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Fine mapping of the major Fusarium head blight resistance QTL *Fhb1* in wheat: Identification of near-isogenic lines with recombinations in the QTL region

Master thesis

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Abstract

Wheat is one of the most important crops. However, the productivity is often limited by the occurrence of pest organisms. Fusarium head blight (FHB), which is mainly caused by *Fusarium graminearum*, is one of the most devastating diseases affecting wheat and other gramineous crop species. Besides negative effects concerning yield and seed quality, the production of mycotoxins poses a hazard to human and animal health. Therefore, the development of resistant cultivars is a declared goal for many breeders and plant scientists. The genetic resistance mechanisms are still largely unknown, despite various studies and intensive breeding strategies against FHB. Numerous quantitative trait loci (QTL) have been identified to be associated with the resistance trait, but none of the underlying genes has been cloned yet. One major QTL, namely *Fhb1* on chromosome 3B, was found to be most promising and has thus been used for marker-assisted breeding. The aim of the master thesis at hand was the selection of near-isogenic lines (NILs) with recombinations in the *Fhb1* region for the fine mapping of the QTL and finally to support gene cloning. Initially, 2080 NILs in the F_2 generation for the *Fhb1* region were screened using two molecular markers (X*GWM493* and X*BARC133*) flanking the *Fhb1* QTL. Thus, 121 lines with recombinations between the two markers were identified with most of the lines heterozygous for one of the markers. In a second selection step in the F_3 generation, 85 NILs were identified to be homozygous and recombinant in the *Fhb1* region. The putative *Fhb1* region of the recombinant lines was further characterized using two additional markers, *UMN10* and *Snp3BS-8*, allowing the separation of the lines in five haplotypes. These lines serve as an essential genetic resource for subsequent fine mapping, in order to narrow down this large QTL region and to facilitate cloning of the *Fhb1* resistance gene(s).

Key words: Fusarium head blight, *Fusarium graminearum*, Quantitative trait loci, *Fhb1,* fine mapping

Abstract deutsch

Weizen ist eine der wichtigsten Kulturpflanzen. Jedoch ist die Ertragsfähigkeit häufig limitiert durch das Auftreten von Schadorganismen. Die Ährenfusariose, vor allem hervorgerufen durch *Fusarium graminearum*, ist eine der verheerendsten Krankheiten von Weizen und anderen Kulturpflanzen. Neben negativen Effekten auf Ertrag und Saatgutqualität, stellt die Produktion von Mykotoxinen eine Gefahr dar für die Gesundheit von Tier und Mensch. Deshalb ist die Entwicklung von resistenten Sorten ein erklärtes Ziel von Züchtern und Pflanzenwissenschaftlern. Die genetischen Resistenzmechanismen sind bis dato unbekannt, trotz vieler Studien und intensiven Züchtungsstrategien gegen die Ährenfusariose. Viele Quantitative Trait Loci (QTL) wurden identifiziert, welche mit der Resistenz assoziiert sind, aber kein verantwortliches Gen wurde bis jetzt kloniert. Ein QTL, nämlich *Fhb1* auf Chromosom 3B, wurde als vielversprechend befunden und für markergestützte Selektion verwendet. Das Ziel dieser Masterarbeit war die Selektion von nah-isogenen Linien (NILs) mit Rekombinationen in der *Fhb1* QTL-Region für die Feinkartierung und um die Genklonierung zu unterstützen. Zunächst wurden 2080 NILs in der F_2 Generation für die *Fhb1*-Region untersucht mit zwei flankierenden molekularen Markern (X*GWM493* und X*BARC133*). Dadurch wurden 121 Linien mit Rekombinationen zwischen diesen Markern identifiziert, die meisten davon waren heterozygot für einen von diesen Markern. In einem zweiten Selektionsprozess in der F_3 Generation wurden 85 NILs identifiziert, welche homozygot und rekombinant in der *Fhb1*-Region sind. Die vermeintliche *Fhb1*-Region der rekombinanten Linien wurde weiter charakterisiert mit Hilfe von zwei weiteren Markern, *UMN10* und *Snp3BS-8*, durch welche sich fünf verschiedene Haplotypen erkennen ließen. Diese Linien stellen eine wichtige genetische Ressource für ein weiteres Feinkartieren dar, um die große QTL-Region zu verkleinern und das Klonieren von Resistenzgenen zu ermöglichen.

Schlüsselwörter: Ährenfusariose*, Fusarium graminearum*, Quantitative Trait Loci, *Fhb1,* Feinkartierung

List of abbreviations

APS – ammonium persulfate

BAC – bacterial artificial chromosome

BC_5F_n – Backcross five filial generation n

bp – base pairs

°C – degree(s) Celsius

CIMMYT – International Maize and Wheat Improvement Center

cm – centimetre

cM – centimorgan

DH – double haploid

dh2O – distilled water

DMSO – dimethyl sulfoxide

EDTA – ethylenediaminetetraacetic acid

FAM – 6-carboxy-fluorescine

HEX – hexachlorocyclopentadiene

MAS – marker-assisted selection

MgCl2 – magnesium chloride

mL – millilitre

mm – millimetre

NaOAc – sodium acetate

NH4OAc – ammonium acetate

NILs – near-isogenic lines

nm – nanometer

PAA – polyacrylamide

PCR – polymerase chain reaction

QTL – quantitative trait loci

rcf – relative centrifugal force

TE buffer – Tris-EDTA buffer

TEMED – tetramethylethylenediamine

USD – United States Dollar

µL – microliter

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1. Introduction

The issues underlying global food security are among the most important in international discussions. Over the past 50 years, the human population has more than doubled and is projected to increase to over 9 billion by 2050 (FAO, 2010). As a result, the growing human population will require a significant increase in agricultural production.

This challenge is made difficult by the fact that productivity of crops is at risk due to the incidence of pests, especially weeds, pathogens and animal pests. Among crops, the total global potential loss due to pests varied from about 50% in wheat to more than 80% in cotton production. Overall, weeds caused the highest potential loss (34%), with animal pests and pathogens being less important (losses of 18 and 16%) (Oerke, 2006). Furthermore, changes in the climatic and environmental conditions under which crops are grown might result in the appearance of new diseases or in higher disease pressure of known pests (Boyd et al., 2013).

Fusarium head blight (FHB) is one of the most serious diseases affecting wheat and barley worldwide. The causative fungi, mostly *Fusarium graminearum* and *Fusarium culmorum*, cause negative effects on grain yield and quality. Furthermore, they can produce several mycotoxins and pose a hazard to both human and animals by the consumption of contaminated products. Therefore, the establishment of resistant crop varieties is a declared goal of several plant breeders and scientists, either by conventional breeding or transgenic approaches. Nevertheless, the production of crop varieties with effective resistance should not impact on other agronomically important crop traits.

In conclusion, breeders and plant scientists all over the world harbour a large responsibility for ensuring global food security. Further research and cooperation between research institutes are indispensable and will lead to the achievement of this objective in future.

1.1 Wheat – origin and genomic characteristics

Bread wheat (*Triticum aestivum*; *Poaceae*) is one of the most important staple crops worldwide. It provides nearly 20% of the world's daily food supply measured by calorie intake, similar to that provided by rice. The yield of wheat has doubled over the last 40 years due to a combination of advanced agronomic practice and improved germplasm through selective breeding (Berkman et al., 2012).

Wheat was domesticated about 10,000 years ago. Since then, it has spread worldwide and became one of the major crops. All *Triticum* species are native to the 'Fertile Crescent', which encompasses the eastern Mediterranean, southeastern Turkey, northern Iraq and western Iran, and its neighbouring regions of the Transcaucasus, and northern Iran (Berkman et al., 2012; Dubcovsky & Dvorak, 2007; Varshney et al., 2006).

The wheat genome is both large and highly complex compared to many other cereal crops, with an estimated size of 17 Gbp. Bread wheat is in fact an allohexaploid, meaning that it consist of three distinct diploid genomes that together function much like any diploid. The diploid donor species diverged about 2.5 – 4.5 million years ago. The three different genomes are termed AA, BB, and DD. Assumedly, two distinct hybridization events led to the production of *Triticum aestivum*. First, *Triticum urartu* (AA) and an unknown relative of *Aegilops speltoides* (BB) are believed to have produced the tetraploid *Triticum turgidum*, followed by hybridization with *Aegilops tauschii* (DD) to produce the hexaploid (Berkman et al., 2012; Chantret et al., 2005; Paux et al., 2006).

The genus *Triticum* consists of six species: *Triticum monococcum* (AA genome); *Triticum urartu* (AA genome); *Triticum turgidum* (AABB genome); *Triticum timopheevii* (AAGG genome); *Triticum aestivum* (AABBDD genome); and *Triticum zhukovskyi* (AAAAGG genome). Of these species, *T. urartu* exists only in its wild form, whereas *T. aestivum* and *T. zhukovskyi* exist only as cultivated forms. The other species, *T. monococcum*, *T. turgidum* and *T. timopheevii*, have both a wild and a domesticated form (Dubcovsky & Dvorak, 2007; Matsuoka, 2011).

The large genome size of wheat and the complex family background have hampered efforts to determine the genetic basis of phenotypic traits. Furthermore, the high proportion of repetitive DNA in the wheat genome complicates genome assembly (Eid et al., 2009).

1.2 Fusarium head blight (FHB)

1.2.1 The pathogen

Fusarium head blight (FHB), also called ear blight or scab, is a major fungal disease affecting several gramineous hosts including wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Osborne & Stein, 2007). It was first discovered in England in the year 1884 where because of the chalky, lifeless appearance of the infected kernels, it was called wheat scab, and later tombstone disease (Clear & Patrick, 2010; Parry et al., 1995). *Fusarium* fungi are ubiquitous in soil from almost any location in any climate worldwide (Leslie & Summerell, 2011).

Up to 17 causal organisms have been associated with the disease and the coexistence of different *Fusarium* spp. in the same field is a normal situation. Among them are: *Fusarium graminearum* (teleomorph: *Gibberella zeae*), *Fusarium culmorum*, *Fusarium poae*, *Fusarium avenaceum* (teleomorph: *Gibberella avenacea*), *Fusarium sporotrichoides*, and *Microdochium nivale* (teleomorph: *Monographella nivalis*). While F. *graminearum* is often the predominant species causing FHB in many of the affected regions, the others can be highly pathogenic as well (Mesterhazy et al., 2005; Osborne & Stein, 2007; Parry et al., 1995; Turkington et al., 2014).

However, only *F. graminearum* was used as inoculum for the master thesis at hand and is thus relevant. Based on the sexual state *Gibberella zeae,* the fungus belongs to the phylum of *Ascomycota*, more precisely to the genus *Gibberella*. In addition to wheat and barley, *F. graminearum* can also cause FHB on rice (*Oryza*), oats (*Avena*) and Gibberella stalk and ear rot disease on maize (*Zea*). The fungus is capable of infecting other plant species without causing disease symptoms. Other host genera for *F. graminearum* include *Agropyron*, *Agrostis*, *Bromus*, *Glycine*, *Lolium*, *Medicago*, *Poa*, *Secale*, *Sorghum*, *Trifolium* etc. (Goswami & Kistler, 2004).

1.2.2 Distribution and impact

Epidemics of FHB are strongly influenced by local and regional environment, physiological status of hosts and pathogen related factors including adaptation and virulence (Osborne & Stein, 2007).

Fusarium graminearum has been indicated wherever wheat is grown and outbreaks have been reported in Asia, Canada, Europe, South and North America. Since 1991, outbreaks of

varying intensity have been common and widespread across much of the eastern half of the United States and Canada (Goswami & Kistler, 2004; McMullen et al., 1997; O'Donnel et al., 2000; Sutton, 1982).

The disease has the ability to completely destroy a potentially high-yielding crop within a few weeks of harvest. In north eastern North Dakota, both yield and quality of wheat were affected and the average yields in barley and wheat dropped 45% from 1992 to 1993 due to FHB epidemics (McMullen et al., 1997).

According to Nganje et al. (2002), the direct and secondary economic losses due to FHB for all crops in the Central United States were estimated to be USD 2.7 billion from 1998 to 2000 alone. In China, FHB is endemic and losses in excess of 1 million metric tons (about 38 million bushels) have been reported (Bai & Shaner, 1994). Actually, the impact of the disease may even increase further due to indirect effects of climate change (West et al., 2012).

1.2.3 Symptoms and negative effects

The symptoms caused by FHB are generally similar in all small grain cereal crops. In case of wheat, brown, dark purple to black necrotic lesions form on the exterior surface of the florets and glumes. The peduncles below the inflorescence may become discoloured brown or purple immediately. The most obvious symptom occurs with time, when the tissue of the inflorescence becomes blighted and appears bleached and tan, while the grain within atrophies. Also, the awns often become deformed or twisted (Goswami & Kistler, 2004; Parry et al., 1995).

The resulting damage from FHB infection is manifold. The disease reduces kernel set and kernel weight, causing grain yield loss. The baking and seed quality is affected as well. By the invasion of the kernel, the fungus destroys the starch granules and cell walls, and affects endosperm storage proteins. Moreover, it lowers market grade due to the discoloured shrivelled 'tombstone' kernels (Buerstmayr et al., 2009; McMullen et al., 1997; Snijders, 2004).

Apart from the above mentioned negative aspects, the major peril due to FHB is the contamination of the crop with toxic fungal secondary metabolites known as mycotoxins (Buerstmayr et al., 2009). A number of trichothecene mycotoxins can be produced including deoxynivalenol (DON) and nivalenol (NIV), as well as zearalenone (ZEA) and moniliformin

(MON), all of which have a range of toxicity to animals (Desjardins, 2006; Leslie & Summerell, 2006; Rotter et al., 1996). Trichothecenes can bind to the 60S ribosomal subunit of eukaryotes, and therefore inhibit protein synthesis and induce apoptosis (Terzi et al., 2013). The most common toxin associated with infected grain by *F. graminearum* is DON (Pestka, 2010; Snijders, 1990).

DON poses a threat to animals and humans as it is known to cause vomiting and feed refusal in non-ruminant animals if exposure levels are high (Snijders, 1990). According to animal experimental studies by Pestka (2010), a chronic low-dose exposure to DON elicits absence of appetite, growth retardation, immunotoxicity as well as impaired reproduction and development resulting from maternal toxicity. Plus, epidemiological studies could help to reveal if relationships exist between consumption of DON and incidence of both gastroenteritis and potential chronic diseases.

For the sake of human health, norms were established regulating the maximum DON levels in food destined for human consumption. The European Commission has proposed limits of 750µg/kg (750 ppb) in cereals and 500µg/kg in cereal-based products such as flour (Champeil et al., 2004).

1.2.4 Infection and life cycle

The infected kernels and head debris of wheat plants return to the soil surface during harvest. The fungus persists and multiplies on that plant debris and overwinters as saprophytic mycelia. Prolonged moist weather during the growing season promotes growth and sporulation of the fungus on crop residues. The produced spores (mostly ascospores) are either windblown or water-splashed onto heads of cereal crops. Wheat is susceptible to infection from the flowering stage (anthesis) up through the soft dough stage of kernel development. Once again, high humidity and frequent rainfalls that coincide with the flowering and early kernel-fill period of the crop favour the infection process. The spores may land on the exposed anthers of the flower and then grow into the kernels, glumes, or other head parts (Goswami & Kistler, 2004; Kugler et al., 2013; McMullen et al., 1997, 2012).

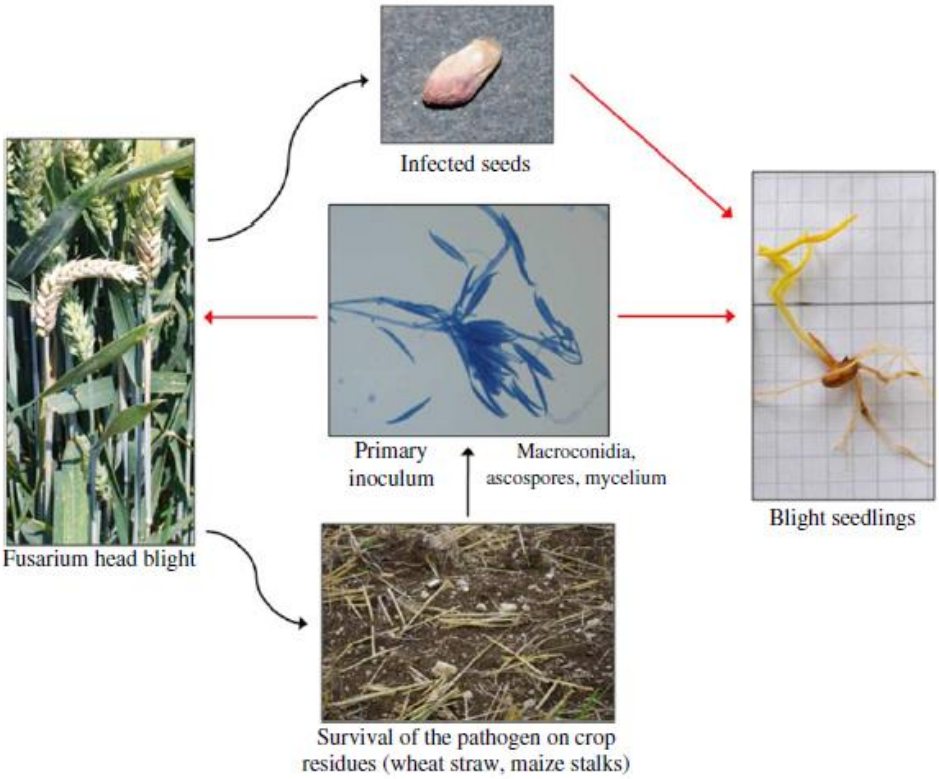


Figure 1: Disease cycle of *Fusarium graminearum.* Black arrows indicate habitats provided by the crop and red arrows indicate infectious activity kept up by habitats (Leplat et al., 2013).

1.2.5 Control

Various studies reveal that agricultural practices play an important role on head blight attacks. Such an important agronomy-based method is crop rotation. Understandably, the severity of FHB depends on the preceding crop, whether that crop is a potential host for the fungus. Besides, the frequency of the crop in the rotation must be considered. It has been shown that, the shorter the rotation, the higher is the frequency of FHB. As a result, FHB is most frequent when the susceptible crop occurs frequently within the rotation (Champeil et al., 2004; McMullen et al., 1997).

Other agricultural measurements, which help to minimize the infection level posed by crop residues and soil, are also relevant. This can be achieved by ploughing or burning excess residues whereby crop residue accumulation and longevity on the soil surface is reduced. Tillage has also an effect on the disease. The presence of *Fusarium* is higher in no-till or reduced-tillage fields than in tilled fields with buried residues (Dill-Macky & Jones, 2000).

Despite some important advances made in fungicide application technology over the past decade, no product can impede the fungus satisfactorily and ensure a full extend protection yet, but they do contribute to significant mean yield and grain test weight increases. Triazole fungicides (tebuconazole, metaconazole, or prothioconazole) are currently the most effective and they are widely used, on their own and in combination (Homdork et al., 2000; Mesterhazy et al., 1996; Paul et al., 2010; Terzi et al., 2013). *Fusarium* species vary in their fungicide susceptibility. *F. graminearum* for example is susceptible to triazoles whereas *F. avenaceum* is more susceptible to strobilurins (Simpson et al., 2001).

However, the application of fungicides will probably always be hampered by some challenges. The success of the application depends on the timing. A short period of flowering during which applications must be made or wet fields are disadvantageous and hinder an efficient control. In addition, uneven fields, low spray volumes and fast ground speeds, or irregular field shapes encourage uneven spray application and thus form an obstacle. Biological control approaches face similar problems (McMullen et al., 2012).

Experiments carried out by Forrer et al. (2014) tested different substances, which interfere with *Fusarium* species. They could show that suspensions or solutions with 1% of Chinese galls (*Galla chinensis*) or 1% of tannic acid, inhibited germination of conidia or mycelium growth of *F. graminearum* by 98%–100% or by 75%–80%.

Chemical and agronomic control measures are only partly effective to control FHB. On account of this, the declared control method of many cereal breeders against *Fusarium* species is the cultivation of resistant varieties, which is also a very cost-effective and ecological measure. Blessedly, the wheat gene pool harbours a large genetic variation. On the other hand, the best regionally adapted and highly productive cultivars are often susceptible to FHB. Breeding for resistance should not impact on important agronomical crop traits, but rather combine resistance and an agronomical high performance within one cultivar. Along with that, the production of a new wheat variety typically takes up to 10 years from when a cross is first made until release (Anderson, 2007; Buerstmayr et al., 2002, 2009; Ruckenbauer et al., 2001).

At the beginning of FHB resistance breeding, the major focus was laid upon finding new resistance sources. But over time, the breeding focus shifted from finding resistance genes

to incorporating resistance into adapted cultivars that seemed to be broadly effective (McMullen et al., 2012).

Appreciable efforts in resistance breeding have been achieved by conventional selection, through repeated testing of wheat lines under induced and natural epidemic conditions (Buerstmayr et al., 2009). The prominent sources of resistance, which have been used in resistance breeding programmes worldwide, are spring wheat genotypes from Asia (e.g. 'Sumai 3', 'Ning 7840', 'Ning 8331') and South America (e.g. 'Frontana') (Liu et al., 2013; Buerstmayr et al., 1999). Unfortunately, these sources are not adapted as they lack agronomic traits needed in modern wheat varieties. In The Netherlands, 'Sumai 3' is susceptible to almost any other disease and is about 3 weeks earlier than elite varieties (Snijders, 2004). Nevertheless, these lines provided a basis for projects to determine the genetic background of FHB resistance (Buerstmayr et al., 2009).

1.3 FHB resistance of wheat

1.3.1 Resistance mechanisms in wheat

Some wheat cultivars are more resistant than others. However, the resistance trait is very complex and further research is still needed to understand the mechanisms involved in detail.

In general, resistance types can be classified as either morphological or physiological (Rudd et al., 2001). Such a morphological trait is plant height. Dwarf types of wheat are more susceptible to FHB infection than tall cultivars (Mesterhazy, 1995). Another feature, which affects disease spread, is the head morphology. It has been shown that awned genotypes with a compact spike are less resistant than awnless genotypes with a lax spike. The flowering date also influences the resistance to FHB (Kollers et al., 2013). However, such morphological resistant types play a minor role in disease manifestation compared to physiological described below (Rudd et al., 2001).

Schroeder & Christensen (1963) were the first who described two most important physiological resistance types (type I and type II resistance) (Buerstmayr et al., 2009). Mesterhazy (1995) reviewed those mechanisms and has extended this classification to five types of varietal resistance so far:

I. Resistance to initial infection

II. Resistance to disease spread within a spike

III. Resistance to kernel infection

IV. Yield tolerance

V. Decomposition or non-accumulation of mycotoxins

Type I resistance remains largely elusive. In fact, this is due to a challenging screening and to the fact that type I resistance is hindered by any differences in type II resistance (Gosman et al., 2010; McMullen et al., 2012; Steiner et al., 2004). Because of food safety concerns associated with DON, there has been an increased interest in resistance traits expressed in grains. But from the outset, most breeding programs focused on type II resistance, and this trait still receives considerable attention (McMullen et al., 2012).

As mentioned before, the most common source of resistance in bread wheat is probably 'Sumai 3', which harbours type II resistance (Osborne & Stein, 2007). This resistance type is generally measured by observing symptoms due to disease spread after some type of point inoculation (Rudd et al., 2001).

However, the complex trait of plant resistance complicates varietal resistance studies (Champeil et al., 2004) and despite much effort, the underlying molecular events during early infection are still poorly understood (Steiner et al., 2009).

Various studies could show that the resistance to FHB is quantitatively inherited and of oligo- to polygenic nature (Bai & Shaner, 1994; Buerstmayr et al., 2009; Mesterhazy, 1995). Therefore, the accurate method to examine resistance to FHB is a QTL (quantitative trait locus/loci) mapping approach (Buerstmayr et al., 2009).

1.3.2 QTL mapping studies and marker-assisted breeding (MAS)

The unknown loci of genes on the chromosomes, which govern a trait, are commonly referred to as QTLs (Jansen, 1996). Those are identified via statistical procedures that integrate genotypic and phenotypic data (Miedaner & Korzun, 2012).

QTL analysis aimed for plant breeding is typically performed in two steps: The first step is the discovery of a QTL. Varying parental lines, which differ for one or more quantitative traits, are hybridized and a segregating population is created. Molecular markers are used to identify QTL regions. The second step is to make use of identified QTL map locations to create resistant plant varieties (Tanksley & Nelson, 1996).

Molecular markers

Molecular markers are specific fragments of DNA with a known location on the chromosome that can be identified within the whole genome and reveal neutral sites of variation (Miedaner & Korzun, 2012). The ideal DNA marker should be highly polymorphic, highly abundant in the genome, co-dominant (refers to the ability to distinguish the heterozygote) and easy to genotype (Ben-Ari & Lavi, 2012).

Since the 1980s, they have had widespread practical applications in wheat. For example, they have been used to confirm identity between parents and progeny, to determine evolutionary relationships and to map genes and to identify QTLs (Bagge et al., 2007). They are very useful tools in plant breeding approaches as one can detect the presence of different alleles and thus help the breeders to select for genes that enhance FHB resistance. The underlying concept is that naturally occurring differences in the DNA sequence of wheat varieties are identified as being genetically linked to a gene that confers resistance to FHB (Anderson, 2007; Collard & Mackill, 2008).

These DNA differences could be due to a difference in the number of repeat units of a sequence (microsatellite or simple sequence repeat; SSR) or a single base pair difference (single nucleotide polymorphism; SNP) (Anderson, 2007).

SSR markers are highly reliable, co-dominant in inheritance, relatively simple and cheap to use and generally highly polymorphic. The only disadvantage of SSRs is that they typically require polyacrylamide gel electrophoresis and give information only about a single locus per assay (Ben-Ari & Lavi, 2012; Collard & Mackill, 2008).

In contrast, SNPs are single locus markers and mostly have a low level of polymorphism. Their mode of inheritance is co-dominant as well. For two reasons they have become the

markers of choice: they are highly abundant and high-throughput technologies of genotyping SNPs are available (Ben-Ari & Lavi, 2012).

The term 'perfect marker' refers to a marker without recombination to the gene of interest (ideally drawn directly from the gene sequence). The availability of such a marker is the most critical point for successful marker-assisted selection (MAS) (Miedaner & Korzun, 2012).

Genetic linkage maps and QTL mapping

For QTL detection, genetic maps need to be established. A genetic map is an abstract model of the linear arrangement of a group of genes and markers, which is based on homologous recombination during meiosis. Recombination in general occurs randomly on chromosomes (Liu, 1997).

The distance between the markers is expressed in centimorgans (cM), which represents the recombination rates between the loci (1 cM = 1% recombination). However, there is no specific relationship between the recombination distance and the physical distance (expressed in base-pairs) because the rate of recombination varies along the length of the chromosome (Kumar, 1999).

Whenever a marker is tightly linked to the target gene, marker and QTL alleles will be associated and as a consequence the genotypic means of the marker will be altered. This can be tested statistically and the likelihood for the presence of a putative QTL can be plotted at the marker positions along the chromosomes, so as to present the evidence for QTLs at the various positions in the genome (Jansen, 1996).

Marker-assisted breeding – a useful tool for plant breeding

The use of DNA markers in plant breeding is called marker-assisted selection (MAS) and is a component of the new discipline of 'molecular breeding' (Collard & Mackill, 2008). MAS could recruit classical breeding methods, because molecular markers allow breeders to predict the presence or absence of specific resistance genes in a wheat variety (Suzuki et al., 2012).

There are many advantages of MAS over conventional phenotypic selection. For instance, the selection of plants can be carried out at the seedling stage. Also, single plants can be selected with MAS. Another benefit is that the total number of lines that need to be tested

can be reduced. Overall, it may save time and effort, as this approach is rather simple (Collard & Mackill, 2008).

One obstacle of MAS is that the utility of 'perfect markers' is restricted by the limited availability of genes that control agronomic characters. Besides, QTL mapping can only be applied for each individual mapping population because different subsets of QTL will be polymorphic in each population, and the linkage phases between the marker and QTL alleles can differ even between closely related genotypes (Bagge et al., 2007).

For this reason, QTL detection and MAS are often carried out separately, despite the fact that hundreds of QTLs have been detected (Lv et al., 2014).

QTL mapping studies for FHB resistance in wheat

Many quantitative resistances are conditioned by a great number of QTL with small effects (Miedaner & Korzun, 2012). Most QTL mapping studies for FHB resistance in wheat have concentrated on infection within the spikes of cultivar 'Sumai 3' and its derivatives (Liu et al., 2008) and indicate that this might also be true for the resistance to FHB.

Since 1992, at least 331 QTL studies have been published on disease resistance in wheat (Miedaner & Korzun, 2012) and more than one hundred FHB resistance QTLs in wheat have been identified (Gunnaiah et al., 2012). In 2009, Buerstmayr et al. reviewed 52 papers published to date. They focussed on QTL regions, which have been detected in more than one mapping population, as this indicates that the QTL is a real effect. Twenty-two such regions were counted, these are among chromosome 1B (two regions), 1D, 2A (2), 2B (2), 2D (2), 3A, 3B (2), 3D, 4B, 4D, 5A, 5B, 6A, 6B, 7A and 7B (2).

Waldron et al. (1999) detected a major FHB resistance QTL on chromosome 3BS via RFLP mapping, originally called *Qfhs.ndsu-3BS* and was later renamed *Fhb1* by Liu et al. (2006). This QTL was confirmed by Anderson et al. (2001) using SSR markers. Independently, this QTL was also verified by a mapping report using a large double haploid (DH) population (resulting from a cross between CM-82036 and Remus) (Buerstmayr et al., 2002).

After numerous studies, the most widely used QTLs for breeding purposes are located on chromosome 3B (namely *Fhb1*) and 5A (namely *Qfhs.ifa-5A*) (Anderson, 2007). More precisely, *Fhb1* is the best validated gene for FHB resistance, which was found in numerous

mapping studies (Anderson, 2007; Steiner et al., 2009). To date, it is the only disease resistance QTL that is routinely used in wheat breeding (Miedaner & Korzun, 2012) and it explains up to 60% of the phenotypic variance for type II FHB resistance (Buerstmayr et al., 2002).

Although its great importance, the corresponding gene(s) for this trait are still not identified and the *Fhb1* region is still quite large. For this reason, fine mapping approaches are needed in order to narrow down QTL regions.

1.3.3 Fine mapping of the QTL *Fhb1*

Fine mapping of *Fhb1* in wheat provides tightly linked markers that can reduce linkage drag associated with marker-assisted selection of *Fhb1* and assists in the cloning of the functional resistance gene (Cuthbert et al., 2006).

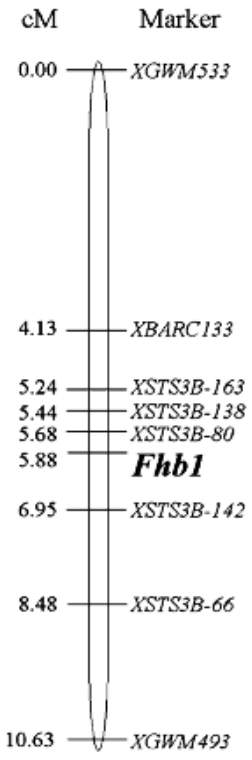


Figure 2: Fine map of the major Fusarium head blight (FHB) resistance QTL *Fhb1* in a Sumai 3*5/Thatcher population (Cuthbert et al., 2006).

A fine mapping strategy is used to estimate the position and effect of a coarsely mapped QTL more accurately by creating a new experimental population. Such a population can be obtained by crossing nearly isogenic lines (NILs) that differ only in the allelic constitution at the short chromosome segment (usually varying from about 10 to 30 cM in length) harbouring the QTL. Because of the absence of other segregating QTLs, the target QTL becomes the major genetic source of variation, and the phenotypic means of the QTL genotypic classes can be statistically differentiated and genotypes recognized accordingly. Appropriate replication and/or progeny testing are generally implemented based upon the heritability of the trait considered. Under such conditions, cM distances between a QTL and the nearby molecular markers can be estimated more precisely (Salvi & Tuberosa, 2005)

QTL analyses performed by Anderson et al. (2001) have positioned *Fhb1* between the markers X*GWM493* and X*GWM533* and the selection for this region made populations more resistant to FHB.

These markers have been routinely used for MAS of *Fhb1*. However, they still define a relatively large chromosome interval for the QTL and more markers closely linked to the QTL would improve the selection efficiency (Bernardo et al., 2012). *Fhb1* was successfully fine mapped on chromosome 3BS within a 1.27 cM interval using a Sumai 3*5/Thatcher mapping population by Cuthbert et al. (2006). Continuing studies could narrow down the *Fhb1* QTL to a 261 kb region with seven putative resistance genes identified in a chromosome 3B BAC library of Chinese Spring. However, the transformation of the candidate genes into susceptible lines did not induce resistance. Besides, a new nearly diagnostic marker was found, X*UMN10* (Liu et al., 2008). Bernardo et al. (2012) suggested two SNP markers for *Fhb1*, which can be used for MAS: X*Snp11* for Chinese and Japanese accessions with *Fhb1* and X*Snp3BS-8* for Sumai 3-related accessions carrying *Fhb1*.

A number of responsible candidate genes have been identified during various studies, though no gene has been validated to date.

Candidate genes for the QTL *Fhb1*

Numerous studies were undertaken to evaluate candidate genes of the major FHB QTL *Fhb1*. Lemmens et al. (2005) concluded that resistance to DON is important in the FHB resistance complex and hypothesized that *Fhb1* either encodes a DON-glucosyltransferase or regulates the expression of such an enzyme.

On the other hand, Gunnaiah et al. (2012) provided evidence that the resistance in *Fhb1* is not due to the detoxification of DON by the glucosyltransferase. Instead, they suggest the involvement of hydroxycinnamic acid amides, flavonoids and lignin monomers in the formation of cell wall appositions. Those parameters play a significant role in restricting the movement of *F. graminearum* in the rachises of plants (Gunnaiah et al., 2012).

By using transcriptome data, it has been reported that wheat resists the spread of *F. graminearum* in the infected spikelets mainly through the activation of the jasmonic acid (JA) defence pathway (Xiao et al., 2013).

1.4 Aim of the master thesis

All corresponding experiments have been performed at the Institute of Biotechnology in Plant Production (IFA-Tulln) of the University of Natural Resources and Life Sciences, Vienna and were part of a seven-year SFB project.

During the past years, a lot of research has been performed to identify the two major Fusarium resistance QTLs *Fhb1* on chromosome 3B and *Qfhs.ifa-5A* on chromosome 5A. However, the corresponding genes are not identified yet. QTL fine mapping approaches can be applied to narrow down QTL regions and to identify candidate genes eventually.

The experiments performed for this master thesis at hand, contributed directly to the fine mapping of the QTL *Fhb1* on chromosome 3B. Three different fields of responsibility can be described:

1.) The identification of recombinant near-isogenic lines with the use of molecular markers flanking the QTL *Fhb1*.

2.) The characterisation of recombinant wheat lines in the *Fhb1* region with additional molecular markers.

3.) A case study, whereas recombinant lines together with control lines were phenotyped for FHB resistance using artificial inoculation.

2. Materials and Methods

In this section the materials and methods, which were used during the practical experiments, are covered.

2.1 Plant material

For QTL fine mapping studies, an appropriate plant mapping population has to be established to facilitate segregation of the QTL in a fixed genetic background. Preliminary work for more than ten years has led to those plant lines, which were used for this master thesis at hand.

2.1.1 Parental lines

The objects of investigation for this master thesis were near-isogenic lines (NILs), which resulted from the initial cross of two parental lines, CM-82036 and Remus.

CM-82036 originates from the cross Sumai#3/ Thornbird-S, was developed in a shuttle breeding program between CIMMYT Mexico and South America and is highly resistant to FHB. Remus, on the other hand, is a spring wheat cultivar, which was developed at the Bavarian State Institute for Agronomy in Freising, Germany. It harbours well-adapted agronomic characters for cultivation in central Europe, but it is highly susceptible to FHB infection (Buerstmayr et al., 2002).

Figure 3: Parental lines CM-82036 (left) and Remus (right) used for this master thesis.

A population of F_1-derived doubled-haploid (DH) lines from the initial cross was used as mapping population and two major FHB resistance QTL, *Fhb1* and *Qfhs.ifa-5A*, were identified (Buerstmayr et al., 2002, 2003).

2.1.2 Near-isogenic lines

For the development of NILs for the *Fhb1* QTL, two different doubled-haploid lines (E2-106-U, E2-62-T), with the genetic background of Remus in the *Fhb1* region, were chosen and five times backcrossed with CM-82036. After each backcross cycle plants were genotyped with SSR markers flanking *Fhb1* and plants heterozygous in the *Fhb1* region were selected and further backcrossed. As a result, the plants of this BC_5population shared the genetic background with CM-82036 to 98%.

Furthermore, four different NILs were included as control lines for phenotyping tests. The genetic background relating to the QTL region on chromosome 3B and 5A is known. Both CM-NIL 38 and CM-NIL 43 exhibit the allele of CM-83036 at the *Fhb1* locus on chromosome 3B. In contrast, CM-NIL 47 and CM-NIL 51 possess Remus alleles at this position.

2.1.3 Establishment of a BC_5F_2 fine mapping population

In the BC_5 generation, four plants (number 8, 16, 26 and 34) heterozygous in the *Fhb1* region were selected, plant 8 and 34 with E2-62-T and 16 and 26 with E2-106-U as donor lines for the susceptible Remus alleles in the *Fhb1* region. In order to develop a large fine mapping population, these four lines were further multiplied. Therefore, twenty seeds per line were planted, genotyped with markers flanking *Fhb1* and again plants heterozygous in the *Fhb1* region were selected. Seeds of the selected lines were harvested and bulked for the four different subpopulations.

This plant material was used for the master thesis at hand, representing a BC_5 population with four subpopulations in a 98% CM-82036 background and for the *Fhb1* region on chromosome 3B in the F_2 generation.

2.1.4 BC_5F_3 fine mapping lines

Several BC_5F_2 lines (identified as heterozygous recombinant) were brought to the next generation, representing a BC_5F_3 population, to select homozygous recombinants. Since one allele was already fixed in the F_2 generation, the heterozygous allele would segregate according to Mendelian rules, resulting in homozygous recombinant F_3 lines. A more detailed

scheme about the development of the plant material used for this master thesis can be seen in figure 4.

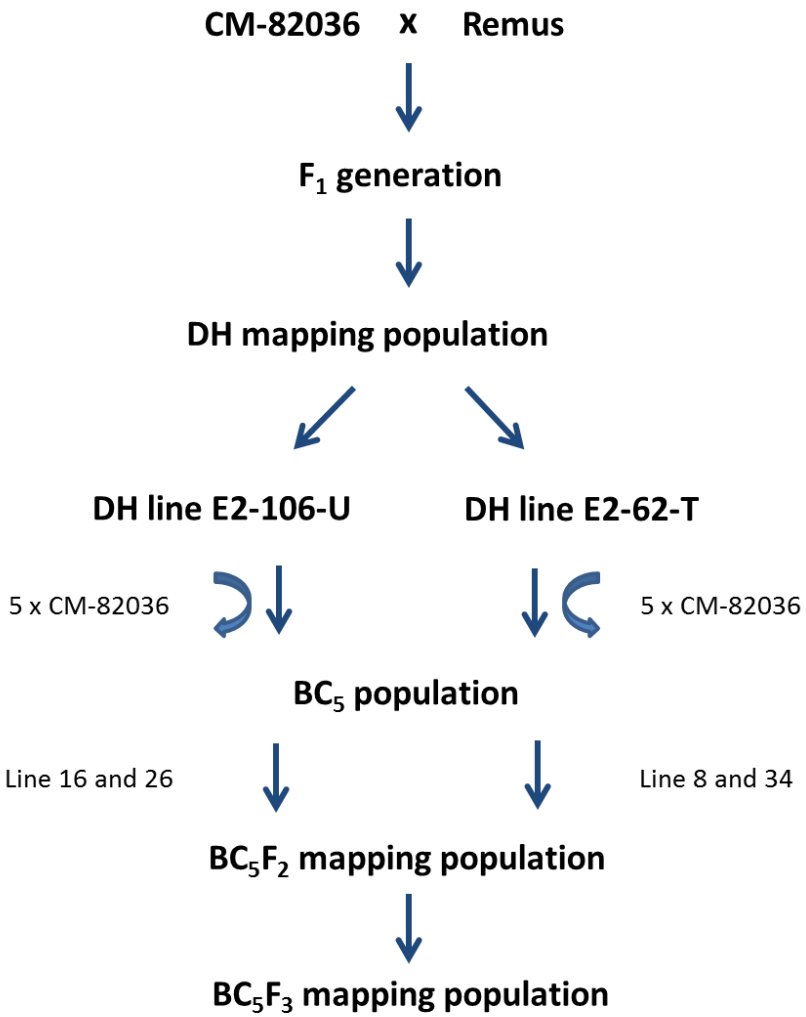


Figure 4: Schematic illustration of the development of the BC_5F_2 and BC_5F_3 plant populations, which were used for the master thesis at hand

2.2 Genotyping of the BC_5F_2 fine mapping population

2.2.1 Glasshouse conditions and leaf sample preparations

In September 2013, seeds of the BC_5F_2 population were planted in the greenhouse with about 520 seeds per subpopulation, resulting in 2080 BC_5F_2 plants. The seeds were grown in 40 well trays (5 x 8), using a mixture of compost and sand as substrate. To ensure simultaneous germination, the trays were put in a vernalisation chamber for 3 days at 4°C.

After 3 weeks leaf samples were taken. At least two fully developed leafs of a minimum length of 10 cm were cut into paper bags and dried in an oven at 36°C for 2 days. The dried leaf samples were stored in a cooling chamber before DNA extraction.

The seeds from the BC_5F_2 population were sown in September and the heads were harvested in January. During the glasshouse phase, an outfall came to pass (about 700 lines) due to mice, birds and the development of infertile heads.

Figure 5: Wheat plants growing in the glass house.

2.2.2 DNA extraction of the BC_5F_2 population

The DNA extraction was performed in 1.2 mL tubes with 8 strips fixed in 96 well racks. In every tube, 5 to 7 small glass beads were filled. Dried leave material was cut into each tube, at which contamination had to be avoided. Two 96 racks at a time were spanned into a Retsch-mill and shaken for 10 minutes, in order to break down the cell wall.

600 mL of freshly made CTAB-Buffer (details can be seen in table 1) were added to the powdered samples under a fume hood. The racks were placed in a water bath at 65°C for 60-90 minutes. After the stripes had reached room temperature, 350 µL chloroform:isoamylalcohol (24:1) were added to each tube under the fume hood. For 5 minutes they had to be shaken by inversion, afterwards the tubes were centrifuged for 10 minutes at 3500 rcf (Sigma 4K15 centrifuge). 300 µL of the top aqueous layer were pipetted off and transferred into new tubes. 300 µL of isopropyl alcohol were added and the tubes

were mixed by gentle inversion. A centrifugation step followed, 8 minutes at about 600 rcf. Since the DNA-pellet sticks to the bottom of the tube, one can pour off the liquid.

100 µL of Wash 1 solution were added to the pellet and mixed for 5 minutes, then the tubes were centrifuged for 8 minutes at about 600 rcf. The liquid was poured off and the washing step was repeated with Wash solution 2. The pellet dried overnight and dissolved the next day with 0.1 M TE buffer. The racks were shaken at room temperature for one day, and then they were stored at 4°C for further uses.

Table 1: Components and amounts of CTAB buffer (100 mL).

Stock	final concentration	final volume of 100 mL
dH_20	-	65 mL
1M Tris-7.5	100 mM	10 mL
5M NaCl[1]	700 mM	14 mL
0.5 M EDTA-(8.0 pH)	50mM	10 mL
CTAB[2]	1%	1 g
14 M BME[3]	140 mM	1 mL

[1] Use freshly made; warm buffer to 60-65°C before adding the CTAB and BME

[2] CTAB = Mixed alkyltrimethyl-ammonium bromide (Sigma M-7635).

[3] Add BME (β-mercaptoethanol) just prior to use, under a fume hood.

2.2.3 DNA quality check and dilution

In order to get a first estimation about the resulting DNA concentrations of the extracted samples, the Spectrophotometer BioSpec-nano was used. Samples with a very high DNA concentration (more than 100 ng/µl DNA) were diluted with 0.1 M TE buffer.

For exact calculations of the concentration and the quality of the samples, a Microplate reader (TECAN spectrafluor plus) was used. Absorptions at 260 nm and 280 nm were measured for all samples.

The quality of the DNA was assessed using both wavelengths, 260 nm and 280 nm (see formula 1). A ratio between 1.8 and 2 indicates good quality of the DNA.

$$DNA\ purity\ level = \frac{A_{260}\ [nm]}{A_{280}\ [nm]}$$

Formula 1: Calculation of the DNA purity level.

A_{260}… Absorption at 260 nm

A_{280}… Absorption at 280 nm

The quantification of the DNA was performed using formula 2. The extracted DNA samples were diluted to a final working DNA concentration of 50 ng/µL. The corresponding amounts of 0.1 M TE 8 buffer had to be added.

$$DNA\ concentration\ [ng/\mu l] = \frac{A_{260}\ \times 50\ \times df}{d\ [cm]}$$

Formula 2: Calculation of the DNA concentration.

A_{260}… Absorption at 260 nm

50… conversion factor, used for double strand DNA

df… dilution factor

d… layer thickness [cm]

2.2.4 PCR amplification with molecular markers flanking the *Fhb1* region

In order to identify wheat lines of the BC_5F_2 population, which have undergone a recombination event within the *Fhb1* region, all plant lines were screened with two molecular markers, namely *GWM493* and *BARC133*. Since those markers border the *Fhb1* region, a recombination event is be detected, whenever the alleles for those two markers differ within the same plant line.

Gradient PCR

A gradient PCR was done to optimize the annealing temperatures of the marker *BARC133*, since the marker was hardly scorable during the initial screenings. 15 different annealing temperatures were tested, starting from 54.2°C up to 61.9°C. Table 2 shows the applied temperatures used for the gradient PCR.

Table 2: Different annealing temperatures for the gradient PCR.

Temperature in [°C]	54,2	54,2	54,4	54,6	55,0	55,4	55,9	56,4	57,0	57,6	58,3	58,9	59,6	60,2	60,8	61,4	61,9

PCR with flanking markers

The two different primer sets (*GWM493* and *BARC133*), which were used for the initial screening are SSR markers, which means one can distinguish different alleles according to the length of fragment which will be amplified during PCR. More detailed information about the primers is presented in table 3.

For the PCR reactions 384 well-plates were used. The total reaction volume of each well represented 10 µL (2µL DNA working solution and 8 µL master mix). The markers for the initial screening were directly labelled. This means, that primers are linked to an infrared dye. The primers become incorporated into the PCR fragments and they can then be detected by different wavelengths (700 or 800 nm) by appropriate devices. Table 4 and 5 provide more information about PCR components and cycle conditions.

Table 3: Information about two SSR markers flanking the *Fhb1* region.

QTL	Marker	Labelling	Forward primer sequence	Reverse primer sequence	References
Fhb1	*BARC133*	direct	5'AGCGCTCGAAAAGTCAG-3'	5'GGCAGGTCCAACTCCAG-3'	Liu et al. (2006)
Fhb1	*GWM493*	direct	5'ACATACAATGGGATTCCAGCAG-3'	5'AACAAACGGTGTTCATGCAAGT-3'	Anderson et al. (2001)

Table 4: Components and amounts of a PCR reaction for directly labelled SSR markers.

PCR components	Stock	Final concentration	Reaction Volume (10 µL)
DNA	50 ng/µL	10 ng/µL	2 µL
PCR-Puffer incl. 1,5 mM MgCl2	10 x	1 x	1 µL
dNTPs	2 mM	0,2 mM	1 µL
R_primer	10 µM	0,2 µM	0,2 µL
F_primer	10 µM	0,2 µM	0,2 µL
GoTAQ	5 µL	0,05 U/µL	0,1 µL
PCR-H_2O			5,5 µL

Table 5: PCR cycle conditions for directly labelled SSR markers.

Steps	Temperature	Time [min]	Runs
1	94 °C	03:00	x1
2	94 °C	01:00	x35
3	60 °C	00:45	
4	72 °C	01:00	
5	72 °C	10:00	x1
6	14 °C	∞	∞

2.2.5 Analysis of the PCR fragments using a LI-COR System (PAGE)

For the separation of the PCR fragments, a polyacrylamide gel electrophoresis (PAGE) was used. The marker screenings were undertaken at three different LI-COR 4200 Fragment Analyzers. The scanner of the device can detect two different wavelengths, 700 nm and 800 nm. As a result, one can load two samples at a time, preconditioned the primer sets are labelled differently. The comb features 64 slots, thus 128 PCR products can be screened in the course of one measurement. Every day, a new gel was prepared.

Gel Pouring

Two glass plates were cleaned with 70% ethanol. Two separation stripes (each 0.25mm thick) were placed between the two glass plates, determining the gel thickness. Clamps fixed the construction.

For obtaining a gel consisting of 7% polyacrylamide, 24 mL of 7 % urea/TBE stock solution, 3.5 mL Long Ranger PAA, 250 µL DMSO, 175 µL APS (10%) and 25 µL TEMED were mixed under the fume hood. The glass plate construction was stabilized in a slanting position and the gel was poured between the two glass plates. A spacer was inserted for creating place at the top 5 mm of the gel, as the comb was inserted there instead afterwards. The polymerisation process took approximately 20 - 30 minutes. Subsequently, the glass plate construction with the gel was washed, the spacer was removed and the comb had to be inserted. A buffer chamber was fixed to the construction and after putting the gel into the LI-COR Analyzer, the chamber was filled with 1 x TBE running buffer. The computer and the device had to be switched on. After the calibration step, one had to wait at least 10 minutes in order to have the device heated up the gel to 48°C before loading the samples.

For the sake of convenience in terms of loading the samples into the wells, the PCR products were mixed with a Fuchsine loading dye. The samples were heated up for 3 minutes at 94°C in order to separate the strands. Then, the PCR plates were cooled on ice and loaded into the wells of the polyacrylamide gel using a 8-channel Hamilton syringe. The loading volume was 0.7 µL.

Gel images and scoring

An electric field was then applied across the gel, resulting into a separation of the fragments depending on their size. A scanner detected the fragments and the computer created an image, where the DNA fragments could be scored. Table 6 shows the applied scoring scheme, whereas parent A is determined as CM-82036 (resistant parent) and parent B as Remus (susceptible cultivar).

Table 6: Scoring scheme of identified alleles.

Sign	Genotypic classes
A	homozygous parent A
B	homozygous parent B
H	heterozygous
C	NOT homozygous A (homozygous B or heterozygous)
D	NOT homozygous B (homozygous A or heterozygous)
-	missing value

2.3 Genotyping of recombinant BC_5F_3 lines

Recombinant BC_5F_2 lines, but still heterozygous for one of the two flanking markers, were brought to the BC_5F_3 generation in order to select the respective recombinant homozygous lines (second selection process) and to characterize them with additional markers. DNA extraction, DNA quality check and dilution and genotyping with markers flanking *Fhb1* were performed similar as for the BC_5F_2 population.

2.3.1. Glasshouse conditions and leaf sample preparation

Wherever applicable, 8 seeds per plant where sown, resulting in 811 plant lines. Since the heterozygote flanking marker should segregate according to Mendelian rules, at least two out of eight sown plants should end up with homozygous recombinant alleles. They were sown in March and harvested in June.

2.3.2 PCR amplification with molecular markers within the *Fhb1* region

For the purpose of a genetic fine mapping, more molecular markers are needed to characterize the *Fhb1* region in recombinant wheat lines. Therefore, one marker (namely *UMN10*) was applied and five other SSR markers were tested, if they are applicable to our plant lines. The M13 tail labelling method was used for those markers, which are explained below.

M13 tail labelling technique for markers within the *Fhb1* region

Figure 6 shows the general outline of the procedure for fluorescent dye labelling of PCR fragments in one reaction, which is performed with three primers: a sequence-specific forward primer with M13 tail at its 5' end, a sequence-specific reverse primer, and the universal fluorescent-labelled M13 primer. The amount of the forward primer should be less than half of the reverse primer. The thermocycling conditions are chosen such that during the first cycles, the forward primer with its M13 sequence is incorporated into the accumulating PCR products. Later, when the forward primer is used up, the annealing temperature is lowered to facilitate annealing of the universal M13 primer. Thus, the universal fluorescent-labelled M13 primer 'takes over' as the forward primer and incorporates the fluorescent dye into the PCR product (Schuelke, 2000).

For visualisation of the PCR products, the infrared dyes (IRD) 700 and 800 were used for the LI-COR system. Information about *UMN10*, the PCR components and PCR cycle conditions for M13-tailed primers can be seen in table7, 8 and 9.

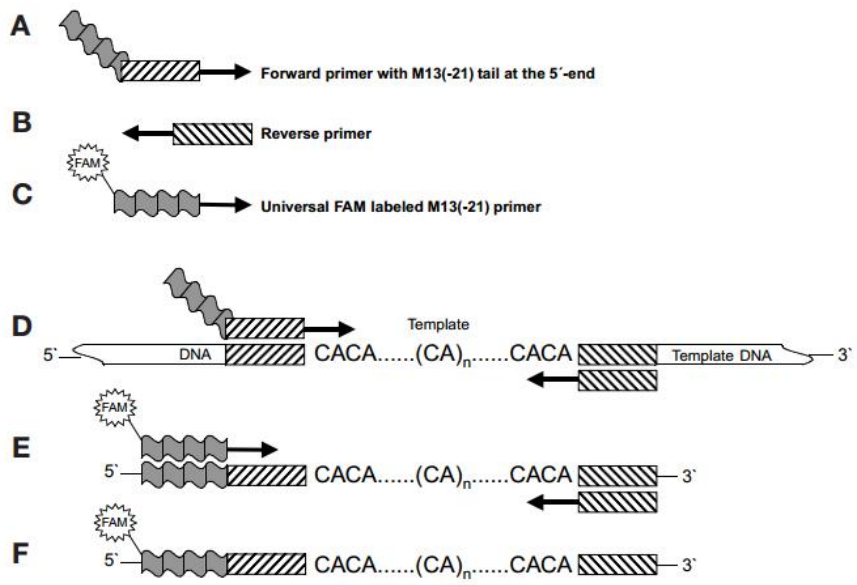


Figure 6: Labelling of the PCR fragments during the PCR with FAM. 'A' shows a sequence-specific forward primer with M13-tail at the 5´-end, 'B' a conventional sequence-specific reverse primer. 'C' shows the FAM labelled M13 primer. During the first cycles, the forward primer with its M13 sequence is incorporated into the produced PCR fragments ('D'). 'E' shows the later cycles. The FAM labelled M13 primer anneals at the M13 sequence of the fragments and substitutes the forward primer. The amplifying and labelling is finished ('F') (Markus Schuelke, 2000).

Table 7: Information about the marker *UMN10*, a marker within the *Fhb1* region.

QTL	Marker	Labelling	Forward primer sequence	Reverse primer sequence	References
Fhb1	*UMN10*	M13	5'CGTGGTTCCACGTCTTCTA-3'	5'TGAAGTTCATGCCACGCATA-3'	Liu et al. (2008)

Table 8: Components and amounts of a PCR reaction for M13-labelled markers.

PCR components	Stock	Final concentration	Reaction Volume (10 µL)
DNA	50 ng/µL	10 ng/µL	2 µL
PCR-Puffer incl. 1,5 mM MgCl2	10 x	1 x	1 µL
dNTPs	2 mM	0,2 mM	1 µL
R_primer	10 µM	0,2 µM	0,2 µL
F_primer	10 µM	0,02 µM	0,02 µL
M13_primer	10 µM	0,18 µM	0,18 µL
GoTAQ	5 µL	0,05 U/µL	0,1 µL
PCR-H_2O			5,5 µL

Table 9: PCR cycle conditions for M13-labelled markers.

Steps	Temperature	Time [min]	Runs
1	95 °C	02:00	x1
2	95 °C	00:50	x6
3	63 °C	01:30	
4	72 °C	01:30	
5	95 °C	00:50	x25
6	51 °C	00:50	
7	72 °C	01:00	
8	72 °C	00:50	x1
9	14 °C	∞	∞

Testing of five different SSR markers

More molecular markers were needed in order to characterize the QTL region more precisely. Unfortunately, the applicability of published molecular markers for the plant population at hand is limited, since they have been designed for a specific cross of different varieties and may not work for other populations. Five different SSR markers (see table 10) were tested. Those markers originate from Hao et al. (2012).

Table 10: Information about tested SSR markers.

QTL	Marker	Labelling	Forward primer sequence	Reverse primer sequence	References
Fhb1	*cfb6011*	M13	5'TTCGTCTCCTTTTGTCACCC-3'	5'GAAAAAGGGAGGAGGTGTCC-3'	Hao et al. (2012)
Fhb1	*cfb6055*	M13	5'ATTTCCCCTCTTGACTTGGG-3'	5'GTTGGAGAGAGCGCGAAG-3'	Hao et al. (2012)
Fhb1	*cfb6058*	M13	5'AGTCACCGAATTGAACGGAG-3'	5'GACGTGAGCCGACTTGAAAC-3'	Hao et al. (2012)
Fhb1	*cfb6061*	M13	5'ACTCCTCCGACTTCAGGTCA-3'	5'GTCCTGGTAATTGTGCAGGC-3'	Hao et al. (2012)
Fhb1	*cfb6067*	M13	5'TATGCCACCAAGTTTCCCTC-3'	5'GGGTCAATTCACCGTGTTTC-3'	Hao et al. (2012)

PCR components and PCR cycle conditions were the same as used for the marker *UMN10*.

2.3.3 Kompetitive Allele Specific PCR (KASP)

In addition to the above mentioned SSR markers, one SNP marker was used for the screening of assumed recombinant lines of the second selection session. As the marker *Snp3BS-8* is not a flanking marker of the *Fhb1* region, it was not used for the initial genotyping. Information about this marker is provided by table 11.

The KASP-analysis was performed using a special KASP kit, provided by the company LGC Genomics. The kit comprises two components: KASP Primer mix (two allele-specific primers and a common reverse primer) and a KASP Master mix (Taq polymerase enzyme, passive reference dye, 5-carboxy-X-rhodamine, succinimidyl ester (ROX), MgCl2, DMSO).

The KASP analyses were performed with a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad). Appropriate white 384 well-plates were used. The plates had to be lightproof to avoid interference while measuring fluorescence. FAM and HEX were used for distinction of the different alleles. PCR components and cycle information can be seen in table 12 and 13.

Table 11: Information about the SNP marker within the *Fhb1* region.

QTL	Marker	Labelling	Forward primer sequence	Reverse primer sequence	References
Fhb1	*Snp3BS-8*	FAM/HEX	5'TAACTCTGAAACAAAGCAGCCT-3'	5'CAGAGGTGAGAAGTTCAATCCA-3'	Bernardo et al. (2012)

Table 12: Components and amounts of the KASP analysis.

PCR components	Stock	Final concentration	Reaction Volume (5 µL)
DNA	20 ng/µL	10 ng/µL	2,5 µL
Mastermix für KASP	2 x	1 x	2,5 µL
Primermix für KASP	2 µM	0,035 µM	0,07 µL
PCR-H_2O	-	-	-

Table 13: Cycle conditions for a KASP analysis using a SNP marker.

Steps	Temperature	Time [min]	Runs
1	94 °C	15:00	x1
2	94 °C	00:20	x10
3	65-57 °C	01:00	
4	94 °C	00:20	x26
5	57 °C	01:00	
6	37 °C	01:00	x1
Picture	-	-	x1

2.4 Data analysis

The gel pictures were scored using Adobe Photoshop CS2 (version 9.0). All allelic data from the gel images were entered into Excel 2010.

In case of the KASP analysis, the created data were transferred from the CFX Manager software (Bio-Rad Laboratories) into Excel 2010 as well.

2.4.1 Chi² Test for segregation distortion of individual markers

In addition, a Chi^2 test was applied for each individual marker to check whether the alleles segregate according to Mendelian rules and be in agreement with the expected numbers. The test was used for the two flanking markers (*GWM493* and *BARC133*). In general, the alleles should segregate according to the split ratio of co-dominant alleles of 1:2:1 (1x A, 2x H, 1x B) in the F_2 generation. The following hypotheses can be claimed:

Table 14: Hypotheses of the Chi^2 test.

Null hypothesis (H_0):	The alleles segregate in the expected 1:2:1 ratio.
Alternative hypothesis (H_a):	The alleles do not segregate in the expected 1:2:1 ratio.

The significance level of the tests was 0.05. If the resulting p-value is less than the significance level, the null hypothesis can be rejected.

The calculations for the Chi^2 test were performed using the following formula:

$$x^2 = \sum_{i=1}^{n} \frac{(O-E)^2}{E}$$

Formula 3: Calculation of Chi^2 value.

O… observed number in a class

E… expected number in a class

n… class of groups

2.4.2 Calculation of the recombination frequencies

In order to evaluate the recombination rates (in centimorgan) of the markers *GWM493*, *BARC133*, *UMN10* and *Snp3BS-8*, the open-source program Carthagene (version 1.3) was used (de Givry et al., 2005). Haldane's and the Kosambi's mapping function were applied. Whereas Haldane's mapping function works well for situations where crossover interference does not occur, the Kosambi's function does involve interferences which increase or

decrease the distance (Liu, 1997). Ambiguous values were excluded from the calculation, as the alleles were not definite. In total, data from 1852 analysed lines for the four markers were used to estimate the genetic distances.

Based on the calculated distances according to the Kosambi's function of Carthagene, a genetic map was created using the program MapChart (version 2.1).

2.5 Phenotyping of the homozygous recombinant lines for the FHB resistance

In the course of this master thesis, a case study was undertaken to reveal the significance of the *Fhb1* phenotyping in terms of type II resistance and to test a specific inoculation method. Three homozygous recombinant lines (line 957, 1469 and 1546), which were identified in the BC_5F_2 generation, were planted in pots with an approximate diameter of 30 cm. At least three kernels per line were sown in one pot. Multiple seed heads were formed due to tillering.

Furthermore, the control lines CM-NIL 38, CM-NIL 43, CM-NIL 47, CM-NIL 51, Remus and CM-82036 were included in the inoculation tests. Seven to ten heads per replication line were inoculated.

Figure 7: Demonstration of the used inoculation method.

These nine lines were inoculated at anthesis stage (two replications per line) with a spore suspension of *Fusarium graminearum* (500.000 spores/mL) by single floret inoculation using a special pipette, which was furnished with a small needle. The florets of two different central spikelets were inoculated, whereas the needle tip of the pipette penetrated the glumes. The inoculum amount was 10µL of spore suspension (5000 conidia per floret).

The heads were humidified with distilled water and covered with a plastic bag for 24 hours to ensure optimal conditions for the fungal infection. Too hot temperatures can interfere with the inoculation success. Therefore, inoculation was only performed at maximum 26°C

air temperature and preferably in the morning to ensure consistent conditions. The plants grew in the glasshouse until anthesis stage. They were then transferred in a canopied grid installation and the inoculation was performed in the open air.

The spread of the disease symptoms from the inoculation site along the head was recorded at 20 days after the inoculation (DAI) by counting the number of infected or bleached spikelets per head. Thus, the incidence of spreading was calculated by the percentage of heads with more symptomatic spikelets than the two inoculated.

3. Results

The following section illustrates the results and final outcomes of this master thesis. The results are demonstrated in a chronological order, which resembles the workflow during the experiments of the master thesis.

Besides the main results and selection of recombinant lines in the *Fhb1* region, investigations with respect to method improvement were carried out as well.

3.1 Genotyping of the BC_5F_2 population with SSR markers flanking *Fhb1*

3.1.1 Gradient PCR for *BARC133*

During the initial screenings, the scoring of the marker *BARC133* was difficult due to unwanted amplified fragments. A gradient PCR was performed to investigate the optimal annealing temperature of the primers.

The PCR products were separated on a PAA gel and scored with '+' for a good, '-' for a bad and '~' for an average result. Factors concerning the evaluation were amplifying of unwanted fragments, visibility and sharpness of the fragments (see figure 8).

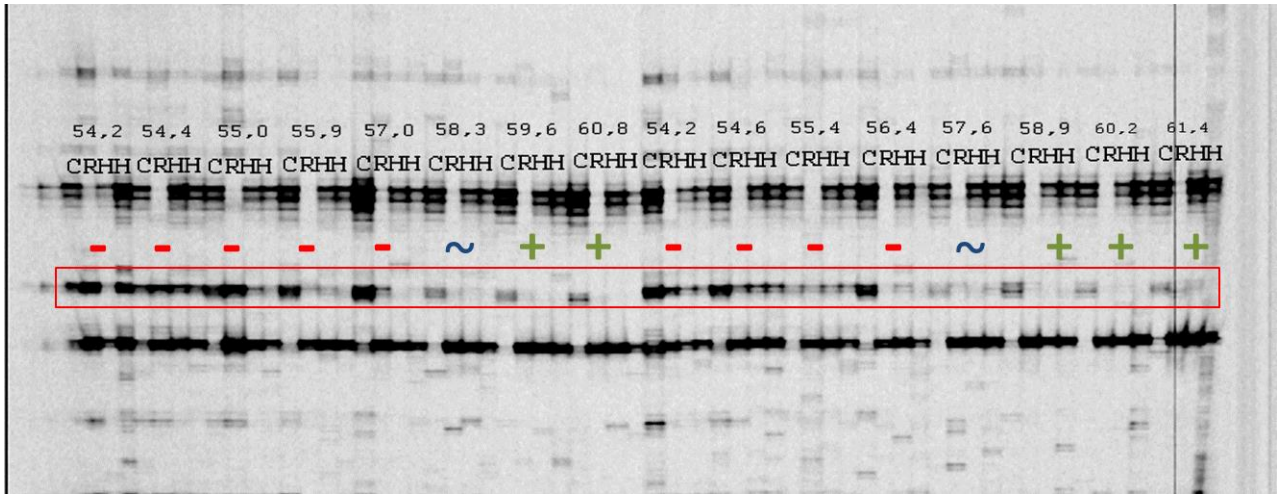


Figure 8: Scoring of gradient PCR fragments. A green plus indicates a good result without unwanted fragments, the blue sign was given for average results and a red minus shows temperatures. 'C' refers to the parental allele of CM-82036, 'R' to the allele of the parental line Remus, 'H' to heterozygous lines. The red, highlighted section shows unwanted bands.

Clearly, the optimal annealing temperature of the marker *BARC133* is between 59.6°C and 61.9°C, as those fragments are very clear and no additional, unwanted fragments are present (see red field in figure 8). The total temperature difference was of 7.7°C.

3.1.2 SSR marker genotyping

Initially, 2080 individual plants were analysed with two flanking markers of the QTL region *Fhb1*, *GWM493* and *BARC133*.

For all the screenings with SSR markers, a gel image was created and the allele type was assessed manually according to the scoring scheme. The band pattern and the corresponding scoring of those two markers can be seen in figure 9 and 10. Both markers amplified polymorphic fragments. To guarantee an optimal differentiation of the allele types, the two parents CM-82036 and Remus were included in the initial analyses.

The fragments of the marker *GWM493* do not vary much in size. As a result, the scoring of this marker was more difficult than the scoring of *BARC133*. Furthermore, stuttering of the microsatellites interfered with the evaluation.

In case of the marker *BARC133*, the scoring was easy after the annealing temperature adjustment during PCR from 55°C to 60°C.

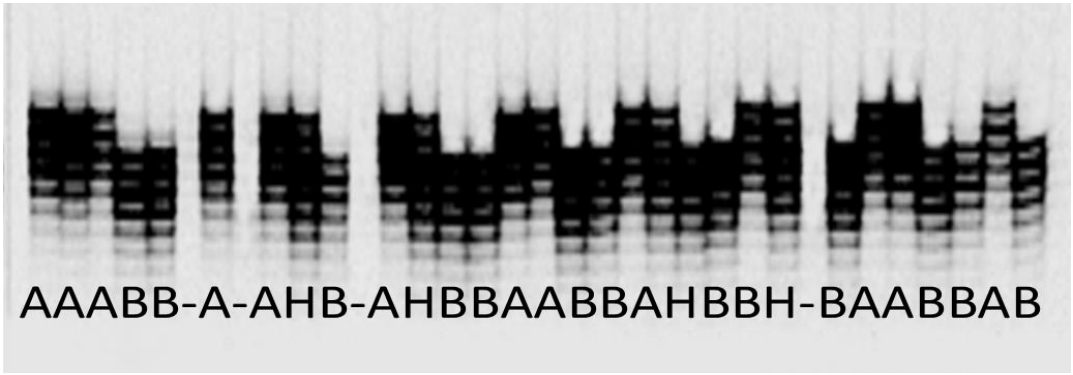


Figure 9: Band pattern and scoring of the SSR marker *GWM493*. The scoring 'A' refers to the allele of CM-82036, 'B' was given whenever alleles of Remus were detected and 'H' was given for heterozygous plant lines.

Figure 10: Band pattern and scoring of the SSR marker *BARC133*. The scoring 'A' refers to the allele of CM-82036, 'B' was given whenever alleles of Remus were detected and 'H' was given for heterozygous plant lines.

The screening of the 2080 F_2 lines resulted in the identification of 119 recombinant lines, which could be used for further analyses and seven lines were found being recombinant and homozygous. Unfortunately, only three homozygous recombinant lines could be used for further studies, since no seed material was available for four lines.

3.2 Genotyping of the BC_5F_3 lines with flanking markers and markers within the *Fhb1* region

Thereafter, the 119 recombinant BC_5F_2 lines, which were still heterozygous for one of the two flanking markers, were brought into the BC_5F_3 generation in order to select the respective recombinant homozygous lines. In general, eight seeds per line were sown, resulting in 812 lines. According to Mendelian segregation rules, two out of eight plants should end up being homozygous recombinant. All lines were screened once again with the two flanking SSR markers (*GWM493* and *BARC133*). Additionally, two more marker were applied, namely *UMN10* and *Snp3BS-8*. At the end of all marker screenings, 85 homozygous recombinant lines could be identified.

3.2.1 SSR marker genotyping within the *Fhb1* region

UMN10 was used for further characterization of the *Fhb1* region. The procedure of the preparation of the PAA gel was the same as for the markers *GWM493* and *BARC133*. The fragment pattern can be seen in figure 11.

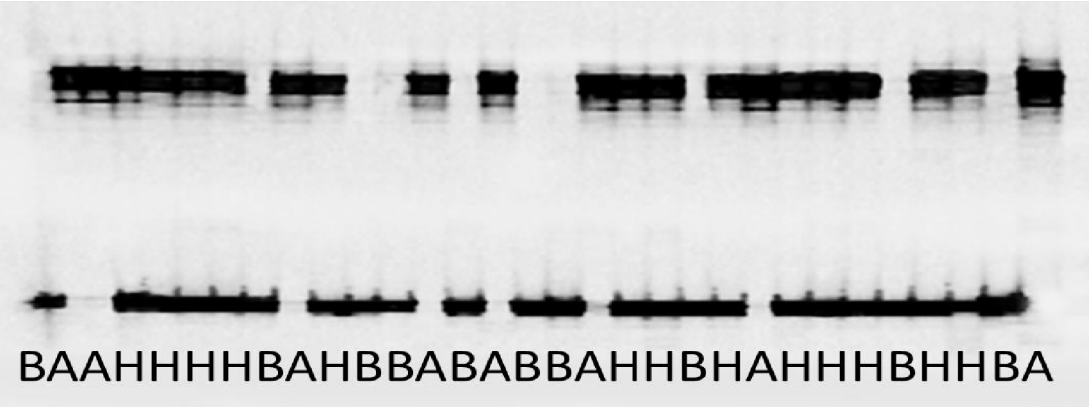


Figure 11: Band pattern and scoring of the marker *UMN10*. The scoring 'A' refers to the allele of CM-82036, 'B' was given whenever alleles of Remus were detected and 'H' was given for heterozygous plant lines.

3.2.2 Testing of five different SSR markers

Five published markers, originating from Hao et al. (2012) were tested on our parental lines CM-82036 and Remus (see figure 12).

Each marker was tested twice on both parents. The fragments from CM-82036 (C) and Remus (R) were arranged in the adjacent neighbourhood, so as to ease the comparison of the fragments on the gel image.

An efficient, co-dominant marker would let to the amplification of two different fragment patterns, which allows the distinction of different alleles. In contrast, a dominant marker serves only for the distinction of one specific allele type, the other allele type is not amplified. In order to make use of the published primers, size differences between the amplified fragments of CM-82036 and Remus should be present for a successful genotyping.

Figure 12 shows the banding pattern for the five different primers. None of them shows a co-dominant marker scheme. Only two markers, namely *cfb6067* and *cfb6011*, show a difference between the fragment pattern of CM-82036 and Remus. Since only one of the parental alleles is present, those markers could eventually serve as dominant markers. Further testing is needed to confirm this assumption.

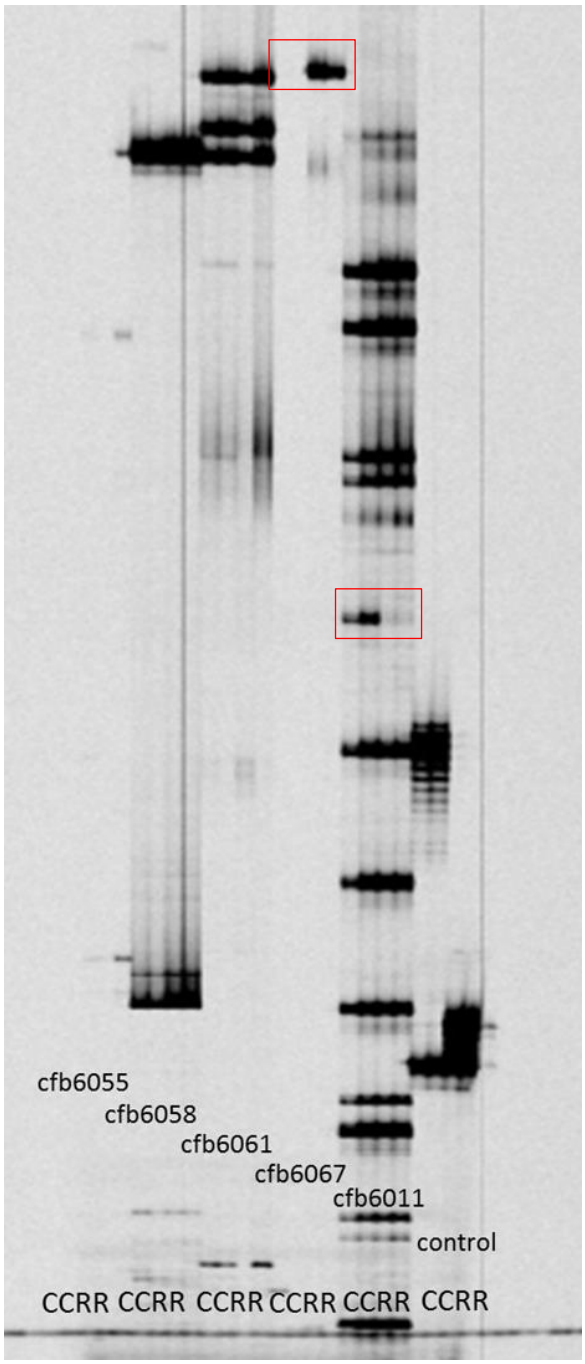


Figure 12: Band pattern of the marker test. The letter 'C' refers to the allelic origin of parent CM-82036 and 'R' for the origin of Remus. The red section shows a difference between the alleles of CM-82036 and Remus.

3.2.3 KASP analysis

Selected homozygous recombinant lines were tested with the KASP marker *Snp3BS-8* (Bernardo et al., 2012). The orange signals indicate the homozygous allele type of CM-82036. In contrast, the blue signals originate from homozygous alleles from the parent Remus. The green signals indicate a heterozygous allelic state. Five NTC's (non-template control) were included during the measurement, representing the black signals in figure 13.

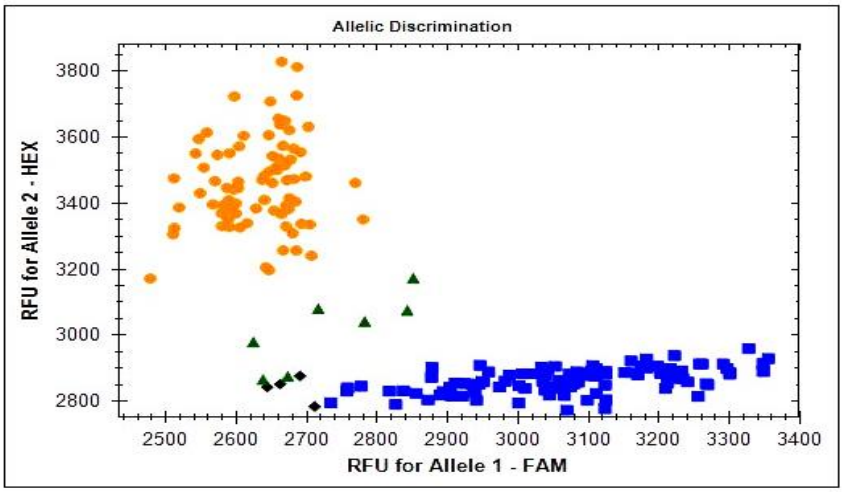


Figure 13: Allelic discrimination of a KASP-analysis for *Snp3BS-8*. The orange dots indicate a homozygote allelic state of parent CM-82036, the blue squares originate from homozygous alleles from parent Remus. The green signals were caused by heterozygous lines and black signals were NTCs.

The 119 lines were successfully genotyped with the marker *Snp3BS-8* and new haplotypes could be identified.

3.3 Chi² tests for segregation distortion of individual markers

A Chi² test was performed in order to check whether the number of observed individuals in a category differ significantly from the number expected individuals. The two flanking markers of *Fhb1* (namely *GWM493* and *BARC133*) were tested.

Table 15 and 16 show the calculated Chi² and p-values for the two above mentioned markers.

Table 15: Results of a Chi² test performed with marker *GWM493*. The letter 'A' stands for the allele type of parent CM-82036, 'B' indicates an allele type of parent Remus and 'H' stands for a heterozygous allelic state.

Marker	Allele	Observed frequency	Expected frequency	Difference	Difference Sq.	Diff. Sq. / Exp fr.
GWM493	**A**	421	463	-42.00	1764.00	3.81
	B	513	463	50.00	2500.00	5.40
	H	918	926	-8.00	64.00	0.07
					Chi2 value:	9.348
					p-value:	0.010

In case of the marker *GWM493*, the Chi^2 value is 9.348. Since the p-value is less than 0.05, the null hypothesis can be rejected and the alternative hypothesis is accepted. This indicates that the alleles do not segregate in an expected manner.

Table 16: Results of a Chi^2 test performed with marker *BARC133*. The letter 'A' stands for the allele type of parent CM-82036, 'B' indicates an allele type of parent Remus and 'H' stands for a heterozygous allelic state.

Marker	Allele	Observed frequency	Expected frequency	Difference	Difference Sq.	Diff. Sq. / Exp fr.
BARC133	**A**	405	463	-58.00	3364.00	7.27
	B	520	463	57.00	3249.00	7.02
	H	927	926	1.00	1.00	0.001
					Chi2 value:	14.285
					p-value:	0.001

The Chi^2 value for the test of *BARC133* is 14.285. A calculated p-value of 0.001 leads to the adoption of the alternative hypothesis, which means, that the observed frequencies differ significantly from the number expected individuals.

3.4 Calculation of the recombination frequencies

The recombination rate was calculated using the software Carthagene (see table 17). In addition, a genetic map of the *Fhb1* QTL on chromosome 3B was established (see figure 14) with four different markers, based on the evaluation of 1852 plant lines. According to Haldane's mapping function, the genetic distance between the two flanking markers is 3.3 cM. Kosambi's mapping function calculated a value of 3.2 cM.

Table 17: Calculated genetic distances in centimorgan (cM) according to Haldane's and Kosambi's mapping function

Number of marker	Marker name	Haldane distance	Kosambi distance
1	BARC133	0.9 cM	0.9 cM
2	UMN10	0.1 cM	0.1 cM
3	SNP8	2.3 cM	2.3 cM
4	GWM493	----------	----------
		3.3 cM	3,2 cM

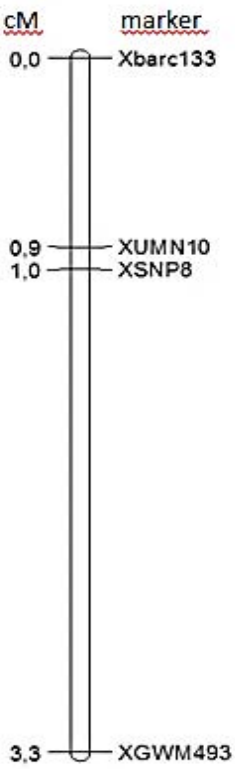
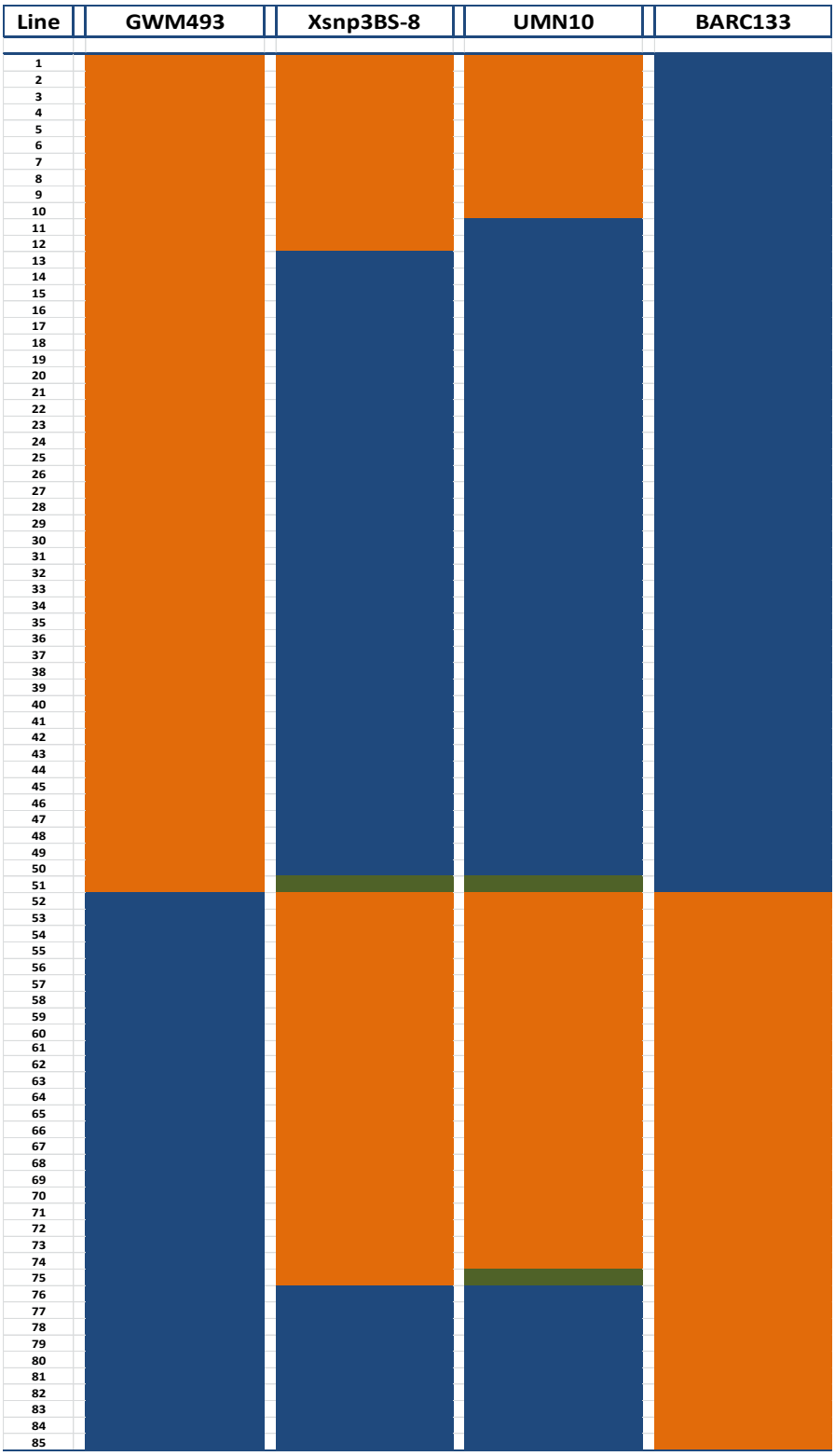


Figure 14: Genetic map of the *Fhb1* region according to Haldane's mapping function using four molecular markers. The unit is centimorgan (cM).

3.5 Lines with recombinations in the *Fhb1* region

At the end of all marker analyses, a list with all homozygous recombinant plant lines was created (see table 18). SSR marker data and KASP marker data were combined. In total, 85 lines were certified as recombinant. These lines form five haplotypes for the markers X*GWM493*, X*Snp3BS*-8, X*UMN10* and X*BARC133*. As expected from the genetic distances, the biggest haplotype groups with 61 and 20 lines respectively are the haplotypes with recombinations between X*GWM493* and X*Snp3BS*-8, and X*UMN10* and X*BARC133*. Two lines belong to the haplotype with recombinations between *XSnp3BS-8* and X*UMN10*. One plant line shows two recombination events at the *Fhb1* region, to be specific between X*GWM493* and X*Snp3BS*-8 and between X*UMN10* and X*BARC133*. The order of the markers in the table reflects the position of the according alleles on the *Fhb1* region.

Table 18: List of selected wheat lines, which are recombinant within the *Fhb1* QTL region. Four different markers were used. The blue colour was given for the parental CM-82036 allele, the orange colour for the parental Remus allele and the green colour shows a heterozygous allelic state.



3.6 Phenotyping results of the homozygous recombinant lines

The incidence of disease spreading and the disease severity were assessed by the percentage of heads with more symptomatic spikelets than the two inoculated. In general, the inoculation trial was partly successful. Two control lines (CM-NIL 51 and Remus) exhibited a high percentage of heads with spread of the disease (up to 100%) in both replications. This is in agreement with our expectations, since these two lines exhibit the allele of the susceptible parent Remus at the *Fhb1* locus. CM-NIL 47 and line 957 show also Remus alleles at the *Fhb1* region and both lines exhibit a very low level of the disease spread (two heads out of 17 for CM-NIL 47 and one out of 9 for line 957). In contrast, the lines 1469, 1546, CM-NIL 38, CM-NIL 43 and CM-82036 harbour the *Fhb1* QTL and indeed, almost no spread of the disease could be scored as can be seen in figure 15.

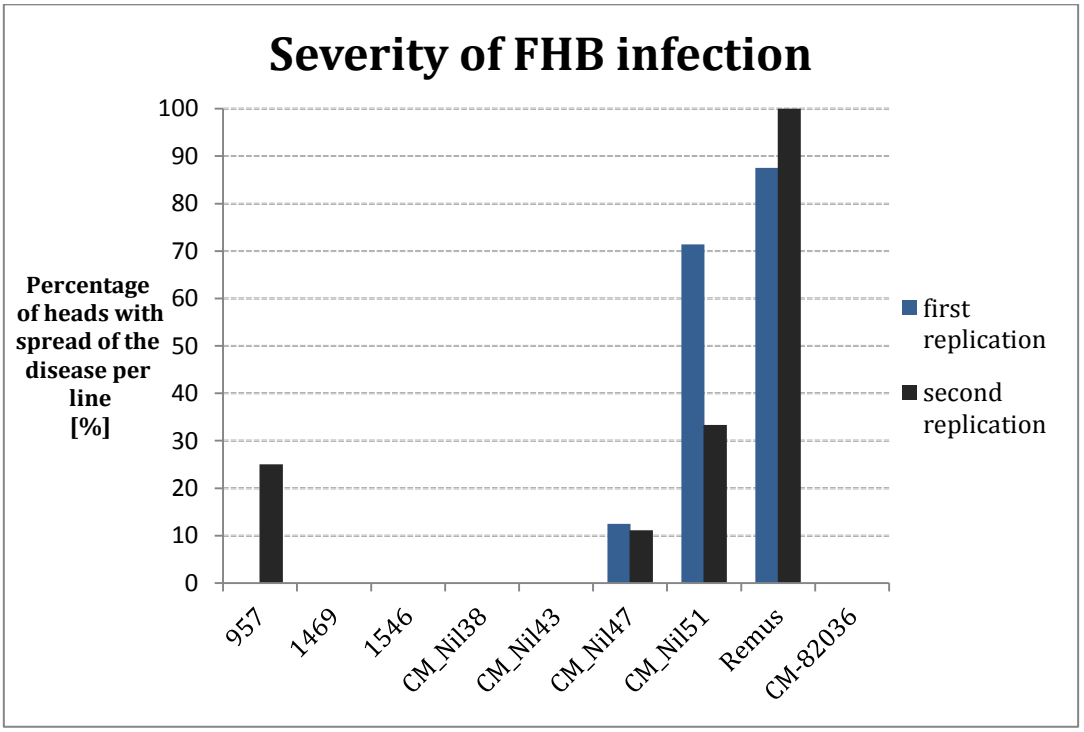


Figure 15: Spreading incidence of artificially inoculated wheat heads in percentage of heads with more than two symptomatic spikelets per line (%). The blue colour refers to the first, the black colour to the second replication.

4. Discussion

4.1 Genotyping of the *Fhb1* region with molecular markers

The characterization of the *Fhb1* region with molecular markers is an integral part of the fine mapping process.

In general, the genotyping with the markers (*GWM493*, *BARC133*, *UMN10* and *Snp3BS-8*) worked well. One main drawback for the SSR markers was the fact that the polyacrylamide gel had to be made freshly every day. Besides, the visual evaluation of the alleles was also time-consuming.

For the flanking markers *GWM493* and *BARC133*, several non-distinctive values were termed 'C' (not homozygous A) or 'D' (not homozygous B) (see table 6). Thus, many ambiguous alleles were not considered for the selection and for the calculation of the genetic map. However, the scoring was double-checked and multiple measurements of lines ensured a confident selection process.

Plus, selected plant lines with the flanking markers were further analysed with the marker *UMN10* and the SNP marker *Snp3BS-8*. By using several markers located in the *Fhb1* region and different methods as well, the identified alleles were verified to a very high extend.

Both SSR and SNP marker approaches feature some major advantages. SSR marker are very reliable, but they need substantial investment of time and money to develop, and adequate numbers for high-density mapping are not available in some crops (Collard & Mackill, 2008).

SNP markers, on the other hand, are highly abundant and the genotyping process is very fast (Ben-Ari & Lavi, 2012). For this reason, I am under the impression that SNP markers are the marker of choice, although the development and application is relatively expensive (Berkman et al., 2012).

4.1.1 Optimization of the PCR protocol for the marker *BARC133*

A gradient PCR was performed for the SSR marker *BARC133* to determine the ideal annealing temperature. Initially, the PCR has been performed at an annealing temperature of 55°C. The scoring was difficult by undesired amplified fragments and diffuse bands, which resulted

from a too low annealing temperature. This temperature is thus a critical step within the PCR, as the primer can also bind to other locations in the genome.

According to the test results (see figure 8), the optimal annealing temperatures is about 58.9 – 61.4°C. The effects caused by altered temperature are drastically within a PCR, since a temperature difference of less than 7°C caused a diverse banding pattern. For QTL analyses, it is very important that the primer binds to the correct chromosome/locus.

4.1.2 Testing of five different SSR markers

Five different SSR markers originating from Hao et al., (2012) were tested on our plant population. As mentioned before, QTL mapping results can only be applied for the individual mapping population (Bagge et al., 2007). This test was performed in order to evaluate, if those published markers are also polymorphic for our parental lines and can therefore be used to further saturate the *Fhb1* region with makers and support fine mapping (see figure 12).

Two out of five markers, namely *cfb6067* and *cfb6011*, showed a difference between the fragment pattern of CM-82036 and Remus. Unfortunately, only one fragment of either CM-82036 or Remus was amplified. Thus, those markers could eventually serve as dominant markers. Nevertheless, further testing is needed to confirm this assumption.

4.1.3 KASP analysis of recombinant wheat lines using *Snp3BS-8*

The KASP method is very fast, once suitable markers are developed. The allele evaluation was very easy and three distinct signals could be detected (homozygous 'A', homozygous 'B' and heterozygous ('H') alleles) (see figure 13). The selected homozygous recombinant lines were verified by this approach, using the SNP marker *Snp3BS-8*. This marker has been developed by Bernardo et al. (2012) and is very close to the nearly diagnostic marker *UMN10* (Liu et al., 2008).

4.2 Analysis of the genotypic data

4.2.1 Chi^2 test for segregation distortion of individual markers

The 2080 F_2 lines were genotyped with the two flanking markers *GWM493* and *BARC133*, 1852 lines could be assigned unambiguously to either homozygous CM-82036 or Remus, or

heterozygous. For these marker data, a Chi^2 test was performed to detect a significant deviation concerning the numbers of expected and observed allele frequencies.

In both cases, the analysed alleles did not segregate according to Mendelian rules. This is due to the fact that ambiguous values had to be excluded from this analysis. In case of *GWM493*, 1852 lines were considered and the p-value is very small, about 0.010. For *BARC133*, 1825 lines were tested as well, resulting in a p-value of 0.001.

The observed numbers of heterozygous lines for both markers equal almost the numbers of the expected alleles (see table 15 and 16). On the other hand, the expected and observed numbers of parental 'A' and 'B' allele types show a difference of about 60 individuals, whereas the observed number of the 'B' allele type exceed the expected for both markers. The observed number of the 'A' type is always less than the expected. This is most probably due to the fact that the discrimination during the visual scoring between 'A' and 'H' alleles was difficult and those values were termed as 'D'. Those ambiguous values were excluded from this analysis, and therefore the biggest variation between observed and expected numbers is mainly caused by 'A' and 'H' allele types.

4.2.2 Recombination rates and genetic distances

Recombination results from crossing over between homologs during meiosis and is fundamental for the development of specific plant material for the fine mapping of QTLs and for the creation of genetic maps. The distance between loci is expressed in centimorgans (cM), named after the *Drosophila* geneticist Thomas Hunt Morgan (Kumar, 1999; Liu, 1997).

The genetic distance was calculated using four molecular markers for this master study at hand (see table 17). The *Fhb1* region, representing the distance between the two flanking markers X*GWM493* and X*BARC133*, is thus 3.3 cM according to Haldane's mapping function. The marker X*UMN10* and X*Snp3BS*-8 are placed closely together, with a genetic distance of 0.1 cM.

Several mapping studies were carried out and have analysed the genetic distances of markers in the *Fhb1* region. Anderson et al. (2001) positioned *Fhb1* between the markers X*GWM493* and X*GWM533* with an approximate genetic distance of 7 cM. Almost the same genetic distance (8 cM) between these two markers was stated by Buerstmayr et al. (2002). Due to further studies, the *Fhb1* region was narrowed down, using the marker X*BARC133*

instead of X*GWM533*. Thus, these values serve only as guide values, since the marker X*GWM533* was not used for this master study at hand.

A fine mapping study, executed by Cuthbert et al. (2006), assigned the distance between the markers X*GWM493* and X*BARC133* and state that it amounts to 6.5 cM using a Sumai 3*5/Thatcher plant population. This value is twice the value which was calculated for the master thesis at hand (3.3 cM). According to Bernardo et al. (2012), the distance between X*GWM493* and X*Snp3BS*-8 is 1.1 cM (the calculated value during this master thesis was 2.3 cM), and the distance between X*Snp3BS*-8 and X*UMN10* is 0.6 cM in a Ning7840/Clark population (0.1 cM in this study).

One possible reason for the deviations of the genetic distances is probably the fact that some ambiguous values needed to be excluded from the data analysis. Especially the genetic distance between *XGWM493* and *XBARC133* is most likely underestimated, because many data points could not be unambiguously evaluated and therefore 228 lines had to be excluded. This fact influenced the ratio of recombinants and non-recombinants and might interfere with an exact calculation of genetic distances. Plus, four homozygous recombinant lines were not incorporated into this analysis, since no seed material for further studies and no marker data for markers within the *Fhb1* region (*XUMN10* and *XSnp3BS-8*) were present. For this reason, the genetic distance is most probably underestimated.

Differences concerning the genetic distance at the *Fhb1* region have also been reported in literature. Cuthbert et al. (2006) listed some possible reasons for this occurrence. It could be due to the difference in population size and structure, genetics of different FHB resistance sources of *Fhb1*, and/or the difference between the generations that were used in the studies.

4.3 Identified recombinant wheat lines for the *Fhb1* fine mapping approach

For QTL fine mapping approaches, only lines are interesting, which have undergone a recombination event in the *Fhb1* region. The recombination process occurs randomly on chromosomes. However, there is also evidence of genetic control and non-randomness of crossing over, such as ‘hot spots’ of recombination or site specific recombination (Liu, 1997).

The starting point for the selection was a BC_5F_2 population, comprising 2080 plants. Two selection steps were involved and at the end, 85 homozygous recombinant plants could be selected (see table 18). These lines form five haplotype. The two biggest haplotype groups are formed by lines with a recombination between the marker *XGWM493* and *XSnp3BS-8*, and between *XUMN10* and *XBARC133*. Two lines with a recombination between the markers *XUMN10* and *XSnp3BS-8* are very valuable and can essentially support gene cloning, once the site of recombination of these lines has been further characterized and the phenotype of these lines is available.

The identification of homozygous recombinant lines between closely linked markers in a F_2 is very rare. AAbb or aaBB plants are formed by the union of two Ab or two aB gametes. Suppose A and B are linked with a recombination frequency of r = 0.1. The probability of a Ab gamete is P(Ab) = ½ r = ½ (0.1) = 0.05. Consequently the probabilities of desired AAbb and aaBB plants are P(AAbb) + P(aaBB) = (P(Ab)*P(Ab)) + (P(aB)*P(aB)) = (0.05*0.05)+ (0.05*0.05) = 0.5%. Considering the about 2000 F_2 plants of our population, only ten homozygous recombinant plants can be expected. In case of a genetic distance of 5 cM, three homozygous recombinant plants can be expected and only one plant, if the genetic distance is 3.3 cM.

After the initial screening of the 2080 F_2 lines with the two flanking markers *GWM493* and *BARC133*, seven homozygous recombinant lines were found. Unfortunately, only three could be used for further analyses, since no seeds were available for the other identified lines due to several reasons (e.g. unfertile heads, mice, birds). The finding of seven homozygous recombinant lines indicates that the genetic distance is probably larger than 3.3 cM. As mentioned before, especially the genetic distance between the two flanking markers *XGWM493* and *XBARC133* was underestimated, since the data from 228 lines including four homozygous recombinant lines were excluded, as no further seed material was present and no marker data were available for the other markers (*UMN10* and *Snp3BS-8*).

In order to gain more homozygous recombinant lines, heterozygous recombinant lines were brought into the F_3 generation. Eight seeds per line were planted and according to Mendelian rules, at two out of eight plants should have homozygous recombinant alleles. In general, the use of eight plants ensured that at least one had the proper alleles and could be used for further studies. Once again, a minor outfall came to pass (29 individual plants

distributed among all lines), but this fact did not affect the numbers of homozygous recombinant lines. However, for 37 out of 119 lines, no homozygous recombinant line could be identified. At the end of all analyses, 85 homozygous recombinant lines were identified (3 from the BC_5F_2 generation and 82 from the BC_5F_3 generation).

4.4 Phenotyping of the recombinant lines for FHB resistance

The inoculation case study was performed to evaluate the success of a special inoculation method to test for resistance against fungal spread, as conferred by *Fhb1*. A needle penetrated the glumes and the spore suspension was placed in the flower. The assumption was that the wounding would promote the fungal growth and infection.

Of the 111 inoculated wheat heads 87% developed some disease symptoms, but for 67% of the inoculated heads the disease symptoms were very weak. Three lines showed spread of the disease, primarily the highly susceptible Remus (up to 100% spreading of inoculated heads). The four near-isogenic control lines have, as the newly developed recombinant lines, CM-82036 as genetic background. In specific, CM-NIL 47 represents the highly resistant CM-82036 with susceptible Remus alleles just in the *Fhb1* region and therefore displays the expected susceptible phenotype of the fine mapping lines. CM-NIL 47 showed a spreading incidence of 11%, just for two heads spread of the disease was observed.

Beside the mentioned control lines, only the recombinant line 957 showed spread of the disease. The inconsistence in spreading of the fungus from the point of inoculation of the control lines suggests that the used inoculation method is not adequate to test for the presence or absence of *Fhb1* in a highly resistant background (see figure 15).

One possible reason would be that this inoculation method is not appropriate. The exact placement of the spore suspension onto the flower cannot be guaranteed.

In general, FHB resistance is strongly influenced by genotype x environment interactions (Miedaner et al., 2001). Another cause is possibly the inoculation circumstances like moisture or temperature. Parry et al. (1995) states that a minimum of 24 hours of moisture after the infection is a prerequisite for an optimal infection. This duration was provided during this experiment. However, maybe a longer period of moist conditions would be more beneficial for fungal growth. In the course of a study performed by Bernardo et al. (2012),

the inoculation process was different. The suspension was injected into the floral cavity between the lemma and palea of a floret in the middle of a spike. Thereafter, inoculated plants were temporarily housed in an enclosed plastic chamber (with 100% humidity) for three days. This approach would perhaps provide better conditions for the fungus.

To exclude the possibility of non germinable spores, the germination of the used spores was controlled visually under the microscope. In every case, the spores were able to germinate to a very high extend.

The fact that not all lines are in flower at the same time complicates the inoculation process (Buerstmayr et al., 1999). The inoculation was performed every two days, but in order to ensure an optimal inoculation time, the plants should be checked every day for the proper point in time.

In general, at least two independent biological experiments (locations or years) are necessary to estimate the repeatability of the resistance evaluation (Buerstmayr et al., 2009). Scientists have had great difficulty achieving reliable and reproducible FHB infection data. Reproducible phenotypic data is essential to create a reliable fine map of QTL candidate genes (Cuthbert et al., 2006).

4.5 Detection of candidate genes

4.5.1 Other methods and approaches

The fine mapping approach serves to identify responsible candidate genes. Once such a gene is detected, their contribution to the resistance trait has to be verified. Such a method is virus-induced gene silencing (VIGS). It works as a natural antiviral defence mechanism, in which host RNA silencing machinery targets and processes the virus derived dsRNA into vsiRNAs (virus- derived siRNAs). They are then recruited to host RISC complexes, which target and inhibit gene expression and protein translation (Zhang et al., 2013).

Nevertheless, a knockdown due to RNAi is incomplete, can vary between experiments and laboratories, has unpredictable off-target effects, and provides only temporary inhibition of gene function. For this reason, the ability to directly link phenotype to genotype is hindered and limits the practical application of RNAi technology (Gaj et al., 2013).

The validation of candidate genes can be obtained by using various approaches. Target-induced local lesions in genomes (TILLING) is a powerful reverse-genetics method to detect induced or natural DNA polymorphisms (Ben-Ari & Lavi, 2012). It combines random chemical mutagenesis with PCR-based screening of genes of interest (Varshney et al., 2006). However, it is also quite expensive (Bagge et al., 2007).

In the past decade, new approach like zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have emerged. They allow scientists to directly manipulate virtually any gene in a diverse range of cell types and organisms. Beside the site-specific nucleases described above, the CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats)/CRISPR-associated (Cas) system is an alternative to ZFNs and TALENs for inducing targeted genetic alterations (Gaj et al., 2013).

4.5.2 Difficulties associated with QTL analyses and MAS

The isolation of relevant genes is impaired by the fact that wheat has a large genome size, comprising three genomes (Bagge et al., 2007). Plus, FHB resistance is a very complex quantitative trait (Terzi et al., 2013).

Collard & Mackill (2008) numerate reasons to explain the low impact of marker-assisted breeding in general. One cause might be that results from studies using molecular markers may not be published or provided by different companies or research groups. The QTL x environment interactions form an obstacle as well, since the extend of those interactions is difficult to predict. Furthermore, the development and use of molecular markers is often very expensive. Plus, there is an 'application gap' between research laboratories and plant breeding institutes.

However, considerable improvements in genetic resistance have been achieved by concentrated breeding efforts that have relied primarily upon repeated field an greenhouse-based screenings (Anderson, 2007).

Marker-based backcrossing of the two QTL *Fhb1* and *Qfhs.ifa-5A* ensured a significant improvement of FHB resistance in European elite winter wheat. Both QTLs are effective and stable within wheat backgrounds and can be used for MAS without any known agronomical penalties. A lot of time and effort was invested from the beginning of QTL mapping to the final introgression of these QTL into practical breeding programs. This was possible because

(i) *Fhb1* is one of those QTL for disease resistance with the highest explained phenotypic variance found to date, (ii) the marker alleles linked to the resistance allele are not present in European and North American elite wheat, and (iii) near-perfect markers are available (Miedaner & Korzun, 2012).

QTL analyses ignore genes with small effects that trigger underpinning quantitative traits. A new promising approach for plant breeders and scientists is genome-wide selection, which estimates marker effects across the whole genome on the target population based on a prediction model developed in the training population. Whole-genome prediction models estimate all marker effects in all loci and determine also small QTL effects (Desta & Ortiz, 2014). This method may also ensure a better understanding of the complex resistance trait and thus facilitates breeding for resistant crop varieties.

5. Conclusion and Outlook

Fine mapping approaches can be seen as important groundwork for constitutive methods. With their help, QTL regions can be narrowed down, which facilitates map-based cloning and eventually helps to identify candidate genes.

The results gained by this master thesis are part of a fine mapping approach of the QTL *Fhb1*. Additional markers have to be established to saturate the genetic map. Furthermore, additional recombinant lines should be obtained by repeated selection. The last part would then be the phenotyping of the detected recombinant lines. By the combination of genetic and phenotypic data, the QTL region of *Fhb1* can thus be narrowed down and subsequent selection can be carried out faster with the help of new flanking markers.

Although many QTL mapping papers (including fine mapping studies) have been published, the gene(s) responsible for *Fhb1* are not identified yet. This underlines the complexity of FHB resistance, that breeding for disease resistance is a challenging field of research and that many approaches are necessary to finally create resistant crop varieties. The collaboration of many research groups and breeding stations would be beneficial for further history in plant breeding.

Complex and challenging questions should not frighten scientists off but rather encourage them to put effort in it to eventually answer them.

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7. Appendix

7.1 Information about chemicals

Table 19: Components and amounts of TE-8 buffer.

Stock solutions	Final concentration	Amounts for end volume (100 mL)
1 M Tris, pH: 8	10 mM	1
0.5 M EDTA, pH: 8	1 mM	0.2
dH2O		98.8

Table 20: Components and amounts of Wash 1.

Stock solutions	Final concentration	Amounts for end volume (100 mL)
dH2O		16
2.5 M NaOAc	200 mM	8
Ethanol absolute	76%	76

Table 21: Components and amounts of Wash 2.

Stock solutions	Final concentration	Amounts for end volume (100 mL)
dH2O		23
1 M NH4OAc	10 mM	1
Ethanol absolute	76%	76

7.2 Genotyping results

Table 22: List of genotyping results of the BC_5F_2 generation (with two flanking markers of *Fhb1*). 'A' stands for the parental allele of CM-82036, 'B' for the parental allele of Remus and 'H' was given for a heterozygous allelic state.

Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133
1	H	H	103	H	H	205	H	H	309	A	A
2	B	B	104	A	A	206	H	H	310	B	B
3	B	B	105	H	H	207	H	H	311	H	H
4	H	H	106	A	A	208	H	H	312	H	H
5	B	H	107	A	H	209	H	H	313	H	H
6	H	H	108	H	H	210	H	H	314	A	A
7	H	H	109	A	A	211	H	H	315	B	B
8	B	H	110	H	H	212	H	H	316	B	B
9	H	B	111	B	B	213	B	B	317	A	A
10	H	H	112	B	B	214	B	B	318	A	H
11	H	H	113	A	A	215	B	B	319	B	B
12	H	B	114	A	A	216	A	A	320	H	H
13	A	H	115	A	A	217	B	B	321	H	H
14	H	H	116	H	B	220	B	B	322	A	A
15	H	H	117	H	H	221	H	H	323	B	B
16	B	B	119	B	B	222	A	A	324	B	B
17	H	H	120	B	B	223	H	H	325	H	H
18	H	H	121	A	H	225	H	H	326	H	H
19	H	H	122	A	A	226	H	H	327	H	H
21	H	H	123	H	H	227	B	B	328	H	A
22	H	H	124	B	B	228	H	H	329	B	B
23	B	B	125	H	H	229	B	B	330	B	B
24	A	A	126	B	B	230	H	H	331	A	A
25	A	H	127	H	H	231	H	H	332	H	B
26	H	H	128	H	H	232	A	A	333	H	H
27	H	H	129	B	B	233	H	H	334	H	H
28	A	H	130	H	H	234	B	B	335	H	H
29	B	B	131	H	H	235	H	A	336	H	H
30	A	A	132	A	A	236	A	A	337	H	H
31	B	H	133	H	H	237	H	H	338	H	H
32	B	B	134	A	H	238	H	H	339	A	H
33	H	H	135	B	B	239	A	A	340	B	B
34	A	A	136	H	H	240	H	H	341	B	B
35	A	A	137	H	H	241	B	B	342	A	A
36	A	A	138	A	H	242	H	H	343	H	H
37	H	H	139	H	H	243	A	A	344	A	H
38	A	H	140	H	H	244	B	B	345	B	B
39	H	H	141	B	B	245	H	B	346	A	A
40	H	H	142	A	H	246	H	H	347	H	H
41	H	H	143	B	B	247	A	A	348	B	B
42	B	B	145	H	H	248	A	H	349	A	A
43	B	B	146	H	H	249	H	H	350	H	H
44	H	H	147	H	A	250	H	H	351	H	H
45	H	H	148	H	H	251	A	A	352	A	H
46	B	B	149	H	H	252	A	A	353	H	H
47	A	A	150	A	A	253	H	H	354	A	A
48	A	A	151	B	H	254	H	H	355	A	A
49	A	A	152	H	H	255	H	H	356	B	B
50	B	B	153	H	H	256	H	H	357	A	A
51	H	H	154	A	A	257	A	A	358	B	B
52	A	H	155	H	H	258	B	B	359	A	H
53	A	A	156	H	H	259	H	H	360	A	A
54	H	H	157	H	B	260	H	H	361	A	A
55	H	H	158	B	B	261	B	B	362	H	H
56	H	H	159	H	H	262	H	B	363	H	H
58	H	H	160	H	H	263	H	H	364	B	B
59	B	B	161	H	H	264	A	A	365	H	B
60	H	H	162	H	H	265	B	H	366	H	H
61	H	H	163	H	H	266	A	A	367	H	H
62	A	A	164	H	H	267	H	H	368	H	H
63	H	H	165	A	A	268	B	B	369	H	H
64	B	H	166	H	H	269	A	A	370	H	H
65	B	B	167	A	A	270	A	A	371	B	B
66	B	B	168	B	B	271	H	H	372	B	B
67	H	H	169	H	H	273	H	A	373	A	A
68	B	B	170	B	B	274	H	H	374	B	B
69	A	A	171	A	H	275	H	H	375	B	A
70	H	H	172	B	B	276	H	H	376	H	H
71	B	B	173	H	H	277	H	H	377	A	A
72	H	H	174	B	B	278	A	H	378	H	H
73	B	H	175	H	H	279	B	B	379	H	H
74	B	B	176	H	H	280	B	B	380	A	A
75	B	B	177	A	A	281	H	H	381	H	A
76	B	B	178	B	B	282	H	H	382	H	H
77	A	A	179	B	B	283	B	B	383	H	H
78	A	H	180	A	A	284	H	H	385	H	H
79	H	H	181	H	B	285	B	B	386	B	B
80	H	H	182	H	H	286	B	B	387	H	H
81	B	B	183	A	A	287	H	H	388	H	H
82	A	H	184	A	A	288	A	A	389	A	A
83	B	B	185	B	B	289	H	H	390	H	H
84	A	A	186	A	A	290	H	H	391	A	A
85	B	B	187	A	A	291	H	H	392	B	B
86	B	B	188	A	H	292	B	B	393	A	A
87	H	H	189	H	H	293	H	H	395	H	H
88	H	H	190	B	B	294	H	H	396	B	B
89	B	B	191	H	H	295	B	B	397	A	H
90	H	H	192	B	B	296	H	A	398	A	A
91	A	H	193	B	B	297	H	A	399	B	B
92	H	B	194	H	H	298	H	H	400	A	A
93	B	B	195	H	H	299	A	A	401	A	H
94	A	H	196	H	H	300	B	B	402	B	B
95	B	B	197	A	A	301	B	B	403	B	B
96	B	B	198	A	A	302	H	H	404	A	H
97	H	H	199	B	H	303	A	A	405	H	H
98	B	B	200	H	H	304	H	H	406	A	A
99	A	A	201	A	A	305	B	B	407	A	A
100	H	H	202	A	H	306	H	A	408	B	B
101	H	H	203	B	H	307	H	H	409	H	H
102	A	A	204	H	H	308	A	H	410	H	H

Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133
411	B	B	512	A	A	612	B	B	718	A	H
412	H	H	513	H	H	613	A	A	719	B	B
413	B	B	514	A	H	614	A	A	720	A	A
414	H	H	515	H	H	615	H	H	721	B	B
415	H	A	516	H	H	616	H	H	722	H	H
416	A	A	517	H	H	618	B	B	723	B	B
417	A	A	518	A	A	619	A	A	724	H	H
418	H	H	519	A	H	620	A	A	725	H	H
419	H	H	520	B	B	621	B	B	726	B	B
420	H	H	521	A	A	622	H	H	727	B	B
421	A	A	522	B	B	623	B	B	728	A	A
422	H	A	523	B	B	624	A	A	729	H	H
424	A	A	524	H	H	625	H	H	730	H	H
425	H	H	525	H	H	626	H	H	731	B	H
426	H	A	526	H	H	627	B	B	733	B	B
427	B	H	527	H	H	628	A	A	734	H	H
428	H	H	528	B	H	629	A	A	735	A	A
429	H	H	529	B	B	630	A	A	736	H	H
430	H	H	530	A	A	631	H	H	737	H	H
431	B	B	531	H	H	632	B	B	738	H	H
432	A	A	532	H	H	633	A	A	739	B	B
433	H	A	533	H	H	634	B	B	740	H	H
434	B	B	534	B	B	635	H	H	741	H	H
435	H	H	535	B	H	636	B	B	742	H	H
436	B	B	536	H	H	637	H	H	743	H	H
437	A	A	537	H	B	638	H	H	744	H	H
438	H	H	538	B	B	639	H	H	745	H	H
439	H	H	539	H	H	640	H	H	746	A	A
440	B	H	540	H	H	641	H	B	747	H	H
441	H	B	541	H	H	642	B	B	748	B	B
442	B	B	542	H	H	643	B	H	749	H	B
443	A	H	543	H	H	644	A	A	750	H	H
444	B	B	544	A	A	646	H	H	751	H	H
445	H	H	545	A	A	647	B	B	752	B	B
446	B	B	546	H	H	648	H	H	753	A	B
447	H	A	547	B	B	649	B	B	754	H	H
448	H	H	548	H	H	651	B	B	755	H	H
449	A	A	549	H	H	652	H	H	756	H	H
450	A	A	550	B	B	653	H	H	757	H	H
451	H	H	551	H	H	655	B	B	758	H	H
452	H	H	552	B	B	656	H	H	759	H	H
453	A	A	553	B	B	657	A	A	760	B	B
454	H	H	554	B	B	658	A	A	761	A	A
455	H	H	555	H	H	659	B	B	762	H	H
456	A	H	556	H	H	660	H	H	763	B	B
457	H	H	557	A	A	661	B	B	764	H	H
458	H	H	558	H	H	662	H	H	765	H	H
459	H	H	559	B	H	663	B	B	766	B	B
460	B	B	560	H	H	664	B	B	767	H	H
461	H	H	561	H	H	665	A	A	768	H	H
462	H	H	562	B	B	666	A	A	769	A	A
463	B	B	563	B	B	667	B	B	770	A	H
464	B	B	564	B	B	668	H	H	771	A	A
465	H	H	565	H	H	669	H	H	772	H	A
466	H	H	566	A	A	670	B	B	773	H	B
467	B	B	567	H	B	671	B	B	774	B	B
468	A	A	568	H	H	672	A	A	775	A	A
469	A	A	569	H	A	673	B	B	777	B	H
470	H	H	570	B	B	674	H	H	779	H	H
471	A	A	571	H	H	675	B	B	780	A	A
472	H	B	572	A	A	676	B	B	781	B	B
473	A	A	573	H	H	677	A	A	782	H	B
474	A	A	574	B	B	678	H	B	783	A	A
475	B	B	575	H	H	679	H	H	784	H	A
476	H	H	576	H	A	680	H	H	785	B	H
477	A	A	577	A	H	681	H	H	786	B	B
478	A	A	578	H	H	682	H	H	787	H	H
479	A	H	579	B	B	684	H	H	788	B	B
480	H	H	580	B	B	685	A	A	789	A	H
481	A	H	581	B	B	686	B	B	790	H	H
482	H	H	582	H	H	687	H	H	791	B	B
483	H	H	583	H	H	688	B	B	792	H	H
484	H	H	584	B	B	689	H	H	793	H	H
485	B	B	585	A	H	690	B	B	794	B	B
486	A	A	586	H	H	691	H	H	795	A	H
487	H	H	587	B	B	692	B	B	796	H	H
488	B	B	588	H	H	693	H	H	797	H	H
489	B	B	589	H	H	694	A	H	798	H	H
490	A	A	590	B	B	695	B	B	800	B	B
491	H	H	591	H	H	696	H	H	801	A	A
492	B	B	592	H	B	697	A	A	802	A	A
493	H	H	593	H	H	698	H	H	803	H	H
494	B	B	594	H	H	699	B	B	804	H	H
495	A	A	595	H	H	700	H	H	805	H	H
496	B	B	596	A	A	701	H	H	806	H	H
497	H	H	597	H	H	702	H	H	807	A	A
498	B	B	598	B	B	703	A	A	808	H	H
499	H	H	599	H	H	704	H	H	809	B	H
500	H	H	600	H	H	705	H	H	810	H	H
501	H	H	601	B	B	706	H	H	811	H	B
502	H	H	602	H	H	708	H	H	814	H	H
503	B	B	603	A	H	709	H	H	815	H	H
504	H	H	604	H	H	710	H	H	816	H	H
505	H	H	605	B	B	711	A	A	817	H	H
506	B	B	606	H	H	712	B	B	818	A	A
507	H	H	607	H	H	713	B	B	819	A	A
508	B	B	608	A	A	714	H	H	821	A	A
509	H	H	609	H	H	715	H	H	822	A	A
510	H	A	610	B	B	716	H	H	823	B	B
511	B	H	611	H	H	717	A	A	824	A	A

Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133
825	B	B	932	H	H	1037	A	A	1141	A	A
827	A	H	933	H	H	1038	H	H	1142	B	B
829	H	H	934	B	B	1039	B	B	1143	B	B
830	H	H	935	H	H	1041	H	H	1144	H	H
831	H	H	936	B	B	1042	A	A	1145	B	B
832	B	B	937	H	H	1043	A	A	1146	H	H
833	A	A	938	H	H	1044	H	H	1147	A	A
834	H	H	939	B	B	1045	A	A	1148	B	B
835	H	H	940	A	A	1046	B	B	1149	B	B
836	A	A	941	B	B	1047	B	B	1150	B	B
837	A	H	942	H	A	1048	H	H	1151	A	A
838	B	B	943	B	B	1049	H	B	1152	H	H
839	B	B	944	H	H	1050	A	A	1153	H	H
841	H	H	945	H	H	1051	H	H	1154	H	H
842	H	H	946	A	A	1053	H	B	1155	A	A
843	H	B	947	A	A	1054	B	B	1156	B	B
844	H	H	948	A	H	1055	H	B	1158	A	A
845	A	A	949	H	H	1056	B	B	1159	B	B
846	H	H	950	H	H	1057	B	B	1160	B	B
849	B	B	951	A	A	1058	H	H	1161	H	H
850	A	A	952	H	H	1059	H	H	1162	B	B
851	H	H	953	A	A	1060	H	B	1163	B	B
853	B	B	954	B	B	1061	B	H	1164	H	H
855	H	H	955	H	H	1062	H	B	1165	H	B
856	H	H	956	B	B	1063	B	B	1166	H	H
857	A	H	957	A	B	1064	H	H	1167	B	B
858	H	H	958	H	H	1065	H	H	1168	H	H
859	H	H	959	H	H	1066	H	B	1169	A	A
860	H	H	960	B	B	1067	H	H	1170	H	H
861	H	H	961	H	H	1068	H	H	1171	H	H
862	A	A	962	H	H	1069	H	B	1172	A	A
863	H	H	963	H	H	1070	B	H	1173	B	B
864	B	B	964	A	H	1071	H	B	1174	H	H
865	H	H	965	A	H	1072	B	B	1175	H	A
866	H	H	966	H	H	1073	B	B	1176	A	A
867	A	A	967	B	B	1074	B	B	1177	B	H
868	B	B	968	A	A	1075	H	H	1178	B	B
869	H	B	969	H	A	1076	B	B	1179	A	A
870	A	A	970	A	H	1077	A	A	1180	H	H
871	B	B	971	H	H	1078	B	B	1181	B	B
872	H	H	972	A	A	1079	H	H	1182	H	B
873	H	H	973	A	A	1080	H	H	1183	H	H
874	A	A	974	B	H	1081	H	H	1184	H	B
875	A	A	975	A	A	1082	H	B	1185	B	B
876	H	H	976	H	H	1083	B	B	1186	B	B
877	H	H	977	H	H	1084	H	H	1187	A	A
878	H	H	978	H	H	1085	B	B	1188	H	H
879	H	H	979	H	H	1086	H	H	1189	H	H
880	B	B	980	H	H	1087	H	H	1190	A	A
881	H	H	981	A	A	1088	B	B	1191	A	A
882	A	H	982	A	A	1089	H	H	1192	H	A
883	B	B	984	H	H	1090	H	H	1193	H	H
884	H	H	985	A	A	1091	A	A	1194	H	H
885	H	H	986	H	H	1092	A	A	1195	H	H
886	B	B	987	A	A	1093	H	H	1196	H	H
887	H	H	988	A	H	1094	H	H	1197	H	H
888	H	A	989	H	H	1095	A	A	1198	A	A
889	B	B	990	H	A	1096	H	H	1199	A	A
890	H	B	991	A	A	1097	A	A	1200	H	H
891	A	A	992	H	B	1098	H	H	1201	H	H
892	H	H	993	B	B	1099	A	A	1202	B	B
893	B	B	994	H	H	1100	H	H	1203	A	A
894	B	B	995	H	H	1101	H	H	1204	A	A
895	H	H	996	H	H	1102	H	H	1205	H	H
896	H	H	997	A	H	1103	H	H	1206	B	B
897	H	H	998	H	H	1104	B	B	1207	B	B
898	A	A	999	B	B	1105	B	B	1208	H	H
899	A	H	1000	B	B	1106	B	B	1209	H	H
900	H	H	1001	B	B	1107	A	A	1210	H	H
901	H	H	1002	B	B	1108	A	A	1211	H	H
902	H	H	1003	H	H	1109	A	A	1212	B	B
903	B	B	1004	H	H	1110	B	B	1213	A	A
904	B	B	1006	B	B	1111	H	H	1215	H	H
905	H	H	1007	H	H	1112	H	A	1216	H	H
906	A	A	1008	B	B	1113	B	B	1217	A	A
907	A	A	1009	H	H	1114	H	H	1218	H	H
908	B	B	1010	A	A	1115	H	H	1219	H	H
909	H	H	1011	H	H	1116	A	A	1220	B	B
910	B	B	1012	H	H	1117	H	H	1222	B	B
911	B	B	1013	H	H	1118	B	B	1223	B	B
912	H	A	1016	B	B	1119	A	A	1224	A	A
913	A	A	1017	H	H	1120	H	H	1225	B	B
914	H	H	1018	H	A	1121	A	H	1227	H	H
915	B	B	1019	B	B	1122	H	H	1228	H	H
916	H	H	1020	H	H	1123	H	H	1229	H	H
917	A	A	1021	H	H	1124	H	H	1230	H	H
918	B	B	1022	A	H	1125	A	A	1231	B	B
919	B	B	1023	H	H	1126	A	A	1232	B	B
920	A	A	1024	H	H	1127	B	H	1233	H	H
921	H	H	1025	H	B	1128	H	H	1234	H	H
922	B	H	1026	H	A	1129	A	H	1235	H	H
923	H	H	1027	A	A	1130	B	B	1236	H	H
924	A	A	1028	H	H	1131	B	B	1237	A	A
925	H	H	1029	B	H	1132	H	H	1238	H	A
926	H	H	1030	H	H	1134	H	H	1239	B	H
927	A	A	1031	B	B	1135	B	B	1240	B	H
928	H	H	1033	A	A	1136	B	H	1241	B	H
929	H	H	1034	A	A	1137	B	H	1242	A	A
930	H	H	1035	H	H	1139	A	A	1243	B	H
931	H	H	1036	H	H	1140	H	H	1244	A	H

Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133
1245	A	A	1354	H	H	1458	B	B	1560	H	H
1246	A	A	1355	B	B	1459	H	H	1561	H	H
1247	B	H	1356	H	H	1460	B	B	1562	H	H
1248	A	A	1357	H	H	1461	B	B	1563	H	H
1249	H	H	1358	H	H	1462	B	B	1564	H	H
1250	H	B	1359	B	B	1463	H	H	1565	A	A
1252	B	B	1360	B	B	1464	A	H	1566	H	A
1253	H	H	1361	H	H	1465	B	B	1567	B	B
1254	H	H	1362	B	B	1466	B	B	1568	H	B
1255	A	A	1363	H	H	1467	A	A	1569	H	H
1256	B	B	1364	H	H	1468	A	A	1570	A	H
1257	H	H	1365	H	H	1469	B	A	1571	H	H
1259	H	B	1366	H	H	1471	B	B	1572	A	A
1260	H	H	1367	H	H	1472	H	H	1573	H	H
1261	H	A	1370	B	B	1473	B	B	1574	B	B
1262	H	H	1371	A	A	1474	B	B	1575	B	B
1263	H	H	1372	B	H	1475	H	H	1576	H	H
1264	B	B	1373	B	B	1476	A	A	1577	A	H
1265	A	H	1374	H	H	1477	B	B	1578	A	A
1266	A	A	1375	B	B	1478	B	B	1579	H	H
1267	H	H	1377	H	H	1479	A	A	1580	H	H
1268	H	H	1378	H	H	1480	B	B	1581	A	A
1269	A	A	1379	B	A	1482	H	H	1582	A	A
1270	H	H	1380	H	H	1483	A	A	1583	B	H
1272	A	A	1381	H	A	1484	H	H	1585	H	H
1273	H	H	1382	B	B	1485	B	B	1587	B	B
1274	A	A	1383	B	B	1486	A	A	1588	A	A
1275	H	H	1384	H	H	1487	H	H	1589	H	H
1276	H	H	1385	H	A	1488	A	A	1590	A	A
1277	B	B	1386	A	H	1489	H	H	1591	A	A
1278	B	H	1387	A	A	1490	H	H	1592	H	B
1279	B	B	1388	H	H	1491	B	B	1594	A	A
1280	A	A	1389	H	H	1492	H	H	1595	B	B
1281	A	A	1390	B	B	1493	A	A	1596	B	B
1282	H	H	1391	H	H	1494	H	H	1597	B	B
1283	B	B	1392	B	H	1495	H	H	1598	H	A
1284	A	A	1393	A	H	1496	H	H	1599	H	H
1285	H	H	1394	H	H	1497	H	H	1600	B	H
1286	A	A	1395	H	H	1498	H	H	1601	H	H
1287	A	H	1396	H	B	1499	B	B	1602	H	H
1288	H	H	1397	B	B	1500	B	B	1603	B	B
1289	H	H	1398	H	H	1501	H	H	1604	B	B
1290	H	H	1399	H	A	1502	A	A	1605	B	B
1291	A	A	1400	A	A	1503	H	H	1606	B	H
1292	B	B	1401	H	H	1504	A	H	1607	H	H
1294	A	A	1402	B	B	1505	B	B	1608	H	H
1295	B	B	1403	B	B	1506	B	B	1609	A	H
1296	A	A	1404	H	H	1507	B	B	1610	H	H
1297	B	B	1405	H	H	1508	H	H	1611	A	A
1298	A	A	1406	H	H	1509	H	H	1612	B	B
1299	A	A	1407	B	B	1510	H	H	1613	H	H
1300	B	B	1408	B	B	1511	B	H	1614	B	B
1301	A	B	1409	H	H	1512	A	A	1615	H	H
1302	H	H	1410	H	H	1513	H	H	1616	H	H
1303	B	B	1411	A	A	1514	A	A	1617	H	H
1305	H	H	1413	H	H	1515	H	A	1618	H	H
1306	A	A	1414	H	H	1516	H	B	1619	B	H
1307	H	H	1415	B	B	1517	A	A	1620	H	H
1308	H	H	1416	B	B	1518	A	A	1621	A	H
1309	B	B	1417	A	A	1519	H	A	1622	A	A
1310	A	A	1418	H	H	1520	H	H	1623	H	H
1311	B	H	1419	H	A	1521	H	H	1624	A	A
1312	A	A	1420	H	H	1522	H	H	1625	A	H
1313	H	H	1421	H	H	1523	A	H	1626	A	A
1314	A	H	1422	H	H	1524	A	A	1627	A	H
1315	A	A	1423	B	B	1525	A	H	1628	H	H
1316	B	B	1424	H	H	1526	B	B	1629	H	H
1317	A	A	1425	H	H	1527	H	B	1630	A	A
1318	B	B	1426	H	H	1528	A	H	1631	B	H
1319	A	A	1427	H	H	1529	A	A	1632	B	B
1320	A	A	1428	B	B	1530	H	H	1633	B	H
1321	H	H	1429	A	A	1531	H	H	1634	H	H
1322	B	B	1430	H	H	1532	H	H	1635	H	B
1323	B	B	1431	H	B	1533	A	H	1636	B	B
1324	B	B	1432	H	H	1534	A	A	1637	H	H
1325	A	A	1433	B	B	1535	B	B	1638	H	H
1326	H	H	1434	A	A	1536	H	H	1639	B	B
1327	A	A	1435	H	H	1537	H	H	1640	A	A
1328	B	B	1436	H	H	1538	H	H	1641	A	A
1329	B	B	1437	H	H	1539	B	B	1642	H	H
1330	H	A	1438	B	H	1540	A	H	1643	H	H
1331	B	B	1439	A	H	1541	H	H	1644	A	A
1333	A	A	1440	H	H	1542	H	H	1645	H	H
1334	H	H	1441	B	H	1543	H	H	1646	B	B
1335	B	B	1442	B	B	1544	B	B	1647	A	A
1336	H	H	1443	B	H	1545	A	A	1648	H	B
1337	B	B	1444	H	H	1546	A	B	1649	H	H
1339	H	H	1445	H	H	1547	H	H	1650	H	H
1340	B	B	1446	A	H	1548	H	H	1651	H	H
1341	A	A	1447	A	H	1549	H	H	1652	H	H
1342	H	H	1448	B	B	1550	B	B	1653	H	H
1343	H	H	1449	H	H	1551	A	A	1654	B	B
1344	H	A	1450	H	H	1552	A	H	1655	B	B
1345	H	H	1451	H	H	1553	H	H	1656	H	B
1346	H	H	1452	A	A	1554	B	B	1657	B	B
1348	A	A	1453	H	H	1555	H	H	1658	H	H
1350	B	B	1454	H	H	1556	H	H	1659	H	B
1351	A	A	1455	B	B	1557	A	A	1660	A	A
1352	H	H	1456	H	H	1558	B	H	1662	H	A
1353	B	B	1457	H	H	1559	A	A	1663	H	A

Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133	Line	GWM493	BARC133
1664	H	H	1770	H	A	1876	H	H	1980	A	A
1665	B	H	1771	H	H	1877	H	H	1981	H	H
1666	B	B	1773	B	B	1879	A	H	1982	B	B
1667	B	B	1774	H	A	1880	A	A	1983	H	H
1668	A	A	1775	H	H	1881	B	B	1984	H	H
1669	H	H	1776	H	H	1882	B	B	1985	H	H
1670	H	H	1777	H	A	1883	H	B	1986	H	H
1671	H	A	1778	H	H	1884	B	B	1987	H	H
1672	B	B	1779	B	B	1885	A	A	1988	A	A
1673	H	H	1780	A	A	1886	A	A	1989	H	H
1674	B	B	1781	A	A	1887	A	B	1991	H	H
1675	A	A	1782	B	B	1888	B	B	1992	A	A
1676	H	A	1783	H	H	1889	B	B	1994	B	B
1677	H	H	1784	B	B	1890	H	H	1995	A	A
1678	A	H	1785	H	B	1891	A	A	1996	H	H
1679	H	H	1786	H	B	1892	B	B	1997	H	H
1680	B	B	1787	H	H	1893	B	B	1998	H	H
1681	B	B	1788	B	B	1894	H	H	1999	H	H
1682	H	A	1789	H	B	1895	B	B	2000	A	A
1683	H	H	1790	A	A	1896	H	H	2001	A	A
1684	H	H	1791	B	B	1897	H	H	2002	A	A
1685	H	H	1792	H	H	1899	H	H	2003	H	H
1686	B	B	1793	A	A	1900	B	H	2004	H	B
1687	A	A	1794	B	B	1901	A	A	2005	B	B
1688	H	H	1795	H	H	1902	H	H	2006	B	B
1690	A	A	1796	H	A	1903	A	A	2007	B	B
1692	H	H	1797	A	A	1904	A	H	2009	B	B
1693	A	A	1798	H	H	1905	H	H	2010	H	H
1694	H	H	1799	H	H	1907	A	A	2011	A	A
1695	H	B	1800	H	H	1908	A	A	2012	H	H
1696	A	H	1801	H	H	1909	B	B	2013	H	H
1697	A	H	1802	B	B	1910	A	A	2014	B	B
1698	H	H	1803	H	H	1911	H	H	2015	A	A
1699	B	B	1804	H	H	1912	A	H	2016	H	B
1700	A	A	1805	A	A	1913	H	H	2017	A	H
1701	A	A	1806	B	B	1914	H	H	2018	B	B
1702	H	H	1807	H	H	1915	B	B	2019	B	B
1703	A	A	1808	A	H	1916	H	H	2020	H	H
1704	A	A	1809	H	H	1917	H	H	2021	A	A
1705	H	H	1810	A	A	1918	A	A	2022	H	H
1706	H	H	1811	H	H	1919	A	A	2023	A	A
1707	A	A	1812	H	H	1920	A	H	2024	A	H
1708	B	B	1813	H	H	1921	A	A	2025	H	H
1709	A	A	1814	A	A	1922	H	H	2026	B	B
1710	H	H	1815	B	B	1923	B	B	2027	H	H
1711	A	H	1816	A	A	1924	H	H	2028	H	H
1712	B	B	1817	H	H	1925	A	A	2029	H	B
1713	B	B	1818	H	H	1926	H	H	2030	B	B
1714	B	B	1819	H	H	1927	H	H	2031	H	B
1715	H	A	1820	H	H	1928	B	H	2032	H	B
1716	A	H	1821	H	H	1930	B	B	2033	H	B
1717	B	B	1822	H	H	1931	B	B	2034	B	B
1718	H	H	1823	B	B	1932	H	H	2035	B	B
1719	B	B	1824	H	H	1933	H	H	2036	H	H
1720	A	A	1825	H	H	1934	H	B	2037	H	B
1721	H	H	1826	B	H	1935	H	H	2038	H	H
1722	B	B	1828	H	H	1936	A	H	2039	A	A
1723	H	H	1829	B	B	1937	H	H	2040	H	H
1724	H	H	1830	H	H	1938	B	B	2041	H	H
1725	H	H	1832	A	A	1939	H	H	2042	H	H
1726	H	B	1833	H	H	1940	H	H	2043	H	H
1728	H	H	1834	H	H	1941	A	A	2044	A	A
1729	H	H	1835	A	H	1942	B	B	2045	A	A
1730	B	B	1836	B	B	1943	H	H	2046	B	B
1731	H	H	1837	B	B	1944	H	H	2047	A	A
1732	B	B	1838	B	B	1945	H	H	2048	H	H
1734	B	B	1839	H	H	1946	A	A	2049	H	H
1735	H	H	1840	A	A	1947	B	B	2050	H	H
1736	H	H	1841	H	H	1948	B	B	2051	B	H
1737	A	H	1842	B	B	1949	B	B	2052	A	A
1739	B	B	1843	B	B	1950	H	H	2053	A	H
1740	H	H	1844	H	H	1951	H	H	2054	H	H
1741	B	B	1845	B	H	1952	H	H	2055	B	B
1742	A	A	1846	B	B	1953	B	B	2056	H	B
1743	H	H	1848	B	B	1954	B	B	2057	H	H
1744	H	H	1850	H	B	1955	H	H	2058	A	A
1745	A	A	1851	H	H	1956	A	A	2059	A	A
1746	A	A	1852	H	H	1957	B	H	2060	B	B
1747	B	B	1853	H	H	1958	A	A	2061	H	H
1748	A	A	1854	A	H	1959	H	H	2063	H	H
1750	H	H	1855	B	B	1960	H	H	2064	A	A
1751	H	H	1857	B	B	1961	H	H	2067	B	B
1752	H	H	1858	B	B	1962	A	A	2068	A	A
1753	H	H	1859	B	B	1963	B	H	2069	A	A
1754	B	B	1860	H	H	1964	B	B	2070	H	H
1755	B	B	1861	H	H	1965	H	H	2071	B	B
1756	H	H	1862	A	A	1966	H	H	2072	H	H
1757	H	B	1863	H	B	1967	B	B	2073	A	H
1758	B	B	1864	B	B	1968	B	H	2074	B	B
1759	H	H	1865	H	A	1969	H	H	2075	A	A
1760	B	B	1866	H	H	1970	H	H	2076	H	H
1761	A	H	1867	A	A	1971	B	B	2077	H	H
1762	H	H	1868	H	H	1972	A	A	2078	H	H
1763	A	A	1869	B	B	1973	B	B	2079	H	H
1764	B	B	1870	H	A	1974	B	B	2080	H	H
1765	B	H	1871	H	H	1975	H	H			
1766	H	H	1872	H	H	1976	H	H			
1767	A	A	1873	A	H	1977	H	H			
1768	B	B	1874	H	H	1978	H	H			
1769	B	H	1875	B	B	1979	A	A			

Table 23: List of genotyping results of the BC_5F_3 lines (with four molecular markers of *Fhb1*). 'A' stands for the parental allele of CM-82036, 'B' for the parental allele of Remus and 'H' was given for a heterozygous allelic state.

Line	GWM493	Xsnp3BS-8	UMN10	BARC133
9_1	A	B	B	B
12_4	A	B	B	B
13_2	A	B	B	B
31_2	B	A	A	A
73_2	B	B	B	A
82_6	A	B	B	B
116_6	A	B	B	B
151_3	B	A	A	A
181_4	A	B	B	B
248_1	A	B	B	B
265_5	B	A	A	A
273_3	B	A	A	A
296_5	B	B	B	A
365_4	A	B	B	B
381_8	B	A	A	A
447_1	B	A	H	A
528_5	B	A	A	A
535_6	B	A	A	A
592_2	A	B	B	B
678_5	A	B	B	B
694_8	A	B	B	B
731_2	B	A	A	A
749_2	A	B	B	B
773_8	A	B	B	B
789_3	A	B	B	B
809_1	B	B	B	A
827_6	A	A	A	B
837_5	A	A	B	B
857_3	A	A	A	B
869_7	A	B	B	B
890_8	A	B	B	B
942_8	B	A	A	A
957_1	A	B	B	B
988_1	A	B	B	B
990_4	B	B	B	A
1022_7	A	H	H	B
1026_6	B	B	B	A
1058_3	A	A	A	B
1067_4	A	B	B	B
1121_4	A	B	B	B
1136_2	B	B	B	A
1137_8	B	A	A	A
1177_7	B	A	A	A
1247_7	B	A	A	A
1250_8	A	B	B	B
1259_7	A	B	B	B
1261_8	B	A	A	A
1265_1	A	B	B	B
1301_6	A	B	B	B
1330_3	B	B	B	A
1344_3	B	A	A	A
1372_4	B	A	A	A
1386_6	A	A	A	B
1441_7	B	A	A	A
1443_5	B	B	B	A
1469_1	B	A	A	A
1516_4	A	B	B	B
1546_1	A	A	A	B
1558_8	B	A	A	A
1566_4	B	A	A	A
1606_2	B	A	A	A
1609_7	A	A	A	B
1627_4	A	B	B	B
1635_1	A	A	A	B
1659_3	A	A	A	B
1663_6	B	A	A	A
1682_1	B	A	A	A
1695_5	A	A	A	B
1711_7	A	B	B	B
1726_8	A	B	B	B
1737_6	A	B	B	B
1757_6	A	B	B	B
1769_6	B	A	A	A
1785_6	A	B	B	B
1786_3	A	B	B	B
1789_3	A	B	B	B
1808_1	A	B	B	B
1835_3	A	A	A	B
1850_7	A	B	B	B
1873_1	A	B	B	B
1883_4	A	B	B	B
1957_7	B	B	B	A
1963_3	B	B	B	A
2016_3	A	B	B	B
2073_2	A	A	B	B