNA of *P. procumbens*, whereas no fragments could be observed by amplification with genomic DNA of *B. vulgaris* (see Table 21 and Table 29). This verifies the wild beet specificity of the selected ORFs.

ORF 801, ORF 802, ORF 803, and ORF 804 are located on super scaffold 21, which is anchored to super contig 1 in section D (Figure 11). ORF 805 and ORF 811 are located on super scaffold 5, which is located in section J. ORF 806 is present on super scaffold 4, which maps with one end to the translocation linesTR520 and TR363, and with the other end only to the translocation line TR363. ORF 807 is located on super scaffold 2, which could be mapped to the lines TR520, TR363, and TR659 in section J of the physical map. ORF 808 is present on one end of super scaffold 13 that is present on the lines TR520, TR363, and TR659 in section K on the physical map. ORF 809 is found on super scaffold 6, which maps to the lines TR520, TR363, and TR659 in section J on the physical map (see Figure 11).

These genes had not been physically mapped before, as their location on the physical map could not been covered by BACs identified by BAC library screening, due to the following reasons: repetitive sequences of BAC-ends, high homology to sugar beet sequences, and an under-representation of specific regions in the BAC library, as it is the case for BAC 62A19 and 150L21 (Capistrano, 2009).

Differences between gDNA and cDNA was determined for the following ORFs: amplification with the primer combinations H44H45 of ORF 801 using cDNA gave visible fragments only for the translocation line TR520 and *P. procumbens* although additional amplicons were obtained for the linesTR363 and TR659 using gDNA. Fragments of the primer combinations H42H43 of ORF 803 using gDNA were determined for *P. procumbens*, the lines TR520, TR363, and TR659. Amplifying cDNA, fragments were determined only for *P. procumbens*, and the translocation lines TR520 and TR363. Using gDNA and the primer combination H113H114 of ORF 807 amplicons were identified on *P. procumbens* and the lines TR520, TR363, and TR659. By amplification with cDNA, fragments were observed only for *P. procumbens* and the translocation line TR520. A weak fragment could be observed only for the translocation line TR363 by cDNA amplification, whereas amplifying gDNA, fragments were determined for *P. procumbens* and the translocation line TR520 with the primer combinations H119H120 of ORF 808. A visible fragment was determined only on the line TR363 by cDNA amplification with H121H122 of ORF 809. Amplifying gDNA with that primer combination, fragments were determined for *P. procumbens* and the translocation line TR363. Regarding ORF 811, by using the primer combination H129H130 with gDNA, amplicons were determined with *P. procumbens* and the lines TR520, TR363, and TR659. Using cDNA, this primer combination amplified only with *P. procumbens*. No difference between amplification on cDNA level compared to gDNA was observed for ORF 802, ORF 804, ORF 805, ORF 806, and ORF 810. All primer combinations of the eleven ORFs amplified at least with one of the different genotypes.

3.3.6.2 Transcriptome Analysis of a Nematode Resistant Translocation Line

The aim of this experiment was to verify the expression of the candidate ORFs, which was previously analyzed by RT-PCR (Figure 15). Therefore, RNA of inoculated root material of the nematode resistant translocation line TR520 (3.2.5) was isolated and RNA-Seq was performed. During this process, mRNA is reversely transcribed to cDNA which subsequently is sequenced. The *de novo* assembly of the short reads was performed with the software Trinity (Grabherr et al., 2011) and consists of 213,385 sequences, of which the longest is 17,309 bp in size (3.2.8). In the following I will refer to these data as the transcriptome data. The transcriptome data were blasted (BLASTn) against the previously identified genomic sequence of each candidate ORF, and the hit sequences of the transcriptome data with the highest score were separated. The selected sequences were analyzed for homology to annotated proteins by tblastx using the non-redundant protein database at the NCBI platform. Table 30 shows the results of the analysis of the predicted ORFs compared to sequences of the transcriptome data.

Table 30: Results of the comparative analysis between the predicted ORFs from candidate genes listed in Table 29 and the transcriptome data using roots of inoculated plants of the translocation line TR520. Numbers of analyzed and predicted exons are given. Alignment score and identity threshold (%) of the sequence analysis between the genomic DNA and the transcriptome data are listed. tblastx analyses were done to identify homology to proteins of other species of the selected mRNA-Seq sequence. Sequence similarity was determined by the CLC bio Genomics Workbench.

ORF	Seq[a]	Exons (pred)	Score	Sequence similarity (%)	tblastx result of mRNA
801	51268_c1_seq4	8 (6)	362-75	100-95	XM_002281947, probable leucine-rich repeat receptor like serine/threonine kinase (*V. vinifera*);
802	34314_c1_seq1	1 (1)	1,098	100	XM_002266989, probable phosphatidylinositol 4-kinase (*V. vinifera*)
803	52364_c0_seq2	11 (18)	677-40	100-98	XM_002516401, map3k delta-1 protein kinase (*R. communis*)
804	No significant hit				
805	39138_c0_seq1	1 (3)	1,884	100	XM_00287779, bZIP[b] family transcription factor (*A. lyrata)*
806	No significant hit				
807	No significant hit				
808	No significant hit				
809	No significant hit				
810	49274_c1_seq1	6 (4)	610-65	100	F-box family-6 (*M. trunculata*)
811	No significant similarity found				

[a]selected sequences of the transcriptome data

For six ORFs, no significant hits from the transcriptome data with high sequence similarity could be determined (Table 30). For generating the transcriptome data, inoculated root material of the translocation line TR520 was used for sequencing (3.2.7). This explains the absence of the six ORFs since they were identified on sequences exclusively located on the translocation line TR363. These results are confirmed by RT-PCR of the ORFs using cDNA of the translocation lines TR520, TR363, TR659, and TR320 (see Table 29).

Differences between the predicted exon-intron structures (exon number, exon size) using the dicotyledonous dataset (3.2.8.3) and the transcriptome data of the translocation line TR520 were found for four ORFs (ORF 801, ORF 803, ORF 805, and ORF 810). For example one exon (1,671 bp) was predicted for ORF 802 and the corresponding sequence from the transcriptome data could be aligned well, with 100% sequence similarity in 796 bp (Suppl. Figure 4), suggesting a smaller exon for ORF 802 than predicted by FGENESH.

Primers were designed from the predicted exons of the ORFs (Table 21, Suppl. Figure 4). These primer sequences were aligned to the assembly of the transcriptome data. As a result significant hits were determined for all primer sequences, which verify that all primers used for RT-PCR (see Table 29, Suppl. Figure 4) are located on exon sequences.

The sequence 52364_c0_seq2 of the transcriptome data showed the highest sequence similarity (98-100%) with ORF 803, and thus it was used for ORF prediction using FGENESH (Softberry FGENESH, 2007). This resulted in a predicted protein sequence of 929 aa and will be denoted in the

following as ORF 803$_{mRNA}$. In Supplementary Figure 6, an alignment of ORF 803 $_{mRNA}$ to the proteins of a MAP3K of *A. thaliana* and to the MAP3K proteins CTR1 and EDR1, which were previously used for alignments with ORF 803 (3.3.6.2), is shown. Pairwise comparisons between the sequences show the overall sequence similarity which was calculated using the CLC bio Genomics Workbench. ORF803$_{mRNA}$ shows a sequence similarity of 48.3% to MAP3K, 54.5% to CTR1 and 34.7% to EDR1. The overall sequence similarity of ORF803$_{mRNA}$ to MAP3K, CTR1 and EDR1 is higher than determined for the predicted protein sequence of ORF 803 (3.3.6.2).

		1	2	3	4
MAP3K_Ath	1		458	518	678
CTR1_Vv	2	54.47		433	583
ORF803$_{mRNA}$	3	48.30	54.47		606
EDR1_Ath	4	34.68	40.81	38.48	

Figure 16: Pairwise comparison of the predicted ORF of the mRNA sequence 52364_c0_seq2 (ORF803$_{mRNA}$) that shows high sequence similarity to ORF 803 (1,131 aa), and of the ENHANCED DISEASE RESISTANCE1 protein (EDR1) of *A. thaliana* (Ath) (ABR45974.1), the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) like protein (XP_002279319.2) of *Vitis vinifera* (Vv) and a mitogen-activated protein kinase kinase kinase (MAP3K) from *A. thaliana* (CAB87658.19). In the upper comparison differences on the amino acid level are given and in the lower comparison, the identity between the proteins is listed in percent. Colors range from minimum values (dark blue) to maximum values (dark red). The comparison was performed on the CLC bio Genomics Workbench.

3.3.7 Analysis of ORF 803

ORFs that are present on the resistant and absent from the susceptible translocation lines are of major interest for further studies. None of the selected ORFs is present only on the resistant translocation lines TR520 and TR363, and absent from the susceptible ones. To find out whether ORFs are differentially expressed, differences between gDNA and cDNA were determined for six ORFs by RT-PCR. ORF 803 was found to be present on the translocation lines TR520, TR363, and TR659 (primer combination H42H43) (Figure 15, Figure 14, Table 29). Interestingly, this sequence is the only that is differentially expressed between the resistant (TR520 and TR363) and the susceptible genotypes (TR659 and TR320). Therefore, this ORF was further investigated.

Figure 17A gives an overview of the predicted exon-intron structure of ORF 803, the promoter region and the primer combinations designed. A number of 18 exons were predicted by the software FGENESH (Softberry FGENESH, 2007) using the dicotyledonous dataset (Figure 17B).

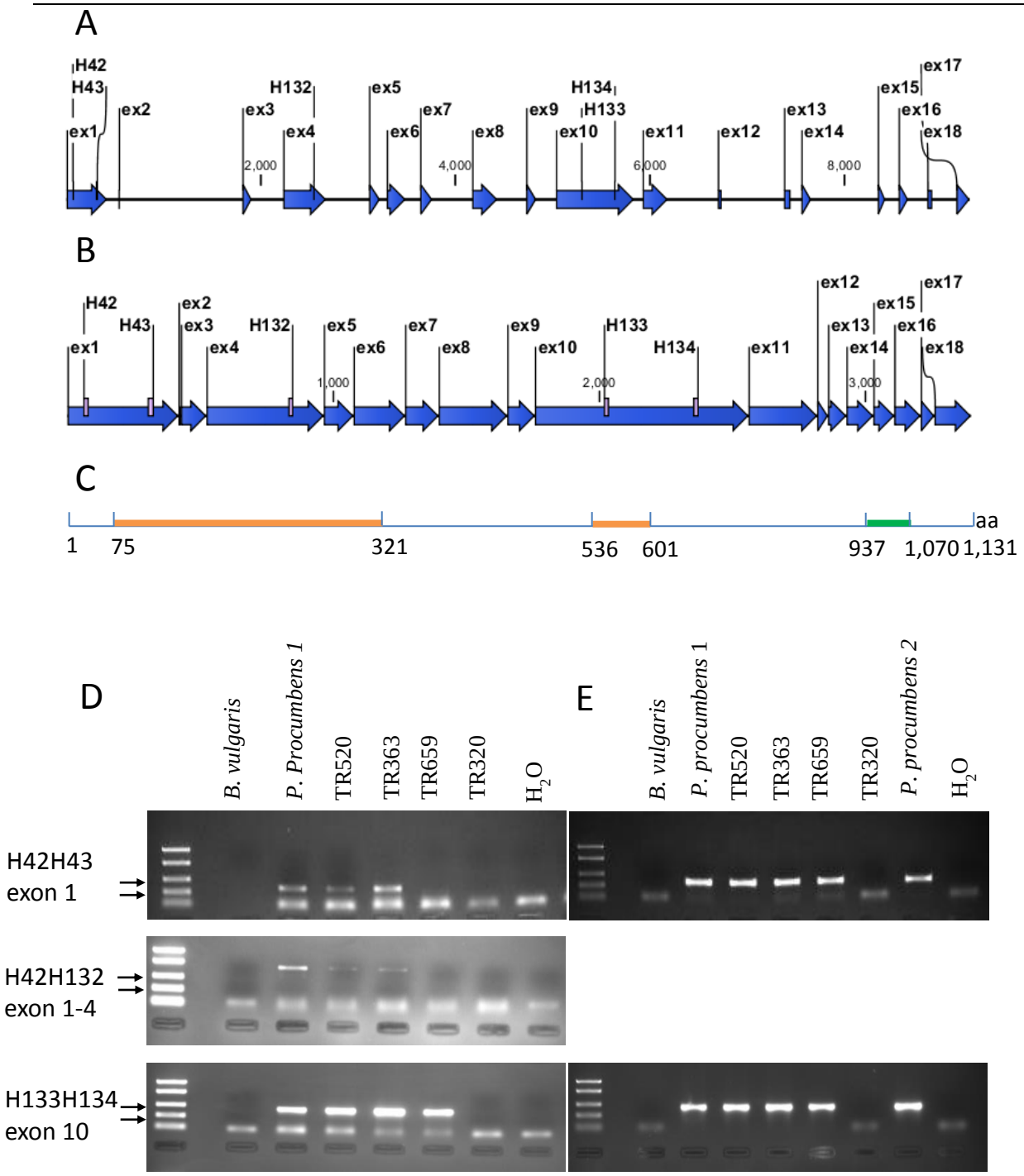

Figure 17: (A) Graphical overview of the predicted exon-intron structure of ORF 803 using FGENESH software. Exons are indicated in blue (as arrows or boxes) and named 'ex1'-'ex18' and the introns are indicated as a line between the exons. A size scale is given in base pair. **(B)** Predicted coding sequence of ORF 803; primer positions are indicated as flags (H42, H43, H132, H133, H134). **(C)** Predicted polypeptide structure in amino acids. The predicted transglutaminase domains are indicated in orange and the predicted kinase domain in green (Table 31). **(D)** Expression analysis of different exon regions of ORF 803 by RT-PCR using cDNA of inoculated root material of *B. vulgaris* (seed code 930176), *P. procumbens* 1 (seed code 35335), and the translocation lines TR520, TR363, TR659, and TR320. **(E)** PCR amplification using genomic DNA of *B. vulgaris* (seed code 930176), *P. procumbens* 1 (seed code 35335), the translocation lines TR520, TR363, TR659, and TR320 and *P. procumbens* 2 (seed code 100001). Expected fragment sizes: H42H43 = 266 bp, H42H132= 790 bp, H133H134 = 357 bp. PCR on genomic DNA with the primer combination H42 (exon1) H132 (exon4) not practical due to long introns. The upper arrow indicates the 400 bp and the lower arrow indicates the 200 bp fragment of the low range DNA ladder. Expected amplicon sizes in bp. Fragments were separated on 1.5% agarose gels.

RT-PCR was additionally performed with the primer combinations H42H132, located on the predicted exon 1 and exon 4, and H133H134 present on predicted exon 10, using cDNA of inoculated root material (3.2.5; Table 21) of *B. vulgaris* (930176), *P. procumbens*1 (35535), and the translocation lines TR520, TR363, TR659, and TR320 (Figure 17 A, B). Amplicons were observed for *P. procumbens* and the lines TR520 and TR363, using the primer combinations H42H43, which amplified exon 1, and H42H132, which amplified from exon 1 to exon 4, (Figure 17 D, E). By amplification with the primer combination H133H134, located on exon 10, in the conserved domain of the protein kinase, amplicons were determined for *P. procumbens*, the resistant translocation lines TR520 and TR363, and the susceptible translocation line TR659 (Figure 17 C, D, E). To summarize, RT-PCR amplicons of the conserved transglutaminase domain were found only in the resistant translocation lines TR520 and TR363 (primer combinations H42H43 and H42H132). The primer combination H133H134 located on exon 10 amplified cDNA from both resistant lines and the susceptible translocation line TR659 (Figure 17 C, D, E). These might indicate differential splicing of the gene. Alternative splicing increases the protein diversity by differential splicing of precursor (pre) mRNA to various mRNA isoforms for example by post-transcriptional regulation such as exon skipping or intron retention (see 3.4) (Syed et al., 2012).

The predicted protein sequence of ORF 803 shows homology to the following proteins: CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) (XP_02279319.2) from *V. vinifera*, ENHANCED DISEASE RESISTANCE1 (EDR1) (ABR45974.1) from *A. thaliana*, and a mitogen activated protein kinase kinase kinase (MAP3K) (CAB8658.1) from *A. thaliana* (see Table 31). All are known to be involved in the regulation of ethylene signaling and stress response (Tang et al., 2005; Huang et al., 2003; Lin et al., 2010). I aligned the predicted protein sequence of ORF 803 with protein sequences of EDR1, CTR1, and a MAP3K of *A. thaliana* because these genes showed the highest identity to ORF 803 and because they are involved in biotic stress response (Supplementary Figure 5). The transglutaminase domain in the N-terminal region of the protein of ORF 803 is similar to EDR1, CTR1, and MAP3K (shown as underlined in the alignment Supplementary Figure 5). The kinase domain in the C-terminal region is highly conserved between MAP3K, CTR1, EDR1, and ORF 803 (shown as bold and underlined; Supplementary Figure 5). The alignment was done using the ClustalW software (European Bioinformatics Institute, 2012).

Table 31: Analysis of the predicted amino acid sequence of ORF 803 using the protein BLAST database for homology search to known proteins from other organisms and conserved domains on the NCBI platform.

Region	Accession no.	Hit description	Score	e-value	Identity	References
1 – 321 aa	XP2279319.2	serine/threonine-protein kinase CTR1-like [*Vitis vinifera*]	686	2e-72	47%	Lin et al., 2008
546 – 1,070 aa			1,232	4e-148	52%	
1 – 321 aa	CAB87658.19	MAP3K delta-1 protein kinase [*Arabidopsis thaliana*]	596	5e-61	42%	Kuykendall et al., 2011
546 – 779 aa			467	7e-45	47%	
909 – 1,070 aa			751	5e-81	83%	
4 – 321 aa	ABR45974.1	EDR1 enhanced disease resistance 1 [*Arabidopsis thaliana*]	407	2e-37	35%	Caldwell and Michelmore 2009
546 – 606 aa			186	4e-11	55%	
919 – 1,070 aa			238	3e-62	72%	

Conserved domains

Region	Accession no.	Hit description	e-value	References
175 – 321 aa	Pfam14381	Transglutaminase-like family	5.70e-47	Tang et al., 2005
536 – 601 aa	Pfam14381	Transglutaminase-like family	6.34e-24	
935 – 1,071 aa	Cd00180	Catalytic domain of protein kinases	4.84e-32	Rodriguez et al., 2010

The predicted amino acid sequence of ORF 803 shows a region of 230 aa (322aa-551 aa) that shows no homology to EDR1, CTR1 or the MAP3K of *A. thaliana* (Supplememtary Figure 5). This region must be further analyzed and verified by cloning the coding sequence of ORF 803.

By pairwise comparison of the predicted protein sequence of ORF 803 to EDR1, CTR1 and the MAP3K using the CLC bio Genomics Workbench, the sequence similarity could be calculated. The predicted protein sequence of ORF 803 shows an overall sequence similarity of 35.48% to CTR1, 24.18% to EDR1, and 34.9% to MAP3K (see Figure 18).

	1	2	3	4
ORF803	1	783	780	925
MAP3K_Ath	34.09	2	454	650
CTR1_Vv	35.48	53.91	3	570
EDR1_Ath	24.18	35.13	40.62	4

Figure 18: Pairwise comparison of predicted sequence of ORF 803 (1,131 aa) and the ENHANCED DISEASE RESISTANCE1 protein (EDR1) of *Arabidopsis thaliana* (Ath) (ABR45974.1), the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) like protein (XP_002279319.2) of *Vitis vinifera* (Vv) and a mitogen-activated protein kinase kinase kinase (MAP3K) from *A. thaliana* (CAB87658.19). In the upper comparison differences on the amino acid level are given and in the lower comparison the identity between the proteins is listed in percent. Colors range from dark blue minimum values to dark red maximum values. The comparison was performed on the CLC bio Genomics Workbench.

3.3.7.1 Promoter Analysis of ORF 803

ORF 803 is located on scaffold 26267 of the 'TR520 *de novo*' assembly, which is incorporated into super contig 1. To analyze the promoter sequence of this candidate gene, the continuous sequence upstream of the ATG until the next gap (3,922 bp) on scaffold 26267 was selected for further analyses (3.2.8.2) of regulatory elements using the PLACE database (Higo et al., 1999; Plant Cis Acting Regulatory Elements, 2012) (Suppl. Table 23). Regulatory elements, which could indicate root specific expression or are related to disease and defense response were selected and are shown in Table 32. Five different elements were identified that could indicate a root specific expression of the gene. Additionally, six different motifs for defense related gene expression were determined. Among them, elicitor responsiveness motifs and binding sites for WRKY transcription factors were identified, which can act as positive or negative regulators in defense responses (Eulgem and Somssich, 2007; Koschmann et al., 2012).

Table 32: Overview of selected root and disease specific regulatory elements present in the promoter region (3,922 bp) of ORF 803 that were identified using the web-based program PLACE (Higo et al., 1999; Plant Cis Acting Regulatory Elements, 2012). Name of regulatory element, sequence, copy number on + and – strand and functions are given. All regulatory elements identified are listed in Suppl. Table 23.

Regulatory element	Sequence	Number of copies[a]	Function	Reference
TATA Box3	TATTAAT	+1,	Transcriptional initiation	Grace et al. (2004)
NODCON 1GM	AAAGAT	5+, -2	Root nodule expression	Sandal et al. (1987)
NODCON 2GM	CTCC	+9, -7	Root nodule expression	Sandal et al. (1987)
OSE2ROOT NODULE	CTCTT	+9, -7	Root nodule expression	Vieweg et al. (2004)
ROOT MOTIF APOX1	ATATT	+19, -21	Root specific expression	Elmayan and Tepfer (1995)
ELRECO REPCRP1	TTGACC	+2, -1	Elicitor responsiveness	Rushton et al. (1996)
NA[b]	GACTTTT	+1	Elicitor responsiveness	Koschmann et al. (2012)
GT1GM SCAM4	GAAAAA	+1, -5	Pathogen-induced gene expression	Park et al. (2004)
MYB1	GTTAGTT	+1, -1	Defence-related gene expression	Chakravarthy et al. (2003)
TAAAG STKST1	TAAAG	+8, -9	Guard cell expression	Plesch et al. (2001)
WRKY71OS	TGAC	+11, -10	Binding site of WRKY transcription factors in pathogenesis related genes	Eulgem et al. (2000)
WBOX ATNPR1	TTGAC	+4, -3	Binding site of WRKY transcription factors in NPR1 gene expression	Yu et al. (2001)

[a] Located on + or – strand; [b]NA: no name specification given in (Koschmann et al., 2012)

3.3.8 Analysis of Previously Proposed Nematode Resistance Gene Candidates from the Resistant Translocation Lines

Tian (2003) used a PCR based approach to amplify NBS-LRR containing RGAs from sugar beet. For primer design, five motifs of the NBS domain were chosen (P-loop, kinase-2, kinase-3a, hydrophobic domain (GLPL), TIR/non-TIR consensus sequences). By amino acid alignment of the four cloned nematode resistance genes *Cre3*, *Mi*, *Gpa2*, *Hero*, and the tomato *Prf*-gene, which confers resistance against *Pseudomonas syringae*, highly conserved regions for each of the five motifs had been determined. Degenerate primer combinations had been designed according to specific codon usage in

sugar beet. The two sequences cZR-3 and cZR-7 had been identified and determined to be present on *P. procumbens*, the translocation lines TR520 and TR363 and absent from *B. vulgaris*. To confirm these results, BLASTn analyses of these sequences to the draft reference assemblies of *B. vulgaris* (RefBeet-0.4) and *P. procumbens* were performed. For all sequences a higher sequence similarity to the sugar beet than to the *P. procumbens* genome (Table 33) was observed. The sequence similarity threshold for cZR-3 and cZR-7 to scaffolds of the *P. procumbens* assembly were 78% and 84%, respectively, and to scaffolds derived from the *B. vulgaris* assembly RefBeet-0.4 100% for both. Additionally, the high alignment score between the *B. vulgaris* and the RGA sequences lead to the assumption that cZR-3 and cZR-7 are derived from the sugar beet genome (Suppl. Table 24). Later studies suggest an involvement of these genes in the *Hs1*$^{pro-1}$-mediated resistance response (Knecht, 2010).

Table 33: Sequence similarity analyses of proposed nematode resistance gene candidates (Menkhaus, 2011; Tian, 2003) to the genomes of *B. vulgaris* (version RefBeet-0.4) and *P. procumbens* by BLASTn.

Query	Hit	Alignment score	E-value	Identities (%)
cZR-3	BV[a]sc[b]53	3,776	0	100
cZR-3	PP[c]sc20754	158	1.05E-82	78
cZR-7	BVsc55	3,402	0	100
cZR-7	PPsc1978	174	3.22E-97	84
BpPIP1	BVsc10	157	5.91E-83	88
BpPIP1	PPsc20220	113	1.37E-56	100

[a]BV: *B. vulgaris*; [b]sc: scaffold; [c]PP: *P. procumbens*

Menkhaus (2011) identified a gene with high sequence similarity to plant aquaporins, which can be divided into seven subfamilies. The gene belongs to the subfamily of plasma membrane intrinsic proteins (PIP) and was denoted as *BpPIP1*. It is expressed in the resistant translocation line TR520 and absent from the *B. vulgaris* genome. The transcript analysis of this gene revealed a constitutive expression and an up-regulation upon nematode infection. Comparative sequence analysis of this sequence to the *B. vulgaris* and *P. procumbens* reference assemblies clearly showed an affiliation to the wild beet genome (Table 33). During the identification of new translocation specific sequences of the WGS approach, the sequence *BpPIP1* could be identified on scaffold 42352 of the 'TR520 *de novo*' assembly (Figure 12). This scaffold overlaps with scaffold 20220 of *P. procumbens* (Suppl. Table 4; Suppl. Table 25) and is integrated into super contig 2-3, linked to the distal side of BAC 74E5 (Figure 11). However, the sequence is absent from the line TR363 because no sequence similarity to the translocation line TR363 could be identified in this region (section H and section E).

3.3.9 Sequence Similarity between the *P. procumbens* Translocations and the Sugar Beet Genome

In a next step, I looked for sugar beet specific sequences that might be present on the translocation. I identified a large *B. vulgaris* insertion within the sequenced BAC 149P7 on super contig 1. The sequence of BAC 149P7 is not contiguous but separated into 18 contigs (Capistrano, 2009). These contigs were blasted against the RefBeet-0.9 and against the *P. procumbens* assembly. A number of 12 contigs show high sequence similarity (99 – 99.9%) to scaffold 711 and two contigs display high sequence homology (96 – 98%) to scaffold 677 of the RefBeet-0.9 assembly. In Supplementary Table 26 the results of the BLASTn analyses of the BAC 149P7 contigs that show significant similarity to sugar beet sequences are given.

The size of scaffold 677 of the *B. vulgaris* RefBeet-0.9 is 161,652 bp, contig 5 and contig 13 of BAC 149P7 show sequence homology from 145,825 bp – 150,445 (4,620 bp). The total size of scaffold 711 of the *B. vulgaris* RefBeet-0.9 is 155,695 bp and the region (1bp- 67,916 bp) homologous to BAC 149P7 is 67,916 bp in size. Contig 1 (24,915 bp) bears the T7-end and contig 18 (7,428 bp) the Sp6-end of BAC 149P7. These contigs, as well as contig 16 (72,145 bp) and contig 17 (2,815 bp), show significant similarity to the *P. procumbens* reference assembly (Suppl. Table 26).

To further analyze the *B. vulgaris* insertions within BAC 149P7, five ORFs were predicted (3.2.8.3) from the 67.9 kb and 4.6 kb sequence of *B. vulgaris* RefBeet-0.9 scaffold 711 and scaffold 677. The search for homology to known proteins from other species using the non-redundant protein database of the NCBI platform revealed similarity to a number of transposons and retro transposons for the five predicted ORFs (Table 34).

Table 34: Open reading frame (ORF) prediction of a 72.5 kbp sequence within the translocation specific BAC clone 149P7 which displays high sequence similarity to the sugar beet genome draft RefBeet-0.9 scaffold 711 and scaffold 677.

ORF	Length (aa)	Accession no.	Description	E-value	Identity
Scaffold 711					
711/1	317	ABD83317.1	Fgenesh protein 73 [*Beta vulgaris*]	2.00E-145	86%
711/2	928	ABA95229.1	retrotransposon protein, Ty3-gypsy [*Oryza sativa* Japonica Group]	4.00E-128	38%
711/3	305	AAY99339.1	Pol polyprotein[a] [*Silene latifolia*]	5.00E-95	50%
711/4	338	CBI26220.3	unnamed protein product [*Vitis vinifera*]	2.00E-117	57%
Scaffold 677					
677/1	985	ABM55245.1	Transposon-like protein [*Beta vulgaris*]	0.0	88%

[a]class of gypsy-like retrotransposons (Kejnovsky et al., 2006)

Moreover, Capistrano (2009) determined a tandem repeat of 59 bp that is repeated 9 times on BAC 149P7. I could confirm the presence of this sequence on contig 15 of BAC149P7 that shows 99.9% sequence similarity to *B. vulgaris* scaffold 711 (RefBeet 0.9). The comparative sequence analysis of the contigs of BAC 149P7 to scaffold 711 revealed that the tandem repeat is present in the *B. vulgaris* genome (data not shown).

3.3.9.1 Sequence Similarity Analyses of Candidate ORFs to the Sugar Beet Genome

In order to investigate sequence similarities of the candidate sequences to the sugar beet genome, ORF 801, ORF 802, and ORF 803 were chosen as they are clustered on scaffold 26267 of the 'TR520 *de novo*' assembly and all show significant similarity to protein kinases (Table 28). BLAST analyses were performed on the CLC bio Genomics Workbench using the reference assembly RefBeet-0.9. Sequence similarities of 82% – 96% between the ORFs and *B. vulgaris* were found (Suppl. Table 27). ORF 801 and ORF 802 have homology to sequences located on scaffold 21 whereas ORF 803 shows homology to scaffold 839 of the RefBeet-0.9 assembly. Thus, the three sequences are located on different scaffolds in the sugar beet assembly. Regarding ORF 801, sequence similarity to sugar beet sequences was determined in all 6 predicted exons with 85% – 93% sequence identity to scaffold 18 (Suppl. Figure 7; Suppl. Table 27). Sequences on the *B. vulgaris* genome showing sequence similarity

to the predicted ORFs will be denoted in the following as e.g. ORF 801_{BV}. The ORF 802 shows a sequence similarity of 85% – 93% to the sugar beet sequence of RefBeet-0.9 scaffold 18. With regard to ORF 803, sequence similarities to the sugar beet sequence of RefBeet-0.9 scaffold 839 were determined on predicted exon 1 and 2, exon 4, and exon 9-16, with a similarity threshold of 83% – 96% (Suppl. Table 27). No significant sequence similarity was found in the 5'- and 3'- regions of ORF 801, ORF 802, and ORF 803 (Suppl. Figure 7), indicating sequence differences also in the promoter region. In order to investigate if the promoter sequence of ORF 803 and ORF 803_{BV} show different regulatory elements, 3,299 bp upstream of the predicted start codon of ORF 803_{BV} on RefBeet-0.9 scaffold 839 were analyzed (3.2.8.2).Nearly all of the elements present in the promoter sequence of ORF 803 were identified with a more or less equal number of copies in the promoter sequence of $ORF803_{BV}$ (Suppl. Table 28). The only regulatory elements present in the promoter sequence of ORF 803 and absent from the *B. vulgaris* promoter sequence are the two elicitor responsiveness elements (Table 32). This result must be regarded carefully since the selected 3,922 bp promoter sequence on RefBeet-0.9 scaffold 839 contains gaps between -2,878 bp and -3,343 bp.

To further analyze the sequence $ORF803_{BV}$ on scaffold 839, the mRNA assembly was mapped against scaffold 839. As a result, the mRNA sequence 52364_c0_seq1 was identified showing high sequence similarity to $ORF803_{BV}$. Comparing the identified mRNA sequence 52364_c0_seq2, which shows high sequence similarity to ORF 803, and 52364_c0_seq1, an overall sequence similarity of 86% was determined (Suppl. Figure 8). This indicates that a gene with 86% sequence similarity to ORF 803 is expressed in the genome of *B. vulgaris*. Indeed, this can be expected, since in the genome of *A. thaliana* a number of approximately 60 MAP3K were identified and regulate numerous processes, including stress and hormonal responses, innate immunity, and morphogenesis (Ichimura et al., 2002; Rodriguez et al., 2010).

3.4 Discussion

The line TR520 has a telomeric translocation of *P. procumbens* chromosome 1, which is attached to the end of chromosome 9 of sugar beet. The position of the translocation within the sugar beet genome had been determined by genetic mapping (Heller et al., 1996) and by fluorescence *in situ* hybridization (FISH) (Desel et al., 2001). The size of the *P. procumbens* translocation of the line TR520 had been estimated at 1,500 kbp via hybridization, using translocation specific probes (Kleine et al., 1998). Kleine et al. (1995) generated the first physical map using YACs from the translocation line A906001. Schulte et al. (2006) established a BAC library of the translocation line TR520 and used previously identified translocation specific YAC and cDNA probes for BAC library screening (Cai et al., 1997; Kleine et al., 1998; Kleine et al., 1995; Salentijn et al., 1995). A BAC and YAC-based physical map of the translocation line TR520 had been presented, which includes nine BACs and six YACs. A minimal tiling path of five BACs (524 kbp) covers 350 kbp shared by the translocation lines TR520 and TR363, which had been determined in the proximal side of the physical map. Additionally, BAC 62A19 had been identified, bearing the previously cloned $Hs1^{pro-1}$ gene (Cai et al., 1997). BAC 68G15 represented the most distal and BAC 33D9 the most proximal BAC on this physical map (Schulte, 2006). According to this map, the translocation breakpoint was identified in BAC 33D9. After analysis of Jäger (2007) and Capistrano (2009), the translocation breakpoint on BAC 33D9 could not be confirmed. My analyses of the BAC sequences to draft genome assemblies of *B. vulgaris* and *P. procumbens* verify an overall sequence similarity of BAC 33D9 to the *P. procumbens* genome of 99.5% (Suppl. Table 5). Capistrano (2009) investigated ~ 1 Mbp translocation-specific BAC sequences, and the physical map was revised. With regard to the sequence-based physical map of this work (Figure 11), which is based on the BAC-based physical map of Capistrano (2009) (Figure 1) five BACs (BAC 3L6, BAC 62A19, BAC 57K15, BAC 39K12, and BAC 33D9) identified by Schulte (2006) are still included and their origin to *P. procumbens* could be confirmed by sequence analysis (Suppl. Table 3 and 5).

After analysis of the WGS data during this study, the physically anchored BAC contigs 1, 2, and 3 (Capistrano, 2009) could be extended in both directions to a total of 1,509 kbp. Additionally, BAC contig 2 and 3 could be linked by overlapping sequences of the WGS approach between YAC end 120 R on the distal side of BAC contig 2 and BAC end 74E5 T7 on the proximal side of BAC contig 3 (Figure 12). Complying with the results of Desel et al. (2001) who showed that YAC end 120 R is located distal to YAC 128, the two BAC contigs were mirrored along the vertical axis. In this way, YAC end 120 R and BAC end 74E5 T7 could be linked (see 3.3.2) and the linked BAC contig 2 and BAC contig 3, which incorporate super scaffolds, was re-named in super contig 2-3 (Figure 12). After these rearrangements, BAC end 89D17 T7 represents the most proximal BAC sequence and 56F10 T7 the most distal BAC sequence of this scaffold (Figure 11).

The new identified sequences of the translocation line TR520 not anchored to the physical map are 716,926 bp. A number of 41 scaffolds and contigs of the 'TR520 *de novo*' assembly were selected and assembled to 13 super scaffolds (Table 25). Additionally, new sequences of 307,886 bp shared by the translocation lines TR520 and TR363 were identified (Table 25).

The total number of sequences of the physical map of the line TR520 adds up to 2,226 kbp. Thus the size estimated by Kleine et al. (1998) (1,500 kbp) was exceeded by 48.4% (see 3.3.3).

For the first time, *P. procumbens* specific sequences were identified exclusively located on the translocation line TR363 and absent from the translocation line TR520. They sum up to 389,063 bp (Table 25). This corroborates to the results of Kleine et al. (1998) who investigated the sizes of the *P. procumbens* translocations by Southern hybridization using the random amplified polymorphic DNA (RAPD) marker X2.1 as probe. The identification of different fragment sizes suggested various translocation sizes between the lines TR520 and TR363. Since my work was based on the selection of sequences shared between the lines TR520 and TR363 in order to identify the nematode resistance

gene *Hs1-2*, an investigation of the *P. procumbens* translocation of the line TR363 is possible for future work by aligning the sequences of the 'TR363 unmapped *de novo*' and the 'TR363 *de novo*' assemblies to the *P. procumbens* genome assembly.

A translocation is a change in the arrangement of genetic material, altering the location of a chromosome segment. Translocations generate novel chromosomes without normal pairing partners and place genes in a new linkage. They occur spontaneously or can be artificially induced by ionizing radiation, mutagens or by transposons (Jacobsen and Schouten, 2007; O'Connor, 2008). Introgression breeding by induced translocations via irradiation has been applied also in wheat, tobacco, oat, and radish (Aung and Thomas, 1976; Caplin and Mann, 1978; Friebe et al., 1996; Kaneko et al., 1992). The translocation lines TR520, TR363, TR659, and TR320 can be defined as intergenomic translocation lines. Also in wheat, several intergenomic translocation lines exist that bear the advantage of transferring resistance to numerous pathogens. Recently, Qi et al. (2011) reported about a novel translocation event between bread wheat and *Dasypyrum villosum* transferring the stem rust resistance gene *Sr52*. Information of typical characteristics on translocation breakpoints in plants are rare. Molnar et al. (2011) analyzed the chromosomal distribution of microsatellite clusters in relation to the intergenomic translocations in the allotetraploids *Aegilops biuncialis* and *A. geniculata.* They mapped translocation breakpoints to SSR-rich chromosomal regions, suggesting that microsatellite sequences may facilitate the formation of chromosomal rearrangements. Gajecka (2008) identified additions of nucleotides, deletions, and duplications at breakpoints in reciprocal translocations in human, but no breakpoint-specific sequence motifs were determined. Therefore, breakpoint analysis for the BAC and WGS sequences in this work was performed in the following way. To investigate the genome specificity of the WGS and the BAC sequences during the assembly, all sequences were compared to the *B. vulgaris* and the *P. procumbens* genome assemblies by BLASTn analysis on the CLC bio Genomics Workbench (Supplementary Tables 3-20). If a sequence would cover a breakpoint, I expected to determine a high sequence similarity to *B. vulgaris* on one side and a high sequence similarity to *P. procumbens* on the other side of a sequence. For all selected WGS sequences of the translocation lines TR520 and TR363 anchored or non-anchored to the physical map, I determined a significant sequence similarity to *P. procumbens* (see Suppl. Tables 4, and 6-20). In this way, I could show that the sequenced BAC 149P7 has a *B. vulgaris* insertion of ~73 kbp (see 3.3.9). For the first time, I demonstrated that the wild beet translocation is interrupted by large insertions from the *B. vulgaris* genome (Suppl. Table 26). Possibly, multiple chromosome breakages have taken place during the chromosome translocation process between wild beet and sugar beet. This renders the identification of the translocation breakpoint(s) less important as there is no single start or end of the translocation. Also, the existence of more than one sugar beet insertion cannot be ruled out.

Comparing the ~ 73 kbp of the *B. vulgaris* insertion and the sequences of 320 predicted ORFs to the NCBI databases resulted also in the identification of repetitive DNA. Such sequences have been studied before in sugar beet and related wild beets (Dechyeva and Schmidt, 2006; Dechyeva et al., 2003; Gao et al., 2000; Gindullis et al., 2001b; Kubis et al., 1997; Schmidt and Metzlaff, 1991; Schmidt and Heslop-Harrison, 1993; Schmidt et al., 1993). Repetitive sequences belonging to various classes were identified and characterized, among them long interspersed elements (LINE) (Kubis et al., 1998), DNA transposons (Jacobs et al., 2004), dispersed repeats belonging to the families pDvul1 and pDvul2, the pRv1 satellite repeat (Menzel et al., 2008), miniature inverted-repeat transposable elements (MITE) (Menzel et al., 2006), Ty1-copia retrotransposons (Schmidt et al., 1995), a novel type of plant non-LTR retrotransposons, designated *B. vulgaris* non-LTR retroelement (BNR) family (Heitkam and Schmidt, 2009) and the *h*AT transposon superfamily (Menzel et al., 2012). Recently, Paesold et al. (2012) published a reference FISH karyotype for *B. vulgaris*. They used the distribution of the satellite subfamilies pBV I-V and pBV VI that are associated with the centromere, the satellite family pEV which is present in intercalary positions and the subtelomeric satellite pAv34 to discriminate all nine sugar beet chromosomes by multicolor FISH.

The identification of repetitive elements specific only for *P. procumbens* was reported by Schmidt et al. (1990). They identified three highly repetitive sequences after restriction and cloning of *Sau*3A fragments of gDNA of *P. procumbens*, pTS1, pTS2, and pTS3, and demonstrated their genome specificity by squash dot hybridization. Later, Schmidt and Heslop-Harrison (1996) used multi-color FISH to physically map members of three *Sau*3A repeat families and determined a tandemly organization. Moreover, the probe pRK643 was identified to hybridize with a short repetitive DNA sequence in *P. procumbens*. By squash dot hybridization with segregating progenies of the resistant fragment addition line PRO1 (Jung and Wricke, 1987) pRK643 could be used as a marker to select resistant plants (Jung and Herrmann, 1991; Jung et al., 1992). Salentijn et al. (1992) identified repetitive DNA markers specific for *P. procumbens* and *P. patellaris* while analyzing translocation lines, fragment addition lines and monosomic addition lines. Two clusters of the DNA satellite family Sat-121 were determined to be clustered in close vicinity to the $Hs1^{pro-1}$ locus (Salentijn et al., 1994). Gindullis et al. (2001b) identified two satellite families, pTS4.1 and pTS5, by applying FISH to the fragment addition line PRO1. Dechyeva et al. (2003) analyzed two repetitive DNA families, pAp4 and pAp22, that are specific for *P. procumbens* by Southern hybridization and FISH of the line PRO1. They demonstrated the application as DNA probes to discriminate parental genomes in interspecific hybrids. Dechyeva and Schmidt (2006) investigated the structure and species-specific diversification of subtelomeric satellite DNA families of the genus *Beta* and related species, by application of Southern blotting and multi-color FISH on extended DNA fibres. Schulte et al. (2006) identified a tandem repeat with an *Alu*I restriction site on the *P. procumbens* translocation of the line TR520 in the sequenced BAC33D9. Grzebelus et al. (2011) identified two insertion polymorphisms of *Vulmar* and *VulMITE* transposons specific for *P. procumbens*.

In this work, 13% of the predicted 320 ORFs showed sequence similarity to repetitive DNA especially to retroelements and transposons. Even though all ORFs were identified on sequences with high sequence similarity to *P. procumbens*, the sequence analysis to the NCBI database revealed sequence similarity to e.g. Ty1/copia retrotransposons or classes of gypsy-like retrotransposons from different organisms such as *B. vulgaris*, *V. vinifera*, *A. thaliana*, or *O. sativa*. The sequence identity thresholds of the predicted ORFs to sequences identified on the NCBI database ranged from 7 to 73% (Suppl. Tables 21 and 22). No sequence similarity was found to any repetitive DNA of *P. procumbens*. An explanation for this might be the low number of database entries of *P. procumbens* retroelements (NCBI: 33 transposable elements) than compared to *B. vulgaris* (NCBI: 144 transposable elements). The sequence analysis of the 73 kbp *B. vulgaris* insertion of BAC 149P7 to the NCBI database revealed amongst others sequence similarity to a *B. vulgaris* transposon with an e-value of 0.0 and an identity threshold of 88% (see Table 34). Recent studies analyzing major chromosomal rearrangements in maize lines, such as translocations and large inversions, revealed pairs of closely linked *Ac* transposable elements in close vicinity at the breakpoints (Zhang et al., 2009). Therefore, a possible role of translocation induction is suggested for the identified *B. vulgaris* transposons within the translocation. Furthermore we can speculate that the other breakpoint(s) have a similar structure.

My main interest was to identify candidate sequences for the *Hs1-2* gene. Therefore, I searched for ORFs with significant sequence similarity to proteins from other organism known to be involved in biotic stress response (see 3.3.6). Until now, eleven major nematode resistance genes have been cloned from different species (see Table 4). Their coding regions have some characteristics in common, like e.g. leucine-rich repeats, and most of them carry a NBS domain with a coiled coil or a toll-interleucin-receptor at their N-termini. Therefore, the candidate sequence for *Hs1-2* was expected to have a similar structure. Efforts have been made to identify similar domains and resistance gene analogues (RGA) on the translocation using degenerate primer combinations (Tian et al., 2004). This resulted in the identification of two RGAs (cZR-3, cZR-7) which were supposed to be located on the *P. procumbens* translocation of the lines TR520 and TR363 (Tian, 2003). Functional analysis in sugar beet hairy roots by overexpressing cZR-3 and cZR-7 showed an decrease in established J4 females compared to the control roots (Tian, 2003). Knecht (2010) suggested an involvement of cZR-3 and cZR-7 in the $Hs1^{pro-1}$ mediated resistance response. Functional analyses in *A. thaliana* resulted in a decrease of

established J4 females. I have searched for sequence similarity of the RGAs cZR-3 and cZR-7 to the genomes of *P. procumbens* and *B. vulgaris*. Based on comparative sequence analysis to the draft assemblies of *B. vulgaris* (RefBeet-0.9) and *P. procumbens*, I demonstrated that cZR-3 and cZR-7 originate from the sugar beet genome and are absent from the *P. procumbens* genome (Table 33). Since no resistance to the BCN has been found in any *B. vulgaris* genotype so far but in *P. procumbens*, I expect the resistance gene(s) must be present on the wild beet genome and are absent from the sugar beet genome (Golden, 1959; Hijner, 1952; Savitsky, 1975).

Capistrano (2009) proposed ORF 702, with sequence similarity to an AVR elicitor response protein and a conserved galactosyltransferase domain, as a potential candidate for the *Hs1-2* gene. Evidence was provided by expression of the gene only in the resistant translocation lines TR520 and TR363 and its location within section A that is shared by the translocation lines TR520 and TR363 and which belongs to super contig 1 (Figure 11). Here, I presented a functional analysis of ORF 702 in two model systems, *A. thaliana* and sugar beet hairy roots (see chapter 2). My data strongly suggest that ORF 702 is not the resistance gene *Hs1-2* because in sugar beet hairy roots and *A. thaliana*, no significant differences in the establishment of J4 females between control and transgenic plants could be determined (see chapter 2.4).

Menkhaus (2011) identified a gene from the resistant translocation line 940043 by analyzing a suppression subtractive hybridization (SSH) library. The SSH library was generated from the resistant sugar beet translocation line (seed code 940043) subtracted by the susceptible sugar beet (seed code 930176) in order to identify genes involved in resistance reactions in regards to nematode infections (Table 20). The gene was denoted as *BpPIP1* and shows high sequence similarity to plant aquaporins, which can be divided into seven subfamilies. *BpPIP1* belongs to the subfamily of plasma membrane intrinsic proteins (PIP) and was proposed to be involved in $Hs1^{pro-1}$-mediated resistance response. Functional analysis was done by overexpression of *BpPIP1* in *A. thaliana* plants and sugar beet hairy roots and a reduction of developed females was described. I determined its location on the translocation of the line TR520. BLASTn analyses of this sequence confirmed the wild beet origin (Table 33), since scaffold 42352 of the 'TR520 *de novo*' assembly could be identified to bear the *BpPIP1* gene. This scaffold is linked to the distal side of BAC 74E5 belonging to sections E and H of the physical map (Figure 11). However, both sections are absent from the resistant line TR363. Therefore, I suggest that *BpPIP1* is not the *Hs1-2* gene because this must be present in a region shared of the two nematode resistant lines TR520 and TR363.

As a result of the WGS analysis, 320 ORFs were identified and eleven ORFs were selected as candidate sequences. ORF 801, ORF 802, ORF 803, ORF 804, and ORF 807 show considerable homology to protein kinases. Therefore, these genes may have putative functions in signaling cascades in resistance pathways such as PTI. Furthermore, the *Rhg1* locus from soybean encodes for an LRR-receptor kinase and is discussed to be involved in resistance reactions against the soybean cyst nematode (*Heterodera glycines*) (Melito et al., 2010; Kandoth et al., 2011) (details see 4.6). Additionally, the *Pto* gene from tomato is an intracellular serine/threonine protein kinase that activates resistance response to *Pseudomonas syringae* infection in tomato (Oh and Martin, 2011). Transcription factors, carrying a basic-helix-loop-helix (bHLH), a basic-leucine zipper (bZIP), or a WRKY domain, are differentially expressed in incompatible interactions of nematodes and tomato as well as soybean (Jones et al., 2011). Therefore, a putative function of ORF 805, which shows sequence similarity to a bZIP transcription factor, may be the involvement in signaling cascades for transcriptional up- or down-regulation of specific genes (Phillips and Hoopes, 2008). Sequence similarity to a spermidine synthase-like protein was identified for ORF 808. A spermidine synthase from *A. thaliana* was shown to interact with an effector protein from *H. schachtii*. According to that interaction, the polyamine oxidase level is increased, which activates cellular antioxidants in syncytia (Hewezi, 2010). ORF 809 shows homology to a caseinolytic protease (clp) and ORF 811 to a metalloprotease. Proteases are involved in many cellular processes like degradation of proteins and cell growth control. Additionally, proteases appear to be relevant in pathogen recognition and induction of defense response (Hoorn and

Jones, 2004). ORF 810 encodes for an F-box protein. These proteins are a part of the SCF (SKP1/CUL1/F-box) complex that functions as ubiquitin E3 ligases. Furthermore, F-box proteins are essential in *A. thaliana* for ethylene, auxin, jasmonate, and gibberellin signaling (Lechner et al., 2006; van den Burg et al., 2008). In conclusion, all selected ORFs show homology to genes involved in plant resistance responses and may therefore be relevant in the sugar beet cyst nematode pathosystem.

The only candidate sequence with similarity to the majority of cloned nematode resistance genes encoding for a NBS domain is ORF 806, showing a predicted amino acid sequence with high sequence similarity to the disease resistance protein RGA2 (resistance gene analogue 2) from *Ricinus communis*. Furthermore, two conserved domains were determined, a NB-ARC domain (pfam00931) with an e-value of 6.94e-64 and an LRR domain with an e-value of 1.25e-03 (Table 28). The putative function of this ORF is effector detection as known for R-genes of the ETI pathway encoding for proteins with NBS and LRR domains (Göhre and Robatzek, 2008). ORF 806 was identified on scaffold 50724 of the 'TR363 unmapped *de novo*' assembly. It could not be integrated to the physical map of the *P. procumbens* translocation of the line TR520 because the sequence originates from the translocation line TR363 and its location on the physical map would be a rough speculation. PCR amplification with genomic DNA confirmed the absence of ORF 806 from the translocation line TR520, which was previously suggested by comparative sequence analyses between the 'TR520 *de novo*' and the 'TR363 unmapped *de novo*' assemblies. RT-PCR revealed an expression of ORF 806 only in *P. procumbens* and the translocation line TR363 (Table 29; Figure 15B). Due to the hypothesis that the *Hs1-2* gene should be located in the overlapping region of the two lines TR520 and TR363, ORF 806 was rejected as candidate sequence for *Hs1-2*.

Seven ORFs (see Table 29) were determined to be present in both resistant lines, TR520 and TR363, as well as in the susceptible line TR659 by sequence analyses of the WGS approach. This could be confirmed by PCR experiments amplifying genomic DNA (Table 29). Concerning the expression analysis by RT-PCR, only four ORFs (ORF 802, ORF 803, ORF 804, and ORF 810) were observed to be expressed in both resistant lines, TR520 and TR363 (Figure 15). Differences between gDNA and cDNA with regard to both resistant (TR520 and TR363) and both susceptible lines (TR659 and TR320) were determined for ORF 801, ORF 803 and ORF 807 (Figure 15B, Table 29). The only sequence being exclusively expressed in both resistant lines is ORF 803, which shows a predicted amino acid sequence with high sequence similarity to a MAP3K. MAP3K stands for mitogen activated protein kinase kinase kinase which is member of the mitogen-activated protein kinase (MAPK) cascade. Kinase signaling cascades are an evolutionarily ancient mechanism of signal transduction, found in eukaryotic cells, and is associated with responses to abiotic and biotic stresses (Hashimoto et al., 2012)

This was the reason why I chose ORF 803 for a detailed analysis. Additionally, the predicted amino acid sequence of ORF 803 showed significant similarity to EDR1 and CTR1 (3.3.6.2). EDR1 and CTR1 represent two of the best studied *A. thaliana* MAP3K and are known to be involved as negative regulators in ethylene-mediated signaling and defense response (Frye and Innes, 1998; Frye et al., 2001; Huang et al., 2003; Lin and Grierson, 2010; Lin et al., 2008; Tang et al., 2005). The role of MAP3K in plant defense response is established. The defense response of PTI in plants relies on the recognition of pathogen specific molecules and is accomplished by receptor kinases following a signaling cascade involving additional kinases, e.g. mitogen associated protein kinases (Göhre and Robatzek, 2008). Li et al. (2006) analyzed MAP kinase cascades required for *Mi*-mediated resistance to aphids, and they determined a decrease in resistance response by silencing of MAP3K. During soybean cyst nematode infection, MAPK are up-regulated in plants carrying the *Rhg1* locus and implicate regulation of soybean cyst nematode resistance via MAPK signaling (Kandoth et al., 2011).

Furthermore, the predicted amino acid sequences of ORF 801 and ORF 802, which show significant sequence similarity to a serine/threonine kinase and a phosphatidylinositol kinase, respectively, are located in close vicinity to ORF 803 on scaffold 26267. Using the trans-membrane prediction software

(TMHMM 2.0), one trans-membrane span was determined. The predicted amino acid sequence of ORF 802 shows homology to phosphatidylinositol kinases that are known to be involved in plant stress responses (Krinke et al., 2007). ORF 801, ORF 802, and ORF 803 form a gene cluster, since this is defined as a set of two or more genes that belong to the same gene family located in close range to each other (Yi et al., 2007). The gene cluster on scaffold 26267 (ORF 801, ORF 802, and ORF 803) of the 'TR520 *de novo*' assembly is integrated into super scaffold 21 which covers BAC 79D15 and 38N19 in section D of the physical map (Figure 11). Altogether, I suggest an involvement of these genes in nematode resistance which is discussed in the following.

Clustering of resistance genes in plants is known for years (Michelmore and Meyers, 1998) and was identified for *Pto*, that confers resistance against *P. syringae* in tomato. *Pto* was the first disease resistance gene cloned from a plant. It encodes for a serine/threonine kinase (Ronald et al., 1992). It is clustered on a single locus with the *Prf* gene that has a NBS-LRR domain. *Pto* acts in concert with *Prf* to activate ETI which leads to programmed cell death such as the hypersensitive response (HR) (Coll et al., 2011; Mucyn et al., 2006). To date, many proteins have been identified to be involved in the *Pto* pathway, such as MAPKKK (MAP3K), MAPKK, chaperones, F-box-LRR proteins, and transcription factors (Oh and Martin, 2011). In wheat, a locus denoted as *Pm21* was transferred from *Haynaldia villosa* as a translocation from the short arm of chromosome 6V to the long arm of chromosome 6A (6VS-6AL) conferring resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*). A serine/threonine protein kinase was cloned from this locus and a decrease in haustorium size was determined by single cell transient expression assay. The *Pm21* locus is discussed to be a cluster of resistance genes (Cao et al., 2011). Furthermore, molecular analysis of the nematode resistance gene *Gpa2*, conferring resistance against the cyst nematode *Globodera pallida* in potato, revealed a R-gene cluster of 115 kb with at least two active genes (van der Vossen et al., 2000). Recently, Afzal et al. (2012) inferred, that the *rhg1* locus, which mediates resistance to the soybean cyst nematode *Heterodera glycines*, might be a multi-gene resistance locus. Three genes were identified within the co-segregating marker-bound region of the *Rhg1* locus, one with sequence similarity to a receptor-like kinase, one laccase, and one predicted sodium/ hydrogen antiporter (for details, see chapter 4.6) (Ruben et al., 2006).

Furthermore, several kinase complexes are involved in plant resistance responses. Plants evolved receptors that receive extracellular signals, such as NBS-LRR receptors or receptor like kinases (RLK), which consist of an extracellular binding domain, a membrane spanning region and a serine/threonine kinase domain (Afzal et al., 2008). PRRs bind to elicitors, so-called PAMPs or microbe- associated molecular patterns (MAMPs), and activate several MAP kinases. (Göhre and Robatzek, 2008). The first identified and best studied PRR is 'flagellin sensing 2' (FLS2), which binds the bacterial flagellin-derived peptide flg22. FLS2 forms heterodimers with several RLK belonging to the SOMATIC-EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK) family, including BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1/SERK3 (BAK1/SERK3) and BAK1-LIKE1/SERK4 (BKK1/SERK4). BAK 1 is an LRR-RLK and is known to function as a co-receptor leading to a regulation of defense related receptors (Chinchilla et al., 2006; Chinchilla et al., 2007; Roux et al., 2011). FLS2 and BAK1 both interact by phosphorylation with the BOTRITYS INDUCED KINASE 1 (BIK1). Interestingly, BIK1 phosphorylates FLS2 and BAK1 (Greeff et al., 2012). The complex of BIK1-FLS2/BAK1 disintegrates after fls2 sensing, suggesting downstream signaling activities of BIK1 (Lu et al., 2010). Another LRR-RLK known to interact with BAK1, EFR (EF-Tu receptor), perceives the signal of bacterial elongation factor EF-Tu peptide elf18. Thus, FLS2 and EFR share some of the downstream signaling components, with EFR being stronger associated with other kinases. This may indicate a preferential interaction of ERF with RLKs other than BAK1, suggesting that additional components are required for signaling (Roux et al., 2011). Kinase complexes such as FLS2/BAK1 or EFR/BAK1 provide evidence for a possible complex formation of ORF 801 that encodes for an LRR serine/threonine receptor kinase with a trans-membrane span, and ORF 803, which encodes for a serine/threonine kinase and a MAP3K.

ORF 801, ORF 802, and ORF 803 were found to be present on the resistance-carrying translocation lines TR520 and TR363 by analysis of the WGS dataset. Their presence or absence from resistant and susceptible translocation lines was verified by PCR. The expression of the candidate sequences was analyzed by RT-PCR. With regard to ORF 802, no differences on cDNA level compared to amplification with gDNA was determined. In contrast, ORF 801 and ORF 803 seem to be differentially expressed because differences were determined by amplification of gDNA and cDNA. RT-PCR amplification indicated that both genes are expressed (see Figure 15). However, as PCR fragments were separated by agarose gel electrophoresis, the measurement of expression is restricted by the detection limits of this system. A high expression of ORF 801 was found in *P. procumbens* and the line TR520, whereas no visible amplicons were obtained for the lines TR363 or TR659. Regarding ORF 803, visible amplicons were obtained for *P. procumbens* and the translocation linesTR520, and TR363, while PCR fragments were absent from the line TR659. In conclusion, differential expression levels could be observed for ORF 801 and ORF 803 but their expression in other genotypes and under different environmental conditions remains to be analyzed by RTq-PCR (see chapter 4.7).

I conclude that ORF 803 is the major candidate sequence for the *Hs1-2* gene due to its sequence similarity to MAP3K and its expression in both resistant translocation lines (TR520, TR363). However, it was also expressed in the susceptible line TR659. Different expression patterns between resistant and susceptible plants were determined by RT-PCR amplification (3.3.6.1). Therefore, I expect ORF 803 to be alternatively spliced. Alternative splicing is a mechanism to increase the protein diversity by generation of various protein isoforms originating from a single gene (Stamm et al., 2005). In *A. thaliana*, 42% of all intron-containing genes are supposed to be alternatively spliced (Filichkin et al., 2010). An amount of approximately 60% – 70% of alternative splicing takes place in translated regions of the mRNA and might have effects on protein stability, signaling activities, and binding properties (Stamm et al., 2005). Alternative splicing events can be classified to four subgroups: during ‘exon skipping’ exons are spliced out of the precursor (pre-) mRNA; ‘selection of alternative 5’ or 3’ splice sites’ is the result of additional splice sites of one end of an exon, and ‘intron retention’ means that introns are incorporated into the mRNA (Keren et al., 2010; Kim et al., 2008).

Ner-Gaon et al. (2004) examined *A. thaliana* EST data from public databases such as TIGR (The Institute for Genomic Research TIGR, 2012) in order to determine different types of alternative splicing. They found intron retention as the major alternative splicing events in *A. thaliana* (30%), which was unexpected, since in human, only 2 – 5% of alternative splicing events are the result of intron retention. Interestingly, transcripts involved in stress and stimuli response were overrepresented among alternatively spliced transcripts, whereas transcripts related to metabolism and cell maintenance were underrepresented (Ner-Gaon et al., 2004). Iida et al. (2004) reported about biotic stresses affecting alternative splicing profiles in *A. thaliana*. The disease resistance gene RESISTANCE TO PSEUDOMONAS SYRINGAE4 (*RPS4*) produces multiple transcripts via alternative splicing. The alternative transcript was up-regulated during resistance response in *A. thaliana* Columbia (Col-0) (Zhang and Gassmann, 2007). Moreover, the wheat transcription factor *Wdreb2* produces three alternatively spliced forms. They are differentially expressed and activated by several abiotic stresses (Egawa et al., 2006). Regarding ORF 803, in contrast to amplification with gDNA, exon 1 and exon 4 could not be amplified using the primer combination H42H43 and H42H132, respectively, by RT-PCR in the susceptible line TR659 (see Figure 17). Several forms of alternative splicing can be discussed for the different transcripts of ORF 803. A possible explanation could be exon skipping of exon 1 and/or exon 4. In that case, the primer combination H42H43 cannot anneal during RT-PCR. Additionally, amplification via RT-PCR of the 790 bp fragment of the primer combination H42H132 is also excluded. Another possibility is the selection of an alternative 5’ splice site in exon 1. In this way, the recognition site for annealing of the primer H42 would be skipped and amplification of the fragments is excluded. A less frequent but a possible alternative splicing event for ORF 803 is the usage of alternative promoters. The 5’ terminal exons can be skipped by using alternative promoters. Interestingly, Koo et al. (2009) reported about the identification of alternative promoters of different transcripts of the rice MAPK gene *OsBWMK1*. They analyzed the expression of *GUS* fusion constructs

of two promoters in transgenic *A. thaliana* and rice plants. The splice variants *OsBWMK1S* and *OsBWMK1L* of *OsBWMK1* are both expressed but the expression level of *OsBWMK1S* was up-regulated in response to JA and SA treatments in contrast to *OsBWMK1L*. This suggests an involvement of the splice variant *OsBWMK1S* in plant defense signaling. A similar regulation might be possible for the different transcripts of ORF 803. Probably, transcripts of ORF 803 that include exon 1 to exon 4, where a major part of the transglutaminase domain is located, are transcribed during stress response in contrast to transcripts with the alternative spliced sites. Since the susceptible translocation lines TR659 and TR320 were produced by gamma irradiation and the generation of alternative spliced mRNAs is regulated by proteins which bind to *cis*-acting sites on the pre-mRNA, a mutation in a specific binding site might also be a possible scenario (David and Manley, 2008).

For future analyses, the sequencing of mate-pair libraries and a following assembly with the identified sequences of the WGS approach, as well as BAC and YAC sequences, is indispensable in order to complete the sequence of the wild beet translocation. Because of the remaining gaps on the translocation, the presence of additional sequences that are shared by the resistant lines TR520 and TR363 cannot be excluded. Thus, the existence of genes with similarity to the cloned nematode resistance genes is still probable.

In summary, I was able to increase the number of sequences determined for the *P. procumbens* translocation of the line TR520 to 2,226 kbp in a short time span of more than 100% compared to the previous identified BAC- and YAC sequences (see Table 24). The determined 2,226 kbp sequences exceed the previous estimated size of the translocation of Kleine et al. (1998) by around 50% and are now grouped into only two large super contigs on the physical map. Another major result is the identification of a large *B. vulgaris* insertion on the *P. procumbens* translocation of the line TR520. For the first time, a cluster of three genes with sequence similarity to kinases that are known to be involved in plant resistance response was identified on a region shared by the resistant translocation lines TR520 and TR363. Via RT-PCR I was able to observe differences between gDNA and cDNA in both resistant and susceptible translocation lines for ORF 803, which shows an overall sequence similarity of 34% on amino acid level to a mitogen activated protein kinase kinase kinase (MAP3K). In addition, I was able to identify ORF 801 and ORF 802, which respectively show high sequence similarity to a receptor kinase and a phosphatidylinositol kinase to be clustered with ORF 803. I expect this cluster of ORFs to be relevant for the resistance to nematode, as I have shown that these candidate sequences hold strong correlations to resistance mechanisms and the presented data that suggest their involvement is stronger than for any previous candidate gene. I suggest concentrating on it for future analysis. A detailed suggestion for future work is described in chapter 4.6.

3.5 References

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4 Closing Discussion

The main focus of my work was to identify a resistance gene, named *Hs1-2*, against *Heterodera schachtii* from two resistant sugar beet translocation lines, TR520 and TR363, which share partly homologous sequences (Jung and Wricke, 1987; Savitsky, 1975; Capistrano, 2009; Kleine et al., 1998; Schulte et al., 2006). The hypotheses were (1) that there is a second nematode resistance gene named *Hs1-2* and it is expected to be located in the region shared by the resistant translocation lines, TR520 and TR363, and absent from the susceptible lines TR659 and TR320 that were identified after irradiation of TR520, and (2) ORF 702 is a candidate for the *Hs1-2* gene (Capistrano, 2009; Schulte, 2006).

4.1 Studies to Select New Nematode Resistant Translocation Lines

For the selection of new translocation lines, I followed two approaches. One relied on crossing between translocation lines and the other relied on irradiation of monosomic addition lines. The purpose was to narrow down regions where *Hs1-2* could be located. Two single plants of the susceptible line TR320 and the resistant translocation line TR363 were crossed in order to identify new recombinant translocation lines resulting from crossing over events. Markers derived from the *P. procumbens* translocations of the lines TR363 and TR320 were used to identify F1 hybrids that carry both translocations. The identified hybrids were then used for seed production via selfing. The offspring was tested for nematode resistance and by PCR analysis using markers for the *P. procumbens* translocations (TR320 and TR363). As a result, no recombination event could be determined by analyzing the 253 F_2 plants.

A total of 4,300 irradiated seeds (400-600 Gy) of monosomic addition lines *P. procumbens* chromosome 1 were sown and 2,871 plants were tested for nematode resistance in the greenhouse. The identified resistant plants (416) were grown in the field in order to pollinate cytoplasmic male sterile (CMS) plants. Pollen of monosomic addition lines (2n=19) carry only 9 chromosomes. Only if the resistance from the monosomic chromosome was translocated to a sugar beet chromosome, resistant diploid (2n=18) plants could arise. Hence, plants derived from seeds harvested on CMS plants were analyzed for nematode resistance and by marker analysis, but no resistant plant carrying 2n=18 chromosomes could be detected.

The purpose of the irradiation experiments with monosomic addition lines of *P. procumbens* chromosome 7, which bears the nematode resistance gene $Hs2^{pro\text{-}7}$, was to induce chromosome breaks via gamma irradiation in order to select new nematode resistant translocation lines. The irradiated 2,947 seeds (400 Gy) were germinated, and 1,139 plants were tested for nematode resistance. The average germination rate of the irradiated chromosome 7 addition lines was quite low and ranged between 1.8% and 58%. All 17 resistant plants identified carried 19 chromosomes after microscopical analysis, and thus, no translocation plant (2n=18) could be identified. In conclusion, no new nematode resistant translocation line was identified in all these three experiments. Thus, they were not further mentioned in my thesis.

As the susceptible lines TR320 and TR659 both bear a modified translocation that was identified after irradiation of seeds of the line TR520, gamma irradiation has been confirmed to be a valuable method for the induction of chromosomal breaks (Capistrano, 2009). For future experiments, I recommend to use a higher amount of plants for crossing experiments in order to increase the chance to identify a crossing over experiment or to induce chromosomal breaks. Hence, these experiments failed to narrow down the region for the *Hs1-2* gene.

4.2 Functional Analysis of ORF 702

In the first chapter of this work, I describe the functional analysis of ORF 702, which was proposed as a candidate sequence for *Hs1-2* by Capistrano (2009) due to several reasons: it is expressed in the resistant translocation lines and in *P. procumbens*, it is located on the physical map in a region shared by the resistant lines TR520 and TR363 (see Figure 11; BAC 7C2 in section A on super contig 1), and it has a sequence homology to an AVR9 elicitor response protein (Capistrano, 2009). Additionally, a conserved galactosyltransferase domain has been determined (Capistrano, 2009). Galactosyltransferases belong to the glycosyltransferase enzymes that catalyze the transfer of monosaccharides (Ross et al., 2001). AVR genes are known to be involved in the gene-for-gene resistance reaction and additionally a glycosyltransferase gene (The Institute for Genomic Research (TIGR), *Solanum lycopersicum* gene index (LeGI) database no. TC166108) was identified by Scaff et al. (2007) to be up-regulated six times more in resistant roots infected with *M. incognita* than in uninfected tomato roots (Sanseverino et al., 2010). The way in which the glycosyltransferase interacts in the resistance reaction to *M. incognita* remains to be investigated, but analyses of Langlois-Meurinne (2005) revealed a major role for expression of a glycosyltransferase in resistance to *Pseudomonas syringae* in *A. thaliana*. They found that the induction of glycosyltransferases is independent of methyljasmonate, but partially dependent on salicylic acid.

The functional analysis of ORF 702 was done by overexpression of ORF 702 under the control of the 35S promoter in sugar beet hairy roots and in *A. thaliana*. The correct orientation and sequence of the cloned CDS of ORF 702 was confirmed by sequencing the plasmid. I determined the expression of the transgene by GUS staining and RT-PCR. As discussed in chapter 3.42.4, this technique has its limitations, and RT-qPCR would be a better choice to analyse different expression levels of transformants. In the end, the data presented in this thesis do not confirm the hypothesis that ORF 702 is a candidate for the *Hs1-2* gene. If I would start over again, I would also generate constructs with the gene-specific promoter of ORF 702 in order to display an expression level and tissue specificity of the transgene in hairy root clones similar to that in resistant translocation lines. Besides that, I suggest to transform resistant sugar beet translocation lines with RNAi constructs for future work. Recently, Lui et al (2012b) reported about the positional cloning of the *Rhg4* gene from soybean, a serine hydroxymethyltransferase, and applied RNAi for functional validation. As discussed in chapter 3.4 resistance reactions often include more than one gene, e.g. as gene clusters or copy number variations (CNV). This possibility cannot be ruled out for the *Hs1-2* gene.

4.3 Revisions of the Translocation Physical Maps

In the second chapter of this work, I describe the WGS approach and subsequent assembly strategies to complete the sequence of the translocation. I sequenced two nematode resistant lines, TR520 and TR363, in collaboration with the IKMB (Institute for Clinical and Molecular Biology, Kiel, Germany). I selected 1,193 kbp new translocation-specific sequences, of which ~40% could be anchored to the physical map. The mapped BACs and the draft assemblies of *P. procumbens* and *B. vulgaris* provided good resources for the assembly, with short reads obtained by WGS sequencing of TR520 and TR363. The major outcome of this work is the identification of new translocation specific sequences. These sequences increase the size of the physical map of the *P. procumbens* translocation of the line TR520 to around 150% of the previous estimate. I extended the physical map of the line TR520 by 1,263 kbp in two ways: chromosome walking and WGS data analyses. Chromosome walking yielded BAC 86F6 and BAC 113H20 at the distal site of the physical map in section A and I, and BAC 56F10 and BAC142D13, which were placed on section D. By analysis of the WGS data I determined new overlapping regions between the two resistant lines TR520 and TR363. Finally, I present ORF 803 as a superior candidate sequence and suggest for future experiments to focus on it due to its high sequence similarity to the MAP3K genes *EDR1* and *CTR1*. Using RT-PCR I was able to detect differences between gDNA and cDNA in resistant and susceptible lines. For the first time, I showed that the translocation from the line TR520 is interrupted by long *B. vulgaris* sequences.

Resequencing of important crops was applied in other studies. My work is the first resequencing experiment with *Beta* species. One major outcome of the other studies was the identification of genetic variations in the form of SNPs, insertions-deletions (InDels), or presence absence variations (PAV) comparable to those in maize inbred lines, analyzed by Lai et al. (2010), and *Sorghum bicolor*, evaluated by Zheng et al. (2011). Resequencing experiments were also applied in order to analyze the effects of domestication, e. g by resequencing of 17 wild relative soybean lines and 14 cultivated soybean lines by Lam et al. (2010). I provide high quality Illumina paired-end reads with >100-fold genome coverage for two nematode resistant sugar beet translocation lines. These data provide a solid background for future experiments. In this work, the data were used for the selection of candidate sequences for nematode resistance on a *P. procumbens* translocation. Besides that, mapping the reads of both translocation lines to the sugar beet genome sequence, which will be published in the near future (The *Beta vulgaris* Resource, 2012), would provide insights into genome wide genetic variations between the doubled haploid line KWS2320 that had been sequenced and the translocation lines. Furthermore, I suggest analyzing structural variations possible due to chromosome rearrangements during the translocation event.

Another major result of this work was the assembly of 13 super scaffolds that are not anchored to the physical map, which include shared sequences of the translocation lines TR520, TR363, and the *P. procumbens* assembly, and sum up to a TR520-specific sequence length of 717 kbp. However, gaps still remain on the physical map. An explanation for this is that repetitive sequences within genomes are still a major challenge to NGS technologies (Zhang et al., 2011). One strategy to handle repeats is to use mate pair information from reads that were sequenced as pairs. A good example is the recent release of the potato genome (The Potato Genome Sequencing Consortium, 2011). The first assembly of the genome (844 Mbp) was generated by combining Illumina and 454 reads. As the potato genome is highly repetitive (62%), contigs were produced with a N50 size of only 697 bp. Reassembling with Illumina mate-pair libraries of 2-10 kbp increased the N50 size to 31,429 bp (The Potato Genome Sequencing Consortium, 2011; Treangen and Salzberg, 2012).

The Illumina HiSeq 2000 sequencer has the lowest cost per Mbp and is therefore often the sequencing platform of choice, even despite its relatively small read size of 100 bp compared to other sequencers (Liu et al., 2012a). An example is the assembly of the 2.4 Gbp genome of the giant panda, which covers 94% of the genome. This assembly was done using only Illumina sequences from 37 libraries of 150 bp to 10 kbp mate pair information (73-fold coverage) (Li et al., 2010).

The present status of the *P. procumbens* translocation of line TR520 is summarized in the following. The total sequence length is 2,226 kbp. The present physical map now includes 20 BACs, 13 of which have been sequenced by Schulte (2006), by Capistrano (2009) and in this work. The BACs and the WGS data were assembled into two super contigs of which BAC 113H20 and super scaffold 25 represent the most distal part and BAC 89D17 and super scaffold 10 represent the most proximal sequences on the physical map (Figure 11). In chapter 3.3.9 I show that the *P. procumbens* translocation of the line TR520 is interrupted by sugar beet sequences. These sequences were identified within BAC 149P7, located in section B on super contig 1.

The *P. procumbens* translocation of the line TR363 sums up to 1,206 kbp (Table 24, Table 25). A number of 406 kbp are exclusively located on the line TR363, whereas 800 kbp are shared between the lines TR520 and TR363. The most distal sequence on the physical map present on the *P. procumbens* translocation of the line TR363 is BAC86F6 located in section A on super contig 1, and the most proximal sequence is BAC-end 158M12 Sp6 (CAU3616) located on section G on super contig 2-3 (Figure 11). Capistrano (2009) mapped the *P. procumbens* translocation of the susceptible line TR320 by comparative analysis of four translocation lines by the sections F, G, and H on the physical map. These sections correspond to BAC contig 3 on the third generation physical map (Capistrano, 2009), which I now integrated into super contig 2-3 (Figure 11). During this work, I assembled super scaffold 13, which maps to the lines TR520, TR659 and TR320 (Table 25) and was placed into the new section

M on the physical map. The most distal sequence present on the translocation of the line TR320 is BAC-end 74E5T7 (CAU3609) and the most proximal sequence is the new super scaffold 13. In total, I identified 662 kbp of new sequence information from the *P. procumbens* translocation of the susceptible line TR320. This result has to be taken with caution, since super scaffold 13 is not anchored to the physical map, and additionally a gap remains between BAC-end 9K7 T7 (CAU3620) and BAC 158M12 in section G on super contig 2-3 (see Figure 11).

Capistrano (2009) mapped the *P. procumbens* translocation of the susceptible line TR659 to sections D, E, F, G, and H by comparative sequence analysis. In this work, I identified new sequences that mapped also to the line TR659. These sequences were placed to the new sections J, K, L, and M. The most distal sequence present on the translocation of the line TR659 is BAC-end 79D15 Sp6 (CAU3601) of section D on super contig1, and the most proximal sequence is super scaffold 13 in section M on super contig 2-3. In conclusion, the total sequence length from the distal to the proximal site of the translocation of the line TR659 is 1,211 kbp. Since the lines TR320 and TR659 were not sequenced, these results have to be observed carefully because the lines were investigated by comparative sequence analyses with selected markers of BAC-ends and scaffolds of the WGS assemblies by PCR, as presented by Capistrano (2009) and in this work (Table 26).

There are still gaps on the recent physical map. The two super contigs are separated by a gap in which 8 super scaffolds were placed by mapping to the four translocation lines (Figure 11). These super scaffolds are not anchored with each other or to any physically mapped BAC or YAC. Since the sequence of the *P. procumbens* translocation of the line TR520 exceeds the previous estimated size of the translocation (1,500 kbp) (Kleine et al., 1998) by 48.4%, it is not possible to calculate the total size of the translocation or the size of the gaps.

Homologous sequences of the lines TR520 and TR363 can be found in sections A, C, D, J, G, and L shown on the physical map (see Figure 11). However, the susceptible line TR320 shares the section G and the line TR659 shares the sections G, J, D and L with TR520 and TR363. I determined sections J and L in this work; they include shared sequence between the lines TR520 and TR363, but these sequences also map to the line TR659, as shown in chapter 3. Homologous sequences that are present of the resistant lines TR520 and TR363 and absent from the susceptible lines TR659 and TR320 can be found in section A and C on the physical map. I identified BAC 86F6 by chromosome walking on the distal side of BAC 7C2. Mapping the sequence of BAC 86F6 to the TR363 assemblies, 7,102 bp could be identified that have a high sequence similarity to sequences of the line TR363. Therefore, section A was extended to a total of 109 kbp whereas section C is 81 kbp in size (Capistrano, 2009). The total size of homologous sequences present only on the resistant lines TR520 and TR363 add up to 190 kbp. The region where the candidate gene *Hs1-2* must be located corresponds to section A and C, as concluded by Capistrano (2009), since these sections are the only sequences present on the resistant lines TR520 and TR363 and absent from the susceptible lines TR659 and TR320. However, the susceptible lines TR659 and TR320 have been generated by gamma irradiation, which increases the possibility of mutations. Moreover, only partial sequence information of the lines TR659 and TR320 is available. Thus, sections D, J, L, and G also provide possible locations for the nematode resistance gene *Hs1-2*.

My major aim was to identify the resistance gene *Hs1-2*. Therefore, I searched all *P. procumbens* specific sequences of the line TR520 for resistance gene candidates. On the translocation, 320 ORFs were predicted, and eleven of those were selected due to their sequence similarity to genes from other organisms known to be involved in resistance responses and due to their location on the physical map shared between the lines TR520 and TR363, as explained in chapter 3.3.6. Their expression in resistant and susceptible plants was investigated by RT-PCR (see Figure 15).

4.4 *Hs1-2* Gene Candidates from Previous Studies

In previous studies, a number of candidates for the *Hs1-2* gene have been proposed. Tian et al. (2003; 2004) proposed the resistance gene analogous (RGA) cZR-3 and cZR-7 to be located on the *P. procumbens* translocation (lines TR520 and TR363). Furthermore, Knecht (2010) suggests an involvement of these genes in the $Hs1^{pro-1}$-mediated resistance response. By comparative sequence analysis of cZR-3 and cZR-7 to the *P. procumbens* and *B. vulgaris* (RefBeet 0.9) assemblies, I could clearly show that these sequences are not present in the genome of *P. procumbens*, but they are identical (100% sequence similarity) to sequences of the *B. vulgaris* genome. I determined 78% sequence similarity to the *P. procumbens* genome for cZR-3 and 84% sequence similarity for cZR-7. Capistrano (2009) proposed ORF 702 as a candidate for *Hs1-2*. In this work the functional analyses of ORF 702 in sugar beet hairy roots and *A. thaliana* show that ORF 702 is not a candidate for *Hs1-2*. Menkhaus (2011) proposed the gene *BpPIP1* to be involved in nematode resistance. He cloned the gene from the translocation of the line TR520 and observed a constitutively expression and an up-regulation upon nematode infection. In chapter 3.3.8 of this work, comparative sequence analysis of this sequence to the *B. vulgaris* and *P. procumbens* reference assemblies clearly showed an affiliation to the wild beet genome. The *BpPIP1* gene could be identified on scaffold 42352 of the translocation line TR520. This scaffold overlaps with scaffold 20220 of *P. procumbens* and is integrated into super contig 2-3, linked to the distal side of BAC 74E5 in section E and H. Both sections are not present on translocation TR363 but on both susceptible translocation lines TR320 and TR659. In conclusion, I could not confirm any of these genes to be the nematode resistance gene *Hs1-2* based on functional analysis of ORF 702 and on sequence analyses of cZR-3, cZR-7 and *BpPIP1*.

4.5 Recent Developments in Sugar Beet Genome Research

In the last months, several achievements of sugar beet research in the fields of genomics, cytogenetics, gene cloning, and mapping have been published. Abou-Elwafa et al. (2012) described a novel major locus, termed *B4*, for bolting control in *B. vulgaris*. Genetic linkage analysis revealed *B4* in a genetic distance to the *B* gene on chromosome 2. Kirchhoff et al. (2012) evaluated the genetic variation for winter hardiness in a panel of *B. vulgaris* for growing sugar beet as a winter crop. Hatlestad et al. (2012) identified a novel phytochrome P450 as the *R* locus described 70 years ago by Keller (1936) that is required to produce red betacyanin pigments in beet. The betalain pathway is unique to the order *Caryophyllales*. They confirmed the role of the gene *CYP76AD1* by virus-induced gene silencing (Hatlestad et al., 2012). At the beginning of this year, Dohm et al. (2012) presented a physical map of *B. vulgaris* that includes 535 contigs, anchored to the chromosomes, 8,361 short 35mer oligonucleotide probes based on ESTs and genomic sequences, and 22,815 BACs. Moreover, they generated an extended genetic map that contains 1,127 single nucleotide polymorphism markers. Comparing the gene order of the physical map with rosid species, the authors could identify syntenic regions involving 1,400-2,700 genes in the sequenced genomes of *A. thaliana*, poplar, grapevine, and cocoa. They showed that *B. vulgaris* has extensive sequence duplications and is an ancient hexaploid species.

Paesold et al. (2012) reported about a reference FISH karyotype for *B. vulgaris*. They used a set of 18 physically anchored BACs to discriminate all nine chromosomes by FISH. In order to find markers for the identification of homoeologous chromosomes of wild *Beta* species (*B. vulgaris* ssp. *maritima*, *B. patula*, *B. corolliflora*, *P. procumbens*), FISH experiments revealed a conserved position of a BAC marker on chromosome 1 that is homoeologous in all four wild species. Because of a different distribution of repetitive elements on the chromosomes, a multicolor FISH procedure that allows the identification of all nine sugar beet chromosomes in one hybridization step with a pool of satellite DNA probes was established. With regard to sugar beet chromosome 9, a considerable distance between the subtelomeric satellite pAv34 and the most distal chromosome arm specific BAC marker by FISH on stretched chromatin fibres was determined, which reveals that this linkage group is not yet terminally closed. Moreover, the subtelomeric satellite pAv34 showed a moderate signal to only one

end of chromosome 9. This result can be helpful for future experiments to complete the sequence of the *P. procumbens* translocation of the lines TR520 and TR363. Additionally, multicolor FISH using chromatin fibres of the translocation line with translocation specific probes and the satellite pAv34 as a probe may provide new insights about the size of the translocation attached to chromosome 9 of sugar beet.

4.6 Recent Findings in Plant-Nematode Interaction

Two important publications were released recently concerning the analyses of nematode resistance genes to *H. glycines* in soybean. Two loci have been investigated since years, *rhg1*, mapped to chromosome 18, and *Rhg4*, mapped to chromosome 8 (Caldwell et al., 1960; Concibido et al., 2004; Matson and Williams, 1965). Liu et al. (2012b) reported about the positional cloning of *Rhg 4* gene from recombinant inbred lines (RIL). They determined the expression of a serine hydroxymethyltransferase (*SHMT*) within syncytia and verified the proposed function by transformation of a susceptible RIL with a 5.1 kbp genomic fragment of *SHMT* including the gene-specific promoter, and by the application of RNAi in hairy roots. Moreover, they suggest a role for *SHMT* to trigger the hypersensitive-response-like programmed cell death of developing feeding sites (Liu et al., 2012b). However, the *Rhg1* locus is discussed to contribute much more than any other loci to soybean cyst nematode resistance and thus approximately 90% of all resistant and commercially cultivated soybean varieties in the central United States bear the *rhg1-b* allele derived from the soybean line PI 88788 (Cook et al., 2012). A 31 kbp fragment identified at the *rhg1-b* locus carries genes encoding an amino acid transporter, an α-synaptosomal-associated (SNAP) protein, and a wound-inducible domain 12 protein. The authors identified one copy of the 31 kbp fragment per haploid genome in susceptible soybean varieties, and detected 10 tandem copies in the *rhg1-b* haplotype. Additionally, overexpression of the single genes was ineffective, but simultaneous overexpression of the genes provided enhanced resistance to *H. glycines* (Cook et al., 2012). Kandoth et al. (2011) analyzed gene expression profiles of developing syncytia using near isogenic lines (NIL) of soybean differing only at the *Rhg1* locus. They detected a number of 1,447 genes that are differentially expressed between the lines. Among others, a MAP3K homolog was up regulated, which implicates mitogen-activated protein signaling in the regulation to *H. glycines*. These results indicate a high complex defense response against root pathogens (Kandoth et al., 2011). Recent publications also discuss the three genes that were identified at the *Rhg1-a* locus, with coding sequences showing similarities to a receptor-like kinase (RLK), a laccase and a predicted sodium/ hydrogen antiporter (Afzal et al., 2012; Ruben et al., 2006). Analysis of the RLK *GmRLK18-1* in transgenic soybean plants revealed that this gene alone is sufficient to provide nearly complete resistance, but the *Rhg4* locus is required to provide full resistance to *H. glycines* type 0 (Srour et al., 2012). These recent results of a plant cyst nematode pathosystem also support the discussion in chapter 3.4 of the involvement of gene clusters, kinases and complex signaling networks in resistance response.

4.7 Outlook

As a major outcome of this work, the sequence of a *P. procumbens* translocation was enormously extended in a short time period by combining conventional Sanger and NGS technologies.

Since the sequencing data of this work provide a solid basis, I suggest the following future experiments. In order to complete the sequence of the *P. procumbens* translocation, sequencing of long mate-pair libraries is recommended. I also propose to use two different assembly strategies: (1) a *de novo* assembly that includes the NGS and the BAC and YAC sequences and (2) mapping of all NGS sequences with high stringency to the *B. vulgaris* reference sequence that will be released in the near future (The *Beta vulgaris* Resource, 2012) prior to a *de novo* assembly with BAC and YAC sequences in order to improve the assembly quality as demonstrated in this work.

Sequencing of the transcriptome (mRNA-Seq) of the susceptible line TR659 and the resistant lines TR520 and TR363 will permit assessment to transcript structure and transcript abundances. I suggest using the susceptible line TR659, since the *P. procumbens* translocation of this line shares a larger part of sequences with the resistant translocation lines TR520 and TR363 than the susceptible line TR320. I recommend employing nematode inoculated root material from different infection time points and non-inoculated root material as control.

Since ORF 803 is located on scaffold 26267 of the 'TR520 *de novo*' assembly that contains gaps, I recommend first to fill the gaps e.g. by primer walking or by assembly with new sequences, resulting from the mate pair library. Subsequently, I suggest a comparative sequence analysis of the genomic and coding sequence of ORF 803 between the resistant lines TR520, TR363, *P. procumbens*, and the susceptible line TR659. In order to investigate a proposed alternative splicing of ORF 803, as discussed above, the usage of alternative promoters is a possibility and therefore sequences (2 – 3 kbp) upstream of the start codon should be included in the sequence analyses.

The expression pattern of ORF 803 in resistant and susceptible lines was analyzed by RT-PCR. Since the measurement of expression is restricted by the detection limits of the agarose gels, I recommend applying RT-qPCR with inoculated and non-inoculated root material harvested at different time points after inoculation of resistant and susceptible lines, as well as *P. procumbens*.

For the functional analyses of the candidate gene, two model systems are available for transformation, (1) sugar beet hairy roots and (2) *A. thaliana*, which is also a host of *H. schachtii*. These systems were used in this work by the functional analysis of ORF 702 (see chapter 2). To investigate if the identified gene cluster coding for protein kinases on scaffold 26267 of the 'TR520 *de novo*' assembly, which is shared by the translocation line TR363, is involved in nematode resistance, first a sequence analyses of ORF 801 and ORF 802 is recommended in the same way as discussed above for ORF 803. Secondly, generation of transgenic hairy roots of *B. vulgaris* or *A. thaliana*, transformed with all genes of the cluster (ORF 801, ORF 802, and ORF 803) is a possibility for verification, as well as RNAi in sugar beet hairy roots of resistant lines. As discussed above and in chapter 3.4, the involvement of more than one gene in resistance response to nematodes in clusters or formation of complexes is possible. If more than one gene from the *P. procumbens* translocation is essential for complete resistance, the production of knock-out mutants by RNAi of the resistant translocation lines TR520 and TR363 is recommended.

Regarding the eleven ORFs selected as candidates for *Hs1-2*, ORF 806 is the only candidate sequence with similarity to the majority of cloned nematode resistance genes carrying an NBS domain. Furthermore, two conserved domains were determined, an NB-ARC domain (pfam00931) with an e-value of 6.94e-64 and an LRR domain with an e-value of 1.25e-03 (Table 28). However, I could show that ORF 806 is located only on the resistant line TR363. To investigate if this sequence plays a role in plant nematode response, I would propose RNAi in hairy roots of the line TR363.

Since previous studies demonstrated that the sugar beet hairy root system yields limited results with regard to functional characterization of nematode resistance genes, studying of whole transformed sugar beet plants is also necessary. Unfortunately, transformation by *A. tumefaciens* to generate whole sugar beet plants takes approximately one year (personal communication, Josef Kraus, KWS, Einbeck, Germany). The technique for sugar beet transformation is hard to establish and currently available as proprietary technology offered only by a small group of private companies and therefore rather expensive. Thus, the transformation of whole sugar beet plants should be considered only after analysis in model systems, such as hairy roots and *Arabidopsis thaliana*, with the advantages of high transformation efficiency and the short time span of the system to generate transformants.

5 Summary

Sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) is a host of the beet cyst nematode (BCN) *Heterodera schachtii* Schmidt, which causes high yield losses. Resistance had been identified in the wild relative *Patellifolia procumbens* and was integrated into the sugar beet genome by a translocation to the end of chromosome 9. Two nematode resistant (TR520 and TR363) and two susceptible (TR659 and TR320), sugar beet translocation lines which share partly homologous sequences from *P. procumbens* chromosome 1, were the focus of my research. I hypothesized that a second gene for nematode resistance (*Hs1-2*) is located on a region shared by both resistance carrying translocations.

The aim of this work was the identification of the *Hs1-2* gene. The work includes a functional analysis of a candidate sequence for the *Hs1-2* gene (ORF 702) which encodes a galactosyltransferase family protein and which had been identified in a previous work. Moreover, new gene candidates have been identified and characterized after whole genome shotgun (WGS) sequencing of the translocation lines TR520 and TR363.

In the first part of this work, I cloned the full-length sequence of ORF 702, which is 1,110 bp (370 aa) in size and consists of 7 exons. The coding sequence was cloned into the binary vector pAM194 under the control of the CaMV 35S promoter and transformed via *Agrobacterium*-mediated transformation into sugar beet hairy roots and *Arabidopsis thaliana*. The expression of the transgene was verified via GUS staining and RT-PCR. I analyzed the nematode development on *in vitro* growing *A. thaliana* T_2 families and sugar beet hairy roots. I observed no significant differences in nematode development between transgenic and control plants. Therefore, ORF 702 was rejected as a candidate for the *Hs1-2* gene.

In the 2nd part of this work, I extended the sequence of the BAC based physical map of the translocation line TR520 by 1,193 kpb. As the extension of the BAC-based physical map by chromosome walking is a tedious work, the translocation lines TR520 and TR363 were sequenced by a WGS approach using an Illumina HiSeq2000 sequencer. This resulted in ~ 87 Gbp raw sequence data (~ 110 fold genome coverage / line). A hybrid assembly was performed, combining short reads of the resistant translocation lines TR520 and TR363, 1,032 kbp BAC and YAC sequences of the physical map of the translocation line TR520, and draft reference assemblies of a sugar beet and a wild beet whole genome sequence. As a result, 477 kbp of new sequences were identified anchored to the physical map. Thus, the total sequence of the physical map adds up to 1,509 kbp. In addition, 13 new super scaffolds were assembled that integrate scaffolds and contigs of *P. procumbens* and the translocations TR520 and TR363 that are not anchored to the physical map. As a major result, the new sequences identified (716 kbp) increase the size of the TR520 translocation to 2,226 kbp, corresponding to ca. 150% of the previously estimated value (1,500 kbp). I was first to be able to demonstrate that the translocation is interrupted by large sequences from the *B. vulgaris* genome. This renders the identification of the translocation breakpoint(s) less important, as there is no single start or end of the translocation. In addition, I was able to identify sequences exclusively located on the TR363 translocation.
Further analyzes of the translocation specific sequences resulted in the prediction of 320 ORFs. I selected eleven ORFs as candidate sequences according to their position on the physical map and to their homology to known genes from other species. Via RT-PCR I was able to observe differences between gDNA and cDNA in both resistant and susceptible translocation lines for ORF 803 which shows an overall sequence similarity of 34% on amino acid level to a mitogen activated protein kinase kinase kinase (MAP3K). In addition, I was able to identify ORF 801 and ORF 802, which respectively show high sequence similarity to a receptor kinase and a phosphatidylinositol kinase to be clustered with ORF 803. I suggest to focus on this cluster of genes, as they may be involved in beet cyst nematode resistance.

6 Zusammenfassung

Die Zuckerrübe (*Beta vulgaris* L.) ist ein Wirt des Rübenzystennematoden *Heterodera schachtii* Schmidt, welcher für hohe Ertragsverluste verantwortlich ist. Resistenz gegen *H. schachtii* konnte in der Wildart *Patellifolia procumbens* festgestellt und durch Translokation an das Ende von Chromosom 9 der Zuckerrübe übertragen werden. Bislang sind zwei nematodenresistente (TR520 und TR363) und zwei anfällige Translokationslinien (TR320 und TR659) bekannt, die teilweise homolog zueinander sind. Die Position eines zweiten Nematodenresistenzgens (*Hs1-2*) wird in einer gemeinsamen Region der beiden resistenten Linien vermutet. Als Ergebnis einer früheren Arbeit war in Bezug auf diese Hypothese der offene Leserahmen (*open reading frame*, ORF) 702 als Kandidatensequenz vorgeschlagen worden.

Das Ziel meiner Arbeit war die Klonierung des *Hs1-2* Gens. Zunächst wurde hierzu eine funktionelle Analyse des ORF 702 durchgeführt und weitere translokationsspezifische Sequenzen und Genkandidaten mittels *whole-genome-shotgun* (WGS) Sequenzierung der Translokationslinien TR520 und TR363 identifiziert.

Im ersten Teil dieser Arbeit präsentiere ich die Volllängensequenz des ORF 702, welcher 1110 bp (370 aa) lang ist und sieben Exons enthält, sowie eine funktionelle Analyse des ORFs in zwei Modelsystemen. Die kodierende Sequenz wurde in den binären Vektor pAM194 unter der Kontrolle des CaMV 35S Promoters kloniert und durch *Agrobacterium* vermittelte Transformation in Zuckerrüben *hairy roots* und in *Arabidopsis thaliana* übertragen. Die Expression des Transgens habe ich über GUS-Färbung und durch RT-PCR untersucht und die Nematodentests mit Zuckerrüben-*hairy root* Klonen und spaltendenden *A. thaliana* T_2 Familien durchgeführt. Hierbei habe ich keine signifikanten Unterschiede in der Nematodenentwicklung zwischen Transgenen- und Kontrollpflanzen festgestellt und deshalb ORF 702 als Kandidat für das *Hs1-2* Gen ausgeschlossen.

Im zweiten Teil meiner Arbeit erweiterte ich die Sequenz der BAC-basierten physischen Karte der Translokationslinie TR520. Da die Ausweitung der BAC-basierten physischen Karte durch *chromosome walking* limitiert ist, wurde eine *whole genome shotgun* (WGS) -Sequenzierung der Translokationslinien TR520 und TR363 mit einem Illumina HiSeq 2000 Sequenzierer durchgeführt. Es wurden ~ 87 Gbp Rohsequenzen produziert mit einer ~110-fachen Genomabdeckung je Translokationslinie. Mit den resultierenden kurzen Sequenzstücken (*short reads*) habe ich eine Hybridassemblierung zusammen mit 1032 kbp BAC- und YAC-Sequenzen, die auf der physischen Karte verankert sind, und vorläufigen *assemblies* eines Zuckerrüben- und eines Wildrübengenoms durchgeführt. In Folge dessen konnten 477 kbp neue Sequenzen, die auf der physischen Karte verankert sind, identifiziert werden. Daraus ergibt sich eine Gesamtlänge von 1509 kbp. Zusätzlich konnte ich 13 neue Sequenz-*scaffolds* assemblieren, in denen Sequenzen von *P. procumbens* und der WGS-Sequenzierung der Tranlokationslinien TR520 und TR363 integriert sind. Die so identifizierten 716 kbp erhöhen die Gesamtlänge der Translokation (Linie TR520) auf 2226 kbp, was etwa 150% des zuvor geschätzten Wertes (1500 kbp) entspricht. Darüber hinaus konnte zum ersten Mal gezeigt werden, dass die Translokation von langen Zuckerrübensequenzen unterbrochen wird. Damit erübrigt sich die Identifizierung des Translokationsbruchpunktes als Referenzpunkt für das proximale Ende der Translokation. Zusätzlich konnten Sequenzen identifiziert werden, die exklusiv nur auf der Translokation der Linie TR363 vorkommen. Weitere Sequenzanalysen ergaben 320 vorhergesagte ORFs, aus denen ich elf ORFs als mögliche Kandidatensequenzen für das *Hs1-2*-Gen entsprechend ihrer Position auf der physikalischen Karte und aufgrund ihrer Sequenzähnlichkeit zu anderen bekannten Resistenzgenen selektierte. ORF 803 liegt auf beiden resistenten Translokationen und auf der anfälligenTranslokationen TR659. Dagegen zeigte die Analyse mittels RT-PCR eine unterschiedliche Expression für die anfälligen und resistenten Translokationslinien. Die Aminosäuresequenz von ORF 803 hat 34% Ähnlichkeit zu einer *mitogen-activated* Protein Kinase Kinase Kinase (MAP3K). Zusätzlich konnte ich ORF 801 und ORF 802 in direkter Nähe zu ORF 803

lokalisieren. Beide weisen eine hohe Sequenzähnlichkeit zu Rezeptorkinasen und Phosphatidylinositolkinasen auf. Diese Anhäufung von Genen innerhalb einer kritischen Region der Translokation könnte auf den *Hs1-2*-Locus hinweisen.

7 Appendix

7.1 Supplementary Tables

Suppl. Table 1: Identification of *cis*-regulatory elements on the promoter sequence of ORF 702 using the web based software PLACE (Higo et al., 1999).

Suppl. Table 2: Overview of the results of the different nematode resistance experiments using diverse clones of the sugar beet hairy root system. Number of analysed Petri dishes (PD) and the average (∅) number of developed J4-females in each experiment is given. The letters A, B, C, D, and E indicate the different experiments. Clone numbers beginning with the same number (e.g. 24_1, 24_2) indicate root clones originating of the same leaf stalk (related clones). "-": clone not tested.

Suppl. Table 3: Results of the BLAST analyses using scaffolds and contigs of super contig 1 and physical mapped BAC clones.

Suppl. Table 4: Results of the BLAST analyses using scaffolds and contigs of super contig 1 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 5: Results of the BLAST analyses using scaffolds and contigs of super contig 2-3 and physical mapped BAC clones.

Suppl. Table 6: Results of the BLAST analyses using scaffolds and contigs of super contig 2-3 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 7: Identification of 22 *P. procumbens* scaffolds via comparative BLAST analyses using TR520 *de novo* and TR363 unmapped *de novo* data

Suppl. Table 8: Results of the BLAST analyses using scaffolds and contigs of super scaffold 1 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 9: Results of the BLAST analyses using scaffolds and contigs of super scaffold 2 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 10: Results of the BLAST analyses using scaffolds and contigs of super scaffold 3 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 11: Results of the BLAST analyses using scaffolds and contigs of super scaffold 4 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 12: Results of the BLAST analyses using scaffolds and contigs of super scaffold 5 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 13: Results of the BLAST analyses using scaffolds and contigs of super scaffold 6 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 14: Results of the BLAST analyses using scaffolds and contigs of super scaffold 7 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 15: Results of the BLAST analyses using scaffolds and contigs of super scaffold 8 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 16: Results of the BLAST analyses using scaffolds and contigs of super scaffold 9 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 17: Results of the BLAST analyses using scaffolds and contigs of super scaffold 10 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 18: Results of the BLAST analyses using scaffolds and contigs of super scaffold 11 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 19: Results of the BLAST analyses using scaffolds and contigs of super scaffold 12 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 20: Results of the BLAST analyses using scaffolds and contigs of super scaffold 13 and the draft assembly of *P. procumbens* and TR363 unmapped *de novo*.

Suppl. Table 21: Open reading frame (ORF) prediction using all TR520 sequences that are not linked to the physical map. Sequence similarity to known genes from other organisms was identified using the non-redundant protein dataset on the NCBI platform. Biological process or functions were identified using the gene ontology (GO) database.

Suppl. Table 22: Open reading frame (ORF) prediction using all TR520 sequences that are linked to the physical map. Sequence similarity to known genes from other organisms was identified using the non-redundant protein dataset on the NCBI platform. Biological process or functions were identified using the gene ontology (GO) database.

Suppl. Table 23: Identification of *cis*-regulatory elements on the promoter sequence of ORF 803 using the web based software PLACE (Higo et al., 1999).

Suppl. Table 24: Results of the BLAST analyses using the cZR genes 3 and 7 (Tian 2004) and the draft genome assemblies of *P. procumbens* and *B. vulgaris* RefBeet 09.

Suppl. Table 25: Results of the BLAST analyses using the *BpPIP1* gene (Menkhaus, 2011) and the draft genome assemblies of *P. procumbens* and *B. vulgaris* RefBeet 09.

Suppl. Table 26: Results of the BLAST analyses using BAC 149P7 and the *B. vulgaris* RefBeet 09 and the *P. procumbens* assembly.

Suppl. Table 27: Results of the BLAST analyses using ORF 801, ORF 802, and ORF 803 and the *B. vulgaris* RefBeet 09 assembly.

Suppl. Table 28: Identification of *cis*-regulatory elements on the promoter sequence of the *B. vulgaris* sequence (scaffold 839; RefBeet 09) showing high similarity to ORF 803 using the web based software PLACE (Higo et al., 1999).

7.2 Supplementary Figures

Suppl. Figure 1: Comparative sequence analysis on amino acid level between ORF 702, the splice variation ORF 702v2, and β-1,3-galactosyltransferases of *V. vinifera* (XP002279252) and *A. thaliana* (NP180179). Equal amino acids are highlighted in grey. The galactosyltransferase domain is underlined. The alignment was generated using ClustalW2 software (European Bioinformatics Institute, 2012).

Suppl. Figure 2: (A) RNA of nematode inoculated roots of *B. vulgaris*, *P. procumbens*, TR520, TR363, TR659, and TR320 (seed codes: 93161, 35535, 940043, 930363, 081933, and 091841) isolated

with the Qiagen RNeasy Mini Kit according to the manufacturer's recommendations. Each RNA sample was quantified on the Nanodrop (ThermoFisher Scientific Inc., Waltham, MA, USA).and 1µg was transcribed to cDNA using the Fermentas First strand cDNA synthesis kit. 'ʎ': 100 ng/ml Lambda DNA was used as concentration marker **(B)** cDNA quality was tested by RT-PCR amplification using the *B. vulgaris* housekeeping gene GAPDH (Primer combination H172H173; Table 1). Low range DNA ladder was used as a marker. The upper arrow indicates 400 bp and the lower arrow 200 bp. Fragments were separated on 1% agarose gels.

Suppl. Figure 3: RNA of nematode inoculated roots of the line TR520 (seed code 940043) 1 day post inoculation (dpi) and 3 dpi was isolated with the Qiagen RNeasy Mini Kit according to the manufacturer's recommendations. 'ʎ': 150 ng/ml Lambda DNA was used as concentration marker. 1µl RNA was separated on 1% agarose gels.

Suppl. Figure 4: (A) Predicted exon-intron structure of scaffold sequences of the WGS approach based on ORF prediction with the software FgeneSH using the dicotyledonous (*A. thaliana*) database. **(B):** Exon-intron structure based on RNA-Seq data of the TR520. Inoculated root material of TR520 was sequenced and assembled via Trinity (Grabherr et al., 2011). These sequences were assembled against the WGS scaffolds and the exon-intron structure was predicted.

Suppl. Figure 5: Amino acid sequence alignment of the predicted sequence of ORF 803 with amino acid sequences of the ENHANCED DISEASE RESISTANCE1 gene (EDR1) of *Arabidopsis thaliana* (Ath) (ABR45974.1), the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) like gene (XP_002279319.2) of *Vitis vinifera* (Vv) and a mitogen-activated protein kinase kinase kinase (MAP3K) from *A. thaliana* (CAB87658.19). The transglutaminase domain (Pfam14381) is underlined (75-321 aa, and 536-601 aa) and the catalytic domain of protein kinases (Cd00180) is highlighted bold and underlined. The alignment was generated using the ClustalW software. '*': identical amino acid; '.': semi-conserved substitutions ; ':': conserved substitutions.

Suppl. Figure 6: Amino acid sequence alignment of the predicted sequence of mRNA sequence 52364_c0_seq2, that shows high sequence similarity to ORF 803, with the amino acid sequences of the ENHANCED DISEASE RESISTANCE1 gene (EDR1) of *Arabidopsis thaliana* (Ath) (ABR45974.1), the CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) like gene (XP_002279319.2) of *Vitis vinifera*.(Vv) and a mitogen-activated protein kinase kinase kinase (MAP3K) from *A. thaliana* (CAB87658.19) The transglutaminse domain (Pfam14381) is underlined (131-336 aa) and the catalytic domain of protein kinases (Cd00192) is highlighted bold and underlined (670-919aa). The alignment was generated using the ClustalW software. '*': identical amino acid; '.': semi-conserved substitutions ;':': conserved

Suppl. Figure 7: Mapping of the ORF s against the sugar beet reference assembly RefBeet-0.9 using BLASTN on the CLC bio Genomics Workbench. Exons are highlighted in green and the matching scaffold of the RefBeet-0.9 assembly is listed on the left side. *B. vulgaris* sequences with significant similarity to the ORF sequence are shown as blue bars. A scale is given in basepair. (A) ORF 801 consists of six exons and maps to *B. vulgaris* sequences of scaffold 18. (B) ORF 802 consists of one exon that maps to *B. vulgaris* sequences on scaffold 18. (C) ORF 803 consists of 18 exons and maps to *B. vulgaris* sequences of scaffold 839. Details about the sequence similarity are given in Suppl. Table 27.

Suppl. Figure 8: Pairwise comparison of the mRNA sequence 52364_c0_seq2 (3,414 bp) that shows high sequence similarity to ORF 803 and the mRNA sequence 52364_c0_seq1 (3,417 bp), which was mapped with high sequence similarity to ORF 803_{BV}. In the upper comparison differences on the amino acid level are given and in the lower comparison the identity between the proteins is listed in percent. Colors range from dark blue, minimum values to dark red, maximum values. The comparison was performed on the CLC bio Genomics Workbench.

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9 Supplementary Data on DVD

File name	Content	Format
Suppl_Figures	Supplementary Figures	.pptx
Suppl_Tables	Supplementary Tables	.xlsx
ORF801gDNA	genomic sequence of ORF 801	.txt
ORF802gDNA	genomic sequence of ORF 802	.txt
ORF803gDNA	genomic sequence of ORF 803	.txt
ORF804gDNA	genomic sequence of ORF 804	.txt
ORF805gDNA	genomic sequence of ORF 805	.txt
ORF806gDNA	genomic sequence of ORF 806	.txt
ORF807gDNA	genomic sequence of ORF 807	.txt
ORF808gDNA	genomic sequence of ORF 808	.txt
ORF809gDNA	genomic sequence of ORF 809	.txt
ORF810gDNA	genomic sequence of ORF 810	.txt
ORF811gDNA	genomic sequence of ORF 811	.txt
ORF801exons_aa	predicted exons and protein sequence of ORF 801	.txt
ORF802 exons_aa	predicted exons and protein sequence of ORF 802	.txt
ORF803 exons_aa	predicted exons and protein sequence of ORF 803	.txt
ORF804 exons_aa	predicted exons and protein sequence of ORF 804	.txt
ORF805 exons_aa	predicted exons and protein sequence of ORF 805	.txt
ORF806 exons_aa	predicted exons and protein sequence of ORF 806	.txt
ORF807 exons_aa	predicted exons and protein sequence of ORF 807	.txt
ORF808 exons_aa	predicted exons and protein sequence of ORF 808	.txt
ORF809 exons_aa	predicted exons and protein sequence of ORF 809	.txt
ORF810 exons_aa	predicted exons and protein sequence of ORF 810	.txt
ORF811 exons_aa	predicted exons and protein sequence of ORF 811	.txt
51268_c1_seq4	transcriptome sequence 51268_c1_seq4	.txt
34314_c1_seq1	transcriptome sequence 34314_c1_seq1	.txt
52364_c0_seq1	transcriptome sequence 52364_c0_seq1	.txt
39138_c0_seq1	transcriptome sequence 39138_c0_seq1	.txt
49274_c1_seq1	transcriptome sequence 49274_c1_seq1	.txt
52364_c0_seq2	transcriptome sequence 52364_c0_seq2	.txt
BAC86F6	Sequence of BAC 86F6	.txt
BAC 142D13 T7	Sequence of BAC end 142D13 T7	.txt
BAC 142D13 Sp6	Sequence of BAC end 142D13 Sp6	.txt
BAC 56F6 T7	Sequence of BAC end 56F6 T7	.txt
BAC 56F6 Sp6	Sequence of BAC end 56F6 Sp6	.txt
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10 Publications and Declaration of own Contribution

The chapters of this thesis are not published. The doctoral student Sarah Christina Jäger contributed to the chapters as follows:

Chapter 2: Functional Analysis of ORF 702 as a Candidate for the Beet Cyst Nematode Resistance Gene *Hs1-2*

Designing the experiments:	75%
Results:	85%
Writing the Manuscript:	100%
Discussion and revision:	100%

Chapter 3: Re-sequencing and hybrid assembly of two nematode resistant *Beta vulgaris* translocation lines

Designing the experiments:	75%
Results:	90%
Writing the Manuscript:	100%
Discussion and revision:	100%

11 Curriculum Vitae

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2007-2012	PhD in Agriculture-Christian Albrechts University, Plant Breeding Institute, Kiel, Germany
2005-2007	Master studies of agricultural sciences
	Master thesis “Physical mapping of a wild beet translocation at chromosome 9 of the sugar beet”
2001-2005	Bachelor studies of agricultural sciences
	Bachelor thesis “Traditional and genetic engineering based approaches for breeding virus resistant agricultural crops”
1988-2001	School education, German Abitur

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