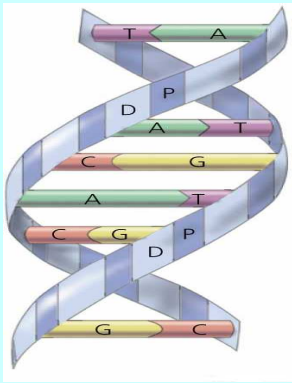
Fine mapping of *Ol-1*, a resistance gene to tomato powdery mildew (*Oidium neolycopersici*)

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Thesis for Master of Sciences in Plant Sciences
Specialization Plant Breeding and Genetic Resources

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Laboratory of Plant Breeding
Wageningen University,
June 23, 2009

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Summary

Tomato powdery mildew caused by *Oidium neolycopersici* is one of the most important diseases causing damage to the tomato plant. It usually affects leaves of the plant causing up to 50% losses in yield (fruit) as a result of loss of vigor (Roberts *et al*., 2002). Previously, all of the cultivars under production were susceptible to the disease. Currently there are some cultivars with resistant genes introgressed from wild relatives of tomato.

Ol-1 is one of the resistant genes obtained from wild relative *Solanum harbochytes* G1. 1560 conferring incomplete resistance (Lindhout *et al*., 1994). This gene was mapped between markers P13M49 and H9A11 on chromosome 6 of tomato (Bai, 2004; Bai, *et al.,* 2005). The objective of this study was to fine map the *Ol-1* gene. The methodology used was first saturating the region of gene of interest by generating more markers in addition to the already existing ones, followed by recombinant screening using disease test. In the mean time, BAC library screening was done in order to fill a gap in BAC clones around the region of *Ol-1* on chromosome 6.

Twenty one molecular markers were developed during the study period in the region of gene of interest and beyond the region. These markers were used in order to genotype plants during recombinant screening using disease test, determine the introgression region between the susceptible parent (Moneymaker) and the donor of *Ol-1* gene (*Solanum habrochites* G1.1560) and dissect *Ol-5* locus from *Ol-1*in the plant material we tested.

Four BAC clones were identified to be positives (overlapping) to the already available ones. Three of them, namely 2G04, 102B23 and 326B14 were found to be positive to 215M16 and one BAC clone, namely 161G17 was found to be overlapping to 123G17. They were confirmed by FISH for their landing on the proposed location.

Molecular markers flanking the introgression region (10.5cM) between Moneymaker and the donor of *Ol-1* gene (*S. habrochites*) were determined. Moreover, it was learnt that *Ol-5* had no influence on the effect of the *Ol-1* gene in the materials we tested as far as resistance to *O. neolycopersici* is concerned.

In this study, after recombinant screening and disease test of BC_1S_2 population, *Ol-1* gene was mapped between markers P13M49 and H9A11 with genetic distance of 0.26 cM on the long arm of chromosome 6 of tomato. The study also found out that marker H9A11 was located on one of the newly identified BAC (161G17) which in turn overlapping to 123G17 making the previous gap between the BACs no more relevant for fine mapping of *Ol-1*. BAC 215M16 have full sequence available while 123G17 has also been sequenced except a small gap (100bp). BAC 161G17 is also partially sequenced. Therefore, as future line of work, more markers will be generated based on the available sequence information of BACs and we will narrow down the *Ol-1* region to a single gene very soon after another recombinant screening.

Key words: Fine mapping, *Ol-1*, tomato, *Oidium neolycopersici*

1. Introduction

1.1 Tomato

Tomato (*Solanum lycopersicum [previously Lycopersicon esculuntum])* is an herbaceous, usually sprawling plant of the *Solanaceae* or nightshade family. It is the most popular fruit world-wide. Botanically speaking it is a fruit, not a vegetable. China is the largest producer, followed by the United States and Turkey (http://www.tomato.org). Tomatoes were first cultivated in 700 AD by Aztecs and Incas. Explorers returning from Mexico introduced the tomato into Europe, where it was first mentioned in 1556. The French called it "the apple of love," the Germans "the apple of paradise" (http://en.wikipedia.org/wiki/Tomato). Tomatoes are used as food in salads, tomato ketchup, pasta, pizza and the likes. They are also known for having natural health components like lycopene, a compound with anti-oxidant property helping in preventing cancer. They are also rich in vitamin A and C.

1.2 Tomato powdery mildew

Powdery mildew in tomato, in general, can be caused by three distinct causative agents. *Levellula taurica* (Lev) is one of the fungi causing the disease in tomato in western region of the U.S. with the host ranges of multiple genera and families (http://nt.ars-grin.gov/fungaldatabases/). The other two fungi causing powdery mildew on tomato are *Oidium lycopersici* and *Oidium neolycopersici*. The two pathogens differ in morphological features and DNA sequence of the internal transcribed spacer regions of the nuclear rRNA genes (Kiss *et al*. 2001), *O. neolycopersici* has apparently become distinct and known to be the powdery mildew causing agent of tomato in Europe. The Australian isolate is named *O. lycopersici* (Kiss *et al*., 2001). The two pathogen species are also different in spore orientation. *O. neolycopersici* produces single spore while *O. lycopersici* bears branched spore during final developmental stage (Jones *et al*., 2001)

1.3 *Oidium neolycopersici*

1.3.1 General

The disease caused by *O. neolycopersici* is severe in crops grown in greenhouses or shade houses causing up to 50% loss of fruit production as a result of loss of

vigor (Roberts *et al*., 2002). It was reported in The Netherlands in 1986 (Paternotte, 1988 cited in Bai, 2004). It is also one of the most fungicide sprayed diseases in tomato (Li, 2005). Almost all tomato cultivars found in the hands of growers in 1996 were susceptible to *O. neolycopersici* (Nunnink, 1996 cited in Huang *et al*., 2000). Jolkowsky and Cohen (2007) reported that among 200 commercial cultivars and lines which were inoculated artificially with conidia of *O. neolycopersici* in greenhouse, none of them found to be resistant. The same authors reported that there were some tested wild species found to be totally immune to the pathogen. Among the resistant wild species, some showed no symptom at all and some produced hypersensitive response few days after inoculation.

Temperature and relative humidity are the major factors influencing the disease development. The disease development is optimum at temperature of 22^{o}C especially 16-24 hours after leaf wetness (Cerkauskas, 2005). High level of nitrogen (fertilizer) and closer spacing are also known to favor the development of the disease as they lead leaf wetness and favorable condition promoting infection. Avoiding high rate of nitrogen application, avoiding closer spacing and watching out not to grow new plants in previously infected lands are some of the cultural practices recommended to reduce the incidence of the disease. Early detection and spraying of chemicals like sulfur and other protectants are also options to control the disease. Wind and rain are the principal dispersing mechanism of the pathogen as the pathogen produces large number of spores that are readily dispersed. Insects like thrips and aphids are also known to spread the fungus. It is reported that *O. neolycopersici* is not a seed borne pathogen (Cerkauskas, 2005).

1.3.2 Origin

No one can locate the origin of *O. neolycopersici* at least until now. But there are some speculations forwarded that the pathogen might have jumped hosts by acquiring one or more pathogenecity factors although it can not be substantiated by evidence (Huang *et al.*, 2000 cited in Jones *et al.*, 2001). The hypothesis that many scholars agree on is that the appearance of *O. neolycopersici* is not a recent phenomenon rather it has just become apparent. The identification of the fungus

from the herbarium specimens dating back to 1947 from Asia substantiates the assumption. However, this may not give true answer why *O. neolycopersici* has become a problem recently and why it is spreading rapidly (Kiss *et al.,* 2001 cited in Jones *et al,* 2001).

1.3.3 Symptoms

The symptom of the disease begins by powdery mildew pustules developing on the upper surface of leaves. The pustules'' size ranges from 1/8 to 1/2 inch in diameter (Roberts *et al.,* 2002). At initial stage, the pustules are circular but later they are fused together to become irregularly wider and cover considerable part of the leaf (Fletcher and Smewin, 1988; Jones *et al.,* 2001). In later stages the infected leaves turn brown and get shriveled remaining attached to the stem with evidence of a white powdery growth of fungal mycelium (Roberts *et al.,* 2002). The disease starts from lower leaves and progress up to the younger leaves. The younger leaves are usually symptomless especially at early stages. While no symptom appears on fruits and roots, petioles and calyx can be affected (Jones *et al.,* 2001).

1.3.4 Host range

O. neolycopersici is a highly polyphagous (Jones *et al.,* 2001) biotrophic fungus (obligate parasite) affecting the upper leaf surface of tomato plants. Tomato being the major host of the pathogen, several plants especially Solanaceous (pepper, egg plant, potato and tobacco) and weeds (nightshade) are also affected by the pathogen (Cerkauskas, 2005). It has been reported that 13 plant families and 80 species were found to be hosts to tomato powdery mildew after testing 26 families of plants (Whipps *et al.,* 1998). The disease is recorded in Europe, North America, South America, Central America, Caribbean, Africa and South East Asia. Until recently, the disease is more concentrated in Eastern Europe than any other continent (CAB International, 2007).

1.3.5 Morphology

As to the morphology of *O. neolycopersici*, the spores have ellipsoidal shape with approximate dimension of 30 µm X 15 µm (Jones *et al.,* 2000). The conidial surface is covered by irregular arrays of ribbon like and rounded projections but

when it germinates germ tubes that are smooth surfaced will appear from the conidia. The tube then will elongate at the tip and will become lobed or "clover-leaf" like in shape. Report from the same authors suggested that the appressorial structure of the fungus used for pressing host cells is commonly found on the junction of three epidermal cells. A tiny infection peg emerges from the center of the 'palm' of the lobed appressorium and penetrates the host plant, leaving an approximate 0.2 µm diameter pore. After successful infection, colonization proceeds as secondary hyphae radiate from both the conidial body and the preceding primary appressorium. Secondary appressorium becomes apparent either singly or in pairs from the hyphae spread over the host surface. The formation of conidiospore, which appear perpendicular to the host surface, commences the completion of the asexual life-cycle of the pathogen. In general, germination of the spore can take 3-5 hours after inoculation (hai). Appressorium differentiation and penetration can take 6-8 hai and 11 hai, respectively (Jones, 2000 cited in Jones *et al.*, 2001).

1.4. Resistance to powdery mildew in tomato

Previously, no commercial cultivar was resistant to *O. neolycopersici* (Lindhout *et al.*, 1994; van der Beek *et al.*, 1994 and Bai *et al.*, 2005). However, resistance has been discovered from different wild species of cultivated tomato (Lindhout *et al.*, 1994). These resistances are conferred by monogenic major genes as well as quantitative trait loci (QTLs). So far six monogenic genes (*Ol-1, ol-2, Ol-3, Ol-4 Ol-5* and *Ol-6*) have been identified from different wild species, except *ol-2* originating from the cultivated tomato *S. lycopersicum var. cerasiforme* (Ciccarese *et al*, 1998 cited in Siciliano and Marte, 2004). Their positions in the genome of respective source plant has been also located. *Ol-1, Ol-3* and *Ol-5* were mapped on the long arm of chromosome 6, which are all derived from *S. habrochaites* (previously *L. hirsutum*). *Ol-4* and *Ol-6* reside on short arm of chromosome 6, with *Ol-4* originating from *S. peruvianum* (previously *Lycopersicum peruvianum)* and *Ol-6* unknown origin. *ol-2* is located in chromosome 4 (Bai, 2004). The resistance response against *O. neolycopersici* infections due to major resistant genes is mainly associated with hypersensitive

response, while papillae formation is the defense mechanism governed by the recessive *ol-2* gene (Bai, 2004; Bai *et al*, 2005).

1.5 Fine mapping of *Ol-1*

Tomato is a simple diploid plant with 12 chromosomes. It is a well known model for both conventional and molecular genetic studies and among the best mapped flowering plants due to several merits it has. Among others, it's simple diploid nature, small genome size, nature of self pollination, precise segregation of large number of monogenic morphological traits (Rick and Yoder, 1988). Its genome size is about 950Mb per haploid nucleus (http://www.plantpath.wisc.edu/GeminivirusResistantTomatoes/MERC/Tomato/Tomato.html). An international consortium of sequencing centers of 11 member countries is carrying out the genomic sequencing of the gene-rcih euchromatic position of the twelve chromosomes. Chromosome 6 is handled to be sequenced in The Netherlands and 56% of the chromosome region has already been sequenced until mid June 2009 (http://www.sgn.cornell.edu/about/tomato_sequencing.pl). There is also a copy of the tomato BAC library which is constructed from *S. lycopersicum* cultivar Heinz 1706 using restriction enzyme *HindIII.* This library is being used for sequencing of the chromosome 6 and used in this study for BAC screening. As the physical map of chromosome 6, there are 28 contigs and 10 singleton BACs were placed (Personal communication).

Ol-1 is one of the genes found to be conferring resistant to *O. neolycopersici* and was introgressed from a wild relative of tomato, *S. habrochaites* G1.1560 (hereafter refers to as G1.1560) (Lindhout *et al*.,1994). As to the genetics, studies showed that *Ol-1* is a dominant gene (van der Breek, *et al.*, 1994) and the resistance mechanisms involved in *Ol-1* to be mainly hypersensitive response (HR) accompanied by accumulation of H_2O_2 and callose (Li *et al*., 2007). As far as mapping of the gene is concerned, efforts have been made precisely map the position of the gene on the chromosome. First, van der Breek and his co-workers mapped the *Ol-1* gene between RFLP markers GP79 and TG153 of chromosome 6 by testing F_2 population of a cross between a susceptible *S. lycopersicum* cultivar Moneymaker (hereafter refers to as MM) and resistant G1.1560 (hereafter

refer to as G1.1560) (van der Breek *et al*, 1994). Then it was mapped between markers SCAF10 and H9A11 on the same region of chromosome 6 after testing F_3 lines and their progenies derived from the cross of MM and G1.1560 (Huang *et al*., 2000). Moreover, *Ol-1* was located between CAPS markers of P13M49 and H9A11 on the long arm of chromosome 6 after testing informative F_2 of MM and G1.1560 and mapping populations derived from a cross of MM and advanced breeding line (ABL) carrying *Ol-1* (Fig.1) (Bai, 2004, Bai, *et al.*, 2005).

In the *Ol-1* region, there were five bacterial artificial chromosomes (BACs). They were C06SLe0123G17 (hereafter refers to as 123G17), C06HBa0215M16 (hereafter refers to as 215M16) , C06SLm0017O21 (hereafter refers to as 17O21), C06HBa0024F02 (hereafter refers to as 24F02) and C6SLm0047M23 (hereafter refers to as 47M23), with their full or BAC-end sequences available. The first three BACs, 123G17, 215M16, 17O21, are in the same contig and the last two, 24F02 and 47M23, are also overlapping one another assuming contig. There was a gap between the two contigs so that the gap needed to be filled.

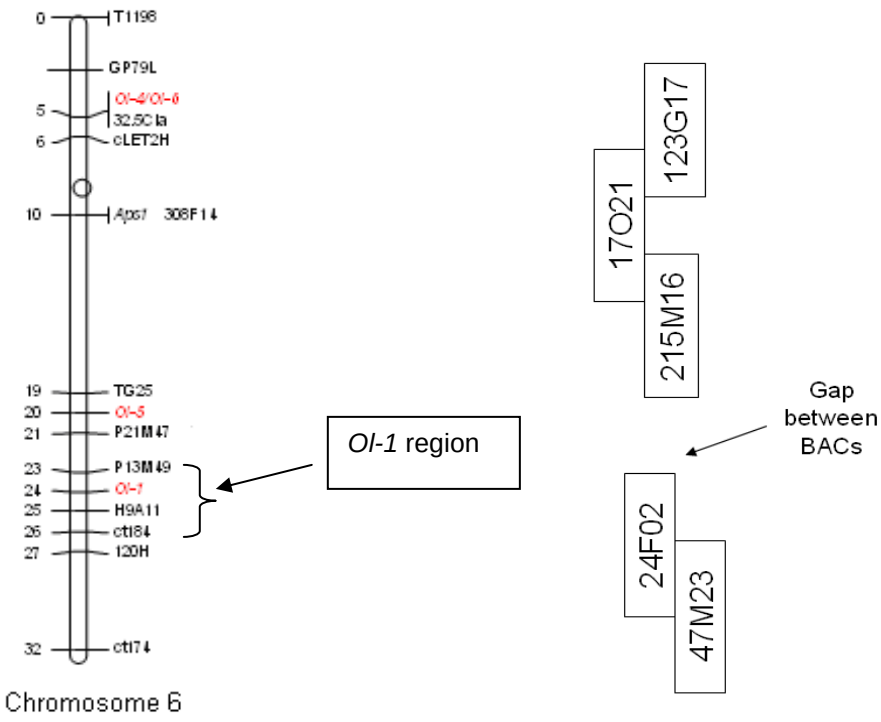

Fig.1. Left: *Ol-1* region flanked by markers P13M49 and H9A11 on chromosome 6 of tomato; right: the five BACs in the region of *Ol-1* with a gap in between.

The aim of this study was, therefore, to fine map *Ol-1* towards map-based cloning of the gene. For this, CAPS markers were generated from the end sequence of the five BACs partially covering the region of *Ol-1*. These BAC-end derived markers were also used to screen the BAC library in order to find positive BACs to fill the gap. Also, a large scale recombinant screening was performed in order to identify recombinants between markers P13M49 and H9A11.

2. Molecular marker development

2.1 Background

Fine mapping involves the identification of markers that are very closely linked to a gene in question. Genetic maps make gene identification, study, isolation and utilization very easy. In plants the utilization of molecular markers in order to make fine mapping is inevitable because other markers such as morphological traits and isozymes are too rare, widely spaced and often difficult to use. In contrary, the advantages of DNA markers include their very large number, a relatively high rate of polymorphism in many populations, usually robust (routine) technologies for scoring, frequent comparability across species, clear dominance or co-dominance in most situations, and no problem of genetic phenomena like penetrance and epistasis (Bennetzen, 2000). Fine mapping has an ultimate goal that is the identification and location of genetic markers that flank the gene under question within one or few centimorgan (cM).

2.2 Material and methods

Primers were designed based on the BAC ends in the region of *Ol-1*. PCR amplification was done using Bioapplied® thermocycler. Amplification conditions were as follows: 35 cycles, each consisting of a 30 second denaturation step at 94°C, a 30 second annealing step at 50 to 60°C depending on the primer type, a 30 to 90 seconds extension step depending on the size of the ampliicon at 72°C. After the 35^{th} cycle, a final extension step was set for 7 minutes at 72 °C.

The amplification products were separated on 1% agarose gel containing ethidium bromide (µl/100 ml gel) added in TBE (Tris-Borate EDTA) buffer, and the DNA bands were observed under UV light and photographed; and reproducible bands were used for further development of CAPS marker. Amplifications which produced dominant markers were not advanced to digestion by restriction enzymes to develop CAPS markers.

After amplification, the amplicons were subject to be cut by different CAPS enzymes in order to find polymorphisms for co-dominant markers. The mixture involved in the digestion was as follows: 5 µl amplicon (DNA), 2 µl buffer and 0.2 µl enzyme. The mixture was incubated overnight in oven at 37^{o}C. All the

enzymes used were active at 37^{o}C. The digested amplicons then were separated on 1.5% agarose gel made from agarose and TBE (Tris-Borate EDTA) containing ethidim bromide (µl/100 ml gel) in order to see if polymorphism exists between the MM and NIL-Ol-1. Bands were observed after photographed by UV transilluminator.

In some cases when restriction digestion did not reveal any polymorphism, the PCR product was gel-extracted (Appendix 1) and sent for sequencing. The amplified DNA was allowed to run on gel of 1% for about 45 minute at 70V. And the DNA fragment was excised from the gel using sharp scalpel carefully not to include the gel as much as possible. The slice of gel was put in PCR tube. Assuming the weight of the gel slice to be 100mg, 300µl QG Buffer was added to the gel. The mixture was then allowed to be incubated at 50^{o}C for about 10 minutes to make the gel melted completely. In two minutes interval during the incubation, the mixture was shaken by hand to mix. 100 µl isopropanol was added and mixed several times by hand. Then the MinElute® column was placed in a 2 ml of collection tube and was centrifuged for one minute at 13000 followed by discarding the flow-through. The MinElute® column was again placed to the collection tube and 500µl of QG buffer was added followed by centrifuging again for one minute at 13000. The flow-through was again discarded and the column was put back in to the same collection tube. In order to wash unwanted debris, the mixture was washed by Buffer PE of 750 µl followed by centrifuging for the third time at 13000 for one minute. The flow-through was again discarded with the collection tube. The remaining residue with the column was subject once more for centrifuging at 13000 for one minute placing in the new clean 1.5 µl microcentrifuge tube so that the DNA would be maintained in the new tube. Finally the DNA was eluted by 10 µl of Buffer EB. The eluted DNA was placed at -20^{o}C. The DNA then was inserted in a pGEM®-T Easy Vector for cloning using T4 DNA ligase and the plasmid was inserted to *E. coli* strain DH5α and grown overnight. Recombinant plasmids were then extracted using the method described by QIAGENE® QIAprep Spin Miniprep Kit (Appendix 2). According to the sequence information and using CAPS designer software new enzymes were tried to find polymorphic pattern (http://www.sgn.cornell.edu/tools/caps_designer/caps_input.pl).

2.3 Result

Twenty one molecular markers were developed (Table1).

Table 1. Primer sequences and PCR conditions of markers developed

No.	Name	Location	Marker type	Primer sequence (5'……>3')	Ann. Temp.	Ext. Time	Restriction Enzyme
1	123G17	*Ol-1*	CAPS	F-TCAAACATTTTTCCCCGTGT R-CACGAGAGTGCCAAAGAGTG	55	1:30	*HypF3I*
2	123G17-1	*Ol-1*	CAPS	F- CAACTGCCTGGCTAAAGCAT R- TTCATATAGCCGGCCTCAAC	55	1:00	*NiaIII*
3	17O21	*Ol-1*	CAPS	F- TCCCCTAACTGGTGCTGAAT R- TGGGTTTCATCACTGGAACA	60	1:00	*Cfr131*
4	at1g 44575	*Ol-1*	CAPS	F- GCCAATGCCAAAGTTGATTT R- CTCATTGATTGGGACACCTG	55	1:30	*RsaI*
5	215M16	*Ol-1*	CAPS	F- GTTTTAGGCCCCTGATCGTT R- GGCGTTAATCTCCGTCTTGA	50	1:00	*DpnI, Hin6I, MspI*
6	PI	*Ol-1*	CAPS	F- AGATTATAAGTTATAACATCC R- ATTTGCAGCCTTCATCTGCT	55	1:00	*HincII*
7	Glu	*Ol-1*	CAPS	F- GCCTGACATGAGGAACGATT R- CGTTTCCTGGTTGATTCCTC	50	1:00	*RsaI*
8	102B23	*Ol-1*	CAPS	F- TAGGACGACATATGTAGAGC R- GTGAAGGACACGTATAATCC	55	0:30	*XbaI*
9	U217233	*Ol-5*	CAPS	F-AGGCATAGCAATTCTATGGATGGG R-TTGGAACGTGCAGCAGATTGTC	55	1:30	*RsaI and HypF3I*
10	57J04	*Ol-5*	CAPS	F- TCCTTGAAATCGCCTAAGGA R- TTGGCTTAAGGGTGTTAGGG	55	1:00	*RsaI and HincII*
11	40F08	*Ol-5*	CAPS	F- TATGCTTGCTTGGACTGTCG R- CTTGATCGGACACAACATGG	55	1:00	*RsaI*
12	116O16	*Ol-5*	CAPS	F- GAAAGTGAGCCATTCCCGTA R- GGCAAGAACAGAAGCAATCA	55	1:00	*HypF3I, Hinc6I*
13	23B17	*Ol-5*	CAPS	F- AAGGTGCATCGAGAATGTCC R- CACACCCACACCATATCCAA	55	1:00	*RsaI*
14	76N05	*Ol-5*	SCAR	F- TCATGGTGCTCCAAGAAACA R- TCACTTTCCATGGGATACCG	55	1:00	Dominant

15	47K24	*Ol-5*	SCAR	F- GATCAATCTCTTGGCCCTCA R- CCCAGCGACGAAGAAAACTA	55	1:00	Dominant
16	123E21	Down stream from *Ol-1*	CAPS	F- ATGCCCTTTTGGTGTTCTTG R- AAGTTTGGCCTTGACACCAG	55	1:30	*RsaI and HypF3I*
17	216P21	Down stream	SCAR	F- AAGTTTGGCCTTGACACCAG R- GTGTCTGGCGGTGGAGTTAT	55	1:00	Dominant
18	177K13	Down stream	SCAR	F-TTGGCACTGGGACATTCATA R-GCGTACACCACATGTTCAGG	55	1:30	Dominant
19	135H21	Down stream	CAPS	F- GAGCGTTGCTGAAAACATCA R- ATGCACGCTGTGTTCAGTTC	55	1:30	*HypF3I*
20	125P18	Down stream	CAPS	F-GAGCGCTCTACCATCTGAGC R-TACGTTTTCTGCTGCCATTG	55	1:30	*RsaI and HypF3I*
21	2C17	Down stream	CAPS	F- ATGCTGCAACTCCACTGATG R- TGACAGAAGCAGCAAAAGGA	55	1:30	*HypF3I*

The markers generated were in three regions in relation to the region of *Ol-1*. Eight were in the region of *Ol-1* gene. Six were developed in the region of *Ol-5* just above *Ol-1* region. The rest six were in the down stream region from *Ol-1*. Not all were CAPS markers as some were dominant markers (Table 1). Co-dominant (CAPS) markers are readily distinguishable markers with difference in band patterns. PCR amplification is done on an already known sequence followed by cutting with special restriction enzymes of this purpose. The digest is then viewed by agarose gel electrophoresis. Dominant markers result when one of the samples (line) is not amplified while the other amplified and characterized by only presence or absence of bands on the gel unlike the co-dominant markers where polymorphism is evidenced by difference in band size.

Markers developed in *Ol-1* region were meant for fine mapping the *Ol-1* gene through recombinant screening and disease test. Markers in the *Ol-5* region were generated to dissect this locus from *Ol-1* locus because there was a suspect from previous study that *Ol-5* might have some influence on the effect of *Ol-1* gene. The downstream markers were generated in order to locate and distinguish the region of introgression of G1.1560 (donor of *Ol-1*) in NIL-Ol-1 with MM as the genetic background.

As far as the introgression region was concerned, after screening MM (susceptible cultivar), NIL-*Ol-1* and G1.1560 (donor of *Ol-1)* using the newly developed markers, the lower (120H21) and upper most (301C21) markers bordering the introgression of G1.1560 in MM were identified in a region of 10.5cM (Fig.2).

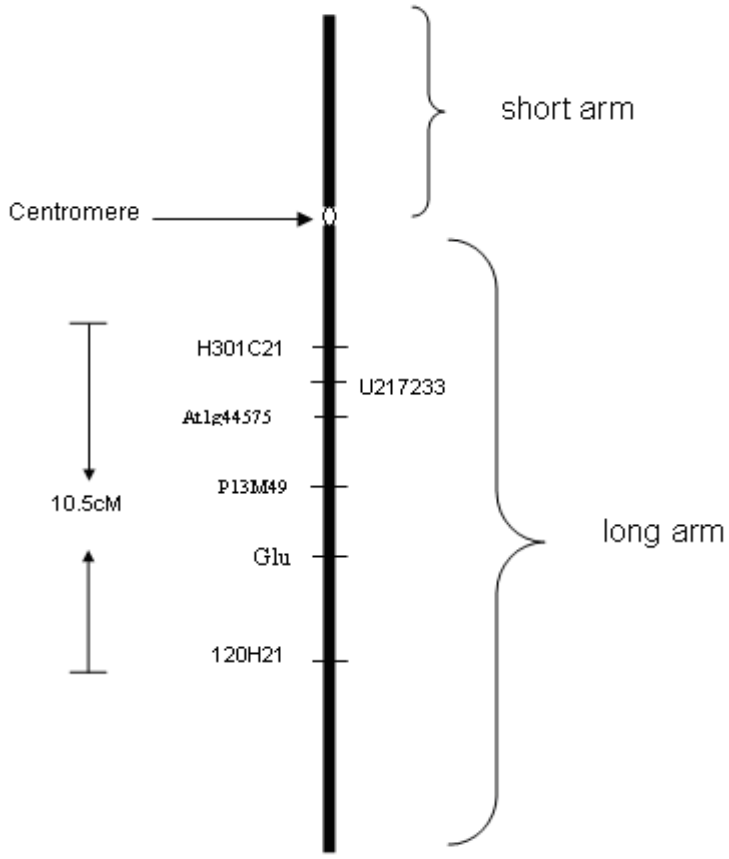

Fig.2. Markers showing the introgression region of G1.1560 in NIL-Ol-1 line on tomato chromosome 6.

3. BAC library screening

3.1 Background

Towards cloning of the *Ol-1* gene by using a map-based cloning approach, markers should be developed sufficiently in the region based on the BAC sequences and should be used for a large-scale recombinant screening. The region where the *Ol-1* gene located was not fully covered by BAC clones, meaning there was a gap in between (Fig.1 and 3).

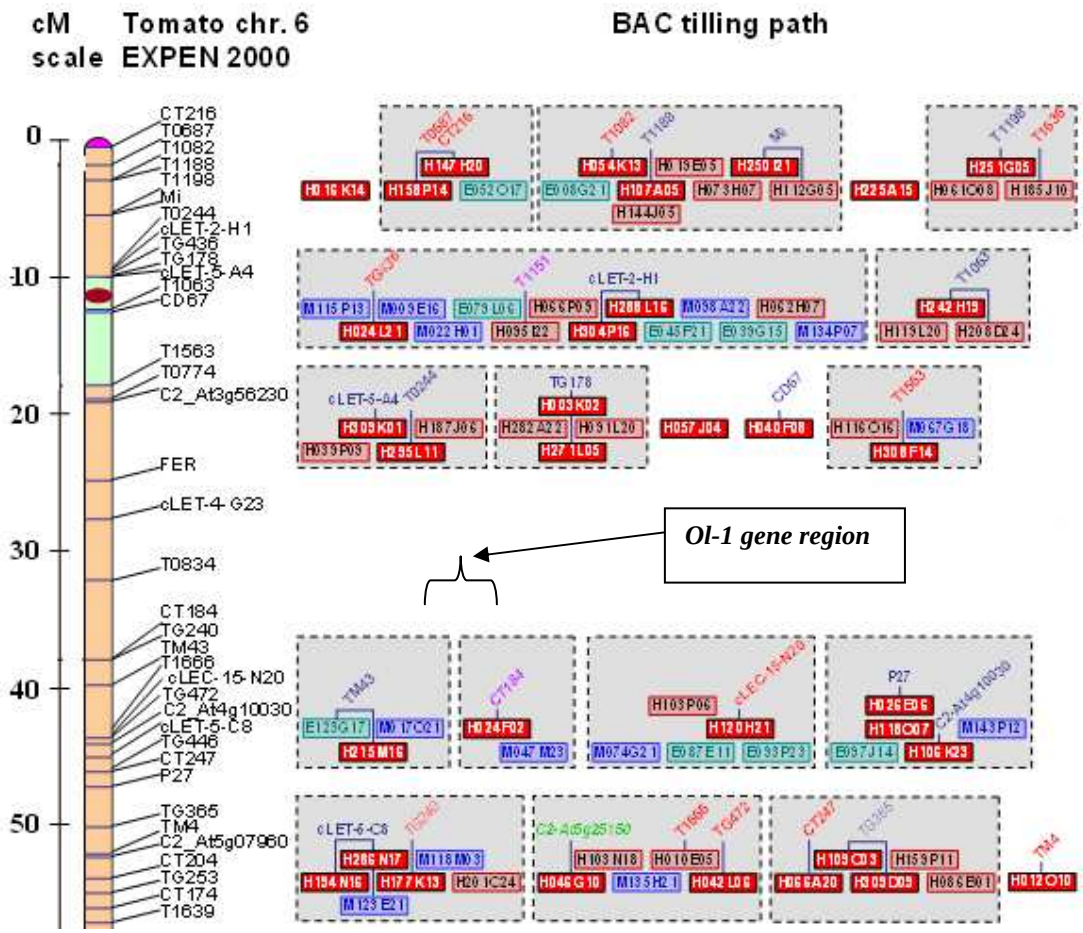

Fig.3. BAC clones on chromosome 6 of tomato showing the gap in *Ol-1* region. *Ol-1* was flanked by 215M16 (picked by marker P13M49) and 24F02 (picked by marker ct-184); see figure 1

From the already available information from SGN (Sol Genomics Network) database BAC clones 47M23 and 24F02 are overlapping. In addition, BACs 215M16, 123G17 and 17O21 are making an overlap too. An investigation made from the online available database information, that 24F02 has nine small contigs

harboring markers *ct184*, *Glu* and *PI*. Contig 1 and 9 were positive for primer of 24F02. 47M23, unlike to the former, was complete contig with marker cleT-2-L2. The two BACs were overlapping in such a way that the *sp6* end of the 24F02 clone is overlapping to 47M23 with 54 kb. Since marker *PI* is already available on the T7 end of BAC 24F02, it was used to screen BAC library. The second contig consisted of BACs 215M16, 123G17 and 17O21. It was found out that the three are overlapping in such a manner that BAC 123G17 and 215M16 were far apart making 17O21 in the middle (Fig. 1).

3.2 Materials and methods

To develop more markers in the region of *Ol-1* and fill the physical map in this region, BAC library screening was done on the available copy of *HindIII* BAC library of *S. lycopersicum* cultivar Heinz 1706 constructed by the lab of Rod Wing (http://sgn.cornell.edu/documents/solanaceae-project/docs/solanaceae-countries.pdf). The vector used to construct the library was pBeloBAC11 and a host bacterium was *E. coli* covering the whole genome of tomato approximately ten times (http://www.genome.arizona.edu/fpc/tomato/). The restriction enzyme for the excision of the genome was *HindIII*.

In our lab, the BAC library had been organized in 336 plates of 384 wells. These plates were pooled to four 96 wells plates (a 384 well plate was merged in one well) in order to make handling and screening easier. Two contigs of BACs were used in order to make screening from two sides. The first contig which was found to the proximal side of centromer consisted three BACs (123G17, 215M16 and 17O21). The second contig positioned relatively to distal from centromer was consisting BACs 24F02 and 47M23.

Primers were designed from the sequence information in Solanaceae Genomics Network (SGN) using the Primer3 program. They were based on the BAC end of 215M16 with forward primer sequence 5'CTTGTGCGGTACGAAAAGGT3' and reverse primer sequence 5'AGGAGTGAAGCCATGTGGAC 3'. For the 123G17, the designed forward and reverse primer sequences were 5'GTTGTGCTTTTAGCGGTGGT3' and 5'CCCTCCTCATATCCCACGTA3',

respectively, in order to pick positive BACs from the BAC pools for extension.

Screening was done based on PCR amplification considering any BAC that could overlap with the candidate BACs would be amplified by the designed primers. The BAC pools were screened by PCR amplification and positive plates were identified. Screening of individual clones was based on colony PCR in such a way that the bacteria colonies harboring the BACs were allowed to grow overnight in a solid medium consisting LB nutrient (20gm/1000ml water) and Agar (12gm/1000ml water). The PCR mixture used for amplification was 20µl: Buffer 2 µl, dNTP and primer 0.5 µl each, super *taq* enzyme 0.2 µl and water 16.8 µl. After adding all the recipes the bacteria was stumped in the PCR mixture with sterile metal stump. PCR amplification was done using a 384 well PTC-200 Peltier Thermal Cycler®. Amplification conditions were as follows: 35 cycles, each consisting of a 30 second denaturation step at 94°C, a 30 second annealing step at 60°C, a 60 seconds extension step at 72°C. After the 35th cycle, a final extension step of 7 minute at 72 °C was performed.

After positive BACs were identified, they were sent for BAC end sequencing. The reaction volume was 20µl consisted of: DETT 4.5µl, dilution buffer (x2.5) 4.5µl, Primer (10µm) 1µl, and BAC DNA 10µl. The sequencing machine was programmed for 75 cycles with 94^{o}C, for 30 seconds, 55 ^{o}C for 10 sec and 60 ^{o}C for 2 min.

3.3 Result

Based on the primers mentioned above, four BAC clones were identified to be positives (overlapping) to already available BACs 215M16 and 123G17. Three of them, namely 2G04, 102B23 and 326B14 were found to be positive to 215M16 and one BAC clone, namely 161G17 was found to be overlapping to 123G17. The sequence of the new BAC clones was obtained (Appendix 3).

The newly proposed putative BACs were sent to Florescence In-situ Hybridization (FISH) to confirm if they are landing on the same location on the chromosome. It was confirmed that the three new BACs (2G04, 102B23 and

326B14) were co-localizing a locus with 215M16 (Appendix 4). BAC 161G17 was not sent to FISH. An attempt to find BACs overlapping to 24F02 was not successful. It was tried with the primer designed based on *PI* marker found on it. No overlapping BAC was found based on this primer at the time.

4. Recombinant line screening

4.1 Material and methods

4.1.1 Plant material

The plant material used in this study was BC_1S_2 population generated from a cross between MM and G1.1560. Two lines (12-7 and 14-4) which were heterozygote for the region of *Ol-1* according to previous study were selected and used for the screening. Total of 1128 seeds (752 from line 12-7 and 376 from line 14-4) were sown and kept in green house with optimum growth condition.

4.1.2 Genotyping test plants

DNA was extracted when seedlings were three weeks old. Harvesting of leaf samples and extraction was done as per described by RETECH protocol 1.4 (Appendix 5). The extracted DNA was amplified by primers P13M49; forward primer 5' TGCTAAGAATCAGAAACCACACCT 3' and reverse primer 5'ACAACAAGCTGATCCACCTAAAGA 3' and ct-184; forward primer 5' TTTCCGTGTATTGCCAACAA 3' and reverse primer 5'ACCAAAGAGTCAATGGATGG 3'. These markers were flanking the gene of interest. Amplification conditions were as follows: 35 cycles, each consisting of a 30 second denaturation step at 94°C, a 30 second annealing step at 56°C. After amplification the product of ct-184 and P13M49 were subject for digestion by the CAPS markers *DdeI* and *XmlI,* respectively overnight at 37°C. The digestion products were separated on 2% agarose gel containing ethidium bromide (µl/100 ml gel) added in TBE (Tris-Borate EDTA) buffer, and the DNA bands were observed under UV light. Genotyping of the plants was done for the two markers (loci) whether they were *hh* (homozygous for allele of resistant line), *eh* (heterozygous) or *ee* (homozygous for allele of susceptible line).

4.1.3 Inoculation

The recombinants were transplanted and inoculated with *O. neolycopersici* conidia when they were one month old. The preparation of the inocula and inoculation procedure was performed as follows: A fully mature spore was harvested along with leaves from susceptible MM tomato plants grown and kept in controlled environment at 20±3°C with 70% humidity. The microspores were

diluted in water and the concentration was calibrated to $2x10^4$ spores per ml of suspension determined by hemocytometer as per described by Bai, 2003. The prepared solution of microspores was sprayed on the recombinant plants by making sure that all the plant parts were sprayed.

4.1.4 Disease scoring

Two weeks after inoculation, disease scoring was done by visual assessment and repeated two times in five days interval. Different severities of the disease were assigned to some numbers (Disease Index) in order to differentiate between the susceptible and resistant ones. The DI was assumed between 0 and 3 where 0= no sporulation, 1= slight sporulation surrounded by necrosis revealing arrest of the spore expansion, 2= moderate sporulation and 3= sporulation was evidenced abundantly (Fig.4). Integers like 0.5, 1.5…were used when necessary.

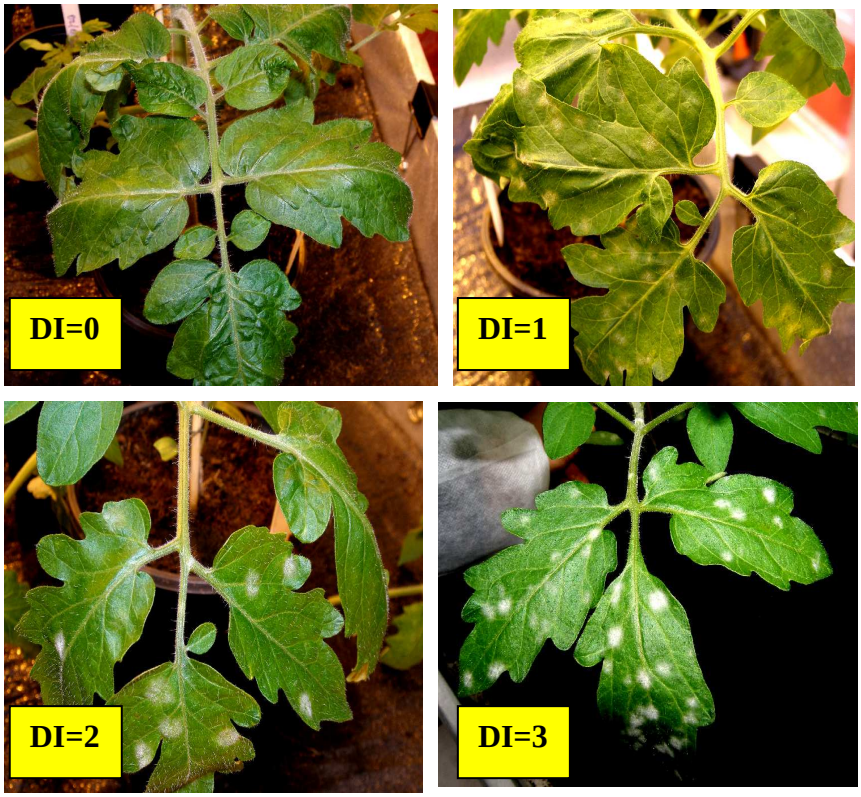

Fig.4 Disease index of *O. neolycopersici* used for disease testing of recombinant tomato plants.

4.2 Result

In total, we screened 1128 BC_2S_1 plants with marker P13M49 and ct-184 (markers flanking *Ol-1*) and obtained 76 recombinants. Then, two newly developed markers within this chromosomal region were applied on these recombinants, which resulted in two valuable recombinants 12-7G-C1 and 12-7H-G1.

12-7G-C1 was informative as it was segregating for P13M49 but homozygous for MM allele at H9A11. This plant was resistant suggesting the *Ol-1* is between P13M49 and H9A11. The other recombinant (12-7H-G1) segregated for ct-184 and *PI* but homozygous for G1.1560 allele at H9A11 and P13M49 substantiating the thought that the gene is above ct-184 (Table 2 and appendix 6).

12-7H-G1 was also informative in order to verify *Ol-5* has no influence on the resistance effect of *Ol-1*. This recombinant was genotyped to be homozygous for MM at the region of *Ol-5* but still resistant (Appendix 6). So, it was an important recombinant line that disproved the suspect that *Ol-5* might have effect on the resistance effect of *Ol-1*(Table 2 and appendix 6).

The rest 74 recombinants between P13M49 and ct-184 were all fixed at two loci, *hh* and *ee*, respectively (Appendix 6). Therefore they were not informative in fine-mapping the *Ol-1* gene. These lines were all *hh* in locus P13M49 and *ee* at H9A11 and *PI*. Except one line all were susceptible after phenotyping suggesting that crossing over occurred between locus P13M49 and H9A11 in such a way that segment harboring the *Ol-1* gene was lost. In contrary the crossover occurred in one recombinant (14-4A-D10) left the *Ol-1* gene in the line as it was evidenced by its resistance phenotype up on disease test suggesting the gene is in between markers P13M49 and H9A11 with genetic distance of 0.26cM.

Table 2. The two informative recombinant lines with respective genotype and disease index

Line	DI*	Markers						
		U217233	**tg-25**	**102B23**	**P13M49**	**H9A11**	**PI**	**ct-184**
12-7G-C1	1	eh	eh	eh	eh	ee	ee	ee
12-7H-G1	0.5		ee	hh	hh	hh	eh	eh

* DI≤1 refers to resistance

Moreover mapping was done based on 190 recombinant plants of BC_1S_2 population generated from a cross between MM and G1.1560 which were heterozygote for the region of *Ol-1*. Thirty plants from one recombinant family (9-9) showed that marker 102B23, which was developed based on BAC end sequence obtained by BAC library screening from Heinz library, to be mapped above marker P13M49 (Appendix 7).

5. General discussion

With a fine map, marker-assisted selection can be very precise. Also, comparisons of map positions can be accurately made with other species to see if they share genes controlling similar traits at particular chromosomal location. In general, fine-mapping a gene is a crucial step towards map-based gene isolation, whereby the researcher can possess the gene for further detail study and subsequent use in crop improvement by gene transformation.

With all the tremendous advantages of fine mapping a gene, it does not mean that one can easily get those markers flanking the target gene. Several problems are associated with the generation of a fine map around any plant gene. First, plants have relatively large genomes. Consequently, most DNA markers are unlinked to any given locus and linked markers may be many cM away from the targeted gene. Second, recombination is relatively rare in plants. In average, a chromosome can undergo one meiotic recombination event per chromosome arm per sexual generation (Benetzen, 2000). Mapping markers to a resolution of one cM requires the investigation of several hundred sexual progenies. Third, marker polymorphism can be low in a mapping population. Whether a particular genetic marker is near the targeted gene can only be determined if there is polymorphism so that populations with low levels of polymorphism will not allow location of most tightly linked markers (Bennetzen, 2000).

As far as *Ol-1* gene is concerned, attempts have been done so far in order to fine map and possibly clone. The results of this experiment are encouraging in such a way that more markers can be generated in between the currently flanking markers that would help find the closest markers flanking the gene.

In this study, even though the main objective of the study was to fine map *Ol-1* gene, we tried to investigate also the probable effect of neighboring *Ol-5* gene on our gene of interest (*Ol-1*). This enabled us to know that *Ol-5* has no effect on the resistance of *Ol-1*. In the mean time the study also determined the introgression region covered by the donor resistant parent (G1.1560) in the background of susceptible commercial cultivar (MM). In our plant population, we determined

the region of introgression by finding bordering markers.

This study also resulted in new positional arrangement of BACs on a contig in in the *Ol-1* region. According to previous knowledge BAC 215M16 believed to be positioned below BAC 123G17 towards *Ol-1* (Fig.1). However, in this study it was found out that the reverse arrangement is logical. This was evidenced when marker H9A11, the lower marker flanking *Ol-1* was located on BAC 161G17 which in turn is positive (overlapping) to BAC 123G17 (Fig. 5).

In previous studies the relative position of markers ct-184 and H9A11 was in doubt, meaning it was not sure whether H9A11 was above or below ct-184. However, after this study it was confirmed that H9A11 to be located above ct-184. This was clearly substantiated by one recombinant plant (12-7H-G1) (Appendix 6).

It is worth mentioning that some recombinants from line 12-7 were found to be non-recombinant up on verification. Twenty four plants out of 400 had been found to be recombinants between P13M49 and ct-184 up on primary screening (data not shown). The recombinant plants were transplanted and allowed to grow for sometime in order to verify after another DNA extraction. In an attempt to compare their genotype after amplification and cutting by same enzyme, they all were found to be non-recombinants. It was thought that some error might have occurred while genotyping or DNA extraction but it was repeated and same result obtained. Moreover, the raw genotype data was checked but no error could be traced. At the moment it is difficult to give precise explanation to tell what happened to the sequences of the plants. However it was noticed that the enzymes cutting the previous sequences could not cut the same to later ones meaning the restriction sites might have been changed. This phenomenon calls for another investigation to find out what is the underlining cause to such events.

6. Conclusion

Ol-1 gene has been mapped between markers P13M49 and H9A11 with genetic distance of 0.26 cM on the long arm of chromosome 6 of tomato. For this and other purposes 21 markers were generated.

Four new BACs were found in the region overlapping to two original BACs. 2G04, 102B23 and 326B14 were overlapping to 215M16. BAC 161G17 was overlapping to 123G17. Interestingly H9A11 was found to be located on this BAC clone (161G17) based on PCR amplification therefore the previous gap between BACs mentioned in figure 1 and 3 is no more relevant as far as fine mapping of *Ol-1* is concerned. Now we have a contig of three BACs with markers flanking the *Ol-1* region (Fig. 5). BAC 215M16 have full sequence available while 123G17 has also been sequenced except a small gap (100bp). BAC 161G17 is also partially sequenced. The exact size of this contig is not known yet, but it should not be more than 150Kb. Luckily, about 70kb of BAC 161G17 and 123G17 are overlapping (Fig. 5) and the sequence of this region is known.

Apart from fine mapping, markers were utilized in order to find border of introgression in our population (NIL-*Ol-1*) between Moneymaker and the donor of *Ol-1* gene (*S. habrochites*). Consequently it was found out that markers 301C21 and 120H21 are bordering the introgression region of 10.5cM on chromosome 6 (Fig. 2).

In addition, it was found out that *Ol-5* has no influence on the resistance effect of *Ol-1*.

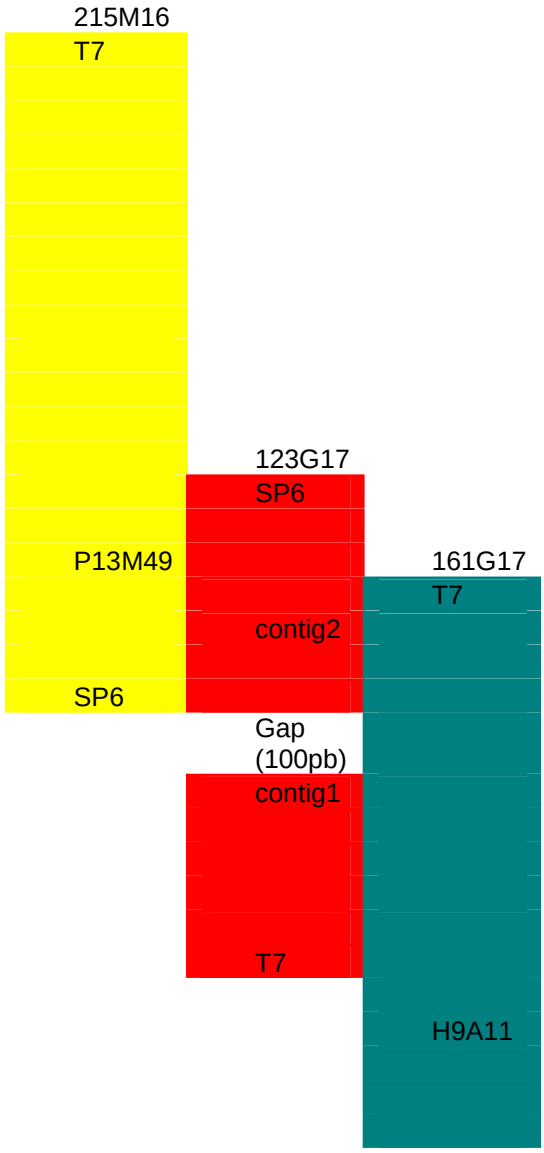

Fig. 5 Contig showing position of marker H9A11 and P13M49 flanking *Ol-1* gene.

7. Future line of work

We will develop more markers based on the sequence information of 161G17-T7, 123G17-T7 and 215M16-Sp6. These markers will be applied to the progeny of the recombinant plant obtained in this study (12-7G-C1) in order to identify recombinant plants between markers P13M49 and H9A11 which would further fine map the region of *Ol-1*. Hopefully we will narrow down the *Ol-1* region to a single gene very soon after the recombinant screening. After narrowing down the region, studies can start to analyze the function of the gene via VIGS or construction of BAC library would be done as a prior step towards cloning.

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Appendices

Appendix 1. MinElute Gel Extraction Kit Protocol

Using a microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 4 kb from standard or low-melt agarose gels in TAE or TBE buffer resulting in high end-concentrations of DNA. Up to 400 mg agarose can be processed per MinElute column.

Important points before starting

■ The yellow color of Buffer QG indicates a pH ≤7.5.

■ Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).

■ All centrifugation steps are carried out at ≥10,000 x *g* in a conventional table-top microcentrifuge at room temperature.

Procedure

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

Minimize the size of the gel slice by removing extra agarose.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg or approximately 100 µl).

For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per spin column is 400 mg; for gel slices >400 mg use more than one MinElute column.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

Note: If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

The adsorption of DNA to the membrane is efficient only at pH ≤7.5. Buffer QG contains a pH indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. Add 1 gel volume of isopropanol to the sample and mix by inverting the tube several times.

For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. Do not centrifuge the sample at this stage.

6. Place a MinElute column in a provided 2 ml collection tube in a suitable rack.

7. To bind DNA, apply the sample to the MinElute column, and centrifuge for 1 min.

For maximum recovery, transfer all traces of sample to the column. The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.

8. Discard the flow-through and place the MinElute column back in the same collection tube.

9. Add 500 µl of Buffer QG to the spin column and centrifuge for 1 min.

10. Discard the flow-through and place the MinElute column back in the same collection tube.

11. To wash, add 750 µl of Buffer PE to the MinElute column and centrifuge for 1 min.

Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.

12. Discard the flow-through and centrifuge the MinElute column for an additional 1 min at ≥10,000 x *g*.

IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

13. Place the MinElute column into a clean 1.5 ml microcentrifuge tube.

14. To elute DNA, add 10 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of the membrane, let the column stand for 1 min, and then centrifuge for 1 min.

IMPORTANT: Ensure that the elution buffer is dispensed directly onto the center of the membrane for complete elution of bound DNA. The average eluate volume is 9 µl from 10 µl elution buffer volume.

Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

15. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 3 (page 15) to identify the dyes according to migration distance and agarose gel percentage and type.

Appendix 2. Recombinant plasmid extraction procedure

- Add 25 ml of bacterial culture in a 50 ml tube
- Centrifuge at 4000 rpm for 10' at 4^{o}C
- Discard the supernatant
- Add 10 ml of P1
- Dissolve the pellet properly
- Add 10 ml P2, turn up and down 6 times and keep for 5 minutes
- Add 14 ml of P3 and turn up and down 6 times and keep for 5 minutes
- Put in an ice for some minutes
- Spin at 4000 rpm and 4^{o}C for 30 minutes
- Discard the supernatant
- Add isopropanol
- Spin at 4000 rpm and 4^{o}C for 30 minutes
- Discard the supernatant
- Add 5ml of ethanol (PE buffer)
- Spin at 4000 rpm and 4^{o}C for 2 minutes
- Discard the supernatant
- Dry the pellet
- Add some water and RNase (200)
- Wait for about 30 minutes
- Determine the conc. of DNA ng/µl
- Dilute the concentration to 100 ng/µl

Appendix 3. End sequences of the newly identified BAC clones

2G04-F trimmed

AAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATG
GTCATAGCTGTGCCTGTGTGAAATNGTTATCCGCTCACAATTCCACAC
AACATACGANCGGGAAGCATANGGTGTAAAGCCTGGGGTGCCTAATG
AGTGANCTAACTCAGATNAATTGCGTNGCGCTCACTGNCCGNTTTCCA
GTCGGGANACCTGTCGTGCCAGNTGNATTAATGAATNGGNCAACGCG
AACCCCTTGTTTTCGNCNGGGACGNCNAGCAATNCTCATGTTNGACAN
NNNANCATCGAATNCCTGCCANTNATCCGNTTATTATCACTTATGNAG
GNGNAGCTACCAGGCGTTTAAGGGCACCAATAACTGCCNTAAAAAAA
TTACGCCCCGCCCTGCCACTCATCGCAGTACTGTTGTAATNCATTAAG
CATTCTGCCGACATGGAAGCCATCACAAACGGNATGATGAACCNGAA
TCGCCAGCGGCATNAGCACCTTGGTCGCCTTGCGTATAATATNTGCCC
ATGGTGAANACGGGGGCGAAGANGNNGACCATNNGGGNCAGGTNTA
AATCANAACNGGNGGAAACNCACCGGGGGNNTGGGCTGAGGACGAA
NAAGNNTGGTCTCNNANNGANCCCNTTTNGGGAAANNANGGNNAGG
NCGNNNNNCCN

2G04-R trimmed

AATCTTGCNTGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAG
CNCGAATTCGCCCNATAGNGAGTCGTATTACAATTCACTGGCCGTCGT
TNTACANNGTCGAGACTGGGAANACCCTGGCGTTACCCAACTTAATCG
CCTNGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGC
CCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCNGAATGGCGAATG
GCGCCTGATGCGGNATAANTCCTGACGCATCTGTGCGGNATCTCACAC
CGCATATGGTGTTTTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAN
GCCAGCCCCGACACCCGCCAACACCCGCTGACGCGAACCCCTTGCGGC
CGCATCGAATATAACTTCGTATAATGTATGCTATACGAAGTNATTAGC
GATGAGGNCGGACGTCCANNGTNCATNCCACGGANNAAAANAAAG

326B14-F trimmed

AATCTTGCNTGCCTGCAGGTCGACTCTAGAGGATCCCCGGGTACCGAG
CNCGAATTCGCCCNATAGNGAGTCGTATTACAATTCACTGGCCGTCGT
TNTACANNGTCGAGACTGGGAANACCCTGGCGTTACCCAACTTAATCG
CCTNGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGC
CCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCNGAATGGCGAATG
GCGCCTGATGCGGNATAANTCCTGACGCATCTGTGCGGNATCTCACAC
CGCATATGGTGTTTTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAN
GCCAGCCCCGACACCCGCCAACACCCGCTGACGCGAACCCCTTGCGGC
CGCATCGAATATAACTTCGTATAATGTATGCTATACGAAGTNATTAGC
GATGAGGNCGGACGTCCANNGTNCATNCCACGGANNAAAANAAAG

326B14-R trimmed

TTATACTTAGGGGGTCATTTGGTAGATGGGATAACGATAATAATCCCG
ATATATCCCATGTGGTTATTTTATCTTGTGATTGGTGATAGGTATTATT
NTGACCCACCACTTTAGCTATAAATGATGGGATATTTNATCCCTTGNT

AGAAGGTAAGACAAAATAATCCAATGGGATATCCTTGTCTATGCCATC
CCATCAANCAAACAGCCCCTAAAGGTATNAAAGGCTACAAGGTTTATT
TTTAGATTGGCGTACTCAAGGAGATTAACTACTTAATTTACTGTTTCTA
ACCTATAGATTNNAATATCTTNTTACGCTTACAATTATGAAATTAAAC
ATATAATATGGGAGGTTCANTTTGAGACTGCCTACTCAAGAGCAGATC
AACTACTTTATTTACTGTNTCTTGCCTTTGGATCCATTATCTACCCTTAT
CATGCCAGTTCCTACTTTAACTATCTGTTGACCTCATCATTCTNNNGCT
ATTNNATAAGGTCAGTTCACCTCGCAANTTCATATCATTATGACCCTC
AGAAAAACAGGCATGCATTACCCNTCACATTTCCNCAAAAAGAAAAA
GAATAGGCTATCAATGGTCAGCTCTCCGCCATTGTCTCTANCCNGGNG
GGGGAAAANGGG

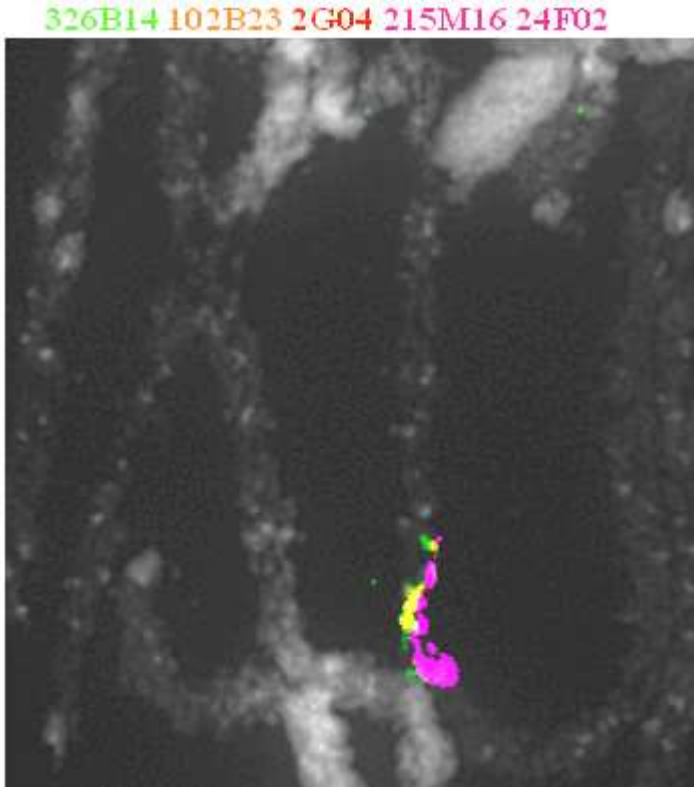
161G17-F (T7)

GCTTGAGCACCATTCAAAACTTGTTGAGCACCCTGTGAATGGTCTGGG
GAGTTTGTGACGGGATTAGGCGGATAAATTGCTTGAAGTCCTGCTTGT
CCACATATAGGGTGGTGACCTTCAACACACTTGAGCGCTGACTTGTGC
TGTTTACCCGAAAGATGTTGTCTCAGAATCTCCCGTGTGTCACATTGCA
CATTGCACAGGTCACACATCAAAGGAATTACAACATTAGAATTNGAA
GGTTCAGTCGCCAGCCTCTTTGCTTTAGAAGCTTTCTTTTTCTTCCTTCC
ACCAACTAATGTCTTACGTTTCACACTGGACCTCTGATTTCCAGGTTGA
TTCATCATGGGCTTATCCGGCGTATCTCGATTTGCATCTTTAGGTGGAT
CAGCTTGTTGTGCATTATTGGTTTTCTGTTGGGTTACTAATTTATTATC
AGAAGATAAAGTATCTAAAGGAATATTTTGTGGTGGATTCTGCTGAGT
CTCTTCTTCCACAAGGAAGTTATCAGGCTTCATGTCTGTTGAAGAAAT
ATTTTGTGGTGGATTCTGCTGAGTCTCTTCTTTCCACAAGCAAGTTATC
AGGCTTCATGTCTGGTTAAAGGAAAATTTTGGTGGTGGATTCTGCTGA
GTCCNCTTCTTCCGNCAAGCAAAGTTATCAGGCTTCATGTCCTGGTTA
AAAGGGAAATATTTTGGNGGGTNGGAATTCCTGGCCTTNGGTTCTNCN
TTCCNTNCCAAACCNANNCCAANNTTCAATNC

161G17 R

AAGCTTTGTTGTTATGTTGTTTGATCTTAAACTTTTAATAAGAGTGTTT
GCATAGATTTTCTTGTATTGTTTGACGAAATATCTAGATAGTATTTTTG
AATTTTAAACCTAATTAATTAAAGACTAACTAAAAAGCAAAGTCCCAA
AGAGACATAGGACCTAGTTTATATTTATAAAATATATTTATATAATTA
ATATATTGTTAATAGAAAATAGAAAATATATTTGTATACATATAAAGT
TATTTAGTTTGATTTTGGTCATTTCAATTATTCGTGACTATTATATNNTT
AAATAAAAATCTACATGTAATTTTAAAAAATAATATAAATAGATTCAG
TATGATTAATTCGTCCGATTTTTCATTTACGTACCAACATTATGGGACA
CCATGTATGCTTCTGCTCTTGTAAATTTCTCATTTATTTCTGAACAATT
ATCATAAAGTAGTTTATTTCTCATTTATTCACCTATATCCACTTAAAAA
AATTTAATTATTCTTCATAGTTATAGTTTGATGATTACAATTAACAACT
ATATATTATATGGAGGAGAGAGGCGAGAGAGACGTAAAAGAGTGGGA
GAAAAGTGAATNGTATATATATATCAGNCAAATAATNGGNATANGCC
ATATATATATATATATGTATGTATGNATNNATATAATCCTGGGGAATTAT
ACNTATACNAANNTGGGGGCCTAAATTTATTACCAAACCCCN

Appendix 4. Newly identified BAC clones as observed by FISH *



* The big purple-pink represents BACs 215M16 and 24F02 to which the newly discovered BACs are overlapping. Light green color represents BAC 326B14, the cinnamon and red colors represent BACs 102B23 and 2G04.

Appendix 5. RETCH protocol 1.4 for DVA extraction (May 2007)

This essentially is a CTAB based method up scaled to multiple 96 racks with tubes.
We make use of titer plate centrifuges and we use multichannels for transferring samples and dispensers for adding liquids to tubes.

Harvesting of leaf material

1Put the 96 micronic tubes in blue holder. Mark position of holder or try to put tubes asymmetrically on the holder so that you will be never puzzled about the original order of the tubes containing samples.

2 Put in every tube of a micronic titre plate two steel balls and put 2 fresh leafdisks (size eppendorf tube lid) in the tubes. While harvesting put your samples on ice.
DO NOT CLOSE THE TUBES UNTIL GRINDING BECAUSE 'CLOSING, OPENING AND RECLOSING' WILL LOOSEN THE CAPS AND CAUSE LEAKAGE DURING MILLING.

(Harvesting and filling the tubes with balls will take you about 45 min per 96 samples.
Using dehydrated material or N_2 frozen (instead of fresh) followed by dry milling will provide excellent material. Material from the –80°C keep in liquid N_2.

DNA extraction

3 Add 2x 250µl CTAB extraction buffer with RNase (per 1 ml CTAB 1 µl RNase (2 mg/ml) using a dispenser and close caps
4 Put the blue holders on the white (bottom) adapter from the RETSCH apparatus put the black adapter on top of your tubes. The protrusion on both the white and black adapter should be in the same orientation. ALWAYS MILL TWO, THUS A BALANCED SET OF PLATES!

5 Mill for 30 secs on 30 r/s speed then change the orientation of the plate and mill another time.

6 Place the tubes and holder in the brown press and tight the nuts (vleugelmoertjes) to prevent popping off the caps during incubation at 65ºC for 60 minutes.

7 Cool tubes on ice (water) to prevent popping off.

8 Work in fumehood. Add 250 µl chloroform isoamyl alcohol (24:1) and mix by inversion for 5 min. (If your samples are still warm the caps will pop off)

9 Separate phases by centrifuging at 3500 RPM for 15 min.

10 Meanwhile take new COSTAR tube holder and fill new tubes with 200µl of Isopropanol.

11 Pipette 200µl water phase to the isopropanol using the multichannel pipet. Cover tubes with 8 strips caps and mix briefly by inversion.

12 Pellet the DNA by centrifuging for 15 min at 3500 RPM

13 Dry pellets for minimally one hour by putting it in the fume hood

14 Dissolve DNA in TE buffer. In most cases the pellet will be dissolved in 100µl.

15 Measure the concentration with the Eppendorf Bio-Photometer or Nanodrop

16 Adjust your DNA at a concentration of 100 ng/µl

17 To check the DNA quality and concentration put 2 µl on agarosegel

If you have -80°C stored material:

Cool down the pins of the Tissue Striker in liquid N_2 and also the tubes. Grind the leaf material and then add 200 µl of CTAB buffer and go on

Composition of the CTAB buffer.

100 ml 1 M TRIS pH 7.5
140 ml 5 M NaCl
20 ml 0.5 M EDTA pH 8.0
740 ml MiliQ H_2O
Add 2% CTAB (dissolve in a 65°C water bath)

Appendix 6. Genotype and disease scoring data of recombinants of BC_1S_2 population

No.	Rec. lines	DI1	DI2	U217233	tg-25	102B23	P13M49	H9A11	PI	ct-184
1	14-4A-A12	2	3	hh	hh	hh	hh	ee	ee	ee
2	14-4A-B8	1.5	3	hh	hh	hh	hh	ee	ee	ee
3	14-4A-B11	1.5	3	hh	hh	hh	hh	ee	ee	ee
4	14-4A-C3	2	3	hh	hh	hh	hh	ee	ee	ee
5	14-4A-C9	1.5	3	hh	hh	hh	hh	ee	ee	ee
6	14-4A-C10	2	3	hh	hh	hh	hh	ee	ee	ee
7	14-4A-D1	1.5	3	hh	hh	hh	hh	ee	ee	ee
8	14-4A-D10	0.5	1	hh	hh	hh	hh	ee	ee	ee
9	14-4A-E1	1.5	3	hh	hh	hh	hh	ee	ee	ee
10	14-4A-E3	1.5	3	hh	hh	hh	hh	ee	ee	ee
11	14-4A-E9	1	3	hh	hh	hh	hh	ee	ee	ee
12	14-4A-E10	1.5	3	hh	hh	hh	hh	ee	ee	ee
13	14-4A-F1	1.5	3	hh	hh	hh	hh	ee	ee	ee
14	14-4A-F7	1.5	3	hh	hh	hh	hh	ee	ee	ee
15	14-4A-G1	1	3	hh	hh	hh	hh	ee	ee	ee
16	14-4A-G5	1	3	hh	hh	hh	hh	ee	ee	ee
17	14-4A-G7	1.5	3	hh	hh	hh	hh	ee	ee	ee
18	14-4A-G8	1	3	hh	hh	hh	hh	ee	ee	ee
19	14-4A-H4	1.5	3	hh	hh	hh	hh	ee	ee	ee
20	14-4A-H6	1	3	hh	hh	hh	hh	ee	ee	ee
21	14-4A-H8	1.5	3	hh	hh	hh	hh	ee	ee	ee
22	14-4B-A2	2	3	hh	hh	hh	hh	ee	ee	ee
23	14-4B-A5	2	3	hh	hh	hh	hh	ee	ee	ee
24	14-4B-B2	2	3	hh	hh	hh	hh	ee	ee	ee
25	14-4B-B6	1.5	3	hh	hh	hh	hh	ee	ee	ee
26	14-4B-B9	2	3	hh	hh	hh	hh	ee	ee	ee
27	14-4B-C2	1.5	3	hh	hh	hh	hh	ee	ee	ee
28	14-4B-C3	2	3	hh	hh	hh	hh	ee	ee	ee
29	14-4B-C4	2	3	hh	hh	hh	hh	ee	ee	ee
30	14-4B-C11	2	3	hh	hh	hh	hh	ee	ee	ee
31	14-4B-D10	1.5	3	hh	hh		hh	ee	ee	ee
32	14-4B-E4	2	3	hh	hh	hh	hh	ee	ee	ee
33	14-4B-F2	1.5	3	hh	hh	hh	hh	ee	ee	ee
34	14-4B-F4	1.5	3	hh	hh	hh	hh	ee	ee	ee
35	14-4B-F5	2	3	hh	hh	hh	hh	ee	ee	ee
36	14-4B-F8	1.5	3	hh	hh	hh	hh	ee	ee	ee
37	14-4B-G1	2	3	hh	hh	hh	hh	ee	ee	ee
38	14-4B-G3	2	3	hh	hh	hh	hh	ee	ee	ee
39	14-4B-G4	1.5	3	hh	hh	hh	hh	ee	ee	ee
40	14-4C-A3	2	3	hh	hh	hh	hh	ee	ee	ee
41	14-4C-A5	1.5	3	hh	hh	hh	hh	ee	ee	ee
42	14-4C-A8	1.5	3	hh	hh	hh	hh	ee	ee	ee

43	14-4C-B8	1.5	3	hh	hh	hh	hh	ee	ee	ee
44	14-4C-B9	1.5	3	hh	hh	hh	hh	ee	ee	ee
45	14-4C-B10	1.5	3	hh	hh	hh	hh	ee	ee	ee
46	14-4C-B11	1.5	3	hh	hh	hh	hh	ee	ee	ee
47	14-4C-C5	1.5	3	hh	hh	hh	hh	ee	ee	ee
48	14-4C-C10	1.5	3	hh	hh	hh	hh	ee	ee	ee
49	14-4C-C11	1.5	3	hh	hh	hh	hh	ee	ee	ee
50	14-4C-D2	1.5	3	hh	hh	hh	hh	ee	ee	ee
51	14-4C-E1	1.5	3	hh	hh	hh	hh	ee	ee	ee
52	14-4C-E11	1.5	3	hh	hh	hh	hh	ee	ee	ee
53	14-4C-E12	1.5	3	hh	hh	hh	hh	ee	ee	ee
54	14-4C-F3	1.5	3	hh	hh	hh	hh	ee	ee	ee
55	14-4C-G3	2	3	hh	hh	hh	hh	ee	ee	ee
56	14-4C-G8	1.5	3	hh	hh	hh	hh	ee	ee	ee
57	14-4D-A3	1.5	3	hh	hh	hh	hh	ee	ee	ee
58	14-4D-A11	2	3	hh	hh	hh	hh	ee	ee	ee
59	14-4D-A12	2	3	hh	hh	hh	hh	ee	ee	ee
60	14-4D-B2	2	3	hh	hh	hh	hh	ee	ee	ee
61	14-4D-C2	2	3	hh	hh	hh	hh	ee	ee	ee
62	14-4D-C10	2	3	hh	hh	hh	hh	ee	ee	ee
63	14-4D-D5	2	3	hh	hh	hh	hh	ee	ee	ee
64	14-4D-D7	2	3	hh	hh		hh	ee	ee	ee
65	14-4D-E2	1.5	3	hh	hh	hh	hh	ee	ee	ee
66	14-4D-E5	1.5	3	hh	hh	hh	hh	ee	ee	ee
67	14-4D-E7	1.5	3	hh	hh	hh	hh	ee	ee	ee
68	14-4D-F1	2	3	hh	hh		hh	ee	ee	ee
69	14-4D-F8	1.5	3	hh	hh	hh	hh	ee	ee	ee
70	14-4D-F9	1.5	3	hh	hh		hh	ee	ee	ee
71	14-4D-G3	2	3	hh	hh		hh	ee	ee	ee
72	14-4D-G7	2	3	ee	hh	hh	hh	ee	ee	ee
73	14-4D-H8	2	3		hh	hh	hh	ee	ee	ee
74	14-4D-H9	2	3		hh	hh	hh	ee	ee	ee
75	12-7G-C1	0.5	1	eh	eh	eh	eh	ee	ee	ee
76	12-7H-G1	0.5	0.5		ee	hh	hh	hh	eh	eh
77	***Ol-1***		1							
78	***MM***		3							

Appendix 7. Genotype and disease scoring data of progenies tested to map marker 102B23

No.	Rec. lines	102B23	P13M49	H9A11	ct184
1	10-1-2001	ee	ee	ee	ee
2	10-1-2010	ee	ee	ee	ee
3	10-1-2011	ee	ee	ee	ee
4	10-1-2012	ee	ee	ee	ee
5	10-1-2013	ee	ee	ee	ee
6	10-1-2014	ee	ee	ee	ee
7	10-1-2015	ee	ee	ee	ee
8	10-1-2016	ee	ee	ee	ee
9	10-1-2017	ee	ee	ee	ee
10	10-1-2018	ee	ee	ee	ee
11	10-1-2019	ee	ee	ee	ee
12	10-1-2002	ee	ee	ee	ee
13	10-1-2020	ee	ee	ee	ee
14	10-1-2021	ee	ee	ee	ee
15	10-1-2022	ee	ee	ee	ee
16	10-1-2023	ee	ee	ee	ee
17	10-1-2024	ee	ee	ee	ee
18	10-1-2025	ee	ee	ee	ee
19	10-1-2026	ee	ee	ee	ee
20	10-1-2027	ee	ee	ee	ee
21	10-1-2028	ee	ee	ee	ee
22	10-1-2029	ee	ee	ee	ee
23	10-1-2003	ee	ee	ee	ee
24	10-1-1930	ee	ee	ee	ee
25	10-1-2004	ee	ee	ee	ee
26	10-1-2005	ee	ee	ee	ee
27	10-1-2006	ee	ee	ee	ee
28	10-1-2007	ee	ee	ee	ee
29	10-1-2008	ee	ee	ee	ee
30	10-1-2009	ee	ee	?	ee
31	14-4-2001	hh	hh	ee	ee
32	14-4-2010	hh	hh	hh	hh
33	14-4-2011	hh	hh	eh	eh
34	14-4-2012	hh	hh	ee	ee
35	14-4-2013	hh	hh	eh	eh
36	14-4-2014	hh	hh	?	ee
37	14-4-2015	hh	hh	hh	hh
38	14-4-2016	hh	hh	eh	eh
39	14-4-2017	hh	hh	eh	eh

40	14-4-2018	hh	hh	eh	eh
41	14-4-2019	hh	hh	hh	hh
42	14-4-2002	hh	hh	eh	hh
43	14-4-2020	hh	hh	hh	hh
44	14-4-2021	hh	hh	eh	eh
45	14-4-2022	hh	hh	eh	eh
46	14-4-2023	hh	hh	ee	ee
47	14-4-2024	hh	hh	eh	eh
48	14-4-2025	hh	hh	eh	eh
49	14-4-2026	hh	hh	hh	hh
50	14-4-2027	hh	hh	eh	eh
51	14-4-2028	hh	hh	eh	eh
52	14-4-2029	hh	hh	eh	eh
53	14-4-2003	hh	hh	ee	ee
54	14-4-1930	hh	hh	eh	eh
55	14-4-2004	hh	hh	eh	eh
56	14-4-2005	hh	hh	eh	eh
57	14-4-2006	hh	hh	hh	hh
58	14-4-2007	hh	hh	eh	eh
59	14-4-2008	hh	hh	ee	ee
60	14-4-2009	hh	hh	eh	eh
61	14-5-2001	hh	hh	ee	ee
62	14-5-2010	hh	hh	ee	ee
63	14-5-2011	hh	hh	ee	ee
64	14-5-2012	hh	hh	eh	eh
65	14-5-2013	hh	hh	ee	ee
66	14-5-2014	hh	hh	hh	hh
67	14-5-2015	?	hh	?	ee
68	14-5-2016	hh	hh	hh	hh
69	14-5-2017	hh	hh	eh	eh
70	14-5-2018	hh	hh	ee	ee
71	14-5-2019	?	hh	eh	eh
72	14-5-2002	hh	hh	eh	eh
73	14-5-2020	hh	hh	eh	eh
74	14-5-2021	hh	hh	ee	ee
75	14-5-2022	hh	hh	ee	ee
76	14-5-2023	hh	hh	ee	ee
77	14-5-2024	hh	hh	hh	hh
78	14-5-2025	hh	hh	eh	eh
79	14-5-2026	hh	hh	ee	ee
80	14-5-2027	hh	hh	eh	eh
81	14-5-2028	hh	hh	eh	eh
82	14-5-2029	hh	hh	eh	eh

83	14-5-2003	hh	hh	eh	eh
84	14-5-1930	hh	hh	ee	ee
85	14-5-2004	hh	hh	ee	ee
86	14-5-2005	hh	hh	hh	hh
87	14-5-2006	hh	hh	eh	eh
88	14-5-2007	hh	hh	eh	eh
89	14-5-2008	hh	hh	ee	ee
90	14-5-2009	hh	hh	eh	eh
91	1-6-2001	hh	hh	hh	hh
92	1-6-2010	hh	hh	hh	hh
93	1-6-2002	hh	hh	hh	hh
94	1-6-2003	hh	hh	hh	hh
95	1-6-2004	hh	hh	hh	hh
96	1-6-2005	hh	hh	hh	hh
97	1-6-2006	hh	hh	hh	hh
98	1-6-2007	hh	hh	hh	hh
99	1-6-2008	hh	hh	hh	hh
100	1-6-2009	hh	hh	hh	hh
101	3-1-2001	hh	hh	hh	hh
102	3-1-2010	hh	hh	hh	hh
103	3-1-2011	hh	hh	hh	hh
104	3-1-2012	hh	hh	hh	hh
105	3-1-2013	hh	hh	hh	hh
106	3-1-2014	hh	hh	hh	hh
107	3-1-2015	hh	hh	hh	hh
108	3-1-2002	hh	hh	hh	hh
109	3-1-2003	hh	hh	hh	hh
110	3-1-2004	hh	hh	hh	hh
111	3-1-2005	hh	hh	hh	hh
112	3-1-2006	hh	hh	hh	hh
113	3-1-2007	hh	hh	hh	hh
114	3-1-2008	hh	hh	hh	hh
115	3-1-2009	hh	hh	hh	hh
116	3-9-2001	hh	hh	hh	hh
117	3-9-2010	hh	hh	hh	hh
118	3-9-2011	hh	hh	hh	hh
119	3-9-2012	hh	hh	hh	hh
120	3-9-2013	hh	hh	hh	hh
121	3-9-2014	hh	hh	hh	hh
122	3-9-2015	hh	hh	hh	hh
123	3-9-2002	hh	hh	hh	hh
124	3-9-2003	hh	hh	hh	hh
125	3-9-2004	hh	hh	hh	hh

126	3-9-2005	hh	hh	hh	hh
127	3-9-2006	hh	hh	hh	hh
128	3-9-2007	hh	hh	hh	hh
129	3-9-2008	hh	hh	hh	hh
130	3-9-2009	hh	hh	hh	hh
131	7-9-2001	eh	eh	eh	eh
132	7-9-2010	eh	eh	eh	eh
133	7-9-2011	eh	eh	eh	eh
134	7-9-2012	eh	eh	?	eh
135	7-9-2013	hh	hh	hh	hh
136	7-9-2014	hh	hh	hh	hh
137	7-9-2015	ee	ee	ee	ee
138	7-9-2016	eh	eh	eh	eh
139	7-9-2017	ee	ee	ee	ee
140	7-9-2018	eh	eh	eh	eh
141	7-9-2019	eh	eh	eh	eh
142	7-9-2002	ee	ee	ee	ee
143	7-9-2020	ee	ee	ee	ee
144	7-9-2021	eh	eh	eh	eh
145	7-9-2022	eh	eh	eh	eh
146	7-9-2023	hh	hh	hh	hh
147	7-9-2024	eh	eh	eh	eh
148	7-9-2025	ee	ee	ee	ee
149	7-9-2026	hh	hh	hh	hh
150	7-9-2027	eh	eh	eh	eh
151	7-9-2028	eh	eh	eh	eh
152	7-9-2029	ee	ee	ee	ee
153	7-9-2003	hh	hh	hh	hh
154	7-9-1930	hh	hh	hh	hh
155	7-9-2004	?	?	?	?
156	7-9-2005	hh	hh	hh	hh
157	7-9-2006	eh	eh	eh	eh
158	7-9-2007	ee	ee	ee	ee
159	7-9-2008	eh	eh	?	eh
160	7-9-2009	eh	eh	eh	eh
161	9-9-2001	ee	eh	eh	eh
162	9-9-2010	ee	eh	eh	eh
163	9-9-2011	ee	eh	eh	eh
164	9-9-2012	ee	hh	hh	hh
165	9-9-2013	?	?	?	?
166	9-9-2014	ee	ee	ee	ee
167	9-9-2015	ee	hh	hh	hh
168	9-9-2016	ee	eh	eh	eh

169	9-9-2017	ee	eh	eh	eh
170	9-9-2018	ee	eh	eh	eh
171	9-9-2019	ee	eh	eh	eh
172	9-9-2002	ee	hh	?	hh
173	9-9-2020	ee	eh	?	ee
174	9-9-2021	ee	ee	ee	ee
175	9-9-2022	ee	hh	hh	hh
176	9-9-2023	ee	hh	hh	hh
177	9-9-2024	ee	eh	eh	eh
178	9-9-2025	ee	ee	ee	ee
179	9-9-2026	ee	eh	eh	eh
180	9-9-2027	ee	hh	hh	hh
181	9-9-2028	ee	ee	ee	ee
182	9-9-2029	ee	eh	eh	eh
183	9-9-2003	ee	hh	hh	hh
184	9-9-1930	ee	eh	eh	eh
185	9-9-2004	ee	hh	hh	hh
186	9-9-2005	ee	?	?	ee
187	9-9-2006	ee	eh	eh	eh
188	9-9-2007	ee	eh	eh	eh
189	9-9-2008	ee	eh	?	eh
190	9-9-2009	ee	ee	ee	ee