GEFÖRDERT VOM

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Zuwendungsempfänger:	Förderkennzeichen:
Institut für Pflanzenbau und Pflanzenzüchtung, Christian-Albrechts-Universität zu Kiel	0315957B
Vorhabenbezeichnung: **Pflanzenbiotechnologie – Verbundvorhaben: "Nutzung von Genen zur Erhöhung der Resistenz und Toleranz der Getreidewurzel gegenüber biotischem und abiotischem Stress (CEREAL-ROOTS)**	
Laufzeit des Vorhabens: 01.07.2011– 30.11.2014	

1 Aufgabenstellung (zu I.)

Wurzelläsionsnematoden der Gattung Pratylenchus sind weltweit wichtige Schädlinge, die verschiedene landwirtschaftliche und gärtnerische Kulturen befallen. Die einzige Möglichkeit, diese Parasiten zu bekämpfen, besteht darin, resistente Kulturpflanzen zu züchten. In Norddeutschland wird seit gut 10 Jahren eine Zunahme an Schädigungen durch Wurzelläsionsnematoden beobachtet. Dabei handelt es sich hauptsächlich um die Arten *Pratylenchus neglectus* und *Pratylenchus penetrans*. Davon betroffen sind im Wesentlichen die landwirtschaftlichen Kulturen Weizen und Gerste.

Am Institut für Pflanzenzüchtung der Christian-Albrechts-Universität zu Kiel wurde 2005 ein Projekt begonnen, welches das Ziel hatte, Gerste-Herkünfte mit Resistenz gegen Wurzelläsionsnematoden zu identifizieren und für die Züchtung zur Verfügung zu stellen. In diesem und in zwei Folgeprojekten konnten erfolgreich aus einem weltweiten Sortiment Herkünfte identifiziert werden, die eine deutlich geringere Befallsintensität zeigen. Vollständige Resistenzen (Immunität) wurden jedoch nicht beobachtet. Die Vererbung ist eindeutig quantitativ, d. h. eine größere Zahl von Genen ist an der Ausprägung beteiligt. Derartige Genregionen (Quantitative Trait Loci ,QTL) wurden am Institut mit Hilfe molekularer Markerkarten im Genom der Gerste lokalisiert und charakterisiert.

Dieses Projekt konnte also auf erste Ergebnisse zurückgreifen, die die genetische Grundlage der Resistenz gegen Wurzelläsionsnematoden bei der Gerste erklären. Die Aufgabe bestand darin, maximal zwei Haupt-QTL mittels molekularer Markerkarten zu kartieren und molekulare Marker als selektierbare Marker für die Selektion in der Gerstenzüchtung verfügbar zu machen. Diese sollten an den Wirtschaftspartner Nordsaat Saatzucht GmbH abgegeben werden. Weiterhin sollten erste Schritte unternommen werden, um die Ursachen der Resistenz zu erforschen. Dazu wurden physiologische Untersuchungen beim Partner Universität Erlangen durchgeführt. Schließlich sollten transgene Gersten, in denen ein Resistenzgen in seiner Expression verändert wurde, auf ihre Resistenz gegen *Pratylenchus neglectus* und *Pratylenchus penetrans* untersucht werden.

Neben umfangreichen Sortimenten an Kulturgersten und Wildgersten, die hinsichtlich Resistenz zuvor charakterisiert worden waren, standen zahlreiche genomische Ressourcen für das Projekt zur Verfügung. Dazu gehört vor allem die Gerste-Referenzsequenz, die im Laufe des Projektes publiziert wurde. Weiterhin gibt es zahlreiche Kartierungspopulationen (DH-Populationen), für die umfangreiche Markerkarten vorliegen. Für die Feinkartierung der QTL sollten daher neben verschiedenen Wiederholungen auch unterschiedliche Kartierungspopulationen herangezogen werden. Damit sollten die Lage der QTL abgesichert werden. Derartige Populationen lagen am Julius Kühn-Institut (Institut für Resistenzforschung und Stresstoleranz, Prof. Ordon), Quedlinburg sowie am IPK Gatersleben vor.

Die quantitative Natur der Resistenz macht es notwendig, Phänotypisierungen unter verschiedenen Umwelten und mit mehreren Wiederholungen durchzuführen. Erschwerend kommt hinzu, dass die Phänotypisierung (Resistenztests im Gewächshaus) langwierig und arbeitsaufwendig ist. Dazu wurde ein Resistenztest am Institut in Kiel entwickelt, der die sichere Erfassung der Resistenz nach einem ca. 10-wöchigen Inokulationsexperiment ermöglicht. Allerdings ist die Zahl der zu testenden Pflanzen mit ca. 250 pro Experiment relativ klein. Damit können bei uns im Jahr max. 1.500 Pflanzen phänotypisiert werden.

2 Eingehende Darstellung (zu II.)

Die Zuwendung wurde für die im Antrag beschriebenen Arbeiten verwendet. Die Ergebnisse sind im Einzelnen unter Kapitel 5 sowie im Anhang beschrieben.

Der zahlenmäßige Nachweis enthält die Ausgaben, die im Rahmen dieses Projektes getätigt worden sind. Dieser gliedert sich in die Posten Beschäftigte E13 (wissenschaftliche Mitarbeiter), Beschäftigte E8 (TA) sowie wissenschaftliche Hilfskräfte, Verbrauchsmaterial, Dienstreisen und Investitionen. In dem Zusammenhang ist es wichtig zu erwähnen, dass auf Grund der sehr arbeitsaufwendigen Resistenztests eine umfangreiche Unterstützung durch wissenschaftliche Hilfskräfte und technische Mitarbeiter notwendig war. Alle Mittel wurden für die geplanten Arbeiten innerhalb des Projektes eingesetzt. Das gilt auch für Arbeiten, die explizit nicht im Antrag genannt worden waren, aber aufgrund des wissenschaftlichen Fortschritts und neuer Erkenntnisse der Gerste-Genomforschung sinnvoll erschienen.

Das Projekt hat Ergebnisse geliefert, die einer wirtschaftlichen Verwertung zugeführt werden können. Dabei handelt es sich um Pflanzenmaterial, welches hinsichtlich seiner Resistenz gegen Wurzelläsionsnematoden charakterisiert worden ist. Resistente DH-Linien wurden bereits an den Wirtschaftspartner Nordsaat Saatzucht GmbH abgegeben. Sie dienen als Quellen für die Resistenzzüchtung. Daneben wurden eine Reihe molekularer Marker entwickelt, die jetzt für die Selektion resistenter Linien genutzt werden können. Dies hat große Bedeutung, weil die Resistenztests sehr aufwendig sind und im Rahmen eines Züchtungsprozesses kaum eingesetzt werden können. Die weitaus größte Bedeutung hat aber die Erkenntnis, dass es QTL-Regionen gibt, die Resistenz gegen beide Pathogene (*P. pratylenchus, P. neglectus*) vermitteln. Dies war vorher nicht bekannt. Für die praktische Sortenzüchtung bedeutet dies, dass Resistenzen nicht mühevoll kombiniert werden müssen, sondern dass sie als ein gemeinsamer Locus betrachtet werden können. Zusammen mit den entwickelten molekularen Markern ergibt sich so die Möglichkeit, doppelt resistente Pflanzen zu züchten.

Die während der Laufzeit des Vorhabens bekannt gewordenen Fortschritte auf dem Gebiet der Resistenzzüchtung, der Genomforschung und der Pflanzenphysiologie wurden laufend in die Projektplanung eingearbeitet. Besonders hervorzuheben ist die *de novo* Sequenz des Gerstegenoms, die während der Laufzeit des Projektes publiziert worden ist. Daraus ergab

sich die Möglichkeit, Sequenz-*contigs* zu identifizieren, die die kritischen QTL-Regionen umfassen. Die verfügbare Gen-Annotation wiederum ermöglichte das Auffinden erster Kandidatengene aus diesen Regionen. Es wurden keine Fremdarbeiten publiziert, die sich mit dem eigentlichen Ziel des Projektes, nämlich der Resistenz gegen Wurzelläsionsnematoden in der Gerste befassen. Daher hat dieses Projekt nach wie vor ein Alleinstellungsmerkmal und leistet Pionierarbeit für zukünftige Forschungen und Entwicklungen.

Veröffentlichungen im Rahmen des Teilprojektes an der CAU-Kiel

Shah S (2014) Genetic mapping and transcriptional analysis of candidate genes for root lesion nematode resistance in barley. Master Thesis, Christian-Albrechts-Universität zu Kiel, Kiel

Galal, A., Sharma, S., Abou-Elwafa, S. F., Sharma, S., Kopisch-Obuch, F., Laubach, E., Perovic, D., Ordon, F., Jung, C. (2014). Comparative QTL Analysis of Root Lesion Nematode Resistance in Barley. Theoretical and Applied Genetics (2014) 127:1399–1407 DOI 10.1007/s00122-014-2307-x

Abou-Elwafa S.F., Xiao K., Jung C. (2014) Candidate Genes for Root Lesion Nematode Resistance in Barley, PLANT 2030 Status Seminar, Potsdam, March 31 - April 2, 2014, oral presentation.

Abou-Elwafa S.F., Galal A., Xiao K., Laubach E., Jung C. (2014) Genetic Fine Mapping of Root Lesion Nematode Resistance QTLs in Barley, PLANT 2030 Status Seminar, Potsdam, March 31 - April 2, 2014, poster presentation.

Abou-Elwafa S.F., Galal A., Xiao K., Laubach E., Jung C. (2014) Genetic Fine Mapping of Root Lesion Nematode Resistance QTLs in Barley, GPZ congress: Genome Research Working Group, Cologne, February 11 – 13, 2014, poster presentation.

Abou-Elwafa S.F., Galal A, Laubach E., Jung C. (2013) Genetic Fine Mapping of QTLs Associated with Root Lesion Nematode Resistance in Barley, PLANT 2030 Status Seminar, Potsdam, March 6 - 8, 2013, poster presentation.

Galal A., Sharma S., Abou-Elwafa S.F., Sharma S., Kopisch-Obuch F., Stein N., Graner A., Laubach E., Ordon F., Jung C. (2012) Genetic Fine Mapping of QTLs Associated with Root Lesion Nematode (RLN) Resistance in Barley, 10. Genome Research Working Group Conference of the GPZ – Plant Stress and Genomics, Halle (Saale), Germany, 18.-20. September 2012, oral presentation

Abou-Elwafa S.F., Galal A., Laubach E., Jung C. (2012) CEREAL ROOTS, PLANT 2030 Status Seminar, Potsdam, March 6 - 8, 2012, poster presentation.

3 Kurzfassung (zu IV.)

Im Teilprojekt der CAU Kiel des Verbundprojektes CEREAL-ROOTS wurden Gerste-Populationen hinsichtlich ihrer Resistenz gegen Wurzelläsionsnematoden (*Pratylenchus penetrans, P. neglectus*) untersucht. Dazu wurde vom Wirtschaftspartner Nordsaat Saatzucht GmbH eine sehr große doppelt-haploiden (DH) Population erzeugt und zur Verfügung gestellt. Diese wurde in einem zuvor etablierten Gewächshaus-Resistenztest auf ihre Resistenz gegen *P. neglectus* untersucht. Mit einer Teilpopulation wurde eine genetische Karte mit molekularen Markern erzeugt, um darin QTL für *P. neglectus*-Resistenz zu lokalisieren. Es zeigte sich, dass von acht QTL zwei gleichzeitig Resistenz gegen *P. neglectus*

und *P. penetrans* vermitteln. Somit besteht in der Gerstezüchtung die Möglichkeit Sorten mit Resistenz gegen beide Schaderreger zu züchten. Dafür wurden molekulare Marker für die Selektion im Rahmen des Zuchtprozesses erzeugt. Die Genotypisierung einer sehr großen Zahl von DH-Linien ermöglichte eine Feinkartierung des QTL-Locus auf Chromosom 6. Diese wiederum stellte den Ausgangspunkt für die Identifizierung von Sequenz-Contigs dar, die den entsprechenden QTL-Locus überstreichen. Dazu wurden jeweils zehn hoch-resistente und hoch-anfällige DH-Linien gemeinsam sequenziert (bulked-sequencing) durch Vergleich mit dem Referenzgenoms wurden Sequenz-contigs assembliert, auf denen erste Kandidatengene für die Resistenz gegen Wurzelläsionsnematoden lokalisiert werden konnten. In Kooperation mit den Projektpartnern in Erlangen und Gießen wurden physiologische Untersuchungen an anfälligen und resistenten DH-Linien durchgeführt und es wurde die Resistenz transgener Gersten getestet, in denen ein Pathogen-Resistenzgen differentiell reguliert wurde. Als Ergebnis der Arbeit stehen resistente DH-Linien für die Gerstezüchtung zur Verfügung. Gleichzeitig wurden molekulare Marker etabliert, die den sehr aufwendigen Resistenztest überflüssig machen. Die Tatsache, dass die resistenten Linien Resistenz gegen beide Schaderreger zeigen, eröffnet neue Perspektiven für die Gerstezüchtung

4 Ergebnisteil

4.1 WP 5: QTL-mapping and transcriptional profiling to identify candidate genes for resistance to *Pratylenchus*

4.1.1 WP 5.1: Identification of QTLs associated with RLN resistance

4.1.1.1 Nematode resistance tests

For genetic mapping of QTLs associated with resistance to the root lesion nematode *P. neglectus* (Project milestone M8), six resistance tests were carried out with 220 DH lines from a Beysehir × Valentina cross and the two parents (**Table 1**). Tests 1-6 (experiment 1-6) had been carried out between July and October 2012. The DH population was produced by the project partner Nordsaat Saatzucht GmbH Saatzucht GmbH.

From 218 DH lines, 18 plants/DH line were grown in six different experiments (3 plants from each DH line in each experiment) on July 2 and August 6, 2012 (**Table 1**). After one day, 6 plants /line were transplanted into 150 cm³ tubes filled with sterilized sand. Ten days later, each plant was inoculated with ca. 1000 *P. neglectus* nematodes, and 7 weeks later (September 3rd to- October 8th, 2012) plants were harvested and roots were chopped and transferred to the misting chamber.

The experimental setup of the resistance test was as follows: The experimental design was a complete randomized block design (RCBD) to minimize the experimental error among genotypes. One experiment was carried out with 220 plants, because this is the maximum number of plants that can be phenotyped in our screening test system. In the misting chamber used for extracting nematodes from roots only 240 plants can be tested at the same time. Altogether, 185 DH lines were tested with 6 replications. All experiments were carried out in a glasshouse with 23° C day and 18° C night temperatures, under long day conditions (16/8 h) with supplementary light (Son-T Agro 400W, Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands).

Becasue some DH lines turned out to be parental type, phenotypic data could be only obtained from 107 DH lines (out of 185 DH lines tested). Therefore, the resistance test was repeated. In the following, only results from the repeated resistance test (experiment 7-12) will be

mentioned. *P. neglectus* experiments 7-12with the Beysehir × Valentina DH population run between November 5th, 2012 and February 18the , 2013. The experimental design (germination and sowing of seeds, nematode inoculation and extraction of nematodes) was essentially as described above.

The results of the experiments 7-12 were as follows: Similarly to the previous resistance test (experiment 1-6), remarkable variations were observed in infection rates between genotypes (**Figure 1**) and between different experiments in each experiment. The average number of nematodes over all six experiments (7-12) was higher (83-2355 nematodes/plant with an average number of 717 nematodes/ plant) than the previous resistance tests (experiments 1-6) (90-1507 nematodes/plant with an average number of 472 nematodes/ plant). The highest infection rate (0-6417 nematodes/plant) was observed in experiment 12 with an average nematode number of 1502 nematodes/plant whereas, the lowest infection rate (0-2640 nematodes/plant) was obtained from experiment 7 with an average number 562 nematodes/plant (**Table 1**).

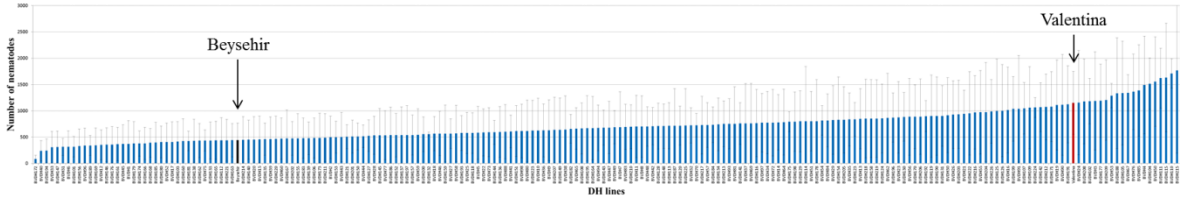

Figure 1: Resistance test with *P. neglectus* of the Beysehir × Valentina DH population (Experiments 7-12). The average number of nematodes from 208 DH lines and the two parents of the mapping population (Beysehir and Valentina) are given over six different experiments. Black and red bars indicate the parents of the population, i.e., Beysehir and Valentina, respectively. Error bars indicate standard deviation.

Table 1: Resistance tests between November 2012 and February 2013 with barley DH lines from the Beysehir × Valentina population.

Experiment No.	Plant Population	Inoculum (species/number of nematodes)	Number of DHs/plants tested	Inoculation date	Root harvest date	Test conditions (pot size)	Nematode infections, mean (range)
Exp. 1	Beysehir × Valentina	*P. neglectus*/ 1000	107	12.07.2012	29.08.2012	150 cm³	490 (110-1662)
Exp. 2	Beysehir × Valentina	*P. neglectus*/ 1000	107	19.07.2012	05.09.2012	150 cm³	307 (0-1104)
Exp. 3	Beysehir × Valentina	*P. neglectus*/ 1000	107	26.07.2012	12.09.2012	150 cm³	634 (0- 4640)
Exp. 4	Beysehir × Valentina	*P. neglectus*/ 1000	107	02.08.2012	19.09.2012	150 cm³	519 (0-2043)
Exp. 5	Beysehir × Valentina	*P. neglectus*/ 1000	107	09.08.2012	26.09.2012	150 cm³	441 (0-2761)
Exp. 6	Beysehir × Valentina	*P. neglectus*/ 1000	107	16.08.2012	03.10.2012	150 cm³	435 (0-2380)
Exp. 7	Beysehir × Valentina	*P. neglectus*/ 1000	207	05.11.2012	14.01.2013	150 cm³	562 (0-2640)
Exp. 8	Beysehir × Valentina	*P. neglectus*/ 1000	207	12.11.2012	21.01.2013	150 cm³	548 (0-2800)
Exp. 9	Beysehir × Valentina	*P. neglectus*/ 1000	207	19.11.2012	28.01.2013	150 cm³	675 (0-3145)
Exp. 10	Beysehir × Valentina	*P. neglectus*/ 1000	207	26.11.2012	04.02.2013	150 cm³	609 (0-5250)
Exp. 11	Beysehir × Valentina	*P. neglectus*/ 1000	207	03.12.2012	11.02.2013	150 cm³	507 (0-2693)
Exp. 12	Beysehir × Valentina	*P. neglectus*/ 1000	207	10.12.2012	18.02.2013	150 cm³	1502 (0-6417)

An analysis of variance (ANOVA) was performed with experiments 7-12 (**where** $\hat{\sigma}_G^{\,2}$, $\hat{\sigma}_{GE}^{\,2}$ and $\hat{\sigma}_e^{\,2}$ are the variance components estimated from the ANOVA (Fehler! Ungültiger Eigenverweis auf Textmarke.) for the genotypic, genotype × experiment and error variance, respectively, with E as the number of experiments and R as the number of replicates.

Table 2), and highly significant variances for number of nematodes extracted from roots were found. Broad-sense heritability was estimated to 0.55 according to Hallauer *et al*. (1981):

$$\hat{h}^2 = \hat{\sigma}_G^{\,2} \Big/ \left(\hat{\sigma}_G^{\,2} + \hat{\sigma}_{GE}^{\,2} \Big/ E + \hat{\sigma}_e^{\,2} \Big/ RE \right)$$

where $\hat{\sigma}_G^{\,2}$, $\hat{\sigma}_{GE}^{\,2}$ and $\hat{\sigma}_e^{\,2}$ are the variance components estimated from the ANOVA (Fehler! Ungültiger Eigenverweis auf Textmarke.) for the genotypic, genotype × experiment and error variance, respectively, with E as the number of experiments and R as the number of replicates.

Table 2: Analysis of variance of number of nematodes extracted from 208 DH lines from the BV population (experiments 7-12).

Source of Variation	SS	DF	MS	F value	P-value	F critical
Genotypes	$1.15E^{+08}$	207	557367.6	2.217	0.000	1.186
Replicates	94306114	5	18861223	75.012	0.000	2.222
Error	$2.6E^{+08}$	1035	251441.8			
Total	4.7E+08	1247				

4.1.1.2 Genetic map construction

Two-hundred and twenty-six Beysehir × Valentina DH lines including the 208 DH lines that have been phenotyped for resistance (experiment 7-12) to the root lesion nematode *P. neglectus* were genotyped (**Table 3**) using amplified fragment length polymorphisms (AFLP) essentially as described by El-Mezawy *et al*. (2002), except that for restriction *PstI* (Fermentas, St. Leon-Rot, Germany) instead of *EcoRI* was used.

Table 3: Summary of genotyping and phenotyping experiments with the Beysehir×Valentina population.

Experiment	Number of DHs
DH lines genotyped by AFLP	226
DH lines genotyped by CAPS and SSRs	226
DH lines tested for *P. neglectus* resistance	208
DH lines tested for *P. neglectus* resistance and genotyped by AFLP	208
DH lines tested for *P. neglectus* resistance and genotyped by AFLP, SSRs and CAPS	208
DH lines fine mapped with 6H-QTL (EBmac0602 and Bmag0613 SSR markers).	1219

Table 4: Polymorphic anchor markers for anchoring the linkage groups in the Beysehir × Valentina population

Marker Designation	Marker type	Linkage group	Primer combination	Detection method
Bmag0770	SSR	1H	CR027-CR028	GE (3% Agarose gel) [a]
Bmac63	SSR	1H	CR111-CR112	GE (3% MetaPhor)
Bmag 0211	SSR	1H	CR345-CR346	GE (3% Agarose gel)
GBM1334	SSR	1H	CR321-CR322	*BfaI* RE [b]/ Agarose gel
Bmag350	SSR	2H	CR277-CR278	GE (3% Agarose gel)
EBmac0684b	SSR	2H	CR303-CR304	GE (4% MetaPhor)
GBM1459	SSR	2H	CR359-CR360	GE (4% Agarose gel)
GBM1309	SSR	2H	CR375-CR376	GE (3% Agarose gel)
(GBM1281	SSR	2H	CR347-CR348	*TseI* RE/ Agarose gel
GBM1214	SSR	2H	CR353-CR354	*BssSI* RE / Agarose gel
Bmag0877	SSR	3H	CR127-CR128	GE (3% MetaPhor)
Bmac0144k	SSR	3H	CR315-CR316	GE (3% MetaPhor)
GBM1405	SSR	3H	CR005-CR006	*NspI* RE/ Agarose gel
Bpb-2433	DArT	3H	SS49-SS50	*BsrI* RE/ Agarose gel
Bmag603	SSR	3H	CR249-CR250	GE (3% Agarose gel)
Bmac0310	SSR	4H	CR137-CR138	GE (3% Agarose gel)
Bmac186	SSR	4H	CR285-CR286	GE (3% MetaPhor)
GBM1465	SSR	4H	CR401-CR402	GE (3% Agarose gel)
EBmatc0003	SSR	5H	CR063-CR064	GE (3% Agarose gel)
Bmac0144l	SSR	5H	CR067-CR068	GE (3% Agarose gel)
GBMS119	SSR	5H	CR087-CR088	GE (3% Agarose gel)
cMWG654	SSR	5H	CR453-CR454	GE (3% MetaPhor)
Bmac0602	SSR	6H	CR099-CR100	*FaiI RE/ Agarose gel*
bpb-0522	DArT	6H	CR021-CR022	GE (2% Agarose gel)
EBmac0806	SSR	6H	CR259-CR260	GE (3% MetaPhor)
Bmag0613	SSR	6H	CR197-CR198	GE (3% Agarose gel)
Bmac0175b	SSR	6H	CR161-CR162	GE (3% Agarose gel)
GBMS180	SSR	6H	CR463-CR464	GE (3% Agarose gel)
EBmac0639	SSR	6H	CR177-CR178	GE (3% Agarose gel)
HvLOX	SSR	6H	CR183-CR184	GE (2% Agarose gel)
GBM1356	SSR	6H	CR105-CR106	GE (3% Agarose gel)
GBM1174	SSR	7H	CR435-CR436	GE (3% Agarose gel)
Bmag206	SSR	7H	CR263-CR264	GE (1% Agarose gel)
bpb-0037	DArT	7H	CR017-CR018	*MwoI* RE/ Agarose gel

[a] GE, gel electrophoresis
[b] RE, restriction enzyme

Pre-amplification was done with primers P01 and M01, and amplification with primers M31–M36 in combination with primers P33–P46 (Vos *et al*. 1995; http://wheat.pw.usda.gov/ggpages/KeygeneAFLPs.html). Oligomers were obtained from MWG Biotech AG (Ebersberg, Germany). Out of 81 AFLP primer combinations, 46 primer combinations were chosen for genotyping the whole population because of their higher polymorphism rates. Their number of polymorphic AFLPs ranged between 7 and 19. In total, they gave rise to 472 polymorphic AFLPs.

The previous RLN resistance QTLs had been mapped mainly with DArT and SSR- based markers (Sharma, Kopisch-Obuch et al. 2011; Galal, Sharma et al. 2014). Therefore, and for a better comparative QTLs analysis which is essential for genetic fine mapping of QTLs, the AFLP-based genetic map was enriched with more sequence-based markers (e.g., SSRs and SSRs- and DArT-derived CAPS markers). A number of 246 previously described DArT and

SSR markers (http://wheat.pw.usda.gov/GG2/index.shtml) were tested for polymorphism between the two parents of the mapping population. Twenty-seven markers could be identified as polymorphic markers based on their electrophoresis banding pattern (**Table 4**).

Markers which produced single DNA amplicons were sequenced and polymorphic markers at the nucleotide level were converted into CAPS (Cleaved Amplified Polymorphic Sequence) markers. Seven SSRs which did not yield polymorphic gragments could be converted into CAPS markers. Thirty-four markers allocated to the seven linkage groups were developed (**Table 4**).

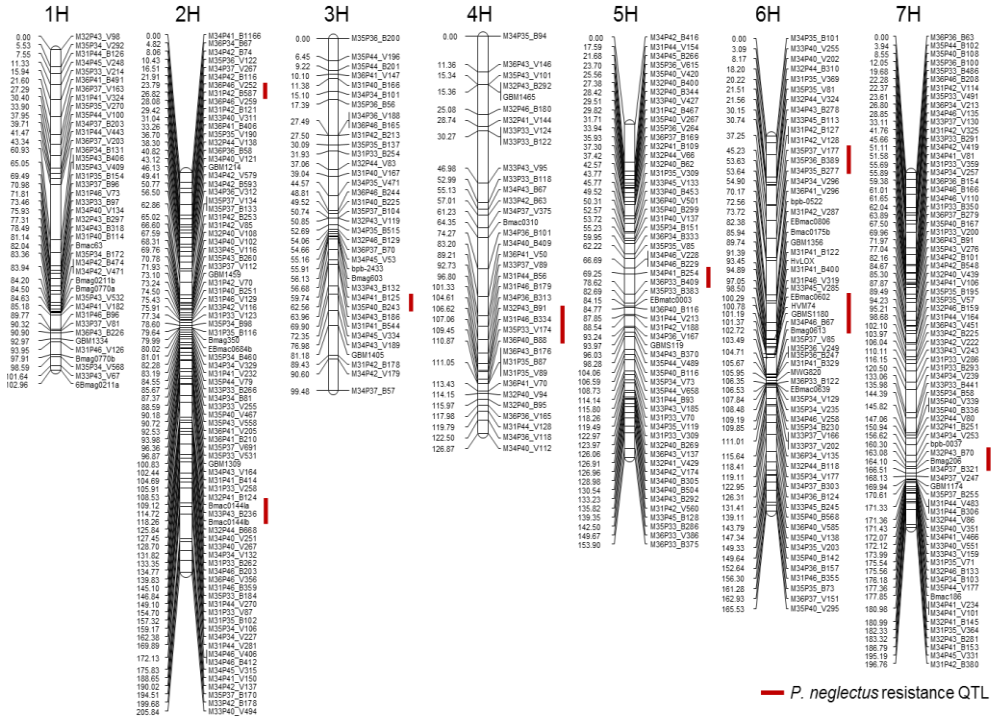

Figure 2: A genetic linkage map of the BV population. The map was constructed with 226 DH lines using 388 AFLPs, SSRs and CAPSs markers. Markers covered 1051 cM on seven linkage groups (1H-7H). Mapping distances are given in centiMorgans (cM) with an average marker density of 2.7 cM/ marker. The location of *P. neglectus* resistance QTLs are indicated in red bars. The bars depict the QTL positions.

A number of 472 AFLPs and 34 SSRs and SSRs- and DArT-derived CAPSs were used to genotype the 226 DH lines from BV population. The genetic linkage map was calculated with the software JoinMap version 4.1 (van Ooijen 2006). The Kosambi mapping function (Kosambi 1943) was used and a minimum LOD score of 3.0 and a maximum recombination frequency of 0.4 were employed. The markers were analyzed by a Chi-square test for goodness of-fit to the expected Mendelian segregation ratios (1:1; $P < 0.01$). Three-hundred and eighty-eight markers (351 AFLPs and 37 SSRs and CAPSs) could be used to construct the genetic linkage map of the BV population. Markers covered 1051 cM on seven linkage groups. The sizes of linkage groups ranged from 99 (LG 3H) to 205 cM (LG 2H) with an average marker density of 2.7 cM/ marker (**Figure 2**).

4.1.1.3 Genetic mapping of *P. neglectus* resistance QTLs in the BV population

QTL analysis was carried out by composite interval mapping using the program PLABQTL version 1.2 (Utz and Melchinger 1996). Genome wide LOD thresholds were determined

empirically with 1,000 permutations for an experiment wise error rate of $\alpha_E = 0.05$ and $\alpha_E = 0.01$.

Using 385 AFLP, SSR and CAPS markers and phenotypic data for resistance to *P. neglectus* from 208 DH lines (experiment 7-12) of the BV DH population, eight QTLs which confer resistance to *P. neglectus* were detected (**Table 5**). These QTLs are located on all chromosomes except 1H (**Table 5**). Five QTLs were significant at $\alpha_E = 0.05$ and 0.01 with LOD scores ranging from 6.36 (*Pne5H-3*) to 12.9 (*Pne6H-4*). Phenotypic variances explained by those significant QTLs ranged between $R^2 = 10.3$ % (*Pne3H-3*) and 24.9 % (*Pne6H-4*). The remaining three QTLs were not significant at $\alpha_E = 0.05$. The adjusted genetic variance explained by all QTLs was 100.2 % (**Table 5**).

Table 5: QTLs for *P. neglectus* resistance by composite interval mapping in the Beysehir×Valentina DH-population. Phenotypic data were from the second resistance test (experiments 7-12). The map was calculated with 385 AFLP, SSR and CAPS markers.

QTL	LG	Flanking markers	Position	Confidence Interval	LOD	R^2 (%)[a]	Additive effect[b]
Pne2H-1	2H	M36P46_V252-M31P42_B587	24	22-26	8.55**	17.2	127
Pne2H-2	2H	Bmac0144la-Bmac0144lb	114	110-118	3.21	4.4	-67
Pne3H-3	3H	M34P41_B125-M35P40_B243	60	58- 62	6.47*	10.3	87
Pne4H-1	4H	M32P43_B91-M36P40_B88	110	106- 112	4.09	5.2	-81
Pne5H-3	5H	M34P41_B254-M36P33_B409	70	66- 74	6.36*	14.9	-129
Pne6H-3	6H	M35P37_V177-M34P35_B277	52	50- 54	7.29**	12.1	111
Pne6H-4	6H	EBmac0602-Bmag0613	100	98-102	12.9**	24.9	-166
Pne7H-4	7H	M32P43_B70-M34P37_B321	164	162-166	5.89*	12.2	-102
Sum						101.2 [c]	

[a] Phenotypic variance explained by the QTL
[b] Additive effects expressed as final nematode counts per plant
[c] Adjusted genetic variance explained by all QTLs
*, **: significant QTLs at αE = 0.05 and at αE = 0.01, respectively.

4.1.2 WP 5.2: Fine mapping of RLN resistance QTL

4.1.2.1 Selection of QTLs for genetic fine mapping

After genetic map construction and identification of QTLs related to resistance to *P. neglectus* in the Beysehir × Valentina DH population, comparative QTL analysis with previously mapped QTLs in different mapping populations (Sharma, Kopisch-Obuch et al. 2011; Galal, Sharma et al. 2014) was carried out to select common QTLs for genetic fine mapping.

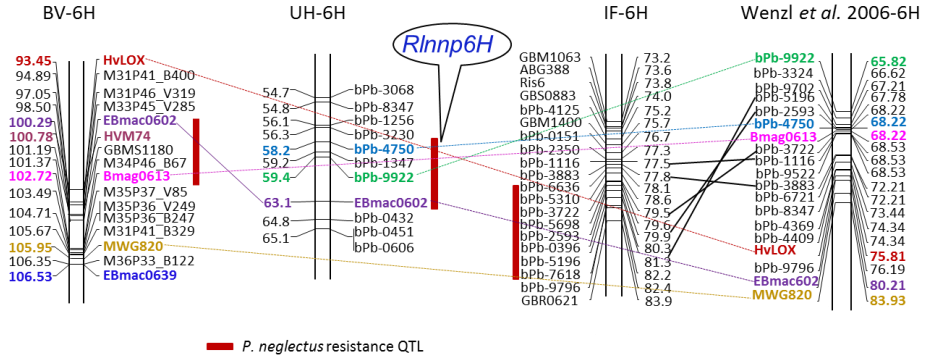

Figure 3: Comparative analysis of the *Pne6H* QTL regions in four mapping populations. EBmac0602 is a flanking marker of the QTL in both BV and UH populations.

One of the flanking markers of QTL *Pne6H* (SSR marker Bmag0613) is closely linked to markers bPb-4750 and bPb-9922 in a distance of 0.00 and 0.60 cM, respectively. These markers are flanking *Pne6H* QTL in the UH population and they are located within the *Pne6H* QTL in the IF population. Marker Bmag0613 is closely linked to 5 markers located within the *Pne6H* QTL in the IF population in a genetic distance of 0.00-0.56 cM. Red bars indicate *P. neglectus* resistance QTLs. The positions of the *P. penetrans* and *P. neglectus* resistance QTLs, which had been calculated in both IF and UH populations, were compared with the positions of the *P. neglectus* resistance QTLs in the BV population. This analysis revealed a co-localization of a major *P. neglectus* resistance QTL *Pne6H-4* in the BV population on chromosome 6H (confidence interval [C.I.] = 98–102, R^2= 24.9%) with a common QTL between both *P. neglectus* and *P. penetrans* resistance QTLs in both IF and UH populations designated *Rlnnp6H* (Galal, Sharma et al. 2014) (**Figure 3**).

Then we compared the genetic positions of *Pne5H* QTL using molecular markers of different populations. The *P. neglectus* resistance QTL *Pne5H-3* in the BV population located on chromosome 5H (C.I. = 66–74, R^2= 14.9%) was co-localized with a common *P. penetrans* and *P. neglectus* resistance QTL in the IF and UH populations designated as *Rlnnp5H* (Galal, Sharma et al. 2014) (**Figure 4**). The SSR marker flanked the BV-5H QTL (EBmatc0003, in red) is closely linked (1.96 cM) to marker *cMWG716a in a barley consensus map (Varshney, Marcel et al. 2007) which located within the IF 5H-QTLs. Moreover, 3 DArT markers which are linked to the *cMWG716a marker (ca. 5 cM, in green) in IF integrated map (Aghnoum et al., 2010) are also located within the IF 5H QTL region. The SSR marker GBMS119 (in blue) which is ca. 10 cM from the BV 5H-QTL is linked to the *cMWG716a in both the consensus and IF integrated map maps (3.64 and 2.49 cM, respectively) and located within IF 5H-QTL. Red and blue bars indicate *P. neglectus P. penetrans* resistance QTLs, respectively.

In conclusion, the positions of *Pne5H* QTL could be precisely mapped in different independent mapping populations.

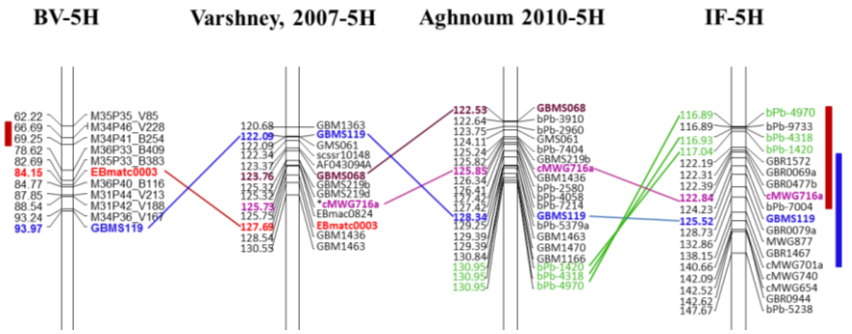

Figure 4: Comparative analysis of the *Pne5H* QTL regions on chromosome 5H in four different mapping populations.

4.1.2.2 Identification of phenotypic and genotypic bulks for bulked segregant analysis

Phenotypic bulks were established by taking the distributional extremes of the Beysehir×Valentina DH population. Two resistant and two susceptible bulks were established:

- the "best bulk" which consists of the most resistant 10 DHs,
- the "good bulk" which consists of the resistant 10 DHs ranked second after the best bulk in infection rate (**Table 6**)
- the "worst bulk" includes the 10 DHs showed the highest infection rate,
- the "bad bulk" includes the 10 DHs ranked second after the worst bulk in infection rate (**Table 6**).

Table 6: Doubled-haploid lines which contributed to the phenotypic bulks used for whole genome resequencing.

The best bulk (83-340 nematodes/plant)		The good bulk (344-380 nematodes/plant)		The worst bulk (1341-2355 nematodes/plant)		The bad bulk (1125-1338 nematodes/plant)	
Seed Code	DH line	Seed Code	DH line	Seed Code	DH line	Seed code	DH line
120848	BVDH172	120842	BVDH166	120743	BVDH67	120867	BVDH191
120552	BVDH40	120845	BVDH169	120516	BVDH4	120536	BVDH24
120870	BVDH194	120524	BVDH12	120752	BVDH76	120884	BVDH208
120545	BVDH33	120822	BVDH146	120562	BVDH50	120531	BVDH19
120823	BVDH147	120852	BVDH176	120761	BVDH85	120515	BVDH3
120543	BVDH31	120837	BVDH161	120787	BVDH111	120853	BVDH177
120518	BVDH6	120557	BVDH45	120791	BVDH115	120885	BVDH209
120777	BVDH101	120513	BVDH1	120786	BVDH110	120565	BVDH53
120832	BVDH156	120855	BVDH179	120891	BVDH215	120864	BVDH188
120865	BVDH189	120850	BVDH174	120780	BVDH104	120781	BVDH105

4.1.2.3 Genetic fine mapping of selected QTLs by whole genome sequencing (WGS)

We decided to adjust our work program according to the enormous progress in sequencing technologies in the past years. Instead of bulk segregant analysis by marker mapping we sequenced phenotypic bulks to directly clone the gene of interest as recently suggested by Takagi *et al*. (2013) (Figure 5).

Genomic DNA extracted from leaf samples of the two phenotypic bulks (**Table 6**) was mixed in an equal ratio to make the bulk-DNA used for sequencing. The sequencing library was constructed from 1.5-2.5 micrograms of DNA and sequenced using Illumina HiSeq 2000 PE. The number of short reads ranged from 297,474,978 reads/lane (the worst bulk) to 340,191,348 reads/lane (the best bulk) with an average read length of 101 bp. The total size of short reads ranged from 28.9 Gb/lane (the worst bulk) to 34.2 Gb/lane (the best bulk) (**Table 7**).

The barley genome sequences which are publically available (a sequence-enriched barley physical map; The International Barley Genome Sequencing Consortium, 2012, http://mips.helmholtz-muenchen.de/plant/barley/) were exploited to generate our own reference sequence using short reads from the two susceptible bulks (S-bulk).

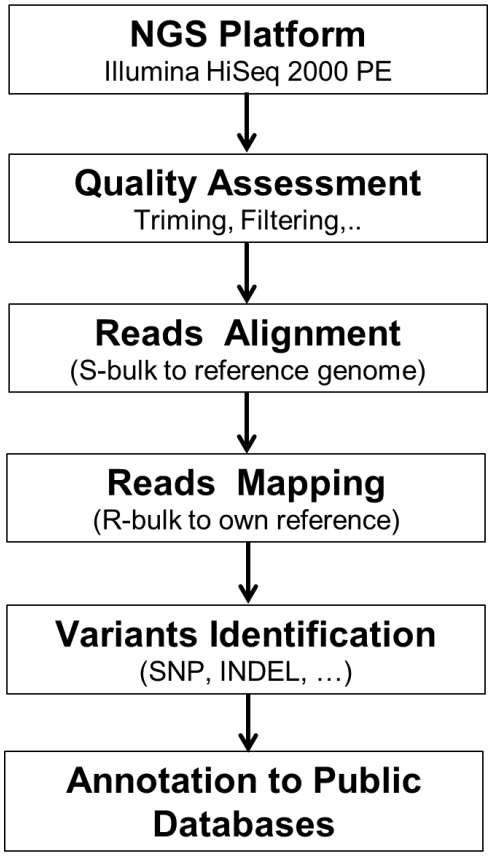

Figure 5: Basic workflow for whole genome sequencing. After library preparation, samples were sequenced using Illumina HiSeq 2000 PE. After quality assessment, short reads from the S-bulk were aligned against the available barley genome sequences and own reference sequence was generated. Short reads from the R-bulk were aligned to the own reference sequence to identify variants between the two phenotypic pools. Variants were filtered, and interesting sequence region was further analyzed and annotated against public databases to identify candidate genes.

The available reference sequences were obtained from the International Barley Sequencing Consortium (http://mips.helmholtz-muenchen.de/plant/barley/), and contigs were assembled according to their order in the physical map using the online bioinformatics tool Galaxy Project (http://galaxyproject.org/). The contig-assembly resulted in a total sequence of 2.136 billion-bp assigned to the seven chromosomes in addition to unassigned sequences referred to as chromosome 0H (**Table 8**). The length of chromosomes ranged from 251.62 Mb (1H) to 340.52 (2H) (**Table 8**).

Table 7: Short reads produced by WGS from four phenotypic bulks.

Lane	Sequence size (Kb)	No. of reads	Avg. length (bp)	Trimmed reads	Trimmed %	Avg. length after trim (bp)
Best bulk	34,202,285	340,191,348	101	326,233,320	95.90	97.6
Worst bulk	28,943,597	297,474,978	101	293,395,574	98.63	98.1
Good bulk	31,636,540	321,654,822	101	317,051,205	98.57	98.0
Bad bulk	29,460,816	302,878,080	101	298,798,166	98.65	98.1

After quality assessment, short reads from the S-bulk were aligned to the assembled barley reference sequences to generate our consensus reference sequence from the consensus sequence using CLC Genomics Workbench v6.5 (CLC Bio). Consensus sequences for all seven chromosomes from the S-bulk were generated. The length of chromosomes of the consensus sequences ranged from 204.51 Mb (1H) to 278.92 Mb (2H) (**Table 8**). The total number of short reads used to generate the consensus reference sequences ranged from 63.6 million reads (1H) to 101.1 million reads (5H) with an average coverage ranged from 23.31 (3H) to 31.93 (5H) (**Table 8**).

Table 8: Number and average coverage of short reads aligned to different barley chromosomes.

Chromosome	Reference length (bp)	Consensus length (bp)	Total read count	Average coverage
0H	45,099,519	39,622,564	19,632,916	42.43
1H	251,619,801	204,511,216	63,598,078	24.64
2H	340,517,144	278,919,508	85,349,851	24.44
3H	302,677,518	242,482,426	72,366,461	23.31
4H	285,183,503	237,929,251	71,306,079	24.36
5H	309,083,703	250,116,269	101,100,583	31.93
6H	266,363,473	216,687,419	66,511,796	24.33
7H	335,273,546	263,953,419	81,073,069	23.58
Total	2,135,818,207			

Short reads from the two resistant bulks (R-bulks) were mapped to the consensus reference sequence and variants between the two bulks were identified using Probablistic algorithm in CLC Genomics Workbench v6.5 (CLC Bio). The number of variants was calculated for each of the seven chromosomes ranging between 2.9 million (1H) and 3.9 million (2H) (**Table 9**). To exclude spurious variants, identified variants were filtered to a frequency of ≥90% which drastically reduced the number of variants between the two bulks (**Table 9**).

Table 9: Identification of sequence variations along different barley chromosomes.

Chromosome	Length (bp)	No. of variants	No. of variants (≥90% frequency)	Variants density	No. of variants (high-intensity criteria)[a]
1H	251,619,801	2,894,586	248,966	0.00099	2764
2H	340,517,144	3,857,448	305,400	0.00089	3860
3H	302,677,518	3,244,618	261,420	0.00086	3181
4H	285,183,503	3,312,157	267,000	0.00094	3473
5H	309,083,703	3,434,195	263,417	0.00085	3876
6H	266,363,473	3,283,755	309,610	0.00116	3418
7H	335,273,546	3,540,492	303,022	0.00090	3652

[a] Frequency ≥90%, count ≥5 and an average quality ≥40

Variants density was considered as an indicator for the degree of divergence between the two bulks and as an evidence for the responsibility of a chromosome to the given phenotype, i.e. the higher the variant density detected on a given chromosome, the higher the probability of that chromosome to be involved in that phenotype. Variants density calculation revealed that chromosome 6H, at which a major QTL (*Rlnnp6H*) was mapped, showed the highest variants density (0.00116) (**Table 9**).

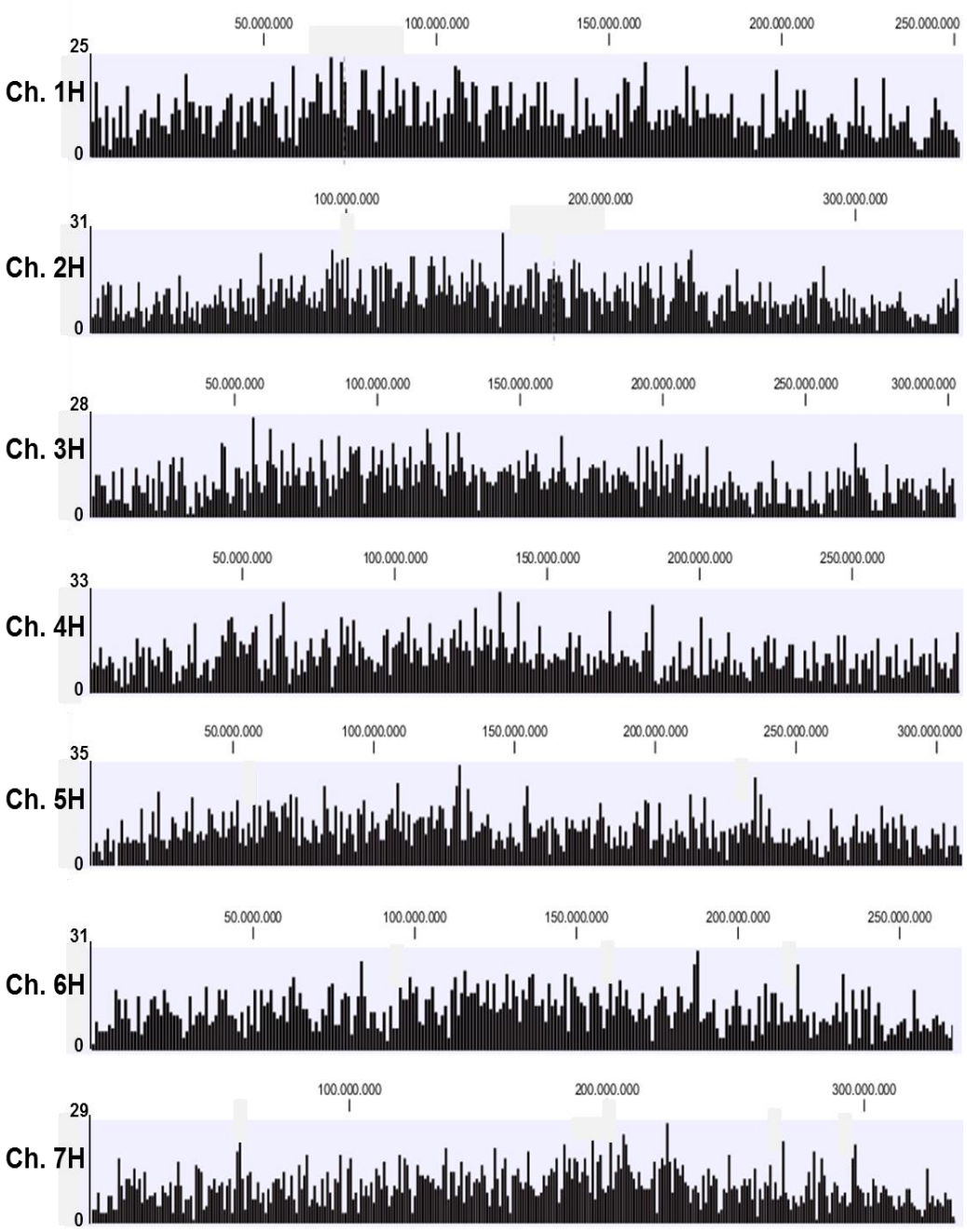

Figure 6: Distribution of variants frequencies along chromosomes. X-axis corresponds to the number of identified variants in a window size of 1 Mb indicated by columns.

To retain the high-confidence variants between the two bulks, identified variants were further filtered using high-intensity criteria, i.e. frequency ≥90%, count ≥5 and an average quality ≥40. Number of high-confidence variants ranged from 2764 (1H) to 3876 (5H) (**Table 9**). Variants frequency along all chromosomes in 1 Mb window size was calculated using CLC Genomics Workbench v6.5 (CLC Bio) for all chromosomes (**Figure 6**).

Variants frequencies calculation revealed a high divergent unique region at position 187-188 Mb of chromosome 6H which shows the highest variants number along the chromosome (**Figure 7**). Furthermore, molecular markers either flanking (EBmac0602, Bmag0613 and bPb-4750) or located within (bPb-6721, bPb-3722, bPb-5310, bPb-6636, bPb-1256, bPb-9522

and GBM1400) the QTL (*Rlnnp6H*) were mapped to the consensus sequence of chromosome 6H. They were located between the position of the mapped markers and the high divergent unique region on chromosome 6H (**Figure 7**). This demonstrates that the most divergent region of chromosome 6H which revealed the highest number of sequence variations between the resistant and susceptible bulks harboring gene(s) conferring resistance to root lesion nematodes.

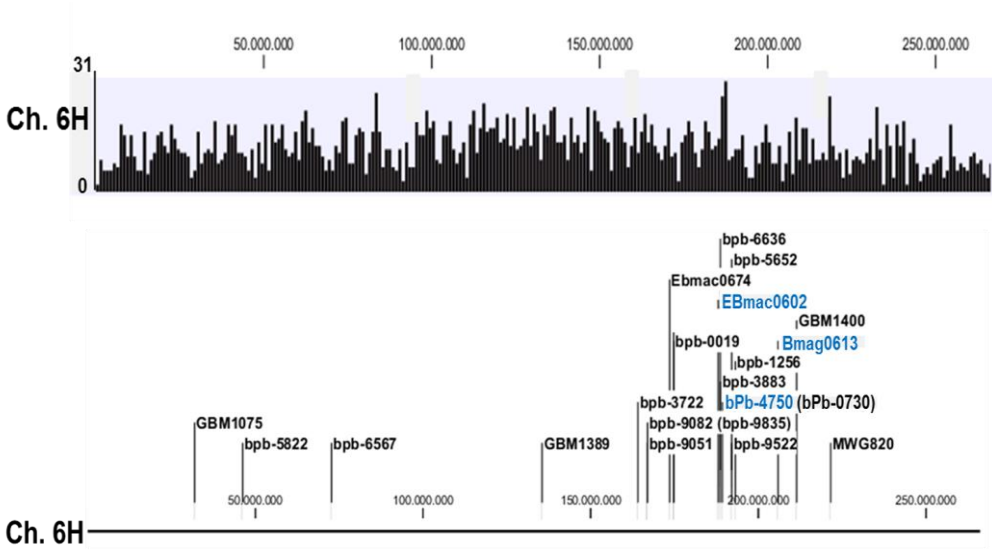

Figure 7: Co-localization of molecular markers from the QTL *Rlnnp6H* on the most divergent region of chromosome 6H. Flanking markers EBmac0602, Bmag0613 and bPb-4750 of the *Rlnnp6H* QTL in the Beysehir×Valentina and Uschi×HHOR populations (in blue) are located within the chromosomal region which showed the highest variants frequency. Markers that are in close proximity to the *Rlnnp6H* flanking markers (bPb-0730, bPb-9522, bPb-3883, bPb-5652 and bPb-6636) are located within the same region.

4.1.2.4 Genetic fine mapping of the *Rlnnp6H* QTL using bulked segregant analysis (BSA)

The *Rlnnp6H* QTL was fine mapped in the following way: two flanking markers that are tightly linked to the QTL were used to genotype the BV-DH population to identify DHs with recombinations within the QTL region. Recombinant DHs were selected and phenotyped (**Figure 8**). In a next step, the recombinant DHs were genotyped using molecular markers mainly located within the 2 Mbp (186-188 Mbp) region on chromosome 6H, because this region was thought to harbor the gene(s) conferring resistance to root lesion nematodes (see **chapter 4.1.2.3**).

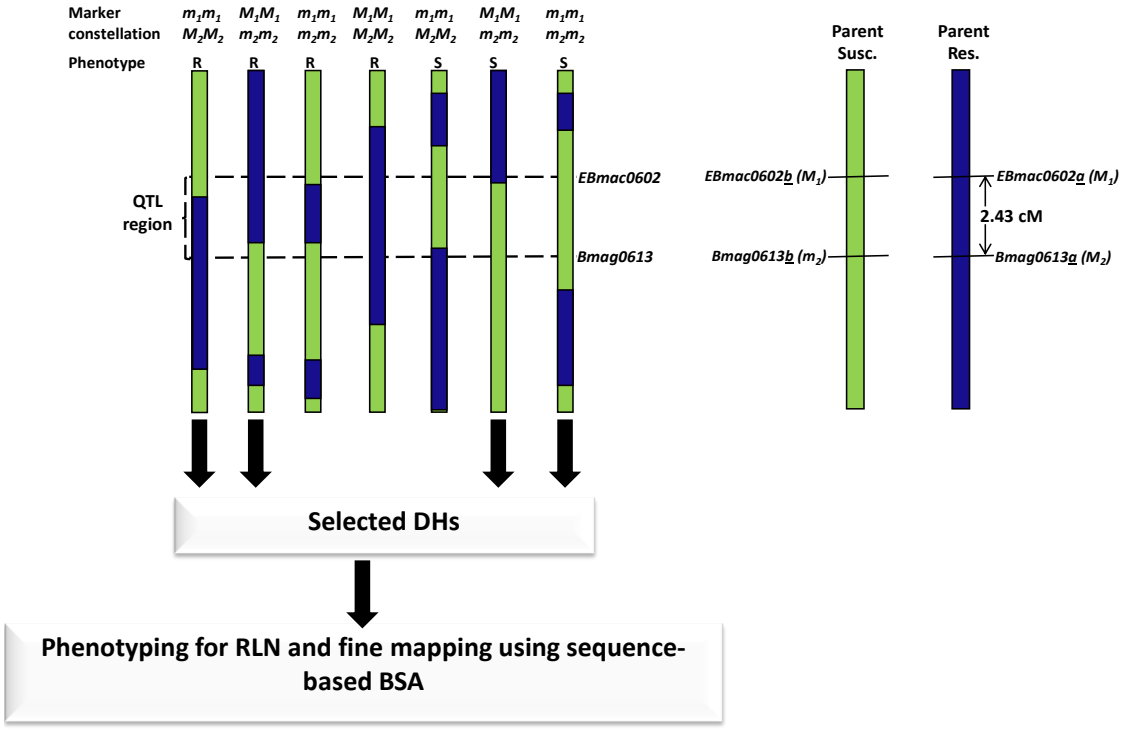

Figure 8: General scheme for the identification of recombinant DH lines for genetic fine mapping of *P. neglectus* resistance. Molecular markers *EBmac0602* and *Bmag0613* are tightly linked to the QTL *Rlnnp6H*, spanning a genetic distance of 2.43 cM.

4.1.2.4.1 Genotyping the BV population with two markers flanking the *Rlnnp6H* QTL

Genomic DNA was isolated from 1219 DH lines of the BV population using the NucleoSpin 96 Plant DNA isolation kit (Macherey and Nagel, Düren, Germany). Two SSR markers flanking the *Rlnnp6H* resistance QTL on chromosome 6H (Bmag0613 and EBmac0602) (**Figure 3**) were used to genotype 1219 DH lines. Recombinant DHs were classified as follows: They carry the Bmag0613 allele from Beysehir 'B'and EBmac0602 allele from Valentina 'V', or *vice versa*. In total, 55 recombinant DH lines were identified (**Table 10**), whereof 40 DH lines were used for fine mapping of *Rlnnp6H* QTL.

Table 10: Recombinant DH lines after genotyping with two SSR markers flanking the *Rlnnp6H* resistance QTL on chromosome 6H (Bmag0613 and EBmac0602). 'V', allele from Valentina; 'B', allele from Beysehir. The respective seed code for each DH line is given.

Seed code	DH line	EBmac0602	EBmag0613	DH line used for fine mapping
130393	BVDH278	V	B	yes
130418	BVDH303	V	B	yes
130420	BVDH305	B	V	yes
130448	BVDH333	V	B	no
130473	BVDH358	B	V	yes
130491	BVDH376	B	V	no
130529	BVDH414	V	B	yes
130534	BVDH419	B	V	yes
130554	BVDH439	V	B	yes

130555	BVDH440	B	V	yes
130578	BVDH463	B	V	yes
130585	BVDH470	B	V	yes
130591	BVDH476	V	B	yes
130724	BVDH609	V	B	yes
130732	BVDH617	B	V	yes
130770	BVDH655	B	V	no
130778	BVDH663	B	V	yes
130798	BVDH683	V	B	yes
130808	BVDH693	V	B	yes
130818	BVDH703	B	V	yes
130843	BVDH728	B	V	yes
130939	BVDH824	V	B	yes
130945	BVDH830	B	V	yes
130962	BVDH847	V	B	yes
130986	BVDH871	V	B	yes
131000	BVDH885	V	B	no
131003	BVDH888	B	V	yes
131013	BVDH898	V	B	yes
131022	BVDH907	V	B	yes
131039	BVDH924	V	B	yes
131087	BVDH972	B	V	yes
133935	BVDH1060	V	B	yes
133937	BVDH1062	V	B	yes
133996	BVDH1121	B	V	yes
134006	BVDH1131	V	B	yes
134020	BVDH1145	B	V	yes
134035	BVDH1160	V	B	yes
134043	BVDH1168	B	V	yes
134047	BVDH1172	B	V	yes
134081	BVDH1206	V	B	yes
134115	BVDH1240	B	V	no
134119	BVDH1244	V	B	no
134157	BVDH1282	V	B	no
134160	BVDH1285	V	B	no
134161	BVDH1286	B	V	no
134184	BVDH1309	V	B	no
134185	BVDH1310	V	B	no
134200	BVDH1325	V	B	yes
134205	BVDH1330	V	B	no
134213	BVDH1338	V	B	no
134261	BVDH1386	B	V	no
134271	BVDH1396	B	V	no

134279	BVDH1404	V	B	yes
134280	BVDH1405	V	B	yes
134297	BVDH1422	V	B	yes

4.1.2.4.2 Resistance tests with recombinant DH lines

Fourty recombinant DHs were used to fine map the *Rlnnp6H* QTL. In order to see the segregation of the markers and subsequently to fine map the QTL region, 40 recombinant DH lines were phenotyped for resistance to *P. neglectus.*

For the resistance test, plants were kept in a glasshouse with 23°C day and 18°C night temperatures, under long day conditions (16h light/8h dark) with supplementary light (Son-T Agro 400W, Koninklijke Philips Electronics N.V., Eindhoven, The Netherlands). Each recombinant DH line was phenotyped in six independent experiments in a complete randomized block design. These six replicates of each recombinant DH lines were further divided into two experiments, each of which had three replicates/ recombinant DH line. This entire resistance test experiment was done as described by Galal et al., 2014.

Seeds of 40 recombinant DH lines and both parental lines were pre-germinated for 24 hours in dark on wet filter paper. These germinated seeds were later transplanted in 150 cm^3 tubes, which were filled with steam sterilized sand. In order to prevent the outgrowth of root and also the movement of nematodes out of the tubes, a 20 µm sieve was placed at the bottom of each tube. All tubes were then transferred to special holders in the glasshouse and were irrigated using an irrigation system described by Keil et al. (2009). Ten days after transplanting, 1,000 nematodes per plant were inoculated using a Muto-syringe. The nematode solution was mixed each time before inoculation to ensure equal number of nematodes in each inoculum.

Seven weeks after inoculation, all plants were uprooted and roots were cut and washed to remove sand. All washed roots along with their identification number were then transferred to a Baermann funnel. In order to extract nematodes from the roots, these Baermann funnels were put in a misting chamber for five days. After five days, the nematode suspensions were collected in bottles and stored at 4°C. For counting the nematodes, two 0.5 ml aliquots from each bottle were loaded sequentially on a nematode counting slide and nematodes were counted under a stereomicroscope at 40-fold magnification.

A fisher's least significance difference (LSD) (α=0.05) value of 417 was observed when the statistical ANOVA test was performed on phenotypic data of all 40 recombinant DH lines. Difference in average number of nematodes from both parental lines was counted for all recombinant DH lines to group them in significant classes based on obtained LSD value. The LSD value classified all recombinant DH lines in three major classes. Out of 40 recombinant DH lines, 9 recombinant DH lines varied significantly from Valentina, hence they were grouped as "resistant", 12 recombinant DH lines varied significantly from Beysehir, hence they were grouped as "susceptible" and remaining 19 recombinant DH lines did not vary significantly from either of the parental lines (**Figure 9**).

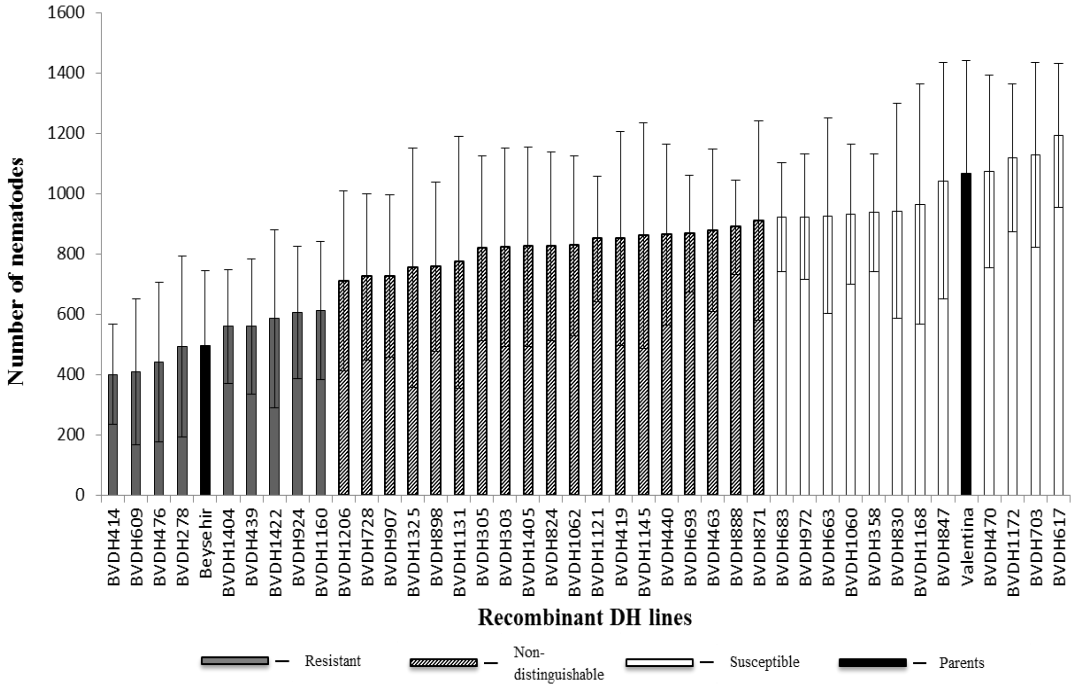

Figure 9: Nematode resistance tests (*P. neglectus*) with 40 recombinant DH lines and both parents (Beysehir and Valentina). Plants were tested in six replicates and nematodes were harvested seven weeks post inoculation. Error bars represent standard error of the mean. Resistant: DH lines which are significantly different from Valentina; Susceptible: DH lines which are significantly different from Beysehir; Non-distinguishable: DH lines which do not differ significantly from either of the parents.

4.1.2.4.3 Genotyping of recombinant DH lines using molecular markers

Sequence variations between the two phenotypic bulks within the *Rlnnp6H* region were selected as molecular markers for genetic fine mapping. For verification, variants flanking primers were used for PCR amplification using DNA of the parental lines Beysehir and Valentina as template. Amplicons were sequenced in both directions using the specific PCR primers as sequencing primers by Sanger sequencing. Confirmed variants were used for marker development, which were then used to genotype the identified recombinant DH lines.

Based on the analysis of DNA sequences and polymorphisms between the parental lines, four polymorphic markers (three CAPS markers and one InDel marker) were developed and used for genotyping 40 recombinant DHs (**Table 11**). Out of these four markers, CAU3856, CAU3857 and CAU3858 were located within the 2 Mb region (186-188 Mbp) of the *Rlnnp6H* QTL mapped after whole genome sequencing. The marker CAU3859 was located within one of the flanking regions of the *Rlnnp6H* QTL mapped in Uschi×HHOR 3073 DH population. All these four markers were located within a region flanked by the markers EBmac0602 (186.867 Mbp) and Bmag0613 (205.719 Mbp).

Table 11: Molecular markers developed for fine-mapping the 2 Mb region (186-188 Mbp) of the *Rlnnp6H* QTL. Primer sequences are given in 5' → 3' direction. For all markers obtained fragments for the parental lines Beysehir and Valentina are given.

Marker name	Marker type (enzyme)	Forward primer		Reverse primer		PCR Product length (bp)	PCR/Restriction fragments (bp)	
		Name	Sequence	Name	Sequence		Beysehir	Valentina
CAU3856	InDel	CR600	CTTCGTC CAGTTGA TGATAGC	CR606	GAAGCAC CTAACAA ACTCGG	1150	1150	1150, 250
CAU3857	CAPS (AatII)	CR706	CCTTAAC TCCAGGA GTTGAG	CR707	GGCAAG GTTTGGG CTTTGCA	1040	490	250
CAU3858	CAPS (DdeI)	CR710	GCACAAC GGCATGG AAAACC	CR711	CCACCAA AAATCAT GCCTCG	730	460, 270	730
CAU3859	CAPS (BsmAI)	CR540	GTATGAT CAAGGTC ACTGCG	CR577	CCAAGTT CTCCAGG ATCATG	1000	520, 220	510, 175, 55

The genotyping results were compared for 9 significantly resistant and 12 significantly susceptible recombinant DH lines. All 9 resistant recombinant lines had the allele from the resistant parent Beysehir for markers CAU3857, CAU3858 and CAU3859. Similarly, all 12 susceptible recombinant DH lines were found to carry the allele from the susceptible parent Valentina for same three markers (**Table 12, Figure 10**). In summary, the fine mapping results showed that three markers, CAU3857, CAU3858 and CAU3859 were completely segregating with the phenotype indicating that the identified 2Mb region of chromosome 6H is likely to harbor gene(s) conferring resistance to root lesion nematodes.

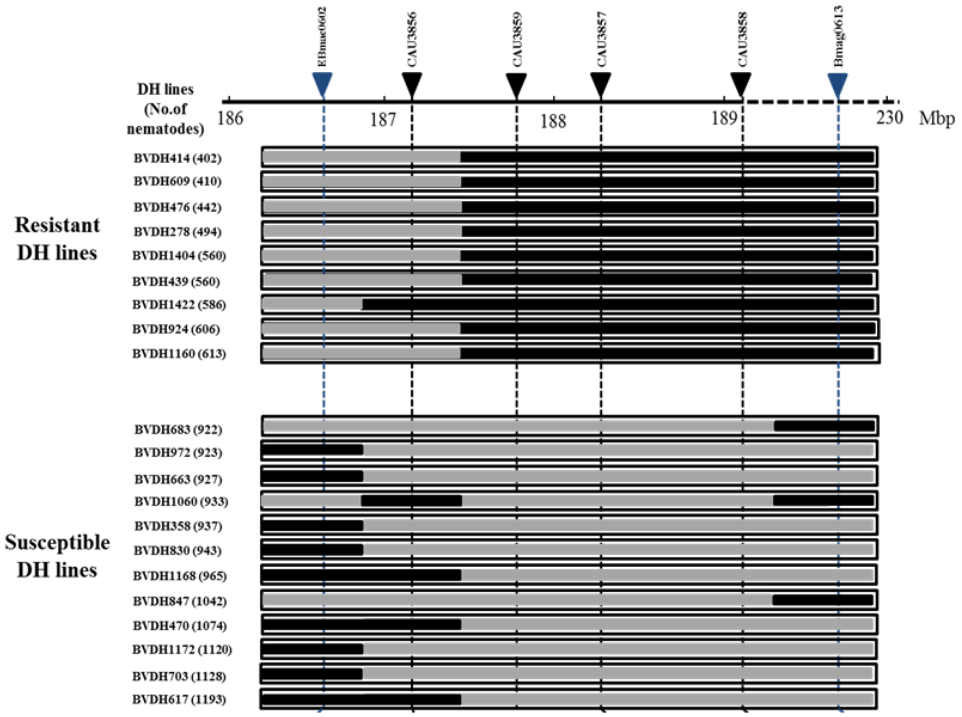

Figure 10: Map of the *Rlnnp6H* locus. Triangles indicate markers flanked (blue) or located within (black) the *Rlnnp6H* QTL and their position on chromosome 6H in mega-basepairs (Mbp). Horizontal bars indicate informative recombinants; black rectangles indicate allele inherited from Beysehir and gray rectangles indicate allele inherited from Valentina.

Table 12: Genotyping 40 recombinant DH lines and parents; 'a': significantly different from Valentina; 'b': significantly different from Beysehir; 'ab': not significantly distinguishable; 'B': allele from resistant parent Beysehir; 'V': allele from susceptible parent Valentina. Markers flanking the *Rlnnp6H* QTL are written in blue.

Groups	Genotype	Seed code	No.of Nematodes	EBmac0602	CAU 3856	CAU 3859	CAU 3857	CAU 3858	Bmag 0613
a	BVDH414	130529	402	V	V	B	B	B	B
a	BVDH609	130724	410	V	V	B	B	B	B
a	BVDH476	130591	442	V	V	B	B	B	B
a	BVDH278	130393	494	V	V	B	B	B	B
a	BVDH1404	134279	560	V	V	B	B	B	B
a	BVDH439	130554	560	V	V	B	B	B	B
a	BVDH1422	130537	586	V	B	B	B	B	B
a	BVDH924	131039	606	V	V	B	B	B	B
a	BVDH1160	134035	613	V	V	B	B	B	B
ab	BVDH1206	134081	711	V	B	B	B	B	B
ab	BVDH728	130843	725	B	B	V	V	V	V
ab	BVDH907	131022	727	V	V	B	B	B	B
ab	BVDH1325	134200	755	V	B	V	V	B	B
ab	BVDH898	131013	758	V	V	B	B	B	B
ab	BVDH1131	134006	773	V	V	B	B	B	B
ab	BVDH305	130420	818	B	V	V	V	V	V
ab	BVDH303	130418	823	V	V	B	B	B	B
ab	BVDH1405	134280	825	V	V	B	B	B	B
ab	BVDH1062	133937	828	V	B	V	V	B	B
ab	BVDH824	130939	832	V	V	B	B	B	B
ab	BVDH1121	133996	850	B	V	B	B	V	V
ab	BVDH419	130534	852	B	V	V	V	B	V
ab	BVDH1145	134020	862	B	V	V	V	V	V
ab	BVDH440	130555	864	B	B	V	V	V	V
ab	BVDH693	130808	867	V	B	B	B	B	B
ab	BVDH463	130578	878	B	V	V	V	V	V
ab	BVDH888	131003	889	B	V	V	V	V	V
ab	BVDH871	130986	911	V	V	B	B	B	B
b	BVDH683	130798	922	V	V	V	V	V	B
b	BVDH972	131087	923	B	V	V	V	V	V
b	BVDH663	130778	927	B	V	V	V	V	V
b	BVDH1060	133935	933	V	B	V	V	V	B
b	BVDH358	130473	937	B	V	V	V	V	V
b	BVDH830	130945	943	B	V	V	V	V	V
b	BVDH1168	134043	965	B	B	V	V	V	V
b	BVDH847	130962	1042	V	V	V	V	V	B
b	BVDH470	130585	1074	B	B	V	V	V	V
b	BVDH1172	134047	1120	B	V	V	V	V	V
b	BVDH703	130818	1128	B	V	V	V	V	V
b	BVDH617	130732	1193	B	B	V	V	V	V

4.1.2.5 Candidate gene identification

The identified region which showed the highest number of variants between the two phenotypic bulks was annotated to the NCBI Reference Sequence (RefSeq) protein database (http://www.ncbi.nlm.nih.gov/refseq) by BLASTX searches. In total, nine gene models could be identified in the 2 Mb region (186-188 Mbp) (**Table 13**).

Table 13: Barley gene models identified within the 2 Mb region (186-188 Mbp) of the *Rlnnp6H* QTL on chromosome 6H.

Gene model (EST)	best homolog identified in other plants	identity (amino acid)	function
MLOC_3095.1	ethylene-responsive transcription factor ERF034 [*Brachypodium distachyon*], XP_003570022.1	75%	involved in secondary wall thickening (Lasserre et al., 2008)
MLOC_10290.2	RING-H2 finger protein ATL79 [*Brachypodium distachyon*], XP_003569979.1	73%	-
MLOC_61035.2	LRR receptor-like serine/threonine-protein kinase [*Aegilops tauschii*], EMT05618.1	91%	LRR receptor-like serine/threonine-protein kinase (aegilops)
MLOC_61720	S-adenosylmethionine synthase 1-like isoform 1 [*Brachypodium distachyon*], XP_003567624.1	97%	methylation cycle (Radchuk et al.,2005)
MLOC_74813.2	trihelix transcription factor GTL1-like isoform X1 [*Setaria italica*], XP_004953287.1	56%	-
AK362483	Short chain dehydrogenase [*Aegilops tauschii*], EMT05529.1	95%	-
MLOC_61036.2	aldehyde dehydrogenase family 3 member F1-like NP_195348.2 [*Brachypodium distachyon*], XP_003569985.1	86%	-
MLOC_240.1	hypothetical protein NP_198938.1 [*Aegilops tauschii*], EMT14896.1	88%	-
MLOC_55679.1	E3 ubiquitin-protein ligase BRE1-like 1-like NP_182022.2 [*Brachypodium distachyon*], XP_003577829.1	81%	HUB1 in disease resistance (Dhawan et al., 2009)

Three out of nine genes identified were selected as candidate genes conferring resistance to root lesion nematodes hence they have been previously described as playing a role in disease resistance. These three genes are: MLOC_3095.1, MLOC_61035.2, and MLOC_55679.1. First RT-PCR experiments revealed that two genes MLOC_3095.1 and MLOC_61035.2 were not expressed. Consequently, we only focused on the gene MLOC_55679.1.

MLOC_55679.1 showed 81% identity (amino acid sequence) to the E3 ubiquitin-protein ligase BRE1-like 1-like and 43% identity to histone monoubiquitination 1 (HUB1) of *Arabidopsis thaliana* (AT2G44960). When the conserved domain structures of MLOC_55679.1 and HUB1 of *Arabidopsis thaliana* were compared, both of them were found to have similar domain structures, a SMC_prok_B domain and a RING domain (**Figure 11**). Literature search confirmed the role of *HUB1* in disease resistance in *Arabidopsis thaliana* and probably its homolog, MLOC_55679.1 was also thought to be involved in conferring resistance.

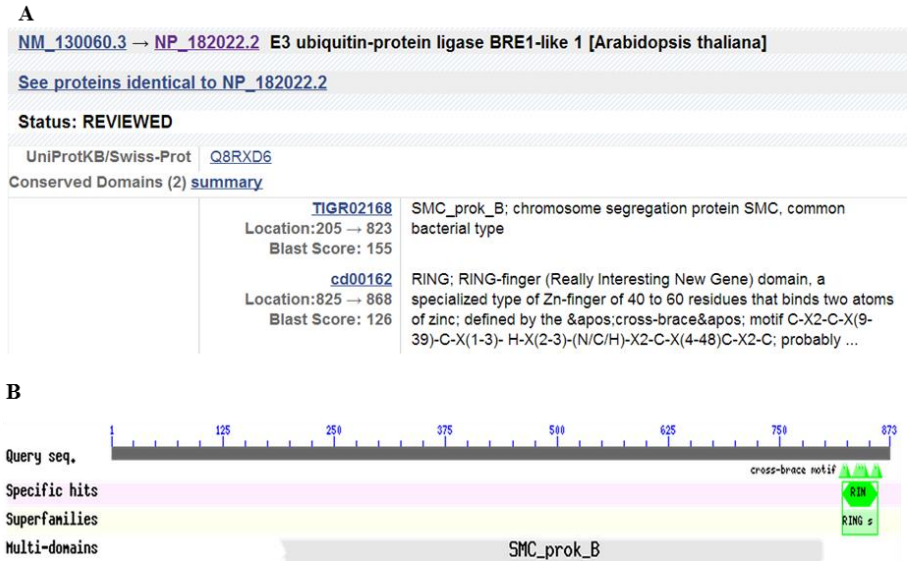

Figure 11: Comparison of conserved domains between HUB1 of *Arabidopsis thaliana* and MLOC_55679 (A) Conserved domains in HUB1 of *Arabidopsis thaliana*. (B) Conserved domains identified in MLOC_55679 using NCBI's CD-search tool (Marchler-Bauer, Lu et al. 2011); Green block: RING domain in MLOC_55679.

The exon intron structure of the gene MLOC_55679 was obtained from the International Barley Sequencing Consortium (IBSC). The genomic DNA sequence of MLOC_55679.1 is 10,714bp in size (Start-Stop), and the coding sequence consists of 19 exons (**Figure 12**) with a corresponding protein sequence length of 873aa.

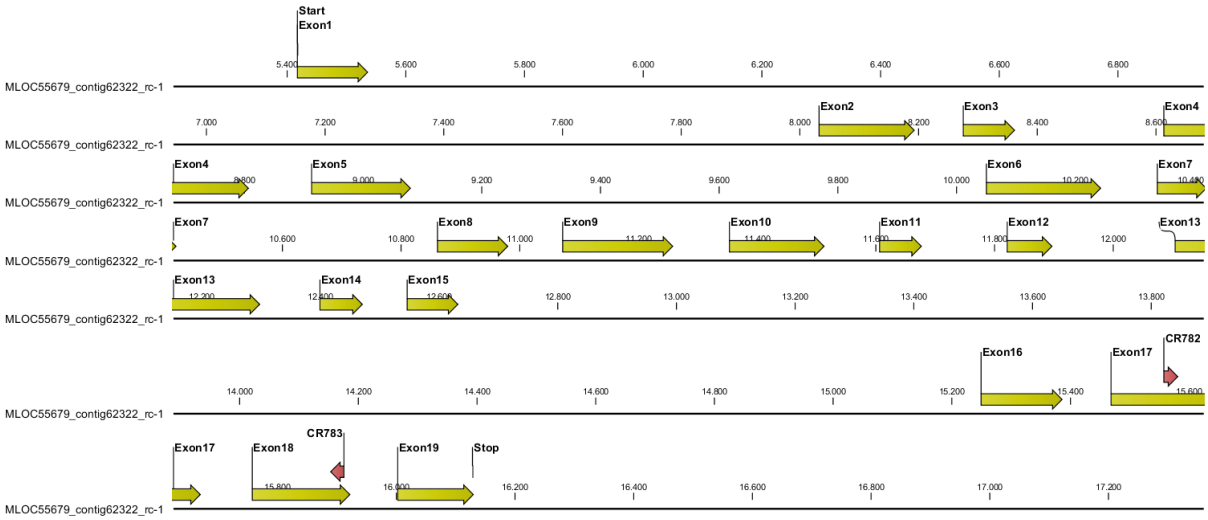

Figure 12: Exon-intron structure and positions of primers designed for RT-qPCR of the MLOC_55679.1 gene. Yellow arrows: exonic regions; red arrows: designed primers. Start and stop positions of coding sequence are given.

4.1.2.6 Expression analysis of candidate genes

For expression analysis, we used the parental lines Beysehir and Valentina, and ten barley accessions with extreme phenotypes; LW95, Igri, LP813, Babylone, and Orza96 (the most resistant 5 accessions), and Tapir, CM4113, BYDV15, IG40177, and IG39918 (the most susceptible 5 accessions) in resistance to *P. neglectus* in a panel of 170 accessions (**Figure 13**). Three plants (biological replicates) from each genotype were inoculated with 10,000 nematodes/plant ten days after sowing. Roots were sampled before inoculating the nematodes and after 7 weeks of inoculation.

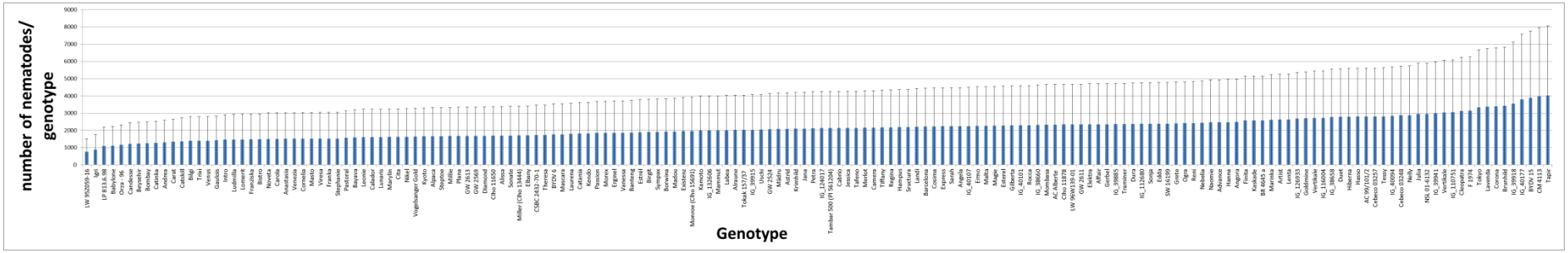

Figure 13: Resistance test of a panel of barley accession with *P. neglectus*. Average number of nematodes extracted from 170 accessions over six different experiments. Error bars indicate standard deviation from the average.

Total RNA was extracted from leaves using the peqGOLD Plant RNA Kit (PeqLab) following the manufacturer's instructions and DNase treated. 1 µg of RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Thermo scientific (Fermentas), St. Leon-Rot, Germany), and the cDNA was diluted ten times for RT-PCR. RT-qPCR was performed using the primer combination CR782/ CR783 (**Table 14**, **Figure 12**) and the Platinum® SYBR® Green qPCR SuperMix-UDG with ROX (Invitrogen, Karlsruhe, Germany) on a CFX96 Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with a final reaction volume of 20 µl. In total the analysis was performed with three biological and three technical replicates. Relative expression of the candidate gene was calculated using the comparative CT (ΔC_T) method and normalized to the geometric mean of *HvActin*.

Table 14: Primers with their targeted exonic regions and other specifications. Primer sequences in 5'→ 3' direction. The PCR product length for the parental DH lines Beysehir and Valentina is given.

Primers	Targeted region	Forward primer	Reverse primer	Product length (bp)
CR570-CR571	*HvActin*	GCCGTGCTTTCCCTCTATG	GCTTCTCCTTGATGTCCCTTA	235
CR782-CR783	MLOC55679_ Exon17-18	GGATGTACAGGGAGAGGTTCAACAG	CGGTCACGGCAAACACCACATTTC	270

The expression of MLOC_55679.1 was analyzed in the parental lines before and after 7 weeks of inoculation. The relative expression data showed that the expression was higher in the susceptible parent Valentina than in the resistant parent Beysehir, indicating higher transcript level of the MLOC_55679.1 gene in the susceptible parent. In non-inoculated plants, the mean relative expression was 0.024 in Beysehir and 0.071 in Valentina. Similarly, in inoculated plants, the relative expression was 0.025 in Beysehir and 0.060 in Valentina (**Figure 14**).

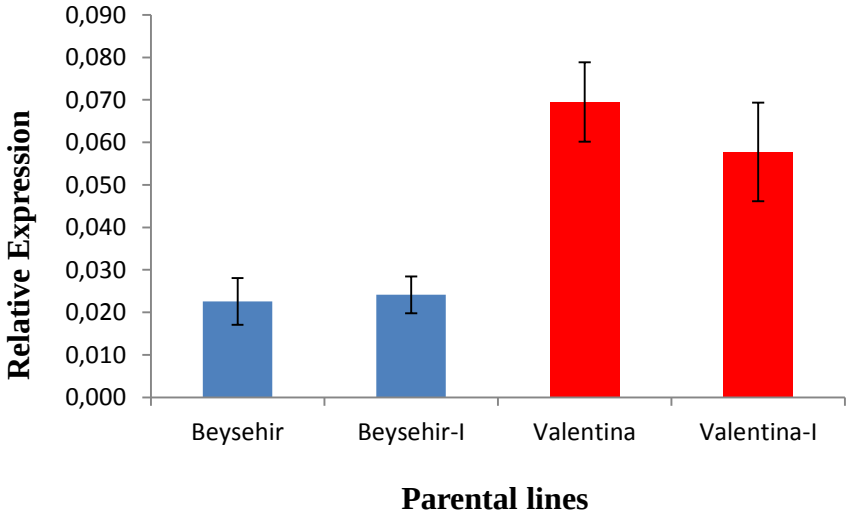

Figure 14: Relative expression of MLOC_55679.1 gene in parental lines Beysehir and Valentina. Relative expression for all samples is mean of three biological and three technical replicates. The vertical bars represent the relative expression of the MLOC_55679.1 gene which was calculated using the ΔC_T method and normalized to the housekeeping gene *HvActin*. Error bars represent standard error of the mean of biological replicates. `I´: inoculated samples collected after 7 weeks of inoculation.

To further extend the expression profile, RT-qPCR analysis was done in five resistant and five susceptible barley accessions. A similar expression pattern was observed in these barley accessions. Though there were some exceptions, the overall pattern suggested a lower expression of MLOC_55679.1 in resistant accessions compared to susceptible accessions. ANOVA test performed on resistant and susceptible accessions showed that there is a significant difference in the transcript level between both groups.

The lowest expression of the candidate gene observed in the resistant accessions (0.025 in 'Igri'-inoculated and 'LW95'-non-inoculated) was much lower compared to the lowest expression observed in susceptible accessions (0.040 in 'IG39918'- inoculated). Similarly, the highest expression analyzed in susceptible accessions (0.119 in 'CM4113'-inoculated) was much higher than that in the resistant accessions (0.082 in 'Orza'-inoculated) (**Figure 15**).

Interestingly, in most of the accessions, the expression of the candidate gene MLOC_55679.1 was higher in inoculated samples than in non-inoculated ones. The resistant accession 'Igri' and the susceptible accession 'IG39918' were the only exceptions having higher expression of the gene in non-inoculated samples. But, when the student's t-test was performed, it suggested that the difference between non-inoculated and inoculated samples from resistant accessions was not statistically significant. However, the difference between non-inoculated and inoculated samples from the susceptible accessions, BYDV15 and CM4113 was significant.

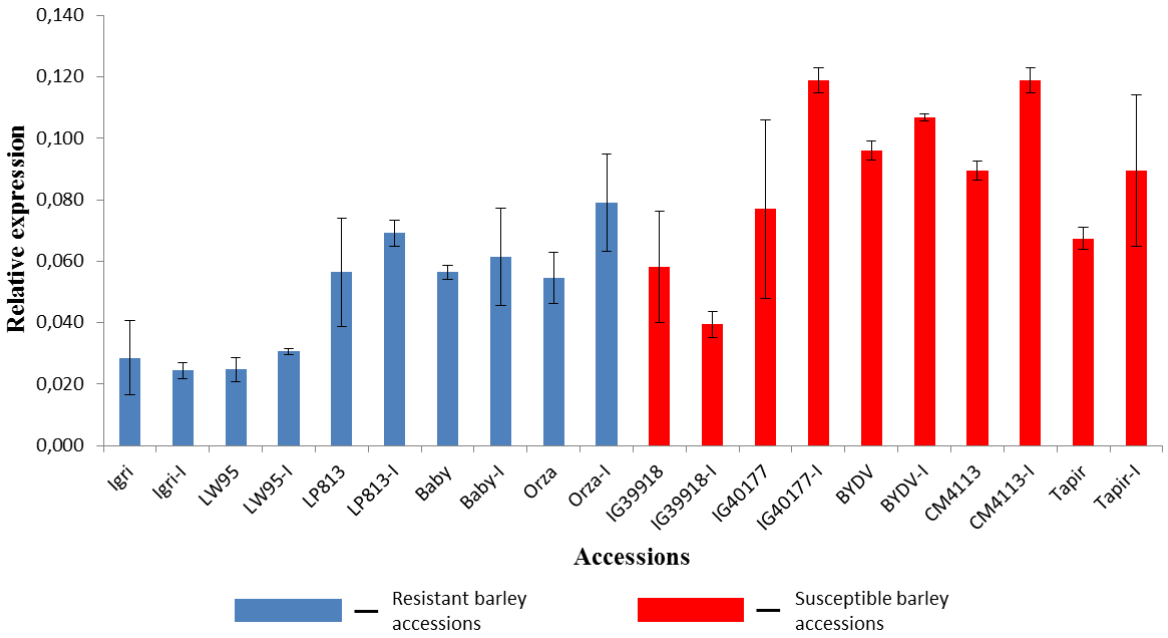

Figure 15: Relative expression of the MLOC_55679.1 gene in resistant and susceptible barley accessions. Relative expression for all samples is mean of three biological and three technical replicates. The vertical bars represent the relative expression of the MLOC_55679.1 gene which was calculated using the ΔC_T method and normalized to the housekeeping gene *HvActin*. Error bars represent standard error of the mean of biological replicates. `I´: inoculated samples collected after 7 weeks of inoculation.

4.2 WP 5.3: Expression profiling of the phenotypic and genotypic bulks

An expression profiling experiment had been planned using the identified phenotypic bulks in collaboration with partner 1 (Prof. Dr. Kogel). In this experiment, RNA from DH lines should be isolated from inoculated and non-inoculated roots. Differentially expressed genes should be identified in a microarray experiment using the Agilent barley 44K microarray (PI1).

As inoculation enhances the expression of a large number of genes related to tissue wounding, analyze the expression profiles between inoculated and non-inoculated bulks can create major problems. Therefore, we decided to abandon the expression profiling experiment.

4.3 WP7: Validation of candidate genes by STARTS and seedling assays

It was intended to use stable root transformation system (STARTS) as high-throughput transformation system for rapid functional analyses of proteins of barley (Imani et al., 2011). Candidate genes from the unbiased and specific approach should be functional validated in plant assays (STARTS) developed from embryogenic calli. Promising genotypes identified in this screen should be further validated in seedling tests under combinations of biotic and abiotic stress.

4.3.1 Evaluation of *CRT1*

T2 plants (segregating for the transgene) from promising *CRT1* knock down transgenic lines (**Table 15**) in addition to the donor genotype (Gold promise) and a control were phenotyped for resistance to the RLN *P. neglectus*. Because of space availability in the greenhouse and the misting chamber, 9-12 individuals from each T2 family were phenotyped. Large variations within each T2 family in terms of number of extracted nematodes were observed. The T2 family L11 showed the highest infection rate with a range of nematode number

between 202 and 2700 nematodes/ plant (**Table 15**). Freeze-dried leaf samples from each individual T2 plant were sent to the partner in Gießen (Prof. Kogel) for identification of the presence or absence of the transgene.

Table 15: Resistance tests of barley CRT1 transgenic lines with the RLN *P. neglectus*.

Genotype	Number of tested plants	Inoculum (species/number of nematodes)	Inoculation date	Root harvest date	Test conditions (pot size	Nematode infection rate (mean, range)
Golden Promise	12	*P. neglectus*/ 1000	16.08.2012	03.10.2012	150 cm³	664 194-1428
Azygous line	9	*P. neglectus*/ 1000	16.08.2012	03.10.2012	150 cm³	1402 422-2354
L11	12	*P. neglectus*/ 1000	16.08.2012	03.10.2012	150 cm³	1047 202-2700
L40	12	*P. neglectus*/ 1000	16.08.2012	03.10.2012	150 cm³	768 0-1603
L55	10	*P. neglectus*/ 1000	16.08.2012	03.10.2012	150 cm³	1205 209-2470

4.4 WP9: Molecular marker development for RLN resistance

The identification of a critical region within the QTL (see 13) by whole genome sequencing between the two phenotypic bulks of the BV population together with a fine mapping experiment by a bulked segregant analysis enabled the development of diagnostic markers for RLN resistance. All markers tightly linked to the QTL can be used as markers. In addition, new primer sequences for easy an cheap markers systems can be quickly derived from the sequenced regions of the QTL. The availability of the whole genome sequencing data from the two phenotypic bulks of the BV population enables the rapid identification of sequence variations between the two phenotypic bulks.

4.5 WP 1: Plant material and genotypes

4.5.1 Developing a large DH population

The project partner 4 (Dr. Laubach, Nordsaat Saatzucht GmbH) produced a large Beysehir × Valentina DH population segregating for *Pratylenchus* resistance. In total, 1445 DH plants have been produced by the project partner 4 (Dr. Laubach, Nordsaat Saatzucht GmbH). These plants have been grown at Nordsaat Saatzucht GmbH and seeds have been produced from 1445 plants. Those DHs were used for the following purposes.

- Two hundred and twenty six (226) DH lines were tested for resistance to *P. neglectus*.
- 226 DH lines were genotyped by AFLP and sequencing-based markers.
- A map was constructed with 388 AFLPs and sequencing-based markers and *P. neglectus* resistance QTLs were mapped.
- 1219 DH lines were for the identification of recombinant DHs required for fine mapping of selected QTLs.

4.5.2 Developing recombinant inbred lines (RILs)

Recombinant inbred lines (RILs) represent unique combinations of parental genotypes and being immortal. Therefore, RILs are very useful in genetic mapping of QTLs related to complex phenotypic traits such as resistance to root lesion nematodes. Recombinant inbred lines from a Beysehir × Valentina cross are being produced to serve as powerful tools for genetic fine mapping by single seed descent (SSD) until an advanced generation (F6) is reached which we expected to be done by end of 2016. During this project, F3 seeds were harvested from 300 F2 plants resulting from a cross between Beysehir and Valentina.

Furthermore, as a backup a cross between Beysehir and Valentina has been initiated. F1 seeds were obtained and 26 viable F1 plants were grown in January 2012. The hybrid nature was confirmed by an SSR marker (CR027-CR028). F2 seeds were harvested from 26 F1 plants. Then, 300 F2 plants were grown to produce F3 seeds using single seed descent (SSD) method. This year, F4 seeds will be harvested from ca. 300 F3 plants.

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6 Anhang

Berichtsblatt

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	15. Abbildungen 15
16. Zusätzliche Angaben	
17. Vorgelegt bei (Titel, Ort, Datum)	

18. Kurzfassung

Im Teilprojekt der CAU Kiel des Verbundprojektes CEREAL-ROOTS wurden Gerste-Populationen hinsichtlich ihrer Resistenz gegen Wurzelläsionsnematoden (*Pratylenchus penetrans, P. neglectus*) untersucht. Dazu wurde vom Wirtschaftspartner Nordsaat Saatzucht GmbH eine sehr große doppelt-haploiden (DH) Population erzeugt und zur Verfügung gestellt. Diese wurde in einem zuvor etablierten Gewächshaus-Resistenztest auf ihre Resistenz gegen *P. neglectus* untersucht. Mit einer Teilpopulation wurde eine genetische Karte mit molekularen Markern erzeugt, um darin QTL für *P. neglectus*-Resistenz zu lokalisieren. Es zeigte sich, dass von acht QTL zwei gleichzeitig Resistenz gegen *P. neglectus* und *P. penetrans* vermitteln. Somit besteht in der Gerstezüchtung die Möglichkeit Sorten mit Resistenz gegen beide Schaderreger zu züchten. Dafür wurden molekulare Marker für die Selektion im Rahmen des Zuchtprozesses erzeugt. Die Genotypisierung einer sehr großen Zahl von DH-Linien ermöglichte eine Feinkartierung des QTL-Locus auf Chromosom 6. Diese wiederum stellte den Ausgangspunkt für die Identifizierung von Sequenz-Contigs dar, die den entsprechenden QTL-Locus überstreichen. Dazu wurden jeweils zehn hoch-resistente und hoch-anfällige DH-Linien gemeinsam sequenziert (bulked-sequencing) durch Vergleich mit dem Referenzgenoms wurden Sequenz-contigs assembliert, auf denen erste Kandidatengene für die Resistenz gegen Wurzelläsionsnematoden lokalisiert werden konnten. In Kooperation mit den Projektpartnern in Erlangen und Gießen wurden physiologische Untersuchungen an anfälligen und resistenten DH-Linien durchgeführt und es wurde die Resistenz transgener Gersten getestet, in denen ein Pathogen-Resistenzgen differentiell reguliert wurde. Als Ergebnis der Arbeit stehen resistente DH-Linien für die Gerstezüchtung zur Verfügung. Gleichzeitig wurden molekulare Marker etabliert, die den sehr aufwendigen Resistenztest überflüssig machen. Die Tatsache, dass die resistenten Linien Resistenz gegen beide Schaderreger zeigen, eröffnet neue Perspektiven für die Gerstezüchtung

19. Schlagwörter Gerste, Resistenz, freilebende Nematoden, Pratylenchus	
20. Verlag	21. Preis