

Looking for the Invisible:

Fine mapping and Characterization
of a
Hybrid Incompatibility
associated with the
L. saligna x *L. sativa* Backcross
Inbred Line[4.1+6.3]

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SUMMARY

Hybrid incompatibilities: a start for evolutionary biologists and a burden to breeders. This study elaborates on a hybrid incompatibility (HI) indicated in a set of Backcross Inbred Lines (BILs) derived from cultivated lettuce *L. sativa* and wild lettuce *L. saligna*, concerning BIL[4.1+6.3]. In BIL[4.1+6.3] a *L. saligna* introgression segment on the bottom of chromosome 6 was always accompanied by a *L. saligna* introgression segment on the top of chromosome 4. BIL[4.1+6.3] resulting from a Transmission Ratio Distortion (TRD) pointed to the presence of a hybrid incompatibility, indicated as HI[4.1+6.3]. PhD-student Erik den Boer identified, fine mapped and characterized HI[4.1+6.3]. HI[4.1+6.3] was mapped at 14.1 cM (14.1-28.2 cM) and 7.9 cM (0-7.9 cM) intervals at chromosome 4.1 (C4.1) and chromosome 6.3 (C6.1), respectively. HI[4.1+6.3] was characterized as a pre-zygotic hybrid incompatibility caused by non-transmission of 4a6b gametophytes. This MSc-study elaborates on fine mapping & characterization of HI[4.1+6.3]. In fine mapping, regions were narrowed down to 5.1 cM (16.1-21.2 cM) and 3.7 cM (0.9-4.6 cM) intervals. In exact terms according to physical positions of the markers, regions are mapped at 7.9-37.9 Mbp and 0-7.8 Mbp for C4.1 and C6.3, respectively. In characterization HI[4.1+6.3] two functionality tests were performed: seed set analysis and vitality testing of the male gametophyte. The functionality tests were designed to supply direct evidence for the non-transmission hypothesis of 4a6b gametophytes. This goal was not met. A new hypothesis was raised describing the presumed character of HI[4.1+6.3]. Taken results of functionality testing together, non-formation of 4a6b gametophytes in meiosis is considered to be the most likely cause for non-transmission of 4a6b gametophytes. Further speculations on HI[4.1+6.3] concern classification of HI[4.1+6.3] as a di-genic sex-independent transmission ratio distortion (si-TRD) caused by an interaction of Joint Fate driven by 4a6b gametophytes. These speculations need to be tested in further research.

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1. INTRODUCTION

Genetic variation is a subject of interest of several scientific disciplines with its own specific objectives. Breeders, for instance, aim to apply genetic variation for commercial purposes. Evolutionary biologists use information about genetic variation in their aim to unravel fundamental evolutionary principles. This research meets both interests by fine mapping and characterizing the hybrid incompatibility in the wild lettuce *Lactuca saligna* x cultivated lettuce *Lactuca sativa* backcross inbred line[4.1+6.3].

HYBRID INCOMPATIBILITY. A clear example of the effect of genetic variation is reflected by offspring of crossing parents. In particular if crossing occurs between two species. Often, hybrids between two species are not viable, sterile or show other deleterious characters. The collective noun for this phenomenon is the 'hybrid incompatibility' (HI) and can often be explained by the Bateson-Dobzhansky-Muller interaction (BDMI) model (Orr, 1996). This model considers the hybrid incompatibility as a side product of speciation. Within species, groups of individuals genetically change from one another due to natural selection. This deviation can cause speciation. If species, arisen from a common ancestor, cross again, the offspring may be confronted with complications, even in later generations. The complications are due to two or more genetically deviated genes interacting improperly causing the hybrid incompatibility.

The deleterious nature of an interspecific hybrid can be recognized by phenotypic symptoms like hybrid necrosis, sterility and low vitality and can cause unusual segregation, also referred as transmission ratio distortions (TRDs), in segregating offspring (F2, RIL, backcross populations). The transmission ratio-distorted loci (TRDL) refer to the loci causing the hybrid incompatibility between a certain combinations of species. Loci underlying these hybrid incompatibilities are identified in several species and turn out to be involved in an array of biological processes (Johnson, 2010).

L. SATIVA X L. SALIGNA BACKCROSS INBRED LINE [4.1+6.3]. The relationship between hybrid incompatibilities and this study is based on crosses between cultivated lettuce *L. sativa* and wild lettuce *L. saligna*. *L. saligna* is a remarkable wild relative of *L. sativa* due to its non-host character to the obligate biotroph *Bremia lactucae Regel* causing downy mildew, which is considered to be the most devastating disease in lettuce

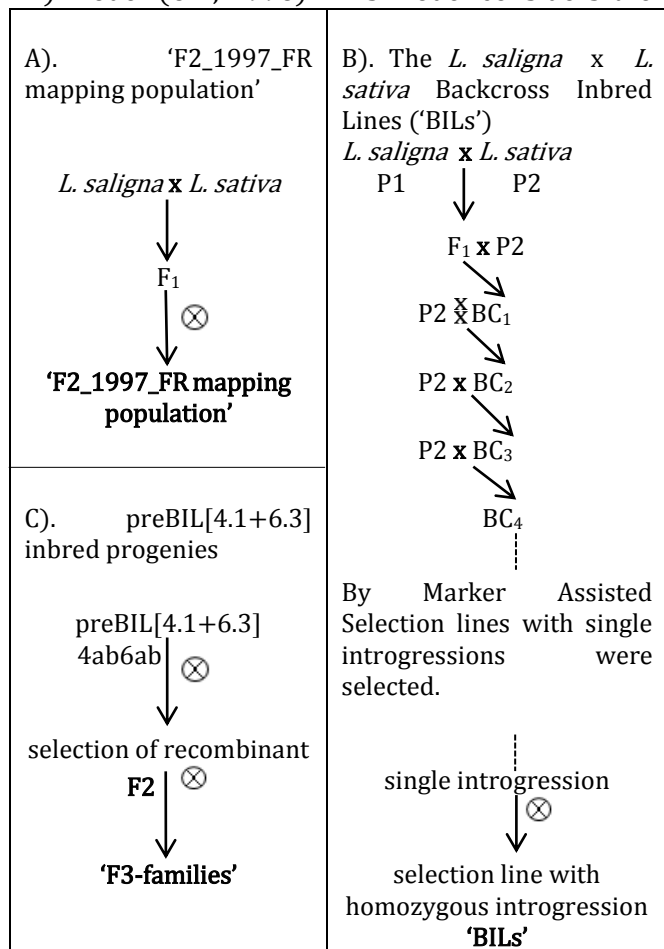


Figure 1. Overview of populations used and/or often referred to in this study. All progenies and plants are derived of the cross *L. saligna* CGN05271 x *L. sativa* cv. Olof A) The F2_1997_FR mapping population consists of 126 plants (Jeuken *et al.*, 2001). B) Backcross Inbred Lines (BILs). This set of lines consists of 28 lines which covers 96% of the *L. saligna* genome. Markers Assisted Selection was used in BC4-5S1-2 (Jeuken *et al.*, 2004). C) The F2 population and F3-families are derived from selfings of the preBIL[4.1+6.3] of the recurrent backcross program of Jeuken *et al.* (2004). 'pre' Refers to the heterozygous introgression segments on chromosome 4.1 and 6.3.

Table 1. Genetic Terminology

Alleles	a = <i>L. sativa</i> b = <i>L. saligna</i>
Genotypes	A = aa homozygous <i>L. sativa</i> B = bb homozygous <i>L. saligna</i> H = ab heterozygous <i>L. sativa L. saligna</i> C = bb <u>or</u> ab homozygous <i>L. saligna or</i> heterozygous <i>L. sativa L. saligna</i> D = aa <u>or</u> ab homozygous <i>L. sativa or</i> heterozygous <i>L. sativa L. saligna</i> - = unknown

The genetic terminology used in this study differentiates alleles (lowercase) and genotypes (uppercase).

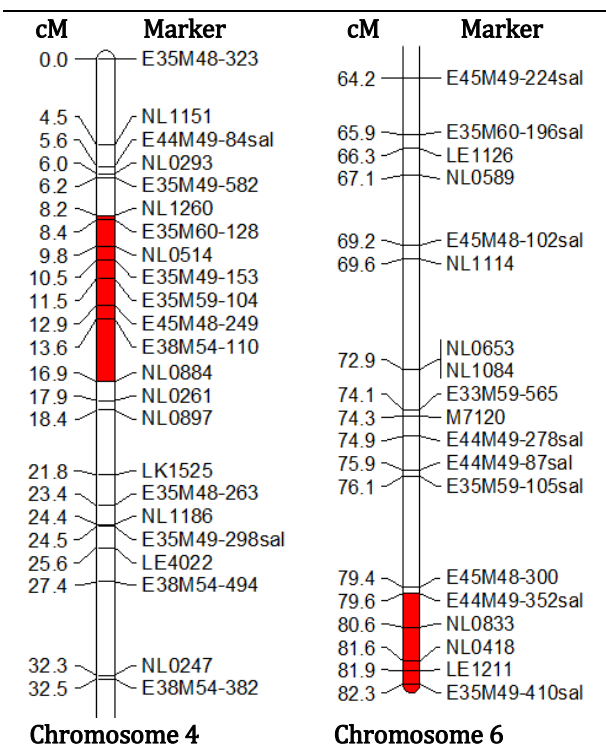


Figure 2. Genetic map position of HI[4.1+6.3] by PhD-student Erik den Boer (PhD thesis concept 2014, chapter 4). The HI regions are indicated in red. Genetic distances are based on mapping population F2_1997_FR.

a segregation analysis of 635 progeny of inbred preBIL[4.1+6.3] plants (Table 2). The progenies were genotyped for chromosome 4.1 and 6.3 with 'aa' indicating a homozygous *L. sativa* allele and 'bb' indicating a homozygous *L. saligna* allele and 'ab' indicating a heterozygous *L. sativa L. saligna* allele (Table 1). General note: lettuce is an autogamous diploid ($2n=18$) plant. The seed germination rate was normal (>95%) and no seedling lethality was observed. In hypothesis testing it was concluded that the loci 4.1 and 6.3 do not segregate independently (Table 2). Moreover, the segregation ratios could not be explained by a post-zygotic incompatibility by the lethality of genotypes 4aa6bb, 4ab6bb and 4aa6ab, not by a pre-zygotic incompatibility by non-transmission of male or female gametophytes with a 4a6b gametophyte genotype and could also not be explained by non-transmission of male or female gametophytes with a 4a6b gametophyte genotype in combination with lethality of genotypes 4aa6bb, 4ab6bb, 4aa6ab. Non-transmission of the 4a6b male and female gametophytes is the only non-rejected hypothesis. Progeny analysis of reciprocal crosses 4ab6ab x 4bb6aa validated the non-transmission hypothesis. However, direct evidence confirming this hypothesis lacks.

production (Bonnier *et al.*, 1991). *L. saligna* has the potential to function as the genetic resource in breeding for downy mildew resistant lettuce. Unfortunately, *L. saligna* is only partly cross-compatible with *L. sativa* due to the genetic distance between these species and belongs, by definition of Harlan and de Wet (Harlan & de Wet, 1971), to the secondary gene pool of *L. sativa* (Zohary, 1991).

A study that indeed indicates the potential of *L. saligna* as a genetic resource for resistance one hand and crossing barriers on the other is done by Jeuken and Lindhout (Jeuken & Lindhout, 2004). Jeuken and Lindhout constructed backcross inbred lines (BILs) of *L. sativa* with introgression segments of *L. saligna* (Figure 1). The set of 28 lines covers 96% the *L. saligna* genome (Appendix 1). The BILs enabled identification of regions associated with the non-host character of *L. saligna*. However, BILs construction encountered identification of TRDLs as well. One of the TRDL observations deals with the bottom of chromosome 6 (hereinafter referred to as C6.3) and the top of chromosome 4 (hereinafter referred to as C4.1). The homozygous *L. saligna* C6.3 introgression did only occur in the presence of a *L. saligna* gene on 4.1 caused by a TRD, pointing to a di-genic hybrid incompatibility. In unpublished work of PhD-student Erik den Boer the di-genic hybrid incompatibility is further defined (PhD thesis concept 2014, chapter 4). The regions were fine mapped to 8 (at 4.1) and 3 (at 6.3) cM intervals by means of 20 markers, according to the genetic maps used by E. den Boer (Figure 2). The hybrid incompatibility was characterized by means of

Table 2. Observed and expected segregation ratios of inbred progeny of preBIL4.1+6.3 (genotype 4ab6ab)

A) Observed Segregation ratios					B) Expected Segregation ratios under independent 1:2:1 Mendelian segregation					C) Expected Segregation ratios under Observed allele frequencies							
C6.3					C6.3					C6.3							
	aa	ab	bb	Tot:		aa	ab	bb	Tot:		aa	ab	bb	Tot:			
C4.1	aa	75	0	0	75	aa	40	79	40	159	aa	33	33	9	75		
	ab	126	130	0	256	C4.1 ab	79	159	79	317	C4.1 ab	113	113	29	255		
	bb	80	151	73	304	bb	40	79	40	159	bb	134	135	35	304		
Tot:					635	Tot:					635	Tot:					634

Table 2 presents the observed and expected segregation ratios of inbred progeny of preBIL[4.1+6.3] (4ab6ab), in which plants are genotyped for the top op chromosome 4 (C4.1) and the bottom of chromosome 6 (C6.3), according to the genetic nomenclature introduced in table 1 (E. den Boer, PhD thesis concept 2014, chapter 4). A). Of the segregating genotypes, three genotypes were absent (4aa6ab, 4aa6bb, 4ab6bb, hereafter indicated as HI-genotypes) and genotype 4ab6ab showed only half of the expected plant numbers (based on independent Mendelian 1:2:1 segregation). B). As a reference, the expected numbers of segregating genotypes under the assumption of independent segregation of two loci with a Mendelian 1:2:1 segregation per locus are shown here. C. As a reference, the expected numbers of segregating genotypes calculated with observed allele frequencies: C4, *L. sativa* allele 'a' = 0.32 and C6, *L. sativa* allele 'a' = 0.66 are shown here.

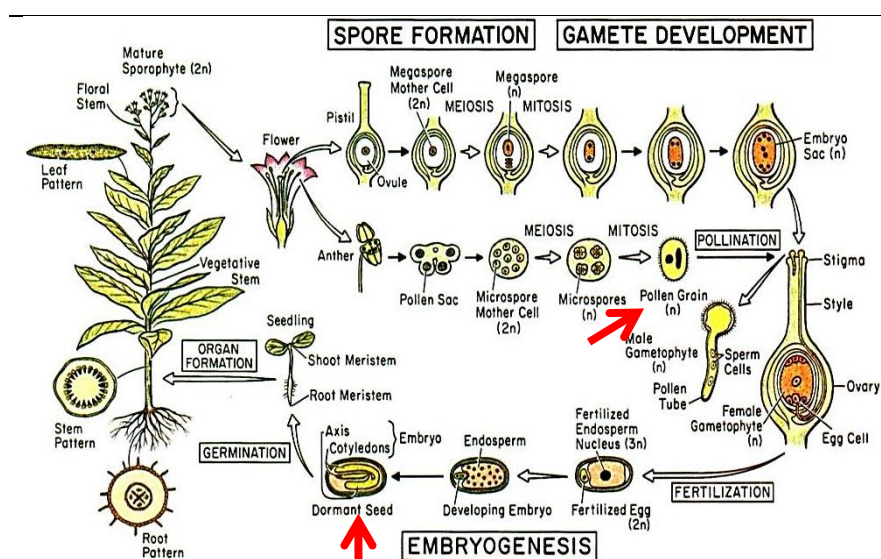


Figure 3. Life Cycle of Angiosperms (Chasan and Walbot, 1993). This model approaches the life cycle of lettuce. Red arrows indicate stages assessed in functionality testing of 4a6b gametophytes concerning the seed set analysis and vitality testing of pollen grains.

Non-transmission of the (male and female) 4a6b gametophyte from one generation to the next indicates the pre-zygotic character of the hybrid incompatibility and points to a complication in the process from meiosis to seed formation (Figure 3). At the moment, it is unclear which developmental process proceeds improperly. In addition, the complexity of the process from meiosis to seed formation is not of ease in revealing the biological reason for non-transmission of 4a6b gametophytes. For instance, formation of 4a6b gametophytes hampers, 4a6b gametophytes are non-vital or non-functional in fertilization or possibly endosperm-formation is obstructed. Non-transmission of 4a6b male and female gametophytes may also be referred to as a sex-independent TRD (*si*-TRD). Examples of *si*-TRDs are available in literature for tomato, rice (Koide *et al.*, 2008; Rick, 1966).

Scope of M.Sc. thesis: Fine mapping and Characterization of *L. sativa* x *L. saligna* BIL[4.1+6.3].

Justification: As mentioned, *L. saligna* is a non-host of downy mildew. Integrating the non-host resistance in cultivated lettuce would offer a solution to fungicide spraying, necessary to ensure lettuce harvest. The International Bremia Evaluation Board (IBEB) yearly mentions the importance of fungicide application at the young plant stage which prevents development of new Bremia races (IBEB, 2011). The chromosomal region 6.3 of *L. saligna* is associated with a strong resistance against downy mildew at the young plant stage, with a relative infection severity of only 10% (Zhang, *et al.*, 2009). Uncoupling the resistance gene(s) and identification of the genes causing the HI would provide a promising perspective.

Current literature only reports on male or female related hybrid incompatibilities or si-TRDs concerning one gene. Elucidation of the HI architecture might extend the fundamental understanding of this unknown mechanism and hybrid incompatibilities in general.

Objectives: This MSc thesis research builds upon the unpublished work of PhD student Erik den Boer (PhD thesis concept 2014, chapter 4) and has two main objectives. Objective 1 is to fine map HI[4.1+6.3]. Markers were developed for the target regions, validated and positioned in the F2_1997_FR mapping population, and enabled fine mapping (Jeuken *et al.*, 2001). Additional fine mapping was done by means of F3 families of recombinant F2 plants identified by E. den Boer. Objective 2 is characterize HI[4.1+6.3] by means a seed set analysis and vitality testing of male gametophytes, aiming to provide direct evidence for the non-transmission hypothesis of 4a6b gametophytes.

2. MATERIAL & METHODS

MATERIAL

Each stage of the research required its specific plant material (primer testing, validation and positioning of the markers, fine mapping, functionality testing). Figure 1 provides an overview of the populations used or often referred to in this study and table 3 provides an overview of the plant material used in this study.

In primer testing, a set of reference lines was used consisting of *L. sativa* cv. Olof, BIL[4.1+6.3] and preBIL[4.1+6.3]. The plants were grown in the greenhouse end of summer under regular growing conditions. DNA was extracted with the NaOH extraction method modified by Koen Pelgrom (Wang *et al.* (1993), Appendix 8).

The F2_1997_FR mapping population was used in positioning, validation of the markers and in fine mapping the HI-genotypes. The F2_1997_FR mapping population derived from the cross *L. saligna* CGN05271 x *L. sativa* cv. Olof and consists of 126 plants (Jeuken *et al.*, (2009), Appendix 1A). The DNA of this plant population is available at the laboratory of Plant Breeding, WUR. In generating new markers data, only DNA samples of recombinant plants were selected (44 DNA samples for C4.1 and 39 for C6.3) (Appendix 2A, 2B).

Additional to the F2_1997_FR mapping population, F₃-families were used in fine mapping. The F₃- families have been derived from inbred preBIL[4.1+6.3] plants, which showed a recombination event in one of the two introgression intervals (project S08 by E den Boer in 2008). ‘pre’ Refers to the heterozygous introgression segments on chromosome 4.1 and 6.3. DNA of the parental F₂-family was not available anymore, but the inbred seeds of the F₂ plants were stored. Selection of (potential) informative plants was based on genotypic information of the parental F₂-family (Appendix 3). To ensure the preBIL[4.1+6.3] F₃ families contained (potential) informative plants, segregation ratios were taken into account by sowing an amount of seeds resulting in a chance >95% that two plants were present with (potential) informative genotype(s). DNA of the F₃-families was isolated with the Kingfisher using sbeadex mini plant kit (LGC Genomics GmbH, Berlin, Germany) and Kingfisher mL magnetic particle processor (Thermo Labsystems, USA) following the manufacturers’ protocol (Stemmer, 2003).

In functionality testing, a set of plants with four genotypes were used in six replications (*L. sativa* cv. Olof, BIL4.1 pv08103, BIL[4.1+6.3] pv09705 and preBIL[4.1+6.3] pv09171). The seeds were sown at 17th of August and transplanted at 5th of September (2013). Pots were placed on a table in the greenhouse. Differential environmental conditions within the greenhouse compartment were taken into account by introduction of six blocks over the horizontal side of the table. First, the plants of each genotype were randomly assigned to one of the blocks, resulting in one block containing four plants with the four distinct genotypes. Within the block, the 4 plants were randomly assigned to its position as well.

Table 3. Plant material used in this study

Plant material	Genotype
Lines:	
<i>L. sativa</i> cv. Olof	4aa6aa
BIL4.1	4bb6aa
BIL[4.1+6.3]	4bb6bb
preBIL[4.1+6.3]	4ab6ab
Populations:	
F2_1997_FR	*
F3 families	*

Plant material accompanied by genotype for chromosome 4 and 6 used in this study.

*Genotype of material is variable; figure 2 elaborates on derivation of material.

METHODS

MARKER DEVELOPMENT

In marker development the following steps were undertaken and are described in detail below: primer design, primer testing, positioning and validation of the markers.

Primer design was adjusted to the availability of genetic information of the lettuce genome. Unfortunately, the complete lettuce genome is not publically available yet. At the moment, a comprehensive physical map is available consisting of aligned scaffolds provided by The Compositae Genome Project (<http://compgenomics.ucdavis.edu>). However, this map still contains some gaps too. The most elaborate genetic map contains 13.943 markers for 12.842 unigenes (Truco et al., 2013). This genetic map is aligned to the physical map of the lettuce genome and publically available via the multi-functional database Gbrowse lattuga v3.2 (http://gviewer.gc.ucdavis.edu/cgi-bin/gbrowse/lattuga_version_3_2/). E. den Boer fine mapped HI[4.1+6.3] on the F2_1997_FR mapping population (PhD thesis concept 2014, chapter 4). The F2_1997_FR map is interconnected with the RIL genetic map (*L. sativa* x *L. serriola*) of Truco by means of overlapping markers. Expressed Sequence Tag (EST) markers of the interrelated RIL genetic map are partly included in Gbrowse as well. The availability of the physical map of the lettuce genome and the interrelated F2_1997_FR map enables primer design based on the physical positions. This advances efficient fine mapping by generating a good spread of markers. The Expressed Sequence Tags (ESTs) retrievable in Gbrowse that were most adjacent to the HI4.1 and 6.3 regions were chosen as flanking ESTs in marker development.

Primers were designed with Primer3Plus (Untergasser et al., 2007). The goal was set to develop one marker per 2 Mbp for the suspected HI-regions. Therefore, one EST was selected per 2 Mbp. Accordingly, two primer pairs were generated based the genomic sequence to which the EST was aligned. Alignment of ESTs to the lettuce genome (available via Gbrowse) enabled strategic positioning of the primers. The exons of *L. sativa* and *L. saligna* were expected to be conserved to a greater extent than the introns. In response, primers were designed to produce either intron-based or exon-based amplicons in PCR (visualized in Figure 4). Design of primers on two neighbouring exons, allowed introns to serve as amplification region. Design of primers within one exon, resulted in exon-based amplicons. Design of intron-based markers was expected to be more successful due to the increased likelihood that primers anneal well and the increased likelihood amplification of polymorphic PCR products. The optimal length of PCR-products in light scanning ranges from 150 to 500 bp (oral communication Marieke Jeuken). Primers were designed with preferred settings for these lengths. The SNP ratio was expected to be 1:100 base pairs (oral communication Koen Pelgrom). Polymorphisms between PCR products of *L. saligna* and *L. sativa* alleles were visualized by means high-resolution-melting curves with the LightScanner® System (Idoha Technology, Salt Lake City, UT, USA).

Primers were tested by means of reference lines. The set of reference lines consisted of *L. sativa* cv. Olof (4aa6aa) and BIL[4.1+6.3] (4bb6bb) in three biological replications and preBIL[4.1+6.3] (4ab6ab) in two biological replications. The reference lines differ only for the alleles on the introgression regions 4.1 and 6.3 and all have a *L. sativa* genomic background. In primer testing, primers were classified as well-performing primers if the following criteria were met: primers produced amplicons in PCR and allele-specific HRM-curves were obtained in light scanning. As mentioned, two primer pairs were generated per locus (per EST). In case two primer pairs were classified as well-performing primers, only the best primer pair was selected and tested for validation on the F2_1997_FR mapping population. As a consequence, not all well performing primers were tested for validation.

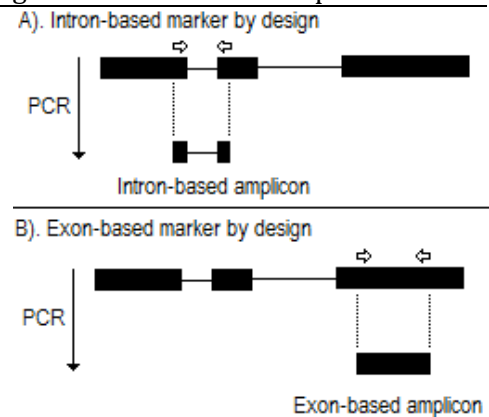


Figure 4. Intron (A) and exon (B)-based Primer Design. An example of an intron-exon structure of a lettuce gene is shown. Black boxes indicate exons, the lines in between indicate introns. The white arrows indicate the strategic positioning and orientation of the primer pairs.

Success of primer design was examined by the success rate of primer design, defined as the percentage of designed primers that were validated at the F2_1997_FR mapping population. The success rate was examined by means of 3 factors: the strategic positioning of the primers (intron- or exon-based primers), the length of the amplicons (150-184, 184-217, 217-250 bp) and the region for which the primer was designed (C4.1 or C6.3). The success rate of primer design was estimated since not all primers that performed well on reference lines were tested for validation of the F2_1997_FR population. Therefore, the influence of the factors could not be statistically tested.

Marker data is generated according to a standard Polymerase Chain Reaction (PCR) protocol suitable for light scanning. Per sample 1 μL DNA ($\pm 10 \text{ ng}/\mu\text{L}$) was used. To the DNA a master mix was added of 5,5 μL Milli-Q, 2 μL buffer, 0,4 μL deoxyribonucleotide triphosphates (dNTP), 1 μL LC Green® Plus DNA binding dye and 1 μL Phire polymerase (Idoha Technology, Salt Lake City, UT, USA). Per sample 20 μL mineral oil was added. The PCR program starts with a hot-start (98°C for 10 seconds) and continues with a cycle of 3 stages (98°C for 10 seconds, 60°C for 10 seconds, 72°C for 30 seconds), 40 times in repetition. The PCR closes off with 30 seconds of 72°C , 94°C , 25°C . The temperature is hold on 10°C till the moment of unloading the plate. High-resolution-melting (HRM-) curves are obtained with the LightScanner® System (Idoha Technology, Salt Lake City, UT, USA). The primers of PCR products that showed polymorphic HRM-curves according to the expectations were selected for validation testing.

Genotyping recombinants of the F2_1997_FR mapping population and F3-families.

Validation of markers on the F2_1997_FR mapping population. As mentioned, the reference lines only differ for the alleles on the introgression regions 4.1 and 6.3 and all have a *L. sativa* genomic background. Therefore, testing the primers in a mixed genomic background of *L. sativa* and *L. saligna* was desirable. Testing the primers on the F2_1997_FR mapping population provided primer testing in a mixed genetic background of *L. sativa* and *L. saligna*. If the patterns of the HRM-curves enabled grouping of the curves into three distinct genotype groups, the data was aligned with existing marker data of the F2_1997_FR mapping population to verify whether the grouping of the HRM-curves was correct. Only the primers that did match these requirements were validated as markers and were used in fine mapping.

Positioning of Markers and Map Construction. Alignment of the developed markers in this study with the existing set of markers was done by hand (in an excel file) according to the following criteria: the probability of mismatching alignments, physical positions of the markers and results in fine mapping (of the F3-families as well).

Maps were constructed with Joinmap4. Joinmap4 calculated the order of the markers and the relative distances between the markers by means of the Monte Carlo Maximum Likelihood (ML) mapping algorithm. By default, Joinmap characterizes a locus with one letter. In adjustment to this, marker data was encoded from 'aa' (a homozygous *L. sativa* allele) to 'A', 'bb' (a homozygous *L. saligna* allele) 'B' and 'ab' (heterozygous *L. sativa L. saligna*) to 'H' (Table 1). The orientation of the chromosomes sticks to the orientation according to the physical map of Gbrowse v3.2. For chromosome 4 this orientation is identical to the genetic F2_1997_FR map, for chromosome 6 this orientation is inverted. Furthermore, this study sticks to the numbering of the chromosomes of F2_1997_FR. Chromosome 4 and 6 according to the F2_1997_FR map resemble linkage group 4 and 8, of the physical map, respectively.

In map construction, the input consisted of existing and newly generated marker data of the F2_1997_FR population. Since new marker data was only generated for subsets of recombinant C4.1 and recombinant C6.3 plants, the initial set of marker data for non-recombinants was extrapolated. Successively, three maps were generated per chromosomal region (C4.1 and C6.3). The first generated map was constructed based on the initial marker data generated by E. den Boer. The second map was based on the extended data set with newly generated marker data. The third map was generated on the extended data set as well with additional imposition of fixed orders. These orders were based on alignment of the markers by hand as described above.

Fine mapping HI[4.1+6.3]. Fine mapping HI[4.1+6.3] was based on exclusion (referred to as the exclusion principle) and has a qualitatively character. Non-transmission of 4a6b gametophytes

confers absence of the genotypes 4aa6ab, 4aa6bb and 4ab6bb (hereafter indicated as HI-genotypes). Sequentially, presence of an 'aa' or 'ab' segment at a recombinant chromosome 4.1 in combination with a non-recombinant 'ab'/'bb' or 'bb' segment (respectively) at the suspected region of chromosome 6.3, causes exclusion of the particular C4 region from the suspected region. The other way around, presence of a recombinant 'ab' or 'bb' segment at C6.3 in combination with a non-recombinant 'aa' or 'aa'/'ab' segment (respectively) at the suspected regions of C4.1, causes exclusion of the particular C6 region from the suspected. In conclusion, the presence of these genotypes indicates the absence of the HI-genes in those specific regions and are therefore excluded from the suspected HI[4.1+6.3] regions.

FUNCTIONALITY TESTING

The non-transmission hypothesis of the 4a6b gametophytes lacks direct evidence: the mechanism on which HI[4.1+6.3] is based is unknown. Functionality testing of (4a6b) gametophytes could provide (more) direct evidence for this hypothesis. The functionality tests were twofold. In vitality testing vitality of male (4a6b) gametophytes was assessed. In a seed set analysis, abortion of female gametophytes and the effects of possibly non-functional male gametophytes on seed set was assessed.

Vitality testing of pollen grains. The vitality of male gametophytes was examined at a specific stage in reproduction as indicated in figure 3 and concerned examination of mature pollen grains. Pollen grains are also referred to as 'pollen', the term non-vital pollen is also referred to as 'aborted pollen'. Vitality testing of pollen grains could provide insight in whether a certain percentage of pollen grains are non-transmitted due to non-vitality of pollen grains with a 4a6b genotype. To test this consideration, four lines were included in vitality testing (Table 4). Test line preBIL[4.1+6.3] was expected to produce 4a6b gametophytes in a 1:4 ratio. This results in 25% non-viable pollen grains under non-vitality of 4a6b gametophytes. Three reference lines were included, namely *L. sativa* cv. Olof, BIL[4.1] and BIL[4.1+6.3] (Table 3). It was assumed that the vitality of pollen grains of the reference were the same since vitality is not expected to be hindered by 4a6b gametophytes or by other disrupted biological processes. Lack of general knowledge (based on experience and literature) on vitality of lettuce pollen grains obliged construction of an assumption on the standard value of vitality of pollen grains. The standard vitality value was estimated on 90% (personal communication Anne Kortstee based on potato tests). Under the hypothesis of non-vitality of 4a6b gametophytes, the vitality of pollen grains of preBIL[4.1+6.3] was expected to be 67.5% (75%/90%).

Table 4. Experimental Design Vitality Testing Male 4a6b Gametophytes

Plant material	Genotype plant material	Expected genotype(s) gametophytes	Expected Vitality**
preBIL[4.1+6.3]*	4ab6ab	4a6a 4a6b 4b6a 4b6b	67.5% (75%/90%)
<i>L. sativa</i> cv. Olof***	4aa6aa	4a6a	90%
BIL4.1***	4bb6aa	4b6a	90%
BIL[4.1+6.3]***	4bb6bb	4b6b	90%

* Non-vitality of male 4a6b gametophytes as cause of non-transmission of 4a6b male gametophytes was tested by means of test line preBIL[4.1+6.3]. ** 25% of the pollen grains of test line preBIL[4.1+6.3] were expected to be non-vital, resulting in 25% lower vitality in comparison with reference lines. *** The vitality of pollen grains of the reference lines was expected to be 90%.

The method used in pollen vitality testing is the Alexander stain (Peterson *et al.*, 2010). This differential staining of aborted and non-aborted pollen is based on two dyes: malachite green and acid fuchsin. Cellulose in the pollen walls reacts with malachite green producing a green colour. Protoplasm reacts with acid fuchsin producing a purple colour. Since non-vital pollen grains lack protoplasm they appear green. In vital pollen, the purple of the protoplasm downs the green colouring pollen cell wall (in appropriate ratio malachite green and acid fuchsin) (Peterson *et al.* (2010), Alexander (1980). The staining solution is prepared by adding

the following constituents in the order given below: 10 mL 95% alcohol, 1 mL malachite green (1% solution in 95% alcohol), 50.5 mL distilled water, 25 mL glycerol, 5 mL acid fuchsin (1% solution in water), 0.5 mL orange G (1% solution in water), 4 mL glacial acetic acid. Add distilled water (4.5 mL) to a total of 100 mL. The alexander stain was prepared by Anne Kortstee. The plant material used should contain mature pollen. At the moment of collecting plant material, lettuce flower heads that did flower that specific day were harvested. The blooming flower heads were recognizable by the opening and closing of the flower head on the day of flowering (in the morning after sun rise). By collecting plant material, one (flowering) flower head was harvested per Eppendorftube and drenched in some drops of Alexander stain. The eppendorftubes were vortexed very well to stimulate release of the pollen grains from the anthers. The flower head was placed on a microscope slide and dissected for extra release of the pollen grains, drops of Alexander stain were added if necessary and a cover-slide was place on the sample. Slides were examined using a Zeiss Axiophot microscope and micrographs were taken by a Zeiss Axiocam ERc 5s camera (Zeiss, Slidrecht, The Netherlands).

In a comprehensive vitality study, the non-transmission of female 4a6b gametophytes due to abortion of 4a6b gametophytes can be confirmed in statistical analysis. However, a pilot was performed first, in order to examine whether the staining worked well on the plant material and whether the assumptions were reasonable. The pilot concerned staining of pollen grains of 6 flower heads: 3 flower heads of *L. sativa* cv. Olof and 3 flowerheads of preBIL[4.1+6.3].

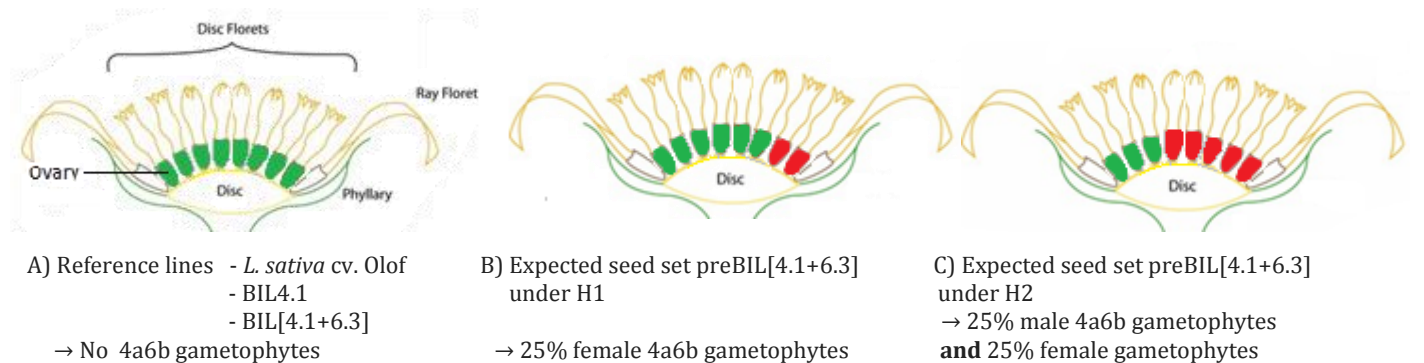


Figure 5. Experimental Design Seed Set Analysis. Functionality of 4a6b gametophytes was assessed by means of test line preBIL[4.1+6.3] and compared with reference lines *L. sativa* cv. Olof, BIL4.1 and BIL[4.1+6.3]. Under H0, seed set of test line preBIL[4.1+6.3] does not deviate from reference lines, producing mature non-aborted seeds only (A). Two hypotheses were tested: hypothesis 1 (H1) and hypothesis 2 (H2). H1: Abortion of female 4a6b gametophytes and compensation of non-functional 4a6b pollen grains by functional pollen cause a reduction of 25% non-aborted seeds in preBIL[4.1+6.3] (B). H2: Abortion of female 4a6b gametophytes and the inability of non-functional 4a6b pollen grains to be compensated by other functional pollen grains causes a reduction of 56,5% non-aborted seeds in preBIL[4.1+6.3] (C). The hypotheses were based on two additional assumptions. Assumption 1: the four lines produce the same total amount of seeds (aborted and non-aborted). Assumption 2: the reference lines resemble a comparable seed set. Aborted and non-aborted seeds are indicated in red and green, respectively. Under H2, on average four or five seeds are aborted per 8 ovaries developed, as an example 5 seeds are indicated in red in figure C.

Seed set analysis. The non-transmission hypothesis of 4a6b gametophytes lacks direct evidence for both male and female gametophytes. This seed set analysis aimed to test whether female 4a6b gametophytes are aborted. The plant material used was identical to the plants of pollen vitality testing (table 3). Functionality of 4a6b gametophytes was assessed by means of test line preBIL[4.1+6.3] and compared with reference lines *L. sativa* cv. Olof, BIL4.1 and BIL[4.1+6.3]. Under H0, seed set of test line preBIL[4.1+6.3] does not deviate from reference lines, producing mature non-aborted seeds only (Figure 5A). By experimental design, two hypotheses were introduced: hypothesis 1 (H1) and hypothesis 2 (H2). H1: Abortion of female 4a6b gametophytes and compensation of non-functional 4a6b pollen grains by functional pollen cause a reduction of 25% non-aborted seeds in preBIL[4.1+6.3] (Figure 5B). H2: Abortion of female 4a6b gametophytes and the inability of non-functional 4a6b pollen grains to be compensated by other functional pollen grains causes a reduction of 56,5% non-aborted seed in preBIL[4.1+6.3] (Figure 5C). The hypotheses were based on two additional assumptions. Assumption 1: the four lines produce the same total (aborted and non-aborted) amount of seeds

(oral communication M. Jeuken). Assumption 2: the reference lines resemble a comparable seed set.

Seeds were collected by labelling flower heads. Per plant, 5 flower heads were labelled at three labelling moments (4, 11 and 18 December 2013). The labelling moments were introduced to take random environmental conditions in account. In total, seeds of 90 flower heads were sampled per genotype (5 flower heads*6 plants per genotype*3 labelling moments) to assure that the hypotheses could be statically tested by means of two-way ANOVA's.

In statistical testing plants were nominated as experimental units. The test variable was the number of seeds. Genotype and labelling were included as explanatory factors. The blocks (1-6) and the plant numbers (1-24) were taken into account as block factors. In the two-way ANOVA, the factors were assessed for interactions and main effects of the factors. In presence of an interaction, main effects of a factor on the y-variate cannot be estimated since the effects are non-additive. In statistical testing we aimed to quantify the genetic effects on seed set, so an interaction of the factor genotype and factor labelling moment is not desirable and should be assessed as well.

SEED WEIGHT

As will be described in results & discussion, differences in seed set among the reference lines provoked additional research to gather more insight in unexpected deviations. Weight of non-aborted seeds of 30 flower heads was used as measure of the total energy input of plants in reproduction. Furthermore, the average weight per seed is estimated and indicates how total seed weight was distributed over non-aborted seeds. In due of the time, only the weight of 30 flower heads per genotype was measured for labelling 1 and 2. For labelling 3 weight measurements were done for non-aborted seeds per 5 flower heads of one plant, which did enable statistical testing. Total seed weight and weight per seed was assessed by means of a one-way ANOVA, including genotype as a factor and either total seed weight or weight per seed as a factor. The plants served as experimental unit. Seed weight was examined for each combination of genotype and labelling moment.

3. RESULTS & DISCUSSION

MARKER DEVELOPMENT.

Results: Based on overlapping markers of the genetic F2_1997_FR map and the genetic RIL map, and alignment of ESTs of the RIL map to the physical map, the HI-regions fine mapped by E. den Boer were mapped to exact positions. This resulted in a HI-region at C4.1 from 0 – 65.5 Mbp and for a HI-region at C6.3 from 0 – 33.5 Mbp. The overlapping EST-markers concerned the ESTs QG_CA_Contig2454 and QGF25D22.yg.ab1 at C4.1 and C6.3, respectively). Unfortunately, no overlapping marker was available for the upper border of the suspected HI-region as fine mapped by E. den Boer. Moreover, the overlapping marker closest to the HI-region C6.3 was overlooked. This marker is positioned at 15.8 Mbp (QGD14014.yg.ab1). This missed opportunity caused marker development over a region twice as big as was necessary.

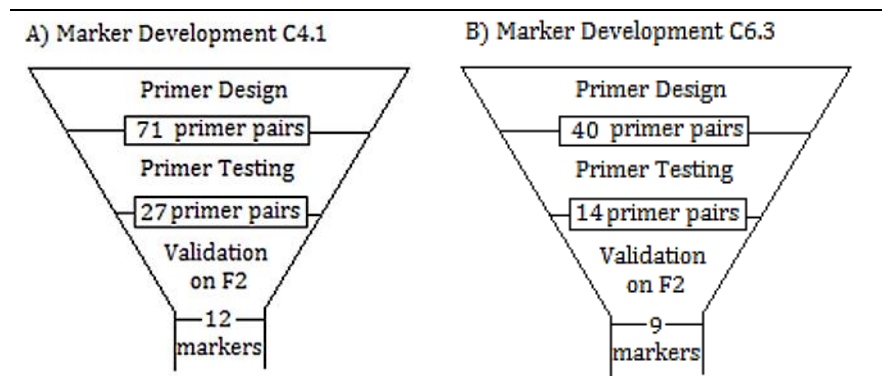


Figure 6. Overview Results Marker Development. The three main steps in marker development were: primer design, primer testing on parental reference lines and validation of the markers on the F2_1997_FR mapping population. A). Twelve markers were developed at chromosome 4.1 out of 71 putative markers (16.9%). B). Nine markers were developed at chromosome 6.3 out of 40 putative markers (22.5%).

Table 5. Overview of Developed Markers

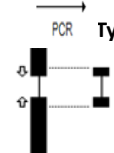
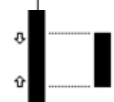
Chromosome	Position on Chromosome (Mbp)*	EST Marker*	Forward Primer	Reverse Primer	Length Amplicon (bp)	Type of amplicon	Map number***	Work Number***
4	2.0	CLS10758.b1_K01.ab1	AGCACCTTGAAACATTGG	GTTCCAATCTCCCGTAGTGC	240	intron-based	4	3
4	7.9	CLLY8178.b1_D05.ab1	GGTAATCAAGCGGAAGTGGA	CCACCAATACACTTGCAAGAAA	211	intron-based	8	9
4	23.9	CLS5115.b1_F07.ab1	GCCACCGTCATTCTCGTATT	GCAAGAACAGCCATGAGTGA	168	exon-based	12	25
4	29.9	CLS_S3_Contig2928	CACCGACACCGCTCTTAAAT	GCACAGCATGAAATTCCTCA	234	exon-based	13	30
4	37.9	CLS_S3_Contig2219	GCAGAAGGACTTGGTGAAGC	CATTGGGGTGCATAAACTG	341	intron-based	15	40
4	41.9	QGG20102.yg.ab1	TTGTAGTGTAATGCGTCATTGG	ACAGCTGTTGACATTTTACTTTTG	215	exon-based	18	43
4	50.0	CLS12433.b1_B09.ab1	CCTGGTGGAGGATTAACCTCG	AACGAATTTGTTCCCATGA	298	intron-based	19	53
4	52.8	CLLY11908.b1_G01.ab1	TTAAGTTCCTCCCTG	TTGGACCAATGTGCTCTTCA	246	intron-based	20	56
4	53.8	CLS_S3_Contig7077	TTCTGTAAAAATGCCACCA	CACCTCCATCGGAACCTTAT	224	intron-based	21	57
4	58.0	CLS_S3_Contig4465	CAGCATCAACTGCATCCAAA	CACCGTAATGGCTACGTCT	230	intron-based	24	62
4	60.4	CLS_S3_Contig5375	CTGTCACTGTGCCTCACACA	TGGAGCATTTTCTCTCTTCA	190	intron-based	26	64
4	61.9	LSS_S3_Contig1360	GATCTTTACGGTGGGTGCAG	ATTCAGTGCCTGTTGCAG	231	intron-based	27	66
6	3.8	CLS_S3_Contig6649	TCGATGGGTGGATTGCTAGT	AAAGGGGTTGCGATGGTTAT	232	intron-based	5	40
6	7.8	Y4646.b1_K09.ab1	TGCAATTAGCATCTGCATCC	CGGAATCCTTGAGGGTACAA	225	exon-based	6	10
6	10.0	CLS_S3_Contig3369	TGGCCTCGGTATCCTATTTG	CGTCATGATCCAGTCTTCA	225	intron-based	7	12
6	14.3	CLS_S3_Contig1511	ATTCCCATTTTCCCTCCATC	GCACAAATGTCGGTGTCTATC	205	intron-based	8	16
6	17.9	CLSM10904.b1_P13.ab1	GGGAAAAGAAATTTGCTCGAC	GCTCATCCCCAGTAATTCCTCA	197	intron-based	9	20
6	22.0	QGA7F22.yg.ab1	GGGAGGAAATGATGTCCTA	TACCGGAGGTTTACCACCAC	173	exon-based	10	23
6	26.0	CLLY9820.b1_G08.ab1	ATGGAAGCTCCAATGGTTTG	CAACATTGAGGGCCAAATCT	161	exon-based	11	28
6	31.9	CLS_S3_Contig4263	GATTGGCTGGGATTTTCAA	CTCTCGGTGGAGCACGTATT	200	intron-based	15	33
6	34.0	CLS_S3_Contig7223	ACCGCATCAAAATCTTCGAC	GGAACCTGTAGTGGGTGA	174	exon-based	18	36

*The physical positions of the markers were obtained from the Gbrowse Lattuga version 3.2. ** Marker names refer to ESTs on which primers are targeted. PCR-products of the markers provide allele-specific HRM-curves and concern homozygous *L. sativa*, homozygous *L. saligna* and heterozygous *L. sativa* *L. saligna* alleles. ***For the purpose of this study, work numbers (used during M.sc. study) and map numbers (corresponding to the map numbers of appendix 4) were assigned to the markers.

The HI-regions were mapped to exact positions delimited by the overlapping markers referred to as flanking markers. A region of ± 5 Mbp was incorporated additional to the delimited regions, referred to as the flanking regions. This resulted in regions of 70 and 38 Mbp for C4.1 and C6.3, respectively.

Sets of two primer pairs per locus (EST) were designed with an even distribution of ± 2 Mbp over the regions defined above (first EST 0-1 Mbp). A number of 71 primer pairs were designed at HI-region C4.1. For one locus, just one primer pair was designed the EST in question. Twenty-seven of 71 primer pairs performed well on reference lines. Primers classified as well performing primers produced amplicons in PCR and provided allele-specific HRM-curves. Twelve of 27 primer pairs were validated on the F2_1997_FR mapping population. From forty primer pairs designed at HI-region C6.3, fourteen primer pairs performed well on reference lines. Nine of the fourteen primer pairs were validated on the F2_1997_FR mapping population (Figure 4). As indicated at material and methods, not all primer pairs that performed well on the reference lines were tested on the F2_1997_FR mapping population. The potential markers not tested at the F2_1997_FR mapping population were, in case of validation, not of added value in fine mapping HI[4.1+6.3] and concern 9 and 4 primer pairs at C4.1 and C6.3 respectively. In total, 21 markers are adjoined to the existing set of markers (Figure 6, Table 5).

Table 6. Success Rate Primer Design

# Primers pairs						
	Chromosome	Primer design	Good performance on Reference lines**	Tested on F2_1997_FR***	Validated on F2_1997_FR population	Estimated success rate of primer design*
	C4.1	71	38 (53.5%)	29	12 (41.4%)	<div><div></div>22%</div>
	C6.3	40	18 (45.0%)	14	9 (64.3%)	<div><div></div>29%</div>
Amplicon Length (bp)						
150-184	C4.1	13	5 (38%)	4	1 (25%)	<div><div></div>10%</div>
	C6.3	12	4 (33%)	4	3 (75%)	<div><div></div>25%</div>
184-217	C4.1	26	12 (46%)	9	4 (44%)	<div><div></div>21%</div>
	C6.3	10	5 (50%)	5	3 (60%)	<div><div></div>30%</div>
217-250	C4.1	29	15 (52%)	13	6 (46%)	<div><div></div>24%</div>
	C6.3	16	8 (50%)	5	3 (60%)	<div><div></div>30%</div>
Type of marker						
 Intron-based	C4.1	43	27 (63%)	21	9 (43%)	<div><div></div>27%</div>
	C6.3	21	10 (48%)	6	5 (83%)	<div><div></div>40%</div>
 Exon-based	C4.1	28	9 (32%)	8	3 (38%)	<div><div></div>12%</div>
	C6.3	19	8 (42%)	8	4 (50%)	<div><div></div>21%</div>

*The 'success rate of primer design' indicates the percentage of primers validated as markers, an estimation is given for the three factors indicated in bold. ** 'Good performance' means amplicons were produced in PCR and allele-specific HRM-curves were observed. *** Since not all well-performing primer pairs were tested on the F2_1997_FR population, an exact value for the success rate could not be given and was estimated instead. The ratio of validated markers was used as an estimator of validation in general. Example: the estimated success rate of primer design for intron-based primers at C4.1 is $(0,43 * 0,63) * 100 = 27\%$. These trends lack statistical underpinning.

Examining the success rate of primer design was done by means of strategic positioning of the primers, target region and amplicon length (Table 6). In examining the influence of amplicon length on the success rate, primer pairs with amplicons lengths >250 bp were regarded as outliers. This concerned six of 111 primer pairs (5.4%) with an amplicon length ranging from 312 – 440 bp. These primers were excluded from analysis since these lengths did not fit the length categories. Examination of the success rate of primer design was estimated and lacks statistical underpinning. Statements resulted from observed trends. The success rate of primer design was slightly more successful for primer targeted at C6.3 than for C4.1 (difference of 7%). The success rate for primers with amplicons of 150-184 bp was 10% less successful than design of primers with amplicon lengths of 184-250 bp. Intron-based markers were estimated to be 15% more successful than exon-based markers.

Discussion: The success rate of primer design seems slightly affected by the target region and amplicon length (ranging from 150-250 bp). The success rate seems most strongly affected by strategic positioning of the markers, with design of intron-based markers to be \pm 15% more successful than exon-based markers. This would meet the expectations. In new rounds marker development, it would be interesting to examine the differences in success rate of intron- and exon-based markers again, to see whether same trends are observed. Since the genomic sequence of *L. saligna* (and other wild relatives of lettuce) are not publically available, one cannot anticipate on deletions and SNPs between these species. Strategic positioning of primers may serve as a trick in efficient marker development. Unfortunately, no articles were found in literature reporting strategic positioning of the primer resulting in exon- or intron-based markers.

POSITIONING OF THE MARKERS

Results: Three maps were generated per chromosomal region (C4.1 and C6.3) and are presented in figure 7. The first generated map was constructed based on the initial marker data generated by E. den Boer. The second map was based on the extended data set with newly generated marker data. The third map was generated on the extended data set as well with additional imposition of fixed orders. The set of maps deviated in length from the initial map of E. den Boer (Figure 2). The marker order differed for the map based on extended marker data with and without imposition of fixed marker orders. However, exact positioning of the fine mapped regions remained the same.

Discussion: The set of maps generated in this study deviated from the initial map generated by E. den Boer. This is probably due to use of another mapping algorithm or similar but different input. It was not possible to compare data input and methods used and the real reason remained unclear. Differential positioning of markers between the maps as mentioned above are not of major interest since the exact positioning of the suspected regions remained the same. New rounds of marker development will be based on the exact positioning. In time, exact maps will take over from relative maps. The maps presented in figure 7 forms a transition between relative and exact maps.

FINE MAPPING HI[4.1+6.3]

Results: HI[4.1+6.3] was fine mapped by means of exclusion. Appendix 4A, 4B, 5, 6A and 6B presents the marker data on which fine mapping was based. In relative terms, the HI-regions were narrowed down from 14.4-28.2 cM to 16.5-19.2 cM at C4.1 (-11.1 cM) and from 0-5.3 to 2.5-2.9 at C6.3 (-4.9 cM). In exact terms, the HI-region at C4.1 was mapped at 7.9-37.9 Mbp and the HI-region at C6.3 0-7.8 Mbp (Figure 7). Progression in fine mapping was most attributable to the F2_1997_FR mapping population. Marker data of F3-families confirmed fine mapping results based on the F2_1997_FR mapping population for C4.1. Moreover, one F3-family was informative in addition to fine mapping in the F2_1997_FR population: one relative marker at the upper border of HI-region was excluded from the suspected region. For C6.3 marker data of F3-families did not confirm earlier results, but the results were not rejected either.

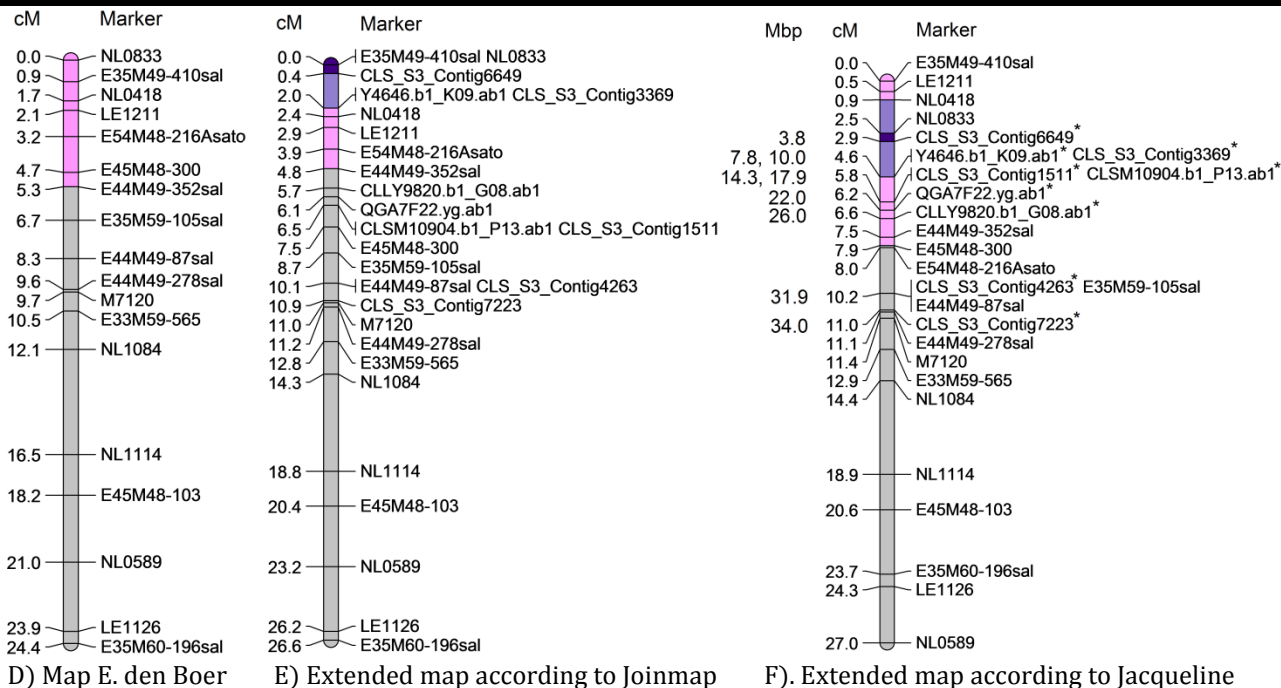
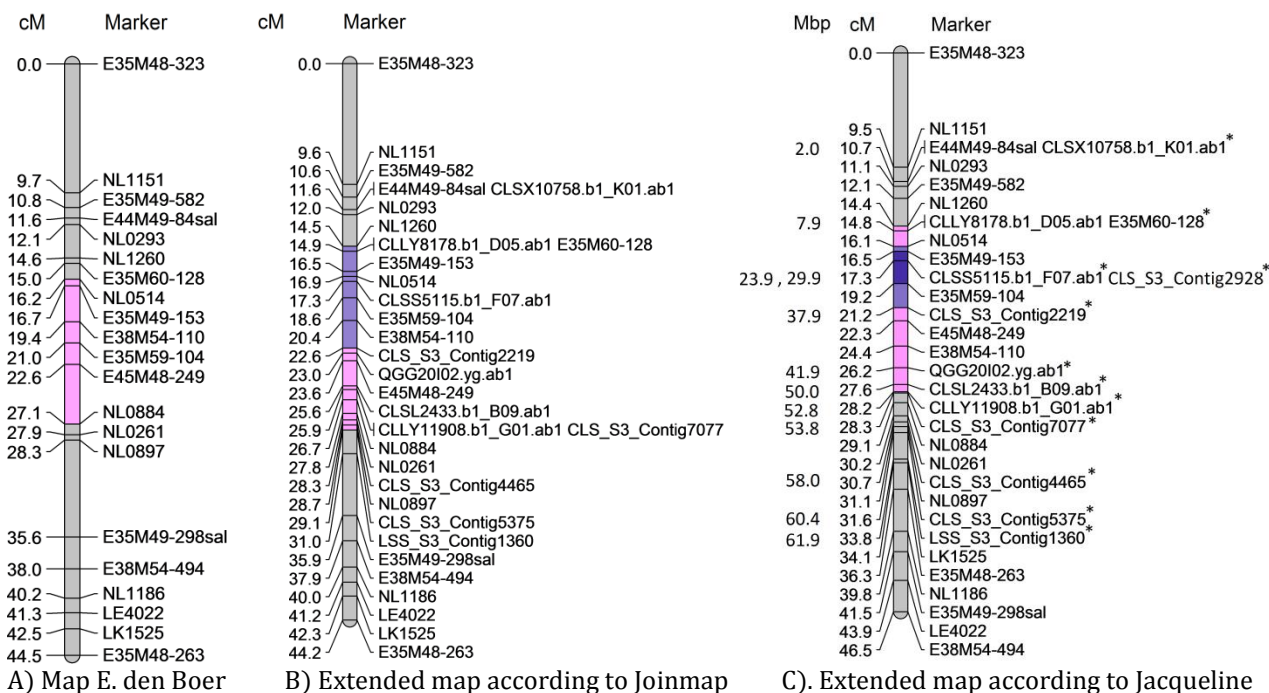


Figure 7. Positioning of markers on F2_1997_FR map. Maps were generated with Joinmap4's Maximum Likelihood (ML) mapping algorithm with extrapolated marker data of the F2_1997_FR map. A, B and C refer to maps of chromosome 4.1, D, E and F to maps of chromosome 6.3. Maps resemble the orientation of the physical map and is identical to the genetic map of C4.1 and inverted for the genetic map of C6.3. Chromosome 4 and 6 resemble linkage group 4 and 8 of the physical map. Map A & D represent the initial map of E. den Boer and deviate in length (cM) from the maps introduced by figure 1. This is probably due to use of another mapping algorithm or similar but different data input. Map B & E are based on extended data with new marker data. Map C & F are generated with additional imposition of fixed orders. These orders were based on positioning of markers by hand by means of following additional criteria: information of physical markers positions (indicated with *, positions incorporated in map), additional genotypic information of F3 families (appendix 5) and results of fine mapping (appendix 6). Light pink indicates initial suspected HI-regions, light grey initial flanking regions. Dark purple indicates suspected regions as narrowed down in this M.sc. study, with in light purple flanking regions. The differences in positioning of markers between the map B and C, E and F (C4.1 and C6.3 respectively) did not affect the physical positioning of the suspected HI-regions. The HI-region at C4.1 was narrowed down from 14.4-28.2 cM to 16.1-21.2 cM (7.9 – 37.9 Mbp). The HI-region at C6.3 was narrowed down from 0 – 7.9 cM to 0.9 – 4.6 cM (0-7.8 Mbp).

Discussion: Progression in fine mapping by means of the F2_1997_FR population was already that successful, that the F3-families (relative to the F2_1997_FR population) could not attribute in fine mapping that much.

Fine mapping by means of the newly developed markers for C6.3 is fully exploited. Two of the newly developed markers of C4.1 still can progress fine mapping. Plant material with informative recombinants is still available too. Plant nr. 109 of the F2_1997_FR population is informative in fine mapping at C6.3. Plant nr. 123 of the F2_1997_FR population and plant nr. pv09345, pv09317, pv09318 of the F3-families are still informative in fine mapping at C4.1. For further research, new markers need to be developed to forward fine mapping. It is advisable to use the informative plants indicated above in further fine mapping.

CANDIDATE GENES.

Results: The EST-markers CLS_S3_Contig2928 and CLSS5115.b1_F07.ab1 are within the suspected regions of C4.1. The EST-marker CLS_S3_Contig6649 are within the suspected region of C6.3. The genomic sequences of these ESTs were put in BLASTn in search for homologous genes. No homologous sequences were identified for CLSS5115.b1_F07.ab1. The genomic sequence of CLS_S3_Contig2928 was homologous for a maximum of 9% to about 10 sequences. CLS_S3_Contig6649 was homologous to genes annotated as a phosphatidylcholine-sterol O-acyltransferase. HI-region C6.3 was narrowed down to an extent it was worthwhile to make an inventory of the ESTs within the region according to Gbrowse Lattuga v3.2. Within the C6.3 region from 0 to 7.9 Mbp 135 ESTs are located (Appendix 7).

Discussion: The annotation of the genomic sequence of the EST markers did not lead to signs that the markers concerned the HI-genes in question. In further investigation, an assessment of the candidate genes at C6.3 for homology with other well characterized genes, may result in a subset of genes with an increased likelihood of being involved in HI[4.1+6.3]. This might accelerate mining for the responsible gene at C6.3 out of the 135 candidate genes.

VITALITY OF POLLEN GRAINS

Results: In the pollen vitality pilot, the pollen vitality appeared to be >95% for the test line preBIL[4.1+6.3] as well as for the reference line *L. sativa* cv. Olof. The observation of highly fertile preBIL[4.1+6.3] pollen did not meet the expectations under non-transmission of male 4a6b gametophytes due to non-vitality. This would induce a 25% lowered pollen vitality of preBIL[4.1+6.3] in comparison with the reference line. Therefore the hypothesis of non-vitality of male 4a6b gametophytes is rejected. The preliminary results did not provoke an incentive to study pollen vitality to a greater extent (Figure 8).

Discussion: Rejection of the non-transmission hypothesis of male 4a6b gametophytes due to abortion created room for two new hypotheses. Absence of non-vital 4a6b gametophytes either implies 4a6b gametophytes are absent and not formed in meiosis or vital male 4a6b gametophytes are present but dysfunctional in a later developmental stage. In the section genetic dissection of HI[4.1+6.3] further implications are discussed.

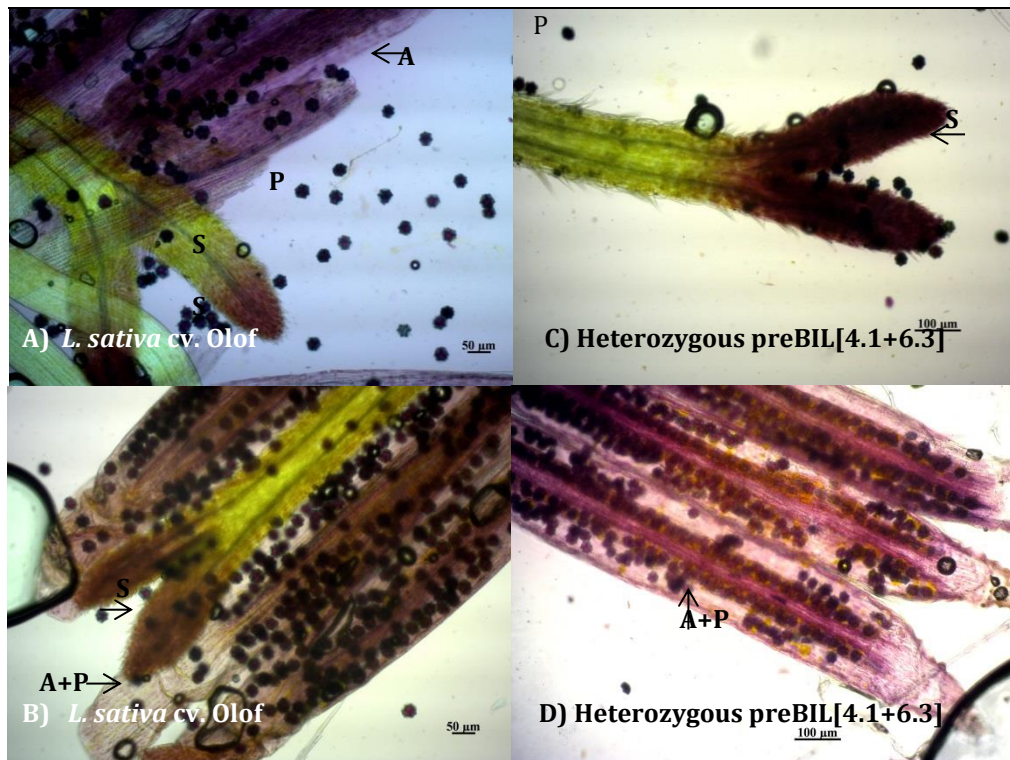


Figure 8. Vitality testing of male 4a6b gametophytes. The figure represents results of the Alexander staining pilot. Viable pollen grains are stained purple, non-viable pollen grains are stained green. 'P' indicates pollen grain, 'A' united anthers, 'S' stigma. Pistles are colored yellow. Anthers are colored purple/brown and house tiny spheres, the pollen grains. Test line preBIL[4.1+6.3] was expected to produce non-vital 4a6b gametophytes (ratio 1:4) resulting in a 25% reduction in vitality of pollen grains in comparison with reference line *L. sativa* cv. Olof. The vitality of pollen grains of *L. sativa* cv. Olof (A and B) and preBIL[4.1+6.3] (C and D) was estimated to be >95%.

Seed set analysis

Labelling. Results: Three labelling moments were included in seed set analysis to account for random environmental effects. At the moment of labeling, 5 flower heads flowering at that day were planned to be labeled. However, this was not always possible for every plant, which made us decide to label flower heads that bloomed the day before if necessary. At labelling moment 3 (18 December 2013), 31 of 120 flower heads were not present, even with inclusion of flower heads flowering the day before. Therefore, plants with missing labelled flower heads were labelled 19 and 20 December again.

Counting. In counting seeds, the strategy was modified several times in adaptation to the results of seed set. Therefore, results and discussion are rather intertwined but disassembled here by citing results and discussion thrice.

Results: Six weeks after labelling, the flower heads were harvested and the seeds counted. The plants were expected to produce mature seeds only. However, it appeared that plants produced mature as well as immature seeds.

Discussion: .. et al. described comparable phenotypes of seeds in Arabidopsis . In their research, the immature seeds were demonstrated to be aborted. This classification was adopted in counting: non-aborted as well as aborted seeds per flower head were counted.

Results: The counts of labelling 2 appeared to deviate substantially from the counts of labelling 1. Count deviated especially for the *L. sativa* cv. Olof plants, which produced on average 8 non-aborted and 7 aborted seeds at labelling moment 1 and on average 2 non-aborted and 10 aborted seeds at labelling moment 2.

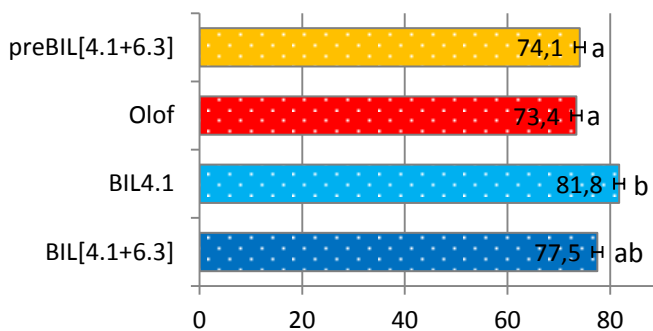
Discussion: This deviation seems explained by the short day length (winter time) and the late developmental stage of the plants (that already withered away). Based on observations of the counts of labelling 2, the counts for labelling 3 were expected to be even more influenced by the shortening of the day length and the developmental stage of the plants. However, the purpose of including labelling

moments was to take in account random environmental circumstances at the moment of labelling by means of a block effect (for instance cloudy rainy weather versus sunny weather), rather than testing for structural effects of these factors in time. Since the structural effects of the developmental stage of the plants and the structural effects of winter time on seed set is not of interest in this analysis labelling 3 was substituted. Instead of the labelled flower heads at labelling moment 3, a random sample of flower heads is taken from the crown of the lettuce plants. These flowers of this flower head did flower earlier (\pm end of November) and in more or less the same developmental stage (oral communication M. Jeuken).

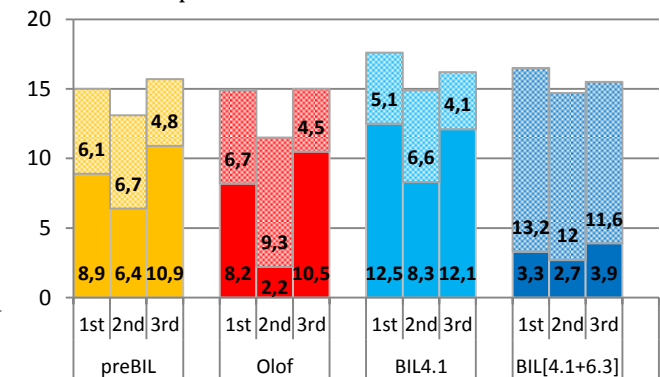
Result: The counts of labelling 3 resembled the counts of labelling 1.

Statistical testing: interactions. Results: In statistical testing, presence of an interaction between the factor labelling and the factor genotype was checked first. For the total amount of seeds (aborted and non-aborted, 5 flower heads) per genotype, the interaction of genotype*labelling was nearly significant ($p=0.051$). For the ratios aborted and non-aborted seeds the interaction of genotype*labelling was highly significant ($p<0.01$). Left out of the data of labelling 2 resulted in absence of the interaction effects.

A) Total amount of seeds (aborted and non-aborted) per 5 flower heads per genotype



C). Overview average amount of aborted and non-aborted seeds per flower head



B) Aborted and non-aborted seeds as percentage of the total amount of seeds per genotype

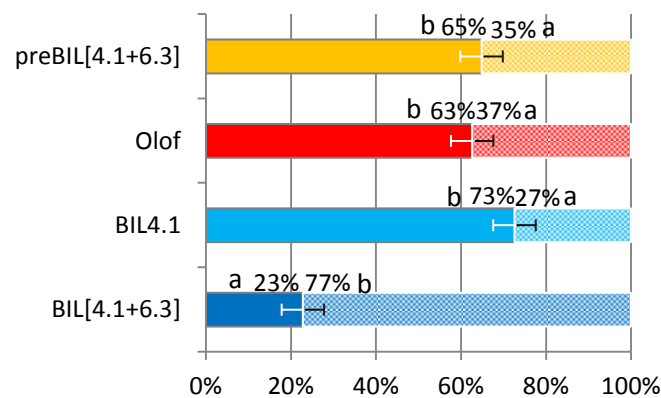


Figure 9. Seed set analysis. Test line preBIL[4.1+6.3] was assessed in comparison with reference lines (*L. sativa* cv.) 'Olof', 'BIL4.1' and 'BIL[4.1+6.3]' by means of two hypotheses. H1: Abortion of female 4a6b gametophytes causes a reduction of 25% non-aborted seeds (assumption: functional pollen grains compensate non-functional 4a6b pollen grains, figure 3B). H2: Abortion of female 4a6b gametophytes combined with the inability of functional pollen grains to compensate non-functional 4a6b pollen grains causes a reduction of 56,5% non-aborted seeds (figure 3C). The hypotheses were based on two additional assumptions. A). Assumption 1: Flower heads of each genotype develop the same fixed number of ovaries. This assumption was rejected in a two-way ANOVA ($\alpha=0,05$). In response, H1 and H2 were tested on the relative amount of aborted

seeds instead of exact amounts. B). Assumption 2: Reference lines produce the same amount of non-aborted seeds. This assumption was rejected too. Solid bars indicate the ratio non-aborted seeds, the dots pattern indicate the ratio aborted seeds. Figure 9A and 9B present combined data of labelling 1 and 3 accompanied by results of statistical testing. 'a' and 'b' indicate significant differences. Figure 9C presents an overview of the seed set data with 'L1', 'L2' and 'L3' indicating Labelling 1, 2 and 3, respectively. Boxes with solid fill indicate non-abortion, 50% fill indicates abortion, the dots pattern indicates total amounts of seeds.

Discussion: As already described above, the number of seeds per genotype for labelling 2 differed substantially from labelling 1 and 3. It is reasonable that factors as day length and the developmental stage of the plants interact with genotypic effects in seed set. Since structural effects of these factors are not of interest in this study data of labelling 2 was left out hypothesis testing. Absence of interaction effects enabled comparison of the main effects of genotype on the seed set of plants. However, the importance of the presence of the interaction should not be played down. One should acknowledge that flower heads of labelling 1 and 3 were probably subjected to adverse effects of winter time too. Further implications on labelling are described in the section below.

Statistical testing: fixed amount of ovaries. Results: Two hypotheses were introduced for statistical testing: H1 and H2 (figure 3). H1 and H2 differ in the assumption whether aborted pollen can (H1) or cannot (H2) be compensated by other viable pollen, resulting in either a 25% or 56,5% reduction in the amount of non-aborted of preBIL[4.1+6.3] plants in comparison with the reference lines. The hypotheses were based on two additional assumptions. Assumption 1: Reference lines develop the same fixed number of ovaries per flower head for each genotype. This assumption was rejected in a two-way ANOVA ($\alpha=0,05$). Not all plants of the reference lines develop the same amount of ovaries per flower head (Figure 9B). BIL4.1 produced more ovaries per flower head than preBIL[4.1+6.3] and *L. sativa* cv. Olof (± 82 ovaries versus ± 74 and ± 73 ovaries per flower head (Figure 9A)).

Discussion: In response to rejection of assumption 1, H1 and H2 were not tested on the exact number of aborted and exact number of non-aborted seeds, but on the ratios of aborted and non-aborted seeds per flower head. The ratios enabled comparison of the amount of aborted and non-aborted seeds per genotype.

Statistical testing: reference lines. Results: Assumption 2 concerned that that the reference lines all produced the same amount of aborted and non-aborted seeds. To our surprise, the ratios of aborted and non-aborted seeds did differ significantly among the reference lines. 37% Abortion of the seeds of *L. sativa* cv. Olof was comparable and not significantly different from 27% abortion of the seeds of BIL4.1. But abortion of 77% of the seeds of BIL[4.1+6.3] was significantly higher than abortion of *L. sativa* cv. Olof and BIL4.1 seeds (Figure 9B).

Discussion: In testing hypothesis, the seed set test line preBIL[4.1+6.3] was aimed to be compared with the reference lines. The fact that the reference lines do not produce 4a6b gametophytes, have the same genetic background as the test line and only do differ for the introgression segments on C4.1 and C6.3 did not guarantee that these reference lines did not differ in seed set. In conclusion, the reference lines did not function as expected and complicated hypothesis testing.

Hypothesis testing & Concluding remarks. Discussion: 65% of the ovaries produced by preBIL[4.1+6.3] reached maturity, 35% of the ovaries were aborted. Since the reference lines cannot be used as a true reference, conclusions cannot be statistically underpinned. Hypothesis 2 results in abortion of 56,5% of the seeds additional to the abortion observed under the reference lines. 'Only' 35% abortion is observed in test line preBIL[4.1+6.3]. It is very unlikely that this hypothesis would hold in statistical testing with good reference lines. Furthermore, hypothesis 1 states 25% of the ovaries are aborted due to 4a6b gametophytes additional to the abortion observed under the reference lines. Apart from BIL[4.1+6.3], the abortion rate for preBIL[4.1+6.3] resembled the abortion rates of the reference lines: additional abortion was not observed. From this perspective, one may conclude female 4a6b gametophytes were not formed in meiosis. Rejection of H1 however, is withdrawn by the possibility that the 35% abortion of seed may include preferential abortion of the 4a6b gametophytes due to winter circumstances. Implications of this hypotheses are discussed in the section genetic dissection of HI[4.1+6.3].

Due to the presumed influence of wintertime, it is advisable to do the seed set analysis over again. In choosing labelling moments, external effects related to the labelling moment should be taken into account. In summer time, abortion due to adverse environmental effects is expected to be substantially lower, which enhances testing of the hypothesized abortion of 4a6b gametophytes on seed set.

Examination of Seed weight.

Results: Against expectations, the seed set of the reference lines differed from each other. In response, seed weight was examined aiming to gain more insight in the cause of these differences. By examining seed weight, weight of non-aborted seeds of 30 flower heads was introduced as measure of the total energy input of plants in reproduction. Furthermore, the average weight per seed was measured and indicates how total seed weight was distributed over non-aborted seeds. Results on weight measurements of non-aborted seeds of 30 flower heads revealed differences in energy input of plants of the four different genotypes (Figure 10A and 10B). BIL[4.1+6.3] puts a substantial lower amount of energy in seed production. Distribution of seed weight differed per genotype as well (Figure 10C). BIL4.1 produced the lightest seeds; *L. sativa* cv. Olof produced the heaviest seeds. An additional observation concerned an overall deviation of labelling 2 for weight of non-aborted seed per 30 flowers and weight per seed. The total energy input is the lowest for labelling 2 in comparison with labelling moment 1 and 3 for all genotypes. Additionally, seed weight per seed is the highest under labelling 2 in comparison with labelling moment 1 and 3 for all genotypes. This trend was most evident for *L. sativa* cv. Olof and less evident for BIL4.1.

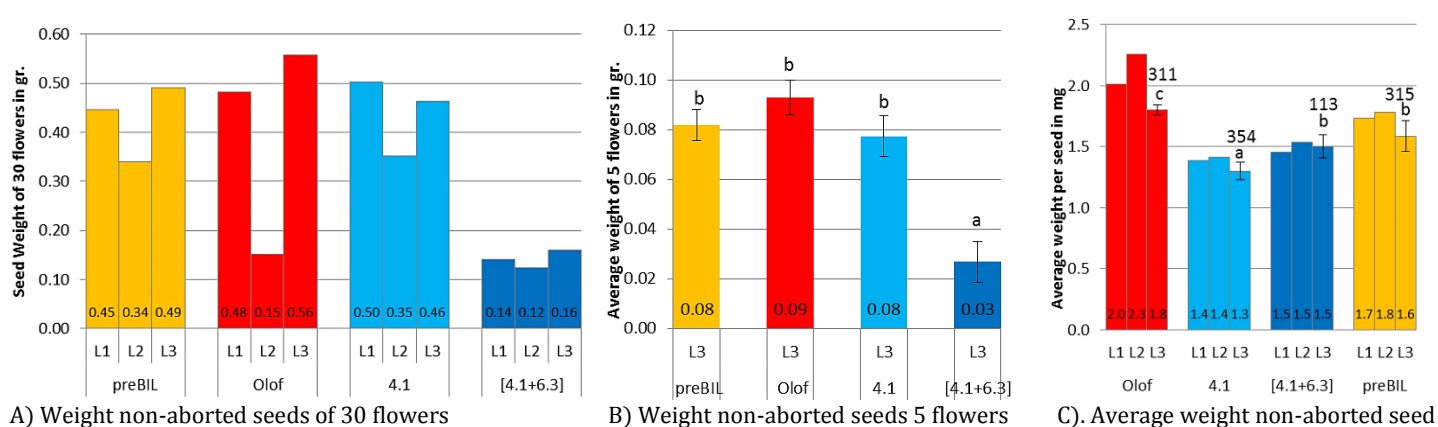


Figure 10. Assessment of Seed weight. 'preBIL'=preBIL[4.1+6.3], 'Olof'= *L. sativa* cv. Olof, '4.1' = BIL4.1, '[4.1+6.3]'= BIL[4.1+6.3]. 'L1', 'L2', 'L3' = Labeling 1, 2, 3 respectively. Statistical differences are indicated by 'a', 'b' and 'c'. Weight of non-aborted seeds of 30 flower heads indicates total energy input in reproduction (A). The average weight per non-aborted seed indicates the distribution of seed weight (C). Statistical testing is based on data of labelling 3. Weight of 5 flower heads was used as measure for total energy input in reproduction to enable statistical testing (B).

Discussion: These data revealed genotype-dependented trade-offs between the energy put in seed production and the weight per seed. Indirectly, the observed varietal organisation in reproduction represents differences in reproduction strategies by the inbred parents *L. sativa* and *L. saligna*. Taking the presumed effects of winter time and developmental stage of the plants on seed set at labelling 2 and the observations of seed weight under labelling 2 in consideration, it seems the trade-off is adaptable to environmental effects and the developmental stage of the plant. Furthermore, plants of different genotypes seem to adapt differently. These kind of relationships are described for many plant species (Baker, 1972).

Interaction between labelling and the amount of non-aborted seeds are already statistically demonstrated. In assessing seed weight, interaction effects between genotype and the trade-off total amount of seeds and weight per seed seem to show up as well but could be not be statistical underpinned. In conclusion, the examination of seed weight revealed that the reference lines differed too in energy input in reproduction and weight per seed in additional to unforeseen differences in abortion rates within the set of reference lines.

Speculations on reference line BIL[4.1+6.3]. The most aberrant line in seed set analysis was BIL[4.1+6.3]. Apart from a moderate weight per seed, BIL[4.1+6.3] is characterized by high rates of abortion (77%). It is unclear whether these high rates of abortion result from an extreme reaction on wintertime or that these high rates of abortion have another genetic reason. BIL[4.1+6.3] is homozygous for its *L. saligna* introgression segments and has a homozygous *L. sativa* background. In reproduction, genetically identical gametophytes are formed. Therefore, distorted segregation ratios

are excluded as a legitimate reason for high abortion ratios. Taking into consideration that plants of BIL[4.1+6.3] reacted minimally in terms of deviated seed set under labelling moment 2, this may indicate the BIL[4.1+6.3] already reacted much earlier on winter circumstances.

Speculations on the Genetic Architecture of HI[4.1+6.3]

Discussion: In the section vitality testing of male gametophytes and in the section seed set analysis, new hypotheses for non-transmission of 4a6b gametophytes were suggested based on outcomes of functionality testing. In this section, all suggested hypotheses are taken together and discussed.

By means of the pollen vitality pilot, the hypothesis of non-transmission of male 4a6b gametophytes caused by non-vitality of the pollen grains was rejected. This either implies male 4a6b gametophytes are not formed in meiosis or implies male 4a6b gametophytes are formed but dysfunctional in later developmental stages. In seed set analysis, the hypothesis (H2) resulting in 56,5% aborted seeds for the test line due to female 4a6b gametophytes and the assumption that male 4a6b gametophytes cannot be compensated by other vital pollen was considered as very unlikely, as only an abortion rate of 35% was observed. It is unclear whether the rate of 35% abortion was caused by environmental conditions (winter time) and the developmental stage of the plants only or that it was caused by a combination of preferential abortion of 4a6b gametophytes and winter circumstances.

Taken together, male 4a6b gametophytes are either not formed in meiosis or dysfunctional in a later developmental stage. The female 4a6b gametophytes are either not formed too or are aborted. In general, it would be more likely that 4a6b gametophytes cause obstruction of one process than 4a6b gametophytes obstructing two different processes. The hypotheses for female and male gametophytes have non-formation of 4a6b gametophytes in meiosis as a common hypothesis. Therefore, non-formation of 4a6b gametophytes in meiosis is considered as the most likely hypothesis for non-transmission of 4a6b gametophytes.

HI[4.1+6.3] classified as a di-genic si-TRD. Elaborating on this hypothesis, no concepts are available that perfectly fit the hypothesis of non-formation of 4a6b gametophytes in meiosis. As mentioned in the introduction, literature does report on sex-independent transmission ratio distortions. The concept of si-TRDs fits the non-transmission hypothesis in the sense male as well as female 4a6b gametophytes are non-transmitted. However, si-TRDs only concern sex independent segregation of one gene. HI[4.1+6.3] concerns sex-independent transmission of two genes. Therefore, the classification of HI[4.1+6.3] as a di-genic si-TRD is introduced here.

4a6b gametophytes and the Interaction of Joint Fate. A transmission ratio distortion caused by malfunction of meiosis relates to meiotic drive. In meiotic drive, an allele or chromosomal segment is preferentially recovered in the next generation of heterozygous parents, resulting in an allele frequency >0.5. In genic drive, there is an interaction between two genetic elements: a drive allele of a drive locus and a target allele of a target locus.

The allele frequencies of double heterozygous preBIL[4.1+6.3] plants from one generation to the next are expected to be 4a/4b, 50:50 and 6a/6b, 50:50. However, the observed allele frequencies were 33:67 for 4a/4b and 67:33 of 6a/6b (calculations derived from expected segregation under non-transmission of 4a6b gametophytes, see figure 1).

The *L. saligna* HI-gene at C6.3 may be denoted as the drive allele, with the *L. sativa* allele at HI-gene C4.1 as target allele since *L. saligna* HI-gene at C6.3 causes HI[4.1+6.3]. However, instead of the *L. saligna* allele of HI-gene at C6.3 (6b) being preferential recovered of this allele in the next generation, the allele frequency drops pairwise with the *L. sativa* allele of HI-gene at C4.1 (4a). Instead of presence of a selfish gene, it is hypothesized one could speak of an interaction of Joint Fate: drive allele 6b and target allele 4a ending up in the same meiotic product cause their own downfall.

4. CONCLUSION

Fine mapping of HI[4.1+6.3] by means of exclusion resulted in HI[4.1+6.3] to be narrowed down to 5.1 cM (16.1-21.2 cM) and 3.7 cM (0.9-4.6 cM) intervals. In exact terms, according to physical positions of the markers, regions are mapped at 7.9-37.9 Mbp and 0-7.8 Mbp for C4.1 and C6.3. Positioning of the markers with Joinmap is done in this study but becomes less important in the course of the time due to the availability of exact positions of the markers beforehand. Based on assessment of the success rate of primer design, success seems increased by strategic positioning of the markers, with intron-based markers being more successful than exon-based markers. In further fine mapping, new markers need to be developed. Informative plant material is still available to forward fine mapping.

In the second part of this study HI[4.1+6.3] was characterized by means of functionality tests. The functionality tests were designed to supply direct evidence for the non-transmission hypothesis of 4a6b gametophytes. This goal was not met. In vitality testing of male gametophytes, non-vitality as cause for non-transmission was rejected. Therefore, male 4a6b gametophytes are not-formed in meiosis or dysfunctional in later developmental stages. In seed set analysis, abortion of female 4a6b gametophytes is not accepted nor rejected. The results of the seed set analysis were overshadowed by effects of winter time. Hypothesis testing was complicated by differential results of the reference lines. Additional observations concerning seed weight extended insight in the differences between the reference lines and the winter time effects. It revealed a trade-off between the amount of energy put in seed production and weight per seed. This trade-off was genotype-dependent and adaptable to environmental effects. Taken results of functionality testing together, the hypothesis of non-formation of 4a6b gametophytes in meiosis was considered to be the most likely hypothesis for non-transmission of 4a6b gametophytes. In describing this hypothesis, HI[4.1+6.3] was classified as a di-genic si-TRD caused by meiotic drive. An interaction of Joint Fate is suggested to explain meiotic drive. These speculations need to be tested in further research. Testing can be done by analyzing tetrads (oral communication R. Niks) of microspores, seed set analysis in summer time and a pollen germination test. Examples of microspore assessments are available in literature. Horner for instance, assessed microspores in a microscopic study of male-fertile and cytoplasmic male-sterile Sunflower (*Helianthus Annuus*) in which abortion of tetrads are observed (Horner, 1977).

5. RECOMMENDATIONS

In further research, it would be interesting to examine the success of primer design again to see whether the same trends are observed. Strategic positioning of the primers may enhance primer design. New markers need to be developed for further fine mapping HI[4.1+6.3]. Informative plant material is still available to forward fine mapping.

In characterization of HI[4.1+6.3], the suggested hypothesis of non-transmission of 4a6b gametophytes caused by meiotic drive needs to be tested. A seed set analysis, an analysis of microspores and a pollen tube germination test might provide the decisive answers. In seed set analysis is advisable to include also wild parent *L. saligna* and to test under optimal seed maturation condition in spring or summer. Analysis of microspores may be preferred over a seed set analysis due to examination in an earlier stage of reproduction.

At last, a literature study on meiotic drive is advisable in order to gain a solid fundamental background on the mechanisms of meiotic drive and the type of genes involved. This would ease judgment whether the identified candidate genes have an increased probability of being involved in HI[4.1+6.3].

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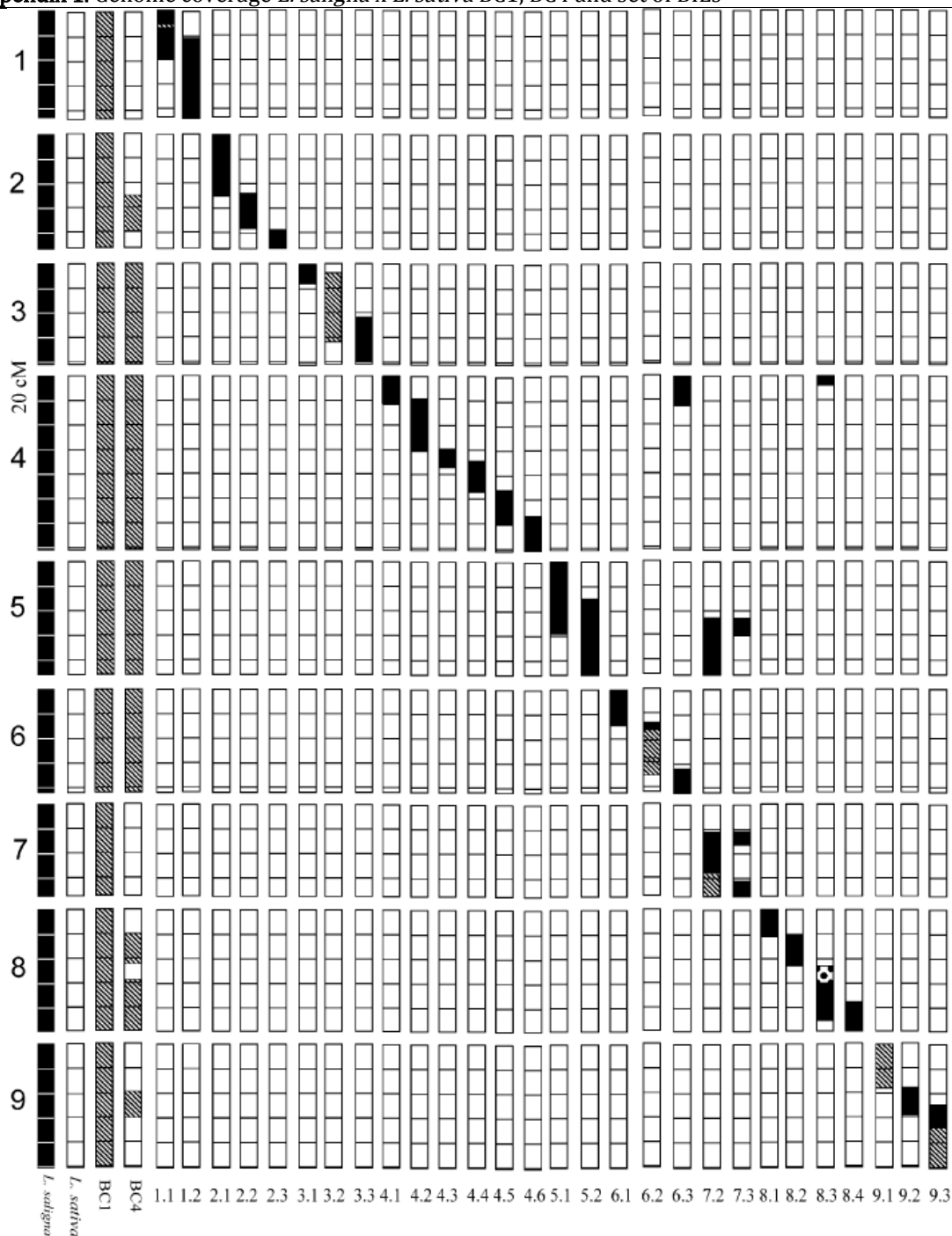
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8. APPENDICES

Appendix 1. Genome coverage *L. saligna* x *L. sativa* BC1, BC4 and set of BILs



This appendix provides an overview of constructed of BILs by Jeuken and Lindhout (2004). The set of 28 *L. saligna* x *L. sativa* BILs (1.1-9.3) covers more than 96% of the *L. saligna* genome. Vertical bars represent the nine chromosomes of lettuce. The chromosomes are segmented in 20-cM intervals that are delimited by horizontal lines. The genomes of the BC1 and BC4 populations and of the 28 backcross lines are indicated in black, white and diagonal stripes: white homozygous *L. sativa* Olof, black homozygous *L. saligna*, diagonal stripes heterozygous. Dot indicates an unknown genotype as not enough markers were analysed in that region. Introgressions of *L. saligna* are shown until the outermost analysed marker. Therefore, this is a minimal representation of the genome coverage. The motive of this M.Sc. thesis arose from the observation that BIL6.3 contains a *L. saligna* introgression segment on chromosome 6 and 4.

Appendix 2A. Overview Genotypic Information Selected Plants of F2_1997_FR Mapping Population for Chromosome 4.1

Genotype*** per plant, with indicated plant number*			*** Mapping Population for Chromosome A1																																															
Marker**	cM		4	5	6	8	11	14	16	19	23	30	31	34	41	42	46	48	57	60	61	64	65	66	69	72	75	81	86	91	92	95	96	97	99	100	108	109	110	113	118	119	123	125	126	133				
E35M48-323	0.0	d	B	d	d	B	d	d	d	B	B	d	B	d	d	d	d	B	d	d	B	-	B	d	-	d	B	-	d	B	d	d	d	-	d	d	d	d	d	-	d	d	d	-	B	d				
NL1151	4.5	B	B	H	H	B	H	H	B	B	B	H	B	H	A	A	A	A	B	H	A	B	B	B	H	B	H	B	B	B	B	H	H	H	B	H	H	H	A	H	B	H	H	H	B	B	B	H		
E44M49-84sal	5.6	c	B	H	H	B	A	H	B	B	B	H	c	-	A	A	A	B	H	A	B	B	c	H	B	A	B	B	B	B	A	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NL0293	5.8	B	B	H	H	B	A	H	B	B	H	B	H	A	A	A	H	B	H	-	B	B	B	H	B	A	B	B	B	B	A	H	H	B	H	H	H	A	H	B	H	H	H	H	B	B	H			
NL0293_WUR	6.0	-	B	H	H	-	-	H	-	B	d	B	H	-	-	-	d	B	-	-	B	c	B	-	B	A	B	B	B	B	A	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
E35M49-582	6.2	H	B	H	c	B	H	H	B	B	H	B	H	A	d	B	d	B	d	d	B	B	B	H	B	A	B	B	B	B	B	d	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NL1260	8.2	H	-	H	H	B	A	H	B	B	H	B	H	A	A	H	H	B	H	A	B	B	B	H	B	A	B	B	B	H	B	A	B	H	B	B	H	H	H	A	H	B	B	H	H	B	B	H		
E35M60-128	8.4	H	B	H	H	B	d	H	B	B	H	B	H	H	H	H	H	B	c	A	B	B	B	H	B	A	B	B	B	H	B	A	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
NL0514	9.8	H	B	B	H	H	-	H	B	B	H	B	H	H	H	H	H	B	H	A	B	B	B	H	B	A	B	B	B	H	B	H	B	H	B	B	B	H	H	H	A	H	B	B	H	H	B	B	H	
E35M49-153	10.5	H	B	B	A	H	A	d	B	B	H	B	H	B	H	H	H	H	B	H	A	B	B	B	H	B	A	B	B	B	H	B	H	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
E35M59-104	11.5	H	B	B	H	H	d	H	B	B	H	B	H	H	H	d	H	B	B	H	B	B	-	H	c	A	B	B	B	H	H	H	c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E45M48-249	12.9	d	B	B	d	d	d	d	B	B	d	d	d	-	B	B	B	B	d	B	B	B	d	B	d	B	B	B	d	d	d	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E38M54-110	13.6	d	B	B	d	d	d	d	-	d	d	B	d	d	d	B	d	-	-	d	B	B	B	-	B	d	B	B	-	d	d	d	B	d	B	B	d	d	d	d	d	B	d	B	d	d	B	B	d	
NL0884	16.9	H	B	B	A	H	A	H	B	H	A	H	A	H	H	H	B	H	B	A	B	B	B	H	A	A	B	B	A	A	H	H	B	H	B	A	A	H	A	H	H	H	H	B	A	H	B	B		
NL0261	17.9	H	B	B	A	H	-	H	B	H	A	H	H	H	H	B	H	B	B	A	B	H	B	H	A	A	A	H	B	A	A	H	H	B	H	B	A	A	H	A	H	H	H	B	A	H	B	B		
NL0897	18.4	H	B	B	A	H	A	H	B	H	A	H	H	H	H	B	H	B	B	A	B	H	B	H	A	A	A	H	H	A	A	H	H	B	H	B	A	A	H	A	H	H	H	B	A	H	B	B		
LK1525	21.8	H	H	B	A	-	A	H	B	H	A	H	H	H	B	H	H	B	A	H	H	H	H	A	A	H	A	H	A	H	H	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
E35M48-263	23.4	d	d	-	d	d	d	B	B	d	d	d	d	d	B	d	d	B	d	-	d	d	d	d	d	d	d	d	d	d	d	d	B	B	B	d	d	d	d	d	d	d	d	d	d	d	d	d	B	
NL1186	24.4	H	H	B	A	B	A	H	B	H	A	H	H	A	B	H	H	B	A	H	H	H	A	A	A	A	H	H	A	H	H	H	B	B	H	A	B	A	H	A	H	A	H	A	H	H	B			
E35M49-298sal	24.5	H	H	-	A	B	A	H	H	H	A	H	H	A	B	H	H	c	A	c	c	c	A	A	A	H	c	A	H	c	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LE4022	25.6	d	d	B	d	-	d	d	-	d	d	d	d	d	B	d	d	B	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
E38M54-494	27.4	d	d	B	d	B	d	d	d	d	d	d	d	d	B	d	d	d	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		

* Selection of recombinant C4.1 plants was based on presented genotypic information and enabled positioning of newly developed markers and fine mapping of HI[4.1+6.3]. Plant numbers in bold were informative in fine mapping. **E. den Boer mapped HI[4.1+6.3] by means of listed markers: the suspected region is marked light pink. Flanking regions are marked light grey. Overlapping marker LK1525 is the closest link between the lower border of the suspected region and the physical map is (marked grey), mapped at 65 Mbp according to Gbrowse Lattuga version 3.2. By our information, no overlapping marker was available between physical map and the F2_1997 FR map for the upper border of the target region. ***Genetic terminology applied as introduced in table 1.

Appendix 2B. Overview Genotypic Information Selected Plants of F2_1997_FR Mapping Population for Chromosome 6.3

		Genotype*** per plant, with indicated plant number*																																								
Marker**	cM	1	2	5	6	15	16	18	27	31	33	36	38	47	49	58	60	62	64	68	79	80	82	83	84	87	89	91	92	94	95	96	97	101	109	117	118	121	123	127		
E35M49-410sal	82.3	A	H	H	A	H	-	H	-	c	A	A	H	H	H	A	A	A	B	c	A	A	c	A	A	A	H	c	c	H	B	-	-	-	-	-	-	-	-	-	-	
LE1211	81.9	A	H	H	A	H	A	H	H	H	A	A	A	H	H	A	A	A	B	H	A	A	H	A	A	A	H	H	H	H	B	-	-	-	-	-	-	-	-	-	-	
NL0418	81.6	A	-	H	A	H	A	H	H	H	A	A	H	H	H	A	A	A	B	H	A	A	H	A	A	A	H	H	H	H	B	A	A	H	H	A	H	H	H	H		
NL0833	80.6	A	-	H	A	H	A	H	H	A	A	A	H	-	H	A	A	A	B	H	A	-	H	A	A	A	H	H	H	-	-	-	H	H	A	A	H	A	H	H	H	
E44M49-352sal	79.6	A	A	H	H	H	A	H	H	A	A	A	H	H	H	A	A	A	B	H	A	A	H	A	A	A	H	H	H	H	c	-	-	-	-	-	-	-	-	-	-	
E45M48-300	79.4	A	A	H	H	H	d	H	H	A	A	A	d	H	H	A	H	A	B	d	d	A	H	A	A	A	H	H	H	c	B	-	-	-	-	-	-	-	-	-	-	
E54M48-216Asato	79.0	-	d	d	-	d	d	d	d	d	d	d	d	d	d	d	d	d	B	d	d	d	d	d	d	d	d	d	d	-	B	d	d	d	d	d	d	d	-	d		
E35M59-105sal	76.1	A	A	c	c	c	A	c	c	A	A	A	c	c	c	A	c	A	c	c	-	c	c	-	A	-	c	c	c	-	c	-	-	-	-	-	-	-	-	-	-	-
E44M49-87sal	75.9	A	A	H	H	H	A	H	c	A	A	A	H	H	B	A	H	A	H	H	A	H	H	A	A	H	H	H	H	H	H	-	-	-	-	-	-	-	-	-	-	-
E44M49-278sal	74.9	H	A	H	H	B	A	H	H	A	A	A	H	H	B	A	H	A	H	H	A	H	H	A	A	H	H	H	H	H	H	-	-	-	-	-	-	-	-	-	-	-
M7120	74.3	H	A	d	d	B	A	d	d	A	A	d	H	H	B	A	H	A	H	H	A	H	H	A	A	H	H	H	H	H	H	-	-	-	-	-	-	-	-	-	-	-
E33M59-565	74.1	d	A	d	d	B	A	d	d	A	A	d	d	d	B	A	-	A	d	H	A	d	d	A	A	d	d	d	d	d	d	d	d	-	H	H	d	H	B	H	H	
NL1084	72.9	H	-	A	H	B	A	H	H	A	H	A	H	H	B	A	H	H	H	B	A	H	H	A	A	H	H	H	H	H	A	A	H	H	H	H	H	H	B	H	H	
NL0653	72.9	H	-	A	H	B	A	H	H	A	H	A	H	H	B	A	H	H	H	B	A	H	H	A	A	H	H	H	H	H	A	A	H	H	H	H	H	H	B	H	H	
NL1114	69.6	H	-	A	H	B	A	B	H	A	H	H	H	H	B	A	H	H	H	B	H	H	H	A	H	B	H	H	A	H	H	A	A	H	H	H	H	H	H	A	A	
E45M48-103	68.4	H	H	A	H	B	A	B	H	A	d	H	H	A	B	A	H	H	H	d	H	H	H	H	H	d	c	H	H	d	H	-	-	-	-	-	-	-	-	-	-	-
NL0589	67.1	H	-	H	H	B	A	B	B	A	H	H	H	A	B	H	H	H	H	H	H	H	A	H	H	H	B	H	H	A	H	H	A	A	H	H	B	H	H	A	A	
LE1126	66.3	H	A	A	H	B	A	B	B	A	H	H	d	A	B	H	H	H	H	H	H	H	H	H	H	B	B	B	A	A	H	-	-	-	-	-	-	-	-	-	-	-
E35M60-196sal	65.9	c	d	A	H	B	H	c	B	A	H	H	c	A	B	H	H	H	H	H	H	d	H	d	H	c	B	B	d	A	H	-	-	-	-	-	-	-	-	-	-	-

* Footnotes of Appendix 1A are applicable for chromosome 6.3. ** Similar footnotes as Appendix 1A. Overlapping marker E45M38-103 (marked in grey), interconnects the genetic and physical map (at 15,8 Mbp). No overlapping marker was available for the upper border of the target region. *** See footnotes Appendix 1A.

Appendix 3. Parental genotypic information of F3-family

Chromosome	Marker**	cM	F3-seeds*										F2-family (plant numbers)			
			pv09315	pv09319	pv09346	pv09350	pv09345	pv09359	pv09317	pv09318	pv09321	pv09322	pv09333	pv09302	pv09303	pv09301
			pv08521.4	pv08521.13	pv08528.13	pv08529.12	pv08528.12	pv08526.3	pv08521.11	pv08521.12	pv08521.15	pv08521.17	pv08524.20	pv08524.6	pv08524.7	pv08524.3
4.1	NL0293	6,0	B	B	B	B	B	B	B	H	H	H	H	H	H	H
4.1	NL0514	9,8	B	B	B	B	B	B	B	H	H	H	H	H	H	H
4.1	NL0884	16,9	H	H	H	H	H	H	H	B	A	A	A	H	d	H
6.3	LE1211	81,9	H	H	H	H	B	B	B	H	H	H	H	A	A	H
6.3	NL0418	81,6	H	H	H	H	B	B	B	H	H	H	H	A	A	H
6.3	M7120_3	74,3	A	H	H	H	B	B	B	H	H	H	H	H	H	B

*The table presents the selection of F3 seeds based on genotypic information of the parental F2-Family (inbred preBIL4.1+6.3) generated by E. den Boer in a recombinant screen. ** Markers indicated in dark grey are within the suspected regions causing HI[4.1+6.3], flanking regions are marked in light grey. Segregation of heterozygous regions in selected recombinant plants may enable fine mapping of HI[4.1+6.3].

*The marker column lists existing and newly developed markers. Physical positions of newly developed markers are indicated. The background colours of the markers indicate progression in fine mapping. E. den Boer narrowed down HI[4.1+6.3] to the regions in light pink, flanking regions are marked light grey. In this study, HI[4.1+6.3] was narrowed down to the regions dark purple, with flanking regions marked light purple. Appendix 6 elaborates on underpinning of fine mapping indicated by bold dashes. For genetic nomenclature see table 1.

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Appendix 4B. Overview of all marker data F2_1997_FR mapping population at C4.1 and C6.3 (part two)

Marker				Plant Number																																								
Chromosome	Number	Name Marker	Mbp	cM	69	70	72	75	79	80	81	82	83	84	86	87	88	89	91	92	94	95	96	97	99	100	101	108	109	110	113	117	118	119	121	123	125	126	127	133				
4	1	E35M48-323	2.0	0.0	-	d	d	B	d	B	-	d	d	d	d	d	d	d	B	d	H	B	H	H	B	H	H	B	H	A	H	B	A	H	H	H	H	B	B	H	H			
4	2	NL1151		4.5	B	H	H	B	A	B	B	H	H	H	B	H	H	H	B	H	B	H	H	B	H	H	B	H	H	A	H	B	A	H	H	H	H	B	B	H	H			
4	3	E44M49-84sal		5.6	B	H	A	B	A	B	B	H	H	H	B	H	C	H	B	A	B	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
4	4	CLSx10758.b1_K01.ab1			u	u	A	B	-	-	B	-	-	-	-	B	-	H	-	u	A	-	B	H	B	H	H	-	u	A	H	B	-	H	H	-	H	-	-	H				
4	5	NL0293		5.8	B	H	A	B	A	B	B	H	H	H	B	H	H	H	B	A	B	H	H	B	H	H	H	B	H	A	H	B	A	H	H	H	H	B	B	H	H			
4	6	NL0293_WUR		6.0	B	C	A	B	A	B	B	H	H	H	B	C	C	-	B	A	B	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
4	7	E35M49-582		6.2	B	H	A	B	A	B	B	H	d	H	B	H	H	H	B	d	B	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
4	8	NL1260		8.2	B	H	A	B	A	B	B	H	H	H	H	H	H	H	H	B	A	B	B	B	B	H	H	B	H	A	H	B	A	H	B	B	H	H	B	H	H			
4	9	CLLY8178.b1_D05.ab1		7.9	B	u	A	B	-	-	B	-	-	-	-	H	-	H	-	u	A	-	B	H	B	B	H	H	-	H	A	H	B	-	H	B	-	H	-	-	H			
4	10	E35M60-128		8.4	B	H	A	B	A	B	B	H	H	H	H	H	H	H	B	A	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
4	11	NL0514		9.8	B	H	A	B	A	B	B	B	H	H	H	H	H	H	H	B	B	B	B	B	B	H	B	H	B	H	A	H	B	A	H	B	H	H	H	B	H	H		
4	12	CLS55115.b1_F07.ab1	23.9		B	H	A	B	-	-	B	-	-	-	H	-	H	-	B	H	-	B	H	B	H	H	-	H	A	H	B	-	H	B	-	H	-	-	-	H				
4	13	CLS_S3_Contig2928	29.9		B	H	A	B	-	-	B	-	-	-	H	-	H	-	B	H	-	B	H	B	B	H	H	-	H	A	H	B	-	H	B	-	H	-	-	-	H			
4	14	E35M49-153	10.5		B	H	A	B	A	B	B	H	H	H	H	H	H	H	B	B	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
4	15	CLS_S3_Contig2219	37.9		B	H	A	B	-	-	B	-	-	-	H	-	H	-	H	H	-	B	B	B	A	H	-	H	H	H	B	-	H	B	-	A	-	-	-	-	B			
4	16	E35M59-104	11.5		C	H	A	B	A	B	B	H	H	H	H	H	H	H	H	H	B	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
4	17	E45M48-249	12.9		B	d	d	B	d	B	B	d	-	d	d	d	d	d	d	d	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
4	18	E38M54-110	13.6		B	d	d	B	d	B	-	d	d	d	d	d	d	d	d	d	B	B	d	B	d	d	-	d	d	d	B	d	d	B	d	d	d	B	d	d				
4	19	QGG20102.yg.ab1	41.9		B	H	A	B	-	-	B	-	-	-	H	-	H	-	H	H	-	B	H	B	A	H	-	H	H	H	B	-	H	B	-	A	-	-	-	B				
4	21	CLS2433.b1_B09.ab1	50.0		d	d	d	B	-	-	B	-	-	-	d	-	d	-	d	d	-	B	d	B	d	d	-	d	d	d	B	-	d	B	-	d	-	-	-	-	B			
4	22	CLLY1908.b1_G01.ab1	52.8		A	H	A	B	-	-	B	-	-	-	A	-	H	-	H	H	-	B	H	B	A	H	-	H	H	H	B	-	H	B	-	A	-	-	-	-	B			
4	23	CLS_S3_Contig7077	53.8		A	H	A	B	-	-	B	-	-	-	A	-	H	-	H	H	-	B	H	B	A	H	-	H	H	H	B	-	H	B	-	A	-	-	-	-	B			
4	24	NL0884	16.9		A	H	A	B	A	B	B	H	H	H	A	H	H	H	H	B	B	H	B	B	A	H	-	B	A	H	H	H	A	H	B	H	A	H	B	H	B			
4	25	NL0261	17.9		A	H	A	B	A	B	B	H	H	H	A	H	H	H	H	B	B	B	B	A	H	-	B	A	H	H	H	A	H	B	H	A	H	B	H	B				
4	26	CLS_S3_Contig4465	58.0		A	H	A	H	-	-	H	-	-	-	A	-	H	-	H	H	-	B	H	B	A	H	-	A	H	H	H	-	H	B	-	A	-	-	-	-	B			
4	27	NL0897	18.4		A	H	A	H	A	B	H	H	H	H	A	H	H	H	H	B	B	H	B	B	A	H	-	B	A	H	H	H	A	H	B	H	A	H	B	H	B			
4	28	CLS_S3_Contig5375	60.4		d	H	A	H	-	-	H	-	-	-	d	-	H	-	d	H	-	B	H	B	d	H	-	d	H	H	H	-	H	B	-	A	-	-	-	-	B			
4	29	LSS_S3_Contig1360	61.9		A	H	A	H	-	-	H	-	-	-	A	-	H	-	H	-	B	H	H	A	H	-	-	A	H	H	H	-	H	H	-	A	-	-	-	-	-	B		
4	30	LK1525	21.8		A	H	A	H	A	B	H	H	H	H	A	H	H	H	H	B	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
4	31	E35M48-263	23.4		d	d	d	d	d	B	d	d	d	d	d	d	d	d	d	d	B	B	B	-	d	d	d	B	d	d	d	d	d	d	d	d	d	d	d	d	B			
4	32	NL1186	24.4		A	H	A	H	A	B	H	H	H	H	A	H	H	H	H	B	H	B	H	A	B	B	A	H	-	B	A	H	A	A	H	-	A	H	H	H	B			
4	33	E35M49-298sal	24.5		A	B	A	H	A	B	C	H	H	H	A	d	B	d	H	C	C	H	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
4	34	LE4022	25.6		d	d	d	d	d	B	d	d	d	d	d	d	d	d	d	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
4	35	E38M54-494	27.4		d	d	d	d	d	B	d	d	d	d	d	d	d	d	d	d	d	-	-	d	d	-	B	d	d	d	d	d	d	d	d	d	d	d	d	-	d	B		
6	1	E35M49-410sal	82.3		H	A	A	C	A	A	C	C	A	A	A	A	A	A	H	C	C	H	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
6	2	LE1211	81.9		H	A	A	B	A	A	H	H	A	A	A	A	A	A	H	H	H	H	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
6	3	NL0418	81.6		H	A	A	B	A	A	H	H	A	A	A	A	A	A	H	H	H	H	B	A	A	A	A	H	A	H	H	B	A	H	H	H	H	A	A	H	H			
6	4	NL0833	80.6		H	-	A	B	A	-	H	H	A	A	A	A	A	A	H	H	H	-	-	H	A	A	A	H	A	A	H	B	A	H	H	A	H	A	A	H	H			
6	5	CLS_S3_Contig6649	3.8		-	-	-	-	A	A	-	H	e	A	-	A	-	-	H	H	H	H	B	A	H	-	H	-	H	-	A	-	A	H	-	A	H	-	A	H	-	H	-	
6	6	CLSY4646.b1_K09.ab1	7.8		-	-	-	-	A	A	-	H	A	A	-	A	-	-	H	H	H	u	B	A	A	-	-	H	-	H	-	A	H	-	H	H	-	H	-	-	H	-		
6	7	CLS_S3_Contig3369	10.0		-	-	-	-	A	A	-	H	A	A	-	A	-	-	H	H	H	H	B	A	A	-	-	H	-	H	-	A	H	-	H	H	-	H	-	-	H	-		
6	8	CLS_S3_Contig1511	14.3		-	-	-	-	A	A	-	H	A	A	-	A	-	-	H	H	H	H	B	u	-	-	-	H	-	H	-	A	H	-	H	H	-	H	-	-	H	-		
6	9	CLSM10904.b1_P13.ab1	17.9		-	-	-	-	A	A	-	H	A	A	-	A	-	-	H	H	H	H	B	A	A	-	-	H	-	H	-	A	H	-	H	H	-	H	-	-	-	H	-	
6	10	QGA7F22.yg.ab1	22.0		-	-	-	-	A	H	-	H	A	A	-	A	-	-	H	H	H	H	B	A	A	-	-	H	-	H	-	A	H	-	H	H	-	H	-	-	-	-	H	-
6	11	CLLY9820.b1_G08.ab1	26.0		-	-	-	-	A	H	-	H	A	A	-	A	-</																											

Appendix 5A. Overview Marker Data F3-families. Informative F3-families at C4.1(1/2)

			pv09321																								pv09345																		
Seed number (F3)			F2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	F2	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
Generation			F2	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F2	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3		
cM	Mbp	Marker	pv08521.15	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv09321	pv08528.12	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345	pv09345		
9,8		NL0293	H																							B																			
	7,9	CLLY8178.b1_D05.ab1		H	A	H	H	A	H	B	H	H	n	H	H	B	H	H	B	H	B	B	B	H	H	B	B																		
	23,9	CLSS5115.b1_F07.ab1		H	A	H	H	A	H	B	H	H	n	H	H	B	H	H	B	H	B	B	B	H	H	B	B		B	B	B	B	B	B	B	n	B	B	B	B	B	B	B	n	
	29,9	CLS_S3_Contig2928		H	A	H	H	A	H	B	H	H	n	H	H	B	H	H	B	H	B	B	B	H	H	B	B		B	B	B	B	B	B	B	n	B	B	B	B	B	B	B	n	
		NL0514	H																							B																			
	37,9	CLS_S3_Contig2219		H	A	H	H	A	H	B	H	H	n	H	H	B	H	H	B	H	B	B	B	H	H	B	B		B	B	H	H	B	H	A	n	A	H	H	H	H	A	A	n	
	41,9	QGG20I02.yg.ab1		H	A	H	H	A	H	B	H	H	n	H	H	B	H	H	B	H	B	B	B	H	H	B	n		B	B	H	H	B	H	A	n	A	H	H	u	H	A	A	n	
	52,8	CLS_S3_Contig8762		H	e	H	H	e	H	e	H	H	n	H	H	e	H	H	e	H	e	e	e	H	H	e	e																		
	50,0	CLSL2433.b1_B09.ab1		H	A	H	H	A	H	B	H	H	n	H	H	B	H	H	B	H	B	B	B	H	H	B	B																		
16,9	52,8	CLLY11908.b1_G01.ab1		A	A	A	A	A	A	A	A	n	A	A	A	A	A	A	A	A	A	A	A	A	A	A																			
	53,8	CLS_S3_Contig7077		A	A	A	A	A	A	A	A	n	A	A	A	A	A	A	A	A	A	A	A	A	A	A																			
		NL0884	A																							H																			
81,6		NL0418	H																							B																			
	3,8	CLS_S3_Contig6649		H	A	A	A	A	H	H	A	A	n	A	A	H	A	A	B	A	H	H	H	A	A	A	A		B	B	B	B	B	B	B	n	B	B	B	B	B	B	B	n	
	7,8	Y4646.b1_K09.ab1																																											
	10,0	CLS_S3_Contig3369		H	A	A	A	A	H	H	A	A	n	A	A	H	A	A	B	A	H	H	H	A	A	A	A																		
	14,3	CLS_S3_Contig1511		H	A	A	A	A	H	H	A	A	n	A	A	H	A	A	B	A	H	H	H	A	A	A	A																		
	17,9	CLSM10904.b1_P13.ab1		H	A	A	A	A	H	H	A	A	n	A	A	H	A	A	B	A	H	H	H	A	A	A	A																		
	22,0	QGA7F22.yg.ab1		H	A	A	A	A	H	H	A	A	n	A	A	H	A	A	B	A	H	H	H	A	A	A	A																		
	26,0	CLLY9820.b1_G08.ab1		H	A	A	A	A	H	H	A	A	n	A	A	H	A	A	B	A	H	H	H	A	A	A	A																		
	74,3		M7120_3	H																							B																		

Per F3-family the genotypic information of the parental line is presented first (delineated in black), the horizontal line indicate the transition from markers of chromosome 4 to 6, fine mapping results are indicated with horizontal dashes, exclamation markers indicate data point that were considered to be unreliable. Markers in pink indicate the HI-regions as mapped by E. den Boer by means of F2_1997_FR. Markers used in the F2 recombinant screen of inbred preBIL[4.1+6.3] progenies are indicated in blue (recombinant screen by E. den Boer). Dark blue indicates the marker was within the suspected region, light blue indicates flanking markers. Markers in dark purple present region as fine mapped in this study with in light purple the flanking markers. These footnotes apply for all subsections of appendix 5.

Appendix 5A. Overview Marker Data F3-families. Informative F3-families at C4.1(2/2)

Appendix 5.11 Overview Data for marker information for F2 and F3

			pv09317																	pv09318																
Seed number (F3)			pv09317	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	pv09318	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Generation			F2	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F2	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3		
			pv08521.11	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv08521.12	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318		
cM	Mbp	Marker	pv08521.11	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv09317	pv08521.12	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318	pv09318		
9,8		NL0293	B																	H	H	H	H	n	B	A	H	A	A	H	B	H	H	n	H	H
	7,9	CLLY8178.b1_D05.ab1																																		
	23,9	CLSS5115.b1_F07.ab1		n	B	B	n	B	B	B	B	B	B	B	B	n	B	B	n		B	B	B	B	B	B	B	B	n	n	B	B	B	B	B	
	29,9	CLS_S3_Contig2928		n	B	B	n	B	B	B	B	n	B	B	B	n	B	B	B	!	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	
		NL0514	B																	H	H	H	H	d	B	A	H	A	A	H	B	H	H	H	H	
	37,9	CLS_S3_Contig2219		n	A	H	n	A	H	A	H	n	B	A	H	n	H	H	n		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	
	41,9	QGG20102.yg.ab1		B	A	H	B	A	H	A	B	A	B	A	H	n	H	H	n		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	
	52,8	CLS_S3_Contig8762																																		
	50,0	CLSL2433.b1_B09.ab1																																		
	52,8	CLLY11908.b1_G01.ab1																																		
	53,8	CLS_S3_Contig7077																																		
16,9		NL0884	H																	B																
81,6		NL0418	B																	H																
	3,8	CLS_S3_Contig6649		n	B	B	n	B	B	B	B	B	B	B	B	n	B	B	B		H	H	H	B	A	A	A	B	B	H	H	A	n	H	H	A
	7,8	Y4646.b1_K09.ab1		n	B	B	n	B	B	B	B	B	B	B	B	n	B	B	B		H	H	H	B	A	A	A	B	B	H	H	A	A	H	H	A
	10,0	CLS_S3_Contig3369																																		
	14,3	CLS_S3_Contig1511																																		
	17,9	CLSM10904.b1_P13.ab1																																		
	22,0	QGA7F22.yg.ab1																																		
	26,0	CLLY9820.b1_G08.ab1																																		
	74,3		M7120_3	B																	H															

For footnotes see appendix 5A(1/2).

Appendix 5B. Overview Marker Data F3-families. Informative F3-families at C4.6.3

[illegible]

For footnotes see appendix 5A(1/2).

Appendix 5C. Overview Marker Data F3-families not informative in fine mapping (1/4)

[illegible]

For footnotes see appendix 5A(1/2).

Appendix 5C. Overview Marker Data F3-families not informative in fine mapping (2/4)

[illegible]

For footnotes see appendix 5A(1/2).

Appendix 5C. Overview Marker Data F3-families not informative in fine mapping (3/4)

[illegible]

For footnotes see appendix 5A(1/2).

Appendix 5C. Overview Marker Data F3-families not informative in fine mapping (4/4)

[illegible]

For footnotes see appendix 5A(1/2).

Appendix 5D. Overview Marker Data of F3-families very likely not informative in fine mapping

			pv09319																	pv09333																								
Seed number (F3)			pv09319	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	pv09333	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
Generation			F2	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F2	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3	F3		
			pv08521.13																	pv08524.20																								
cM	Mbp	Marker	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09319	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333	pv09333		
9,8		NL0293	B																	H																								
	7,9	CLLY8178.b1_D05.ab1																																										
	23,9	CLSS5115.b1_F07.ab1		B	B	B	B	B	B	B	B	B	A	A	n	n	n	n	n	A		A	H	B	H	H	H	B	H	A	H	A	H	H	H	A	A	H	c	B	A	A	H	A
	29,9	CLS_S3_Contig2928		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B			A	H	B	H	H	H	B	H	A	H	A	H	H	H	A	A	H	B	B	A	A	n	A
		NL0514	B																	H																								
	37,9	CLS_S3_Contig2219		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B																									
	41,9	QGG20102.yg.ab1		B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B			A	H	B	H	H	H	B	n	A	H	A	H	H	H	A	A	H	B	B	A	A	H	A
	52,8	CLS_S3_Contig8762																																										
	50,0	CLSL2433.b1_B09.ab1																																										
	52,8	CLLY11908.b1_G01.ab1																																										
	53,8	CLS_S3_Contig7077																																										
16,9		NL0884	H																	A																								
81,6		NL0418	H																	H																								
74,3	3,8	CLS_S3_Contig6649		B	H	A	H	A	A	H	A	H	H	B	H	H	B	H	B		A	A	A	A	A	A	A	A	n	A	A	A	A	A	A	n	A	A	A	n	n	n	n	
	7,8	Y4646.b1_K09.ab1		B	H	A	H	A	A	H	A	H	H	B	H	H	B	c	B		n	A	A	B	B	B	n	B	n	A	A	B	n	n	n	n	B	n	H	A	A	n	n	
	10,0	CLS_S3_Contig3369																																										
	14,3	CLS_S3_Contig1511																																										
	17,9	CLSM10904.b1_P13.ab1																																										
	22,0	QGA7F22.yg.ab1																																										
	26,0	CLLY9820.b1_G08.ab1																																										
		M7120_3	H																	H																								

For footnotes see appendix 5A(1/2).

Appendix 6A. Overview of informative plants in Fine Mapping C4.1

Population					F2_1997_FR mapping population**										F3-Family***							
					Replications of plants										2x	5x	7x	4x	4x	5x	3x	
Map					Plant Number																	
Chromosome	Number	Markers*	Mbp	cM	41	95	48	75	113	30	69	123	42	92	pV09321	pV09321	pV09345	pV09345	pV09317	pV09317	pV09318	
4	4	CLSx10758.b1_K01.ab1	2.0		A	H	B	B	B	B	H	u	H	A	A	-	-	-	-	-	-	-
4	5	NL0293		5.8	A	H	B	B	B	B	H	B	H	A	A	-	-	-	-	-	-	A
4	6	E35M49-582		6.2	A	H	B	B	-	H	B	-	d	d	-	-	-	-	-	-	-	
4	7	NL1260		8.2	A	B	B	B	B	B	H	B	H	H	A	-	-	-	-	-	-	
4	8	CLLY8178.b1_D05.ab1	7.9		H	B	B	B	B	B	H	B	H	H	A	H	B	-	-	-	A	
4	9	E35M60-128		8.4	H	B	B	B	-	H	B	-	H	A	-	-	-	-	-	-	-	
4	10	NL0514		9.8	H	B	B	B	B	B	H	B	H	H	H	-	-	-	-	-	A	
4	11	E35M49-153		10.5	H	B	B	B	-	H	B	-	H	H	-	-	-	-	-	-	-	
4	12	CLSS5115.b1_F07.ab1	23.9		H	B	B	B	B	B	H	B	H	H	H	H	B	B	B	B	B	
4	13	CLS_S3_Contig2928	29.9		H	B	B	B	B	B	H	B	H	H	H	H	B	B	B	B	B	
4	14	E35M59-104		11.5	H	c	B	B	-	H	c	-	d	H	-	-	-	-	-	-	-	
4	15	CLS_S3_Contig2219	37.9		H	B	B	B	B	H	B	A	B	H	H	H	B	H	A	A	H	
4	16	E45M48-249		12.9	-	B	B	B	-	d	B	-	B	d	-	-	-	-	-	-	-	
4	17	E38M54-110		13.6	d	B	-	B	B	d	B	d	B	d	-	-	-	-	-	-	-	
4	18	QGG20I02.yg.ab1	41.9		H	B	B	B	B	H	B	A	B	H	H	B	H	A	A	H	B	
4	19	CLS2433.b1_B09.ab1	50.0		d	B	B	B	B	d	d	d	B	d	H	B	-	-	-	-	-	
4	20	CLLY11908.b1_G01.ab1	52.8		H	B	B	B	B	u	A	A	B	H	A	A	-	-	-	-	-	
4	21	CLS_S3_Contig7077	53.8		H	B	B	B	B	A	A	A	B	H	A	A	-	-	-	-	-	
4	22	NL0884		16.9	H	B	B	B	B	H	A	A	A	B	H	-	-	-	-	-	-	
4	23	NL0261		17.9	H	B	B	B	B	H	A	A	A	B	H	-	-	-	-	-	-	
4	24	CLS_S3_Contig4465		58.0	H	B	B	B	B	H	A	A	A	B	H	-	-	-	-	-	-	
4	25	NL0897		18.4	H	B	B	B	B	H	A	A	A	B	H	-	-	-	-	-	-	
4	26	CLS_S3_Contig5375		60.4	H	B	B	B	B	A	d	A	B	H	-	-	-	-	-	-	-	
4	27	LSS_S3_Contig1360		61.9	H	B	B	B	B	H	A	A	A	B	H	-	-	-	-	-	-	
4	28	LK1525		21.8	H	B	B	B	B	-	A	A	-	B	H	-	-	-	-	-	-	
4	29	E35M48-263		23.4	d	B	d	d	d	d	d	d	B	d	-	-	-	-	-	-	-	
4	30	NL1186		24.4	A	H	H	H	H	A	A	A	B	H	-	-	-	-	-	-	-	
4	31	E35M49-298sal		24.5	A	H	H	H	-	A	A	-	B	c	-	-	-	-	-	-	-	

6	1	E35M49-410sal		82.3	H	B	c	c	-	c	H	-	H	c	-	-	-	-	-	-	-
6	2	LE1211		81.9	H	B	B	B	-	H	H	-	H	H	-	-	-	-	-	-	-
6	3	NL0418		81.6	H	B	B	B	B	H	H	H	H	H	-	-	-	-	-	-	-
6	4	NL0833		80.6	H	-	B	B	B	B	H	H	H	H	H	-	-	-	-	-	-
6	5	CLS_S3_Contig6649	3.8		-	B	-	-	-	-	-	H	-	H	H	H	B	B	B	B	B
6	6	Y4646.b1_K09.ab1	7.8		-	B	-	-	-	-	-	H	-	H	-	-	B	B	B	B	B
6	7	CLS_S3_Contig3369	10.0		-	B	-	-	-	-	-	H	-	H	H	H	-	-	-	-	-
6	8	CLS_S3_Contig1511	14.3		-	B	-	-	-	-	-	H	-	H	H	H	-	-	-	-	-
6	9	CLSM10904.b1_P13.ab1	17.9		-	B	-	-	-	-	-	H	-	H	H	H	-	-	-	-	-
6	10	QGA7F22.yg.ab1	22.0		-	B	-	-	-	-	-	H	-	H	H	H	-	-	-	-	-
6	11	CLLY9820.b1_G08.ab1	26.0		-	c	-	-	-	-	-	B	-	H	H	H	-	-	-	-	-
6	12	E44M49-352sal	79.6		H	c	B	B	-	H	H	-	H	H	-	-	-	-	-	-	-
6	13	E45M48-300	79.4		d	B	B	B	B	-	H	H	-	H	H	-	-	-	-	-	-
6	14	E54M48-216Asato	79.0		d	B	B	B	B	B	d	d	-	d	d	-	-	-	-	-	-
6	15	CLS_S3_Contig4263	31.9		-	H	-	-	-	-	-	B	-	H	-	-	-	-	-	-	-
6	16	E35M59-105sal	76.1		c	c	c	c	-	c	c	-	c	c	-	-	-	-	-	-	-
6	17	E44M49-87sal	75.9		H	H	B	B	-	H	H	-	H	H	-	-	-	-	-	-	-
6	18	CLS_S3_Contig7223	34.0		-	H	-	-	-	-	-	B	-	H	-	-	-	-	-	-	-
6	19	E44M49-278sal	74.9		H	H	B	B	-	H	H	-	H	H	-	-	-	-	-	-	-
6	20	M7120	74.3		H	H	B	B	B	-	d	H	-	H	H	-	-	-	-	-	-

Summary of Analysis****

Map		Presence of non-transmission genotypes with plant numbers		
Number	Marker	4A6H	4A6B	4H6B
1	CLSx10758.b1_K01.ab1	3 (F2: 41, 42 92)	1 (F3, pv..18)	1 (F2: 95)
7	NL0514	0	1 (F3, pv..18)	0
9	CLS_S3_Contig2928	0	0	0
12	CLS_S3_Contig2219	1 (F2: 123)	8 (F3: pv..17, pv..45)	2 (F3: pv..45, pv..17)
28	E35M49-298sal	4 (F2: 30, 69 F3: pv..21)	8 (F3: pv..17, pv..45)	6 (F2: 95, 48, 75, 113)

General notes – Non-transmission of 4a6b gametophytes confers absence of genotypes 4a6h, 4a6b and 4h6b (non-transmitted genotypes). Fine mapping was based on exclusion of recombinant regions in presence of non-transmitted genotypes and is indicated by bold dashes. The background colours of the markers indicate progression in fine mapping. E. den Boer narrowed down HI[4.1+6.3] to the regions in light pink, flanking regions are marked light grey. In this study, HI[4.1+6.3] was narrowed down to the regions dark purple, with flanking regions marked light purple. *The marker column lists existing and newly developed markers. Physical positions of newly developed markers are indicated. ** The

F2_1997_FR mapping population was used in positioning and validation of the markers and fine mapping. Informative plants are presented in the table. *** The C6.3 segments of F3-family plants are expected to be non-recombinant based on extrapolation of the genotypic information of the parental F2-family (Appendix 2). Plants with an identical genotype descending from the same parent are indicated once, the number of replications is listed. ****Summary of Analysis summarizes fine mapping results by means of the exclusion principle. At the suspected regions, 4a6h, 4a6b, 4h6b are absent (purple line). The markers in light purple visualize the borders of the regions arisen due to presence of one or more non-transmitted genotypes. Presence of the listed genotypes was enumerated up to the outer markers in light grey.

Appendix 6B. Overview of informative plants in Fine Mapping C6.3

Population					F2_1997_FR mapping population**					F3-Family**
					Replications					7
					Plant Number					pv09303
Chromosome	Map Number	Markers*	Mbp	cM	79	89	117	123	109	
4	4	CLSx10758.b1_K01.ab1	2.0		-	-	-	-	-	-
4	5	NL0293		5.8	-	-	-	-	-	-
4	6	E35M49-582		6.2	-	-	-	-	-	-
4	7	NL1260		8.2	-	-	-	-	-	-
4	8	CLLY8178.b1_D05.ab1	7.9		-	-	-	-	-	-
4	9	E35M60-128		8.4	A	H	-	-	-	-
4	10	NL0514		9.8	A	H	A	H	A	-
4	11	E35M49-153		10.5	A	H	-	-	-	-
4	12	CLS55115.b1_F07.ab1	23.9		-	-	-	H	A	-
4	13	CLS_S3_Contig2928	29.9		-	-	-	H	A	A
4	14	E35M59-104		11.5	A	H	-	-	-	-
4	15	CLS_S3_Contig2219	37.9		-	-	-	A	H	A
4	16	E45M48-249		12.9	d	d	-	-	-	-
4	17	E38M54-110		13.6	d	d	d	d	d	-
4	18	QGG20102.yg.ab1	41.9		-	-	-	A	H	A
4	19	CLS12433.b1_B09.ab1	50.0		-	-	-	d	d	A
4	20	CLLY11908.b1_G01.ab1	52.8		-	-	-	A	H	A
4	21	CLS_S3_Contig7077	53.8		-	-	-	A	H	A
4	22	NL0884		16.9	A	H	A	A	H	-
4	23	NL0261		17.9	A	H	A	A	H	-
4	24	CLS_S3_Contig4465	58.0		-	-	-	A	H	-
4	25	NL0897		18.4	A	H	A	A	H	-
4	26	CLS_S3_Contig5375	60.4		-	-	-	A	H	-
4	27	LSS_S3_Contig1360	61.9		-	-	-	A	H	-
4	28	LK1525		21.8	A	H	-	-	-	-
4	29	E35M48-263		23.4	d	d	d	d	d	-
4	30	NL1186		24.4	A	H	A	A	H	-
4	31	E35M49-298sal		24.5	A	d	-	-	-	-
6	1	E35M49-410sal		82.3	A	H	-	-	-	-
6	2	LE1211		81.9	A	H	-	-	-	-
6	3	NL0418		81.6	A	H	A	H	H	-
6	4	NL0833		80.6	A	H	A	H	A	-
6	5	CLS_S3_Contig6649	3.8		A	H	A	H	A	A
6	6	Y4646.b1_K09.ab1	7.8		A	H	A	H	H	-
6	7	CLS_S3_Contig3369	10.0		A	H	A	H	H	A
6	8	CLS_S3_Contig1511	14.3		A	H	A	H	H	A
6	9	CLSM10904.b1_P13.ab1	17.9		A	H	A	H	H	A
6	10	QGA7F22.yg.ab1	22.0		A	H	A	H	H	H
6	11	CLLY9820.b1_G08.ab1	26.0		A	H	A	B	H	H
6	12	E44M49-352sal		79.6	A	H	-	-	-	-
6	13	E45M48-300		79.4	d	H	-	-	-	-
6	14	E54M48-216Asato		79.0	d	d	d	-	d	-
6	15	CLS_S3_Contig4263	31.9		A	H	H	B	H	-
6	16	E35M59-105sal		76.1	-	c	-	-	-	-
6	17	E44M49-87sal		75.9	A	H	-	-	-	-
6	18	CLS_S3_Contig7223	34.0		A	H	H	B	H	-
6	19	E44M49-278sal		74.9	A	H	-	-	-	-
6	20	M7120		74.3	A	H	-	-	-	-
6	21	E33M59-565		74.1	A	d	H	B	H	-
6	22	NL1084		72.9	A	H	H	B	H	-
6	23	NL0653		72.9	A	H	H	B	H	-
6	24	NL1114		69.6	H	B	H	H	H	-
6	25	E45M48-103		68.4	H	c	-	-	-	-
6	26	NL0589		67.1	H	B	H	H	H	-

Summary of Analysis****

Map Number	Marker	Presence of genotypes		
		4A6H	4A6B	4H6B
1	E35M49-410sal	1 (F2: 109)	0	0
3	NL0418	1 (F2: 109)	0	0
5	CLS_S3_Contig6649	0	0	0
6	Y4646.b1_K09.ab1	1 (F2:109)	0	0
26	NL0589	4 (F2: 79, 117 F3: pv..03)	0	2 (F2: 89, 123)

For footnotes, see Appendix 4A. Apply footnotes of Appendix 4A to chromosome 6.3.

Appendix 7. Candidate Genes Chromosome 6.3

Number	EST*	Number	EST	Number	EST
1	CLSM2709.b1_I05.ab1	47	CLSS6320.b1_P19.ab1	93	QGI13K05.yg.ab1
2	CLS_S3_Contig4708	48	CLS_S3_Contig7342	94	CLSM6134.b1_L22.ab1
3	CLS_S3_Contig10776	49	CLSM8557.b1_J03.ab1	95	CLSS8242.b1_D21.ab1
4	QGC10P17.yg.ab1	50	CLS_S3_Contig355	96	CLS_S3_Contig4799
5	QGD3f09.yg.ab1	51	QGE10A22.yg.ab1	97	CLX13391.b1_N12.ab1
6	CLPZ4514.b1_D02.ab1	52	CLVZ527.b1_N11.ab1	98	CLS_S3_Contig9579
7	CLSM8201.b1_B11.ab1	53	CLSX4337.b1_B05.ab1	99	CLS_S3_Contig2319
8	CLS_S3_Contig4570	54	QGD12D08.yg.ab1	100	QGG22F05.yg.ab1
9	CLS_S3_Contig4227	55	CLS_S3_Contig9365	101	CLS_S3_Contig7060
10	CLS_S3_Contig5273	56	CLS_S3_Contig4513	102	CLSM11485.b2_J16.ab1
11	QGD10A08.yg.ab1	57	QGI8N02.yg.ab1	103	CLS_S3_Contig996
12	CLV_S1_Contig2001	58	CLS_S3_Contig6649	104	CLSX5507.b1_F09.ab1
13	CLS_S3_Contig10060	59	CLSM4397.b1_J19.ab1	105	CLS_S3_Contig4534
14	CLSM10028.b1_G11.ab1	60	CLS_S3_Contig6701	106	CLSX7934.b1_K16.ab1
15	CLS_S3_Contig7127	61	CLSY7119.b1_M04.ab1	107	CLS_S3_Contig10864
16	CLPX2587.b1_F24.ab1	62	CLSM9884.b1_G24.ab1	108	CLS_S3_Contig4794
17	CLS_S3_Contig10372	63	CLS_S3_Contig7164	109	QGG21K06.yg.ab1
18	CLS_S3_Contig4843	64	CLPY5827.b1_E17.ab1	110	QGI1N09.yg.ab1
19	QGB10G14.yg.ab1	65	CLS_S3_Contig9033	111	CLSS12007.b1_N01.ab1
20	CLS_S3_Contig8464	66	QGI10D22.yg.ab1	112	CLSS3954.b1_D05.ab1
21	CLSM13260.b1_G04.ab1	67	CLS_S3_Contig4846	113	QGB23B17.yg.ab1
22	CLS_S3_Contig2753	68	CLR_S1_Contig2873	114	CLSM652.b2_G20.ab1
23	CLS_S3_Contig8890	69	CLS_S3_Contig616	115	CLSY3053.b1_J20.ab1
24	CLSY3830.b1_L22.ab1	70	CLS_S3_Contig6018	116	CLSM2869.b1_J21.ab1
25	CLSX4330.b1_D03.ab1	71	CLPX5624.b1_O14.ab1	117	QGA4b05.yg.ab1
26	QGE17P21.yg.ab1	72	CLS_S3_Contig2169	118	CLV_S1_Contig2268
27	CLS_S3_Contig7925	73	QGD12L06.yg.ab1	119	CLS_S3_Contig4483
28	QGC14G22.yg.ab1	74	QGI7O02.yg.ab1	120	CLS_S3_Contig2940
29	CLS_S3_Contig3346	75	CLSM19724.b1_H11.ab1	121	QGB19F01.yg.ab1
30	CLS_S3_Contig11198	76	CLS_S3_Contig1289	122	CLS_S3_Contig1766
31	QGH12A23.yg.ab1	77	CLS_S3_Contig9385	123	CLRZ570.b1_D23.ab1
32	CLS_S3_Contig4259	78	CLSM10034.b1_C13.ab1	124	CLS_S3_Contig11322
33	CLS_S3_Contig10506	79	QGC5N03.yg.ab1	125	CLSY5096.b1_P01.ab1
34	QGD13P21.yg.ab1	80	CLS_S3_Contig7325	126	QGH8L03.yg.ab1
35	CLSM11902.b1_L24.ab1	81	CLLZ1986.b1_C17.ab1	127	CLRZ533.b1_J13.ab1
36	CLS_S3_Contig7473	82	CLSM5654.b1_K22.ab1	128	QGG34H14.yg.ab1
37	CLSX727.b1_N14.ab1	83	CLS_S3_Contig8059	129	QGG6M10.yg.ab1
38	CLR_S1_Contig402	84	CLS_S3_Contig8187	130	CLS_S3_Contig3147
39	QGD10H11.yg.ab1	85	CLSS2753.b1_A17.ab1	131	CLSY5404.b1_G07.ab1
40	CLS_S3_Contig7432	86	CLVX8691.b1_E14.ab1	132	CLS_S3_Contig9900
41	CLS_S3_Contig9196	87	CLRZ4472.b1_O14.ab1	133	CLP_S1_Contig1976
42	CLSM8573.b1_J07.ab1	88	CLS_S3_Contig2207	134	CLR5903.b2_N11.ab1
43	QGB20P18.yg.ab1	89	CLS_S3_Contig1468	135	CLLY10127.b1_N11.ab1
44	CLS_S3_Contig9435	90	CLSM3195.b1_F07.ab1		CLSY4646.b1_K09.ab1
45	QGB25J15.yg.ab1	91	QGC5B06.yg.ab1		
46	CLS_S3_Contig4426	92	CLSM17878.b1_K06.ab1		

*According to Gbrowse Lattuga version 3.2, 135 genes are located within the suspected HI-region at chromosome 6. Marker CLS_S3_Contig6649 (number 58, indicated in dark purple) is the only EST-marker within the suspected region. As a reference, flanking marker CLSY4646.b1_K09.ab1 is included, indicated in light purple.

Appendix 8. Protocol DNA extraction with NaOH

Method according to Wang H *et al.* modified by Koen Pelgrom

Preparation

- A scheme in which samples are linked to the tubes of destination (plant 1 in tube A1, plant 2 in tube B1 etc.). Organise the plant samples extremely logically.
- Bucket with ice
- Tweezers
- '8 in a row'- tubes and a box to store them.
- NaOH 0.5M (breaks down the plant material, DNA will be released)
- Tris 100mM (buffer, slows down the degradation of the plant material by NaOH)
- Pipets for 20 μ L (repetitive), 5 μ L (8-canal) and 100 μ L (8-canal)

Greenhouse: collect leaf material

- Mark the tubes in order to prevent confounding of the tubes
- Keep the box with tubes on ice.
- Collect a small piece of leaf (0.5 cm²) per sample, preferably the first leaf and not the cotyledon.
- Lab
- Spin down at full speed (4500 rpm).
- Check whether all your samples are at the bottom of the tubes, if not, use your tweezers to do so and spin down again.
- Add 20 μ L NaOH 0.5M per sample. There is no need to spin down the sample again: the NaOH will go down in the tissue striker. Don't waste much time between adding the NaOH and the next step (the NaOH is breaking down your sample).
- Place your box with tubes in the Tissue striker.
- Check whether the striker is clean, otherwise rinse with water.
- In case you sampled less than 96 plants, make sure that the amount of tubes is equal for the left side and the right side of the box or fill the box with empty tubes. Keep balance.
- Strike for 5 minutes at full speed.
- Rinse the striker with water after use.
- Check whether all your samples are grinded, if not, grind manually by using a pipet point.
- Immediately add 20 μ L Tris 100mM per sample.
- Spin down
- Check whether your samples are at the bottom of the tubes.
- Prepare a plate by filling the tubes with 200 μ L Tris.
- Add 5 μ L liquid of the leaf sample to the prepared plate with 200 μ L Tris. Don't pipet up the grinded leaf, but the liquid!
- Mix your leaf sample with the Tris by pipetting up and down with a volume of 100 μ L.

Storage

When you store the DNA in the -20°C freezer, the DNA quality is good for at least 4 weeks.

When you store the DNA in the refrigerator, the DNA quality will be degraded after a week (the NaOH will break down the DNA as well).

Use in PCR

use 1 μ L DNA per PCR sample
